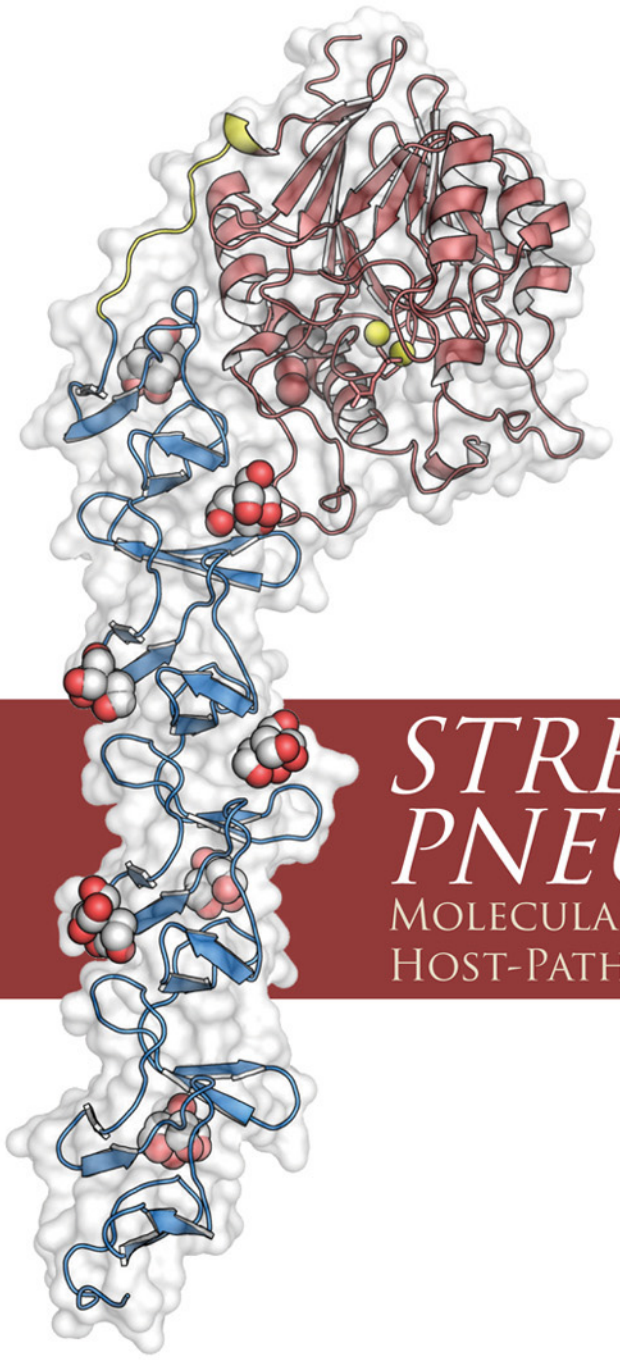
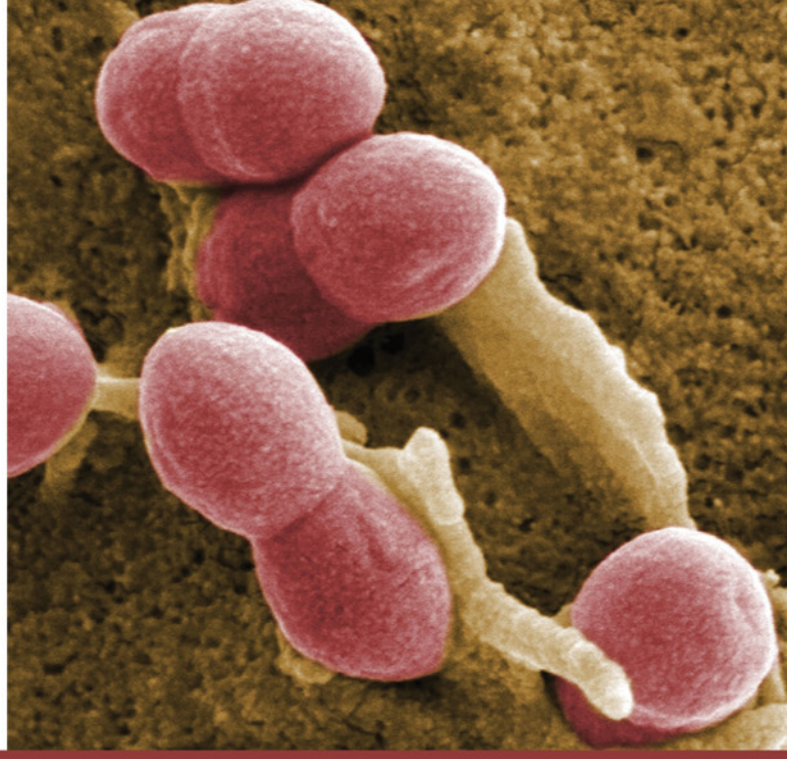
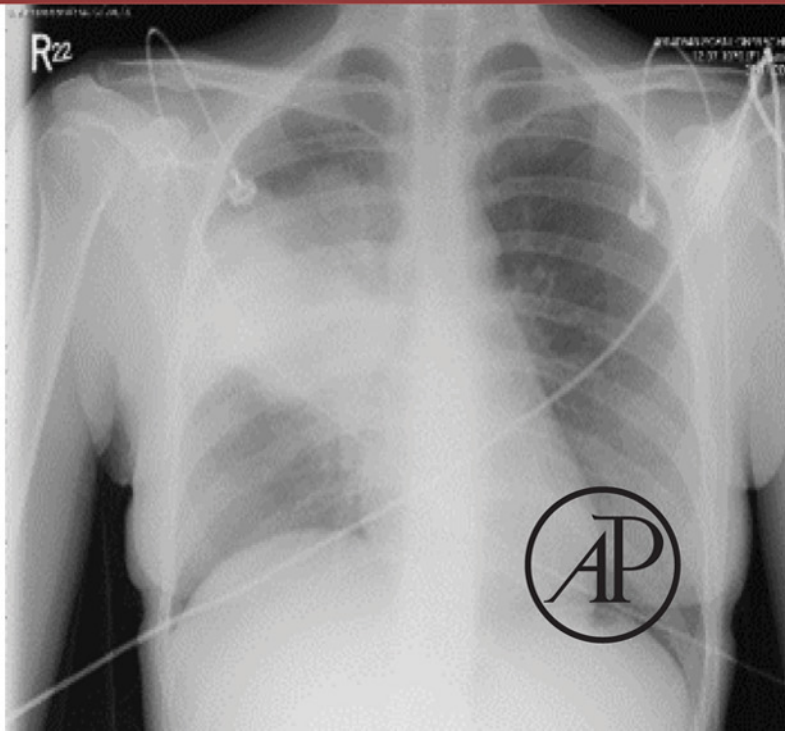




STREPTOCOCCUS PNEUMONIAE

Molecular Mechanisms of Host-Pathogen Interactions



Edited by

Jeremy Brown

Sven Hammerschmidt

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STREPTOCOCCUS PNEUMONIAE

Molecular Mechanisms of Host–pathogen Interactions

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Preface

Streptococcus pneumoniae (the pneumococcus) has been and continues to be among the chief causes of human misery and death. It is capable of a broad swath of disease manifestations, including otitis media, the more serious community-acquired pneumonia, and devastating illnesses such as septicemia and meningitis. As a commensal colonizer of the nasopharynx, the pneumococcus is the prototypical opportunistic pathogen, but it is also capable of causing disease in previously seemingly healthy individuals. While the overall attack rate for the pneumococcus is low, so many individuals are colonized that the global burden of disease is enormous. Primarily affected are infants whose immune system has not yet developed the capacity to ward off infection, the elderly whose immune system is waning, and those who are immunocompromised. Most often age and immunosuppression overlap, with malnourished and smoke-exposed children and the elderly with multiple underlying medical conditions being at greatest risk. Epidemiological studies suggest that the number of children who succumb to pneumococcal disease exceeds 650,000 annually worldwide. For the elderly, the case-fatality rate for pneumococcal pneumonia is 10–15%, climbing approximately 10% with every decade of life after 65 years. As a result, and despite effective antibiotics, the mortality rate for an 85-year-old with pneumococcal pneumonia is 30–45%, reinforcing Sir William Osler's early-twentieth-century observation that the pneumococcus is the "old man's friend" and that this pathogen is still highly relevant in the twenty-first century. Importantly, the morbidity and socioeconomic cost associated with nonlethal pneumococcal infections is also very large. For these reasons, research on basic pneumococcal biology, disease pathogenesis, and interactions with the host continues to be vital for human health, and is probably undersupported given the global burden of pneumococcal disease in both developing and industrial countries.

The human effort to prevent pneumococcal disease has also directly and indirectly led to some of our greatest biological discoveries. Pneumococci were used to obtain evidence of genetic recombination by Griffith in 1928; identification of DNA as the transforming principle by Avery, Macleod, and McCarthy in 1944; and the discovery of antibody-mediated opsonization by Neufeld (1902, 1904, and 1910), the latter being the basis of many of today's vaccines. The pneumococcus remains the subject of continued intense research, with considerable progress having been made in our understanding of the molecular basis of pneumococcal biology. It is probably one of the most studied single bacterial pathogens, and rightly so given its importance for human disease. Important discoveries that are relevant not just for the pneumococcus, but more broadly for bacterial pathogens, continue today despite more than 120 years of research on the pneumococcus. For example, recent studies suggest that the pneumococcus uses epigenetics to regulate the expression of its virulence genes, the first description of this for a bacterium, and that the pneumococcus may cause cardiac damage during pneumonia. In addition, the pneumococcus has been at the forefront of the bacterial

genome sequencing revolution, with sequences now available for thousands of strains, as well as in investigating the cell biology of bacterial infection and defining the role of cellular immune responses in preventing mucosal infections by extracellular pathogens.

In this textbook we present what we believe is an exciting, up-to-date description of the epidemiology, evolution, microbiology, pathogenesis, immunology, and cell biology of the pneumococcus. We have strived to do so with a focus on the molecular mechanisms responsible. The chapters are written by recognized experts in their respective areas, and we are extremely thankful for their willingness to participate. The sheer number of important new revelations in recent years about the biology of pneumococcus and the pathogenesis of pneumococcal infection have made the need for a new textbook obvious. By presenting up-to-date reviews of a wide range of different areas, this book allows the reader a thorough overview of the biology of this important pathogen and its capacity to modulate the host immune response. The textbook is most obviously of importance for researchers working on the pneumococcus, but it is also of interest for anyone working in the field of bacterial pathogenesis or involved in caring for patients with pneumococcal infection. We hope that readers will obtain a greater appreciation of the tremendous accomplishments made in understanding how the pneumococcus causes disease, how it adapts to the host environment, how we as the host protect ourselves against it, and the challenges that face current and future generations of investigators as we strive to fully understand the biology of the complex interactions between the pneumococcus and ourselves.

As editors, it has been our privilege to read each chapter and therefore obtain a broad overview of what we know and, perhaps more importantly, what we don't know about the pneumococcus. This has allowed us to think about what might be the important questions to address in order to better understand how and why the pneumococcus is such a successful pathogen. We have summarized some of these questions in Table 1. These are very much our personal views of areas that could be important for future research, and are not meant to be exhaustive; other questions we have not included or considered will be equally important. Many of these questions are probably self-evident to researchers in the field, but others may be less so. We hope that you as a reader will find them stimulating and perhaps the basis of potential future research projects. Below, we have discussed in more detail important aspects of pneumococcal biology underpinning why we feel some of these potential research questions are important.

Much attention has been paid to the role of the polysaccharide capsule for pneumococcal biology, and deservedly so, as the capsule is both the principal virulence determinant of the pneumococcus and the target antigen for currently licensed vaccines. The capsule protects the bacteria from entrapment in mucus during colonization, opsonophagocytosis by neutrophils and macrophages, and by killing by neutrophil-extracellular traps. Although antibodies against the capsule are highly protective, the pneumococcus has more than 90 biochemically and immunologically distinct capsule types, providing a considerable amount of surface antigenic variation. Importantly, extensive epidemiological and experimental evidence indicates that different capsule types have distinct propensities to cause invasive disease. Serotypes with lower numbers, that is, 1, 2, 3, 4, were those first isolated from patients as they are (or used to be) frequent causes of invasive infections. Capsular serotypes divide into three groups: those where colonization events are more frequently associated with invasive infection (e.g., serotypes 1, 5, 7F, and 14); those that are less likely to cause invasive infection per colonization event but are common causes of

TABLE 1 Important Questions About *Streptococcus pneumoniae* Biology and Potential Areas for Future Research

How does capsular serotype affect different aspects of pneumococcal biology? What are the molecular basis for and relationships between capsule structure, underlying protein virulence factors, and functional interactions with the host?
Why does the pneumococcus have a polysaccharide capsule when other nasopharyngeal bacterial commensals seem to cope perfectly well without one?
What are the effects of the respiratory tract microbiome on development of pneumococcal colonization and disease?
Do respiratory viruses other than influenza increase pneumococcal virulence? If so, by what mechanisms?
How can "wet biology" catch up with the explosion of genome sequence data? Can we devise much better methods of rapidly ascertaining gene function?
Why does the pneumococcus have such variation in genome content and such a large accessory genome? What are the effects of this on pneumococcal biology?
What is the minimum genome requirement for a bacterium to be phenotypically a pneumococcus?
Why is the pneumococcus a frequent cause of fatal disease, whereas *S. mitis* (its closest genetic relation) is only a rare cause of infections?
Why does the pneumococcus have so many surface adhesins? Is this due to redundancy or the need for pneumococci to sequentially interact with host cells?
What is the functional significance of the large differences in gene function between strains for some genes? Is this a biologically important effect, and if so how can we overcome its role in confounding data obtained with mutants?
How is pneumococcal virulence regulated? Is there a "master regulator" of virulence? What environmental signals stimulate an invasive phenotype?
What is the role of redundancy of nutrient acquisition—for example, carbohydrates and cations?
Why do many pneumococcal protein virulence factors have multiple functions? Which functions are actually relevant during colonization and disease?
How can we explain serotype- and strain-dependent colonization/virulence phenotypes? What is it about the pneumococcal nasopharyngeal colonization strategy that drives development of invasive disease?
Most murine infection data have been used in an innate immune setting; what are the effects of an adaptive immune response on pneumococcal and host determinants of successful infection?
What are the major host factors causing the marked bipolar age distribution of pneumococcal infection, with most disease affecting infants or the elderly?
Why is pneumococcal infection more prevalent in patients with some chronic diseases?
What are the main antigen targets and mechanisms of naturally acquired adaptive immunity to the pneumococcus?
How do epigenetic differences in humans influence their susceptibility to pneumococcal infections, and which are the host genetic determinants favoring invasive infections?
What are the molecular mechanisms used by pneumococci to breach the respiratory epithelial barrier, and which route is exploited by this extracellular pathogen during actual disease?
How do the physiology and gene expression profile of the pneumococcus change in the various environmental conditions (i.e., at different anatomical sites) the bacteria encounters during actual infection?

infections as they are highly prevalent as nasopharyngeal commensals (e.g., 6A and 19F); and finally, those serotypes that are rare both as commensals and as causes of infection (e.g., 88, 89, and 90). These data suggest that the physiological properties of different capsular serotypes have considerable influence on a range of pneumococcal interactions with the host, including those necessary for colonization or for bacterial survival during more invasive disease. We have only really just begun to assess the molecular basis for how capsular serotype affects multiple areas of pneumococcal biology, and this remains an important area for future research.

However, capsular serotype alone does not fully explain virulence, as evidenced by multiple studies demonstrating that isogenic capsule switching only sometimes confers virulence to a previously nonvirulent strain, and in some instances may even reduce virulence. A striking observation from genome sequencing data is the sheer amount of genetic variation among pneumococcal strains, with a core genome that is estimated to be only about 50% of the genome of a specific strain. Hence there are considerable differences in the protein content between strains that will contribute toward capsular serotype-independent effects on pneumococcal biology; moreover, we do not yet understand why there are such large variations in genetic content between pneumococci or how this may influence disease pathogenesis and pneumococcal ecology.

Multiple core and noncore protein virulence determinants such as the pore-forming toxin pneumolysin and adhesins like the pili, respectively, have been investigated and shown to have major roles during infection. We can surmise that the physiological properties and limitations imposed on the pneumococcus by its particular capsular serotype could be complemented or overcome by protein determinants. For example, a strain expressing a capsular serotype that is relatively inefficient at blocking complement deposition could boost its ability to evade complement-mediated immunity by expressing sufficient levels of proteins such as PspC (CbpA) or PspA, which bind the complement inhibitor factor H or prevent bacterial recognition by C-reactive protein, respectively. Likewise, a strain with a capsular serotype that inhibits epithelial cell adhesion could express greater levels of compensatory protein adhesins, often involved in recruiting host extracellular matrix or serum proteins, to overcome this deficit. This would explain why switching capsule types does not always result in a virulent strain, as the required complement of proteins for that specific capsular serotype may not be encoded in the genome of the recipient strain. The compensatory properties addressing the restrictions imposed by capsular type may not always be dependent on a single protein, but instead could be characterized by the necessity to reach a certain activity threshold. For example, for a strain from a capsular serotype that tends to prevent adhesion, high levels of expression of a single powerful adhesin or the collective effects of lower levels of expression of multiple adhesins could both overcome the limitations imposed by the capsule. With evolutionary pressure to minimize bacterial expression of superfluous products, a reasonable presumption is that pathogenic pneumococci carry the minimal compensatory factors necessary to adequately complement their specific capsule type. As such, loss of any one protein virulence factor could be sufficient to drop the bacteria below the required threshold for virulence and would lead to an attenuated mutant that is unable to cause severe disease during infection. This model potentially explains why experimental deletion of any one of the multiple known adhesins or proteins that inhibit complement activity tend to have a strong attenuated phenotype in the laboratory. Overall, the existing data suggest that a complex interplay between capsular type and the panoply of protein virulence determinants expressed by each strain will combine to influence each strain's ability to cause invasive disease. Further effort

is needed to clarify how infection phenotypes are affected by the interaction of protein virulence determinants with different capsular serotypes and by the effects of genome variation. Additional levels of complexity are created by the effects of phase variation and the transcriptional response to environmental signals on capsule and surface protein expression. In contrast to other bacterial pathogens, a single major pneumococcal transcriptional regulator of virulence has not been identified, and the environmental signals important during the development of invasive disease remain unknown. Together, these factors that influence subtle differences in phenotypes between and within pneumococcal strains create major challenges in truly understanding how the pneumococcus subverts the host defense and is able to cause disease.

Another important area for understanding pneumococcal biology and disease concerns the interactions between strains of pneumococci, pneumococci and viruses, and pneumococci and other bacteria that commonly infect or colonize the respiratory tract. While viral infection has long been appreciated as enhancing susceptibility to pneumococcal disease, recent work has described a range of often seemingly contradictory mechanisms that might be involved. These include increased or depressed inflammatory responses, and unexpected effects of viruses in acting as a signal for pneumococcal biofilms to disperse from what may be immunoquiescent and avirulent biofilms in the nasopharynx. Complex interactions with other bacterial pathogens such as *Haemophilus influenzae* have also been described. These interactions are most obviously relevant for colonization of the nasopharynx; however, they also important for disease. For example, in chronic otitis media, antibiotic-resistant *H. influenzae* assist pneumococci to form robust interspecies biofilms as well as to resist antimicrobial killing due to their secretion of β-lactamases. Considerably more work is necessary to fully define the potential consequences of *S. pneumoniae* interactions with other microbes during colonization and disease. Furthermore, new evidence suggests that the lower airways are not sterile, with a resident lung microbiome that is substantially disrupted in patients with chronic lung disease, a group who are particularly susceptible to pneumococcal pneumonia; how the lung microbiome in health and disease impacts pneumococcal lung infection and its progression to pneumonia remains an important unanswered question.

Molecular epidemiology studies now document the rapid expansion of specific capsular-switched clones that occurred after introduction of the conjugate vaccine. These demonstrate that the capsule type and protein/genetic background interactions discussed above are not just theoretical considerations but instead key aspects for our understanding of pneumococcal biology. While the current vaccine formulations have reduced disease, sufficient evidence now exists to indicate that the pneumococcus has the capacity to evolve around the restraints imposed by these vaccines. Novel preventive strategies will be required to stay ahead of the pneumococcus; for example, a protein-based vaccine offers the potential for broad non-capsule-type−based protection against pneumococcus. This is being actively explored in ongoing preclinical clinical phase I and phase II studies. A better understanding of the role of pneumococcal proteins in the disease process and as targets for naturally acquired immunity will identify potential targets for novel therapeutic interventions.

As can be seen by the above discussion and our list of questions, despite the many advances in our knowledge of pneumococcal biology we are still far from fully understanding *S. pneumoniae* and its capacity to cause human infection. We hope this book will provide a stimulating summary of existing knowledge on important areas of pneumococcal biology and serve as a springboard for the future research that is required if we are to prevent the terrible toll the pneumococcus continues to make on human health.

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SECTION A

STREPTOCOCCUS PNEUMONIAE EPIDEMIOLOGY AND VACCINES

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CHAPTER

1

Molecular Epidemiology of *Streptococcus pneumoniae*

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ETIOLOGICAL DIAGNOSIS OF PNEUMOCOCCAL INFECTIONS

Pneumococci are a leading cause of pneumonia and an important cause of meningitis, bacteremia, sepsis, otitis media, rhinitis, and sinusitis [1]. Classically, the etiological diagnosis of these infections has been done by growing the microorganism from suitable patient samples.

Identification of *Streptococcus pneumoniae* from culture depends on observation of the morphologic characteristics of both the bacteria and the colonies, as well as on three other main phenotypic characteristics, including catalase negativity, bile solubility, and optochin susceptibility. Susceptibility to optochin is a mainstay for the identification of pneumococci due to the ease of performance of the test, the basis of which is optochin's inhibition of the pneumococcal ATPase, a characteristic that is not generally shared by other *viridians* streptococci [2]. However, the emergence of optochin-resistant variants [3] has brought into question the validity of using this sole test for the presumptive identification of pneumococci. The specificity of the bile solubility test remains high, and it is the most accurate single test for the identification of *S. pneumoniae* [3]. The bile solubility phenotype is due to the activation of the major autolytic enzyme (an *N*-acetylmuramyl-L-alanine amidase encoded by the *lytA* gene), which can also be achieved by sodium deoxycholate. A few pneumococcal isolates were found to be insoluble in sodium deoxycholate, which has been ascribed to alterations in the major autolysin [4], but the overwhelming majority of pneumococci remain bile soluble, making it an extremely accurate test for pneumococcal identification.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF) is bringing a fundamental shift in the routine

Streptococcus pneumoniae.
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identification of microbial pathogens in clinical microbiology laboratories [5]. In spite of MALDI-TOF's success in streamlining and providing consistently accurate identification, even with previously problematic organisms, the success of currently available systems in identifying *S. pneumoniae* has been poor [5]. The mass profile of MALDI-TOF systems deployed in clinical microbiology laboratories is generated primarily by ribosomal proteins facilitating the alignment with current taxonomical classifications. However, *S. pneumoniae* is a clade within the evolutionarily related *mitis* group of streptococci, with which it can share many characteristics, including similar ribosomal proteins [6]. Therefore, distinction by MALDI-TOF between *S. pneumoniae* and its less pathogenic relatives of the *mitis* group is difficult. Recently, it was argued that making this distinction could be possible using a more detailed analysis of the mass profiles [7], and this was followed by a publication reporting the success of a commercially available system in distinguishing *S. pneumoniae* from other species of the *mitis* group [8]. These encouraging developments may result in simplification of the routine identification of *S. pneumoniae* in clinical microbiology laboratories.

The ability to produce a capsular polysaccharide (CPS) is also a hallmark of pneumococci. The capsule can be visualized by several microscopy techniques, but in pneumococci the presence of a CPS is usually detected using specific sera [9]. The Statens Serum Institut in Copenhagen, Denmark, is the most frequent source of sera to identify pneumococcal capsules. They provide an "omni-serum" that reacts with all known pneumococcal capsules and that may be useful in the identification of pneumococci, as well as specific sera that react only with particular polysaccharides or groups of polysaccharides [9]. Non-encapsulated pneumococci are known and have frequently been associated with conjunctivitis outbreaks [10]. However, since the production of a CPS is such a defining trait of pneumococci, these have been subject to particularly stringent tests to confirm their identification as *S. pneumoniae* [10].

The identification of the pneumococcal CPS by the *Quellung* effect or Neufeld test, using specific rabbit sera, is a proven technique that has been used since the early days of pneumococcal serotyping [9]. However, this technique requires specific expertise, so more recently, the Statens Serum Institut has made a latex agglutination test available, which allows a more streamlined procedure for serotyping pneumococci [11]. To further simplify this process, several "genetic serotyping" schemes have been developed to identify particular characteristics of the *cps* loci. In spite of the multitude of approaches, those more widely adopted are based on PCR amplification of specific serogroup or serotype genes [12,13]. In fact, both conventional and real-time PCR procedures have been developed, and a great diversity of schemes have been proposed to accommodate the differences in prevalence of the various serotypes in different geographic regions [13–15]. Although genetic serotyping has made serotyping available to a greater number of laboratories and has helped to clarify unclear reactions, phenotypic methods remain the gold standard for pneumococcal serotyping [15], and reflecting this, hybrid approaches involving both PCR and monoclonal antibodies have also been developed [16]. Perhaps the clearest examples of this are isolates in which the capsular locus contains point mutations or insertions leading to the absence of expression of a CPS (van der Linden and Ramirez, unpublished data) but that would be assigned a serotype according to genetic serotyping schemes.

Newer methodologies relying on the detection of microbial components are becoming increasingly important in the diagnosis of pneumococcal infections [17,18]. The immunochromatographic detection of C polysaccharide (teichoic acid) in urine has greatly improved the diagnosis of pneumococcal pneumonia in

adults, although in children the high frequency of pneumococcal carriage results in inadequate specificity of the test [18]. The test is also validated for use in CSF, leading to enhanced etiological diagnosis of meningitis [18]. There is increasing evidence for the usefulness of the test in detecting pneumococci in pleural fluid in both children and adults [19], but there is much less information regarding its use in bronchoalveolar lavage [20], in nasopharyngeal aspirates [21], or in blood culture media, where it can be of use in detecting pneumococci [22] which are no longer viable.

More recently, the detection of pneumococcal DNA has been used for diagnostics. For this purpose, the amplification by PCR of fragments of genes specific to *S. pneumoniae*, such as *lytA*, *ply*, *psaA*, *cpsA* (*wzg*), or *spn9802* [17,18] has been used. Real-time PCR methodologies have been shown to be more sensitive than conventional PCR, but other variations, including detection of the PCR products with beads, microarrays, or size fractionation were also developed [23]. Combining DNA amplification for identification with the genetic serotyping approaches discussed above allows the identification of the serotype of the strain without the necessity of culture [14]. The use of these methodologies in parapneumonic effusions or empyema is well documented and greatly enhances the etiologic diagnostic yield over culture [14,19].

There is great interest in using these methodologies to detect pneumococci in the blood for cases of pneumonia in both children and adults [23–25]. However, the high carriage rate of pneumococci in children could be an important confounder by detecting the circulation of pneumococcal DNA in healthy carriers [25]. Two studies have specifically addressed this issue, with contradictory results [26,27]. Other studies indicate that the estimated pneumococcal load in blood is correlated with disease severity and could potentially be used to distinguish between colonization and infection [23,24].

A similar approach has been advocated in the case of the respiratory specimens that are more frequently available, such as sputum. The value of sputum in the diagnosis of pneumonia has been amply discussed [18], even in the context of conventional culture methods. While it may be argued that the absence of pneumococci could potentially exclude it as an etiological agent, a hypothesis that certainly warrants further studies, its detection could be attributed to either infection or asymptomatic carriage [18]. In adults, the quantification of pneumococci or pneumococcal DNA in sputum has been proposed to distinguish between colonization and disease [28], but this may be complicated by the variability of the assays and the lack of clear criteria for defining cutoff values, even in good-quality samples [18]. In children, similar approaches have been suggested [29], but the diagnostic value of this approach is further called into question by the fact that many children are colonized by pneumococci at very high densities. Monitoring two key host markers, C-reactive protein and procalcitonin, appears to increase the specificity of PCR assays in the diagnosis of pneumococcal lower respiratory tract infection [23]. In spite of these uncertainties, several commercially available assays already offer the detection of pneumococcal DNA for diagnostic purposes [23].

Although traditional microbiological methods, including the more recent antigen detection methods, will remain the mainstay in many laboratories for the diagnosis of pneumococcal infections, newer molecular methods will undoubtedly become increasingly important. The increased adoption of molecular tests will depend on clarifying the relevance for identifying infection of detecting evidence of the presence of bacterial products in human samples. This will be particularly complicated for respiratory specimens, where debate remains ongoing, even for the more traditional approaches. Given our increasing understanding of the relationships between multiple

pathogens in the upper respiratory tract that may condition their ability to cause infections, molecular approaches that detect multiple pathogens will undoubtedly become increasingly important in the etiological diagnosis of respiratory tract infections [18,23].

SOME BASIC CONCEPTS IN MOLECULAR EPIDEMIOLOGY

Molecular epidemiology was defined as "the study of the distribution and determinants of infectious diseases that utilizes molecular biology methods" [30]. This is a particularly rich definition that encompasses several distinct aspects. A frequent goal of molecular epidemiology studies is to distinguish isolates from the same species and identify particular clones. The goal may be to identify the pathogen's sources or route of transmission in the context of outbreak investigations, or to identify particularly virulent or emerging new clones and document their spread, in more sustained surveillance efforts. When focusing on individual humans instead of human populations, molecular methods have highlighted the diversity existing in a bacterial species asymptomatically colonizing a single individual. These populations have been shown to be dynamic over time, and to respond to changes triggered by alterations in their niche, such as the acquisition of other bacteria or viruses, antibiotic consumption, or the development of naturally or vaccine-induced immunity. When focusing on human populations, these studies can be used to identify and evaluate the distribution of important "determinants" in the context of infection, such as virulence factors or genes conferring antimicrobial resistance. Another important application is to monitor the distribution of current or potential vaccine components by directly determining the presence and variability of these factors or of the genes encoding them. This approach has been used to evaluate the potential benefits of vaccination and to monitor the effects of vaccination on bacterial populations and particularly on pneumococci.

The shift toward typing methods using nucleic acid sequence information has led to suggestions for convergence between the fields of molecular epidemiology and evolutionary genetics, drawing on methods from population genetics and phylogenetic analysis. Using the information already available and the tools from population genetics, one could derive a better understanding of the mechanisms underlying the evolution and dynamics of bacterial populations in order to recognize and predict the consequences of human-imposed selective pressures, such as antimicrobial use or vaccination. This has been particularly relevant to the case of multilocus sequence typing (MLST), but important issues regarding strain sampling and how this affects the estimation of parameters (such as rates of mutation and recombination) have been raised. This has exposed differences in the objectives, sampling strategies, and design of most molecular epidemiology studies and those required for an unbiased study of microbial diversity and evolution.

At the heart of molecular epidemiology lie the concepts of *isolate*, *strain*, and *clone*. In spite of being amply used in the literature, there is no universally accepted definition for any of these terms. *Isolate* has been taken to refer to "a population of microbial cells in pure culture derived from a single colony on an isolation plate and identified to the species level" [30]. However, this definition is almost indistinguishable from that of *strain* from different authors: "[A] strain is made up of the descendants of a single isolation in pure culture and usually made up of a succession of cultures ultimately derived from an initial single colony" [31]. Both definitions are unambiguous and easy to agree upon, since they only imply the isolation from a particular site at a

particular time that is then propagated as an axenic culture in the laboratory. Note that no implication is made about the identity of the subsequent subcultures at the genetic or biochemical level. Confirming previous phenotypic observations, genomic studies showed that the propagation in the laboratory of a single colony, isolated many years ago, may result in the accumulation of differences between the descendants of this original isolation, so that different stocks of the same *strain* can now correspond to different genetic contents. These definitions do not correspond to a natural entity, since both imply the isolation in axenic culture of a single individual of a natural population. Riley reserves the term *strain* for "an isolate or group of isolates exhibiting phenotypic and/or genotypic traits belonging to the same lineage, distinct from those of other isolates of the same species" [30]. Although this could correspond to a natural entity, we will see that this definition has many points in common with what is commonly referred to as a *clone*. Even though the difference between an *isolate* and a *strain* may be taken to comprise the amount of information available regarding its characterization, we would argue that these terms, as they are currently used in molecular epidemiology, are interchangeable.

Clone, on the other hand, is a more natural concept. The definition of *clone* from the Oxford Dictionary as "an organism or cell, or group of organisms or cells, produced asexually from one ancestor or stock, to which they are genetically identical" (www.oxforddictionaries.com) finds a strong correspondence in the reality of bacteria that reproduce by binary fission and may therefore be expected to be clonal. However, when we independently isolate bacteria from nature we cannot know if these bacteria descend from a common ancestor, but must infer this kinship. Reflecting this, Riley defines *clone* as "an isolate or group of isolates descending from a common precursor strain by nonsexual reproduction exhibiting phenotypic or genotypic traits characterized by a strain-typing method to belong to the same group" [30]. Other definitions also emphasize common ancestry [31,32], but Riley's definition specifically states that these similarities are defined by a given "strain-typing method." The definition opens up the possibility that *clones* identified by one method may be further subdivided by more discriminatory methods. The possibility of conflicting clones identified by different typing methods is not discussed by Riley, and he makes no attempt to harmonize findings of different typing methods. Other definitions emphasize a polyphasic approach ("so many identical phenotypic and genotypic traits that the most likely explanation for this identity is a common origin" [31]) so that a consistent identification of *clones* is achieved.

Most definitions of *clone* omit the important aspect of time [30–32]. As can be seen in Figure 1.1, a number of currently recognizable *clones* can be traced back to a more recent or more distant common ancestor, and nested clones can be defined. For instance, in Figure 1.1, although the extant strains (g) and (h) share a more recent common ancestor (c), all extant strains share the more distant common ancestor (a). On the other hand, evidence is accumulating that horizontal gene transfer plays an important role in bacterial evolution, particularly in naturally transformable bacteria such as pneumococci (see Chapters 5 and 7). When this happens, particular fragments of the genome may have a different ancestry from the majority of the genome, potentially reflecting a different evolutionary history, and having been subject to distinct selective pressures. Although not frequently used, the term *meroclone* has been proposed to describe these strains [32]. In Figure 1.1, strains (i) and (j) are representatives of a *meroclone*. These strains share the most recent common ancestor (d) but differ from each other in that an ancestor of strain (j) incorporated in its genome DNA from strain (k) of a

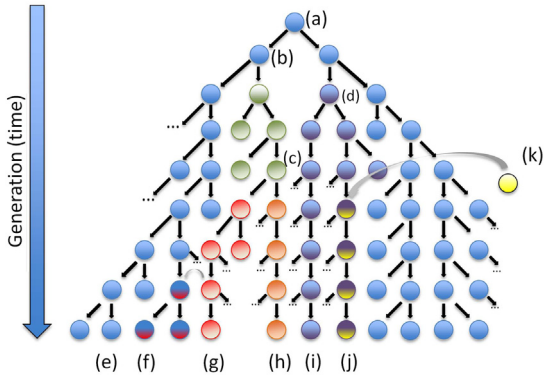

FIGURE 1.1 **Bacterial clonal evolution.** Each color represents a distinct genotype. Black arrows represent lines of descent by binary fission. Ellipses denote lines of descent that are not represented in the figure. Gray arrows represent horizontal exchange of DNA. When a mutation occurs, the strains are represented by circles of a single color, different from that of its ancestor. When DNA is incorporated into a strain, the resulting strain is represented by a circle of the colors of the two strains involved. Extant strains are represented at the bottom of the figure. Letters (a), (b), (c), and (d) identify ancestral strains. Letters (e), (f), (g), (h), (i), and (j) identify extant strains. The letter (k) represents a strain of a different species that acted as a DNA donor to the ancestral strain of the extant (j) strain. See text for a more detailed discussion of the indicated letters.

different species. In a similar manner, strains (e) and (f) are also representatives of a *meroclone*, but in this case the DNA donor belongs to the same species as the recipient. Particular cases of the latter are the "capsular switching events" of pneumococci that result from the replacement of the native capsular locus with an exogenous one originating from other isolates of *S. pneumoniae*. However, in pneumococci, recombination can also occur with DNA from other species, as is the case with the *pbp* genes conferring resistance to penicillin (Figure 1.2B). The critical question then becomes how much change, either through mutation or recombination, can be tolerated to identify two strains as representing the same *clone* or *meroclone*? An answer to this question would partly reflect the amount of time one would allow to have elapsed since the strains shared a common ancestor. It is clear that any answer is therefore dependent on subjective criteria that may not be universally accepted. This problem was solved by defining arbitrary cutoff values for each typing method, for instance, by linking single-locus variants (SLVs) when typing by MLST or the 80% threshold to define clones in pulsed-field gel electrophoresis (PFGE) (see next section), which have gained wide acceptance in the community. However, the advent of whole genome sequencing (WGS) is raising the question once again. For instance: When using WGS data, should the difference in gene content be considered, or should one focus on a common genomic scaffold? The most frequently taken approaches to the analysis of WGS data are focusing on the genome sections common to all strains analyzed, and attempting to specifically exclude recognizable recombination events. The rationale is to identify a set of directly comparable sequences that can inform us of the line of vertical descent on the genomic scaffold of the clones. But even considering only these regions, should a single nucleotide polymorphism (SNP), out of the 2.2 Mb of the pneumococcal genome, be enough to differentiate two clones? An answer to this question is further complicated by the recognition that specific SNPs and the acquisition of particular genetic elements can result in dramatic phenotype changes. Currently, no satisfactory, universally applicable, and commonly agreed criteria exist to distinguish clones.

SEROTYPING AND MOLECULAR TYPING OF *S. PNEUMONIAE*

Serotyping

Distinction among pneumococcal strains based on serotype emerged early in the study of *S. pneumoniae* because of its importance for the therapy of these infections [1], and later

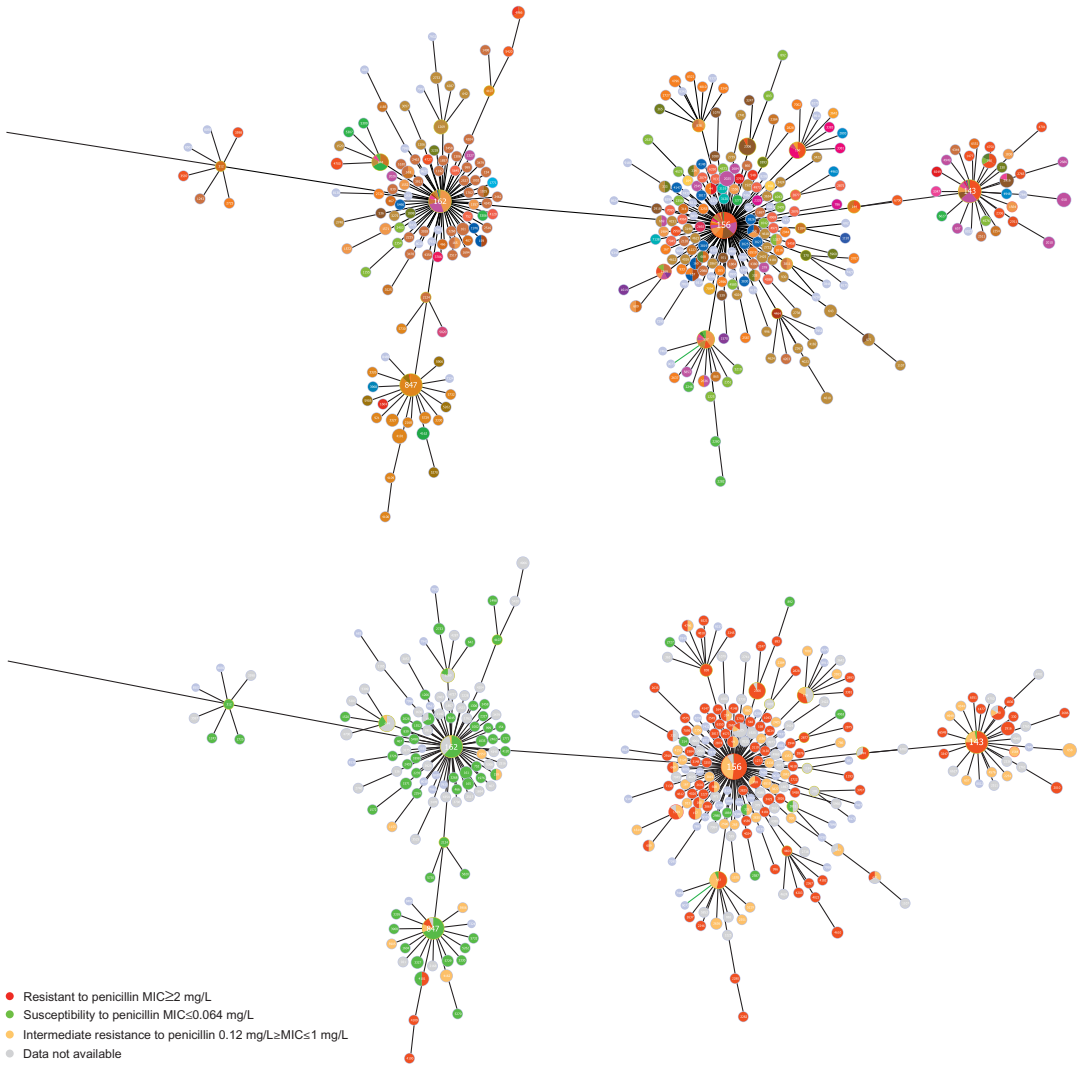

FIGURE 1.2 **Integrating metadata with MLST using PHYLOViZ: the example of the ST156 and ST162 subgroups on the largest CC in *S. pneumoniae*.** The data used to create the figure was the public data available at the pubmlst.org website in January 2014. The size of each circle is proportional to the number of strains with that particular ST on a logarithmic scale. STs that are SLVs of each other are linked by straight lines. The figures show ST162, ST156, ST143, and ST847 and their immediate descendants according to goeBURST. (A) Each color represents a country. Whenever strains of the same ST were recovered in multiple countries, the circle is divided into fractions corresponding to the relative abundance in the database of strains isolated in different countries. The founder genotypes, located at the center of star-like arrangements, are STs that are more frequently represented by strains isolated in multiple countries than their putative descendant STs. This is compatible with a widespread geographic dissemination of a few clones followed by local diversification. (B) The colors represent penicillin susceptibility: susceptible (green) MIC ≤ 0.064 mg/L; intermediate (orange) 0.12 mg/L $\leq$ MIC $\leq$ 1 mg/L; resistant (red) MIC $\geq$ 2 mg/L. ST162 (on the left), representing mostly penicillin-susceptible strains, is surrounded by SLVs that are also mostly penicillin-susceptible. In contrast, ST156 (on the right) and its SLVs represent mostly penicillin-nonsusceptible strains. This is compatible with the acquisition of exogenous DNA encoding resistance-conferring *pbps* by ST156 that were then passed on to its progeny.

because of the development of vaccines targeting the CPS (see Chapter 3). The total number of pneumococcal serotypes recognized has increased in recent years, and given the genetic plasticity of pneumococci and the renewed interest in the diversity of the CPSs, it is likely that new pneumococcal CPS will be identified in the future (see Chapter 9). In spite of CPS diversity, a more restricted number of serotypes cause the majority of human infections, reducing the potential discriminatory power of serotyping as a typing method. It was recognized early that different pneumococcal serotypes had a different epidemiologies and even different spectra of disease [1]. These initial studies were followed by more recent ones that attributed different invasive disease potentials to different serotypes [33], strengthening the usefulness of serotyping for pneumococcal typing. With the advent of CPS conjugate vaccines that target 7, 10, and now 13 pneumococcal serotypes, serotyping became the primary tool to evaluate the efficacy of the newly introduced vaccines, to monitor the remaining disease, and the potential replacement of the serotypes included in the vaccine with other serotypes [34,35].

Although serotyping will remain important in distinguishing pneumococcal strains and in evaluating the impact of available vaccines, the development of antimicrobial resistance in only a fraction of the isolates expressing certain serotypes led to an interest in being able to distinguish isolates of the same serotype [36] (see Chapter 2). The development of molecular typing methods that could do this, such as PFGE profiling and later MLST, revolutionized our knowledge of the population biology of *S. pneumoniae*.

Pulsed-Field Gel Electrophoresis

PFGE typing of pneumococci is generally based on comparing the profiles generated by pulsed-field electrophoresis of the digestion of the total DNA of a strain using SmaI. Clones are identified by visual comparison of the profiles using arbitrarily defined rules, or by defining cutoff values in software-generated dendrograms (usually an 80% cutoff value in a UPGMA dendrogram constructed using the Dice coefficient) [37]. Initially, PFGE was the dominant method used to type pneumococci. Its application to the study of bacteria recovered from individuals in close contact revealed that they were colonized by the same clones, indicating that there is transmission and circulation of the same clones in these groups. Furthermore, PFGE was applied to more longitudinal surveillance efforts, leading to the realization that a few antibiotic-resistant clones had disseminated globally and were responsible for most resistant isolates recovered worldwide. These studies also highlighted the great diversity of PFGE-defined clones in some serotypes in contrast to others that were very homogeneous, with a few clones accounting for most isolates [38,39], and uncovered the first evidence of capsular switching, even before vaccine introduction [40,41]. Although PFGE was very successful, the method does require specialized expertise and the analysis of its results in large-scale studies needs appropriate software [42]. Furthermore, the comparison of PFGE results across laboratories is not straightforward, and the adoption of MLST, which has partially overcome these limitations (although with lower discriminatory power), resulted in its increasing use in molecular epidemiology studies.

Multilocus Sequence Typing

MLST is based on the sequence of internal fragments of seven housekeeping genes. Unique sequences of each of these fragments are taken to identify alleles at each of the seven loci. Sets of seven unique allele numbers define sequence types (STs). Attesting to the popularity of MLST, in December 2014 the public

database (http://pubmlst.org/spneumoniae/) had over 24,300 isolates representing 9939 distinct STs. Although already with PFGE the worldwide dissemination of successful pneumococcal clones susceptible to most antimicrobials was apparent (www.sph.emory.edu/PMEN/), this became even clearer with the advent of MLST. For instance, according to pubmlst.org, strains representing ST9 have been found in 16 countries. The use of both PFGE and MLST also allowed for refinement of the evaluation of the invasive disease potential of certain serotypes. Although capsular type is recognized as a major pneumococcal virulence factor and a major determinant of invasiveness, these methods allowed the identification of particularly invasive clones among those sharing the same serotype [33].

Analysis of MLST data has most frequently been done using eBURST [43]. This method disregards the sequence information and constructs an unrooted tree representation of the relationship of the isolates analyzed, based on the number of differences in the allelic profile, assigning isolates to clonal complexes (CCs). The main advantage of eBURST is that it implements a simple model for the emergence of CCs [43]: A given genotype increases in frequency in the population as a consequence of a fitness advantage or of random genetic drift, becoming a founder clone in the population. This increase is accompanied by a gradual diversification of that genotype by mutation and recombination, forming a cluster of strains which are phylogenetically closely related. Such diversification of the "founding" genotype is reflected in the appearance of STs differing in the DNA sequence of only one housekeeping gene from the founder genotype—an SLV. Further diversification of those SLVs will result in the appearance of variants in other loci: double-locus variants (DLVs), triple-locus variants (TLVs), and so on. Upon application of the eBURST algorithm to an entire data set, the result is a forest, a disjointed set of trees, where each tree corresponds to a CC. Although a particular ST may have many SLVs, the application of a set of rules based on the model just described results in the representation of a single SLV link [44] that joins all the isolates in a CC. Thus, by considering only SLV links, eBURST does not aim at linking the entire population, but instead identifies different CCs. However, it is important to note that by linking STs that are SLVs of at least one other member of a CC, the eBURST rules may link STs that have no alleles in common in the same CC, so the CC does not necessarily reflect a group of genetically closely related isolates.

The final eBURST forest provides a hypothetical pattern of descent for the strains analyzed, illustrating the possible phylogenetic relationships between STs. Reliance on the comparison of allelic profiles buffers eBURST against the possibility of the introduction of multiple sequence changes in a *locus* by a single recombination event, a particularly useful characteristic when analyzing a highly recombinogenic species such as *S. pneumoniae*.

Recently, in order to guarantee an optimal solution for eBURST, a novel algorithm based on the original eBURST was proposed and named goeBURST [45]. This algorithm guarantees that the chosen tree fully complies with the eBURST rules and also extends the eBURST rules by including as a last tie-break rule the assigned ST number (ID). Although this last tie-breaker is rarely reached, this criterion is necessary to provide a consistent and unique solution to the tree construction problem. This is guaranteed due to the uniqueness and stability of ST ID. As implemented, lower ST IDs take precedence over higher ST IDs. The rationale for this choice was that, assuming a growing database with data from several contributing international studies, the more common STs are sampled first and will have lower ST IDs than those identified in subsequent studies. Other novel features of

the goeBURST algorithm include the quality assessment of each link by the level of the tie-break rules reached and the ability to create CC at the DLV or TLV level, which may prove useful in some species [45].

A minimum spanning tree (MST) is a tree that connects all entries in such a way that the summed distance of all links on the tree is the shortest (minimum). In a biological context, the MST principle and the maximum parsimony principle share the idea that evolution should be explained with as few events as possible. The main difference between the two is that parsimony methods allow the introduction of hypothetical samples that are created to construct the internal nodes of the tree, whereas the real samples from the data set are represented as the leaves of the tree. Those hypothetical samples are assumed to be common ancestors of the current population that can no longer be sampled. MSTs and eBURST/goeBURST for the analysis of MLST data have frequently been considered distinct methodologies but, as they are currently applied, both are instances of graphic matroids [44], and an expansion of goeBURST allowing the creation of MSTs is implemented in the freely available software PHYLOViZ [44] (www.phyloviz.net).

Another important aspect of molecular epidemiology studies is the integration of information from several sources, such as phenotypic or epidemiological data. This may involve representing phenotypic data such as penicillin resistance, which involves the acquisition of foreign DNA in pneumococci (see Chapter 2), on a tree constructed using MLST data. This is possible using PHYLOViZ [44]. The software can use locally stored private databases, but it also interacts with various public MLST databases, directly retrieving the available public data. The user can then provide metadata to be overlaid onto an MLST-based tree. Figure 1.2 shows two examples of the integration of metadata using PHYLOViZ and the publicly available data at pubmlst.org. In Figure 1.2A the distribution of each ST in different countries is plotted, and in Figure 1.2B the classification regarding penicillin susceptibility is shown. The figures show ST162, ST156, ST143, and ST847 and their immediate descendants according to goeBURST. Although data submission to pubmlst.org is certainly biased, it is evident from Figure 1.2A that the STs identified as subfounders are geographically widespread, whereas their SLVs seem to have a more restricted geographic distribution. In Figure 1.2B, one can follow the development of penicillin resistance (through the acquisition of foreign DNA—see Chapter 2) in the ST156 clone, an SLV of the ST162 clone, and its subsequent diversification.

Since MLST is sequence based, these data have been used to infer phylogenies using classical methodologies and to support population dynamics studies [46], as well as to determine important parameters in the evolution of *S. pneumoniae* [47]. The concatenated sequences of multiple housekeeping genes have also proved useful in identifying and defining bacterial species as sequence clusters, an approach usually designated multilocus sequence analysis (MLSA) [48]. Specific genes are frequently used in MLSA that are different from those chosen for MLST schemes, with the specific aim of typing isolates of the same species; the usefulness of such an MLSA scheme to identify and distinguish species of the genus *Streptococcus* has already been shown [48]. However, it is also known that pneumococci can be distinguished from closely related species of the *mitis* group by the divergence of their MLST profiles (http://pubmlst.org/spneumoniae/).

While the WGS approaches discussed below are gaining increasing momentum in molecular epidemiology studies, MLST continues to be the most frequently employed approach in the study of *S. pneumoniae* and may continue to play an important role in the selection of isolates for WGS studies. MLST will remain the foremost typing method to characterize

pneumococci until suitable and standard tools are developed to allow a more widespread adoption of WGS.

Other Typing Methods

Other typing methods have been applied to *S. pneumoniae*, but their use has been more limited. A good example of this is multilocus variable number of tandem repeat analysis (MLVA). Although the method was developed for pneumococci and there are even two online databases (http://www.mlva.net/spneumoniae/ and http://www.mlva.eu/recherche.php?type=spneumoniae), the method was not widely adopted by the community. The variability of single genes was also explored, but none of these became established as an accepted typing method. These studies have been motivated mostly by determining the variability of surface exposed proteins to evaluate the feasibility of using them as components of future vaccines.

Whole Genome Sequencing

The advent of next-generation sequencing technologies that promise to rapidly deliver draft whole genome sequences at an affordable price is expected to revolutionize clinical microbiology [49], and molecular epidemiology in particular [50]. The wide adoption of these technologies will require the development of standards and frameworks to analyze WGS data and to report the results in order to produce the desired reproducibility and common language necessary for typing [42].

Currently, a myriad of highly technical software tools are available to translate raw data into meaningfully assembled and annotated fragments of the genome of interest. This software is not user friendly, requiring expertise that is not yet available in all laboratories interested in molecular epidemiology. Since these steps will produce the sequence from which further analysis will be done, they are critical in determining the quality of the inferences made. The current diversity of methods makes comparison across studies difficult and still baffles novices in the field. But even if such pipelines for assembly and annotation were available, it is still not clear which will become the mainstream analysis methodologies for subsequent analyses.

One approach that is being advocated is the analysis of whole genome sequence data on a gene-by-gene approach, similar to MLST [51]. This approach has the advantage that MLST is well known in the field of bacterial molecular epidemiology and can draw upon analysis methodologies that have been specifically developed with MLST data in mind [44]. In this case, one would use the allelic information of all the loci common to the strains being analyzed, or even extend the analysis to include loci that are not present in all strains, by encoding an absent locus as a different allele, to create disjoint trees using the eBURST rules or a fully connected MST. PHYLOViZ is capable of performing both by expanding the eBURST rules to the number of loci being considered [44]. The commercially available software Bionumerics (Applied Maths, Ghent, Belgium) and Ridom SeqSphere+ (Ridom GmbH, Münster, Germany) also create MSTs using WGS data.

Another approach to WGS data is the SNP discovery that has been used with great success to distinguish very closely related isolates [42,50]. However, both approaches focus mostly on the core genome (the fraction of genes present in all or most bacterial isolates of the same species), while the accessory genome (the fraction of genes present in only some or even a single isolate) is mostly disregarded [50–53]. The importance of proteins encoded by the accessory genome for the host–pathogen interaction, for instance through the acquisition of pathogenicity islands that may encode

multiple virulence factors, highlights its significance in the context of the molecular epidemiology of bacterial pathogens and stresses the importance of developing methods that take them into account.

In *S. pneumoniae*, WGS has been used to study the evolution of a successful antimicrobial-resistant clone [54], to detect and characterize capsular switching events [54–56], to explore intra-host pneumococcal evolution [57], and to define the pneumococcal pan-genome [53]. All of these studies make use of highly specialized bioinformatics tools that are not easily accessible to the molecular epidemiology community. WGS will probably be the future of bacterial typing, including pneumococci, but while there are no standards or tools that can be used easily by researchers with limited bioinformatics skills, more traditional typing methods such as MLST will remain the mainstay for the characterization of pneumococcal clones.

CLONES OF *S. PNEUMONIAE*

Typing of pneumococci led to the surprising recognition that most antibiotic-resistant pneumococci before the introduction of conjugate vaccines belonged to a limited number of clones that were dispersed worldwide. Since at that time clonal identification relied mostly on PFGE clustering that produced groups that were arbitrarily named, this resulted in efforts to standardize the nomenclature, and the Pneumococcal Molecular Epidemiology Network (PMEN) was formed [34]. Subsequently, a number of resistant clones were identified, such as Spain^{23F}-1, Spain^{9V}-3, Hungary^{19A}-6, or Taiwan^{19F}-14. The names reflect the serotype and the first country where a strain representing that particular clone was identified, with the number being attributed sequentially. With the wider adoption of MLST, PMEN recommended that these clones should be known by their ST number, for instance Spain^{9V}-ST156. Since then, it has become clear that isolates representing these clones are sometimes found to express different serotypes, such as 9V, 14, and 23F in the case of Spain^{9V}-ST156, and currently clones are frequently identified simply by their ST number.

PMEN's initial efforts were directed mainly toward resistant isolates and, as a consequence, included mostly clones expressing serotypes that were later included in the seven-valent conjugate vaccine (PCV7). Among the first 16 recognized clones, only serotypes included in PCV7 and serotype 19A were found [36]. Later, the importance and global spread of fully susceptible clones was recognized by PMEN, which led to the acknowledgment of clones such as Sweden^{1}-ST304 and Netherlands^{3}-ST180, which are important causes of invasive pneumococcal disease (IPD) worldwide.

One of the major questions that arose when vaccines were introduced was whether the well-known circulating clones, such as Spain^{9V}-ST156 or Spain^{23F}-ST81, would persist by simply changing their capsular locus and hence escape vaccine pressure. Careful surveillance in the post-PCV7 period revealed that although "capsular switching" did occur, it was much less frequent than anticipated and did not lead to the overwhelming persistence of the previously prevalent and widely disseminated clones. Perhaps the best example of this is the dynamics of the clones expressing serotype 19A in the post-PCV7 period: Isolates expressing serotype 19A increased significantly in the post-PCV7 period, and the possibility of capsular switching events was immediately put forward. Indeed, capsular switching was detected in one of the initial studies, and although it did involve a serotype included in PCV7, it was not a particularly dominant genotype, but rather the acquisition by members of ST695 expressing serotype 4 of the 19A capsular locus, and of its flanking *pbp* genes, potentially donated by a ST199 isolate [58]. In a single event, the ST695 genetic background was able to escape vaccine pressure and

become intermediately resistant to penicillin, and the ST695[19A] variant certainly increased in prevalence, but a great diversity of STs and CCs was noted among isolates expressing serotype 19A [58,59]. In both Portugal and the United States, this included representatives of previously widely disseminated resistant clones expressing vaccine serotypes such as Spain[9V]-ST156 and Spain[23F]-ST81, and it is not clear why these did not become dominant clones expressing the 19A serotype. However, there is circumstantial evidence that most isolates expressing serotype 19A could have originated within other serotypes. For instance, the dominant clone in the United States is Netherlands[15B]-ST199 and related STs, followed by STs 320/271, which are closely related to Taiwan[19F]-ST236, both of which were found before the introduction of PCV7, and only then by the ST695 isolates resulting from capsular switching in the post-PCV7 period [58]. On the other hand, in Portugal a different clone, the Denmark[14]-ST230 clone, is the most prevalent. This clone had also been detected before the introduction of PCV7, although isolates representing the dominant clones found in the United States were also identified [59]. The reasons some clones were so successful in some geographic areas and not in others are not clear. Taken together, the evidence so far indicates that capsular switching has been ongoing among pneumococci and that it probably played a significant role in generating some of the currently successful clones in particular serotypes, but most of these clones emerged before the introduction of pneumococcal conjugate vaccines (PCVs), and vaccination has not led to the overwhelming selection of novel capsular-switched variants [34,56].

It is difficult to say whether PCV7 has directly influenced the clonal composition of the pneumococcal population in addition to selecting against clones expressing the PCV7 serotypes. Most existing evidence indicates that vaccination does not distinguish between clones expressing the same serotype, but vaccination together with other selective pressures may result in the preferential selection of particular clones. For instance, in Portugal, where PCVs have been used in the private market but with high vaccination coverage in infants and young children, the remaining infections caused by isolates expressing serotypes included in PCV7 are due to isolates that represent antimicrobial-resistant clones, whereas susceptible isolates expressing these serotypes have declined in importance [35,60]. The significance of antimicrobial resistance was also highlighted in the emergence of the successful lineages expressing serotype 19A [58].

Clones that persist in the population over long periods of time would, of course, be expected to accumulate changes in their genomes. This was specifically demonstrated for representatives of the Spain[23F]-ST81 clone isolated since 1984 using WGS [54]. It had been known for a long time that this clone could express multiple serotypes (3, 6A, 14, 15B, 19F, 19A, 23F), so it was not surprising to find that not all accumulated change was due to mutations, but that recombination with DNA from other pneumococcal clones was responsible for a significant share of this diversification. Most of the recognized hotspots of recombination involve regions encoding important virulence factors, including surface-exposed antigens that would be expected to be under selective pressure to escape the immune response. Examples of the latter are the capsular locus and the loci encoding PspA, PsrP, and PspC [54]. This demonstrates the importance of recombination in the adaptation of pneumococcal clones to changing selective pressure and illustrates why the term *meroclone* is preferable when referring to pneumococcal clones. It is also important to note that the timeframe for the generation of *meroclones* in pneumococci may be much shorter than the 30 years found in the characterization of the Spain[23F]-ST81 clone, as was shown by the identification

of frequent recombination occurring among strains isolated during a 7-month period from a patient with a chronic infection [57].

Not only was the Spain^{23F}-ST81 clone a frequent recipient of exogenous DNA, but it also acted frequently as a donor of DNA for other clones [61]. This is perhaps unsurprising, since, everything else being equal, one would expect that the more prevalent clones in the population would, simply by force of their frequency, act regularly as DNA donors. Using information from WGS, it was shown that the Spain^{23F}-ST81 clone was a frequent donor of modified *pbp* genes conferring resistance to β-lactams, and also of regions encoding genes associated with increased colonization and virulence such as the ICE*Sp*23FST81 element or the ΦMM1 phage. As has been documented in other cases [55,57], multiple fragments of the same donor were found in a given strain. This suggests that even a single contact between donor and recipient strains can result in multiple recombination events scattered around the genome, so that representatives of a *meroclone* can have variable but significant noncontiguous fractions of their genome changed. Multiple successive transformation events can result in the incorporation of several DNA fragments from distinct sources, creating *meroclones* with complex and diverse ancestries.

As mentioned previously, the clonal variability within serotypes is not consistent. Two extreme examples are serotype 19A, described above, which is represented by a large number of clones with significant geographic variability, and serotype 7F, which is represented mostly by one clone (ST191) with a wide and uniform geographic dispersion. Isolates expressing serotype 7F were shown to have alterations in the *ply* gene encoding pneumolysin, a major virulence factor of pneumococci, resulting in lower hemolytic activity. This translates the dominance of ST191 among serotype 7F and the stable property of carrying this allele. Given the multifaceted roles of pneumolysin (see Chapter 14), the consequences of carrying these alleles for the virulence of these clones is not fully understood. Another important clone that carries an altered *ply* allele is one of the dominant clones among serotype 1 isolates: ST306. This clone carries a nonhemolytic allele of pneumolysin. It was suggested that isolates expressing serotype 1 could be divided into three major lineages with distinct geographic distributions: a South American lineage (ST615 and related STs), an African and Israeli lineage (ST217 and related STs), and a European and North American lineage (ST306 and related STs) [39]. Mouse models of infection indicate that the European and North American lineage has lower virulence than the other two lineages [62]. However, IPD due to serotype 1 mostly representing ST306 can be very frequent in some European countries [35,63], indicating that, in spite of the animal model results, this lineage is capable of causing a significant burden of IPD in human populations. Isolates of serotype 1 and ST306 in particular, are also known to cause outbreaks of IPD, an unusual characteristic shared by only a few serotypes and clones. Serotype 1 was among the first serotypes to be identified and was a major cause of IPD in all early studies. No systematic studies of the evolution of the clones expressing this serotype are available, but repeated *in vitro* attempts to transform representatives of the major lineages were unsuccessful, suggesting that these may be impaired in the necessary machinery [62]. A similar impairment in transformation was suggested for isolates of ST180 and related STs expressing serotype 3 [64], potentially explaining the few diversifying recombinations detected in this lineage. A similar situation occurring among the serotype 1 clones would indicate that these have remained excluded from frequent exchange of DNA with other pneumococci.

S. pneumoniae is a highly diverse species, expressing multiple CPSs, themselves associated with various clones. In spite of the dominant

role played by the capsular type in the relationship with the human host, it is clear that the diversity between clones expressing the same serotype is critical in defining virulence [33,64] as well as antimicrobial resistance [36]. These properties justify the significance of identifying clones for understanding pneumococcal evolution. The importance of horizontal gene exchange in *S. pneumoniae* is an additional challenge in understanding its clonal dynamics, and the advent of WGS will certainly offer new insights into its consequences and the nature of the *meroclones* within this bacterial population.

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CHAPTER

2

Antibiotic Resistance of Pneumococci

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INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) has been recognized as an important human pathogen for over 100 years and continues to be a major cause of morbidity and mortality worldwide. It can asymptomatically colonize the nasopharynx and can cause a wide variety of diseases, ranging from mild infections to serious lower respiratory infections, as well as life-threatening invasive infections such as meningitis. It is the most common bacterial cause of acute otitis media and pneumonia and an important cause of childhood mortality. Despite the availability of vaccines and antibiotics, a 2010 report estimated that *S. pneumoniae* is still responsible for approximately 1.3 million deaths annually, particularly among young children and the elderly [1].

Infections caused by *S. pneumoniae* were for many years traditionally treated with penicillin or ampicillin, to which this species was exquisitely sensitive when penicillin was first introduced in the 1940s. However, resistance, first seen in the 1960s, has continued to increase throughout the world in more recent decades. The emergence of resistance to penicillin and other β-lactam antibiotics in pneumococci in the 1980s and 1990s led to increased use of macrolides, fluoroquinolones, and other non-β-lactam antibiotics for pneumococcal infections. Efforts to treat pneumococcal disease in both adults and children have been complicated by this increasing resistance to antimicrobials. The increase in antimicrobial resistance rates is due in part to the selective pressures associated with the widespread use of antibiotics [1] and the clonal expansion and spread of multiresistant *S. pneumoniae*.

More recently, changes in antimicrobial use and the introduction of the pneumococcal conjugate vaccine (PCV) have markedly altered the resistance patterns of *S. pneumoniae* in some countries.

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This chapter will focus on the risk factors and clinical relevance of resistance as well as detection of antibiotic resistance in pneumococci, mechanisms of resistance, and changes in drug resistance patterns in the era of conjugate vaccines.

RISK FACTORS FOR RESISTANCE

While many risks have been described for the isolation of antibiotic-resistant pneumococci, most of them can be linked to an increased risk of antimicrobial exposure, given the selective pressure of antibiotics in elimination of susceptible strains, thus increasing the relative likelihood of detection of resistant organisms (Table 2.1).

Many studies have identified young age as a risk for detection of resistant pneumococci. As resistant strains emerge in a population, it is in children that they will, in most instances, emerge [2,3]; even in mature epidemics where resistant strains have circulated for over 30 years, resistance remains more common in children [5]. Compared to younger adults, resistance may be higher in the elderly [5], and in circumstances where a drug is not licensed for general use in children, such as fluoroquinolones, resistance first emerges in adults [6].

Nosocomial acquisition is a significant risk for isolation of resistant pneumococci, given the density of antimicrobial use in that setting. The first fully penicillin-resistant and multiply resistant pneumococci were isolated from children hospitalized with measles [3], and in the hospital setting even resistance among pneumococci to fluoroquinolones has been described in children treated for multidrug-resistant (MDR) tuberculosis [4]. Recent hospitalization is also a risk for infection with multiply resistant pneumococci [5].

Children in rural settings generally have less access to antibiotics and therefore have less resistant strains [5,7], while in some large cities, where poorer children live in the city center with less access to care and more affluent children live in the suburbs, there may be more resistance outside the city [8].

Exposure to antibiotics has been directly related to resistance in pneumococci at the national level [13]; even in countries of similar socioeconomic status, patterns of antibiotic use predict resistance [14,15]. Resistance is also related to exposure at the level of the individual [9,10]. Even individual treatment for malaria with a related antimicrobial such as fansidar may lead to increased risk of cotrimoxazole-resistant pneumococci in exposed infants [11]. There are data to suggest that increased duration of exposure to antibiotics in an individual child is a risk for acquisition of resistant strains [12]. Day care is also a risk for acquisition of resistant pneumococci [16]. Persons infected with HIV have a greater risk of resistance for several reasons, including exposure to antimicrobial prophylaxis [5,17], and HIV-infected women are at greater risk of acquiring resistant pneumococci from their children [18].

TABLE 2.1 Risk Factors for Resistance

Risk factor	References
Age—children	[2–4]
Age—elderly (fluoroquinolone resistance)	[5,6]
Hospitalization	[3–5]
Urbanization	[5,7,8]
Exposure to antibiotics—individual level	[9–12]
Exposure to antibiotics—national level	[13–15]
Day care attendance	[16]
HIV	[5,17,18]
Lack PCV vaccination	[19,20]
Bacterial clonal structure	[21]

Certain clones of pneumococci are global in their distribution, and the occurrence of these clones is a particular risk for the emergence of resistance in the population [21]. If the mechanism of resistant strain acquisition is mutation within a susceptible population, rather than the acquisition of a pneumococcal strain already harboring a gene conferring resistance, then the rate of acquisition may be high, especially for simple mutations that do not confer fitness costs—thus cotrimoxazole resistance is readily acquired by pneumococci [11], while resistance to linezolid is not [22].

Children exposed to conjugate vaccine, as well as adults living in countries where these vaccines are routinely administered to children, are at lower risk for pneumococcal infections due to resistant strains, as described in the section "Vaccines and Resistance."

CLINICAL RELEVANCE OF RESISTANCE

The clinical relevance of antimicrobial resistance in pneumococci is explained by pharmacodynamic principles, whereby strains fail to respond to therapy if the concentration of drugs at the site of infection does not exceed the minimum concentration able to inhibit the growth of the organism. In the treatment of pneumonia, high concentrations of intravenous penicillin have not been associated with clinical failure in either children or adults [23], while macrolide resistance [24] will lead to failure as sufficient drug concentrations to overcome resistance cannot be achieved. For the treatment of pneumococcal otitis media, the same principles apply, with successful therapy given high doses of oral amoxicillin [25], but inability to clear the infection using azithromycin [25], cotrimoxazole [26], or oral cephalosporins [27], which are less active than amoxicillin. For the treatment of pneumococcal meningitis, the cerebrospinal fluid CSF penetration of penicillin will not allow the successful management of even intermediately resistant strains [28], so therapy requires the use of extended-spectrum cephalosporins, plus vancomycin if there is resistance to the cephalosporin [29].

The unprecedented global increase of *S. pneumoniae* carrying resistance to penicillin, macrolides, and tetracyclines initiated the development of fluoroquinolones with increased effectiveness to pneumococci. Moxifloxacin and levofloxacin belong to the class of so-called "respiratory" fluoroquinolones. Several international guidelines have long implemented the use of these fluoroquinolones in treating community-acquired pneumonia (CAP) [30,31]. In some geographic regions of the world, fluoroquinolones continue to be the drugs most often used to treat CAP. Respiratory fluoroquinolones may still show *in vitro* activity despite the presence of ciprofloxacin resistance. However, animal experiments have shown that treatment of strains resistant to ciprofloxacin but susceptible to either levofloxacin or moxifloxacin frequently led to selection of complete resistance to all fluoroquinolones and consequent treatment failure [32]. In 2002, Davidson et al. [33] published clinical data describing the empiric treatment of four patients with fluoroquinolones that resulted in complete treatment failure in all four cases, resulting in death in two of the cases.

DETECTION OF RESISTANCE

Determination of antimicrobial susceptibility is essential not only to guide antimicrobial treatment in a specific patient but also, from a general perspective, for compiling data for antimicrobial guidance. Even though we can now identify pneumococci and many resistances based upon genetic features, bacterial culture and phenotypic susceptibility tests remain the gold standard approaches in clinical laboratories.

In the clinical setting, criteria for assessing resistance in *S. pneumoniae* are standardized by specific methods and interpretations developed by a variety of professional bodies, including the Clinical and Laboratory Standards Institute (CLSI), the British Society for Antimicrobial Chemotherapy, and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [34]. For some antibiotics, such as penicillin, defining resistance is a complex issue. Because the breakpoints are determined on the basis of microbiological, pharmacological, and clinical outcome data, and since patterns of resistance to antimicrobial drugs continue to evolve, changes to breakpoints can occur during the lifetime of an antibiotic. A good example is CLSI's revised breakpoints for penicillin, adopted in January 2008 to redefine the susceptibility of meningeal and non-meningeal pneumococcal isolates [35].

Culture of clinical specimens and antibiotic susceptibility testing are often slow, taking up to 48 h, and are often negative due to prior antibiotic use before sampling or autolysis of the organism. Rapid tests, based mainly on immunological or molecular techniques, have gained importance for detection of bacteria and antibacterial resistance over the last two decades. PCR has been shown to be a useful tool for the rapid identification of *S. pneumoniae* from both clinical specimens and bacterial isolates [36,37]. The increased use of molecular tests such as PCR for the diagnosis of bacterial infections has led in turn to an increased demand for antibiotic susceptibility testing using molecular methods. However, unlike phenotypic testing for antibiotic susceptibility, which examines all resistance mechanisms for a particular antibiotic simultaneously, molecular testing can detect only known resistance mechanisms. A variety of assays have been described to detect the presence of specific resistance genes in pneumococcal isolates as well as directly from clinical specimens [36–40]. The majority of these assays are PCR based [36,37], although sequencing approaches and microarrays have also been used [39,40].

MECHANISMS OF RESISTANCE

β-Lactam Resistance

Penicillin resistance was demonstrated in laboratory mutants soon after the introduction of penicillin G into clinical use in the 1940s, but was not reported in clinical strains until 20 years later when investigators in Boston reported penicillin resistance in 2 of 200 strains [41]. Initially, the observation was not considered relevant, until a report by Hansman and Bullen [42] describing a penicillin-resistant strain (minimum inhibitory concentration [MIC] 0.6 mg/L) isolated in Australia from the sputum of a patient with hypogammaglobulinemia. Subsequently, resistant strains were identified in New Guinea and Australia, and in 1974 the first clinical infection due to a penicillin-nonsusceptible strain was reported in the United States [43]. In 1977 pneumococci resistant to penicillin began to appear in South Africa, and in 1978 the first multidrug-resistant pneumococci were documented in Johannesburg, South Africa [3]. Between and after these initial reports, detection of penicillin-resistant pneumococci among clinical isolates began to be reported with increasing frequency in the clinical and microbiological setting. Today, penicillin-resistant strains are encountered in all countries in which adequate surveys are conducted, and an increasing number of countries are reporting a high prevalence of penicillin-nonsusceptible pneumococci. Recombination appears to be an essential mechanism in the evolution of β-lactam resistance in nature, and the resultant clonal spread of resistant strains plays an enormous role in the global increase in β-lactam resistance [21].

β-Lactam antibiotics inhibit the growth of pneumococci by inactivation of cell wall–synthesizing penicillin-binding proteins (PBPs). β-Lactam resistance in pneumococci occurs by acquisition of *pbp* genes encoding cell-wall PBPs with decreased affinities for these antimicrobials. Six PBPs have been identified in *S. pneumoniae*: PBPs 1a, 1b, 2a, 2b, 2x, and 3, of which PBP2x and PBP2b have been confirmed to be essential for cell growth [44]. Resistance to β-lactams is complex and involves a multifactorial process (Figure 2.1).

Depending on the selecting β-lactam, different combinations of *pbp* genes and mutations within these *pbp* genes are involved in conferring resistance. Little data exists for the role of PBPs 1b, 2a, and 3 as resistance determinants; altered PBPs 2x, 2b, and 1a are the major players in the development of β-lactam resistance in most clinical isolates. The altered PBPs

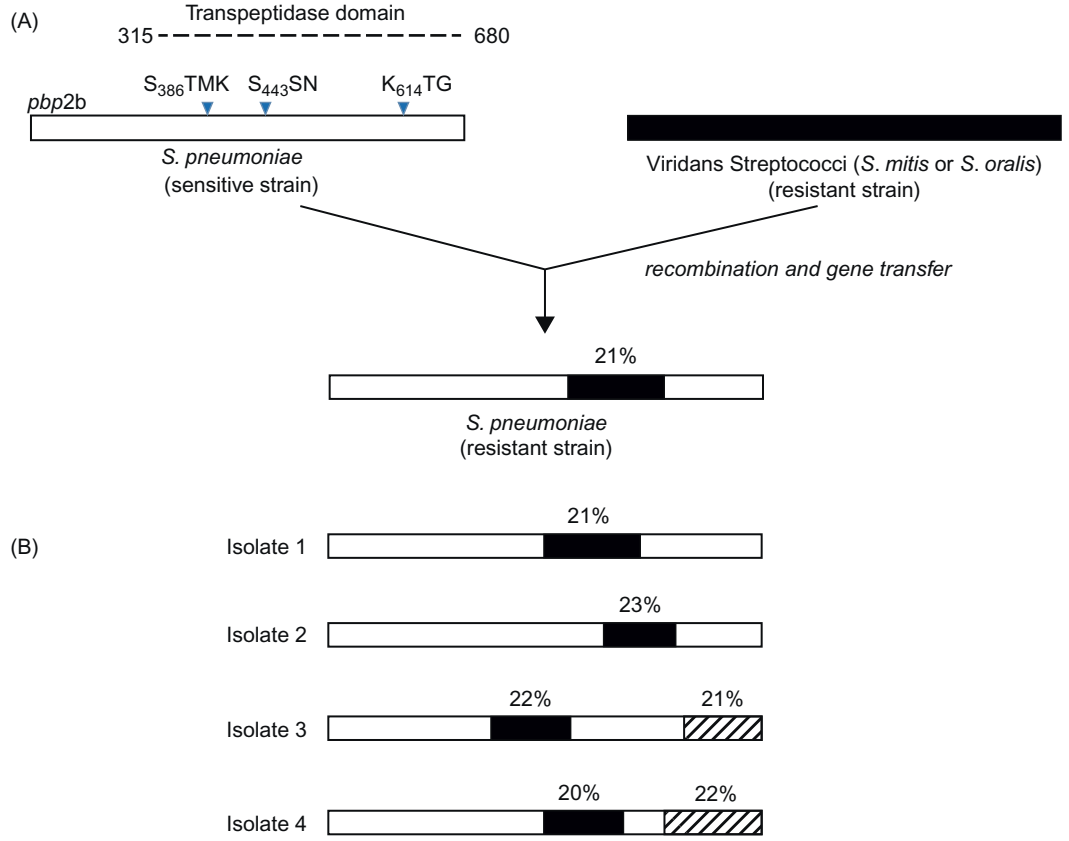

FIGURE 2.1 Schematic representation of evolution of mosaic *pbp*2b gene in *S. pneumoniae* (A) and some examples of isolate's *pbp*2b genes showing sequence diversity (B). Penicillin-sensitive *S. pneumoniae* can incorporate regions of altered *pbp* genes from commensal streptococci by transformation and homologous recombination. A combination of intra- and interspecies gene transfer, along with additional secondary mutations, results in a mosaic structure of *pbp* genes in the pneumococcal population. The open rectangles show regions where sequences are similar to those in penicillin-sensitive pneumococci ("sensitive blocks"). The location of the transpeptidase domain is shown by the dashed line in (A), and the active site motifs are marked by black triangles. The solid and hatched rectangles are regions that are highly divergent ("resistant block"). Black and white regions' sequences differ by approximately 20% on the DNA level.

TABLE 2.2 Molecular Mechanisms of Antibiotic Resistance in *S. pneumoniae*

Antibiotic	Mechanisms
β-Lactams (penicillin and cephalosporins)	Mutations in *pbp* genes (primarily *pbp*2x, *pbp*2b and *pbp*1a)
	Mutations in *mur*M
	Mutations in other genes include: *pdg*A, *cia*H/*cia*R, *stk*P
Macrolides	*erm* (23S rRNA methyltransferases) (primarily *erm*(B))
	mef-mediated efflux (*mef*(A) or *mef*(E))
	Mutations in 23S rRNA or L4 or L22 ribosomal protein genes
Fluoroquinolones	Mutations in DNA gyrase (primarily *gyr*A) and/or topoisomerase IV genes (primarily *par*C)
	PmrA-mediated efflux
Tetracycline	Ribosomal protection proteins: primarily Tet(M) but also more rarely Tet(O)
Rifampin	Mutations in *rpo*B RNA polymerase gene
Chloramphenicol	Inactivation of chloramphenicol by CAT enzyme
Trimethoprim–sulfamethoxazole	Mutations in DHFR
	Mutations in DHPS
Ketolides	Mutations in 23S rRNA or L4 or L22 ribosomal protein genes
	erm(B) with deletion or mutation in leader sequence
Oxazolidinones	Mutations in 23S rRNA
	Deletions in L4 ribosomal protein gene

in clinical isolates are invariably encoded by genes with a mosaic structure and can undergo inter- and intraspecies recombination so that parts of the genes are replaced by allelic variants that differ by up to 20% in the DNA sequence [45]. Mosaic sequences of *pbp* genes are very difficult to classify and organize. In general, the resistance profile of particular isolates results from interactions between various combinations of altered PBPs, in conjunction with a functional murMN operon which encodes enzymes involved in the synthesis of branched-structured muropeptides. Several other genes have been implicated in β-lactam resistance in selected clinical isolates that contribute to resistance in addition to mutations in *pbp* genes [45], although certain combinations of these three altered *pbp* genes alone appear to confer resistance (Table 2.2).

Resistance to penicillin is generally associated with some degree of nonsusceptibility to other β-lactam antibiotics and vice versa. Mutations in *pbp2x* confer low-grade penicillin resistance and may be sufficient for the cell to become nonsusceptible to oral cephalosporins. Alterations in *pbp2b* result in even higher MICs to penicillin [46], while changes in *pbp1a* are required for high-level penicillin resistance and extended-spectrum cephalosporin resistance [47]. Isolates with very high levels of penicillin resistance (MICs ≥ 8 mg/L) require changes in all three PBPs (i.e., 1a, 2b, and 2x) and

sometimes in additional non-PBP resistance determinants such as MurM [48].

Resistance rates reported for amoxicillin are relatively low (<5%) as a result of the favorable pharmacodynamic properties of this agent. Generally, MICs to amoxicillin are equal to, or two to four times less than, the MIC of penicillin. In the past there have been numerous reports of strains with amoxicillin MICs (4–16 mg/L) higher than penicillin MICs (2–8 mg/L) [49,50]. In particular, PBP2b appears to play a significant role in mediating the expression of this resistance phenotype [47,51]. In addition to typical changes in *pbp1a* and *pbp2x*, these strains have unique mutations in the 590–641 region of the *pbp2b* gene, in close proximity to the active binding site [50].

Resistance to cephalosporins may develop with mutations in the *pbp1a* and *pbp2x* genes, and the close linkage of these two genes on the chromosome is conducive to the transfer of both genes in a single transformation step. PBP2b is not a target for cephalosporins, and so would remain unaltered in isolates expressing cephalosporin resistance and susceptibility to penicillin [47]. Most, but not all, extended-spectrum cephalosporin–resistant strains are also penicillin-resistant and, as with amoxicillin, the MICs of cefotaxime and ceftriaxone are usually lower than the MICs of penicillin. Newer antibiotics such as ceftaroline and ceftobiprole appear to be more active and have greater affinity for altered *pbp* genes, allowing it to be active against strains with elevated MICs to other β-lactams [52,53]. In the early 1990s in the United States, pneumococci with high-level cefotaxime and ceftriaxone (2–32 mg/L) resistance were detected, and this high-level resistance was due to alterations in PBPs 1a and 2x [47]. The cephalosporin MICs were in excess of the MICs of penicillin for these isolates, and specific point mutations ($Thr_{550}Ala$) in the *pbp2x* gene were associated with this phenotype [47]. These cephalosporin-resistant strains emerged within a few preexisting clones and demonstrate that point mutations as well as recombinational events are important in the development of resistance to β-lactam antibiotics in pneumococci.

Macrolide Resistance

The macrolides (e.g., erythromycin, clarithromycin, and azithromycin) have been used extensively worldwide to treat community-acquired respiratory tract infections, and their use has led to increased rates of resistance in *S. pneumoniae* and even clinical treatment failure in several cases. Macrolide resistance rates in clinical isolates of *S. pneumoniae* vary greatly among countries; in the majority of regions, macrolide resistance is more prevalent than resistance to penicillin. Erythromycin resistance rates range from about 15% in Latin America to as high as 80% recorded among isolates in the Far East [54]; these differences probably reflect, in part, the variation in antibiotic prescribing behavior between different countries.

Macrolides are microbiostatic agents that inhibit bacterial protein synthesis by binding to the 23S ribosomal RNA (rRNA). Pneumococcal macrolide resistance is mediated by two major mechanisms: target modification and drug efflux (Table 2.2).

Target Modification

In *S. pneumoniae*, the *erm*(B) gene, encoding a 23S RNA methylase, is a major resistance determinant and the prevalent mechanism in some Asian, European, Middle Eastern, and African countries. Expression of the *erm*(B) gene results in the dimethylation of the adenine residue at position 2058 on the 23S rRNA, reducing the affinity of the macrolide to the 23S binding site. This methylation confers, in the majority of pneumococci, constitutive high-level resistance to 14-, 15-, and 16-member macrolides, as well as resistance to lincosamides and streptogramins (MLS_B phenotype). *Erm*(B) resistance can

be expressed by pneumococci either constitutively (cMLS$_B$ phenotype) or inducibly (iMLS$_B$ phenotype) [55]. Rarely a methylase encoded by *erm*(A) subclass *erm*(TR) has also been shown to confer MLS$_B$ resistance [56].

In pneumococci, Tn*916*-family transposons with various insertions are the basis of most *erm* (B)-carrying mobile genetic elements. A number of Tn*916* derivatives carrying *erm*(B) have been described (Tn*1545*, Tn*3872*, Tn*6002*, and Tn*6003*), and the *tet*(M) gene is typically also carried by these elements [57]. Most macrolide-resistant *S. pneumoniae* are therefore also resistant to tetracycline; however, some recent studies have shown Tn*916*-related elements where the *tet* (M) gene is present in a silent form [58].

Other less common target modifications are point mutations in domains II and V of 23S rRNA and in the genes encoding riboproteins L4 and L22. These mutations have been shown to confer macrolide resistance and have been documented in clinical isolates from widely distributed global sites [59].

Efflux Pumps

Efflux-mediated erythromycin resistance is associated with a low-level resistance pattern affecting only 14- and 15-membered macrolides, but not lincosamides or streptogramins (M phenotype). The M phenotype isolates are predominant in the United States, Canada, and some Asian and European countries. Active efflux is encoded by *mef*-class genes, which include several variants: the abundant *mef*(A) and *mef*(E), which share 90% sequence identity, and the rare variant *mef*(I), which has only been described in two Italian clinical strains [60].

In pneumococci, the three subclasses of *mef* are carried on a number of similar but distinct genetic elements. *Mef*(A) is located on the defective transposon Tn*1207.1*, or the closely related Tn*1207.3*, whereas *mef*(E) is typically carried on the mega (macrolide efflux genetic assembly) element. The *mef*(I) gene exhibits 91.4% and 93.6% homologies to the *mef*(A) gene of Tn*1207.1* and the *mef*(E) gene of the mega element, respectively [60], and is carried on a nonmobile composite structure, designated the 5216IQ complex [61].

Dual Phenotype

In recent years, the presence of both the *erm* (B) and the *mef* genes in *S. pneumoniae* clinical isolates has increasingly been recognized, particularly in Asian countries but also in Europe, South Africa, and the United States. The PROTEKT study reported a 12% global prevalence of macrolide-resistant isolates positive for both *erm*(B) and *mef*(A) in 2003–2004 [54].

The majority of dual-positive isolates exhibit multidrug resistance and are clonal lineages of Taiwan^{19F}-14, mostly multilocus sequence types 320, 271, and 236. It appears that the global increase in macrolide-resistant strains carrying both the *erm*(B) and *mef* genes is being driven in part by the diversification and expansion of this Taiwan^{19F}-14 clone following the introduction of conjugate vaccine. This was especially true of the major 19A ST320 variant in the United States, which became the single most common IPD-causing genetic complex in the United States prior to PCV13 implementation.

Fluoroquinolone Resistance

Surveillance studies suggest that at least 1% of clinical isolates are resistant to levofloxacin, moxifloxacin, or gemifloxacin in the United States [62]. However, a study in assisted living facilities found 6% of all colonizing pneumococci to exhibit resistance to quinolones [63]. In Canada it was recently suggested that there is increased fluoroquinolone resistance in the so-called replacement strains that have emerged since the widespread use of conjugate pneumococcal vaccine, such as serotypes 19A, 35B, and 11A [64]. In Croatia and Hong Kong, 4–13% of all pneumococci were reported resistant to fluoroquinolones, and a study from Asia in 2009 and 2010 found up

to 4% prevalence of resistance to newer fluoroquinolones [65]. Within countries that report increasing incidence of resistance, the proportion of resistant isolates is much higher among older subjects and patients with chronic lung disease, a patient population that is frequently exposed to fluoroquinolones [66]. The feared global rise of fluoroquinolone-resistant pneumococci in comparison to macrolide resistance has not yet materialized. This may be explained by the fact that children, who are the main reservoir of pneumococci, are not generally treated with fluoroquinolones but with macrolides. This is supported by a recent study from South Africa showing a rise in fluoroquinolone resistance in pneumococci isolated from children treated with fluoroquinolones due to MDR tuberculosis [67]. The type of fluoroquinolone used may also play a role in resistance: A study from Germany found no fluoroquinolone-resistant isolates and only 1.2% first-step mutants, compared to 16.2% of isolates recovered from US nursing home residents and 6.4% from non–nursing home residents [68]. In contrast to the United States, moxifloxacin (not affected by efflux) is by far the most frequently used fluoroquinolone in Germany compared to levofloxacin (http://www.pharmacytimes.com/publications/issue/2009/2009-05/RxFocusTop200Drugs-0509). EUCAST no longer recommends pneumococcal breakpoints for ciprofloxacin because selection for resistant pneumococci has been described frequently during fluoroquinolone treatment, and therefore ciprofloxacin is no longer considered an antibiotic with sufficient activity against pneumococci [69,70].

Molecular Mechanisms of Fluoroquinolone Resistance

Fluoroquinolones belong to a relatively new class of synthetic antibiotics. After penetration into bacteria, the quinolones bind to type II topoisomerase enzymes (i.e., DNA gyrase and topoisomerase IV) that govern the twisting and knotting of double-stranded DNA. These two enzymes are essential for DNA replication and cell division. Specifically, each of the enzymes consists of their respective subunits, which are structurally related to each other. Both enzymes are tetrameric with pairs of two different subunits: the *gyr*A and *gyr*B subunits of DNA gyrase are respectively homologous with the *par*C and *par*E subunits of type IV topoisomerase.

The quinolone resistance–determining regions (QRDRs) are the binding sites of FQ to the respective subunits of the two enzymes. Generally, after binding these sites, quinolones block the enzymatic activity so that bacterial replication cannot take place. The specific site of action of a quinolone is determined by the avidity with which it binds each enzyme. For example, ciprofloxacin prefers binding to topoisomerase IV, whereas levofloxacin binds more avidly with topoisomerase IV but also exhibits avidity to DNA gyrase. Moxifloxacin binds with higher avidity to DNA gyrase than to topoisomerase IV. Gemifloxacin binds with both.

Mutations in QRDRs

Mutations that lead to conformational changes in the fluoroquinolone-binding enzymes can confer complete resistance in pneumococci to fluoroquinolones. These mutations are seen mostly in the QRDR of *gyr*A and *par*C; the catalytic subunits of type II topoisomerase enzymes and to a lesser extend in the QRDR of *par*E und *gyr*B, the energy-providing subunits. Pneumococci carrying a single mutation in just one of the two enzymes ("first-step mutation") are mostly susceptible to fluoroquinolones. Mutations conferring resistance occur in a stepwise fashion, with mutations observed in either *par*C or *gyr*A (depending on the selecting fluoroquinolone). Strains usually become fully fluoroquinolone resistant with the acquisition of a second mutation in the other of the target genes (*gyr*A and/or *par*C). Mutations in *par*E and *gyr*B may contribute to resistance in some isolates but appear to have limited effect when present alone.

Several mutations have been described in these enzymes, but only a few have been shown by *in vitro* studies to confer resistance: S81F, Y, C, or I, and E85K in *gyr*A; E474K in *gyr*B; A63T, S79F, Y, or L, and D83G or N in *par*C; and E474K and D435N or H in *par*E [71]. Other frequently described mutations are K137N in *par*C and I460V in *par*E, which appear not to contribute to fluoroquinolone resistance because they are commonly found in susceptible strains, and no evidence exists for their conferring fluoroquinolone resistance *in vitro*. Pletz et al. [72] found a Q118K in *gyr*A together with S79F in *par*C in a fluoroquinolone-resistant isolate resulting in treatment failure.

Efflux Pump

A fluoroquinolone efflux pump is mediated by the membrane ABC-transporter protein PmrA and some unknown factors. In contrast to the *mef*A gene conferring macrolide resistance, the efflux mechanisms in fluoroquinolone resistance are poorly characterized. They are not encoded by resistance genes but are thought to be over-expressed in 8–45% of pneumococcal strains [68]. Little is known about the mechanism of expression regulation of PmrA, but the efflux pump can be blocked by the plant alkaloid reserpine and, to a lesser degree, by verapamil [73]. Interestingly, both substances are licensed drugs for the treatment of hypertension in humans.

Currently, detection of this efflux pump is based on phenotypic features, where a twofold increase in MIC to ciprofloxacin in the presence of reserpine at 10 mg/L suggests the presence of a pump. To date, no highly resistant isolate has been found with efflux being the only mechanism of resistance. Fluoroquinolones with a small molecule size (e.g., ciprofloxacin) seem to be affected to a greater extent than larger molecules such as moxifloxacin. It has previously been observed that phenotypic ciprofloxacin resistance can be selected more frequently from isolates with an efflux phenotype [74]. The efflux pump inhibitor reserpine, and to a lesser degree verapamil, can prevent the selection of ciprofloxacin-resistant isolates by reduction of the mutation ratio, particularly in strains with an efflux phenotype. Efflux may not confer complete resistance but may be able to lower intracellular fluoroquinolones to sublethal concentrations, fostering the occurrence of QRDR mutations [75].

Horizontal Gene Transfer and the Clonal Concept

In contrast to β-lactam resistance, horizontal gene transfer and the role recombination plays in the evolution of fluoroquinolone resistance are uncertain. Both intra- and interspecies transfer of fluoroquinolone resistance loci have been found to occur *in vivo*, but the frequency of such events appears to be rare. *In vitro* models report a higher frequency of recombination of QRDRs between viridans group streptococci and *S. pneumoniae* compared to that of spontaneous mutations [76]; however, this level of recombination does not appear to be replicated in *vivo* [77]. Published studies addressing this question of recombination found evidence for horizontal gene transfer in 0–11% of fluoroquinolone-resistant isolates; interestingly, this ratio seems to be higher in respiratory isolates than in invasive isolates [78,79].

Fluoroquinolone resistance has been reported in a number of international pneumococcal clones that have been associated with the evolution of resistance to penicillin and macrolides [80]. However, the role that clonal spread plays in the increase of fluoroquinolone resistance is controversial, with studies placing different degrees of significance on its importance. The increased prevalence of levofloxacin resistance that was reported from Hong Kong between 1995 and 2001 was suggested to be associated with the dissemination of strains related to the Spain^{23F}-1 clone. However, several studies have shown that clonal spread does not play a significant role in

the increase of fluoroquinolone resistance [80,81]. Data on levofloxacin-resistant pneumococci from 25 countries analyzed as part of the PROTEKT study (1999–2000) showed the majority were genetically unrelated, although 34% belonged to the Spain^{23F}-1 clone [80]. These studies suggest that both clonal dissemination and the emergence of newly resistant strains contribute to the spread of fluoroquinolone resistance.

Tetracycline Resistance

One class of antimicrobial agents previously used often in clinical practice is the tetracyclines, which are broad-spectrum bacteriostatic drugs shown to be active against pneumococci. Reflecting patterns of past usage, in some countries reported rates of nonsusceptibility to tetracyclines remain the most frequently observed resistance phenotype [82]. In *S. pneumoniae*, tetracycline resistance is due to the protection of the bacterial 30S ribosome subunit against antibiotic binding by the TetM or TetO [83] proteins, with the *tet*(M) gene being far more common than the *tet*(O)gene in pneumococci. In streptococci, *tet*(M) is usually associated with highly mobile conjugative transposons of the T*n916*–T*n1545* type and large composite structures like T*n5253* and T*n3872*. A recent study discovered the oldest known examples of two different Tn*916*-like, *tet*(M)-containing elements identified among pneumococci dated from 1967 and 1968 [82]. These transposons often carry other resistance genes, such as *erm*(B) coding for resistance to macrolides, lincosamides, and type B streptogramins, which explains the persistence of tetracycline resistance (these transposons continue to be selected by macrolides). Comparison of *tet*(M) sequences in MDR isolates reveal a high degree of allelic variation. There is evidence of clonal distribution of selected alleles as well as horizontal movement of the mobile elements carrying *tet*(M) [84].

Rifampin Resistance

The use of rifampin combined with either β-lactam antibiotics or vancomycin has been recommended for the treatment of meningitis caused by multiresistant pneumococci. Rifampin has been used in combined therapy to treat tuberculosis and resistant staphylococci, and it is extensively used in the prophylaxis of *Neisseria meningitidis* and *Haemophilus influenzae* type b exposure. The prevalence of rifampin resistance among pneumococcal isolates is low at present, with reported rates varying between 0.1% and 1.5%. Rifampin resistance has been described in several bacterial species and is caused by an alteration of the β subunit of RNA polymerase, the target for the antibiotic. Resistance to rifampin in pneumococci has been linked to mutations in clusters N, I, II, and III of the *rpo*B gene, which encodes the β subunit [85].

Chloramphenicol Resistance

Resistance to chloramphenicol in *S. pneumoniae* is due to the acetylation of the antibiotic by the production of a chloramphenicol acetyltransferase (CAT). The cat gene in pneumococcal isolates is carried on the conjugative transposon *Tn*5253, a composite transposon consisting of the tetracycline resistance transposon, *Tn*5251, and *Tn*5252, which carries the chloramphenicol resistance determinant. Chloramphenicol-resistant strains have been shown to contain sequences homologous to cat_{pC194} and other flanking sequences from *Staphylococcus aureus* plasmid pC194 [86].

Trimethoprim–Sulfamethoxazole Resistance

Trimethoprim and sulfamethoxazole are used extensively in combination as the drug cotrimoxazole. Cotrimoxazole has been used in

the treatment of a range of *S. pneumoniae* diseases, especially in children, because it is inexpensive and generally effective. Resistance to cotrimoxazole has increased dramatically in many regions of the world; recent surveillance studies show rates ranging from 19% in Europe to around 50% associated with HIV infection in Africa, and greater than 60% in Asia. Resistance to cotrimoxazole is often associated with resistance to other antibiotics, especially to penicillin. Trimethoprim resistance in pneumococci has been reported to result from a single amino acid substitution (Ile-100→Leu) in the dihydrofolate reductase (DHFR) protein [87] and often associated with mosaic alleles. Additional mutations have also been reported that seem to enhance resistance and modulate the effects of existing alterations on the affinity of DHFR for its natural substrates [88]. In many cases, resistance to sulfonamides is associated with chromosomal mutations within the gene encoding dihydropteroate synthase (DHPS). Different studies have reported the occurrence of single and/or multiple amino acid mutations in the DHPS of sulfonamide-resistant clinical isolates of *S. pneumoniae* [89]. The use of fansidar therapy for malaria in Africa has been shown to increase cotrimoxazole resistance in pneumococci.

Ketolides Resistance

Ketolides are a new class of semisynthetic agents derived from erythromycin A, designed to overcome macrolide resistance against *S. pneumoniae*. Ketolides bind to a secondary region on domain II of the 23S rRNA subunit and therefore have a stronger binding affinity for the ribosome. Telithromycin was the first ketolide drug approved for clinical use; however, safety issues have limited the clinical utility of this drug. Both cethromycin (ABT-773) and solithromycin (CEM-101), a novel fluoroketolide, have shown improved activity against macrolide-resistant as well as telithromycin-intermediate and telithromycin-resistant organisms [90,91]. This enhanced potency shows promise for future clinical use for these compounds, subject to pharmacokinetic/pharmacodynamic, toxicity, and animal infection model studies.

High-level telithromycin resistance in *S. pneumoniae* has been experimentally generated by mutations in domain II or V of the 23S rRNA gene and ribosomal proteins L4 and L22, and is easily created from a macrolide-resistant strain by deletion or mutation of the region upstream of *erm*(B) [92]. In contrast, clinical telithromycin resistance in *S. pneumoniae* remains rare. Farrell and Felmingham reported that among a worldwide collection of 13,874 *S. pneumoniae* isolates, isolated between 1999 and 2003, only 10 were resistant, with MICs greater than or equal to 4 μg/mL, and all contained *erm*(B) gene [93]. Mutations in 23S rRNA, L4, and L22 have also been found in clinical telithromycin-resistant isolates [94], and a combination of mutated genes can result in a higher telithromycin resistance than mutation of only one gene [95]. Wolter et al. [96] demonstrated that *erm*(B) with a deletion in the leader sequence was responsible for high-level telithromycin resistance in a strain isolated in Canada in 2007.

Oxazolidinone Resistance

Linezolid is the first in the class oxazolidinone that was approved for clinical use in 2000 for the treatment of nosocomial and community-acquired pneumonia. Linezolid binds to the 50S subunit of the bacterial ribosome via interactions with the central loop segment of domain V of 23S rRNA to block the formation of protein synthesis initiation complexes. To date, linezolid-nonsusceptible pneumococcal strains are extremely rare [93]. Recent data from the US

LEADER and global ZAAPS surveillance systems show no linezolid-nonsusceptible isolates among 2150 *S. pneumoniae* isolates tested in 2011 [113,114]. Reports of nonsusceptibility to linezolid have been sporadic among clinical isolates of staphylococci and enterococci, and resistance has been found to be conferred by mutations in domain V of 23S rRNA [99]. In pneumococci, Wolter et al. [100] have described two clinical isolates with decreased susceptibility to linezolid (MICs 4 mg/L), which were found to contain 6-bp deletions in the gene encoding the riboprotein L4. The L4 deletions were also found to confer a novel mechanism of simultaneous resistance to macrolides, oxazolidinones, and chloramphenicol. A more recent study identified two additional linezolid-nonsusceptible pneumococci from the United States within the Centers for Disease Control and Prevention (CDC) Active Bacterial Core Surveillance (ABCs) program with mutations and deletions within the *rplD* gene [22]. Whole genome sequencing of linezolid-resistant laboratory-generated mutants have also revealed a role in resistance for a 23S rRNA methyltransferase (spr0333) and for the ABC proteins PatA and PatB [101]. A proteomic and transcriptomic screen suggested increased energy requirement needs associated with the burden of resistance in these laboratory-derived mutants [102].

Second-generation oxazolidinones like tedizolid, which is a protein synthesis inhibitor, are in clinical development for the treatment of Gram positive infections. Tedizolid has demonstrated potent *in vitro* activity against penicillin-resistant *S. pneumoniae*, including linezolid-resistant strains [103].

Streptogramin Resistance

Quinupristin–dalfopristin is a 30:70 combination of a type B and a type A streptogramin. The two components target the late and early stages of bacterial protein synthesis, respectively, and thus have a synergistic inhibitory effect. Resistance to quinupristin–dalfopristin among Gram positive cocci has been very uncommon. Two clinical isolates among 8837 (0.02%) *S. pneumoniae* isolates were discovered in 2001–2002 with MICs of 4 μg/mL. Each had a five-amino-acid tandem duplication (RTAHI) in the L22 ribosomal protein gene (rplV), preventing synergistic ribosomal binding of the streptogramin combination [104] (Table 2.2).

ROLE OF CLONES IN RESISTANCE

The increase in antibiotic resistance and the introduction of conjugate vaccines have focused attention on the epidemiology of *S. pneumoniae*. Molecular typing data from numerous studies over the past few decades has added to our knowledge by showing that, although there is considerable diversity among resistant strains within most serotypes, a small number of highly successful clones have emerged within various countries and in some cases have achieved massive geographical spread [21]. In response to this, the Pneumococcal Molecular Epidemiology Network (PMEN) was established in 1997 with the aim of standardizing nomenclature and classification of pneumococcal clones worldwide. At present, PMEN has documented 43 international clones, 26 of which are MDR. The best characterized and most widespread of these international clones is the Spain^{23F}-1 or PMEN1, originally described in Spain during the 1980s. Intercontinental spread of this clone to the United States was described in 1991 and shortly thereafter in the United Kingdom, South Africa, Hungary, and South America [105]. By the late 1990s it was estimated that approximately 40% of penicillin-nonsusceptible pneumococci circulating in the United States were members of this clone, and

while strains belonging to this genotype continue to be isolated today in many countries all over the world, their prevalence has decreased in countries where conjugate vaccines have been introduced [106,107]. Recent studies looking at whole genome sequencing of pneumococci representing PMEN1 show that there is a considerable amount of genetic diversity within this lineage [105,108,109]. This diversity, which results largely from hundreds of recombination events, indicates rapid genomic evolution and presumably allowed rapid response to selective pressures such as those imposed by vaccine and antibiotic use [108].

Clonal analyses of large surveillance collections of pneumococci have revealed the remarkable dominance of a small number of clones among the antimicrobial-resistant population. As these clones have spread globally, they have been exposed to new selective pressures applied by regional variations in the use of different antibiotics. This has led to the further selection of strains belonging to these clones with varying antimicrobial resistance patterns. These resistant clones have also been exposed more recently to conjugate vaccines, and shifts in both serotype and clonal types have been documented [106,107,110]. In the United States, for example, serotype 19A strains have been identified as the main cause of serotype replacement in both carriage and invasive disease since PCV7 introduction; this has coincided with a significant increase in penicillin resistance and multidrug resistance among 19A clinical strains [106,107,110]. The majority of penicillin-resistant 19A strains belonged to emerging clonal complex 320 (CC320), which is descended from MDR Taiwan^{19F}-14 (PMEN14). In 1999, prior to PCV7 introduction, only CC199 and three minor clones were apparent among 19A strains. In 2005 post PCV, 11 clonal complexes were detected, including ST695 capsular variants of serotype 4 [110].

VACCINES AND RESISTANCE

Introduction of PCV Has Led to Direct and Indirect Protection Against Antimicrobial Resistance

The direct reduction of antibiotic-resistant invasive pneumococcal disease in PCV recipients compared to controls was demonstrated in a double blind clinical trial of a nine-valent PCV in South Africa [19]. The subsequent widespread vaccination of infants—the main reservoir of pneumococci—has reduced not only the incidence of invasive infections in the vaccinated population but also the proportion of colonized children, at least for the serotypes contained in PCV7, PCV10, and PCV13. The decrease in colonized children has subsequently interrupted the typical infection chain infants-to-parents or infants-to-grandparents and therefore protected non-vaccinated adults [115]. This initially led to dramatic reductions in the prevalence of antimicrobial resistance among pneumococcal isolates in all ages [20]. Continued exposure of replacement strains of pneumococci belonging to serotypes not included in the vaccine has, however, led to subsequent increases in the proportion of resistant strains among pneumococci causing disease, even though the absolute number of these infections has been reduced [112].

The opposed effects of increased antimicrobial usage and the herd protection effects of PCV can be summarized as follows: Resistant clones expressing vaccine serotypes are diminished, while resistance rates within non-vaccine serotypes continue to increase [112]. In Germany, macrolide resistance in IPD peaked in 2005 (32% in children and 19% in adults) and decreased to 15% and 13%, respectively, in 2008—only 18 months after implementation of PCV7 [113]. Similar observations were made in the United States [20]. Thereafter a rise in multidrug-resistant pneumococci, especially

due to the rise of serotype 19A (which is now contained in PCV13) occurred after the introduction of PCV7 [110,112].

Post PCV13 there is limited data on the incidence of multiply resistant serotype 19A strains; however, one study from Italy shows that this is decreasing [113]. It is a reasonable assumption that post PCV13 there will be a further reduction in incidence of resistant pneumococci as serotype 19A circulation is interrupted, with, however, emergence of resistance in non-PCV13 strains likely [114]. In the absence of a reduction in serotype 19A and limited herd protection of the elderly immediately post PCV10 in Finland, the proportion of antimicrobial resistance among pneumococci actually increased [115], emphasizing the need for continued surveillance of resistance post PCV introduction.

CONCLUDING REMARKS

Multiply resistant pneumococcus continues to have a global distribution. Antimicrobial resistance within the pneumococcal population emerges and is maintained through a complex interplay of many factors. Attempts to reduce the burden of resistance in this pathogen are frustrated by widespread empiric therapy for respiratory infections. Both appropriate and inappropriate antibiotic use continue to select resistance in this pathogen. Although the conjugate vaccine has reduced the burden of resistance in vaccine serotype isolates, continued antibiotic exposure is leading to the emergence of resistance in non-vaccine types. While the introduction of higher-valency vaccines like PCV13 has the potential to further reduce the problem of antimicrobial resistance, continued surveillance of emergence of resistance in non-vaccine types is essential.

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CHAPTER

3

Pneumococcal Vaccination and Consequences

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Vaccination is the best tool to prevent infectious diseases. Every year vaccines save thousands of lives worldwide, especially in young children, the most vulnerable age group affected by infections. Vaccines not only diminish personal risks but also, through herd immunity, protect entire communities against infectious diseases. Pneumococcal disease stands out among infectious diseases as a major global public health problem. Its etiological agent, *Streptococcus pneumoniae* (pneumococcus), is the most common cause of bacterial community-acquired pneumonia (CAP) and meningitis in developed countries, and is often associated with sequelae and death [1,2]. More than 800,000 children are reported to have died of pneumococcal disease in developing countries annually, the majority as a result of pneumonia [3]. In addition, *S. pneumoniae* is regarded as the leading cause of acute otitis media (AOM) among children in developed countries [4] and the pathogen most frequently isolated from elderly patients with CAP [5]. Due to the large impact of pneumococcal disease on individuals and society, widespread vaccination against this serious condition appears to be a necessary and highly recommendable preventive action.

The development of an effective vaccine against pneumococcus has long challenged researchers. The first clinical trial of a whole-cell pneumococcal vaccine was conducted by Wright et al. in 1911 [6]. Some years later, in 1945 [7], the first clinical trial using a capsular polysaccharide was completed. However, it was not until 1977 and 1983 that progressively improved polysaccharide vaccines covering 14 pneumococcal serotypes and 23 serotypes (PPSV23), respectively, were approved for licensure [8,9].

The antibody response produced by polysaccharide vaccines protects against the virulent action of the capsule that reduces the phagocytic uptake of bacteria by macrophages and neutrophils. By binding to the capsule, anti-polysaccharide antibodies promote complement

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the Fc-dependent opsonophagocytosis [10]. Nonetheless, the antibody response induced by all these vaccines was very low in younger children [11,12], the main population group affected by the disease and the primary group associated with the transmission of *S. pneumoniae* in the community. Due to their low capacity to induce a Th1 response, children less than 24 months of age respond poorly to most polysaccharide antigens [13]. Since PPSV23 is composed entirely of polysaccharide and induces a T-cell–independent response without immunological memory, there is also no anamnestic response to revaccination [14]. These immunological characteristics explain the fact that PPSV23 is unable to induce an effective immune response in younger children.

Drawbacks of PPSV23 were overcome in 2000, when a pneumococcal conjugate vaccine (PCV7) against seven of the most frequent serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) associated with invasive disease was introduced for use in children under 5 years in the United States. Conjugate vaccines, composed of polysaccharides covalently linked to protein carriers, induce a T-cell–dependent response producing immunoglobulin G and memory B-cells. This T-cell–dependent response primes the immune system to natural exposure and booster vaccination [15]. It is noteworthy that the large clinical trial that supported PCV7 licensure demonstrated significant protection not only against pediatric invasive pneumococcal disease (IPD), especially bacteremia, but also against pneumonia and otitis media [16–18]. In light of PCV7's effectiveness over the following years, in 2007 the World Health Organization (WHO) recommended its inclusion in national immunization programs in developing countries with high rates of childhood mortality. Post-licensure surveillance across countries has documented significant reductions in PCV7-type IPD and carriage, particularly in the age group targeted for vaccination (direct effects), as well as in non-vaccinated groups (indirect effects) [19–22]. Even so, while rates of PCV7-type IPD have declined, rates of IPD caused by non-PCV7 serotypes have simultaneously increased in many settings [23,24]. Response to these epidemiological dynamics has recently led to the licensure of two new PCVs: the 10-valent PCV (PCV10, which includes the seven serotypes of PCV7 and serotypes 1, 5, and 7F) [25] in 2009, and the 13-valent PCV (PCV13, covering PCV10 serotypes and additional serotypes 3, 6A, and 19A) [26] in 2010. Table 3.1 shows the main characteristics of the current pneumococcal vaccines.

The progressive spread of PCVs has led to a dramatic fall in IPD incidence both in vaccinated children (direct effects) and in the non-vaccinated population (indirect effects). Additionally, a shift has been observed in the main serotypes detected in nasopharyngeal carriers and in active disease. This replacement phenomenon has been associated with changes in the clinical manifestation of disease and in overall rates of antimicrobial resistance to pneumococcus.

EFFECT OF THE VACCINE ON PNEUMOCOCCAL CARRIAGE

Pneumococci are mainly found as a normal component of commensal microflora of the nasopharynx, particularly in healthy young children. Pneumococcal carriage is a dynamic event and the first step toward causing disease [33]. The duration of the carriage state is variable and ranges from less than 1 week to more than 30 weeks depending on the serotype age of the carrier [34,35]. There are other factors that influence acquisition and duration of carriage status such as crowding, season, host immunological factors, passive smoking, breast-feeding, recent use of antimicrobials, and co-infection with other respiratory pathogens including co-colonization

TABLE 3.1 Main Characteristics of Current Pneumococcal Vaccines

Vaccine	Included serotypes	Manufacturer	Year of licensure	Vaccine recommendation
PCV7 (Prevnar®)	4, 6B, 9V, 14, 18C, 19F, 23F	Wyeth (now Pfizer)	2000	All children aged ≤23 Children aged 24–59 months if they are at high risk for pneumococcal infection caused by an underlying medical condition [27]
PCV10 (Synflorix™)	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F	GlaxoSmith Kline	2009	Infants (<12 months of age) [28]
PCV13 (Prevnar®13)	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F	Pfizer	2010	All children aged 2–59 months [29] Children aged 60 months–18 years who are at increased risk for pneumococcal disease [30] Adults aged ≥65 years [31] Adults aged ≥19 years with immunocompromising conditions, functional or anatomic asplenia, CSF leaks, or cochlear implants [32]
PPSV23 (Pneumovax®23)	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F	Merck & Co.	1983	Adults aged ≥50 years [31] Children aged ≥6 years who are at increased risk for pneumococcal disease [30]

with multiple serotypes [36–40]. Above all these factors, vaccination is a crucial action that affects carriage status.

Conjugate vaccines protect against disease and against pneumococcal nasopharyngeal colonization, preventing acquisition of a new pneumococcal strain and transmission between humans. However, it should be noted that the protection is specific to the capsular types included in the vaccines. As a consequence, decreased carriage of vaccine types leaves the ecological niche of the nasopharynx open to being filled by capsular types not included in the vaccine, a phenomenon known as serotype replacement.

The first report of serotype replacement was published in 1996, based on a double-blind, placebo-controlled randomized trial in Gambian infants immunized with a 5-valent conjugate vaccine. This study proved that carriage of vaccine types had significantly declined after immunization. On the other hand, it showed a significant increase in non-vaccine types, and there was almost no change in the overall prevalence of pneumococcal disease [41]. Other randomized trials with a 9-valent conjugate vaccine from Gambia [42], South Africa [43], and Israel [44], and with PCV7 in the US Navajo population [45] and in the Netherlands [46], also documented replacement in carriage. Given the randomized trial design of these studies, reported results strongly suggest a significant association between replacement and vaccination. Concurrently, diverse observational studies subsequent to PCV7 commercialization observed a significant decrease in PCV7 serotypes in parallel to an increase of non-PCV7 in carriers [47–52] in countries

including the United States, Norway, Portugal, Spain, and South Korea, among others.

Various hypotheses have been proposed to explain the increase in non-vaccine serotypes after vaccination. First, an unmasking effect was postulated as a reason for replacement, since it is well known that simultaneous carriage of different pneumococci (co-colonization) can occur [53]. The important limitations of traditional serotyping methods for simultaneous detection of different serotypes has been presented as an alternative explanation of the phenomenon: If vaccination reduced vaccine types, the increased detection of non-vaccine serotypes would then be attributable to improved performance by serotyping techniques and not to any real increase in the acquisition of non-vaccine types. However, this hypothesis appears not to be plausible because new molecular capsular methods that allow simultaneous detection of different serotypes have shown an actual increase in non-vaccine types together with a decrease in vaccine types [54,55]. Second, there is wide consensus that capsular switching combined with co-colonization may explain the increase of non-vaccine types in the era of conjugate vaccines [56–58]. Capsular switching is a phenomenon whereby an isolate of *S. pneumoniae* undergoes homologous recombination with DNA from its environment and replaces its capsule cassette with that of another *S. pneumoniae* serotype, in effect keeping all other genome-encoded virulence determinants but now evading antibodies against its capsule serotype. Along these lines, co-colonization is associated with potential horizontal gene transfer [56]. An example of capsular switching is shown in Figure 3.1. Considerable documentation indicates that a clonal type of pneumococci that previously expressed a vaccine capsular type can turn into a non-vaccine type by capsular switching [57]. This process would be closely correlated with the emergence of

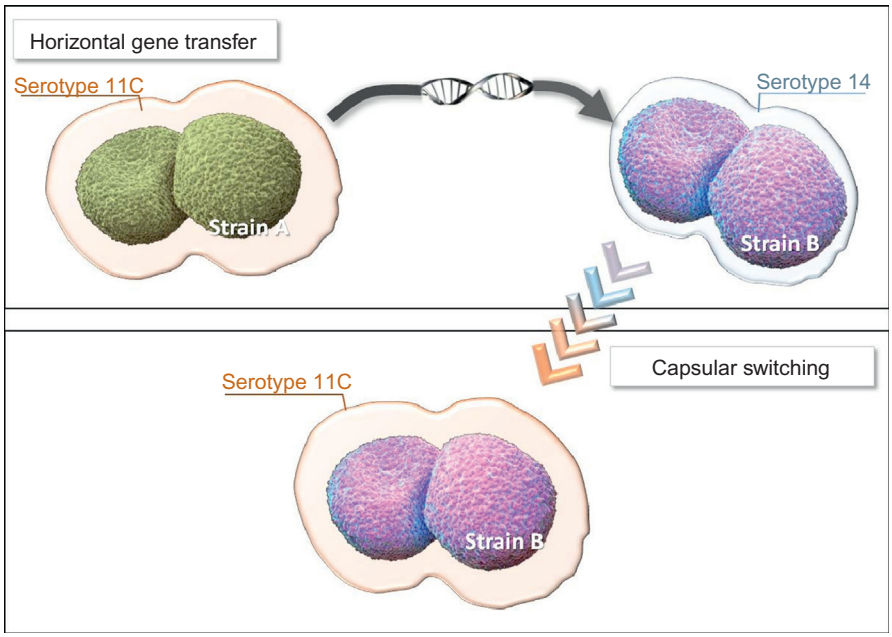

FIGURE 3.1 Representation of capsular switching. *Source: Adapted from www.sapiensmedicus.org.*

well-known international multidrug-resistant clones expressing non-vaccine serotypes [58]. A third possibility is simply that the ecological niche left open by immunization allows for serotypes that would normally be outcompeted by vaccine serotypes to be present for longer and at higher titers.

Nevertheless, how capsular switching and ecological replacement could affect vaccine effectiveness remains unclear due to the complicated interactions between capsular type and other virulence-determinant genes. For this reason, surveillance of non-vaccine serotypes and carriage is important to determinate future vaccination strategies.

EFFECT OF THE VACCINE ON THE DISEASE

The epidemiology of pneumococcal disease has dramatically changed since the introduction of conjugate vaccines. The burden of disease caused by vaccine serotypes (especially the seven included in the first available conjugate vaccine) has fallen considerably, and currently the disease is caused mostly by other serotypes such as serotype 1, 7F, 19A, and others [59]. Shifting serotypes have been associated with changes in the clinical manifestations of disease, in the incidence rates for the different age groups, and in overall antimicrobial resistance rates of invasive pneumococci. The impact of conjugate vaccines has been proved in both mucosa disease (otitis and sinusitis) and invasive disease both on the target population and on non-vaccinated population by herd immunity.

Impact of Conjugate Vaccines on Mucosal Infection

Conjugate vaccines reduce vaccine serotypes in the nasopharynx and consequently decrease local pneumococci spread that can cause AOM or sinusitis to adjacent mucosa. It has been reported that PCV7 was associated with a 40% decrease in pediatric ambulatory visits and antibiotic prescriptions attributable to AOM [60]. Similarly, a Finnish randomized clinical trial found 57% efficacy of PCV7 in preventing culture-confirmed vaccine serotype AOM episodes and an overall net decrease in AOM of 34% by any pneumococcal serotype [61]. Worth noting is that subsequent replacement serotypes and lack of efficacy in recurrent AOM were reported by the same authors [62]. Interestingly, an important proportion of AOM are mixed infections involving *S. pneumoniae* and non-typable *Haemophilus influenzae* (NTHi) as the main pathogens. In addition, co-infection with respiratory viruses (especially influenza virus) is a well-known factor that enables pneumococci dissemination. Conjugate vaccines affect these pathogen interactions and have become an important preventive tool against AOM. Similarly, combined maternal influenza vaccine and infant PCV have been reported to confer protection against AOM in the first year of life, which appears to be higher than that by PCV7 vaccine alone [63]. It is interesting that due to the role played by NTHi, in combination with pneumococcus, the 10-valent conjugate vaccine includes an *H. influenzae*–derived protein D as carrier, aiming to achieve further protection against this co-infection [64].

Effect of Conjugate Vaccines on IPD

PCV was initially introduced in wealthy countries. Since 2000, the use of PCVs has increased globally. Recommendations for PCV use from WHO and funding from public–private partnerships have resulted in the progressive inclusion of PCV into national immunization programs, especially in middle- and low-income countries.

PCVs have been shown to be safe and effective against IPD across a spectrum of populations [16,65], though the recent introduction of PCV10 and PCV13 does not yet allow unanimous conclusions about their effects. There are a large number of effectiveness and impact studies available on the benefits of PCV7 implementation, mostly from developed countries. Among them, diverse clinical studies have documented rates of efficacy against IPD of over 90% for this vaccine [66]. Similarly, a large-scale efficacy trial in the Northern California Kaiser Permanente system [17] reported a rate of 97.4% against IPD caused by vaccine serotypes and 89% against all IPD regardless of serotype. According to O'Brien et al. [67], in Native American populations the primary efficacy of PCV7 against vaccine types was 77%.

PCV7 has not been widely tested in low- and middle-income countries, but clinical trials evaluating a 9-valent pneumococcal vaccine (PCV9, including serotypes 1 and 5 in addition to those in PCV7) performed in specific settings may offer some insight into PCV efficacy in those countries. In particular, PCV9 clinical trials showed the vaccine to be efficacious in reducing mortality in Gambian children [68] and in reducing the incidence of lower respiratory tract infection in HIV-infected South African children [69].

The significant impact of PCV7 on IPD across all ages following its inclusion in pediatric immunization programs has been widely acknowledged. A substantial reduction in rates of IPD has been observed since the inclusion of PCV7 in the childhood immunization schedule of the United States in 2000 [19,70]. Although the impact of PCV7 on IPD caused by vaccine types has been very consistent across countries, the overall impact of PCV has varied among different populations, depending on serotype distribution and rates of vaccine coverage [71–74]. Moreover, vaccine schedules adopted in various countries may differ from one another. Table 3.2 summarizes the principal findings on the impact of PCV7 on IPD from different studies. The greatest impact was found in the United States, where the incidence of IPD dropped dramatically in both children and adults following introduction of PCV7. A similar pattern was observed in Canada and Australia, countries where the vaccine serotypes were responsible for the majority of IPD cases before PCV7 introduction. In other areas, such as Europe, the impact has varied across countries.

In addition to the direct protection conferred upon immunized individuals, vaccines also have the potential to produce an indirect protective effect on non-vaccinated populations and in all age groups, an effect known as herd immunity. Studies in the United States and United Kingdom have demonstrated significant reductions in the incidence of IPD in populations not directly vaccinated. A surveillance study [79] conducted in eight areas of the United States comparing IPD cases in adults 50 years of age and older, before (1998–1999) and after (2002–2003) the routine use of PCV7 in children, indicated that the use of conjugate vaccine in children also benefited the adults included in the study, showing a decline of 28% in IPD among adults aged 50 and older. In England and Wales [80], PCV7 was introduced in 2006 with a vaccination schedule of 2, 4, and 13 months, and catch-up vaccination for children aged up to 2 years. The rate of invasive disease caused by vaccine serotypes among persons aged 65 and older decreased by 81%, to 28.2 cases per 100,000 in 2009–2010.

Neonates and children too young to have received PCV also may be protected through herd effects. In a population-based study of infants aged 0–90 days residing in eight US states identified through ABCs found that mean rates of IPD decreased 40%, from 11.8 to 7.2 per 100,000 live births, following PCV7 introduction [81].

Although previous studies found that the PCV7 vaccine was highly effective in the

TABLE 3.2 Impact of IPD Rates Caused by PCV7 and Non-PCV7 Serotypes

Study [ref], country	PCV vaccination policy, year of introduction/schedule	Age group	Pre-vaccination			Post-vaccination			% Change		
				Rates/100.000			Rates/100.000				
			Years	PCV7 types	Non-PCV7 types	Years	PCV7 types	Non-PCV7 types	PCV7 types	Non-PCV7 types	Overall
NORTH AMERICA											
Pilishvili [19], USA	PCV7, 2000/3 + 1	<5 years	1998–1999	81.9	6.8	2007	0.4	10.3	−99.5	+51	−81.5
		All ages		15.5	6.1		1.0	7.9	−93.5	+29	−45
Whitney [75], USA	PCV7, 2000/3 + 1	<2 years	1998–1999	156.1	12.4	2001	33.6	15.7	−78.5	+26.6	−68.6
Singleton [23], USA (Alaska)	PCV7, 2000/3 + 1	Non-native <2 years	1998–1999	101.3	23.6	2004–2006	2.3	39.0	−97.7	+65.2	−67.8
		Non-native All ages		8.9	6.1		1.3	8.7	−85.4	+42.6	–33.5
Kellner [76], Canada	PCV7, 2002/2 + 1	<2 years	1998–2001	66.4	11.3	2003–2007	9.0	8.0	−86.4	−29.2	−77
AUSTRALIA											
Lehmann [77], Australia	PCV7 + PPSV23, 2001/3 + 1 (Aboriginal children) PCV7/2005/3 + 0 (non-Aboriginal children)	Non-Aboriginal <2 years Non-Aboriginal All ages	1997–2001	61.2 5.3	9.1 1.7	2005–2007	6.6 2.2	13.9 2.3	−89.2 −58.5	+52.7 +35.2	−67.2 −36.0
EUROPE											
Verstrheim [78], Norway	PCV7, 2006/2 + 1	<1 year	2004–2005	40.5	15.8	2007	3.4	17.0	−91.6	+7.6	−51.8
		1 year		53.7	9.7		24.3	17.3	−54.7	+78.4	−45.3
Hanquet [21], Belgium	PCV7, 2004 (partially reimbursed); 2007 (free for <2 years)/3 + 1	<2 years	2002–2003	92.9	31.6	2008	4.0	75.8	−95.7	+139.9	−36.5
Muñoz-Almagro [24], Spain	PCV7, 2001 (private market)/3 + 1	<2 years	1997–2001	26.8	5.6	2002–2006	16.1	35.2	−39.9	+528.5	+58.3
		2–4 years		6.8	4.5		9.2	17.3	+35.3	+284.4	+135

decline of IPD cases caused by the included serotypes, cases associated with non-PCV7 serotypes increased post-PCV7 introduction (serotype replacement) [82–84]. In the United States, the emergence of serotype 19A, as an increasingly important cause of invasive infection was observed in all age groups after the introduction of PCV7 [19].

Two years after the universal introduction of PCV7, a study from Alaska indicated an increase in the IPD rate caused by non-vaccine serotypes among Alaskan Native children less than 2 years of age [20]. Results documented a 140% increase of IPD caused by non-PCV7 serotypes among children aged less than 2 years during 2004–2006 compared with the pre-vaccine period (1995–2000). However, during this same period, there was a 96% decrease in the rate of IPD caused by serotypes included in the vaccine. Several other studies have confirmed a rise in the rate of IPD caused by non-vaccine serotypes since the introduction of PCV7 [33].

Serotype replacement has also been documented among adults. Studies conducted in countries using PCV7 observed a marked decrease in serotypes included in PCV7 causing IPD in older adults [75,79,80,85]. The decrease of PCV7 serotypes has been associated with an increase in the frequency of non-PCV7 serotypes included in PCV13 (1, 3, 5, 7F, 6A, and 19A) [71,72,81,82].

It is expected that the limitations of PCV7 in preventing these non-vaccine emergent serotypes could be resolved in part by the implementation of PCV13. A published review article on serotype distribution worldwide before the introduction of new conjugate vaccines found that serotypes included in PCV13 caused more than 70% of all IPD episodes worldwide, with rates ranging from 74% in Asia to 88% in Europe [86]. This potential range of coverage is markedly higher than that reported for PCV7 in Asia and Europe (30% and 59%, respectively) before its introduction in 2000 [87] (Figure 3.2).

Recent data confirm that PCV13 shows high effectiveness in preventing. In the United Kingdom, Andrews et al. [88] reported PCV13 vaccine effectiveness of 75% after two doses before age 12 months or one dose after 12 months (95% CI 58–84). Vaccine effectiveness was 90% (34–98) for the PCV7 serotypes and 73% (55–84) for the six additional serotypes included in PCV13. A study performed in Alaskan Native children aged less than 5 years indicated early declines in the incidence of IPD since vaccine introduction [89]. These data were consistent with the decrease of pneumococcal hospital admissions in children younger than 5 years, as well as in some adult age groups observed after the introduction of PCV13 in the United States [90]. Likewise, in Madrid and England two studies have shown a reduction in pediatric incidence of IPD after changing the childhood vaccination schedule from PCV7 to PCV13 [91,92]. Finally, a Danish study reported a significant decline in IPD incidence, especially in children younger than 2 years, shortly after the shift from PCV7 to PCV13 in the national immunization program. This decline was accompanied by a global decline in pneumococcal-related mortality among non-vaccinated persons [93].

Impact on Clinical Manifestations

The impact of PCV7 varies according to clinical manifestations. While a decrease in the proportion of pneumococcal bacteremia [84,91,94–96] was consistent in the majority of data, results for meningitis [97–100] and especially for pneumonia are more heterogeneous [84,100–103]. However, the positive results of PCV13 are more consistent for all clinical manifestations. See Table 3.3.

EFFECT OF PCV IN BACTEREMIA AND MENINGITIS

In the United States, the incidence rate of bacteremia decreased in all age groups, whereas the rate of pneumococcal meningitis

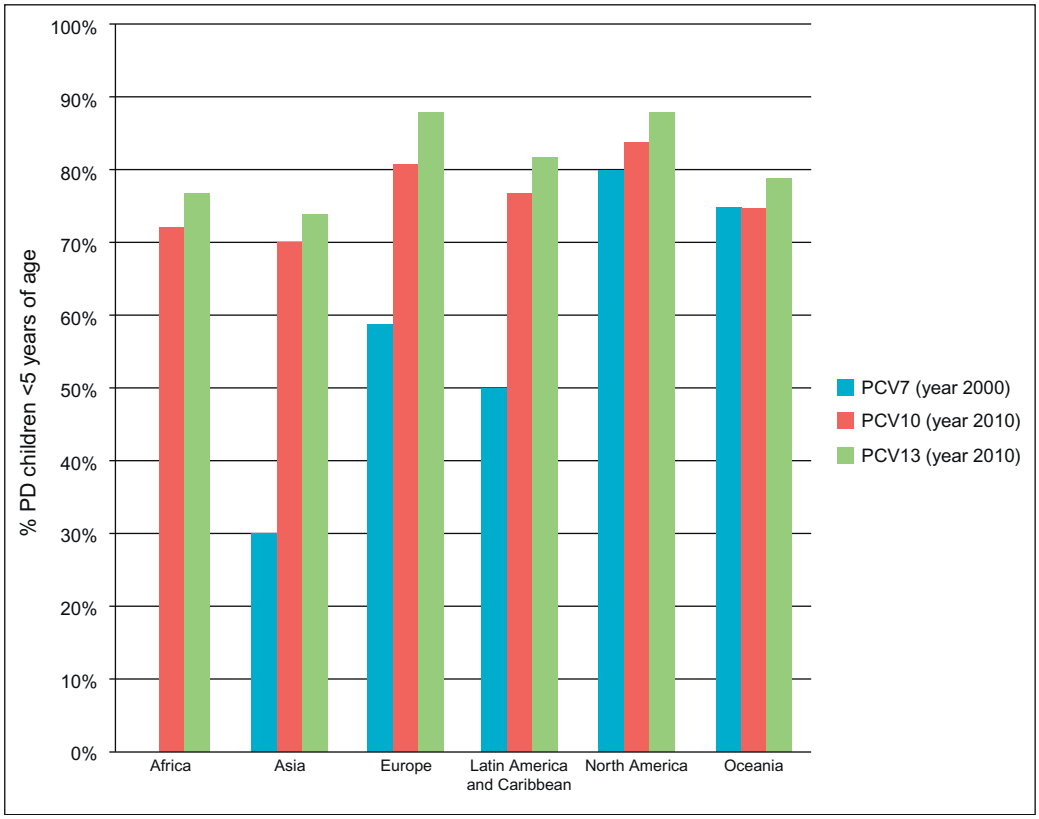

FIGURE 3.2 **Percentage of IPD in children under 5 years of age due to serotypes in existing PCV formulations.** PCV7 data correspond to the pre-vaccination period (before 2000); extracted from Hausdorff et al. [87]. PCV10 and PCV13 data correspond to the post-PCV7 period (2010); extracted from Johnson et al. [86]. PCV7 serotypes include 4, 6B, 9V, 14, 18C, 19F, and 23F. PCV10 adds serotypes 1, 5, and 7F. PCV13 adds serotypes 3, 6A, and 19A.

caused by non-PCV7 types increased for all age groups after PCV7 introduction [19]. According to reported data, the overall rates of bacteremia and meningitis declined significantly among all age groups, with the exception of meningitis among adults, for which reductions in disease caused by PCV7 types were offset by increases in disease caused by non-PCV7 types. These findings are in line with those documenting reductions in meningitis previously reported by Hsu et al. [82].

Similar results were described in England and Wales, where the incidence of pneumococcal meningitis fell by nearly half in children younger than 5 years [80]. In contrast, the vaccine had no overall effect of decreasing cases of pneumococcal meningitis in children under 5 in France, though it did show a benefit in those under 2 [98]. The effect on bacteremia in that country was also modest, affecting the distribution of bacteremia but not its frequency [96]. In Spain since the introduction of PCV7, rates of bacteremia caused by vaccines included in the vaccine have decreased significantly [94], at rates similar to data previously reported in the United States [95]. With respect to PCV13,

TABLE 3.3 Effect of Conjugate Vaccines on Carriage and IPD

Effect of conjugated vaccines		Vaccine	Country	Reference
Carriage	• Reduction of VS and increase of NVS after vaccination	PCV7	USA	[39]
		PCV7	USA	[41]
	• Reduction of VS, increase of NVS and global reduction after vaccination	PCV7	The Netherlands	[40]
		PCV13	Canada	[104]
INVASIVE PNEUMOCOCCAL DISEASE				
Pneumonia	• Increase in pneumonia rates after vaccination	PCV7	Canada	[105]
		PCV7	Spain	[100]
	• Increase in empyema after vaccination	PCV7	USA	[102]
		PCV7	Spain	[103]
		PCV7	UK	[106]
	• Decrease in empyema after vaccination	PCV13	UK	[106]
	• Decrease in pneumonia after vaccination	PCV13	Nicaragua	[107]
Meningitis	• No significance differences after vaccination	PCV7	Spain	[100]
	• Reduction in meningitis rates in children ≤2 years and increase in children >2 years	PCV7	France	[98]
	• Reduction in Meningitis rates	PCV7	USA	[108]
		PCV7	USA	[97]
		PCV13	Spain	[91]
		PCV13	France	[99]
Bacteremia	• Reduction in the incidence of bacteremia after vaccination	PCV7	Canada	[84]
		PCV7	Spain	[94]
		PCV7	USA	[95]
		PV13	Spain	[91]
	• Not significance differences	PCV7	France	[96]
		PCV7	Spain	[100]

NVS, non-vaccine serotypes; VS, vaccine serotypes; PCV-7, 7-valent PCV; PCV-13, 13-valent PCV.

Levy et al. [99] reported a significant decrease of 27.4% in episodes of pneumococcal meningitis in children after PCV13 implementation in France, from 2009 to 2012. In Madrid, Picazo et al. [91] reported a reduction of bacteremic pneumonia and meningitis after PCV13, due mainly to a decrease in episodes caused by serotypes 1 and 19A.

EFFECT OF PCV IN PNEUMONIA

Since PCV7 introduction, an increase in pneumonia has been described by several

authors [102,103,106]. This observation may relate to changes in the circulating serotypes and their ability to cause different clinical syndromes. Furthermore, the significant increase of empyema due to epidemic serotypes not included in PCV7 has been of special concern in some geographical areas [100,102]. These epidemic serotypes, such as serotypes 1, 5, and others (e.g., serotype 19A) also related to an increase of empyema, are included in PCV13. Consequently, the new vaccine has shown a positive impact in preventing these clinical manifestations [106,107].

IMPACT ON ANTIMICROBIAL RESISTANCE

Following the initial detection of penicillin-nonsusceptible *S. pneumoniae* (PNSP) in a few geographic regions (e.g., South Africa, Australia, Spain) in the 1970s, resistance to penicillin spread rapidly worldwide [109]. Before the introduction of PCV7, in some regions such as Asia, France, and Spain, the rate of PNSP was greater than 50% [109]. These PNSP strains corresponded predominantly to PCV7 serotypes.

After the introduction of PCV7, studies performed in various countries reported a decrease in the prevalence of PNSP, together with a rise of penicillin resistance among non-vaccine serogroups [110–112]. Data from ABC areas in the United States from 1998 to 2008 showed that during 2007–2008, serotypes in PCV13 but not PCV7 caused 78–97% of penicillin-nonsusceptible IPD, depending on age [111]. Authors highlighted that the main serotype related to antimicrobial resistance was serotype 19A. The emergence of multiresistant serotype 19A was widely reported in the years before the introduction of PCV13 [58,113]. It is noteworthy that this emergence was also reported in countries such as South Korea [114] and Israel [115], without widespread use of PCV7. The introduction of PCV13 appears to be an important tool to combat the worldwide problem of antimicrobial resistance.

PNEUMOCOCCAL CONJUGATE VACCINATION FOR OLDER ADULTS

The prevalence of pneumococcal carriage decreases as children grow, with a prevalence of about 10% in adults [116], due to immune system maturation and progressive natural pneumococcal exposure. Nevertheless, different risk factors such as chronic diseases, smoking, or alcohol consumption raise the possibility of developing pneumococcal disease [117]. In addition, the presence of young children in the household increases the probability of adult colonization [118]. This fact would explain the link between routine childhood vaccination and the decline in the incidence of pneumococcal diseases in non-vaccinated adults [119].

In the United States, before introduction of PCV13 for children, PPSV23 was recommended for all adults 65 years or older and adults under 65 with risk factors [120]. However, its effectiveness has not been demonstrated against IPD, especially in high-risk populations [121]. In December 2011, PCV13 was approved in the United States for use in adults aged 50 or older for the prevention of pneumonia and invasive disease caused by the 13 *S. pneumoniae* serotypes contained in the vaccine [122]. Immunogenicity studies that compared antibody responses to PCV13 with antibody responses to PPSV23 showed the superior response to a single dose of PCV13 and how this vaccine primed the immune system for a booster response to subsequent vaccination with either vaccine [123].

These results are consistent with those found in a recent clinical trial involving adults aged 60–64. The authors reported that an initial PCV13 dose increased the anti-pneumococcal response to

a subsequent administration of PPSV23 for many of the serotypes common to both vaccines [124]. Moreover, Chen et al. [125] studied the sequential PCV13–PPSV23 vaccination for certain at-risk adults with immunocompromising conditions, concluding that this strategy seems to be a cost-effective vaccine policy.

In June 2014, the results of a randomized placebo-controlled trial were presented to the Advisory Committee on Immunization Practices (ACIP), showing the efficacy of PCV13 against CAP among adults aged 65 and older [31]. As a result, the recommended PCV13 vaccination of adults was elevated as a Category A recommendation. ACIP recommends that all adults 65 years of age or older, as well as adults with immunocompromising conditions, functional or anatomic asplenia, CSF leaks, or cochlear implants with no previous vaccination, receive a dose of PCV13 followed by a dose of PPSV23 6 to 12 months later. In the case of adults who have not received PCV13 and who have previously received one or more doses of PPSV23, they should receive a dose of PCV13 at least 1 year after receipt of the most recent PPSV23 dose. For those who require additional doses of PPSV23, the first such dose should be given no sooner than 8 weeks after PCV13 and at least 5 years after the most recent dose of PPSV23 [31].

However, despite recent data on the efficacy of PCV13 in adults, there is no real consensus supporting use of PCV in adults in geographical areas in which children receive PCV. A recent study performed in the United Kingdom assessed the cost-effectiveness of vaccinating 65 and older and at-risk adults with either PPSV23 or PCV13. The conclusion of the study was that PPSV23 was cost-effective when compared to both PCV13 and no vaccination [126].

On the other hand, Smith et al., in the United States, analyzed the cost-effectiveness of adult vaccination strategies using PCV13 versus PPSV23. Both were better strategies in comparison with no vaccination, while routine use of PCV13 was the most economically efficient. However, when childhood vaccination's indirect effects were modeled, PPSV23 were favored [127].

CONCLUSION

Despite serotype replacement, PCVs have drastically reduced the burden of pneumococcal disease in children and adults, and they continue to make important contributions to public health.

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CHAPTER

4

Vaccine Potential of Pneumococcal Proteins

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INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) is a major human pathogen that causes a broad spectrum of diseases, ranging from less serious infections such as sinusitis, conjunctivitis, and otitis media, to potentially fatal diseases such as pneumonia, bacteremia, and meningitis. Pneumococcus accounts for more deaths worldwide than any other single pathogen. In developing countries, an estimated 1.1 million children under 5 years of age die each year from pneumonia (~20% of all deaths in this age group), and *S. pneumoniae* is the single most common cause. Even in developed countries, where antimicrobial therapy is readily accessible, morbidity and mortality are substantial, outbreaks of infection with "hypervirulent" strains are emerging, and antibiotic resistance is increasing. In these countries, deaths from pneumococcal disease occur primarily among the elderly, with case-fatality rates of 30–40% for bacteremia. Furthermore, less serious but highly prevalent infections such as otitis media and sinusitis have a massive impact on health care costs in developed countries. The continuing problem of pneumococcal disease is partly attributable to the rate at which this organism is acquiring resistance to multiple antimicrobials and the rapid global spread of highly resistant clones [1]. The problem is exacerbated by major shortcomings associated with the current capsular-based vaccines, including cost, strictly serotype-specific protection, and incomplete serotype coverage. Widespread use of conjugate vaccines has massively reduced invasive disease caused by included serotypes, but this has been offset to varying extents by replacement carriage and disease caused by non-vaccine serotypes [2]. Serotype replacement can occur either by unmasking of non-vaccine type strains previously present in the population but at lower frequencies, or, more worryingly, by genetic recombination resulting in capsule serotype switching, such that highly invasive strains which previously expressed capsular serotypes covered by the vaccine now express capsules for which the vaccine provides no protection whatsoever. A further issue is that the existing conjugate

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vaccines have unproven efficacy against pneumococcal meningitis, and do not appear to provide significant protection against otitis media [3].

The suboptimal overall efficacy of existing pneumococcal vaccination strategies is in part attributable to our incomplete understanding of the biology of pneumococcal disease. Nevertheless, vaccination still represents the best prospect for managing pneumococcal disease in the twenty-first century, with due consideration to the lessons learnt from the past. In this context, concerted global efforts are focused on accelerating the development of alternative pneumococcal vaccine strategies that address the shortcomings of existing approaches, without compromising efficacy. One of these approaches involves the development of vaccines based on pneumococcal proteins that contribute to pathogenesis and are *common* to all serotypes [4–7].

Rational protein vaccine design requires a systematic assessment of the precise role and relative contribution of candidate proteins to disease pathogenesis in order to understand how protection might be mediated. The contribution of a range of pneumococcal virulence proteins to pathogenesis of disease has been investigated in detail over the last 30 years. The virulence proteins which have received the greatest attention to date are the thiol-activated toxin pneumolysin (Ply) [8], two choline-binding surface proteins called pneumococcal surface protein A (PspA) [9,10] and choline-binding protein A (CbpA) (also referred to as PspC or SpsA) [11,12], a metal-binding lipoprotein called pneumococcal surface antigen A (PsaA) [13,14], and iron uptake and acquisition proteins PiuA and PiaA [15,16]. These proteins have diverse biological activities, and analysis of pneumococci with single and multiple mutations in the respective genes indicates that they may act at different stages of the pathogenic process [9,13,15,17–20]. Consequently, combinations of these proteins have been proposed for inclusion in a broadly protective pneumococcal protein vaccine. Many additional candidate proteins have also been identified and appraised for inclusion in multicomponent pneumococcal protein vaccine formulations. These include a family of newly characterized surface proteins, the poly-histidine triad (Pht) proteins, comprised of four members: PhtA, PhtB, PhtD, and PhtE [21–25]. Other pneumococcal proteins that have been shown from animal models to have vaccine potential include Pili (RrgA, RrgB, RrgC), neuraminidase A (NanA), autolysin (LytA), ClpP, GlpO, PotD, StkP, and PcsB. Available data on each of these candidates is described in detail below.

NEXT-GENERATION PNEUMOCOCCAL VACCINE CANDIDATES AND STRATEGIES

As indicated above, current global efforts are directed toward the development of highly efficacious, non-serotype-dependent alternative vaccines against all forms of pneumococcal disease. One such strategy involves the use of protein antigens common to all pneumococcal serotypes. Such a vaccine would confer the following additional advantages:

1. Proteins, being T-cell–dependent antigens, should be highly immunogenic and are likely to elicit immunological memory in human infants.
2. Proteins can be engineered for high-level expression at relatively low cost, and formulation is likely to be simpler, and thereby vaccines more affordable.
3. Given the reported failure of conjugate vaccines to protect against acute otitis media [3], some of the proteins could provide much better protection against otitis media than existing conjugate vaccine formulations.
4. A highly efficacious protein antigen could also be used as a carrier in a

polysaccharide–protein conjugate vaccine formulation, thereby potentially requiring the conjugation of fewer polysaccharides while resulting in an overall increase in magnitude of protection.

Pneumolysin

One of the best-characterized pneumococcal protein vaccine candidates is Ply, a 53-kDa toxin produced by all clinical isolates of *S. pneumoniae*. Ply belongs to a family of thiol-activated cytolysins, referred to as cholesterol-dependent cytolysins, produced by several Gram positive bacteria [26]. Ply was the first pneumococcal protein to be proposed as a vaccine antigen, as demonstrated by the finding that immunization of mice with highly purified toxin provided protection against intranasal challenge with virulent pneumococci [8]. Ply antibody-mediated protection is presumed to be due largely to neutralization of free toxin. While a proportion of Ply appears to be associated with the cell wall fraction [27,28], there is no evidence of a role for anti-Ply in opsonophagocytic clearance. Although native pneumolysin is a protective immunogen in mice, it is not suitable as a human vaccine antigen, because of its toxicity. To overcome this, mutations have been introduced into the pneumolysin gene in regions essential for its cytotoxic and/or complement activation properties, resulting in expression of nontoxic but immunogenic and protective pneumolysoids, which are easily purified from recombinant *Escherichia coli* expression systems [29–33]. Ply is a highly conserved protein, and extensive analysis of genes from a wide range of *S. pneumoniae* serotypes has detected negligible variation in deduced amino acid sequences, auguring well for broad coverage. Indeed, immunization of mice with a pneumolysoid carrying the $Trp_{433} \rightarrow Phe$ mutation (designated PdB) provided a significant degree of protection against all nine serotypes of *S. pneumoniae* that were tested [29]. Another pneumolysoid, PdT, with three point mutations ($Cys_{428} \rightarrow Gly$, $Trp_{433} \rightarrow Phe$, and $Asp_{385} \rightarrow Asn$) that totally abolish cytolytic activity and complement activation, provided a significant degree of protection against two highly virulent serotype 1 isolates [34]. PdB and related pneumolysoids have also been shown to be effective carriers of capsular polysaccharide (CPS) in experimental conjugate vaccines [33,35,36]. These vaccines elicit anti-CPS and anti-Ply titers that are highly protective against challenge with *S. pneumoniae* belonging to homologous as well as heterologous serotypes, thereby avoiding one of the principal shortcomings of current conjugate vaccines, namely selection for non-vaccine capsular serotypes.

Humans mount an effective antibody response to pneumolysin as a result of natural exposure to *S. pneumoniae*, and purified human anti-pneumolysin IgG also passively protects mice from challenge with virulent pneumococci [37], suggesting that pneumolysoids will be immunogenic in humans. Indeed, recent studies have demonstrated the safety and immunogenicity of various toxoid derivatives of Ply in healthy toddlers and adults [38–40]. However, it was evident from preclinical studies that pneumolysoid may not provide a sufficient degree of protection to be an effective stand-alone human vaccine antigen, especially against the most virulent pneumococcal strains. As mentioned above, antibodies to pneumolysin are presumed to impart protection by neutralization of the biological properties of the toxin, thereby impeding the kinetics of infection, rather than by stimulating opsonophagocytic clearance of the invading bacteria. Thus, protein-based vaccines combining pneumolysoid with pneumococcal surface proteins capable of eliciting opsonic antibodies, such as PspA and PspC (discussed below), would be expected to be more effective. Indeed, immunization of mice with combinations of these proteins was shown to provide significantly increased protection compared to immunization with each

protein alone in mouse models of pneumococcal carriage, otitis media, pneumonia, sepsis, and meningitis [5–7,32,41].

Choline-Binding Proteins

The pneumococcal cell surface is decorated with a family of virulence-associated surface proteins referred to as choline-binding proteins (CBPs), which are tethered noncovalently to phosphocholine (ChoP) present in the teichoic and lipoteichoic acid residues of the cell wall [9–12,42,43]. With a few exceptions, CBPs are characterized by a highly charged, variable N-terminus, followed by a proline-rich region and a C-terminal region consisting of repeats of up to 11 highly conserved 20 amino acid choline-binding residues, which bind noncovalently to cell wall ChoP [44].

Pneumococcal Surface Protein A

PspA is the first characterized [9] and most studied CBP, and is expressed by all clinical *S. pneumoniae* strains analyzed from seven Western countries [45]. The molecular size of PspA varies from 67 to 99 kDa [46]. The N-terminal alpha-helical half of the molecule has an anti-parallel coiled coil structure and is highly variable between *S. pneumoniae* strains [46,47]. The proline-rich region of the PspA molecule contains about 80 amino acid residues, consisting of 40% proline residues, mostly interspersed by a 33 amino acid, highly conserved, non-proline-containing block (Figure 4.1). The N-terminal alpha-helical half, the proline-rich region, and the non-proline-containing block are all exposed on the pneumococcal surface [48] and elicit cross-reactive protection against fatal pneumococcal sepsis in mice [49]. Ninety-eight percent of PspAs exist in two cross-protective PspA families [45,46]; thus, it has been suggested that a PspA vaccine may require no more than three PspA molecules to protect against most *S. pneumoniae* [4,50]. Immunization of humans and mice with recombinant PspA generated broadly cross-reactive antibodies that protected mice from fatal sepsis with pneumococci bearing heterologous PspA [51,52]. Importantly, children produce antibodies to the conserved proline-rich region of PspA upon exposure to pneumococci [53], suggesting that this region alone has the potential to provide broadly cross-reactive antibodies that can protect against heterologous pneumococcal strains. Relative resistance to pneumococcal infection in children coincides with the development of anti-PspA antibodies, suggesting these antibodies are protective [54–56].

Protection against pneumococcal infection is largely mediated by a mechanism involving complement-dependent opsonophagocytic clearance. PspA interferes with this process and slows the clearance of pneumococci from the blood of infected mice [9,57] by blocking deposition of C1q (triggered through the classical pathway) to the pneumococcal surface, and antibody to PspA enhances complement deposition [58,59]. Another mechanism used by PspA to protect pneumococci from the host immune system involves binding specifically and strongly to lactoferrin, an iron-sequestering protein found mostly at the host mucosa [60], via region 2 of its alpha-helical domain [47,61]. Recombinant PspA protects pneumococci against the bactericidal effects of apolactoferrin (the iron-depleted form of lactoferrin) [62], and apolactoferrin-mediated killing of pneumococci is increased in the presence of antibodies to PspA [62].

PspA seems to hold great promise, in combination with other proteins, for inclusion in next-generation pneumococcal protein vaccines against nasal colonization, otitis media, and invasive pneumococcal disease. Indeed, available comparative immunogenicity and protection studies from humans, mice, and rabbits support this notion [5–7,51,53,63–65]. Furthermore, as demonstrated for pneumolysoids, PspA can also be an effective carrier of CPS when tested in mouse models [66,67].

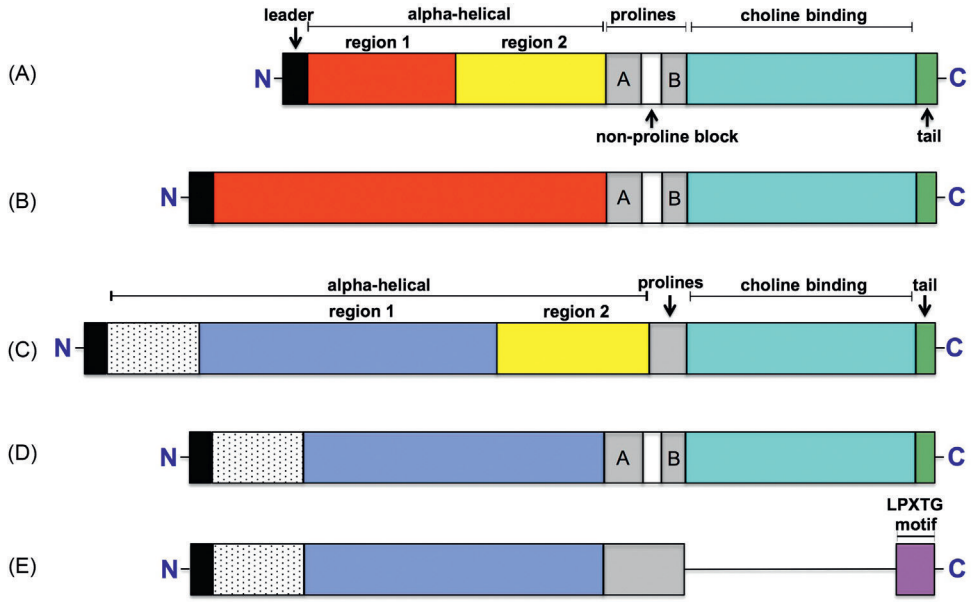

FIGURE 4.1 **Schematic representation of the modular organization of various PspA and PspC molecules of *S. pneumoniae*.** (A) Family 1 PspA; (B) Family 2 PspA; (C) Clade A PspC; (D) Clade B PspC; and (E) Hic. Similar colors depict homologous regions. Boxes with dotted patterns indicate the hyper-variable N-terminal regions of PspC proteins. Hic is distinguished from PspC by a characteristic LPXTG C-terminal cell wall anchoring motif. The protection-eliciting epitopes of PspA and PspC are located principally in the N-terminal alpha-helical and proline-rich domains.

Pneumococcal Surface Protein C

Pneumococcal surface protein C (PspC) is a polymorphic CBP contemporaneously described by multiple research groups, and annotated differently, perhaps based on the different functional properties associated with its multi-domain allelic structure [68,69] (Figure 4.1). Accordingly, it is also referred to as CbpA based on its strong binding to choline immobilized on a solid matrix [12], and as *S. pneumoniae* secretory immunoglobulin A binding protein (SpsA), based on binding to the secretory component of human IgA [11,70]. A distinct protein with significant homology to the N-terminal regions of several PspC proteins has been named Hic (factor *H*—binding *i*nhibitor of *c*omplement) as it binds to human factor H, thereby inhibiting the alternative pathway of complement activation [71,72]. The *hic* gene is essentially an allele of *pspC*, as the genes are located at the same chromosomal position and a given pneumococcal strain has either one or the other gene, but not both [73]. However, Hic is anchored to the cell wall by means of an LPXTG motif instead of via noncovalent binding to cell wall ChoP mediated by the choline-binding domain of PspC [71,73] (Figure 4.1).

PspC is present in approximately 75% of all *S. pneumoniae* strains, and its N-terminal alpha-helical domain is highly variable in both size and sequence among different strains [11,12,68,71]. Nevertheless, the structure of the N-terminal alpha-helical domains of some PspC molecules are very similar to portions of the alpha-helical domain of some PspA molecules, the proline-rich domains of PspC and PspA are highly homologous, and the choline-binding domains of the PspC of most pneumococci are indistinguishable from those of PspA [11,68,74].

PspC mediates adherence to cytokine-activated lung cells, and plays a major role in colonization of the nasopharynx in infant rat [12] and adult mouse colonization and pneumonia [75] models. PspC interacts with the human polymeric immunoglobulin receptor, thereby facilitating invasion of the mucosa [76]. Furthermore, PspC is involved in invasion of the cerebrospinal fluid [75] as well as adherence to the vascular endothelium of the blood–brain barrier [77] by binding to the laminin receptor via its cell-binding domains R1 and R2 [78]. Moreover, much higher levels of complement were deposited on the surface (and hence enhanced clearance from the blood) of pneumococci deficient in both PspA and PspC than in those lacking one or the other molecule [58,79]. This is understandable, given the fact that a large proportion of the complement deposited on invading pneumococci after triggering of the classical pathway (due to PspA blocking C1q deposition) is dependent on the amplification loop of the alternative pathway (inhibited by PspC binding to factor H).

Immunization with PspC was highly protective in mice following systemic challenge, and the protection imparted was mediated by antibodies that were cross-reactive with PspA domains [41,68]. However, subcutaneous immunization of mice with the factor H–binding fragment of PspC was only protective after intravenous challenge with a homologous strain [80]. Intranasal immunization with PspC was also protective against carriage [17], but not against pneumonia [81]. Importantly, when mice were immunized with a combination of PdB and PspA, PdB and PspC, or PspA and PspC, additive (and sometimes synergistic), protection was imparted against systemic challenge, depending on the pneumococcal challenge strain or the mouse strain [5–7,41,63]. Moreover, vaccination with a PspC (CbpA) peptide–pneumolysoid fusion protein (YPT-L460D-NEEK), comprised of protection-eliciting components of CbpA against colonization (YPT) and meningitis (NEEK) fused to pneumolysoid (L460D), was protective in mouse models of pneumococcal carriage, otitis media, pneumonia, bacteremia, meningitis, and meningococcal sepsis [32].

Autolysin (LytA)

The major pneumococcal autolysin, LytA, an *N*-acetyl-muramoyl-L-alanyl amidase, was one of the first CBPs to be extensively characterized [82,83].

Immunization of mice with purified LytA elicits a significant protective response against fatal pneumococcal challenge [84]. Interestingly, in that study, while the degrees of protection in mice immunized with either LytA or Ply toxoid (PdA) were similar, additive protection was not observed when the two proteins were co-administered. Moreover, immunization with LytA did not protect against challenge with a Ply-negative pneumococcus, suggesting that the protective role of LytA antibodies was primarily via preventing the release of Ply [84]. Nevertheless, in a chinchilla otitis media model where the role of Ply is more limited, LytA had a significant role in preventing middle ear inflammation [85]. Recently, intranasal immunization of mice with LytA was shown to elicit cross-protective antibodies against colonization and systemic disease by multiple serotypes [86]. However, allelic variation in the sequence of LytA has also been reported [87], which may limit the utility of this protein as a vaccine candidate.

Pneumococcal Choline-Binding Protein

Pneumococcal choline-binding protein (PcpA) is a 79 kDa CBP with a typical C-terminal choline-binding domain consisting of 11 identical motifs of 20 amino acids plus a tail of 19 amino acids [44]. The N-terminal region of PcpA is marked by the presence of two tandem arrays of five characteristic amphipatic leucine-rich repeats of 22–26 amino acids in length, a feature that suggests a role in

protein–protein and protein–lipid interactions. A role for PcpA in the adherence of pneumococci to human nasopharyngeal and lung epithelial cells has been demonstrated, and this can be inhibited by human anti-PcpA antibodies [88].

Immunization with PcpA can elicit statistically significant protection in murine models of pneumonia and sepsis [89]. A role for anti-PcpA antibodies in protection against otitis media is suggested by the finding that otitis-prone children either failed to show anti-PcpA IgG antibody titer rises or the rises were significantly less than the non-otitis-prone children [90]. Moreover, antibody levels to PcpA increased over time in children after natural exposure to pneumococcal colonization and acute otitis media [91]. Formulations of monovalent PcpA and PcpA plus histidine triad protein D (PhtD) bivalent protein vaccines showed promising safety and immunogenicity profiles in adults [92]. A trivalent PcpA, PhtD, and detoxified pneumolysin (PlyD1) also protected against lethal pneumonia in an infant murine model, suggesting that PcpA is a promising additional pneumococcal protein vaccine candidate [93].

Lipoproteins

The pneumococcal genome includes over 30 putative lipoproteins, with a prolipoprotein signal peptidase II (LxxC) recognition motif [94], which directs covalent attachment of a diacyl glycerol moiety to the N-terminal cysteine residue of the mature protein, anchoring it to the outer leaflet of the plasma membrane. Thus, they are located beneath the cell wall and the capsule in *S. pneumoniae*. These lipoproteins have diverse functions, the most common being substrate-binding components of ATP binding cassette (ABC) transport systems, and many are important for growth and survival of the pneumococcus *in vitro* and *in vivo*. Indeed, the most abundant class of putative virulence genes detected in *S. pneumoniae* using signature-tagged mutagenesis appeared to encode transporters, the majority of which were ABC permeases [95].

Pneumococcal Surface Antigen A

PsaA is a 37 kDa lipoprotein [13,96] belonging to the cluster A-I family of bacterial transporters of the essential metal ions Mn^{2+}, Zn^{2+}, and Fe^{2+} [14,97]. It is the solute-binding component of an ABC cation permease encoded by the *psaBCA* locus of *S. pneumoniae* [14]. All of the physiological data collected to date indicate that PsaA is involved in the transport of Mn^{2}, and growth of a *psaA* mutant showed an absolute requirement for additional Mn^{2+} [14,98,99]. PsaA plays a key role in the resistance of pneumococci to oxidative stress, possibly because Mn^{2+} is required for protection from reactive oxygen intermediates and a Mn^{2+}-dependent superoxide dismutase, SodA, protects the cell from superoxide [100]. Mutants of *psaA* exhibit greatly reduced virulence in systemic as well as in respiratory tract and otitis media models of infection [13,98,99], probably due to the hypersensitivity to killing by H_2O_2 and superoxide [99,101,102]. Taken together, the data show that PsaA is an important virulence factor with a significant role in *S. pneumoniae* pathogenesis.

Comparative sequence analyses indicate that the gene encoding PsaA is highly conserved among diverse capsular serotypes of *S. pneumoniae* [103], making it an attractive protein vaccine candidate. The dimensions of PsaA (~7 nm at its longest axis) [104] are such that if it is indeed anchored to the outer face of the cell membrane via its N-terminal lipid moiety, it is unlikely to be exposed on the cell surface to any significant extent, which in turn implies that PsaA is unlikely to elicit opsonic antibodies. However, this does not necessarily preclude its utility as a vaccine target, since exogenous antibody may diffuse through the capsule and cell wall layers and

inhibit the biological function of the lipoprotein. Indeed, PsaA was a protective immunogen against colonization and systemic disease in some mouse models [4,63,105,106]. However, in other studies, immunization with PsaA elicited only marginal protection and was less efficacious than pneumolysoid in an intraperitoneal challenge model [6,107]. Flow cytometry using exogenous specific antibodies showed PspA was readily detectable on the surface of twelve *S. pneumoniae* strains, whereas PsaA was not [108]. This directly correlated with the protective efficacy of either active or passive immunization with the respective protein or antibody; significant protection against systemic challenge was achieved using PspA or anti-PspA, but not using PsaA or anti-PsaA. Accessibility of PsaA to exogenous antibody may well be influenced by the thickness of the capsule, which varies from strain to strain and is increased during invasive infection [109]. In contrast, pneumococci colonizing the nasopharynx may down-regulate capsule expression, thereby facilitating interaction between surface adhesins and the host mucosa. Consistent with this hypothesis, several studies have shown that intranasal immunization of mice with PsaA and strong mucosal adjuvants such as CTB or cdiGMP significantly reduces the level of nasopharyngeal carriage of *S. pneumoniae* [63,110,111]. A lesser, but still significant, reduction in susceptibility to carriage was also achieved by subcutaneous immunization of mice with synthetic lipidated multi-antigenic PsaA peptides [112]. Thus, at least in the nasopharynx, PsaA appears to be accessible to exogenous antibody.

PiuA and PiaA

PiuA (*p*neumococcal *i*ron *u*ptake A) and PiaA (*p*neumococcal *i*ron *a*cquisition A) are lipoprotein components of two separate iron uptake ABC transporter operons, *piu* and *pia*, of *S. pneumoniae*. Originally termed Pit1A (*p*neumococcal *i*ron *t*ransport 1A) and Pit2A, respectively, these proteins were first characterized by Brown et al. [15] following identification during a signature-tagged mutagenesis screen [95]. Mutagenesis studies demonstrate that PiuA and PiaA are required for bacterial growth and full virulence in both systemic and pulmonary models of infection [15]. PiuA and PiaA are highly conserved across serotypes, and are present in all strains tested so far, making these proteins potential candidates for inclusion in a protein-based pneumococcal vaccine. Indeed, active and passive immunization with either PiuA or PiaA (and a combination of the two) was protective against systemic infection with highly virulent *S. pneumoniae* in mice [16,113]. The passive protection data suggest that protection afforded by these proteins was, at least in part, antibody-mediated; this was confirmed to occur via complement-dependent and -independent bacterial opsonophagocytosis rather than inhibition of iron transport [114]. The presence of elevated levels of antibody to PiuA and PiaA in convalescent-phase sera of patients with pneumococcal septicemia, and the demonstration that the immune response elicited is serotype-independent [115], suggests that PiuA and PiaA are promising vaccine candidates.

Other Lipoprotein Vaccine Candidates

PotD of *S. pneumoniae* is the solute-binding lipoprotein of an ABC transporter complex (PotABCD) for polyamines, an important nutrient of pneumococci [116]. It is highly conserved and is essential for virulence in murine models of pneumonia and sepsis [116]. Active immunization with PotD and passive transfer of anti-PotD antisera protects mice against colonization, pneumonia, and sepsis [117,118]. AliA is an additional lipoprotein vaccine candidate that is discussed below.

Pht Proteins

Proteins belonging to the Pht family are characterized by five to six repeated histidine triad

(HxxHxH) motifs in their sequences. These proteins were first identified and described by Adamou et al. [21] utilizing whole genome random sequence scanning for prediction of surface-localized proteins. By using the deduced amino acid sequence of the prototype Pht protein, PhtA, three other family members, designated PhtB, PhtD, and PhtE, were identified. The molecular mass of the proteins is 90.1, 92.1, 93.7, and 114.6 kDa, respectively, and each possesses an LxxC lipoprotein motif at the N-terminus.

Interestingly, the genes encoding Pht proteins are arranged in tandem pairs, with *phtA* and *phtB* co-transcribed in the opposite orientation from *phtD* and *phtE* and separated by several other genes and operons in the pneumococcal genome. This genetic arrangement suggests functional redundancy and may indicate complementary or compensatory roles for these proteins in the biology of the pneumococcus. Indeed, in murine models of sepsis and pneumonia using *pht* mutants, mutagenesis of all four genes was required to completely abolish virulence relative to the wild type [23]. Genotypic and phenotypic analyses show that *pht* genes and their corresponding products are highly conserved across serotypes, with PhtD, PhtE, PhtB, and PhtA present in 100%, 97%, 81%, and 62%, respectively, of all pneumococcal strains tested [22,119]. A role for Pht proteins as Mn^{2+} and Zn^{2+} scavengers and/or in Zn^{2+} homeostasis has been proposed, particularly in ion-restricted environments [24,119,120].

Active immunization of mice with recombinant PhtA, PhtB, and/or PhtE was highly protective against nasopharyngeal colonization [121], pneumonia [22], and sepsis [21,22,121,122] after challenge with capsular types 3, 4, 6A, and 6B strains. Passive immunization studies [22] suggested that antibody-mediated opsonophagocytosis might be the major mechanism of protection after vaccination with PhtB and PhtE. Of the four proteins, PhtD is considered the most promising vaccine candidate due to its superior protective efficacy against nasopharyngeal colonization, its having the highest level of conservation among pneumococcal strains [119,123], and its additive protective effect as a vaccine when administered with CPSs [124]. Moreover, a trivalent PcpA, PhtD, and detoxified Ply formulation was protective against lethal pneumonia in an infant murine model [93]. However, another study showed only modest protection afforded by vaccination with PhtB or PhtE singly or in combination against intraperitoneal challenge with a highly virulent capsular type 2 (D39) strain and a clinical type 6A (blood) isolate compared to vaccination with PspA, PspC, and/or pneumolysin toxoid PdB [7]. Furthermore, the capacity of PhtD to protect against colonization or systemic challenge with a virulent serotype 6A strain was modest [25]. These findings imply that rational decisions regarding the formulation of multi-component pneumococcal protein vaccines will require rigorous comparisons of individual antigens and all possible combinations thereof, using multiple mouse infection models and challenge strains. Interestingly, recent clinical studies have demonstrated the safety and immunogenicity of PhtD in combination with detoxified Ply and PcpA [39,40,92].

Sortase-Dependent Surface Proteins and Other Secreted Proteins

S. pneumoniae produces a distinct set of surface proteins, referred to as sortase-dependent proteins due to a characteristic LPXTG signature motif, usually at their C-termini, which is cleaved before covalent anchoring to the pneumococcal cell wall. The best-characterized of these proteins is neuraminidase A (NanA) [125,126]. These proteins function mostly as hydrolytic enzymes, some of which degrade host glycoconjugates or extracellular matrix [94]. For instance, NanA cleaves terminal sialic acid residues from host glycoproteins, glycolipids, mucins, and oligosaccharides, thereby enabling pneumococcal attachment to host epithelial and endothelial surfaces [127]. NanA

mutants are attenuated for colonization of the nasopharynx, development of otitis media, invasion of the lungs, and crossing of the blood–brain barrier in various animal models [19,75,128–131]. Immunization with native or recombinant NanA is protective in chinchilla nasopharyngeal colonization [132] and otitis media [133] models and against fatal sepsis in juvenile mice [134].

Another well-characterized sortase-dependent pneumococcal surface protein is immunoglobulin A1 (IgA1) protease, a cell-associated and secreted Zn-metalloproteinase that hydrolyzes human IgA1. Unlike most sortase-dependent surface proteins, the IgA1 protease sortase motif is atypically located near the amino- rather than the carboxy-terminus, and it is highly heterogeneous, ranging in size from 135 to 215 kDa [135–137]. IgA1 protease increases bacterial attachment to host cells due to IgA1 protease hydrolysis of type-specific IgA1 [138] and is required for successful colonization and infection by *S. pneumoniae* [139], indicating that it is a potential pneumococcal protein vaccine candidate. Indeed, intranasal immunization of mice with a combination of IgA1 protease and streptococcal lipoprotein A anchored to the surface of Gram positive enhancer matrix particles was protective against fatal pneumococcal pneumonia [140].

Fimbriae, or pili, are another class of sortase-dependent proteins, first demonstrated on the pneumococcal surface in 2006 [141]. Pili consist of the proteins RrgB (the backbone) and two to three clusters of RrgA (the major subunit), and individual RrgC (anchor) molecules on the pilus surface [142,143]. Only about 20–30% of pneumococci express pili [144,145], and their role in pneumococcal virulence is controversial [141,144,145]. When used as an immunogen, RrgA protected mice against lethal challenge with pneumococci that produce pili [145–147].

Recently Characterized Protein Vaccine Candidates

The increasing number of fully sequenced pneumococcal genomes has facilitated the use of novel strategies for genomic-scale discovery and appraisal of potential pneumococcal protein antigens, specific examples of which are discussed below.

PcsB and StkP

A study using a genomic surface display library approach (ANTIGENome technology) [148,149] compared serum antibodies of exposed, but not infected, individuals with those of convalescing patients. In this manner, two lead vaccine candidates, a protein required for cell wall separation of group B streptococcus (PcsB) [150], and serine/threonine protein kinase (StkP) [151], were identified [152]. PcsB is a murein hydrolase with a predicted CHAP domain at its C-terminus, and is involved in hydrolysis of peptidoglycan [153]. StkP positively controls virulence and competence, and is a global regulator of gene expression in *S. pneumoniae* [151,154]. PcsB and StkP are highly conserved among clinical isolates and were cross-protective when used as vaccines against four different serotypes in murine lethal sepsis and pneumonia models. These two proteins also elicited a Th17-immune response in humans [155], and higher antibody titers against PcsB (and PdB) were associated with absence of pneumococcal colonization [156]. Immunization of neonatal mice with a combination of PcsB, StkP, PsaA, and PspA is protective against pneumococcal pneumonia and bacteremia [157], highlighting the potential of a combination of PcsB and StkP with other proteins as immunogens against invasive pneumococcal disease.

AliA and GlpO

Another powerful technique used for novel antigen discovery employs *in vivo* transcriptomic

comparison to identify putative virulence factors whose genes are specifically up-regulated during particular stages of pneumococcal disease. This led to the identification of an oligopeptide ABC transporter (AliA) [158] with a signal peptidase II recognition sequence characteristic of lipoproteins, which contributed significantly to the development of bacteremia in mice [113]. Immunization of mice with recombinant AliA conferred significant protection against pneumococcal sepsis [113], providing the first evidence that AliA is a promising protein vaccine candidate against systemic disease. Using the same strategy, one previously uncharacterized protein, alpha-glycerophosphate oxidase (GlpO), was found to contribute significantly to progression of pneumococci from the blood to the brain *in vivo* [159]. A *glpO* mutant also induced markedly reduced meningeal inflammation and brain pathology compared to wild type, despite similar levels of bacteremia. Immunization of mice with GlpO protected against invasive pneumococcal disease, and provided additive protection when formulated with pneumolysin toxoid PdT. The finding that nonclassical metabolic proteins such as GlpO are potential vaccine candidates is interesting, and is an emerging concept that calls for a detailed analysis of the vaccine potential of such hitherto overlooked proteins [160].

Whole Cell Vaccines

Apart from the subunit vaccine approach to address the shortcomings of capsular-based pneumococcal vaccines, an alternative vaccine strategy that is being considered involves the use of whole, attenuated (or killed) unencapsulated pneumococci. A whole-cell killed unencapsulated pneumococcal vaccine administered intranasally with a mucosal adjuvant was highly efficacious in preventing nasopharyngeal colonization in mice, and also protected rats against invasive disease by encapsulated pneumococci [161]. Likewise, intranasal or intraperitoneal administration of live, attenuated (due to either lack of capsule or auxotrophy) pneumococci (without adjuvant) elicited broadly protective mucosal and systemic antibodies in mice against subsequent pneumococcal challenge [162–164]. Protection against nasopharyngeal colonization was shown to be dependent on IL-17A-secreting, antigen-specific CD4+ (T_H-17) T cells [165–168]. Such vaccines contain large numbers of surface-exposed protein antigens, inducing antibodies or Th17 responses to multiple antigens and thereby potentially improving strain coverage. However, despite this, vaccination with live attenuated *S. pneumoniae* does not necessarily induce cross-protective immunity against heterologous strains [164]; the reasons for this remain unclear.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this chapter we have provided an overview (summarized in Table 4.1) of the diverse array of pneumococcal proteins, or derivatives thereof, that have individually shown promise as components of vaccines capable of eliciting robust and broad-based protection against pneumococci regardless of capsular serotype. We have also summarized evidence supporting development of multi-component protein vaccines, which have significantly enhanced protective efficacy relative to individual antigens. Such protein combination vaccines elicit immune responses that attack the pneumococcus at multiple stages in the pathogenic process. They also provide an opportunity to include antigens that elicit improved protection against diseases such as meningitis and otitis media, against which the existing conjugate vaccines have uncertain efficacy. There is, of course, a limit to the number of protein components that could be included in an economically viable formulation. Thus, there is merit in construction of fusion proteins comprising important

TABLE 4.1 Pneumococcal Virulence Proteins and Other Antigens Demonstrated to Be Protective in Animal Models

	Proven protective efficacy against					
Antigen	**Colonization**	**Otitis media**	**Pneumonia**	**Sepsis**	**Meningitis**	**Example references**
CBPS						
PspA	+	+	+	+	ND[c]	[5–7,65]
PspC	+	+	–	+	+	[4,7,17,32,41,68]
LytA	+	+	–	+	ND	[84–86]
PcpA	+	+	+	+	ND	[89–91,93]
LIPOPROTEINS						
PsaA	+	+	–	–	ND	[4,63,106,110,111,155]
PiuA, PiaA	ND	ND	–	+	ND	[16,113,115]
PotD	+	–	+	+	ND	[117,118]
AliA	ND	ND	–	+	ND	[113,158]
LPXTG PROTEINS						
NanA	+	+	ND	+	ND	[132–134]
IgA1 protease	+	ND	+	+	ND	[139,140]
Pili (RrgA)	+	ND	+	+	ND	[145–147]
OTHER PROTEIN CANDIDATES/APPROACHES						
Ply	+[a]	–[b]	+	+	+	[5–7,32,33,156]
PhtA/B/D/E	+	–	+	+	–	[7,21,22,93,121,122]
PcsB	+	ND	+	+	ND	[152,155,156]
StkP	+	ND	+	+	ND	[152,155]
GlpO	ND	ND	ND	+	+	[159]
Whole cell vaccines	+	ND	ND	+	ND	[161,162,164–166]

[a] *Shown to provide significant protection in at least one study.*
[b] *No protective effects reported.*
[c] *ND, not determined.*

protection-eliciting domains or epitopes from multiple virulence factors (e.g., the PspC/CbpA loop-peptide-pneumolysoid fusion described by Mann et al.) [32]. Nevertheless, the various vaccine formulations currently under late pre-clinical or clinical development may not necessarily represent the best possible combinations of antigens, owing to a complex patent landscape affecting commercial freedom to operate. This issue may well have restricted the range of combination protein formulations that have undergone rigorous comparative efficacy studies in a full range of animal models. Thus, there is still a need for continued comparative immunogenicity studies of combinations, including existing and newly identified candidate antigens, to identify the optimal formulation, which might then be developed through appropriately brokered partnerships.

The pathway to licensure of pneumococcal protein vaccines is potentially complicated by the ready availability of the conjugate vaccines, which provide robust protection against invasive disease caused by included serotypes. Thus, initial deployment will probably be as adjuncts to a conjugate vaccine, after confirmation that inclusion of the protein components did not reduce the anti-capsular responses. Of course, if such a roll-out occurred in regions where conjugates have been in use for some years, then included serotypes will be very rare, so efficacy will be largely be attributable to the non-serotype-dependent protection elicited by the protein components (a *de facto* placebo-controlled trial). Such post-licensure data may eventually provide a sufficiently robust evidence base to enable licensure of stand-alone combination protein vaccine formulations. An alternative approach, first proposed more than 20 years ago, would be to deploy at least some of the protective pneumococcal protein antigens as carriers for CPS in novel conjugate vaccines. The success of pneumolysoid-CPS [33,35,36] and PspA-CPS [66,67] conjugate formulations in animal models provides excellent "proof of concept" for this strategy. Moreover, licensure could initially be sought on the basis of serological analysis of the anti-capsular responses, for which correlates of protection against IPD are now well established.

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SECTION B

GENETICS AND FUNCTIONAL GENOMICS OF *STREPTOCOCCUS PNEUMONIAE*

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CHAPTER

5

Genomics, Genetic Variation, and Regions of Differences

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STREPTOCOCCUS PNEUMONIAE COMPARATIVE GENOMICS

High-throughput sequencing technologies applied to bacterial pathogens provide an information-rich environment for the study of bacterial evolution, epidemiology, and pathogenesis [1]. Since publication of its first whole genome sequences in 2001 [2,3], *Streptococcus pneumoniae* has received a great deal of attention from a genomics perspective, with more than 4000 genomes sequenced to date, most of them in draft form, and many more to come. Comparative genomics analyses range from the comparison of assembled and annotated genomes to the mapping of unannotated raw sequence data to a reference genome.

A recent analysis of 240 *S. pneumoniae* isolates of the PMEN1 (Spain^{23F}-1) multidrug-resistant lineage has provided the ability to distinguish accumulated base substitutions from polymorphisms generated by recombination of imported DNA [4]. A phylogeny constructed using only vertically inherited single nucleotide polymorphisms (SNPs) (regions affected by recombination events were removed) proved to be a superior estimation of the evolution of the PMEN1 clone based on the correlation between the distance from the root of the tree and the date of isolation of each strain. By comparing this more accurate phylogeny to the dates and locations of isolation, the authors were able to pinpoint the likely origins of different clades and to discern information about the geographic

Streptococcus pneumoniae.
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spread of the clone [5]. The Croucher et al. [4] study of closely related isolates from 22 countries revealed capsule switching events and acquisition of antibiotic resistance determinants in response to clinical interventions over short timescales. For instance, it identified 10 cases where PMEN1 isolates escaped pressure applied by the PCV7 vaccine by switching to the expression of non-vaccine serotypes. Analysis of integrative and conjugative mobile elements revealed selection for determinants of resistance to drugs commonly used for treatment of upper respiratory tract infections.

In a separate study, the genomes of 426 isolates from a genetically diverse, historical, and global collection covering the years 1937–2007 were sequenced in order to track the rapid increase of penicillin resistance [6]. A particular focus on PMEN1 revealed that its likely ancestor is one of the earliest known penicillin-nonsusceptible strains, isolated in 1967 in Australia. The study further indicated that PMEN1 is a very efficient donor of penicillin and other antibiotic resistance genes to many genotypically diverse *S. pneumoniae* lineages. This led the authors to designate PMEN1 a "paradigm for genetic success."

In-depth genomic analysis of the PMEN1 clone provided a detailed overview of the worldwide distribution and evolution of a set of closely related isolates that successfully adapted to host and therapeutic pressures. An alternate way of studying pneumococcal evolution is to compare unrelated isolates recovered over time from a specific geographical location. We took this approach in order to characterize the genomic diversity of *S. pneumoniae* clinical isolates within the Atlanta, Georgia, metropolitan area. Our study focused on the generation of 147 whole genome sequences, including 121 invasive and 10 carriage isolates from Atlanta as well as 16 invasive isolates from outside of Atlanta. The genomes encompassed 22 serotypes, 86 multilocus sequence typing (MLST) types, resistance to 10 antibiotics, and 10 disease outcomes. The collection included 29 strains belonging to the MLST-based clonal complex CC320, a multidrug-resistant complex responsible for the global emergence of non-vaccine serotype 19A in the years following the introduction of PCV7. The predicted founder of the complex, ST320, was a serotype 19A clone carrying dual macrolide resistance determinants (Mega and *erm*(B)) [7]. The clone represented a 19F-19A capsule switch and horizontal acquisition of multiple antibiotic resistance mechanisms, suggesting that vaccine and antibiotic pressures influenced its emergence [8].

The 29 CC320 whole genome sequences in our study (20 Atlanta invasive, 1 Atlanta carriage, and 8 invasive isolates from states outside of Georgia), together with publicly available CC320 genomes TCH8431/19A (ST320) and Taiwan^{19F}-14 (ST236), were subjected to whole genome multiple sequence alignment using the Mugsy software [9] with default parameters. The alignment in MAF format was then filtered with Phylomark [10] to extract and concatenate the core nucleotides, including SNPs, and to construct a neighbor-joining phylogenetic tree using MEGA v6.06 [11]. Of the 31 CC320 isolates, 18 were serotype 19F and 13 were 19A. In order to eliminate the influence of capsule switching on the phylogeny, we deleted the capsule locus from the genome sequence of the Taiwan^{19F}-14 isolate such that the capsule locus would no longer be part of the core alignment. The genomes clustered into three clades representing each of the three subgroups in CC320: ST236, ST271, and ST320 (Figure 5.1). Clade 236 consisted exclusively of serotype 19F isolates belonging to the CC320 subgroup founded by ST236. Clade 236 isolates were identified in the pre- and post-vaccine eras, despite vaccine pressure against serotype 19F due to PCV7. This could be explained by antibiotic pressure selecting for the multidrug-resistant phenotype. Clade 236 isolates, with exception of one pre-PCV7 era isolate (GA13499), were resistant to

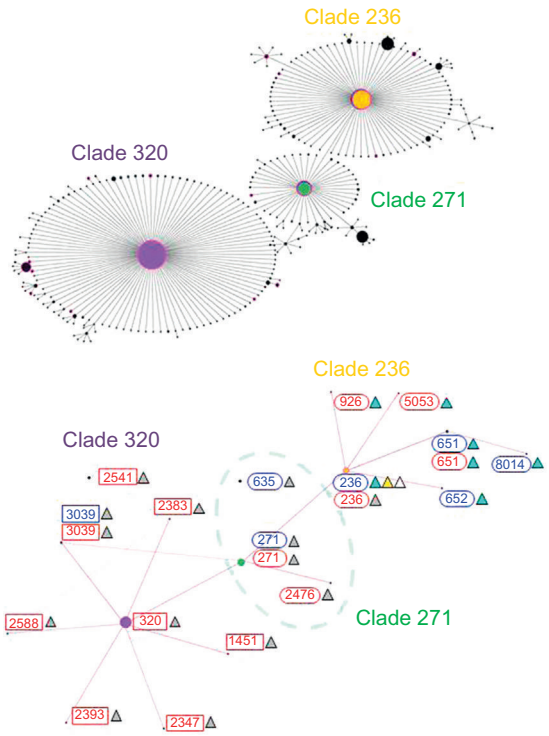

FIGURE 5.1 Comparison of clonal complex 320 (CC320) multilocus sequence types in the Atlanta genome collection with all known CC320 sequence types. Top panel: eBURST analysis of clonal complex CC320 in the spneumoniae.mlst.net database (as of November 2014). CC320 is composed of three subgroups, or clades, each named after the predicted founding sequence type (ST) of the clade. Each dot represents a unique ST with a diameter proportional to the number of representatives in the database. The founding ST is located at the center of each clade. Clade 320 was founded by ST320 (purple), clade 271 was founded by ST271 (green), and clade 236 was founded by ST236 (orange). Lines connecting each ST represent a single locus variation between the two types. Sequence types circled (pink) are represented in the Atlanta genome collection. Bottom panel: eBURST analysis of CC320 in the Atlanta pneumococcal genome collection. Sequence types and clades are color-coded as above. Numbers indicate the ST number of each type. Boxed ST numbers indicate that at least one member of the ST was serotype 19A. Numbers enclosed by a rounded rectangle contain a serotype 19F isolate. ST connected by a pink line were single locus variants. Blue numbers indicate that an isolate of that ST was isolated prior to the introduction of PCV7 in the Atlanta metropolitan area (i.e., prior to November 2000). Red indicates that the ST was isolated post-PCV7. Triangles indicate the presence or absence of mobile elements encoding macrolide resistance: gray, Tn*2010*; light blue, Tn*2009*; yellow, Mega; open, susceptible isolate without macrolide resistance element.

erythromycin. Each resistant isolate contained macrolide efflux genes *mef*(E) and *mel*, encoded on the macrolide efflux genetic assembly (Mega) integrated directly into the pneumococcal chromosome or nested within a Tn*916*-like element, either Tn*2009* or Tn*2010*. Tn*2010* also contained the *erm*-type methylase gene *erm*(B) (Figure 5.1). Interestingly, Tn*2010* was identified only in post-PCV7 isolates in clade 236 (Figure 5.1). The earliest isolated CC320 strain in this genome collection was GA04375, a 19F, ST236 isolate from 1995. GA04375 did not contain the Mega- and *erm*(B)-containing transposon Tn*2010* that is commonly found in ST320. It contained instead the Mega element integrated into the RNA methyltransferase gene (*rumA*) located at the left junction of the pneumococcal pathogenicity island-1 (PPI-1, [12]), which was partially deleted. The genome of GA04375 clustered closely with serotype 19F isolates from 1999, GA13499 (ST236) and 3063-00 (ST652). Strain 3063-00 contained Mega integrated the DNA-3-methyladenine glycosidase gene (TIGR4 annotation, SP_0108) instead of *rumA*. GA13499 was sensitive to macrolides and contained no macrolide resistance determinant. This demonstrated the independent acquisition of macrolide resistance by closely related isolates prior to PCV7 introduction. CC320 strains isolated after 2000 were mostly 19A, and all CC320 19A isolates contained Tn*2010*, suggesting that the clone acquired Tn*2010* prior to the 19F to 19A serotype switch. This is supported by the observation that Tn*2010* is inserted into the same locus and with identical junction sequences within the chromosome, regardless of serotype. The core analyses of CC320 isolates revealed the influence of antibiotic pressure, vaccine pressure, and the passage of time on the evolution of pneumococcal clones. It will be interesting to see if CC320 emerges with a new serotype in the post-PCV13 era.

Clade 271 correlated to the ST271 subgroup of CC320 and included only macrolide-resistant serotype 19F isolates (Figure 5.1). Unlike clade 236, clade 271 isolates all contained Tn*2010*, including those dating to the pre-PCV7 era. Clade 320 correlated to the CC320 subgroup founded by ST320. Clade 320 represents a PCV7 escape clone, containing mechanisms to avoid antibiotic and vaccine pressures (Figure 5.1). All clade 320 isolates were serotype 19A, indicating a serotype switch, and dual-macrolide resistance determinants were encoded on Tn*2010* (Figure 5.2). Tn*916*-like elements harboring macrolide resistance elements were in the Atlanta population prior to the appearance of ST320 in Atlanta, and indeed globally. It is believed that the clone developed in Asia and was subsequently disseminated worldwide. Epidemiology data supports this theory [13]. However, our sampling of CC320 in Atlanta isolates reveals CC320 clonal diversity in Atlanta similar to that observed around the world. This suggests that the ingredients for the ST320 superbug evolution exist in local populations globally and raises the possibility that convergent evolution could result in similar clones developing independently in local pneumococcal populations. Does this mean that under the correct selective pressures, the formation of the superbug, or a similar dominant clone, was inevitable and will happen again? Significantly, while many of the precursors of ST320 were present in Atlanta prior to PCV7, serotype 19A ST320, with its characteristic dual macrolide resistance determinants encoded by Tn*2010*, did not appear in Atlanta until a single isolate was identified post-PCV7 in 2003 (unpublished). The earliest ST320 isolate in the genome study was from 2004 (Figure 5.2).

Because the ST320 clone emerged globally, including in locations with poor or no PCV7 coverage, antibiotic resistance was thought to be the major selective force driving its emergence in these populations. However, this did not explain why 19A displaced multidrug-resistant 19F in non-vaccinated populations. Recent findings have suggested that the ST320 clone was a better colonizer of the nasopharynx than its progenitor ST236 [13]. The increased fitness was not explained by the capsule difference [13]. This suggested that ST236 had acquired, by transformation and recombination, genes involved in colonization as well as the 19A capsule locus. Thus, it appears the evolution of ST320 was a result of multiple selective pressures.

Another geographically restricted study focused on more than 3000 carriage isolates from a 2.4 km^2 refugee camp at the border of Thailand and Myanmar [14]. Hierarchical clustering of genomes based on sequence similarity revealed clusters that roughly corresponded to MLST-based clonal complexes. This study revealed that among the 3085 carriage isolates sequenced, the largest capsule phenotype group (512 isolates) was composed of non-typable *S. pneumoniae*. These non-typable isolates appear to act as a reservoir of recombinant DNA, especially drug resistance determinants, for the different *S. pneumoniae* lineages; and given that these non-typable isolates are not targeted by current polysaccharide vaccines, they will continue to be carried. The study also identified hotspots of recombination within the *S. pneumoniae* genome. These indicate that there are a limited number of genes in which diversity accumulates as a consequence of recombination. It is likely that host and therapeutic pressures underlie this phenomenon. Indeed, the six most prominent hotspots among the refugee camp isolates harbored genes encoding cell surface antigens (*pspA* and *pspC*) and genes associated with resistance to antibiotics (*pbp1a*, *pbp2b*, *pbp2x*, and *folA*).

These applications of whole genome sequencing and analysis to different collections of *S. pneumoniae* illustrated the power of

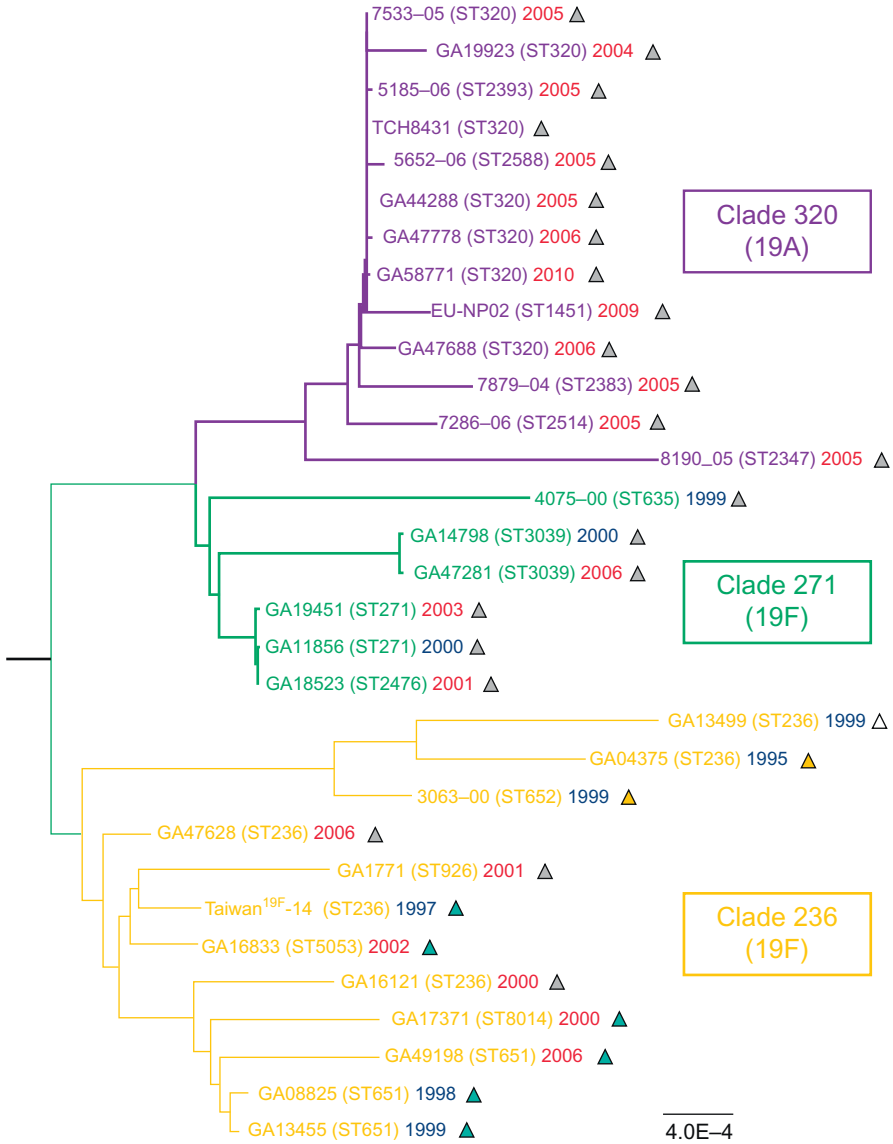

FIGURE 5.2 Core genome phylogeny of CC320 isolates from metropolitan Atlanta. Clades are color-coded as in Figure 5.1. Purple, clade 320; green, clade 271; orange, clade 236. Each clade harbors a single serotype, which is indicated in parentheses within the boxed clade labels. Branch tips of the tree are labeled with strain names, followed by ST (in parentheses) and year of isolation. Years are color-coded to distinguish between strains isolated prior to and after the introduction of PCV7 in the Atlanta metropolitan area (blue and red, respectively). Triangles represent the presence of macrolide resistance elements and are color-coded as in Figure 5.1: gray, Tn*2010*; light blue, Tn*2009*; yellow, Mega; open, susceptible isolate without macrolide resistance determinant.

genomics in providing critical insights into the biology of this species. Current sequencing efforts are focused on filling in the pneumococcal space of genomic diversity, geographical origin, and time (evolution). For instance, the ongoing global pneumococcal sequencing project (GPS, http://news.emory.edu/stories/2013/03/video_pnemonia_genome/) aims to sequence the genome of 20,000 pneumococcal strains isolated before and after the introduction of vaccines in developing countries. The team aims to better characterize vaccine escape and devise next-generation vaccines that avoid that escape.

S. *pneumoniae* Pan-Genome

S. pneumoniae displays extensive genomic diversity. This is reflected in the analysis of its pan-genome, the entire repertoire of genes accessible to the *S. pneumoniae* species, which was determined to be much larger than the genome of any individual strain or isolate [15–18]. In fact, the *S. pneumoniae* pan-genome was defined as open, its size increasing logarithmically, meaning that extrapolation based on the 44 genomes sequenced in 2010 suggested that every new genome sequenced contributed new genes to the species, and the trend indicated that a very large number of genomes would have to be sequenced to fully characterize the entire gene repertoire [15,16].

A pan-genome analysis we performed based on 158 isolates from the Atlanta metropolitan area and other publicly available genomes confirmed the trend based on 44 genomes (Figure 5.3). The new genes power law regression equation for 158 genomes was:

$$y = 269.3229 \pm 2.7959x^{(-0.9821 \pm 0.0028)}$$

This formula allows for extrapolation that can be used to predict the number of new genes that would be identified given increasing numbers of additional genomes sequenced; this is shown in Table 5.1. It suggests that after 500 genomes sequenced, every other genome will provide a new gene on average (~0.5 new gene per genome); after 1000 genomes every third genome will still provide a new gene; and so on. This, of course, depends on the randomness of sampling for strains to be sequenced. Thus, the pan-genome of *S. pneumoniae* is still predicted to be extremely large. This has broad implications for the biology of *S. pneumoniae*. The core genome (shared by all strains) typically includes genes responsible for the basic aspects of the biology of the species and its major phenotypic traits. By contrast, dispensable genes (shared by a subset of the strains) contribute to the species diversity and might encode supplementary biochemical pathways and functions that are not essential for bacterial growth but which confer selective advantages, such as adaptation to different niches, antibiotic resistance, or colonization of a new host [19]. Donati et al. [15] predicted that a fast-growing pan-genome, with strains that are quickly diversifying by integrating new genes, indicates that the species is exploring novel evolutionary possibilities. They postulate that the *S. pneumoniae* species is possibly adapted to its current ecological niche but remains open to the acquisition of new genes while maintaining stability.

Van Tonder et al. [20] used a Bayesian approach to estimate a bacterial core genome that, unlike the classical pan-genome analysis described above, does not require that every single isolate sequenced harbors all core genes. This accommodates for the possible presence of rare strain variants that may be missing some genes that would otherwise be considered core. Application of the model to 336 *S. pneumoniae* genomes encompassing 39 serotypes, 32 countries, and 90 years of isolation estimated the presence of 948 core genes. Another method, based on clusters of orthologous genes, also applied by van Tonder et al. predicted 1194 core genes. Generally speaking, differences in core gene counts arise from the

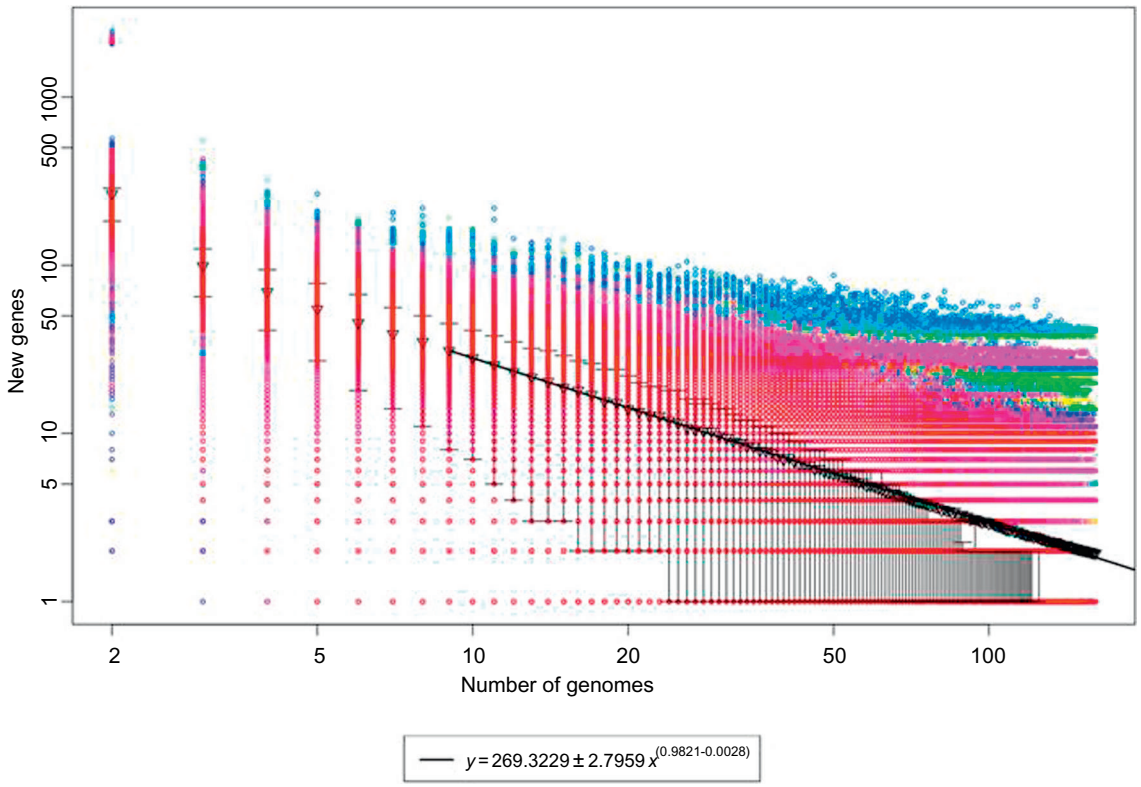

FIGURE 5.3 **New gene discovery graph from the pan-genome analysis of 158 *S. pneumoniae* isolates.** For each reported number of genomes (n), the circles represent the number of new genes found in different randomly chosen combinations. Triangles show the mean values for each distribution. The curve and equation represent a power law regression for new genes discovered that was fitted to the means of new gene counts (triangles) for each value of n.

use of different data sets (the more genomes analyzed, the smaller the core genome), different models, and varying methods for alignment and different cutoffs, including percent identity and whether or not alignment had to occur over the full length of the genes (estimated core genome model) or half of the gene length [17].

Dispensable Genome and Mobile Elements

The concept of what constitutes a pneumococcal clone (or strain, or isolate) has evolved with our ability to look into the genetics behind pneumococcal evolution with increasing resolution. Capsule serotyping was insufficient as a means to infer phylogeny. Several typing methods were improvements, but not until MLST provided a standardized and accessible method whereby isolates could be reliably typed and compared to typed isolates from any location [21]. MLST demonstrated that a single sequence type can exist with many different capsule serotypes due to serotype switching [8,22]. Comparative genomics revealed a surprisingly large amount of variation within the genomes of "identical" clones as defined by MLST.

TABLE 5.1 Use of a Pan-Genome Analysis to Extrapolate the Number of New Genes That Would Be Identified Given Increasing Numbers of Additional Genomes Sequenced

Number of genomes sequenced	Estimated number of new genes identified per genome	Estimated number of genomes needed to find a new gene
158	1.87	0.54
200	1.48	0.68
500	0.60	1.66
1000	0.30	3.28
5000	0.06	15.94
10,000	0.03	31.49
20,000	0.02	62.20
50,000	0.01	152.96

Variability between individuals within a single clonal complex is due primarily to variations in the gene content of their dispensable genomes, that is, those genes not essential for survival and pathogenicity of the pneumococcus. The dispensable genome accounts for approximately one-quarter of a pneumococcal genome [15]. The contents of the dispensable genome of a pneumococcal clone are heavily influenced by horizontal gene transfer of material from other pneumococci, closely related commensal streptococci, and/or more distantly related bacteria. The pneumococcus is extremely adept at acquiring novel DNA through transformation and recombination, allowing virtually unrestricted flow of gene content within pneumococcal populations. Frequent recombination events lead to constant shuffling of the dispensable genome and often to swapping of large regions of the core genomes. This process has created genotypic heterogeneity within pneumococcal populations [22]. Successful clones emerge from the milieu of genotypes due to selective environmental and host-related pressures. Antibiotic pressure selects for genotypes that include antibiotic resistance genes. Vaccine pressure suppresses targeted capsule genotypes, thus promoting the emergence of non-vaccine capsule genotypes. The pneumococcal capsule conjugate vaccines (PCV7, PCV13) selected for non-vaccine capsule serotypes including serotype switch "escape" mutants [8].

Transformation and recombination can also be a genome-stabilizing factor. There is no SOS response system encoded within the core genome of *S. pneumoniae* to repair damaged DNA [23]. Instead, pneumococci repair damaged DNA by allele replacement through recombination with undamaged DNA acquired by transformation [24]. The "sharing" of DNA between pneumococcal cells to repair randomly located DNA lesions can have a homogenizing effect on the pneumococcal genome.

Clonal diversity is also limited by the recombination-dependent phenomenon of soft selective sweeps. Interspecies transformation events have been demonstrated to transfer fragments ranging in size from 0.4 to 235 kb [25]. Transformation of genes providing a selective advantage can be linked to neutral or even detrimental genes. The closer two genes are on the chromosome, the more likely they are to be transferred concomitantly and the stronger the selective pressure on the indirectly selected gene.

In the Atlanta genome collection, soft sweeps driven by macrolide resistance are apparent. Tn*5253*-like conjugative transposons carry the chloramphenicol acetyltransferase gene (*cat*), conferring resistance to chloramphenicol. In all instances, Tn*5253*-like elements were inserted into the ribosome maturation protein *ylqF* (TIGR4 annotation, SP_1154) [26]. Nested in the Tn*5253*-like elements were Tn*916*-like elements carrying the tetracycline resistance gene *tet*(M). Inserted in conserved loci of the Tn*916*-like elements were various macrolide resistance elements including Mega and the *erm*(B)-containing elements Omega and Tn*917*. Macrolide-resistant Tn*916*-like elements associated with Tn*5253*-like elements included Tn*6002* (Omega), Tn*2009* (Mega), Tn*2010* (Omega and Mega), or Tn*3872* (Tn*917*). This indicates that selection for recombination of the Tn*916*-like elements, or fragments thereof, into larger elements allows interconversion between Tn*916* and the macrolide-resistant version of Tn*916*. Selection for these recombination events by macrolide exposure also provides a soft selective sweep for chloramphenicol and tetracycline resistance.

The efficiency of homologous recombination depends upon suitable regions of homology between donor and recipient cells. This becomes a barrier to horizontal gene transfer between distantly related bacteria. Transfer between species lacking extensive homology with pneumococcal chromosomes is facilitated by mobile DNA elements such as insertion sequences, phages, and integrative and conjugative transposons (ICE). Mobile elements are excised, transferred, and integrated in a target sequence–dependent manner, thus bypassing the need for homology between donor and recipient. These elements often carry "cargo" genes, which may come from distantly related bacteria and which may be beneficial to the pneumococcus. Once integrated into a pneumococcal chromosome, the mobile element and its novel gene cargo can be disseminated within the pneumococcal population by transformation and recombination. Thus, mobile elements are a means of expansion of the pneumococcal pan-genome through additions of novel gene content to the accessory genome.

In conclusion, *S. pneumoniae* has a large and growing pan-genome. Horizontal gene transfer is mediated by transformation and recombination, and by the movement of mobile elements. The continual shuffling of gene content within the pan-genome and the occasional acquisition of novel DNA from non-pneumococcal bacteria have led to tremendous variation in the genetic background of pneumococcal clones circulating in a population. Selective forces including antibiotics, vaccines, inter- and intraspecies competition, and host defenses pull the previously existing rare strains, from the milieu of pneumococcal genotypes, that are most suitably adapted to deal with the environmental challenges at hand at any given time. Widespread selective pressure, such as antibiotic usage and vaccination, subsequently promote the clonal propagation and dissemination of successful clones. Future genomic studies will aid in improving our understanding of the relative roles played by genetic variability in local and global pneumococcal populations.

VARIATION AND VIRULENCE

S. pneumoniae (the pneumococcus) colonizes the human nasopharynx and in some cases can cause diseases such otitis media, pneumonia, and meningitis. The pneumococcus produces a range of colonization and virulence factors including a polysaccharide capsule, surface proteins and enzymes, and the cytoplasmic toxin pneumolysin. In terms of ability to cause disease, not all pneumococci are equal. Some strains or serotypes are rarely associated with disease, while others are often associated with invasive disease [27,28]. The ability of pneumococci to cause disease in humans is related to

the genetic content of the organism, such that the presence or absence of virulence genes and/or variation in the sequence of virulence genes dictates the virulence of the strain. Several screens have been conducted to identify genes important in pathogenesis of infection. These include signature-tagged mutagenesis in animal models of infection and colonization [29–32] as well as TnSeq-based screening [33]. Microarray-based studies and whole genome sequencing have been used in attempts to determine the complement of genes required to define the ability to cause invasive disease [27,34,35]. These studies assume that there is an essential core genome and that the differing virulence of pneumococcal strains is determined by a set of accessory genes in the pneumococcal chromosome. It has proved difficult to associate the ability to cause invasive disease with particular genetic loci. The capsule locus is essential for virulence, but not all capsulated strains cause disease. Serotypes associated with the highest rates of invasive disease are 1, 4, and 7F. However, there may also be differences in ability to cause invasive disease among clonal types of the same serotype [27]. Blomberg et al. [27] conclude that the accessory regions required for invasive disease may be redundant as no unique pattern distinguishes the most invasive pneumococcal clones from others. Gene content may also be reflected in different regulatory pathways within strains of pneumococci. In addition to variation in gene content there is also variation in the sequence of individual genes known to be important for virulence, such that SNPs may define the virulence profile of some strains. By understanding the effect of these sequence variations on the ability of pneumococci to cause disease, it may be possible to define more subtle mutations (rather than presence and absence of genes) that allow pneumococci to vary in invasive potential. Some of these key genes and the biological effects of variation in sequence of these virulence factors are considered here.

Capsule

The polysaccharide capsule is the most important virulence factor in the pneumococcus and is the basis for serotyping of pneumococci. There are 94 known serotypes [36–40]. The genes for the biosynthesis of 93 of the capsule types are found in the same location in the pneumococcal chromosome, between the *dexB* and *aliA* genes. The exception to this is serotype 37, which is synthesized from a single gene elsewhere in the chromosome. One of the most striking features of the pneumococcal capsule locus is its huge genetic divergence, as only a few genes are conserved among the different clusters [36,41]. The capsule protects the pneumococcus from phagocytosis [42]. Antibody to cell wall constituents binds to the surface of the pneumococcus and in turn binds complement components. Presence of the capsule prevents iC3b and the Fc of immunoglobulins bound to the bacterium from interacting with their receptors on the surface of phagocytic cells, with the result that the bacteria cannot be taken up and killed by the phagocyte [43]. The capsule is also crucial for colonization as it prevents removal by mucus [44] and can also restrict autolysis and reduce exposure to antibiotics [45]. Pneumococci lacking a polysaccharide capsule can be isolated from the upper respiratory tract of humans [46]. These strains are often referred to as nontypable and are usually associated with asymptomatic carriage but are also associated with outbreaks of conjunctivitis [47] and occasionally with invasive disease [48,49]. Non-typable strains can be divided into two groups. Group I are those with a disrupted or nonfunctional capsule locus and Group II are those isolates that contain genes not found in normal capsular types [50]. Group II can be further divided

into NCC1 and NCC2. NCC1 isolates have the *pspK* gene present at the site of the capsule locus [51]. The *pspK* gene codes for a novel pneumococcal surface protein that may play a role in colonization [52]. The *pspK* gene has also been named novel surface protein gene A (*nspA*) [53]. The *nspA* gene is present along with a variety of intact and disruptive IS elements. The *nspA* gene itself shows high levels of conservation in some areas, with a hypervariable repeat region: no two isolates are identical [53]. NCC2 isolates have both the *aliB*-like ORF1 and *aliB*-like ORF-2 genes [50,51,53]. Analysis of population structure shows that *nspA* is not restricted to a single lineage of closely related pneumococci but is found in distantly related isolates. The presence of this gene in strains isolated from distant geographical locations suggests that strains carrying this gene are successful [53].

Surface Proteins

Analysis of the genome sequence of *S. pneumoniae* strain TIGR4 [3] identified 70 genes for proteins predicted to be exposed at the cell surface. These proteins are surface attached by one of three mechanisms: peptidoglycan anchor motif (LPXTG), choline-binding motif, or lipid-attachment motif [54]. The LPXTG motif allows the enzyme sortase-A to covalently link the protein to the bacterial cell wall by linkage of the threonine of the motif to the pentaglycine linkage of peptidoglycan in the pneumococcal cell wall [55]. The number of LPXTG proteins can differ between strains, and several of these proteins are known to be associated with the virulence of the organism. Key LPXTG-anchored proteins are neuraminidase A (NanA), serine protease PrtA and hyaluronidase.

Neuraminidase cleaves *N*-acetyl neuraminic acid from glycolipids, lipoproteins, and oligosaccharides in host cells, which may unmask binding sites for the organism. NanA plays a role in colonization and development of otitis media in a chinchilla model [56]. Loss of sialic acid as a result of neuraminidase activity accompanies the spread of pneumococci along the eustachian tube to the middle ear [56]. NanA plays an important role in biofilm formation, and sialic acid released by the action of NanA may be an important signal in regulation of pneumococcal virulence [57]. The *nanA* gene is present in all clinical isolates [58–60]. The *nanA* gene shows high sequence diversity that may be important in avoidance of the host immune response [58]. The original cloning of the *nanA* gene from *S. pneumoniae* strain R36a (NCTC 10319) isolated an enzymatically active clone [61]. Subsequent sequence analysis showed this clone was not complete, lacking 233 amino acids from the C-terminus. Interestingly, in the original genome sequencing project of TIGR4 the *nanA* sequence is annotated as a pseudo-gene due to the presence of an 11 base pair deletion that results in a changed reading frame and termination of the gene at amino acid 804 (of a possible 1035) [3]. However, the enzymatic portion of the protein is intact and can be isolated from the pneumococcus. The absence of the C-terminal part of the protein means that the LPXTG anchor is missing and the enzyme is not linked to the cell wall. This may be important in the pathogenesis of disease as NanA is important in binding pneumococci to human cells, including those of the blood–brain barrier [62,63], and lack of surface anchoring may compromise this function.

Hyaluronidase breaks down the hyaluronic acid component of mammalian connective tissue and extracellular matrix and is produced by clinical isolates of pneumococci [64]. The degradation of hyaluronic acid may aid bacterial spread and colonization. Hyaluronidase may also potentiate pulmonary inflammation by complex interaction with chemokines and cytokines. TNFα and IL-1β are able to induce the production of hyaluronic acid by fibroblasts [65], which can then promote further cytokine

secretion by binding to CD44 on host cells. The system is further complicated by the ability of IL-1 to release host hyaluronidase. Breakdown products of hyaluronic acid stimulate chemokine production by macrophages [66], which increases cell recruitment and inflammation. Some serotype 3, ST180 strains contain an SNP at position 376 of the hyaluronidase coding sequence, which results in a stop codon and truncation of the protein after 125 amino acids. These strains produce no active hyaluronidase.

The *prtA* gene has been confirmed in all pneumococcal isolates tested [67]. PrtA is a serine protease and is required for full virulence in animal models; vaccination with the protein provides protection from infectious challenge [67]. PrtA plays a role in the killing of *S. pneumoniae* by apolactoferin [68]. Expression of *prtA* is co-regulated with a number of other virulence genes, including those encoding the pilus and pneumolysin genes, by the transcriptional factor PsaR [69]. Regulation of *prtA* expression by PsaR has also been demonstrated to be oppositely repressed and stimulated by manganese or zinc [70].

Three proteins from TIGR4 have LPXTG-like motifs; these are the pilin proteins (SP_0462, SP_0463, and SP_0464), which are linked to each other by specific pilus-sortase enzymes [71]. These genes are part of the *rlrA* pathogenicity islet [72] and are transcribed together on the same transcript by the adjacent transcriptional regulator. The *rlrA* pathogenicity islet codes for the production of the pneumococcal pilus; this genetic locus is present in less than 20% of clinical strains [73]. A second pilus type, which is involved in adherence of pneumococci to epithelial cells, has been identified, and some strains can express both types [74].

Choline-binding proteins (CBPs) are anchored to the cell surface via the interaction of repeat domains of the protein with choline residues present in the pneumococcal cell wall. Teichoic and lipoteichoic acids in the cell wall are decorated with phosphorylcholine residues that anchor the CBPs to the pneumococcal cell. These proteins have repeated sequences of approximately 20 amino acids (choline-binding module), usually present in the C-terminal region of the protein. Two to twelve modules form the choline-binding domain that is attached to phosphorylcholine in the cell wall in a noncovalent manner. CBPs may have various enzymatic activities or may have binding properties to allow binding to host cells or extracellular matrix [54]. Analysis of the genome sequences of pneumococcal strains R6 [75] and TIGR4 [3] predict 12 CBPs in R6 and 15 in TIGR4 [76]. Several CBPs are associated with the ability to bind to host proteins.

The genome of *S. pneumoniae* contains approximately 40 genes predicted to code for lipoproteins [3,54], many of which are involved in virulence as part of nutrient uptake transporters. Cation ABC transporters have major effects on pneumococcal virulence, with loss of PsaA manganese transporter lipoprotein or combined loss of AdcA and AdcAII zinc or the PiaA and PiuA iron ABC transporter lipoproteins, resulting in strains of greatly reduced virulence [77–82]. The mechanism of lipoprotein attachment to the bacterial cell membrane and processing is conserved among bacteria. Prolipoproteins are secreted by the general secretory pathway and then are covalently linked to the cell membrane by the enzyme diacylglyceryl transferase (Lgt) [82]. A type II lipoprotein signal peptidase (Lsp) then cleaves the N-terminal signal peptide adjacent to the "lipobox" cysteine residue to form the mature lipoprotein [83]. Deletion of the *lgt* gene from pneumococcus has widespread effects on ABC transporter functions that collectively prevent the mutant from establishing invasive infection [84].

Pneumolysin

Pneumolysin (Ply) is a 53-kDa pore-forming toxin made by almost all clinical

isolates of the pneumococcus; it is expressed during the log phase of growth [85]. There are at least 16 different naturally occurring variants of Ply including allele 5 Ply, which is expressed in specific strains of serotypes 1 and 8 pneumococci [86–88]. It has been demonstrated that both human and murine mononuclear cells exposed to *S. pneumoniae* that express fully lytic toxin produce IL-1β, and the production of this cytokine depends on the NOD-like receptor family, pyrin domain containing 3 (NALP3) inflammasome [89]. Strains expressing the nonhemolytic allele 5 of the toxin did not stimulate IL-1β production. NLRP3 activation was beneficial for mice during pneumonia caused by pneumococcal strains expressing fully active toxin due to cytokine production and maintenance of the pulmonary microvascular barrier. Thus, polymorphisms in the pneumolysin protein may substantially affect recognition of bacteria by the innate immune system. Pneumolysin is produced by virtually all clinical isolates of the pneumococcus [90], and analysis of the pneumolysin gene sequence from 121 clinical isolates identified 14 protein alleles [87], some of which are associated with lack of hemolytic activity of the toxin. Some clinical strains were shown to have insertions of either a section of duplicated sequence or transposon IS1515 [87,91], suggesting that pneumolysin is not absolutely essential for the pneumococcus to be able to cause infection. Although thought to be released only when pneumococci undergo autolysis [92], Ply can be released independently of the major autolysin [93]. Ply has been shown to be exported to the cell wall [94,95]. Ply plays several roles in infection. The toxin appears to have no role in inflammation associated with meningitis [96–98] but does have a role in deafness associated with meningitis [98] and in bacteremia [99] and pneumonia [100]. Ply has been suggested to play a role in damage to the blood brain barrier as it his responsible for the majority of cytotoxicity in brain microvascular endothelial cells exposed to *S. pneumoniae in vitro* [101].

Two-Component Systems and Regulation of Virulence

Bacterial adaptation to the external environment is often mediated by two-component systems (TCS). A typical TCS is composed of a membrane-bound sensor histidine protein kinase (HK) and a cognate response regulator (RR), which is usually a DN-binding protein. On stimulation by an appropriate signal the HK is auto-phosphorylated on a conserved histidine. The phosphate group is then relayed to an aspartate residue in the RR. The availability of pneumococcal genome sequences reveals 13 HK:RR pairs and a single "orphan" RR with no associated HK [102,103]. Genetic studies have been conducted to define the roles of these systems in virulence. Lange et al. [102] analyzed the effect of gene deletions on the virulence of serotype 3 and serotype 22 pneumococci in a mouse model of systemic infection and found no effect on virulence. Throup et al. [103] used a mouse model of pneumonia and demonstrated a role in infection for most of the TCS in a serotype 3 strain (0100993). Thus the role of TCS in virulence is dependent on the genetic background of the bacterial strain as well as route of infection. This finding was confirmed by Blue and Mitchell [104], who found that deletion of the TCS09 system had no effect on the virulence of strain D39 in murine models of pneumonia and bacteremia; the same mutation in strain 0100993 caused attenuation in the pneumonia model but not in the systemic disease model. There are many other examples of different effects of regulatory genes on virulence depending on the strain of pneumococcus studied. It is becoming increasingly clear that to understand these regulatory processes we need to study the control of bacterial gene

expression in the *in vivo* environment as well as the detailed regulatory pathways involved in the processes of bacterial colonization and development of disease in the host. The use of RNA sequencing to analyze bacterial transcriptomics will allow these detailed relationships to be dissected.

Pneumococcal Strain Variation During Infection

The pneumococcus can undergo genetic changes during the course of an infection. Two isolates of a serotype 3, ST180 were taken from a patient with pneumococcal meningitis [105]. One isolate was grown from the blood and the other from a sample of cerebrospinal fluid (CSF). The two strains were compared by microarray and RNA sequencing, which showed that they had different expression profiles, including a marked up-regulation in the expression of the PatAB transporter in the strain isolated from CSF. Whole genome sequencing identified an SNP present in the regulatory region of the PatAB gene that was associated with changes in gene expression. When the two strains were compared in an animal model of disease, they showed different profiles, with the strain isolated from human blood growing better in mouse blood, while the strain from CSF grew less well in blood but reached higher numbers in the brain. Thus, one SNP can have a marked effect on the virulence of *S. pneumoniae*. The role of recombination also plays a key role in the evolution of pneumococci. Croucher et al. [4] used high-throughput sequencing to analyze 240 isolates of the PMEN-1 (Spain^{23F}-1) strain, and more than 700 recombination events were detected, which frequently affected major antigens, including 10 capsule switch events, one of which accompanied a population shift as vaccine escape serotype 19A isolates emerged in the United States after the introduction of the conjugate vaccine. The evolution of antibiotic resistance was observed to occur on multiple occasions. The study shows how genomic plasticity within the pneumococcus can permit adaptation to clinical interventions on very short timescales.

The environment within patients infected with pneumococcus can vary not only according to clinical interventions (antibiotics, etc.) but also due to underlying conditions. For example, children with sickle cell disease (SCD) have a 600-fold increased risk of invasive pneumococcal disease [106]. The increased risk of infection is due to functional asplenia and complement deficiency, and patients also have altered plasma levels of zinc, iron, amino acids, and carbohydrates [107,108]. These patients are routinely vaccinated and prescribed prophylactic antibiotics. Analysis of strains from carriage and disease in the general population and those isolated from patients with SCD shows that strains from SCD patients have specific adaptations [109]. As well as the expected adaptation to antibiotics and vaccination, strains from SCD had undergone gene loss and intragenic recombination to produce mosaic genes. These events had occurred in four key groups of genes responsible for penicillin resistance, capsule biosynthesis, metabolic pathways, and metal ion uptake. The mutations in genes involved in metal ion uptake suggest that these mutations are beneficial in the SCD host and can only be tolerated in this host environment but not in the normal host. Use of a library of Tn-seq mutants in wild type and SCD mice identified genes involved with aspects of SCD pathophysiology in humans, such as abnormal iron homeostasis, purine metabolism, and complement function. One of the six genes identified by Tn-seq analysis is involved in iron uptake into the pneumococcus. The iron transport complex is immunogenic, and loss of this protein may be advantageous in avoiding the immune response. The iron transport process is probably not required or may

even be detrimental to the bacterium in an iron-rich SCD host. The ability of the pneumococcus to thrive despite the loss of antigenic proteins could compromise protective immunity in specific host environments and could influence targets for new protein-based vaccination in SCD patients. The study of Carter et al. [109] also highlights how analysis of bacterial genome sequences from particular disease groups may yield information on the selective conditions within patients suffering from the disease (in this case SCD).

S. *PNEUMONIAE* AND CLOSE RELATIVES

The pathogenic potential of *S. pneumoniae* is intriguing in the light of its closest relatives, such as *S. mitis* and *S. oralis*, that are rarely associated with disease and are representatives of the commensal microbiota of the upper respiratory tract of humans. Recently, *S. pseudopneumoniae* has been added as a new species, a group of bacteria previously referred to as atypical pneumococci [110–112]. These species are part of the Mitis group of streptococci [113–115]. They are naturally competent for genetic transformation; that is, they can take up DNA and incorporate it into their genome via homologous recombination. It is this property that is the major driving force for genomic diversity within a single species, resulting in a large accessory genome. The molecular mechanism of competence development, a quorum-sensing process based on secretion of the competence-stimulating peptide CSP and its recognition by the TCS ComCD, which is present in all genomes of the above-mentioned species, is well understood (for review, see [116]). However, it is not known under which *in vivo* conditions competence develops, and how frequent gene transfer occurs within and between species. Genomic comparison revealed that in several *S. mitis* and *S. oralis* strains, not all of the 22 essential competence genes described in *S. pneumoniae* are functional or are even absent [117], an indication of fighting genome instability. Nevertheless, *S. mitis* NCTC10712 and *S. oralis* Uo5, which are included in this list, are transformable under laboratory conditions, and it should be noted also that most clinical isolates of *S. pneumoniae* do not develop competence under laboratory conditions, although they contain the entire equipment for competence.

S. pneumoniae inhabits the nasopharynx, whereas oral streptococci reside in the oral cavity. However, signs of interspecies gene transfer, which are obvious from genomic data, indicate that at least occasionally DNA from other species is available. The fact that pneumococcal genomes are significantly larger, approximately 2.1 Mb compared to those of most *S. mitis* or *S. oralis* genomes (~2 Mb), documents a larger accessory genome. Genetic transformation under laboratory conditions results in considerable sequence exchange. After four successive steps of transformation, over 3% of the recipient *S. pneumoniae* genome was replaced by donor *S. mitis* DNA [118]. The recombination events resulted in deletion of one gene, the replacement of a functional gene copy that was fragmented in the recipient strain, and the acquisition of genes not present in the pneumococcal population. The 36 recombination events, spanning between approximately 100 nucleotides up to over 10 kb, clustered in 16 regions throughout the genome. Apparently, gene transfer from *S. mitis* to *S. pneumoniae* also occurs under natural conditions. Phylogenetic trees obtained from all predicted genes among 35 *Streptococcus* spp. revealed clustering of *S. pneumoniae* genes among *S. mitis* genes, which was interpreted as gene transfer from *S. mitis* to *S. pneumoniae* [117]. The size of the regions affected spanned between 116 and 10,600 bp, in agreement with the results reported in the *in vitro* experiment [118].

S. pseudopneumoniae, *S. mitis*, and *S. oralis* are the closest relatives of *S. pneumoniae*. The

finished genomes of members of *S. mitis* B6 [119] and *S. oralis* Uo5 [120] are preferentially used in the following analysis. Despite the relative close relatedness of these species, the overall arrangement of the *S. pneumoniae* genome reveals a striking arrangement, termed X-alignment, when compared to *S. mitis* B6 or *S. oralis* Uo5 (Figure 5.4). Throughout the genome, sequences that are symmetrically inverted with respect to the position of the replication origin or terminus alternate with those that have the same positioning. It is not clear what causes this phenomenon. It has been suggested that inversions might be linked to the replication or termination processes [122]. In *S. mitis* B6, several breakpoints are associated with insertion elements ISSmi1, but the relevance of this observation is not clear [119].

Genomic Comparison—An Overall View

A clear distinction among *S. pneumoniae*, *S. mitis*, and *S. oralis* (Figure 5.5) is obtained by MLST [114,115], which has become the gold standard for analyzing pathogenic bacteria [124] and which is based on sequence comparison of housekeeping genes. MLSA data derived from *S. pseudopneumoniae* strains places this species between *S. pneumoniae* and *S. mitis* [111], but according to MLST analysis the type strain of *S. pseudopneumoniae* as well as strain IS7493, the genome of which is available [125], are found among the *S. mitis* group (Figure 5.5). More genomes of *S. pseudopneumoniae* will be required to clarify the phylogenetic relationship on a genomic basis.

Genomic hybridization of oral streptococci using oligonucleotides based on the *S. pneumoniae* R6 and TIGR4 as well as *S. mitis* B6 sequences revealed an almost smooth transition between these species (Figure 5.6). The explanation is a large accessory genome that circulates among these species but which becomes apparent only by whole genome analysis [126,127] and not by using individual genes that are part of the common core genome.

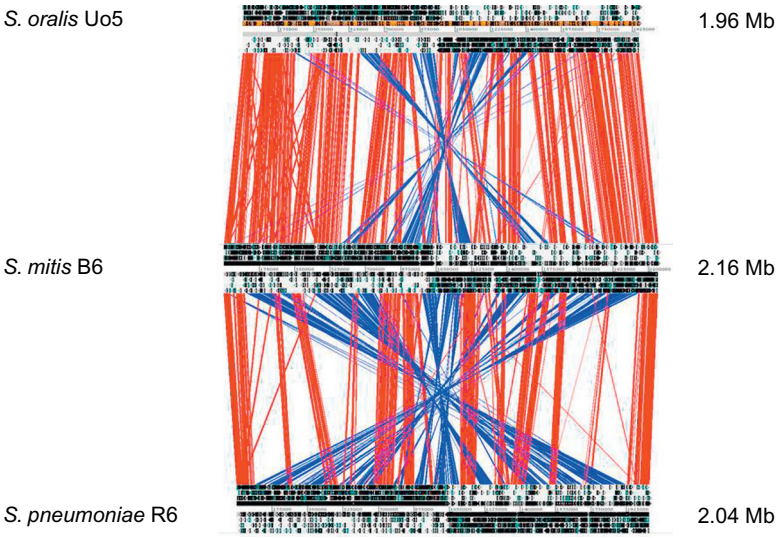

FIGURE 5.4 Genome alignments of the *S. pneumoniae* R6 genome with those of *S. oralis* Uo5 and *S. mitis* B6. The alignments are displayed using the ACT program [121]. Red areas mark regions of the same orientation in both species, blue indicates regions implicated in the X-alignment. Only regions greater than 1 kb are shown.

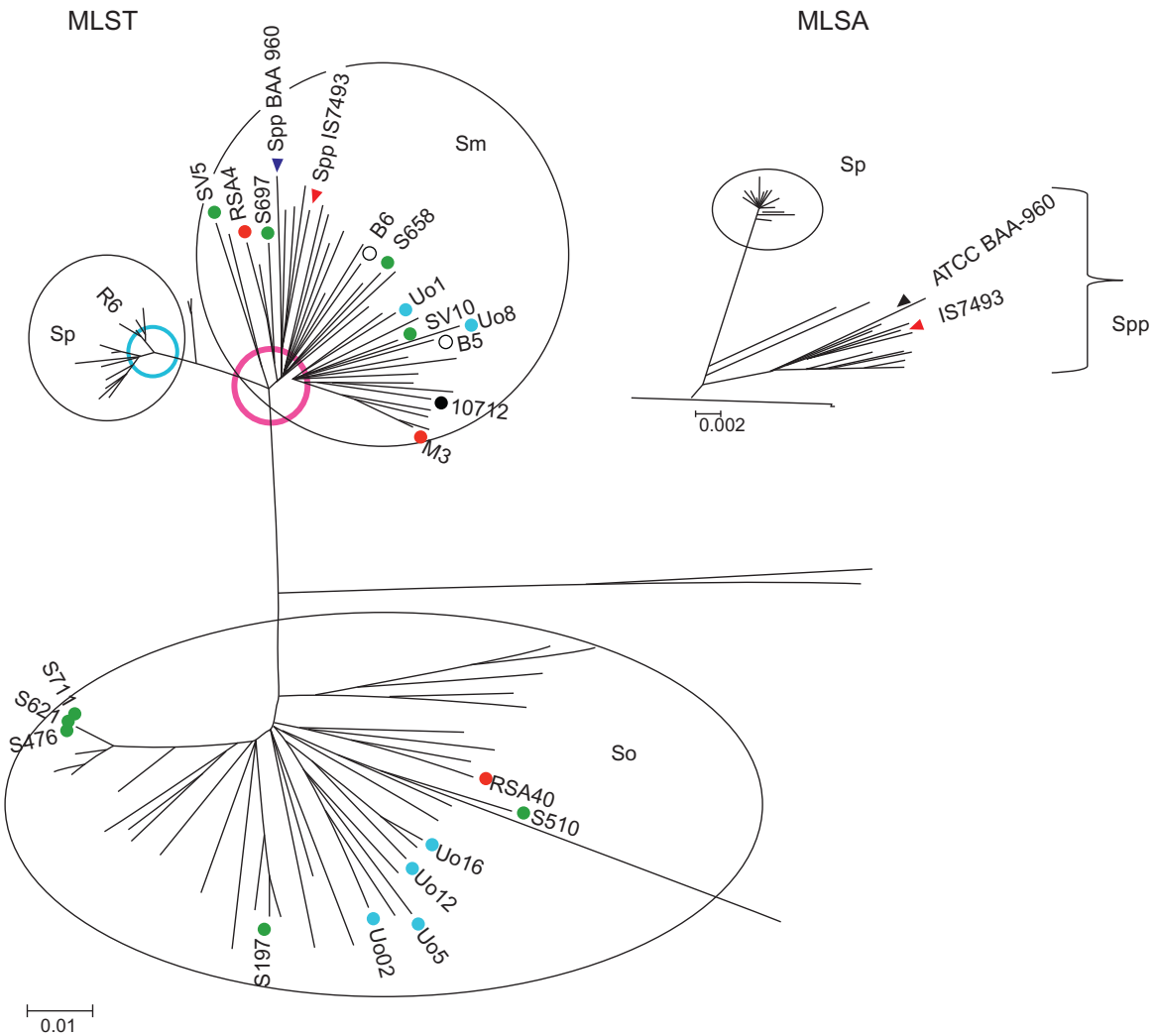

FIGURE 5.5 **Phylogenetic tree of *Streptococcus* spp.** Left: The concatenated sequences of loci used for MLST of *Streptococcus* spp. [115] except that of *ddl* were used for tree construction with the MEGA3.1 program [123]. Sequences were treated as protein coding data. The bootstrap test of phylogeny was chosen as the principal phylogenetic analysis method with the minimum evolution algorithm applied. For bootstrapping the default option of 1050 replicate calculations was chosen. For all other parameters the default options given by the program were used. The color of the dots marks the origin of the strains used in the hybridization experiment shown in Figure 5.6. Red, South Africa; green, Spain; white, Germany; light blue, Hungary; black, reference strains *S. pneumoniae* R6 and *S. mitis* NCTC10712. Sp: *S. pneumoniae*; Sm: *S. mitis*; So: *S. oralis*. For comparison, the type strain of *S. pseudopneumoniae* ATCC BAA-960 and *S. pseudopneumoniae* IS7493 whose genomes are available are included (triangles). Right: multilocus sequence analysis, MLSA [113]. The bracket shows *S. pseudopneumoniae*; Sp: *S. pneumoniae*; others: uncertain. The triangles mark the two *S. pseudopneumoniae* strains used in the MLST tree on the left.

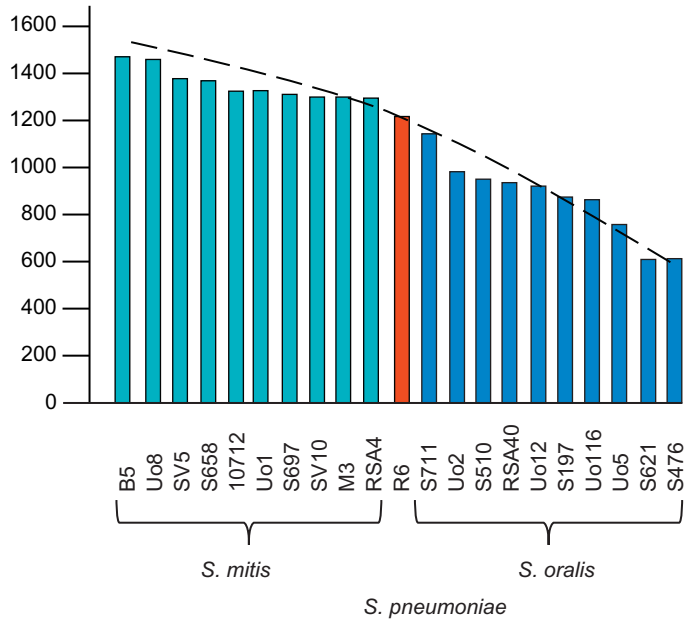

FIGURE 5.6 **Genomic hybridization analysis of *Streptococcus* spp. using a *S. mitis* B6-specific oligonucleotide microarray.** Phage-related gene clusters and mobile elements were not considered. The number of genes giving positive hybridization signals is indicated. The microarray data were evaluated as described [119].

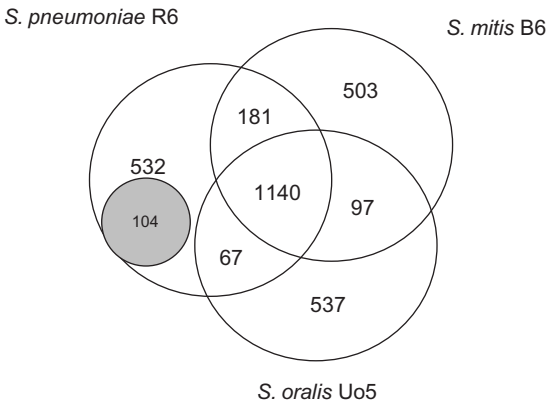

FIGURE 5.7 **Core genomes of *S. pneumoniae* R6, *S. mitis* B6, and *S. oralis* Uo5.** The numbers represent proteins that are 60% identical with a coverage of 70%. When all whole genomes listed in the NCBI microbial genome data base are included, the *S. pneumoniae*-specific proteins drop to 104 (gray circle).

The core genome derived from three strains—*S. pneumoniae* R6, *S. mitis* B6 and *S. oralis* Uo5—includes approximately 60% of the deduced proteins (Figure 5.7), and between 461 and 537 protein encoding genes represent the accessory genome specific to each strain. When the genome of *S. pseudopneumoniae* IS7493 is included, the number of common genes drops to 1105. This number is drastically reduced if more strains of one species are used. When all 26 complete *S. pneumoniae* genomes listed in the NCBI microbial genome database are included, only 104 proteins remain specifically associated with *S. pneumoniae*, with 72 genes being associated with 15 clusters of 2–12 genes. Among them are mostly genes encoding for sugar uptake and utilization systems that are probably a reflection of the special ecological niche

acquired by this species, in addition to components related to its pathogenicity potential, as outlined in the next section. It is clear that with the growing number of genomes all these numbers will need to be adjusted over time.

During the evolution of *S. pneumoniae*, the MLST tree points to two crucial events. One (red circle in Figure 5.5) reveals a common ancestor of both *S. mitis* and *S. pneumoniae*, with *S. pneumoniae* representing one lineage in a cluster of *S. mitis* strains. In fact, each *S. mitis* is approximately as distantly related from each other as from *S. pneumoniae*, and the problem of defining species is obvious. Diversification within the *S. pneumoniae* lineage (blue circle in Figure 5.5) occurred later. It is possible that this second process also reflects the growing population of humans, suggesting that *S. mitis* or *S. oralis* had evolved already in primates. In this context it is curious that specialized serotype 3 clones of *S. pneumoniae* were found in diseased wild chimpanzees in the Thaï National Park, Ivory Coast, which were distinct from human isolates described so far [128,129]. Moreover, *S. oralis* and *S. mitis* could be isolated from primates held in captivity (own unpublished results), suggesting that these oral streptococci might have evolved before *S. pneumoniae* had conquered humans as their optimal host. A curious example of host expansion is the occurrence of type 3 pneumococci that have lost some virulence-associated genes in racing horses [130].

It has been proposed that the three species *S. mitis*, *S. pneumoniae*, and *S. pseudopneumoniae*, arose from an ancient bacterial population that included all *S. pneumoniae*-specific genes [114]. This model was supported recently by genomic analysis of 35 *Streptococcus* spp. Genomic comparison revealed that the average genetic distance from the type strain *S. oralis* ATCC35037 is slightly but significantly larger for *S. pneumoniae* than for *S. mitis*, indicating that the common ancestor was a pneumococcus-like species [117]. The authors provided evidence that interspecies gene transfer occurred mainly unidirectionally from *S. mitis* to *S. pneumoniae*. This includes the import of genes involved in capsular biosynthesis from different groups of streptococci [117], an explanation of the astounding biochemical diversity of the capsule. Similarly, mosaic genes encoding penicillin target enzymes (penicillin-binding proteins) that occur in penicillin-resistant *S. pneumoniae* include sequences that are found in *S. mitis* and *S. oralis* [131,132]. These blocks are larger in *S. mitis* compared to *S. pneumoniae* strains, indicating that they evolved in sensitive *S. mitis* prior to being transferred *to S. pneumoniae* [115]. On the other hand, more genes are decayed in the genome of *S. pneumoniae* R6 or TIGR4 (48 and 62) versus 20 in *S. mitis* B6, excluding IS elements [119], some of them affecting important functions including amino acid biosynthesis, as shown in Figure 5.8. It has been suggested that this functional reduction signifies a "route of no return," that is, fixes *S. pneumoniae* into a current pathogenic lifestyle [119].

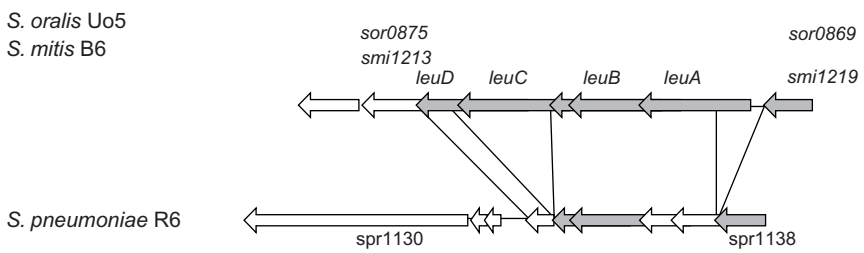

FIGURE 5.8 **Decay of the leucine gene cluster in *S. pneumoniae*.** The leucine gene cluster of *S. mitis* B6 and *S. oralis* Uo5 is shown on top; gray: intact genes.

Virulence Genes of *S. pneumoniae* in *S. mitis* and *S. oralis*

One of the key questions is: Why are oral streptococci not pathogenic; that is, what is specific for *S. pneumoniae* to make it a pathogen?

The choline-containing wall and lipo-teichoic acid (WTA and LTA) have long been believed to be specific features of *S. pneumoniae*. The genes involved in biosynthesis of TA molecules are well conserved in *S. pseudopneumoniae* [125] as well as in *S. mitis*, whereas *S. oralis* strains contain a different cluster, indicating a different TA repeat structure, which is also present in *S. mitis* M3 [133]. All these species also contain CBPs which are associated with TAs by hydrophobic interaction. However, their number is highly variable and differs even among strains of the same species, including *S. pneumoniae* [134]. *S. mitis* B6, with 22 CBPs, represents an unusual example of gene expansion and diversification through gene duplication and recombination events [119]. In contrast, only six CBPs are found in *S. oralis* Uo5, including those that play a principal role in cell physiology: *lytB*, *lytC*, *cbpF*, two paralogues of *cbpD*, in addition to a CBP of unusual repeat structure at the position of *spr0583*/SP_0666, suggesting that they represent the minimum set of physiologically relevant CBPs, and that expansion of CBPs has taken place later in evolution.

The number of LPXTG cell surface proteins that frequently contain repeat motifs predicted to be arranged in coiled-coil structures [119] varies largely not only between species but also within a species. For example, 12 LPXTG proteins are annotated in *S. pneumoniae* R6, 18 in *S. mitis* B6, 17 in *S. pseudopneumoniae* IS7493, and 20 in *S. oralis* Uo5. Many LPXTG proteins of *S. pneumoniae* are found in close relatives as well, the commensal species *S. mitis* [119] and *S. pseudopneumoniae* [111], strongly suggesting that they are important in these commensal species for colonization and interaction with host cells. Also, the *S. oralis* Uo5 genome contains a large number of *S. pneumoniae* homologues [135], similar to other *S. oralis* strains, as suggested from genomic hybridization on a special microarray covering cell-surface proteins and other virulence factors of *S. pneumoniae* R6/TIGR4 and *S. mitis* B6 [135]. The number of homologues detected by microarray analysis, however, represents only a minimal number due to sequence variation of the gene and insufficient coverage by the oligonucleotides. According to genomic analysis, only three LPXTG proteins are common to the four species: the pullulanase gene *pulA* (*spr0247*), an endo-beta-*N*-acetylglucosaminidase (*spr0440*), and an LPXTG protein of unknown function and of different length depending on the number of repeats (*spr0075*). It is curious that LPXTG protein-encoding genes are frequently found in tandem or in close vicinity, indicating either hotspots of recombination or diversification after duplication.

One special example of genome expansion by interspecies gene transfer is the huge serine-rich protein (named MonX, *mon*ster, in *S. mitis* B6 and PsrP, pneumococcal serine-rich repeat protein, in *S. pneumoniae*) and associated genes encoding components involved in export and glycosylation. Serine-rich proteins are common among Gram-positive bacteria [136], but in the Mitis group highly similar clusters occur that differ mainly in the number of glycosyltransferases in the center of the cluster which is greater than 25 kb (Figure 5.9). The reported length of MonX/PsrP varies from 2100 amino acids (aa) to over 4700 aa. However, due to the repeat sequences, the assembly of genome sequencing data is problematic, and Southern blots may be required to confirm its size. In *S. gordonii* it has been described as a platelet-binding protein probably important for oral colonization [137].

There are several other examples of exceptionally large islands specifically associated with only a few strains in one or more species. The *cylM* island (14 kb), encoding for a cytolysin, is

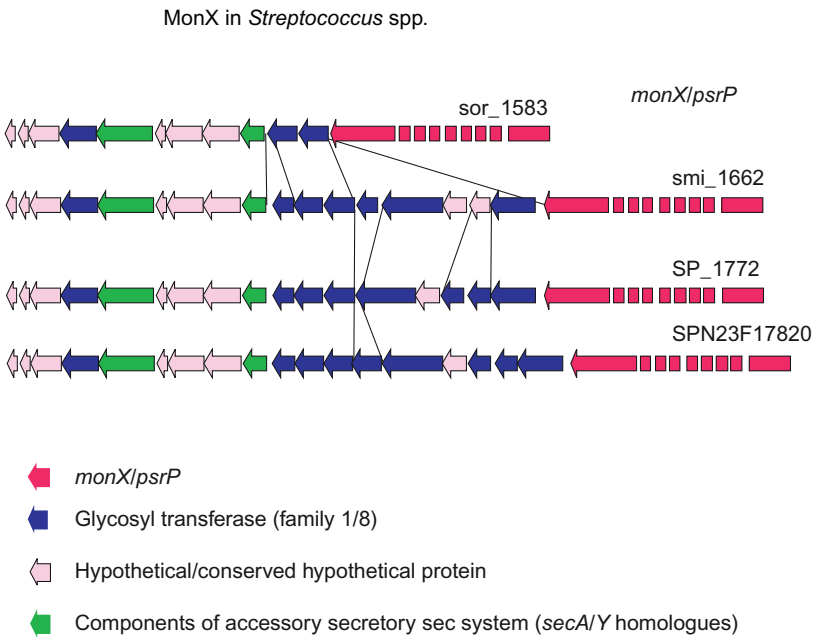

FIGURE 5.9 **The *monX/psrP* cluster in *Streptococcus* spp.** Red, *monX/psrP*; blue, glycosyltransferases; green, components of the accessory sec system (*secA/Y*); pink, hypothetical proteins. Lines indicate regions of similarity.

found exclusively in four *S. pneumoniae* strains. Related genes are common among *Enterococcus*, but not found in other *Streptococcus* spp. Unique to *S. oralis* Uo5 are genes associated with the ESA6 secretion pathway (>45 kb), which is common in mycobacteria [138]. Another island (26 kb), which includes components of the Vtype ATPase, is common among different *Streptococcus* spp. including *S. pneumoniae* TIGR4 [139]. Important in view of antibiotics resistance are Tn*916*-like elements containing the tetracycline resistance determinant *tetM* as mentioned in the previous section, and which occasionally includes erythromycin resistance genes as well (Figure 5.10). *TetM* in *S. mitis* B6 is located on Tn*5801*, which is almost identical to the one described in *S. aureus*, a rare example of interspecies gene transfer between these two species.

Taken together, only a few genes and genomic islands appear to be specifically associated with *S. pneumoniae* in addition to the highly variable capsule cluster: the pneumolysin-autolysin *ply-lytA* island, the CBPs *pspA*, *pcpA*, *pspC* and its variant *hic*, together with the two-component regulatory system TCS06, and the hyaluronidase *hlyA*. In fact, no hyaluronidase activity has been found in *S. mitis* strains [114], but it is present in *S. oralis*. *S. pseudopneumoniae* IS7493 also harbors *ply* and *lytA* in close proximity, but genes in between differ completely from the *S. pneumoniae* island. Occasional isolates of *S. mitis* harbor the Ply gene [114,119,140,141], and the analysis of some *ply*-containing *S. mitis* strains again revealed a genomic environment distinct from that in *S. pneumoniae* [127]. The presence of *hlyA* together with the expansion of sugar-utilizing systems in *S. pneumoniae* might be linked to the conquest of a special ecological niche.

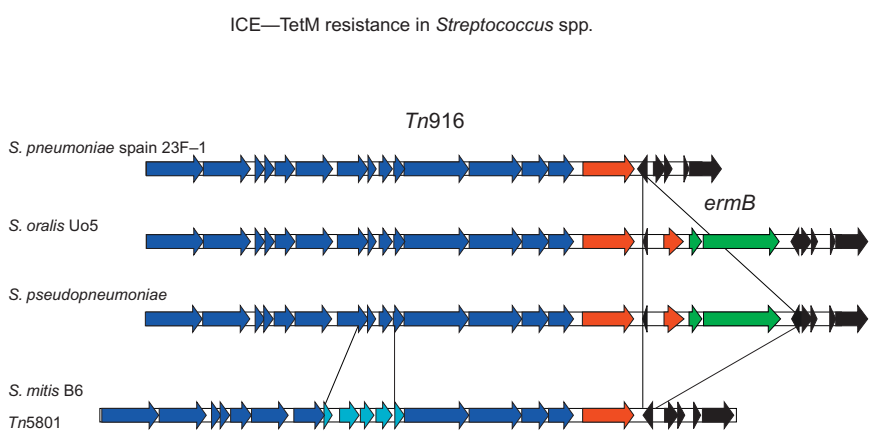

FIGURE 5.10 **ICE elements carrying the tetracycline resistance gene *tetM* in *Streptococcus* spp.** Red, *tetM*; green, *erm* (B); light blue, *S. mitis* B6-specific.

Acknowledgments

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CHAPTER

6

Regulatory Strategies of the Pneumococcus

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TWO-COMPONENT REGULATORS

Transcriptional responses to extrinsic environmental signals require that information from outside the cell be passed to machinery controlling the abundance of gene transcripts inside of the cell. Often bacteria must adapt to the presence of deleterious molecules whose transport into a cell would result in harm or disruption of vital processes; the most common strategy used by bacteria to transduce information while maintaining compartmentalization of molecules is the use of *T*wo-*C*omponent *S*ignal transduction *S*ystems (TCSSs). TCSSs are comprised of a membrane-bound, sensor histidine kinase (HK) and a cytoplasmic response regulator (RR) protein. In general, HKs have an extracellular domain that binds to a signaling molecule outside the cell. This extracellular binding event leads to effects in the cytoplasmic compartment through a phosphorylation cascade. Autophosphorylation of a histidine residue in the intracellular domain of HKs is followed by the transfer of phosphate to the cognate RR. Most RR proteins have DNA-binding domains that recognize specific sequences in promoters of regulated genes. Phosphorylation of the RR then leads to a change in transcription of target promoters through altered DNA binding and/or interaction with RNA polymerase subunits. The regulatory circuit of TCSSs is then reset through the dephosphorylation of the RR, usually by phosphatase activity within the HK protein.

Early genomic analysis of the TIGR4 genome indicated that the pneumococcus possesses one orphaned RR encoding gene (orphaned due to the lack of a closely linked HK-encoding gene) and 13 TCSSs, which were given the nonredundant labels TCS01–TCS13 (with the cognate HK or RR for each system likewise labeled HK01 and RR01, HK02 and RR02, etc.) [1,2]. Researchers attempting to introduce nonfunctional mutations in each of the TCSSs found that 8 of the 13 TCSSs are necessary for full virulence and that the gene encoding RR02 is essential [2]. Despite these clear links to virulence, surprisingly little is known about the majority of TCSSs in pneumococcal biology. The signal that binds to the HK is known for

Streptococcus pneumoniae.
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only two of these systems (TCS12 and 13, ComDE and BlpRH), and in both cases, it was a peptide pheromone that was discovered due to close genetic linkage to the TCSS that senses it. Several of the TCSSs remain unstudied beyond their contribution to virulence.

This section will discuss the role of several of the best-studied TCSSs, concentrating on the role they play in pneumococcal physiology, virulence, and regulation of key genes. In many cases, microarray analysis has been used to more fully define the regulons of pneumococcal TCSSs; however, a discussion of the global regulons for individual mutants is beyond the scope of this chapter and can be found in the primary literature. The two-component systems will be referred to mainly by their TCS number as originally described, although several TCSSs are referred to by multiple names in the literature.

TCS02 (MicAB, VicRH, WalRK) and Cell Shape

Genomic identification of all the pneumococcal TCSSs led to early studies of TCS02 because of the essentiality of the gene encoding RR02, the only essential TCSS gene. Mutations in HK02 were readily recovered, and HK02 mutants in two different serotypes (D39 and a serotype 6 strain) have drastically attenuated abilities to cause pneumonia and survive in the blood [3]. Despite attenuation, no major virulence genes are altered in these strains; the use of alternative genetic tools has revealed roles for TCS02 in competence signaling and maintaining cell shape during division.

Certain environmental signals greatly alter the natural transformability of pneumococci, and one of these signals is microaerobiosis; oxygen limitation in pneumococci leads to an almost complete block of natural competence. Null mutations in HK02 allow for increased expression of competence genes under O_2-limiting conditions; however, strains with these mutations remain non-transformable [4]. Mutating the phosphate-accepting residue in RR02 so that it is no longer phosphorylated by HK02 leads to both increased transcript abundance of competence genes and transformability under low-oxygen conditions. Despite contributing to an important physiologic aspect of pneumococcal biology, the link between HK02, the phospho-status of RR02, and competence does not explain the essential nature of RR02 because both hyper-competent and competence-deficient mutants outside of HK02 are readily recoverable.

Since congenic null mutations in RR02 are unrecoverable, a different strategy was used to explore how a lack of RR02 led to cell death. By using a pneumococcal strain in which RR02 was ectopically expressed, and therefore could be depleted by removing the inducer from the medium, it was observed that reducing the amount of RR02 in the cell leads to major alterations in cell morphology and to the concomitant decrease in expression of several genes [5]. Constitutive expression of one such gene, *pcsB*, allowed RR02 to be deleted. PcsB is a cell-wall hydrolase that is important in septum resolution during cell division and is also encoded by an essential gene. The diminished expression of *pcsB* in the absence of RR02 explains why deletions were non-viable and why depletions of RR02 led to altered cell morphology. HK02 is localized in punctae at the sites of new cell division, and in addition to regulating *pcsB* it has been shown that over-expression of RR02 can alter membrane lipids, increasing the length of fatty acyl chains, probably through altered regulation of fatty acid synthesis genes [6,7]. Together, these observations suggest that TCS02 plays a vital role in cell shape determination through multiple regulatory interactions.

TCS03 (LiaRS) and Antibiotics

The TCS03 system has been studied best in *Bacillus subtilis* (known as LiaRS), in which it

responds to cell-wall damage (as by lysozyme) and to cell wall–active antibiotics that target lipid II, the undecaprenylated intermediate in peptidoglycan synthesis. Bacitracin, nisin, and tunicamycin all induce TCS03 expression, whereas cycloserine, ampicillin, and vancomycin do not [8]. Interestingly, although antibiotics induce TCS03 activity, no traditional antibiotic-resistance genes, such as those encoding antibiotic efflux pumps, are activated by TCS03. This situation suggests that TCS03 does not sense antibiotics directly, but rather senses an intermediate in cell-wall biogenesis that is perturbed specifically by lipid II–interacting antibiotics or other cell-wall stresses that feed back to a common intermediate.

In addition to antibiotic-induced stress, TCS03 also plays a role during natural competence. Competent pneumococci release cell-wall hydrolases that attack non-competent cells, leading to lysis of non-competent organisms and release of DNA that can then be taken up by the competent bacteria; this process is termed fratricide. Competent bacteria are protected from their own hydrolases through the production of an immunity protein. Hydrolases produced by competent cells stimulate TCS03 activity in mutants lacking the correct immunity protein, suggesting that TCS03 is important in competition with other bacteria that release lytic enzymes, and especially in those with diverse strains for which the TCS03-containing pneumococcus does not possess the requisite immunity proteins for protection [8].

TCS04 (PnpRS) and Strain-Specific Regulation

Initial studies of TCS04 attempted to link this system with phosphate sensing because of homology between TCS04 and the PhoPR TCSS of *Bacillus subtilis* and because of linkage to a phosphate import system. Varying the phosphate concentration does not affect TCS04 transcription, suggesting that pneumococcal TCS04 has adapted to sense a signal other than phosphate [9]. Inactivating TCS04 in D39, TIGR4, and a serotype 3 strain led to very little overlap in the regulons controlled by TCS04; this was mirrored in variable effects on virulence [10]. In the absence of a functional TCS04, only TIGR4 is attenuated after intranasal infection, both in the lung and in invasion into the bloodstream. In the D39 background, only a systemic infection is attenuated; and in the serotype 3 strain, virulence is relatively unaffected. Uniquely in the TIGR4 strain, loss of RR04 decreases the transcript abundance of the *pcsA-C* operon, which is implicated in adhesion and virulence [10]. The control of different genes in different strains provides a mechanism for strain-specific effects on virulence and highlights the importance of multiple strain comparisons when studying transcriptional responses.

TCS05 (CiaRH) and Adapting to Lytic Stress

TCS05, along with TCS02 and TCS12, is one of the best-studied TCSSs in Firmicutes and specifically in the pneumococcus. Due in large part to its importance as a human pathogen, many studies on pneumococcal biology have focused on autolysis, a process in which cell-wall hydrolytic enzymes are activated, leading to breakdown of peptidoglycan and bacterial lysis in the absence of active cell-wall synthesis. Autolysis is stimulated by several signals, including antibiotics, bile salts, competence, and cell-wall stress, and the repression of autolysis is a common adaptation that allows pneumococci to evade the sterilizing effects of antibiotics during infection. TCS05 was initially identified in a screen for mutants that were resistant to the cell wall–active antibiotic cefotaxime. The mutant cells were resistant to lysis but were also almost completely non-competent [11]. The mutation

was identified as being a constitutively active HK05 allele. Subsequent studies have identified major roles for TCS05 in resisting autolysis during competence, antibiotic treatment, and cell-wall stress.

After constitutively active HK05 alleles were linked to a reduction in transformation efficiency, the role of TCS05 in competence induction and exit was studied. A combination of microarray studies of TCS05 mutants and solid-phase DNA-binding assays with purified RR05 revealed direct regulation of cell-wall polymer synthesis, including regulation of and binding to genes for peptidoglycan and teichoic acid biosynthesis. Although transcript abundance of competence-related genes is affected by mutations in TCS05, no binding of RR05 to competence loci was observed [12].

Unlike constitutive activation of HK05 alleles, null mutations of TCS05 lead to constitutively expressed competence genes. For instance, the absence of the *comE* gene (encoding the Competence-*S*timulating *P*eptide [CSP] preprotein) leads to a loss of competence in TCS05-null mutants, and supplying exogenous CSP again induces competence [13], suggesting that constitutive competence in TCS05-null mutants is due to overproduction of CSP and not due to loss of repression of *com* genes in the absence of TCS05. On the basis of the deduced RR05-binding site (TTTAAG-5 bp-(A/T)TTAAG), it was predicted that TCS05 controlled the expression of several small RNA (sRNA)−encoding genes, and it was hypothesized that these RNAs linked TCS05 regulation to competence [14]. Indeed, null mutations in multiple sRNAs, as well as mutations of base pairs that were predicted to hybridize to the sRNAs in the 5′ UTR of the *comCDE* operon, greatly reduce the effects of constitutively active HK05 alleles on competence [15]. Taken together, these observations support a model whereby signaling through TCS05 drives the expression of several sRNAs whose targets are competence genes, and it is through the action of these sRNAs on target transcripts that competence is repressed.

Though sRNAs provide a direct link between TCS05 and competence, other phenotypes of TCS05 mutants are due to direct regulation of stress response genes. The best-described instance of TCS05 driving the expression of a stress response gene leading to virulence-associated phenotypes is the regulation of *htrA*, encoding the main DegP homolog in the pneumococcus. After investigations of the role of TCSSs in acute disease, it was found that only TCS05 is necessary in a model of pneumococcal carriage in the nasopharynx of infant rats [16]. Microarray analysis indicates that, in the absence of TCS05, the *htrA* gene is significantly underexpressed. Ectopic expression of *htrA* relieves the colonization defect in a TCS05 mutant and impairs its ability to respond to temperature and oxidative stress [17]. Deletion of *htrA* also attenuates pneumococcal carriage, suggesting that the main role of TCS05 in carriage and stress response is induction of stress response genes, the most important of which is *htrA*.

TCS05 is well established as being necessary for avoidance of autolytic stress responses, and activation of TCS05 leads to repression of competence. The need to balance avoidance of lysis and control of competence has likely limited the types of mutations in TCS05 that are tolerated in nature. Overactivation of TCS05, as in the case of laboratory-isolated constitutive HK05 alleles, can protect pneumococci from lysis in the presence of cell wall−active antibiotics, at the cost of a significant reduction in competence. In clinical isolates of pneumococcus that are either resistant or tolerant to antibiotics, mutations occur in HK05 that, when transferred to sensitive strains, both increase resistance to drugs (albeit at lower levels than laboratory-derived mutations) and allow for inducible competence [18]. This suggests that although mutation of TCS05 is a mechanism of resistance in nature, loss of competence

constrains the types of mutations that can survive in a population.

TCS06 (CbpRS) and Control of Surface Virulence Factors

Attachment and adhesion of pneumococci involve important virulence proteins that are required for multiple stages of pneumococcal disease. Although studies of the role of TCS06 in global gene regulation have found few genes to be affected by the loss of TCS06, it is interesting to note that TCS06 appears to control the expression of two major virulence factors CbpA and PspA, in a strain-specific manner. In the D39 parental background, a deletion in RR06 leads to loss of CbpA, causing a decrease in adhesion to airway epithelial cell lines [19]. In the TIGR4 genetic background, null RR06 mutations do not affect CbpA expression, but deleting HK06, over-expressing RR06, or over-expressing both RR06 and HK06 together increases CbpA expression [20]. In addition, over-expression of RR06 or TCS06 leads to down-regulation of *pspA*, suggesting that although control of specific genes is not conserved, TCS06 regulates attachment and adhesion of multiple pneumococcal strains.

TCS12 (ComDE) and Competence

The mechanism for genetic competence has been studied in detail and is conserved in many Firmicutes, including the pneumococcus and related oral streptococci. For a detailed review of competence regulation see Ref. [21]. In the pneumococcus, competence is triggered by the secretion of a *comC*-derived peptide pheromone termed CSP. After reaching threshold concentrations, CSP is sensed by HK12, which then phosphorylates RR12, driving a burst of expression of several early competence genes, including those of the *comAB* operon (CSP-secretion machinery), the *comCDE* operon, *comX1*, *comX2*, and *comW* [22]. RR12 binds to a direct repeat found in the promoter region of early genes characterized by a CAnTT-16-CAnTT motif. ComX1 and X2 are redundant alternative sigma factors that bind to the conserved octamer TACGAATA; ComW expression stabilizes the ComX proteins, allowing them to drive the expression of late competence genes [23]. Late competence genes include those needed for the DNA-processing and -import apparatus (*cgl* and *cel*), DNA stabilization (*ssbB*), and recombination (*recA*).

A third class of genes was described as being dependent on CSP stimulation but displayed no early burst of transcription (and thus is unlikely to be regulated directly by TCS12) and was not dependent on ComX. This class of genes includes the TCS05 and TCS05-controlled *htrA* gene [22]. As was discussed earlier, TCS05 is thought to be involved in competence, specifically in exiting the competent state, and HtrA is thought to play a role by degrading signaling peptides (such as CSP) through its protease activity. Altogether in the TIGR4 background, 188 genes are differentially regulated by CSP stimulation; surprisingly, of 124 CSP-regulated genes that were deleted, more than half (67) had no discernible role in natural transformation. The contribution of these genes to competence may be niche-specific, or they may contribute to secondary attributes that do not greatly affect transformation efficiency. Supporting the idea that some CSP-induced genes are niche specific, environmental signals that suppress natural competence, such as pH, seem to involve HK12, as escape mutants are no longer sensitive to pH and microaerobiosis [24].

Although major virulence genes are absent from the list of genes differentially regulated by CSP induction, several experimental links between competence signaling and virulence have been described. RR12-null mutants are attenuated in pneumonia and bacteremia/sepsis models of virulence [25]. This observation is

partially explained by decreased expression of the DNA-import pilus, which contributes to adhesion under certain conditions and is also important in sepsis/bacteremia after intraperitoneal infection. During colonization of the nasopharynx, constitutive HK12 mutants and HK12-over-expression mutants are outcompeted by wild-type strains; however, HK12-null mutants or RR12 mutants in which the resistance cassette was designed to minimize HK12 expression, colonized better than wild-type strains [26]. The TCS12 mutation (deletion of both HK12 and RR12) had very little effect on colonization. The colonization phenotypes of competence-regulatory mutants suggest that misregulation of part of the *com* system is more deleterious to pneumococcal carriage than is complete loss of regulation.

TCS13 (BlpRS, AgrCA) and the Preservation of Self

The *blp* locus in pneumococci is a highly variable region that encodes several bacteriocin biosynthetic operons, their cognate immunity proteins, and the quorum-sensing TCS13. Bacteriocins are small, modified bactericidal peptides produced by many organisms that are thought to kill closely associated competing bacteria. Bacteriocin-producing strains are protected from the bactericidal effects of their own bacteriocins through the production of an immunity protein. Immunity proteins are generally specific to their cognate bacteriocins. The production of bacteriocins from the *blp* locus is controlled by TCS13 and the peptide pheromone produced from processing the *blpC* gene product [27]. Similar to competence signaling, the BlpC-derived peptide is exported by an ATP-utilizing export apparatus linked closely to TCS13 and *blpC*. After reaching sufficient concentration, the BlpC peptide is sensed by HK13, leading to RR13-induced expression of the *blp* locus and production of several bacteriocins that presumably kill unrelated strains in the same niche as pneumococci [27]. Closely related pneumococci that have the same allele of the TCS13 can sense BlpC peptide and produce bacteriocins as well as the immunity proteins that will protect them from bacteriocins produced by related neighboring strains.

Competition among organisms that colonize the nasopharynx and upper respiratory tract of humans is thought to be fierce. The human host rapidly decreases the amount of colonizing flora as we move from the oral cavity, which is highly colonized, down the respiratory tract; the lungs are thought to be kept relatively sterile. The nasopharynx, which is the main niche where pneumococcus is found, is generally colonized at lower bacterial density than other niches such as the gut, underscoring the importance of competition between the diverse bacterial inhabitants. The pneumococcus is thought to use the Blp system as a means of competing with nasopharyngeal flora, including divergent pneumococci. Indeed, several alleles of TCS13 have been revealed by genomic sequence analysis in pneumococci, and the BlpC peptide produced by one allele is not sensed by other TCS13/BlpC alleles [27,28]. Therefore, the *blp* locus is thought to be central in discriminating between closely related pneumococcal strains, providing a mechanism whereby a restricted genetic lineage of pneumococci can both sense itself and deploy an arsenal of bacteriocins to eliminate more-divergent competing organisms.

STAND-ALONE REGULATORS

Unlike two-component regulators, so-called stand-alone regulators have both the DNA-binding activity and signal recognition domains in one protein, although in many cases how stand-alone regulators sense signals is unknown. These regulators generally function autonomously from other regulatory proteins;

however, some have evolved to use co-regulators to integrate multiple signals. These regulators generally control dedicated metabolic pathways whose genes are organized into operons. Often the transcriptional regulator responds to end products, inputs, or intermediates from the reactions carried out by enzymes encoded by the operon and only regulate a small number of genes; for instance, one class of familiar stand-alone regulators is linked to carbohydrate utilization operons such as maltose or lactose repressors. These regulators bind to the carbohydrate molecules that are metabolized by enzymes within the operon, controlling the amount of enzymes produced in direct response to carbohydrate availability. Other stand-alone regulators control many genes throughout the genome, coordinating more complex phenotypes. In some cases, the signal controlling these regulators remains elusive because they control genes with disparate predicted functions and contain sensory domains without known specificity. Several stand-alone global regulators contribute to virulence in the pneumococcus.

CcpA and Global Regulation in Response to Carbohydrate Availability

CcpA, or catabolite control protein A, is the main global regulator of carbon catabolite repression in Firmicutes (for a review of CcpA function and carbon catabolite repression see [29]). CcpA binds to *C*atabolite *R*esponse *E*lements (CREs) in the promoter of regulated genes; binding to CRE sites is altered by association with a phosphorylated form of the high-affinity carbohydrate-import accessory protein Hpr or by association with the metabolites fructose-1,6-bisphosphate or glucose-6-phosphate. In this way, CcpA can sense the import machinery status through Hpr-P and the intracellular metabolite pool status through binding to glycolytic intermediates. In general, increasing availability of glucose leads to repression of CcpA-controlled genes; however, transcription of a small subset of genes is induced by CcpA-CRE interactions. Microarray analysis of CcpA-null mutations in the D39 background, comparing mutants and wild-type bacteria grown in glucose or galactose, revealed that 14–19% of the predicted ORFs are differentially regulated in a CcpA-dependent manner under these conditions [30]. Less than half of those transcriptional units have a conserved CRE site in the promoter region, suggesting that CcpA controls a regulon with many secondary regulators such as TCS07 and TCS12, which are both affected by CcpA. The majority of CcpA-controlled transcripts are more abundant in the mutant strain and predicted to be involved in metabolic processes, confirming that in pneumococcus as in other Firmicutes, CcpA is largely a repressor of transcription when bound to CRE sites [30].

During virulence, CcpA plays a critical role in maintaining the pathogenicity of the pneumococcus. Null mutations in the D39 or TIGR4 backgrounds lead to attenuation in a sepsis model for D39 and defects in colonization, pneumonia, and blood survival in TIGR4 [31,32]. Attenuation of virulence due to a loss of CcpA likely involves two distinct mechanisms. First, CcpA affects the expression of important virulence factors such as NanA, NanB, and PcpA, which participate in adhesion, remodeling of host receptors, and invasion past tissue barriers [30]. Second, the core metabolism of the pneumococcus is altered in a CcpA mutant: Different fermentation products are produced, growth is slower, and biomass accumulation is decreased [30]. This alteration of pneumococcal physiology, the incorrect production of virulence and adhesion molecules, and inefficient use of a preferred growth substrate such as glucose likely combine to cause the attenuation seen in different infected tissue types.

Pilus, RlrA, Mga-spn (MgrA), and What to Do with New DNA

The discussion of transcriptional regulators thus far has focused on regulators conserved among the majority of sequenced pneumococcal isolates. Natural competence leads to the rapid exchange and introduction of new DNA and is a main mechanism leading to generation of distinct pneumococcal strains. Clustered, regularly interspaced, short palindromic repeat (CRISPR) loci, a major bacterial defense against phage, are not present in the pneumococcus, highlighting the strong selective pressure for CRISPR loss and retention of robust genetic exchange. Despite the obvious advantages of being able to acquire new gene functions, the introduction of foreign DNA into a recipient genome presents several problems. Notably, unless the donor DNA is derived from a highly related organism, the recipient is unlikely to contain regulators able to control expression of new genes. Not only is unregulated transcription inefficient and wasteful of cellular resources, but in the case of pathogens, the uncontrolled production of antigens can also lead to attenuation of virulence through increased immune recognition or altered tropism. In the pneumococcus, several pathogenicity islands are present in only a subset of strains, raising the possibility of recent acquisition of these genes in certain pneumococcal lineages. The pilus locus, present in a third of isolates, is one such region: Multiple regulators have converged on this locus to control its expression, using regulatory networks already programmed for pneumococcal virulence [33].

The *rlr* locus, encoding the pilus structural genes, sortase genes for pilus biogenesis at the cell surface, and a gene encoding a dedicated transcriptional regulatory protein, is important for adhesion and invasion of respiratory epithelial cells and contributes to pneumonia and nasopharyngeal colonization. The expression of the pilus is dependent on the regulatory protein RlrA, encoded by the transcriptional regulator gene in the *rlr* locus [34]. In the absence of RlrA, pili are not expressed. RlrA itself is a member of the RofA-like family of regulators important in coordinating transcription of virulence factors in other streptococci. RlrA expression increases transcription from four promoters throughout the *rlr* locus, suggesting that RlrA is a transcriptional activator [34]. RlrA also activates transcription of its own promoter; this autoregulation of *rlrA* leads to two distinct populations of pneumococci, high- and low-level pilus expressers [35]. Although the signal for RlrA activity is unknown (as it is in most RofA family members), growth in normal medium yields small populations of genetically identical pneumococci that are preadapted for conditions in which pilus expression is needed.

If steady-state regulation of the pilus locus leads to two populations of pilus-expressing bacteria, then there remains the question of how further choices about pilus expression are made during virulence. The Mga family of regulators is widespread and centrally important in the regulation of virulence genes in several pathogenic streptococci. Studies of the pneumococcal Mga-like protein Mga-spn (MgrA) link this transcriptional regulator to repression of the pilus locus in the piliated TIGR4 background [34]. It is unclear whether repression of the *rlr* locus is due to Mga-spn repression of the *rlrA* gene or of all promoters in this locus; however, the result of Mga-spn expression in the pneumococcus is reduced pilus expression, suggesting that Mga-spn signaling can bias the distribution of pilus expressers toward the non-piliated end of the spectrum. In the non-piliated strain R6, the Mga-spn protein activates a four-gene operon that is divergently transcribed from the *mga-spn* locus [36]. The function of this operon is not known, but its existence suggests that Mga-spn has adapted to regulate strain-specific genes in piliated and non-piliated pneumococci.

In addition to RlrA and Mga-spn, TCS03, TCS06, and the metalloregulator merR are linked to regulation of the pilus locus [37]. Remarkably, null mutations in HK03, HK06, and merR all increase pilus expression in an *rlrA*-dependent manner, suggesting that all repress *rlrA* expression [37]. Therefore, pilus expression is predicted to remain low until an appropriate signal allows for RlrA production, which leads to increased *rlr* locus transcription. TCS06, as already described, is dedicated to the control of virulence genes in the non-piliated D39 background, and Mga family members appear to be dedicated to virulence regulation in other streptococci, so after acquisition of the *rlr* locus, it appears that pneumococci put the pilus under the control of regulatory mechanisms that would tune its expression with that of other important virulence adaptations. TCS03 and MerR appear to be dedicated to environmental stress responses, perhaps allowing for fine tuning of pilus expression in specific virulence niches in which those signals (e.g., cell-wall and oxidative stress) are likely encountered. Taken together, it is apparent that the pneumococcus quickly adapts foreign DNA to its own endogenous transcriptional control mechanisms.

METAL-DEPENDENT REGULATION

A common theme in bacterial pathogens is the tight regulatory control of metal influx and efflux systems to maintain optimal intracellular levels in the midst of varying environmental bioavailability. The ability to precisely control the uptake and efflux of metals is a critical aspect of pneumococcal pathogenesis. Although metals are necessary cofactors in many cellular proteins, either excess or limiting amounts of metals can be detrimental to the cell. Metal concentrations and bioavailability also vary greatly at different body sites, particularly between the mucosa and the bloodstream, as well as between a naïve and an infected host [38]. For example, the concentration of zinc is more than 10-fold greater in lung tissue than in serum. Pneumococci are adept at thriving in both these environments and, hence, have the requisite means for nutrient acquisition in these niches. As such, the pneumococci encode a number of metalloregulators that precisely control both uptake and efflux of metals, as well as numerous accessory loci at various stages of infection. Information gleaned from global transcriptional profiling of pneumococci under metal-limiting and metal-replete conditions have provided considerable insight into how the pneumococcus adapts to the dynamic nature of transition metal bioavailability. Of particular note is the observation that many of the pneumococcal metalloregulatory proteins can bind multiple metals under physiologically relevant conditions, which results in altered binding affinities for their cognate promoters [39]. It is important to consider not only the overall intracellular and extracellular metal content but also the ratios of transition metals to each other. It should also be stressed that the bioavailable pool of metal within the cell can vary considerably from the total cellular content. Such factors are critical for understanding the specificity and potential cross talk between metal-dependent regulatory factors.

Manganese

One of the most important elements for pneumococci to acquire is manganese, which plays roles in the antioxidant defenses, metabolic pathways, and capsule production of the pneumococcus [40]. The importance of manganese uptake is underscored by the efficiency of its import, maintaining intracellular levels equivalent to those of zinc, even when the former is present in 50-fold excess in the media [41]. Manganese plays a number of roles in pneumococcus, as does the manganese-dependent superoxide dismutase, providing defense against superoxide radicals.

Manganese concentrations vary substantially between the bloodstream and mucosa, and levels in the bloodstream are altered during pneumococcal infection [38]. Uptake of manganese is mediated by the PsaBCA transporter, directly regulated by the PsaR repressor [40]. The PsaR protein is a member of the DtxR/MntR family of metalloregulatory proteins, which typically function to mediate control of iron or manganese uptake. Binding of PsaR to the respective promoters is dependent upon manganese; however, its release is mediated by zinc [42]. Competition for manganese and zinc also extends to uptake, with both manganese and zinc binding the PsaABC complex, but only manganese being translocated [43]. Hence, there is considerable cross talk between these two transition metals in pneumococcal biology.

Global transcriptional profiling of the PsaR knockout indicates that regulation extends beyond the PsaABC manganese uptake system in pneumococcus. In addition to the PsaABC complex, evidence suggests that PsaR regulates a number of important adhesion molecules, including the choline-binding protein CbpA and the regulator RlrA, which mediates pilus expression [40]. The genes controlled by PsaR and the role of PsaR in pneumococcal pathogenesis also appear to vary significantly between the various strains of pneumococci [44]. Although able to effectively colonize the nasopharynx, mutants defective in the PsaR regulator show dramatically reduced burden in murine lungs during infection [40]. This deficiency underscores the central importance of manganese in pneumococcus.

Copper

Pneumococci also encode an operon dedicated to the efflux of copper from the bacterial cytoplasm. Transcriptional control of the *cop* operon in pneumococci is under the tight regulatory control of CopY, which functions as a repressor of the *cop* operon by binding the promoter region of the operon [45]. Data obtained from other organisms show that once CopY binds copper, its affinity for DNA is greatly decreased, thus allowing transcription of the operon [46]. The affinity of CopY for copper has been estimated to be in the zeptomolar range (10^{-21}), as determined in *Escherichia coli*, equivalent to less than one molecule of copper per bacterium, supporting the case for free intracellular copper being extremely detrimental to bacteria [47]. In accordance with this, the *cop* operon is highly up-regulated in response to copper stress [45]. Although no copper-dependent proteins are known to exist in pneumococci, intracellular copper is found at ratios of 1:10 in respect to the amounts of manganese and zinc, potentially due to non-cognate import via another metal-uptake system. Interestingly, this regulator also appears to have interactions with zinc, as the *cop* operon is repressed by supplementing the media with zinc [45]. Hence, the pneumococcus appears to benefit from having the appropriate ratios of copper and zinc.

Zinc

Another central player in metal-dependent gene regulation in pneumococcus is zinc. Transcriptional regulation in response to zinc is a complex process in pneumococcus, with multiple regulators responding to perturbation of zinc availability. Whereas other Gram positive organisms such as *B. subtilis* and other streptococcal species encode a Fur-family regulator, Zur, the pneumococcus relies upon AdcR, which binds zinc and controls a number of important cellular processes [48]. This protein was the first member of the MarR family of repressors to be implicated in metal sensing. This regulator has both repressive and activating functions, mediated by direct DNA binding, and functions as a homodimer [49]. Similar to what is observed with PsaR, AdcR

also controls the expression of its corresponding uptake system, AdcBCA, a Zn^{2+}-dependent ABC-uptake system, as well as that of AdcAII, a Zn^{2+}-binding lipoprotein [49,50]. One class of proteins controlled by this regulator includes the pneumococcal histidine triad (Pht) proteins, which partially block complement deposition on the bacterial surface [51]. The precise control of these surface-exposed proteins is crucial because constitutive expression could lead to enhanced immune recognition and bacterial clearance. Acknowledgment of this importance is evidenced by the interest in developing the Pht protein family as a component of a protein-based vaccine [52].

Pneumococcus also encodes SczA, a TetR family regulator that is responsive to fluctuations in intracellular zinc concentrations. SczA binds zinc and activates transcription of an adjacent operon encoding a MerR regulator, a putative zinc-dependent alcohol dehydrogenase, and the zinc-efflux protein CzcD [48]. It is thought that this system is primarily operative during high-zinc conditions to mediate expression of the downstream zinc-efflux pump CzcD. The existence of both SczA and AdcR raises the question of why pneumococcus would encode two seemingly redundant zinc-dependent regulators. Biochemical data suggest that these two regulators have distinct affinities for zinc, whereby they would be expected to have non-overlapping functions in regulation based on the precise intracellular concentrations of zinc. This situation would allow pneumococci to have more refined transcriptional control over a greater range of intracellular zinc concentrations, underscoring the importance for the pneumococcus of maintaining precise control of this critical element.

Iron

The pneumococcus also orchestrates distinct transcriptional responses to control the uptake of iron from various sources within the human host. Many of the studies of iron-dependent gene regulation in pneumococci have focused on RitR, originally identified as being an orphan RR during the initial characterization of the TCSSs of the pneumococcus [1,2,53]. Transcriptional profiling has identified approximately 50 genes having significantly altered transcript abundance when this regulator is absent. These include multiple classes of proteins involved in iron uptake (PiuA), iron-storage peroxide-resistance proteins, and an iron-binding alcohol dehydrogenase [54,55]. Consistent with its role in iron homeostasis, RitR is preferentially expressed under iron-replete conditions [56]. The expression of SpxB, which generates H_2O_2, is also higher in the *ritR* mutant under iron-replete conditions. Deletion of *ritR* also appears to have some role in the expression of the manganese importer PsaA, although whether this is a function of direct control or an indirect effect mediated by the pleiotropic effects of the knockout remains unknown. In the D39 strain background, deletion of *ritR* led to enhanced bacterial outgrowth, potentially due to upregulation of the iron-uptake systems [57], a result that is in contrast with previous findings, potentially due to intrastrain variation, as a serotype 3 isolate was used in the initial virulence determinations [2]. This is another example of strain to strain variation for the contribution of a regulatory system to pathogenesis.

Calcium

The importance of calcium in signaling is well characterized in eukaryotes, but the role of this element in microbial signaling remains only partially understood. One of the central roles played by calcium in pneumococci is the regulatory control of various stress-induced chaperones, including DnaK and GroEL, through the activity of the HrcA repressor. Interestingly, there does not appear to be any

direct binding between HrcA and calcium; rather, the hydrophobicity of the protein is enhanced under increasing concentrations of calcium, which is thought to facilitate the interaction of the repressor with GroEL, which is required for DNA binding and, hence, repression of the cognate chaperone loci [58].

Pneumococci encode a dedicated calcium efflux system, CaxP/MgtA, which experimental data indicate functions as a calcium/magnesium antiporter [59,60]. Although no protein-based regulator of this system has been identified, RNA-seq and sequence analysis indicate that a regulatory RNA may exist immediately upstream [59,61]. However, this predicted riboswitch is not homologous to characterized divalent cation−sensing regulatory RNAs upstream of MgtA; therefore, how this system is controlled at the transcriptional level remains uncharacterized. Given the importance of HrcA and CaxP/MgtA in pneumococcal pathogenesis, a clearer mechanistic understanding is warranted.

OXIDATIVE STRESS REGULONS

The pneumococcus produces prodigious quantities of hydrogen peroxide via the action of the pyruvate oxidase SpxB, lactate oxidase, and carbamoyl phosphate synthase (CarB). The strategies used by pneumococcus to deal with oxidative stress are likely to be distinct among the streptococci, as many of the proteins characterized in other species have no discernible homologs in the pneumococci [62]. Because pneumococcus must resist endogenously produced oxygen radicals generated through metabolism as well as oxidative damage from host sources, understanding the regulatory pathways involved in this response is of critical importance. Transcriptional control of *spxB* is mediated by SpxR, which was identified in a genetic screen for regulators of *spxB* that also implicated *cis*-acting repeat domains and belongs to the helix-turn-helix GntR regulator family [63]. The precise cues sensed by SpxR to control *spxB* expression remain unknown. There also appear to be posttranslational effects on SpxB expression that are mediated by intracellular manganese abundance [64]. There is a considerable gap in our knowledge regarding the signals sensed by this regulator and the control of this process in pneumococci.

The pneumococcus encodes three putative MerR transcriptional regulators, which can sense a myriad of physiological changes, including those involving heavy metals, antibiotics, and oxidative stress [65]. Thus far, only one member of this family has been characterized in the pneumococcus, Sp1856, also referred to as NmlR on the basis of homology to the *Neisseria* protein of similar function [65]. Both the gene itself and its main regulatory target, an alcohol dehydrogenase immediately downstream of the regulator, are required for bacterial resistance to nitro-oxidative stress [66]. This regulator may function as a general aldehyde-responsive system, potentially protecting the bacteria from these reactive groups generated during metabolism [67]. The precise mechanism underlying how MerR/NmlR senses these groups remains unknown, although evidence suggests that the reduction of only a single cysteine to a thiol is required to mediate DNA binding [68]. Deletion of this system led to defects in systemic virulence in the D39 strain background, yet had minimal effects on virulence in the TIGR4 background, although both bacterial and mouse strain differences among the studies could have contributed to this discrepancy [37,66].

REGULATORY RNAs

Pneumococci encode not only protein factors but also numerous sRNAs, with only a small subset having an experimentally determined functional role. Regulatory RNAs targeting gene

expression in streptococci can function at both the transcriptional and translational levels [69] in myriad ways, including binding the promoter regions of genes, mediating transcript stability, and directly interacting with proteins to modulate activity. The number of sRNAs putatively identified in pneumococci and other bacterial pathogens has greatly expanded in recent years because of studies using high-throughput sequencing technologies and more robust bioinformatics predictions. A number of techniques for identifying sRNAs have been developed and applied to various strains of pneumococcus, including bioinformatics approaches, tiled microarrays, and RNA sequencing–based approaches [70–72]. However, there is an overall paucity of data for direct regulatory links between the sRNAs and their cellular targets. Predictions made by computational methods such as Target RNA have met with some success, though all predictions must be experimentally confirmed due to the possibility of false positives.

Given the amount of the genome dedicated to these putative regulators, there has been great interest in understanding the functional role of these sRNAs during pneumococcal infection. Transposon mutagenesis coupled with next-generation sequencing (Tn-Seq) has proved fruitful in the putative identification of sRNAs involved in various aspects of pneumococcal disease, including a number of sRNAs predicted to influence bacterial fitness in the nasopharynx, lungs, and bloodstream [61]. Although the precise functional roles of these sRNAs in virulence is unclear, the sheer number of sRNAs predicted to be attenuated in murine models (28 in lung models, 26 in nasal colonization models, 18 in blood models) implies that sRNA-mediated regulatory pathways are an indispensable aspect of how pneumococcus survives within the human host. In addition to transposon-based screens, targeted deletions of multiple sRNAs display prominent virulence defects [61].

Probably the best-characterized noncoding RNA family in the pneumococci are a set of five genes that are specifically controlled by the CiaRH (TCS05) two-component regulatory system and were the first sRNAs described in the pneumococcus. These sRNAs were initially detected and designated as being cia-dependent small RNAs (csRNAs) [73]. Deletion of these sRNAs has pleiotropic effects on pneumococcal physiology, including autolysis and the development of competence [14]. Interestingly, a number of the pleiotropic phenotypes initially attributed to the CiaRH two-component system, including resistance to β-lactam antibiotics and competence development, could be attributed to the csRNAs. By using a combination of computation approaches and transcriptional fusions, the link between CiaR and competence has been established as being csRNA-mediated control of *comC* [15]. Such a regulatory strategy would allow for the cell to fine tune the regulatory cascades initiated by two-component systems to be more specific to a particular stimulus, and may partially explain some of the variation in the regulatory networks under the control of the two-component systems. Given the repertoire of sRNAs encoded by pneumococci, they are likely to play a number of diverse roles in the cell, although the precise functions of only a few have been studied in detail.

INTERSPECIES SIGNALING AND REGULATION

In nature, no individual bacterium is an island, but rather just one player in a dynamic, complex community consisting of multiple species interacting both competitively and synergistically. It stands to reason that pneumococci respond not only to environmental cues but also to signals produced by other bacteria within this shared niche. One means by which interspecies communication could influence pneumococcal gene regulation is through the

luxS quorum-sensing system, which is utilized by both Gram negative and Gram positive bacterial species, including many inhabitants of the nasopharynx that frequently co-colonize alongside the pneumococcus. This widespread system responds to autoinducer-2 (AI-2), which is synthesized by LuxS, an *S*-ribosylhomocysteine lyase. In the pneumococcus, the luxS system plays important roles in biofilm formation, competence, and virulence [74]. Due to the prevalent nature of this signaling system, the pneumococci could respond to AI-2 produced by other bacterial species, similar to what has been observed with other colonizers of the nasal passages [75]. Such signaling could enhance biofilm formation by both species, thus being mutually beneficial, similar to what is observed under *in vitro* co-culture experiments with the pneumococcus and other bacterial species resulting in enhanced biofilm production [76]. Another system relies upon short hydrophobic peptides and Rgg regulators to mediate interspecies communication between various species of streptococci [77]. Although this bidirectional signaling and accompanying transcriptional response has not been demonstrated in pneumococci, the rgg homolog, short hydrophobic peptide, and binding site in the genome are conserved, indicating that this system is likely to be active among pneumococci as well. Hence, pneumococci have the capacity to both sense extracellular signals from diverse bacterial species and adjust their transcriptional response appropriately.

ROLES OF REGULATORS IN DISTINCT HOST PATHOGENESIS MODELS

One of the hallmarks of pneumococcal infection is the diversity of disease manifestations corresponding to that of the many tissue types in which this organism can thrive. Robust murine models exist for nasal colonization, sinusitis, otitis media, pneumonia, sepsis, and meningitis. Our understanding of the contribution of the various regulatory cascades on pneumococcal virulence has been greatly aided by both global transcriptional profiling of invasive disease, signature-tagged mutagenesis, and Tn-seq screens. These screens have demonstrated the importance of various regulators and the associated pathways in the various host niches. An overview of the current understanding of these regulators and their roles in infection models is summarized in Figure 6.1, with predictions summarized from a number of genetic screens [78–82].

CHALLENGES OF CROSS TALK AND DIVERSITY

A major challenge to understanding the role of the various regulatory cascades on pneumococcal disease is the amount of genetic diversity among pneumococci. Being naturally competent, the pneumococcus is adept at incorporating and recombining environmental DNA. Individual strains of pneumococci typically encode approximately 2200 genes out of the estimated 4700 genes available in the pneumococcal supragenome [83]. This diversity substantially complicates the applicability of observations made in laboratory isolates of genetically diverse strains driven by the frequent recombination events observed in pneumococcal population genetics. All sequenced pneumococci encode the 13 two-component systems and the metalloregulators described in this chapter. However, the contribution of these systems to virulence and their regulatory targets can vary considerably between strains. The most obvious scenario is the situation whereby the operons under control of the respective regulators are absent from a subset of strains, which can easily be the situation given the inherent diversity of pneumococci. Putting our understanding of pneumococcal gene regulation in the context of the broader

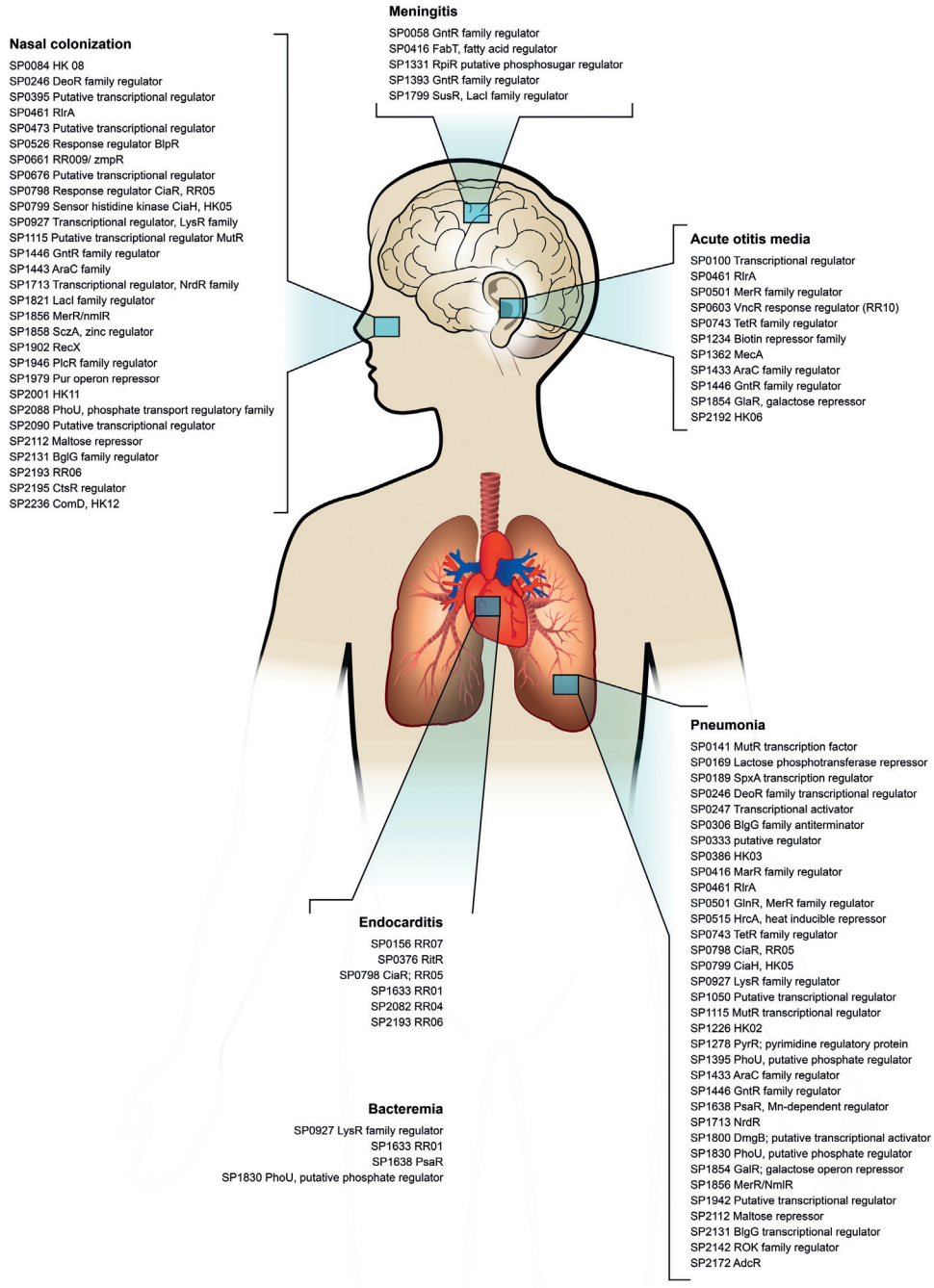

FIGURE 6.1 **Pneumococcal regulators predicted from genetic screens to play roles in various host niches.** The TIGR4 annotations for the individual genes are listed as well as the putative function or predicted transcriptional family. Predictions of contributions to infection were taken from references indicated in the main text.

pneumococcal population apart from the few relatively well-characterized laboratory isolates remains a fundamental challenge in the field.

The attractiveness of regulatory networks as antimicrobial targets remains high due to the drastic attenuation observed in regulatory mutants; however, as was discussed earlier in this section, within infected tissues, individual virulence genes are controlled by multiple regulatory networks. Whereas genetic inactivation of a regulator can cause arrest of disease progression at any step, resulting in attenuation, when treating a disease such as pneumonia, otitis media, or meningitis it is necessary to target a regulator that is required for the disease, and not a regulator that is involved in earlier steps in disease progression but which is dispensable once the pneumococcus arrives at its ultimate destination. Complex interactive networks typically characterize bacterial regulatory networks, mechanisms of increased stringency or promiscuity to either limit or enhance cross talk with other pathways [84,85]. The pneumococcus must have the capacity to integrate multiple, competing environmental cues into an appropriate cellular response. Many of the regulatory cascades characterized thus far have been established under tightly controlled *in vitro* conditions with a single stimulus. This is not representative of what occurs naturally, as within the human host the pneumococci would need to adjust their lifestyle according to multiple, dynamic signals from the host environment. One mechanism by which this can occur is when multiple HKs can phosphorylate the same RR, as is observed in *B. subtilis* [86]. In the pneumococcus, the kinase of TCS02 phosphorylates not only the cognate RR, but also the RitR orphan RR [87]. It is also becoming increasingly evident that tight control of the phosphorylation level of the RRs is tightly controlled to limit potential cross talk [88]. Potential competition of multiple RRs at a given locus will be dependent upon the dynamics of both promoter binding and phosphorylation kinetics, as well as the relative expression levels of the individual regulators, further complicating our understanding of competition and synergy in regulatory pathways. Thus we must understand the hierarchy of regulation, how multiple signals are integrated on a genomic level to control genes, and in the case of individual genes controlled by multiple regulators, what interactions at the promoter lead to ultimate control of the gene.

CONCLUDING REMARKS

It is evident from both the conservation and the predicted roles in the various models of pneumococcal infection that transcriptional regulators play a critical role in disease development by the pneumococcus. The continued challenge remains a precise understanding of the genes under control of these regulators, as well as the extent of cross talk between these systems. While gene deletions and profiling efforts have provided considerable insight into these networks, in many cases the precise signals these systems are responding to remain unknown. As the pneumococcus typically resides as a commensal in the nasopharynx, understanding the precise signals and the corresponding transcriptional response that incite invasive pneumococcal disease is vital for a mechanistic understanding of the virulence strategies deployed by this major human pathogen.

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CHAPTER

7

Pneumococcal Genetic Transformation During Colonization and Biofilm Formation

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INTRODUCTION

Based on the high incidence of pneumonia in the late nineteenth century, and the early identification of what we now refer to as *Streptococcus pneumoniae* (the pneumococcus) as a major cause of this infection, the pneumococcus has played a prominent role in our understanding of basic principles in pathogenesis, immunology, diagnosis, treatment, and prevention of bacterial disease. However, the pneumococcus has also played a central role in the development of modern molecular biology. Early in the twentieth century, Stryker identified rough, unencapsulated colonies after serial passage that were avirulent in mice [1]. While studying this phenomenon, Fredrick Griffith was the first to observe and describe natural genetic transformation of pneumococci in 1928. He found that when mice were injected with a mixture of live, unencapsulated (rough) type II pneumococci and heat-killed, encapsulated (smooth) type I pneumococci (either of which by itself failed to kill mice), the mice died and live encapsulated organisms with type I capsule were recovered from the blood. He named this phenomenon "transformation" [2]. Griffith's experiments were soon confirmed by Oswald Avery, who over the next several years investigated this phenomenon in detail, and in 1944, together with Colin MacLeod and Maclyn McCarty, proved that DNA was the "transforming principle"—the biochemical basis of inheritance [3]. It was later shown that

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incorporation of foreign DNA occurs entirely by transformation as pneumococci lack systems for conjugation or transduction, and over the next almost 90 years the mechanisms of competence induction and transformation have been thoroughly studied. A description of our current knowledge will be presented in the first section of this chapter.

More recently, the optimal environment in which transformation occurs has become a major topic of interest. Spontaneous transformation of *S. pneumoniae* strains has usually been reported to occur at extremely low frequencies *in vivo* [2,4]. This is due to the fact that, historically, transformation has been studied primarily in the context of infections, such as sepsis and pneumonia, in considerable contrast to the epidemiological evidence suggesting that genetic exchange of pneumococci, such as the acquisition of antibiotic resistance, occurs mainly during colonization in children, where high carriage rates and exposure to antibiotics favors the selection of drug-resistant strains [5,6]. This information, combined with studies over approximately the last 8 years that have provided evidence that colonizing pneumococci form multicellular communities or biofilms (which will be covered in detail in Chapters 13 and 16), has focused recent studies of transformation on biofilm cultures and the pneumococcal colonization niche, the nasopharynx. This interesting trajectory of transformation research will be covered in the next section of this chapter.

Finally, the implications of transformation and horizontal gene transfer for bacterial evolution and adaptation are far-reaching. The rapid emergence and spread of antibiotic resistance and capsular switching are the most commonly recognized manifestations of this process [7,8]; these will be described briefly in the last section of this chapter; a more comprehensive description of the evolution and genomic variation in pneumococci is provided in Chapter 5.

MECHANISMS OF PNEUMOCOCCAL TRANSFORMATION

Induction of Competence

Since the early experiments by Griffith and Avery, the detailed mechanism of competence initiation, DNA uptake, and integration in the bacterial chromosome has been well studied in *S. pneumoniae* (for recent reviews see [9–11]). It is difficult to discuss the role and implications of transformation during biofilm growth and colonization without providing at least a cursory description of these important findings obtained from efforts lead by Sanford Lacks, Alexander Tomasz, Donald Morrison, Leiv Havarstein, Jean-Pierre Claverys, and others in the field.

Natural transformation is different from other types of genetic exchange in that it is initiated by the recipient strain and is a natural part of the physiology of pneumococci. A cartoon of the general mechanisms involved in competence induction, DNA uptake, and homologous recombination is depicted in Figure 7.1. The initial induction of competence is not fully clear, but is likely due to environmental signals provided in the context of biofilm formation in the colonizing environment in the nasopharynx. Induction activates the transcription of two operons: *comAB*, encoding an ATP-binding cassette (ABC) transporter [12], and *comCDE*, encoding one of two major variants of ComC, a pre-pheromone or competence-stimulating peptide (CSP) [13,14], the ComC/CSP-receptor histidine kinase ComD, and ComE, its cognate response regulator in the ComDE two-component system [15]. CSP is exported through the ComAB transporter, which actively cleaves off a leader/signal peptide at a Gly-Gly site to secrete the mature form of the peptide (Figure 7.1, step 1). CSP can now bind in an autocrine or potentially paracrine fashion to

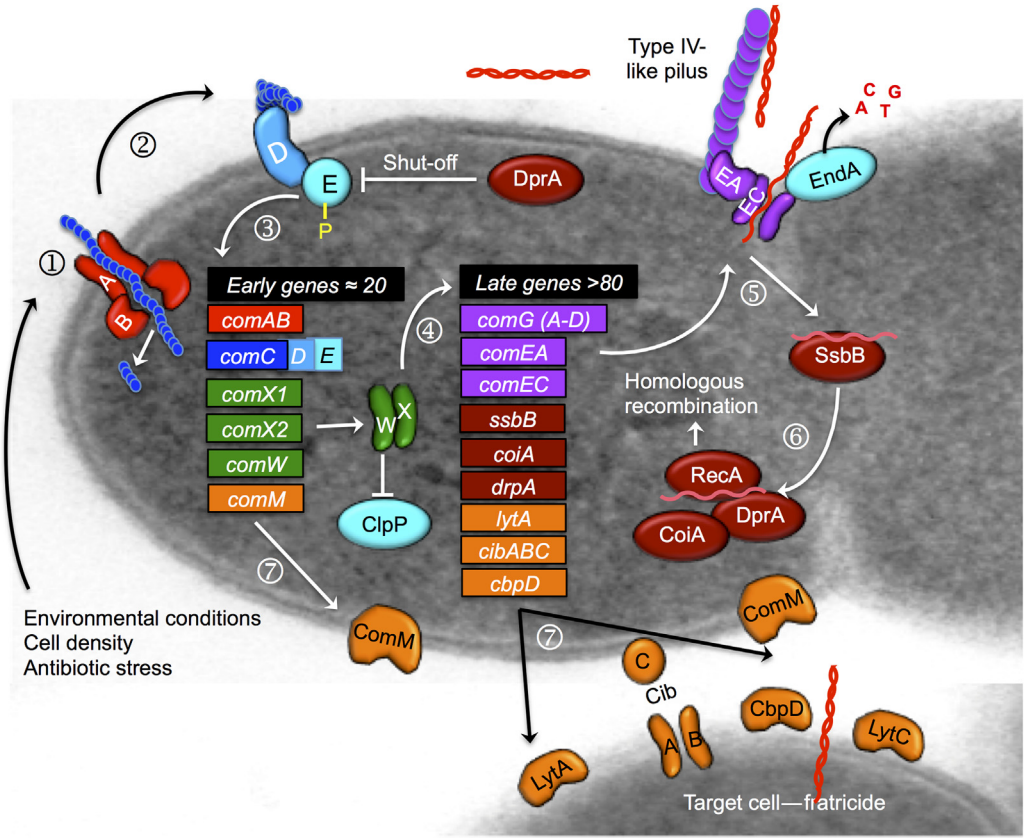

FIGURE 7.1 **The mechanisms of competence and fratricide.** ① Competence is activated by environmental signals including cell density and environmental stressors. This activates the expression of the ComAB ABC transporter as well as the ComC pheromone and the ComDE two-component system. The ComC competence peptide is secreted through the ComAB ABC transporter, which cleaves of the signal sequence during export. ② Mature CSP binds in an autocrine fashion to the histidine kinase ComD, which, through a conformational transformation, phosphorylates the response regulator ComE. ③ Phosphorylated ComE binds to a 9 bp sequence in the promoter sequence of approximately 20 early competence genes to activate their expression. ④ The early competence protein ComX makes a complex with ComW that protects it from proteolysis, and the ComX can now activate the more than 80 late competence genes by binding to a combox in the promoter region of these genes. ⑤ The ComGA-GD operon forms a type-IV–like pilus in the septum region of the pneumococcal cell, with ComGC forming the major building block of the pilus. The pilus binds DNA in the environment and transfers it to ComEA, which feeds it to the transporter ComEC. ComEC transports the DNA into the bacterial cell after EndA has degraded the dsDNA to ssDNA. ⑥ ssDNA will be sequestered by SsbB and will first be replaced by the DprA, which will load it onto RecA in a process that requires both CoiA and RadA for incorporation into the chromosome through homologous recombination. ⑦ Fratricide is activated. The murein hydrolases CbpD and LytA are induced during competence and will, together with LytC, which is constitutively expressed, attack non-competent target cells. In parallel, the bacteriocin-like molecules CibA and B will be released to act as trigger factors for target cell lysis. The competent cell is protected from the effect of these molecules by the expression of the immunity proteins ComM and CibC.

ComD that is expressed in the bacterial membrane. This binding will lead to a conformational change in the intracellular domain of ComD that results in phosphorylation of a His-residue in ComE (Figure 7.1, step 2). In its phosphorylated state, ComE will be able to bind to a 9 bp imperfect repeat sequence present in the promoter region of approximately 20 early competence genes and induce their expression. These include the promoters for *comAB* and *comCDE*, providing a positive feedback loop, as well as the genes encoding the alternative sigma factors *comX1* and *comX2*, and the ComX activator and stabilizer *comW*, which are all required for competence (Figure 7.1, step 3). Phosphorylated ComE also induces expression of *comM*, the immunity protein involved in fratricide (see first paragraph on page 133) and an additional seven operons not required for competence, including bacteriocins, enzymes, and transporters [16,17].

Activation of DNA Uptake and Homologous Recombination

Expression of ComX activates the expression of the more than 80 late competence genes, of which only a minority are directly required for competence [16]. These include the DNA uptake and homologous recombination machinery (Figure 7.1, step 4). The DNA uptake machinery contains the *comGA-GD* operon that encodes a type IV-like pilus, which is expressed in the septum region of the bacterial cell and is involved in binding of dsDNA from the environment [18]. Once bound to the surface, dsDNA is thought to be transferred to the ComEA receptor before being degraded into ssDNA by the EndA endonuclease on the outside of the cell [19]. The ssDNA is then imported through the ComEC pore [11] (Figure 7.1, step 5). Once inside the cell, the ssDNA binds to the single strand binding proteins SsbA and SsbB; the latter protein is a late competence gene and appears to be more important in protecting the DNA from degradation by forming an eclipse complex, providing DNA for the recombination machinery, and also potentially providing a DNA pool for sequential recombination events [20]. Transformation requires the general recombinase RecA, and although specific proteins exist to load RecA with DNA under normal circumstances, the late competence gene DprA is required for transformation and is specifically involved in loading RecA with ssDNA [21], after which RecA integrates the DNA in the chromosome through homologous recombination. This process is further facilitated by the competence-specific protein CoiA and the DNA repair protein RadA through mechanisms that are so far unknown [22,23] (Figure 7.1, step 6).

Fratricide

In natural ecosystems, exogenous DNA used for pneumococcal transformation most likely originates from incompletely degraded DNA fragments released from dead cells in the immediate vicinity, and also makes up part of the extracellular matrix of many biofilms [24]. The pneumococcus additionally possesses a genetic program, fratricide, whereby development of competence in a subpopulation of the bacteria triggers cell lysis and DNA release from surrounding non-competent bacteria [25,26] (Figure 7.1, step 7). Early experiments showed that high concentrations of choline could inhibit cell lysis, suggesting a role for choline-binding proteins in the process. This led to the identification of three proteins involved in cell lysis of sibling cells associated with degradation of the cell wall: LytC, the pneumococcal lysozyme that is constitutively expressed; LytA, the major autolysin that is constitutively expressed but is up-regulated upon competence induction; and CbpD, a murein hydrolase that is exclusively expressed during competence [26]. Although all three

proteins appear to be involved in the process, CbpD is essential for the lysis of non-competent cells [27]. The attacker cells also induce an early competence gene, *comM*, that produces an integral membrane protein that acts as an immunity protein and protects the attacker cell against lysis [28]. In conjunction with the three choline-binding proteins, competent cells also produce the bacteriocin-like molecules encoded by *cibAB*. This operon is expressed with the late competence genes and is responsible for fratricide on agar plates, but not in liquid medium. The genes are co-transcribed with *cibC*, which confers immunity against lysis of the expressing cell [26]. The specific role of CibA and CibB *in vivo* are not well defined, but it is thought that they act as trigger factors for fratricide in conjunction with the murein hydrolases.

Regulation of Competence

Being an activation cascade, the competence process is highly regulated. This complex regulation has been reviewed in more detail elsewhere [10]. Here we will only highlight the main aspects of regulation and the molecules that are associated with colonization and biofilm formation (see later). Although the specific activation mechanism is unclear, pneumococcal competence is activated by high cell densities that may occur in biofilms under antibiotic or other stress conditions, and is influenced by the two-component system CiaRH and the Ser/Thr kinase StkP, where the latter is also known to influence biofilm formation [29,30] (Figure 7.1, step 1). Environmental conditions will also impact competence induction and efficiency (see next section). One of the most fascinating aspects of natural competence of pneumococci in liquid culture is its rapid activation in the early logarithmic growth phase and its equally rapid deactivation within approximately 30 min [31]. Neither the signals involved in activation nor those involved in deactivation are completely clear. However, recent data indicate that DprA is directly responsible for the shut-off of competence by inhibiting ComE-driven transcription [32,33] (Figure 7.1, step 3). As mentioned earlier ComW stabilizes the alternative sigma factor ComX by protecting it from protease cleavage by ClpP [34] (Figure 7.1, step 4). Finally, the chaperone/protease HtrA is required for full competence induction [35]. Although the specific role for ClpP and HtrA have not been specifically identified during pneumococcal biofilm formation, mutants lacking these factors have shown an increased and decreased ability, respectively, to form biofilms in other streptococcal species [36]. As will be described in detail in the next section, this data implies that competence and biofilm formation are closely connected and that there is potential cross talk and overlap between the pathways inducing either phenotype.

COMPETENCE AND BIOFILM FORMATION

Effect of Bacterial and Environmental Factors on Competence and Transformation

It is important to recognize that most studies investigating competence *in vitro* have relied on a small subset of hyper-competent rough laboratory strains descended from the original Avery experiments and that clinical strains have frequently been found to lack natural competence under these same conditions. Yet natural transformation is known to occur in *S. pneumoniae in vivo*, based on its mosaic genomes and its ability to acquire genetic elements such as antibiotic resistance cassettes. Therefore, the influence of bacterial and host factors on competence induction and efficiency has been studied in culture systems for a long time. The

lack of competence in clinical, encapsulated isolates may well be due to the fact that transformation efficiency decreases with increasing capsule expression [37]. Therefore, attempts to characterize the natural transformability of clinical isolates have uniformly required the artificial induction of competence using exogenous addition of high concentrations of synthetic CSP [38].

With regard to environmental factors, studies of transformation in liquid medium have shown that transformation is temperature-dependent, with its highest efficiency between 30°C and 34°C, decreasing linearly as the temperature increases or decreases [39]. Similarly, competence induction is higher in the presence of oxygen and repressed under oxygen-limiting conditions [40]. It has been shown that ion and nutrient concentrations are important variables during competence development in planktonic pneumococcal cultures [41,42]. Although competence can be induced at various pHs, it requires an initial culture pH above 7, and transformation efficiency increases as the initial pH becomes more alkaline, suggesting that the critical CSP level for inducing competence may be pH dependent [41]. See Table 7.1 for a compilation of the role of environmental conditions in transformation. Interestingly, these optimal environmental conditions *in vitro* correspond very well with those present in the nasopharynx, which, together with the presence of DNA as part of the matrix of biofilms, makes biofilm colonization an optimal environment for genetic exchange.

The Requirement for Competence to Form Biofilms

Pneumococcal biofilm formation was first observed associated with disease states both in the middle ear and in patients with chronic rhinosinusitis [43,44]. Biofilm formation *in vitro* will be presented in more detail in Chapter 13, and biofilm formation during disease states will be presented in depth in Chapter 16. The role of competence during biofilm formation has not been consistent between studies, most probably depending on the model system used. However, it is clear that competence influences biofilm formation and that biofilm formation is associated with competence activation. Early biofilm models on abiotic surfaces showed a requirement for an active competence system, as competence mutants failed to form biofilms and CSP was required for optimal biofilm formation [45]. Later studies by the same investigators showed that the impact of competence induction for biofilm formation

TABLE 7.1 Environmental Factors Involved in Competence and Biofilm Formation

Optimal transformation factors	Conditions during biofilm colonization	Conditions during sepsis
Competence	Constitutively up-regulated	Transient
Presence of epithelial cells	Present	Absent
Decreased capsule	Low capsule expression	High capsule expression
30–34°C temperature	Low temperature (34°C)	High temperature (37°C)
Presence of oxygen	Higher O_2 exposure	Low O_2 exposure
Low nutrient availability	Low availability	High availability
High DNA availability	High	Low

depended on the model system, with static models requiring addition of CSP, whereas continuous-flow models showed no impact of CSP addition [46]. Other investigators, including our laboratory, have not seen any requirement for exogenous competence induction to form biofilms [38,47–49]; however, Vidal et al. observed that lack of intrinsic competence in the form of a ComC-negative strain made pneumococci unable to form early biofilms on epithelial cells but formed equally good biofilms on abiotic surfaces [49]. This suggests tightly interlaced pathways of competence activation and biofilm formation, which is also observed in many other streptococcal species including *S. mutans* and *S. pyogenes*.

Regulation of Competence in Biofilms

Recently, work from our laboratory demonstrated that pneumococci organize into biofilms attached to the mucosal epithelium and encased in a self-produced polymeric matrix during nasopharyngeal colonization [50]. This was the first time biofilm formation during asymptomatic colonization had been demonstrated *in vivo*. Taking into account the role of environmental factors on competence induction, as described earlier as well as what is known about the nasopharyngeal environment, we recapitulated a biofilm model system *in vitro* with close resemblance to the *in vivo* situation [50] (Figure 7.2A). First, epithelial cells were used for the first time as a substratum, as pneumococci never form biofilms under natural conditions on abiotic surfaces. This strategy has since been used by other investigators [49]. Furthermore, the biofilms were grown at 34°C, a temperature more conducive to transformation than 37°C and also the temperature within the human nasopharynx at ambient temperature [51]. The biofilms were grown in the presence of oxygen, at an initial pH of 7.4, in a nutrient-limiting medium, mimicking the conditions in the nasopharynx. This model system

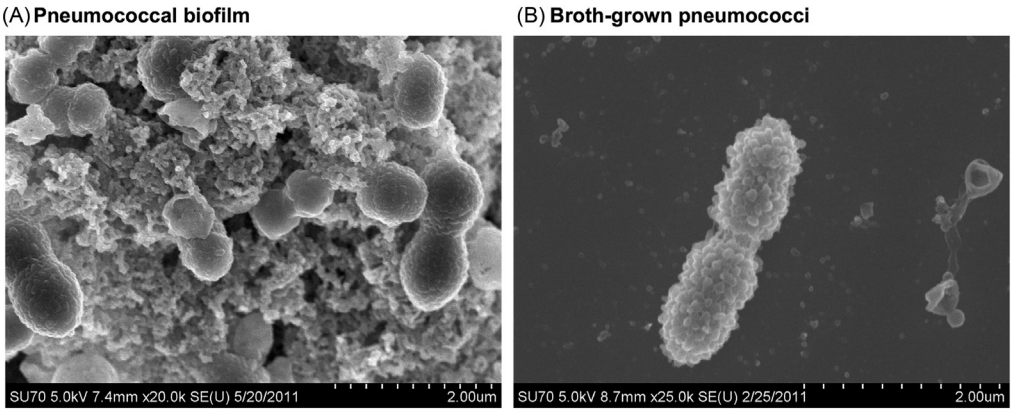

FIGURE 7.2 Depiction of biofilm and planktonic organisms by microscopy. The image shows high-resolution scanning electron micrographs of (A) a pneumococcal biofilm made from the D39 Avery strain and (B) a diplococcus of the same strain growth to mid-logarithmic phase in liquid media (planktonic organism). The biofilm image shows a maturing biofilm with a well-organized architecture consisting of bacteria with little capsule expression and a matrix (small globular debris) consisting of lysed bacterial cells. Capsule is not involved in matrix production as unencapsulated strains make equal amounts of matrix with similar structure. The low expression of capsule on the biofilm bacteria is evident when looking at the planktonic organism, which is heavily encapsulated (shown by small blobs of material covering the organism).

showed a denser biofilm growth with more intricate architecture, which was primarily associated with the presence of an epithelial cell substratum but was further improved by the lower temperature and lower nutrient conditions, as biofilms grown on glass, at 37°C, or in rich media, produced less-structured biofilms with lower biomass. Furthermore, capsule has been shown to be down-regulated during biofilm formation on abiotic surfaces [52], and using a model with epithelial cells as substratum we showed that the down-regulation of capsule was even greater than had been described earlier (Figure 7.2), which may be related to the interaction with epithelial cells that has also been shown to promote capsule down-regulation [53]. These results indicate that bacteria–host cell interactions are important for the ability to form a biofilm that structurally and functionally supports transformation.

These biofilms showed a constitutive up-regulation of *comD* and *comX* [47], similar to the up-regulation of competence genes observed in biofilm models produced by several other investigators [38,45,54]. This up-regulation is most likely associated with a shifting subpopulation of genetically competent organisms within the dynamic biofilm, making the biofilm as a whole constantly competent. As our model system is different and our studies were more prolonged than those reported earlier (up to 96 h), it is important to point out that constitutive up-regulation does not occur during a phase of rapidly changing cell density, and is not limited in time, instead providing increased opportunities over a prolonged period of time for genetic recombination, similar to what would occur during colonization.

Transformation of Pneumococci in Biofilms

Both our group and Havarstein's group have described gene transfer in biofilm models [38,47]. In our experiments, when biofilms were exposed to a high concentration (1 μg/mL) of chromosomal DNA harboring erythromycin or penicillin resistance genes, biofilm bacteria could efficiently incorporate this DNA in their chromosome (Figure 7.3A). Transfer was most effective if DNA was added during the initial phase of biofilm formation and the biofilms were left to mature for 48 h. No exogenous CSP was required for effective incorporation of the marked DNA. Wei and Havarstein showed a similar level of transfer in 4-h biofilm cultures treated with CSP, but showed a decreased genetic exchange with time [38]. Their study also showed that CbpD, involved in the release of target-cell DNA during fratricide, is essential for transformation in early stage biofilms.

Similarly, in our studies, co-culture in biofilms of two heterologous strains of different serotypes with erythromycin and penicillin resistance, respectively, resulted in a natural transformation efficiency of approximately 10^{-3} (one double-resistant clone per 1000 total colonies) after 48 h of biofilm formation. A similar co-culture in liquid medium yielded no double-resistant transformants, which was not surprising as the strains were encapsulated (Figure 7.3A). In the studies by Wei et al., co-culture of donor and target cells for fratricide yielded higher genetic transfer at the 4 h time point in the presence of CSP and declined over the next 8 h.

In our studies we further characterized the role of environmental factors for natural transformation observed in our biofilm model [47] (Table 7.1). The constant presence of CSP did not significantly increase the already elevated biofilm transformation efficiency when biofilms were grown in nutrient-poor chemically defined media. However, biofilms formed in the nutrient-rich Todd–Hewitt medium with yeast extract had lower biomass and showed both increased biofilm formation and transformation efficiency after exogenous addition of

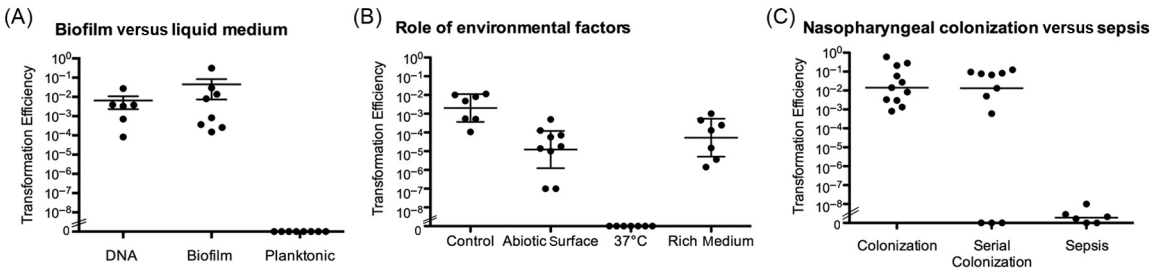

FIGURE 7.3 Transformation efficiency in biofilm cultures and during nasopharyngeal colonization. Transformation efficiency of antibiotic resistance elements between *S. pneumoniae* strains SP670 (PenR) and D39-C08P2 (ErmR). (A) "DNA" represents the uptake and incorporation of D39-C08P2 DNA by SP670 pneumococcal biofilms. DNA was added with each change of medium for the first 24 h, and the biofilms were grown for an additional 24 h before transformation efficiency was measured. "Biofilm" indicates that the strains were grown together under the conditions specified in the text (on epithelial cells, at 34°C, and in chemically defined medium) for 48 h in the absence of CSP before transformation efficiency was measured. "Planktonic" indicates that the two strains were seeded at the same concentration in one tube containing liquid medium and grown until mid-logarithmic phase before transformation efficiency was measured. Transformation efficiency was measured as the number of double–antibiotic resistant colonies divided by total recovery of bacteria from each condition. (B) A dual-strain biofilm culture was grown under different environmental conditions, where "control" indicates the optimal conditions used in figure (A), "abiotic surface" indicates biofilm growth in the absence of epithelial cells, "37°C" indicates growth at 37°C rather than 34°C, and "rich medium" indicates growth of biofilms in THY medium rather than chemically defined medium. Transformation efficiency was measured as indicated above. (C) In this figure, mice were co-colonized with both strains at the same time, colonized sequentially with one strain 48 h prior to the second strain, or both strains were used for co-infection in a septicemia model in mice. Transformation efficiency was measured as indicated above. *Source: Adapted from [47].*

CSP, supporting the earlier studies in which addition of CSP could increase biofilm stability [45,46]. Transformation efficiency *in vitro* on epithelial cells was significantly improved (~2 $\log_{10}$) compared with biofilms produced on plastic surfaces (Figure 7.3B). The most important experimental variable for transformation was temperature: Biofilm growth at 34°C yielded high transformation efficiency, whereas growth at 37°C resulted in no transformation efficiency for most strain combinations (Figure 7.2B). Competence induction from the increased oxygen concentration in the nasopharynx may likewise contribute to increased transformation efficiency in colonizing pneumococci, although that was not assessed.

While no single environmental parameter appears to account for the entire difference observed between planktonic and biofilm bacteria, our current knowledge suggests that the combined environmental conditions found during colonization of the nasopharynx, and associated with a developing biofilm, generate distinct physiological signals that together are optimal for both biofilm formation and competence induction, leading to increased horizontal gene transfer.

COMPETENCE AND NASOPHARYNGEAL COLONIZATION

The Epidemiologic Evidence

An in-depth look at nasopharyngeal colonization will be presented in Chapter 15. Here we will focus primarily on what is known about competence induction during colonization. Epidemiologic studies suggest that pneumococci colonizing the nasopharynx, rather than bacteria isolated from invasive disease,

are the source for much of the horizontal transfer of resistance genes and potentially other fitness traits between strains [55,56]. Based on the effective colonization of the nasopharyngeal tract of children starting in the first month of life, and the equally effective spread of strains between individuals at this age, the nasopharynx is an optimal niche for transformation and evolution of fitness. Likewise, colonization of the upper respiratory tract exposes the bacteria to lower temperatures (closer to 34°C), an atmospheric pressure of oxygen, and a nutritionally challenging environment—conditions that were shown above to favor both biofilm formation and natural transformation both in liquid media and in biofilm cultures (Figure 7.2, Table 7.1). Furthermore, pneumococci regulate capsule expression, with transparent variants expressing a thinner capsule layer predominating over capsule-expressing opaque variants during colonization [57]. As capsule expression inhibits transformation *in vitro*, this capsule down-regulation during colonization and biofilm formation likely contributes to the observed increase in transformation efficiency.

Exchange of genetic material in the nasopharynx requires colonization with diverse organisms from which donor DNA can be obtained. This may be facilitated by simultaneous carriage of multiple strains of pneumococci (co-colonization). Co-colonization is a clinical reality that was first observed by Gundel in 1933 [58] but has since been documented in relatively few studies, and is most likely under-reported as most epidemiological studies have relied on serotyping individual colonies isolated from culture. The few studies that have specifically investigated co-colonization have shown that between 9% and 49% of colonized individuals carry at least two different strains and have found up to six separate pneumococcal strains in the same individual [5,8,59,60]. The levels of co-colonization are most likely closer to the higher end of the range, as detection of pneumococci in most studies using culture shows a co-colonization of around 10%, whereas detection through PCR-based techniques shows much higher levels [60].

Experimental Evidence

Direct evidence for transformation during colonization was first described in an experiment by Elena Ottolenghi-Nightingale in 1972, in which streptomycin-resistant colonies were recovered from a healthy individual who was colonized experimentally with pneumococci and sprayed in the pharynx with extracted chromosomal DNA from a streptomycin-resistant type III isolate [61]. Until recently, no mechanistic studies have addressed transformation capability during asymptomatic nasopharyngeal colonization. As a continuation of our biofilm transformation studies, our laboratory also evaluated the efficiency of transformation during colonization of mice [47]. In our animal studies, we showed that transformation efficiency of mice co-colonized with two strains of different serotypes, each carrying a different antibiotic marker, resulted in a dramatically increased frequency of double-resistant transformants (10^{-2}) than what we recovered from the blood of mice injected intravenously with the same strains (10^{-8} to 10^{-9}) (Figure 7.3C). This constitutes an impressive 10^6-fold higher transformation efficiency in the nasopharynx. Importantly, the transformation efficiency in the nasopharynx did not change if the animals were colonized with both strains at the same time or colonized sequentially with the two strains 48 h apart, suggesting that an incoming strain has the ability to integrate into already established colonizing biofilms and integrate DNA from that biofilm colonization. Future studies

are needed to establish the specific mechanisms involved in transformation *in vivo* and the host factors that may influence transformation efficiency and pressure for horizontal gene transfer.

TRANSFORMATION: THE DRIVING FORCE OF PNEUMOCOCCAL EVOLUTION

Horizontal gene transfer mediated by transformation in the nasopharynx is critical to bacterial evolution, facilitating and accelerating the fitness of pneumococci for colonization, as well as adapting them to environmental stresses and clinical intervention, such as antibiotics and vaccines [7,8,62,63]. Although the concepts of genome variability are presented in detail in Chapter 5, a short presentation of the downstream consequences of transformation during nasopharyngeal biofilm colonization will be presented here.

The high frequency of natural transformation during colonization is thought to explain current epidemiological typing data indicating high genome plasticity within lineages, resulting in adaptation to environmental stressors [63,64]. The ability of pneumococci to adapt to the host environment can be observed through capsule switching events [7,65], as well as exchange of other virulence factors, such as the extensive recombination events of IgA1 proteases observed between pneumococci, but also between closely related species such as *S. mitis* and *S. oralis* [66]. In general, pneumococcal transformation has been associated with certain mobile regions, or hotspots, of the genome associated with transposases; mobile regions originating from other pneumococci as well as from closely related species [62,63]. This is supported by a study that followed the same strain during a chronic infection and identified sequential recombination events associated with contiguous genomic blocks [67].

Although capsule switching was observed before the introduction of the conjugate vaccine in 2000, there is some recent evidence suggesting that capsule switching through transformation has occurred over a very short time [64]. Although this adaptation to the capsule vaccine has not been enough to retain the infectiousness of pneumococci, it is likely to act to increase the fitness of the remaining circulating pneumococcal strains to obtain a new equilibrium with the nasopharyngeal microbiota. Similarly, antibiotic pressure has become a major driving force for horizontal gene transfer [7,65,68]. *S. pneumoniae* antimicrobial resistance is widely distributed among the 96 different serotypes where molecular epidemiology surveys show identical or highly related restriction fragment length polymorphism (RFLP) patterns for penicillin-binding protein genes (responsible for resistance to penicillin), with the remaining genetic background being greatly heterogeneous [8,56,59]. In an elegant study by Brueggemann's laboratory, the evolution of penicillin resistance was studied in a population of strains collected from 1937 to 2007. The results indicated a major increase in penicillin resistance over the last 30 years and identified the multidrug-resistant PMEN1 clone as an especially successful clone that has been able to spread its DNA to other lineages [69]. Finally, a recent study of 3085 genome sequences showed that non-encapsulated strains constitute a disproportionally large pool of DNA for adaptation and may drive evolutionary development of the encapsulated strains. This is interesting as we recently showed that non-encapsulated strains can be protected by encapsulated strains during co-colonization [47] and that non-typable or non-encapsulated isolates are detected nearly 10-fold more often in the nasopharynx of multiply colonized individuals [70].

CONCLUDING REMARKS

Although our understanding of competence and genetic exchange in pneumococci has come a long way over the past 90 years, we still have more to learn about how the molecules induced during competence orchestrate uptake and incorporation of exogenous DNA and how this is regulated by the environment these organisms live in. As competence is closely related to biofilm formation and colonization, dissection of these factors in relevant model systems will be of major importance for a better understanding not only of competence and horizontal spread but also of pneumococcal pathogenesis. Similarly, understanding these interactions within colonizing biofilm communities will provide critical information to develop novel treatment and preventive strategies in the face of the global increase in antibiotic resistance and vaccine escape.

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SECTION C

STREPTOCOCCUS PNEUMONIAE BIOLOGY

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CHAPTER

8

The Pneumococcal Cell Wall

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INTRODUCTION

Bacteria surround their cytoplasmic membrane with a complex cell wall that has two main functions [1]: it protects the cell from bursting due to the internal turgor of several atmospheres, and it maintains the shape of the cell. Several important classes of antibiotics, such as β-lactams and glycopeptides, and components of the immune system, such as cationic antimicrobial peptides and lysozyme, target essential bacterial cell-wall components and their biosynthetic pathways, causing cell lysis.

Like other Gram-positive Firmicutes, *Streptococcus pneumoniae* has a thick, multilayered cell wall composed mainly of peptidoglycan (PG) and teichoic acids (TAs). The latter are either covalently attached to PG (wall teichoic acid, WTA) or anchored to the cytoplasmic membrane (lipoteichoic acid, LTA). Most of the more than 90 distinct, serotype-specific capsular polysaccharides are covalently linked to the PG to protect the bacterial cell from lethal components of the immune system, enabling this pathogen to survive within the host organism and to cause disease [2]. The PG and TAs also anchor cell-surface proteins involved in transport processes and bacteria–host interactions. In addition, the PG-linked pili form long filamentous protein assemblies that extend far from the cell surface to interact with the host matrix or cells.

In this chapter we focus on pneumococcal PG and TAs, which have unique features and have been the focus of intense research. Pneumococci share the basic PG structure with other Firmicutes and, like other pathogens, modify their PG to gain resistance to antibacterial enzymes. Unlike those in many species, pneumococcal TAs have an unusually complex structure in their repeating unit, which is the same for LTA and WTA. In addition, pneumococci load their TAs with phosphocholine residues, which are rarely found in bacteria and which serve as anchors for a

Streptococcus pneumoniae.
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class of cell-surface proteins, the choline-binding proteins. Remarkably, pneumococci have a unique growth requirement for choline [3], which is solely used to decorate the cell surface TAs, illustrating the importance of these molecules in the lifestyle of this bacterium.

S. pneumoniae has an oval cell shape and grows in pairs or short chains of cells, prompting its former name, "diplococcus." It became a model bacterium to study growth and morphology in elongated cocci. Recent work has identified cell morphogenesis proteins and regulators that control cell-wall synthesis and hydrolysis in pneumococci. Although the precise mechanisms of cell-wall growth and cell division are poorly understood, the available data support a model according to which pneumococci grow by incorporating new cell wall at a central zone at mid-cell [4]. The data also suggest that cell-wall synthases and some of their regulators form a single, membrane-bound complex that is coordinated from inside the cell and that switches from an elongating to a dividing mode of cell-wall synthesis in the cell cycle. Growing bacteria constantly synthesize, remodel, and remove parts of their cell wall, and these processes affect the fine structure of the cell wall. Therefore, knowledge of cell-wall structure can help to understand how bacteria synthesize and enlarge their cell wall during growth.

COMPOSITION OF PNEUMOCOCCAL PG

Basic PG Structure and Modifications

Pneumococci have a PG structure typical for Firmicutes, made of glycan chains of alternating *N*-acetylglucosamine (Glc*N*Ac), *N*-acetylmuramic acid (Mur*N*Ac), and peptides linked to Mur*N*Ac. The peptides are synthesized as a pentapeptide with the sequence L-Ala–D-(γ)Glu–L-Lys–D-Ala–D-Ala. Most of the D-Glu residues become amidated by MurT/GatD at C (α) to D-Gln, but a few percent of D-Glu residues escape this modification and are found in the cell-wall PG [5,6] (Figure 8.1). The peptides may also be modified by MurM/MurN with an L-Ser–L-Ala or L-Ala–L-Ala dipeptide ("branch") linked to the ε-amino group of the lysine stem residue [7]. Most strains have a small percentage of branched peptides in their PG, but these are abundant in certain lineages of β-lactam-resistant strains. Shortly after synthesis, the peptides are either utilized to form cross-links connecting adjacent glycan chains, or they are trimmed to remove the D-Ala residues at positions 4 and 5 by the DD-carboxypeptidase PBP3 (DacA) and the LD-carboxypeptidase LdcB (DacB). Peptide cross-linking by DD-transpeptidases (penicillin-binding proteins, PBPs) leads to dimeric tetra-pentapeptides or trimeric tetra-tetra-pentapeptides (with or without the dipeptide branch), and these are trimmed to corresponding tripeptide versions, the tetra-tripeptide and tetra-tetra-tripeptide. If present at all, tetramers or higher peptide oligomers are of very low abundance. Hence, the most abundant peptides in mature PG are tripeptide monomers, tetra-tripeptide dimers, and tetra-tetra-tripeptide trimers, which can have a D-Glu residue instead of D-Gln due to incomplete amidation, and which can carry one or more dipeptide branches that may form an interpeptide bridge in cross-links [5].

The *N*-acetylamino sugars in the glycan chains are also subject to modification reactions. Some of the Glc*N*Ac residues are deacetylated to glucosamine (Glc*N*) by the PG deacetylase PgdA [8]. Many Mur*N*Ac residues become acetylated at C6-OH by the PG acetyltransferase Adr, which for unknown reasons is required to express high β-lactam resistance [9]. PG *O*-acetylation competes with the attachment of WTA and capsular polysaccharides by LCP phosphotransferases, which occurs at the same position of Mur*N*Ac [10,11]. All these modifications and substituents

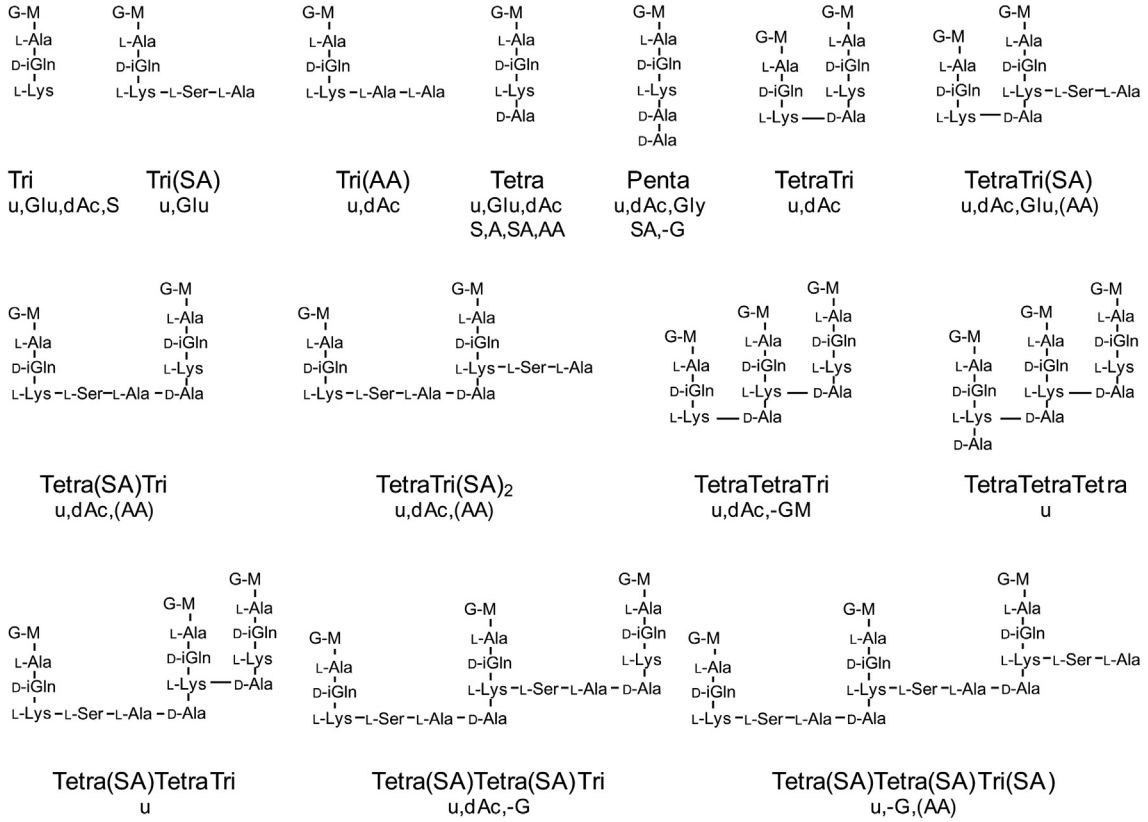

FIGURE 8.1 **Examples of muropeptides released from PG in *S. pneumoniae*.** Shown are the basic, unmodified structures. A 'u' indicates that the unmodified muropeptide is present in PG. Modifications found for each muropeptide are denoted below their name: Glu, glutamate instead of glutamine at position 2; dAc, glucosamine instead of *N*-acetylglucosamine; S, additional L-serine residue at L-Lys; A, additional L-alanine residue at L-Lys; SA, additional L-serine–L-alanine dipeptide at L-Lys; AA, additional L-alanine–L-alanine dipeptide at L-Lys; -G, missing *N*-acetylglucosamine; (AA), L-alanine–L-alanine instead of L-serine–L-alanine; -GM, missing *N*-acetylglucosamine–*N*-acetylmuramic acid disaccharide. G, *N*-acetylglucosamine; M, *N*-acetylmuramic acid.

at the glycan chain contribute to the resistance of the cell to lysozyme, an antibacterial host enzyme with cleavage preference to unmodified PG glycan chains. Hence, these modifications are abundant in pathogenic bacteria that are able to resist lysozyme.

Analysis of PG Composition

The pneumococcal cell wall can be isolated by boiling cells in the presence of sodium dodecyl sulfate, followed by further purification steps to remove contaminating nucleic acids and proteins [5,6]. The resulting cell wall contains mainly PG and covalently linked WTA. Hydrolysis of the WTA by treatment with hydrofluoric acid (HF) yields the pure, high-molecular-weight PG, which can be analyzed by different techniques. Both the cell wall and the PG can be analyzed by solid-state nuclear magnetic resonance (NMR) spectroscopy, which can be adapted to measure the ^{31}P resonances of the phosphate groups in the

WTA, or to obtain the $^{13}C-^{13}C$ correlation spectrum showing in cell-wall samples mainly the resonances from the cell-wall sugar residues, and in PG samples the residues from the amino acid and sugar residues. Solid-state NMR can be used to determine the main components of the cell wall and PG, but also to measure the binding of a protein to cell wall components [12,13].

For a more detailed analysis of the composition of high-molecular-weight PG, it must be degraded to soluble fragments, which are analyzed by high-pressure liquid chromatography (HPLC), mass spectrometry (MS), and other analytical techniques. The peptides can be released from cell wall by the amidase LytA, followed by HPLC analysis of the peptide composition. This method has been used to investigate the extent of peptide branching in β-lactam-resistant strains, revealing the requirement of the branching enzymes (MurM and MurN) for expression of high β-lactam resistance [7]. The muramidase cellosyl can be used to degrade pneumococcal PG, yielding muropeptides, the disaccharide peptide fragments. These can be separated by HPLC, which revealed 50 structurally distinct muropeptides differing in their degree of oligomerization (monomer, dimer, trimer) and the presence or absence of modifications either in the glycosyl or the peptide moiety (D-Glu, peptide branch, *N*-deacetylated Glc*N*, *O*-acetylated Mur*N*Ac, and missing sugars) (Figure 8.1) [5]. Peptide and muropeptide analysis of several strains showed that in pneumococci about half of the peptides are not cross-linked; the rest are mainly dimers and trimers. Hence the degree of PG cross-linking in pneumococci is similar to that in the Gram-negative *Escherichia coli* and significantly lower than in *Staphylococcus aureus*, a species with hyper-cross-linked PG [14]. HPLC analysis can also reveal specificity of PG modifying enzymes. Branched peptides are more often found in cross-linked muropeptides as compared to monomers, indicating that at least some of the cross-linking enzymes (PBPs) prefer branched substrates. An opposite effect is seen with the unamidated D-Gln (D-Glu) residues. These are found mainly in monomers, indicating that the PBPs might prefer fully amidated substrates; this was recently confirmed with purified enzymes [15]. Glc*N*Ac or Glc*N*Ac-Mur*N*Ac residues are found missing mainly in trimeric, but not monomeric or dimeric muropeptides, suggesting that the enzymes responsible for these losses, the LytB *N*-acetylglucosaminidase and the LytA amidase, are mainly acting on PG with peptide trimers. These might accumulate at the cell division sites where LytB is required to split the PG for daughter cell separation.

The glycan chains can be released from PG by LytA, followed by chemical acetylation of Glc*N* residues and separation of glycan chains from the peptides by size-exclusion chromatography. Remarkably, the pneumococcal glycan chains are relatively long, exceeding 25 disaccharide units; they can be digested with lysozyme to shorter chains with 2−9 disaccharide units [5]. Hence, pneumococci have long glycan chains in their PG, which is similar to *Bacillus subtilis* and unlike *S. aureus*, a species with particularly short glycan chains [14].

SYNTHESIS AND HYDROLYSIS OF PG

PG synthesis involves more than 20 enzymes acting in different compartments of the bacterial cell, the cytoplasm, the cytoplasmic membrane, and the cell wall [4,16].

The PG Biosynthesis Pathway

The cytoplasmic steps lead to the nucleotide sugar-linked precursors UDP-Glc*N*Ac and UDP-Mur*N*Ac catalyzed by GlmU, MurA, and MurB, followed by the addition of the pentapeptide to UDP-Mur*N*Ac by the amino acid ligases MurC−MurF. D-amino acids are produced by the racemases Alr (for D-Ala) and

MurI (for D-Glu), and the D-Ala–D-Ala dipeptide is synthesized by the ligase Ddl. The resulting UDP-Mur*N*Ac pentapepide is transferred to the membrane-bound lipid carrier undecaprenyl phosphate by MraY, leading to undecaprenyl pyrophosphoryl-Mur*N*Ac pentapeptide (lipid I), which is subsequently converted to lipid II by the transfer of a Glc*N*Ac residue from UDP-Glc*N*Ac by MurG.

Lipid II is then modified by two reactions. The essential MurT/GatD complex amidates the α-carboxy group of D-Glu (position 2 of the stem peptide) [17], and the Fem-type transferases MurM and MurN add an L-Ser–L-Ala or L-Ala–L-Ala branch to the ε-amino group of Lys (position 3) [7,18]. Neither modification is quantitative, and in particular the extent of branches depends strongly on the strain background, whereby strongly branched PG is found in certain lineages of penicillin-resistant strains [19]. Lipid II is transported across the cytoplasmic membrane by FtsW/RodA [20] and incorporated into the cell wall by GTase and TPase reactions performed by PBPs [1]. Modifications such as *N*-deacetylation or *O*-acetylation of the glycan chains occur at either lipid II or the already polymerized (nascent) PG.

Penicillin-Binding Proteins

S. pneumoniae contains six PBPs, which are responsible for PG synthesis from lipid II and which are the primary targets of β-lactam antibiotics. The three class A PBPs, PBP1a, PBP1b, and PBP2a, are bifunctional enzymes. They polymerize the glycan chains by their N-terminal glycosyltransferase (GTase) domain and perform peptide cross-linking by the C-terminal DD-transpeptidase (TPase) domain. None of the class A PBPs is essential; however, double mutants lacking PBP1a and PBP2a are not viable [21]. Purified PBP1a and PBP2a are active *in vitro* with lipid II as substrate [15]. Interestingly, while both PBPs polymerized the amidated and unamidated lipid II by GTase reactions, they have efficient cross-linking activity only with amidated lipid II. Hence, the essentiality of the amidating enzymes, MurT/GatD, appears to be caused by the inability of PBPs to utilize the unmodified substrate.

S. pneumoniae has two essential class B PBPs, the monofunctional TPases PBP2x and PBP2b, responsible for septal and peripheral PG synthesis, respectively [22,23]. Consistent with their function in different growth phases, the depletion of class B PBPs causes cells to become either short, lentil-shaped (PBP2b) or elongated, lemon-shaped (PBP2x) [22]. In addition, genetic evidence and peptide composition analysis indicated an important role for branched peptides in the function of PBP2b and PBP2x.

PBP3 is a class C PBP with DD-carboxypeptidase (CPase) activity; it trims pentapeptides to tetrapeptides [24]. The LD-CPase LdcB (also called DacB) trims tetrapeptides to tripeptides, which are abundant in PG, and is not a member of the PBP family of proteins [25]. Cells lacking PBP3 or DacB produce aberrant morphology and display defects in septum placement, indicating an important function for peptide trimming in regulating cell wall growth [26,27].

PBP2x and Septum PG Synthesis

PBP2x has a short cytoplasmic region of 27 amino acids, followed by a single membrane spanning segment, which anchors PBP2x to the cytoplasmic membrane. The crystal structure of the extracellular part shows distinct domains, whereby the TPase domain locates between an N-terminal non-catalytic domain and two C-terminal PASTA (for PBP And Serine/Threonine kinase Associated) domains [28]. The N-terminal domain has an elongated, "sugar tongue"–like structure and may function as a pedestal or as a protein–protein interaction domain [29]. The central transpeptidase domain contains three conserved motifs including the active site Ser337.

The mid-cell localization of PBP2x has been demonstrated by immunofluorescence microscopy and GFP-tagging [30,31]. A more recent study showed that PBP2x arrives at the septum together with PBP1a and StkP, and that mid-cell localization of PBP2x is independent of its catalytic transpeptidase domain, but depends on the PASTA domains [23]. In the case of the serine/threonine kinase StkP, the four extracellular PASTA domains are important in the regulation of septal cell-wall synthesis, presumably by targeting the protein to mid-cell via direct interaction with nascent, non-cross-linked septal PG [32,33].

The PBP2x PASTA domains are connected to the TPase domain via a flexible linker and have a highly conserved α/β/β/β fold, originating probably from a gene duplication event. The possible low-affinity binding to PG is supported by the crystal structure of acylated PBP2x showing the association of a cefuroxime molecule, a β-lactam mimicking the D-Ala–D-Ala termini of PG pentapeptide, to the first PASTA domain [34].

Interestingly, the α-helix of the second PASTA domain is essential for the enzymatic binding of β-lactam antibiotics, indicating a role of this region in the activation of the PBP [35]. Indeed, work in *E. coli* has recently demonstrated the role of non-catalytic domains in the activation of class A PBPs [36]. A GFP-PBP2x version with an A707D substitution in the α-helix of the second PASTA domain was unstable and did not localize at mid-cell, suggesting that A707 is of structural or/and functional importance [37].

PBP2x interacts with StkP in pull-down assays, and both seem to be present in the same membrane-associated complex; complex formation was inhibited in the presence of cell-wall fragments [31]. Another recent study reported the formation of stable membrane protein complexes consisting of late division proteins DivIB, DivIC, FtsL, PBP2x, and FtsW [38]. These data indicate a specific interaction between PBP2x and DivIB, perhaps mediated by the transmembrane regions of both proteins, and of PBP2x with the large extracellular loop between transmembrane segments 7 and 8 of the lipid II transporter FtsW [38]. Insights into the role of the N-terminal domain came from domain swapping and mutational analysis of PBP2x and PBP2b [39]. These results revealed that both the cytoplasmic and transmembrane domains of PBP2x and PBP2b are needed for correct functioning of these enzymes, suggesting possible interactions with divisome proteins such as DivIB and FtsW [39].

PG Hydrolases

Enlargement and remodeling of the PG during bacterial growth and division require both PG synthases and hydrolases. Indeed, PG hydrolases essential for cell elongation and daughter cell separation have been identified in *E. coli* and other species. There are specific PG hydrolases for almost every bond in the PG [4,40]. In this section, the PG hydrolases important for pneumococcal growth and morphology will be discussed.

Roles of PcsB and LytB in Septum Synthesis, Remodeling, and Cleavage

The extracellular protein PcsB localizes to the division site and is required for proper septum synthesis and cell separation [41]. The multidomain protein consists of four parts: an N-terminal signal peptide followed by a coiled-coil (CC) region with two leucine zipper motifs and the C-terminal catalytic CHAP (for Cysteine, Histidine-dependent Amidohydrolase/Peptidase) domain. Although CHAP domains are found in many PG endopeptidases, a purified PcsB protein showed no PG hydrolase activity [42]; the only evidence for possible hydrolase activity came from an in-gel PG clearing assay (zymogram) [43]. PcsB requires activation by the

membrane-bound ABC transporter FtsEX; hence, depletion of FtsEX causes morphological changes similar to depletion of PcsB [41]. Activation occurs by direct interaction, whereby PcsB interacts with two extracellular loops of FtsX via its N-terminal CC region [41,44]. Interestingly, a possible activation mechanism has been suggested based on the recently solved crystal structure of PcsB [43], according to which PcsB forms an inactive homodimer in which the V-shaped CC domain of each monomer blocks the catalytic domain of the other. It was suggested that FtsEX induces conformational changes in PcsB to unlock this mutual inhibition. However, the precise role of PcsB in septum synthesis has yet to be established.

LytB is an endo-β-*N*-acetylglucosaminidase with a signal peptide, a TA choline-binding domain with 14−18 imperfect repeats, and a C-terminal catalytic domain [45]. LytB acts at the end of the cell division process to separate the daughter cells, as cells lacking LytB grow in long chains of deeply constricted, non-separated cells [45]. GFP-LytB localizes preferentially at the poles of the cells when added to cells, and purified LytB degrades pneumococcal PG. The exogenous LytB disperses the chains of *lytB* cells containing choline-loaded TA, but not chains of ethanolamine-grown cells, indicating that the presence of ethanolamine in the TA does not activate LytB [45].

The autolysins LytA, LytC, and CbpD are PG hydrolases with a choline-binding domain. They are required for antibiotic-induced autolysis and fratricide during genetic transformation; they are described in detail in Chapter 11.

GROWTH AND CELL DIVISION

Pneumococci have an ovoid cell shape and, unlike true cocci such as *S. aureus*, employ an elongative mode of cell growth that proceeds and overlaps cell division, resulting in the characteristic prolate cell poles. Elongation and division are intimately linked, and new cell-wall material is incorporated at a central growth zone, consistent with the mid-cell localization of all PG synthases, PBP1a, PBP1b, PBP2a, PBP2x, and PBP2b [46].

The current model for cell growth and division of ovococci is based on the results of early ultrastructure studies [47]. Shortly after completion of the new cross-wall, each of the two daughter cells forms a new equatorial ring defining the new mid-cell growth zone. New hemispheres are synthesized by peripheral growth characterized by simultaneous elongation and constriction, until the new hemisphere has the same length as the old one, followed by cross-wall synthesis to close the septum. A recent study confirms this model using super-resolution three-dimensional structured illumination fluorescence microscopy of cells with labeled PG synthesis sites [48]. Recently, a single, large machinery responsible for both peripheral and septal growth was proposed, combining proteins of the elongasome and divisome [4]. A central question is how septal and peripheral growth are regulated and spatiotemporally coordinated to achieve the typical ovococcal shape. Currently it is assumed that the pneumococcal PG synthesis proteins have roles similar to their homologs in rod-shaped bacteria [4].

The elongasome multiprotein complex synthesizes the peripheral cell wall and contains PBP2b, PBP1a, MreC, MreD, GpsB, RodA, and RodZ [4]. Pneumococci lack the actin-like MreB, which organizes elongation in most rods. MreC and MreD localize at the PG insertion sites and are essential; depletion of *mreCD* results in the formation of round cells and lysis [49]. This study also showed that MreCD directs peripheral PG synthesis, presumably by localizing and/or activating PBP1a.

The divisome is a multiprotein complex that carries out cell division, including the cross-wall synthesis. The essential key protein of the divisome is the tubulin-like GTPase FtsZ, which orchestrates cell division. It forms the

mid-cell Z-ring to which other division proteins assemble in *E. coli* in a hierarchical manner (reviewed in [50]). The essential FtsA, an actin homolog, anchors FtsZ to the membrane and stabilizes the Z-ring. FtsZ interacts also with ZapA and FtsK, and FtsA interacts with PBP2x and ZapA [31,51,52]. Several other proteins, including ZapA, EzrA, and SepF, regulate Z-ring dynamics and/or membrane anchoring. The trimeric FtsQ (DivIB)/FtsL/FtsB (DivIC) complex, DivIVA, the lipid II flippase FstW, and the two PG synthases PBP2x and PBP1a all localize to the Z-ring to form the mature divisome [32,53,54].

Regulation of Cell-Wall Growth

The regulation of bacterial cell-wall synthesis during growth and cell division is still poorly understood, involving in pneumococci at least four proteins: DivIVA, GpsB, and StkP/PhpP. DivIVA is important for the formation and maturation of the cell poles [55]. It is simultaneously present as a ring at the division site and as dots at the cell poles [55]; it interacts with itself, FtsZ, FtsA, ZapA, EzrA, and PcsB. GpsB was initially identified in *B. subtilis*, in which it shuttles PBP1 from peripheral to septal PG synthesis sites [56]. Although not essential, GpsB contributes to peripheral and septal PG synthesis in pneumococci [57]. However, a more recent study suggests that pneumococcal GpsB is essential [30]. Depletion of GpsB results in greatly elongated cells with unsegregated nucleoids and unconstricted rings of FtsZ, PBP2x, PBP1a, and MreC [30]. GpsB mainly co-localizes with FtsZ at division sites, where it was suggested to mediate septal ring closure [30]. The pneumococcal serine/threonine kinase StkP and its cognate phosphatase PhpP localize to the division sites. StkP requires its extracellular PASTA domains and the cytoplasmic kinase domain for mid-cell localization [32,58]. StkP arrives at mid-cell before DivIVA, but shortly after FtsA, and remains there longer than FtsA [32]. StkP and PhpP are responsible for the coordination of cell growth and cell division, probably through reversible phosphorylation of cell division proteins. StkP might signal the status of the cell wall to other cell division proteins and could be required to switch from peripheral to septal PG synthesis.

CHEMICAL COMPOSITION OF PNEUMOCOCCAL TAs

Compared to the TAs of many other Gram-positive bacteria, pneumococcal TAs are unique in regard to two major aspects: an extraordinary complexity, including the presence of unusual phosphorylcholine (*P*Cho) residues [59], and an identical chemical structure within their repeating units [60]. In contrast to the strain-specific capsular polysaccharides (see Chapter 9), pneumococcal LTA (originally named *pneumococcal lipocarbohydrate* or *pneumococcal F-antigen* [61]) and WTA (originally named *pneumococcal C-polysaccharide* [62]) are common pneumococcal antigens with highly conserved structures [63].

The complex composition of pneumococcal LTAs, including linkage analysis, was initially investigated in the early 1990s by analyzing partial structures of LTA obtained from strain R6 after alkaline treatment or treatment with HF (48%) as well as the de-*O*-acylated, *N*-acetylated oligomeric LTA. The HF treatment resulted in the formation of β-D-glucopyranosyl-(1→3)-α-2-acetamido-4-amino-2,4,6-trideoxygalactosyl-(1→4)-α-D-*N*-acetyl-galactosaminyl-(1→3)-β-D-*N*-acetyl-galactosaminyl-(1→1)-ribitol(β-D-Glc*p*-(1→3)-α-AATGal*p*-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→1)-ribitol) as the major fragment and, in the case of LTA, β-D-Glc*p*-(1→3)-β-AATGal*p*-(1→4)-α-D-Gal*p*NAc-(1→3)-glycerol as well, which was thought to be the deacylated lipid anchor. Alkaline

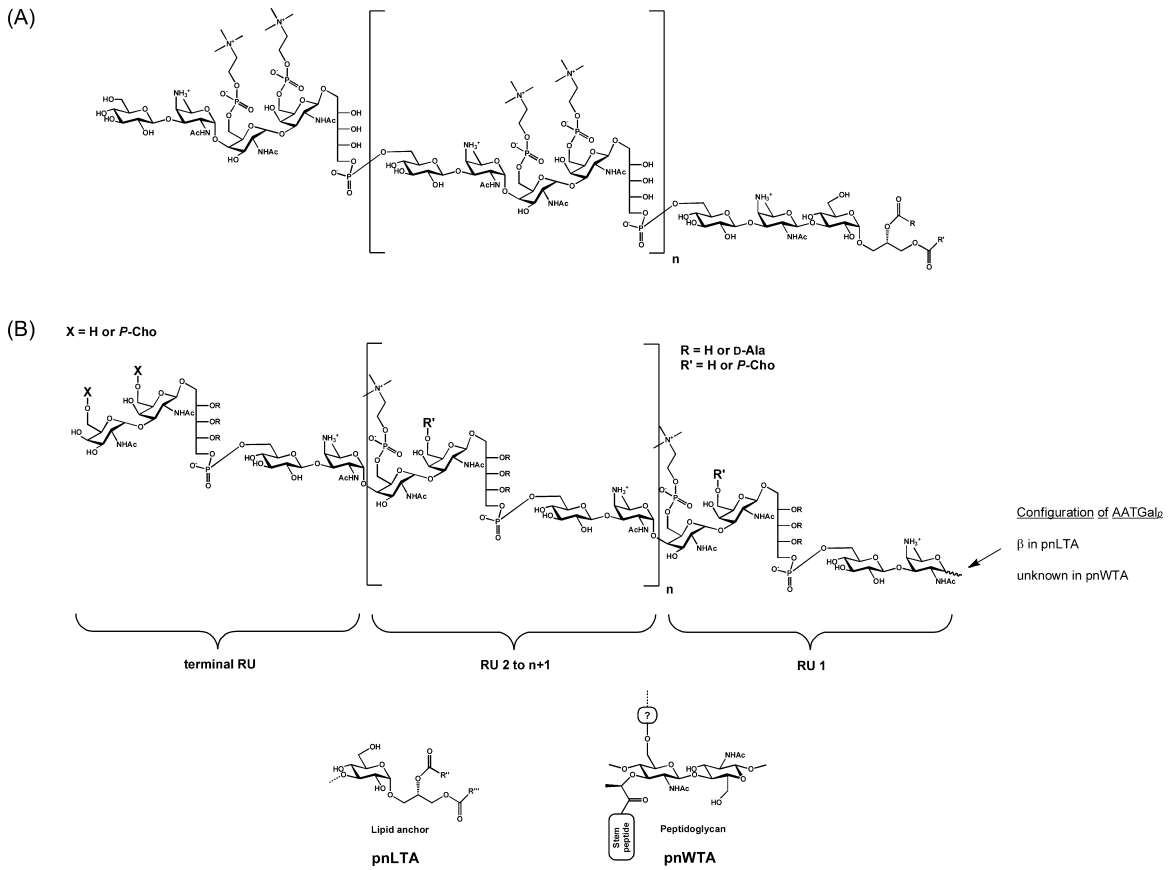

FIGURE 8.2 **Structure of pneumococcal TAs.** (A) Initially proposed LTA structure [60]. *n*, number of repeating units ($n = 2-8$); R, R′, alkyl or alkenyl residues of fatty acid chains in the lipid anchor. (B) Current structural model of *S. pneumoniae* LTA and WTA. RUs of both TA types contain the same *pseudo*-pentasaccharide building blocks (6-*O*-*P*Cho-α-Gal*p*NAc−(6-*O*-*P*Cho-) β-Gal*p*NAc−ribitol-5-*P*−β-Glc*p*−α-AATGal*p*), whereby the terminal RU can occur with or without 6-*O*-*P*Cho substitution on both Gal*p*NAc. Mono-*P*Cho-substituted TAs lack the *P*Cho at β-Gal*p*NAc (R′), as shown for two strains (Table 8.2). In LTA, the first repeating unit is β-1-linked to the lipid anchor (α-D-Glc*p*-(1→3)-DAG); the configuration of the respective linkage in WTA to PG is not known. All other RUs are α-glycosidically linked to the previous one. Hydroxyl groups of ribitol can be substituted by D-alanine (D-Ala; R). *n*, number of repeating units (n = 2-6; Table 8.2); R″, R‴, alkyl or alkenyl residues of fatty acid chains in the DAG lipid anchor.

hydrolysis resulted in the same major molecule, but with *P*Cho residues at the O6 position of both Gal*p*NAc residues. Oxidization of *N*-acetylated LTA with $NaIO_4$ followed by β-elimination clearly indicated a phosphodiester bond between O5 of ribitol and O6 of the glycopyranosyl residue of the adjacent unit. Mass spectrometric investigation of the delipidated and derivated oligomeric LTA revealed a chain length of 5−7 repeating units. These findings led to the initial structural model for LTA (Figure 8.2A) and to the hypothesis that the isolated *pseudo*-pentasaccharides are the biosynthetic repeating units, with β-D-Glc*p* and ribitol-5-phosphate as the respective termini [60,64]. The first repeat is bound to the lipid anchor β-D-Glc*p*-(1→3)-β-AATGal*p*-(1→4)-α-D-Gal*p*NAc-(1→3)-DAG (DAG: diacylglycerol).

The original model has been revised and completed stepwise within the last few years, using additional analytical methods and high-resolution NMR and MS. The revisions concerned the terminal part of the sugar polysaccharide and the structure of the biosynthetical repeating unit; they were prompted by the identification of ambiguous structural and immunological features in the original model. First, the original model was unable to explain the described cross-reactivity of LTA with anti-Forssman antigen antibodies (requiring a terminal α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→) disaccharide motif) [64–66]. Second, the originally defined trisaccharide-DAG biosynthetic lipid anchor (β-D-Glc*p*-(1→3)-β-AATGal*p*-(1→3)-α-D-Glc*p*(1→3)-DAG) has never been identified in *S. pneumoniae* cell-wall lipid extracts. Instead, the major glycolipids extracted from pneumococcal cell walls are α-D-Glc*p*-(1→3)-DAG and α-D-Gal*p*-(1→2)-α-D-Glc*p*-(1→3)-DAG [67,68].

The structural moiety of the Forssman antigenic disaccharide (α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→1)-ribitol) was detected for the first time when LTA from strains R6 and R36A was treated by HF and analyzed by MALDI-TOF MS without any further purification procedure used in the initial study. With the *P*Cho substitution of Gal*p*NAc, which has been removed by the analytical procedure, the non-lipid terminus of LTA consists of 6-*O*-*P*Cho-α-D-Gal*p*NAc-(1→3)-6-*O*-*P*Cho-β-D-Gal*p*NAc [69]. In the early studies, this partial structure was only defined as being within the repeating unit [60,64]. These observations led to a revised model of the LTA structure, and especially of the nature of the biosynthetical repeating unit. In this model, the α-D-Glc*p*-(1→3)-DAG was defined as the lipid anchor (now representing a common pneumococcal cell wall glycolipid), which resulted in a "shift" of the *pseudo*-pentameric repeating unit, now starting with AATGal*p* and ending with 6-*O*-*P*Cho-substituted α-D-Gal*p*NAc. Recent NMR studies using hydrazine-treated LTA have confirmed the presence of this Forssman disaccharide [70]. Furthermore, it was shown that in serotype 4 strain Fp23 (TIGR4Δ*cps*) the *P*Cho content of the terminus is pH dependent. Whereas under physiological growth conditions both Gal*p*NAc residues at the terminus bear *P*Cho substituents, slightly acidic conditions (pH 6.5) led to a terminal Forssman disaccharide without any *P*Cho. The enzyme responsible for the removal of the terminal *P*Cho residues is likely the pneumococcal phosphorylcholine esterase (Pce), which is capable of releasing 15–30% of *P*Cho residues from TA [71,72].

In contrast to pneumococcal LTA, staphylococcal LTA has glycerol phosphate (Gro-*P*)-containing repeating units that are substituted with D-Ala or α-*N*-acetyl-D-glucosamine (α-D-Glc*p*NAc) [73,74]. The alanine substituents in the repeating units of the LTA polymer were thought to play essential roles in the biological activity of LTAs [73,74], especially with respect to their pro-inflammatory potency (compare last part of this chapter). Alanine was absent in the initial structural models of pneumococcal LTA, but was later predicted to be present from genome wide searches for the corresponding biosynthetic genes. Indeed, an improved, pH-controlled isolation procedure yielded pure LTA in which the ribitol residue was substituted with D-Ala in a strain-specific manner, as shown by NMR analysis [70,75]. Serotype 4 strain Fp23 contains D-Ala substituents [70,75], whereas serotype 2 strain R6 [75] was found to be unsubstituted. Indeed, the *dlt* operon, the gene products of which are responsible for D-Ala substitution of TAs, contained a premature stop codon in strain R6 but was fully functional in strains D39 and Rx, explaining the absence of D-Ala in TAs of R6 [76]. The D-Ala substituents in Fp23 were present in equal amounts at positions 2-*O*, 3-*O*,

and 4-*O* of the ribitol unit [75], but it is unclear if this reflects the natural situation or if it is the result of a D-alanyl ester migration via a cyclic *ortho*-ester intermediate during sample preparation. The proposed presence of an α-D-Glc*p*NAc residue at the ribitol [75] could not be confirmed in a later work [70], and genomic studies have so far not identified any candidate enzymes responsible for such a substitution [77].

In contrast to LTA, chemical analysis of the WTA structure in *S. pneumoniae* turned out to be more difficult, because classical procedures for WTA isolation (mild acid or alkaline treatment) have been proven inappropriate. The anchoring of the WTA to the PG is resistant to alkaline treatment, which hydrolyzes the phosphodiester bonds between the repeating units. Furthermore, mild acid hydrolysis leads to a cleavage of the glycosidic bond between Gal*p*NAc and ribitol [78]. Trichloroacetic acid releases almost intact WTA chains, but hydrolysis is not complete or reproducible [60,79–81], and the agent also partially hydrolyzes the phosphodiester bonds between the repeating units, resulting in a heterogeneous mixture of oligosaccharides (N. Gisch, unpublished). A recent new enzymatic approach (using LytA amidase and cellosyl muramidase) proved to be more efficient for the isolation of WTA than the chemical procedures [5]. Interestingly, this procedure yielded pure WTA chains without a bound PG disaccharide that might be expected, suggesting that the enzymatic treatment had hydrolyzed the bond connecting the WTA chain to a hypothetical linkage unit or Mur*N*Ac in PG. Importantly, the isolated chains consisted of predominantly 6–7 *pseudo*-pentameric units with AATGal*p* and 6-*O*-*P*Cho-substituted α-D-Gal*p*NAc at the respective termini, thus representing the same biosynthetic repeating unit as the revised model for LTA described above [69,70]. Overall, the structure of WTA appears conserved for most serotypes tested, with the dephosphorylated backbone structure of the WTA repeat being the *pseudo*-pentameric unit β-D-Glc*p*-(1→3)-α-AATGal*p*-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→1)-ribitol, which is identical to the repeat in LTA. The only known structural modification within the RU has been reported for the WTA of a serotype 5 strain. Here the β-D-Glc*p* was replaced by a β-D-Gal*p* [82]. This altered structure could be due to the presence of a different glycosyltransferase, SP70585_0164, that in the serotype 5 strain replaces the glycosyltransferase Spr0091, which has been proposed to add the glucose residue to TAs in other strains [77] (for more detail see next part of this chapter; all protein and gene names in this chapter are for strain R6) (Table 8.1).

The chain lengths of the LTA and WTA seem to be very similar and consistent among strains. All investigated LTA (from serotypes 2, 3, 4, and 6B) contained 4–8 repeating units, with 6 and 7 repeats predominant. The shorter chain length of LTA of strains R6 (2 repeats) and Fp23 (2–3 repeats) [75] were the result of erroneous NMR integration caused by misinterpretation of integral signals due to aggregate formation of LTAs in aqueous solvent systems [70]. For WTA, much less data about chain length is available, and only serotype 2 strains have been investigated so far [5,78]. Nonetheless, the reported predominant chain lengths vary between 5 and 7 repeats (Table 8.2).

The degree of *P*Cho substituents per repeating unit within the TAs is less conserved. Some strains are substituted in position 6 of both Gal*p*NAc residues by two *P*Cho substituents, but others contain only one. Notably, in all strains with available data for both TA types, the *P*Cho content per repeat is the same (Table 8.2).

The specific position of the *P*Cho residue in mono-substituted strains has only been investigated in serotype 2 strains (Rx1 [78] and CSR SCS2 [84]), and was determined to be attached to the α-D-Gal*p*NAc. Whether the mono-*P*Cho

TABLE 8.1 Cell-Wall Synthesis and Cell Morphogenesis Proteins of *S. pneumoniae*

Function/category	Protein[a]	Role/remarks[b]
PG SYNTHESIS AND MODIFICATION		
Formation of UDP-Mur*N*Ac-pentapeptide	• GcaD (GlmU) • Alr (DadX), MurI • Ddl, MurA1, MurA2, MurB, MurC, MurD, MurE, MurF	• UDP-Glc*N*Ac pyrophosphorylase • Racemases, formation of D-Ala and D-Glu • Amino acid ligases, synthesis of D-Ala–D-Ala and the peptide stem at UDP-Mur*N*Ac
Transport lipid	• UppS • UppP	• Undecaprenyl diphosphate synthase • Undecaprenyl diphosphate phosphatase
Formation and modification of lipid II	• MraY, MurG • MurT/GatD • MurM, MurN	• Sugar transferases for lipid I/II synthesis • Amidation of D-Glu to D-Gln • Amino acid transferases, Ser–Ala branch
PG synthesis from lipid II	• RodA, FtsW • PBP1a, PBP1b, PBP2a • PBP2a, PBP2x	• Lipid II flippases • GTase-TPase, PG synthases • TPase, PG synthases
PG modification	• PgdA, Adr	• Glycan chain deacetylation/*O*-acetylation
Hydrolysis of PG	• PBP3 (DacA), LdcB (DacB) • PcsB, LytB • LytA, LytC, CbpD	• Trimming of pentapeptides to tetra/tripeptides • Septum synthesis and cleavage • Autolysis, fratricide
TA SYNTHESIS		
Coline uptake, incorporation into TA and removal	• LicA, LicB, LicC • LicD1, LicD2 • Pce	• Choline uptake and activation • Phosphocholine incorporation into TA • Removal of phosphocholine from TA
Synthesis and lipid modification of AATGal	• Spr0092, Spr1654 • Spr1665	• Synthesis of UDP-AATGal from UDP-Glc*N*Ac (hypothetical) • Synthesis of upr-AATGal (hypothetical)
Synthesis and polymerization of TA repeating unit	• Spr0091, Spr1223, Spr1224 • Spr1225 • Spr1222	• Hypothetical glycosyltransferases • Hypothetical phosphotransferase • Hypothetical repeating unit polymerase
TA flippase	• TacF	• Transport of TA subunits across the cell membrane
Attachment of TA and CPS to cell wall	• LytR, Psr, Cps2A,	• Phosphotransferases for the attachment of cell wall polymers to PG
CELL ELONGATION AND DIVISION PROTEINS		
Elongation	• MreC, MreD, RodZ, GpsB • PBP1a, PBP2b, RodA	• Morphogenesis proteins of unknown function • PG synthases and flippase for elongation
Cell division	• FtsZ, FtsA, ZapA, ZapB, SepF, EzrA • FtsK • DivIB, DivIC, FtsL • PBP1a, PBP2x, FtsW • PcsB, LytB, FteE, FtsX • DivIVA, GpsB	• Early division proteins, Z-ring formation (FtsZ), its regulation and membrane anchoring • Chromosome segregation during division • Unknown function in septal PG synthesis • PG synthases and flippase for cell division • PG hydrolases; activation of PcsB (FtsEX) • Septum synthesis/completion
Signaling/control	• StkP • PhpP	• Ser/Thr kinase, control of cell division • Phosphatase, control of cell division

[a] *The Spr protein names are for strain R6.*
[b] *See text for detailed discussion and references.*

TABLE 8.2 Reported Chain Length and Phosphocholine Content Per Repeat for Pneumococcal TAs

	LTA		WTA	
Pneumococcal strain[a]	**No. of RU**	***P*Cho/RU**	**No. of RU**	***P*Cho/RU**
Serotype 1[b]				1 [80]
Serotype 2				
R6	6–8 [78], 5–7 [69], 7–8 [83]	2 [69,78,83]	1–8 [5]	2 [78,83]
R36	5–7 [69]	2 [69]		
R36A				2 [81]
D39	5–7 [69]	2 [69]		
D39Δ*cps*	4–7 [70]	2 [70]	5–6[c]	2[c]
Rx1	6–7 [78], 7–8 [83]	1 [78,83]	5–9 [78]	1 [78,83]
CSR SCS2				1 [82]
Serotype 3				
Wu2	5–8 [69]	2 [69]		
Serotype 4				
TIGR4	5–8 [69]	2 [69]		
Fp23 (TIGR4Δ*cps*)	4–7 [70]	2 [70]		2[c]
Serotype 5[b]				2 [82]
Serotype 6B[b]				2 [82]
MX-73 HIM	5–7 [69]	2 [69]		
Serotype 7F[b]				2 [82]
Serotype 9A[b]				1 [80]
Serotype 14[b]				2 [82]
Serotype 18B[b]				1 [84]
Serotype 18C[b]				1 [80]
Serotype 23F[b]				1 [80]
Serotype 32A[b]				1 [84]
Serotype 32F[b]				1 [84]

[a] *The Danish nomenclature is used for serotype designation.*
[b] *No strain specified.*
[c] *N. Gisch, unpublished.*

substitution is a result of a post-biosynthetic removal of one residue or, more likely, caused by an incomplete biosynthesis, remains to be determined. The structural models for LTA and WTA summarizing all the above-mentioned observations are depicted in Figure 8.2B. Since the connection of the WTA to the PG has not been clarified yet, the anomeric configuration of the terminal AATGal*p* remains to be determined. Based on evidence from chemical hydrolysis experiments, WTAs are likely linked to the PG via a phosphodiester bond to the C6 hydroxyl group of the Mur*N*Ac [60].

BIOSYNTHESIS OF PNEUMOCOCCAL TAs

S. pneumoniae is the only known bacterial species depending on exogenous choline for growth. Choline is exclusively metabolized to decorate the TA chains [3]. The biosynthesis of the repeating unit of TAs has recently been deduced based on a bioinformatic analysis of the *S. pneumoniae* R6 genome, the structural details of the correct biosynthetic RU of TAs (above), and known mutations in choline-independent pneumococcal mutant strains [77,83,85–87]. The predicted pathway is summarized in Figure 8.3; it requires the products of at least 16 known and hypothetical genes, organized in four different chromosomal regions [77]. Because the structural revisions of TAs showed AATGal*p* at the start of the biosynthetic repeating unit, the first membrane step is most likely the transfer of AATGal*p*-1-phosphate from UDP-AATGal*p* to an undecaprenyl phosphate lipid anchor. Here, the anomeric linkage (α or β) of AATGal*p* is not known. The transfer is predicted to be performed by Spr1655, which shares 44% sequence identity with WcfS of *Bacteroides fragilis*, which catalyzes this reaction as part of the exopolysaccharide synthesis [88]. UDP-AATGal*p* is synthesized by the conversion of UDP-Glc*pN*Ac (via UDP-4-keto-6-deoxy-Glc*pN*Ac), presumably by Spr0092 and Spr1654 [77]. Afterwards, glucose, ribitol-phosphate, and the two Gal*pN*Ac residues are added sequentially. The predicted glycosyltransferase gene *spr0091* is essential for pneumococcal growth [89]. That Spr0091, and not the other putative glycosyltransferases Spr1123 and Spr1124, transfers the glucose [77] is supported by the fact that the pneumococcal serotype 5 strain 70585, in which Spr0091 is replaced by SP70585_0164 [90], bears a Gal residue in the repeating units of its WTA instead of the Glc [82]. LicD3 (Spr1125) catalyzes the transfer of ribitol-phosphate from CDP-ribitol to the glucose, followed by the transfer of the two Gal*pN*Ac residues by the predicted glycosyltransferases Spr1123 and Spr1124. CDP-ribitol is synthesized from ribulose-phosphate via ribitol-phosphate by TarJ (Spr1149) and TarI (Spr1148) [91]. The proposed role of LicD3 in the ribitol-phosphate transfer is corroborated by genetic data, using a choline-independent *S. pneumoniae* strain (R6Cho$^-$), in which the whole *lic3* gene region (genes *spr1221–spr1225*) has been replaced by *S. oralis* DNA [92].

*P*Cho residues are incorporated on the O6 positions of both the Gal*pN*Ac residues by LicD1 (Spr1151) and LicD2 (Spr1152) to form the complete repeating unit building block (framed red in Figure 8.3). LicD1 is essential and likely incorporates *P*Cho to the α-D-Gal*pN*Ac; LicD2 is not essential and transfers another *P*Cho to the β-D-Gal*pN*Ac residue. Strain Rx1 lacked the *P*Cho modification at β-D-Gal*pN*Ac and incorporated only half the amount of choline into its cell wall compared to the parental strain R6 [67,83], similar to the R6*licD2* mutant [93]. Interestingly, the *licD2* mutant was impaired in its adherence to human alveolar cells and shows reduced virulence in the intraperitoneal mouse model, indicating that both *P*Cho residues are required for full virulence [93].

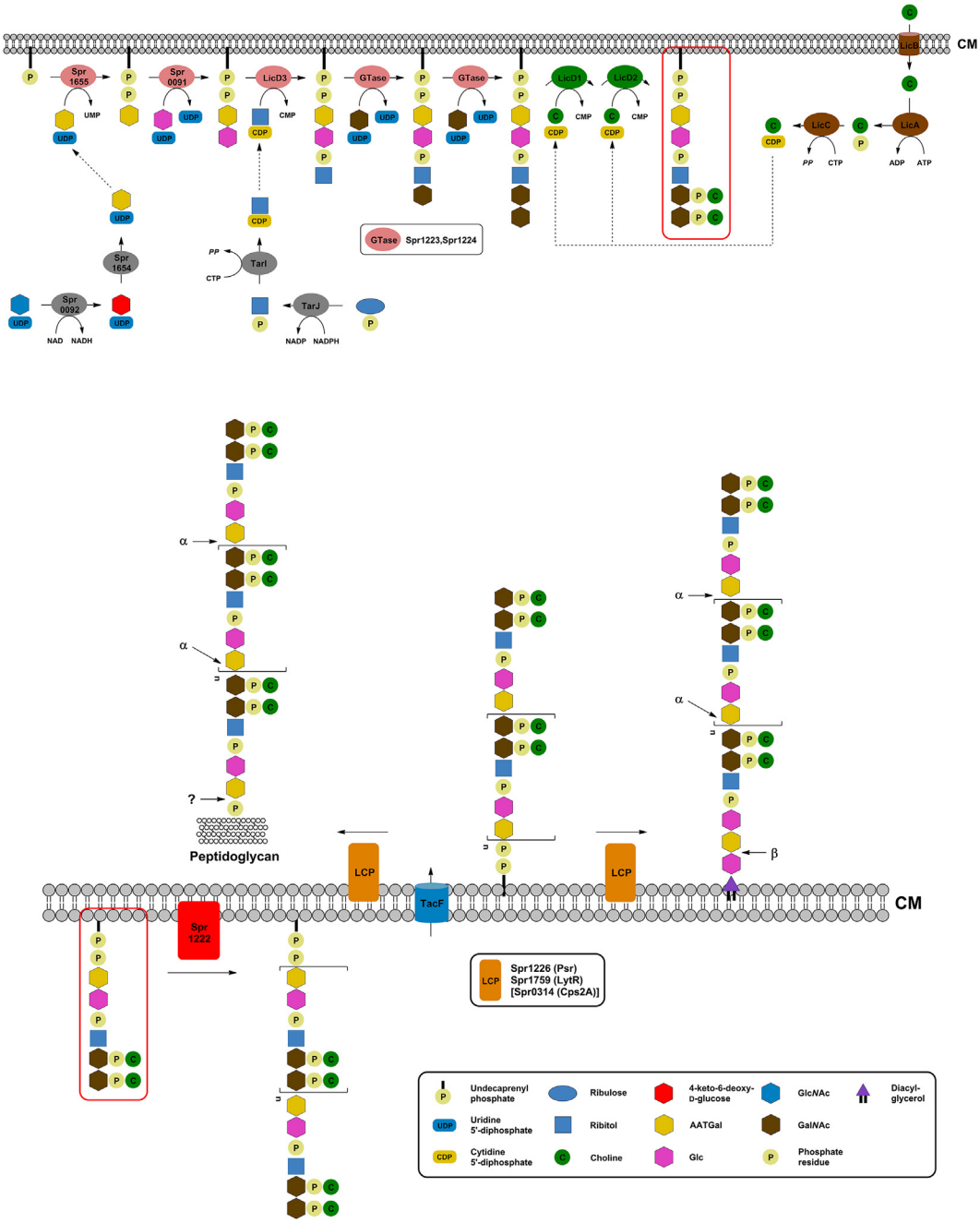

FIGURE 8.3 **Proposed pathway for TA biosynthesis and cell wall incorporation in *S. pneumoniae*.** The gene and protein names are for strain R6. AATGal, 2-acetamido-4-amino-2,4,6-trideoxygalactose; CM, cytoplasmic membrane; CMP, cytidine-monophosphate; Gal*N*Ac, *N*-acetylgalactosamine; Glc, glucose; Glc*N*Ac, *N*-acetylglucosamine; GTase, glycosyltransferase; LCP, LytR-Cps2A-Psr family protein; UMP, uridine-monophosphate. *Source: Modified from [77].*

Three proteins are involved in the uptake and activation of exogenous choline: LicA, LicB, and LicC. LicB is a predicted integral membrane transporter for choline uptake [94]. In the cytoplasm, choline becomes phosphorylated by the choline kinase LicA [95], and then activated by the cytidylyl transferase LicC to CDP-choline [96,97], which is the substrate for LicD1 and LicD2.

Several important questions concerning TA biosynthesis remain to be answered. For example, how are the RUs polymerized, and where does this process take place? It was hypothesized that the integral membrane protein Spr1222 catalyzes this reaction on the cytosolic side of the membrane [77], but experimental proof is still lacking. The TA precursor or chain has to be translocated to the outer side of the membrane, a process probably catalyzed by TacF (Spr1150), which belongs to the family of transmembrane transporters (flippases) with 14 predicted transmembrane helices; it is an essential gene [77,85]. Interestingly, just a single point mutation in *tacF* renders strain R6Chi independent for exogenous choline. Therefore, the choline dependency of pneumococcal growth seems to be caused by the specificity of TacF for *P*Cho-loaded TA chains, ensuring the loading of the cell wall with *P*Cho. Presumably, the mutation in *tacF* altered this transport specificity, thus allowing the transport of *P*Cho-loaded and unloaded TA chains, explaining the growth of the mutant in the presence and absence of exogenous choline [85].

The undecaprenyl diphosphate–linked TA chains are transferred to the lipid anchor to form the LTA or to the PG to form the WTA. In particular, LytR (Spr1759) and Psr (Spr1226), both representing phosphotransferases, are suggested as being involved in these processes [10,77]. LytR and Psr belong together with their homolog Cps2A to the LytR-Cps2A-Psr (LCP) protein family. A recent study showed that all three LCP homologs have semi-redundant roles in retaining capsular polysaccharide at the cell wall, and perhaps they also attach TA precursor chains to PG and the glycolipid anchor [10].

In *S. aureus* the lack of all members of the LytR-CpsA-Psr gene family leads to the secretion of cell-wall TAs to the extracellular medium, causing a significant drop in cell-wall phosphate level [98]. The precise role of each of these enzymes, as well as the biochemical mechanisms of the attachment reactions, have yet to be defined.

The lipid anchor of LTA, α-D-Glc*p*-(1→3)-DAG, is produced by the glucosyltransferase Spr0982, which attaches a Glc to DAG [99]. Formation of the second major pneumococcal glycolipid α-D-Gal*p*-(1→2)-α-D-Glc*p*-(1→3)-DAG requires the glycosyltransferase CpoA [100,101]. Interestingly, two piperacillin-resistant laboratory mutants of *S. pneumoniae* R6 have mutations in the *cpoA* gene and a normal cell-wall choline content [102], suggesting that TAs are present in similar amounts and that the *cpoA* mutations do not affect TA biosynthesis [101].

INTERACTIONS OF PNEUMOCOCCAL CELL-WALL COMPONENTS WITH HOST FACTORS

The innate immune system initially recognizes *S. pneumoniae* by several pattern-recognition receptors, which control the host's defense mechanisms, including transmembrane Toll-like receptors (TLRs), cytosolic NOD-like receptors (NLRs), DNA sensors, scavenger receptors, and C-type lectins [103,104]. In the following, the interactions of TAs and pneumococcal PG with the host immune system are summarized.

The inflammatory potency of LTAs has been discussed in a controversial manner over the last two decades. Besides several other bacterial cell wall components, LTAs have long been

thought to activate specifically the Toll-like receptor 2 (TLR2) [105,106]. In this regard, the LTAs of *S. aureus* and *S. pneumoniae* have been investigated and compared most extensively [105,107]. A significant contribution to clarifying the general immunostimulatory potency of LTAs came from an *S. aureus* SA113Δ*lgt* strain that was deficient in the lipidation of the prelipoproteins and, most importantly, showed significant attenuation in immune activation and growth [108]. Subsequent studies demonstrated that lipoproteins (LPs) are the predominant TLR2 stimuli in LTA preparations of *S. aureus* and not the LTA itself [106].

The mechanism of this immune activation has been specified further in recent years: The signaling induced by triacylated LPs occurs via a TLR2/TLR1-heterodimer (proven with a hTLR2-hTLR1-Pam_3CSK_4 co-crystal [109]), whereas diacylated LPs signal via a TLR2/TLR6-heterodimer (as shown by solving an mTLR2-mTLR6-Pam_2CSK_4 co-crystal [110]). Experiments using a mouse pneumonia model and leukocytes from IL-1 receptor-associated kinase-4-deficient patients showed that TLR2- and IL-1 receptor-associated kinase-4-mediated inflammatory responses to *S. pneumoniae* are dependent on the surface expression of pneumococcal lipoproteins [111]. Recently the absence of TLR2-stimulating activity in the LTA preparation of a pneumococcal Δ*lgt* mutant (of strain D39Δ*cps*) has been proven; in contrast to the staphylococcal LTA from SA113Δ*lgt*, this preparation had residual immunostimulatory properties in human mononuclear cells (hMNCs) [70]. This was consistent with the stimulation of hMNCs by a synthetic short-chain analog of pneumococcal LTA [112]. The receptor(s) and the mechanism through which LTA in general stimulates the innate immune system are only rarely investigated. The proposed C-type lectin pathway of complement should be considered with caution because most studies have not used LTA preparations from Δ*lgt* mutants [113–116]. However, mouse infection models pointed out an important role of the classical pathway of complement activation in the innate host immune response to *S. pneumoniae* [117], as well as an amplification function of the alternative pathway [118]. In addition, mice deficient in the mannose-binding protein-associated serine protease 2 are highly susceptible to pneumococcal infection due to a defect in *S. pneumoniae* opsonization and clearance [119]. Mice deficient in ficolin A and/or ficolin B displayed increased susceptibility to *S. pneumoniae* infection, thus substantiating the importance of the lectin pathway in immune response to pneumococcal infections [120]. Moreover, it has been shown that mouse ficolin A, human L-ficolin, and CL-K1 recognize the pneumococcal cell surface and trigger activation of the lectin complement pathway [119]. L-ficolin is able to interact with the synthetic short-chain analog of LTA and with the synthetic *pseudo*-pentamer β-D-Glc*p*-(1→3)-α-AATGal*p*-(1→4)-(6-*O*-*P*Cho)-α-D-Gal*p*NAc-(1→3)-(6-*O*-*P*Cho)-β-D-Gal*p*NAc-(1→1)-ribitol [121], a partial structure of pneumococcal TAs [112]. As reported in this study, L-ficolin interacts through its fibrinogen-recognition domain with the *P*Cho substituents of these two synthetic compounds.

The specific recognition of (pneumococcal) PG by NLRs, followed by induction of the inflammatory cascades, is another ongoing and intensively investigated area of the mammalian innate immune response. However, the exact mechanisms of activation and regulation of the different protein complexes formed by NLRs, the so-called "inflammasomes" [122], have not been elucidated completely. The PG of nearly all bacteria, including *S. pneumoniae* [123], is recognized by NOD2 [124]. This process involves phagocytosis, lysozyme-dependent cell-wall digestion in macrophages, and pneumolysin (Ply)-mediated delivery of pneumococcal PG fragments into the cytosol [125].

In recent years it has become increasingly clear that the NLRP3 inflammasome has a key function in PG recognition [126,127]. $NLRP3^{-/-}$

mice are significantly more susceptible to pneumococcal pneumonia than wild-type mice [128,129], since NLRP3 controls the bacterial clearance [128] and is required for maintenance of the epithelial/endothelial barrier in the lung [129]. However, NLRP3$^{-/-}$ cells and mice are still capable of producing significant amounts of IL-1β after infection with *S. pneumoniae* [128–130], indicating that other inflammasomes—recognizing distinct pneumococcal cell-wall components—might contribute to the recognition of pneumococci by the innate immune system as well.

CONCLUDING REMARKS

The last few years have seen a substantial increase in knowledge of cell-wall synthesis, processing or modifying enzymes in *S. pneumoniae*. This progress was made possible by new, powerful tools for the structural analysis of pneumococcal cell wall components, especially PG and TAs, and by novel methods to study pneumococcal cell biology. However, the impressive level of complexity associated with the biosynthesis of pneumococcal cell wall requires further research. We know little about how the TA-containing PG is synthesized to increase the cell surface of a growing and dividing cell, and how cell-wall synthesis is regulated and coordinated with other cellular processes. The role of the LytR-CpsA-Psr family enzymes in attaching the TA precursor chains and the capsule to the PG, and the mechanism of this crucial reaction in maintaining cell-wall stability, and hence bacterial viability, need to be investigated at the molecular level. Furthermore, we need to elucidate the specific molecular requirements for the anchoring of choline-binding proteins at the TAs. In addition, the interaction of pneumococcal cell-wall components with the innate immune system is currently a widely discussed topic. The recognition of PG partial structures by NLRs and TLR2 stimulation by LPs is now widely accepted, but the interaction of pneumococcal TAs with the complement system is only rarely investigated. With new tools available, we anticipate that many outstanding questions on the physiology and biological interactions of the pneumococcal cell wall will be answered within the coming years.

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CHAPTER

9

Capsule Structure, Synthesis, and Regulation

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INTRODUCTION

A wide variety of pathogenic bacteria possess polysaccharide extracellular capsule structures, including *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. The role of the capsule structures is generally similar in these pathogens, namely evasion of the host immune system. Capsule structures can be split into two main groups—a "proper" capsule (capsular polysaccharide, CPS) in which the polysaccharide material is attached to the cell, or an exopolysaccharide (EPS) in which the polysaccharide is secreted into the medium. The pneumococcus possesses a CPS which in most strains is covalently attached to the cell wall by the enzyme CpsA [1]; the function of the pneumococcal capsule is discussed in detail in Chapter 21. In addition to the attachment to the cell wall, we and others have seen significant secretion of pneumococcal CPS polysaccharide into the extracellular milieu [2], and this may also have a significant role during infection, but not (unlike in some other bacteria) biofilm formation [3].

S. PNEUMONIAE CPS SEROTYPES

Many bacterial pathogens possess limited capsular serotypes, with, for example, *S. pyogenes* having just one. In contrast, more than 90 distinct CPS serotypes have been identified in the pneumococcus to date. Each CPS serotype has a unique capsule gene locus, polysaccharide chemical structure, and serological property. For all but two pneumococcal CPS serotypes, biosynthesis follows a Wzy-dependent mechanism, as described later. Two strains, serotypes 3 and 37, do not follow the norm but utilize a synthase-dependent mechanism, also described later. In general, the structures of synthase-dependent CPS are simpler than their Wzy-dependent counterparts, as are the genes responsible for their biosynthesis (Figures 9.1B, 9.2B and C). The polymers comprising Wzy-dependent CPS are normally composed of repeating units of three or four monosaccharides. Which monosaccharides, their order, the glycosidic linkages between them, and the presence or absence of branches dictate CPS serotype. Conversely,

Streptococcus pneumoniae.
DOI: http://dx.doi.org/10.1016/B978-0-12-410530-0.00009-0

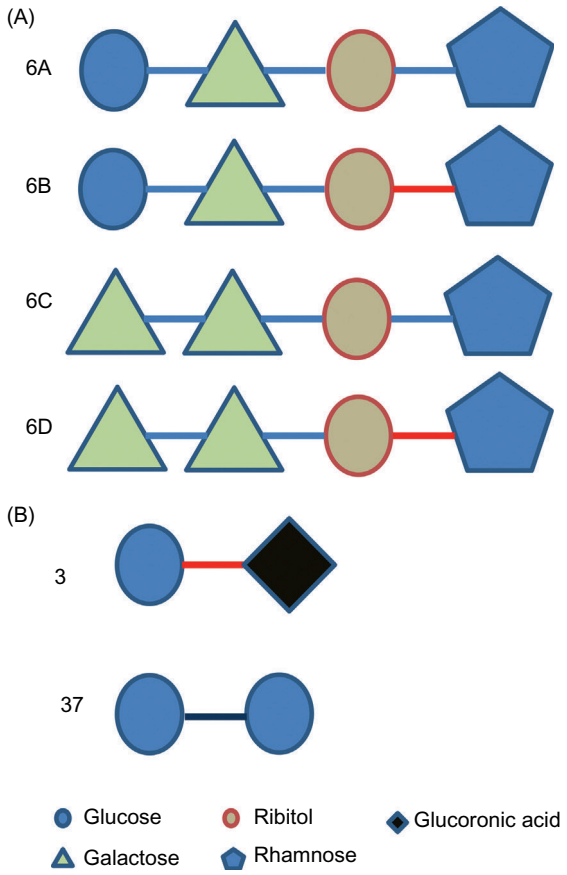

FIGURE 9.1 **Representative structure of pneumococcal capsules.** (A) Structures of Wzy-dependent capsules from serogroup 6 strains, representing the small variation within members of the serogroup. Differences in linkages between sugars are highlighted by colored lines. (B) Comparatively, the structure of synthase-dependent capsules from serotype 3 and 37 are much simpler structures.

synthase-dependent capsules are normally composed of one (serotype 37) or two sugars (serotype 3).

Some structurally similar CPSs show a degree of serologic cross-reactivity to serotype-specific polyclonal antibodies and are thus grouped into one individual serogroup. An example of this is serogroup 6, of which there are now six different members [4] (four of which are summarized in Figure 9.1A). Members of particular serogroups can generally be distinguished from each other through the use of monoclonal antibodies. Single nucleotide [5] changes within the capsule loci of both serogroup 6 and 9 serotypes can be responsible for these serological differences. This large number of capsular serotypes creates significant antigenic diversity, which may enable the pathogen to evade the adaptive immune response. This has been illustrated by the identification of strains that have switched capsular serotype by genetic recombination of the *cps* locus, which has allowed these strains to evade the adaptive immunity induced by the current pneumococcal vaccines, which target a limited number of specific capsular serotypes (described in Chapter 3). Interestingly, a recent study has described additional mechanisms of variation in CPS structure that may allow immune evasion [6]. Serotype 11D differs from serotype 11A by a single codon change in WcrL, the glycosyltransferase which adds the fourth sugar of the repeating unit of the polysaccharide chain. As well as changing serotype specificity, this codon change enables WcrL to act as a bispecific glycosyltransferase, adding either Glc or GlcNAc and resulting in a CPS with a mixture of two different repeating unit structures [6]. These data suggest that CPS structure, and therefore serotype specificity, can be more readily manipulated than perhaps it was first thought. Similar effects may be seen with other glycosyltransferases, resulting in more variation in CPS structure and subsequent immune evasion.

THE CAPSULE GENE LOCUS

The locus responsible for CPS biosynthesis (Figure 9.2) is located at the same chromosomal location in all serotypes, between *dexB* and *aliA* on the chromosome. The sole exception is serotype 37. Biosynthesis of serotype 37 CPS relies on one gene, *tts*, located outside the CPS locus on the chromosome, with a cryptic serotype 33F

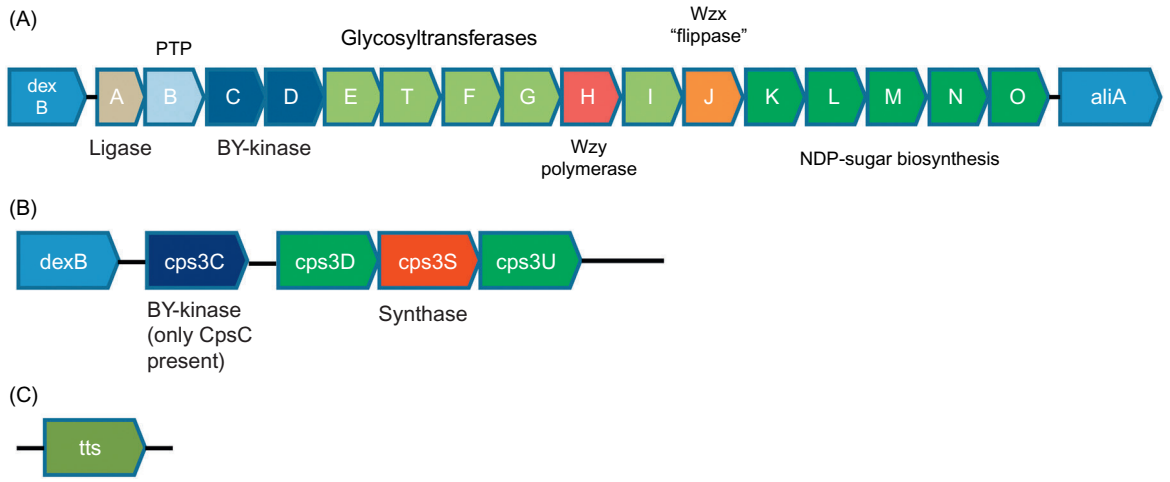

FIGURE 9.2 **Examples of capsule locus.** (A) Capsule locus from serotype 2 D39 (Wzy-dependent), (B) from serotype 3 (synthase-dependent), and (C) from serotype 37. Only genes that do not possess mutations and/or are functional are shown for serotype 3. In serotype 3 only *cps3D* and *cps3S* are essential for capsule production. For serotype 37, *tts* is the only gene responsible for capsule production; this is found at a site distant to the capsule locus.

locus present at the CPS locus [7,8]. The original nomenclature of CPS genes was *cpsA*, *cpsB*, etc. However, in recent times, a new nomenclature has also been used in order to avoid the same enzyme being called different names between serotypes due to gene deletions and insertions.

For Wzy-dependent loci, the CPS locus begins with four highly conserved loci: *cpsA*, *cpsB*, *cpsC*, and *cpsD* (*wzg*, *wzh*, *wzd*, and *wze*). CpsA has been proposed to act as a transcriptional regulator of CPS biosynthesis in a range of streptococci [9], with evidence of it binding to DNA. However, a recent study has suggested that CpsA is the long-sought ligase responsible for ligation of CPS to the cell wall [1]. The three other genes form a much-studied tyrosine phosphorylation regulatory cascade. As discussed in detail later, these genes are involved in the regulation of the biosynthesis of Wzy-dependent capsule. While analysis of *cpsA* sequences has illustrated that they comprise a single cluster of related sequences, the sequences of *cpsB*, *cpsC*, and *cpsD* form two distinct sequence clusters. Interestingly, a survey of epidemiological data found that one cluster was associated with carriage, while the other was associated with invasive disease [10]. The genes immediately downstream of *cpsA−D* are responsible for encoding the initiating glucose phosphate transferase (*cpsE*/*wchA*), the polysaccharide polymerase (*wzy*), and the flippase (*wzx*) required for CPS production. Following these, the genes are serotype specific, encoding proteins such as glycosyl and acetyltransferases, along with enzymes responsible for the synthesis of nucleotide-activated sugar precursors.

While the *cps* locus for serotype 3 is present at the same location and possesses the same 5′ region as other serotypes, all but *cpsC* contain mutations [11]. Additionally, there is evidence the genes are not transcribed, although more work is still required to confirm this [11]. A number of genes in the 3′ end of the locus also contain truncations, and only two genes in the locus are required for serotype 3 CPS expression. These include *cps3d* (*ugd*), which encodes the UDP-Glc dehydrogenase responsible for oxidizing UDP-Glc to form UDP-GlcUA [12]. The other essential enzyme is the synthase

encoded by *cps3s* (*wchE*). As with Wzy-dependent loci, the genes responsible for serotype 3 synthesis are transcribed as operons [13].

BIOSYNTHESIS OF CPS

As noted earlier, the CPS of *S. pneumoniae* is synthesized by one of two mechanisms—either Wzy-dependent or synthase dependent—while the ABC transporter-dependent mechanism seen in other bacteria is absent [14]. Both mechanisms are conserved throughout bacteria, and are seen in a variety of both Gram positive and Gram negative bacteria. There are, however, significant differences between the two, summarized in Table 9.1.

Wzy-Dependent Biosynthesis

Wzy-dependent synthesis begins with sugar repeat units assembled on the inner side of the cell membrane, followed by transport to the outside of the membrane by the flippase, and then subsequent polymerization by the namesake Wzy polymerase. The initiation of synthesis begins with the reversible transfer of sugar phosphate to undecaprenol phosphate (UndP) via a pyrophosphate linkage, resulting in the formation of an UndPP-sugar. Interestingly, UndPP is also utilized in the formation of peptidoglycan. In most pneumococcal serotypes, the initiating sugar is Glc-1-P, and the reaction is catalyzed by the glycosyltransferase CpsE, an integral membrane protein. Subsequent additional monosaccharide subunits, either identical or different, are then added to the growing oligosaccharide chain, with these processes being undertaken by additional glycosyltransferases encoded by the *cps* locus.

The growing chain is then flipped to the outer face of the membrane by the appropriately named enzyme, the flippase Wzx [15]. Once on the other side, the Wzy polymerase then polymerizes these oligosaccharide repeat units in a non-processive manner by blockwise addition of repeats to the non-reducing end of single repeat units. The CPS then needs to undergo one final step—attachment to the cell. As with most Gram positive bacteria, in the pneumococcus CPS is covalently attached to the cell, probably to peptidoglycan. This process is undertaken by CpsA, transcribed from the

TABLE 9.1 Significant Differences Between Wzy-Dependent and Synthase-Dependent Capsule Biosynthesis

Wzy-dependent	Synthase
CPS covalently attached to cell wall	CPS connected via phosphatidylglycerol anchor or through interactions with synthase enzyme
Different enzymes for:	Synthase enzyme responsible for:
a. Initiation (CpsE) **b.** Polymerization (Wzy) **c.** Transport (Wzx)	**a.** Initiation **b.** Polymerization **c.** Transport of polymer
Polymers can be complex—contain multiple sugars/linkages	Polymers generally simpler
Blockwise addition of discrete repeat units	Sugars added to chain while it is still being synthesized
Regulated by phosphotyrosine regulon (CpsBCD)	Phosphotyrosine regulation either not expressed or mutated

conserved 5′ region of the CPS locus [1]. The site and the nature of the exact linkage formed to attach the CPS has yet to be reported.

Synthase-Dependent Capsule Biosynthesis

Synthase CPS biosynthesis, as used by serotypes 3 and 37, is a significantly different process compared to that described earlier. Unlike Wzy-dependent CPS, such CPSs are not found covalently attached to the cell but rather are connected to the cell via a phosphatidylglycerol membrane anchor or through interactions with the synthase enzyme [16]. In synthase-dependent CPS biosynthesis, a single enzyme is responsible for (1) initiation of biosynthesis, (2) polymerization of the polysaccharides, and (3) transport of the polymer. These enzymes are the synthases, and they are integral membrane proteins. The polymers comprising these polysaccharides are also generally much simpler than their Wzy counterparts. For example, serotype 3 is composed of a single disaccharide repeat unit consisting of glucose and glucuronic acid. The other significant difference is that while Wzy-dependent uses blockwise addition of discrete repeat units, in the synthase-dependent system, individual sugars are added to the growing chain on the non-reducing end while it is still being synthesized [17].

The biosynthesis of serotype 3 CPS begins with the transfer of glucose from UDP-Glc to the phosphatidylglycerol membrane anchor [16]. Synthesis subsequently continues with the alternating addition of glucoronic acid and glucose from their relevant NDP-sugars, with the pace of this reaction regulated by the availability of the sugars, as described later. An important switch is observed when the length is approximately eight sugars in length, with this resulting in binding of the lipid-linked sugars to the synthase [18]. This binding brings about the processive synthesis of high-molecular-weight polymers, and additionally results in the changing of the orientation of the polysaccharide, enabling it to protrude through the cell membrane. This is another difference from Wzy-dependent capsule synthesis. The capsule remains attached to the cell via interactions with the phosphatidylglycerol or through interactions with the synthase, rather than being bound to the peptidoglycan [19]. While many details concerning the mechanism of type 37 CPS biosynthesis are unknown, the synthase encoded by *tts* is essential [8]. Indeed, introduction of this protein into other bacteria results in production of a homologous CPS [7]. Type 37 is the only homopolysaccharide CPS described to date in the pneumococcus; it can use either UDP-Glc or UDP-Gal as a substrate, suggesting that the synthase has bifunctional activity [7].

REGULATION OF CAPSULE BIOSYNTHESIS

General Considerations for Regulation of CPS Biosynthesis

As CPS is a critical factor for the virulence of the pneumococcus [20], control of CPS expression is essential for successful colonization and for causing disease. During initial colonization, CPS expression is required in order to escape from the mucus [21]. However, expression subsequently needs to be down-regulated such that adhesins, including the choline-binding protein CbpA (PspC) [22], can be exposed and enable the pneumococci to adhere to and colonize the nasopharynx. Invasive disease then requires a switch to increased CPS expression so that the pathogen can avoid killing by host phagocytes and can establish infection. Several factors have been described that play a role in the regulation of CPS, as described later and summarized in Figure 9.3. However, much remains unknown about how CPS levels are controlled by the pneumococcus during infection at different anatomical sites.

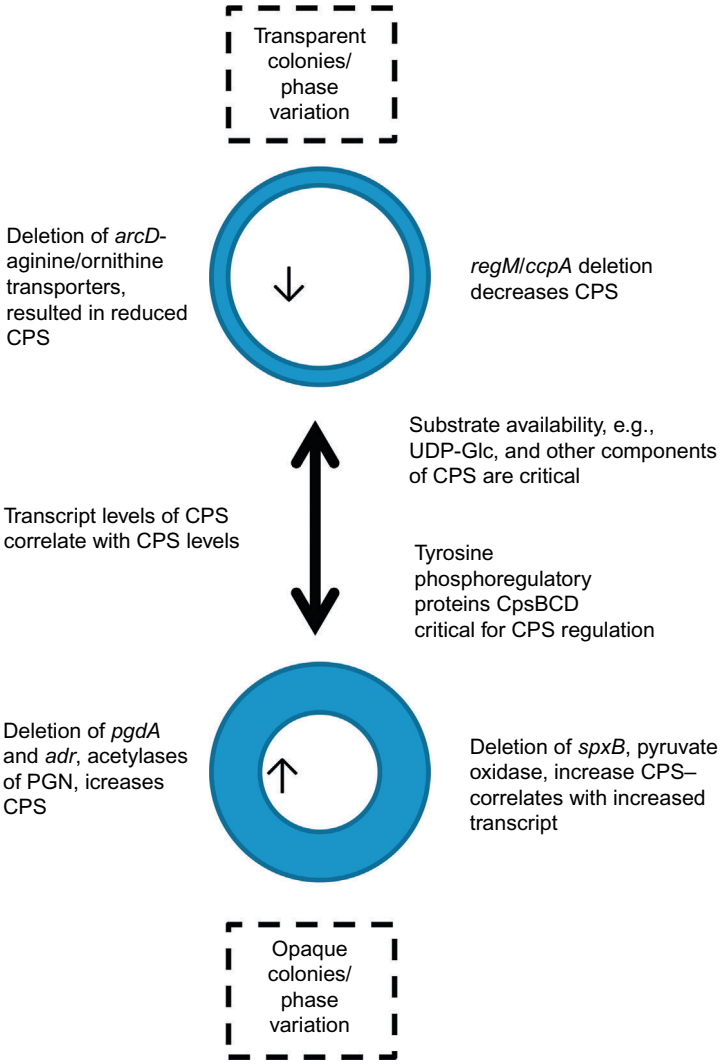

FIGURE 9.3 Effects on capsule expression. Schematic illustrating a number of different factors that play a role in the regulation of capsule biosynthesis in the pneumococcus.

One as yet undefined mechanism of regulation that has received much attention is phase variation. In the 1990s, Weiser et al. [23] described the observation of two pneumococcal colony morphologies, designated opaque and transparent. Recent important data show that genetic rearrangements of a Type I restriction-modification system causes epigenetic modifications that correlate with opaque / transparent phenotypes [54]. While the mechanism by which controls the switch between these two phases occur is not known, opaque and transparent phases show a number of well-characterized phenotypic differences, including the level of CPS expression. The transparent phenotype, which predominates during asymptomatic colonization of the nasopharynx, has lower levels of CPS, likely enabling it to have greater exposure of adhesins critical to adhere to epithelial cells. On the other hand, the opaque phenotype, with enhanced invasive disease potential, shows relatively increased CPS

expression for improved evasion of host immune responses.

A number of studies have found specific regulators and proteins that play key roles in CPS regulation. Included in these is RegM (also known as the catabolite control protein A), which can influence the level of CPS [24]. A study investigating the effect of modifications of peptidoglycan on lysozyme resistance also found interesting effects on CPS levels. Mutations in both PgdA, which deacetylates GlcNac, and Adr, which acetylates MurNAc, both resulted in 5–8-fold increases in the synthesis of CPS. It is interesting to hypothesize that the effect of mutating PgdA, which would lead to a less positively charged cell wall, could affect the attachment of anionic molecules such as the type 4 capsule [25].

A recent study in which the *cpsA* promoter was altered has shown that manipulating levels of CPS locus transcript manipulates the level of CPS produced [26]. Increased transcription led to increased CPS, while decreased transcript-reduced CPS levels. Interestingly, both strains showed loss of virulence in a mouse model of disease, likely due to their inability to regulate levels of CPS and adapt to the various environmental niches. These data suggest that regulating capsule locus mRNA levels is a likely mechanism used to regulate levels of CPS expression. Another study that fits with this hypothesis concentrated on the pyruvate oxidase SpxB, which is responsible for the ability of the pneumococcus to produce and be resistant to hydrogen peroxide. Deletion of *spxB* from the pneumococcus resulted in increased CPS production, correlating with increased transcription of the capsule locus itself [27]. However, a study investigating the mRNA expression of a multitude of virulence factors in different *in vivo* niches showed that the expression of *cpsA*, the first gene of the *cps* locus, did not vary between the nasopharynx, lungs, and blood [28], despite transparent colonies (less capsule) predominating in the nasopharynx, while opaque (more capsule) was more prevalent at sites of invasive disease. This suggests that regulation of opaque/transparent phase variation is independent of *cps* locus expression, and helps to illustrate the complexity behind the regulation of CPS levels in the pneumococcus.

Another study investigated the impact of *arcD* on CPS levels [29]. ArcD is an arginine/ornithine antiporter transporter that forms one component of the arginine deiminase system involved in arginine catabolism, and thus plays a role in the virulence of a number of pathogenic bacteria, including the pneumococcus. In D39 *S. pneumoniae*, mutation of *arcD* leads to decreased invasive disease, while the strain displayed an increased capacity to colonize the nasopharynx. This appeared to correlate with the *arcD* mutation being associated with a decreased level of CPS, which, while rendering it more susceptible to host phagocytes, enabled it to adhere to host cells, and thus presumably to possess an enhanced colonization capacity. How ArgD affected CPS levels is not known and did not seem to be related to reduced intracellular argninine levels [29].

Regulation by Tyrosine Phosphoregulon CpsBCD

The best-studied mechanism of CPS regulation in Wzy-dependent capsules is the tyrosine phosphoregulon encoded by *cpsB*, *cpsC*, and *cpsD* [30]. Indeed, this is a promising target for development of novel antivirulence antimicrobials [31]. These genes, present within the CPS locus, encode CpsB, a protein tyrosine phosphatase (PTP), and CpsC and CpsD, which together form an active bacterial tyrosine kinase. All three are important for complete CPS production [32], as well as for the ability of the pneumococcus to colonize and cause invasive disease [2,32].

CpsB is a PTP, from the family of polymerase and histidinol phosphatase [30]. This family comprises a range of proteins with diverse function, including DNA polymerases

[33] and histidinol phosphatases [34]. Studies on the role of CpsB in CPS regulation have produced divergent results. Some studies report that mutants in *cpsB* show reduced CPS levels [2,30,32], while others suggest that this is not the case [35]. What is clear is that the tyrosine phosphatase functions to dephosphorylate CpsD, with increased phosphorylation of CpsD evident in *cpsB* mutants [36]. Interestingly, a recent study has shown that an inhibitor of CpsB phosphatase activity, FQE, results in lower levels of CPS, and increased adherence to macrophages, suggesting that CpsB may be a novel target for the development of antivirulence antimicrobials [37].

The importance of CpsD for the level of CPS production is more widely accepted, with multiple studies suggesting mutation of *cpsD* results in non-encapsulated pneumococci unable to cause invasive disease [32,35]. The BY-kinase family of tyrosine kinases, to which CpsD belongs, are characterized by the presence of Walker A and Walker B motifs, and show homology to the MinD family of proteins [38]. Unlike in Gram negative bacteria, in the pneumococcus and other Gram positive bacteria such as *S. aureus*, the BY-kinase is split in two: a membrane protein CpsC and a cytosolic component CpsD. CpsC is predicted to encode two transmembrane segments and is a member of the polysaccharide co-polymerase family of proteins [39]. CpsC acts as a transmembrane activator, with the activity of the cytoplasmic BY-kinase component CpsD dependent on its interacting with CpsC [35]. This appears to be specifically dependent on interaction with the C-terminal cytoplasmic region [40]. Interestingly, mutations in the extracellular loop of CpsC result in lower levels of tyrosine phosphorylation of CpsD, and lower levels of CPS, suggesting that these are important in the regulation of kinase activity [41].

While the contribution of the CpsBCD system to CPS regulation is not disputed, how this regulation occurs is poorly understood. Indeed, confusion as to the effect of mutations in CpsB have resulted in a lack of understanding as to whether it is the phosphorylated or non-phosphorylated form of the BY-kinase that is important for optimal capsule biosynthesis. Indeed, similar discrepancies have been seen in homologous systems in other bacteria as well [42,43]. Structures of BY-kinases from *E. coli* and *S. aureus* have shown that the non-phosphorylated form is an octomer that becomes disrupted by phosphorylation [44,45]. It seems likely that additional targets of CpsD and CpsB outside the CPS locus may play important roles in CPS regulation. In *E. coli*, *Bacillus subtilis*, and *S. aureus*, tyrosine phosphorylation can modulate UGD activity, which is important for CPS biosynthesis, as described earlier, and therefore potentially CPS expression [46–49]. Whether similar mechanisms of regulation exist in the pneumococcus is still not apparent; however, a number of pneumococcal strains do possess UGDs critical for encapsulation. Recent phosphotyrosine proteomes in a range of bacteria have identified a large number of tyrosine phosphorylated proteins, with a total of up to 342 identified in *E. coli* alone [50]. This suggests that significantly more work is required in order to identify substrates of CpsB and CpsD and to understand their role in the regulation of CPS.

Substrate Limitation

Serotype 3 and 37 capsules do not possess active BY-kinases or PTPs, so regulation by this mechanism does not occur in these serotypes. Instead, sugar metabolism plays a key role in regulation of serotype 3 CPS biosynthesis. Mutation of a phosphoglucomutase, which resides outside the CPS locus and is required for the production of UDP-Glc, results in a strain that produces less than 10% of wild-type CPS [13]. Thus, it would appear that regulation of the activity of this enzyme, and thus availability of a monosaccharide sugar

component of the CPS, is crucial for complete encapsulation. Indeed, further research has shown how mutations in UDP-glucose dehydrogenase (Cps3D) in serotype 3 strains also modulate capsule synthesis [51]. Additionally, serotype 3 polymer size can be directly linked to the concentration of UDP-GlcUA [52]. Indeed, concentration of UDP-GlcUA can be very low in the cell, while UDP-Glu can be present in mM quantities. Research *in vitro* has illustrated how the growing CPS polysaccharide chain can be displaced from the synthase when only one substrate is present or when the concentration of one substrate is present at low levels [53]. With UDP-glucose present in many serotypes of *S. pneumoniae*, it is conceivable that regulation of its synthesis is critical across a number of strains. As discussed earlier, in strains with Wzy-dependent capsules, this may be a result of tyrosine phosphorylation modulation of the activity of the UGD. However, further research is required in order to confirm this.

CONCLUSIONS

With CPS widely recognized as the single most critical factor for the pathogenesis of the pneumococcus, greater understanding of CPS structure, biosynthesis, and regulation is essential so that we can develop new methods to inhibit the virulence of this major human pathogen. This is particularly required to combat the ability of the pathogen to undergo vaccine escape by serotype conversion, and for understanding how CPS levels are manipulated by the bacteria to maximize successful infection.

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CHAPTER

10

Streptococcus pneumoniae Lipoproteins and ABC Transporters

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INTRODUCTION

ATP-binding cassette (ABC) transporters are a major category of bacterial protein structures associated with the cell membrane; they are involved in the import or export of a wide range of substrates, including micronutrients, sugars, amino acids and peptides, antibiotics, and antimicrobial peptides. There are at least 68 *Streptococcus pneumoniae* ABC transporters, most of which are conserved between all strains. Many ABC transporters have important effects on pathogen–host interactions.

GENERAL FEATURES OF ABC TRANSPORTER PROTEIN AND LIPOPROTEIN ORGANIZATION

The superfamily of ABC transporters hydrolyze ATP as a source of energy for unidirectional translocation of a specific substrate across the phospholipid bilayer of the cell membrane. The majority of *S. pneumoniae* ABC transporters are importers, transporting a wide range of substrates into the cytoplasm, often against the concentration gradient, but 21 are exporters involved in peptide, antimicrobial peptide, or drug efflux from the cytoplasm. All ABC transporters share a common architecture composed of four domains: two transmembrane domains (TMDs, often termed permeases) and two nucleotide-binding domains (NBDs, also termed ATPases). The NBD contains an ATP-binding signature motif LSGGQ, which can be used to identify NBD containing proteins in protein databases (Table 10.1). Each NBD has two additional characteristic motifs referred to as Walker A (GxxxxGK-T/S) and Walker B (ILLAD-E). Dimerization of the NBD creates the nucleotide-binding site between the Walker A and B motifs of one monomer and the LSGGQ motif of the second monomer. The bound ATP is hydrolyzed to provide the

Streptococcus pneumoniae.
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TABLE 10.1 ABC Importers Identified in Different Pneumococcal Strains (TIGR4/D39/R6) [2,7,8]

Specificity	Lipoprotein			Permease		ATP-binding protein			Regulatory element	Identified by virulence screens (reference)
	Name	Lipobox	Gene number	Name	Gene number	Name	ATP-binding motif	Gene number		
AMINO ACIDS AND PEPTIDES										
Aspartate[b]		LFAC-	SP0111/SPD0108/spr0100				LSGGEQ-	SP0112/SPD0109/spr0101		
Methionine[a]		LAAC-	SP0148/SPD0150/spr0146	MetP	SP0152/SPD0154/spr0150	MetN	LSGGQ-	SP0151/SPD0153/spr0149		Yes [17]
	MetQ	LAAC-	SP0149/SPD0151/spr0147							
Glutamine[b]					SP0453/SPD0412/spr0409		LSGGQ-	SP0452/SPD0411/spr0408		
Glutamine[b]			SP0609/SPD0530/spr0534		SP0607/SPD0528/spr0532		LSGGQ-	SP0610/SPD0531/spr0535		
					SP0608/SPD0529/spr0533					
Glutamine[b]					SP0823/SPD0719/spr0727		LSGGQ-	SP0824/SPD0720/spr0728		Yes [17]
Glutamine[b]	AatB	LVAC-	SP1500/SPD1328/spr1353		SP1502/SPD1330/spr1355		LSGGQ	SP1501/SPD1329/spr1354		Yes [17]
Glutamine Glutamate[a]					SP1241/SPD1098/spr1120		LSGGQ-	SP1242/SPD1099/spr1121		
Amino acids Glutamine[b]	GlnH		SP0708/SPD0615/spr0621		SP0710/SPD0617/spr0623	GlnQ	LSGGQ-	SP0709/SPD0616/spr0622		Yes [18,19]
					SP0711/SPD0618/spr0624					
Amino acids[b]					SP1461/SPD1290/spr1315			SP1460/SPD1289/spr1314		
Branched-chain amino acid[a]	LivJ	LAAC-	SP0749/SPD0652/spr0659	LivH	SP0750/SPD0653/spr0660	LivG	LSYGQ-	SP0752/SPD0655/spr0662		Yes [17]
				LivM	SP0751/SPD0654/spr0661	LivF	LSGGE-	SP0753/SPD0656/spr0663		

Choline[b]				ProWX	SP1860/SPD1642/spr677	ProV	LSGG-	SP1861/SPD1643/spr678	
Oligopeptides[b]		LVAC-	Absent/SPD1170/spr1194		Absent/SPD1168/spr1192		SGGQ-	Absent/SPD1167/spr1191	
					Absent/SPD1169/spr1193				
Oligopeptides[b]	AliA	LAAC-	SP0366/SPD0334/spr0327						
Oligopeptides[b]	AliB	LSAC-	SP1527/SPD1357/spr1382						
Oligopeptides[a]	AmiA	LAAC-	SP1891/SPD1671/spr1707	AmiD	SP1889/SPD1669/spr1705	AmiF	SGGQ-	SP1887/SPD1667/spr1703	Yes [20]
				AmiC	SP1890/SPD1670/spr1706	AmiE	SGG-	SP1888/SPD1668/spr1704	
Polyamine[a]	PotD		SP1386/SPD1218/spr1243	PotC	SP1387/SPD1219/spr1244	PotA	SGGQ-	SP1389/SPD1221/spr1246	Yes [19]
				PotB	SP1388/SPD1220/spr1245				
METAL IONS									
Iron(III)[a]	PitA		SP0243/SPD0226/spr0223	PitC	SP0240/SPD0223/spr0220	PitD	LSGGQ-	SP0242/SPD0225/spr0222	Yes [20]
			SP0244/SPD0227/spr0224	PitB	SP0241/SPD0224/spr0221				
Iron[a]	PiaA	LAMC-	SP1032/SPD0915/spr0934	PiaB	SP1033/SPD0916/spr0935	PiaC	LSGGQ-	SP1035/SPD0918/spr0938	Yes [20]
					SP1034/SPD0917/spr0936				
Iron[b]		LAAC-	SP1826/SPD1609/spr1645		SP1824/SPD1607/spr1643			SP1825/SPD1608/spr1644	
Iron[a]	PiuA	LGAC-	SP1872/SPD1652/spr1687	PiuB	SP1869/SPD1649/spr1684	PiuC	LSGGQ-	SP1871/SPD1651/spr1686	Yes [18,21]
					SP1870/SPD1650/spr1685				

(*Continued*)

TABLE 10.1 (Continued)

Specificity	Lipoprotein			Permease		ATP-binding protein			Regulatory element	Identified by virulence screens (reference)
	Name	Lipobox	Gene number	Name	Gene number	Name	ATP-binding motif	Gene number		
Manganese[a]	PsaA	LVAC-	SP1650/SPD1463/spr1494	PsaC	SP1649/SPD1462/spr1493	PsaB	LSGGQ	SP1648/SPD1461/spr1492	PsaR/SP1638/SPD1450/spr1480	Yes [21]
Zinc[a]	AdcAII	LGAC-	SP1002/SPD0888/spr0906							
Zinc[a]	AdcA	LVAC-	SP2169/SPD1997/spr1975	AdcB	SP2170/SPD1998/spr1976	AdcC	LSGGQ	SP2171/SPD1999/spr1977	AdcR/SP2172/SPD2000/spr1978	Yes [20–22]
Cobalt[b]		LAAGC-	SP0482/SPD0433/spr0429		SP0484/SPD0435/spr0431	CbiO	LSGGQ-	SP0483/SPD0434/spr0430		
Cobalt[b]							LSGGQ-	SP1438/SPD1267/spr1293		
Cobalt[b]				EcfT	SP2219/SPD2046/spr2024	EcfA1	LSGGQ-	SP2220/SPD2047/spr2025		
						EcfA2	LSGGQ-	SP2221/SPD2048/spr2026		
Natrium[b]					SP0523/SPD0465/spr0460		ESGQ-	SP0522:SPD0464/spr0459		
Phosphate[b]	PstS1	LVGC-	SP1400/SPD1232/spr1257	PstC	SP1398/SPD1230/spr1255	PstB	LSGGQ-	SP1396/SPD1228/spr1253	PhoU/SP1395/SPD1227/spr1252	Yes [20]
				PstA	SP1399/SPD1231/spr1256	PstB	LSGGQ-	SP1397/SPD1229/spr1254		
Phosphate[b]	PstS2	LVGC-	SP2084/SPD1910/spr1895		SP2085/SPD1911/spr1896		LSGGQ-	SP2087/SPD1913/spr1898	PhoU/SP2088/SPD1914/spr1899	Yes [20]
					SP2086/SPD1912/spr1897					

CARBOHYDRATES											
Sugar[b]		LAAC-	SP0092/SPD0090/ spr0083		SP0090/SPD0088/ spr0081						Yes [20,22]
					SP0091/SPD0089/ spr0082						
Sugar[b]						MsmK	LSGGL-	SP1580/SPD1409/ spr1437			Yes [18−21]
N-acetylmannosamine, *N*-acetylneuraminic acid[b]		LAAC-	SP1683/SPD1495/ spr1527		SP1681/SPD1493/ spr1525						
					SP1682/SPD1494/ spr1526						
Maltooligosaccharides[a]	MalX	LVAC-	SP2108/SPD1934/ spr1918	MalC	SP2109/SPD1935/ spr1919					MalR/ SP2112/ SPD1938/ spr1922	Yes [20]
				MalD	SP2110/SPD1936/ spr1920						
Galactose[b]	TmpC	LAAC-	SP0845/SPD0739/ spr0747		SP0847/SPD0741/ spr0749		LSGGNQ-	SP0846/SPD0740/ spr0748			
					SP0848/SPD0742/ spr0750						
Raffinose[b]	RafE	LGAC-	SP1897/SPD1677/ spr1712	RafG	SP1895/SPD1675/ spr1710						
				RafF	SP1896/SPD1676/ spr1711						
N-acetylmannosamine, *N*-acetylneuraminic acid[b]		LVAC-	SP1690/SPD1502/ spr1534		SP1688/SPD1500/ apr1532						
					SP1689/SPD1501: spr1533						
Fructoligosaccharide[b]	SusX	LAAC-	SP1796SPD1585/ spr1620	SusT1	SP1797/SPD1583/ spr1618					SusR/ SP1799/ SPD1586/ spr1021	
				SusT2	SP1798/SPD1584/ spr1619						
NO IDENTIFIED SUBSTRATES											
		LMAC-	SP0136/SPD0139/ spr0136				LSGGQ-	SP0137/SPD0140/ spr0137			
			SP0638/SPD0556/ spr0559		SP0637/SPD0555/ spr0558		LSLGQ-	SP0636/SPD0554/ spr0557			

(*Continued*)

TABLE 10.1 (Continued)

Specificity	Lipoprotein			Permease		ATP-binding protein			Regulatory element	Identified by virulence screens (reference)
	Name	Lipobox	Gene number	Name	Gene number	Name	ATP-binding motif	Gene number		
				FtsX (plain)	SP0757/SPD0660/spr0667	FtsE		SP0756/SPD0659/spr0666		
	SrlA	LAGC-	SP0771/SPD0672/spr0679				LSGGQ-	SP0770/SPD0671/spr0678		
					SP1063/absent/absent		LSGGQ-	SP1062/absent/absent		Yes [17]
			SP1069/SPD0954/spr0975		SP1070/SPD0955/spr0976	PhnK	LSGGQ-	SP1071/SPD0956/spr0977		Yes [17]
					SP1357/SPD1191/spr1215		LSLGQ-	SP1358/SPD1192/spr1216		
					SP1716/SPD1527/spr1561		LSKGNQ-	SP1717/SPD1528/spr1562		Yes [18,20]
		LVAC	SP2197/SPD2025/spr2003		SP2198/SPD2026/spr2004		FSGGQ-	SP2196/SPD2024/spr2002	SP2195/SPD2023/spr2001	Yes [17]
					SP2231/SPD2058/spr2036		LSGGE-	SP2230/SPD2057/spr2035		Yes [17,20]

[a] *OR by bioinformatic analyses.*

[b] *And regulator element when known. Gene numbers for TIGR4/D39/R6 are indicated. "Orphan" substrate-binding proteins (SBPs) and ATPases are shown in Table 10.3. http://www.expasy.org/, http://blast.ncbi.nlm.nih.gov/Blast.cgi consulted from May to November 2013.*

Lipoproteins, permeases, and ATP-binding proteins are listed, as well as their substrate specificity experimentally determined.

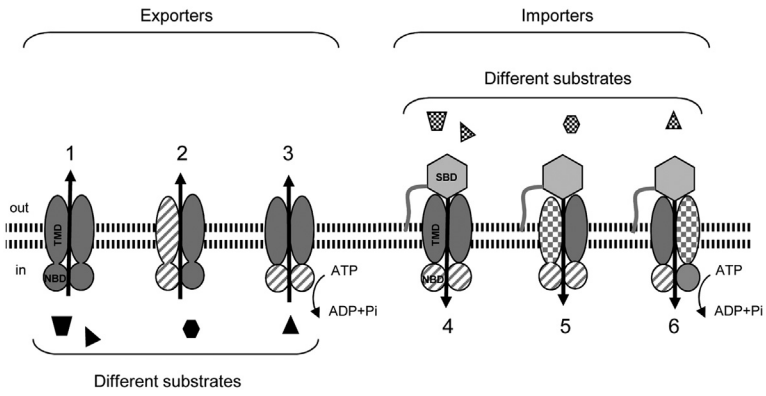
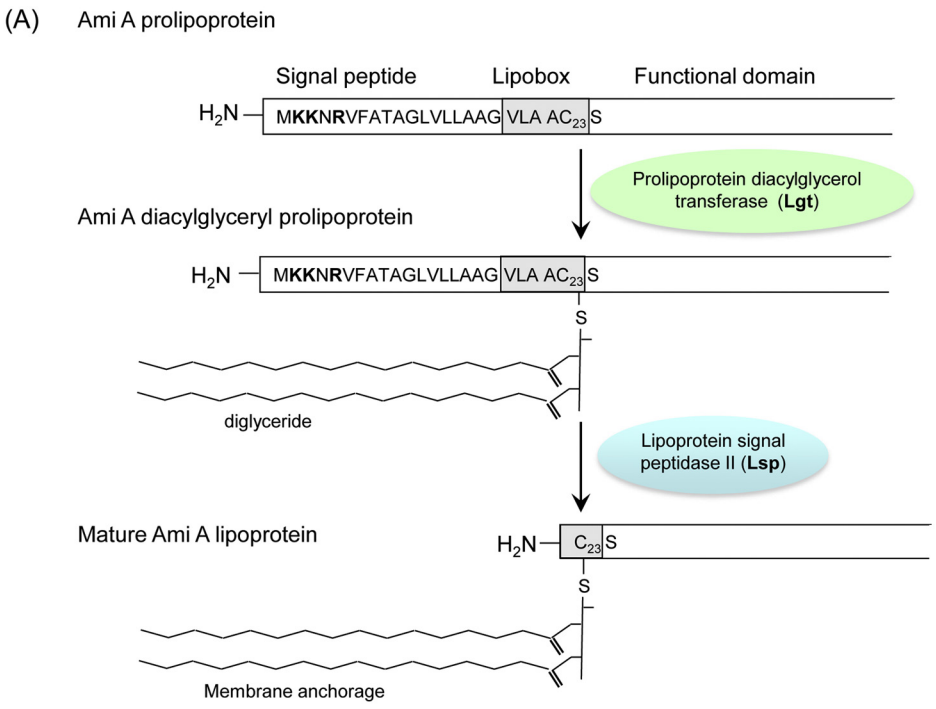


FIGURE 10.1 **Typical conformations of ABC exporters (1-3) and ABC importers (4-6):** 1, homo-dimer of a single polypeptide chains including the transmembrane domain (TMD, oval shape) and the nucleotide binding domain (NBD, circles); 2, hetero-dimer of two separate polypeptide chains including the TMD and NBD; 3 and 4, homo-dimer of two separate polypeptides each encoding the TMD or NBD; 5, hetero-dimer of TMD and homodimer of NBD; 6, hetero-dimer of TMD and NBD. SBD (hexagon) represents the substrate binding protein, usually a lipoprotein. The internal pictogram illustrates the homo or hetero-dimeric forms and gene structure.

energy required for transport through conformational changes in structure of the TMDs. The TMD subunits of the ABC transporters are integral membrane proteins, generally composed of 5–10 transmembrane helices. They form the pore through which the ABC transporter substrate is transported across the cell membrane. For detailed reviews of ABC transporter features, see [1]. Each of the TMD and NBD domains can be composed of a single protein or by two separate proteins. Export ABC transporters are frequently organized as homodimeric or heterodimeric forms of single polypeptide chains, which encode both the TMD and NBD, although other configurations also exist (Figure 10.1). In contrast, import ABC transporters often have two separate proteins forming a homo- or heterodimeric permease and one homo- or heterodimeric ATPase protein (Figure 10.1).

Import ABC transporters also contain a substrate-binding protein (SBP), a lipoprotein that is attached to the outer surface of the cell membrane by covalent linkage to phospholipid. SBPs make up the majority, but not all, of the *S. pneumoniae* lipoproteins [2–4]. The SBP helps confer substrate specificity for import ABC transporters, and therefore can be used to identify the specific function of a given ABC transporter [5,6]. Probable lipoproteins consist of typical protein structural motifs, including an N-terminus signal peptide containing an n-region of positively charged amino acids, a hydrophobic h-region sequence, and a lipobox motif (defined by the LAAC sequence) that precedes the cysteine required for covalent attachment to phospholipid [3] (Figure 10.2). Similar to other bacteria, in *S. pneumoniae* two enzymes are responsible for the maturation of lipoproteins (Figure 10.2). After biosynthesis, the N-terminal signal peptide allows export of the prolipoproteins from the cell cytosol across the cell membrane [9]. The prolipoprotein is then covalently linked via the cysteine of the N-terminal lipobox to membrane phospholipid diglyceride, catalyzed by the diacylglyceryl transferase enzyme, which is encoded by *lgt* [10]. Then a type II signal peptidase (encoded by *lsp*) cleaves the N-terminal signal peptide proximal to the cysteine/diglyceride bond, generating a mature lipid-anchored lipoprotein [4]. Bioinformatics has identified more than 50 *S. pneumoniae* lipoproteins in serotype 2 and 4 strains (Table 10.2). About 30 *S. pneumoniae* lipoproteins are potentially involved in an ABC transport uptake system, and 21 have various cellular functions unrelated to uptake ABC transporters. A portion of the *S. pneumoniae* lipoproteins identified by bioinformatics have been



(B)

Protein	n-region sequence (number of aa)	h-region sequence (number of aa)	Lipobox (c-region) –1 –2 –3 –4 Cn* +2
AmiA	M**KKNR** (5)	VFATAGLVLLAAG (13)	V L A A C23 S
AliB	M**KKSKSK** (7)	YLTLAGLVLGTGV (13)	L L S A C25 G
PsaA	M**KK** (3)	LGTLLVLFLSAI (12)	I L V A C20 A
	MN**KM**K**K** (6)	VLMTMFGLVMLP (12)	L L F A C 23 N
PiaA	M**KNK** (4)	FFLIA (5)	I L A M C14 V
AdcAII	MV**K**ELFM**KK**(9)	QNLFLVLLSVFLL (13)	C L G A C27 Q
PstS	M**KKR**K**K** (6)	LALSLIAFWLTA (12)	C L V G C23 S

* Number of the lipidated cysteine

FIGURE 10.2 **General conformation of a lipoprotein.** (A): Lipoprotein biosynthesis in *S. pneumoniae*. The prolipoprotein is anchored to the membrane via the thioester linkage of diglyceride to the cysteine of the lipobox. Then the peptide signal is cleaved by the lipoprotein signal peptidase generating the mature lipoprotein. The general representation of the lipoprotein is that of *S. pneumoniae* AmiA where C23 becomes C1 in the mature lipoprotein form. (B) Examples of signal peptide sequences of pneumococcal lipoproteins. The positively charged n-terminal region amino acids (bold), the hydrophobic h-region sequence and the lipobox containing the lipidated cysteine are shown.

experimentally confirmed using changes in surface location or protein size in *lgt*- or *lsp*-mutant strains. These include the ABC transporter SBPs PsaA, PiuA, PiaA, MetQ, AdcA, AdcAII, MalX, PstS, and the non-ABC transporter lipoproteins SlrA and PpmA [4,6,10,11].

A classification for bacterial SBPs has been proposed based on structural alignments of the

TABLE 10.2 Lipoproteins Identified in TIGR4 and/or in D39 Pneumococcal Strains Based on Their Lipobox Motif

ABC transporter-associated lipoproteins (SBPs)		
Name/lipobox	**Gene number**	**Substrate**
LFAC-	SP0112/SPD0109/spr0101	Aspartate
LAAC-	SP0148/SPD0150/spr0146	Methionine
LAAC-	SP0149/SPD0151/spr0147	Methionine
aatB/LVAC-	SP1500/SPD1328/spr1353	Amino acids
LivJ/LAAC-	SP0749/SPD0652/spr0659	Branched-chain amino acid
LVAC-	ND/SPD1170/spr1194	Oligopeptides
AliA/LAAC-	SP0366/SPD0334/spr0327	Oligopeptides
AliB/LSAC-	SP1527/SPD1357/spr1382	Oligopeptides
AmiA/LAAC-	SP1891/SPD1671/spr1707	Oligopeptides
PiaA/LAMC-	SP1032/SPD0915/spr0934	Iron
LAAC-	SP1826/SPD1609/spr1645	Iron
PiuA/LGAC-	SP1872/SPD1652/spr1687	Iron
PsaA/LVAC-	SP1650/SPD1463/spr1494	Manganese
AdcAII/LGAC-	SP1002/SPD0888/spr0906	Zinc
AdcA/LVAC-	SP2169/SPD1997/spr1975	Zinc
LAAGC-	SP0482/SPD0433/spr0429	Cobalt
PstS1/LVGC-	SP1400/SPD1232/spr1257	Phosphate
PstS2/LVGC-	SP2084/SPD1910/spr1895	Phosphate
LAAC-	SP0092/SPD0090/spr0083	Sugar
LAAC-	SP1683/SPD1495/spr1527	Sugar
malX/LVAC-	SP2108/SPD1934/spr1918	Maltooligosaccharides
tmpC/LAAC-	SP0845/SPD0739/spr0747	Galactose
LVAC-	SP1690/SPD1502/spr1534	*N*-Acetylmannosamine
susX/LAAC-	SP1796/SPD1585/spr1620	Fructoligosaccharide
rafE/LGAC-	SP1897/SPD1677/spr1712	Raffinose
LMAC-	SP0136/SPD0139/spr0136	?
srlA/LAGC-	SP0771/SPD0672/spr0679	?
LVAC-	SP2197/SPD2025/spr2003	?
Likely ABC transporter-associated lipoproteins		Hypothetical substrate
LAAV-	SP0629/SPD0549/spr0554	?
LVAC-	SP0858/SPD0750/spr0759	Oligopeptides

(*Continued*)

TABLE 10.2 (Continued)

ABC transporter-associated lipoproteins (SBPs)		
Name/lipobox	**Gene number**	**Substrate**
LAAC-	SP0899/SPD0792/spr0799	?
LVAC-	SP1394/SPD1226/spr1251	Amino acids
LVAC-	SP2144/SPD1972/spr1952	?
Non-ABC transporter lipoproteins		
Name/lipobox	**Gene number**	**Hypothetical function**
LAAC-	SP2037/SPD1846/spr1848	PTS system transporter subunit IIB
LAAC-	SP1197/SPD1057/spr1080	PTS system transporter subunit IIB
LVAC-	SP0283/SPD0263/spr0260	PTS system mannose-specific transporter subunit IIC
LVAC-	SP0474/SPD0424/spr0421	PTS system cellobiose-specific transporter subunit IIC
LGAC-	SP0716/SPD0622/spr0628	Transcriptional regulator, TENA/THI-4 family protein
LLIC-	SP2131/SPD1961/spr1940	BglG family transcriptional regulator: sugar sensor
LMAC-	SP1000/SPD0886/spr0904	Thioredoxin family protein
LALC-	SP0262/SPD0244/spr0241	Thioredoxin family protein
LVGA-	SP1996/SPD1793/spr1810	Universal stress protein family protein
LVAC-	absent/SPD1773/absent	OxaA-like protein precursor
LVAC-	SP0280/SPD0260/spr0256	Ribosomal small subunit pseudouridine synthase A
LVAC-	absent/SPD1597/absent	Tryptophan synthase subunit beta
LLVC-	SP1602/SPD1427/spr1455	Phna protein: phosphonate metabolism
MgtC/LAAC-	SP1823/SPD1606/spr1641	Manganese binding
LTGC	SP1975/absent/absent	Membrane protein insertase YidC 1
MTAC	SP2041/absent/absent	Membrane protein insertase YidC 2
LVAC-	SP0468/absent/absent	Sortase C
ppmA/LAAC-	SP0981/SPD0868/spr0884	Foldase protein
LVAC-	absent/SPD0184/spr0179	Hypothetical protein
LAAC-	absent/SPD0313/absent	Hypothetical protein
LVAC-	absent/SPD0198/absent	Hypothetical protein

The lipoprotein name, lipobox motif, and predicted substrate-binding specificity are presented. Gene numbers for TIGR4/D39/R6 are indicated. The names of the genes or proteins are written in italics or in roman respectively in front of gene numbers (?): No identified substrate. ND: Not determined http://www.expasy.org/, http://blast.ncbi.nlm.nih.gov/Blast.cgi consulted from May to November 2013.

available crystal structures [12]. For *S. pneumoniae* only four 3D-structures of SBP lipoproteins are available; three metal-ion uptake SBPs found in all *S. pneumoniae* (PsaA [13], AdcAII [14], and PiaA [15]), and the fucose-binding protein FcsSBP specifically expressed in a serotype 3 strain (SP3-BS71) but not the TIGR4 or D39 strains [16]. According to their structural features, PsaA, AdcAII, and PiaA were classified in cluster A SBPs [12], characterized by a single rigid α helix between two globular domains, which only show small movements on substrate binding. FcsSBP belongs to the cluster D SBPs, which includes slightly larger SBPs (above 40 kDa) with a wide range of substrates and hinge regions consisting of two short strands. Whatever the compound transported, SBPs bind their substrate with nano- to micromolar affinities and deliver them to their associated permease. Thus, they trigger ABC transporter specificity and govern the first step for substrate uptake, potentially acting as a bottleneck for the import of substrates into *S. pneumoniae*. In contrast, substrate specificity of export ABC transporters is provided by the TMDs/permease(s) [1].

GENETIC ORGANIZATION OF ABC TRANSPORTERS

ABC transporter exporters are generally encoded for by no more than two genes, usually arranged in tandem (Table 10.3). *S. pneumoniae* ABC transporter substrate importers are generally encoded by several genes arranged in an operon. Depending on the ABC transporter, up to six genes can be found within a single operon encoding the different elements of a given ABC transporter; one encoding an SBP, one or two encoding permeases, one or two encoding ATPases, and often with a regulatory element included, for example, the pstABCSphoU (SP1395-1400) phosphate transporter operon (Table 10.1 and Figure 10.3). More frequently, the operon consists of three to four genes that suffice for the synthesis of the whole ABC transporter (genes for one SBP, one or two permeases, one ATPase) without an obvious regulator. The order of the genes encoding the SBPs, TMDs, NBDs, and regulator components varies between ABC transporters.

There are several cases of *S. pneumoniae* ABC importers for which no lipoprotein gene is associated with the permease/ATPase tandem. For instance, 30% of the permease/ATPase genes predicted to encode amino acid uptake ABC transporters are not associated with a recognized SBP (Table 10.1). Conversely, at least five predicted lipoproteins from TIGR4 strain are "orphan" SBPs (Tables 10.2 and 10.4), with no genetic or functional data to link them to specific permease/ATPases. Two of these "orphan" SBPs are predicted to bind amino acids or oligopeptides and therefore could match some of the orphan permease/ATPase pairs that bioinformatics suggests are specific for amino acids. In addition, orphan SBPs could replace an SBP expressed by a complete ABC transporter unit. For example, the SBPs AliA (SP0366) and AliB (SP1527) may associate with the Ami oligopeptide-specific ABC transporter permeases and ATPases encoded by the *amiACDEF* operon [23,24], and the zinc-specific lipoprotein AdcAII uses the AdcB and AdcC permease/ATPase system usually associated with the zinc-specific SBP AdcA to compensate for deletion of *adcA* [5]. For many sugar uptake ABC transporters, the majority of their corresponding potential operons consist of one gene encoding the SBP and two genes encoding permeases (e.g., SP0090-0092, SP1681-1683, SP1688-1690, SP1895-1897, SP1796-1798, and SP2108-2010) (Table 10.1 and Figure 10.3). A single orphan gene encoding an ABC protein (*msmk* SP1580) probably provides the ATPAse component for these sugar uptake ABC transporters, including the maltotetraose (SP2108-2110), raffinose (SP1895-1897), and fructooligosaccharide (SP1796-1798) specific ABC transporters [25,26]. The identity of the ABC protein powering the other lipoprotein/permease tandems for

TABLE 10.3 ABC Exporters Identified in Different Pneumococcal Strains [2,49]

Combined TMD and NBD polypeptides

Drug specificity	Name	ATP-binding motif	Gene number	Identified by virulence screens (reference)
Competence factor[a]	ComA	MSLGQ	SP0042/SPD0049/Spr0043	
	ComB	LISQ-	SP0043/SPD0050/Spr0044	
Drug[a]		LSGGE-	SP1434/SPD1263/spr1289	Yes [20]
		LSGGE-	SP1435/SPD1264/spr1290	
Multidrug[a]	PatA	LSGIM-	SP2073/SPD1900/spr1885	
	PatB	FSGGQ-	SP2075/SPD1902/spr1887	
Drug[b]			SP1839/SPD1621/spr1656	
		LSGGEQ-	SP1840/SPD1622/spr1657	
Macrolide[b]	*mefE*	SLYQQ-	SP0168/SPD0950/spr0971	
Bacteriocin[b]		LSGGE-	SP1957/SPD1755/spr1773	
Bacteriocin[b]	*lanE*	FSGGQ-	SP1953/SPD1752/spr1770	
Drug[b]	*lanT*	LSGGE-	SP1987/SPD1784/spr1801	
Antimicrobial peptides[b]		LSGGEQ-	SP1341/SPD1176/spr1202	
		LSGGQ-	SP1342/SPD1177/spr1203	
Drug[a]		LSLG-	SP1704/SPD1514/spr1546	
			SP1705/SPD1516/spr1547	

Separate TMD and NBD polypetides

Drug specificity	Permease/TMD gene number	ATPase/NMD		Identified by virulence screens (reference)
		ATP-binding motif	NBD gene number	
Drug[b]	SP0686/SPD0596/spr0601	LSGGE-	SP0687/SPD0597/spr0602	Yes [20]
Drug[a]	SP1116/SPD1000/spr1023	LSGGQ-	SP1114/SPD0998/spr1021	
Drug[a]	SP1919/SPD1722/spr1736	LSVGQ-	SP1918/SPD1721/spr1734	
Antimicrobial peptides[b]	SP1652/SPD1465/spr1496	LSGGEQ-	SP1653/SPD1466/spr1497	Yes [20]

Exporter with two-component system								
	Permease/TBD		ATPase/NBD					
Drug specificity	Name	Gene number	Name	ATP-binding motif	Gene number	Histidine kinase	Response regulator	Identified by virulence screens (reference)
Antimicrobial peptides[a]		SP0385/SPD0350/spr0342				SP0386/SPD0351/spr0343	SP0387/SPD0352/spr0344	
Drug[b]		Vexp1SP0599/Vexp3SPD0521/spr0524			Vexp2SP0600/SPD0522/spr0525	*vncS*-SP0604/SPD0525/spr0528	VncR-SP0603/SPD0524/spr0527	
		SP0601/SPD0523/spr0526		LSGGQ-				
Bacteriocin[a]		BlpBSP0529/SPD0471/spr0466		BlpALSGGQ-	SP0530pseudo/SPD0472/spr0468	BlpHSP0527/SPD0469/spr0464	BlpRSP0526/SPD0468/spr0463	Yes [20]
Antimicrobial peptides[a]		SP0787/SPD0688/spr0695		LSGGQ-	SP0786/SPD0687/spr0694	SP0785/SPD0686/spr0693		
Antimicrobial peptides[a]		SP0913/SPD0805/spr0813		ISGGQ-	SP0912/SPD0804/spr0812	SP1632/SPD1445/spr1473	SP1633/SPD1446/spr1474	Yes [20]
Antimicrobial peptides[a]				LSKENK-	SP1715/SPD1525/spr1559	SP1714/SPD1524/spr1558		Yes [18,20]
Multidrug[b]		*ccmA*SP2002/SPD1800/spr1816		LSGGQ-	SP2003/SPD1801/spr1817	SP2001/SPD1799/spr1815	SP2000/SPD1798/spr1814	Yes [20]

[a] *Identified substrate.*

[b] *bioinformatic determination. http://www.expasy.org/, http://blast.ncbi.nlm.nih.gov/Blast.cgi consulted from May to November 2013.*

Permeases and ATP-binding proteins are listed as well as histidine kinase and response regulator for the two component systems. Gene numbers for TIGR4/D39/R6 are indicated. In front of the gene numbers the names of the genes or proteins are written in italics or normal respectively, and the amino acid sequence of the typical LSGGQ ATP-binding motif.

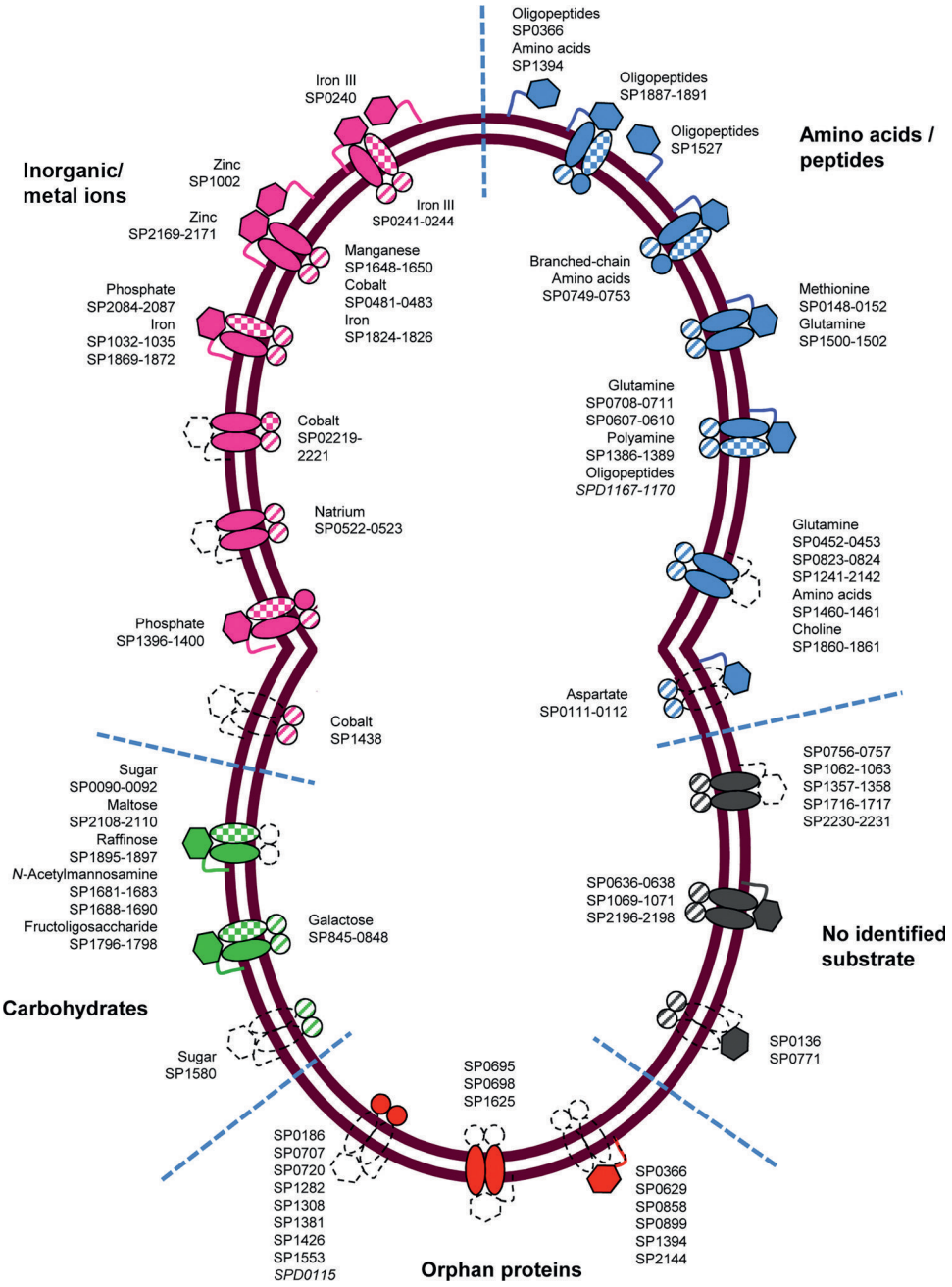


FIGURE 10.3 **Overview of ABC importers in *Streptococcus pneumoniae*.** Gene numbers (SPxxxx) are for the TIGR4 strain genome, except when written in italics (*SPDxxxx*) when they are for the D39 strain genome. Lipoproteins are depicted as diamonds, permeases as ovals, and ATP-binding proteins as circles. The internal pictogram illustrates the homo or hetero-dimeric forms. Empty dotted lines indicate unidentified partners. The ABC transporters sharing the same general organisation are gathered under a single icon provided they transport the same class of compound. ABC importers specific for amino acids and peptides are in blue, ABC importers specific for inorganic/metal ions are in pink, ABC importers specific for carbohydrates are in green, ABC importers for which no substrate has been identified are in grey and 'orphan proteins' are in red. Substrate specificity was provided by experimental and/or bioinformatics data.

TABLE 10.4 Orphan ATP-Binding and Permease Proteins

Orphan proteins	
ATPASE	
uvrA/LSSGE/LSGGE-	SP0186/SPD0176/spr0171
SLGMRK-	SP0707/SPD0614/spr0619
mglA/LSSGE-	SP0720/SPD0626/spr0632[e]
LSGGE/LSTKGE-	SP1282/SPD1137/spr1161
ISGGQ-	SP1308pseudo/SPD1160/spr1183
LVDGQ-	SP1381/SPD1214/spr1238
LSKTG-	SP1426/SPD1255/spr1281
chvD/LSSGL/LSGGE-	SP1553/SPD1385/spr1412
msmK/LSGGL-	SP1580/SPD1409/spr1437[a]
LSSGQ-	ND/SPD0115/spr0108
PERMEASE	
	SP0695/SPD0604/spr0608
	SP0698/SPD0605/spr06010
	SP1625/SPD1438/spr1466[b]
LIPOPROTEINS	
LAAV-	SP0629/SPD0549/spr0554
LVAC-	SP0858/SPD0750/spr0759[c]
LAAC-	SP0899/SPD0792/spr0799
LVAC-	SP1394/SPD1226/spr1251[d]
LVAC-	SP2144/SPD1972/spr1952

ATP-binding proteins are identified according to their LSGGQ sequence. Gene names are in italics. The amino acid sequence of the typical LSGGQ ATP-binding motif is indicated in front of the gene numbers for TIGR4/D39/R6. Hypothetical substrate: a: sugar, b: cadmium, c: oligopeptides, d: amino acids, e: identified by virulence screen. http://blast.ncbi.nlm.nih.gov/Blast.cgi consulted from May to November 2013.

sugar import has not been clarified [27]. At least an additional thirteen orphan genes encoding putative permease or ATPase proteins are present in the TIGR4 and/or D39 *S. pneumoniae* genomes (Table 10.4). Bioinformatic analysis suggests that three of the corresponding proteins have six transmembrane helices and are potential orphan permeases (SP0695, SP0686, and SP1625); one contains a transmembrane helix and a large soluble domain that has the feature of an ATPase (SPD0115); the rest encode putative ATPases, three of which (SP0186, SP1282, and SP1553) are about twice the molecular weight larger of single ATPase and contain two LSGGQ, Walker A, and Walker B motifs, suggesting they encode ATPase dimers. However, without identification of a permease partner, these proteins cannot be considered part of an ABC transporter

with any certainty. The overall biological relevance of incomplete gene organization of ABC transporters is not clear.

FUNCTIONS OF *S. PNEUMONIAE* IMPORT ABC TRANSPORTERS

S. pneumoniae import ABC transporters are involved in the bacterial uptake of a surprisingly wide variety of substrates (Table 10.1 and Figure 10.3). Major substrate categories include cations (important protein cofactors), sugars, phosphate, peptides, and amino acids (all sources of energy and/or building blocks for basic biosynthesis pathways); overall, the putative substrates would be predicted to be essential for bacterial growth, especially under stress conditions. Identifying the substrate for a specific import ABC transporter can be inferred by bioinformatic analysis (Table 10.1), but this can be misleading and requires experimental support, which is now available for many *S. pneumoniae* import ABC transporters.

Function can be indicated by the effects of deletion of the genes encoding the SBP or other ABC transporter components on growth in restricted media. For example, poor growth in media with restricted availability of cations that was partly or fully restored after supplementation with a specific cation has identified the specificity of the Psa ABC transporter for manganese, Piu, Pia, and Pit ABC transporters for iron, and AdcA and AdcAII ABC transporters for zinc [5,28–31]. Similarly, monitoring the growth of bacteria in various culture conditions has shown that AmiA is specific for oligopeptides [32], PotD (SP1386) for polyamine [33], SP1241-1242 for glutamine [7], and MalX (SP2108) for maltooligosaccharides [34]. More precise confirmation of import ABC transporter specificity can be provided using purified recombinant SBPs or uptake transport assays. For example, thermal shift assays or the thermal stability of recombinant SBP upon the addition of a putative substrate can give relative affinities and dissociation constants (Kd) of SBPs for different substrates. Using these methodologies, the affinities of PiaA, PsaA, SP0149, MalX, AdcAII, and the FcsSBP for iron, manganese, methionine, maltotetraose, zinc, and fucose, respectively, have been determined [6,14–16,34,35]. In addition, uptake of radioactive substrate or fluorescent probes by mutant compared to wild-type strains has confirmed the roles of the PstSCAB, Liv, Pia/Piu/Pit, and AdcA/AdcAII systems for uptake of phosphate, branched amino acids, iron, and zinc, respectively [5,36–40].

The substrates for around two-thirds of import *S. pneumoniae* ABC transporters have been identified (Table 10.2 and Figure 10.3); they include cations, amino acids, oligopeptides, various sugars, and polyamines, confirming that import ABC transporters are largely involved in micronutrient acquisition. However, additional functions have also been identified. For example, one of the more abundant ABC transporter SBPs, PsaA, may be an adhesin, binding to E-cadherin [41], as well as a manganese transporter [29]; it is not clear whether this is a direct effect of PsaA or indirect due to effects on intracellular manganese levels affecting gene transcription [42]. Many *S. pneumoniae* surface proteins have multiple roles, and hence other ABC transporter SBPs could also have additional functions independent of their substrate-binding and import roles. In addition, import ABC transporter substrates frequently have roles independent of their effects on bacterial growth. For example, lack of manganese due to loss of the Psa ABC transporter increases *S. pneumoniae* sensitivity to oxidative stress as manganese is a cofactor for superoxide dismutase. Other examples include the requirement for manganese and zinc uptake for full competence, and the PstS phosphate ABC transporter for resistance to penicillin [42–47]. Furthermore, many of the substrates for import ABC transporter are signals that control regulators (e.g., methionine, manganese, iron), thereby resulting in more widespread effects of substrate

import by ABC transporters via effects on transcription of unrelated genes [42,48]. These effects are likely to contribute to the phenotypes observed for *S. pneumoniae* strains containing mutations affecting ABC transporters in murine models of infection.

FUNCTIONS OF *S. PNEUMONIAE* EXPORT ABC TRANSPORTERS

The described functions and the range of potential substrates for the *S. pneumoniae* export ABC transporters are relatively poorly defined; in general, they secrete peptides and potentially toxic substances from the bacterial membrane and/or cytosol. Four functions have been ascertained for *S. pneumoniae* export ABC transporters (Table 10.3 and Figure 10.4):

1. Conferring bacterial resistance to toxic peptides (termed bacteriocins) and antibiotics produced by other bacteria, potentially assisting *S. pneumoniae* competition versus other commensal bacteria in the nasopharynx. For example, mutation of SP0912-0913 export ABC transporters increases *S. pneumoniae* sensitivity to nisin, a bacteriocin produced by *Lactococcus lactis*, and mutation of SP0385-0387, SP0912-0913, and SP1714-1715 increases sensitivity to the antibiotic bacitracin (produced by *Bacillus licheniformis*) [49]. Conversely, the BlpAB (SP0529-0530) export ABC transporter secretes bacteriocins that are produced by *S. pneumoniae* as discussed below.
2. Mutation of the *S. pneumoniae* SP0785-0787 export ABC transporter increases sensitivity to LL37, an antimicrobial peptide produced by neutrophils and epithelial cells that destabilizes bacterial cell membranes [49]. Hence, export ABC transporters can increase *S. pneumoniae* resistance to host antimicrobial peptides, which would help bacterial evasion of mucosal or phagocyte-mediated immunity. This may explain why some export ABC transporters have been identified by signature-tagged mutagenesis screens for virulence genes (Table 10.3).
3. Export of antibiotics by ABC transporters is also one mechanism causing antibiotic resistance for *S. pneumoniae*. For example, the PatA-PatB (SP2073-75) heterodimer, the most studied ABC exporter in *S pneumoniae*, causes efflux of linezolid [50], acriflavin [51], and the fluoroquinolones ciprofloxacin and norfloxacin [52–56]. Another export ABC transporter encoded by SP1114-1116 also confers tolerance to linezolid, with over-expression of this transporter increasing of resistance to linezolid [57], and resistance to vancomycin has been attributed to the *vex123* genes (SP0059-61) [58].
4. Secretion of small signaling peptides by export ABC transporters is a component of at least two *S. pneumoniae* quorum sensing systems. One is the well-described competence system, regulating uptake and integration of foreign DNA into the *S. pneumoniae* chromosome; this is activated by the competence-stimulating peptide, which is secreted by the ComA/B (SP0042-43) ABC transporter system [59]. The second controls expression of the *S. pneumoniae* Blp bacteriocin secretion system, which causes intraspecies competition between different *S. pneumoniae* strains. The export ABC transporter BlpAB (SP0529-0530) secretes the peptide pheromone BlpC; when extracellular concentrations of BlpC are high enough, they activate a two-component sensor kinase system that in turn increases expression of different bacteriocins (termed pneumocins), which are then also secreted via BlpAB [60,61]. Genetic variation within the *blp* system confers resistance/susceptibility to specific pneumocins, thereby promoting intrastrain competition [60].

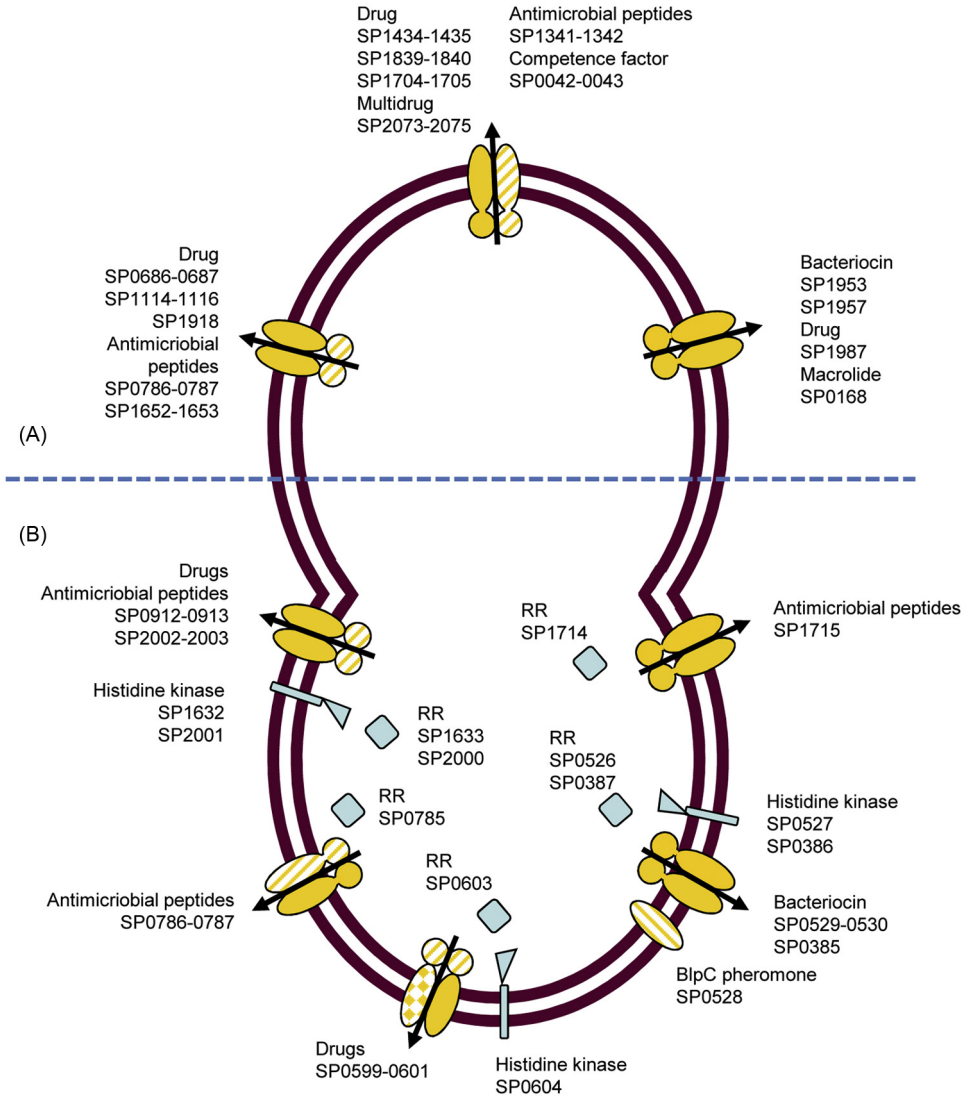


FIGURE 10.4 **Overview of ABC drug exporter in *Streptococcus pneumoniae*.** Gene numbers (SPxxxx) are for the TIGR4 strain genome. Permeases are depicted as ovals, ATP-binding proteins as circles, histidine kinases as a rectangle and response regulators as a square. The internal pictogram illustrates the homo or hetero-dimeric forms. The ABC transporters sharing the same general organisation are gathered under a single icon provided they transport the same class of compound. Substrate specificity was provided by experimental and/or bioinformatics data. ABC exporters specific for drugs are in orange. Two component systems (histidine kinase and RR (response regulator) are in blue). A: ABC exporter, B: ABC exporters associated with two component systems.

A puzzling feature of export ABC transporters is that an individual exporter may be able to secrete a range of structurally dissimilar substrates. For example, the PatA-PatB export ABC transporter increases *S. pneumoniae* secretion of fluoroquinolones, ethidium bromide, the dye Hoechst 33342, and acriflavine [62]. Recognition of similar physical properties such as high

cationic charge or hydrophobicity that are conserved between diverse substrates might enable a given transporter to export very different substrates; this promiscuity may also explain why export ABC transporters can be involved in resistance to antibiotics that are not likely to be products of nasopharyngeal commensals.

REGULATION OF ABC TRANSPORTERS

As discussed above, import ABC transporter operons can be associated with genes that encode putative regulators, usually a repressor located upstream of the genes encoding the ABC transporter structural elements (Table 10.1). Two examples that have been extensively documented are AdcR and the Adc zinc uptake ABC transporter, and PsaR and the Psa manganese uptake ABC transporter [63]. The regulatory element represses ABC transporter expression by binding to specific DNA palindromic sequences located on the operon's promoter region, and thereby also regulates its own expression. Generally, the ABC transporter substrate binds to the repressor, triggering a conformational modification that stabilizes DNA binding and represses gene expression. As a consequence, reduced intracellular availability of the substrate results in increased expression of the ABC transporter.

ABC transporter drug exporters can be regulated by two-component histidine kinase and response regulator systems encoded by genes that are frequently present in the same operon as the permease/ATPase tandem, as described for *Bacillus subtilis* [64] (Table 10.3 and Figure 10.4). Detection of the antimicrobial agent by the sensor histidine kinase activates a response regulator, which in turn up-regulates the expression of the ABC transporter through binding to its target DNA sequences. This process triggers antimicrobial export and hence increases bacterial resistance. Transcriptomic analysis after treatment with antimicrobial peptides [49] indicates two-component systems could control expression of at least five *S. pneumoniae* drug exporters (SP0912-13, SP0529-30, SP0786-87, SP1715, and SP2002-03) (Table 10.3). Expression of the Blp bacteriocin system is controlled by quorum sensing of BlpC by the histidine kinase (SP0527), which then activates the response regulator (SP0526) to up-regulate expression of the ABC transporter BlpBA, thereby increasing secretion of pneumocins [60].

REDUNDANCY OF *S. PNEUMONIAE* ABC TRANSPORTER FUNCTION

Loss of an individual import ABC transporter may have only weak or undetectable effects on *S. pneumoniae* phenotype due to redundancy. At the simplest level, this is due to compensation by other import ABC transporters with the same substrate. For example, there are three *S. pneumoniae* iron-specific ABC transporters: Pia, Piu, and Pit systems [65,66]. In the serotype 3 strain, background mutation of a single transporter had only mild effects on phenotype, and in fact the TIGR4 genome seems to contain a mutation creating a stop codon affecting the third gene of the *pit* operon [2,38]. However, dual mutation of the Pia and Piu iron transporters has marked effects on *S. pneumoniae* growth, iron uptake, and virulence, suggesting that loss of just one of these iron transporters is compensated for by the other [39]. Similar redundancy has been described for the Adc and AdcAII zinc uptake SBPs [5], the AliA, AliB, and AmiA oligopeptide uptake ABC transporters in a nasopharyngeal colonization model [23,67], and is likely for the six potential *S. pneumoniae* glutamine uptake ABC transporters [7]. Redundancy may also occur through a degree of SBP substrate promiscuity. For example, sugar uptake ABC transporters may have several substrates [27], and the manganese-specific lipoprotein

PsaA may also transport zinc, especially under zinc-mediated stress conditions or in strains where the expression of the zinc-specific lipoproteins AdcA and AdcAII is suppressed [5,40]. Similar "leak" of cations or amino acids may occur through other *S. pneumoniae* ABC uptake systems [37]. However, interpretation of data showing substrate "leak" through an ABC transporter needs some caution, as it may be an experimental artifact due to the large substrate concentrations used *in vitro* soliciting low-affinity transport systems, which does not reflect ABC transporter function in the host. A third type of redundancy occurs when an import ABC transporter is only one mechanism for acquiring a specific substrate. For example, there are multiple *S. pneumoniae* sugar uptake ABC transporters, but in addition there are multiple phosphotransferase system (PTS) sugar importers. As a result, the *sus* sucrose ABC transporter is redundant with the sucrose *scr* PTS transporter [68], and similar situations occur with the import of other sugars [69]. Similarly, the methionine-specific ABC transporter SP0149 contributes only marginally to virulence and *S. pneumoniae* pneumococcal growth under methionine-restricted conditions as methionine synthesis by the MetEF system (SP0586) compensates for lack of methonine uptake [37].

An important observation from these studies is that a given ABC transporter is often more dominant than others that transport the same substrate. For instance, for *S. pneumoniae* iron transporter loss of PiaA has stronger effects on phenotype *in vitro* and in mouse models than loss of PiuA and PitA [38], and for glutamine uptake ABC transporter loss of SP1241 has more marked effects in mouse models of disease than loss of SP0452 [7]. However, the relative dominance of a given ABC transporter for a particular substrate only reflects the conditions in which it has been tested, and these are unlikely to reflect all the potential environmental conditions faced by *S. pneumoniae* during colonization and invasive disease. Different ABC transporters may be dominant under specific conditions that have not been tested, and functional redundancy may be necessary for the ability of *S. pneumoniae* to adjust rapidly to growth in different host niches.

ROLE IN VIRULENCE

Investigations of *S. pneumoniae* virulence attributes have tended to concentrate on the capsule and cell-wall proteins (Chapters 9 and 11) [70]. However, the host environment is characterized by restricted availability of many nutrients required for bacterial growth, suggesting that import ABC transporters could affect *S. pneumoniae* growth and survival in the host. The exact concentrations of specific nutrients will vary with anatomical site, and are also affected by inflammation. For example, free iron concentrations are around 138 μM in the blood but 74 μM in the lung, and zinc concentrations in the normal nasopharynx are 41 μM but increase to 214 μM during infection [71]. These patterns of nutrient availability would be predicted to dictate the relative importance of specific import ABC transporters during infection at different sites. Even before their substrate specificity had been described, screens for *S. pneumoniae* virulence determinants confirmed an important role for a range of ABC transporters during infection. ABC transporter components were a predominant group among virulence genes identified using signature-tagged mutagenesis or similar methodologies for screening large numbers of insertional mutants for loss of virulence in animal models [17–20] (Table 10.2). Microarray analysis of *S. pneumoniae* gene expression at different disease sites (e.g., nasopharyngeal carriage, pneumonia, bacteremia, meningitis, or in cell culture models of bacterial/epithelial interactions) also demonstrated expression of ABC transporters during infection [21,22,72]. Taken together, these screens indicate 29 different ABC transporters with a role during

infection; these include import ABC transporters that promote uptake of all substrate classes, and export ABC transporters that might protect *S. pneumoniae* against antimicrobial peptides. High gene expression levels during infection does not necessarily reflect a requirement for virulence for the corresponding protein, and only 10 genes were identified by both virulence screens and expression studies: the ATPase and permeases of an oligopeptide ABC transporter (SP1888-1890), the permease of the zinc ABC transporter SP2170, the lipoproteins of an iron and a maltose importer (SP1872 and SP210, respectively), the ATPases for the sugar importer SP1580 and the antimicrobial peptide exporter SP1715, and the ABC transporter SP1716-1717 (unknown substrate). Why all the genes within an operon encoding an ABC transporter do not necessarily show similar changes in expression *in vivo* remains unclear. The above studies show the advantages of high-throughput identification for global analysis of virulence functions but do not provide detailed data on the role of an individual ABC transporter during infection.

Global information on the role of lipoproteins, including SBPs, for *S. pneumoniae* virulence was obtained using mutants with deletions of the *lsp* (encoding the type II signal peptidase) or *lgt* gene (encoding the prolipoprotein diacylglyceryl transferase), and also indicates an important role for uptake ABC transporters during infection [4,10]. The *lgt* and *lsp* deletion strains have multiple phenotypes indicative of impaired uptake ABC transporter function, including reduction of intracellular cation concentrations, increased sensitivity to oxidative stress, and poor growth in blood. These mutants also have reduced virulence in different murine infection models, including pneumonia, septicemia, and nasopharyngeal colonization [4,10]. Deletion of *lgt* has the greatest effect on virulence, perhaps as deletion of *lsp* still allows attachment of prolipoproteins to the bacterial cell membrane, whereas loss of Lgt results in almost no retention of prolipoprotein [4,10]. These data support an important role for ABC transporters during infection, but are confounded by the effects of the *lgt* and *lsp* deletions on non-SBP lipoproteins as well as SBPs.

More precise evaluations of the role of an uptake ABC transporter during disease development can be achieved by investigating mutant strains affecting individual transporters or combinations of ABC transporters with related functions in murine models of infection. Metal uptake ABC transporters have been studied the most intensively for their effects on *S. pneumoniae* virulence, although the involvement of ABC transporters specific for sugars, amino acids, phosphate, and polyamines has also been characterized. The Psa ABC manganese ABC transporter is highly expressed during infection [22] and is required for growth in low-manganese conditions such as those found at various infection sites in humans [71], protection against oxidative stress [44], gene regulation [42], and possibly adherence to epithelial cells [73,74]. As a consequence, deletion of either the complete *psaABC* operon or the manganese SBP PsaA has marked affects on *S. pneumoniae* virulence [30,43,74,75] in different murine models of infection, and PsaA is recognized as one of the major *S. pneumoniae* virulence factors. Other cation uptake ABC transporters that are involved in virulence include the Piu, Pia, and Pit iron transporters, and the AdcA and AdcAII zinc transporters [5,38,39]. Similar studies have shown significant roles for virulence in murine models of sepsis, nasopharyngeal colonization, pneumonia, and/or meningitis for the polyamine transporter PotD (SP1386) [33], glutamine transporters SP1241-1242 and SP0610 [7,37,76], branch-chained amino acid transporter Liv [37], various sugar transporters [25,37,68], and the Ami and Ali oligopeptide uptake transporters [67]. In contrast to Psa, redundancy is often important for other uptake ABC transporters, with loss of a single transporter having small effects on virulence, but combined deletion of two SBP lipoproteins (e.g., PiaA/PiuA or

AdcA/AdcAII) having profound effects on virulence [5,39,48]. Studies on iron uptake ABC transporter also show some infection site specificity, with loss of the Pit iron transporter having the greatest effect on systemic virulence and loss of the Piu iron transporter the greatest effect on pneumonia. Similarly, the *sus* ABC transporter and *scr* PTS sucrose uptake systems are differentially expressed during infection, with *sus* dominant during pneumonia and *scr* during nasopharyngeal colonization in mouse models [68]. How the loss of an individual ABC transporter can affect virulence is poorly characterized; this is often assumed to be due to poor bacterial growth under infection conditions, but the other consequences of loss of ABC transporter functions, such as increased sensitivity to oxidative stress or zinc toxicity, or effects on gene regulation, are also likely to be relevant for some uptake ABC transporters [42,43]. Several studies have shown that mutation affecting some ABC transporters has only moderate or even no effect on virulence in murine models of disease progression or target organ colony forming units, despite readily detectable effects using the highly sensitive mixed infection technique [6,37]. For example, mutants with impaired methionine uptake transporter function are able to cause levels of infection similar to the wild-type strain when given as a pure inoculums, despite consistently low recovery in mixed infections compared to wild type [6,17,37]. These data are surprising given the low levels of methionine found in nasal secretions (and therefore perhaps the nasopharynx) [77], and may reflect either the effects of redundancy (e.g., due to methionine synthesis) [6] or very specific roles for a given transporter during infection that are not properly tested by noncompetitive assessment of virulence.

ABC transporter SBPs are attached to the external surface of the cell membrane, and the large number of SBPs means they represent a significant component of the *S. pneumoniae* extracellular protein envelope. The importance of SBPs for importer ABC transporter function means many SBPs are necessary for *S. pneumoniae* survival and growth during infection, and they are usually highly conserved between strains [66]. Hence it is perhaps not surprising that the host immune system exploits lipoproteins, including SBPs, both as potential key mediators of the inflammatory response to *S. pneumoniae* through recognition by Toll-like receptor 2 [78], and as common targets for cellular and humoral adaptive immune responses (see Chapters 4 and 22).

CONCLUSIONS AND UNANSWERED QUESTIONS

The existing data on *S. pneumoniae* ABC transporters has identified the substrates for a significant proportion, and shown important functional roles for many at different anatomical sites of infection. Overall, *S. pneumoniae* ABC transporters are a vital component of bacterial physiology, essential for establishing infection at all anatomical sites. However, there are still many questions about *S. pneumoniae* ABC transporters that need to be addressed. These include identifying the substrates for all ABC transporters, better definition of ABC transporter function(s) and regulation at different sites and time points during infection (both singularly and in combination with other transport and synthesis mechanisms), how "orphan" or partial ABC transporter systems interact with other ABC transporter components, and detailed structure/function analyses of specific transporters to characterize the molecular mechanisms underlying substrate specificity.

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CHAPTER

11

Structure and Function of Choline-Binding Proteins

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INTRODUCTION: THE PNEUMOCOCCAL SURFACE PROTEIN FAMILIES

The pneumococcal cell wall contains a multilayered murein (peptidoglycan) with a covalently attached teichoic acid (TA), and a membrane-bound lipoteichoic acid (LTA). The peptides in the murein are cross-linked either directly or via an interpeptide bridge. The repeating units of TA and LTA have identical chemical structures, and so far this is a unique property of pneumococci [1]. The structure of the repeating unit in pneumococcal TA and LTA is of unusual chemical complexity and contains choline, an amino alcohol that is abundant in eukaryotes but only rarely found in bacteria [2,3].

Three main groups of surface proteins have been identified in *Streptococcus pneumoniae*: (1) around 50 lipoproteins (LPs), (2) up to 18 LPXTG consensus sequence-carrying proteins that are covalently linked via sortase to the cell-wall peptidoglycan, and (3) up to 16 choline-binding proteins (CBPs). In addition to the main three groups of surface proteins, the cell envelope of pneumococci is decorated with another cluster of proteins that lack a classic leader peptide and membrane-anchoring motifs; these are termed nonclassical surface proteins (NCSP).

Surface proteins carrying an LPXTG motif are covalently anchored via sortase to the cell-wall peptidoglycan. Eighteen and 15 proteins with peptidoglycan anchoring the LPXTG-like motif have been indentified in TIGR4 and R6 strains, respectively [4–6]. The available

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structural and topological information shows that pneumococcal LPXTG proteins are mostly modular proteins with enzymatic or adhesive functions. Adhesins help the pathogen to attach to host cells and tissues by recognizing host molecules, in a step that is critical for successfully establishing an infection [6].

The LPs are modified at their N-terminal region with the addition of an *N*-acyl diacylglyceryl group that anchors them to the membrane. The biosynthetic pathway is ubiquitous in bacteria, reflecting the essential role of these LPs in bacterial survival. Genome sequencing projects in combination with informatic analyses predict up to 46 LPs out of more of 2000 coding genes reported in *S. pneumoniae* genomes [4,7,8]. Interestingly, most of LPs seem to have a role in bacterial fitness [9]. In the TIGR4 genome, of the 46 predicted LPs [4,8] 26 are ABC transporters and six are involved in substrate binding and transformation, which are essential for maintaining the fitness of *S. pneumoniae*, an obligate commensal that is forced to scavenge host nutrients due to its incomplete biosynthetic pathways [10]. Six LPs are involved in protein folding or activation of cell surface molecules, such as sortase (StrC-3), which was shown to be specifically involved in pilus formation [11]. Recently, the role of two pneumococcal LPs, Etrx1 and Etrx2, in extracellular oxidative stress resistance has been reported [12].

NCSPs are described primarily as cytoplasmic proteins with intracellular roles that are not involved in host–pathogen interactions. However, once NCSPs are transported by unknown mechanisms to the cell surface, they display moonlighting functions and frequently act as adhesins, playing a relevant role in subverting the physiologic function of host-derived proteins [10]. NCSP binding to host molecules, such as fibronectin (PavA) or plasminogen (Enolase), promotes pneumococcal invasion and spread of the infection [13,14]. Recently, phosphoglycerate kinase has been reported to be an NCSP able to bind plasminogen and its activator [15]. Due to the lack of secretory signals or cell-wall anchoring domains, it is difficult to detect these NCSPs. Future research and technological advances in direct surface protein detection will presumably uncover new NCSPs displaying different moonlighting roles.

A unique characteristic of *S. pneumoniae* is its nutritional requirement for choline [16], which is taken up from the growth medium [17] and incorporated into the repeating units of TAs and LTAs [18]. CBP family members share a modular organization (Figure 11.1) consisting of a biologically active module and a choline-binding module (CBM), placed at the N- or C-terminus, which anchors these proteins to the cell envelope through a noncovalent interaction with such choline residues. This family of proteins is present in pneumococci and their relatives, as well as in some of their associated bacteriophages [2,19]. Of the approximately 15 CBP members (depending on the strain), the X-ray crystal structure of the complete protein is available for only three (Pce, CbpF, and LytC). Three-dimensional structures of different domains have been reported for five other CBPs.

THE CBP FAMILY IN PNEUMOCOCCI AND THEIR RELATIVES

Phosphorylcholine (PC) is a small zwitterionic amino alcohol expressed by several microbes across taxonomic kingdoms that infect humans and other eukaryotic hosts. PC is not required for growth in most prokaryotes, but it is an essential nutrient in eukaryotes. *S. pneumoniae* cannot synthesize choline, and thus is dependent on this amino alcohol for growth. Nevertheless, pneumococci can also grow without choline in the presence of a structural analogue such as ethanolamine,

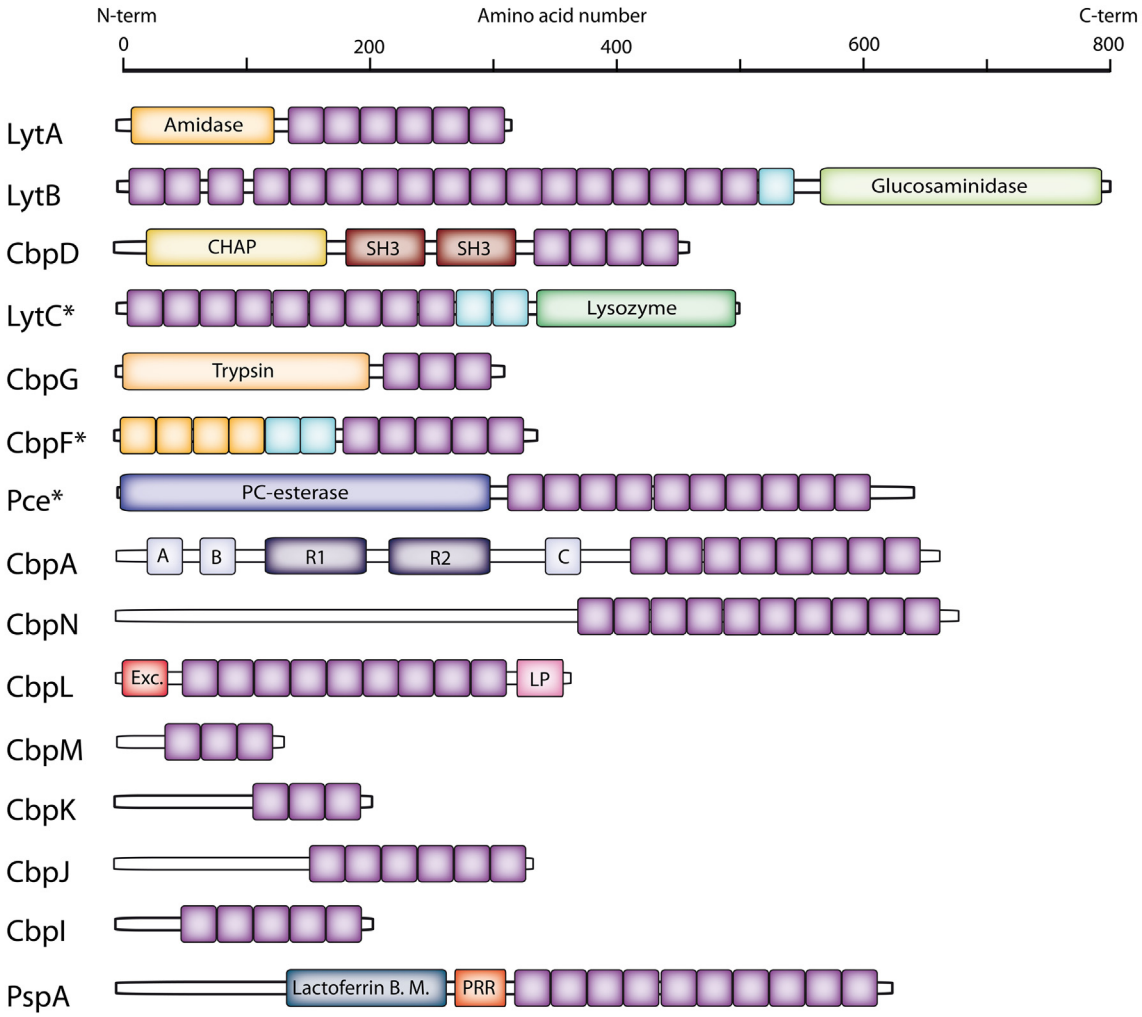

FIGURE 11.1 **The modular nature of CBPs.** CBP family members share an N- or C-terminal CBM made up of homologous repeats of about 20 amino acid residues (magenta boxes) that facilitate the anchorage of these proteins to the cell envelope. Some of these repeats present non-canonical sequences (colored in orange and light blue). In addition, CBPs present a catalytic or functional module displaying various activities (labeled; see text for details). The CbpL protein presents two small domains referred to as Excalibur (Exc) and Lipoprot (LP). Asterisks indicate proteins for which the complete three-dimensional structure is available.

although they form long chains and are unable to autolyze or undergo transformation. Choline is transported into the cell from the environment, transformed to PC, and incorporated in the TA or LTA through the genes clustered in the *lic* operons [20]. The number of PC groups in the cell-wall repeating unit and the amount of TA itself are both variable [21]. These PC groups are targeted by the C-reactive protein (CRP), an acute-phase serum protein that shows a rapid increase in response to infection or injury, leading to complement activation

and protection against pneumococcal infection *in vivo*. It is worth noting that expression of PC can affect epithelial cell adhesion and immune recognition. In addition, pneumococcal strains without PC in the TAs are unable to colonize the upper respiratory tract in mice and are less virulent in a murine sepsis model [22].

The number of CBPs ranges from 13 to 16 depending on the pneumococcal strain. The task of unequivocally assigning each CBP is complicated since some of them are differently named by different authors; also in addition, the annotation differs in regard to the first two sequenced genomes, the unencapsulated laboratory R6, and the clinical TIGR4 strains [5,23].

Reported functions for CBPs are summarized in Table 11.1. Overall, CBPs play important roles in cell-wall physiology, some stages of the colonization process, and the interaction with host cells leading to the disease transition. In fact, several CBPs have been identified as virulence factors in a large-scale sequence-tagged mutagenesis study in mouse models of sepsis and pneumonia using the TIGR4 strain [45].

Conservation of CBPs in Different Pneumococcal and Relative Strains

Currently, the genus *Streptococcus* is divided into six groups: Mutans, Pyogenic, Salivarius, Anginosus, Sanguinis, and Mitis, although the separation of the last two is controversial. In fact, the line separating certain species within streptococci belonging to the oral bacterial community is not sharp and continuous, due to the fact that many members of these groups are naturally transformable as a result of interspecies gene transfer and recombination events [46].

Relevant genomic data from different pneumococcal strains are available at NCBI (http://www.ncbi.nlm.nih.gov/genomes/browse). Choline residues have also been identified in the cell wall of other closely related, commensal streptococci such as *S. mitis* and *S. oralis*; consequently, some CBPs have been identified. In a further study, a detailed comparison of CBPs among R6, TIGR4, and four other clinical pneumococcal isolates, together with two strains of *S. mitis*, was published [23]. A complete analysis of the cell surface proteins of *S. pneumoniae*, *S. mitis*, and *S. oralis* strains using an oligonucleotide microarray covering 29 CBP genes has also been carried out. The results demonstrated that many related surface proteins, CBPs among them, were common in these three species [47]. In addition, some respiratory pathogens able to colonize mucosal surfaces also display PC on their cell surface (e.g., *Haemophilus influenzae*, *S. pseudopneumoniae*, *Neisseria* spp., and *Pseudomonas aeruginosa*).

In general, genes encoding CBPs are polymorphic, but some of them are exacerbated in this polymorphism. Examples of this feature are *pspA* and *pspC* genes; one variant of the latter gene product has been described as Hic, which contains an LPXTG motif instead of the CBM [48]. In addition, the case of LytB polymorphism is interesting since a survey of several *S. mitis* strains revealed noticeable variation in the number of choline-binding repeats (CBRs) as well as in their amino acid sequences [49]. Although LytB is a well-conserved protein among related streptococci, different lengths and arrangements of the substrate-recognizing module could point to a fine tuning to optimize binding to biochemically different TAs.

Currently, huge genome sequencing projects are in progress, and different bacteria are included, although this effort is mainly focused on certain pathogens. Specifically, there are 27 pneumococcal strains that have been completely sequenced and 275 clinical pneumococcal isolates partially sequenced (last accessed October 25, 2014).

From a global comparison of the particular content of 10 representative CBPs among pneumococcus and the more closely related species, it is clearly seen that each species has a set of

TABLE 11.1 Identified CBPs in *S. pneumoniae*

CBPs			
Protein name	**UniProtKB locus**	**Function/reference**	**PDB entry codes**
CbpI	Q9KGY8-SP_0069-CbpI (211 aa)	Putative adhesin [4,5,24].	
PspA	Q97T39-SP_0117-PspA (744 aa) Q8DRI0-spr0121-PspA (619 aa)	Virulence factor. Binds lactoferrin and inhibits complement activation [4,7,25–27].	2PMS[a]
CpbF[b]	Q97SI4-SP_0377-CpCp-CbpC (340 aa) Q8DR52-spr0337-CbpF (338 aa)	Regulatory function for autolysis by inhibiting autolysin LytC [4,7,28,29].	2V04, 2V05, 2VYU, 2X8M, 2X8O, 2X8P
CbpJ	Q9KGY7-SP_0378-CbpJ (332 aa)	Putative adhesin [4,5,24].	
CbpG	Q97SH5-SP_0390-CbpG (285 aa)	Unknown physiological function [4]. Serine protease activity.	
CbpK	Q9KGY9-SP_0391-CbpF (340 aa) Q8DR39-spr0351-PcpC (294 aa)	Putative adhesin [4,7,24].	
CbpL	Q97RW9-SP_0667 (332 aa) Q8CZ16-spr0583 (329 aa)	Putative adhesin [4,5,7].	
Pce[b]	Q9KGZ1-SP_0930-CbpE (627 aa) Q8DQ62-spr0831-CbpE-LytD-Pce (627 aa)	Teichoic acid phosphorylcholine esterase. Critical function in virulence and invasiveness [4,5,7,24,30].	2BIB, 1WRA
LytB	P59205-SP_0965-LytB (658 aa) P59206-spr0867-LytB (702 aa)	*N*-acetylglucosaminidase activity involved in separation of daughter cells. Role in colonization [4,7,31,86].	4Q2W
PspC-Like	SP_1417	PspC-related protein [4].	
LytC[b]	Q2MGF6-SP_1573-LytC (490 aa) Q8DP07-spr1431-LytC (501 aa)	Lysozyme activity. Role in colonization. Virulence factor involved in fratricide (competence-programmed mechanism). Binding to extracellular DNA to form biofilms [24,32–34a].	2WW5, 2WWC, 2WWD

(*Continued*)

TABLE 11.1 (Continued)

CBPs			
Protein name	**UniProtKB locus**	**Function/reference**	**PDB entry codes**
LytA	P06653-SP_1937-LytA (318 aa) Q7ZAK4-spr1754-LytA (318 aa)	*N*-acetylmuramoyl-L-alanine amidase. Virulence. Release of Ply and inflammatory PG and TA from bacterial cell wall. Cell-wall growth and turnover. Participates in fratricide [34–38].	2BML, 1GVM, 1H8G, 1HCX , 4IVV
CbpA	Q97N74-SP_2190-PspC-SpsA-CbpA	Major adhesin. Immunoglobulin A inactivation [5,27,38–41].	1W9R
	Q8DN05-spr1995-CbpA-PcpA	Binds factor H, binds the ectodomain of the pIgR, called secretory component, and also vitronectin [42].	
CbpM	Q8DP99-spr1274 (129aa)	Putative adhesin [5,7,43].	3HIA
PcpA	Q97NB5-SP_2136-Pcpa (621 aa) Q8DN38-spr1945-Pcpa (690 aa)	Putative adhesin [23,44].	
CbpD	Q9KGZ2-SP_2201-CbpD (448 aa) Q8DMZ4-spr2006-CbpD (448 aa)	Adherence and nasopharyngeal colonization [24]. Participates in fratricide [89,90].	

[a] *PDB entry 2PMS refers to UnitProtKB entry Q54972, corresponding to PspA from pneumococcal Rx1 strain [25], which exhibits 100% sequence identity with PspA from R6 strain (UniProtKB entry Q8DRI0).*
[b] *CBPs for which complete three-dimensional structure (CBM and functional modules) is available.*

CBPs, although only LytB, Pce, and CbpD are present in all of them. But even within the same species, every single strain may possess its own particular CBPs. Another interesting point is that LytA is present only in pneumococcus, *S. pseudopneumoniae*, and some strains of Mitis group. It is worth noting that some proteins that are not CBPs in pneumococcus may appear as CBPs in some of these related species, such as β-galactosidase of *S. mitis* or neuraminidase of *S. pseudopneumoniae*.

The CBP family has interesting traits from an evolutionary point of view: (1) These proteins are surface exposed and thus subjected to the selection of sequence variants to evade immunological responses; (2) they often appear in a tandem arrangement, most likely as a result of gene duplication events, and truncated versions are frequently encountered in the genomes; (3) choline has acted to exert strong selective pressure reducing the evolutionary drift of the CBMs; (4) a plausible difference between pathogenic and commensal species might consist in the evolutionary events referred to the CBPs (e.g., incorporation of *pspC* and *pspA* genes into *S. pneumoniae*).

The reason for the large difference in virulence potential among these members of the Mitis group is not fully understood. Many gene products have been described as *S. pneumoniae* virulence factors on the basis of mouse infection models. However, most of them are present in

the first completed *S. mitis* genome of strain B6. Thus, only a few are apparently pneumococcal-specific components, including the capsular cluster, genes encoding surface proteins such as the CBPs PspC, PspA, and PcpA, the hyaluronidase HlyA, and a genomic island that contains *ply* plus *lytA*, encoding the potent pneumolysin and the major autolysin. The genes *ply* and *lytA* have been identified in several *S. mitis*, and *lytA* gene is frequently part of prophages.

Genomes of *S. pneumoniae* clones vary by over 10%, and probably less than 50% of all genes are common to all *S. pneumoniae* strains [50]. Similarly, the accessory genome of *S. mitis* B6 has been estimated to constitute over 40% of all coding sequences [51].

LytA is noteworthy since this amidase is a virulence factor that is almost unique to the pneumococcus. LytA's Amidase_2 domain (Pfam code PF01510) is present in 3179 bacterial isolates (953 Firmicutes, 1615 Proteobacteria, 358 Actinobacteria, and 126 Bacteroides), 291 phages, and 154 eukaryotic cells, whereas CW_binding_1 domain (Pfam code PF01473), corresponding to the CBR, has been found in 530 bacterial isolates (498 Firmicutes, 26 Actinobacteria, and 6 Proteobacteria) and 16 phages (all of them from streptococci) (last accessed November 1, 2013). Available sequences from databanks suggest that acquisition of the *lytA* gene took place in an early step in the appearance of the Mitis group, and different alleles have continually evolved to their current versions in modern pneumococcal strains, which are especially adapted to the pathogenicity of these bacteria.

STRUCTURAL BASIS OF CHOLINE RECOGNITION BY CBPs

The Main Brick in Choline Recognition, the Structure of the CBR

The CBMs are built up from a variable number of CBRs, which contain approximately 20 residues and a high content of aromatic amino acids. The typical CBR follows a consensus GWXK-X_{4-5}-WYY-Φ-X_{3-5}GXMX_{2-3} motif, where X is any residue and Φ is a hydrophobic residue. The 3D structure of CBR presents β-hairpin (12−14 residues) followed by a loop 8−10 residues long (Figure 11.2A). In most cases, CBRs are arranged in a ββ-3 solenoid superhelix (Figure 11.2B) in which choline molecules are recognized at the interface between two consecutive repeats. Three aromatic residues (usually two tryptophan residues from one repeat and one tyrosine residue from the next repeat) stabilize the choline moieties by cation−π interactions (Figure 11.2C). In addition, a hydrophobic residue (methionine or leucine) is usually located at the bottom of the pocket.

Molecular Recognition of Choline by Non-Canonical Binding Sites

Although the canonical binding pocket is widespread among CBPs, variations in the number of aromatic residues building the site are also observed. The combination of the canonical with the non-canonical sites endows each CBP with a specific cell-wall recognition pattern that might tune the enzyme's localization and its regulation [28,33,52].

Non-Canonical Sites with Two or Three Aromatic Residues

The simplest example of divergence from the canonical architecture involves the formation of a choline-binding site by only two aromatic residues from a single repeat (Figure 11.2D). A structural example of this divergence is found in Pce (PDB code 2BIB), which shows a BIS-TRIS molecule (a choline analogue) stabilized by cation−π interaction with two aromatic residues, the first (Trp323) coming from a β-strand and the second (Tyr333) from the loop (Figure 11.2D). Another

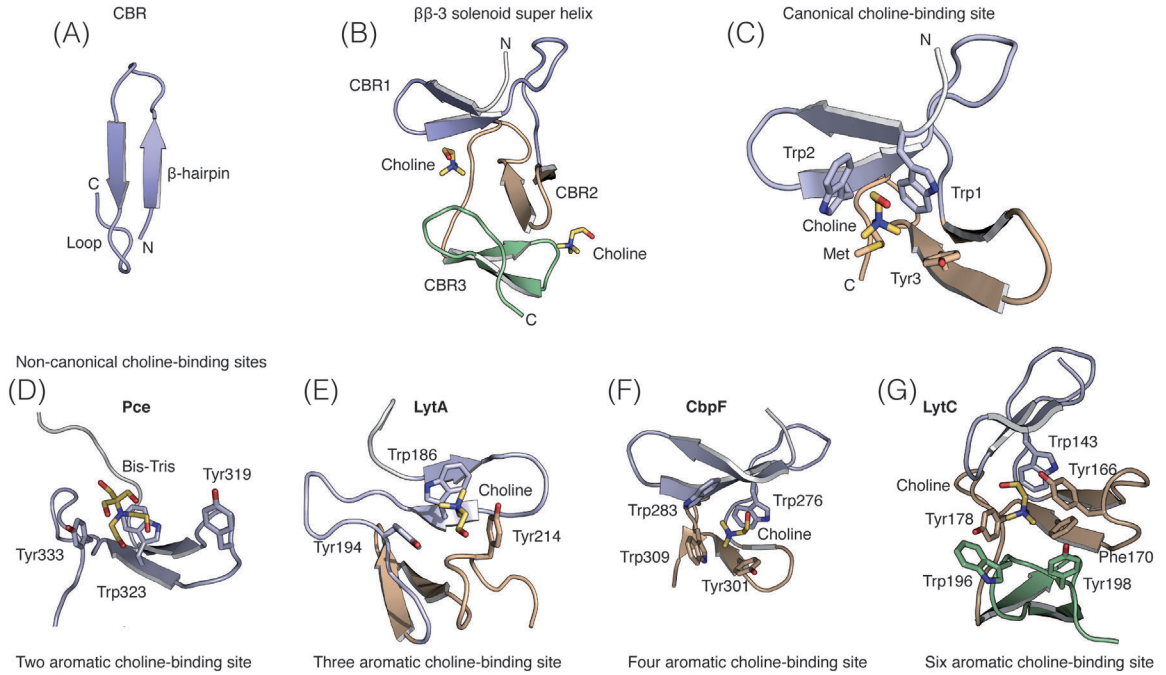

FIGURE 11.2 **The structural framework for choline recognition in CBPs.** (A) Three-dimensional structure of a CBR. (B) CBRs are usually arranged in a ββ-3 solenoid superhelix, with the choline-binding sites located at the interface of two consecutive repeats. (C) Three-dimensional structure of a canonical choline-binding site. (D–G) Three-dimensional structure of non-canonical choline-binding sites built with different numbers of aromatic residues.

Tyr residue (Tyr319) is found in the turn of the beta hairpin but does not directly interact with the ligand. Other examples of divergence from the canonical architecture are found in the CBM of LytA (PDB code 4YWT) that presents a binding site composed of three aromatic residues, but with only one coming from a β-strand, while the other two come from loops (Figure 11.2E).

Non-Canonical Sites with Four and Six Aromatic Residues

More complex variations of the canonical site appear in the form of a four-aromatic binding site. These sites appear at the C-terminus of Pce and CbpF and are formed by a standard CBR followed by a nonhomologous segment that adopts a β-hairpin motif (Figure 11.2F).

The largest reported choline-binding site structure contains six aromatic residues and is produced by three consecutive repeats (Figure 11.2G). Choline binding is stabilized by cation–π interactions with a tryptophan residue located in the first β-strand from the first repeat, a tyrosine located in the second repeat β-hairpin connection, a phenylalanine placed in the second β-strand from the second repeat, a tyrosine located in the second repeat loop, and, finally, a tryptophan and a tyrosine, both from the third repeat loop. This site contains a characteristic Gly-Tyr-Met-Ala (GYMA) sequence located in the third repeat loop

that could serve to recognize and anchor the *N*-acetyl glucosamine moieties of the TAs, thus providing strong multivalent recognition and attachment to the cell wall [33]. Sequence analysis reveals that GYMA sites are, at least, found in LytC and LytB, both having the CBM at the N-terminal position.

Structural Plasticity of CBR: Functional Implications

CBRs are usually arranged in a ββ-3 solenoid showing a 120° counterclockwise rotation superhelix arrangement. This structural arrangement is independent of the number of aromatic residues involved in choline stabilization. However, mutations and/or insertions inside the repeat affect the superhelical geometry, preclude choline binding, and promote specific structural changes in CBPs that are critical for their specific function. The presence of an extra β-strand with charged residues in the p10 repeat of LytC creates a three-stranded anti-parallel β-sheet that breaks the superhelix rotation. The p11 repeat also changes its structure, favoring the stabilization of the catalytic domain and orienting its active site toward the CBM, resulting in the characteristic hook-like conformation of LytC (Figure 11.3F), which is critical in its physiological function (see "Autolysin LytC" section).

In CbpF, insertions in the canonical repeats result in a completely different three-dimensional structure. Its N-terminal domain is built up by six non-consensus CBRs providing a disc-shaped conformation for this domain (Figure 11.3D) crucial in its regulatory role (see "Choline-Binding Protein F" section). Another structural variation is observed in Cpl-1, a CBP encoded by pneumococcal phage Cp-1, in which the last two repeats (named p5 and p6), together with the C-terminal tail, are folded in an extended six-stranded β-sheet which prevents choline binding and promotes intermodular interactions with the catalytic domain (Figure 11.4A).

Although the main role of CBRs is choline binding, the structural examples reported up to now indicate great plasticity for these repeats, thus allowing different structural and functional roles in each CBP.

THREE-DIMENSIONAL STRUCTURES OF CBPs AND THEIR IMPLICATIONS IN PATHOGENESIS AND VIRULENCE

Autolysin LytA

LytA amidase has been well studied and represents the paradigm of autolytic enzymes [54]. LytA is responsible for cellular autolysis, through which it mediates release of toxic substances—such as the pore-forming toxin pneumolysin and cell-wall degradation products—that damage endothelial and epithelial barriers and allow pneumococci to gain access to the bloodstream and disseminate through the body [55]. Interestingly, it has been shown that pneumococci are protected from the lytic activity of LytA during exponential growth, but not in the stationary phase [56]. Recent results confirm this point, and a model explaining how this phenomenon occurs has been proposed [57]. LytA is structurally organized as a two-module protein (Figure 11.1) with an N-terminal module (residues 1−174) that catalyzes the cleavage of the *N*-acetylmuramoyl-L-alanine bond to the pneumococcal peptidoglycan backbone [58], and a C-terminal CBM. The C-terminal module (residues 175−301), responsible for cell-wall binding, is formed by a tandem of six repeats.

Up to now, there is no structural information for the full-length protein, whereas the crystal structures of the CBM from LytA (C-LytA) [35,36] and for the catalytic domain

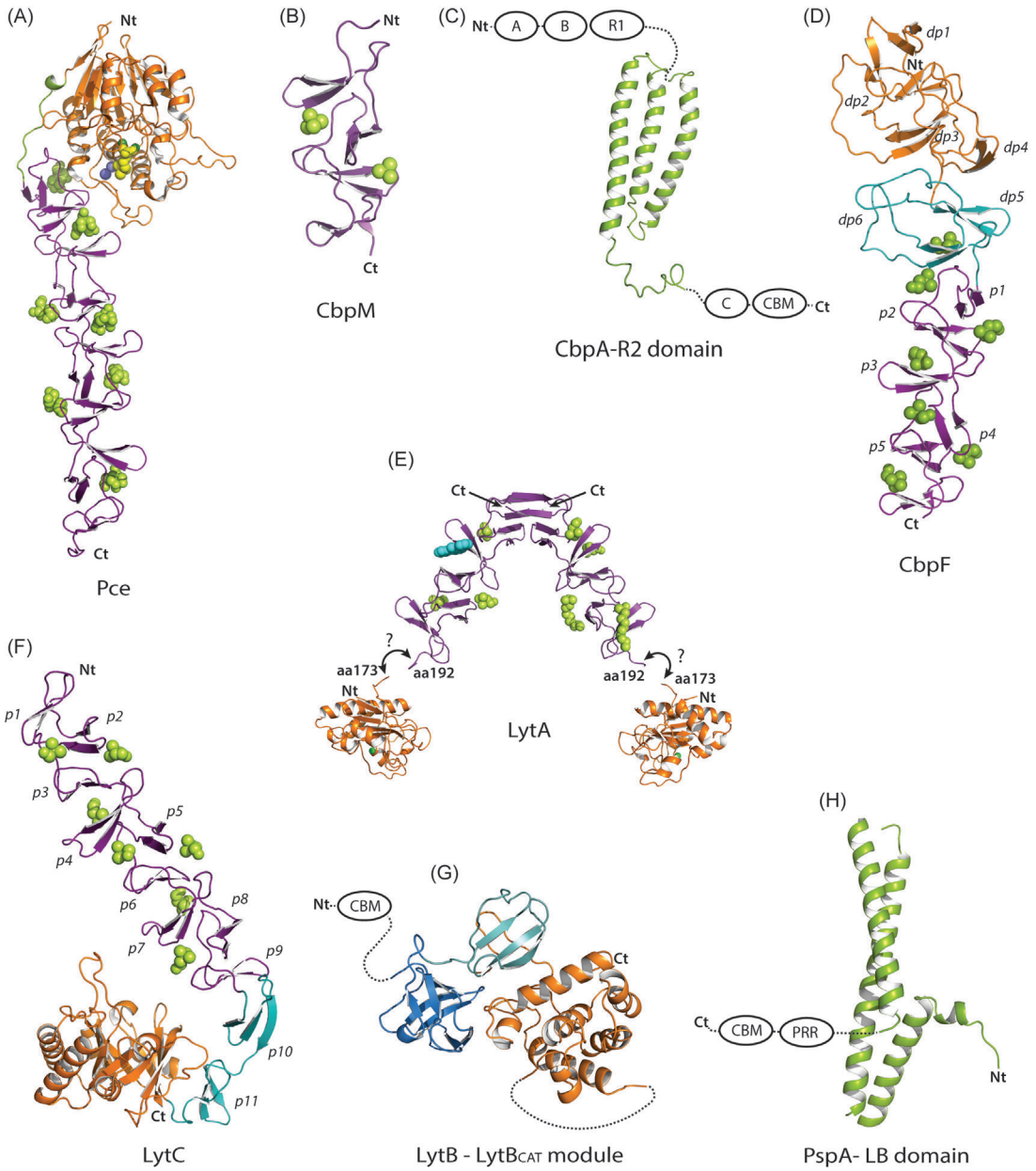

FIGURE 11.3 **Three-dimensional structures of CBPs.** (A) Ribbon diagram of full-length Pce (PDB code 2BIB), showing the CBM in violet, the catalytic module in orange, and the linker in green. Choline analogs are depicted as green spheres and the (2,2′:6′,2″-terpyridine)-platinum(II) used for C-LytA phasing is represented as cyan spheres. This color code is maintained in all panels. Reaction products (PC) at the active site is depicted as yellow spheres. (B) Crystal structure of CbpM (PDB code 3HIA). (C) Three-dimensional structure of the R2 domain of CbpA (PDB code 1W9R). (D) Ribbon diagram of CbpF with CBM in violet, linker domain in cyan, and N-terminal domain in orange (PDB entry 2V04). Consensus (p1−p5) and non-consensus (dp1−dp6) CBRs are labeled. (E) Three-dimensional structures of the CBM of LytA (C-LytA) (PDB entry 1HCX) and the catalytic module of LytA (PDB code 4IVV). The dimeric arrangement is based on crystallographic and SAXS studies [53]. (F) Three-dimensional structure of pneumococcal autolysin LytC (PDB entry 2WWD). Repeats p10 and p11 presenting a different structural arrangement are colored in cyan. (G) Three-dimensional structure of the catalytic module of LytB. The glycoside hydrolase family 73 module is colored in orange, the WW module in cyan, and the SH3b module in dark blue. (H) Structure of the LB domain of PspA.

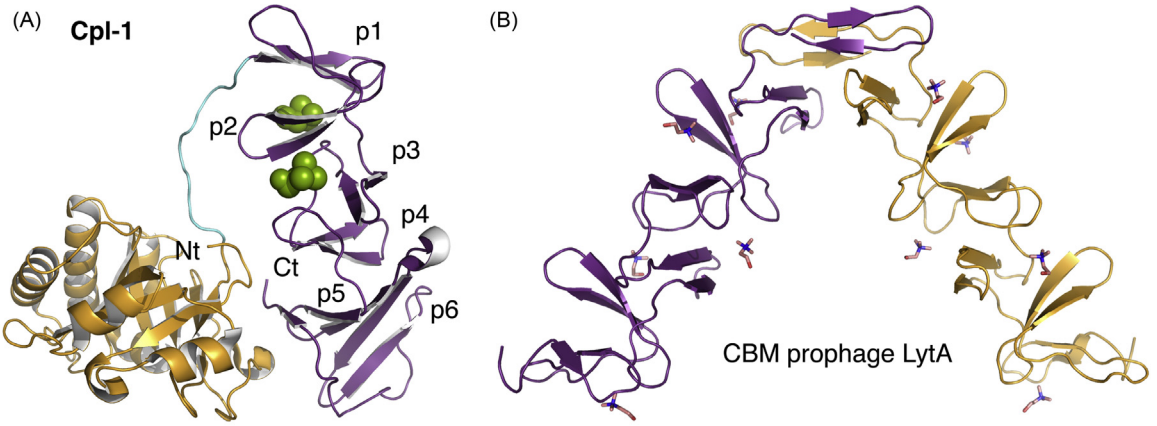

FIGURE 11.4 **Three-dimensional structures of phage CBPs.** (A) Three-dimensional structure of full-length Cpl-1 endolysin in complex with choline (green spheres) (PDB code 1OBA). The lysozyme module (GH25 family) is colored in orange, the linker in cyan, and the CBM in violet. The first four repeats (p1−p4) are folded in a superhelical arrangement, allowing choline binding, while the final repeats (p5−p6) and the C-terminal tail are folded in a six-stranded β-sheet that allows intermodular interactions with the catalytic module. (B) Structure of the CBM of a phage LytA-like protein (PDB code 4IWT). As observed in LytA, the CBM forms a dimer through interactions of the C-terminal repeats. Choline molecules represented in sticks.

[53] have been reported [35,36]. C-LytA presents a single regular fold formed by a superhelical arrangement of the CBRs and the C-terminal tail (Figure 11.3E). These modules dimerize through interactions of the terminal repeats, producing a boomerang-like structure (Figure 11.3E). This dimer is observed not only for the CBM alone but also for the complete protein in solution [53,59].

The structure of the catalytic domain of LytA (Figure 11.3E) reveals a prominent Y-shaped binding crevice with a Zn^{++}-containing active site localized at the bottom of the branch point [53]. Two branches would be responsible for recognition of the glycan chain, while the remaining branch would accommodate the peptide stem. Site-directed mutagenesis was employed to identify catalytic residues and to map the potential substrate-interacting residues lining the binding crevice for the lytic activity of LytA.

Despite the numerous studies on LytA, several basic features of this protein still remain elusive. It is unclear what regulates its lytic activity, why lysis is triggered in a predictable timeframe following entry into stationary phase, how autolysis is connected to penicillin-induced lysis [56], how conversion of an inactive E-form to the active C-form of LytA [60] is produced, and how LytA is targeted to the cell wall. Therefore, the elucidation of the three-dimensional structure of the full-length LytA could provide valuable insights to clarify these questions.

Pneumococcal Surface Protein A

Pneumococcal surface protein A (PspA) is a CBP [61] that localizes on the surface of the cell wall of pneumococci [62]. Its molecular mass ranges between 67 and 98 kDa in various pneumococcal strains. PspA is structurally organized in four distinct regions: an N-terminal functional module/domain (composed of 288 residues in the Rx1 strain of *S. pneumoniae*), a proline-rich region (83 residues), a CBM built from a stretch of ten CBRs, and finally a C-terminal tail of 17 hydrophobic residues [63]

(Figure 11.3H). The N-terminal functional domain is thought to extend from the cell wall and even to protrude outside of the capsule. The N-terminal half of mature PspA is predicted to be entirely α-helical [64]. A lactoferrin-binding region of PspA has been localized within residues 168–288 of this α-helical domain [65,66].

The only structural information reported for PspA consists of the crystal structure of a complex of the lactoferrin-binding domain of PspA with the N-lobe of human lactoferrin, a component of the innate immune system [25]. The lactoferrin-binding domain of PspA consists of four α-helices connected by mobile loops. At the N-terminus, there is a short α-helix followed by three long amphipathic helices with numerous hydrophobic interactions between neighboring anti-parallel helices. The structure of the complex revealed direct and specific interactions between the negatively charged surface of PspA helices and the highly cationic moiety of lactoferrin. Binding of PspA should block surface accessibility of this bactericidal peptide, preventing it from penetrating the bacterial membrane [25].

Choline-Binding Protein A

CbpA (also referred to as PspC, SpsA, and PbcA) is one of the principal pneumococcal adhesins. The sequence of the CbpA N-terminus (residues 39–514) [4] exhibits numerous repeats of the leucine zipper motif [67] clustered within five domains termed A, B, R1, R2, and C [68] (Figure 11.1). Domains A, B, and C are 21–25 residues in length and are predicted to form coiled-coil dimers. The 110-residue-long, "repeated" domains R1 and R2 (78% identical) [69] are the adhesion domains of CbpA [69–71]. The C-terminal domain of CbpA is formed by a variable number of CBRs depending on the strain (from 4 to 10).

To avoid complement-mediated bacterial lysis, pneumococci recruit the central complement regulators factor H and C4b-binding protein. The major factor H–binding protein of *S. pneumoniae* is CbpA (PspC) and is termed Hic (factor H-binding inhibitor of complement) in another subset of strains [39,42,72].

CbpA R domains bind the extracellular immunoglobulin-like domains of polymeric immunoglobulin receptor (pIgR) during invasion of human nasopharyngeal epithelial (NE) cells [69,73]. CbpA binds specifically to an extracellular domain of pIgR [71,74] and hijacks the endocytosis machinery to translocate pneumococci across NE cells into the bloodstream [40]. Whereas the complete CbpA structure remains unreported, the solution structure of domain R2 of CbpA has been determined using NMR spectroscopy [40] (Figure 11.3C). This structure was also used to model that for the R1 domain. The R domains, composed of 12 imperfect copies of the leucine zipper heptad motif, adopt a unique 3-α-helix, raft-like structure. Each pair of α-helices is anti-parallel, and conserved residues in the loop between helices 1 and 2 exhibit a novel "tyrosine fork" structure that is involved in binding pIgR.

PC Esterase Pce

The teichoic-acid PC esterase, Pce or CbpE, was first described in 1974 [75], showing that the enzyme is capable of removing a limited number of PC residues from pneumococcal cell walls. The molecular architecture of Pce includes a catalytic module localized at the N-terminal region of the protein (312 residues), a C-terminal CBM with 10 homologous repeating units (205 residues), and a long C-terminal tail of 85 residues (Figure 11.1).

Structural information has been reported for the catalytic domain [76] and for the complete

Pce without the C-terminal tail [30]. The crystal structure of the full-length protein (Figure 11.3A) is comprised of the catalytic module and the CBM, which are joined by a small linker. The catalytic module folds into an αβ/βα sandwich similar to that of metallo-β-lactamases. The CBM is formed by 10 repeats (p1−p10), following a left-handed superhelical fold (Figure 11.3A). The active site is located at the interface of the β-sheets in the N-terminal module, where a long groove contains two Zn^{2+} ions placed at the bottom of a deep hole.

Pce shows a novel structural arrangement in its constituent modules. Despite strong differences in the sizes and shapes of the modules, the overall structure seems to be quite rigid. The Pce structural framework is determined by three main factors: (1) a short linker on the surface of the catalytic module; (2) the presence of a very long loop in the catalytic module (residues 36−61), L36-61, that strongly interacts with the first three repeating units of the CBM; and (3) two structural Ca^{2+} ions reinforcing the L36-61 conformation.

One of the most striking features of Pce was the earlier observation, both *in vivo* [62] and *in vitro* [77,78], that the enzyme is able to liberate only a limited number of choline residues from the pneumococcal cell wall, representing about 30% of the total PC content. The modular arrangement in Pce configures the active site of the full-length protein in such a way that only residues located at the end of the TA chains are accessible to the catalytic center. Therefore, Pce would be involved in specifically releasing only those PC terminal residues relevant for cell−cell interactions but retaining the choline residues of the cell wall that are important for normal cell growth and maintenance of CBPs attached to the bacterial envelope [30].

Selective modification of the distribution of the PC moieties on the bacterial surface by Pce should impair the ability of components of the host response targeting PC, such as human CRP, to efficiently bind the bacteria, and would provide a mechanism for pneumococci to escape the attack by the host defense system. Moreover, the remaining exposed PC residues would be used to bind to platelet-activating factor (PAF) receptors to promote invasion. Thus, fine regulation of PC decoration on the bacterial surface, mediated by Pce activity, may favor both infection and colonization by *S. pneumoniae*. In addition, it has been demonstrated [30] that Pce is able to hydrolyze PAF, a pivotal second messenger of the inflammatory processes, suggesting that this enzyme has other functions during infection.

Choline-Binding Protein F

CbpF is one of the most abundant proteins in the pneumococcal cell wall. The crystal structure of CbpF in complex with choline has been reported [28]. CbpF displays a novel modular structure comprised of both consensus (p1−p5) and non-consensus CBRs (dp1−dp6) (Figures 11.1 and 11.3D) distributed along its length, which dramatically alters its shape and binding ability, organizing the protein in two-well defined modules. The N-terminal module (N-CbpF) displays a disc-shaped conformation, while the C-terminal module (C-CbpF) follows the superhelical fold (Figure 11.3D). Seven choline molecules were found attached to the C-terminal domain. Remarkably, the repeats building the N-terminal module are highly modified, by both additional residues and mutations, at various positions of the consensus motif, and do not fulfill the choline-binding requirements. Variations in the sequences of the CBRs were also responsible for the modular arrangement in CbpF [28].

Experimental assays proved that CbpF can inhibit the activity of pneumococcal autolysin LytC, both *in vitro* and *in vivo*. Interestingly,

C-CbpF did not alter the enzymatic activity of LytC, strongly suggesting that the N-CbpF might play a critical role in this specific inhibition. Remarkably, the inhibitory effect is not limited to LytC lysozyme, but is also displayed by other pneumococcal phage lysozymes such as Cpl-1 and Cpl-7, sharing the same catalytic module as LytC. This inhibitory effect could somehow be related with cell-wall binding through its N-terminal domain; this hypothesis is supported by results from surface plasmon resonance experiments [28].

A study of the CbpF orthologs in other pneumococcal strains revealed the existence of proteins CbpC and CbpJ in the TIGR4 strain. Further analyses of known pneumococcal genomes revealed the existence of several proteins that are likely to show the same architecture as CbpF. These proteins might constitute a new CbpF-like subfamily within the large CBP family of proteins having a typical CBM and an N-terminal region formed by a series of non-consensus repeats. Most of the residues involved in both the N-terminal structural framework and the intermodular interactions are preserved in CbpF-like subfamily, suggesting that these proteins could exhibit regulatory functions similar to those of CbpF.

The 3D structure of CbpM, presenting only CBRs, has been deposited (Figure 11.3B), but no structural characterization has been reported.

Autolysin LytC

LytC lysozyme is gaining more attention, mainly due to its significant role in cellular fratricide [34]. It has been demonstrated that, during the competent state, pneumococci turn on the expression of proteinaceous toxins (LytA, LytC, and CbpD), which kill and lyse non-competent pneumococcal sister cells, as well as other bacteria from closely related species [79]. Fratricide contributes to virulence by exacerbating an infection through the release of virulence factors and inflammatory mediators. CbpD causes some lysis of target cells on its own, but its effect is multiplied several-fold by its activation of LytA and LytC [80]. As lysis coincides with competence, DNA released from target cells is taken up by competent attacker cells, resulting in increased efficiency of gene transfer and therefore also in antibiotic resistance [81]. One of the most striking features concerning LytC was the observation that the relatively high concentration of LytC detected in the medium of non-competent pneumococcal cultures in the exponential phase of growth was not harmful to the cells, demonstrating that LytC is inactive or highly regulated under these circumstances [80]. It was also shown that the presence of CbpD, which harbors a CHAP (cysteine, histidine-dependent amidohydrolase/peptidase) domain involved in murein stem-peptide cleavage, was required to activate the LytC lysozyme during fratricide. However, the nature of this activation mechanism remained elusive until the crystal structure of full-length LytC was reported in a ternary complex with choline and a peptidoglycan fragment [33] (Figure 11.3F). LytC consists of an N-terminal CBM (residues 1−267) and a C-terminal catalytic module (residues 268−468) formed by a single structural domain, showing the irregular $((\beta/\alpha)_5\beta_3)$ barrel typical of the glycosyl hydrolase family 25 (GH25) [82]. Eleven CBRs (p1−p11) build the CBM. While the first nine repeats (p1−p9) are arranged in the typical superhelical left-handed fold involved in choline binding, the last two repeats (p10, p11) are critical in the modular arrangement of LytC. The 3D structure of these repeats differs from that of the preceding repeats, and breaks the linear orientation of the CBM as it orients the LytC active site toward the anchoring module. Experimental results suggest that the catalytic properties of LytC, such as specific activity and optimal temperature at 30°C, arise not only from the nature of the given active site, but also from its specific modular arrangement [33].

Furthermore, the unusual hook-shaped conformation of LytC, with the active site oriented toward the CBM (Figure 11.3F), can explain its activation by CbpD in the fratricide process. The crystal structure of the LytC-choline-peptidoglycan complex indicates that long peptide stems and/or cross-linked peptidoglycan chains should be deleterious to the hydrolytic activity by steric hindrance by both a LytC-specific loop and by the entire CBM. Therefore, prior cleavage of the peptide stems performed by the CHAP domain of CbpD should facilitate hydrolysis of the non-cross-linked peptidoglycan chains by LytC, thus explaining the activation of LytC observed in fratricide [33].

LytB

LytB consists of an immature polypeptide chain of 658 residues containing a cleavable signal peptide 23 residues long [83]. The mature protein is built by an N-terminal CBM containing 18 CBRs, followed by a catalytic module that has glucosaminidase activity [31,84]. LytB plays an essential role in cell separation, as suggested by the phenotype of *lytB* mutants that present long chains of pneumococcal cells [83,84]. During separation of daughter cells, LytB acts together with LytA, which contributes moderately to the process [85]. The *lytA*/*lytB* double mutant exhibits a more exacerbated phenotype in which even longer chains, with more than 100 cells, were observed [32,84]. In agreement with this role, LytB fused to green fluorescent protein was shown to bind to the cell poles, supporting the idea that LytB accumulates in this region, where it will assist cell-wall separation during division. Cell-wall localization of LytB has been proposed to happen by interaction of the enzyme with specific receptors positioned at the polar region on the pneumococcal surface [31]. Beyond the role in daughter cell separation and its derived implications in cell dissemination and pathogenesis, recent work has reported the direct contribution of LytB in virulence [86] by its participation in: (1) attachment to human nasopharyngeal and murine alveolar macrophage cells; (2) nasopharyngeal colonization; (3) adhesion and invasion into human lung epithelial cells; (4) decreasing the binding of the key complement component C3b; and (5) evasion of phagocytosis by alveolar macrophages and neutrophils. In this work, LytB is also reported to contribute to pneumococcal sepsis and pneumonia [86]. In addition, it has been reported that an antiserum produced against LytB significantly protected mice from lethal challenge by some pneumococcal strains [87]. LytB is therefore a very promising candidate for the design of new antibiotics or a conjugate vaccine.

The 3D structure of the catalytic module of LytB ($LytB_{CAT}$, residues 375−658) has recently been reported [88] (Figure 11.3G). It consists of three structurally independent modules that pack against each other: an SH3b module ($LytB_{SH3b}$, residues 385−450), followed by a WW domain−like module ($LytB_{WW}$, residues 451−493), and a glycoside hydrolase family 73 (GH73) module ($LytB_{GH73}$, residues 494−658). Structural analysis suggests that both $LytB_{SH3b}$ and $LytB_{WW}$ could participate in substrate binding. The structure of $LytB_{CAT}$ shows a "T-shaped" groove formed by residues from the three modules. This putative substrate-binding pocket consists of a channel at the surface of the $LytB_{GH73}$ module that contains the active site and connects with a cleft formed by $LytB_{SH3b}$ and $LytB_{WW}$ modules. The authors suggest that both SH3b and WW modules work together with the catalytic GH73 module during substrate recognition, and are required for proper peptidoglycan hydrolysis by LytB [88]. The role of the CBM in this process remains to be elucidated, as no structure has been reported for the module alone or within the full-length protein.

FUNCTIONAL CHARACTERIZATION OF OTHER CBPs

Of the 16 CBPs identified so far in the pneumococcus, eight have been functionally and structurally characterized. There is no structural information about the remaining eight members; but functional information has been reported for the peptidase CbpD and, in less extensive form, for the putative serine protease CbpG. The other six CBPs (CbpI, CbpJ, CbpK, CbpL, CbpN, and CbpM) have been reported to work as adhesins, but their functions remain poorly characterized.

CbpD

CbpD is a secreted murein hydrolase, part of the competence regulon and a key component in fratricide [89,90]. During this process, competent pneumococci make use of CbpD to kill and lyse their non-competent siblings and closely related bacterial species such as *S. mitis* and *S. oralis* [79]. CbpD consists of an N-terminal CHAP domain, two central SH3b modules, and a C-terminal CBM formed by four CBRs. The CHAP domain belongs to a family of cysteine- and histidine-dependent amidohydrolases/peptidases, functioning as either an *N*-acetylmuramoyl-L-Ala amidase, which disrupts the *N*-acetylmuramyl-L-Ala bond, or an endopeptidase, which cleaves within the peptide moiety of peptidoglycan [91]. The SH3b modules specifically bind to the peptidoglycan portion of the cell wall [91], and the CBM interacts with the TAs of the pneumococcal cell wall, directing CbpD binding to the bacterial division zone [91].

In liquid cultures, lysis of non-competent cells is conducted by CbpD that acts in concert with two other murein hydrolases, LytA and LytC, which also play key roles in the fratricide mechanism in *S. pneumoniae* and are constitutively synthesized by non-competent cells [80,90]. Target cells are first attacked by CbpD, which binds and digests the peptidoglycan at their septal regions. This causes cell-wall damage that activates LytA and LytC, which results in a more extensive lysis of non-competent cells than is achieved by CbpD on its own [80]. In this process, activation of LytC by CbpD has been proposed to occur by the action of the peptidase against the peptide cross-linkers of the peptidoglycan molecule, whose rupture would provide a more accessible substrate for the autolysin, thereby triggering its hydrolytic ability [33]. Competent pneumococci protect themselves against CbpD by producing ComM, an integral membrane protein that neutralizes the action of CbpD by a yet-unknown mechanism [92]. CbpD and its protective counterpart ComM have been proposed to constitute a predatory mechanism directed against related strains and species that have evolved to increase the ability of competent pneumococci to acquire homologous DNA [79,81]. Indeed, it has been shown that competent pneumococci expressing CbpD acquire transforming DNA from their non-competent biofilm neighbors much more efficiently than competent pneumococci lacking a functional *cbpD* gene [81].

CbpG

CbpG is a putative serine protease with adhesin function involved in virulence in both mucosal colonization and sepsis [24,93]. CbpG is built by an N-terminal domain exhibiting 47% sequence identity with trypsin-like serine proteases, and by a CBM [4,24] that very likely contains three CBRs. A truncated CbpG form lacking the CBM has also been identified in clinical strains of *S. pneumoniae*, in which case the protein cannot be retained in the cell surface and is delivered to the medium [93]. CbpG

protease activity has been found in cell extracts from the pneumococcal TIGR4 strain, which were able to proteolyze casein and fibronectin. Heterologous expression of CbpG conferred protease activity against casein and fibronectin in *Lactobacillus casei*, and against casein in 293T eukaryotic cells, supporting its proposed catalytic activity [93]. In addition, CbpG has been shown to participate in adherence to several types of epithelial cells via its CBM, as well as in colonization and invasion using mouse models infected intratracheally and intravenously [93]. Interestingly, CbpG-vaccinated mice have been shown to have protection against colonization and systemic infection [93].

CBPs IN BACTERIOPHAGES

Bacteriophages, or phages, have played a central role in the shaping of natural populations of bacteria. In recent years, phages have again come to prominence due to the emergence of antibiotic resistance [94]. The importance of phages is increasingly evident from their role in the dynamics underlying the coevolution with bacteria and the subsequent implications for virulence.

Pneumococcal phages were first isolated in 1975 from throat swab samples, and since then have been identified from various sources and locations. Most of these phages are temperate, that is, integrated in the chromosome as prophages; in fact, it is now clear that more than half of all clinical pneumococcal isolates are lysogenic [95]. Only a few pneumococcal phages belonging to the Dp-1 and Cp families have been reported to be lytic phages to date.

Two different types of lytic enzymes (called endolysins) have been found in pneumococcal phages: All the reported temperate phages (e.g., HB-3, MM1, EJ-1, or VO1) as well as the lytic phages Dp-1 and ω-1 harbor amidases, whereas phages belonging to the Cp family encode lysozymes. All the bacterial and phage murein hydrolases, with the exception of Cpl-7, are CBPs and, consequently, share homologous CBMs located at the C-terminal region of the enzymes [54].

It is noteworthy that all typical pneumococcal isolates reported to date, as well as their prophages, contain the 957 bp *lytA* alleles (including the termination codon) coding for a 318-residue-long amidase that share a high degree of similarity, whereas the atypical pneumococci contained *lytA* alleles very different from those of typical strains. A characteristic signature of atypical *lytA* alleles (951 bp) is the presence of a 6 bp deletion (ACAGGC) located in the sixth CBR of the wild-type LytA amidase. It has been demonstrated that this two-residue deletion was responsible for the inhibitory effect of deoxycholate on the enzymatic activity of the lytic amidases from atypical pneumococci. Two temperate bacteriophages of *S. mitis*, B6 and HER, also encode a $LytA_{Spn}$-like endolysin of 318 residues, whereas the EJ − 1 inducible prophage isolated from *S. mitis* 101/87 harbors a gene (*ejl*) with the characteristic deletion of *lytA* alleles belonging to *Streptococcus* Mitis Group (SMG). Interestingly, the Pal amidase (amidase_5), coded by phage Dp-1, is a natural chimeric enzyme of intergeneric origin and is homologous to the corresponding murein hydrolase coded by the *S. mitis* phage SM1 [96].

Modular Arrangement of Endolysins

All known pneumococcal endolysins display a bimodular structure, formed by an N-terminal catalytic module with either amidase or lysozyme activities, and a C-terminal cell-wall anchoring module [54,97]. In all cases, with the exception of Cpl-7, the cell-wall binding module consists of a CBM that confers on these proteins the ability to specifically bind

the pneumococcal cell wall in a choline-dependent manner [54,97]. Examples of reported phage lytic amidases are Pal (from Dp-1 bacteriophage), Ejl (from EJ-1 bacteriophage), Hbl (from HB-3 bacteriophage), Mml (from MM1 bacteriophage), and LytA-VOl (from VO1 bacteriophage) [54]. With the exception of Pal, all these endolysins exhibit very high levels of sequence identity with full-length pneumococcal LytA (85.8−90.9%). Despite the high similarity in sequence and secondary structure, the minor differences seem to significantly affect Ejl properties compared to LytA [98]. In the case of Pal, the greatest similarity with LytA is restricted to the C-terminal region, exhibiting 64.6% identity with the CBM of the autolysin, while the N-terminal catalytic domain resembles the murein hydrolase produced by lactococcal phage BK5-T, with 41.9% sequence identity [99]. Biophysical studies of Pal have shown that the catalytic and cell-wall anchoring modules are two interdependent cooperative regions, but exhibit strong interactions between them [100]. Despite the high level of similarity between C-Pal and C-LytA polypeptides, the properties of the CBM of Pal bear a much stronger resemblance to those reported for Cpl-1 endolysin, with which it exhibits 68% sequence identity. Pal has been reported to have 2−3 choline-binding sites (Cpl-1 has 3 sites) and an IC_{50} by choline of 2 mM, which is identical to the one reported for Cpl-1 and 12 times lower than that reported for LytA [53,100].

In the group of endolysins with lysozyme activity including Cpl-1, Cpl-7, and Cpl-9 (from bacteriophages Cp-1, Cpl-7, and Cp-9, respectively [19,97]), all of them belong to the Cp family. They all have an N-terminal catalytic module belonging to the glycosyl hydrolase family 25, whose sequence is highly conserved in these three enzymes [97]. Cpl-1 and Cpl-9 are almost identical proteins (96.8% full-length sequence identity), and hence their structures are expected to be the same. However, Cpl-7 has a totally different cell-wall binding module consisting of three tandem repeats of 48 residues, which allows the endolysin to bind the cell wall in a choline-independent manner [97].

Three-Dimensional Structure of Phage CBPs

Up to now, only the 3D structures of the complete Cpl-1 lysozyme [82] and that of the CBM of a prophage LytA amidase [57] have been reported. The structure of modular Cpl-1 (Figure 11.4A) consists of an N-terminal catalytic module (residues 1−188) and a C-terminal CBM (residues 200−339), joined by an anionic linker (residues 189−199) [82]. The catalytic module consists of a flattened ellipsoid that folds into an irregular $((\beta/\alpha)_5\beta_3)$ barrel. The CBM is built up by six CBRs (p1−p6). The first four repeats (p1−p4) are folded in a superhelical arrangement providing two canonical choline-binding sites. An additional choline-binding site is located at the top of the CBM, and is formed by three aromatic residues (Trp-210, Phe-218, and Tyr-238). The other two repeats (p5−p6) and the C-terminus fold as an anti-parallel-like six-stranded β-sheet. The relative position of the catalytic module and the CBMs is restrained both by the interactions of the catalytic module with the end of the CBM, and by the tightness of the linker. Small-angle X-ray scattering (SAXS) studies in solution reveal the existence of a certain flexibility between the catalytic module and the CBM, which would facilitate the endolysin attachment to the cell wall and the subsequent recognition and hydrolysis of the substrate [101].

The substrate binding site of Cpl-1 consists of a long cleft located at the C-terminus of the catalytic barrel. Crystallographic and molecular dynamic studies have shown that this cleft

can accommodate a peptidoglycan substrate of five saccharide units going from position −2 to position +3 [102]. Among residues lining active site, Tyr127 plays a determinant role in modulating substrate binding. In the absence of ligand, Tyr127 blocks the entrance to the active site, moving out upon the ligand binding and allowing access to the substrate [102]. Based on these findings, a model of how Cpl-1 recognizes the murein molecule was proposed, suggesting that Cpl-1 digests its substrate by a processive mechanism [102].

The structure of the CBM of a phage LytA-like protein [53] (Figure 11.4B) is larger than the truncated forms of the CBM of pneumococcal LytA, which were previously reported. However, as expected from the 93% sequence identity and 97% similarity between endogenous and prophage CBMs, the overall structure of this phage C-LytA was similar to that of endogenous LytA CBM, and also displays a similar dimeric arrangement (Figure 11.4). As mentioned above, the *lytA* gene is present in all pneumococcal clinical isolates and their temperate phages analyzed to date. This means that it forms part of the core genome, and the LytA protein must fulfill some essential physiological role. Nevertheless, *lytA* genes are absent in many SMG species and their prophages, which suggests an accessory role for LytA in these bacteria.

Biomedical Implications of Endolysins: Enzybiotics

The increasing number of antibiotic-resistant bacterial strains and newly emerging viruses has prompted a new exploration of alternate modes of therapy for infectious diseases. Phages and their gene products offer promising new therapeutic alternatives. Among several advantages over antibiotics, a useful aspect of phage therapy in fighting bacterial infections is the minimal perturbation to the human microbiome.

Phage endolysins act by breaking up the cell wall from the inside, but can also lyse from outside when reaching the corresponding host peptidoglycan, in a species- or genus-specific manner [103]. Endolysins are also called enzybiotics, a hybrid word from *enzyme* and *antibiotic*. Recently, the concept of enzybiotics has been suggested to be extended and refer to all the enzymes, regardless of their origin, that exhibit antibacterial and/or antifungal activity (see the website http://www.phibiotics.org/index.php?nav=home). Endolysins have been shown to be very effective at killing Gram positive bacteria through very tight binding to their cell-wall substrates [104].

Various animal models have been set up with pneumococci, *Streptococcus pyogenes*, or group B streptococci, and the effectiveness of endolysins against a variety of Gram positive pathogens has been demonstrated [105]. Focusing on pneumococci, the successfully proven enzymes (Cpl-1, Pal, and also the bacterial LytA) contained the CBM that allowed a rapid and specific recognition of choline-containing substrate. Interestingly, Cpl-7 lysozyme constitutes the only exception to this rule, since its cell-wall binding module is built by three identical repeats unrelated to the CBM of CBPs [106]. Recently, it has been reported that an improved variant of this lysozyme, Cpl-7S, displays important novelties in the field: (1) broad specificity, as its enzymatic activity is not restricted to pneumococci and efficiently kills other Gram positive bacteria such as *S. pyogenes*; (2) bactericidal effect, under appropriate conditions, against Gram negative bacteria; (3) validated results on a relatively new animal model, zebrafish embryos, where mortality rates after treatment with *S. pneumoniae* or *S. pyogenes* were fully reversed by a single dose of Cpl-7S; (4) tailor-made protein engineering to suggest a generalized method to enhance the lethal activity of current and future enzybiotics [107].

Overall, enzybiotics have great potential as effective antibacterials, and may have several applications in the veterinary and food sectors, in plant protection against phytopathogens, in diagnostics, or in treatment of human pathogen infections.

CONCLUSIONS AND PERSPECTIVES

CBP is a very important group of surface-exposed proteins that are directly linked to the pneumococcal family and associated phages. Information reported up to now indicates that CBPs play essential roles in virulence, colonization, and also in host–pathogen interactions. Structural characterization of some its members reveal that modular arrangement is critical in understanding the physiological functions of these proteins. The structural plasticity of the CBRs allows a plethora of slight modifications in each CBP, providing specific features concerning aspects such as localization on the surface, choline-binding pattern, and specific catalytic activities, among others. It is expected that structural information on the remaining surface proteins will allow deciphering of the interactions orchestrating the union of different proteins and the assembly of modular proteins. Considering the relevant role that these proteins play in the infective process and also as new vaccine candidates and bactericidal agents (enzybiotics), this information will provide invaluable help in tackling pneumococcal infections.

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CHAPTER

12

Non-Adhesive Surface Proteins of *Streptococcus pneumoniae*

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INTRODUCTION

Surface proteins of *Streptococcus pneumoniae* (the surfeome) play an important role in the interaction with the human host, as these are the primary points of contact with the host cells and the immune system. Many well-studied pneumococcal surface proteins will be discussed in other chapters. Here we will focus on a subset of surface proteins of pneumococcus, namely those with non-adhesive, mostly unknown function.

IDENTIFICATION OF SURFACE PROTEINS

Identification of surface-expressed proteins of *S. pneumoniae* has long relied on classical biochemical methods and/or immunolabeling. With the advent of the genomics era, bioinformatic approaches have been introduced, usually relying on the identification of specific recognition sites in the predicted amino acid sequence of a given protein. More recently, proteomics-based approaches have been utilized for various microorganisms including the pneumococcus. Increasingly in these types of experiments, surface proteins are identified by liquid chromatography–mass spectrometry following "surface-shaving" of live cells using proteases [1,2] or, alternatively, by characterization of membrane-derived extracellular vesicles [3].

TYPES OF SURFACE PROTEINS

Pneumococcal cell-surface proteins can be classified into different groups based on their

Streptococcus pneumoniae.
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mode of attachment to the cell. These include: choline-binding proteins (CBPs), lipoproteins, LPxTG proteins, the pneumococcal histidine triad (Pht) family of proteins, and non-classical surface proteins.

Choline-Binding Proteins

One of the features on the pneumococcal cell surface is phosphorylcholine (ChoP), which is a constituent of both cell-wall-associated teichoic acids and membrane-associated lipoteichoic acids [4]. CBPs bind noncovalently to ChoP through choline-binding domains located at the C-terminus of the proteins. *S. pneumoniae* encodes 10–15 CBPs, depending on the pneumococcal strain. The structure and function of CBPs are covered in Chapter 11.

Lipoproteins

Lipoproteins are synthesized as precursors with N-terminal signal peptides containing a characteristic recognition sequence for lipid modification at the C-terminal end. This so-called lipobox motif directs the covalent attachment to a lipid component of the bacterial cell membrane of the N-terminal Cys residue of the mature protein, anchoring the protein in the plasma membrane beneath the capsule and cell wall.

LPxTG-Anchored Proteins

This group of proteins is covalently linked to the peptidoglycan backbone of the bacterial cell wall by a sortase transpeptidase that recognizes the carboxy (C)-terminal amino-acid sequence LPxTG, where x denotes any amino acid. The pneumococcal genome contains at least 18 LPxTG-anchored proteins and several sortases, some of which are encoded close to their targets on the genome (e.g., the pili-encoding cluster) [5].

Pht Family Proteins

The recently recognized Pht family consists of four members: PhtA (91.5 kDa), PhtB (92.1 kDa), PhtD (93.5 kDa), and PhtE (114.6 kDa). All members share extensive peptide sequence identity and contain five (PhtA, PhtB, PhtD) or six (PhtE) characteristic histidine-triad motifs (HxxHxH), in addition to α-helical coiled-coil regions, a proline-rich region, and two repeats of 61 amino acids [6]. They contain a type II signal sequence for secretion but are not lipoproteins nor attached to the membrane, nor do they contain choline-binding domains or LPxTG motifs. A three-amino-acid region (Q27-H28-R29) was found to be important in attachment of PhtD to the surface, as deletion of this region resulted in loss of attachment of the protein to the cell surface. However, these residues are not fully conserved among the different Pht proteins, suggesting either that the attachment signal is protein specific or, more likely, that amino acids with similar physiochemical properties are sufficient for secretion [7]. During growth of the bacterium, Pht proteins are released in the medium, but whether this is a result of proteolytic cleavage or noncovalent bonding remains unclear.

Non-Classical Surface Proteins

There are several proteins that do not possess the classical features of bacterial surface proteins (i.e., signal peptides and anchor motifs). They are intracellular proteins, normally functioning within the cell, that are relocated to the surface via unknown methods and play a role in interaction with the host. These proteins, also called "moonlighting proteins" [8], constitute a new class of virulence factors [9]. Whether surface exposure is the result of active transport or whether they are derived from lysed adjacent cells remains unclear. Several of these proteins have been identified by classical and proteomics-based approaches, but their identification by bioinformatics tools is difficult as no properties can

be assigned to them that predict their localization, although they all appear to be highly expressed proteins.

ROLES OF SURFACE PROTEINS

Below, we will discuss a selection of surface proteins in more detail, focusing on those whose predicted function in pneumococcal pathogenesis is not directly related to cellular adhesion (Table 12.1).

Lipoproteins

Lipoproteins are an important class of membrane-bound protein that typically represent approximately 2% of the bacterial proteome. They have diverse functions such as adhesion, substrate transport, protein folding, and bacterial fitness. Depending on the strain, there are 42–47 lipoproteins found in *S. pneumoniae* [8,10–12]. In the TIGR4 genome, among the 46 predicted lipoproteins, 26 are components of ABC transporters, which are covered in Chapter 10. In addition to these ABC components, several other lipoproteins are predicted to play a role in nutrient acquisition and transformation, processes essential for pneumococcal fitness, among which the *N*-acetylmannosamine-6-phosphate 2-epimerases NanE and NanE2, encoded by SP1330 and SP1685, respectively [12]. Six lipoproteins are involved in processes of protein folding or activation of cell-surface molecules: two SpoIIIJ family proteins (OxaA1/2), which are required for the insertion of integral membrane

TABLE 12.1 Non-Adhesive Surface Proteins of *S. pneumoniae* Described in This Chapter

TIGR4 locus[a]	Protein	Description
LIPOPROTEINS		
SP0191		Hypothetical protein
SP0198		Putative D-stereospecific aminopeptidase
SP0468	StrD	Sortase
SP0629		Hypothetical protein
SP0659	Etrx1	Thioredoxin family protein
SP0771	SlrA	Streptococcal lipoprotein rotamase A
SP0859		Hypothetical protein
SP0899		Hypothetical protein
SP0981	PpmA	Putative proteinase maturation protein A
SP1000	Etrx2	Thioredoxin family protein
SP1330	NanE	*N*-Acetylmannosamine-6-phosphate 2-epimerase
SP1685	NanE2	*N*-Acetylmannosamine-6-phosphate 2-epimerase
SP1916		PAP2 family protein
SP1945		Hypothetical protein
SP1975	OxaA2	SpoIIIJ family protein
SP2041	OxaA1	SpoIIIJ family protein

(*Continued*)

TABLE 12.1 (Continued)

TIGR4 locus[a]	Protein	Description
LPXTG-ANCHORED PROTEINS		
SP0057	StrH	Beta-*N*-acetylhexosaminidase
SP0071	ZmpC	Zinc metalloprotease
SP0082	PavB	Pneumococcal adherence and virulence factor B
SP0268	SpuA	Alkaline amylopullulanase
SP0314	Hyl	Hyaluronate lyase
SP0368	Eng	Endo-alpha-*N*-acetylgalactosaminidase
SP0498	EndoD	Endo-beta-*N*-acetylglucosaminidase
SP0641	PrtA	Serine protease
SP0648	BgaA	Beta-galactosidase
SP0664	ZmpB	Zinc metalloprotease
SP1154	Iga/ZmpA	IgA1 protease
SP1693	NanA	Neuraminidase
SP1833	PfbA	Plasmin- and fibronectin-binding protein A
SP1955		Hypothetical protein
spr0075	PfbB	Plasmin- and fibronectin-binding protein B
Pht FAMILY PROTEINS		
SP1003	PhtD	Pneumococcal histidine triad protein D
SP1004	PhtE	Pneumococcal histidine triad protein E
SP1174	PhtB	Pneumococcal histidine triad protein B
SP1175	PhtA	Pneumococcal histidine triad protein A
NON-CLASSICAL SURFACE PROTEINS		
SP0499	PGK	Phosphoglycerate kinase
SP0966	PavA	Pneumococcal adherence and virulence factor A
SP1128	EnoA	Enolase
SP1489	Tuf	Elongation factor Tu
SP1572	PppA	Pneumococcal protective protein A
SP1954	SFP	Subtilisin family protein serine protease
SP2012	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
SP2239	HtrA	Serine protease

[a] *If no ortholog is present in TIGR4 genome, R6 locus is given.*

proteins into the membrane [13]; the sortase StrD, involved in the formation of the pilus [14]; and the two peptidyl-prolyl isomerases (PPIases) SlrA and PpmA [15]. Thus far, functional PPIase activity has only been demonstrated for SlrA, homologous to members of the cyclophilin family. Both SlrA and PpmA have been shown to contribute to pneumococcal colonization and adherence, albeit in a strain-specific manner [15,16]. Furthermore, pneumococci lacking *slrA*, but not *ppmA*, were found to be significantly reduced in virulence in a mouse otitis media model, while deletion of both had an additive effect on attenuation [17]. Two surface-exposed thioredoxin family proteins, Etrx1 and Etrx2, together with their methionine sulfoxide reductase *Sp*MsrAB2, reduce methionine sulfoxides (MetSO) that are formed when methionine residues are oxidized through reactive oxygen species. Etrx1 and Etrx2 compensate each other, and a double mutant caused accumulation of oxidized SpMsrAB2 *in vivo*. Mutants were more susceptible to bacterial uptake by macrophages and to killing by H_2O_2 or free MetSO [18]. The roles of the remaining lipoproteins are as yet uncharacterized.

LPxTG-Anchored Proteins

In *S. pneumoniae* TIGR4, this class of cell-surface attached proteins consists of seven carbohydrate-active enzymes, four proteases, one mucin-specific adhesin, two adhesins with fibronectin-binding ability, one protein of unidentified function, three pilus-associated proteins (see Chapter 17 for discussion of pili function), and the large serine-rich repeat protein PsrP, which is a lung cell adhesin [19,20].

Hyaluronate Lyase

Hyaluronate lyase (Hyl, also called SpnHL) is part of a broad group of enzymes called hyaluronidases that depolymerize hyaluronic acid, an important component of the host extracellular matrix (ECM) and connective tissue; it is produced by virtually all pneumococcal strains [21]. Full-length Hyl is 107 kDa, and the enzyme is anchored to the cell surface as well as released by *S. pneumoniae* to surrounding host tissues during infection to facilitate bacterial invasion. No significant impact on virulence was found in a mouse intraperitoneal infection model using a single *hyl* mutant; however, when a double mutant including *ply* was tested, virulence was significantly decreased [22]. The exact role of Hyl in pneumococcal pathogenesis has yet to be clarified; however, it is known that *S. pneumoniae* can use hyaluronic acid, either from the host or from other bacterial species, as a carbon source [23].

ECM-Binding Proteins

Two fibronection-binding proteins were identified using bioinformatic analysis of LPxTG anchor-encoding proteins in the sequenced genomes of TIGR4 and R6: PavB and PfbA. PavB, an adhesin protein, named using the same nomenclature as for PavA (see below), has four SSURE (streptococcal surface repeat) domains. Over-expressed in *Escherichia coli*, the protein was shown to bind fibronectin, specifically with the third SSURE repeat, but not collagen or submaxillary mucin [24,25]. Its role in virulence was later determined by Jensch et al. in a mouse pneumonia model, where it was shown that a *pavB*-mutant was outcompeted by the wild type and showed delayed transmigration to the lung [26].

Plasmin- and fibronectin-binding protein A (PfbA) was found to be expressed on the pneumococcal cell surface, and a *pfbA* mutant displayed attenuated adherence to lung and laryngeal epithelial cells, and was also more prone to be phagocytosed. The over-expressed protein was able to bind to fibronectin, plasmin, plasminogen, and human serum albumin [27]. Surface localization was confirmed using immunofluorescence microscopy. Structural analysis suggests that the fibronectin-binding region is

located at a short, disordered region at amino acids 571–607, while plasminogen binding is probably mediated by surface-exposed lysine residues in the region from amino acid 150 to 607 [28,29]. Another plasminogen- and fibonectin-binding protein has been identified in R6, designated PfbB, which has a significant role in mediating pneumococcal adhesion to respiratory epithelial cells [30]. No ortholog of PfbB is present in the TIGR4 genome. It is thought that pneumococcal binding to the ECM and hyaluronate degradation may play a role in pneumococcal disease progression by enabling access to the vascular endothelium that composes the alveoli–capillary barrier [31].

Carbohydrate-Active Enzymes

To survive in the human host, *S. pneumoniae* has developed a rich repertoire of surface proteins to liberate carbohydrates from its environment. Not only do these surface proteins function in nutrient uptake, they also play a role in virulence through immune evasion or adherence to the epithelial cell layer, and they may also function in biofilm formation [32]. Their appearance in high-throughput animal model screen studies to identify proteins associated with virulence and pathogenicity comes as no surprise, as they are essential for survival within the host [33–35]. Surface-exposed exoglycosidases such as StrH (*N*-acetylglucosaminidase), BgaA (β-galactosidase), and NanA (neuraminidase) have been found to be involved in deglycosylating N-linked glycans that are available in the host, acting in a sequential manner [36,37], thereby enabling the pneumococcus to grow on these host-derived carbohydrates. Their deglycosylating activities have also been suggested to be involved in escaping phagocytic killing by neutrophils. The proposed mechanism of action, deglycosylation of serum glycoconjugates, reduces the deposition of complement component C3 on the bacterial surface, thereby limiting phagocytic killing [38].

Two neuraminidases, encoded by *nanA* and *nanB*, have been described for *S. pneumonia* [39,40]. Virtually all clinical isolates examined to date produce an enzyme with neuraminidase activity. A *nanB* homolog, *nanC*, has also been identified in the pneumococcal genome [12], but its expression and activity have not been described to date. Of the three neuraminidases, NanA (~108 kDA) is the only one with a C-terminus containing the LPxTG anchoring motif. Loss of NanA has been shown to impair pneumococcal persistence in the nasopharynx and middle ear in a chinchilla infection model [41]. Furthermore, a *nanA* deletion mutant was cleared from the nasopharynx, trachea, and lungs within 12 h post-infection, while the *nanB* mutant persisted but never increased in either the nasopharynx, trachea, or lungs [42].

Other surface-exposed carbohydrate-active enzymes include SpuA (pullulanase A), EndoD (endo-β-1,4-*N*-acetylglucosamidase), and Eng (endo-α-*N*-acetylgalactosaminidase). SpuA is required for growth of *S. pneumoniae* on host glycogen by hydrolyzing the alpha 1,6 bonds in the glycogen branch points [43], and is a factor required for virulence of the pneumococcus, specifically for survival in the lungs or in the blood. Interestingly, an effect on colonization was not observed [34,43,44]. EndoD is responsible for cleaving the chitobiose core of N-linked glycans, and it has been suggested to play a role in virulence based on its role in other pathogens [45]. The O-glycosidase Eng is responsible for cleaving sialylated core-1 O-linked glycans in concert with NanA to provide nutrients to the host but perhaps also to expose glycosylated receptors for adherence. A mutant of Eng showed a reduced ability to adhere to human epithelial cells, and was much less able to colonize the nasopharynx in a mouse model. These findings suggest that the functionality of Eng is important for colonization [46].

Surface-Exposed Proteases

Serine Proteases

Surface-exposed serine proteases such as HtrA (high temperature requirement A protein) and PrtA (cell wall–associated serine protease A) have been identified in many bacteria and play a known role in virulence [47–54]. Recently, the role in virulence of another surface-exposed serine protease, subtilase family protein (SFP), was elucidated [55]. HtrA plays a role in quality control of surface exported proteins; additionally, it plays a role in competence [51,56,57] by degrading CSP, the competence-stimulating peptide [56]. HtrA also controls the activity of the bacteriocin pneumocin by disruption of the peptide processing and secretion machinery, and not by degradation of the peptide pheromone BlpC [49,52]. During cell division, HtrA was found to be predominantly located at the equators and septa of dividing pneumococcal cells, suggesting that it plays a general role in cell division by quality control of proteins exported by the Sec translocase in *S. pneumoniae* [54]. Virulence of an *htrA* mutant was drastically reduced in animal models of pneumonia and bacteremia [51,55], which is not surprising as loss of HtrA will result in a fitness defect. Mutants of PrtA were attenuated in intraperitoneal infection [47], while in a pneumonia model a high-dose infection with D39Δ*prtA* induced significantly less lung inflammation without influencing bacterial loads [55], suggesting that PrtA plays a role in immunomodulation. A similar phenotype was observed with an SFP mutant, where colony-forming unit (CFU) count and inflammation were significantly lower in the lungs after a low-dose infection [55].

Zinc Metalloproteases

S. pneumoniae possesses two to four zinc metalloproteinases, depending on the strain. Best-characterized of these is IgA1 protease (also designated ZmpA), which cleaves human IgA1 [58], including secretory IgA1, in the hinge region, thus interfering with the functions of IgA antibodies by eliminating the Fc-mediating effector function. IgA1 protease has a molecular mass of approximately 215 kDa and contains a signal peptide, an LPxTG motif for anchoring, and a zinc-binding motif [59,60]. Interestingly, Weiser et al. showed that human IgA1 antibodies enhanced bacterial attachment to respiratory epithelial cells in culture, but only when cleaved by IgA1 protease [61]. Due to the specificity of this zinc metalloprotease for human IgA1, direct demonstration of the role of this protease in bacterial survival *in vivo* has proven difficult. Recently, however, Janoff et al. provided the first *in vivo* evidence that IgA1 protease can inhibit IgA-dependent killing of *S. pneumoniae*, using novel IgA1 and IgA2 human monoclonal antibodies (hMAbs) specific for the pneumococcal capsule [62]. They showed that IgA1 protease–resistant IgA2, but not IgA1 protease–sensitive IgA1, supported survival of mice infected with wild-type pneumococci, and that IgA1 hMAbs protected mice against an IgA1-protease mutant but not against the wild type [62]. The second zinc metalloprotease identified is ZmpB. A *zmpB* knockout was shown to be attenuated in virulence in a murine infection model of pneumonia, where CFU counts of the mutant were approximately 1000-fold lower in blood than the wild type; however, no differences were observed in the airways and lung tissue. In an intravenous challenge model, CFU counts were 33-fold less for the mutant compared to the wild type. No difference was observed in complement-deficient mice, suggesting that ZmpB does not play a role in complement-mediated killing; however, presence of ZmpB was associated with increased production of tumor necrosis factor alpha, a pro-inflammatory cytokine. Pro-inflammatory cytokines could potentially induce expression of pneumococcal receptor proteins of the host, thereby increasing adhesion [63]. Furthermore, ZmpB was recently shown to interact with

collagen IV, as were the LPxTG-anchored proteins PrtA, NanA, and spr1806 [64]. The third zinc metalloprotease, incorrectly annotated in the *S. pneumoniae* TIGR4 genome as an IgA protease, is ZmpC. Several biological activities for ZmpC have been described: cleavage of human matrix metalloproteinase 9 (MMP-9) [65], inhibition of neutrophil extravasation during pneumococcal pneumonia by degradation of the N-terminal domain of P-selectin glycoprotein 1 [66], and induction of ectodomain shedding of the membrane-associated mucin MUC16 [67]. Furthermore, ZmpC-induced removal of MUC16 from the epithelium led to loss of the glycocalyx barrier function and enhanced internalization of the bacterium [67].

The *zmpC* gene was identified in 21% of the isolates in a collection of 542 invasive pneumococcal strains collected over a 10-year period in the Netherlands [68]. In the study by Cremers et al., patients who smoked were more often infected with a pneumococcus carrying *zmpC*, and rates of sepsis and ICU admission were higher in patients infected with *zmpC*-positive strains; however, whether ZmpC is associated with a more severe course of disease, or is associated with smoking, remains to be elucidated. In an analysis of 218 invasive isolates, Oggioni and coworkers looked at the prevalence of these zinc metalloproteases. ZmpB- and IgA1 protease–encoding genes were present in all the strains; however, *zmpC* was found in only 18% of the isolates. The gene encoding ZmpD was present in 49% of the isolates examined. A co-occurrence of *zmpC* with *zmpD* was noted [69].

Pht Family Proteins

Ogunniyi et al. showed that Pht proteins are required for inhibiting complement deposition on the pneumococcal surface by recruiting complement regulator factor H [70]. In a sepsis and pneumonia model of infection, avirulence was observed when mutagenesis of all four genes was used relative to the wild type, suggesting significant functional redundancy among the Pht proteins [70]. PhtD, PhtE, PhtB, and PhtA are present in 100%, 97%, 81%, and 62% of the strains, respectively, and analysis of 107 pneumococcal strains showed little variability in sequence conservation for PhtD [71]. Recently, Pht proteins were found to contribute to adherence in a strain- and serotype-specific manner [72] and to play a role in maintaining zinc homeostasis [71]. Expression of the Pht genes is induced by zinc, and they are under the control of AdcR [70,73]. Each of the Pht proteins is capable of delivering zinc to the *adcAII*-encoded zinc importer, but for optimal fitness all four are required; the reason for this redundancy is not known [74].

Non-Classical Surface Proteins

The serine proteases HtrA and SFP, described above, lack known surface-attachment motifs, and could therefore also be classified as non-classical surface proteins. The most well-known proteins included in this class are the adhesin PavA and the glycolytic enzymes enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

PavA

PavA, named after its role in *p*neumococcal *a*dhesion and *v*irulence, is a fibronectin-binding protein, similar to the fibronectin-binding protein FBP54 of *S. pyogenes* [75]. In addition, in its role in binding fibronectin mediating adherence to epithelial cells, it also plays a role in protecting pneumococci against recognition and actin cytoskeleton-dependent phagocytosis by dendritic cells [76], modulating inflammation [77], and maintaining colonization and development of sepsis [78]. Although its role in adherence could not restored by exogenously added PavA when using a *pavA* knockout strain, the influence on phagocytosis and cytokine induction

was restored when a *pavA* knockout strain was reassociated with exogenously provided PavA protein [77].

Glycolytic Enzymes

Enolase (Eno), an anchorless surface protein, is a plasminogen-binding protein [79] facilitating transmigration of pneumococci through the basement membrane. It was also found that this 47 kDa protein exhibits α-enolase activity and is necessary for viability, as it may be an essential enzyme of the glycolytic pathway [79]. By use of immunoelectron microscopy, Eno was detected not only in the cytoplasm, but also on the surface of encapsulated and unencapsulated pneumococci [80]. In particular, it is the α-enolase located on the surface that is responsible for the recruitment of proteolytic activity [81]. Recruitment of plasminogen to the pneumococcal surface has broad functional benefits, contributing to pneumococcal adherence and murine nasopharyngeal colonization [80,82].

Similar to Eno, GAPDH is a plasmin(ogen)-binding protein located in the cytoplasm as well as on the surface [83]. However, GAPDH exhibits a significantly lower affinity for plasminogen, but has a high affinity for plasmin [83]. Recently, GAPDH was found to bind both hemoglobin and heme, suggesting an additional role for this protein in iron acquisition by *S. pneumoniae* [84]. A third protein in this class is phosphoglycerate kinase (PGK), a widespread two-domain enzyme that is a major component of the glycolytic pathway, and has been shown to be surface-exposed in a number of streptococcal species [85–87].

Other Non-Classical Surface Proteins

Elongation factor Tu (Tuf) plays a major role in translation and has been found to be located in the cytoplasm as well as on the surface. Recently, Tuf was shown to also bind factor H, factor H-like protein 1, complement factor H-related protein 1, and also plasminogen. It is likely to be involved in complement escape, and possibly also in ECM degradation [88]. Another non-classical surface protein, PppA, was identified using ion-exchange chromatography and SDS-page analysis of PBS washes of intact pneumococci, followed by N-terminal amino acid sequencing of the major bands detected on gel. It was named after its role as a vaccinate candidate, pneumococcal protective protein A, and is most likely a non-heme iron-containing ferritin protein [89]. It has frequently been used as a vaccine candidate for oral vaccination in various compositions [90].

PROTEOMIC DETECTION OF SURFACE PROTEINS

In general, surface proteins of *S. pneumoniae* can be easily identified from their sequence: For example, the presence of LPxTG motifs, CBP motifs, membrane-spanning domains, and signal sequences are all telltale signs of surface localization. These markers obviously do not function for moonlighting proteins that have an intracellular role but are also found on the surface. To detect these non-classical surface proteins, high-throughput proteomics approaches can be used whereby proteinaceous structures on the surface of the pneumococcus are cleaved off using a protease treatment, purified, and analyzed by LC/MS analysis [1,2]. In total, 16 isolates were analyzed, resulting in a 254-protein pan-surface proteome of *S. pneumoniae*. This method identified the common surface proteins, but interestingly, several intracellular proteins were also found, such as the proteins described above, but also proteins such as chaperone ClpP, pyruvate kinase PykF, and trigger factor tig. ClpP has been found to be transported to the cell wall after heat shock [91], but the role of others has not been defined.

Recently, a remarkable observation was made on the production of membrane vesicles from the plasma membrane by live pneumococci [3]. Although Gram negative outer membrane

vesicles are well known for their role in immunomodulation and virulence ([92] and references therein), the role of these plasma membrane vesicles in pneumococcal pathogenesis is unknown. Proteomic analysis of these vesicles revealed that, like their Gram negative counterparts, they are enriched in lipoproteins and multi-transmembrane proteins, and they are immunogenic, protecting against infection in an animal model [3], although the mechanism behind the protective effect is currently unknown. Further studies are needed to investigate this phenomenon, as in other Gram positive pathogens they are thought to play an important role in pathogenesis ([3] and references therein).

CONCLUDING REMARKS

Pneumococcal surface proteins play critical roles in various aspects of pathogenesis, such as bacterial fitness, nutrient acquisition, and pathogen–host interaction (e.g., adhesion or immune evasion). Clearly, different surface proteins have complementary, but sometimes also redundant functions. Given their localization on the surface, such proteins are considered prime candidates for novel vaccination strategies and, our ever-expanding knowledge of the (three-dimensional) structure and function of these proteins may also improve future treatments or may help us assess the virulence potential of pneumococci currently circulating in the population.

A good example is the study by Cremers et al., wherein first steps are taken to couple the presence of ZmpC to clinical presentation of invasive pneumococcal disease. As programs are now being formed to incorporate whole genome sequencing (WGS) into molecular epidemiology surveillance programs and routine diagnostics, this knowledge can, for instance, be used to adjust treatment or type of patient care. Another application of WGS could be prediction of the effects of vaccination on the pneumococcal population and the inherent change in expression of surface-exposed virulence factors. As rapid replacement takes place by non-vaccine-type pneumococcal strains, the virulence potential of these new strains may be predicted, owing to our extensive knowledge of pneumococcal pathogenesis.

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CHAPTER

13

Biofilm Formation Under *In Vitro* Conditions

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Streptococcus pneumoniae is an extremely diverse species in terms of both its antigenic surface composition and the association of a given strain to either invasive or non-invasive disease. Despite its impact on global morbidity and mortality, *S. pneumoniae* is part of the normal commensal microflora of the human nasopharynx [1]. Although most colonized individuals are asymptomatic, carriers are the principal reservoir for transmission of *S. pneumoniae* in the community. In a small proportion of carriers, which nevertheless translates into globally significant numbers, *S. pneumoniae* invades from its nasopharyngeal beachhead to cause disease, for example, by aspiration into the lungs to cause pneumonia, by direct invasion of the blood, or by ascension of the eustachian tube to access the middle ear and cause otitis media (OM) [2]. The molecular basis for this variation in pathogenic phenotype is poorly understood. However, the ability to form a biofilm on host mucosal surfaces is increasingly being recognized as a critical event in the pathogenesis of pneumococcal disease [3]. The attempt to bridge observations in human samples to *in vitro* models was performed by correlating gene expression profiles and quorum sensing (QS) in experimental infection models in mice with biofilm growth in microtiter plates [4]. This work fostered many further investigations by providing a reference for the relevance of pneumococcal biofilm models in mimicking aspects of infection.

Biofilms, and in particular pneumococcal biofilms, have been covered by excellent reviews focusing on both *in vitro* aspects of pneumococcal biofilms [5,6] and the importance of pneumococcal biofilms during infection of the host [7–10]. The following paragraphs focus on some of the aspects investigated so far on pneumococcal *in vitro* biofilm models, and we attempt to summarize the main aspects.

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PNEUMOCOCCAL BIOFILM MODELS

Bacteria living in biofilms are phenotypically distinct from their planktonic counterparts, and so far much of our understanding of biofilm physiology and micro-ecology originates from experiments using *in vitro* biofilm models. Various *in vitro* models have been developed for pneumococcal biofilm growth. The first published biofilm model was a Sorbarod biofilm [11]. A Sorbarod consists of a paper sleeve containing compacted cellulose fibers placed in a silicone tube and connected via a plastic adaptor to a sterile glass tube. This simple model of biofilm was used to establish a continuous bacterial culture for over 12 h and to test its susceptibility to various antibiotics. This model system also allowed the finding that capsule-off mutations are selected in biofilms [12].

The continuous-flow reactor, established in 2004, is one of the most popular biofilm model systems. This "open" system, which allows continual replenishment of fresh nutrients, permits the formation of biofilm under a wide range of flow rates and nutrient conditions over extended periods of incubation [13,14]. This model is commonly used to study the development of mature biofilms and assess changes in the growth environment or specific genetic mutations to the structure of mature biofilms, detachment from biofilms, spatial and temporal gene expression, and, most importantly, the distribution of extracellular polymeric substances. Still, *in vitro* biofilms cannot be considered universally valid as an *in vitro* model for disease. The group of Carlos Orihuela clearly showed that using a continuous-flow-through line model, sessile bacteria were highly attenuated in experimental invasive disease models, possibly suggesting a better correlation to bacteria during colonization [15].

In addition to these systems, two static microtiter models were set up exploiting either high inocula in poor media [6,16] or low inocula in rich media [4,17] using plastic supports. This technique simplifies biofilm formation and quantification, allowing analyses of high numbers of laboratory samples. The use of plastic supports permits the study of early biofilm stages up to 24 h; however, if the growth medium is replenished daily, biofilms can also be maintained for up to 3–4 days. Moscoso et al. determined that the optimal conditions for biofilm formation of *S. pneumoniae* on abiotic surfaces were obtained when polyvinyl chloride plates were used [16]. In addition, they found that either a chemically defined (Cden or CDM) or semisynthetic (C) medium supported strong biofilm formation, whereas growth in a complex medium, such as CAT or Todd–Hewitt broth, resulted in weak biofilms. In C medium, the number of adherent cells reached a maximum after 8 h of incubation at 34°C. Oggioni et al. performed a biofilm time course experiment using the rich tryptic soy broth (TSB) media, where low-inoculum bacteria were grown on flat-bottom polystyrene tissue culture plates in the presence or absence of pneumococcal competence-stimulating peptide (CSP) at various concentrations (0–300 ng/mL). After the initial background attachment of a few cells upon inoculation, pneumococci attached quite abruptly to the solid support during the late exponential phase of growth. The stability of biofilm in this model was found to be dependent on a functional competence system, which, in response to exogenous CSP added to the medium, allowed for the formation of stable biofilms; this was also observed at 24 h [4]. CSP concentration for biofilm formation showed a narrow optimum condition in a range similar to that of inducing maximal competence in planktonic cells. The biofilm-competence dependence was not confirmed for microtiter models based on a more steady state of growth as continuous culture biofilm or the microtiter model in poor medium [18].

In vitro biofilm formation using microtiter plates was more recently evaluated at 6, 12, 18, and 24 h of incubation in TSB media in presence of glucose. The authors found that biofilm growth was also independent of exogenously added stimulating peptide and medium replacement. Instead, the best results were obtained after 18 h in the presence of 1% glucose. The authors proposed a correlation of the findings with the clinical biofilm disease common in hyperglycemic patients, where high concentrations of glucose in the blood can worsen systemic bacterial infection [19]. More solid are investigations that link distinct aspects of *in vitro* biofilms to disease and that describe lack of correlation to invasive disease potential [20,21].

Lately, Marks et al. emphasized the importance of using epithelial cells as a support for streptococcal biofilm formation, providing a better platform for bacteria to form a more mature and structured biofilm. Fixed tissue cultures are able to maintain the adhesive ability of the cells, and streptococcal biofilm formed on this substrate shares the same morphology and architecture with biofilm formed in the nasopharynx of infected mice. Moreover, biofilms formed on epithelial cells have the ability to initiate faster attachment than the glass support; after 6 h a specialized architecture matrix-like formation could be detected [22].

Selection of the appropriate *in vitro* biofilm model system depends not only on the preferences of the investigators and the resources available, but most importantly on the issues to be investigated. The primary advantage of the microtiter plates method is that its relatively high throughput capacity enables screens for mutants defective in attachment and evaluation of the effects of different treatments or compounds on attachment or biofilm formation. However, this method is less well suited to studies of biofilm structure or of antimicrobial resistance properties. On the other hand, flow cells are capable of creating a uniform and constant environment for *in vitro* purposes. Furthermore, the regulatory processes of biofilm elaboration are cyclical and dynamic, and the external stimuli, normally present in the host, trigger alterations in the expression of a subset of genes required for biofilm formation; thus, the current state of technology is still a distant representation of the dynamics of the host environment *in vivo*.

QS AND BIOFILM

Bacteria survive in their natural environment through the development of complex multicellular communities, not by operating as individual cells [23]. Communication between bacterial cells is an essential prerequisite for the successful development of these communities. This communication is achieved through secretion and detection of small chemical signaling molecules, a mechanism known as "QS" [24,25]. Different types of QS have been described that allow bacteria to sense distinct stimuli and respond with appropriate phenotypic changes. Two QS systems used by the pathogenic bacterium *S. pneumoniae* have been characterized so far. These include a peptide pheromone (CSP)-based system and, more recently, a system dependent on the metabolic enzyme LuxS, which synthesizes the autoinducer-2 (AI-2) signaling molecule. Both of these systems influence bacterial surface attachment (biofilm formation) and the inter- and intraspecies transfer of genetic material.

Pneumococcal competence is regulated by a ribosomally synthesized peptide pheromone called CSP, encoded by *comC* [26]. CSP is a polypeptide of 41 amino acids, which is synthesized as a pro-peptide and is subsequently exported and processed by a dedicated ABC transporter, ComAB. CSP secreted into the extracellular environment is sensed by a Two Component Signal Transduction System (TCSTS) composed of the ComD histidine kinase and its cognate response regulator, ComE. ComE is responsible for direct

transcriptional activation of its own operon (*comCDE*), of the genes responsible for CSP secretion (*comAB*), and of the alternative sigma factor ComX. ComX, in turn, is responsible for activating the expression of a second set of approximately 40 late competence genes, encoding diverse functions required for DNA uptake and recombination [27], as well as bacteriocins and cell-wall lysins capable of killing and releasing DNA from non-competent pneumococci (a phenomenon referred to as bacterial fratricide [28]).

The stability of pneumococcal biofilms is dependent on a functional competence system. It has been demonstrated that the addition of exogenous CSP stimulates the formation of stable biofilms after 24 h of *in vitro* growth [4,18,29–31]. The CSP concentration for biofilm formation showed a narrow optimal range, similar to that required for inducing maximal competence in planktonic cells. Quantitative gene expression analysis has shown comparable gene expression profiles for bacteria grown *in vitro* biofilms to those residing in the lungs of infected mice [4]. Furthermore, intranasal treatment with CSP increases the severity of pneumonia, a disease correlated with up-regulated pneumococcal competence gene expression, while intravenous treatment with CSP reduces the severity of sepsis, a disease correlated with low expression of competence genes and in which the pneumococcus grows in a planktonic state [4]. This landmark paper demonstrated that biofilm-grown bacteria preferentially induced pneumonia and meningitis, while planktonic cells more efficiently induced sepsis. Furthermore, the effect of CSP on pneumococcal growth behavior showed that signaling events profoundly influence the state of pneumococci in their environmental niche [4,18,29–31]. The importance of competence in biofilm formation was finally confirmed and clearly shown *in vivo* when Marks et al. demonstrated that genetic exchange and natural transformation occur in nasopharyngeal colonization, where bacteria are in a biofilm-like state, and *in vitro* biofilm on pre-fixed tissue culture. It is clear that biofilm growth creates an optimal environment for genetic exchange [22].

Finally, a QS system used by both Gram positive and Gram negative bacteria has been identified, which represents a "universal language." This system involves the synthesis of a molecule called AI-2. The metabolic enzyme LuxS (*S*-ribosyl-homocysteine lyase) synthesizes AI-2 as a by-product of the conversion of *S*-ribosyl-homocysteine into homocysteine, an integral reaction of the activated methyl cycle. Homologs of LuxS have been found in almost all bacterial species, suggesting a key role in interspecies communication. LuxS-dependent QS in pneumococci has received significantly less attention compared to the CSP-mediated system. The first study on pneumococcal luxS system, was conducted by Stroeher et al., who examined the effect of *luxS* mutagenesis on pneumococcal virulence [32]. The *luxS* deletion strain was less invasive than the parental strain, and showed multiple changes in protein expression patterns were observed. However the importance of the AI-2 molecule *in vivo* could not be confirmed since the mutant was unable to respond to exogenous AI-2 released by the wild type strain. Another study analyzed the transcriptional profile of a *luxS* deletion strain, observing pronounced effects on transcription in the early log phase of growth, revealing a function for LuxS in gene regulation that is not dependent upon high cell density [33]. Recently, two independent groups have investigated the impact of LuxS-mediated AI-2 QS, and the effect of exogenous iron (Fe(III)), on biofilm formation by *S. pneumoniae* [31,34,35]. Supplementation of pneumococci with Fe(III) was found to strongly enhance biofilm formation *in vitro*, while chelation of residual Fe(III) in the growth medium was inhibitory. Importantly, Fe(III) supplementation was found to result in up-regulated expression of *luxS* in pneumococci. A *luxS*-deletion mutant failed to form a biofilm, even with Fe(III) supplementation, whereas a derivative over-expressing *luxS*

exhibited enhanced biofilm formation capacity, and could form a robust biofilm without added Fe(III). Release of extracellular DNA, an important component of the pneumococcal biofilm matrix [16], was also found to be directly related to *luxS* expression. Similarly, genetic competence, as measured by transformation frequency and level of expression of competence genes and the murein hydrolase *cbpD* (associated with fratricide-dependent DNA release), were all directly related to *luxS* expression levels. Moreover, mutagenesis of *cbpD* blocked biofilm formation. Moreover the importance of fratricide in biofilm formation was recently demonstrated by Wei and Havarstein [36]. They determined that the fratricide mechanism enhances the efficiency of gene exchange between adherent pneumococci, and that *cbpD*, one of the major fratricins expressed on competent pneumococci, acquired transforming DNA from their noncompetent biofilm neighbors much more efficiently than pneumococci lacking a functional *cbpD* gene. They found that transformability is not only related to the fratricide mechanism, but is strongly affected by the stage of biofilm formation. In the early stage of biofilm formation (4 h), the cells become competent when treated with CSP, and only a small fraction of the cells were transformed in 8 h-old biofilm [36]. Together, these studies have demonstrated that competence, fratricide, and biofilm formation are closely linked in pneumococci, and that LuxS-mediated QS is central to the regulation of these processes.

Vidal et al. investigated a link between these two QS systems in the formation of pneumococcal biofilm [34]. Using the abiotic surfaces, immobilized human respiratory epithelial cells (HRECs), and the bioreactor system, they found that *S. pneumoniae* biofilm was regulated by the two QS systems. However, while LuxS has been shown to be able to regulate biofilm formation in both the bioreactor and HREC, the competence system was unable to regulate biofilm formation in the first 8 h of incubation on abiotic surfaces. To further investigate whether QS regulated biofilm autolysis in the bioreactor system, *comC* and *LuxS* mutants were tested at 24 and 48 h post-inoculum. The biofilm formation of *luxS* and *ComC* was found to be significantly different in the two models: At 48 h post-inoculums, wild-type strains were still viable; however, in both mutants lysis occurred at the earlier time point of 24 h. Thus, this paper established for the first time the critical importance of *luxS* and competence QS in *S. pneumoniae* biofilm formed on tissue cultures [34].

One striking feature is that in almost all organisms, QS systems have been associated with biofilm formation. The cell–cell signaling systems have therefore become one of the principal objects of study in biofilm research and one of the most studied targets for species–specific biofilm inhibition. An important drawback of cell–cell signaling as a drug target is the fact that it may represent a double-edged sword, as shown in animal models for pneumococci and staphylococci, where treatment is observed to be beneficial to one disease condition but shows undesirable side effects in another [4,37].

BIOFILM SPECIFIC GENE AND PROTEIN EXPRESSION

Biofilm formation is essential in *S. pneumoniae* pathogenesis; it protects the pathogen against antimicrobials or host immune responses. Bacteria living in biofilms are phenotypically distinct from their planktonic counterparts, and thus the antigen profile available for host recognition is altered in biofilm, with implications for adaptive immunity.

The main focus in the study of pneumococcal biofilms has been on the bacterial mechanisms by which *S. pneumoniae* switches from planktonic to sessile life. However, recently, Sanchez et al. explored the various host responses to sessile and/or planktonic pneumococci [38]. They showed that a dramatic change occurred

between planktonic and mature biofilms at the protein level; most of the proteins in biofilm exhibited diminished production compared to planktonic cells, and, most importantly, these growth-phase-dependent changes were able to alter the immunoreactivity of pneumococci. Sanchez et al. examined the antigenic profiles of biofilm and planktonic pneumococcal cell lysates by testing their reactivity with convalescent sera from humans who had confirmed pneumococcal pneumonia and sera from mice immunized with killed *S. pneumoniae* biofilm pneumococci. A strong reaction occurred in the planktonic cell lysates when in contact with convalescent sera, whereas an opposite effect was found in sessile cells. On the other hand, sera obtained from biofilm-immunized mice strongly recognized proteins in the biofilm cell lysates. This indicated lack of an appropriate immune response against pneumococcal invasive disease (or biofilm), even in the presence of prior bacterial colonization [38].

The distinct phenotypes observed in this work were also found due to differential expression patterns of genes between sessile and planktonic cells. Microarray analysis performed on early biofilms and planktonic cells showed the exclusive expression of genes of certain functional categories such as isoprenoid biosynthesis, cell-wall biosynthesis, and purine and pyrimidine nucleotide metabolic pathways in sessile cells, and the expression of transcription regulator genes in planktonic cells [39].

It is now clear that this natural disposition of bacteria within the biofilm is a function of a complex and coordinated consortium. The genetic and molecular diversity within the bacteria in the biofilm community and their planktonic counterpart make these heterogeneous populations especially difficult to study. So despite the great number of scientific works on multiple aspects of pneumococcal biofilm, the events leading to biofilm formation, maintenance and dissolution are not or only sparingly described and further analyses are needed.

THE PNEUMOCOCCAL BIOFILM MATRIX

Sessile pneumococcal communities are thought, like other bacterial sessile communities, to produce specific polymeric extracellular compounds, which make up the matrix of these structures. This has so far been investigated in clinical samples and *in vitro* biofilm by microscopy and a few other in-depth analyses (Figure 13.1). Unfortunately, data on the extracellular matrix of the most frequently laboratory-grown sessile communities—bacterial colonies on agar plates—is completely missing. One of the first pneumococcal biofilm papers investigated in detail (by microscopy) the extracellular matrix of pneumococcal biofilm grown in a continuous culture device [14]. Microscopic detection with fluorescently labeled lectins showed reactivity of wheat germ agglutinin with the extracellular

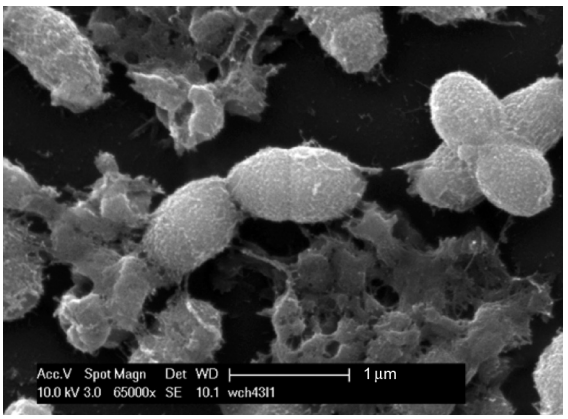

FIGURE 13.1 Scanning electron microscopy of early pneumococcal biofilm showing intact cells and thick extracellular matrix.

polymeric matrix of the biofilm, indicating that it contained the amino sugar *N*-acetylglucosamine, also abundant in bacterial cell walls [14].

In a very elegant work, Domenech et al. confirmed the presence of extracellular polysaccharide different from the capsule by staining the biofilms with Calcofluor dye and four types of lectins conjugated to Alexa fluorophores as well as incubation with glycoside hydrolases. Only polysaccharides from biofilms, not planktonic cells, bound to the Calcofluor dye. The presence of Glc*p*(1−4) and GlcNAc(1−4) in its deacylated form was further confirmed by GC-MS techniques [40]. They therefore concluded that biofilm strain R6 synthesizes an exopolysaccharide containing residues of glucose beta (1→4) and *N*-acetylglucosamine beta (1→4), with possible glucose/galactose alpha (1→6) branches. The group of Luanne Hall-Stoodley published a series of papers on the microscopic examination of pneumococcal biofilms [3,41,42]. They confirmed the presence of carbohydrates in the extracellular matrix, but without defining the exact nature of those carbohydrates, as they used a lectin cocktail. They were also able to show, in accordance with others, the down-regulation of the capsule operon, but interestingly and specifically they also showed that capsule immune-staining of cells in biofilm was brightest in towers compared to adherent cells [41]. Moscoso et al. [16] first identified the importance of DNA as a component of the extracellular matrix. Other groups confirmed this finding, both by microscopic staining of DNA and by confirming that DNAse treatment was able to destructure the biofilms [41]. Still, at least to some extent, these studies do not satisfy the search for a component which would be specific to the extracellular matrix of pneumococcal sessile cultures. It is thus unresolved at the present stage whether such a component exists or if the extracellular matrix is composed exclusively of DNA and other sugars containing *N*-acetylglucosamine. Our personal opinion is that further investigation is needed to resolve the chemical nature of the extracellular matrix of sessile pneumococcal communities.

RESISTANCE TO AND IN BIOFILMS

One of the main challenges posed by biofilms is related to the difficulty of their removal and the killing of bacteria in these structures. There are three distinct principal features that make biofilms more resistant: low or absent growth of part of the biofilm community; the three-dimensional aspects of biofilms, which can pose challenges to diffusion of bioactive compounds; and the specific nature of the extracellular polymeric matrix, which contributes to protection of bacterial cells.

This latter aspect has been recently addressed by Hakansson et al. [43], who analyzed survival of planktonic and biofilm-derived pneumococcal cells in the environment. Their data clearly showed that planktonic cells of two pneumococcal strains survived significantly less well, both on plastic and on the skin of hands than did biofilm-derived cells [43]. This highlights aspects of clear medical relevance, as pneumococcal transmission in children is highly likely to occur by several paths, including saliva contamination of toys and objects. Interestingly, the work included a demonstration that the pneumococci contaminating fomites not only remained viable, but also were able to remain infectious in an experimental mouse carriage model [43]. This work shows very clearly how the matrix of pneumococcal biofilms is able to protect bacteria from desiccation and how this can directly impact on transmission of disease.

More difficult is to address antimicrobial drug resistance. There are two main points in antimicrobial susceptibility testing which are nearly always completely misunderstood by experimental workers investigating biofilm and resistance. The first is that it is not the

concentration of the drug able to kill a bacterium that defines whether the strain is resistant or susceptible; rather, it is whether this concentration can be reached within the human host at the site of infection during therapeutic use of the drug. The most critical aspect thus lies in the correlation of the data obtained to clinical relevance. This is exemplified by the great effort put into the definition of clinical breakpoints that define whether a given susceptibility profile confers resistance or susceptibility. The second aspect is that standard protocols for antimicrobial susceptibility testing, defined by regulatory authorities such as CLSI in the United States or EUCAST in Europe, are designed to test the susceptibility of bacteria, not the efficacy of antibiotics. Many papers on biofilms incur this error as they perform efficacy tests of antibiotics on biofilms, without defining standard parameters and protocols. In the context of biofilm resistance testing it is furthermore overlooked that (normal) bacterial colonies on agar are by definition sessile biofilm communities on a solid–air interface and that disc diffusion is an internationally accepted test under these conditions. What differentiates standardized Kirby–Bauer tests from the "biofilm literature" is that in the former test the inhibition of growth of biofilms, while the latter test the killing of biofilms. This discrepancy between standard testing and the "biofilm literature" does not add to clarity in the field.

Given these premises, few papers meet the criteria to provide qualified insight into antimicrobial susceptibility of pneumococcal biofilms. One of these is the early work on cellulose filter–grown biofilms of an uncharacterized pneumococcal clinical isolate and the investigation into susceptibility to β-lactams [11]. This investigation found no significant difference between susceptibility values tested by conventional broth microdilution assays (MIC and MBC) and Etest on planktonic pneumococci compared to the biofilm-eradicating concentration calculated on the biofilm or the MBC tested on biofilm-effluent bacteria. The authors concluded that in their particular experimental setup, "biofilms confer no protection against the activities of the antibiotics tested on this particular strain of *S. pneumoniae*" [11]. Del Prado et al. evaluated amoxicillin, erythromycin, and levofloxacin for their impact on pre-grown biofilms in microtiter plates. In contrast to the work on cellulose filters, in this model system only a weak or no reduction of the counts of biofilm-associated bacteria could be observed [44].

Recently the group of Van Bambeke published a pharmacodynamics investigation into the activity of clinically relevant antibiotics on pneumococcal biofilms, importantly taking into account both of cell viability and biomass and using a very wide range of drug concentrations [45]. On 2-day-old biofilms, they observe a reduction of viability ranging from 30% to 80%, while on 11-day-old biofilms reduction did not exceed 10–60%, depending on the drugs tested. The observed reduction of biomass was even lower, ranging from 50% on young biofilms to 20% or less on old biofilms [45]. An important lesson from this work is that differences among antibiotic classes are not major in their efficacy in reducing the viability and mass of pneumococcal biofilms. Interestingly, this work compiled a table with the activities of the antibiotics on biofilms at concentrations found in epithelial lining fluid with the specific aim of providing indications for the clinical relevance of the pharmacodynamic data obtained [45]. In follow-up work, the same group, working on a large strain collection, found that there was no correlation between biofilm formation and intrinsic susceptibility or expression of resistance determinants in pneumococci [46].

In summary, the impact of biofilm formation appears to have potentially an important impact on transmission of pneumococci, as resistance to desiccation and survival on fomites is increased. On the other hand,

various models of *in vitro* biofilms show different levels of reduction of antimicrobial drug susceptibility. Despite attempts to correlate *in vitro* biofilm models and infections [4,15], we have as yet no clear indication of which *in vitro* model mimics best what extent what actually happens in the host in the different aspects of pneumococcal disease. More effort must be put into finding a clear correlation between *in vitro* data and clinical relevance [47]. Taking this into account, data appear to indicate that, as for other bacterial infections with a biofilm-like component, pneumococcus may be more difficult to treat, but encouragingly no major differences were observed between drug classes, allowing the clinician to choose the appropriate drug based on standard susceptibility criteria.

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CHAPTER

14

Pneumolysin

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HISTORY

Pneumococci were first reported to produce a hemolysin in 1905 [1]. Since this report, numerous studies have been carried out using crude preparations of the toxin [2–6] that demonstrated that the protein is toxic to animals, susceptible to oxidation (a process that could be reversed by treatment with thiol-reducing agents), antigenic, and reversibly inactivated by cholesterol. The sensitivity of the toxin to oxidation and reactivation with thiol-reducing agents led to pneumolysin being termed a "thiol-activated toxin." The family of thiol-activated toxins [7] are produced by four genera of Gram positive bacteria. In the light of more recent findings regarding the structure and function of these proteins (see below), they have been renamed the cholesterol-binding or cholesterol-dependent cytolysins (CDCs) [8]. More recently, the production of CDCs by Gram negative bacteria has been demonstrated [9].

Purification of pneumolysin to homogeneity was reported in the early 1970s [10,11]. The availability of purified protein allowed the biological effects of the toxin on a range of cell types to be determined. Some of the early studies showed that the toxin had biological effects on polymorphonuclear leukocytes that were elicited at concentrations of the toxin that did not cause cell lysis [12]. Bacterial cell fractionation experiments demonstrated that the toxin was present in the bacterial cytoplasm but is not secreted [13], a finding that has important implications for understanding the role of the toxin in disease processes. The toxin is thought to be released only when the bacteria undergo lysis, for example, due to autolysis, spontaneous bacterial cell death, degradation in the phagolysosome, or antibiotic therapy. More recently, it has been shown that pneumolysin may be attached to the pneumococcal cell wall, but a mechanism of active export has not been identified [14,15].

Pneumolysin purified from the pneumococcus is a protective immunogen against pneumococcal disease in mouse models of pneumonia [16]. This important study led to many other studies to develop protein-based vaccines to prevent pneumococcal disease in humans. The pneumolysin

Streptococcus pneumoniae.
DOI: http://dx.doi.org/10.1016/B978-0-12-410530-0.00014-4

gene (*ply*) was first cloned in 1986 [17] and sequenced in 1987 [18]. Analysis of the gene sequence predicted that pneumolysin consists of a single 53 kDa polypeptide chain. Pneumolysin is produced by virtually all clinical isolates of the pneumococcus [19]. Analysis of the *ply* gene sequence from 121 clinical isolates identified 14 protein alleles [20], some of which are associated with lack of hemolytic activity by the toxin. Nonhemolytic alleles affect the ability of pneumococci to activate the inflammasome (discussed elsewhere in this chapter), and are associated with serotypes known to cause disease outbreaks (serotypes 1 and 8) [20]. Some clinical strains have insertions in the *ply* gene of either a section of duplicated sequence or the transposon IS1515 [20,21], suggesting that pneumolysin is not absolutely essential for the pneumococcus to be able to cause infection.

As pneumolysin hemolytic activity is abolished by oxidation and restored by thiol reduction, it was thought for many years that the activation process involved reduction of an internal intramolecular disulfide bond [7]. Pneumolysin was the first member of the "thiol-activated" toxins to be sequenced; analysis of the primary amino acid sequence showed the presence of only a single cysteine residue toward the C-terminal end of the protein (amino acid position 428 out of 471) in a region of 11 amino acids that is conserved among many of this family of toxins [22]. This conserved sequence (ECTGLAWEWWR) plays an important role in the hemolytic activity of the toxin, as determined by site-directed mutagenesis experiments. However, Saunders et al. showed in 1989 that the cysteine residue is not essential for activity and can be replaced by alanine with no effect on the lytic activity of the toxin [23]. This led to the suggested change in the nomenclature for this group of toxins from thiol-activated toxins to cholesterol-binding or CDC.

Although pneumolysin was shown to be a protective immunogen in mouse models of infection in 1993 [16], it was only after 20 years that the first stage of using Ply as a vaccine in humans was achieved with the testing of a pneumolysin genetic toxoid in phase I clinical trials in humans in 2013 [24].

STRUCTURE AND FUNCTION OF PNEUMOLYSIN

Pneumolysin is a single polypeptide chain composed of 471 amino acids [18]. No three-dimensional structure is available for pneumolysin as all attempts to crystallize the protein have so far been unsuccessful. However, crystal structures are available for other CDCs, and it has been possible to model the structure of pneumolysin based on that of perfringolysin, with which it shares 60% sequence similarity [25]. The homology model shows that Ply is long and rod-shaped, with overall dimensions of 11 nm × 5 nm × 3 nm, and consists of four distinct domains (Figure 14.1). Structural

FIGURE 14.1 **Model of the structure of pneumolysin.** The protein has four domains. Domain 1 is shown in blue, domain 2 in gray, domain 3 in red, and domain 4 in green. The conserved undecapeptide sequence is highlighted in pink.

domains 1–3 (D1–3) form the N-terminal part of the molecule, while domain 4 (residues 360–469) forms the C-terminal domain. The polypeptide backbone weaves in and out of domains 1–3 (domain1 [residues 6–21, 58–147, 198–243, 319–342], domain 2 [residues 22–57, 343–359], and domain 3 [residues 148–197, 244–318]), making these three domains physically inseparable and distinct from domain 4.

Membrane binding of the intact toxin is mediated through domain 4, and isolated domain 4 is capable of binding to membranes and competing with the full-length toxin [26]. Domain 4 also contains the undecapeptide sequence that contains the single cysteine residue present in the toxin. Once pneumolysin has bound to the cell membrane it can have several effects depending on the concentration of the protein. At relatively high concentrations, the toxin forms pores in the cell membrane, leading to lysis of the cell. The accepted dogma for this process is monomeric toxin binding to the cell that can assemble into a prepore structure consisting of 30–50 monomers. Three-dimensional studies of pneumolysin by cryo-electron microscopy show that the monomers then undergo a major conformational change that allows membrane insertion and pore formation [27,28]. In addition to forming large pores, pneumolysin forms smaller lesions that can consist of incomplete oligomers or "arc" structures [29].

Perturbation of membrane integrity has biological effects, ranging from induction of apoptosis and cell death to more subtle changes in cell signaling. The toxin can also have biological effects at sublytic concentrations, and even isolated domain 4 of the protein can elicit effects on human cells [30]. At sublytic concentrations Ply has several effects on eukaryotic cell signaling. Pneumolysin induces early membrane depolarization, and micropores form in the plasma membrane. These micropores lead to calcium influx and activation of rac and rho GTPases, including rac-1 and the rho-associated kinase [31]. These changes cause cytoskeletal rearrangement in the affected cell, and changes in cell morphology have been attributed to Ply-induced changes to the actin cytoskeleton. For example, rearrangement of the actin cytoskeleton and microtubule stabilization (as indicated by increased acetylation and bundling) can be observed in astrocytes [32,33]. Although these effects were seen in the absence of observable pores, pore-forming ability was required, as nonlytic mutants did not induce these cytoskeletal changes. Cholesterol was also required for microtubule stabilization as these effects were abolished in cholesterol-depleted cells or when using cholesterol-treated Ply. Recently, the mechanism of Ply-dependent cytoskeletal remodeling has been further elucidated, as it has been shown that Ply has the ability to directly bind actin *in vitro*. While domains 1, 2, and 3 and full-length Ply bind strongly to actin, the nonlytic deletion mutant has reduced binding activity, suggesting that the refolding and/or conformational changes that occur in domains 1 to 3 during pore formation are required for actin binding [34].

ROLE OF PNEUMOLYSIN IN PATHOGENESIS

The cytotoxic and hemolytic effects of pneumolysin have been well characterized through over a 100 years of rigorous investigation and research [5,35,36]. However, it wasn't until the 1980s, when James Paton et al. performed immunization studies with a partially inactivated pneumolysin toxoid, that the importance of pneumolysin to virulence and pathogenesis was demonstrated [16]. This paper described evidence of moderate protection against invasive pneumococcal disease in toxoid-immunized mice. A number of pneumolysoid toxins have since been developed, and have shown promise as pneumococcal vaccine candidates, offering protection against

both pneumococcal carriage [37] and invasive disease [38,39]. These studies have highlighted the immunogenic nature of pneumolysin, and many of the most interesting effects of the toxin and its interactions with host cells that have since been elucidated occur at sublytic concentrations or even in the absence of hemolytic activity. In recent years, the importance of such effects in the context of pathogenesis has become apparent, particularly with regard to interactions with cells of the immune system.

The first defining studies that singled out pneumolysin as a key pneumococcal virulence factor were performed with Ply-deficient bacteria on a serotype 2 (D39) background. This strain, designated PLN-A, was found to be approximately 100-fold attenuated in virulence in intranasal infection models in mice [40], and also significantly impaired in virulence when administered intravenously [41]. Furthermore, in a lobar pneumonia model utilizing an intratracheal inoculation route, PLN-A was approximately 10-fold less virulent that its wild-type parent, demonstrating a significantly decreased ability to grow in the lung and to penetrate the alveolar epithelial cell barrier [42]. The subtle differences in virulence attenuation in different infection models likely results from the different inoculation routes used, and thus points to an ability of pneumolysin to affect immune responses and disease outcomes at different sites within the host.

INFLAMMATION AND INNATE IMMUNE RECOGNITION OF PNEUMOLYSIN

The pro-inflammatory effects of pneumolysin are well documented, and stimulation of TNF, IL-1, IL-8, and IL-6 production has been demonstrated in a range of host cells including dendritic cells, macrophages, epithelial cells, and endothelial cells [43–47]. This process is thought to contribute both to generation of anti-pneumococcal immunity and to enhanced pathology. For example, pneumolysin induces nitric oxide, a key component of antimicrobial immunity but also a significant contributor to tissue pathology. Production by macrophages occurs via an IFNγ-dependent pathway that involves up-regulation of *cox-2* gene expression [43,48].

Pneumolysin-induced production of CXCL8 and IL-6 in cells of the upper airways has been documented by several groups, and MAPK, NFκB, and TLR-4 signaling pathways have been implicated [49]. Intriguingly, the degree of induction of cytokine release by pneumolysin appears to be limited by the polysaccharide capsule [50]. In a murine model of nasopharyngeal carriage, suppression of the CXCL8 homologs CXCL2 and MIP-2 by capsule was observed, but this was only discernible in the absence of pneumolysin. Thus, capsule may play a role in limiting the pro-inflammatory effects of pneumolysin *in vivo*. The mechanisms of recognition of pneumococci by the innate immune system remains to be comprehensively elucidated, but several pattern recognition receptors including Toll-like receptors 2 and 4 have been implicated [44,51].

Toll-Like Receptors

TLR4 has been implicated in mediating inflammatory responses to pneumolysin. This link was initially made by Malley et al., who showed that pneumolysin-induced production of IL-6 and TNF-α by macrophages required expression of TLR4 and the adaptor protein MyD88, and that TLR4-deficient mice had enhanced susceptibility to fatal infection following pneumococcal colonization [51]. The mechanistic explanation for this observation appeared to be that pneumolysin binding to TLR4 enhances host-mediated apoptosis, which aids in bacterial clearance. Pneumolysin has been shown to physically interact with

TLR4 in a solid-phase binding assay, and this could mediate signaling in macrophages and epithelial cells [52]. The interaction of pneumolysin with TLR4 is thought to stimulate caspase-1 activation and the production of associated cytokines, including IL-1α, IL-1β, and IL-18 [53]. Pneumolysin-induced caspase-1 activation has subsequently been shown to be dependent on the AIM2 and NOD-like receptor family, pyrin domain−containing 3 (NLRP3) inflammasomes [54].

S. pneumoniae expressing a mutated pneumolysin protein lacking both hemolytic and complement-activating activity is more virulent than pneumococcus in which the pneumolysin gene was deleted [55]. The additional pneumolysin-driven function that gives rise to this effect may be TLR-4 mediated, as a pneumococcal mutant lacking hemolytic activity has previously been shown to activate TLR-4-dependent responses [51]. However, in contrast to the above data, several groups have found no or only minimal effects of pneumococci on TLR4 signaling [44,56−58], while others have suggested that both TLR-2 and TLR-4 might play a role [45,59].

The role, therefore, of TLR-4 in pneumolysin interactions with innate immune signaling pathways remains contentious, not least due to the difficulty of producing purified toxin free of bacterial lipopolysaccharide (LPS), a major TLR4 agonist and, thus, potential confounder. LPS concentrations as low as 10 pg/mL (or 0.1 endotoxin units EU/mL) can induce dendritic cell cytokine expression, including IL-6 and TNF-α, and in combination with toxin this level could be as low as 5 pg/mL and still elicit an effect [60]. Therefore, to prevent artificial and misleading conclusions about the effects of pneumolysin on TLR4 would require almost complete elimination of LPS in the purified toxin preparations. In experiments with highly purified LPS free pneumolysin, no role for TLR4 was observed in toxin-induced maturation of dendritic cells or in induction of cytokine production [44]. However endotoxin-free pneumolysin alone was still capable of inducing co-stimulatory molecule expression on dendritic cells and enhanced TLR-agonist-induced cytokine secretion, including IL-12, IL-23, IL-6, IL-1β, IL-1α, and TNF-α. The synergistic effect of pneumolysin on TLR-agonist-induced cytokine responses was entirely TLR-4 independent. These data suggest pneumolysin-dependent cytokine stimulation may occur through a mechanism(s) separate from TLR4 stimulation.

Inflammasome

An important observation was that pneumolysin-induced enhancement of IL-1β production required NLRP3, highlighting the potential role of the toxin as an activator of the inflammasome. This appears to be a key step in the development of anti-pneumococcal immunity, as NLRP3-deficient mice fail to control bacterial numbers during pneumococcal pneumonia. Inflammasome signaling is also important for the promotion of IL-17A responses, which have been implicated in resistance to both pneumococcal carriage [61] and pneumonia [62], and pneumolysin-deficient pneumococci induced significantly less IL-17A (and IFN-γ) production in mice than wild-type bacteria. Further support for a key role for NLRP3 in anti-pneumococcal immunity comes from the observation that pneumococcal serotypes that are associated with increased invasiveness, and that produce pneumolysin with reduced hemolytic activity, often fail to induce inflammasome activation [58,63]. These include serotypes 1, 7F, and 8, all of which have been associated with high degrees of bacterial invasiveness and poor clinical outcomes [64,65].

Inflammasome activation is clearly associated with robust immune responses and improved control of bacterial numbers, but a potential consequence of activation of any inflammatory pathway is bystander tissue

damage. In murine models of pneumococcal meningitis, activation of the inflammasome adaptor molecule ASC and sensor NLRP3 is associated with increased pathology and clinical disease score [66]. Inhibition of this pathway by use of inhibitors of IL-1β, IL-18, or cathepsin B led to an improved clinical course and decreased brain inflammation. Furthermore, pathology was significantly reduced during infection with pneumolysin-deficient bacteria incapable of inflammasome activation [66] or in mice deficient in ASC or NLRP3 [67]. In meningitis patients, cerebrospinal fluid levels of IL-1β and IL-18 were correlated with complications and unfavorable outcome [67], and persistence of pneumolysin in patients is associated with mortality [68]. These findings raise hope that inflammasome pathways might offer a target for adjunctive therapy in meningitis.

The inflammasome also influences outcomes in pneumococcal lung infection. In a pneumococcal pneumonia model, ASC-deficient mice displayed increased bacterial dissemination and lethality compared to NLRP3-deficient mice, despite significantly attenuated cytokine responses in both mouse strains [69]. The mechanistic explanation for this observation may be the greater degree of attenuation of IL-17, GM-CSF, and integrin-αM responses coupled to reduced adaptive immune response signaling in the ASC-deficient mice [69].

A study by Littmann et al. has demonstrated that, in contrast to the pneumolysin effects on inflammasome activation discussed above, pneumolysin can also inhibit the induction of inflammatory pathways in human dendritic cells [70]. Using serotype 4 (TIGR4) pneumococci with and without *ply* deletion, the authors demonstrated that pneumolysin enhances pneumococcal uptake by dendritic cells by 50%. However, the enhanced uptake was associated with increased dendritic cell caspase-dependent apoptosis, and this process was pneumolysin dependent. Furthermore, *ply* expression was associated with down-regulation of the dendritic cell cytokine response, decreased inflammasome activation, and decreased expression of dendritic cell co-stimulatory molecules. These results are in apparent contradiction to the growing body of evidence of a role for pneumolysin in inflammasome activation, and the authors argue that the findings reflect differences between human and murine signaling pathways. However, a number of studies have reported similar responses to pneumolysin in murine and human cells [44,58,66]. The reasons for the divergent observation of the effects of pneumolysin on inflammasome activity remain unclear.

Other Host Cell Signaling Pathways

Pneumolysin can activate immune cells in an entirely TLR- and inflammasome-independent manner. One described mechanism requires the pore-forming properties of the toxin, whereby sublytic concentrations of pneumolysin activate p38 mitogen-activated protein kinase (p38-MAPK) due to osmotic stress [46]. Cells protected from osmotic stress by use of high molecular weight dextran do not activate p38-MAPK in response to pneumolysin, suggesting that osmosensing is a general mechanism for activating immune responses [46]. Macrophages exposed to sublytic concentrations of toxin also show activation of phosphoinositide-3-kinase, and this is required for recruitment of inflammatory macrophages to the site of infection, perhaps due to stimulation of IL-8 release from epithelial cells [46]. Levels of IL-8 release increase substantially when the response to pneumolysin synergizes with that to other respiratory pathogens or commensals. In one well-characterized example, pore formation by pneumolysin allows peptidoglycan from *Haemophilus influenzae* to enter epithelial cells and stimulate immunity by binding to the

cytoplasmic pattern-recognition receptor Nod1 [46]. In addition, pneumolysin can affect lipid mediators of inflammation through activation of phospholipase A in pulmonary epithelium. This is thought to contribute to pathology through degradation of membrane phospholipids, which in turn damage lung tissue via the release of free fatty acids and lysophosphatides [71]. Furthermore, release of arachidonic acid promotes neutrophil chemotaxis and respiratory burst [72,73]. Indeed, transendothelial migration of neutrophils in response to pneumococci has been shown to be pneumolysin-dependent. Pneumolysin induces increased neutrophil superoxide [74], leukotriene B4 and prostaglandin E2 production [75,76], and increased synthesis and release of matrix metalloproteinases 8 and 9 [77].

CONSEQUENCES OF THE EFFECTS OF PNEUMOLYSIN ON INFLAMMATION

The ability of pneumolysin to induce expression of a range of pro-inflammatory molecules might be assumed to be detrimental to pathogen survival. However, many of the inflammatory responses induced by pneumolysin are thought to be inappropriate, either in magnitude or in direction, and thus fail to assist resolution of infection. For example, pneumolysin reduces killing of pneumococci by neutrophils *in vitro* [78] and aids bacterial dissemination *in vivo* by breaking epithelial tight junctions and increasing alveolar permeability, hastening the development of bacteremia [42,79,80]. A major complication of pneumococcal pneumonia is pulmonary permeability edema, characterized by endothelial hyper-permeability, and can even occur several days after bacteria have been cleared from the lungs. Hyper-permeability is associated with the presence of pneumolysin [81], which increases permeability by reducing stable and dynamic microtubule content and by modulating expression of VE-cadherin [82]. Increases in endothelium permeability are associated with activation of protein-kinases, alteration of the RhoA/Rac 1 balance, and increased phosphorylation of myosin light chain. The level of arginase-1 is also increased by pneumolysin treatment. Inhibition of PKC reduces the activation of arginase by pneumolysin and also decreases toxin-induced capillary leak. Pneumolysin also reduces local systemic blood pressure in the lung by increasing vascular resistance, and this is associated with increased levels of platelet-activating factor (PAF) in the lung [83]. Inhibition of the PAF response by use of PAF-receptor knockout mice or pharmacological blockade of the PAF receptor reduced the pressure response. These observations identify potential targets for clinical intervention to prevent respiratory failure caused by pneumococcal pneumonia.

Pneumolysin is a potent inducer of host cell apoptosis, but the significance of this for disease outcomes and pathogenesis is not always straightforward to decipher. In the context of pneumococcal meningitis, it is well established that pneumolysin-induced apoptosis is associated with pathology and unfavorable outcomes [48,84], but in the case of pneumonia, apoptosis in the respiratory tract may actually be associated with resistance to severe disease [52,85]. Macrophage and epithelial cell apoptosis in the lung benefit the host by enhanced bacterial clearance in carriage and subclinical infection [52,85,86] and by down-regulation of the inflammatory response during clinical infection [87].

The attractive hypothesis that emerged from these observations is that pneumolysin-induced cytotoxicity represents a conserved host response linking detection of infection with apoptosis to remove infected cells. Pathogenesis results as pathogen-driven (pore formation and calcium influx) [46] and host-driven (pattern-recognition receptor−mediated detection of pneumolysin) [44,51] apoptosis

combine, resulting in high sensitivity to the induction of apoptotic pathways and perturbation of normal cellular homeostasis.

PNEUMOLYSIN AND COMPLEMENT

One of the first of the effects of pneumolysin on the immune system to be identified was the ability to activate the classical pathway of complement, even in the absence of pneumolysin-specific antibodies [88,89]. This ability is not dependent on either pore formation or binding of cholesterol [88], and it allows the pneumococcus to deplete host complement and induce inflammation that aids bacterial dissemination. Pneumolysin decreases opsonization of pneumococci, both *in vitro* and *in vivo* [90]. *In vivo*, this activity contributes both to pneumococcal persistence in the lung and to replication in the blood [42]. The importance of the complement-activating activity of pneumolysin is further underscored by the observation that complement is essential for pneumococcal clearance and that both the classical and alternative pathways are required [90]. The complicated relationship between pneumococci and complement pathways is discussed in detail elsewhere in this volume (Chapter 21).

PNEUMOLYSIN AND T CELL IMMUNITY

T cells are a key aspect of the anti-pneumococcal immune response, and T cell depletion reduces pneumococcal clearance in colonization and pneumonia models [57,91,92]. The link between pneumolysin and T cell immunity is still only partially understood, but the existing data show that pneumolysin is important for both T cell recruitment and T cell cytokine production [91,93,94]. T cells accumulate in the lungs during pneumococcal pneumonia in mice, peaking at the point at which pneumococcal proliferation ceases. This accumulation of T cells is significantly reduced and delayed in infections with pneumolysin-deficient pneumococci [92]. More recently, a role for pneumolysin in induction of memory T cell responses has been identified [30], with demonstration of proliferation of human CD4 + T cells isolated from blood or nasal-associated lymphoid tissue in response to stimulation with domain 4 of pneumolysin. Furthermore, a memory Th17 response was elicited in response to pneumolysin that was more marked in cells isolated from carriage-negative children than in carriage-positives, suggesting that pneumolysin might induce Th17 immunity that provides protection against future colonization. However, pneumolysin has separately been shown to inhibit lymphocyte proliferation and cytokine production at high concentrations [78]. Overall, it is likely that during infection, pneumolysin will have either a net activating or a net inhibitory effect on T cell responses and inflammatory responses in general, depending on its concentration. The range of effects on inflammation and immunity described in this chapter is summarized in Table 14.1.

PNEUMOLYSIN AND THE EQUILIBRIUM BETWEEN PATHOGEN AND HOST

It is clear, then, that pneumolysin is a key stimulator of host immunity as well as a major virulence factor for *S. pneumoniae*. This dichotomy is most apparent in the phenotype of serotype 3 pneumolysin mutants. A serotype 3 pneumococcal strain engineered to produce reduced levels of pneumolysin was attenuated in virulence due to altered immune stimulation and enhanced bacterial clearance [95]. The altered immune response during infection with this mutant was characterized by reduced

TABLE 14.1 Host Immune Responses to Pneumolysin

Cell or effector molecule	Effect	References
Macrophage	Cytokine production	[44]
	Nitric oxide production	[43]
	TLR4 activation	[51–53]
	MAPK and PI3K signaling	[46]
	Apoptosis	[52,85,86]
Dendritic cell	Cytokine production	[44]
	NLRP3 and ASC inflammasome activation	[44,54,58,66]
	Co-stimulatory molecule expression	[44]
	Apoptosis	[70]
Epithelial cell	Cytokine production	[45,46,49]
	Phospholipase A activation	[71]
	Apoptosis	[52,85,86]
	TLR4 activation	[49,52]
Endothelial cell	Cytokine production	[47]
	Hyper-permeability	[81,82]
Neutrophil	Chemotaxis	[72]
	Transmigration	[74]
	Leukotriene and prostaglandin production	[75,76]
	Matrix metalloproteinase production	[77]
Complement	Classical pathway activation	[88,89,90]
T cells	Early protective role in pneumococcal infection	[91]
	Memory response	[30]
	Chemotaxis	[91,92]
	TH17 induction	[30]
	Inhibition of cytokine production and proliferation	[78]

Key findings in the field of pneumolysin interactions with the immune system. The list is by no means exhaustive, but highlights early or key papers in each area.

levels of pro-inflammatory cytokine and neutrophil influx to the lungs but increased levels of the immunomodulatory cytokine IL-10 and increased infiltrating T cell numbers. This suggests that pneumolysin may affect the balance of inflammatory and immune regulatory pathways of the host during infection, something discussed in detail below. However, in stark contrast to the effects of *reduced* pneumolysin production in serotype 3, when the *ply* gene

was deleted, markedly *increased* virulence was observed [95].

Exchange of the hemolytic pneumolysin of serotype 3 with the ahemolytic pneumolysin of serotype 8 altered the immune-stimulating activity and behavior in a mouse model of nasopharyngeal colonization of both serotypes. Pneumococci expressing ahemolytic pneumolysin induced diminished innate and adaptive immune responses and were carried at a higher density in the nasopharynx of mice than pneumococci expressing hemolytic pneumolysin [96]. These data show that pneumolysin affects pneumococcal colonization and virulence irrespective of capsular type. In a study by Alexander et al., mice that had been immunized with pneumolysin were significantly protected from disease caused by nine pneumococcal strains, although no protection was afforded against a tenth [97]. The presence of pneumolysin may tip the balance in favor of either host or pathogen depending on the site of infection, timing, immunological state of the host, and bacterial density. This complexity likely explains the very different outcomes often observed in infection models using pneumolysin mutants or pneumolysin-deficient pneumococci. For example, in contrast to the colonization models described above, pneumolysin deficiency clearly benefits the host in models of pneumonia, where pneumolysin-deficient bacteria are rapidly cleared [79,92].

That pneumolysin is detrimental to host outcome in established infection is evidenced by an elegant study in which outcomes in bacteremia were compared between wild-type infections, pneumolysin-deficient infections, and mixed infections containing both pneumolysin-sufficient and pneumolysin-deficient bacteria [98]. Wild-type bacteria grew exponentially in blood up to 10^{10} colony-forming units (CFU) per mL, at which point the animals died. By comparison, when pneumolysin-deficient bacteria were used, bacterial numbers plateaued at 10^7 CFU per mL and then remained constant for several days. The crucial observation that in a mixed infection the pneumolysin-deficient bacteria behaved like wild type suggests that pneumolysin effects in the context of bacteremia can be mediated at a distance. The authors concluded that during the first few hours of bacteremia, pneumolysin plays a crucial role by preventing the initiation of host immune responses. This theory suggests that upon early bacterial death, either due to host killing or bacterial-induced self-lysis, pneumolysin release hampers generation of immune responses, allowing remaining viable pneumococci to flourish.

In infection experiments using pneumococcal mutants lacking either the pore-forming or complement-activating activity of pneumolysin, both functions were found to play an important role in the induction of host responses [99]. Infection with either mutant led to delayed histological changes in the lung as compared to infection with an isogenic wild-type strain. In the absence of complement-activating activity, T cell infiltration of the lung was reduced, while in the absence of pore-forming activity, neutrophil influx was compromised. Furthermore, the same study observed early rises in bacterial numbers in the lungs following infection with pneumolysin mutants as compared to the isogenic wild-type strain. Thus, pneumolysin expression might aid the host in early infection by kick-starting immune responses, but it is clearly detrimental in established infection, where it drives excessive inflammation that compromises epithelial barrier integrity and aids bacterial dissemination.

THE ROLE OF PNEUMOLYSIN IN PNEUMOCOCCAL CARRIAGE

The relationship between pneumolysin hemolytic activity and virulence is further confounded by the observation that many serotypes known to produce ahemolytic pneumolysin or

toxin with substantially reduced hemolytic activity are also those associated with the highest degree of invasiveness. These include serotypes 1, 7, and 8 [20,63,64]; serotypes 1 and 8 in particular have been associated with epidemics of pneumococcal disease [20]. Intriguingly, however, these same serotypes are rarely found in carriage and are thought to be poor colonizers [64,65]. Thus, pneumolysin might be important for prolonged colonization of the nasopharynx, and the high incidence of invasive disease seen with serotypes 1, 7, and 8 might reflect a failure to induce responses conducive to non-invasive carriage. It is clear that nasopharyngeal colonization with *S. pneumoniae* is a prerequisite for invasive disease; thus, understanding the bacterial and host factors that control is are key to developing appropriate strategies for disease monitoring and elimination.

In support of such a model, recent papers have highlighted the importance of pneumolysin for prolonged nasopharyngeal colonization in mice [45,100]. In a model in which wild-type serotype 2 (D39) pneumococci stably colonize the nasopharynx for more than four weeks without clearance or progression to invasive disease, isogenic pneumolysin-deficient bacteria fail to establish prolonged colonization and are cleared within 7–14 days [45,79,100]. This is somewhat in contradiction to an earlier study that reported reduced adherence to epithelial cells by pneumolysin-deficient bacteria but no defect in a murine pneumococcal carriage model [71]. This discrepancy may be explained by the different pneumococcal serotypes used in the two studies or the differences in the mouse model. The model used in the Rubins study showed gradually decreasing bacterial numbers in nasal wash over four weeks of colonization, even with wild-type pneumococci, while the Richards study showed stable bacterial numbers over the same time period in nasopharyngeal homogenates.

Recently, identification of a novel pneumolysin interaction with host cells during colonization has shed some light on the role of the toxin in mediating long-term pneumococcal carriage, and has also provided an explanation for why animal infection models using pneumolysin mutants have produced seemingly conflicting findings. Purified pneumolysin and serotype 2 pneumococci, but not PLN-A, induce production of the immune modulatory cytokine TGFβ1 from epithelial cells of both the nasopharynx and lungs [45]. This process requires TLR signaling, and dependence on potassium efflux and phagosomal rupture implicate the NLRP3 inflammasome. Importantly, the host TGFβ1 responses to pneumolysin showed a unique dose response, whereby low or intermediate doses of bacteria or toxin induced increased TGFβ1 production in a dose-dependent manner, but high concentrations of bacteria or toxin led to an attenuated TGFβ1 response but increased IFN-γ production.

This finding has important implications for our understanding of host responses to the pneumococcus in the nasopharynx. In the study by Neill et al., a comparison of carriage density and duration following nasopharyngeal colonization by low or high pneumococcal numbers revealed that while low-density colonization leads to prolonged carriage, high-density colonization or pneumolysin-deficient bacteria result in only transient carriage. The mechanistic explanation for these findings was an altered balance of pro- and anti-inflammatory immune responses following high-density or pneumolysin-deficient pneumococcal colonization. Prolonged carriage was found to be crucially dependent upon induction of TGFβ1 and associated T regulatory cell responses that act to limit pro-inflammatory cytokine production and neutrophil infiltration, and thus allow maintenance of stable pneumococcal numbers in the nasopharynx. Pneumolysin-deficient bacteria fail to induce TGFβ1 and T regulatory cell responses, and consequently are cleared from the upper airways. Similarly, high-density colonization induces a pro-inflammatory response

in airway epithelial cells, overriding the TGFβ1 response and leading to bacterial clearance. Supporting this concept, following human experimental pneumococcal challenge via nasal instillation [101,102], levels of TGFβ1 in nasal wash at 48 h post-challenge correlated with successful establishment of pneumococcal carriage [45]. Furthermore, T regulatory cell numbers and inhibitory capacity have been shown to be elevated in children with pneumococcal carriage [103,104].

The hypothesis emerging from these collective observations is that invasive pneumococcal disease results from a failure to induce or maintain appropriate T regulatory responses in the nasopharynx. TGFβ1- and T regulatory cell–driven responses act to induce a degree of immune tolerance to pneumococcal colonization of the upper airways. The consequence of this is preservation of the airway epithelial cell barrier, which prevents bacterial dissemination within the host. The price is that pneumococcal colonization is not cleared and prolonged carriage can occur. When this normal pathway breaks down, for example, following high-density pneumococcal colonization or colonization with pneumolysin-deficient pneumococci, inflammatory pathways are activated and bacteria are either cleared or else disseminate due to inflammatory tissue damage and cause pneumonia and invasive disease. The described role of T regulatory cells in pneumococcal carriage is summarized in Figure 14.2. A similar role for regulatory pathways in limiting inflammation to prevent pneumococcal spread has been described in pneumonia [105].

In support of this hypothesis, when TGFβ1 is experimentally inhibited following low-density pneumococcal colonization in mice, stable carriage is not established; instead,

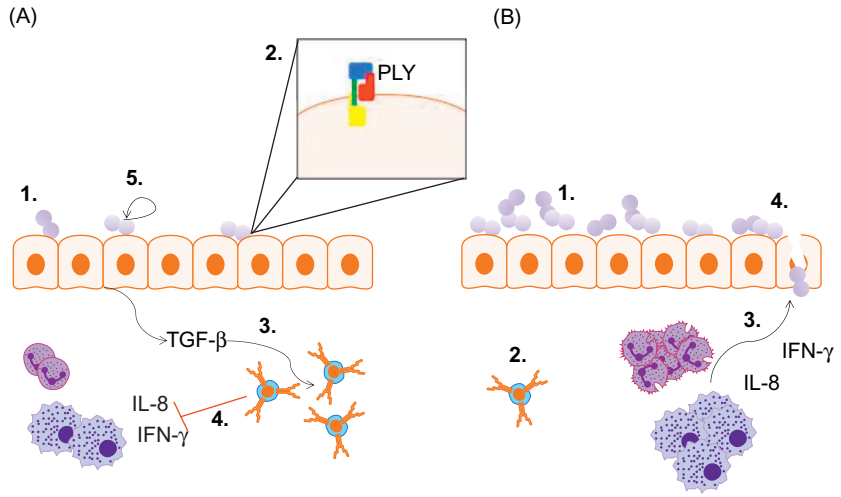

FIGURE 14.2 **Transforming growth factor β-driven responses to pneumococcal colonization of the nasopharynx.** (A) When low-density colonization of the nasopharyngeal epithelium occurs (1), pneumolysin-dependent signaling induced by pore formation or interaction with an unidentified receptor (2) triggers release of TGF-β. TGF-β-induced T regulatory cell responses limit proinflammatory cytokine production (4) and thus prevents excessive neutrophil or macrophage activity that might damage local tissues. In the absence of strong inflammatory responses, bacterial carriage is maintained at low density (5). (B) High density colonization (1) induces only weak TGF-β and T regulatory responses (2), allowing IL-8- and IFN-γ-driven inflammation to fight the infection (3). Macrophages and neutrophils phagocytose the colonized bacteria, but bystander tissue damage (4) may provide a route for bacterial dissemination in susceptible individuals.

clearance occurs within 7–14 days. Clearance is preceded by a period in which increased dissemination of pneumococci from nasopharynx to lung is observed, suggesting that there is a fine line between immune responses that clear colonization and those that are damaging and worsen prognosis. This might explain why experimental models with pneumolysin-deficient pneumococci display increased or decreased virulence in the hands of different laboratories, and also why pneumococcal serotypes expressing pneumolysin with altered hemolytic activity are associated with a high degree of invasiveness but are only infrequently found in carriage.

THE USE OF PNEUMOLYSIN AS A VACCINE

It was first demonstrated in the 1990s that vaccination with pneumolysin could protect mice from challenge with several different capsular types of pneumococci [16]. Because native pneumolysin is too toxic to be used as a vaccine, a toxoid form of the protein is required for use in humans. Chemical processes such as formaldehyde treatment can make toxoid forms of pneumolysin; in attempts to retain the native structure of the protein, several genetic toxoids have been created and tested in animals and are now being used for early trials in humans. One mutant version of the toxin was constructed by introduction of a single amino acid deletion at position 146 (Δ146) [106]. The mutation in this toxoid prevents the protein from forming pores in cell membranes, prevents the inflammatory effects of native pneumolysin, and markedly reduces toxicity in animals. Mice vaccinated with this toxoid generated high levels of neutralizing antibody and were significantly protected from challenge with virulent pneumococci. Ply is also able to act as a powerful mucosal adjuvant when genetically coupled to other proteins such as pneumococcal surface protein A (PspA) [107]. Mice developed high levels of serum IgG and mucosal IgA to PsaA when coupled to the N-terminal end of pneumolysin. Pneumolysin has also been fused to PspA, and vaccination with this fusion protein elicited protective immune reposes in animals [108]. A structural biology approach was used to design another toxoid version of Ply, termed PlyD1. This toxoid contains three mutations (T65C, G293C, and C428A). PlyD1 has been used in phase I clinical trials in humans, where all dose levels used in adults were safe and immunogenic [24]. A detoxified version of pneumolysin has also been developed by GlaxoSmithKline and tested along with the PhtD protein in a phase I clinical trial in humans [109].

CONCLUDING REMARKS

The dual role of pneumolysin as a key virulence factor of *S. pneumoniae* and as a powerful initiator and potentiator of host responses has made it an intriguing protein to study, with new functions and roles still being discovered. The past couple of decades have seen significant progress in our understanding of its structure, function, and role in pathogenesis, and have led to the identification of myriad and often surprising interactions with host cells. Unraveling which of these effects are important during actual infection remains a challenging task. Furthermore, after many years of effort, finally, pneumolysin toxoids have the serious potential to form the basis of the next generation of broad-coverage pneumococcal vaccines. This is down to its role as a key immunogen, stimulating adaptive immunity but also its potent adjuvant activity through its effects on multiple immune cell populations (summarized in Figure 14.3). Further research is still necessary in order to fully understand and harness the potential of this fascinating toxin, for both novel vaccines and adjunct therapies.

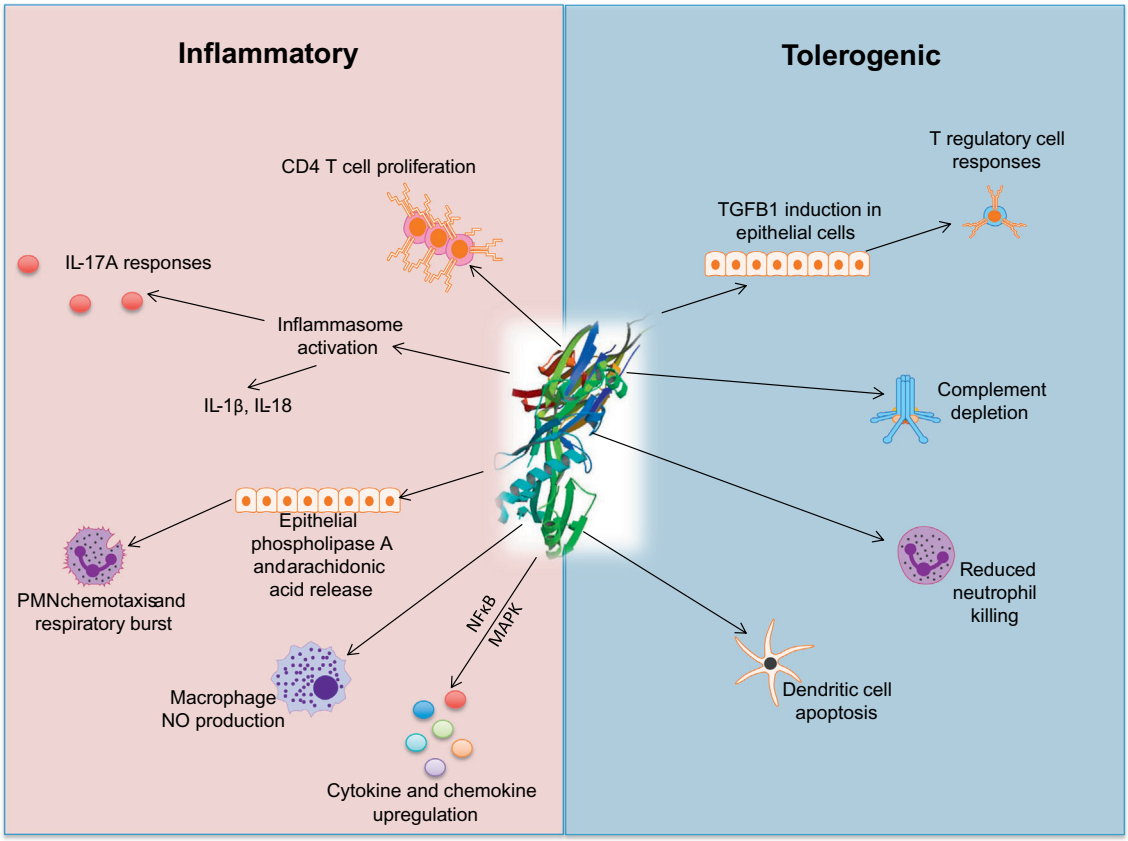

FIGURE 14.3 Inflammatory and tolerogenic host responses to pneumolysin.

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SECTION D

PNEUMOCOCCAL INTERACTIONS WITH THE HOST

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CHAPTER

15

Nasopharyngeal Colonization with *Streptococcus pneumoniae*

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INTRODUCTION

To survive in the human population, *Streptococcus pneumoniae* has evolved to colonize the mucosal surfaces of the upper respiratory tract. From there, the pneumococcus can then successfully spread to other susceptible hosts. The success of the pneumococcus in surviving and persisting in the human population is clearly demonstrated by the approximately 1.9–5.8 billion people estimated to be colonized with *S. pneumoniae* at any given time (inferred from [1,2]). Despite the fact that the pneumococcus is notorious for its ability to cause severe invasive disease, the majority of colonized individuals will not develop clinical symptoms. This strongly suggests that colonization actually represents the primary selective force for pneumococcal evolution and implies that many of the host–pathogen interactions observed during pneumococcal disease *must* be viewed within the context of asymptomatic colonization. In this chapter we will discuss the dynamics and mechanisms of pneumococcal colonization of the upper respiratory tract and subsequent transmission to a new host. Although we will also briefly touch upon how pneumococcal colonization perturbs mucosal homeostasis and how this affects immune signaling, disease development, and bacterial clearance, these aspects are primarily discussed in other chapters.

NATURAL BARRIERS TO PNEUMOCOCCAL COLONIZATION

Pneumococcal transmission occurs via contact with the respiratory secretions of colonized individuals [3]. It is not clear whether

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pneumococcal transmission occurs directly via the inhalation of bacteria, or indirectly via contact with contaminated surfaces. Indeed, while *S. pneumoniae* does not remain viable for an extended period of time on contaminated hands, contact with objects carrying pneumococci in the form of desiccation-resistant biofilms may still play a role in pneumococcal transmission [4–6]. Following initial entry into the airways, *S. pneumoniae* must overcome several hurdles before it can establish stable mucosal colonization in the upper respiratory tract. These hurdles, and the mechanisms by which the pneumococcus overcomes them, are discussed in detail below.

Mucus Barrier

Within the upper respiratory tract, the nasopharynx is the primary niche of pneumococcal colonization [7,8]. The nasopharynx is lined with the same ciliated pseudostratified columnar epithelium present in the nasal cavity. These ciliated epithelial cells are covered by a 0.5–10 μm thick mucus layer. This mucus layer is produced by surface goblet cells which are dispersed throughout the epithelial layer, as well as by goblet cells present in clefts that extend down into the underlying basement membrane and lamina propria. This mucus layer represents a major barrier to successful pneumococcal colonization. Specifically, the mucus layer consists of an upper viscous gel that contains antimicrobial compounds, traps larger particles including bacteria, and transports these, through ciliary beating, toward the pharynx for removal via the oral-fecal route [9]. The mucosal fluid also contains various polymer-like mucin glycoproteins, with both shared and unique features (reviewed in [10]). Many of these mucins are extensively covered with negatively charged *N*-acetylneuraminic acid (sialic acid) [11,12], which can bind to positively charged particles (e.g., bacteria) with high avidity, preventing these particles from crossing the mucus barrier, and thus facilitating their removal via mucociliary clearance. To overcome these adhesive restrictions, the pneumococcus expresses one of more than 90 polysaccharide capsules, a layer of polysaccharide molecules which is primarily covalently attached to the peptidoglycan cell wall. Whereas the pneumococcal capsule is well known for its role in providing protection to complement and phagocytic clearance during invasive pneumococcal disease, its contribution toward colonization has remained unclear for a long time. In light of the central role of colonization in the pneumococcal life cycle, it is likely that the role of capsule extends beyond resistance to complement and phagocytic killing. Pneumococcal capsules are immunologically highly distinct. However, they share one unique feature: None of them are positively charged. Indeed, with the exception of serotypes 7F, 7A, 15, 33F, 37, and 1, which have a net neutral charge, the other polysaccharide capsules are negatively charged due the presence of uronic acid, phosphate, and pyruvate [13]. A study by Weiser and colleagues identified an important role of the capsule during colonization; they found that the capsule mediates translocation of the pneumococci across the negatively charged mucus layer [14]. This facilitated subsequent pneumococcal adherence to epithelial cells and establishment of colonization. Subsequent work by Li et al. showed that the capsular serotype is also the most important factor in determining the bacterial surface charge and that this is strongly correlated to serotype distribution in carriage [15]. To aid in this process, *S. pneumoniae* expresses several exoglycosidases (e.g., NanA, NanB, and NanC), which cleave sialic acid residues [16] and thus further negate the adhesive properties of the mucins.

Although expression of the polysaccharide capsule is important for evading the adhesive effects of the mucus layer, the presence of

capsule inhibits adherence to epithelial cells [17]. Indeed, it has long been known that "transparent" phenotypes adhere better to epithelial cells than "opaque" phenotypes, which produce one- to sixfold more capsule than transparent colonies [18]. Strikingly, and through a mechanism which has not yet been fully elucidated, Hammerschmidt et al. showed that pneumococci in close proximity to epithelial cells shed their capsule [19], which likely allows increased access of surface-exposed pneumococcal adhesins to host receptors on epithelial cells, and adherence to and invasion of epithelial cells.

During transport across the mucus layer, cilia beating by epithelial cells is inhibited by the pneumococcal pore-forming toxin pneumolysin (Ply) [20], suggesting an essential role for this virulence factor in colonization. It was therefore surprising that deletion of Ply did not attenuate pneumococcal colonization of mice in a study by Rubins et al. [21]. However, a subsequent study by Kadioglu et al. showed that Ply-deficient strains were indeed less able to colonize the nasopharynx, but also found that this effect was dependent not only on the capsule type but also on the genetic background [22]. Perhaps this should not come as a surprise, considering the above-described importance of the capsule in the evasion of mucus entrapment, as well as other factors that might be encoded on the genome.

Antimicrobial Compounds

The peptidoglycan cell wall and its covalently bound teichoic acids and polysaccharide capsule further promote pneumococcal survival by conferring resistance to the many antimicrobial compounds present in normal mucus [23]. One of the principal antimicrobial compounds is lysozyme, which is present in very high concentrations in airway mucus [24]. Human lysozyme is a muramidase which enzymatically targets the bond between *N*-acetyl glucosamine and *N*-acetyl muramic acid residues on peptidoglycan, resulting in cell wall degradation. Lysozyme can also directly kill both Gram positive and Gram negative bacteria via its cationic antimicrobial peptide motif, which has been shown to occur independent of its muramidase activity [25]. To evade enzymatic cell wall degradation by lysozyme, *S. pneumoniae* produces two enzymes: the peptidoglycan *N*-acetylglucosamine deacetylase PgdA [26] and the *O*-acetyltransferase Adr [27]. A study by Davis et al. found that these enzymes act in synergy in modifying the targeting moieties on the pneumococcal peptidoglycan, rendering its peptidoglycan less recognizable by mucosal lysozyme. While this was associated with a decrease in fitness, the net effects of peptidoglycan modification provided a strong *in vivo* survival advantage to *S. pneumoniae* in the context of high concentrations of lysozyme M [28]. Weiser and colleagues also elegantly showed that cleavage of pneumococcal peptidoglycan in the presence of active Ply induces intracellular immune signaling via the cytosolic innate immune receptor nucleotide-binding oligomerization domain-containing protein 2 (Nod2). This leads to the expression of the chemokine CCL2, which facilitates recruitment of mononuclear phagocytes and the subsequent induction of specific immunity [29]. Lactoferrin is another compound which is abundantly present in the mucus layer and has antimicrobial activity against *S. pneumoniae*.

Lactoferrin is a multifunctional antimicrobial glycoprotein best known for its ability to sequester free iron [30]. Hammerschmidt et al. first reported that lactoferrin is bound by the choline-binding protein pneumococcal surface protein A (PspA), one of the few pneumococcal proteins to extend beyond the polysaccharide capsule [31]. Subsequent studies confirmed that binding of PspA to the iron-depleted (mucosal) form of lactoferrin strongly reduced bactericidal activity, which could be blocked by neutralizing antibodies to PspA [32].

Antibodies

Secretory antibodies form an additional barrier against colonization by *S. pneumoniae*. Although a reduction in vaccine serotype pneumococcal strains was observed soon after the introduction of conjugate pneumococcal polysaccharide vaccines [33], the exact mechanisms by which vaccine-induced immunity protected against colonization remained unclear. Recent work by Weiser's group showed that the presence of vaccine-induced agglutinating IgG and IgA prevented pneumococci from establishing colonization in a mouse model of pneumococcal transmission [34]. As local IgA is strongly induced by pneumococcal colonization [35], it presents an important barrier to overcome in order to establish colonization. Consequently, some *S. pneumoniae* strains have evolved evasion strategies by expressing IgA1 protease, a surface protein that recognizes and cleaves IgA1 [36], thereby preventing agglutination and facilitating transmission [34]. Thus far, no IgG proteases have been identified in *S. pneumoniae*. We speculate that this may be due to the fact that pneumococcal polysaccharide conjugate vaccines (PCVs) have constituted an important evolutionary driving force against colonization only for the last one to two decades. PCVs are known to induce very high mucosal levels of serotype-specific IgG, which are typically much higher than IgG levels found in children after natural colonization [37].

Niche Competition

Another major barrier to successful *S. pneumoniae* colonization is competition for resources (i.e., space and nutrients) from other colonizing bacteria. Indeed, this competition can even occur within the same species between different pneumococcal serotypes. For example, in a mouse model of *S. pneumoniae* colonization, mice colonized with a serotype 6B strain were less susceptible to "super-colonization" by a 23F strain [38]. The relationship between pneumococcal colonization and colonization by other bacterial species within the nasopharynx is complex. There have been several studies that have investigated the direct relationship between *S. pneumoniae* and other prominent bacterial members of the respiratory tract. *S. pneumoniae* is thought to disrupt the colonization of at least some other competing bacteria in the nasopharynx via the production of hydrogen peroxide [39]. However, Lysenko and colleagues showed that *S. pneumoniae* was less able to colonize the murine nasopharynx when co-administered with *Haemophilus influenzae* [40]. This was largely due to the ability of *H. influenzae* to stimulate complement-dependent phagocytic killing of *S. pneumoniae* [40]. While the extent to which *H. influenzae* inhibits pneumococcal colonization depends, at least in part, on the serotype of the colonizing pneumococcal strain [41], these data clearly demonstrate that the local microbiome is a key determinant in the success of *S. pneumoniae* colonization. With the advent of high-throughput next-generations sequencing techniques, researchers have now begun to elucidate the complex dynamics of the microbiome and to define the presence of key constituents of the microbiota that predict susceptibility and/or resistance to pneumococcal colonization [42,43]. For instance, recent work by Bogaert's group demonstrates how breastfeeding affects temporal changes in the nasopharyngeal microbiome in infants from 6 weeks of age up to the age of 2 years, including *S. pneumoniae* [44,45]. These studies identified a strong correlation between breastfeeding and early colonization by *Moraxella* and/or *Dolosigranulum*/*Corynebacterium*. Moreover, microbiomes dominated by these species were more stable over time and were associated with fewer respiratory infections. Conversely, the detection of *S. pneumoniae* was associated with an absence of clear and/or stable microbiome patterns [43,46]. Although these associative findings still need to be validated in follow-up studies, it is clear that next-generation methodologies will

play an important role in our understanding of the complex host–microbial dynamics that determine asymptomatic pneumococcal colonization and the transition to pneumococcal disease.

DYNAMICS OF PNEUMOCOCCAL COLONIZATION

Should *S. pneumoniae* be able to overcome all these barriers in the nasopharynx, the bacteria can firmly attach to epithelial cells, after which they may replicate locally and form biofilms or, infrequently, translocate across the epithelial layer and become invasive (see Chapter 23). It is only in the last few years that the importance of biofilm formation in pneumococcal biology has been acknowledged and more widely studied. Despite the essential role of biofilms in pneumococcal colonization and transmission, the exact mechanisms by which biofilms contribute to pneumococcal biology will not be discussed here but in Chapters 7, 13, and 16.

The onset of the first colonization event with *S. pneumoniae* is strongly dependent on many factors, including geographical location and socioeconomic conditions, but it typically occurs around the sixth month of life. However, in some populations, such as Indigenous Australians, newborns are colonized as early as 3 weeks of age [47]. Several risk factors have been associated with increased nasopharyngeal colonization rates of *S. pneumoniae*, including having older siblings and attending day care [48], recent exposure to antibiotics [49], and seasonality [50]. The contribution to pneumococcal carriage of respiratory viruses circulating during the winter season is further discussed further below.

Colonization can occur with a single pneumococcal strain, although multiple studies have shown that carriage with more than one strain also occurs [51,52]. The duration of colonization in children is inversely related to age, with children under the age of 1 being colonized for longer than older children and adults [53,54]. This age-dependent difference has been attributed primarily to the cumulative development of immunological memory in response to repeated colonization events with antigenically distinct pneumococcal strains (reviewed in [55]). After the fifth year of life, nasopharyngeal carriage, as determined by the recovery of viable pneumococci, typically drops to a steady 10%, although improved molecular detection methods suggest that carriage in adults may actually be much higher [8]. It is therefore possible that adults are not completely impervious to pneumococcal colonization, but rather that they restrict the density of *S. pneumoniae* in the nasopharynx through acquired immunity. Whether the benefits of repeated boosting of local immunological memory by pneumococcal colonization outweighs the intrinsic risk of progression to disease remains an ongoing and relevant topic of discussion, particularly in light of the introduction of vaccines that reduce colonization.

THE ROLE OF VIRUSES IN PNEUMOCOCCAL COLONIZATION

The density of pneumococci within the nasopharynx can by influenced by numerous different socioeconomic, immunological, and microbiological factors [56–58]. One of the key factors that can increase the density of pneumococci in the upper respiratory tract is coinfection with a respiratory virus [59–66]. This synergistic relationship has been best described in regard to the influenza virus [59–64]. Here we summarize some of the key suggested mechanisms by which influenza virus may increase the load of *S. pneumoniae* in the nasopharynx, and assess the relevance of these mechanisms to other respiratory viruses.

Infection with influenza virus disrupts the integrity of the epithelial cell layer within the

respiratory tract [67]. In doing so, the virus exposes components of the basement membrane or/and extracellular matrix to which *S. pneumoniae* can adhere [68–71]. For example, during the 1968 Hong Kong influenza virus pandemic, autopsies performed on patients who succumbed to bacterial pneumonia showed that bacteria adhered to sites where influenza A virus (IAV) had damaged the respiratory epithelium [72]. Similar observations have been recorded in mice infected with IAV strain A/Mexico/4108/09 (Mex09; H1N1) and *S. pneumoniae* [73]. It is therefore tempting to speculate that in the nasopharynx, cellular destruction by influenza virus facilitates an increased density of pneumococci. However, while this may be true for other areas of the respiratory tract, an association between virus-induced damage and pneumococcal adherence has yet to be shown in the nasopharynx. Indeed, recent evidence suggests that IAV infection in mice does not result in extensive tissue damage in the nasal cavity [64]. Rather, a greater factor influencing the density of pneumococcal colonization during IAV infection is the availability of free sialic acid [64].

As described above, *S. pneumoniae* possesses three different neuraminidases (NanA, NanB, and NanC). In addition to helping to penetrate the mucus layer, these neuraminidases remove terminal sialic acid residues from host glycoproteins and expose receptors for pneumococcal adherence [74–76]. During IAV infection, the viral neuraminidase (NA) also cleaves off host sialic acid residues in order to facilitate the release of newly made virus particles. The sialidase activity of the viral NA, working in concert with bacterial neuraminidases, may then expose cryptic receptors for pneumococcal adherence [71,77,78]. For example, in chinchillas increased nasopharyngeal titers of *S. pneumoniae* following IAV infection correlated with IAV-induced changes in lectin staining patterns in the nasopharynx [78]. The presence of a viral NA also increases the amount of free sialic acid available. This too increases pneumococcal adherence and replication in the nasal cavity [64,79]. The role of free sialic acid in pneumococcal colonization has been clearly demonstrated by two separate studies [64,79]. First, intranasal administration of sialic acid to mice increases pneumococcal counts in the nasopharynx and increases translocation of *S. pneumoniae* to the lung [79]. Second, while influenza virus increases both the availability of sialic acid and the density of *S. pneumoniae* colonization in a murine model, this effect is annulled when the pneumococci are unable to catabolize sialic acid [64]. This not demonstrates only the importance of sialic acid in pneumococcal colonization, but also suggests that free sialic acid provides an important carbon source for bacterial growth in the nasal cavity [64,79,80]. These free sialic acids are likely to be derived from the mucin Muc5ac, as mice that lack Muc5ac do not display an influenza virus–induced increase in the density of pneumococcal carriage [64].

The immune response to IAV in the upper respiratory tract may also help facilitate an increased density of pneumococcal colonization. Specifically, upon colonization of the nasopharynx, pneumococci trigger type I interferon (IFN) production [62]. Co-infection with influenza virus results in a synergistic increase in the levels of type I IFNs in the nasopharynx [62]. This increased level of type I IFNs then blocks macrophage recruitment by decreasing the production of the chemokine CCL2 [62]. IAV can also deleteriously affect macrophage functionality by down-regulating the alveolar macrophage scavenger receptor MACRO [81]. Accordingly, upon influenza virus infection there is a significant increase in the density of colonizing pneumococci [62]. However, this increase was only observed when the pneumococci possessed a functional Ply [62], suggesting the important role of specific pneumococcal virulence factors

in viral–bacterial interactions. It is possible that some pneumococcal strains/lineages may have evolved to depend on the presence of external cofactors such as viral co-infections to facilitate their colonization, while other lineages may be more capable of independent colonization.

The above studies point to the diversity and complexity of mechanisms that may result in increased pneumococcal nasopharyngeal loads in the presence of influenza virus. However, it is important to note that this ability to increase pneumococcal titers in the nasopharynx is not unique to the influenza virus. Viruses including rhinovirus [82] and respiratory syncytial virus (RSV) [83,84] increase either pneumococcal adherence to nasal epithelial cells *in vitro* or the density of pneumococcal colonization *in vivo*. In the case of rhinovirus, increased pneumococcal adherence to nasal epithelial cells is associated with an increase in the gene and protein expression of fibronectin [85], suggesting that at least some of the mechanisms described for *S. pneumoniae* and IAV may be applicable to other respiratory viruses. Indeed, given that the nonspecific triggering of type I IFNs is sufficient to increase pneumococcal colonization densities in mice [62], it is also possible that any virus that stimulates a robust type I IFN response is also able to increase pneumococcal colonization by blocking macrophage recruitment, as has been described for influenza virus [62]. RSV is also thought to have its own distinct method of facilitating pneumococcal adherence and replication. *S. pneumoniae* binds directly to the RSV protein G, expressed on the surface of infected cells [83,86]. This mechanism has been associated with an increased incidence of pneumococcal bacteremia in RSV-infected mice [83]. However, while it is plausible that a similar mechanism occurs in the nasopharynx to increase pneumococcal binding and replication, this has yet to be shown *in vivo*. In sum, the interaction between viruses and pneumococci in the nasal cavity is complex and there still remain many unanswered questions. Nevertheless, it is clear that co-infection with specific respiratory viruses can significantly affect the growth of pneumococci in the upper respiratory tract.

VIRAL INFECTIONS AND PNEUMOCOCCAL TRANSMISSION

Given that viral infections can significantly influence pneumococcal colonization, it is somewhat unsurprising that co-infection with certain respiratory viruses is also thought to facilitate pneumococcal transmission. Perhaps one of the first indicators of this synergistic relationship was the 1918 Spanish influenza virus pandemic, during which it was noted that "every patient with influenza must be considered a potential source of pneumococcus infection for his neighbor" [87]. A study in the 1970s also implicated a concurrent viral infection of the respiratory tract in the spread of *S. pneumoniae* among family members [88]. Additionally, Ujiie and colleagues showed that infection with the 2009 pandemic H1N1 virus was linked to the horizontal spread of *S. pneumonia* [89], while others have demonstrated that an upper respiratory tract infection is associated with an increased rate of pneumococcal carriage in adults [90]. However, perhaps the strongest evidence for a role for influenza virus in pneumococcal transmission comes from more recent experimental studies. In 2010, it was demonstrated that co-infection with influenza virus was absolutely necessary for the transmission of pneumococci between co-housed infant mice [60]. Specifically, in order for pneumococcal transmission to occur, both the colonized "index" mice and co-housed, naïve "contact" mice needed to be co-infected with influenza virus [60]. The role of influenza virus in pneumococcal transmission was not restricted to a specific viral strain, as both

H1N1 and H3N2 viruses facilitated the spread of pneumococci [59]. Rather, the ability of influenza virus to increase the spread of pneumococci may be related to the ability of IAV to increase the pneumococcal load in the nasal cavity of the index mice and induce inflammation in the nasal cavity of the contact mice [59]. Interestingly, recent studies in TLR2-deficient mice suggested that inflammation and increased bacterial shedding are not necessarily mutually exclusive events, as increased inflammation in infant $tlr2^{-/-}$ mice is responsible for increased pneumococcal shedding and transmission [91]. How inflammation may facilitate pneumococcal transmission remains unclear. It is possible that this reflects the ability of a pro-inflammatory response to up-regulate receptors for pneumococcal adherence (such as the platelet-activating factor receptor) or expose components of the basement for pneumococcal adherence [68,92,93]. Alternatively, it is possible IAV facilitates pneumococcal transmission among co-housed mice by inducing the release of bacteria from biofilms in the upper respiratory tract [94,95]. Within the nasopharynx, *S. pneumoniae* often exists with highly structured, complex biofilm communities [96,97]. Not only can IAV disperse pneumococci from these biofilms, but these dispersed pneumococci are more virulent *in vivo* and display markedly different expression profiles when compared to bacteria still present in a biofilm [94,95]. It is therefore tempting to speculate that these IAV-dispersed pneumococci are more adept at transmission due to (for example) the differential expression of specific pneumococcal virulence factors or increased survival time *ex vivo* [95].

McCullers and colleagues have also demonstrated an important role for influenza virus in pneumococcal transmission using a ferret model of disease [61]. Ferrets are traditionally considered the most accurate small animal model of influenza virus infection, as ferrets can be infected with human strains of influenza and infection accurately mimics the symptoms of human disease [98]. McCullers and colleagues showed that influenza virus coinfection facilitated pneumococcal transmission between co-housed ferrets, even when there was 10 m of separation between the index and contact animals [61]. Influenza virus infection in the contact animals appears to be the most important factor in pneumococcal transmission, as only when the contact animals were virally infected could pneumococcal transmission occur over 10 m of separation [61]. Consistent with this observation, virus-induced increases in pneumococcal titers in the index animals alone was not sufficient for transmission, as influenza virus increased the nasopharyngeal load of both pneumococcal strains BHN97 and BHN54, but only BHN97 was able to transmit between ferrets [61]. However, despite these important findings, there still remain many questions to be answered regarding the role of viruses in pneumococcal transmission. For example, is the association between influenza virus infection and pneumococcal transmission virus-specific, or does a similar synergy exist between pneumococci and other respiratory viruses? Certainly, epidemiological evidence would suggest that other respiratory viruses facilitate nasopharyngeal pneumococcal acquisition [99,100], but this remains to be proven in a defined experimental setting. Do viruses play a more significant role in pneumococcal transmission in particular subsets of the population (e.g., those at the extremes of age)? Does the role of respiratory viruses in pneumococcal transmission vary between pneumococcal strains? We have found that influenza virus facilitates the transmission of at least three different strains of pneumococci ([60] and unpublished data), although the data of McCullers and colleagues [61] clearly indicates that there is strain-to-strain variation in pneumococcal transmission. This is also relevant from a more ecological perspective, as pneumococcal strains

which are intrinsically more likely to colonize compared to more "virulent" strains may be more dependent on external cofactors such as viral infections to facilitate spread to a new host. These and other questions need to be answered not only experimentally, but also by sound clinical and epidemiological studies. Ultimately, it must be remembered that transmission and colonization marks the first step toward disease. Thus, if we are able to understand and prevent both transmission and colonization, we will be making significant progress toward reducing the worldwide burden of pneumococcal disease.

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CHAPTER

16

Pneumococcal Biofilms and Bacterial Persistence During Otitis Media Infections

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THE BIOFILM CONCEPT

Biofilms are generically referred to as multicellular microbial surface-adherent communities; they were described in an early review as featuring "a forest of protruding linear macromolecules such as pili, lipopolysaccharide O antigen or teichoic acid, and exopolysaccharides" [1]. Over time, this definition has been restated more specifically to include encasement of the community within an extracellular polymeric matrix material that originates from the bacterial community [2–4]. In regard to chronic infections, the definition has been extended to include "persistent pathology" [4].

The formation of biofilms may be an important survival factor and may play an evolutionary role, enabling organisms to adapt to changing nutritional and environmental conditions collectively instead of as single cells. Importantly, whether in water-handling systems, streams, ship hulls, or in chronic infections, the primary contribution of a biofilm was in permitting the microbes within to resist shear forces, environmental stresses or microbicides, and/or host immune effectors [5]. Biofilms are also resistant to many host defense mechanisms. The biofilm architecture seems to play a critical role in limiting the penetration of leukocytes and their products into the biofilm and also in decreasing the phagocytic capacity of host cells in a process called "frustrated phagocytosis." This phenomenon is best illustrated by *Pseudomonas aeruginosa* infections of cystic fibrosis patients, whereby leukocytes attempt to clear biofilms but end up releasing reactive oxygen species into the immediate environment, causing self-damage to lung tissue. Another major resistance mechanism of bacterial biofilms is reduced susceptibility to antimicrobial agents [6,7]. There are two main methods of determining bacterial antibiotic sensitivities: minimum

Streptococcus pneumoniae.
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inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC). For planktonic cultures, the MIC is defined as the minimum amount of antibiotic necessary to inhibit visible growth of bacteria following an overnight incubation. For biofilm communities, the MBEC is generally defined as the minimum concentration of antibiotic necessary to reduce biofilm bacteria to a level that is at or below the limit of detection. The MBEC can be 500−1500 times higher than MIC, even if individual cells are highly susceptible [3]. One study found that an antibiotic can penetrate into the core of a *P. aeruginosa* biofilm within hours at concentrations that would be bactericidal for planktonic forms [8]. However, even at high doses the antibiotic was unsuccessful at killing all biofilm bacteria. Although most bacterial antibiotic resistance is genetically based, biofilm resistance may be physiological. One explanation is that the biofilm structure represents a physical barrier that reduces penetration of antibiotics. Alternatively, the antibiotic may be adsorbed by biofilm matrix components (e.g., extracellular polysaccharides) and prevented from accessing the core. It is possible that metabolically active bacteria encased in a developing biofilm encode and up-regulate efflux pumps that drive out the antibiotic. Conversely, perhaps antibiotics that target metabolically active or dividing cells are ineffective against biofilm bacteria that have reduced divisional and metabolic rates [9]. Slow-growing or non-growing bacteria are known to be less susceptible to antibiotic treatment. Thus, biofilm-mediated diseases can be difficult to treat with antibiotics and can represent a long-lasting burden to the patient.

PNEUMOCOCCAL BIOFILMS

Streptococcus pneumoniae (pneumococcus) is an extremely diverse species that includes more than 90 different capsular serotypes. Pneumococci can cause a variety of disease presentations, including localized infections in the airway mucosa and systemic invasive infections such as sepsis and meningitis. Notably, gene expression and roles for specific virulence factors between localized disease and carriage are consistent with those observed in biofilm models [10,11]. Pneumococci found within biofilms are predominantly variants expressing less capsular polysaccharide, whether transparent phase variants or mutants [12−14]. These variants within biofilms are less virulent in terms of invasive disease, but are more readily adapted to colonization and persistence at mucosal surfaces [9,15]. As with other pathogens, the growth of pneumococci within biofilm communities confers an inherent resistance to clearance by antibiotics [16]; this is complicated further by the frequent occurrence of different strains/species within biofilm communities that can confer resistance by multiple mechanisms [17,18]. The determinants of pneumococcal biofilm formation and maturation appear to be somewhat strain-dependent [19]; for example, the Rrg pilus [11] and pneumococcal serine-rich repeat adhesion [9] have been shown to be determinants of pneumococcal biofilm formation in different lineages. There is also recent evidence supporting roles for pneumolysin [20] and a non-capsular carbohydrate matrix [21] in pneumococcal biofilm formation.

Pneumococcal biofilm development and structural integrity is dependent on many different factors, including the surface composition on which initial attachment occurs. Most *in vitro* studies focus on bacterial biofilm formation grown on abiotic surfaces (e.g., glass cover slips and polystyrene plates) under static conditions for short periods of time [22,23]. A common method of assessing the total biomass of a biofilm is by crystal violet staining of the cellular material. While a good indicator of biofilm integrity, the method does not convey total bacterial burden. Quantitative studies that enumerate total bacteria counts are more

informative. It is important to note that initial biofilm formation *in vitro* is not necessarily dependent on inoculum size because bacterial density usually peaks 12–16 h post-inoculation. In one study, the strength of biofilms formed on abiotic surfaces was assessed by crystal violet staining and was found to be associated with the culture medium used. Specifically, the authors found that growth of pneumococci in less complex media resulted in the formation of strong biofilms, whereas growth in complex media (e.g., THY) resulted in the formation of weaker biofilms that were sensitive to shear forces and were easily washed away [24]. Interestingly, supplementation of culture medium with a sugar source tended to promote biofilm formation by many bacterial species. For pneumococci, growth in THY media supplemented with 1% glucose exhibited increased biofilm formation [19]. Another way to study bacterial biofilms is by using a continuous-flow biofilm approach coupled with confocal scanning laser microscopy. This method is used to assess how well biofilms form under shear stress, which may more closely mimic what occurs *in vivo* [25]. Following a short establishment period, a pump is used to flow fresh culture medium over the biofilm at a constant rate for a set period. The biofilm can be visualized with live/dead staining to examine viability or with antibodies that target bacterial components in the extracellular matrix. While this method is useful in assessing biofilm attachment and integrity, it is essential to understand that biofilms formed *in vitro* may not directly relate to biofilms formed *in vivo*. A more physiologically relevant *in vitro* model of biofilm formation involves growth of pneumococci directly on eukaryotic epithelial cells. Pneumococcal biofilms grown on human respiratory epithelial cells had increased biomass and tended to form earlier than those grown on abiotic surfaces [26,27]. They also had architecture, matrix formation, and bacterial organization similar to biofilms formed *in vivo* [27]. Indeed, studying bacterial biofilms formed *in vivo* is the most relevant way to understand their role in pathogenesis. Many animal models have been adapted to study biofilm-mediated diseases; these include rats, mice, and chinchillas. One study assessed the ability of a pneumococcal strain to colonize the mouse nasopharynx. The authors found that biofilms that formed on the nasopharyngeal mucosal surfaces were encased in a matrix with ciliated cells and leukocytes and were more resistant to antibiotic treatment than the cells recovered from nasal wash [15,27]. Thus, bacterial biofilm attachment, development, and integrity are dependent on many conditions, including nutrient availability, presence of shear forces, and the biotic or abiotic environment.

The Switch from Planktonic to Biofilm Phenotype

In general, bacteria exist as two phenotypes: planktonic and biofilm. Planktonic growth consists of free-floating, non-adherent cells that may exist in small clusters or chains and are mostly associated with acute infections. Such infections are generally easily treatable since planktonic cells are normally sensitive to antibiotics at very low concentrations. Biofilm formation is initiated when planktonic cells attach to surfaces in moist, nutritious environments, such as in water-handling systems, streams, ship hulls, or on other natural materials. When bacteria form a biofilm within the human host on indwelling medical devices (e.g., catheters and pacemakers) or on host tissue (e.g., heart valves, bladder, and middle ears), the infection can become resistant to antimicrobial treatment and develop into a chronic state. Environmental factors (e.g., shear stress) can slough off cells on the outside of biofilms and facilitate dispersal. Of concern is that established biofilms on indwelling medical devices

can shed planktonic bacteria that can reenter the blood to cause septicemia, explore new niches, and establish new biofilms, thus putting the patient at constant risk of reinfection. In the case of the pneumococcus, biofilms formed within the nasopharynx may be sloughed during sneezing and/or the secretion of mucus and spread on fomites, such as toys in a day care setting. In comparison to planktonic bacteria, those within biofilm particles are desiccation-resistant, allowing the pneumococcus to persist on these fomites for prolonged periods of time.

The switch from planktonic growth to biofilm growth may represent a survival strategy for pneumococci in nutritionally limited, or changing, environments. A good example of a constantly changing environment is the natural reservoir for pneumococci: the human nasopharyngeal airway surface. In addition to high aeration, there are changes in pH, osmolarity, nutrient availability, and temperature. Additionally, there are many host factors present, such as antimicrobial peptides (e.g., lysozyme) and other innate immune factors. During growth, pneumococci alter expression of proteins that can affect persistence and virulence. Adaptation from the planktonic to biofilm phenotype may involve significant regulation of proteins involved in biosynthesis, metabolism, and virulence [13,23,28]. Additionally, live/dead staining of biofilms coupled with confocal scanning laser microscopy shows many dead and metabolically inactive cells, suggesting that after a biofilm is established, pneumococci adopt a metabolically dormant or reduced state [23]. A proteomic analysis of the planktonic and biofilm bacterial growth phenotypes identified more than 100 proteins that were differentially expressed [23]. Specifically, proteins involved in carbohydrate, pyruvate, and arginine metabolism, as well as three ABC transporter systems, were up-regulated in the biofilms. Proteins involved in the glycolytic pathway, translation, and transcription were mostly down-regulated, suggesting that alternative metabolic pathways are used to help pneumococci adapt to biofilm growth [14,23]. Thus, biofilm formation is dependent on many factors, but may help planktonic bacteria overcome environmental and host factors [24,29].

Bacterial Components Important for Biofilm Development

There are three main stages of pneumococcal biofilm development: initial attachment, aggregation formation, and biofilm maturation. Scanning electron microscopy studies of pneumococcal biofilms formed on polystyrene plates found that individual cells were interconnected by small, thin filaments and a mesh-like net [24,30]. During the different stages of biofilm development, pneumococci produced many bacterial products that played significant roles and were incorporated into the biofilm matrix [14,22,24]. Many studies have assessed the cellular and molecular composition of the biofilm matrix and have found that in general, biofilm composition includes a mixture of biopolymers known as extracellular polymeric substances (EPS), including DNA, proteins, and polysaccharides. Together, these matrix materials may comprise more than 90% of the total biomass of the biofilm, and they have many functions. EPSs aid in initial adhesion, aggregation formation, cohesion, water retention, protection (e.g., adsorption of antimicrobial agents), and providing a source of extracellular DNA and energy [22,27,29]. EPS matrix composition and integrity may differ depending on the environment on which biofilms attach and mature, the microorganisms present, temperature, nutrient availability, and shear forces [29].

The importance of extracellular DNA in initial biofilm attachment is well known [31]. During pneumococcal growth, DNA is released

into the environment in a fratricidal LytA- and LytC-associated manner, creating a reservoir of genes that are readily available for horizontal gene transfer, which can increase genetic and phenotypic variability in biofilm bacteria [24,30]. Importantly, this can promote distribution of antibiotic resistance genes. Biofilm growth, but not planktonic growth, is impaired in the presence of DNAse I, suggesting that extracellular DNA is a major biofilm matrix constituent that is important for structural integrity [21,24]. Detection of extracellular DNA within biofilms can be achieved by specific fluorescent DNA staining methods (e.g., propidium iodide, STYO-9, ethidium bromide and 4′,6-diamidino-2-phenylindole), followed by examination with confocal scanning laser microscopy [22]. The molecule DDAO (7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)) can be very useful because it does not penetrate intact cell membranes, such as planktonic cells, which increases the sensitivity of extracellular DNA staining of biofilms [32].

During pneumococcal biofilm development, many proteins are produced that are incorporated as structural constituents of the intercellular matrix. The integrity of biofilms is altered when treated with proteases [11,24]. A continuous-culture biofilm system found that *de novo* protein biosynthesis of more than 700 proteins was observed in 9-day-old biofilms that were absent in planktonic cells [14]. Following attachment, the pneumococcal serine-rich repeat protein and the enzyme pyruvate oxidase SpxB were found to be important for aggregate formation [15]. Other important proteins included choline-binding proteins (e.g., CbpA, PspC, and PcpA) that function to anchor teichoic acids of the cell envelope and are involved in adhesion to laminin and invasion of host cells [24]. The pneumococcal surface protein A (PspA) was also involved in adhesion. The murein hydrolases LytB glucosaminidase and LytC lysozyme were important in establishing nasopharyngeal colonization, while LytA *N*-acetylmuramoyl-L-alanine amidase was important for cell division, autolysis, and release of DNA in stationary phase [33]. All LytA, LytB, LytC, CbpA, PcpA, and PspA mutants had decreased capacities to form biofilms *in vitro* [11,24,27], although another study found that LytA, CbpA, LuxS, and pneumolysin were less critical *in vivo* within the mouse nasopharynx [15]. Interestingly, it was shown that extracellular DNA co-localizes with LytC in the intercellular matrix, suggesting that DNA−protein interactions can increase the structural integrity of the biofilm [30].

Pneumolysin is a cholesterol-dependent pore-forming toxin and important virulence factor in pneumococci that may also play an important role in early biofilm assembly. Pneumococcal strains deficient in pneumolysin produced less biofilm than their isogenic wild-type parent [20]. Microscopy studies of biofilms formed on both polystyrene and human respiratory epithelial cells under static and continuous-flow conditions assessed toxin localization. It was found that the toxin was expressed on the bacterial surface and was also incorporated into the extracellular matrix. Interestingly, the pore-forming activity of pneumolysin may not play a significant role in biofilm formation. A strain that produced a nonhemolytic variant of the toxin remained fully capable of forming biofilms [20]. The mRNA level of pneumolysin is affected by the LuxS quorum-signaling system [34]

Pneumococci express an enzyme called neuraminidase (NanA) on the bacterial surface that functions to specifically cleave eukaryotic terminal sialic acid moieties from host respiratory glycoconjugates. The cleaved sialic acid serves as a nutritional source for pneumococci, and the asialyl moieties serve as new binding sites, increasing adherence [35]. NanA may play a significant role in biofilm formation. Recent work has found that NanA-deficient mutants of encapsulated and unencapsulated

strains had reduced capacities to form biofilms *in vitro* [36]. Treatment of the biofilms with oseltamivir, a small-molecule inhibitor of NanA, was effective at reducing biofilm biomass [36]. Similarly, high concentrations of the sialic acids *N*-acetylneuraminic acid and its analog 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid were effective at inhibiting biofilm formation by 50% [36]. Together, these studies suggest that although NanA may not be essential to the process, it can promote bacterial adhesion and biofilm formation.

Pneumococcal biofilms contain extracellular polysaccharides that are different from the capsular polysaccharide. Although non-typeable pneumococci do not produce capsular polysaccharide, a biofilm produced by the non-typeable strain R6 was positively stained with the fluorescently labeled lectins wheat germ agglutinin (WGA; specific for *N*-acetylglucosamine and sialic acid) and soybean agglutinin (SBA; specific for galactose and *N*-acetylgalactosamine), suggesting the presence of non-capsule extracellular polysaccharides [22,30]. Planktonic R6 cells were negative for WGA and SBA staining, suggesting the lectins were specific for biofilm-associated extracellular polysaccharides. Identification of this extracellular polysaccharide may help understand how biofilms are held together. However, another study pointed out that glycosylated proteins (e.g., pneumococcal serine-rich repeat protein) can confound identification of extracellular polysaccharides via lectin staining [30].

Role of Capsule in Biofilm

The dogma in pneumococcal pathobiology is that expression of a protective capsular polysaccharide is essential for virulence. In the past few years, new work has revealed that this may not be the case for otitis media (OM). There have been increased reports of unencapsulated (non-typeable) pneumococci recovered from infection sites. The conventional serotyping method is the Quellung reaction, whereby pneumococci are mixed with polyclonal rabbit anti-capsule typing serum and examined under the microscope. If the bacteria appear swollen, this is a positive reaction. However, Quellung reactions are subjective and laborious and cannot always detect new, or slightly different, serotypes. Identification of non-typeable pneumococci is dependent on a non-reaction. This presents a challenge with accurate identification because a non-reaction could mean the pneumococcus is expressing a novel capsule type that is not recognized by conventional serotyping methods. New molecular diagnostic PCR-based tests that sequence parts of the capsule locus are more sensitive and accurate at serotyping. Homologous recombination of capsule synthesis genes represents a major mechanism for increasing genetic variation and capsule diversity in pneumococcal populations. Previous studies have found that capsule structure can be significantly altered by minimal genetic changes in the capsule synthesis locus [37]. Thus, identification of non-typeable strains using Quellung may not always mean the strain is unencapsulated.

Pneumococcal strains can spontaneously switch between two different phenotypes (opaque and transparent) via a mechanism known as phase variation, by an epigenetic mechanism that has only very recently been described [37,38]. Briefly, changes in DNA methylation pattern results in either up-regulation or down-regulation of capsular polysaccharide biosynthesis. Increased capsule production is associated with the opaque phenotype, while decreased capsule production is associated with the transparent phenotype. During invasive infections, pneumococci increase capsule production to help avoid immune detection of surface proteins, decrease complement deposition, and inhibit phagocytosis [39]. However, relative to unencapsulated

strain controls, expression of capsular polysaccharide can impair biofilm development by 30–60%, possibly due to an inhibitory effect on adherence [14,24]. Phase variation research in our lab has found that opaque variants of pneumococcal strain EF3030 were better able to cause middle ear infections than transparent variants, but they had a diminished ability to form biofilms *in vitro* [40].

Since capsule production represents a metabolic burden to the bacteria, adaptation to the biofilm phenotype (e.g., reduction of capsule expression) may be advantageous in nutritionally restricted environments. To establish colonization, pneumococci down-regulate capsule production to help expose normally masked proteins involved in adhesion (e.g., PspA) and thereby promote binding to mucosal surfaces [24]. Pneumococci representing serotypes 3 and 37 use a synthase-dependent mechanism to produce their capsular polysaccharides, resulting in a heavily encapsulated phenotype [41]. One study found that when serotype 3 pneumococci reduced the amount of capsule on the surface and thus the colony size, bacterial adhesion was increased, which directly contributed to increased biofilm formation *in vitro* [30]. Specifically, the small colony variants were better able to form clusters and had increased adherence relative to typical serotype 3 clones. The advantage of reduced capsule expression has been demonstrated most elegantly using non-typeable pneumococci. Due to the absence of capsular polysaccharide, non-typeable pneumococci have maximal exposure of adhesion proteins and can form stronger, more antibiotic-resistant biofilms than encapsulated strains. Targeted disruption or deletion of the capsule synthesis genes in an encapsulated isolate can generate an isogenic capsule-null variant. Crystal violet staining of biofilms formed by isogenic encapsulated/unencapsulated pairs found that the unencapsulated variants had increased biomass compared to their isogenic parent on abiotic surfaces [24]. Despite having maximal exposure of adhesion proteins and being hyperadhesive, non-typeable pneumococci are highly avirulent *in vivo*, suggesting that reduced capsule expression is only advantageous during colonization or when the bacteria are in a metabolically reduced or dormant state [42].

Yet evidence also suggests that capsular serotype may also play an important role in biofilm formation. When clinical isolates were tested for their capacity to form biofilms *in vitro*, serotypes 19A and 19F had increased biomass compared to other serogroup 19 members [30]. Interestingly, serogroup 6 members share some structural homology with the serotype 19A and 19F capsule and were similarly able to form biofilms. These findings suggested that disaccharides composed of glucose linked to α-L-rhamnose may play a role in initial biofilm attachment. Unlike serogroups 19 and 6, serogroup 18 members had a diminished capacity to form biofilms and the uncommon serotype 24F was unable to form a biofilm [30]. A better way to assess the individual contribution of capsular serotype to biofilm formation is to eliminate potentially confounding genetic and phenotypic variation. To achieve this, generation of isogenic capsule switch variants in an identical genetic background is particularly useful, especially when the genetic backbone is sequenced. Unpublished work from our lab has found that, relative to the invasive isolate TIGR4, capsule switch variants in the TIGR4 genetic background expressing serotypes 6A, 15C, 19F, and 23F have increased capacities to form biofilms *in vitro* on polystyrene surfaces as well as on human respiratory epithelial cells. We have found that, unlike TIGR4, the variants undergo phase variation. This is particularly the case with strain TIGR-19F. It will be interesting to enrich transparent and opaque populations of each variant and examine the contribution of each phenotype to *in vitro* biofilm formation.

Quorum-Sensing Systems, Competence, and Genetic Exchange in Biofilms

Pneumococci encode several quorum-sensing (QS) systems that regulate bacterial communities to coordinate a population-type behavior and alter gene expression at very specific cell densities. The formation, maturation, and dispersal of biofilms has long been thought to be controlled or influenced by production and sensing of diffusible quorum signals. Quorum signals interact with specific receptors to regulate the expression of genes involved in bacterial competence and a number of other virulence-associated factors. Since biofilm bacteria are engaged in close contact for long periods of time, this close association increases the likelihood that quorum signaling is efficacious.

As a species, *S. pneumoniae* is highly adaptable to changing environments, and the switch from planktonic to biofilm growth may be linked to competence gene regulation. The competence QS system produces pheromones called competence-stimulating peptides (CSPs), which facilitate bacterial competence to uptake DNA and facilitate genetic exchange in a density-dependent manner. The functionality of the system is dependent on the gene products encoded by the *comAB* and *comCDE* genetic loci. Following a signaling cascade, not only is CSP produced, but approximately 6% of the pneumococcal genome is induced [43]. Since only a few genes are necessary for genetic exchange, this suggests that the ComQS system plays a broader role in pneumococcal biology.

The pheromone CSP may play a huge role in the promotion of pneumococcal biofilm formation and genetic exchange. CSP is encoded by the *comC* gene, of which many allelic variants have been identified. The most common alleles are *comC1* and *comC2*, which produce CSP-1 and CSP-2, respectively. It is important to note that interpherotype communication does not occur. Strains can only respond to the pherotype they produce. The contribution of CSP to the planktonic and biofilm phenotypes has been recently studied. Addition of CSP increased early biofilm formation on abiotic surfaces in a *comC*-dependent manner as assessed by biofilm scraping and counting of colony-forming units [10,44]. Another study investigated whether CSP pherotype influenced biofilm formation; it found that clinical isolates encoding *comC1* were better able to form biofilms than those encoding *comC2* [43]. Isogenic strains that expressed either the *comC1* or *comC2* allele were tested for their ability to form biofilms *in vitro*. Microscopy studies found that the *comC1* variants had denser biofilms, suggesting that CSP-1 may play a more significant role in biofilm formation than CSP-2.

Pneumococci have evolved and retained complex inducible autocompetence and autotransformation mechanisms that facilitate the ability to readily exchange DNA. Advantageous traits that provide a substantial survival benefit to pneumococci can be readily distributed within a biofilm community. Horizontal gene transfer (e.g., exchange of antibiotic resistance genes) with other strains and species is a major mechanism of creating genetic and phenotypic diversity in bacterial populations. High-density pneumococcal cultures that experience reduced nutrient availability and will undergo autolysis, resulting in an increase of extracellular DNA in the environment, which can directly promote genetic exchange. Interestingly, pneumococcal strains encoding *comC1* were found to be more transformable [43]. Thus, autoinduction of competence and autotransformation systems may promote horizontal gene transfer in biofilms during periods of stress and can facilitate rapid adaptation to new environmental conditions.

The Com and LuxS/autoinducer-2 (AI-2) quorum-signaling/sensing systems regulate virulence, persistence in murine carriage, and early biofilm formation on abiotic surfaces [10,26,34]. One study found that LuxS can affect early biofilm assembly on abiotic surfaces via regulation of two major virulence genes: *lytA* (autolysin) and *ply* (pneumolysin) [26,34,45]. The enzyme *S*-ribosylhomocysteine lyase (LuxS) produces the quorum signal homoserine lactone AI-2, a metabolic by-product of the activated methyl cycle [46,47]. Another molecule in the activated methyl cycle is *S*-adenosyl-L-methionine (SAM), which donates a methyl group to methionine recycling, biosynthesis of AI-2, and other reactions [48]. Studies using quorum signal inhibitors have been successful in inhibition of biofilm formation *in vitro*. Sinefungin, a natural nucleoside and a structural analog of SAM, is known to have antiviral, antifungal, and antiprotozoal activities [48]. Growth of pneumococci in the presence of sinefungin resulted in a decrease in biofilm biomass, total bacteria counts, AI-2 production, and *luxS* gene expression. However, there was little effect against established biofilms, suggesting this inhibitor is only effective against initial attachment and early biofilm assembly.

BIOFILM FORMATION BY OTOPATHOGENS

A large body of work over the past 15 years has demonstrated that all of the major pathogens associated with OM have the capacity to form biofilms. In a number of studies from our laboratory and others, determinants of biofilm formation, maturation, and persistence have been identified. As in other bacterial infections, the contribution of biofilms formed by these species to antimicrobial resistance has also been demonstrated [25].

EVIDENCE FOR BACTERIAL BIOFILMS IN OM

Biofilms are important to many persistent infections and have been proposed to be involved in some manner in the majority of bacterial infections [49]. The first microscopic evidence of an association between biofilms and chronic disease was shown with *Staphylococcus aureus* in a chronic wound model of infection [50]. Interestingly, biofilms are associated with many chronic diseases (e.g., chronic sinusitis, cystic fibrosis, Legionnaire's disease, and chronic OM), representing up to 80% of all infections [51]. The idea that biofilms contribute to OM infection was first advanced by Bill Costerton, based on the chronic and recurrent nature of the infection [3] (Figure 16.1).

The first strong evidence for a role for a persistent, non-growing, or slowly growing microbial community in most of these cases was provided by Garth Ehrlich and colleagues in a series of landmark papers [52–57]. In these studies, bacteria and/or bacterial components were detected using PCR-based molecular methodology in the vast majority of samples from patients with chronic/recurrent OM. In later work, opportunists associated with OM were shown to form and persist within microbial communities within the middle-ear chamber in the chinchilla animal infection model (Figure 16.2) [47,58]. Microbial communities were directly observed using confocal laser scanning microscopy with fluorescent probes on tissue samples from patients undergoing tympanostomy for recurrent OM infections [59]. Additionally, polymicrobial biofilms containing pneumococcus, *Haemophilus influenzae*, and *Moraxella catarrhalis* have been found to various degrees in association with adenoids and mucosal epithelia in children with recurrent or chronic middle ear infections and chronic rhinosinusitis [47,60]. Another study

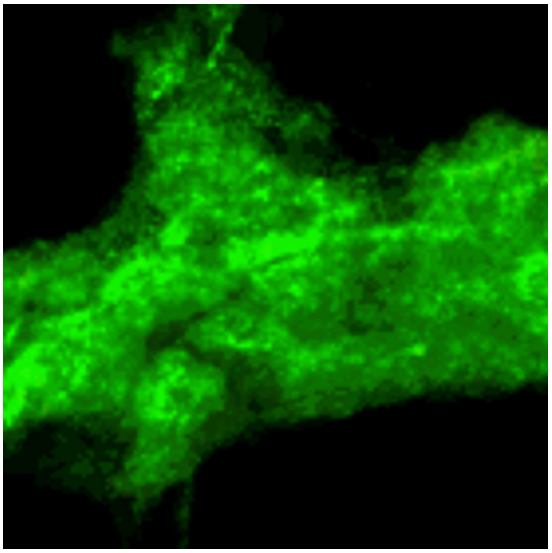

FIGURE 16.1 **Pneumococcal community from an experimentally infected chinchilla.** Animal was infected with *S. pneumoniae* strain TIGR4 for 24 h, and then euthanized. The exudate material within the middle ear chamber was cryosectioned and stained with a polyclonal rabbit antiserum and fluorescent secondary antibody, and visualized by confocal laser scanning microscopy.

assessed middle ear mucosal biopsies for otopathogens in children with chronic OM or OM with effusion and found pneumococcal biofilms formed on, as well as intracellularly in, the middle ear mucosa [61].

Bacterial biofilms promote persistence during recurrent or chronic OM infections [62]. Experimental OM infections in the chinchilla animal model revealed that many bacterial pathogens form biofilms in the middle ear spaces [63,64]. In one study, chinchillas were infected via the transbullar route with pneumococcal strain TIGR4 [47]. Several days later, middle ear tissue was excised, cryosectioned, and examined via immunofluorescent staining of surface-associated proteins. This experiment clearly showed that pneumococci could form biofilms in the middle ear of chinchillas. Our laboratory has recently adapted a BALB/c mouse animal model of experimental OM.

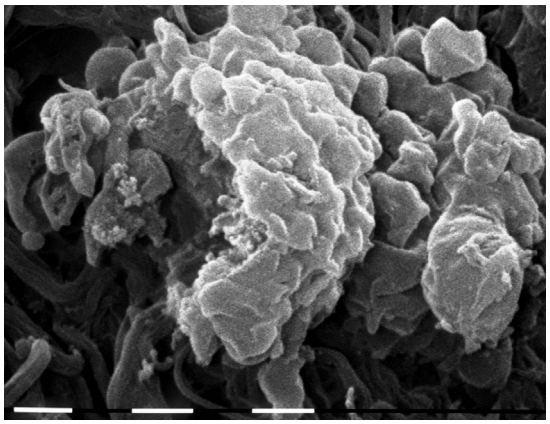

FIGURE 16.2 Scanning electron microscopy image of pneumococcal biofilm from an experimentally infected chinchilla.

Following intranasal infection, we can detect bacteria in the middle ear [40]. Although exciting, all the studies in the mouse and chinchilla animal models used the well-studied laboratory strains TIGR4 and EF3030. TIGR4 is highly virulent and causes bacteremia in mice [9], while EF3030 is well adapted for colonization and rarely causes invasive disease. We wondered whether the capacity of pneumococci to cause OM was broadly applicable to many strains. Preliminary studies have revealed that clinical strains from children with OM can colonize mice as well as EF3030 and ascend to the middle ear spaces (unpublished observation).

INTRODUCTION TO OM

OM, or inflammation of the middle ear, is a very common clinical diagnosis in young children. It is the leading cause for physician visits, with nearly 80% of children experiencing at least one episode by 3 years of age [65]. It is also a leading reason for antibiotic prescriptions [66] and represents an annual economic burden of direct and indirect costs of $3–5 billion in the United States [67]. OM infections can be

subdivided into two general types: acute otitis media (AOM) or otitis media with effusion (OME). AOM is the predominant type; it is characterized by rapid onset of purulent middle ear effusion behind a bulging tympanic membrane, with symptoms of fever, otalgia, otorrhoea, and/or irritability. OME is characterized as the presence of middle ear effusion without signs or symptoms of acute infection. Parents and clinicians can even miss diagnosis since some symptoms are not outwardly apparent. This is worrisome because impaired hearing due to OM can have serious socioeconomic implications, such as contribution to conductive hearing loss, which can affect a child's behavior, education, and development of language skills [52,57,67]. Conventional microbiology has depended on Koch's postulates to identify a single organism as the etiological agent of infectious disease. Unfortunately, most chronic OME infections have long been recognized as "culture-negative" cases for which no microbe could be readily cultured from effusions or other clinical samples. Novel molecular-based diagnostic tests, particularly the PCR-based test, can detect bacterial genetic material in culture-negative samples, leading to more accurate diagnoses [53].

OM arises when viruses or bacterial opportunists that reside in the nasopharynx infect the middle ear chamber via the eustachian tube. The eustachian tube is an anatomical structure that connects the back of the nose to the middle ear; it serves two main functions: First, it helps equalize the air pressure in the middle ear spaces to that of outside air. Second, it permits drainage of fluid built up inside the middle ear. Equalization of air pressure can insufflate nasopharyngeal microorganisms into the middle ear spaces, which can colonize the middle ear mucosa, establish an infection, and evade the mucociliary immune responses, resulting in inflammation. Eustachian tube dysfunction can also contribute to OM infections. Blockage or alteration of function can arise due to pollutants, allergies, or nasal infections caused by viruses. This can lead to fluid buildup, improper fluid drainage, and retention of infectious agents and inflammatory mediators in the middle ear. Young children often have recurrent or chronic AOM or OME. One explanation for the recurrent nature of this type of infection is that in young children it could be physiological. In children, the eustachian tube anatomy is more lateral than that of an adult, which can permit nasopharyngeal opportunists to more easily access to the middle ear spaces.

The most commonly isolated organisms from OM specimens are pneumococci, nontypeable *H. influenzae* (NTHi), and *M. catarrhalis* [62,68]. Accordingly, clinicians tend to prescribe antimicrobial agents that are effective against all three species. β-lactam antibiotics (e.g., amoxicillin and penicillin) are the most commonly prescribed medications [66]. However, overprescription and excessive use of antibiotics for the treatment of AOM in children not at risk of developing complications has promoted the emergence of antibiotic-resistant strains, further complicating treatment. Although somewhat effective, antibiotic treatment alone does not always clear infections that cause chronic OME. This is because chronic OME is considered a biofilm-mediated disease, whereby the established biofilm sloughs off free-floating planktonic cells that cause a low but constant state of infection and inflammation. If the infection worsens, fluid builds up behind the tympanic membrane due to impaired drainage, causing pain. Chronic OME is usually treated with surgical insertion of a tympanostomy tube.

Polymicrobial OM Infections

Recent studies have found that OM and other upper respiratory tract infections can be caused by co-infection by more than one pathogen [69,70]. Pneumococci and NTHi are

leading causes of bacterial AOM, accounting for 60–70% of clinical episodes, and are often found together in the context of polymicrobial biofilms [62,68]. Prior work from our group and others has demonstrated that biofilms are an important component in the course of OM disease. One study found that AOM caused by pneumococcus alone was more severe than AOM caused by a polymicrobial infection with NTHi [70]. This study also found an association between serotype and disease severity. Virulent serotypes (e.g., 19A) tended to cause more serious cases of AOM, while less virulent serotypes were often found in polymicrobial infections with NTHi. The interspecies interactions or molecular mechanisms that can explain this observation are not currently clear. What is clear is that polymicrobial biofilms can provide a huge benefit to pneumococci. *In vivo* studies using the chinchilla animal model found that pneumococcal biofilm formation and persistence in OM are promoted during co-infection with NTHi [63]. Additionally, many clinical strains of NTHi and *M. catarrhalis* can produce β-lactamase, an enzyme that breaks down β-lactam antibiotics. Recent studies of polymicrobial biofilms have determined that NTHi and *M. catarrhalis* can passively protect pneumococci from amoxicillin treatment *in vitro* and *in vivo* in a β-lactamase-dependent manner [16,18,25,63].

It is well documented that the incidence of pneumococcal disease coincides with influenza A virus (IAV) infections. The IAV replicates in the upper respiratory tract epithelium and alters the mucosal surface in a way that is mutually beneficial for both the virus and pneumococci. Specifically, the virus produces an enzyme called neuraminidase that specifically cleaves eukaryotic terminal sialic acid moieties from host respiratory glycoconjugates, permitting the virus to access the cell surface for infection and replication. The cleaved sialic acid serves as a source of nutrition for pneumococci, and the asialyl moieties serve as new binding sites. Most pneumococcal strains encode several exoglycosidases that can sequentially act to cleave glycosyl moieties for increased adherence [35]. Epidemiological studies have found a strong association between co-infection with IAV and increased pneumococcal ascension of the eustachian tube to cause middle ear infections. Recent work from our lab has assessed the contribution of IAV co-infection to pneumococcal pathology and how viral predisposition affects middle ear infections by pneumococcal subpopulations [40]. Our studies found that, following an IAV infection, the bacterial burden and inflammatory response in the middle ear is increased in co-infected mice [40]. Histopathology staining of ear sections from mice infected with pneumococcus alone revealed that colonization may not be as asymptomatic as previously thought.

Recent work from our lab also investigated the role of pneumococcal phase variation in the context of co-infection with IAV. Enriched populations of opaque- or transparent-phase variants were assessed for their ability to form biofilms *in vitro* as well as their capacity to colonize and cause disease in our experimental OM mouse model of infection. Conventionally, strains in the opaque phase are thought to be better fit to cause disease than strains in the transparent phase. Our studies found that the opaque phase had decreased biofilm viability, decreased adherence to epithelial cells, and decreased capacity to colonize the mouse nasopharynx. Interestingly, following IAV co-infection, both phase variants were able to colonize the nasopharynx and cause middle ear infections at a similar level [40]. Therefore, co-infection with IAV was independent of phase variation. Although this is in contrast to what other studies have found, it is important to note that those studies used more invasive strains derived from blood isolates. We attribute the difference in our findings to our murine infection model strain, EF3030. This strain is uniquely adapted to colonize the

nasopharyngeal mucosa without causing invasive disease, persisting for at least 30 days (unpublished observation). Thus, the distinction between the opaque and transparent phases of colonizing strains may differ from invasive strains. Encouragingly, future phase variation research may be more definitive since the molecular basis of phase switching has been described [38]. Together, these results reveal that polymicrobial infections can increase pneumococcal disease burden in our experimental mouse model of infection in a phase-independent manner. Our findings are also consistent with clinical and epidemiological data indicating that most OM infections are polymicrobial in nature.

SUMMARY

Bacterial biofilms are well designed to evade antimicrobial treatment, changing environmental conditions, and host immune responses. These bacterial communities are associated with many persistent and chronic diseases. The very common pediatric disease OM represents a good example of a chronic infection. Following eustachian tube dysfunction, bacterial opportunists that reside in the nasopharynx can ascend to the middle ear spaces and establish colonization of the middle ear mucosa. Pneumococci are one of the most frequently isolated organisms from OM ear effusions, highlighting the need to develop more efficacious therapeutic interventional and preventive strategies against this significant human pathogen. Understanding how pneumococci can escape immune responses during middle ear infections is an important area that should be studied. Some studies have found that pneumococci can reside intracellularly in the middle ear mucosa, which presents a problem for efficient bacterial eradication. Successful treatment of OM infections may be challenged by the increased biofilm forming capacity of pneumococci. Additionally, the ever-increasing distribution of antibiotic-resistant genes among bacterial populations and the passive antibiotic protection afforded to pneumococci by other otopathogens further complicate treatment.

Future vaccine development may be fundamental in decreasing the incidence of bacterial OM and disease burden it causes. Recent work in pneumococcal biology has discovered hybrid serotypes that may be useful as vaccine candidates [37]. Inclusion of hybrid serotype capsular polysaccharides could decrease the number of serotypes included in the vaccine composition, yet target multiple serotypes. Alternatively, a protein-based vaccine that targets conserved epitopes in pneumococci, *H. influenzae*, *M. catarrhalis*, and even common upper respiratory tract viruses may be useful. A good candidate epitope would be a protein that is conserved in both the planktonic and biofilm phenotypes. Another good target would be proteins involved in sugar and arginine metabolism that are up-regulated in pneumococcal biofilms [23]. Since quorum-signaling/sensing systems play a huge role in the regulation of many proteins involved in pneumococcal biofilm formation, inhibition of quorum signals may be useful. Studies using quorum signal inhibitors have been successful in dispersing biofilms and contributing to inhibition of biofilm maturation *in vitro* [48]. Ideally, future therapeutic strategies will focus on prevention of bacterial biofilm assembly, maturation, and dispersal.

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CHAPTER

17

Pneumococcal Pili and Adhesins

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INTRODUCTION

Pneumococcal carriage and infection is a complex interplay between pathogen- and host-specific factors. *Streptococcus pneumoniae* utilizes numerous strategies to colonize and conquer many different niches in the human body. Secreted and surface-attached proteins play a crucial role in pneumococcal pathogenesis. They facilitate pneumococcal adhesion to, invasion into, and transmigration through host tissues and matrices; sequester complement regulatory proteins for immune evasion; exhibit toxic activity; protect against oxidative stress; transport nutrients and ions; and are involved in competence, biofilm formation, and quorum sensing [1–7]. Importantly, during establishment and transition from asymptomatic carriage to invasive pneumococcal disease, certain virulence factors are differentially expressed and therefore contribute to the success of *S. pneumoniae* in distinct locations in the human host [8–10]. *S. pneumoniae* expresses the intracellular toxin pneumolysin, which is released and elicits multiple functions in the human host, and possesses several surface-exposed proteins and high-molecular-weight pili appendages that perform a variety of physiological functions and can also serve as virulence factors.

CLASSIFICATION AND DISTRIBUTION OF PNEUMOCOCCAL SURFACE-EXPOSED PROTEINS

The pneumococcal cell envelope is decorated with proteins that differ in their modular structure, function, and export and

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surface-attachment mechanism. Five main groups of surface proteins are distinguished in *S. pneumoniae*: (1) transmembrane proteins; (2) membrane-anchored proteins, the so-called lipoproteins; (3) peptidoglycan-anchored proteins; (4) noncovalently attached choline-binding proteins (CBPs); and (5) non-classical surface proteins (NCSPs) (Figure 17.1).

Transmembrane proteins consist of a cytoplasmic domain, one or more transmembrane domain(s), and an extracellular domain. They are involved mainly in substrate transport (nutrients, ions, metabolites, peptide pheromones), energy metabolism, and antibiotic resistance [4,11–14]. In addition, transmembrane proteins, such as two-component regulatory systems (TCSs) and eukaryote-like Ser/Thr protein kinases (StkP), sense and transduce environmental and nutritional signals, thereby regulating the pathogen's stress response, competence, and expression of virulence determinants [15–18]. Two-component systems are evolutionarily highly conserved signal transduction pathways; they are usually composed of a membrane-bound sensor histidine kinase (HK) and a cytoplasmic cognate response regulator (RR) [19]. RRs can elicit multiple functions. They either regulate gene expression through their activity as DNA-binding transcriptional regulators or by interacting with downstream signaling molecules, or they control protein function through direct protein–protein interaction [19,20]. In *S. pneumoniae*, 13 HK:RR pairs and one unpaired ("orphan") RR have been identified so far. The adaptation of *S. pneumoniae* to different sites of the human body during carriage and infection is strongly associated with virulence gene expression regulation by TCSs. The TCSs TCS02 (VicKR, WalKR), TCS04 (PnpRS), TCS05 (CiaRH), TCS09 (ZmpSR), TCS12 (ComDE), TCS13 (BlpRH), and the orphan RR (RR14, RitR) have been shown to be directly involved in pneumococcal pathogenesis [15]. Importantly, TCS effector functions can be pleiotropic, strain-specific, and dependent on the infection site; TCS pathways can also influence each other by functional cross talk [15,21]. For instance, *pneumococcal surface protein A* (*pspA*) was demonstrated to be regulated *in vitro* by both TCS02 and TCS06, which also regulate the expression of *pneumococcal surface protein C* (*pspC*) [22,23]. In addition, *tcs* networks might also be influenced by other transcriptional regulators such as CcpA, a key regulator of carbohydrate metabolism, and SmrC, a putative regulator of *pneumococcal phosphorylcholine esterase pce* (also referred to as *cbpE*) [24,25]. Interestingly, the orphan RR, RitR, which regulates the expression of the *piu* iron uptake operon and is involved in oxidative stress response and sugar metabolism, was recently shown to be phosphorylated by the membrane-bound Ser/Thr protein kinase (StkP), providing new insights into regulatory networks in *S. pneumoniae* [26,27]. However, the impact of two-component systems on pneumococcal virulence observed under *in vitro* and *in vivo* conditions still needs further investigation. For instance, the recently identified *pneumococcal adhesin PavB* (*pneumococcal adherence and virulence factor B*) was found to be in close proximity to the downstream-located *tcs08*, and might be regulated by this *tcs*, which could explain the observed attenuation of a Δ*tcs08* mutant in a mouse respiratory tract infection model [28,29].

Lipoproteins possess an N-terminal exportation signal peptide (LX_1X_2C) (Figure 17.1), also referred to as lipobox, which is recognized by the signal peptidase II (Lsp). After extracellular translocation, the indispensable cysteine residue of the lipobox is covalently linked to diacylglycerol in the cytoplasmic membrane through the action of the lipoprotein diacylglyceroltransferase (Lgt), and thereafter the signal peptide is cleaved off by the lipoprotein signal peptidase (Lsp) [30]. Most of the identified lipoproteins in *S. pneumoniae* have important roles in physiological processes such as substrate uptake, signal transduction, antibiotic and oxidative stress resistance, and protein folding or activation. In addition, lipoproteins

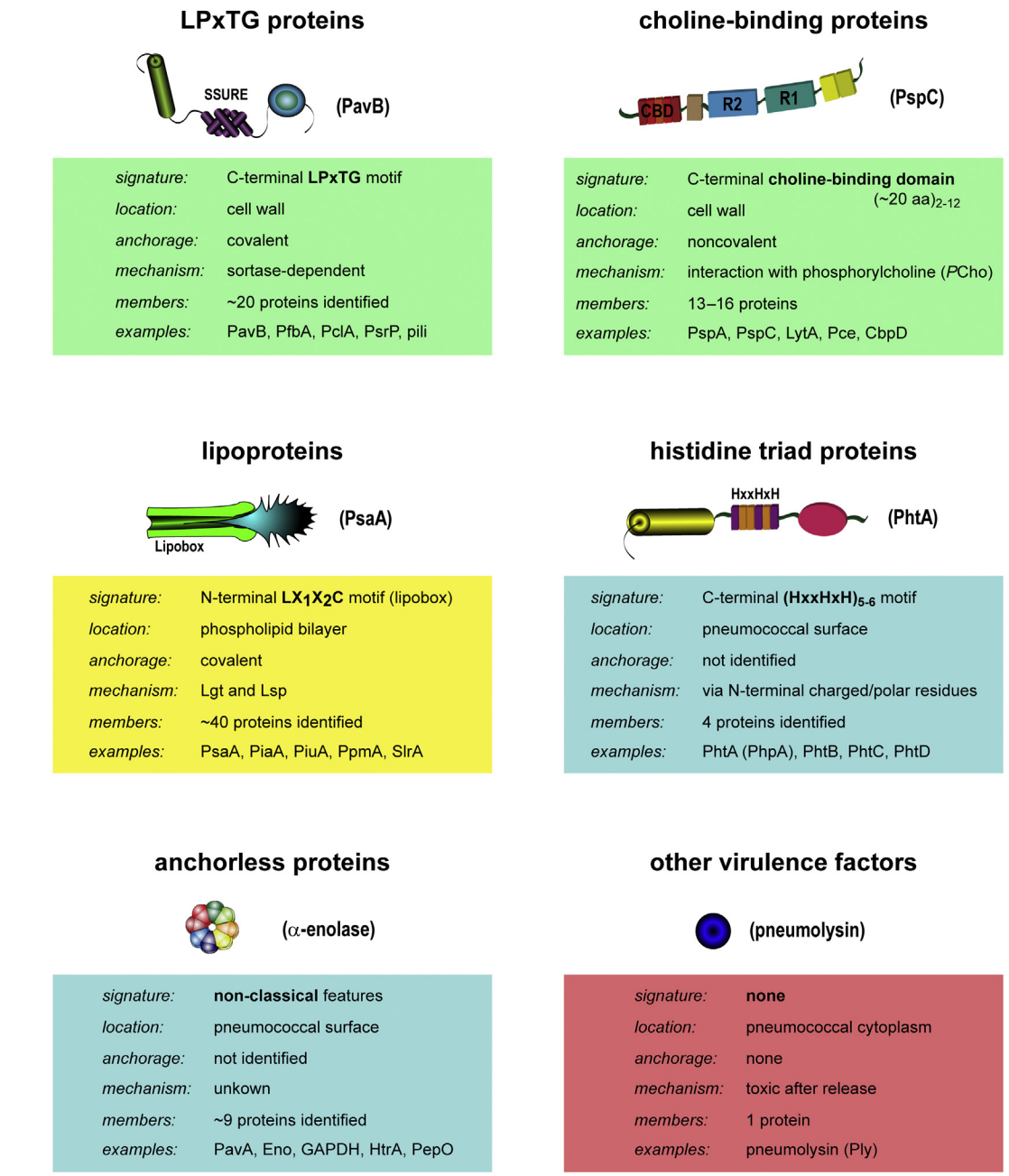


FIGURE 17.1 Classification and distribution of pneumococcal surface proteins.

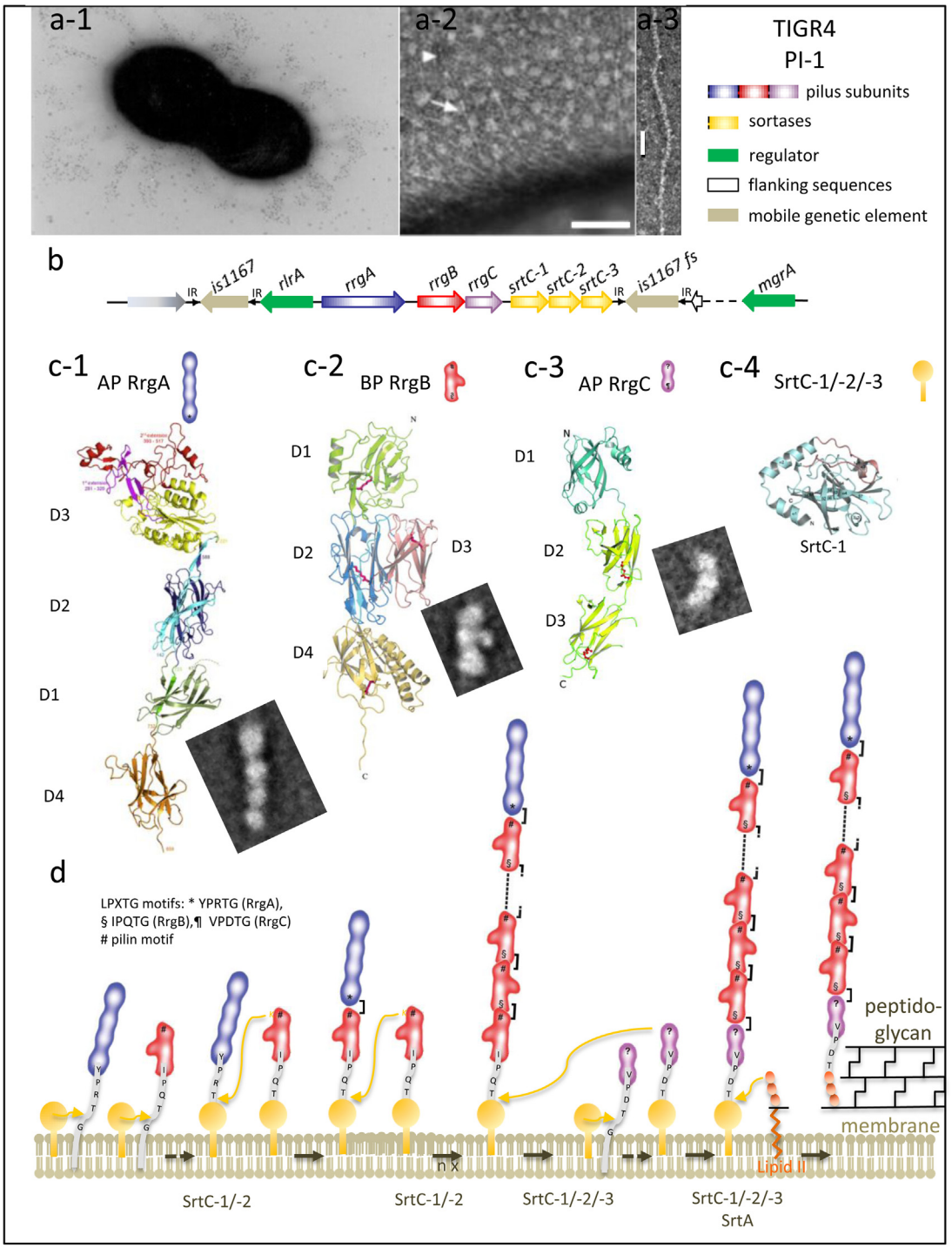
contribute directly to pneumococcal virulence by promoting colonization, invasion into, and survival in the bloodstream, such as the peptidyl-prolylisomerases SlrA and PpmA, as well as the thiol-disulfide oxidoreductases Etrx1 and Etrx2 [6,30–32]. The highly conserved pneumococcal surface adhesin A (PsaA) is a metal ion-binding (Mn^{2+} and, with lower affinity, Zn^{2+}) lipoprotein belonging to an ATP-binding cassette (ABC) transporter system encoded by the *psaBCAD* operon and controlled by the PsaR regulon and TCS04 [33–35]. In addition to its metal ion-binding activity, PsaA was associated with pneumococcal tolerance to penicillin and was demonstrated to interact with E-cadherin, the major determinant of epithelial barrier integrity [36,37] (Figure 17.4B).

Sortase-anchored proteins contain a classical N-terminal signal peptide and a C-terminal cell wall sorting signal composed of an LPxTG motif ("x" is any amino acid [aa] residue), followed by a hydrophobic membrane-spanning stretch of approximately 20 aa and a short positively charged tail (Figure 17.1). After translocation and cleavage of the signal peptide the immature protein is retained in the cytoplasmic membrane via its C-terminal hydrophobic stretch. Sortase A recognizes and cleaves the LPxTG motif between threonine and glycine, generating an enzyme–protein intermediate, which is attacked by the pentapeptide bridge of lipid II, a peptidoglycan biosynthesis precursor. The resulting lipid II-protein precursor is then incorporated into the peptidoglyan envelope by penicillin-binding proteins to generate a mature surface-anchored protein [38]. Sortase enzymes are classified into six distinct subfamilies [39]. Sortase A belongs to class A sortases, which are membrane-bound housekeeping sortases present in all Gram-positive bacteria, linking surface proteins covalently to the cell-wall peptidoglycan [38,39]. *S. pneumoniae* possesses an arsenal of sortase-anchored lytic enzymes, such as hyaluronidases (SpnHL), glycosyl hydrolyases (neuraminidase A, NanA, β-galactosidase, BgaA, Exo-β-*N*-acetylglucosaminidase, StrH, pullulanase, SpuA, endo-β-*N*-acetylglucosaminidase, EndoD, and Endo-α-*N*-acetylgalactosaminidase, Eng), and proteases (zinc metalloproteases, serine protease PrtA), with a broad substrate specificity enabling the degradation of human glycoproteins and glycosaminoglycans present in body fluids and cellular surfaces [31,40,41]. This can unmask potential binding sites for the pneumococcus, promoting colonization; cause direct damage to the human host, facilitating pneumococcal tissue invasion and dissemination; and aid in pneumococcal evasion of the human immune response [31,42,43]. In addition, several LPxTG-anchored surface proteins have been implicated in pneumococcal adhesion and colonization, such as PavB, pneumococcal collagen-like protein A (PclA), plasmin- and fibronectin-binding protein A (PfbA), and BgaA, as well as biofilm formation, such as the pathogenicity island (PI)-encoded pneumococcal serine-rich repeat protein (PsrP) (Figure 17.4B) [28,44–47]. Importantly, class C sortases catalyze the assembly and anchorage of pili, which are large multi-subunit hair-like fibers on the surface of *S. pneumoniae* and other Gram-positive bacteria [38,39,48]. In pneumococci, pilus-associated sortases are encoded in an operon together with the pilus subunits within PIs; two pilus-encoding PIs have been identified so far [49–51]. Both pilus types have been implicated in pneumococcal pathogenesis, since they protrude from the polysaccharide capsule, facilitating long-distance adhesion to host structures such as cellular receptors and proteins of the extracellular matrix (ECM) [49,50,52].

Two characteristic features are unique to *S. pneumoniae*: its nutritional dependence on choline and, associated with this, the expression of CBPs [53]. Choline, taken up from the environment, is converted to phosphorylcholine (*P*Cho) and incorporated into teichoic acid chains, which are then translocated across the

cytoplasmic membrane [54,55]. On the pneumococcal surface, *P*Cho residues serve as anchors for CBPs and are often required for the functional activity of peptidoglycan hydrolases, representing key enzymes in cell-wall homeostasis [53,56–58]. CBPs consist of a choline-binding domain (CBD), which is composed of at least two repetitive units of approximately 20 aa residues [31]. The CBD is mainly located at the C-terminal end, with the exception of the peptidoglycan hydrolases LytB and LytC, and is sometimes preceded by a proline-rich linker region, as in PspC and PspA [31,59–62]. Functional diversity of CBPs in *S. pneumoniae* is based on the biologically active domain; depending on the strain, pneumococci express a variable number of CBPs (Figure 17.1) [31,53,61]. Several CBPs have been implicated in pneumococcal virulence, including the cell-wall hydrolases LytA and LytC, Pce, PspC and PspA, and the CBPs CbpD and CbpG (Figure 17.4B) [31,53,63]. The murein hydrolases LytA, LytC, and CbpD are responsible for pneumococcal autolysis and fratricide, resulting in release of highly inflammatory cell-wall degradation products, intracellular toxins, and DNA. This promotes inflammation and damages tissue barriers, facilitates dissemination of pneumococci within the human host, and allows for the transfer of genes (e.g., antibiotic resistance and virulence genes) in a bacterial community [9,64–68]. Pce (CbpE) removes *P*Cho located at the ends of surface-exposed teichoic acid chains. It is proposed that Pce liberates only those *P*Cho residues relevant for binding of C-reactive protein (CRP) for immune evasion, without affecting *P*Cho residues important for the attachment and activity of CBPs as well as for the interaction with the PAF receptor, facilitating pneumococcal adhesion and invasion [69]. PspC is regarded as one of the most important adhesins in *S. pneumoniae*; also it is designated as *S. pneumoniae* secretory IgA (sIgA)-binding protein (SpsA), choline-binding protein A (CbpA), or factor H-binding inhibitor of complement (Hic), reflecting its various biological activities. The PspC family is highly polymorphic, but its members share a common modular organization: a signal peptide of 37 aa residues, the functional N-terminal domain, a proline-rich linker region, and the C-terminal surface-anchoring domain. Over 40 *pspC* alleles have been identified so far, divided into 11 major subtypes in two different subgroups. The classical PspC proteins (subtypes 1–6) possess a CBD and constitute subgroup 1. The second subgroup is represented by atypical or PspC-like proteins (subtypes 7–11), such as Hic (PspC11), which contain a C-terminal sorting signal and are covalently linked to the peptidoglycan [60]. Either one or two perfect copies of an approximately 110-amino-acid-long α-helical domain, referred to as repeat domains 1 and 2 (R1 and R2), can be found in classical PspC proteins [60,70]. Each R domain consists of three anti-parallel, slightly angled α-helices, which adopt a raft-like structure [70].

The surface of *S. pneumoniae* is further decorated with proteins that do not possess classical secretory and anchoring motifs. These NCSPs are often enzymes of metabolic pathways which exert additional biological functions ("moonlighting"). To date, eight pneumococcal surface-exposed enzymes with adhesive functions and significant impact on pneumococcal virulence have been described, including enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the serine protease HtrA, as well as the recently identified proteins NADH oxidase (NOX), phosphoglycerate kinase (PGK), and pneumococcal endopeptidase O (PepO) (Figures 17.1 and 17.4B) [71–76]. Another NCSP contributing to pneumococcal pathogenesis is the pneumococcal adherence and virulence factor A (PavA) (Figure 17.1). PavA was first identified as an adhesin for immobilized fibronectin *in vitro* (Figure 17.4B) and has recently been shown to prevent pneumococcal phagocytosis by dendritic cells, negatively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LPXTG motifs: * YPRTG (RrgA),
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affecting the development of an adaptive immune response [77,78]. In addition, PavA is essential for colonization of the upper respiratory tract and progression to invasive disease in mouse infection models [79,80]. However, it is suggested that PavA modulates expression or function of important virulence factors rather than being directly involved in adherence and invasion [80].

MOLECULAR ARCHITECTURE AND ASSEMBLY OF PNEUMOCOCCAL PILI AS UNIQUE CELL WALL–ANCHORED COVALENT POLYMERS

Various types of long, filamentous surface appendages, pili (Latin for "hairs") have been identified in Gram-negative and Gram-positive bacteria as fulfilling manifold functions during bacterial life cycles (such as host cell invasion, biofilm formation, cell aggregation, DNA transfer) [81]. Importantly, their role as adhesive organelles is crucial to the survival of pathogenic bacteria, which must attach to specific host cells and ECM proteins for colonization and to establish an infection. While many pili in Gram-negative bacteria, which are typically formed by noncovalent interactions between pilin subunits, have been studied in detail over the last decades, the majority of Gram-positive pili, although first observed in *Corynebacterium renale* in 1968, have been discovered only recently [81–84]. Pioneering studies on Gram-positive pili of *Corynebacterium diphtheriae* were initiated by Schneewind and coworkers [85]. In contrast to Gram-negative pili, they are surface polymers assembled from covalently cross-linked pilin subunits and attached to the cell-wall peptidoglycan.

Studies of the major human pathogen *S. pneumoniae* have revealed two types of sortase-assembled pili important for host–pathogen interaction and adhesion. The main pilus (pilus-1) was first described in 2006 and found to be virulence-related in a clinical serotype 4 pneumococcal strain [50]. In 2008, a second pilus (pilus-2) was discovered in pneumococci [49]. Pilus-1 and -2 are encoded by so called PIs and protrude from the bacterial surface as long, polymeric structures (Figures 17.2A and 17.3A), strengthening the

FIGURE 17.2 **Electronmicroscopic visualization, genome organization, and assembly of pilus-1.** (A) *S. pneumoniae* pilus-1: (A-1) Identification of pilus-1 by immunogold electron microscopy (EM) of major pilin RrgB in pneumococcal strain TIGR4 (applying mouse polyclonal anti-RrgB antiserum). (A-2) Negative stain transmission EM (TEM) of TIGR4 bacterial surface, illustrating variability in pilus number and preparation-dependent width variation (arrow and arrowhead, respectively); (A-3) TEM image of isolated individual TIGR4 pilus (negatively stained). Scale bars: 100 nm. (B) PI-1 genomic region of *S. pneumoniae* strain TIGR4: schematic representation of PI-1 genetic elements encoding proteins with different functional roles. (C) TIGR4 pilus-1 building blocks (ancillary proteins APs [RrgA and RrgC], backbone protein BP [RrgB]) and PI-1 encoded sortases (SrtC-1–3); schematic drawings are given for easier assignment in pilus assembly model. (D) Ribbon diagrams of pilins (C1–C3) indicate subunit domain organization: Two RrgA extended arms and an integrin I-like domain, important for host interaction, are shown in red/magenta and yellow, respectively (C-1). Intramolecular isopeptide bond stabilization occurring in RrgA, RrgB, and RrgC is depicted in red for RrgB domains D1–D4 (C-2) and RrgC domains D2/D3 (C-3). Ribbon diagram of SrtC-1 (C-4): Access to the active site is controlled by a flexible lid (shown in pink). EM of purified recombinant pilus constituents RrgA, RrgB, and RrgC (negative stained, TEM averages) are shown for comparison (C-1–C-3). (D) Assembly model of TIGR4 heterotrimeric pilus-1: Sec pathway secreted pilins (RrgA, RrgB, RrgC) are retained in the membrane and processed by membrane-bound sortases. Pilin-specific sortases cleave sorting signals of BP RrgB and AP RrgA between T/G of the respective motif, resulting in an acyl-enzyme intermediate. AP-BP intermolecular isopeptide bond formation is generated by the nucleophilic attack of a lysine side chain residue of the RrgB pilin motif. Polymerization of pilus-1 is catalyzed by the addition of further BP RrgB subunits. Pilus-1 assembly is terminated by sortase-mediated incorporation of an anchor AP RrgC. The assembled pilus-1 is finally covalently attached to the pneumococcal cell wall via a lipid II intermediate. Described sortase activities involved in individual steps of pilus-1 assembly are stated. *Source: Adapted from [49,87–89]. Ribbon diagrams C-1–C-4 are kindly provided by Drs. A. M. di Guilmi and A. Dessen.*

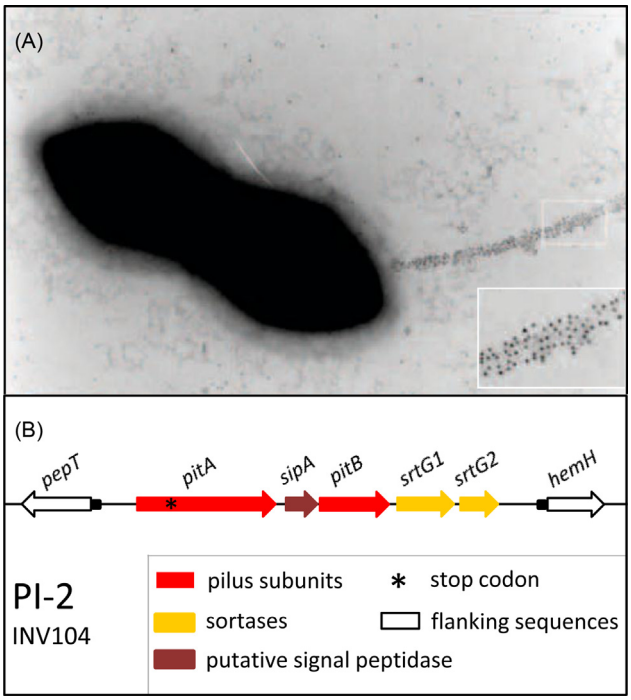


FIGURE 17.3 Electron microscopic visualization and genome organization of *S. pneumoniae* pilus-2. (A) Identification of pilus-2 by immunogold-EM of major pilin PitB in pneumococcal strain PN110 (applying mouse polyclonal anti-PitB antiserum). Inset displays an enlarged part of the surface appendage. (B) PI-2 genomic region of *S. pneumoniae* strain INV104: schematic representation of PI-2 genetic elements encoding proteins with different functional roles. *Source: Adapted from [49].*

interaction with the host. Cell wall–anchored pneumococcal pili belong to the class of "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs), that mediate bacterial binding to host serum and ECM proteins, contributing to the highly dynamic process of host–pathogen interaction and modulation of underlying immune responses (see section "Impact of Pneumococcal Adhesins on Carriage and Invasive Disease"). Very recently—in addition to pilus-1 and -2—a Type IV-like pilus structure was discovered in *S. pneumoniae*, with a functional role during natural transformation [86].

Pneumococcal Pilus-1 and Pilus-2 Gene Operons

The main *S. pneumoniae* pilus (pilus-1) is encoded by a 12-kb PI known as rlrA islet or PI-1 [50,51] (Figure 17.2B). PI-1 contains seven genes, of which *rrgA*, *rrgB*, and *rrgC* encode LPXTG-containing pilus subunits; three sortases (*srtC-1*, *srtC-2*, *and srtC-3*) required for pilus assembly; and a positive transcriptional RofA-like regulator, *RlrA*. A transcriptional repressor, *MgrA*, is located outside the PI-1 gene locus. PI-1 is flanked by insertion sequence elements (IS1167) containing inverted repeats that are characteristic of mobile genetic elements, suggesting horizontal gene transfer of pilus-encoding genetic elements potentially deriving from one ancestral region [50,84]. The second pneumococcal pilus (pilus-2) was found in emerging serotypes characterized by a 6.6-kb genetic island (PI-2) flanked by putative insertion sites and containing five genes (Figure 17.3B): *pitA* and *pitB*, encoding pilus-2 subunits; two for sortase-like proteins (*srtG-1* and *srtG-2*); and one for a putative signal

peptidase (*sipA*) [49]. Pili constitute an important factor in pneumococcal colonization; however, occurrence of pili seems to be restricted among the 94 known pneumococcal serotypes. Within a global collection of *S. pneumoniae* isolates, PI-1 was found in approximately 30% overall and in approximately 50% of antibiotic-resistant strains; the frequency of PI-2 is approximately 16% in a global collection of clinical isolates [49,90,91]. The observed spread of certain pilus-containing pneumococcal clones indicates a pilus-related selective fitness advantage. Expression and assembly of pneumococcal pili is a tightly regulated complex process that is intertwined with other regulatory networks existing in the pneumococcus [89,92]. Interestingly, pilus-1 expression was found to be bistable, and data from a mouse model of infection indicate its specific regulation during host colonization [93–96].

S. pneumoniae Pilus Building Blocks—Optimized Structures Guaranteeing Pilus Function and Stability

The discovery of pili in major Gram-positive human pathogens like pathogenic streptococci (*S. pyogenes* [GAS], *S. agalactiae* [GBS], and *S. pneumoniae*) has stimulated intensive research activity to study their role as microbial virulence factors, which in turn proposed the pili structures as novel targets for therapeutic interventions or application in vaccine formulations [84].

PI-1- and PI-2-encoded pilus subunits are assembled into surface-located polymeric structures by specialized sortase transpeptidase activity (see section "Assembly of Pneumococcal Pili by Sortases"). The final localization of individual subunits within the pilus, as, for example, major/backbone pilin (BP) or minor/ancillary pilin (AP) subunits with adhesive or anchor roles, is defined by conserved genetic requirements, such as the pilin motif, the E-Box domain, and the cell-wall sorting signal (LPXTG or variants thereof) [84]. After reporting the first crystal structure of a pilin protein from a Gram-positive *Streptococcus* in 2007, an increasing amount of structural data on pilus building blocks has appeared, leading to novel molecular models of pilus assembly mechanisms and respective functional roles [97]. Pneumococcal PI-1 encodes three structural pilins: BP subunit RrgB, forming the pilus backbone structure, and two AP subunits (RrgA and RrgC), which are involved in host cell adhesion and anchoring the pilus to the bacterial cell wall, respectively (see section "Native Architecture of Pneumococcal Pilus 1"). *S. pneumoniae* TIGR4 RrgB is composed of four domains, designated D1–D4 (Figure 17.2C-2). After structural studies of truncated forms of RrgB (D2-4), a recombinant crystal structure of RrgB encompassing the four domains D1–D4 (residues 30–628) showed that D1–D4 assemble in immunoglobulin (Ig)-like domains, with common CNA-B topology for D1, D3, and D4, while the D2 domain harbors a CNA-A topology [98–101]. Domain stabilization by autocatalytically formed, internal Lys-Asn isopeptide bonds, as shown for the first time in GAS major pilin Spy0128 by Kang and coworkers in 2007, was detected for RrgB D4, D3, and D2 domains [98–102]. Structural investigations of RrgB, including the sorting motif IPQTG (aa 628–632), revealed the presence of a D1-stabilizing isopeptide bond, suggesting an underlying interaction of the C-terminal IPQTG of one RrgB subunit with the N-terminal flexible D1 domain of a successive RrgB subunit as prerequisite for proper domain organization for intramolecular bond formation [100,101]. Importantly, this indicates that certain structural features of pilus-building blocks most likely take place upon interaction of individual subunits during assembly of the polymeric pilus structure and are getting lost from studies using distant and individual recombinant domains. Backbone RrgB

intramolecular isopeptide bond stabilization (like for PI-1 AP) implies a fundamental role in resisting mechanical stress, while pili are involved in host cell attachment. Crystal packing RrgB data revealed a head-to-tail organization involving D4−D1 sortase-mediated intermolecular isopeptide linkage between two consecutive RrgB subunits [101]. A similar RrgB backbone organization was proposed by fitting experiments of the partial RrgB crystal structure into the assembled pilus and confirmed by quantitative electron microscopy techniques studying the architecture of native TIGR4 pili [87,98] (see section "Native Architecture of Pneumococcal Pilus 1"). High-resolution crystal structure of recombinant RrgA adhesin (residues 39−868) of the TIGR4 pneumococcal strain displayed an elongated molecule consisting of four domains (D1−D4) arranged as "beads on a string" [103] (Figure 17.2C-1). Interestingly, residues forming the tertiary structure of D1 and D2 are interspersed throughout the RrgA primary amino acid sequence, leading to a D4-D1-D2-D3 semilinear domain arrangement. C-terminal domain D4, together with D1 and D2, are likely to form a stalk to present the largest domain, D3, at the distal tip of the assembled TIGR4 pilus (see section "Native Architecture of Pneumococcal Pilus 1"). Part of the D3 domain displays high similarity to the eukaryotic collagen-binding integrin I domain, with an associated MIDAS subdomain that, together with neighboring extended arm structures, might mediate specific interactions with the host, and suggests an adhesion mechanism shared with eukaryotic proteins. The RrgA stalk region displays domains reminiscent of IgG-like domains (D1 and D4) and characteristic of CNA-like folds (D2). The elongated, recombinant RrgA molecule is stabilized by two intramolecular isopeptide bonds in domain D2 (Lys^{191}-Asn^{695}) and D4 (Lys^{742}-Asn^{854}). The stabilized RrgA modular composition containing elements of both eukaryotic and prokaryotic systems, structured in a complex fold that shows high similarity to other streptococcal pilus adhesins, suggests a common co-evolved principle to guarantee optimal interaction with the host during microbial infection. Data on clade I and II RrgA variants indicate that sequence diversity caused by immunologic pressure is restricted to the D3 head region, maintaining proper adhesive and functional D3 epitopes conserved, whereas stalk domains D4-D1-D2 are mostly invariant. In both variants, full-length, properly folded RrgA seems to be essential for RrgA-mediated pilus binding abilities [104]. The last component of heterotrimeric TIGR4 pilus-1, the AP RrgC (residues 22−368, with three stabilizing mutations) was described very recently as a rod-like structure containing three independent domains, D1-D3 (Figure 17.2C-3) [105]. IgG-like D2 and D3 are stabilized by two intradomain isopeptide bonds (Lys^{155}-Asn^{252} and Lys^{264}-Asn^{354}, respectively). Sortase-mediated incorporation of cell-wall anchor molecule RrgC at the proximal end of pilus-1 is considered the final step of the assembly process (see sections "Native Architecture of Pneumococcal Pilus 1" and "Assembly of Pneumococcal Pili by Sortases").

PitB was characterized as a major pilin of the pneumococcal pilus-2 (Figure 17.3), polymerization of which requires PI-2-encoded sortase SrtG1 and the signal peptidase-like protein SipA [49]. Further, PI-2 genes, encoding a hypothetical ancillary protein PitA and putative sortase SrtG2, are considered pseudogenes that may have lost the ability to encode functional proteins. Recombinant PitB contains two stabilizing intramolecular isopeptide bonds (Lys^{63}-Asn^{214} and Lys^{243}-Asn^{372}); additional high-resolution structural data on PI-2 gene products are missing as yet [106]. *In vitro* studies using various cell lines suggest that in addition to pilus-1, pilus-2 is also involved in host cell adherence, although potential individual roles during host−pathogen interaction need further investigation [49].

Native Architecture of Pneumococcal Pilus 1

Investigation of native pili (*S. pneumoniae* TIGR4 pilus-1) showed that they form approximately 6 nm wide flexible filaments that can be over 1 μm long (Figure 17.2A). They are composed of RrgB monomers (containing necessary pilin and LPXTG sequence motifs) as implied by immunoelectron microscopy, genetic studies, and *in vitro* RrgB polymerization [50,107–110]. Results from transmission and scanning transmission electron microscopy have provided direct experimental evidence that the native pneumococcal pilus shaft is composed exclusively of covalently linked, slightly overlapping RrgB subunits oriented head-to-tail [87]. One RrgB monomer every approximately 10.2 nm on average was calculated to be incorporated into the pilus backbone, which implies the association of more than 100 RrgB subunits within a thin filament longer than 1 μm. Studies unraveling the localization of AP RrgA and RrgC within the heterotrimeric pneumococcal pilus-1 by immunoelectron microscopy have proposed both local association of RrgA and RrgC to the pilus shaft and their incorporation within the RrgB backbone [50,52,107–109]. Using quantitative electron microscopy techniques with direct visualization of highly specific primary antibodies to locate minor pilins within the pilus backbone at increased resolution has identified RrgA and RrgC position only at the end of the pilus fiber. The structural polarity of the RrgB subunit within the backbone revealed RrgA and RrgC as present exclusively at opposite ends of the pilus shaft, tip and base, respectively, compatible with roles as adhesin and anchor to the cell-wall surface [52,87,105]. An equimolar RrgA/RrgC content is also suggested by LeMieux et al., who found both molecules present in similar quantities in pili of all sizes in cell-wall extracts [107,111]. Additionally, pili formed by a TIGR4Δ*rrgC* genetic background detach more easily from the bacteria, supporting the role of RrgC as anchor to the cell wall [87].

Assembly of Pneumococcal Pili by Sortases

Sortase-mediated Gram-positive pilus assembly has been studied in detail, and general principles are applicable for heterotrimeric pneumococcal pilus-1 (pilus-1 assembly model [Figure 17.2C-4 and D]) [88,112]. PI-2 BP PitB polymerization was described as SrtG1/SipA dependent, although the description of a detailed pilus-2 assembly model needs further investigation [49]. After cytoplasmic expression of the P-1 subunits and Sec-dependent secretion, pilins are anchored to the membrane by C-terminal hydrophobic stretches. Pilus assembly occurs in the extracellular space by sortases that recognize LPxTG cell-wall sorting motifs (or variants thereof) near the C-terminal of individual subunits. Sortase cysteine-transpeptidase activity mediates cleavage between threonine and glycine and catalyzes the subsequent ligation of the new C-terminus to a lysine ε-amino group of the next subunit to be incorporated into the pilus. PI-1, TIGR4-specific sortases SrtC-1, SrtC-2, and SrtC-3 diverge in sequence from the housekeeping sortase (SrtA) and exhibit functional redundancy concerning pilus assembly [48,109,110,113–115]. Polymerization of the major subunit RrgB that contains an IPQTG sorting motif and the conserved DVVDAHVYPKN pilin motif (with the ε-amino group of Lys^{183}) was described as being catalyzed by SrtC-1 and SrtC-2, resulting in covalent intersubunit linkages of the pilus backbone structure [48,101,109,110,113,114] (Figure 17.2D). Incorporation of ancillary protein RrgA at the pilus tip (RrgA sorting motif: YPRTG↔Lys^{183} of subsequent RrgB backbone subunit) is suggested by redundant SrtC-1 and SrtC-2 activity [48,101,109]. Attachment of anchor AP RrgC to the pilus shaft has been proposed for all three sortases, SrtC-1–3 (RrgB CWSS IPQTG↔Lys^{X} of RrgC) [48,101,109,113,115]. The respective RrgC

nucleophilic Lys residue involved in covalent linkage with the most proximal BP RrgB IPQTG sorting motif of the pre-formed pilus-1 is yet to be identified. Very recently, structural studies of pilus-1 anchoring by RrgC to a pneumococcal strain R6 cell wall revealed a major function of the housekeeping sortase, SrtA, with some background activity from SrtC-1 [105]. In addition to SrtA and SrtC-1, an earlier report also proposed SrtC-2 and -3 are involved in pilus-1 cell-wall anchoring and describes a topological role for SrtC-3 in proper pilin presentation at the cell surface [109,113]. Interestingly, structures of SrtC-1−3—unlike non-pilin sortases—all exhibit a flexible lid that shields the active site, implying an underlying activation mechanism by their specific substrates (LPxTG-like motifs of RrgA, RrgB, and RrgC, respectively) for pilus assembly [110,113,114] Figure 17.2C-4. Finally, the pre-assembled heterotrimeric pilus-1 is supposed to be anchored to the pneumococcal cell wall via the AP RrgC by the nucleophilic attack of lipid II at the RrgC−sortase intermediate. Despite a variety of data using different experimental setups, central questions around native pilus-1 and pilus-2 assembly need further investigation (e.g., assembly principles of pneumococcal pili at the membrane, regulation of pilus length, native sortase specificity, and potential redundancy during pilus assembly).

IMPACT OF PNEUMOCOCCAL ADHESINS ON CARRIAGE AND INVASIVE DISEASE

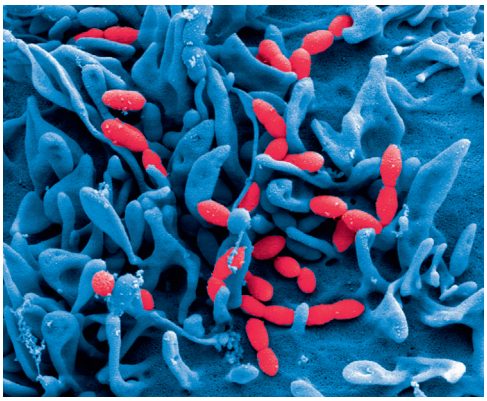
Bacterial surface-exposed proteins with adhesive functions are called adhesins. A general characteristic of adhesins is the targeting of host structures, such as components of the eukaryotic cell membrane, ECM, or body fluids (Figure 17.4). Adhesins mediate either loose attachment or tight binding of bacteria to the host structures. Most Gram-positive bacteria including *S. pneumoniae* express multiple adhesins that bind to either the same or distinct host molecules, and their expression strongly correlates with the adaptation of the bacterium to different host milieus and to different stages of an infection. The interaction of adhesins with host components can modulate host signaling cascades and immune responses, and regulate the expression and secretion of further bacterial virulence determinants, influencing both the course of infection and outcome of disease. Therefore, adhesins have been recognized as promising non-polysaccharide antigen candidates to develop serotype-independent vaccines with broad strain coverage.

Pneumococcal Adhesins Interacting with ECM Components

A prerequisite for successful colonization and progression to invasive disease is the intimate contact between the pathogen and its host. After acquisition, *S. pneumoniae* must surmount several lines of defense of the human body to reach the nasopharyngeal epithelium for colonization and to translocate to the lungs and into other sterile niches in later stages of infection. The "mucociliary escalator" removes inhaled particles like irritants, bacteria, and viruses from airway surfaces; it is composed of a mucus-covered ciliated epithelium. *S. pneumoniae* circumvents mucociliary clearance by expressing surface-associated enzymes that degrade mucosal components, releasing pneumolysin, which distorts ciliary beating, and binding to the ECM of the respiratory epithelium.

S. pneumoniae actively disrupts the mucus layer by expressing surface-associated glycosyl hydrolases (NanA, BgaA, StrH, and Eng) [40,41,116]. These enzymes remove sugar moieties from secreted mucins and other mucosal glycoconjugates to reduce mucus viscosity and to provide nutrients and energy. Membrane-tethered

(A)



(B)

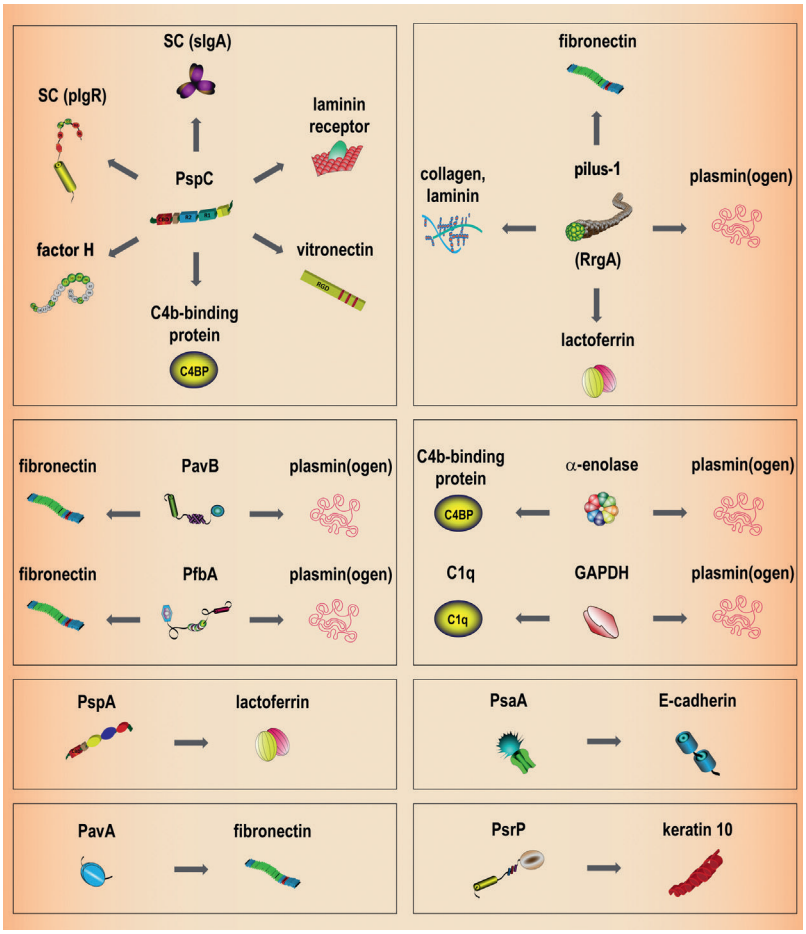


FIGURE 17.4 **Interaction of *S. pneumoniae* with human host structures.** (A) Field emission scanning electron microscopy of pneumococcal adherence to bronchial epithelial cells (Detroit562). (B) Pneumococcal surface proteins directly interacting with human ECM components, soluble plasma proteins, including complement components, and/or cellular receptors on the respiratory epithelium.

glycosaminoglycans and membrane-spanning mucins are part of the apical extracellular membrane; they are degraded by the pneumococcal hyaluronan lyase SpnHL and zinc metalloproteinase C (ZmpC), respectively [117,118]. In addition, *S. pneumoniae* loosely attaches to low-molecular-weight oligosaccharides and glycosaminoglycans via lectin interactions mediated directly by CBPs and by mucin-binding protein MucBP, and most likely indirectly by pyruvate oxidase SpxB, methionine sulfoxide reductase MsrAB1, and the peptide permeases AliA (PlpA) and AmiA [6,119–124]. However, to ensure strong adhesion to and translocation through epithelial and endothelial tissue barriers, pneumococci express surface-exposed adhesins that recognize glycoproteins present in the ECM or on cell surfaces (MSCRAMMs) [125]. Pneumococcal MSCRAMMs belong to the four main classes of surface-exposed proteins. They are encoded either by the core genome, and are thus present in the majority of pneumococcal strains, or by the flexible genome and are found in highly variable genomic regions such as RD10-(*psrP-secY2A2*) and PIs encoding pneumococcal pili (see section "Molecular Architecture and Assembly of Pneumococcal Pili as Unique Cell Wall–Anchored Covalent Polymers"). Pneumococcal MSCRAMMs show high levels of affinity and specificity for their ECM ligands and often display functional redundancy, which reinforces the efficiency of pathogen–host interactions. Similar to other human pathogens, pneumococci exploit ECM proteins as molecular bridges to link themselves to eukaryotic surface-expressed cell receptors to ensure tight adherence.

PavA, an NCSP, was identified as the first pneumococcal adhesin for immobilized fibronectin *in vitro* [77,126] (Figure 17.4B). The protein shares high sequence identity with FbpA of *S. gordonii* and Fbp54 of *S. pyogenes* (85% and 67%, respectively), and a homolog was recently identified in *Enterococcus faecalis* showing 51% sequence identity to PavA [77,127–129]. Binding of fibronectin by PavA is mediated by two heparin-binding domains (HBDs) within the fibronectin molecule: weak or less specific interactions with the N-terminal HBD, and specific, high-affinity interactions with the C-terminal HBD within fibronectin [126]. The fibronectin-binding region was mapped to the C-terminal part of PavA. However, pneumococcal mutants deficient in PavA expression retained up to 50% of wild-type activity to bind to fibronectin, suggesting that pneumococci express further fibronectin-binding protein(s) [77,80].

Genome analysis allowed the identification of ORF SP0082 in *S. pneumoniae* TIGR4 as a surface protein with binding activity to fibronectin [130]. The encoded protein was termed PavB and consists of an LPNTG-containing C-terminal cell-wall sorting signal and several copies of a conserved repeat, designated the streptococcal surface repeat (SSURE) domain. Each SSURE domain consists of approximately 150 aa. Homologs to PavB were found in *S. mitis*, *S. agalactiae*, and *S. gordonii*, but not in other human pathogens. Sequence alignment identified three types of repeats (first, core, and last SSURE), and bioinformatic analysis of the *pavB* locus in various pneumococcal strains revealed a variable number of repeats (e.g., five in *S. pneumoniae* G54 and TIGR4, seven in D39, and nine SSURE domains in R6, R800, and 35A), resulting in different molecular weights of the PavB protein [28]. The core repeats of PavB were able to bind individually to immobilized fibronectin and plasminogen *in vitro*, but high-affinity binding was only observed with increasing number of SSUREs. By binding to ECM fibronectin and/or by directly interacting with a yet-unknown receptor present on respiratory cells (Figure 17.4B), PavB significantly contributed to pneumococcal colonization and infection of the respiratory tract in a mouse infection model [28].

Whole genome analysis for LPxTG-containing proteins in *S. pneumoniae* R6 has identified SPR1652 as a conserved surface-

exposed protein interacting with fibronectin and plasmin(ogen), and is therefore designated as PfbA (Figure 17.4B). The protein also directly binds to human respiratory cells and protects pneumococci against phagocytosis under *in vitro* conditions [45]. In addition, the pilus-1 tip protein RrgA was shown to interact with immobilized fibronectin, collagen I, and laminin *in vitro* [108] (Figure 17.4B). Interestingly, it has been proposed that, via a basic region (BR) in the D3 domain, RrgA recognizes glycosaminoglycan chains directly associated with fibronectin and laminin rather than the adhesive glycoproteins themselves [103]. The CbpG has been suggested to function as pneumococcal trypsin-like serine protease involved in pneumococcal pathogenesis. In its surface-associated form, CbpG is suggested to facilitate pneumococcal colonization of the upper respiratory tract, whereas secreted CbpG degrades ECM-deposited fibronectin to promote invasive disease such as pneumonia and bacteremia [131,132]. Recently, pneumococcal endopeptidase PepO, a 72-kDa protein with predicted metalloendopeptidase activity, was identified as a novel NCSP that binds to fibronectin, plasminogen, and to a yet-unknown cellular receptor, thereby promoting pneumococcal adherence to and invasion into respiratory epithelial and endothelial cells under *in vitro* conditions [76].

S. pneumoniae was also demonstrated to recruit the human glycoprotein vitronectin to its surface [133]. In addition, it was reported that pneumococci bind to multimeric, host cell–bound vitronectin, which promotes pneumococcal adherence to and invasion into human epithelial and endothelial cells. Vitronectin-mediated invasion of *S. pneumoniae* was shown to depend on the engagement of the integrin $\alpha_v\beta_3$ receptor and the activation of intracellular signaling pathways, involving integrin-linked kinase, phosphatidylinositol 3-kinase (PI3K), and protein-kinase B (Akt), culminating in cytoskeletal rearrangements. Recruitment of vitronectin and vitronectin-mediated adherence and invasion of pneumococci was effectively inhibited by heparin, suggesting that *S. pneumoniae* interacts with one or several HBDs within the vitronectin molecule [134]. Recently, classical PspC was identified as a pneumococcal adhesin for vitronectin (Figure 17.4B). It was suggested that two interconnected R domains within the mature PspC protein are required for efficient vitronectin binding. Importantly, secretory IgA (see sections "Pneumococcal Adhesins Directly Engaging Host Cell Receptors" and "Pneumococcal Adhesins Modulating Innate Immune Responses"), and vitronectin did not completely compete for binding to PspC, indicating that neither human glycoproteins shares the identical binding epitope within PspC. The PspC-binding region was localized to the C-terminal HBD of vitronectin and a region N-terminally located to the identified HBD [135]. In addition, vitronectin-binding activity for Hic, a sortase-anchored, non-classical PspC protein, has recently been described. Despite the sequential and structural differences from classical PspC, the PspC-like protein Hic specifically interacted with vitronectin with affinity similar to PspC proteins containing two linked R domains. Binding studies confirmed that factor H binds to the very N-terminal region of Hic showing high sequence homology to classical PspC proteins, while vitronectin recognizes an adjacent region in the N-terminal region of Hic [136].

S. pneumoniae was also shown to utilize host cell–bound thrombospondin-1 (TSP-1) for adhesion to endothelial and respiratory epithelial cells, which might facilitate pneumococcal colonization and tissue invasion. Binding of TSP-1 to host cells was inhibited by heparin, heparan sulfate, and heparitinase treatment of the cell surface, but not by anti-integrin antibodies, suggesting that TSP-1-mediated pneumococcal adherence to host cells involves cell surface–associated glycosaminoglycans rather than integrin receptors. It was suggested that a component associated to peptidoglycan is the

bacterial adhesin for TSP-1, strongly indicating that pneumococcal surface-exposed protein(s) might specifically interact with TSP-1, which needs further investigation [137,138].

Factor H is an abundant glycoprotein with a molecular mass of approximately 150 kDa, mainly synthesized by hepatocytes and secreted into the bloodstream at concentrations up to 500 μg/mL. The mature protein consists of 20 CCPs (complement control protein repeats), also termed short consensus repeats (SCRs). Factor H interacts with several host factors, including C3b (via SCR1-4, SCR19-20, and possibly SCR12-14), heparin, and other glycosaminoglycans (via SCR7, SCR20, and possibly SCR12-14), and with cell membrane receptors (e.g., CR3 and other integrins via an RGD motif in SCR4) [139,140]. In plasma, factor H is an important complement regulatory protein (see section "Pneumococcal Adhesins Modulating Innate Immune Responses") but can also interact with components of the ECM [139–141]. To date, the multifunctional PspC protein is the only known factor H-binding protein of pneumococci, and two binding sites for PspC were identified in factor H (Figure 17.4B). The major PspC-binding region is located in SCR8-11 and a second, minor site in SCR19-20 [142,143]. The factor H-binding region of classical, choline-bound PspC was mapped to a 121-aa sequence in the N-terminal part of the mature PspC protein. Since the N-terminal region of Hic, the LPSTG-anchored PspC11 protein expressed by serotype 3 pneumococci, shows considerable sequence homology with the factor H-binding site in classical PspC proteins, it was suggested that this region is also involved in the Hic-factor H interaction [60,142,144]. Factor H bound to the pneumococcal surface was demonstrated to promote pneumococcal adherence and invasion into endothelial cells via the glycosaminoglycan-binding site in SCR20 of factor H as well as into epithelial cells via the interaction of an RGD motif in SCR4 with integrin receptors [145].

Once pneumococci have crossed the respiratory epithelium, via either the transcellular or intercellular route, they interact with ECM proteins in the subendothelial basement membrane, such as collagens and laminins. It was previously demonstrated that *S. pneumoniae* binds collagen I, II, IV, and laminin. RrgA was identified as an adhesin for collagen I and laminin (Figure 17.4B). Binding to collagen I is supposed to be mediated by the D3 domain within RrgA, which shows high structural similarity to the collagen-binding integrin I domain found in eukaryotic proteins such as von Willebrand factor and integrin α-chains [103]. More recently, pneumococci have also been reported to interact with collagen VI [146]. However, pneumococcal adhesins recognizing collagen II, IV, and VI have not yet been determined.

ZmpC is one of four paralogous zinc metalloproteinases (Zmps) identified in *S. pneumoniae*, with homologs in other streptococci and Gram-positive commensals of the human respiratory tract. In contrast to ubiquitously expressed ZmpA (IgA1 protease), ZmpC can be found in less than 20% of pneumococcal strains, and the presence of the *zmpC* gene has been correlated with pneumococcal disease severity and progression [147]. Secreted ZmpC was demonstrated to remove membrane-tethered mucin MUC16, which is expressed on the apical surface of the mucosal epithelium, including the respiratory tract. Shedding of MUC16 by ZmpC abrogated the epithelial barrier function and enhanced pneumococcal internalization *in vitro* [117]. ZmpC was also shown to cleave and activate human matrix metalloproteinase 9 (MMP-9) [147]. MMP-9 degrades ECM proteins (collagens IV, V, VI, X, and XIV; vitronectin; fibronectin; elastin; and entactin), plasminogen, cytokines, chemokines, and cell-surface receptors [148]. Both shedding of MUC16 and activation of MMP-9 by ZmpC may therefore promote pneumococcal tissue invasion and progression to severe invasive disease such as pneumonia and meningitis.

Pneumococcal Adhesins Recruiting Host-Derived Proteolytic Activity

As mentioned above, *S. pneumoniae* expresses several surface-exposed proteins that interact with and proteolytically cleave components of the ECM. In addition, pneumococci engage the human plasmin(ogen) system to promote adherence, invasion, and bacterial spread across fibrin deposits and extracellular matrices. Plasminogen (Plg) is a zymogen secreted primarily by hepatocytes; it circulates in the bloodstream as Glu-plasminogen (791 aa, ~92 kDa), which can be modified by digestion with plasmin to a series of N-terminally truncated plasminogen molecules, called Lys-plasminogen [149]. Both Glu- and Lys-plasminogen can be cleaved by tissue- and urokinase-type plasminogen activator (tPA and uPA) to generate the active serine protease plasmin, in which the heavy chain is composed of five homologous Kringle domains (K1−5), linked with the C-terminal protease domain (light chain) by two disulfide bonds [149]. Mini-plasminogen can be generated by proteolytic cleavage of plasminogen; it contains K5 and the protease domain, though its physiological function is still elusive. Except for K3, Kringle domains 1, 2, 4, and 5 contain lysine-binding sites with differing affinities for free lysines and lysine-like compounds (K1 > K4 > K5 > K2) [149]. Generation and activity of plasmin(ogen) is regulated at different levels: plasminogen gene expression, Plg receptors, Plg activators, Plg activator receptors, and plasminogen activator inhibitors, as well as plasmin inhibitors and plasmin-degrading proteases. The main physiological role of plasmin is the degradation of fibrin clots. In addition, plasmin cleaves ECM proteins, including fibronectin, thrombospondin, and laminin, as well as cellular receptors (e.g., E-cadherin), proMMPs, hormones, and components of the complement system [150,151]. Moreover, plasmin facilitates cell migration in tissues and triggers signaling cascades in a variety of cell types, including immune cells, endothelial and epithelial cells, platelets, and fibroblasts. Finally, plasmin stimulates platelet aggregation and production of cytokines, reactive oxygen species (ROS), and other soluble mediators, and thereby contributes to chemotaxis, inflammation, and wound healing [151].

Recruitment of the plasmin(ogen) system by *S. pneumoniae* is mediated by specialized classical, surface-anchored proteins or by cytoplasmic proteins, often metabolic enzymes, localized to the pneumococcal surface. Alpha-enolase and GAPDH have been identified as major plasmin(ogen)-binding proteins of *S. pneumoniae* (Figure 17.4B). Both proteins are intracellular glycolytic enzymes that are tethered to the pneumococcal surface by yet-unknown mechanisms [71,72]. In contrast to other bacterial enolases, which are homodimeric, the pneumococcal enolase is composed of a tetramer of dimers [152]. Initially, two adjacent C-terminal lysine residues (^{433}KK^{434}) have been identified as plasminogen-binding sites. However, these residues are supposed to be buried in the enolase octamer. A second internal nine-residue motif with two lysines (^{248}FYDKERKVY^{256}), which is exposed on the octamer surface, has been suggested as the primary binding site for plasminogen [72,152]. The binding site of enolase was mapped to Kringle domains 1−3 (lysine-binding site 1, LBS1) within plasmin(ogen) [153,154]. Pneumococcal enolase displays a higher affinity for plasminogen than for plasmin [153]. Importantly, deletion of enolase-mediated plasminogen-binding significantly reduced the virulence of *S. pneumoniae* in a mouse infection model [154]. Plasminogen binding to GAPDH is suggested to be mediated by a four-residue motif of two C-terminal lysine resides (KIAK), which is common among streptococcal surface-exposed GAPDHs. In contrast to enolase, pneumococcal GAPDH preferentially binds to plasmin rather than to plasminogen [71]. There is growing knowledge about pneumococcal surface-anchored proteins that recruit plasminogen. CbpE (Pce) was shown to bind the LBS1 of plasminogen, and three internal lysine residues

(K^{259}, K^{267}, and K^{319}) within the catalytic domain of Pce have been implicated in this interaction [155]. The sortase-anchored proteins PavB and PfbA were identified as fibronectin- and also as plasmin(ogen)-binding proteins [28] (Figure 17.4B). PfbA showed higher affinity for plasmin than for plasminogen [45]. To date, the mechanisms of plasmin(ogen) binding to PfbA and PavB have not been resolved. Recently, the cytoplasmic enzymes endopeptidase O (PepO) and PGK have been described as surface-exposed plasminogen-binding adhesins of *S. pneumoniae*. A lysine analog significantly reduced Plg binding to PepO, indicating that lysine residues within PepO mediate the interaction with plasminogen [76]. The interaction of PGK with Plg has been studied in more detail. Similar to Pce, PGK recognizes lysine-binding domain 1 of plasminogen. By peptide-spot array, two Plg-binding sites within PGK have been identified, ^{13}GKKV^{16} and ^{53}RAIL^{56}, and other structurally proximate residues are suggested to be important. The plasminogen-binding site has been located to the N-terminal domain but opposite to the catalytic center of PGK. Interestingly, PGK also binds tissue-type plasminogen activator (tPA), but not to urokinase-type PA, and PGK-bound tPA was shown to be able to cleave plasminogen to generate the active protease plasmin [75].

The human plasmin(ogen) system plays a pivotal role not only in fibrinolysis and inhibition of clot formation but also in ECM remodeling, inflammation, and complement control. It is therefore an attractive target of pathogenic bacteria, including *S. pneumoniae*. Plasmin, either directly recruited to the pneumococcal surface or generated from surface-bound plasminogen by host-derived Plg activators, can facilitate fibrinolysis, prevent formation of new thrombi, and promote the escape of pneumococci entrapped in fibrin clots for survival and dissemination in the bloodstream. Furthermore, plasmin and fibrin degradation products are potent stimulators of pro-inflammatory immune responses, including production of ROS, cytokines, and chemokines, and secretion of MMPs, thereby modulating leukocyte recruitment, extravasation, and migration [151]. An excessive activation of the immune system may render the host unable to effectively eliminate pneumococci from infected sites of the body. Plasmin also degrades essential components of the human complement and, when bound to the pneumococcal surface, can provide pneumococci the capacity to evade the innate immune system (see section "Pneumococcal Adhesins Modulating Innate Immune Responses") [150,151]. At tissue barriers, cell-surface bound, but not bacterial-bound, plasminogen has been shown to increase the adherence of *S. pneumoniae* [156]. In addition, plasmin bound to the pneumococcal surface results in degradation of ECM proteins, either directly or by activation of MMPs, which leads to ECM breakdown and thus helps *S. pneumoniae* to migrate through the ECM and basement membrane and to disseminate within the human host [72,150]. Furthermore, plasmin degrades cadherins in intercellular junctions, which was shown to promote translocation of pneumococci through tissue barriers via the pericellular route *in vitro* [157].

Pneumococcal Adhesins Directly Engaging Host Cell Receptors

S. pneumoniae expresses several adhesins that directly engage receptors on human epithelial and endothelial cells for adherence and invasion. PsaA was the first putative adhesin described in *S. pneumoniae*, since adherence of pneumococci and fluorospheres coated with recombinant PsaA to human nasopharyngeal epithelial cells was shown to be inhibited by anti-PsaA antibodies and purified PsaA peptides [33]. In a consecutive study, human E-cadherin, a single-pass transmembrane cadherin superfamily protein forming adherens junctions at the apical-lateral surface of

epithelial tissues, has been identified as a receptor for PsaA under *in vitro* conditions [36] (Figure 17.4B). Deletion of *psaA* significantly impaired nasopharyngeal colonization in a mouse infection model [33]. In the host, *S. pneumoniae* must adapt to different concentrations of the essential trace element Mn^{2+} (saliva > lungs > blood). At low Mn^{2+} levels, the transcriptional regulator PsaR was shown to activate the expression of the *psaBCA* gene complex and the *rlrA* pathogenicity islet, which encodes for type 1 pilus components (see section "Molecular Architecture and Assembly of Pneumococcal Pili as Unique Cell Wall–Anchored Covalent Polymers") and *pcpA* (pneumococcal choline-binding protein A) [34,36]. Thus, when pneumococci translocate from the nasopharynx into the lungs, the expression of the gene complex would be increased, facilitating pneumococcal adhesion to the lung tissue through PsaA, RrgA, and PcpA. In addition, mutation in *psaR* significantly reduced pneumococcal virulence in a murine bacteremia model and increased pneumococcal susceptibility to oxidative stress; thus the *psaR* regulon contributes to survival of *S. pneumoniae* in the blood [158,159]. Flamingo cadherin, a G protein-coupled receptor containing nine cadherin domains, can be found in adherens junctions such as E-cadherin and is essential in early neuronal differentiation and planar cell polarity of epithelia [160,161]. This protein was proposed as a receptor on respiratory cells for the pneumococcal non-classical, surface-exposed fructose bisphosphate aldolase. The interaction of pneumococcal aldolase with Flamingo cadherin significantly contributed to pneumococcal nasopharyngeal and lung colonization in mice [162].

The extracellular region of the hpIgR consists of five Ig-like domains (D1-5, "secretory component (SC)," ~80 kDa) and a non-Ig-like domain connecting the SC to the transmembrane region. The hpIgR is expressed at the basolateral surface of epithelial cells in mucous membranes and exocrine glands; it transports Ig complexes to the apical surface. Pneumococci engage unloaded hpIgR for adherence, invasion, and transcytosis through the respiratory epithelium. The Ig-like domains D3 and D4 have been shown to specifically bind to the YPT motif present in each R domain of PspC [163,164] (Figure 17.4B). Invasion of pneumococci into respiratory cells via the PspC-hpIgR interaction required activation of the small GTPase Cdc42, protein tyrosine kinases Src, and focal adhesion kinase, as well as PI3K and Akt, which results in actin cytoskeleton rearrangements. In addition, the MAPK and JNK pathways are involved in host cell invasion by pneumococci [165]. PspC also directly binds to the 67-kDa laminin receptor (LR), which is expressed in many different tissues including the respiratory tract and blood–brain barrier (Figure 17.4B). In a murine meningitis model, the interaction of PspC with LR was required for adherence of *S. pneumoniae* to brain microvascular endothelial cells and development of meningitis. The seven-residue motif EPRNEEK, which is exposed at the loop connecting helix 2 and helix 3 in the triple-helical R domain of PspC, was found to be critical for PspC binding to LR. Importantly, this motif is conserved in R2 but to a lesser extent in the R1 domains of the various PspC proteins, suggesting an allele-dependent interaction of PspC with LR [166]. Interestingly, another surface protein that binds to the SC has recently been described: Pneumococcal surface protein K (PspK) is only expressed by a certain clade of non-typable, non-encapsulated *S. pneumoniae* isolates. The original capsule locus of these isolates lacks all capsule sequences, despite having *ali*-like open-reading frames, but contains an insertion sequence and the *pspK* open-reading frame, which encodes for a 534-aa polypeptide. PspK is composed of an N-terminal signal peptide, a long α-helical region (aa 40–475), and a C-terminal LPSTG cell-wall sorting signal. In part, the α-helical

domain shows remarkable sequence identity to the R domains of PspC (67% and 76% identical to R1 and R2, respectively, of PspC in *S. pneumoniae* TIGR4). Similarly to PspC, the PspK R-like domain (aa 203–280) consists of a YPT motif shown to mediate binding of PspK to the SC of sIgA [167,168]. In addition, PspK increased pneumococcal adherence and colonization of the nasopharynx in mice [167].

Recently, PsrP was identified as a novel adhesin required for pneumococcal attachment and persistence in the lung, but not essential for nasopharyngeal colonization and survival in blood [169]. PsrP is, at approximately 2300 kDa, the largest bacterial protein known; it contains a signal peptide, a BR between two serine-rich repeat domains (SRR1: ~49 aa, and SRR2: ~4319 aa), and a C-terminal LPxTG motif [170]. The N-terminal part of BR within PsrP was shown to be responsible for pneumococcal aggregation and biofilm formation *in vivo* [47]. In contrast, the C-terminal part of BR interacts with keratin-10 on lung epithelial cells [171] (Figure 17.4B). Keratin-10 belongs to the keratin family of intermediate filaments and usually constitutes the cytoskeleton of epithelia. However, expression of keratin-10 was also demonstrated on the apical surface of lung epithelia and increased with aging, which may account for the high incidence of respiratory, especially pneumococcal, disease in the elderly [171,172].

Pneumococcal Adhesins Interacting with Unknown Ligands on the Respiratory Epithelium

In many cases, pneumococcal surface-exposed proteins have been implicated in adherence and colonization of respiratory epithelia, though the specific receptors have not always been identified. For instance, PavB and PfbA were shown to bind fibronectin and plasminogen, but both also seem to directly interact with respiratory cells, thereby contributing to pneumococcal colonization and airway infection [28,45]. Deletion of *pclA*, encoding another LPxTG-anchored surface protein, resulted in reduced adherence to and invasion of nasopharyngeal and lung cells. This was only observed for encapsulated strains, suggesting that the 265-kDa protein can protrude and function outside the capsule, which masks adhesins of smaller size [44]. The pilus-2 backbone protein PitB was also shown to adhere directly to yet-unknown receptors on respiratory epithelial cells [49]. In an infant rat model, pneumococci deficient in expression of the CbpD, CbpE (Pce), and CbpG showed reduced capacity to colonize the nasopharynx. In addition, CbpE and CbpG displayed significantly decreased adherence to nasopharyngeal epithelial cells *in vitro*, indicating that they play a role in adhesion and colonization [131]. *PcpA*, which is highly prevalent in clinical isolates and is controlled by the regulator PsaR, has previously been shown to be required for biofilm formation *in vitro* and to elicit protective immune responses during human nasopharyngeal colonization, and lung and systemic infection [173–177]. Recently, PcpA was demonstrated to mediate pneumococcal adherence to nasopharyngeal and lung epithelial cells, and anti-PcpA antibodies efficiently blocked this interaction, suggesting that PcpA might act as an adhesin [178].

The non-classical surface-exposed pneumococcal histidine triad (Pht) proteins, PhtA, PhtB, PhtD, and PhtE (Figure 17.1), have been implicated in colonization and induction of protective immune responses [179,180]. Pneumococci lacking all four Pht proteins have shown a marked reduction in adherence to nasopharyngeal and lung epithelial cells, suggesting a redundant function of the Pht proteins. In addition, the impact of the individual Pht proteins on pneumococcal adherence seemed to depend strongly on the respective genetic background (*phtD* is present in 100%, *phtE* in 97%, *phtB* in 81%, and *phtA* in 62% of

pneumococcal strains [181]), capsular serotype, and transcriptional regulation, and could be, at least in part, compensated by other surface adhesins [179].

The pentose phosphate pathway enzyme 6-phosphogluconate-dehydrogenase (6PGD) has been proposed to be a surface-located protein involved in pneumococcal adherence to lung epithelial cells *in vitro* and eliciting protective immune response in mice. 6PGD is a pneumococcal lectin, suggesting that it might bind to glycoconjugates on respiratory epithelial cells, though it cannot be excluded that 6PGD interacts with a cellular receptor independent of its lectin activity, as demonstrated for PsrP [182]. Recently, NOX and endopeptidase O (PepO) have been found to "moonlight" on the pneumococcal surface. NOX, which usually catalyzes the intracellular conversion of free oxygen to water, bound to lung epithelial cells *in vitro*, an interaction which could be inhibited by anti-NOX antibodies. Peptide screening using phage display revealed several putative binding candidates for NOX, including laminin subunit α-5, collagen XI, and chondroitin 4 sulfotransferase [74]. PepO has been shown to facilitate pneumococcal adherence and invasion into lung epithelial and endothelial cells; however, a specific receptor remains elusive [76].

Pneumococcal Adhesins Modulating Innate Immune Responses

After acquisition, *S. pneumoniae* not only needs to adhere to the human nasopharyngeal epithelium to establish itself and/or to cause disease by disseminating into deeper tissue layers or the bloodstream; the pathogen also has to evade or suppress host immune responses to prolong its survival in the different host niches encountered. The innate immune system, as the first line of defense, is responsible for pathogen recognition and elimination, and for activation of adaptive immune responses against the pathogenic intruder. Soluble factors such as antimicrobial peptides and complement proteins, cellular components (i.e., leukocytes), as well as tissue barriers and their anatomical features are part of the human innate ("nonspecific") immune system. In order to negotiate the host's defense mechanisms, *S. pneumoniae* has evolved sophisticated strategies involving sequestration or degradation and inactivation of soluble host factors, inhibition of phagocytosis, and modulation of phagocytes' functions. Pneumococci circumvent the deleterious effects of siderophores, activated complement proteins, and neutrophil extracellular traps, and efficiently prevent phagocytosis by producing a thick polysaccharide capsule, minimizing their chain length, forming biofilms, undergoing autolysis, and invading erythrocytes [183–188].

The human complement system is composed of more than 30 different proteins that are mainly synthesized by the liver, circulate in blood and tissue fluids or are present on cell surfaces, and either become activated or serve as regulators and inhibitors. Three distinct cascades of complement activation can be triggered, starting with the binding of an inactive complement component to foreign or self-molecules, its activation by proteolytic cleavage, progressing by activation of downstream components, and culminating in a common terminal pathway. The classical pathway is initiated by antigen-bound Igs or pentraxins such as CRP and serum amyloid P; the lectin pathway is induced by carbohydrate structures on foreign or modified self-proteins; and the alternative pathway is activated nonspecifically by spontaneous hydrolysis of complement component C3, and amplifies the other two pathways [189]. Tight regulation of complement activity in terms of time and space is accomplished by a series of soluble or membrane-bound regulators, including complement receptors, and inhibitors that control complement activation, propagation, and severity, and direct complement effectors to their

target sites [189,190]. *S. pneumoniae* is recognized by all three complement cascades but has evolved several means, besides production of a thick capsule and secretion of pneumolysin, to minimize or block complement activation and progression at different levels [191–193].

S. pneumoniae directly inhibits activation of the complement by the expression of surface-exposed virulence factors. Pce was suggested to remove *P*Cho residues relevant for binding of CRP to the pneumococcal surface [69]. PspA interferes with the classical pathway by competing with CRP for binding to *P*Cho, and with the alternative pathway by a yet-unknown mechanism [193–195]. Recently, the three pneumococcal glycosyl hydrolases NanA, BgA, and StrH were demonstrated to inhibit surface deposition of C3, probably by deglycosylating a complement component upstream of C3, and opsonophagocytotic killing [43]. Moreover, pneumococcal EndoD was shown to deglycosylate IgG synergistically with NanA, BgA, and StrH, consequently inhibiting binding of C1q to the antibody and blocking complement activation [40,196]. In addition, pneumococci recruit either plasmin or its zymogen to their surface, which is converted into active plasmin by use of host plasminogen activators (see section "Pneumococcal Adhesins Recruiting Host-Derived Proteolytic Activity"). Plasminogen enhances the cofactor activity of factor H for factor I-mediated cleavage of C3b into its inactive form, and plasmin degrades C3, C3b, and C5, thereby inhibiting the formation of both complement convertases [197].

Pneumococci also impede complement effector functions of the C3 convertase by sequestration of the complement regulatory proteins C4b-binding protein (C4BP) and factor H, respectively. C4BP is the major soluble regulator of the classical and lectin pathways by inhibiting the formation and accelerating the decay of the C3 and C5 convertase, as well as acting as a cofactor for factor I-mediated cleavage of C3b and C4b [198,199]. C4BP is a glycoprotein of approximately 570 kDa, mainly synthesized by the liver and circulating in plasma at a concentration of about 200 μg/mL. The major isoform of C4BP consists of seven identical α-chains and one β-chain, which are linked by disulfide bonds in their C-terminal parts. Each α-chain is composed of eight CCPs, and the β-chain contains three CCPs [198]. PspC was shown to bind to the interface of CCP1 and CCP2 in the α-chain without interfering with C4b-binding, therefore retaining C4BP's inhibitory function and cofactor activity (Figure 17.4B). C4BP binding was demonstrated to be dependent on the PspC allele, showing high variability in C4BP-binding activity among the different pneumococcal strains; the PspC4.4 allele was suggested as the major ligand for C4BP [200]. However, recruitment of C4BP to *pspC* mutants was not totally abolished, indicating the presence of another surface-exposed C4BP-binding protein(s). Recently, C4BP was found to interact with pneumococcal enolase and to remain functionally active. Interestingly, two binding sites for enolase were identified in C4BP, CCP1/CCP2 and CCP8 [201].

Factor H is the major fluid-phase regulator of the alternative pathway of complement, as it inhibits the binding of factor B to C3b, assists factor I in degrading C3b, inhibits the assembly, and accelerates the decay of the alternative C3 convertase [190]. The N-terminal part of the multifunctional PspC protein interacts with two binding sites in factor H (SCR8-11, as the major binding site, and SCR19-20) (see section "Pneumococcal Adhesins Interacting with the Extracellular Matrix Components") [142,143] (Figure 17.4B). Factor H bound to the pneumococcal surface via PspC was shown to provide resistance to complement, with opsonophagocytosis significantly promoting pneumococcal pathogenesis [143,202–204].

Human lactoferrin is a monomeric, globular glycoprotein of the transferrin family and is highly abundant in musocal secretions (e.g., milk, tears, saliva, and nasal secretions) and phagocytic cells. Lactoferrin displays a high

affinity for iron(III); hololactoferrin carries two iron ions, whereas apolactoferrin is iron-free [205]. Lactoferrin is part of the innate immune system as it inhibits bacterial growth by iron depletion, enhancing the activity of host lysozyme, and by disruption of Gram-negative cell membranes. In addition, lactoferrin induces the expansion of and cytokine release by phagocytes. In addition, lactoferrin modulates adaptive immune responses by promoting the maturation of T cells and B cells [205–207]. PspA was shown to bind lactoferrin in a strain-dependent manner [208] (Figure 17.4B). The mature PspA protein consists of an N-terminal α-helical domain, a proline-rich region, a CBD, and a short C-terminal tail. The *pspA* gene locus is highly polymorphic, and PspA proteins are divided into at least six clades belonging to three different PspA families [209]. The lactoferrin-binding region in family 1 PspA protein from *S. pneumoniae* R1x was mapped to a negatively charged stretch consisting of residues 167–288, which is located in the C-terminal part of the α-helical domain adjacent to the proline-rich region [210]. Interestingly, family 1 PspA proteins bind with a higher affinity to lactoferrin than family 2 PspA [211]. It was suggested that specific binding of lactoferrin to surface-exposed PspA provides an iron source for pneumococci at mucosal surfaces. In addition, PspA may protect from the bactericidal action of lactoferrin by inhibiting its proteolytic cleavage into lactoferricin, an N-terminal peptide of lactoferrin with enhanced antimicrobial activity but without iron-binding capacity, and preventing access to the pneumococcal cell membrane [205,208,212]. Lactoferrin has also been demonstrated to be a substrate for pneumococcal surface-exposed lytic enzymes. The glycosyl hydrolases NanA, BgaA, and StrH were shown to sequentially deglycosylate lactoferrin, which does not influence its binding to iron and lysozyme but increases the susceptibility to proteolytic degradation [41].

The SC serves as a receptor for polymeric Igs (IgM and IgA) and usually mediates their translocation into secretions. At the apical surface, proteolytic cleavage releases either free SC or SC-bound polymeric Igs, which are called secretory IgM (sIgM) and IgA (sIgA), respectively. SC is considered a link between innate (natural) and adaptive immune responses at mucosal surfaces, since it neutralizes pathogens, either by lectin–glycan or protein–protein interactions, and inhibits their adherence to the epithelium, a property that is retained in natural as well as antigen-specific sIgA [213,214]. SC scavenges *S. pneumoniae* by low-affinity binding of lectin-like adhesins, such as neuraminidase A and B, to the various N-glycosylation sites identified in SC [214,215]. In contrast, PspC specifically binds to the interface of domains D3 and D4 in SC (Figure 17.4B). Pneumococci are not able to directly circumvent binding of SC to their surface, but they express surface-exposed proteases that modulate immune functions of SC and sIgA, which aids in escape from immune defense mechanisms and promotes pathogenesis. Free and bound SC were shown to be substrates for NanA, BgaA, and StrH [41]. Since SC stabilizes sIgA against proteases and glycosylation of SC is absolutely required for localization of sIgA within the mucus, deglycosylation of SC by pneumococcal glycosyl hydrolases may result in diffused distribution of sIgA in the respiratory tract and increased susceptibility of sIgA to proteolytic cleavage [216,217]. Secretory IgA, consisting of two IgA monomers connected by a J chain and the SC, neutralizes and agglutinates invading infectious agents via its respective Fab portions and initiates their phagocytosis via IgA-Fcα receptor interactions [218]. Mucous IgA contains two subclasses of N-glycosylated IgA, IgA1 and IgA2, whose main structural difference is a longer, protease-sensitive hinge region in IgA1 that additionally contains several O-glycosylation sites [213]. IgA1 dominates in the respiratory tract, whereas IgA2 is more abundant in the gastrointestinal and genitourinary tract [219].

Pneumococci produce an IgA1 protease, which contains a zinc-binding consensus sequence found in other bacterial metalloproteinases; it is also referred to as pneumococcal zinc metalloproteinase A (ZmpA) [220]. ZmpA specifically cleaves IgA1, but not IgA2, at its hinge region, releasing the Fcα effector domain from the antigen-binding Fab fragments [221]. This was shown to inhibit phagocytic killing of pneumococci both *in vitro* and *in vivo* [222]. Moreover, surface-bound specific IgA1-Fab fragments might shield pneumococci from intact antibody molecules and complement proteins, thus maintaining survival of *S. pneumoniae* within the host. In addition, capsule type−specific Fab fragments derived from IgA1 protease treatment significantly enhanced pneumococcal adherence to nasopharyngeal epithelial cells *in vitro*, which might promote pneumococcal epithelial colonization and invasiveness *in vivo* [223].

Interaction with the innate immune system was also described for pilus-1 surface appendages: Domain 3 of RrgA, especially if present as part of an oligomer, was assigned a role as TLR2 agonist, inducing TNFα response after mouse intraperitoneal infection [224]. In addition, it was shown recently that RrgA, which contains an RGD motif, directly interacts with complement receptor CR3 expressed on neutrophils and lymphocytes [104,225]. Engagement of CR3 by RrgA-expressing pneumococci promoted phagocytosis by macrophages was shown to prolong pneumococcal survival in the phagocytes and to activate lymphocytes, such as alveolar macrophages, to migrate from respiratory epithelial tissues to the capillaries, likely contributing to systemic dissemination of pneumococci and exacerbation of disease [225].

CONCLUSIONS AND PERSPECTIVES

S. pneumoniae is an extracellular pathogen that has evolved sophisticated strategies to encounter the host and cause disease. Its ability to efficiently colonize the human nasopharynx depends on the interaction of pneumococcal surface-exposed adhesins and pili with cellular receptors and/or host proteins, which promote attachment of pneumococci to mucosal epithelial cells. Adherence to human host cells may trigger the activation of further virulence determinants involved in invasion, fitness, and immune evasion of pneumococci. On the host cell side the pathogen−host interaction via adhesins may induce signal cascades and immune responses such as cytokine release and complement activation, which in turn provoke significant inflammatory processes. Thus, preventing infections and reducing the clinical severity of pneumococcal diseases may depend directly on the ability to target important pneumococcal pathophysiological players and processes. Due to the development of new anti-virulence drugs and the almost simultaneous emergence of respective antibiotic resistances in *S. pneumoniae*, the rational design of a protein-based vaccine with expanded strain and serotype coverage can serve as an alternative strategy in the battle against pneumococcal infections showing advantages over existing polysaccharide and traditional conjugate vaccination strategies. Since adhesins identified in *S. pneumoniae*, including PspC, PspA, PavB, PcpA, pili, and PsaA, can be found in a majority of clinically relevant pneumococcal strains and serotypes, they are considered promising vaccine candidates, especially if applied in combination. Either administered mucosally or systemically, individually simultaneously, or heterologously expressed by recombinant *Salmonella* or *Lactococcus lactis* strains, these adhesins are able to elicit strong immune responses and increase survival in animal models. Thus, current clinical trials in healthy adults investigate the safety and efficacy of protein-based pneumococcal vaccines [63,226,227] (Table 17.1).

TABLE 17.1 Pneumococcal Surface-Exposed Adhesins Discussed in this Chapter

Protein	Name/characteristic(s)	Human host target	References	Section(s)
LIPOPROTEINS				
PsaA	Pneumococcal surface adhesin A	E-cadherin	(33, 34, 36, 37, 158, 159)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Directly Engaging Host Cell Receptors"
SlrA	Streptococcal lipoprotein rotamase A	n.d.	(30, 31, 228)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"
PpmA	Putative proteinase maturation protein A	n.d.	(30, 31, 229)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"
Etrx1 (TlpA)	thiol-disulfide oxidoreductase	n.d.	(6, 30, 31)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"
Etrx2	thiol-disulfide oxidoreductase	n.d.	(6, 30, 31)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"
SORTASE-ANCHORED PROTEINS				
NanA	neuraminidase	N-linked glycans in IgA1, lactoferrin, SC, α1-acid glycoprotein	(31, 40–43, 230)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Extracellular Matrix Components"; "Pneumococcal Adhesins Modulating Innate Immune Responses"
BgaA	β-galactosidase		(31, 40, 41, 43)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Extracellular Matrix Components"; "Pneumococcal Adhesins Modulating Innate Immune Responses"
StrH	exo-β-N-acetyl-glucosaminidase		(31, 40, 41, 43)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Extracellular Matrix Components"; "Pneumococcal Adhesins Modulating Innate Immune Responses"
EndoD	endo-β-N-acetyl-glucosaminidase	transferrin, IgG; fetuin	(31, 196)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Modulating Innate Immune Responses"
Eng	endo-α-N-acetyl-galactosaminidase	O-linked glycans	(31, 116)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Extracellular Matrix Components"
SpnHL	hyaluronan lyase	hyaluronan, (un-) sulfated chondroitin	(31, 118)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Extracellular Matrix Components"

(*Continued*)

TABLE 17.1 (Continued)

Protein	Name/characteristic(s)	Human host target	References	Section(s)
ZmpA	zinc metalloproteinase A (IgA1 protease)	IgA1	(31, 221, 222, 231, 232)	"Pneumococcal Adhesins Interacting with the Extracellular Matrix Components"; "Pneumococcal Adhesins Modulating Innate Immune Responses"
ZmpC	zinc metalloproteinase C	n.d.	(31, 117, 147, 231)	"Pneumococcal Adhesins Interacting with Extracellular Matrix Components"
PrtA	serine protease	lactoferrin	(31, 73, 233, 234)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"
PavB	Pneumococcal adherence and virulence factor B	Fn, Pl(g); cell receptor	(28, 31)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Extracellular Matrix Components"; "Pneumococcal Adhesins Recruiting Host-Derived Proteolytic Activity"; "Pneumococcal Adhesins Interacting with Unknown Ligands on the Respiratory Epithelium"
PfbA	Plasmin- and fibronectin-binding protein A	Fn, Pl(g); cell receptor	(31, 45)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Extracellular Matrix Components"; "Pneumococcal Adhesins Recruiting Host-Derived Proteolytic Activity"; "Pneumococcal Adhesins Interacting with Unknown Ligands on the Respiratory Epithelium"
RrgA	Rlr-regulated gene A, pilus islet 1	Fn, Col(I), Ln; integrins (CR3), TLR2	(102, 103, 107, 224, 225)	"Molecular Architecture and Assembly of Pneumococcal Pili as Unique Cell Wall-Anchored Covalent Polymers"; "Pneumococcal Adhesins Interacting with Extracellular Matrix Components"; "Pneumococcal Adhesins Directly Engaging Host Cell Receptors"; "Pneumococcal Adhesins Interacting with Unknown Ligands on the Respiratory Epithelium"
PitB	pilus islet 2	cell receptor	(49)	"Molecular Architecture and Assembly of Pneumococcal Pili as Unique Cell Wall-Anchored Covalent Polymers"; "Pneumococcal Adhesins Interacting with Unknown Ligands on the Respiratory Epithelium"
PclA	Pneumococcal collagen-like protein A	cell receptor	(31, 44)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Unknown Ligands on the Respiratory Epithelium"

(*Continued*)

TABLE 17.1 (Continued)

Protein	Name/characteristic(s)	Human host target	References	Section(s)
PsrP	Pneumococcal serine-rich repeat protein	Keratin 10	(31, 47, 169–172, 235)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Extracellular Matrix Components"; "Pneumococcal Adhesins Directly Engaging Host Cell Receptors"
Hic	Factor H-binding inhibitor of complement (PspC11 allele)	FH, Vn	(136, 143, 144)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Extracellular Matrix Components"; "Pneumococcal Adhesins Modulating Innate Immune Responses"
MucBP	Mucin-binding protein	mucins	(31, 120)	"Pneumococcal Adhesins Interacting with Extracellular Matrix Components"
CHOLINE-BINDING PROTEINS				
LytA	N-acetylmuramoyl-L-alanine amidase	n.d.	(9, 31)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"
LytB	N-acetylglucosaminidase	n.d.	(31, 66)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"
LytC	N-acetyl-muramidase	n.d.	(31, 66)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"
CbpD	Choline-binding protein D, murein hydrolase	n.d.	(31, 131)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Unknown Ligands on the Respiratory Epithelium"
CbpE (Pce)	Choline-binding protein E, phosphorylcholine esterase	Pl(g)	(31, 69, 131, 155)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Recruiting Host-Derived Proteolytic Activity"; "Pneumococcal Adhesins Interacting with Unknown Ligands on the Respiratory Epithelium"
PspA	Pneumococcal surface protein A	lactoferrin	(31, 208, 210–212)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Modulating Innate Immune Responses"
PspC	Pneumococcal surface protein C (also known as CbpA, SpsA, and Hic)	SC (sIgA), FH, C4BP, Vn; SC (pIgR), laminin receptor ('67LR')	(9, 31, 142, 145, 163–165, 185, 200, 202–204)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Extracellular Matrix Components"; "Pneumococcal Adhesins Directly Engaging Host Cell Receptors"; "Pneumococcal Adhesins Modulating Innate Immune Responses"

(*Continued*)

TABLE 17.1 (Continued)

Protein	Name/characteristic(s)	Human host target	References	Section(s)
CbpG	Choline-binding protein G	Fn; cell receptor	(131, 132)	"Pneumococcal Adhesins Interacting with Unknown Ligands on the Respiratory Epithelium"
PcpA	Pneumococcal choline-binding protein A	cell receptor	(31, 178)	"Pneumococcal Adhesins Directly Engaging Host Cell Receptors"; "Pneumococcal Adhesins Interacting with Unknown Ligands on the Respiratory Epithelium"
NON-CLASSICAL SURFACE PROTEINS				
Enolase	Phosphopyruvate hydratase	Pl(g); C4BP	(31, 72, 152–154, 156, 201)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Recruiting Host-Derived Proteolytic Activity"; "Pneumococcal Adhesins Modulating Innate Immune Responses"
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Pl(g)	(31, 71)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Recruiting Host-Derived Proteolytic Activity"
6PGD	6-phosphogluconate dehydrogenase	cell receptor	(31, 182)	"Pneumococcal Adhesins Interacting with Unknown Ligands on the Respiratory Epithelium"
PGK	Phosphoglycerate kinase	Plg & tPA	(31, 75)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Recruiting Host-Derived Proteolytic Activity"
Aldolase	Fructosebisphosphate aldolase	flamingo cadherin	(162)	"Pneumococcal Adhesins Directly Engaging Host Cell Receptors"
PepO	Pneumococcal endopeptidase O	Pl(g); Fn; cell receptor	(76)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Extracellular MatrixComponents"; "Pneumococcal Adhesins Recruiting Host-Derived Proteolytic Activity"; "Pneumococcal Adhesins Interacting with Unknown Ligands on the Respiratory Epithelium"
NOX	NADH oxidase	cell receptor	(74)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Unknown Ligands on the Respiratory Epithelium"
HtrA	serine protease, heat shock protein	denatured proteins	(31, 73)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"

(*Continued*)

TABLE 17.1 (Continued)

Protein	Name/characteristic(s)	Human host target	References	Section(s)
PavA	Pneumococcal adherence and virulence factor B	Fn	(77–80, 126)	"Classification and Distribution of Pneumococcal Surface-Exposed Proteins"; "Pneumococcal Adhesins Interacting with Extracellular Matrix Components"
Pht (D, E,B,A)	Pneumococcal histidine triad protein	FH (low affinity); cell receptor	(179, 180)	"Pneumococcal Adhesins Interacting withUnknown Ligands on the Respiratory Epithelium"

Abbreviations: C4BP, C4b-binding protein; Col, collagen; CR, complement receptor; FH, Factor H; Fn, fibronectin; Ig, immunoglobulin; Ln, laminin; n.d., not determined; pIgR, polymeric Ig receptor; Pl(g), plasmin(ogen); SC, secretory component; TLR, toll-like receptor; tPA, tissue plasminogen activator; Vn, vitronectin.

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CHAPTER

18

Exploitation of Host Signal Transduction Pathways Induced by *Streptococcus pneumoniae*

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INTRODUCTION

The elucidation of pneumococcal pathogenesis strengthened the axiom that severity and progress of infection depends on both the bacterial virulence factor repertoire and host response mechanisms. The broad spectrum of effects of pneumococcal factors on eukaryotic cells is initiated by host cell signaling events, and these are currently the focus of intensive investigation. Recent studies have revealed the complexity and multilayered interconnection of signaling pathways induced by different modes of pneumococcal–cell interactions and by secreted pneumococcal effector molecules like pneumolysin (Ply) [1–3]. Ply belongs to the family of cholesterol-dependent pore-forming cytolysins; it has lytic effects on many mammalian cell types [4]. In addition, at sublytic concentrations Ply also has numerous other effects, including complement activation and induction of pro-inflammatory mediators [3].

A variety of surface-exposed adhesins ensure efficient and intimate contact between pneumococci and different host tissues, especially the nasopharyngeal epithelium [1,2]. Pneumococci express a remarkable number of different pneumococcal proteins that recruit the adhesive glycoprotein fibronectin (Fn) as a molecular bridge linking pneumcocci to a eukaryotic receptor, including the pneumococcal adhesion and virulence factors A and B (PavA, PavB), the pneumococcal plasminogen- and fibronectin-binding protein A (PfbA), the choline-binding protein G (CbpG), and the adhesive factor of type 1 pilus RrgA [1,5,6]. Similarly, pneumococci

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interact with the matricellular protein thrombospondin-1 (TSP-1) and bind vitronectin (Vn) via the pneumococcal surface protein C (PspC) to facilitate adherence to host cells [7,8,8a]. Extracellular matrix (ECM) glycoproteins are exploited by *Streptococcus pneumoniae* as bridging molecules to bind to integrin receptors via Arg-Gly-Asp (RGD)-sequences present in the host glycoproteins. This leads to manipulation of host cell signal transduction cascades, thereby promoting dissemination and invasive infections [7]. Pneumococci have a similar strategy to improve cell adhesion through recruitment of serum components such as complement factor H (FH) and C1q. Although these complement components are serum proteins, they can also bind to eukaryotic receptors, thereby mediating pneumococcal attachment and triggering various signaling events [9–12]. Surface-exposed adhesins are also involved in the manipulation of the host fibrinolysis cascade. Striking examples are the pneumococcal endopeptidase O (PepO) and the glycolytic enzymes enolase, glyceraldehyde-3-phospho-dehydrogenase (GAPDH), and phosphoglycerate kinase (PGK). These non-classical surface proteins, also referred to as "moonlighting" proteins, subvert plasminogen and its cognate activators to accelerate pneumococcal-driven tissue degradation, complement evasion, and cell surface attachment [11,13–19,19a]. Although these moonlighting protein effectors are likely to be involved in induction of host cell signaling (e.g., via their interaction with receptors for plasminogen activators), these pathways have not been investigated as yet.

To provide a structured overview of current knowledge of pneumococci-induced signal transduction cascades and the subsequent functional and morphological host cell changes/alterations, three different modes of triggering signal transduction stimulation have been defined: (1) via a direct interaction between pneumococcal adhesins and specific host cell receptors, (2) via an indirect binding promoted by host serum proteins or ECM proteins, and (3) by the multifunctional cytotoxin and cytolysin Ply.

DIRECT INTERACTION BETWEEN ADHESIVE MOLECULES AND EUKARYOTIC CELL-SURFACE RECEPTORS

The adhesive properties of *S. pneumoniae* depend on adhesive proteins and also on non-proteinaceous molecules [1,2]. One of the earliest described receptors implicated in pneumococcal adhesion, intracellular invasion, and transcytosis through endothelial cells was the platelet-activating factor receptor (PAFR) [20]. The physiological ligand for PAFR is platelet-activating factor (PAF)—a phospholipid containing phosphorylcholine moities (*P*Cho). The *P*Cho of pneumococcal teichoic and lipoteichoic acids mimics the bioactive determinant of PAF [20] and serves as non-proteinaceous ligand for PAFR. Pneumococcal opaque or transparent phase variants reflect the amount of *P*Cho-containing teichoic acids on the bacterial surface relative to capsular polysaccharide. As a consequence, *P*Cho-mediated interaction with PAFR, as well as bacterial adhesion via surface-exposed choline-binding proteins (CBPs) such as PspC, depend on phase variation [21]. Although there have been several reports describing signaling pathways involved in PAFR interaction with its natural ligand PAF, detailed studies are lacking for pneumococcal *P*Cho. Nevertheless, the function of PAFR as a G protein-coupled receptor implies the involvement of GTPase-mediated activation of phospholipases C, D, and A2, and activation of protein kinase C (PKC) and tyrosine kinases (Figure 18.1A; Table 18.1 [30]). A further report also suggests a role for the scaffold/adaptor protein β-arrestin 1 as a cofactor involved in pneumococcus-PAFR-mediated interplay [22], thereby tethering the ligand-occupied G protein receptor PAFR to the vesicular trafficking system. Activation of the extracellular signal-regulated kinase 1 (ERK1)/ERK2 has also been detected as a predominant signaling pattern in pneumococcus–PAFR interaction [22].

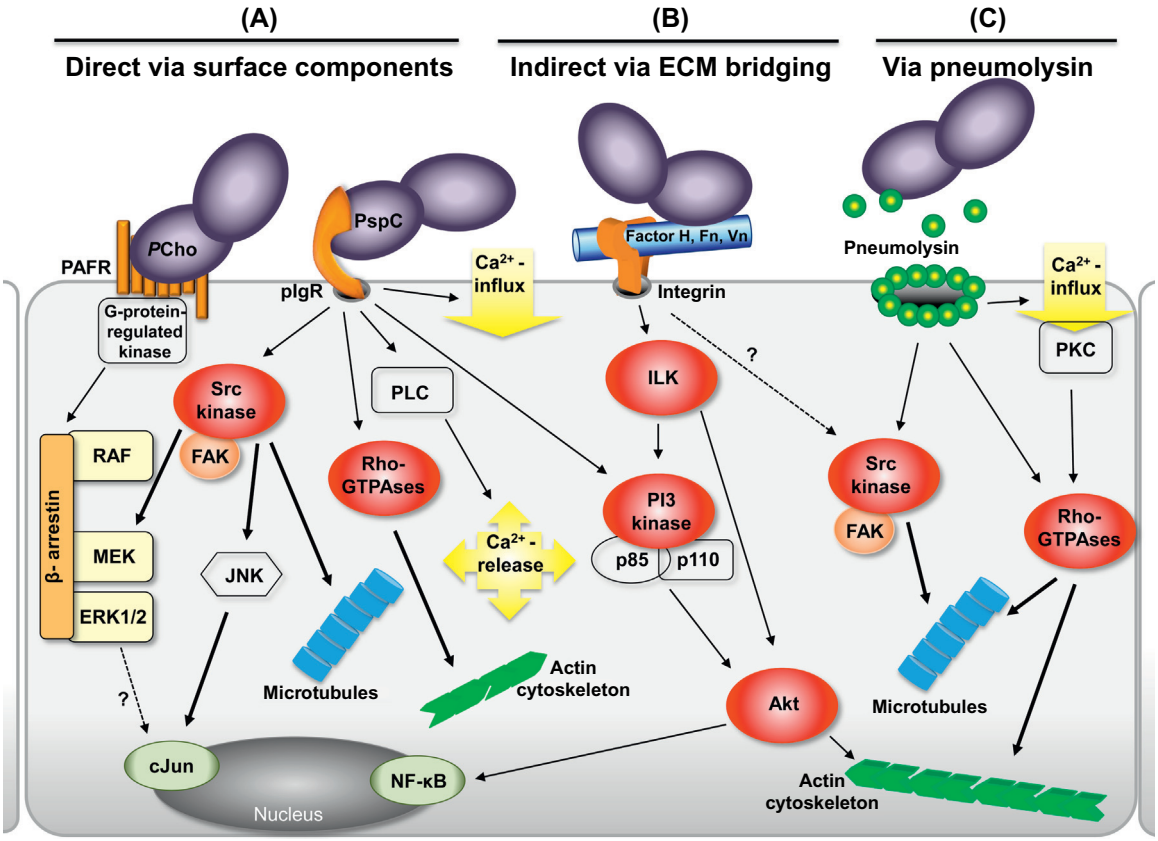

FIGURE 18.1 **Schematic model of signal transduction induced by different modes of pneumococcus–host interaction.** (A) Direct induction via surface adhesins and cell-surface receptors. (B) Indirect induction via ECM proteins bridging bacterial surface adhesins with integrin receptors. (C) Signal transduction induced by Ply. Key molecules involved in pneumococcus-induced signal transduction appear in red; thicker dark arrows indicate downstream signaling events.

TABLE 18.1 Pneumococcal Factors Inducing Host Signaling

Factor/Adhesin	Host receptor	Involved signal component	Reference
PCho	PAFR	GTPases, ERK1/2, β-arrestin	[20,22,23]
PspC	pIgR	PI3K, CDC42, Src, Akt, ERK1/2, JNK1/2	[10,24,25]
	Via Vn β1, β3, β5 integrins	ILK, PI3K, Akt	[7,26]
PspC/Hic	Via Factor H $\alpha_M\beta 2$ integrin/CR3	ILK, PI3K, Akt	[9]
PLY	TLR4	RhoA, Rac1, Myd88, MAPK, NFκB	[27–29]

Most publications on receptor-mediated interaction of pneumococci with host tissues describe host interactions with the *P*Cho moities of teichoic acids or cell-wall proteins such as PspC, which are noncovalently associated to *P*Cho molecules. Only a few reports have been published on the function in pneumococcal adherence of other classes of surface proteins such as lipoproteins and sortase-anchored proteins. Examples include the substrate-binding lipoprotein for an ABC manganese transporter; PsaA a typical example of a direct ligand-receptor interaction. PsaA interacts with the epithelial transmembrane E-cadherin, thereby inducing pneumococcal uptake and stimulating release of several cytokines [31,32]. A further example of a direct receptor contact is binding of the serine-rich cell-wall protein PsrP to keratin-10 on human lung epithelial cells [33]. However, detailed information about the associated underlying signaling events is lacking. In contrast, the most comprehensively characterized pneumococcal adhesin receptor–mediated inductions of signaling cascades are those caused by PspC (also referred to as CbpA or SpsA). PspC was identified as a major surface-expressed virulence factor [9,24,34–38] that, like PspA, the binding protein for the iron-sequestering human lactoferrin, is one of 15 pneumococcal CBPs [39]. CBPs are highly abundant on the pneumococcal cell surface; they are noncovalently associated to the cell surface by interacting with the *P*Cho of teichoic acids [40]. PspC interacts with host cells by binding the polymeric immunoglobulin receptor (pIgR), and also binds to cell-bound FH, an important regulatory component of the complement system, and to the ECM glycoprotein Vn [26,41–43]. The effects of PspC interactions with these host proteins are described in more detail below.

PspC, a Multifunctional Adhesin Triggering Host Cell Signaling

PspC is a multifunctional CBP that is one of the most important pneumococcal adhesins. Among various functions attributed to PspC, its human-specific interactions with the pIgR, also known as the secretory components (SCs), and the FH have been extensively characterized at the molecular level [9,10,24,34–36,44]. The PspC–pIgR interaction is mediated via the hexapeptide motifs located within the PspC repeat domains R1 or R2, recognizing human-specific amino acids within the D3 and D4 ectodomains of pIgR, whereas FH binding is localized to the very N-terminal part of PspC [36,44–46]. Interestingly, *S. pneumoniae* is the only bacterium known to require interactions with human pIgR for virulence; this interaction is critical for pneumococcal translocation from the nasopharynx to the lungs or blood to cause infection [35,36]. Even though pneumococci are typically extracellular pathogens, cell adherence mediated via pIgR results in pneumococcal uptake into host cells, but also involves additional host cell receptors including PAFR, as discussed above [22].

A common concept is that intimate bacteria–cell contact effectively induces outside-in signaling in the host cell. Hence, pneumococcal adherence to host cells via PspC–pIgR interaction initiates the activation of numerous cellular signaling molecules that facilitate bacterial uptake by the host cell (Figure 18.1A; Table 18.1 [10,24]). This includes but is not limited to phospho-inositide 3-kinase (PI3-kinase) and one of its downstream effector molecules, the serine-threonine kinase Akt (also referred to as protein kinase B). PI3-kinase and Akt are implicated in a variety of cellular functions such as regulation of the actin cytoskeleton, vesicle trafficking, and survival [47], and are targeted by a variety of pathogenic bacteria and viruses [48]. Not surprisingly, this pathway is also crucial for the uptake of species related to the pneumococcus, including *S. agalactiae* (group B streptococci, GBS) and *S. pyogenes* (group A streptococci, GAS) by host epithelial cells [49,50]. One remarkable exception is invasion of endothelial cells by the tissue-invasive M3-type GAS strain, which is

independent of PI3-kinase activity but requires GTPase Rac1 and Src family tyrosine kinase, suggesting a strain- and cell type–specific effect [51]. An important role for the PI3-kinase/Akt pathway during pneumococci–host cell interactions is supported by the observation that engagement of pIgR by multifunctional PspC protein induces, in a time-dependent manner, phosphorylation of the PI3-kinase p85α subunit and Akt. Furthermore, inhibition of these signaling molecules with specific pharmacological inhibitors causes a significant reduction in bacterial internalization into host cells [10]. Strikingly, uptake of pneumococci is a relatively rare event and is not necessary for activation of epithelial intracellular signaling, as the adherence of pneumococci alone is sufficient [10]. This observation might help explain the benefit for pneumococci of triggering intracellular signaling pathways—alternating host cell morphology may be profitable for the bacteria by facilitating access to adhesin receptors and opening up paracellular junctions.

Pneumococcal infection via the PspC–pIgR interaction triggers a broad subset of downstream signaling molecules such as Src family tyrosine kinases and mitogen-activated protein kinases (MAPKs). PspC–pIgR-mediated adherence especially activates ERK1 (p44 MAPK) and ERK2 (p42 MAPK) and the c-Jun NH2 terminal kinases (JNK1/2; Figure 18.1B; Table 18.1). It is noteworthy that PI3-kinase and Src kinase are independently activated during pneumococcal infection of pIgR-expressing cells, and simultaneous inactivation of these signal molecules has synergistic effects on reducing bacterial uptake by host cells [24]. Both Src family tyrosine kinase and MAPKs are critical signal transducers modulating a wide variety of cellular functions [52], and are targeted by numerous bacterial pathogens, highlighting their importance for infection processes [48]. Degree of encapsulation is an important determining factor for pneumococcal-induced signaling involving Src family tyrosine kinases and MAPK. Unencapsulated pneumococci induce JNK-, MAPK-, and AP-1-dependent IL-8 release by lung epithelial BEAS-2B cells [25]. Moreover, the unencapsulated R6x strain also induces p38 MAPK- and JNK-mediated caspase-dependent apoptosis in human endothelial cells, while the encapsulated strain D39 induces necrotic cell death [53]. Interestingly, the PspC–pIgR-mediated pneumococcal uptake by epithelial cells also requires the Src family tyrosine kinase and ERK 1/2 and JNK-MAPK. Inhibition of each of these pathways resulted in a significant decrease of pneumococcal uptake by pIgR-expressing cells [24]. Activation of ERK1/2 and JNK depends on Src family tyrosine kinase activity, with significant reduction of activation of ERK1/2 in response to pneumococci in cells over-expressing a dominant-negative, kinase-inactive version of Src (Src K297M) [24]. Furthermore, experiments performed using inhibitors revealed that Src family tyrosine kinase is activated upstream of the JNK pathway when pneumococci adhere to cells via the PspC–pIgR mechanism.

The host cell cytoskeleton is comprised of microtubules, actin filaments, and intermediate filaments, and is the most commonly described target for pathogen-mediated intracellular effects. Src family tyrosine kinases are important key mediators for the induction of cytoskeleton-dependent changes and cell morphology changes after pneumococcal infection. Importantly, host cell cytoskeleton dynamics plays a key role during pneumococcal ingestion by host epithelial cells via the PspC–pIgR mechanism [10]. The disruption of host cell cytoskeleton network organization by pharmacological inhibitors such as nocodazole (inhibiting polymerization of microtubules) or cytochalasin D (disrupting actin filaments) dramatically inhibits pneumococcal uptake by pIgR-expressing cells. In general, bacterial pathogens do not directly interfere with the actin filaments; however, they manipulate the cytoskeleton organization and control

polymerization of actin filaments by modulating cellular regulators of this process, such as the small Rho family of GTPases (Figure 18.1 [54,55]). A striking example involves the type-3 secretion system (T3SS) effector proteins SopE and SopE2 of *Salmonella enterica* activating the small GTPases Cdc42 and Rac in target cells, thereby generating actin-rich membrane ruffles to facilitate engulfment and internalization of the bacteria [56]. Other pathogens such as *Vibrio cholera* or *Bacillus anthracis* secrete toxins that enter the host and modulate signaling cascades, while pathogens such as enterohemorrhagic *Escherichia coli* employ both T3SS-mediated effector proteins and toxin to facilitate internalization [57]. Apparently, pneumococci also exploit small GTPases, in particular Cdc42, a key GTPase regulating PspC−pIgR-mediated pneumococcal internalization by host epithelial cells (Figure 18.1A [10]). Pneumococcal Ply has also been identified as a trigger molecule for activation of the small GTPases RhoA and Rac1 in neuronal cells, resulting in formation of cellular protrusions such as filopodia, stress fibers, and lamellipodia (Figure 18.3 [27]). The unencapsulated pneumococcal R6 strain also activates Rac1 in bronchial epithelial cells via TLR1/2 [25], and the lack of pIgR expression by these cells suggests this is a PspC−pIgR−independent activation.

Release of Calcium from Intracellular Stores upon PspC-Mediated Signaling

Signal transduction cascades are highly complex and tightly regulated pathways resulting in activation or deactivation of various effector proteins, secondary messengers, and other downstream signaling pathways. In general, most of the cellular signaling cascades are directly or indirectly dependent upon transient or sustained elevations in the intracellular calcium concentration [59]. Pathogenic bacteria including pneumococci have developed multiple mechanisms to influence host cells by modulating the calcium signals. For example, the pore-forming toxin listeriolysin O from *Listeria monocytogenes* has been identified as a *sine qua non* of calcium mobilization [60], and association of *Campylobacter jejuni* and *Salmonella typhimurium* with host cells induces calcium signaling that results in cytoskeleton rearrangements and bacterial internalization [61,62]. For pneumococci, Ply induction of NFκB activation and synthesis of IL-8 in neutrophils is dependent on calcium influx (Figure 18.1 [28]). *S. pneumoniae* infection of pIgR-expressing host cells also induces, in a phospholipase C-dependent manner, release of calcium from intracellular stores [34]. This effect depends on PspC, since no calcium mobilization was detected when a PspC-deficient strain was used [34,63]. Strikingly and contrary to the observation that increased intracellular calcium concentration facilitates bacterial internalization by host cells, elevated calcium levels seem to restrict pneumococcal uptake [34]. This is perhaps because pneumococci are predominantly extracellular pathogens, restricting their uptake by modulating the intracellular calcium levels within the host.

RECRUITMENT OF ECM-PROTEINS FOR INDIRECT BACTERIAL−HOST CELL CONTACT

The Role of FH in PspC-Induced Signaling

The complement system is a central component of the innate immune response and represents the first line of immune defense against invading pathogens. In order to escape complement attack and to establish infection, many pathogens bind inhibitors of the complement system to their cell surface [64,65]. To date several pathogenic microorganism including

S. pneumoniae have been identified as recruiting FH [41,64,65], a 150-kDa soluble glycoprotein that is a central fluid phase inhibitor of the alternative complement pathway. FH associates with host cell membranes via recognition of polyanionic components such as glycoaminoglycans (GAGs) and sialic acid [42]. FH is bound to the pneumococcal surface via PspC or the PspC-like protein Hic and acts as a molecular bridge to mediate adherence to host epithelial and endothelial cells. This process requires a concerted liaison of host epithelial cell surface GAGs, integrin(s), and host cell signaling pathways (Figure 18.1B; Table 18.1 [9]). Interestingly, recruitment of factor H-like protein1 (FHL-1, a splicing variant of FH consisting of the first seven domains) by *S. pyogenes* also promotes bacterial invasion of host epithelial cells [66].

Integrin CD11b/CD18 (also termed CR3, MAC1, or $\alpha_M\beta_2$) functions as an FH receptor on human polymorphonuclear leukocytes (PMNs), and FH promotes adhesion of PMNs to GAGs [67]. CR3 was identified as a PMN receptor for pneumococci coated with FH, whereas epithelial cells seem to express a different repertoire of FH receptors [9]. The existence of an RGD sequence in the fourth short consensus repeat (SCR4) of FH and the ability of Pra1, an integrin-binding protein of *Candida albicans*, to inhibit FH-mediated adherence to epithelial cells suggests that integrin(s) may be the receptor for FH-mediated binding of pneumococci to epithelial cells. In addition, the heparin-binding sites in the SCRs of bacteria-bound FH seem to be involved in pneumococcal attachment to host cell GAGs [9].

Whereas PspC–pIgR-mediated pneumococcal uptake by host cells requires both host cell actin microfilaments and microtubules, FH-mediated pneumococcal ingestion by host epithelial cells only needs actin microfilaments [9]. Similar to the PspC–pIgR-mediated signaling, protein tyrosine kinases and PI3-kinase have an essential role in FH-promoted uptake by host cells. In contrast, Rho family GTPases are not relevant for this specific mechanism [9]. However, despite the importance of FH recruitment by various pathogens, including *S. pneumoniae* and *S. pyogenes*, our understanding of bacteria-bound FH-mediated host cell signaling pathways is incomplete. Dissection of FH-mediated outside-inside and inside-outside signaling events during infection of eukaryotic cells is an important area for future research.

Targeting of Integrins by Pneumococci

Integrin-mediated signaling probably occurs primarily at the basal region of polarized epithelial cells [7,68], which is initially inaccessible to bacteria colonizing the nasopharyngeal cavity. Pneumococci penetrate ECM-forming epithelial barriers via recruitment of host-derived proteolytic activity of plasmin(ogen) and bacterial surface-expressed zinc metalloproteases. In addition, the cytolytic and cytotoxic activities of the pore-forming toxin Ply and cell surface–shaving enzymes like neuraminidase and hyaluronidase unmask cell-surface receptors, which are then accessible for pneumococcal adhesins [2,13,69,70].

In addition to FH and C1q (the first protein of the classical complement pathway), pneumococci also exploit other ECM glycoproteins such as human TSP-1, Vn, or Fn as molecular bridges to facilitate bacterial adhesion to host cell surfaces. Pneumococci preferentially bind multimerized Vn, and so far PspC is the sole surface protein known to interact specifically with Vn [7,26]. Although several pathogenic bacteria interact with Vn [71], the impact of this interaction on host cell infection and induced signaling transduction cascades has only been analyzed in detail for the human-specific species *Neisseria meningitidis* and *S. pneumoniae* [7,72]. Although five pneumococcal Fn-binding proteins have been identified so far [1], the effects of Fn-mediated adherence on pneumococci-induced

signaling remain unresolved. However, a plethora of studies have demonstrated in detail Fn-mediated cell adherence and induced signaling for several other bacterial pathogens, including *S. pyogenes* and *S. aureus* [73,74], which, like pneumococci, express several Fn-binding proteins [75]. Fn binds preferentially to α5β1 integrins, and not surprisingly these studies revealed remarkable similarities in the induced signal transduction molecules.

Activation of β1, β3, and β5-integrin receptors by pneumococci binding to host cell–bound Vn involves the N-terminal RGD site and induces signal molecules of the focal adhesion complex (Figure 18.1B [7]). Similarly, the indirect engagement of α5β1 integrin receptors via Fn-binding protein-producing Gram positive pathogens such as *S. aureus* or *S. pyogenes* induces bacterial invasion and signal cascades in host cells [76,77]. The outer surface protein C (Opc) of the Gram negative *N. meningitidis* interacts with both Vn and Fn, leading to attachment of meningococci to endothelial αvβ3 integrins, representing the main Vn receptor, and to α5β1-integrin, representing the main Fn receptor, respectively [78]. After Fn-mediated attachment of *S. aureus* and *S. pyogenes*, integrin-mediated signaling induces integrin receptor clustering and the concerted assembly of intracellular protein complexes [77,79]. This induced outside-inside signaling has been shown to promote internalization of pathogens by host cells [80,81]. Nevertheless, several studies have highlighted the important role of both chemical ligand-induced and mechanical surface tension–induced activation and clustering of α5β1 integrins [82,83] based on the structural properties of fibrillizing Fn dimers. A similar β3 integrin-mediated assembly of integrins may also be stimulated by multimerized Vn [7]; however, integrin clustering has not been observed in Vn-mediated pneumococcal uptake. Nevertheless, Vn-mediated binding of pneumococci to β3 integrins does induce cytoskeleton rearrangements, triggering pneumococcal uptake and the formation of extracellular microspikes in nasopharyngeal epithelial cells (Figure 18.2 [7]). These needle-like structures belong to the filopodia subtype and contain the actin-bundling protein fascein and actin-binding proteins like profilin and frabin [7,84,85]. Similar to FH-mediated pneumococcal uptake, Vn-induced internalization exclusively engages the actin cytoskeleton network [7]. Fn-mediated internalization of the Gram negative pathogen *C. jejuni* involves α5β1 integrins at fibrillar adhesion sites and also leads to filopodia and microspike formation in fibroblasts, indicating cytoskeleton rearrangements similar to those reported for pneumococcal interactions with Vn [86]. Various ligand-activated conformational states of α5β1 integrins may stimulate distinct intracellular signals [87,88], suggesting that β3 integrin-mediated signaling initiated by pneumococci binding to Vn might also reflect a specific subtype of integrin activation and downstream signaling induction.

ILK as Multiplayer of Integrin-Mediated Signaling

The integrin-linked kinase (ILK) is multifunctional scaffold protein that is implicated in integrin-dependent cytoskeletal reorganization and other cellular activities such as activation of glycogen synthase kinase-3, focal adhesion kinase (FAK), PI3K, protein kinase B (Akt), c-Src, or Ras/Raf pathways [7,68,80,89,90]. The intracellular connection of ILKs with most integrin heterodimers [91] suggests that ILKs may participate in integrin-mediated bacterial invasion. Indeed, signal transduction via ILK is required for αvβ3 integrin–dependent pneumococcal internalization via engagement of integrin-bound Vn, and also for Fn-induced uptake of *S. pyogenes* (Figure 18.1B [7,68,92]). Along with PI3K activation, phosphorylation of ILK appears to be another indispensable

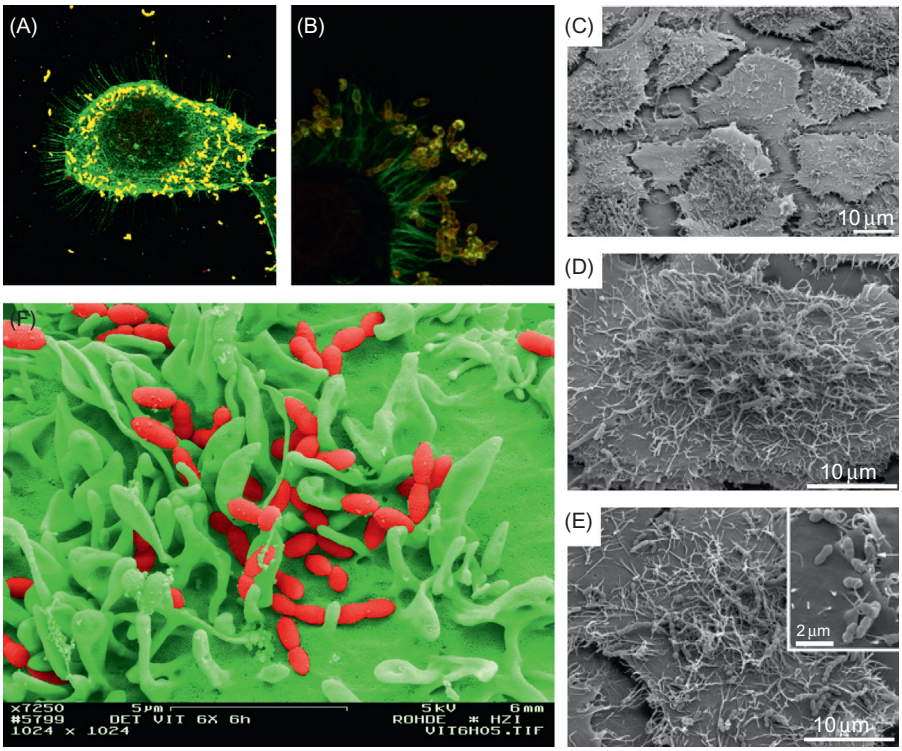

FIGURE 18.2 Vn-mediated pneumococcal adherence induces microspike formation by human nasopharyngeal Detroit 562 cells. Differential immune fluorescence staining after *in vitro* cell culture infection visualized adhered pneumococci in yellow (Alexa568/Alexa488 stain) and actin cytoskeleton in green (Phalloidin-Alexa488). (A) The microspikes appear as needle-like structures at the cell border. (B) Higher magnification elucidates entrapment of bacteria in microspikes. (C–E) Scanning electron micrographs illustrate generation of filopodia-like microspikes on the cell surface in response to Vn-mediated pneumococcal adherence at different magnifications. (F) Colorization of a 7250-fold magnification visualizes bacteria in red and microspike structures on the cell surface in green. *Source: Figures were partly taken from a publication by our group in* Journal of Cell Sciences *in 2009 [7].*

prerequisite for Vn-mediated pneumococcal uptake by host cells [7,50,68,92]. Similarly, ILK-dependent phosphorylation of Akt is required for integrin-mediated uptake of both pneumococci and GAS. It has been shown that recruitment and phosphorylation of the focal adhesion molecule paxillin via ILK and the dual kinase complex FAK and c-Src are required for *S. pyogenes* uptake after Fn binding via M1 protein and for caveolae-mediated *S. pyogenes* internalization via F/Sfb1 protein [92]. Recruitment and phosphorylation of FAK and c-Src are associated with recruitment of focal contact–associated proteins to the site of bacterial attachment; these tyrosine kinases are also commonly essential for Fn-mediated uptake of *S. aureus* and *C. jejuni* [77,86], as well as both Fn- and Vn-dependent integrin-mediated internalization of *N. meningitidis* into brain endothelium [72]. Integrin clustering at focal and fibrillar adhesion sites is not yet reported for Vn-mediated pneumococcal uptake, and the impact of FAK, c-Src, and paxillin in this signaling mechanism requires further investigation [7,92].

PLY: A MULTIFUNCTIONAL EFFECTOR OF EUKARYOTIC SIGNALING

Signal transduction pathways are also stimulated by the cholesterol-dependent pore-forming cytolysin Ply, which induces various responses in host epithelium, in vascular endothelial cells, and in immune cells as well [3]. Ply is produced as a pore-forming toxin by all known clinical isolates of *S. pneumoniae*, regardless of serotype and genotype. The toxin generates pores in the eukaryotic cell membrane, thereby promoting influx of large osmotic molecules, which provokes cell lysis [93]. However, in addition to its lytic effects on many mammalian cell types, Ply has numerous other effects at sublytic concentrations, including complement activation and induction of pro-inflammatory mediators [3,94,94a]. Ply is now known to signal via myeloid differentiation marker 88 (MyD88); it induces the release of tumor necrosis factor alpha and interleukin 6 ([29,95]). Transcriptome analyses have demonstrated that Ply induces expression of many host genes, for example, upregulating expression of more than 140 genes in the monocytic cell line THP-1 [96]. Micropore formation induced by sublytic concentrations of Ply also leads to various cell responses, including substantial calcium influx. This is followed by activation of Rac1 and RhoA GTPases and further downstream kinases, and subsequent actin cytoskeleton rearrangement that mediates radical changes of cell morphology in epithelial cells, including induction of focal adhesions (Figure 18.3 [3,27,58]). In contrast to actin cytoskeleton rearrangements, pathogen-induced signaling effects on microtubule stabilization have rarely been described. Remarkable results point to the function of Ply in reducing stable and dynamic microtubule content and inducing an increase in myosin light chain phosphorylation [97]. Src family tyrosine kinase activity has been identified as a key signal mediating microtubule stabilization, accompanied by strong impairment of mitochondrial trafficking

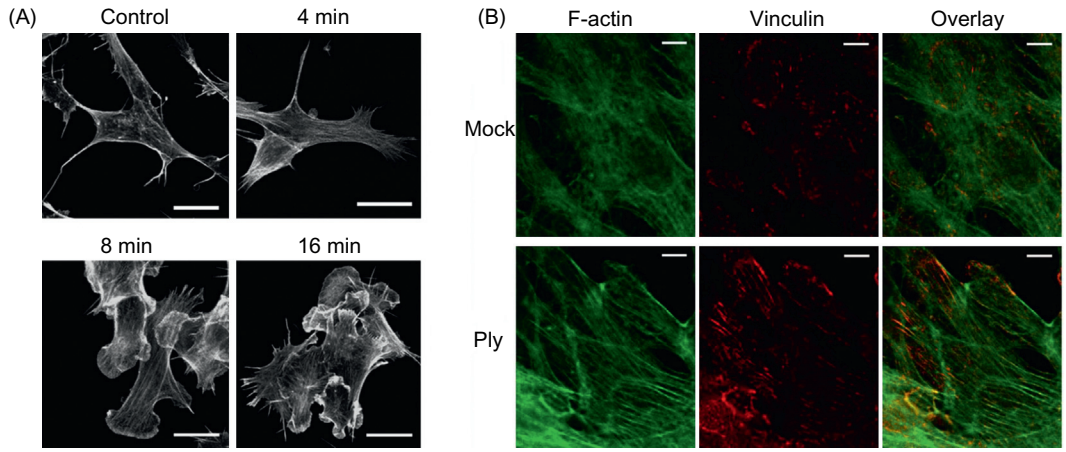

FIGURE 18.3 Induction of cytoskeletal protrusions and focal contacts by Ply. (A) Phalloidin staining of human neuroblastoma cells after treatment with Ply for different time points revealed filopodia, lamellipodia, and stress fiber formation (scale bar: 10 μm). (B) Staining of F-actin (green) and vinculin (red) of primary astrocytes visualizes induction of focal adhesions by Ply (scale bar: 10 μm). *Source: (A) The figure is reprinted from [27] with permission of the copyright holder. Copyright (2007) by National Academy of Sciences. (B) The figure is reprinted from [58] with permission of the copyright holder.*

[27]. The p38-MAPK and NFkB pathways are also activated in epithelial cells after incubation with sublytic amounts of Ply (Figure 18.1C [27,93,98]). Similar signaling events have been reported for vascular endothelial cells, thereby increasing the permeability of endothelial layers and modulating VE-cadherin expression. In contrast to calcium-independent microtubule bundling [3], endothelial hyper-permeability is strongly dependent on increased calcium influx [97]. Early signaling events such as PKC activation, perturbation of RhoA/Rac1 balance, and increased expression and activation of arginase enzyme at later time points contribute to Ply's effects on endothelium [97]. Ply-mediated signaling induction results in enhanced secretion of chemokines and cytokines such as IL8, which in turn enhances recruitment of neutrophils and collateral damage to inflamed tissue. A contribution of Ply in NFκB activation via TLR4 sensing has also been reported to account for subsequent cytokine release [99]. Remarkably, there are at least 16 different Ply variants at the protein level which differ in the presence and strength of hemolytic activity and other biological effects [100]. These variants might present valuable tools to study Ply-triggered signaling pathways. In summary, Ply acts as a versatile cytotoxic and cytolytic effector molecule, simultaneously triggering various signaling pathways in order to modify cell morphology, cell trafficking, and various branches of immune response [3].

CONCLUDING REMARKS

Three different strategies of pneumococci-induced host signaling cascades can be distinguished. The first mechanism represents a direct adhesion receptor−initiated signal transduction, while the second requires a host matrix bridging molecule that links pneumococci indirectly with a host cellular receptor. A third mechanism is induced by the secreted toxin Ply. Strikingly, PspC has multiple effects via both the first and second mechanisms, making this cell-wall protein a central component during the interactions of pneumococci with host cells. Several phosphorylation events in the host cells such as the *P*Cho-PAFR, PspC-pIgR, and the FH/Vn integrin−mediated signaling have been investigated in detail. In contrast, the impact of Fn-mediated pneumococcal adherence to and internalization into host cells on signal transduction pathways remain elusive. However, current knowledge indicates that pneumococcal−host interactions are able to trigger two different major signaling profiles. The first is initiated by both direct adhesin−receptor interaction and Ply-dependent effects and involves Src family tyrosine kinases as key mediators forwarding signals to further kinases, small GTPases, and transcription factors. The second is activated via integrin-bound matrix molecules interacting with pneumococci, and resembles the prototype of "integrin-mediated" signaling involving ILK and Akt as crucial mediators. Although the three different mechanisms of interaction with host cells differ in the key molecules initially activated, the consequences for host cell response seem to be similar. With the exception of some cell type−specific reactions, for example, type and amount of cytokine expression, most of the induced signaling pathways induce tremendous cytoskeletal rearrangements, leading to cellular morphological changes, thereby promoting pneumococcal uptake. Comparing pneumococcal-induced signaling profiles with those for other Gram positive bacteria such as *S. pyogenes* has highlighted the high degree of similarity regarding the key molecules and downstream signaling hierarchies involved. This high degree of coincidence might reflect the fundamental benefit of the similar yet diverse strategies evolved by pneumococci and other pathogens to interfere with host signaling for bacterial survival and dissemination in host tissues during disease pathogenesis.

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CHAPTER

19

Mechanisms of Predisposition to Pneumonia: Infants, the Elderly, and Viral Infections

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INTRODUCTION

Pneumococcal disease is opportunistic in nature, with the greatest incidence of pneumonia and invasive pneumococcal disease (IPD) occurring in infants (<2 years) and the elderly (>65 years) [1]. For young infants, susceptibility to infection is largely the result of a naïve immune system. For otherwise healthy young children and adults, mutations in genes that result in defects of either the innate or adaptive immune system are at fault for severe recurring infection. For the elderly, disease is most often the result of underlying morbidities that enhance susceptibility to infection and a waning innate and adaptive immune system. At all ages and in all groups, preceding respiratory tract viral infections are a major contributing factor to pneumococcal disease. During the 1918 Spanish influenza outbreak, young adults who succumbed to infection were often infected with *Streptococcus pneumoniae* [2]. Likewise, human immunodeficiency virus (HIV) is a serious risk factor for *S. pneumoniae*, with approximately 91,000 pneumococcal-related deaths in children worldwide associated with HIV infection occurring annually [3]. Beginning at 55 years of age, the incidence and severity of pneumococcal infection climbs, such that in the United States an individual older than 65 years of age is 42-fold more likely to die as a result of pneumococcal infection than a young adult 18–34 years of age [1]. In the United States, the case fatality rate for individuals over 65 years of age is

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approximately 20%; for those over 85 years of age it may be as high as 60% [4]. Importantly, in underdeveloped countries the mortality rates for pneumococcal infection in children, the elderly, and those who are immunocompromised are considerably higher [3,5]. Herein we discuss the molecular mechanisms that underlie enhanced susceptibility to IPD. We focus on those that impact the very young, those who are immunocompromised due to genetic mutations, the elderly, and the contributory role of preceding viral infection.

DISEASE IN CHILDREN

Susceptibility of Children to IPD

While the pattern is changing with the wider application of conjugate vaccines, the peak incidence of IPD occurs in the first 2 years of life. Infection is uncommon in neonates and in infants for the first several months, with incidence rising rapidly thereafter, before dropping sharply after age 2 years. This pattern fits neatly with the following observations: Protective anti-capsular polysaccharide (CPS) IgG antibodies are transferred across the placenta from mother to fetus, these antibodies disappear from the infant's circulation according to the known half-life of IgG (23–27 days), and robust production of such antibodies by the infant is delayed until about age 2 years. Certain details, however, show that the story is somewhat more complicated. First, failure of infants under 2 to respond to CPS is relative, as studies have recently shown that infants at 12 months can generate protective responses to a substantial degree, with 21–98% of infants responding to various CPS serotype antigens [6]. Second, the drop in IPD incidence around age 2 appears to precede the detection of anti-CPS antibodies in the sera of most children, suggesting that while antibody immunity to CPS is sufficient to prevent IPD, other factors also play important roles [7]. Antibodies to conserved antigens, including lipoteichoic acid (LTA), cell-wall components, and pneumococcal proteins might also be involved in developing protection. These factors also play a role in providing the appropriate inflammatory milieu for anti-CPS antibody production, for example, through stimulation of Toll-like receptors (TLRs).

The type-specific protective effect of anti-CPS antibody has been known for some time from studies of adaptive transfer of human-derived IgG in mice, and is directly confirmed by the human experience with CPS-based vaccines. Specifically, the use of vaccines containing CPS of the most common serotypes causing IPD clearly reduces the incidence of invasive disease caused by those serotypes. CPS is a pneumococcal virulence factor that inhibits phagocytosis by preventing or altering complement deposition [7]. In some cases, particular serotypes also demonstrate low intrinsic antigenicity for antibody production. Encapsulated bacteria are approximately 10^5-fold more virulent than corresponding unencapsulated variants. Anti-CPS antibodies, along with complement, increase phagocytosis of the organism through opsonization [8], a process of enhanced phagocytosis mediated by cell-surface receptors for antibody (i.e., Fc receptor) and complement (i.e., complement receptor). Notably, complement levels in infants do not appear to be developmentally regulated, with levels in infants similar to adult values.

CPS stimulates B lymphocytes in a T cell-independent type II (TI-2) manner, leading to a short-lived response which does not produce immunological memory [9]. While antibodies against the cell-wall polysaccharide (also known as C polysaccharide) are induced by all serotypes of *S. pneumoniae*, these antibodies are not protective, highlighting the importance of

type-specific responses. The successful development and implementation of protein-conjugate CPS vaccines has revolutionized prevention strategies for diseases caused by encapsulated bacteria, including the pneumococcus. Such vaccines overcome the age-dependent block in developing anti-CPS antibodies in children less than 2 years, presumably by engaging memory T cells specific for the carrier protein to which the CPS is conjugated. In essence, the antibody response is converted from T cell-independent to T cell-dependent. However, one should refrain from concluding that these two types of stimulation result in identical B cell responses, as qualitatively different antibody responses to type-specific CPS and corresponding conjugate vaccine have been demonstrated in animal models and in human children [10].

The nature of the hyporesponsiveness of infant B cells to CPS and its regulation appear to be complex. Most likely, effective antibody responses to TI-2 antigens depend on cytokine production as well as cell surface receptor−ligand interaction provided by bystander T cells, natural killer (NK) cells, and other B cells, and these responses require maturation of the human immune response through cross-reactive or nonspecific means in order to be optimally effective. Animal studies indicate an important role for TLR signaling [11,12], which is consistent with the finding of genetic susceptibility to IPD in patients with defective TLR signaling, as discussed below. According to these observations, the age-dependent delay in generating anti-CPS antibodies may be due to an intrinsic reduced capacity of B cells to generate a TI-2 response against CPS. Lower cell-surface expression of complement receptor 2 (also known as CD21) has also been implicated as a mechanism by which normal infants under age 2 fail to respond. CD21, along with CD19 and CD81, form the B cell receptor complex for antigen in concert with cell surface antigen-specific IgM. The adaptive significance of the delay in response to CPS has been debated, with opinion favoring a period of immunological adjustment to the gut microbiome as an explanation.

Genetic Mutations in Innate and Adaptive Immunity and IPD

The paradigm of anti-CPS antibody plus complement-mediated opsonization as the primary mechanism of protection against IPD correlates well with observations in patients with mutations in genes affecting innate and adaptive immunity. Infection with encapsulated bacteria, including pneumococcus, is a hallmark of well-defined antibody deficiency syndromes, including X-linked (Bruton's) agammaglobulinemia, the hyper-IgM syndromes, and common variable immune deficiency. Susceptibility to pneumococcal and other bacterial infections begins to manifest itself when maternal antibodies wane after the first several months of life. Antibody replacement therapy fully protects these patients from further invasive disease thereafter. Available replacement antibody preparations contain adequate titers of anti-CPS antibodies. This is due to widespread immunization and natural infections of the donor population. Anti-CPS titers in patient sera correlate with total IgG levels achieved after replacement, and no specific alteration in dosing is indicated to prevent infections.

Individuals with mutations leading to complement deficiencies may present with repeated infection even earlier in life, as these proteins do not cross the placenta and levels are not developmentally regulated. The important role of complement component C3 in antibody production and opsonization is consistent with the observation that C3 deficiency is associated with increased susceptibility to infections with pyogenic bacteria, including the pneumococcus. The traditional recommendation is that individuals experiencing two or

more invasive bacterial infections in childhood be screened for antibody and complement deficiency. In the current era of effective conjugate vaccines against *Haemophilus influenzae* type b and pneumococcus, it must be asked whether any child suffering from even a single invasive infectious illness with an encapsulated organism should be screened for these conditions [13]. Testing for total IgG and complement are inexpensive and readily available, and corresponding treatments are effective and potentially life saving.

Susceptibility to IPD due to mutations in genes involved in host defense is not limited to defects in humoral immunity. Indeed, inherited susceptibility to IPD is a hallmark of certain pathways of innate immunity [14]. In particular, IPD is seen in the scenario of mutation in two molecules critically important in TLR signaling in myeloid-derived cells, including macrophages, monocytes, and dendritic cells: the adaptor protein MyD88 [15] and the associated interleukin (IL)-1 receptor–associated kinase-4, i.e., IRAK-4 [16]. Initially described in families with multiple members expressing susceptibility to IPD, isolated individuals with IPD but without family history are now being shown to have alterations in innate immunity as well [17]. In keeping with the theme of infancy-dependent susceptibility, individuals with innate immune defects leading to IPD appear to become less susceptible with increasing age, which has been ascribed to the emergence of antibody-mediated, antigen-specific adaptive immunity. However, a recent report demonstrates that even with antibiotic prophylaxis and repeated vaccination with conjugate vaccines, subjects with IRAK-4 deficiency may still be subject to fatal IPD [18]. This may relate not only to intrinsic innate immune pathway defects in phagocytic cell activation, but also to "cross talk" with the adaptive immune system, such as failure to sustain the B cell response against CPS [19].

Type-Specific Protective Immunity and Anti-Polysaccharide Antibody Deficiency

Antibody responses to pneumococcal vaccination have long been used as a probe of the immune system in individuals with recurrent infections of the upper and lower respiratory tract [20,21]. Clinical observations have suggested the presence of a subgroup of individuals older than 2 years with impaired ability to mount a robust anti-CPS response despite otherwise normal immune responses, including normal total IgG levels and normal response to T cell-dependent protein antigens, such as diphtheria and tetanus toxoids. It has been proposed that such individuals represent a distinct clinical syndrome, variously labeled specific antibody deficiency (SAD), SAD with normal immunoglobulins (SADNI), or specific anti-polysaccharide antibody deficiency (SAPAD or SPAD) [22]. The identification of this condition required the adaptation of previously available serological tests for pneumococcal CPS, in particular, pre-absorption of patient sera with cross-reactive cell-wall polysaccharide and CPS. According to one commonly cited definition, "a normal response to vaccination with pneumococcal polysaccharide vaccine is the generation of protective titers (defined as $\geq$1.3 micrograms/mL) to more than 50% of serotypes tested in children between 2–5 years, and more than 70% of serotypes in patients $\geq$6 years" [23]. Thus, individuals falling outside this range can be defined as having SAD. By this definition, therefore, the diagnosis of SAD does not apply to subjects under 2 years. In various reports, patients meeting the definition of SAD correspond to anywhere between 5% and 10%, and as high as 23% of those referred for subspecialty evaluation of recurrent infections. Treatment options are not fully standardized, but approaches include immunization with conjugate vaccine, prophylactic antibiotics, and immunoglobulin replacement therapy. There is

some anecdotal evidence that, at least in children, SAD may disappear over time, suggesting that SAD represents a prolonged state of the developmentally regulated hyporesponsiveness to polysaccharide antigens usually seen in infants. A need for further investigation into measurement of antibody avidity in addition to antibody amount has recently been suggested. Patients with recurrent pneumococcal disease were identified who had normal antibody titers but reduced avidity for vaccine antigens [24]. This finding suggests an even more sophisticated approach to anti-pneumococcal immunity may be warranted.

The introduction of the highly effective 7-valent protein-conjugated pneumococcal polysaccharide vaccine in 2000 and the 13-valent formulation in 2010 (PCV13) has clearly reduced the incidence of IPD caused by vaccine serotypes. The overall reduction in IPD incidence has been mitigated somewhat by a compensatory increase in disease caused by non-vaccine serotypes [25]. A logical and effective response to this problem has been the development of conjugate vaccines containing increased numbers of CPS serotypes, explaining the move from a 7- to 13-valent formulation. An alternative approach is the development of vaccines based on conserved pneumococcal protein antigens, such as genetically detoxified versions of pneumolysin (e.g., L460D), pneumococcal histidine triad D (PhtD), choline-binding protein A (CbpA), or recombinant fusion constructs of these proteins. Such vaccines have shown activity in animal models of pneumococcal challenge, and studies in humans are planned [26,27]. As noted previously, such studies suggest that anti-CPS immunity, while sufficient, is not necessary for prevention of IPD. Likewise, they provide evidence for additional antigen-specific immune responses during infection that may provide sources of cellular "help" and cytokines during the TI-2 response to CPS.

ADVANCED AGE AND ENHANCED SUSCEPTIBILITY TO *S. PNEUMONIAE*

It is estimated that by the year 2050 more than 2 billion individuals will be greater than 65 years of age worldwide [28]. This represents a tremendous challenge to public health as advanced age is associated with increased incidence and severity of infectious disease, with lower respiratory tract infections the leading cause of infectious death for the elderly. The US Centers for Disease Control and World Health Organization both estimate that the mortality rate of adults with pneumococcal pneumonia averages 10–20% and may exceed 50% in high-risk groups such as those in a nursing home setting [4,29,30]. Importantly, immunocompetent healthy adults rarely succumb to primary pneumococcal pneumonia, suggesting that underlying medical conditions enable *S. pneumoniae* to overcome the host defense and cause opportunistic disease. Along such lines, it is well established that those with underlying age-associated morbidities such as chronic obstructive pulmonary disease, cardiovascular disease, and diabetes are at increased risk for IPD [29,31]. Thus, obtaining an understanding of why the elderly are more susceptible to pneumococcal infection and identifying new prophylactic options is critical to offset the tremendous associated morbidity and mortality.

Numerous age-related changes in both the innate and adaptive immune systems have been documented that can contribute to the enhanced susceptibility of the elderly to pneumococcal pneumonia. These include a less effective mucociliary escalator and diminished cough reflex that reduce clearance of aspirated bacteria [32], diminished responsiveness by macrophages and monocytes to TLR ligands such as pathogen-associated molecular patterns [11,12], defects in innate immune cell

chemotaxis and their killing of bacteria [33], impaired dendritic cell function [34], a decline in T cell responses and a switch to memory T cell [35], and impaired B cell antibody production and reduced antibody avidity [36]. Thus, advanced age has simultaneous and detrimental effects on multiple host defense mechanisms, and these defects are especially increased in those who are frail.

Herein we have chosen to focus on the new observation that chronic low-grade age-associated inflammation, both prior to and following pneumococcal pneumonia, enhances the susceptibility of the elderly to pneumococcal disease. Low-grade sterile inflammation is concomitant with advanced age, the result of a lifetime's exposure to environmental toxins (e.g., cigarette smoke), chronic infectious disease (e.g., cytomegalovirus infection, periodontal disease), the presence of underlying morbidities (e.g., diabetes, cardiovascular disease), and naturally age-occurring phenomena such as cellular senescence. Age-associated inflammation is characterized by low but persistently elevated levels of tissue and serum pro-inflammatory cytokines such as tumor necrosis factor (TNF)α and IL-6, and activation of the pro-inflammatory transcription factor nuclear factor kappa B (NFκB) within cells [37–39]. These pro-inflammatory factors and regulators are elevated and active, respectively, within individuals who have underlying morbidities that are known risk factors for pneumonia [40]. Thus, we propose and present below the notion that chronic low-grade inflammation is a common feature and by itself a risk factor for pneumococcal pneumonia in the elderly.

Chronic Inflammation Is Linked to Increased Susceptibility to Pneumonia

It is firmly established that aging is associated with chronic low-grade inflammation, a condition named by Franceschi et al. as "inflamm-aging" [38,39]. Multiple investigators including ourselves have shown that inflamm-aging occurs in the lungs of elderly humans and aged mice, the latter being the most commonly used age experimental model. Lungs from aged humans and mice have increased basal levels of NFκB activation [41], senescent cells that secrete pro-inflammatory factors [42,43], higher levels of tissue cytokines including TNFα [43], and are frequently marked by the presence of neutrophils despite the absence of an infection [43,44]. A causal link between chronic low-grade inflammation and susceptibility of the elderly to community-acquired pneumonia (CAP) is supported by a positive correlation between elevated serum TNFα and IL-6 levels with a greater incidence of this disease in otherwise healthy 70- to 79-year-old seniors over a 6.5-year period [40]. Directly implicating TNFα as a key cytokine that at low and persistent exposure enhances susceptibility to pneumonia, we have shown that young mice (4–5 months) infused with age-relevant physiological levels of TNFα for 5 days using a subcutaneously implanted osmotic pump had 100-fold more *S. pneumoniae* in their lungs and 10-fold more bacteria in their blood 1 day post-intranasal challenge than mice receiving the control carrier solution [45].

Age-Associated Inflammation Enhances Permissivity for Pneumococcal Adhesion

One reason chronic inflammation enhances susceptibility to pneumonia is that it induces expression of host proteins that are co-opted by *S. pneumoniae* for bacterial attachment to lung epithelial cells. As part of the studies that substantiated the latter, polymeric immunoglobulin receptor and platelet-activating factor receptor, proteins bound by the pneumococcal adhesin CbpA and bacterial cell wall phosphorylcholine, were shown to be elevated in the lungs of

healthy young mice implanted with osmotic pumps that delivered age-relevant physiological levels of TNFα [45]. These proteins are NFκB-regulated and are expressed in response to cell exposure to pro-inflammatory stimuli such as TNFα [46,47]. What is more, we have shown that healthy elderly humans and aged mice had basally elevated levels of laminin receptor, polymeric immunoglobulin receptor, and platelet-activating factor receptor in their lungs than young controls [43,45]. Laminin receptor is a second ligand for CbpA [48]. *S. pneumoniae* not only attaches to these protein but also co-opts the associated cell signaling and cell trafficking molecules to translocate through epithelial cells to the basolateral surface and gain access to vascular endothelial cells that line the lung capillaries. Importantly, the increase in bacterial adhesion when cells are exposed to pro-inflammatory cytokines is not modest, with exposure to TNFα increasing pneumococcal adhesion by as much as 25-fold [46,49].

Cellular senescence, the age-related phenomenon whereby DNA-damaged cells stop replicating and assume a pro-inflammatory phenotype (not to be confused with immunosenescence), also contributes to permissiveness for pneumococcal attachment. Senescent human and mouse lung epithelial cells express not only increased levels of laminin receptor, but also of keratin 10, a normally intracellular protein that is now recognized to also be targeted for extracellular adhesion by the pneumococcal adhesin PsrP [43]. What is more, the pro-inflammatory cytokines secreted by senescent cells (e.g., IL-1a, IL-6, IL-8) affect normal neighboring cells in a paracrine manner, increasing their expression of platelet-activating factor receptor and increasing their permissiveness for pneumococcal adhesion [43]. Supporting a role for senescent cells in the development of pneumonia, treatment of aged mice with rapamycin, an mTOR inhibitor that confers resistance to cellular senescence, resulted in a dramatic decrease in lung levels of laminin receptor, platelet-activating factor receptor, and keratin 10 in the lungs of aged mice and enhanced their survival post-infection [50]. Senescent cells are also recognized to be a major contributor to the pro-inflammatory lung exacerbations associated with chronic obstructive pulmonary disease [51]. As indicated above, this disease is a major risk factor for pneumococcal pneumonia. Figure 19.1 illustrates how cellular senescence and the low-grade but chronic pro-inflammatory microenvironment that occurs during advanced age enhances permissiveness for pneumococcal adhesion to lung cells.

Age-Dependent Macrophage Dysfunction

Paradoxically, whereas the elderly experience sterile chronic low-grade inflammation, innate immune cells from elderly subjects and aged animals also exhibit an inability to respond to infection with a robust acute pro-inflammatory response [11,12]. TLRs are host surface proteins that recognize various microbial components such as pneumococcal LTA, Gram negative lipopolysaccharide (LPS), and yeast zymosan. Following TLR engagement, an intracellular signaling cascade ensues that ultimately results in activation of the transcription factor NFκB and mitogen-activated protein kinase (MAPK) activation. Activation of NFκB and MAPK together leads toward the robust production of the pro-inflammatory cytokines, chemokines, and antimicrobial peptides that are necessary to keep infection in control [52]. In particular regard to the pneumococcus, TLR2 heterodimerizes with TLR1 to detect LTA, a component of the pneumococcal bacterial cell wall [53,54], TLR4 detects the pneumococcal toxin pneumolysin [55], and TLR9 resides within endosomes, where it recognizes unmethylated CpG DNA following bacterial internalization [56]. Studies with knockout mice indicate that the loss of TLR2 or TLR4

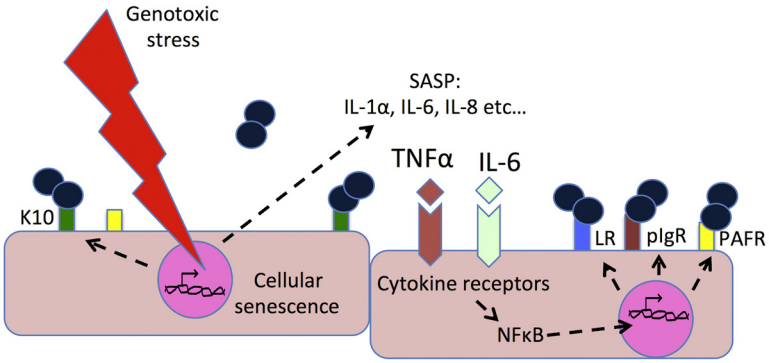

FIGURE 19.1 **Cellular senescence and the pro-inflammatory microenvironment present during advanced age induce expression of pneumococcal ligands.** DNA damage due to environmental exposures and telomere shortening can trigger cellular senescence. Senescent lung epithelial cells express elevated levels of keratin-10 (K10), which is the ligand for the pneumococcal adhesin PsrP. Pro-inflammatory factors produced by senescent lung cells (SASP) and in the tissue as a result of underlying morbidity (i.e., IL-6 and TNFα) also result in NFκB activation and increased expression of laminin receptor (LR), polymeric immunoglobulin receptor (pIgR), and platelet-activating factor receptor (PAFR). These are targeted by the pneumococcal adhesin CbpA and cell-wall phosphorylcholine, respectively.

does not significantly influence mouse survival following infection. In contrast, TLR9 and Myd88 deficient mice are exquisitely susceptible to pneumococcal infection, emphasizing not only the redundant roles for TLRs, but also an essential role for Toll/IL-1 receptor signaling in host survival [54]. In agreement with studies using gene-deficient mice, humans with loss of function mutations in key TLR signaling molecules such as MyD88, NEMO, and IRAK-4 (as discussed above) show enhanced susceptibility to pneumococcal infection, often resulting in IPD.

Macrophages and monocytes isolated from the elderly and aged animals have a dramatically reduced capacity to kill bacteria and fail to respond to TLR ligands with a robust pro-inflammatory cytokine response [57–61]. We have shown an age-dependent inability of alveolar macrophages (AM) from aged mice to control *S. pneumoniae* replication within the lungs of mice and sharply diminished cytokine production by alveolar macrophages from aged mice following *ex vivo* challenge with *S. pneumoniae* [62]. This occurred during the first 24 h of infection and in mice that had never been exposed to *S. pneumoniae*, thus ruling out age-related defects in the adaptive arm of the immune system. Consistent with multiple other reports examining the reasons for macrophage dysfunction [57–61], analysis of AM from aged mice indicated that their poor response to *S. pneumoniae* was the result of weak NFκB and MAPK (i.e., p38, JNK) activation following bacterial exposure [62]. Importantly, levels of TLRs 1, 2, and 4 on the surface of aged AM were not negatively affected by age, albeit other reports suggest that levels of TLRs are diminished with advanced age on human peripheral monocytes [12]. This, at least for AM, suggests that the age-dependent decrease in NFκB and MAPK activation was due to alterations in intracellular cell signaling. Recently, Mahbub et al. have shown that macrophages derived from the bone marrow of aged mice do not show age-related defects in their cytokine response to bacterial

products [63]. This suggests that age-dependent macrophage dysfunction is not intrinsic but instead a result of cells residing within an altered aged microenvironment.

Chronic Low-Grade Inflammation Suppresses Macrophage Function

In a phenomena called LPS tolerance, macrophages exposed to LPS become anergic to subsequent bacterial challenge and are unable to produce cytokines *de novo*. This is the result of increased levels of intracellular A20, a key and central homeostatic suppressor of both the NFκB and MAPK activation pathways, that is up-regulated by TNFα-induced GSK3 kinase activity (Figure 19.2A) [64]. Importantly, studies examining age-related changes in A20 levels have shown that this suppressor of inflammation is dramatically elevated in the lungs and alveolar macrophages of aged versus younger animals (Figure 19.2B). What is more, *ex vivo* exposure of alveolar macrophages to TNFα overnight resulted in their up-regulation of A20 and decreased ability to produce *de novo* IL-6 in response to *S. pneumoniae*. Finally, induction of A20 using a lentivirus construct also decreased the ability of primary alveolar macrophages to respond to pneumococcal challenge [65].

A20 is an ubiquitin editing enzyme that blocks TRAF6 activation (i.e., polyubiquitination) through disruption of the ubiquitin complexes that activate TRAF6 [66]. Briefly, TRAF6 is a shared signal transducer in the TNF receptor family, Toll-like/IL-1 receptor family, and T cell receptor pro-inflammatory cell signaling pathways that is upstream of MAPK and NFκB (Figure 19.2A). Macrophages from aged mice known to have elevated A20 levels failed to polyubiquitinate TRAF6 following exposure to *S. pneumoniae* [65]. Thus, one potential reason macrophages from the elderly fail to respond to pneumococci is that elevated TNFα during advance age triggers a homeostatic A20 response that suppresses the ability of these cells to respond to novel TLR stimulation, such as exposure to pneumococcal products.

Aging Impacts B Cell Function and Development of Protective Immunity

In the United States, two vaccines are approved for vaccination of adults against *S. pneumoniae*. The first, licensed in 1983, is composed of purified polysaccharides of the 23 most common CPSs that cause disease (PPV23) and is currently recommended only for those in clinical risk groups. The second, licensed for adults in 2010, is a protein conjugate vaccine composed of 13 capsule types conjugated to diphtheria CRM_{197} (PCV13). The protective efficacy of PPV23 among the elderly is estimated to be 55–70% against bacteremia and meningitis, but it is unclear whether the vaccine reduces the incidence of pneumonia [30,67,68]. The efficacy of PCV13 against pneumonia in the elderly remains unclear, and clinical trials are ongoing, though it is highly effective against IPD for the included capsule types in children. Thus, for the elderly the current focus is on identifying a vaccine formulation that is broadly protective against more than 13 capsule types and protects against pneumonia, the most common disease iteration of *S. pneumoniae* in the elderly.

Importantly, a similar anergizing response to TNFα has been reported for B cells, with high levels of TNFα levels in resting B cells negatively correlating with their poor response [69]. Aged mice and humans have documented decreases in immunoglobulin class switch (e.g., IgM to IgG), decreases in the enzyme AID (i.e., activation-induced cytidine deaminase), and decreases in the transcription factor E47, which is required for proliferation and passage through developmental checkpoints. Thus, both class switching and affinity maturation

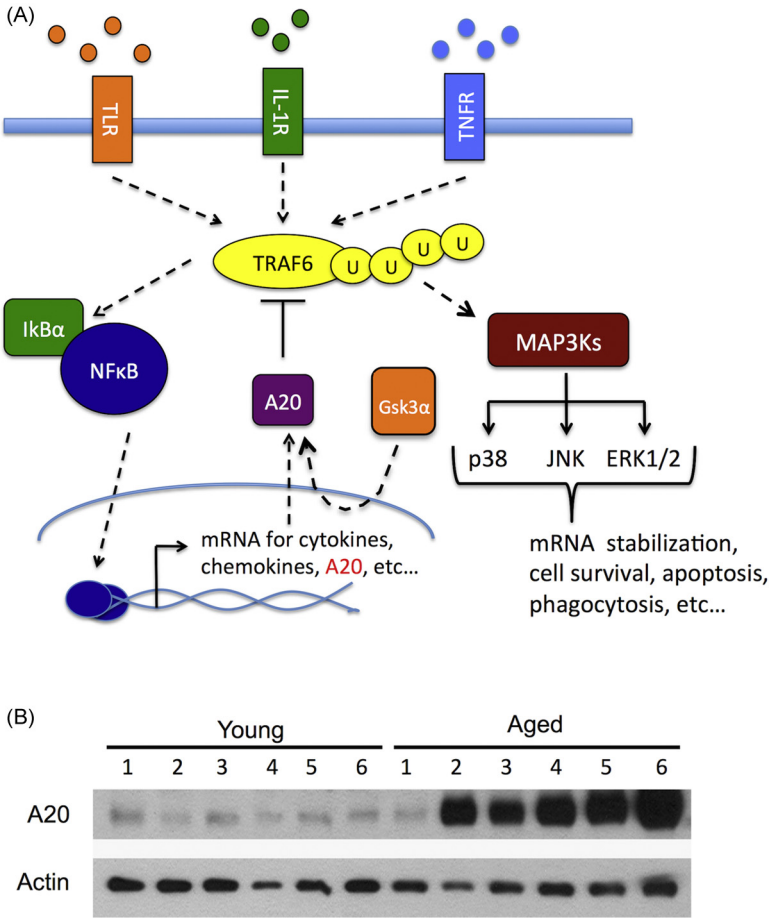

FIGURE 19.2 **The TRAF6 pro-inflammatory axis and age-related up-regulation of A20.** (A) Simplified schematic of how assorted pro-inflammatory stimuli, including those received from the Toll-like receptor (TLR), interleukin-1 receptor (IL-1R), and tumor necrosis factor (TNF) receptor families (i.e., cytokines and pathogen products) leads to NFκB and MAPK activation through TRAF6 polyubiquitination. Note that NFκB activation results in the expression and production of A20, which in turn suppresses TRAF6 by blocking its ubiquitination. Thus, A20 serves as a negative regulator of cell activation and inhibits the pro-inflammatory response. (B) Western blot for A20 levels in whole lung samples from individual young (4–5 months) and aged (19–21 months) healthy C57BL/6 mice. Elevated A20 levels inhibit the ability of a macrophage to respond to *S. pneumoniae*.

are decreased in elderly responses. Similar to what is reported for alveolar macrophages, pre-incubation of B cells with TNFα decreased both young and old B cell responses to LPS, with B cell function capable of being restored by the addition of antibody against TNFα. It was subsequently determined that incubation of B cells with TNFα induced TTP, a physiological regulator of mRNA stability for AID [69]. TNFα has also been shown to negatively regulate the survival of B cell precursors within the bone marrow [70].

IMPACT OF VIRAL INFECTIONS

Pneumonia in the Compromised Host

The natural host niche of the pneumococcus is the human nasopharynx. More than 90 serotypes of *S. pneumoniae* can transiently colonize the nasopharynx; these colonizing strains serve as a reservoir for transmission to other susceptible hosts. During the period immediately after colonization, pneumonia and invasive disease may occur. However, numerous layered and overlapping defenses normally protect the host from the development of disease. Pneumonia, when it occurs, is therefore almost always in a person that has their defenses impaired by one or more intrinsic or extrinsic factors. As detailed earlier in this chapter, altered immunity at the extremes of age is a major risk factor for pneumococcal pneumonia. In addition, certain disease states predispose to pneumonia through dysfunction of immune responses (e.g., sickle cell disease, nephrotic syndrome), others through disruption of physical barriers or physiologic limitations of clearance from the lungs (e.g., cerebral palsy, chronic obstructive pulmonary disease, chronic smoking) [71], and as a result of chronic low-grade inflammation that develops. In persons without preexisting medical comorbidities, however, viral co-infections are the most common factors that lead to a compromised state, allowing *S. pneumoniae* to cause serious disease [72]. Although epidemiologic evidence suggests that numerous viruses can be co-pathogens with the pneumococcus, the best-studied interactions at this point are with HIV and influenza viruses.

Immunity to *S. pneumoniae*

In order to initiate an infection of the lung, *S. pneumoniae* must either be directly inhaled from the environment or transmigrate from a colonizing site in the nasopharynx [73]. Several physical and physiologic barriers act in concert to keep pathogenic bacteria out of the lung. Production of a mucus layer with sialylated proteins and antibacterial defensin molecules that can bind and neutralize infectious agents, coupled with ciliary action, serve to trap and move bacteria out of the airways. The cough reflex may potentiate this removal. The tendency of bacteria to form biofilms in the nasopharynx may regulate release of individual bacteria cells, contributing to localization in the upper respiratory tract. When small inocula of *S. pneumoniae* escape these physical barriers, however, the next line of defense is the alveolar macrophage. Alveolar macrophages perform dual roles in the lung: They can efficiently phagocytize bacteria, with the aid of complement as an opsonin, while simultaneously acting as a negative regulator of inflammatory responses [74]. This maintains a homeostasis in the lung so that antibacterial defenses, which can damage the host if not controlled, are not repeatedly activated with frequent, small exposures to bacteria.

When large infectious doses of bacteria are encountered that overwhelm the clearance capacity of alveolar macrophages, these cells and epithelial cells within the lungs secrete cytokines and chemokines to initiate innate responses [75]. NK cells are activated and secrete TNFα and chemokines such as the macrophage inflammatory protein 2, leading to an influx of neutrophils into the lungs. Inflammatory monocytes are also recruited from the peripheral blood to differentiate into macrophages that can act as antigen-presenting cells. Neutrophils act as the primary phagocytic cell during pneumococcal pneumonia, but are inefficient when only complement is available as an opsonin. Several days into the infection, the adaptive response, which includes pneumococcus-specific CD4+ T cells and B cells, begins to develop. The production of antigen-specific antibody recognizing the particular pneumococcal capsule of

the infecting strain allows efficient opsonization of the infecting bacteria and rapid, neutrophil-mediated clearance. A return to homeostasis mediated by macrophages of a wound-healing phenotype ensues, accompanied by a shift in the cytokine milieu to a more anti-inflammatory environment characterized by maturation of CD4+ T cell memory responses and durable immunity. Viral infections may disrupt any or several of these protective defenses, depending on the timing of the co-infection, allowing immune escape of *S. pneumoniae* or enhanced pathogenicity of the resulting disease.

Epidemiology of Pneumococcal Co-infection in HIV

The burden of many infections, including CAP, is greatly increased in HIV-infected persons. Pneumococcal disease is more than 40-fold as common in HIV-infected children in high-prevalence areas in Africa, and was 9–13 times greater in US children in the era prior to the use of highly active antiretroviral therapy (HAART) [76]. Similar disparities are seen in adults, with the incidence of bacteremia more than 80-fold as high in HIV-infected persons in the United States prior to HAART. Use of HAART reduces this difference by more than half [76], while pneumococcal vaccine reduces it by about 2.5-fold [77]. HAART also decreases the incidence of CAP, including that caused by *S. pneumoniae*. In the HAART era, patients who develop pneumococcal disease tend to have associated chronic medical conditions and higher mortality, and severe disease is associated with smoking and alcohol abuse [78]. In HAART-naïve patients in Africa, CAP was associated with IV drug use, fewer comorbid chronic illnesses, and worse respiratory disease [79]. *S. pneumoniae* is the most common cause of CAP in HIV-infected persons. In patients with CD4+ T cells counts greater than 200 per mm^3 of blood, *S. pneumoniae* dominates as the major etiology, while *Pneumocystis jirovecii* is slightly more common in persons with CD4+ T cell counts less than or equal to 200 per mm^3 [80]. Finally, HIV-infected persons have significantly higher mortality rates from CAP than non-HIV-infected persons when adjustments are made for comorbidities [79].

Immune Defects in HIV Co-Infection with *S. pneumoniae*

The primary immune defect in HIV-infected persons is a massive depletion of CD4+ T cells. CD4+ T cells have roles in controlling colonization, coordinating adaptive immune responses to IPD, and maintaining homeostasis in the lung. Loss of Th17-committed CD4+ T cells, which produce IL-17 and IL-22, impacts defense against colonization, as these cells are chiefly responsible for clearance of *S. pneumoniae* from the nasopharynx through recruitment of neutrophils and production of antimicrobial β-defensins [81]. Colonization may be impacted earlier than pulmonary immunity, as CD4+ T cell depletion is accelerated at mucosal surfaces [82]. During early HIV infection, there is a skewing in the peripheral blood away from Th17 and toward the Th1 subset of T cells [81,83]. These cells produce IFN-γ, have impaired proliferation in response to antigen, and fail to up-regulate the co-stimulatory molecule CD154, which allows cross talk with antigen-presenting cells [82,83]. Thus, memory responses to *S. pneumoniae* in the lung are impaired, allowing infection even prior to depletion of CD4+ T cells and development of acquired immunodeficiency syndrome, i.e., AIDS. Although the relative frequency of antigen-specific CD4+ T cells is higher in bronchoalveolar lavage fluid than in the peripheral blood, there remains a relative deficiency of such cells compared to non-HIV-infected persons.

Finally, increased immunosenescence, not to be confused with cellular senescence, which is a non-replicating state incurred by DNA damage, of CD4+ T regulatory cells leads to a loss of homeostatic responses associated with increased autoimmunity and an inability to return to normal levels of inflammation following a pulmonary infection [81,83]. In HIV-infected persons who have advanced disease and low CD4+ T cell counts and who are not on HAART, there is a progressive loss of control of colonization and invasion of *S. pneumoniae*, coupled with an inability to appropriately resolve severe infections. Interestingly, many other host defenses are normal, even in profoundly depleted persons infected with HIV. Alveolar macrophage responses are normal, and complement deposition for opsonization is unaffected [84,85]. Pneumococcal-specific immunoglobulin levels are normal in the BAL, despite a relative decrease in frequency due to immune activation and increased overall IgG levels [86]. However, opsonic function is impaired in bronchoalveolar lavage [87], and reduced IL-8 responses are present, which may impact recruitment of neutrophils to the lung during pneumonia [88].

Epidemiology of Pneumococcal Co-Infection with Influenza

It is now recognized that a high proportion of CAP is caused by co-infections, contributing to millions of ambulatory care visits for pneumonia and thousands of deaths each year worldwide. Although influenza is a major public health threat on its own, bacterial co-infections complicating influenza contribute greatly by exacerbating disease severity. Detailed descriptions of fatal cases date as far back as the eighteenth century (reviewed in [73]), indicating that viral–bacterial co-infections have been recognized as being prevalent for hundreds of years. This interaction came to the forefront of scientific study during the "Spanish Flu" pandemic in 1918–1919, during which more than 95% of the more than 50 million deaths were complicated by a bacterial co-infection [2]. Approximately 50–70% of severe or fatal cases in the 1957 H2N2 and 1968 H3N2 pandemics, and nearly one-third of those in the 2009 H1N1 pandemic, had bacterial complications [73]. Furthermore, when a bacterial co-infection was identified in recent studies during the 2009 H1N1 pandemic, mortality was high despite appropriate antibiotic use in the majority of cases [89,90]. *S. pneumoniae* remains the most frequently identified bacterial pathogen associated with influenza infections and the most common cause of CAP despite use of the pneumococcal conjugate vaccine in children and adults. There is little systematic surveillance of bacterial co-infections during seasonal influenza, but this continued threat to public health has led to increased research on the co-pathogenesis of pneumonia due to influenza viruses and bacterial pathogens (reviewed in [73,91]).

Immune Defects in Influenza Co-Infection with *S. pneumoniae*

Respiratory tract damage accumulates during viral infections and primes the damaged and undamaged areas for bacterial colonization due to disrupted mechanical clearance mechanisms and exposed receptors. The host depends on the mucociliary apparatus in the lung and nasal passages to clear invading pathogens, but viral insults can damage the respiratory epithelium and inhibit this mode of removal [92]. Receptors (e.g., platelet-activating factor receptor) permissive of attachment of bacterial invaders become exposed in these inflamed areas, as defined by autopsy studies in humans and *in vivo* infections in mice [1,93]. Additional adhesion sites in the lung appear as the viral lesions begin to heal. The pneumococcus and other

bacteria can utilize bacterial adhesins to bind exposed laminin, type I and IV collagen, and fibrin/fibrinogen deposition in areas of incomplete healing. Injured or differentiating cells also provide new sites on apical receptors (e.g., asialylated glycans or integrins) for bacterial pathogens such as the pneumococcus (reviewed in [94]). This increased attachment within the lung, trachea, and nasopharyngeal surfaces may be mediated, at least in part, by viral neuraminidase activity [95], which facilitates bacterial adherence by exposing host cell receptors, disrupting sialylated mucins, and providing a catabolic substrate for bacterial growth in the form of free sialic acids.

During the early stages of co-infection, the capacity of alveolar macrophages to phagocytize and eliminate small inocula of bacteria is compromised through profound depletion of these airway-resident cells [96]. Inhibition of acute pro-inflammatory cytokines through impairment of NK responses and direct suppression of chemokines mediated by the antiviral state promoted by type I interferon depress the normal phagocytic activity of macrophages and neutrophils [97,98]. Together, these impairments allow escape from early innate immunity and outgrowth of bacteria in the lungs. Bacterial cytotoxins, like the pneumococcal pneumolysin, are known to influence host inflammation and may work in concert with viral cytotoxins. These bacterial factors may intensify the cell death and inflammatory signaling resulting from pores formed by the influenza cytotoxic protein PB1-F2 [99]. Viruses and bacteria also activate many of the same cytokines, inflammatory cells, and pattern-recognition receptors (e.g., TLR4), which can synergize during co-infections and generate inflammation [100]. Interference with immune responses occurs through various manners, such as by viral expression of multifunctional proteins like the influenza virus NS-1 and PB1-F2 [99]. Depending on the stage of influenza, the innate, cellular, and anergic responses may differentially synergize. Production of interferon-γ increases during influenza resolution and can down-regulate bacterial scavenger receptors (e.g., MARCO) on macrophages, leaving phagocytic cells suppressed and cytokine profiles altered [3,101]. Additional pro-inflammatory (e.g., IL-1β, TNFα, IL-6, and IL-12) and anti-inflammatory cytokines (e.g., IL-10) are induced, and further compound downstream events such as macrophage and neutrophil recruitment and dendritic cell function during influenza-pneumococcal coinfection are promoted [97,102]. Thus, the host is in a relative state of immune dysregulation, with heightened inflammatory and anti-inflammatory responses in response to this rapidly progressive infection (reviewed in [73,91]).

As the vigorous antiviral inflammatory response begins to subside, a new state of innate immune activation that may alter responsiveness to new pathogenic insults is reached. The lung becomes repopulated with resident AM as recruited macrophages proliferate and differentiate. In an attempt to return the lung to homeostasis, wound-healing processes coordinate an anti-inflammatory response characterized by IL-10 and suppress pathogen recognition systems [103]. During the recovery phase, the host becomes immunologically desensitized both locally and systemically, which can last for several weeks, prolonging the opportunity for bacterial invasion. The degree and length of this suppression is viral strain−dependent, and occurs through diverse mechanisms. For instance, AM with high expression of homeostatic moieties such as CD200R, a regulatory anti-inflammatory ligand, become desensitized when expression of CD200 on apoptotic immune cells increases, opening the airways to bacterial invasion [104]. In conjunction, absence of CD200R in mice inhibits bacterial outgrowth and prevents migration of bacteria to exogenous sites, such as the blood, in influenza-infected mice.

Impact of Viral Co-Infection on Transmission

There is a relationship between colonization density in the nasopharynx and ease of transmission. Respiratory viruses such as influenza virus appear to enhance transmission potential in high-prevalence settings [105]. Both HIV and influenza viruses have been shown to increase nasopharynx colonization levels of *S. pneumoniae*, suggesting that they might influence person-to-person spread through this mechanism [106]. Careful studies in mother–child pairs in Africa suggest that transmission does appear to be enhanced by HIV infection in close contact settings, but no evidence of more widespread transmission was found [107,108]. Exploration of influenza transmission in animal models [109] and modeling of human incidence data [110] suggest that effects on susceptibility of the recipient are stronger than effects on transmission from the donor. Further controlled studies in family settings need to be done to better understand the impact of these and other viruses on pneumococcal colonization density and transmission.

OVERVIEW

Control of pneumococcal infections is a complex and multilayered process; it requires the development and maintenance of a robust and capable immune system, and these are often absent at the extremes of age. In healthy adults, and not excluding those with underlying conditions, viruses can interfere with many of these host defense mechanisms in a manner that can increase the incidence or augment the severity of disease. For those with immune defects, complementation strategies, such as administration of immunoglobulins or prophylactic antibiotic therapy, are the current strategies to protect against disease. For individuals with HIV, immunization before the development of AIDS is recommended to ensure sufficient CD4 activity. Further exacerbating susceptibility to infection is the chronic inflammatory state in the aged host. Inflamm-aging results in increased bacterial ligand expression, decreased alveolar macrophage function, and diminished B cell responsiveness. These deficits occur on top of the other age-related defects not discussed herein, such as those described for neutrophils, macrophage and monocyte killing, and in T cell activation and response. Finally, viral respiratory tract infections are a major facilitator of serious infections in persons who do not have other underlying comorbidities that enhance the likelihood of disease. Prevention and treatment in the setting of co-infection is complicated by the need to control multiple pathogens, and by the complex and interrelated effects on the immune system.

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CHAPTER

20

Mechanisms Causing the Inflammatory Response to *Streptococcus pneumoniae*

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INTRODUCTION

Pneumococcus can infect almost every tissue of the human host, with consequences ranging from next to nothing, through devastating inflammation, to severe illness, disability, and death. The extremes of this response range are seen in the upper respiratory tract mucosal surface, which is uniquely adapted to manage colonization and stable carriage with a controlled inflammatory response, and in the meninges, where even the presence of dead pneumococci can cause death in the host. The concept of tolerance as a defense strategy rather than maintaining sterility at huge cost is important to understand inflammatory responses to pneumococcus [1].

This chapter will consider mechanisms causing the inflammatory response in various tissues, drawing out common themes and important differences as well as the translational impact of this knowledge in both the prevention and treatment of pneumococcal disease. We will first consider the clinical features of these compartmentalized responses, then the host and pathogen factors that permit compartmentalization to occur—from the tightly regulated extent that is seen in human carriage through mucosal disease to the lungs, blood, and even the brain.

Compartmentalization of Immune Responses to Pneumococcus

Humans are (almost) the sole pneumococcal host. Apart from very rare exceptions, such as race horses, almost all transmission occurs by contact with the respiratory secretions of colonized young children. Whether this is predominantly smeared hand to hand, picked up from shared working surfaces or water bottles, or spread mainly by coughing, sneezing, and inhalation is relatively unimportant as the experience of exposure of the respiratory mucosa to pneumococcus is both universal and repeated [2]. Given this ubiquitous exposure, all humans have adapted respiratory mucosal surfaces able to interact with pneumococcus; this usually results in innate and acquired, humoral, and cellular responses, which are described initially

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in the section on the nasopharynx, then developed and adapted to explain the different patterns of inflammation in other tissues.

Otitis media and sinusitis are the most common illnesses caused by pneumococcus (see Chapter 16). Development of these diseases involves only a small anatomical migration in terms of distance traveled by the bacteria, with minimal change in host histology but an inflammatory response that is painful and induces temporary deafness. The development of otitis media is dependent on exposure intensity, co-infection (particularly with viruses), and maturity. Maturation of the skull shape to optimally ventilate the middle ear and sinuses may play a role, and the development of capsule and protein-responsive mucosal immunity reduces disease with age.

The lung is not a sterile space [3], and indeed small numbers of aspirated pneumococci can be removed by macrophages with no evident inflammation and with measurable lung immune priming benefit. Pneumococcal pneumonia is initially a mucosal infection, but pneumococcal outgrowth results in a classical series of inflammatory responses to consolidate the respiratory airspaces of the lung. Pulmonary parenchyma is uniquely protected by immune mechanisms and adapted both to aggressively defend the airspace from infection and to completely resolve this inflammatory response and protect the tissue.

Bacteremia with *Streptococcus pneumoniae* is always symptomatic. Patients with pneumococcemia exhibit fever, rigors, and often suffer the consequence of metastatic spread of infection to bone, joints, muscle, and even heart valves. The mechanisms of inflammation are common to other bacterial sepsis and dysregulated inflammation results in a syndrome leading to multi-organ failure [4].

Finally, translocation of pneumococci to the cerebrospinal fluid (CSF) results in overwhelming inflammation, with collapse of blood–brain barrier integrity and neutrophil influx even if the bacteria are dead. Hippocampal apoptosis and neuronal necrosis are compartmentally unique features of this response that lead to pathogen-specific brain damage [5].

In conclusion, pneumococcal infection results in different degrees of inflammatory responses and tissue damage at each compartment as tolerance capacity varies between infection sites (Figure 20.1).

Pathogen-Specific Virulence Factors That Facilitate Compartmentalization of Response

S. pneumoniae possesses an armamentarium of virulence factors that it deploys in a coordinated fashion to enable its survival and propagation within multiple niches in its human host (summarized in Table 20.1) [6]. The most important attributes of a successful virulence phenotype are initial colonization, adherence, and translocation through epithelia; direct toxin-mediated tissue damage; subversion of host immune responses, particularly complement-mediated opsonophagocytosis; and resistance to conditions of nutrient deficiency.

Tissue Specificity of Virulence Factors

The dynamic nature of virulence factor expression during the progression of pneumococcal infection has been demonstrated with increasing levels of sophistication in recent years. Orihuela et al. demonstrated that not only were particular virulence factors involved in tissue-specific replication, but distinct factors were required to allow transition between body sites [7]. For example, pneumolysin was required for replication in the lungs and both translocation to, and survival in, the bloodstream. PspC (also referred to as CbpA or choline-binding protein A) contributes to transition both from upper to lower respiratory tracts and from blood to CSF by critical binding of the PAF receptor, but is redundant for tissue

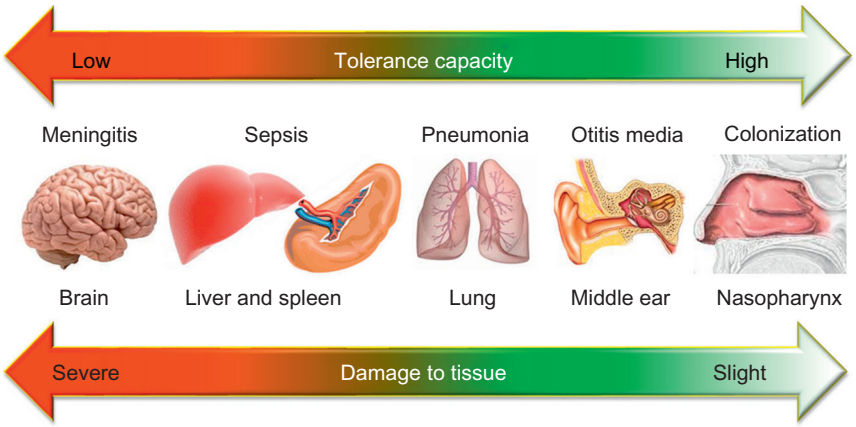

FIGURE 20.1 **Tissue tolerance to pneumococcal infection.** Tolerance is a host defense strategy that reduces the negative impact (tissue damage) of pathogen infection or exacerbated immune response against them. Different from resistance by the immune system, which protects the infected host by reducing its pathogen burden, tolerance does not affect pathogen burden. The nasopharynx therefore has the highest tolerance to pneumococcus and the lowest susceptible to tissue damage, allowing bacterial replication and maintenance of carriage for weeks. On the other hand, the brain has the lowest tolerance level and high susceptibility to tissue damage during meningitis. *Source: Figure modified from [1].*

TABLE 20.1 Pneumococcal Virulence Factors Grouped According to Main Function in Pneumonia

Virulence factor	Main function in pneumococcal disease
RESISTANCE TO OPSONOPHAGOCYTOSIS	
Polysaccharide capsule	Resistance to opsonophagocytosis by inhibition of classical and alternative complement pathways; reduces trapping by NETs; inhibits mucus binding, promoting transit to epithelial surface.
Pneumococcal surface protein A (PspA)	Limits C3b deposition by blocking formation of alternative pathway C3 convertase; inhibits bacteriocidal actions of apolactoferrin.
Pneumococcal surface protein C (PspC)[a]	Limits C3b formation by binding factor H; initiates invasion through binding human polymeric immunoglobulin receptor.
IgA protease	Cleaves IgA—surface-bound Fab fragments limit opsonophagocytosis and expose phosphorylchoine that promotes adherence by binding PAFR.
Polyhistidine triad (PhtA, B, D, and E)	Reduction of complement deposition via factor H recruitment.
Endonuclease A (EndA)	Degradation of DNA in NETs favoring subsequent invasion.
DEGRADATION OF EXTRACELLULAR MATRIX	
Neuraminidase	Removes terminal sialic acid residues from cell surface glycopeptides, promoting adherence; confers resistance to complement deposition.
B-galactoseide (BgaA) and B-N-acetylglucsaminidase (StrH)	Expose glycopeptides for pneumococcal epithelial binding; reduce C3b deposition.

(*Continued*)

TABLE 20.1 (Continued)

Virulence factor	Main function in pneumococcal disease
Hyaluronate lyase	Degrades hyaluronan in the extracellular matrix, facilitating bacterial spread and tissue invasion.
Enolase	Binds plasminogen, promoting transmigration through extracellular matrix; contributes to complement evasion by binding complement inhibitor C4b-binding protein.
Pullulanase (SpuA)	Glycogen degradation enzyme required for full virulence; mechanism of action uncertain.
EPITHELIAL ADHESION	
Pili	Promotes epithelial adherence and tissue invasion; associated with resistance to intracellular killing within macrophages.
Pneumococcal serine-rich protein (PsrP)	Adhesin required for bacterial persistence in lungs.
Sortase A (StrA)	Anchors cell-surface proteins, promoting bacterial epithelial adherence.
Pneumococcal adhesion and virulence A (PavA)	Binds fibronectin, facilitating stable colonization.
Pneumococcal adhesion and virulence B (PavB)	Binds fibronectin and plasminogen; promotes colonization and lung transmigration.
Pneumococcal choline-binding protein A (PcpA)	Surface protein induced by low manganese concentrations; contributes to epithelial adherence.
TISSUE DAMAGE AND PRO-INFLAMMATORY RESPONSE	
Pneumolysin	Cytolysis; complement activation; induction of host inflammatory response via multiple pathways; inhibition of phagocyte respiratory burst and ciliary beating on epithelium.
Autolysin (LytA)	Induces autolysis by peptidoglycan cleavage—release of pneumolysin and inflammatory cell-wall components (e.g., teichoic acids).
Phosphorylcholine (ChoP)	Binds PAFR on nasopharyngeal epithelial cells and activates host cell signaling pathways.
Lipoteichoic acid	Induces pro-inflammatory response, platelet and coagulation pathway activation via TLR2, and probably TLR4 and PAFR binding.
RESISTANCE TO NUTRIENT DEFICIENCY AND OXIDATIVE STRESS	
Pneumococcal surface antigen A (PsaA)	Mediates divalent metal ion uptake—required for resistance to oxidative stress; regulates expression of bacterial adhesins.
Pneumococcal iron acquisition A (PiaA) and iron uptake A (PiuA)	Lipoprotein components of ABC transporters that acquire iron for bacterial growth.
Maganese superoxide dismutase (SodA)	Confers protection against extracellular oxidative stress.
ATP-dependent caseinolytic protease (ClpP)	Confers resistance to oxidative stress following macrophage phagocytosis.

(*Continued*)

TABLE 20.1 (Continued)

Virulence factor	Main function in pneumococcal disease
BACTERIAL COMPETITION AND COOPERATION	
Biofilm and competence	Differential regulation of virulence factors by CSP in bacteremia and pneumonia; production correlates with expression of biofilm.
Bacteriocin	Mediates intraspecies competition between co-colonizing pneumococcal strains in the nasopharynx.
Pryruvate oxidase (SpxB)	Inhibits co-colonizing bacteria via hydrogen peroxide production.

[a] *Also known as choline-binding protein A (CbpA).*
NET, neutrophil extracellular trap; ABC, ATP-binding cassette; NLRP3, NOD-like receptor family pyrin containing 3; CSP, competence-stimulating peptide.

replication. Similarly, the novel bacterial adhesin PsrP is required for bacterial invasion from the lungs, but not for either nasopharyngeal colonization or survival in the bloodstream.

Emerging transcriptomic data give a more complete picture of the coordinated expression of virulence determinants. Two main patterns of *in vivo* gene expression by *S. pneumoniae* have been described: the first relating to bacteria in the bloodstream, characterized by increased expression of pneumolysin and PspA; and the second of bacteria isolated from tissues (i.e., lungs and brain), showing increased expression of neuraminidases, metalloproteinases, and oxidative stress and competence genes [8]. More recently, Ogunniyi et al. described differences in the transcriptomic profile between pneumococci obtained from the nasopharynx, lung, and blood following intranasal infection [9]. The relevance of selected differentially expressed genes was confirmed by targeted mutagenesis, which rendered organisms completely avirulent or significantly attenuated for virulence in a specific host niche. For example, the ATP-binding cassette-iron transporter component, pneumococcal iron uptake A (PiaA), was among the genes up-regulated in the blood, and ΔpiaA mutants were avirulent. Furthermore, immunization with recombinant PiuA was protective against sepsis.

NASOPHARYNX: CARRIAGE, INFLAMMATION, AND CLEARANCE

The Importance of Carriage

Carriage is important as the prerequisite for infection, the primary reservoir for transmission, and the predominant source of immunizing exposure and immunological boosting against pneumococcal infection in both children and adults. Infants are colonized by pneumococci early in life and experience multiple episodes of carriage throughout the early years of life. Almost all episodes of carriage are benign and do not result in disease [10,11].

Rates of pneumococcal carriage and disease progressively fall through childhood and early adult life as a result of progressive acquisition of humoral and cellular immunity [12]. Serum levels of capsule-specific immunoglobulin initially increase in proportion to pneumococcal exposure, and this is protective against invasive pneumococcal disease [13]. The dramatic fall in pneumococcal carriage rate at the end of infancy precedes the development of protective levels of IgG [14] and supports a role for cellular mucosal immunity in protection from carriage. Among adults, carriage, outbreak exposure and disease are associated with increased serum anti-

capsular IgG and subsequent protection from disease caused by the same pneumococcal serotype.

Experimental human pneumococcal carriage studies [15] confirmed the beneficial immunizing effect of carriage [10]. Experimental carriage resulted in increased immunoglobulin and antigen-specific T cell [16] responses at the respiratory mucosa and blood as well as protection against reacquisition of carriage by the same strain [10]. Furthermore, pneumococcal intranasal inoculation results in increased mucosal antibody response in the nasopharynx and lung compartments, even in the absence of subsequent carriage [17].

Carriage Onset

Initial steps of colonization depend on binding of the pneumococcus to host epithelium and evasion of host defense, particularly anticapsule antibody-mediated agglutination at the mucosa [18]. The usual response to the presence of the pneumococcus in the nasopharynx is an initial controlled inflammatory response followed by increased cellular infiltration that leads to clearance of carriage and subsequent acquisition of protective immunity.

Infection of the upper respiratory tract by respiratory virus such as influenza alters the epithelium by decreasing mucociliary velocity, increasing epithelial denudation exposing basement membranes, and modulation of chemokine and innate defenses. It is known that increased inflammatory cytokines such as type I interferon promotes increased pneumococcal colonization during influenza virus co-infection [19].

T Regulatory versus Th17 Responses During Carriage

Murine models have demonstrated that regulation of pneumococcal carriage is critically dependent on the balance between Th17-mediated inflammation and anti-inflammatory regulatory T cells (Treg). In humans, maturation of the Treg subset occurs in childhood along with the development of humoral and Th17 responses in the mucosal, lymphoid, and circulating T cell populations. The association of pneumococcal carriage with an increased frequency of adenoidal Treg secreting IL-10 but not IL-17 suggests that Treg in nasal-associated lymphoid tissue (NALT) may contribute to the persistence of pneumococcus in children [20]. More information about the role of cell-mediated immunity during carriage can be found in Chapter 22.

The suppression of inflammatory responses by pneumococcus may be important to preserve the balance of flora and lessen tissue damage during carriage. Immune tolerance profile, characterized by elevated TGF-β1 and high nasopharyngeal Treg numbers seems to be crucial for establishment and maintenance of carriage [21], while antigen-specific CD4 T cell (Th17) response leads to carriage clearance.

Carriage Clearance

Clearance of pneumococci from the nasopharynx is associated with antibody-dependent and antibody-independent mechanisms [22,23]. Protection against pneumococcal colonization (and possibly also mucosal disease) may derive, at least in part, from the development of $CD4^+$ IL-17A-producing T cells that recognize pneumococcal antigens that are expressed in the course of colonization [24]. Secretion of IL-17A from these cells may thus recruit professional phagocytes (neutrophils or macrophages) to the site of colonization and help reduce the duration of carriage [25]. Although neutrophils are the first cell type to be recruited after colonization, the peak of their presence in the nasopharynx does not correlate with initiation of clearance, and their depletion does not seem to affect bacterial burden during carriage in murine models. Weiser and colleagues have

recently reported a critical role for the sustained presence of macrophages in pneumococcal clearance [25].

Carriage Leading to Disease: The Dysregulation of Immune Tolerance

Long-term noninvasive carriage is beneficial to both pathogen and host. Carriage seems to be beneficial and works as a natural boosting mechanism to sustain protective immunity against disease in adults [10]. Dysregulation of the fine balance between immune tolerance and inflammatory response in the host could lead to the development of subsequent disease. This mechanism may be particularly important in the context of vulnerable groups such as the elderly, who do not sustain carriage, and could explain the paradox in which high incidence of pneumococcal disease is associated with low carriage rates in this group [26].

Increased production of inflammatory mediators has been described in the elderly, a condition termed "inflamm-aging" [27]. The presence of a balanced response between T cell-derived IFN-gamma and IL-10 as well as Treg cells in NALT has been associated with carriage persistence in children [20]. The unbalanced inflammatory response and lower levels of Treg cells in the elderly could explain how they might fail to sustain carriage. As carriage provides a mechanism for natural boosting of the protective immunity, lack of carriage may be detrimental in the elderly and contribute to the observed high susceptibility to disease.

Lung: Micro-Aspiration, Sterility, and Maintenance of Immune Defense

Pneumococci descend from the colonized human nasopharynx through the vocal cords by aspiration of respiratory secretion [28]. This process is easily viewed at bronchoscopy and is assisted by both gravity and the air flow of inspiration. Pneumococci may impact in large airways, particularly at bifurcations, where the mucosal surface is very similar to the nasopharynx.

The lung has evolved a range of innate strategies to restrict bacterial growth. Lactoferrin secreted by the airway epithelium has direct bacteriocidal effects on the pneumococcus, and by sequestering iron, depletes the environment of this key bacterial nutrient—a strategy referred to as "nutritional immunity." Lysozyme is a highly effective anti-pneumococcal agent secreted from submucosal glands; it is present in high concentrations in the lower airway. The human antimicrobial peptides called human β-defensins (hBD1−hBD4) and the human cathelicidin-related antimicrobial peptide-LL37 (or LL37 for short) act synergistically with lysozyme and secreted phospholipases A2 ($sPLA_2$) to lyse bacteria, as well as limiting growth by restricting bacterial nutrient uptake. Two collectins, surfactant proteins A and D (SPA, SPD), in addition to being important opsonins, exert direct antimicrobial effects against pneumococci by altering cell permeability and by interfering with nutrient uptake. Lymph node richness and submucosal lymphoid tissue defense are enhanced at carinae, but on arrival in the alveolus, pneumococci meet alveolar macrophages for the first time.

Alveolar macrophages are professional phagocytes and antigen-presenting cells capable of multiple episodes of phagocytosis and of cytokine regulation of the alveolar milieu. A non-inflamed interstitium is critical for gas transfer, as the distance from air space to circulating erythrocytes is only a few microns. In health, therefore, the alveolar macrophage maintains a non-inflammatory state both by ingestion of pathogen-associated material and by the production of anti-inflammatory mediators such as TGFβ and IL-10.

Human alveolar macrophages (HAM) ingest pneumococci in an opsonin-dependent manner using both immunoglobulin (predominantly IgG against capsular polysaccharide) and complement, which facilitate binding to the fcR and complement receptor, respectively. In the

absence of capsule-specific antibodies, opsonophagocytosis of *S. pneumoniae* is predominantly complement mediated. The polysaccharide capsule inhibits both the classical and alternative pathways through distinct mechanisms, limiting the deposition of the C3b/iC3b on the bacterial surface [29]. The highly negatively charged capsule also sterically inhibits the interaction between deposited C3b and complement receptors. Additionally, some nonspecific binding to scavenger receptors occurs at a much lower efficiency.

HAMs can ingest a small number (probably not >3) of pneumococci without eliciting an inflammatory response. When HAMs have ingested several pneumococci, they can be observed *in vitro* to have larger numbers of bacteria bound to the external surface, but ingestion slows and cytokine production, predominantly NFκB-mediated pro-inflammatory cytokine, takes over [30]. This cytokine signal amplifies similar epithelial responses, and neutrophils are then recruited to result in the inflammatory response described below. This pulmonary inflammatory response to pneumococci is critical, carefully regulated, and minimally altered even in HIV infection.

Neutrophils are poised for attack but are contained in the pulmonary vasculature until drawn into the airspace by integrin-mediated diapedesis [31]. Neutrophils are restricted in their passage through pulmonary capillaries by their stoichiometry (stiffness). Neutrophils are not found in uninflamed airspaces but become the dominant phagocyte when pneumonia occurs.

PNEUMOCOCCAL PNEUMONIA: THE PERFECT PARADIGM OF INFLAMMATION AND RESOLUTION

A dramatic switch occurs from tolerance of low numbers of bacteria in the lower airway to an active immune response found in pneumonia. These stages and their regulatory mechanisms were classically described as red and white hepatization, but are now much more completely understood as a result of murine models, and genetic and transcriptomic studies.

The Acute Inflammatory Stage

Bacteria can detect a favorable change in their environment and exploit it to multiply rapidly, with potentially deleterious effects for the host; to match this, the host must respond rapidly. Most deaths from pneumococcal pneumonia occur soon after the onset of symptoms. This timeframe is too short for a new pneumococcal-specific adaptive immune response to contribute, and the host must therefore rely on a rapid amplification of innate responses and adaptive responses already primed. The early response cells of the alveolus are the epithelium and the alveolar macrophage, which must sound an alarm of sufficient clarity to overcome the Treg [32] and alveolar macrophage [33] maintenance of normal quiescent lung homeostasis.

Recognition and Signaling Pathways

S. pneumoniae components can bind Toll-like receptors (TLRs), which span the cell wall of alveolar macrophages and epithelial cells. Lipoteichoic acid is a constituent of the outer face of the cytoplasmic membrane of Gram positive bacteria and is recognized by TLR-2 but not TLR-4. Certain DNA motifs from pneumococci can bind to TLR-9. Several groups have demonstrated that key immune responses are only triggered by the simultaneous binding of host-derived products containing so-called damage-associated molecular patterns (DAMPs), along with pathogen-derived ligands called pathogen-derived molecular patterns (PAMPs) [34]. DAMPs include hyaluronic acid, host DNA, and

uric acid, among other components. This requirement for two signals may go some way toward explaining why in some circumstances pneumococci can be recognized by TLRs without eliciting a pro-inflammatory response. TLR expression on macrophages and epithelial cells can be up-regulated during acute infection to facilitate better recognition of pathogen, but following influenza infection TLR expression is significantly reduced, rendering the lung susceptible to bacterial super-infection [35].

Epithelium

The lung epithelium is an important regulator and effector tissue. Epithelial cells orchestrate the innate response to local damage, set the threshold for this response, actively contribute to inhibiting excess bacterial growth, and return the system to its homeostatic state [36]. The epithelium itself is highly plastic, and many studies have shown that it can rapidly scale up its production of the antimicrobial effector molecules discussed in the previous section. For example, changes in levels of SPA and SPD modulates the functions of antigen-presenting cells such that the dynamics of neutrophil and T cell recruitment are altered. Indeed several groups have shown that augmenting the innate immune response by stimulating the epithelium with microbial products allows a potentially lethal inoculum of pneumococci to be overcome [37]. When a more potent reaction is required, epithelial responses to intact pneumococci include production of soluble innate factors including CXCL8 and up-regulation of the platelet-activating factor receptor (PAFR) [38]. The CXCL8 signal recruits neutrophils to the lung from the blood to tackle pneumococci, but epithelial binding of pneumococcal cell-wall phosphorylcholine by the PAFR accelerates bacterial invasion. This example is typical of each phase of the host response to pneumococcus, where a well-adapted host response has in many cases been abrogated by pathogen counter-evolution.

Alveolar Macrophage

The alveolar macrophage has roles in pathogen detection, early alarm signaling, and phagocytosis, followed by antigen presentation, neutrophil and lymphocyte recruitment, and coordination of the resolution of inflammation (Figure 20.2). Macrophage behavior in the healthy alveolus is essentially anti-inflammatory. This is, in large part, due to the inhibitory consequences of close physical interaction between alveolar macrophages and the airway epithelium. CD200 receptor (CD200R) on the macrophage surface binds the CD200 ligand on the surface of the epithelium. Alveolar macrophages are induced to express very high levels of CD200R by high local levels of IL10 and TGF-β, which are expressed on and secreted by the epithelium [40]. Another receptor expressed at high levels on alveolar macrophages is signal regulatory protein alpha, which, via its interaction with SPA and SPD, renders the cell quiescent. Moreover, the uniquely high levels of GM-CSF and SPD to which alveolar macrophages are exposed lead to a dramatic reduction in their ability to present antigen in comparison to peritoneal counterparts. Despite these restraints, macrophages can still recognize and phagocytose pneumococci, but this does not result in an escalation of inflammation while in their quiescent state. If bacterial ingestion exceeds more than single numbers per macrophage *in vitro*, active phagocytosis is reduced and cytokine production increases. What is not clear is how macrophages become unbound by this suppression in the context of pneumonia. One possibility is that physical damage to the epithelium (e.g., due to lytic viruses such as influenza) leads macrophages to become detached from the CD200 interaction releasing them from suppression [41]. In this context the combined TLR signaling of pneumococcal PAMPs and DAMPs released from the lysed epithelium leads macrophages,

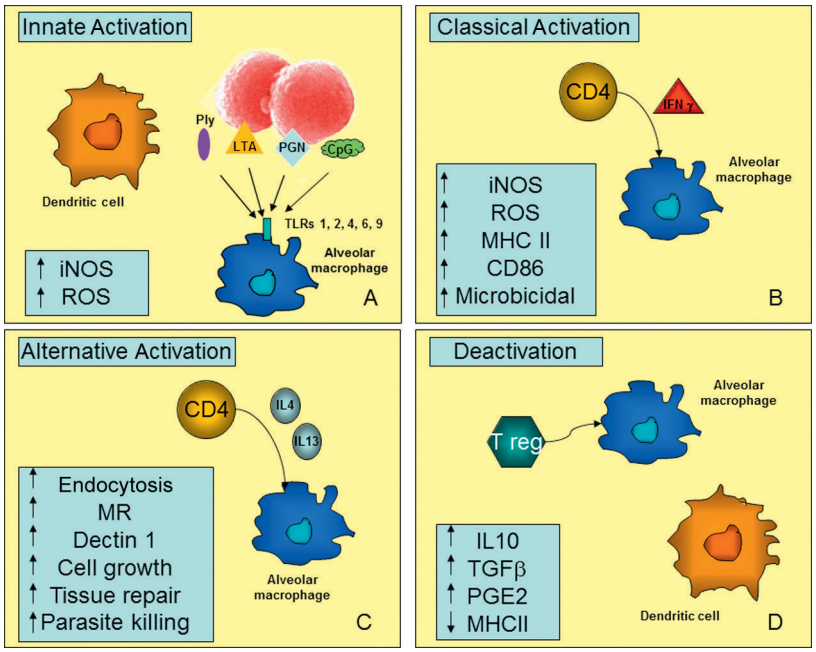

FIGURE 20.2 **Macrophage activation states.** Macrophages are activated in several patterns dependent on the antigenic stimulus, the receptor activated, co-receptor stimulation, and the cytokine milieu. (A) Innate activation results when pattern recognition, typically by TLRs, results in increased production of reactive nitrogen species by inducible nitric oxide synthase (iNOS) and reactive oxygen species (ROS). (B) Classical activation results when activated $CD4^+$ lymphocytes (T helper 1 type) producing gamma interferon (IFN-γ) interact with macrophages. This interaction results in increased antigen presentation, increased co-receptor expression (including CD86), and increased microbicidal activity in addition to the features of innate activation. (C) Alternative activation results from T helper 2-type activation of macrophages by CD4 lymphocytes. This alternative activation pattern results in parasite killing as well as increased endocytosis, mannose receptor (MR) expression, fibrosis, and tissue repair. (D) Deactivation results from the effect of regulatory T cells (Treg). Inhibitory factors, including interleukin 10 (IL-10) and transforming growth factor beta (TGF-β), result in down-regulation of pro-inflammatory cytokines by the macrophage, prostaglandin E2 (PGE2) production, and decreased antigen expression, finally leading to apoptosis. *Source: Figure reproduced with permission from [39].*

released from the restraints imposed by the epithelium, to become activated. In the activated state, the phagocytosis of pneumococci leads to recognition by cytoplasmic nucleotide-binding oligomerization domain–like receptors [42] and NF-κB-transduced up-regulation of multiple pro-inflammatory genes. The result of phagocytosis in this context is dramatic increases in the production of pro-inflammatory cytokines such as TNF-α, IL1-β, IL6, and the neutrophil-recruiting chemokine CXCL8, along with increased expression of a range of receptors for pathogen recognition [43]. Levels of pro-inflammatory cytokines seem to be similar when patients with pneumococcal pneumonia are compared with pneumonia caused by atypical pathogens, but the use of corticosteroids had little effect on cytokine levels in the context of pneumococcal pneumonia.

Neutrophils

The essential output of the epithelial and macrophage signaling pathways described above is the rapid recruitment of large numbers of these professional phagocytes. Neutrophils

respond to CXCL8 by up-regulating integrins [44] in order to bind endothelium and migrate into the alveolar space [45]. Neutrophils circulate in the pulmonary microvasculature at three times the concentration in peripheral venous blood, owing to the stoichiometry of the phagocytes (stiff and large) compared to the microvasculature (narrow and compressed). This allows very rapid adhesion, migration, and activation of neutrophils in response to local pulmonary epithelial signals [45].

The chief immune effector function in pneumococcal pneumonia is neutrophil-mediated phagocytosis. Phagocytosis of non-opsonized pneumococci occurs via a range of molecules including the PAFR, SPA, scavenger receptor-A, and macrophage receptor with collagenous structure (MARCO) [46]. However, these mechanisms of phagocytosis are inefficient compared to that of opsonized bacteria via FcR, particularly FcγR (IgG receptor) and complement-receptor binding. The complement system is vital to pneumococcal defense, and accordingly many pneumococcal virulence factors, including pneumococcal surface protein C (PspC) binding of factor H, have evolved specifically to subvert it. In addition to complement, other key opsonins include C-reactive protein, which binds teichoic acid and lipoteichoic acid of all *S. pneumonia* serotypes and is secreted by epithelial cells in the lower airway [47]. SPA and SPD also bind opsonically to pneumococci and enhance neutrophil and macrophage uptake and killing.

Adaptive Immune Response

In the heat of the inflammatory response, alveolar macrophages may transfer antigen to dendritic cells or migrate directly to the regional lymph node, where cognate responses are developed with naïve T cells to allow proliferation and production of appropriate IgG (Figure 20.3). This acquired response develops over weeks in naïve individuals, but owing to the immunological priming achieved by carriage exposures, boosted immune responses can normally occur within days of infection, resulting in high IgG levels in serum and exudative lung fluid. Dendritic cells play a key role at the interface between the innate and adaptive immune responses. Their phagocytosis of pneumococci leads to interactions with NK cells [49], pro-inflammatory cytokine release, and presentation of antigen to T cells. Pneumococci subvert these functions by the potent inhibition of dendritic cell phagocytosis by pneumococcal adherence and virulence factor A (PavA). Moreover, the migration of dendritic cells from sites of infection to lymph nodes has recently been associated with deleterious effects and seems to facilitate pneumococcal dissemination [50].

Alveolar immunoglobulin (both IgG and IgA) responsive to both pneumococcal capsule and pneumococcal proteins can be measured in most adults [17,51]. Alveolar macrophages only exhibit full opsonophagocytic killing potential against pneumococcus in the presence of both cognate immunoglobulin and complement [52]. Antigen-specific T cells responsive to pneumococcal antigens have been found in bronchoalveolar lavage from all healthy adults examined, but their exact function is not known [16]. The Th17 subset has been shown to be increased by pneumococcal colonization and is assumed to mediate pneumococcal killing by recruitment of neutrophils. In the context of pneumococcal bacteremia, marginal zone macrophages in the spleen that express the specific intercellular adhesion molecule-grabbing non-integrin receptor 1 (SIGNR1) are vital for the initiation of IgM responses in early infection.

The Control of Infection and Inflammation and Resolution

Despite the innate and adaptive responses described above, if patients are untreated, the bacteria are not contained and at least half of

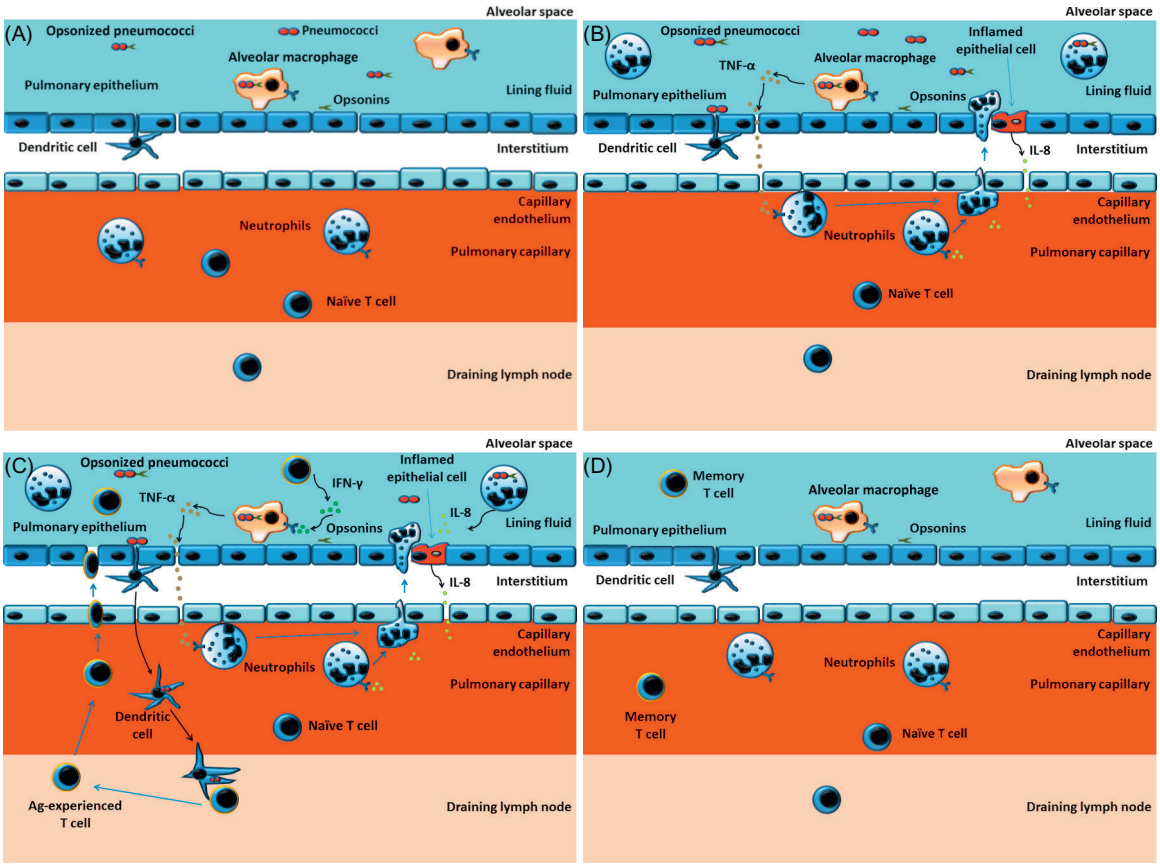

FIGURE 20.3 **Pneumococcal clearance in the lung.** Host defense in the lower respiratory tract is mediated by alveolar macrophages. (A) During early infection where the bacterial load is low, resident alveolar macrophages efficiently kill and phagocytise opsonized pneumococci in a quiescent manner, effectively preventing bacteria–dendritic cell interaction, and hence inhibiting initiation of T cell-mediated inflammatory responses. (B) In situations where bacterial load exceeds the capability for macrophages to perform effective opsonophagocytosis, neutrophils are recruited following secretion of TNF-α by alveolar macrophages and/or IL-8 by epithelial cells. (C) T cells are recruited following successful antigen presentation in the draining lymph nodes by pulmonary dendritic cells. These cells secrete IFN-γ, which activates macrophages to kill internalized pneumococci and also promotes further TNF-α production by alveolar macrophages. (D) Following clearance of pneumococci from the lungs, neutrophils, some macrophages, and T cells undergo rapid apoptosis. Surviving T cells remain in the alveoli as resident effector memory cells. *Source: Figure reproduced with permission from [48].*

patients die. The key to de-escalation of the inflammatory response to the pneumococcus is the cessation of bacterial metabolism and replication, which is most successfully achieved using antibiotics.

Recent work has described the importance of macrophage apoptosis in the evolution of an acute inflammatory response [53]. Altered alveolar macrophage apoptosis results in impaired alveolar defense. Pneumococci induce macrophage apoptosis by pneumolysin-dependent mechanisms (caspase dependent and caspase independent), but delayed apoptosis is required for evolution of an effective inflammatory response. As control is achieved over the invading bacterial

population, macrophage phenotype changes again to support repair, and macrophage apoptotic mechanisms allow the non-inflammatory resolution of some of the inflammatory exudates. Further, effective neutrophil apoptosis pathways allow alveolar damage to be minimized even in the context of severe bacterial infection. At the height of the pneumonic illness, the alveolar space is choked with serum, organized inflammatory debris, bacterial DNA, and cellular debris. The process of macrophage efferocytosis (literally "burying the dead") allows restoration of normal pulmonary architecture and respiratory function [54]. To facilitate the return to homeostatic numbers, expanded population of activated macrophages and dendritic cells in the pneumonic lung is depleted by the direct cytotoxic activity of γδTcells [55].

SEPSIS: CHAOTIC INFLAMMATION AND A THREAT TO THE HOST

Pneumococcal proteins that interact with PAF and polymeric Ig receptors allow bacteria to cross epithelial barriers. Pneumococcal virulence factors damage host epithelial integrity, allowing plasma extravasation and neutrophil migration, but also facilitate direct bacterial invasion of the circulation. Pneumococci expressing pneumolysin have an early growth advantage in blood, the mechanism of which has been described in elegant detail (see Chapter 14). It is therefore no surprise that pneumococci invade the circulation in a manner dependent on both host response and pathogen virulence factors. Sepsis, however, results in an emergency for both the pathogen and the host.

Pneumococci that invade the circulation are suddenly exposed to high concentrations of immunoglobulin and large numbers of neutrophils. Extracellular polysaccharide capsule expressed by *S. pneumoniae* potently inhibits phagocytosis and is essential for the organism's virulence, particularly in sepsis. In contrast to bacteria in the mucosal niche, invading bacteria in the blood must up-regulate capsule production rapidly (the opaque phase) in order to protect their surface from phagocyte binding and hence survive [56]. Pneumococcal adherence to mucosal surfaces is dependent on surface proteins, and so thin polysaccharide capsule through which protein can protrude (the transparent phase) provides a colonization advantage [57]. The mechanisms whereby pneumococci alter the degree of expression of the capsule to adapt to particular host niches are yet to be fully elucidated, but may relate to changes in oxygen tension. The 94 antigenically distinct capsular serotypes differ markedly in their potential to cause invasive disease in proportion to their relative resistance to phagocytosis. Capsular serotype is therefore an independent determinant of the outcome of invasive pneumococcal disease.

Acquired responses to pneumococcal capsule and innate/acquired responses to pneumococcal proteins modulate the host inflammatory responses to pneumococcal bacteremia. The capsular responses are elicited by the uptake of pneumococcal debris, apoptotic phagocytes, and cellular debris by the macrophages of the reticulo-endothelial system, predominantly resident in the liver and spleen. This process is opsonin dependent, and altered complement biology explains the high mortality from invasive pneumococcal disease seen in patients with the functional hyposplenism of sickle cell disease.

Protein responses include responses to pneumolysin (see Chapter 14), surface proteins, and others. For example, PspA is an important virulence determinant universally expressed in clinical pneumococcal isolates [58]. It inhibits the bactericidal activity of the secreted innate immune protein apolactoferrin and reduces complement-dependent phagocytosis by inhibiting the formation and/or

function of the alternative pathway C3 convertase. Immunization using recombinant PspA protein elicits protection against pneumonia, sepsis, and nasopharyngeal colonization in mice [59]. Moreover, passive transfer of human antibodies raised against recombinant PspA is also protective [60]. A wide range of PspA-based immunization strategies are currently in various stages of clinical development. When present, pilus is encoded for by three structural proteins (RrgA, RrgB, and RrgC) and three associated sortases. The RrgA component is the main determinant of adhesion, and also invokes a host inflammatory response via TLR2. RrgA is also implicated in the systemic invasion of pneumococci; pneumococci expressing RrgA are preferentially phagocytosed by macrophages and show prolonged intracellular survival and higher rates of early bacteremia.

The host responses to pneumococcal bacteremia are extreme, characterized by very high levels of pro-inflammatory cytokine, altered endothelial integrity and adhesive properties, severe systemic disruption of vessel tone, and altered coagulation cascades. If untreated, these changes ultimately lead to multi-organ failure. A full discussion of the inflammatory cascade in sepsis is beyond the scope of this chapter but has been reviewed elsewhere [61]. What is important in this context is that the exquisite control of the anti-inflammatory response in carriage, or the moderated inflammation, controlled macrophage apoptosis, and efferocytotic response of pneumonia are missing in sepsis.

Pneumococcal sepsis is a medical emergency that requires urgent antibiotic treatment and supportive care to ameliorate the systemic effects of excessive pro-inflammatory response. A promising novel therapeutic approach for pneumococcal sepsis focuses on augmenting pathogen and pathogen debris clearance by neutrophils and macrophages. The immune-activating peptide P4, which incidentally is a surface-expressed moiety of the pneumococcal virulence factor PsaA, augments the response to passive immunotherapy by up-regulating fcR and hence enhancing the ability of neutrophils and macrophages to phagocytose opsonized pneumococci [62,63]. This may remove some of the pro-inflammatory signal and certainly has dramatic effects in reducing death from sepsis in murine models.

MENINGITIS: AVOIDING DEATH IN A DESPERATE SITUATION

Pneumococcus in the CSF is profoundly pro-inflammatory. The full syndrome of pneumococcal meningitis can be reproduced in a rabbit model by injection of dead bacteria in to the cisternal space. The syndrome is therefore almost entirely dependent on host response; it is characterized by very high CSF cytokine levels (much higher than in other bacterial meningitis cases) and neutrophil infiltrate, as well as focal necrosis and hippocampal apoptosis. Resolving or partially treated meningitis may have a predominantly lymphocytic picture.

Studies of human CSF are few but demonstrate that many thousands of proteins are present in a pattern suggestive of blood–brain barrier breakdown and local production. Total protein numbers and concentrations were not associated with clinical outcome in a large case series, but complement depletion was seen to be lower in fatal cases with CSF studied by mass spectrometry [64]. This suggests a dysregulated response typical of sepsis.

In the same study, CSF samples were searched using proteomic techniques for evidence of apoptosis and necrosis [65]. Given the potential therapeutic potential of membrane-stabilizing agents, it was disappointing that there was no evidence to support the hypothesis generated in the rabbit model that hippocampal or neuronal apoptosis was a major contributor to human mortality. Steroid therapy has been attempted in a number of studies.

Steroid reduced the cytokine load in pneumococcal meningitis, but again, this reduction had no association with clinical outcome. There is therapeutic optimism to be gained from the observation that a dramatic, effective reduction in pneumolysin concentration in CSF was associated with favorable outcome; pneumolysin antagonists are the subject of current research.

TRANSLATIONAL SIGNIFICANCE OF COMPARTMENTAL DIFFERENCES IN IMMUNE RESPONSE

A strategy to eliminate pneumococcus by vaccination must include prevention of carriage [66]. Pneumococcal conjugate vaccines reduce carriage in a dramatic fashion associated with the serum and mucosal production of high levels of anti-capsular immunoglobulin. In addition, pneumococcal carriage in humans is itself protective against recolonization and produces anti-capsular immunoglobulin, anti-protein immunoglobulin, and antigen-specific Th17 responses [10,16,17]. Modern vaccines must now focus on anti-protein immunoglobulin responses and Th17 responses in order to gain advantage beyond that obtained from conjugate vaccines [67].

In the treatment of pneumonia and sepsis, antibiotics and supportive therapy have resulted in stable outcomes for many years. Improved outcome in pneumonia and sepsis will require adjuvant therapy to ameliorate the adverse effects of inappropriate inflammatory responses and to augment effective responses such as opsonophagocytic removal of pro-inflammatory debris [4].

Improved outcomes in meningitis are likely to come with improved immune-modulatory therapy, particularly if targeted to pneumococcal toxins such as pneumolysin.

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CHAPTER

21

Streptococcus pneumoniae Interactions with Macrophages and Mechanisms of Immune Evasion

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INTRODUCTION

Streptococcus pneumoniae persistence and survival within a host will require evasion of the dominant immune mediators targeting extracellular bacteria at each site of infection. Within the respiratory tract the main mechanisms that either prevent *S. pneumoniae* infection or kill *S. pneumoniae* are the physical defenses and soluble antibacterial components of the mucosal layer, resident nasopharyngeal or alveolar macrophages, and neutrophils and monocytes recruited to the site of infection by the inflammatory response. During systemic infection, *S. pneumoniae* are killed by resident macrophage subsets within the liver and spleen (part of the reticuloendothelial system) and circulating neutrophils. In order to kill invading *S. pneumoniae*, phagocytes need to be able to identify the bacteria, and this is enhanced by soluble components of the alveolar lining fluid and blood, especially by complement and antibody. The inflammatory response to bacterial infection causes an acute phase response, characterized by increased production of immune proteins and also by effects on micronutrient availability that can inhibit bacterial replication. In this chapter we will address *S. pneumoniae* interactions with each of these aspects of the immune response. In particular, we will discuss the complex interactions of *S. pneumoniae* with macrophages, which are key during airway infection, and complement-mediated immunity, which is critically important for development of septicemia and, to a lesser extent, pneumonia.

S. PNEUMONIAE INTERACTIONS WITH PHYSICAL AND MUCOSAL SOLUBLE IMMUNE MEDIATORS

Initial infection of mucosal surfaces needs to overcome local epithelial immunity. A major component of epithelial immunity is the

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enhanced physical clearance of bacteria from the epithelial layer by production of a negatively charged mucus layer, supported in the lungs by motile cilia that continually expel mucus from the lungs. Most *S. pneumoniae* capsular serotypes have an overall negative charge, which ensures that electrostatic forces inhibit the *S. pneumoniae* from interacting with mucus and can evade mucus-dependent clearance from the upper respiratory tract [1]. This effect of the capsule on mucus-dependent clearance from the upper airways would be predicted to have a similar effect within the bronchial tree, but this has not been investigated. In addition, *S. pneumoniae* neuraminidases aid colonization, probably by reducing the overall negative charge of mucus by removing sialic acid residues [2]. Infection of respiratory epithelium with *S. pneumoniae* results in a relatively slow impairment of mucociliary clearance. This effect is only partially dependent on the cytotoxin pneumolysin [3], and seems to be the consequence of bacterial-induced disorganization of F-actin and epithelial architecture by an unknown mechanism [4]. *S. pneumoniae* adhesins will also counteract mucociliary clearance by increasing bacterial binding to the underlying epithelium; these are discussed in detail in Chapter 17. Another major component of mucosal immunity involves soluble antibacterial products present in the epithelial lining fluid. These include antimicrobial peptides (AMPs), lactoferrin, and the enzyme lysozyme. The wide range of these mucosal antimicrobial factors suggests they are an important component of host defense, but this redundancy also makes it difficult to identify the role of individual components in immunity to *S. pneumoniae*. Several *S. pneumoniae* factors have been described that inhibit these mucosal immune effectors. Inclusion of D-alanine into the teichoic acid (dependent on the *dlt* operon) component of the *S. pneumoniae* cell wall reduces cell surface negative charge (in contrast to the effects of the capsule) and thereby inhibits bacterial killing by host cationic AMPs [5]. Export ABC transporters may have a similar effect by rapidly removing AMPs from the bacterial cytosol (see Chapter 10), although how this may inhibit AMPs when their mode of action requires insertion into the bacterial membrane from the extracellular environment is not clear [6]. The enzymes PdgA and Adr alter *S. pneumoniae* peptidoglycan structure and make it more resistant to lysozyme degradation, and in combination these enzymes improve *S. pneumoniae* fitness during colonization [7]. The mucosal protein lactoferrin reduces iron availability within airway lining fluid, thus preventing bacterial growth, and also has a direct antibacterial effect, predominantly mediated by its N-terminal 11 amino acid cleavage product called lactoferricin. Release of lactoferricin from apolactoferrin is increased by the *S. pneumoniae* serine protease PrtA, an example of the host subverting bacterial products for the host's advantage [8]. To counteract lactoferrin, the *S. pneumoniae* cell-wall protein PspA binds to lactoferricin through electrostatic interaction, thereby blocking bacterial killing by lactoferricin [9,10]. Overall, a range of *S. pneumoniae* factors have been described that prevent different elements of mucosal immunity. However, for many of these mechanisms, how necessary they are for establishing actual infection at mucosal sites has not been clearly defined.

S. PNEUMONIAE INTERACTIONS WITH THE MACROPHAGE

Within the respiratory tract, resident airway macrophages (alveolar macrophages) are the dominant immune effector during the initial stages of infection, regulating clearance of bacteria that evade mucosal immune defenses. As a consequence, *S. pneumoniae*/macrophage interactions have a major influence on whether

invading bacteria are able to establish pneumonia or are rapidly cleared from the lungs [11]. Splenic and liver macrophage subsets are also essential for *S. pneumoniae* clearance from the systemic circulation, without which *S. pneumoniae* can cause rapidly progressive septicemia [12]. Macrophage differentiation reflects specific anatomical imprinting so that macrophages are adapted to perform the homeostatic functions unique to the tissue in which they reside [13]. Activation of tissue macrophages such as alveolar macrophages allows adaptation to specific roles such as those required for antibacterial host defense. Macrophages recognize opsonized *S. pneumoniae* via complement and Fcγ receptors and non-opsonized bacteria through a variety of receptors, including scavenger receptors (e.g., macrophage receptor with collagenous structure [MARCO]) and lectin receptors (e.g., SIGN-R1). Inflammation can be initiated by recognition of *S. pneumoniae* ligands by toll-like receptors (TLRs) and cytoplasmic receptors such as nucleotide-binding oligomerization domain−containing proteins (see Chapter 20). Macrophages have three main functions in relation to *S. pneumoniae* infections: generating an inflammatory response to the presence of *S. pneumoniae* for cellular activation and recruitment; killing of phagocytosed bacteria; and antigen presentation. The first two will be discussed in detail below; antigen presentation is part of the adaptive immune response and will not be discussed.

Macrophage Activation in Response to *S. pneumoniae*

Macrophage killing mechanisms and inflammatory responses are highly dependent on macrophage activation and polarization [13]. A range of pattern recognition receptors (PRR) contribute to macrophage activation in response to *S. pneumoniae*. TLR2 (stimulated by lipoproteins and possibly lipoteichoic acid or other cell wall), TLR4 (which may recognize the cholesterol-dependent cytolysin pneumolysin), and TLR9 (responding to unmethylated CpG dinucleotides found in prokaryotic DNA) all contribute to the inflammatory response and macrophage activation by *S. pneumoniae*. However, individually the effect of each PRR is modest, whereas loss of the adaptor protein MyD88, which mediates responses from different TLR receptors, has profound effects on the inflammatory response to *S. pneumoniae* [14,15]. The specific roles of TLRs in directly priming antimicrobial responses, as compared to indirectly via pro-inflammatory cytokine expression, are unclear. TLR4 acts synergistically with TLR2 to activate macrophages and stimulate the clearance of low doses of *S. pneumoniae* from the murine lung [15,16], suggesting that TLR4 may have a particular role in priming alveolar macrophages' antimicrobial responses. Intracellular degradation of peptidoglycan by lysozyme results in recognition by the intracellular PRR Nod2 and also helps activate macrophages in response to *S. pneumoniae*. In this case an additional antimicrobial strategy is activated involving the release of CCL2 and the recruitment of CCR2+ monocytes, which may have an enhanced antimicrobial capacity compared to resident airway macrophages [17]. The inflammasome contributes to macrophage activation, and *S. pneumoniae* may actively increase inflammasome activity by pneumolysin stimulating the Nod-like receptor (NLR)P3 and also by mechanisms involving the AIM2 (absent in melanoma2) NLR, which both result in caspase-1 dependent IL-1β release [18,19]. Optimal stimulation of the inflammasome requires bacterial pore formation [18]. Since considerable redundancy exists, several PRRs are probably involved in activating macrophage antimicrobial response to a range of different *S. pneumoniae* ligands; subtle modifications in the structure or amount of

pro-inflammatory bacterial factors will influence macrophage responses, and therefore are likely to account for some of the variations in infection phenotypes between *S. pneumoniae* strains. For example, certain hypervirulent epidemic strains such as the ST306 serotype 1 strains or the ST53 serotype 8 strains express pneumolysin variants that lack cytolytic activity, thereby reducing inflammasome activation [18]. These strains are more invasive, with higher levels of early bacteremia in murine models of pneumonia, although reduced mortality overall [20], suggesting that failure to activate the inflammasome enables ST306 serotype 1 strains or the ST53 serotype 8 strains to escape immune surveillance, but conversely the subsequent reduced IL-1β-dependent inflammation results in better survival.

Canonical Phagolysomal Macrophage Killing Mechanisms

Intracellular killing of ingested bacteria in differentiated macrophages, such as the alveolar macrophages, is facilitated by the extensive dynamic surface membrane able to ingest bacteria, by a large lysosomal compartment able to fuse with phagosomes containing bacteria and by a high density of mitochondria able to generate energy for bacterial ingestion and killing [21]. Differentiated macrophages are, however, less able to generate certain antimicrobial reactive oxygen species (ROS) than neutrophils, since they lack the myeloperoxidase enzyme. Instead, macrophages generate ROS from alternative systems to the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, such as from mitochondria [22]. However, *S. pneumoniae* generate hydrogen peroxide through their own metabolism, via pyruvate oxidase (encoded by *spxB*), lactate oxidase (encoded by *lox*, which converts lactate to pyruvate), and via carbamoyl phosphate synthase (encoded by *carB*) [23,24]. As a consequence, *S. pneumoniae* has multiple adaptations to withstand oxidative stress, and ROS generated by the NADPH oxidase system does not make a significant contribution to *S. pneumoniae* killing in phagocytes [25,26]. *S. pneumoniae*'s mechanisms of preventing oxidative stress include [21]:

1. SpxB, which allows acetyl phosphate to be converted to ATP, preventing ATP depletion during oxidative stress
2. an NADH oxidase (encoded by *nox*), which converts O_2 to H_2O, limiting its availability for conversion to superoxide
3. a manganese superoxide dismutase (SodA)
4. the Psa ATP-binding cassette importer required for manganese uptake, a cofactor for SodA activity
5. a putative glutathione peroxidase PsaD, which together with the Psa lipoprotein component PsaA alters redox status and pyruvate metabolism to limit hydrogen peroxide production via SpxB
6. a class III alcohol dehydrogenase (encoded by *adhC*) that provides resistance to hydrogen peroxide, potentially by generating reduced glutathione, required for the PsaD glutathione peroxidase as *S. pneumoniae* is unable to generate glutathione via its own metabolism
7. a range of less well-characterized mechanisms, including the Clbp protease, a putative alkylhydroperoxidase , the transcriptional regulator Rgg (for resistance against superoxide but not hydrogen peroxide), the heat shock–induced serine protease HtrA (that provides resistance against hydrogen peroxide but not superoxide), and possibly TlpA, a predicted thioredoxin-like protein.

Despite this, macrophages are able to kill a substantial proportion of *S. pneumoniae* within a few hours of phagocytosis. Alternative killing mechanisms to ROS include nitric oxide (NO), generated by inducible nitric oxide synthase, and reactive nitrogen species (RNS), formed by the combination of NO and ROS. Mice that lack inducible nitric oxide synthase (iNOS/NOS2) are

less able to clear *S. pneumoniae* from the lung [25], suggesting that NO is required for macrophage-mediated killing of *S. pneumoniae*. Furthermore, pneumolysin and pneumococcal cell wall stimulates macrophage NO production, and this contributes to antimicrobial killing [27,28]. However, human macrophages may generate lower levels of NO than murine macrophages, and differentiated tissue macrophages less than monocytes [21]. More potent antimicrobial effects may result when NO reacts to form *S*-nitrosothiols, such as *S*-nitrosoglutathione (GSNO), or combines with ROS to generate reactive nitrogen species (RNS), an example of which is peroxynitrite, formed between NO and superoxide. As is the case with ROS, *S. pneumoniae* have developed adaptations to combat the effects of NO or RNS. PspC inhibits NO production and antimicrobial killing by poorly understood mechanisms [29], and the ClpP protease and AdhC alcohol dehydrogenase (an *S*-nitrosoglutathione reductase), which reduce resistance to oxidative stress, also inhibit NO-mediated bacterial killing, implicating these factors as contributing to resistance to RNS [21]. Macrophages lack enzymes equivalent to neutrophil cationic granule proteases; instead, they activate lysozyme and various pH-dependent proteases to ensure degradation of bacteria. Hence, PdgA- and Adr-mediated alterations to *S. pneumoniae* peptidoglycan to make it more resistant to lysozyme [7] could help inhibit macrophage killing, although this has not been confirmed experimentally. However, little is known about how macrophage proteases contribute to killing of *S. pneumoniae* or whether there are bacterial adaptations to these mechanisms enabling resistance.

Apoptosis-Associated Killing

Since differentiated macrophages such as alveolar macrophages have a restricted capacity to engage canonical intracellular killing relative to other phagocytes, the lack of significant intracellular persistence by *S. pneumoniae* suggests that additional mechanisms contribute to bacterial clearance when the canonical phagolysosomal killing mechanisms are exhausted. One potential mechanism for *S. pneumoniae* killing is macrophage apoptosis, a proven killing mechanism for persistent intracellular bacteria such as *Mycobacterium tuberculosis*. Macrophages challenged with *S. pneumoniae* undergo apoptosis in direct correlation with the intracellular bacterial burden and levels of macrophage nitrosative stress [27,30]. That macrophage apoptosis is a host response is supported by data suggesting that TLR4 signaling, *S. pneumoniae* opsonization, or infection with less-virulent unencapsulated strains all enhance induction of apoptosis [27,31]. The molecular pathway leading to apoptosis induction involves permeabilization of the phagolysosome and activation of the lysosomal protease cathepsin D, which in turn leads to decreased translation and enhanced degradation by ubiquitination of a key macrophage anti-apoptotic factor, the Bcl-2 family member Mcl-1 (Figure 21.1) [32]. Cathepsin D activation allows Mcl-1 to preferentially interact with its E3 ubiquitin ligase Mule rather than with Hsp70, favoring Mcl-1 ubiquitination and degradation. The short half-life of Mcl-1 and its dynamic regulation enhance macrophage survival during ingestion and killing of *S. pneumoniae* by canonical phagolysosomal mechanisms through an initial phase of transcriptional Mcl-1 upregulation, followed by activation of apoptosis when canonical phagolysosomal killing is exhausted through reduced translation and proteasomal degradation of Mcl-1 [33].

Macrophage apoptosis enhances *S. pneumoniae* clearance by macrophages in murine models of pneumonia, particularly in low-dose bacterial challenge when resident alveolar macrophages are able to control infection, and reduces *S. pneumoniae* invasion of the blood from the lung [32,33]. Apoptosis also helps

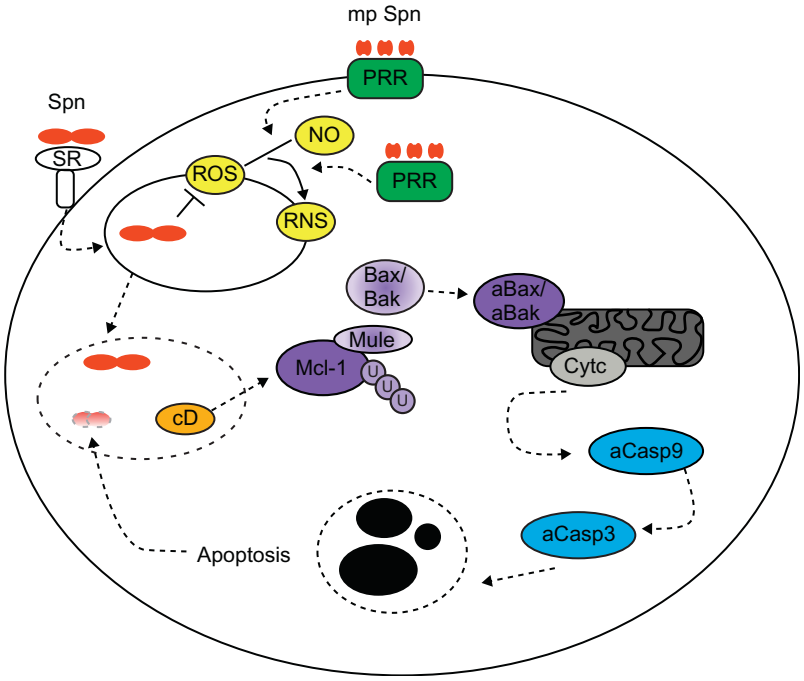


FIGURE 21.1 **Apoptosis-associated killing of *S. pneumoniae*.** *S. pneumoniae* (Spn) interact with a range of surface receptors (SR), which recognize both opsonized and non-opsonized bacteria and mediate their internalization into phagolysosomes. Microbial products of *S. pneumoniae* (mpSpn) are recognized by various surface-expressed and intracellular PRR, including Toll-like receptors and nucleotide-binding oligomerization domain (Nod)-like receptor (NLR)-containing inflammasomes. These stimulate pro-inflammatory cytokine responses and increased expression of the SR involved in phagocytosis, but also generation of microbicidal molecules such as ROS, nitric oxide (NO), and the products generated by reaction of ROS and NO, reactive nitrogen species (RNS). Intracellular *S. pneumoniae* produce a variety of factors that help them resist ROS and potentially other microbicidal factors generated by the macrophage. Ultimately, phagolysosomes containing residual *S. pneumoniae* undergo membrane permeabilization, and activation of the lysosomal protease cathepsin D (cD) results in enhanced ubiquitination (U) and proteasomal degradation of the anti-apoptotic Bcl-2 family member myeloid cell leukemia sequence 1 (Mcl-1) when Mcl-1 interacts with the Mcl-1 ubiquitin ligase E3 (Mule). Degradation of Mcl-1 favors activation of pro-apoptotic factors that ultimately induce activation of the pro-apoptotic factors Bcl-2 associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak), which interact with the mitochondrion (aBax/aBak) to induce mitochondrial outer membrane permeabilization and cytochrome c (Cytc) release. Cytc results in formation of a molecular complex that activates caspase 9 (aCasp9), which in turn activates caspase 3 (aCasp3) and induces downstream features of apoptosis such as nuclear fragmentation. The induction of apoptosis results in cell death but also killing of the residual *S. pneumoniae*.

down-regulate the inflammatory response [33], inhibiting alternative macrophage death pathways such as necroptosis and reducing ER stress [21]. *S. pneumoniae* interactions with macrophages do not seem to result in induction of pyroptosis, a cell death mechanism associated with decreased bacterial clearance and enhanced inflammatory lung injury, perhaps due to *S. pneumoniae* induction of the Chitinase 3-like-1 protein in the lungs [34]. Although macrophage apoptosis appears to involve host sensing of pneumolysin, it does not require pneumolysin pore formation [21]. Hence, pneumolysin pore formation and inflammasome activation could be considered a mechanism by which *S. pneumoniae* tips the immune response

toward inflammation and limits apoptosis-associated clearance, enabling rather than preventing tissue invasion. Inhibition of phagocytosis by the *S. pneumoniae* capsule or surface proteins affecting complement activation (see below) will also limit macrophage apoptosis-associated killing, since it appears to be triggered purely by intracellular bacteria [30].

S. PNEUMONIAE INTERACTIONS WITH COMPLEMENT

Overview

The complement system consists of a series of plasma and cell-surface proteins that assist host immunity by identifying and opsonizing invading microorganisms, contributing to the inflammatory response, and links the innate and adaptive immune response by promoting antigen recognition and improving antibody efficacy. The complement system is an example of a biochemical proteinase cascade; complement proteins circulate as inactive zymogens which, when activated, act as proteases that activate the next complement protein of the cascade. Complement is organized into three pathways known as the classical, mannose-binding lectin (MBL), and alternative pathways (Figure 21.2). When activated, all three pathways form a C3 convertase complex on the target cell surface, which cleaves the central complement component C3 (186 kDa) into the active molecules C3b (177 kDa) and C3a (9 kDa). Conversion of C3 into C3b exposes a highly reactive thioester moiety, resulting in covalent binding of C3b to hydroxyl-containing molecules on the pathogen cell surface. C3b and its degradation product iC3b are opsonins that strongly stimulate phagocytosis by neutrophils and macrophages via complement receptors (CR) 1, 3, 4, and the complement receptor of the IgG superfamily (CRIg); this is the major mechanism by which complement activity assists the killing of *S. pneumoniae*. Human erythrocytes also express surface CR1, and can bind C3b-opsonized *S. pneumoniae*, which are then removed in the reticuloendothelial system, with the erythrocyte re-entering the circulation [35]. Conversion of C3 to C3b exposes binding sites for the alternative complement pathway proteins factor B and properdin, which stabilizes the resulting C3bBb convertase and thereby amplifies the complement response. C3b also forms a C5 convertase that cleaves C5 to produce C5a and C5b. C3a and C5a are potent chemoattractants and activators of neutrophils, monocytes, and macrophages, which accentuate the inflammatory response and improve phagocytosis activity. In addition, C5b initiates assembly of the later components of the complement cascade (C6, C7, C8, and C9) into the membrane attack complex (MAC), which forms a pore in the cell membrane and can result in bacterial cell lysis. However, *S. pneumoniae* is thought to be resistant to lysis by the MAC. Complement activation also up-regulates the B-cell response to pathogen antigens 10,000-fold [36–39], thereby considerably improving the adaptive immune response to the target pathogen. Inappropriate complement activation will kill host cells, and hence the complement system is tightly regulated by a series of host proteins. Important regulators for *S. pneumoniae* interactions with complement include the C4b binding protein (C4BP), which prevents classical pathway activity by promoting dissociation of the classical pathway C3 convertase, and factor H (FH), which inhibits alternative pathway activity.

Complement Recognition of *S. pneumoniae*

The importance of complement for immunity to *S. pneumoniae* is demonstrated by the massively increased incidence of *S. pneumoniae* infections in patients with inherited complement deficiencies. For example, serum from

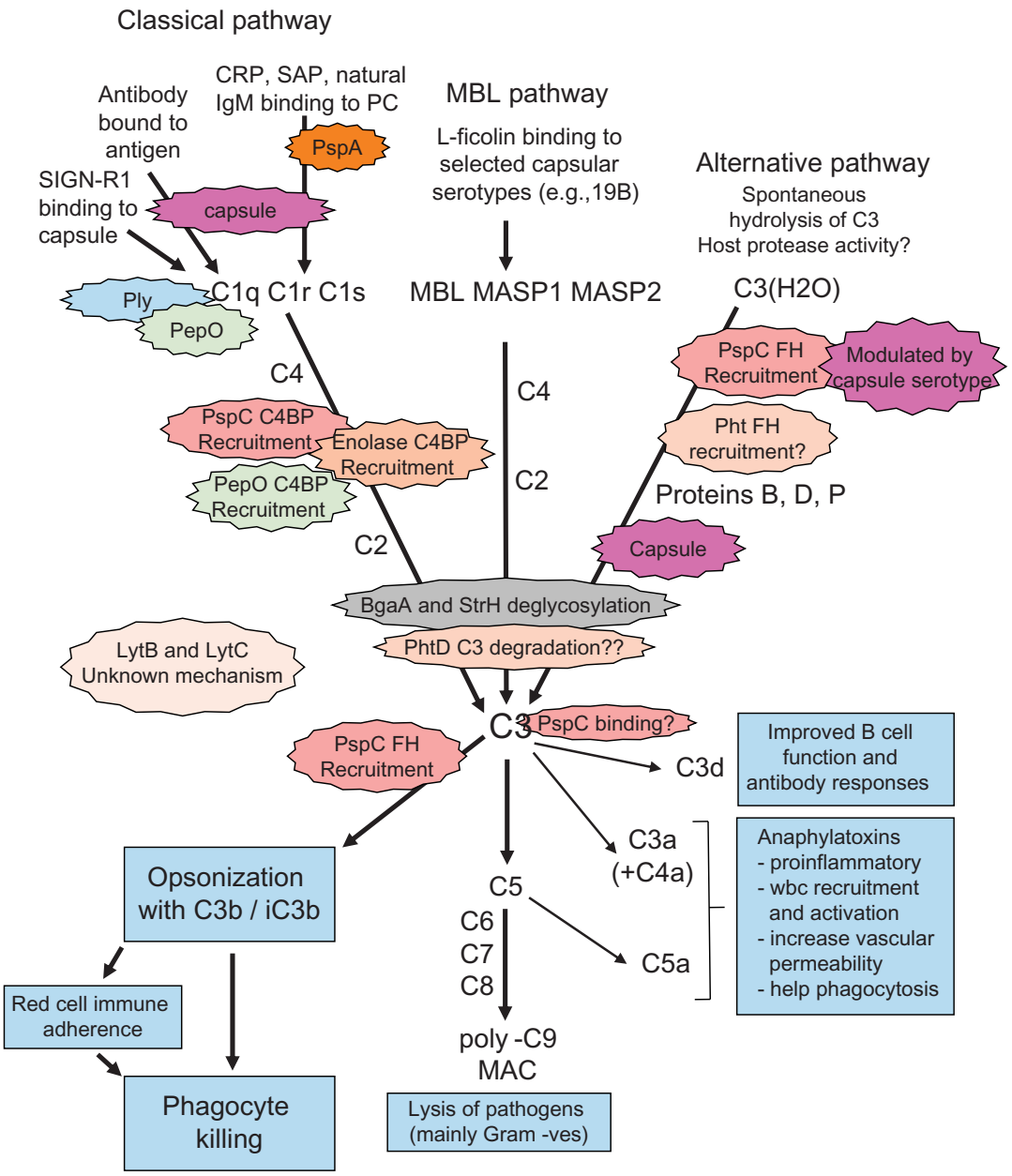


FIGURE 21.2 **Mechanisms of complement identification of *S. pneumoniae* and the bacterial factors that prevent complement activation.** Each complement pathway is delineated, with the major components in bold. The mechanisms of initiation by *S. pneumoniae* are indicated at the top and major immune effects of complement activation are shown in the light blue boxes. The *S. pneumoniae* factors interfering with complement activity are shown in colored balloons at their recognized sites of action.

subjects with an inherited homozygous deficiency of the classical pathway component C2 has a reduced ability to support complement-dependent phagocytosis of *S. pneumoniae* [40], and as a consequence the subjects suffer recurrent episodes of *S. pneumoniae* pneumonia, meningitis, or septicemia [41]. Mouse models have confirmed essential roles for complement for innate immunity to *S. pneumoniae* pneumonia, sepsis, meningitis, and otitis media [42–44]. Although complement has a significant role during mucosal infection, it is particularly important in preventing bacterial replication within the blood, and complement-deficient mice are highly susceptible to *S. pneumoniae* septicemia [42,45]. Mice with genetic deficiencies affecting the classical, alternative, and MBL pathways are all more susceptible to *S. pneumoniae* infections [42,46–48], suggesting that each of these pathways has a role in complement activity against *S. pneumoniae*. The classical pathway is activated when C1q binds directly to pathogen surfaces or to antibody complexed with antigen, either natural IgM binding to cell-wall phosphocholine (PC) [42,49] or acquired antibody to multiple different antigens. The serum proteins C-reactive protein (CRP) or serum amyloid P (SAP) also bind to PC and can be recognized by C1q to activate the classical pathway [50,51]. In addition, a C-type lectin, SIGN-R1, expressed by splenic macrophages binds capsular polysaccharide and C1q, thereby activating the classical pathway on bacteria attached to the macrophage surface [52]. The MBL pathway is similar to the classical pathway, with MBL or ficolins replacing C1q and binding directly to sugar residues on the cell surface. MBL does not seem to bind to *S. pneumoniae*, but the MBL pathway can be involved in complement recognition of at least some strains of *S. pneumoniae* due to ficolin binding to capsular monosaccharides, or possibly pneumolysin rather than via MBL [47,48,53–55]. The alternative pathway is activated by spontaneous C3 hydrolysis and possibly host proteases, and can therefore potentially be activated by any pathogen, including *S. pneumoniae*.

S. *pneumoniae* Inhibition of Complement Activity

Similar to other important extracellular pathogens such as *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Neisseria meningitides*, *S. pneumoniae* has multiple mechanisms of inhibiting complement activity. These mechanisms include direct inhibition of complement activation, degradation of complement components, alteration of the physical properties of the bacteria to reduce the consequences of complement activation, and subversion of normal host mechanisms for regulating complement. The main *S. pneumoniae* factors affecting complement are described below, and shown in Table 21.1 and Figure 21.2.

S. pneumoniae *Growth Characteristics and Physical Properties*

Commensal streptococci often grow in chains consisting of multiple bacteria, whereas *S. pneumoniae* is usually found as pairs or single bacteria. Growth as diplococcic rather than as chains helps reduce *S. pneumoniae*'s susceptibility to complement because complement activation is a stochastic process that is relatively hard to initiate, yet once a nidus of complement has bound to a bacterium, it then forms a focus for propagation and amplification of complement activation [56]. Hence, activation on one bacterium of a chain allows complement binding to spread to the rest of the bacteria within the chain, whereas growth as diplococcic minimizes the number of additional bacteria affected. Similarly, a smaller surface area would reduce the chance of complement activation against a specific bacterium, which is perhaps why

TABLE 21.1 *S. pneumoniae* Factors Required for Evasion of Major Host Immune Effector Mechanisms

S. pneumoniae factor	Host target	Mechanism
Capsule	Mucosa	Inhibition of adhesion to mucus by electrostatic repulsion
	Complement	Inhibition of classical pathway by preventing direct C1q binding, natural IgM and CRP binding to PC, and specific antibody binding
	Complement	Inhibition of the alternative pathway, mechanism unknown
	Complement	Modifies PspC interactions with FH (related to serotype invasive potential)
	Antibody	Inhibition of antibody binding to subcapsular antigens
	Phagocytes	Reduced opsonization with complement and antibody
	Phagocytes	Direct inhibition of phagocytosis, mechanism unknown
	Phagocytes	Prevention of entrapment in NETs
Pneumolysin	Mucosa	Inhibiting ciliary beat frequency
	Complement	Diverting complement activity by direct activation of the classical pathway
	Phagocytes	Lysis of phagocytes by pore formation in the cell membrane
	Phagocytes	Inhibition of the oxidative burst and neutrophil killing
	Phagocytes	Inhibition of neutrophil chemotaxis
	Phagocytes	Induction of macrophage apoptosis (enhances *S. pneumoniae* killing)
	APR	Binds to CRP
	Dendritic cells	Inhibits inflammatory response and inflammasome activation, promotes apoptosis
PspA	Mucosa	Binding of lactoferricin to prevent bacterial killing
	Phagocytes	Binding of lactoferricin to prevent bacterial killing
	Complement	Prevention of classical pathway by inhibition of C1q binding
PspC	Mucosa	Adhesin and translocation through epithelial layers
	Complement	FH binding and inhibition of alternative pathway activity
	Complement	C4BP binding and inhibition of classical pathway activity (selected strains)
	Complement	C3 binding?
	Phagocyte	Inhibition of RNS killing mechanisms
Neuraminidase NanA, NanB	Mucosa	Cleavage of sialic acid, reducing mucus negative charge
	Complement	Deglycosylation of complement proteins? (theoretical)
Beta-galactosidase BgaA	Complement	Deglycosylation of complement proteins

(*Continued*)

TABLE 21.1 (Continued)

S. pneumoniae factor	Host target	Mechanism
N-Acetylglucosaminidase StrH	Complement	Deglycosylation of complement proteins
dlt operon	Mucosa	Reducing cell wall negative charge inhibiting AMPs
	Phagocytes	Reducing cell wall negative charge inhibiting killing by NET AMPs?
PdgA and Adr	Mucosa	Make peptidoglycan more resistant to lysozyme degradation
	Phagocyte	Increased resistance to phagosomal lysozyme?
ZmpA/IgA_1 protease	Antibody	Degradation of IgA1
ZmpC	Phagocytes	Degradation of neutrophil expressed P-selectin glycoprotein 1
Export ABC transporters	Mucosa	Removal of AMPs from the bacterial cytosol
	Phagocytes	Removal of AMPs from the bacterial cytosol; inhibition of killing by NET AMPs?
Import ABC transporters	Growth	Acquisition of micronutrients with restricted availability in the host
	Phagocytes	PsaA-mediated manganese uptake, a cofactor for SodA and avoidance of oxidative killing mechanisms?
LytB and LytC	Complement	Cell-wall hydrolases, mechanism(s) of complement inhibition unknown
EndA	Phagocytes	Breakdown of DNA components of NETs
Pht proteins	Complement	Degradation of C3 (PhtD?)
	Complement	FH binding and inhibition of alternative pathway activity
Enolase	Complement	C4BP binding and inhibition of classical pathway activity
PepO	Complement	Binding of C1q diverts classical pathway activity
	Complement	C4BP binding and inhibition of classical pathway activity
Growth as diplococci	Complement	Inhibit complement deposition on bacterial surface?
Growth in a biofilm	Complement	Inhibit complement deposition on bacterial surface?
	Antibody	Inhibit antibody binding to the bacterial surface?
	Phagocytes	Reduced opsonization, direct inhibition of phagocytosis
INHIBITION OF ROS/RNS		
SodA	Phagocytes	Avoidance of oxidative killing mechanisms?
Nox NADH oxidase	Phagocyte	Converts O_2 to H_2O, avoidance of oxidative killing mechanisms?
SpxB	Phagocyte	Converts acetyl phosphate to ATP, preventing ATP depletion during oxidative stress
AdhC class III alcohol dehydrogenase	Phagocyte	Generating reduced glutathione for resistance to hydrogen peroxide and oxidative killing mechanisms; inhibition of RNS
PsaD, Rgg, HtrA, TlpA	Phagocyte	Increase resistance to oxidative killing mechanisms
ClpP	Phagocyte	Increase resistance to oxidative and RNS killing mechanisms

pathogenic bacteria including *S. pneumoniae* often have relatively small diameters. Growth in a biofilm also protects *S. pneumoniae* against both classical and alternative pathway complement activity, inhibiting binding of C1q and CRP to cell-wall PC and promoting FH binding (see below) to the bacteria [57].

Capsule

The *S. pneumoniae* capsule is vital for *S. pneumoniae* virulence. Although unencapsulated strains are found as nasopharyngeal colonizers, they do not cause *S. pneumoniae* septicemia in humans; in animal models unencapsulated mutants are unable to cause sustained septicemia and have a reduced ability to colonize the nasopharynx [58,59]. Although the capsule has multiple effects on host–*S. pneumoniae* interactions (Table 21.1), one of its major affects is to reduce bacterial sensitivity to complement. Unencapsulated *S. pneumoniae* are highly susceptible to complement, and transparent-phase variants (thin capsule) also have increased opsonization with complement compared to opaque-phase variants (thick capsule) [59]. Hence the capsule inhibits complement activity, despite being the target for complement activation through SIGN-1, SAP, or naturally occurring and vaccine-induced antibody binding to the capsule [51,52]. As the capsule forms an extracellular layer surrounding the bacterium, it can block the interactions of host proteins with *S. pneumoniae* cell-wall components; indeed, it reduces classical pathway complement activity against *S. pneumoniae* by preventing CRP and natural antibody access to cell-wall PC, and blocks acquired antibody access to subcapsular protein antigens [59]. In addition, it can prevent alternative pathway activity, though the mechanism is not clear [59]. The effects of the capsule on complement sensitivity have been shown to vary markedly among different capsular serotypes expressed on the same strain background [60,61]. Importantly, serotype complement sensitivity correlates closely with the relative invasiveness of different *S. pneumoniae* capsular serotypes, when defined as the chance of causing invasive infection per colonization event [60]; hence, capsule-dependent complement resistance seems to be vital for the development of invasive infections such as septicemia. The MBL pathway seems to play a role in recognition of some strains of *S. pneumonia*, and this depends on the capsule structure, with ficolin binding to capsular serotype 11A, 19B, and 19C due to the presence of specific carbohydrate epitopes [54,55]. This is a mechanism by which virulence can vary between closely related serotypes, with ficolin-resistant strains more likely to cause invasive disease than ficolin-sensitive strains [54].

Cell Wall and Other S. pneumoniae *Proteins*

The powerful effects of capsular serotype on *S. pneumoniae* complement resistance might be expected to dominate capsule-independent causes of variations in complement sensitivity. However, data using strains with different genetic backgrounds but expressing the same capsular serotype show that strain background also causes marked differences in complement sensitivity independent of capsular serotype, even after controlling for phase variation and capsular thickness [62]. This non-capsule-dependent variation in complement resistance demonstrates that other *S. pneumoniae* factors must influence complement resistance. Indeed, an increasing number of protein-dependent complement evasion mechanisms have been described (Figure 21.1); these are discussed below.

PspC

The abundant choline-binding cell-wall protein PspC (also known as Hic or CbpA) binds to FH, a negative regulator of alternative pathway activity [60,63,64]. FH binding inhibits complement activity against *S. pneumoniae* by increasing the rate of C3

degradation to iC3b, reducing C3b binding by causing dissociation of factor B (Bf) from the C3 convertase, and by inhibiting C3bBb formation by preferentially binding C3b. FH binding is an example of a pathogen subverting the host's own complement regulation system to avoid complement attack, a strategy used by multiple other bacterial pathogens. The degree of FH binding to *S. pneumoniae* depends on capsular serotype; it inversely correlates with complement sensitivity and directly correlates with invasive potential [60]. This suggests that certain capsular serotypes are more likely to cause invasive disease as they allow greater FH binding to PspC and thereby are more resistant to complement activity. Hence the capsule can modify the activity of cell-wall virulence factors to potentially contribute toward strain variation in virulence. Of note is that minor changes in capsule structure may have major effects on FH binding; for example, serotypes 19A and 19F differ in structure by a single bond in the trisaccharide repeating unit yet have significant differences in FH binding and complement sensitivity [60]. FH binds to an N-terminal site between amino acid residues 38–158 of PspC [65]. The amino acid structure of PspC is highly variable between *S. pneumoniae* strains [66], and the degree of FH binding can vary between strains with the same capsule serotype [60]; whether this is due to the allelic variation in PspC or additional contributions for some *S. pneumoniae* strains from other putative FH binding proteins (see below) is not known. Additional mechanisms of PspC-mediated complement resistance have been described by single reports. These include binding of C4-binding protein, the negative regulator of the classical pathway, by some PspC allelic variants, thereby inhibiting classical pathway activity [67]; and PspC binding to thioester-disrupted C3, a form of C3 not recognized by neutrophils [68].

PspA

PspA is another choline-binding protein that is highly expressed by *S. pneumoniae* during infection and affects complement deposition by both the classical and alternative pathways [45,69,70]. These effects maybe caused by PspA inhibiting CRP binding to *S. pneumoniae* PC [70], thereby directly preventing classical pathway activity and indirectly inhibiting alternative pathway activity. The effects of PspA on virulence are reduced in complement-deficient mice, demonstrating that its ability to inhibit complement does contribute toward *S. pneumoniae* virulence [45].

PNEUMOLYSIN

Data obtained using mouse models of infection show that the effects of pneumolysin in assisting *S. pneumoniae* virulence are partially reduced in mice deficient in C1q [45], demonstrating that pneumolysin inhibits classical pathway activity. However, in contrast to other *S. pneumoniae* factors that facilitate complement evasion, pneumolysin actually increases complement activation by binding to the Fc portion of IgG or to CRP, thereby initiating the classical pathway, or by binding to ficolin to activate the MBL pathway [53,71,72]. This may help complement evasion as pneumolysin is released extracellularly and so could be diverting complement activity away from *S. pneumoniae*; which of the above-described mechanisms are actually responsible for pneumolysin-mediated complement evasion requires further clarification.

OTHER PROTEINS

The effects of the capsule, PspA, PspC, and pneumolysin on complement are reasonably well defined and have been shown to be relevant in models of disease. In addition, there are increasing numbers of additional *S. pneumoniae* cell-surface proteins that have been shown to affect complement activity,

including pneumococcal histidine triad (Pht) proteins, exoglycosidases, cell-wall hydrolases, and PepO. Pht proteins (PhtA, B, C, and D) are a family of cell-wall proteins that share extensive peptide sequence identity and have characteristic histidine-containing motifs whose roles in pneumococcal virulence are poorly understood. PhtB may show C3 degradation activity in human serum [73], and loss of all four PhtD proteins seems to increase complement deposition on *S. pneumoniae*, perhaps due to reduced FH binding [74]. The major pneumococcal neuraminidase NanA and two other surface-associated exoglycosidases, the beta-galactosidase BgaA and the *N*-acetylglucosaminidase StrH, together reduce *S. pneumoniae* oposonization with C3b/iC3b, perhaps because they cause deglycosylation of unspecified complement proteins and so inhibit their function [75]. Mutation of the genes encoding the cell-wall hydrolases LytB and LytC results in increased complement deposition on *S. pneumoniae*, but whether this is a direct effect of loss of LytB/LytC complement inhibition or due to secondary effects on other cell-wall components is not known [76]. The protein PepO reduces complement activity against *S. pneumoniae* when secreted by binding C1q to divert classical pathway activation, similar to the effects of pneumolysin [77]. In addition, when expressed as a cell-surface protein, PepO binds to the classical pathway inhibitor C4BP, as does another cell-surface protein, enolase [77,78], to prevent classical pathway activation.

The relative importance of each of these mechanisms for complement evasion and how they interact with each other is largely unclear. Synergistic effects of PspA and pneumolysin on complement inhibition have been shown *in vitro* and in animal models [45], but the relative contributions of other anti-complement mechanisms for complement evasion is not known and is likely to vary between *S. pneumoniae* strains due to variable expression levels, allelic variations in protein structure, or differences in protein interactions with the capsule or other complement-inhibitory proteins.

S. PNEUMONIAE INTERACTIONS WITH ANTIBODY

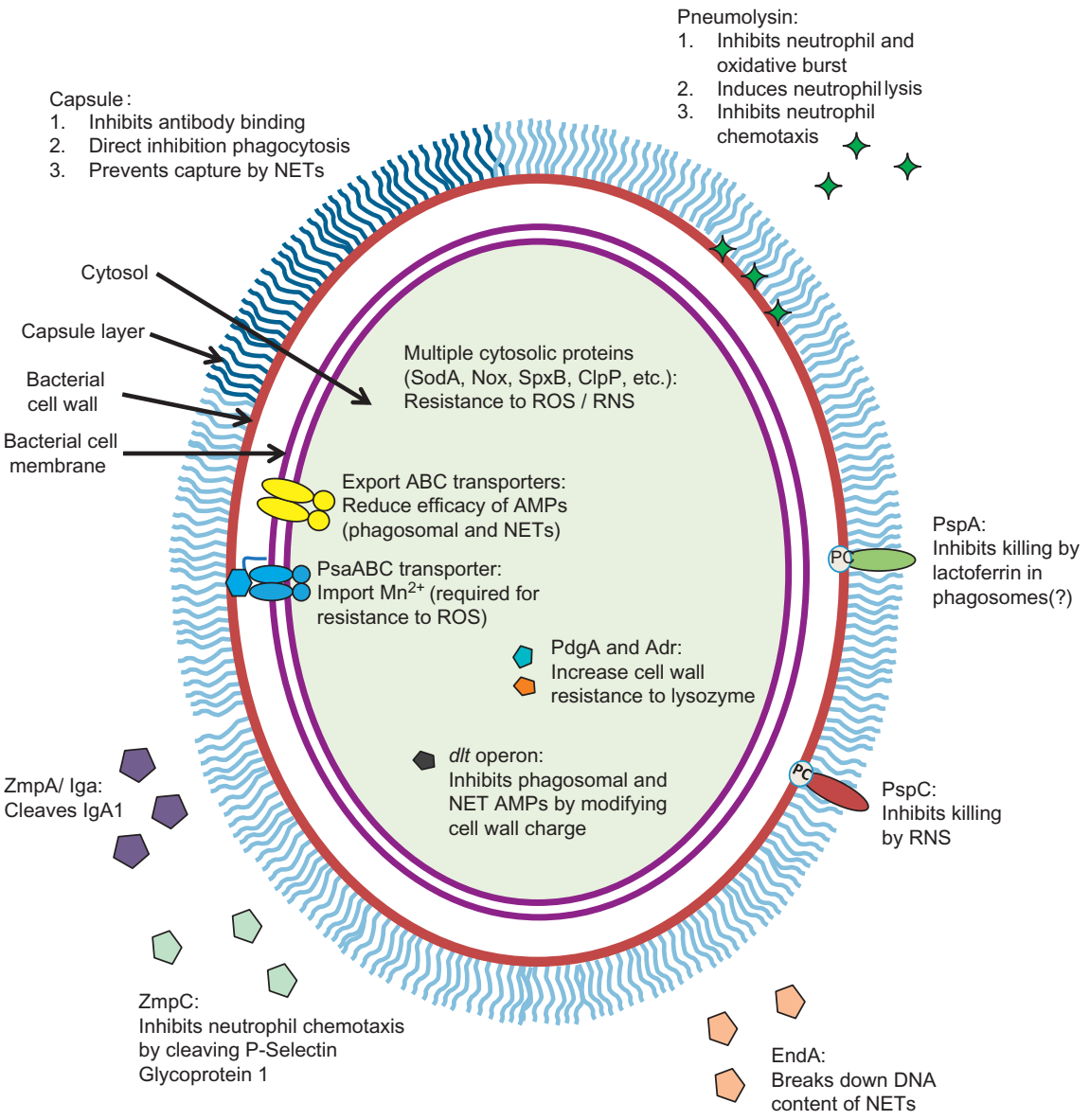
Antibody is a major mechanism of acquired or vaccine-induced immunity to *S. pneumoniae* that can prevent nasopharyngeal colonization and respiratory tract and invasive infections, and would therefore be expected to be a target for *S. pneumoniae* immune evasion. As the effects of antibody are partially dependent on complement and complement boosts adaptive immune responses, *S. pneumoniae*'s mechanisms of inhibiting complement activity described above will also reduce the efficacy of antibody-mediated immunity. In addition, the capsule reduces antibody recognition of *S. pneumoniae* subcapsular antigens, thereby preventing antibody-mediated complement activation, Fcγ-mediated phagocytosis, or agglutination by natural IgM targeting cell-wall PC or acquired antibody against protein antigens (e.g., cell-wall proteins or lipoproteins) [42,59]. This is perhaps one reason why antibodies to capsular antigen seem to induce a more powerful protective immunity than antibodies to protein antigens. *S. pneumoniae* also expresses a zinc metalloproteinase, termed ZmpA/IgA_1 protease, which specifically cleaves IgA_1 at the hinge region to prevent IgA_1-mediated oposonization [79]. Together, IgA_1 and ZmpA promote *S. pneumoniae* adherence in the upper respiratory tract, perhaps because a non-opsonic coating of IgA_1 Fab fragments counteracts the negative charge of the capsule and reduces electrostatic repulsion from the epithelial layer [80]. Finally, pneumolysin lyses or induces apoptosis of host cells and can inhibit dendritic cell maturation and inflammatory response [81], and both of these effects could in theory reduce antibody responses to *S. pneumoniae*, for example, through lysis of

lymphocytes and/or by preventing effective dendritic cell antigen presentation.

S. PNEUMONIAE INTERACTIONS WITH NEUTROPHILS

Severe *S. pneumoniae* infection results in rapid recruitment of large numbers of neutrophils (PMNs) to the site of infection from the blood, and circulating neutrophils will also engage with *S. pneumoniae* during septicemia (Figure 21.3). The importance of neutrophils for immunity to *S. pneumoniae* is supported by a possible increased incidence of IPD in patients who are neutropenic, and has been experimentally confirmed using animal models [12,82]. However, the contribution of recruited neutrophils to bacterial killing is partially counterbalanced by their contribution toward tissue damage through the release of ROS and proteinases, and alveolar consolidation and therefore hypoxia during pneumonia. Recruited neutrophils can phagocytose *S. pneumoniae* within minutes, but this is highly dependent on opsonization with complement and/or antibody, and unopsonized *S. pneumoniae* are relatively resistant to neutrophil phagocytosis [26,40,59]. Hence, the major mechanisms by which *S. pneumoniae* prevents neutrophil-mediated killing are the capsule and the *S. pneumoniae* proteins that prevent opsonization with complement or antibody, as discussed above. Consequently, the degree of susceptibility to neutrophil phagocytosis correlates very closely with sensitivity to opsonization with complement for both capsular switched strains expressing different capsular serotypes on the same genetic background (TIGR4) and also clinical strains [60,62]. The capsule also prevents neutrophil phagocytosis of unopsonized *S. pneumoniae* directly by undefined mechanism(s), and the strength of this effect correlates with capsule thickness [59,83]; this is presumably through blocking bacterial interactions with non-opsonic phagocytic receptors. An interesting mechanism of neutrophil evasion by some strains of *S. pneumoniae* is through secretion of the zinc metalloproteinase ZmpC. This protease reduces neutrophil recruitment to sites of *S. pneumoniae* infection by degrading neutrophil-expressed P-selectin glycoprotein 1 (PSGL-1), a protein required for engagement with P-selectin during neutrophil extravasation from the blood [84].

Once phagocytosed, an *S. pneumoniae*-containing phagosome fuses with intracellular granules containing AMPs (e.g., α-defensins), serine proteases (elastase, cathepsin G, and proteinase 3), lysozyme, lactoferrin, and ROS to form a phagolysosome. Hence, in theory the *S. pneumoniae* components discussed above that inhibit AMPs (the *dlt* operon, export ABC transporters) and lactoferrin (PspA) or increase resistance to lysozyme (PdgA and Adr) would reduce the bacteria's sensitivity to phagocyte killing mechanisms, but this has not been investigated (Table 21.1). In addition, as discussed above, *S. pneumoniae* has a wide range of protective mechanisms against ROS and is relatively resistant to ROS-mediated killing mechanisms; neutrophils from subjects with chronic granulomatous disease (who have reduced ROS generation due to inherited defects of the NADPH-oxidase complex) are able to kill *S. pneumoniae* as efficiently as neutrophils from healthy individuals [26]. Instead, neutrophil-dependent killing of *S. pneumoniae* is dependent on the serine proteases elastase, cathepsin G, and proteinase 3 [26]. No *S. pneumoniae* factors that inhibit serine protease-dependent killing have been described, and most ingested *S. pneumoniae* are killed rapidly by neutrophils, bringing into question whether there is much advantage to the bacteria in avoiding neutrophil killing by ROS, AMPs, lactoferrin, or lysozyme. However, *S. pneumoniae* do seem to be resistant to killing by neutrophil extracellular traps (NETs), an extracellular lattice work of bacteriocidal fibers containing



+ THE MECHANISMS OF INHIBITION OF COMPLEMENT ACTIVITY (Fig. 21.2)

FIGURE 21.3 **Described mechanisms by which *S. pneumoniae* evades neutrophil-mediated immunity.** The main bacterial factors affecting neutrophil-dependent immunity and their cellular localization are shown. PC = cell-wall phosphocholine component. NETs = neutrophil extracellular traps. AMPs = antimicrobial peptides. ROS = reactive oxygen species. RNS = reactive nitrogen species.

DNA, histones, and granule proteins released by neutrophil degranulation. *S. pneumoniae* resistance to NET-dependent killing could be mediated by the factors that help the bacteria avoid AMP killing, such as the *dlt* operon [85]. In addition, the negative charge of most *S. pneumoniae* capsules can prevent entrapment in NETs [85], and *S. pneumoniae* produces an endonuclease EndA that degrades the DNA component of NET filaments [86,87]. In addition to its role in complement evasion, pneumolysin may help evade neutrophil-mediated immunity by several other mechanisms. High concentrations of pneumolysin are cytotoxic toward neutrophils and other phagocytes, and so could prevent *S. pneumoniae* clearance by directly killing the phagocytes [88,89], but these levels of pneumolysin may not be achieved during infection. At very low non-cytotoxic concentrations (≤1 hemolytic unit [2 ng] per million neutrophils), pneumolysin also has functional effects on neutrophils that might prevent bacterial clearance, including inhibiting the respiratory burst and neutrophil killing of opsonized *S. pneumoniae* and reducing neutrophil chemotaxis [90]. The exact mechanisms responsible for these effects have not been described.

THE INFLAMMATORY RESPONSE AND THE ACUTE PHASE RESPONSE

S. pneumoniae infections are characteristically highly pro-inflammatory, with rapid and large increases in pro-inflammatory cytokines and chemokines at the site of significant infection. One consequence of the inflammatory response is activation of the acute phase response (APR), mediated by IL-6 acting on the liver [91]. The APR causes rapid increases of serum proteins such as CRP and SAP that are involved in recognition of *S. pneumoniae* by the complement system [51]. The increase in CRP during the APR will increase complement activity against *S. pneumoniae* and may also directly promote phagocytosis, and binding of pneumolysin to CRP could potentially inhibit this effect. Another consequence of the APR is an increase in iron sequestration and changes to other cation concentrations in the blood, which inhibits bacterial growth as many basic bacterial biochemical processes require iron and cations. *S. pneumoniae* has a large number of import ABC transporters (see Chapter 10) that obtain micronutrients and thereby assist the bacteria to overcome the effects of the APR on bacterial growth. For example, the Pia and Piu iron uptake ABC transporters allow growth in iron-deficient conditions and are essential for full virulence in mouse models of infection [92]. Many extracellular bacteria can modulate the inflammatory response; for example, *S. pyogenes* produces an enzyme SpyCEP that inactivates the cytokine IL-8 and thereby prevents neutrophil recruitment to the site of infection. In contrast, there are few described mechanisms by which *S. pneumoniae* directly modulates inflammation for its advantage. The one exception are data suggesting pneumolysin inhibits dendritic cell inflammatory responses and induces dendritic cell apoptosis, a process that requires pore formation [81], which would be predicted to reduce inflammation caused by *S. pneumoniae*. However, these data seem to contradict the data discussed above on pneumolysin promotion of macrophage inflammasome activation [18,19].

GENERAL ASPECTS OF *S. PNEUMONIAE* INTERACTIONS WITH IMMUNE MEDIATORS

Several general observations can be drawn from an overview of the *S. pneumoniae* factors that influence host immune responses. First, the same virulence factor frequently has multiple roles. This may not be so surprising for the capsule since this could inhibit many different

host interactions due to its location surrounding the bacteria. However, many protein virulence factors have multiple and really quite different roles including pneumolysin, PspA, and PspC. These can, as exemplified by pneumolysin, involve roles both as immune evasion factors and as stimuli for innate immune responses. Perhaps genetic variation between strains and/or the host will tip the balance in favor or against optimal host responses. The multiple roles for a single protein create a problem for the researcher in identifying the relative importance of each function at each stage or site of infection. Second, some immune functions are targeted by multiple *S. pneumoniae* factors, most obviously complement- and phagocyte-mediated immunity (Table 21.1 and Figure 21.1). The relative contribution and whether there is significant synergy between various virulence factors has, in the main, not been assessed, and for many of the described mechanisms of *S. pneumoniae* immune evasion it is not clear how important they actually are during infection *in vivo*. A third observation is that there are some important areas of host immunity that have not yet been shown to be affected by *S. pneumoniae* virulence factors, despite being targeted by other extracellular pathogens; examples include degradation of IgG, preventing phagocytosis by targeting actin polymerization or preventing phagolysomal fusion, or inhibiting inflammatory responses by degrading cytokines or chemokines. Finally, important general questions remain about *S. pneumoniae* virulence. For example, why does a nasopharyngeal commensal like *S. pneumoniae* possess powerful mechanisms of evading complement- and phagocyte-mediated immunity, whereas other nasopharyngeal commensals seem to cope without these? These mechanisms contribute greatly to the virulence of *S. pneumoniae* during invasive infections, but would not have evolved unless there was an evolutionary advantage during nasopharyngeal colonization. An additional question is why, despite the array of *S. pneumoniae* virulence factors, pneumonia and invasive disease is relatively rare compared to colonization events? The host manages to prevent bacteria replicating in the distal airway or blood in most cases, and only rarely do host defences fail but why this happens is poorly understood. The answers to these questions are fundamental for understanding why a mainly commensal bacterium can on occasion behave as an aggressive pathogen.

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CHAPTER

22

Cell-Mediated Immunity to the Pneumococcus

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There is a substantial literature describing naturally acquired immune responses to noncapsular pneumococcal antigens; alongside this literature, work toward the development of vaccines containing such antigens has been going on for some time. However, until relatively recently, research into antibacterial T cell-mediated immunity has mostly focused on type 1 responses to intracellular organisms such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*, and specifically T cell interferon gamma–mediated enhancement of macrophage intracellular bacterial killing.

This has changed for pneumococcus with publications from several groups describing a role for T cell mucosal immunity in the upper and lower respiratory tracts in murine models, and the subsequent recognition of the potentially important role of TH17 pathways, recently designated type 3 cell-mediated immunity. This work has, in turn, impacted upon applied vaccinology, with several groups and companies now considering TH17 responses to be of potential importance in the design of novel vaccines for the induction of mucosal responses and thus effects on transmission and possibly disease as well.

CLASSICAL CELL-MEDIATED IMMUNITY TO BACTERIA

The well-established paradigm of types 1 and 2 specific immunity, long described in both mice and humans, has recently been enriched not only by the description of T regulatory and TH17 cells and elucidation of their importance for the function of protective immunity and the pathophysiology of autoimmunity, but also by the description of several distinct phenotypes of cytotoxic (NK) and helper innate lymphoid cells that do not express the T cell receptor. In this context, three main types of cell-mediated effector immunity have now been proposed [1].

With regard to antibacterial cell-mediated immunity, the classic example is perhaps the

Streptococcus pneumoniae.
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intracellular pathogen *M. tuberculosis*, first understood in the context of delayed-type hypersensitivity as manifested by the positive tuberculin skin test. That this is type 1 immunity associated with interferon gamma–augmented killing by macrophages is now axiomatic for host protection against this and related bacteria, and this knowledge has been translated into diagnostic methodologies and has driven vaccine development strategies. Infections with other mycobacterial and bacterial species including *Listeria* and *Salmonella*, which can evade these protective pathways and survive inside human cells, have been observed to occur more frequently in humans with specific defects in this branch of the protective immune response [2].

TH1 cells support generation of B cell production of immunoglobulin responses and are thus important both for naturally occurring and vaccine-induced humoral specific immunity to pneumococcus, and in particular the enhanced protective effects of protein polysaccharide conjugate vaccines as compared to pure capsular polysaccharide vaccines. However, the concept that T cells might directly protect the host against pneumococcal and other extracellular bacterial infections in an antibody-independent fashion is comparatively recent. Certainly, from the host's perspective, there are clear potential advantages to having two distinct mechanisms of specific immunity—T cells and antibodies—targeting distinct epitopes of the antigens expressed by a potentially pathogenic species like the pneumococcus [3].

IMMUNODEFICIENCY AND PNEUMOCOCCAL DISEASE

Some characteristics of individuals and groups who are predisposed to pneumococcal infection suggest that antibody responses may not be the only effectors of specific immunity.

Both invasive infection (of normally sterile sites) and upper and lower respiratory tract mucosal surface infections caused by pneumococcus are particularly seen at the extremes of age. While this observation does not *per se* preferentially specifically implicate either humoral or cellular specific immunity, it is compatible with both, as immunological studies and the clinical spectrum of diseases that afflict infants and the elderly point to reduced size, quality, and longevity of both these arms of immunity [4,5]. However, rates of pneumococcal colonization differ between these two age groups. While pneumococcal infection in young children occurs in the context of a superabundance of upper respiratory carriage, although data are few, carriage rates among the elderly, who suffer frequently from both pneumonia and invasive infection, are much lower than in the very young [6]. Given this difference, comparative analysis of neonatal mucosal immunity and immunosenescence may shed further light on the immunopathogenesis of pneumococcal disease [7].

Pneumococcal infections are prominent in individuals with a range of syndromic immunodeficiencies, the underlying immunological phenotypes and genetic defects of which have been progressively elucidated. In addition to more or less pure antibody deficiencies like Bruton's agammaglobulinemia and to combined cellular and humoral deficiencies, these also include complement component and related innate opsonic defects. Pneumococcal infections are also seen in patients with neutropenia. These examples reconfirm the roles played by antibodies, other opsonins, and phagocytes in the control and prevention of invasive and progressive upper and lower respiratory tract pneumococcal infections.

Use of such "experiments of nature" to demonstrate whether antibody-independent T cell

immunity is important in either the regulation of pneumococcal colonization or direct prevention of disease is complicated by the fact that—apart from antibody responses to pure polysaccharide antigens, which form at most only a part of naturally occurring specific immunity to pneumococcus—many aspects including the size, quality, and longevity of B cell responses are highly T cell dependent. This means that patients with deficient or defective T cells may necessarily also have impaired B cell responses as well. There is even descriptive evidence to suggest that B cell responses are often suboptimal in patients with thymic hypoplasia (di George syndrome, 22q11.2 deletion) [8], even including responses to T-independent antigens [9]. Recent elucidation of defects in type 3/TH17 cell-mediated immunity in the context of hyper-IgE syndromes (reviewed in [10])—in which predisposition to pneumococcal pneumonia is reported [11] alongside prominent problems with *Staphylococcus aureus*, *Candida Albicans*, and, in the autosomal recessive forms, viral infections—may point to its importance in all of these infections. However, these patients often have demonstrable B cell dysfunction as well, including reduced B memory cells and short-lived IgG responses to vaccines, so that gammaglobulin replacement therapy is often used with apparent benefit. As the specific genotypes and immunological phenotypes of this group of patients become more clearly elucidated, it may become possible to deduce more precisely the relative importance of different aspects of specific immunity for the control of pneumococcal and other infections.

A close look at the association between acquired $CD4^+$ T cell deficiency and pneumococcal infection and/or colonization is informative in this regard. Incidence rates of pneumococcal infection are much higher in individuals with HIV infection [12]. The central immunological deficit in HIV is thought to be the consequence of progressive depletion of $CD4^+$ T cells. The consequent immunodysregulation—again—has profound consequences for both T and B cell immunity. Patients continue to have increased risk of pneumococcal disease, even long after initiation of anti-retroviral therapy [12]. There is evidence that although B cell recovery takes longer than normalization of circulating T and B cell numbers [13], aspects of $CD4^+$ and $CD8^+$ T cell function fail to normalize even after prolonged treatment [14,15].

Rates of pneumococcal colonization are higher in HIV infection [16], and density of pneumococcus measured by PCR is higher in HIV patients with pneumococcal pneumonia, not only in sputum (expectorated from the lower respiratory tract) but also in nasopharyngeal samples [17]. As at the extremes of age, this may be telling us something about the relative importance of systemic and mucosal immunity in the pathogenesis of pneumonia and invasive infection. One study, which also demonstrated higher rates of pneumococcal carriage in symptomatic HIV-infected adults both before and after institution of anti-retroviral therapy, was associated with a demonstrable defect in pneumococcal-specific interferon gamma production *in vitro* [18].

Thus, as with several primary immunodeficiencies, the clinical and immunological phenotypes of HIV infection are compatible with a predisposition to pneumococcal infection, due at least in part to B cell-independent T cell dysfunction, although the evidence in humans to date is not conclusive.

EVIDENCE FOR CELL-MEDIATED IMMUNITY TO PNEUMOCOCCUS IN MICE

As discussed above, there is an inherent difficulty in evaluating a role of cell-mediated immunity independent of antibodies in humans, since defects that affect T cells are associated with antibody production deficiencies as well. Whereas it is clear that patients with autosomal

dominant hyperimmunoglobulin E are unable to generate memory TH17 cells and are at significantly increased risk of pneumococcal infection, their genetic defect also clearly impacts other aspects of their immune function, essentially preventing conclusions regarding any causal link between TH17 deficiency and susceptibility to pneumococcus. In contrast, studies in mice, either "knocked out" for specific genes or adoptively transferred with specific T cell populations, allow for a more direct evaluation of the impact of either innate or acquired T cells on pneumococcal immunity, albeit in a mammal that is not naturally susceptible to pneumococcal colonization or infection.

Studies in Naïve Mice

The earliest studies evaluating a possible role for T cells in controlling pneumococcus focused on their role in naïve mice, that is, mice that had neither been previously exposed to pneumococcus nor immunized with pneumococcal antigens. These studies have, with some exceptions noted below, focused primarily on pulmonary infection rather than colonization. It was shown that pneumococcal pulmonary infection in mice results in a rapid infiltration with T cells, causing bronchiolar inflammation, predominantly in areas more densely infected with the bacterium [19]. Subsequently, it was shown that mice deficient in the major histocompatibility complex (MHC) class II, and therefore essentially deficient in $CD4^+$ T cells, were much more susceptible to pneumococcal pneumonia than their wild-type counterparts, implicating a role for these T cells in innate resistance to pneumococcal infection [20]. These innate responses appeared to be dependent on the presence of pneumolysin, the major pneumococcal toxin, since a bacterial strain rendered deficient in the toxin by mutagenesis did not induce the same response.

More recently, investigators demonstrated that in a similar mouse model of pulmonary infection and using the same pneumococcal strain, $CD4^+$ T cells appeared to play a deleterious role during early phases of infection, with a *higher* mortality rate in wild-type compared to MHCII-deficient mice [21]. Consistent with the absence of $CD4^+$ T cells, MHCII-deficient mice had lower levels of classic pro-inflammatory cytokines in serum, including lower levels of TNF-α, IL-6, and IL-1β, as well as lower serum levels of chemokines MIP-1β and MIP-2. The reasons for the discrepancies between the studies have not been elucidated, despite efforts by these authors to explore potential experimental differences between the two studies.

Complicating the picture further, another group demonstrated a role for $CD8^+$ T cells in resistance to pneumococcal type 3 pulmonary infection [22]. In contrast to the studies above, here mice defective in $CD8^+$ but not $CD4^+$ T cells exhibited significantly greater mortality than wild-type control mice; bacterial dissemination and lung inflammation were also significantly greater in the absence of $CD8^+$ T cells. Consistent with these findings, there was no increased mortality in mice deficient in IL-17A, a product of $CD4^+$ T cells (and other cell types). Depletion of the relevant cell type by administration of monoclonal antibodies provided results that were consistent with the findings in the knockout mice.

Regulatory T cells also appear to play an important role in invasive pneumococcal disease. Taking advantage of the relative susceptibilities of different mouse genotypes to pneumococcal pulmonary infection, investigators demonstrated that inhibition of TGF-β1 increased susceptibility in Balb/c mice otherwise usually relatively resistant to infection, whereas passive transfer of regulatory T cells to more susceptible mice (CBA/Ca) was associated with increased survival [23].

Overall, these studies provide further evidence of the complexity of the role of T cells in

innate resistance to pneumococcal infection in mice and raise the daunting, but not unreasonable, possibility that immune mechanisms of protection against pneumococcal sepsis may differ among pneumococcal strains, different serotypes, and routes of infection.

With respect to colonization, intraperitoneal administration of a TGF-β1 inhibitor to naïve mice just prior to intranasal pneumococcal inoculation reduced both the density and the duration of carriage, and increased neutrophil influx into the nasopharynx [24]. Interestingly, this reduction in carriage was associated with an increased risk of translocation of pneumococci to the lungs, which was not observed in animals that received PBS instead of the inhibitor. These studies thus suggest the possibility that regulatory T cells may contribute to prolonged duration of carriage in mice, while at the same time limiting the risk of pulmonary translocation. A possible role for IL-10 is also suggested by studies comparing the innate susceptibility of infant versus adult mice to pneumococcal colonization. Infant mice, whose monocytes secrete significantly more IL-10 following stimulation with pneumococcus than do cells from adult mice, are also significantly more susceptible to pneumococcal colonization, and at much higher densities [25]. Even if this relationship were ultimately shown to be causal, many cells can produce IL-10; thus, it is of course possible that cells other than regulatory T cells may be responsible for secretion of this cytokine and the associated increased susceptibility of neonatal mice.

Studies in Immune Mice

With respect to acquired immunity to pneumococcus, it is helpful to distinguish between studies of resistance to pneumococcal colonization versus infection. For resistance to colonization, clues to the possible role of T cells were obtained through immunologic studies of mice following immunization with a candidate pneumococcal whole cell vaccine (WCV) [26] or after nasopharyngeal exposure to live pneumococci [27,28].

In these studies, it was noted that protection did not appear to be dependent on the generation of anticapsular immunity. With WCV, this was obvious since the strain from which the vaccine is derived is unencapsulated; similarly, with live exposure to pneumococcus, equal protection was noted following exposure to homologous or heterologous capsular serotypes [26]. Furthermore, it was noted that protection against colonization by either of these two exposures in animals appeared to reflect what is observed as children age [29,30], namely that, instead of becoming resistant to initial colonization, immunized or exposed mice had a reduction in the duration and density of carriage.

Experiments in knockout mice confirmed that protection by either the WCV or live exposure to pneumococci conferred robust antibody-independent, $CD4^+$ T cell-dependent protection against carriage [26–28]. Adoptive transfer of immune, but not naïve, $CD4^+$ T cells conferred protection against carriage in RAG-deficient mice (i.e., mice congenitally lacking any B or T cells), implying that these $CD4^+$ T cells are not only necessary but also sufficient for protection against carriage in mice [30]. The IL-17A $CD4^+$ T cell lineage was then shown to be the effector arm, as IL-17A receptor-deficient or neutrophil-depleted mice were not protected by the WCV [30]. Secretion of IL-17A from these cells is thought to recruit professional phagocytes (macrophages or neutrophils, depending on the history of previous exposure to pneumococcal antigens [31]) to the site of colonization, activate these cells, and reduce density as well as duration of carriage.

Using mice whose $CD4^+$ T cell can recognize only a specific OVA epitope, it was subsequently demonstrated that the protection generated by immunization with the WCV is antigen-specific [32], strongly suggesting the

possibility of immunizing with specific pneumococcal antigens to generate the same type of protection. Further studies of acquired resistance to colonization in mice have thus evaluated more defined immunogens than the WCV, such as the conserved zwitterionic cell-wall pneumococcal polysaccharide [33] or combinations of pneumococcal surface proteins [34,35]. Proteomic screens of pneumococcal antigens in mice were subsequently performed, using immune cells from mice exposed to the WCV [31,36]. From these studies, a number of conserved pneumococcal protein antigens that elicit robust TH17 responses following exposure to either WCV or live pneumococci have been identified. When these antigens were then used as immunogens in mice, they conferred protection against pneumococcal carriage [31,36]; as discussed below, a vaccine combining three such antigens is currently undergoing phase II clinical trials for evaluation of an effect against carriage in a model of intentional human exposure.

With respect to acquired immunity to pneumococcal infection, data on a possible role for cell-mediated immunity are sparse. One study evaluated the impact of depletion of $CD4^+$ T cells following nasopharyngeal pneumococcal exposure [37]. In mice, intranasal pneumococcal inoculation confers protection against subsequent invasive pulmonary challenge by homologous and heterologous challenges [37,38]. Depletion of $CD4^+$ T cells after the primary exposure did not reduce subsequent protection against invasive disease challenge; in contrast, previously nasally exposed μMT mice (deficient in immunoglobulins) are not protected against invasive pulmonary challenge. Thus, these experiments did not reveal a major role for $CD4^+$ T cells in protection against pneumococcal invasive disease in mice.

In summary, there is substantial evidence in mice that acquired immunity to pneumococcal colonization can result from the generation of antigen-specific $CD4^+$ T cell- and IL-17A-dependent responses to pneumococcal antigens. Next we will examine the evidence in support of similar mechanisms in humans.

EVIDENCE FOR CELL-MEDIATED IMMUNITY TO PNEUMOCOCCUS IN HUMANS

Demonstration of circulating antibodies to several non-capsular pneumococcal antigens at increasing frequency and concentration with increasing age in early childhood [39,40], as well as the presence of antibody-secreting cells to such antigens in adenoidal tissues of children [41], have been recognized for some time. Notwithstanding the demonstrated effectiveness of capsular antigen−containing conjugate vaccines against both disease and carriage, it is also now accepted that naturally acquired immunity is likely to include responses to non-capsular antigens [42,43]. These observations have fueled the search for potential cross-serotype protective vaccines that might mimic such immunity, be manufactured more cheaply and easily, and evade the emerging problem of serotype replacement of vaccine types in highly immunized populations.

An association in children between the presence of serum and salivary IgG antibodies to some pneumococcal proteins and the absence of carriage was shown alongside greater adenoidal cell production of such antibodies following antigen stimulation *in vitro*. Such antibody production appears to be dependent upon T cell-derived interferon gamma and interleukin-10 [44]. However, such associations do not prove the existence or size of a functional role of antibody in the control or clearance of carriage [40]. In a similarly designed observational study of children undergoing adenoidectomy, lower $CD4^+$ T cell proliferative responses were seen in peripheral blood mononuclear cells stimulated with pneumolysin and a detoxified mutant of the

same antigen in those who had pneumococcus isolated from their nasopharynges at the time of operation, while such differences in T cell proliferation rates were not observed between culture-positive and -negative children when adenoidal instead of blood cells were cultured and stimulated with the same pneumococcal protein antigens [45]. A study of West African adults showed demonstrable T-proliferative responses to pneumococcal antigens in peripheral blood mononuclear cells in 60% of subjects, with evidence of both resting and effector memory but, in this setting, no positive or negative association with intercurrent pneumococcal carriage [46]. Studies in adults have demonstrated rapid T cell interferon gamma production in response to pneumococcal antigens and have characterized pneumococcus-specific effector T cells responsive to an immunodominant HLA-DR-restricted epitope in the conserved pneumococcal serine threonine kinase StkP [47,48]; attempts to map T cell epitopes in the vaccine candidate antigen PsaA have also been made [49].

It appears that some pneumococcal antigens may promote activation of innate immune cells including antigen-presenting cells and thus indirectly promote specific immune responses [50]. Bacterial lipoproteins, which are present in many bacterial species and bind to TLR2 receptors, appear to be important for inflammatory responses to pneumococcus [51]. While augmenting naïve B and T cell responses, they appear to down-regulate memory responses in association with enhanced IL10 production via a pathway that may involving binding to TLR2-expressing antigen-presenting cells, resulting in B7h-ICOS binding and IL10-producing T regulatory cells [52]. This suggests that pneumococcus may have evolved strategies to down-regulate host mucosal T cell responses through TLR2 signaling. This may help explain why colonization can persist and how generation of specific naturally acquired immunity sufficient to clear colonization is often followed by colonization on reexposure at a later date, especially in childhood. A further pediatric study demonstrated higher numbers of T regulatory cells in the adenoids of children carrying pneumococcus, suggesting that the presence of these cells and associated IL10 production might be partly responsible for the high frequency, density, and duration of pneumococcal colonization in young children [53], while a study in infants in Papua New Guinea suggested that early pneumococcal colonization may down-regulate subsequent cell-mediated immune responses [54].

Demonstrating directly that T cell immunity *per se* matters in children is hampered not only by the ubiquity of T cell−B cell interactions, as discussed above, but also by the necessarily observational nature of clinical studies, since pneumococcus, although a common commensal, is also a significant pathogen in this age group, making challenge studies untenable. However, experimental induction of nasal carriage by nasal inoculation has been done in adults [24]. Experiments done this way suggest that the induction of transforming growth factor β-1 production (by epithelial cells and fibroblasts) and IL10 are associated with establishment of stable colonization in adult volunteers, observations supported by experiments in mice and *in vitro* [55]. Conversely, deliberate nasal inoculation with pneumococci has been shown to increase the numbers of TH17 cells in both blood and bronchoalveolar lavage fluid in adult volunteers [56] and to protect against subsequent rechallenge [57].

Stimulation of human mononuclear cells from blood and tonsils with pneumococcal protein antigens elicits an IL17 response, and IL17 promotes neutrophil-mediated killing of pneumococci, even in the absence of antibodies and complement [30]. A comparative study that examined IL17 responses in peripheral blood to pneumococcal antigens in both children and adults from both Sweden and Bangladesh demonstrated greater responses in adults than

in children in the former country, but no age-related differences in the latter. It also demonstrated larger IL17 responses in children after proven or suspected pneumococcal pneumonia [58]. A further study in children has shown stimulation of *ex vivo* TH17 memory cell proliferative responses in tonsil-derived mononuclear leukocytes, which were greater in those without pneumococcal carriage at time of surgery, while TH1 responses did not differ [59].

Accordingly, whether nasal colonization with pneumococci is established and persists or is eliminated may have as much to do with the balance of T cell activity—specifically T regulatory cells and associated TGF-b1 and IL10 release versus TH17 cells and associated IL17 and IL22 release—and related innate cell activity as with the presence or absence of mucosal anti-pneumococcal antibodies.

NOVEL PNEUMOCOCCAL VACCINES AND CELL-MEDIATED IMMUNITY TO PNEUMOCOCCUS

An interesting translational consequence of these scientific findings has been their implication for development strategies for novel pneumococcal vaccines for the prevention of pneumococcal colonization. Indeed, a major advantage for novel pneumococcal vaccine development is the ease with which an impact on carriage can be measured in infants and children, compared to an impact on disease. Whereas it is estimated that more than 200,000 children would need to be enrolled in a study to demonstrate non-inferiority of a candidate non-capsular pneumococcal vaccine compared to a pneumococcal conjugate vaccine, a much smaller number (estimates vary between 200 and 400 children) would be required to evaluate a possible impact on carriage.

Following demonstration of protection using good manufacturing practice (GMP)-grade material in mice [60] and a favorable safety profile in rabbits, the pneumococcal WCV has been taken forward for trials in humans. The WCV has completed a phase I trial in healthy adult volunteers in the United States and is now undergoing phase II evaluation in children in Kenya. A three-component pneumococcal protein vaccine developed by Genocea Biosciences based on a proteomic screen [61] has also completed a phase I clinical trial in healthy adult volunteers, and is currently being evaluated in a phase II trial in Liverpool, with a controlled intentional intranasal exposure to pneumococcus. Results from these two clinical trials should provide important insights regarding not only the ability to generate TH17 responses in adults and children, but also whether these immunization strategies can have an impact on carriage.

Just as the paradigm of protective specific immunity to pneumococcus is emerging as a combination of B and T cell-mediated effector responses, so too the approach of would-be vaccine developers is moving progressively toward both antibody and cell-mediated responses for optimal effectiveness.

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CHAPTER

23

Pneumococcal Invasion: Development of Bacteremia and Meningitis

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Streptococcus pneumoniae (the pneumococcus) is carried asymptomatically in the nasopharynx in up to 60% of the population [1]. While it most commonly causes upper respiratory tract infection, it is the leading cause of bacteremia and meningitis worldwide. In the United States, despite the implementation of childhood vaccination programs and effective antimicrobial agents, it is estimated that the pneumococcus is responsible for 175,000 cases of pneumonia (case fatality rate 5–7%), >50,000 cases of bacteremia (case fatality rate 20%), and 3000–6000 cases of meningitis (case fatality rate ~30%) annually [2]. In developing countries, the World Health Organization (WHO) estimates that case fatality rates range from 5% to 20% for bacteremia, and from 40% to >50% for meningitis [3]. It is estimated about half of the survivors of pneumococcal meningitis develop long-term sequelae, including hearing loss, focal neurological deficits, or cognitive impairment [4]. Thus, the pneumococcus is arguably the greatest single etiology of invasive bacterial disease in the world.

Invasive pneumococcal disease (IPD), defined as isolation of *S. pneumoniae* from a normally sterile site (e.g., blood, cerebrospinal fluid (CSF), joint, pleural, or pericardial fluid), is seen particularly in young children and the elderly or in patients with underlying conditions, including human immunodeficiency virus infection, sickle cell disease, hemolytic-uremic syndrome, and dialysis [5]. Table 23.1 shows the virulence determinants that are believed to play major roles in driving pneumococcal infection from benign to invasive disease, such as sepsis and meningitis. Each body site is recognized as having a distinct and specific set of virulence determinants that accelerate infection, with only some overlap between sites. This complicates the search for a vaccine protection strategy that seeks a single protein antigen for all infected organs. The profound efficacy of the capsule-based vaccines derives from their ability to rapidly clear the bloodstream of bacteria [3]. However, they are limited by the emergence of non-vaccine serotypes [2]. A further complication for vaccine design has recently been recognized on the side of the host. It appears that various high-risk hosts may select for different virulence determinants

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TABLE 23.1 Pneumococcal Virulence Factors and Their Principal Roles in IPD

Virulence factor	Main role in disease
LOWER RESPIRATORY TRACT TO BLOOD	
NanA neuraminidase A	Cleaves terminal sugars from human glycoconjugates to unmask carbohydrate receptors for bacterial adhesion
PCho phosphorylcholine	Binds to platelet-activating factor receptor (PAFr) on alveolar epithelium for bacterial translocation
SpxB pyruvate oxidase	Produces H_2O_2 that kills host cells
Ply pneumolysin	Cholesterol-dependent, pore-forming cytotoxin that lyses host cells and activates the classical complement pathway
EndA endonuclease A	Degrades DNA scaffold of NETs
BLOOD TO CENTRAL NERVOUS SYSTEM	
NanA neuraminidase A	Promotes invasion of the BBB by activating brain endothelial cells
CbpA choline-binding protein A	Binds to LR on brain endothelial cells
PCho phosphorylcholine	Binds to PAFr on epithelia and endothelia for bacterial translocation
LytA autolysin	Digests the bacterial cell wall, results in the release of cell-wall fragments
Ply pneumolysin	Cholesterol-dependent, pore-forming cytotoxin that triggers brain endothelial and neuronal cell death
GlpO glycerophosphate oxidase	Generates H_2O_2, resulting in brain endothelial cell death

based on how their underlying disease changes their metabolism and host environment. For instance, children with sickle cell disease have a 600-fold increased risk of potentially fatal IPD compared with the general population, despite having similar rates of colonization [6]. The increased risk of IPD is related to hyposplenism, complement deficiency, and chronic vascular inflammation that promotes up-regulation of the endothelial ligand for pneumococcal invasion [7,8]. To counteract this risk, these children receive antibiotic prophylaxis and vaccines. This sets up a situation in which the naturally competent pneumococcus is constantly under selective pressure. The highly plastic pneumococcal genome adapts to both therapeutic pressures and the host environment to survive better in the sickle cell niche, a host that is highly prevalent in malaria-endemic regions of the world. Comparing the genomic content of hundreds of isolates from healthy children versus sickle cell patients by deep genomic sequencing, it was found that invasive isolates prevalent in sickle cell disease differed by metabolic genes, metal responsive elements (such as iron), and other changes suited to the altered host [9]. While all strains colonized healthy and sickle cell hosts well, only some strains could advance to invasive disease, and their repertoire of virulence determinants for this step differed significantly. As a consequence of these differences in virulence factors needed for invasive disease progression in the two settings, antigens that would be effective vaccines in healthy humans were rendered irrelevant to

protection in the high-risk sickle cell host. Thus, the requirements for IPD (and for a vaccine to prevent invasive disease) can be profoundly different depending on the host.

UPPER RESPIRATORY TRACT COLONIZATION

The human nasopharynx is the major ecological niche for *S. pneumoniae*. In the nasopharynx, the pneumococcus undergoes a process called phase variation, expressing a thick, negatively charged capsule that repels the sialic acid residues of mucus to prevent the bacterium from being trapped [10]. Once the pneumococcus reaches the mucosal epithelial surface, the bacterium down-regulates its capsule expression, expresses a thinner capsule, and possesses other characteristics that promote adhesion to host tissues and biofilm formation [11,12]. *S. pneumoniae* also expresses surface-associated exoglycosidases, including NanA (neuraminidase), BgaA (β-glucosidase), and SrtH (β-*N*-glucosaminidase), which are capable of deglycosylating mucous glyconjugates, thereby decreasing mucous viscosity and preventing entrapment, and also exposing receptors for bacterial adhesion [12,13].

The host pulmonary mucosal surface actively produces antimicrobial agents, such as secretory IgA (sIgA), to interfere with microbial adhesion and invasion of the mucosa [14]. The pneumococcus counteracts sIgA by secreting IgA1 protease, which cleaves specifically at the heavy chain of sIgA, inhibiting IgA-mediated opsonization, and hence allowing bacterial adhesion to the nasopharynx [15].

The pneumococcal surface is decorated with numerous surface proteins that are essential for nasopharygeal colonization. One of the surface proteins, choline-binding protein A (CbpA, also known as PspC or SpsA), binds to the ectodomains of the polymeric immunoglobulin receptor (pIgR), which is expressed exclusively in the upper respiratory tract [16]. The binding occurs through conserved hexapeptide motifs Y/RRNYPT localized in two N-terminal repeated domains of CbpA with two of the five immunoglobulin (Ig)-like ectodomains, namely D3 and D4, of the pIgR [17–19]. Following the attachment, a cascade of kinases and GTPases, such as Cdc42, phosphatidylinositol 3-kinase (PI3K), and protein kinase B (Akt), is activated, leading to the internalization and translocation of the bacteria across the nasopharyngeal epithelial cells [16,17,20,21]. Interestingly, the CbpA-pIgR interaction also simultaneously triggers a cellular defense mechanism in which the intracellular calcium levels are elevated in a phospholipase C-dependent manner, which then diminishes bacterial invasion of host epithelial cells [22].

In addition, some clinical isolates, particularly penicillin-nonsusceptible pneumococcal isolates (PNSP), possess a pilus-like structure to promote pneumococcal adhesion to an unknown host cell receptor [23,24]. The pneumococcal serine-rich repeat protein (PsrP) promotes bacterial aggregation *in vivo* in the nasopharynx and lungs of mice, and influences biofilm formation [25].

PROGRESSION FROM PNEUMONIA TO BACTEREMIA

In the lower respiratory tract, pneumococci rapidly multiply in the alveolus and spread in a contiguous manner through the pores of Kohn to establish classical lobar pneumonia. Within this niche, the pneumococcal neuraminidase, NanA, cleaves terminal sialic acid from alveolar epithelial cell glycoconjugates, revealing carbohydrates such as *N*-acetylgalactosamine β1-3 galactose, that are recognized receptors for bacterial binding [26]. Following adherence to alveolar cells, pneumococcal replication drives the initiation of host innate immunity. In a rabbit pneumonia model, inoculation of purified pneumococcal peptidoglycan-teichoic acid cell-

wall complex (CW) leads to the development of pneumonia, with serum components, neutrophils, and fibrin detected in the bronchoalveolar lavage fluid [27]. The interaction of CW with Toll-like receptor 2 (TLR2), an innate immune receptor expressed on the surface of mammalian cells, leads to the secretion of various cytokines (e.g., TNF-α, IL-1, IL-6, IL-10, IL-12), and induces procoagulant activity on human endothelial cells [28–30]. Pneumolysin, a pore-forming toxin that binds to all cells, is a potent mechanism of injury that intensifies the inflammatory response [26]. This response, one of the most intense and most classically studied in medicine, may remain localized to the lung and resolve without permanent lung damage or it may progress to IPD by seeding the bloodstream.

In healthy individuals, the transition from pneumonia to IPD signifies a major increase in the risk of morbidity and mortality. Most respiratory pathogens have developed their own specialized cadre of virulence determinants to enable this translocation, including pili and fimbriae. Pili have recently been recognized on ~20% of pneumococcal isolates, and they are suspected of contributing to adherence in the respiratory tract and thereby increasing the opportunity for invasion rather than partaking in the translocation itself [31]. The pneumococcus is an example of biological simplicity in the process of invasion, and an understanding of the pneumococcal invasion scheme has proved to be important since it is a process shared by virtually all respiratory pathogens (including the major invasive pathogens of children: *S. pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis*, oral flora and even mycoplasma). This common invasion mechanism has been termed *innate invasion* to reflect its subversion of innate immunity [32]. The basic mechanism involves the display of phosphorylcholine (PCho) on a component of the bacterial surface: cell wall, protein, or lipid depending on the pathogen [33–35]. Pneumococcus adds PCho to its cell-wall teichoic acid and lipoteichoic acid in a phase-variable fashion [11,36]. PCho enables bacterial recognition of the platelet-activating factor receptor (PAFr) by molecular mimicry of the PCho component of the chemokine PAF [37,38]. During infection, the expression of PAFr on the host cell plasma membrane is up-regulated in a CCAAT/enhancer-binding protein δ (C/EBPδ)-dependent manner [39]. The binding of bacterial PCho to PAFr results in activation of β-arrestin-mediated endocytosis of the bacteria, which allows the bacteria to traverse the cytoplasm to the basolateral membrane of pulmonary epithelial cells and then into the blood [37,40]. PAFr-deficient mice and wild-type mice treated with PAFr antagonists demonstrate reduced bacterial translocation across the pulmonary endothelium and delayed bacteremia [40]. This innate invasion interaction is competitively counteracted by PCho-directed innate host defense mechanisms, such as PCho in surfactant, which is a direct competitive antagonist, and C-reactive protein (CRP), which is an acute-phase reactant that binds to PCho and enhances bacterial clearance by macrophages [41].

The maintenance of epithelial barrier integrity by the host is an important factor preventing the translocation of the pneumococcus from the lung into the bloodstream. A recent study revealed that type I interferon (IFN) leads to down-regulation of the PAFr and up-regulation of tight junction proteins, thus limiting the progression of infection from the lung into the bloodstream [42]. Barrier integrity is also regulated by inflammatory responses to early bacterial colonization. Detection of bacteria by pattern recognition receptors (PRRs) leads to the activation of the p38 MAPK and TGF-β signaling cascades, resulting in the down-regulation of tight junction proteins such as claudin 7 and claudin 10 via the transcriptional regulator Snail1, thereby facilitating transepithelial invasion by the bacteria [43–45]. The production of type I interferons and the down-

regulation of tight junction proteins due to inflammatory signaling not only affect colonization but also potentially contribute to invasion of the blood–brain barrier (BBB).

Bacterial translocation into the blood can also be enhanced by damage to the pulmonary epithelium. The pneumococcus produces the cytolytic toxin pneumolysin and copious amounts of hydrogen peroxide (H_2O_2), both of which disrupt the alveolar epithelium and lead to the leakage of erythrocytes into the alveolar space. Pneumolysin is a potent cytotoxin that binds to cholesterol on host plasma membranes and oligomerizes to form a large transmembrane pore [46]. Pneumolysin also induces proinflammatory cytokine production, activates complement, and induces the secretion of the procoagulant von Willebrand factor (vWF) from endothelial cells [47,48]. The pneumococcus, via a pyruvate oxidase encoded by *spxB*, also produces millimolar amounts of H_2O_2 by converting pyruvate to acetyl phosphate and H_2O_2. *S. pneumoniae* lacking *spxB* demonstrate reduced virulence in a pneumonia model [49].

Clinically, a critical determinant of progression of pneumonia to IPD is the amount of damage to the alveolar epithelium, with increased damage enabling more rapid and extensive invasion to the blood. Two clinically important settings for enhanced invasion are preexisting viral infection or an overactive host inflammatory response. There is a striking clinical association of influenza with secondary invasive pneumococcal pneumonia. Modeling in animals has shown that strains of influenza that damage the lung, even though not causing significant morbidity on their own, facilitate the spread of bacteria that subsequently enter the lung [50]. As the pulmonary epithelium is left denuded by the virus, bacteria can readily gain access to the underlying connective tissue and vasculature to accelerate secondary pneumonia and bacteremia.

Invasion can also be accelerated at later stages of the infection when the alveolar space is packed with lysed erythrocytes and neutrophils. Neutrophils are essential to kill *S. pneumoniae* via opsonophagocytosis and non-oxidative mechanisms involving serine proteases [51]. An *in vitro* study showed that *S. pneumoniae* α-enolase recruits neutrophils and binds to their myoblast antigen 24.1D5 to trigger the release of neutrophil extracellular traps (NETs) containing antimicrobial proteins bound to a DNA scaffold [52]. Interestingly, pneumococci counterattack by degrading NETs via an endonuclease encoded by *endA* [53]. Although neutrophils are essential for the clearance of bacteria from the infected lungs, the intense inflammation induced by the pneumococcal–neutrophil interaction contributes to lung damage [1]. An experimental pneumonia model showed that neutropenia may attenuate lung damage and improve disease outcome [54].

BLOODSTREAM SURVIVAL

Once pneumococci have breached the pulmonary epithelial barrier and invaded the bloodstream, the bacteria encounter both classical and alternative complement pathways, which opsonize the bacteria to promote phagocytosis. In order to evade opsonophagocytosis, pneumococci undergo phase variation, a spontaneous, coordinated conversion of expression of many genes such that the bacteria become more encapsulated and show decreased expression of adhesins and surface decoration by PCho [11]. The negatively charged capsule strongly inhibits the deposition of complement, CRP, mannose-binding proteins, and antibodies on the bacterial surface, thus limiting phagocytosis of the bacteria [14]. Recently it has been suggested that some pneumococci evade the lectin-dependent pathway of complement activation and recognition by ficolin-2 by mutating the *O*-acetyltransferase gene, *wcjE*, in the capsule synthesis locus, resulting in a highly invasive strain [55].

Pneumococcal surface proteins, such as PspA, CbpA, and pneumolysin, target specific complement components. PspA inhibits the activation and deposition of complement protein C3b through the classical pathway [56]. CbpA binds to factor H in the blood and subsequently prevents complement attack by inhibition of C3b, limiting opsonophagocytosis [56]. Pneumolysin activates the classical complement pathway by binding to the Fc portion of IgG, resulting in depletion of complement factors and greater bacterial bloodstream survival [57]. In murine bacteremic infection, in the absence of pneumolysin, pneumococci are either cleared from the bloodstream or develop chronic bacteremia [14].

Nitric oxide (NO) is synthesized from arginine by host NO synthase. A recent study found that clinical pneumococcal isolates with argininosuccinate synthase (*argG*) and argininosuccinate lyase (*argH*), both arginine biosynthesis genes, demonstrated resistance to oxidative stress and increased bloodstream and CSF survival [58]. These pneumococcal arginine biosynthesis enzymes compete with NO synthase for substrate, and hence reduce NO production and increase bacterial survival [58].

BACTEREMIA AND SEPSIS

The clinical manifestations of pneumococcal bacteremia are highly variable, depending on several factors such as the health status of the patient, the causative strain, and initiation of treatment [59]. If the host immune response fails to eliminate *S. pneumoniae* from the bloodstream, sepsis can occur. This life-threatening condition is defined as an uncontrolled hyperinflammatory cytokine response that can be accompanied by organ dysfunction (severe sepsis) and/or hypotension or hyperlactatemia (septic shock) and may ultimately lead to death of the patient. One of the major hallmarks is acute organ dysfunction, mostly of the cardiovascular and respiratory systems, but the brain, liver, and kidneys are also often affected. This is due to the response of immune cells to bacteria and bacterial components (e.g., lipoteichoic acid, bacterial toxins), producing profuse amounts of immune mediators including cytokines, chemokines, prostaglandins, reactive oxygen species, and lipid mediators [60,61]. The release of these components leads to the activation of leukocytes, lymphocytes, and endothelial cells. Stimulation of coagulation by tissue factor, a transmembrane glycoprotein expressed by many cell types, together with reduced activity of endogenous anticoagulant pathways and impaired fibrinolysis, provokes microvascular thrombosis. Thrombus formation results in hypoperfusion of the tissue, which is exacerbated by hypotension, red blood cell deformability, and vasodilatation [59]. The damage caused by intra- and extravascular phagocytic cells in combination with hypoperfusion, loss of barrier function of the endothelium, and hypoxia ultimately leads to organ failure [60].

CENTRAL NERVOUS SYSTEM INVASION

In the presence of sustained high-grade bacteremia, pneumococci bind to the cerebral capillary endothelium and invade into the fluid-filled subarachnoid space around the brain. The bacteria take advantage of the limited host defense mechanisms in the CSF, and efficient bacterial replication results in the recruitment of highly activated leukocytes, a hallmark of bacterial meningitis [62]. During meningitis, inflammation is not limited to the CSF and meninges surrounding the brain, but also affects the brain parenchyma and the ventricles, and disseminates along the spinal cord. In principle, there are three potential entry sites into the CSF (Figure 23.1A): (1) the blood−CSF barrier in the subarachnoid space that is formed

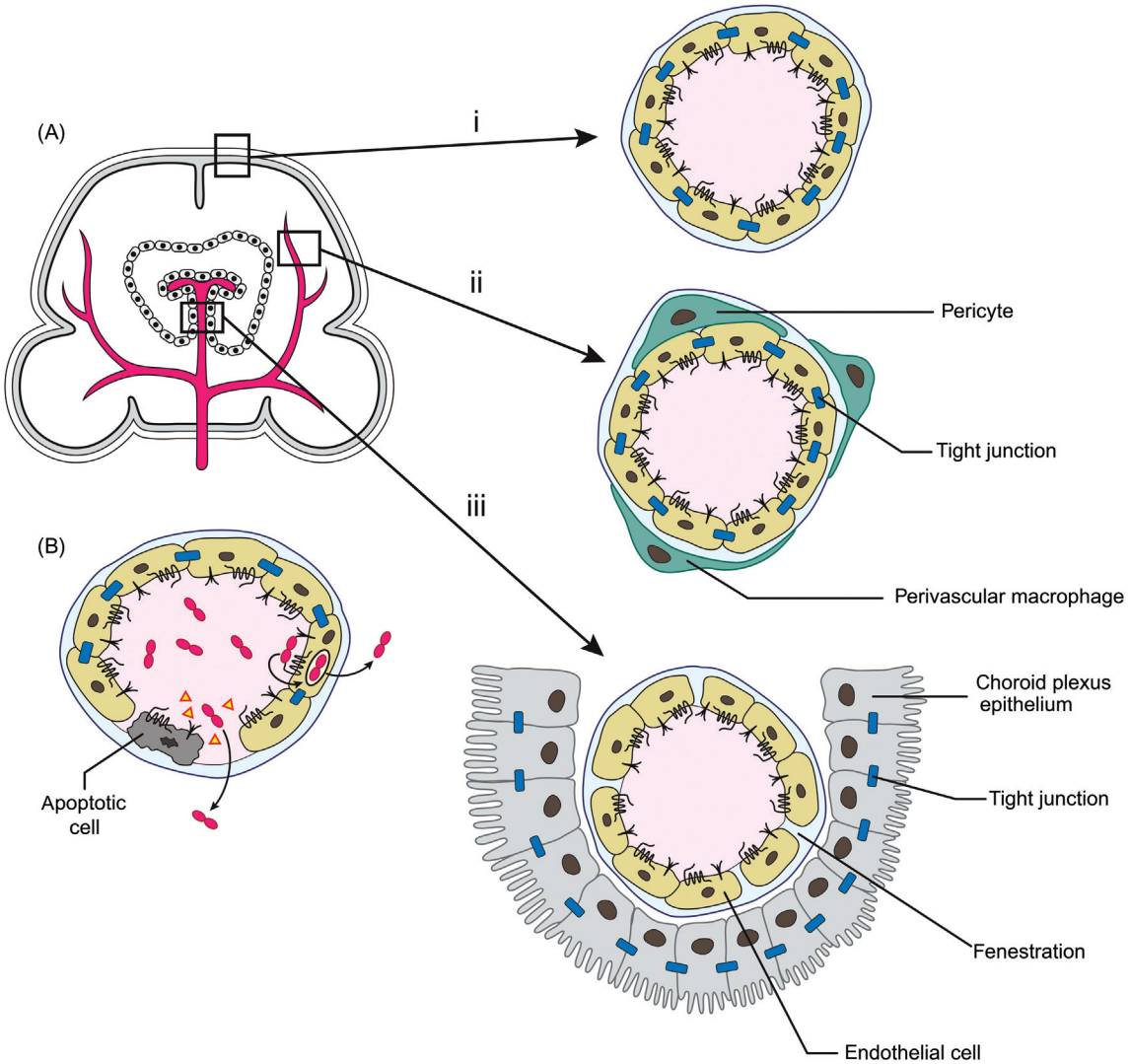

FIGURE 23.1 **Interaction of pneumococci with the BBB.** (A) Schematic illustration of: (i) The blood–CSF barrier in the subarachnoid space is formed by tight junctions between endothelial cells. (ii) The BBB of the cerebral cortex is composed of endothelial cells that form tight junctions, and they are in contact with foot processes of astrocytes and pericytes. (iii) The blood–CSF barrier in the choroid plexus is made up of fenestrated capillaries in close contact with ependymal epithelial cells that are interconnected by tight junctions. (B) Schematic illustration of the pneumococcal *innate invasion* of the brain endothelial cells. Pneumococcal CbpA binds to laminin receptor (Y), activating the cell and up-regulating PAFr (M). Cell-wall PCho binds to PAFr, followed by bacterial endocytosis and traversal of the cell in vacuole. The pneumococci also produce pneumolysin (yellow triangles), which disrupts barrier integrity by pore formation, triggering of the host's apoptotic response and allowing bacterial penetration.

by tight junctions between endothelial cells; (2) the BBB of the cerebral cortex, which consists of capillary endothelial cells that exhibit very tight junctions and are in close contact with pericytes and astrocytes; and (3) the blood–CSF barrier in the choroid plexus, which is composed of fenestrated capillaries in close contact with tight junctions of ependymal epithelial cells. The precise anatomical sites for pneumococci invasion into the CSF are still unclear and in many cases controversial. A recent study using a mouse meningitis model suggested that subarachnoid vessels could be the initial site of invasion, as evidenced by the detection of adherent bacteria at the vessels at very early time points after intravenous challenge [63].

Meningitis-causing pathogens invade the central nervous system via a common adherence step targeting laminin receptor (LR). The initial interaction between blood-borne pathogen and the BBB that sets up translocation is shared by neurotropic bacteria, viruses, and prions and involves a family of antigenically related ligands that bind to LR. Pneumococcal CbpA, meningococcal PilQ and PorA, and OmpP2 of *H. influenzae* bind to the C-terminus of endothelial 37/67-kDa LR (Figure 23.1B) [64]. The LR-binding site on CbpA is a highly conserved, surface-exposed loop, and antibodies to this epitope bind to the LR-ligands of the other meningeal pathogens and block passage of all three bacteria from blood to the CSF. In addition, CbpA contains a motif that binds to the pIgR [17]. These discoveries shed light on the possibility of generating a broadly protective vaccine against bacterial nasopharyngeal colonization, pneumonia, sepsis, and meningitis by inhibiting the interaction of bacterial adhesins with LR and pIgR [65]. In addition to LR binding, pneumococcal NanA has been suggested to promote adhesion to brain endothelial cells via its laminin G-like lectin domain [66]. Recent findings have suggested that the pneumococcus binds to pIgR and platelet endothelial cell adhesion molecule-1 (PECAM-1, also known as CD31) complex on the surface of brain microvascular endothelial cells; however, the requirement for this interaction in bacterial internalization and traversal from the endothelial cells is unknown [67,68].

Following the establishment of firm bacterial adhesion to LR on the vascular wall, the underlying endothelial cells are activated, resulting in the up-regulation of PAFr expression. PAFr then binds to the PCho on the pneumococcal cell wall, leading to the activation of β-arrestin and triggering of bacterial endocytosis via a clathrin- or caveolae-dependent pathway [69,70]. After internalization, the majority of pneumococci are killed in the lysosome, but a small population evades degradation and translocates transcellularly into the CSF [71]. Intriguingly, the host proteasome-ubiquitin system has been demonstrated to be essential for efficient killing of the internalized pneumococci *in vitro* and *in vivo* [72]. PAFr-deficient mice, CbpA knockout bacteria, and PCho-deficient *S. pneumoniae* show drastically reduced virulence in various animal meningitis models [40,73,74].

Pneumococci may also invade the CSF by disrupting the integrity of the BBB, either by cytotoxicity of bacterial virulence factors or by bystander damage from an exuberant host immune response. The pneumococcal pneumolysin and autolysin are important for invasion of the CSF by activating endothelial cell p38 mitogen-activated protein kinase and promoting apoptosis [75,76]. Another recently identified pneumococcal surface protein, α-glycerophosphate oxidase (GlpO), is also capable of disrupting endothelia *in vitro* via generation of H_2O_2 [77]. Infection of animals with GlpO-deficient bacteria yields a significant reduction in meningeal inflammation and brain pathology.

CNS INFLAMMATORY RESPONSE

The tight junctions of the BBB make the brain an immune-privileged organ by preventing

access of blood cells and serum innate immune components. Microglia, the brain resident macrophages, in combination with meningeal and perivascular macrophages, form the first line of defense against bacterial invasion in the brain and are believed to play an important role in the immune response. Microglia can be found in all regions of the brain. In contrast to astrocytes and oligodendrocytes, microglia are derived from the yolk sac and populate the brain prior to its vasculogenesis. These cells express pattern recognition receptors (PRRs), which function to detect various bacterial pathogen–associated molecular patterns such as cell-wall fragments, lipoteichoic acids, or peptidoglycan (Figure 23.2).

The most important PRRs in the brain during pneumococcal meningitis are TLR2, TLR4, and Nod-like receptors (NLRs) [14]. TLR2 is known to recognize cell wall, lipoteichoic acid, and lipoproteins [28,78], whereas TLR4 detects pneumolysin [79]. A mouse model of pneumococcal meningitis revealed elevated blood and CSF bacterial titers, enhanced inflammation, and increased disease severity in TLR2-deficient mice [80,81]. TLR2 signaling is enhanced by its coreceptor CD14 and by LPS-binding protein. Infection of TLR2/CD14 double knockout mice resulted in significantly increased CSF bacterial loads and disease severity [82]. Although TLR4 deletion in mice did not show a strong phenotype compared to wild-type animals, infection of TLR2/TLR4 double deficient mice revealed an increased disease severity compared to wild-type or single knockout mice, suggesting that both receptors are needed to mount a robust

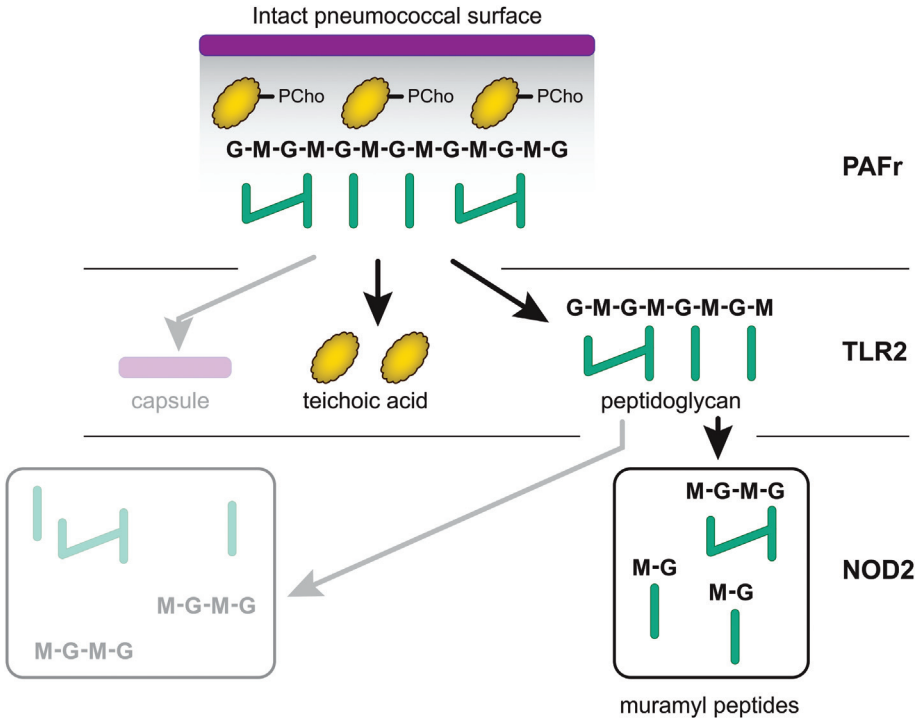

FIGURE 23.2 **Recognition of pneumococcal cell-wall components by the innate immune system.** The pneumococcal cell wall is recognized by the host innate immune system at multiple levels. PCho on the intact cell wall binds to PAFr and facilitates invasion of host cells and transmigration across barriers of both the intact bacteria and released cell-wall fragments. Smaller fragments of peptidoglycan and teichoic acid are recognized by TLR2 and evoke a strong inflammatory response, while the capsular polysaccharide is relatively inert (faded image). Muramyl peptides interact with NOD2 within cells, but dissociation of the carbohydrate backbone from the stem peptides destroys inflammatory activity (faded image).

inflammatory response against *S. pneumoniae*. The involvement of TLR9, a receptor that is activated by CpG repeats in bacterial DNA, has only been indirectly studied using TLR2/TLR4/TLR9 triple deficient mice. Infection of the triple deficient mice did not show any difference in bacterial load or survival compared to the TLR2/TLR4 double knockout mice, leading to the assumption that TLR9 plays only a small role in the detection of *S. pneumoniae* in the CNS [83].

Besides the TLR family of PRRs, another group of PRRs is involved in sensing of pneumococcal products: the family of NLRs that sense muramyl peptides from peptidoglycan. Among them, the cytosolic NOD2 receptor has been shown to be important for the initiation and progression of a lethal inflammatory response during pneumococcal meningitis, since NOD2-deficient mice displayed less inflammation, demyelination, and astrogliosis [84]. Some NLRs activate the assembly of large caspase-1-activating complexes called inflammasomes after recognition of danger-associated molecular patterns. Inflammasomes are intracellular macromolecular complexes consisting of an inflammasome sensor molecule, the adaptor ASC, and caspase-1. The assembly of this complex leads to activation of the highly proinflammatory cytokines IL-1 and IL-18 [85]. NLPR3 is the best-characterized inflammasome; it consists of the sensor NLPR3, ASC, and caspase-1. Activation of this inflammasome depends on pneumolysin, which causes the release of ATP from host cells. Loss of ATP leads to secretion of cathepsin B, which in turn activates the inflammasome [86]. Two recent studies using NLPR3- and ASC-deficient mice showed that these two proteins play an important role in regulation of the inflammatory response and brain damage during pneumococcal meningitis, since knockout animals showed decreased disease severity compared to wild-type animals [86,87].

Recognition of *S. pneumoniae* by PRRs on microglia leads to the induction of a strong inflammatory response, resulting in the production of pro-inflammatory cytokines and chemokines. Cytokines are produced by a variety of cells including activated astrocytes, microglia, monocytes, neurons, and endothelial cells within the CNS; they act as key regulators of the immune response by switching on and off entire cascades of events. A recent study revealed that activation of microglia and astrocytes was detectable just 1 h after infection with *S. pneumoniae*, leading to the conclusion that the innate immune system of the CNS responds to the bacteria even when they are in the blood and not yet within the brain [63]. Animal studies investigating the development of pneumococcal meningitis during sepsis in mice revealed that neuronal damage in the hippocampus occurred even prior to the entry of the bacteria into the CNS [88]. Sepsis-associated neuronal damage was also observed when cell wall was injected intravenously into wild-type mice. Damage was reduced in TLR2- or NOD2-knockout mice or mice overexpressing anti-inflammatory IL-10 in macrophages, leading to the conclusion that neuronal damage occurs across the BBB from intravascular inflammation, even in the absence of meningitis [88]. It is envisioned that generation of cytokines in the vascular space activates microglia and astrocytes in the CNS. Although the precise pathway is unknown, the cytokines could activate the cerebral endothelium, which in turn activates CNS cells, or some inflammatory cytokines and cells could traverse the barrier and directly act on CNS defenses.

In the CSF of patients with pneumococcal meningitis, high levels of the pro-inflammatory cytokines including TNF-α, IL-1 beta, IFN-γ, IL-2, IL-6, IL-12, as well as the anti-inflammatory cytokines IL-10 and TGF-β, are detectable [89]. Not only living bacteria but also bacterial components such as cell wall are known to induce the production of pro-inflammatory cytokines in the CSF. Peptidoglycan and teichoic acid, but not the capsular polysaccharide of the pneumococcus, have been identified as causing inflammation and

inciting the entire symptom complex of disease [90], including pneumococcal meningitis, pneumonia, and otitis media [91]. This observation is important, especially in the clinical setting, since antibiotic treatment causes rapid lysis and thereby release of a burst of pro-inflammatory cell-wall fragments that transiently exacerbates symptoms of meningitis even as bacteria are killed [90,91].

Chemokines, a subgroup of cytokines with chemotactic properties, are secreted by infiltrating leukocytes and tissue cells in the presence of cytokines such as IL-1, TNF-α, and IFN-γ, endotoxins and phorbol esters; they function to generate a gradient that navigates effector lymphocytes to the site of infection. During pneumococcal meningitis, many chemoattractants have been shown to be elevated in the CSF of patients, such as CXCL8 (IL-8), CCL3 (MIP-1α), and CCL2 (MCP-1) [89].

After the bacterial pathogen is recognized and defense mechanisms in the CNS are activated, the recruitment of highly activated leukocytes, mostly polymorphonuclear leukocytes, is the next important step during pneumococcal meningitis. Therefore, leukocytes have to be attracted by chemokines to the BBB, where they cross this barrier in a process that involves interaction with several surface adhesion molecules on the surface of the vascular endothelium. Leukocytes roll along the endothelium when endothelial P-selectin and E-selectin interact with leukocyte CD15 sialyl Lewis-X carbohydrate moieties, or when leukocyte L-selectin binds endothelial carbohydrate moieties. To arrest the motion of the leukocyte, the leukocyte integrin $\alpha M\beta 2$ CD18/CD11b (Mac-1) binds endothelial intercellular adhesion molecule 1 (ICAM-1, CD 54). Once stopped, the leukocytes diapedese through tight junctions between BBB endothelial cells, leading to CSF pleocytosis [92]. In animal studies where leukocyte invasion was blocked using intravascular antibodies against either ICAM-1 or CD-18, antibody-treated animals showed decreased leukocyte numbers within the brain, reduced inflammation, and less tissue damage [93,94]. Further, fucoidin, a polysaccharide known to inhibit L-selectin, has been shown to inhibit pleocytosis and to attenuate the inflammatory response to *S. pneumonia* [95].

The rapid migration of leukocytes from the blood to the site of infection within the brain comes at a cost. Invading leukocytes generate toxic agents such as reactive oxygen species, reactive nitrogen species, and myeloperoxidase, leading to bystander damage of cortical and subcortical structures [14]. Activated microglia within the brain have been shown to produce large amounts of pro-inflammatory cytokines as well as reactive oxygen and nitrogen species that have both neuroprotective roles by killing pathogens and neurotoxic roles by causing tissue damage in murine meningitis models [14]. In addition, activated leukocytes produce matrix metalloproteases (MMPs), Zn^{2+}- and Ca^{2+}-dependent endopeptidases that play a crucial role in the degradation and remodeling of extracellular matrix components such as type IV collagen, fibronectin, laminin, and proteoglycans. They have been shown to contribute to inflammation by releasing and activating soluble cytokines and their receptors [14]. MMPs are produced by a variety of cells including neutrophils, glial cells, neurons, vascular smooth muscle cells, and endothelial cells; they have been demonstrated to play a major role in BBB breakdown and leukocyte pleocytosis during bacterial meningitis [14,96]. MMP-8 and MMP-9 have been shown to be present at high levels in the CSF of children with bacterial meningitis, and high MMP-9 levels are a risk factor for development of neurological sequelae [97]. In a rat model of meningococcal meningitis, MMP inhibition significantly reduced BBB disruption but failed to reduce leukocyte pleocytosis [98]. Two recent studies using inhibitors against several MMPs (e.g., MMP-1, 8, 13, TACE) revealed that the drugs successfully reduced proinflammatory cytokine production and mortality rates and that inhibition of MMP-2 and MMP-9 prevented

cognitive impairment following pneumococcal meningitis [99,100].

As a strong inflammatory response progresses, vasogenic brain edema, increased intracranial pressure, cerebral herniation, and reduced cerebral blood flow may occur [91]. Impairment of neuronal function can lead to increased release of excitatory amino acids and thus to metabolic disturbances and cellular edema. Cerebral ischemia and hypo-/hypertension are provoked by vasoconstriction and loss of autoregulation of cerebral bloodflow decreases CNS perfusion.

To reduce the excessive inflammatory response within the brain of patients suffering from pneumococcal meningitis, antibiotic treatment is generally accompanied by anti-inflammatory therapy. Administration of a steroid or non-steroidal anti-inflammatory agent during the most intense cytolysis as bacteria die has strong beneficial effects in animal models and has shown recognizable benefits in children [101–103].

NEURONAL INJURY

A study in Great Britain evaluated over 1500 children at the age of 5 who had meningitis in their first year of life and reported that 25.8% had hearing problems, 15.6% speech or language problems, 13.7% ocular or visual disorders, 11.9% behavioral disorders, 8.1% neuromotor disabilities, 7.5% learning disabilities, and 7.3% seizure disorders [104]. A retrospective study on the incidence and spectrum of pneumococcal meningitis in adults revealed that almost 75% of the patients developed meningitis-associated intracranial complications such as seizures, diffuse brain swelling, cerebrovascular complications (arterial and venous), brain edema, hydrocephalus, and hearing loss [105].

The hippocampus, especially the dentate gyrus, is the most prominent region where neuronal damage occurs during meningitis. Although bacteria and leukocytes are not physically in contact with this region of the brain, the hippocampus is surrounded by interstitial fluid that allows diffusion of bacterial toxins and immune system components into the parenchyma. In rabbit and infant mouse models of meningitis, apoptotic neuronal damage occurs in the dentate gyrus and can also be found in 70% of human autopsy cases [106]. Necrosis of neurons occurs mainly in the neocortex and the CA1–CA4 sectors of the hippocampus. During bacterial meningitis, several factors such as hypoxia, neurotoxic bacterial products, and mediators of the host response combine to cause neuronal damage that can be necrotic and apoptotic (Figure 23.3). *S. pneumoniae* has been shown to cause apoptosis of neurons and endothelial cells in two temporally and mechanistically distinct waves [107]. During the early phase of meningitis, pneumococcal toxins such as pneumolysin and H_2O_2 lead to an increase in reactive oxygen species and calcium flux, resulting in mitochondrial dysfunction and release of apoptosis-inducing factor (AIF). AIF then induces the subsequent caspase-independent cell death of neurons [108]. The second wave of neuronal damage occurs approximately 24 h after infection and involves cell-wall/TLR2-mediated induction of caspase-dependent apoptosis [109]. After TLR2 engagement, p53 and ATM act together to initiate the release of cytochrome c, which is needed to promote the oligomerization of Apaf-1 into an apoptosome. The apoptosome then recruits and activates caspase-9, which in turn activates caspase-3. Treatment of rabbits with the broad-spectrum caspase inhibitor z-VAD-fmk reduces caspase-dependent apoptosis in the hippocampus but does not resolve all damage as it does not control caspase-independent cell death [109]. Examination of

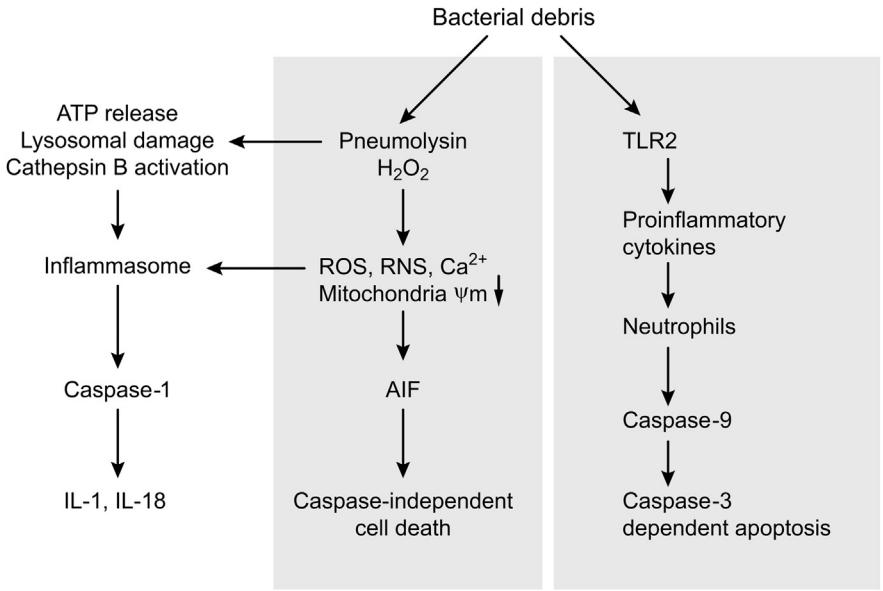

FIGURE 23.3 **Bacterial components induce neuronal damage.** During the course of infection, several mechanisms lead to neuronal injury. Release of pneumolysin and hydrogen peroxide by the bacteria leads to the production of reactive oxygen and nitrogen species (ROS, RNS), resulting in activation of AIF and subsequent caspase-independent cell death. Recognition of bacterial debris by TLR2 prompts the production of pro-inflammatory cytokines, resulting in neutrophil recruitment to the brain and neuronal death due to activation of caspase-3-dependent apoptosis. Pneumolysin also activates the NLPR3 inflammasome, causing the release of the pro-inflammatory cytokines IL-1 and IL-18.

other mechanisms to control cell death included a study of p53/Puma-mediated apoptosis during pneumococcal infection [110]. Although the absence of pro-apoptotic mediators in knockout mice attenuated brain damage, survival was not always increased. This revealed that in knockout mice, global inhibition of apoptosis protected the brain, but less apoptosis of neutrophils prolonged their presence in sites of inflammation, leading to abscess formation and failure to clear the infection.

CONCLUSION

The pneumococcus is the prototypical example of an invasive extracellular pathogen. It causes very significant mortality worldwide, a problem only partially addressed by current vaccines. The basic cell biology of the invasive process for pneumococcus is now known to apply to virtually all respiratory pathogens (Figure 23.4). Pneumococci translocate from the lung to the blood by innate invasion, a process by which cell wall-bound phosphorylcholine binds to the chemokine receptor PAFr. Activation of PAFr leads to endocytosis and translocation of bacterial cargo in a vacuole across epithelial or endothelial barriers. As the bacteria circulate in the blood, adherence to the BBB is mediated by the binding of CbpA to LR. In turn, this up-regulates PAFr and enables translocation. Once in peripheral organs, inflammation is induced by cell wall recognition by TLR2 and cytolysis by the toxin pneumolysin. A characteristically intense inflammatory response involves a brisk influx of

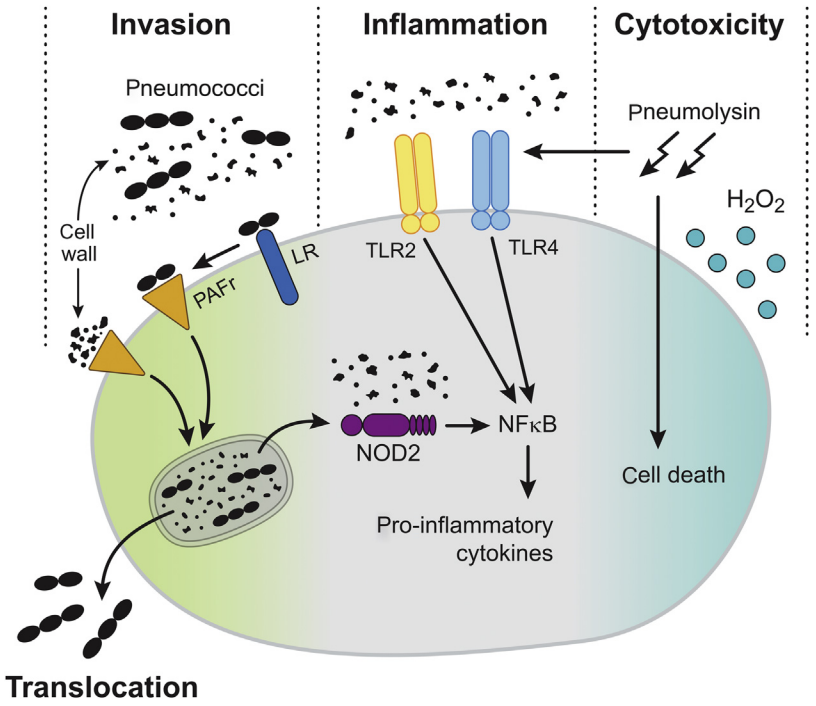

FIGURE 23.4 **Hallmarks of pneumococcal interactions with host cells.** ***Invasion:*** *S. pneumoniae* CbpA binds to the LR to initiate adherence. Then the PCho on the bacterial cell wall binds to PAFr and induces endocytosis of the bacteria or cell-wall fragments into a vacuole and translocation across the cellular barrier. ***Inflammation:*** Recognition of pneumococcal cell-wall components by the pattern recognition receptors TLR2 and NOD2 results in activation of the transcription factor NF-κB and subsequent release of pro-inflammatory cytokines. This is amplified by TLR4 recognition of pneumolysin. ***Cytotoxicity:*** Release of hydrogen peroxide (H_2O_2) and pneumolysin by the bacteria causes death of the host cell by pore formation and oxidative damage.

neutrophils that not only kills bacteria but also exacerbates host cell damage by necrosis and apoptosis. Interventions targeting the steps in invasion serve as current targets for efforts to design vaccines and adjuvants to antibiotics that would improve the breadth of prevention and outcome.

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