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Streptococcus pneumoniae: virulence factors and variation

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Abstract

Streptococcus pneumoniae is a major pathogen of humans, causing diseases such as pneumonia and meningitis. The organism produces several virulence factors that are involved in the disease process. The molecular basis of the action of some of these virulence factors is being elucidated. The advent of whole genome sequencing combined with biological studies has demonstrated that genome variation is important in the ability of pneumococci to interact with the host. This review discusses the biological activity of several pneumococcal virulence factors, and describes how genome variation may impact on the ability of pneumococci to cause disease.

Keywords: Disease, genome, infection, pneumococcus, review, virulence
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Introduction

Streptococcus pneumoniae (the pneumococcus) colonizes the human nasopharynx and can also cause diseases, including otitis media, pneumonia, bacteraemia, and meningitis. The organism produces a range of colonization and virulence factors, including the polysaccharide capsule, surface proteins and enzymes, and the toxin pneumolysin (PLY). The role played by virulence factors in disease has been partly elucidated. It is clear from analysis of the increasing number of whole genome sequences that strains of pneumococci differ in their ability to produce virulence factors. Understanding the role played by virulence factors allows an understanding of the pathogenesis of infection and can be used to identify possible points of intervention for treatment or vaccination. Understanding the distribution of virulence factors among strains may also allow associations to be made between these factors and the ability to cause different types of disease. There are many pneumococcal virulence factors, and this review will focus on the capsule, the toxin, and surface-associated proteins.

Capsule

The polysaccharide capsule is probably the most important virulence factor of the pneumococcus. The role of the capsule in virulence stems from its antiphagocytic activity [1]. Antibody to cell wall constituents of the pneumococcus becomes attached to the surface of the organism, and in turn binds complement. The presence of the capsule prevents iC3b and the Fc of immunoglobulins on the bacterial cell surface from interacting with their receptors on the surface of phagocytic cells, with the result that the organisms remain extracellular [2]. The capsule is also crucial for colonization, prevents mechanical removal by mucus [3], and can also restrict autolysis and reduce exposure to antibiotics [4].

PLY

PLY, probably the most widely studied pneumococcal protein virulence factor, belongs to the family of pore-forming toxins produced by more than 20 species of Gram-positive bacteria, originally described as haemolysins. PLY is a 53-kDa protein made by almost all clinical isolates of the pneumococcus and expressed during the late log phase of growth [5]. Although thought to be released only when cells undergo autolysis [6], PLY can be released independently of the major autolysin [7]. Initially, the toxin binds to membrane cholesterol; it then forms large pores (up to 30 nm in diameter) by the oligomerization of up to 50 toxin monomers [8]. PLY also mediates other effects at sublytic concentrations, including

activation of the classic complement pathway, despite the absence of specific antibody [9].

The role of PLY in the pathogenesis of infection has been studied in animal models, using mutants of the pneumococcus in which the gene for the toxin has been interrupted or deleted. Early experiments with PLY$^-$ strains showed that lack of the toxin reduces the virulence of the organism in both intranasal and systemic routes of infection [10]. When instilled into the mouse lung, PLY$^-$ pneumococci induce much less inflammation [11,12]. Cell recruitment in the lung in response to respiratory tract infections with PLY$^-$ mutants is delayed and reduced in comparison with infection with wild-type bacteria [12]. Neutrophil responses were the most affected, and the redistribution of T-lymphocytes and B-lymphocytes in and around inflamed bronchioles was also delayed and reduced. After intranasal infection, lack of PLY also causes reduced numbers of bacteria in the nasopharynx, trachea, and lungs [13], although Rubins *et al.* [14] showed that PLY has no role in colonization. At lower concentrations, the toxin has effects on the immune system, including induction of the proinflammatory response, and production of reactive oxygen intermediates and other mediators (reviewed in [15]). These effects are potentially mediated by the ability of PLY to interact with the lipopolysaccharide receptor, Toll-like receptor 4 (TLR4). Macrophages from TLR4-deficient mice do not mount an inflammatory response to PLY. Moreover, these TLR4-deficient mice are more easily colonized and show higher susceptibility to pneumococcal sepsis [16]. This increased susceptibility is dependent on the presence of PLY. In addition, a mutant version of PLY in which cytotoxic and complement-activating ability have been removed (PdT) is still active in these assays, suggesting that the TLR4 interaction is distinct from these activities [16]. It has also been demonstrated, using a solid-phase assay, that PLY physically interacts with TLR4 [17]. Protection from colonization with the pneumococcus has been shown to require both TLR4 [16] and interleukin (IL)-17 [18], and both effects appear to be dependent on PLY. IL-17 is a cytokine produced mainly from a novel subset of T-cells termed Th17 cells, and plays an important role in host defence against a number of pathogens (reviewed in [19]). It acts to augment neutrophil production and emigration to sites of infection [20]. Th17 cell development requires the proinflammatory cytokines IL-6 and IL-1β as well as transforming growth factor-β [21–23]. PLY has been demonstrated to stimulate IL-17 production from splenocytes of mice immunized with a combination of pneumococcal proteins [24], but it is not clear whether this is a direct effect or requires prior stimulation or co-stimulation with proinflammatory cytokines, as has been shown for pertussis immunization [25].

The role of PLY in infections outside of the respiratory tract is more controversial. PLY appears not to play a role in the inflammation associated with otitis media in the chinchilla model [26]. Intracisternal injection of PLY into rabbit brain causes a rapid inflammatory response, but in this study there was no evidence for a contribution of the toxin to the inflammation caused by the whole organism, as determined by a comparison of wild-type and PLY$^-$ pneumococci [27]. The lack of effect of PLY on inflammation of the brain has been confirmed in a mouse model [28]. Similar results with regard to inflammation were seen in a guinea pig model of infection [29]. Use of wild-type and PLY$^-$ organisms in this system showed there to be no difference in the levels of inflammation induced. The PLY$^-$ organism does induce less protein influx into the cerebrospinal fluid (CSF), less ultrastructural damage to the cochlea of infected animals, and less associated hearing loss [29].

PLY has been shown to play a key role in brain damage induced during pneumococcal meningitis in the rabbit model of infection [30]. Also in the rabbit model of pneumococcal meningitis, the levels of PLY released have been measured, and shown to be about 20 ng/mL CSF [31]. This is similar to the level of toxin found in the CSF of patients suffering from pneumococcal meningitis, which ranges from about 1 to 180 ng/mL CSF. The level of toxin released into the CSF during antibiotic treatment is dependent on the type of antibiotic used. Much more PLY is found in the CSF if ceftriaxone is used to treat the infection in rabbits than if a non-bacteriolytic antibody, such as rifampin, is used [31]. This emphasizes the importance of bacterial lysis in the release of PLY, and has important consequences when considering the treatment of pneumococcal meningitis.

The two main activities of PLY are its lytic activity and its ability to activate complement, and regions of the protein responsible for these activities have been identified [32]. The use of mutants that vary in these activities has shown that the contributions of the two activities vary according to the type of infection. The region of the toxin that interacts with TLR4 remains to be defined.

In summary, PLY plays several roles in infection. The toxin appears to have no role in the inflammation associated with meningitis [27–29], but has a role in deafness associated with meningitis [28], and in bacteraemia [33] and pneumonia [10]. Where PLY does have a role, the contributions of complement activation and the lytic activities of the toxin to the disease process differ according to the type of infection. A comparison of strains with point mutations that abolish the complement activation and lytic activities of PLY with a toxin-negative mutant suggests that PLY has other activities

that contribute to the disease process. These activities await further characterization.

LPXTG-anchored Surface Proteins

Analysis of the pneumococcal genome sequence indicates the presence of some 17 putative LPXTG-anchored proteins, although this number may vary between strains [34]. The LPXTG motif is normally near the C-terminus of the protein, but in some of the pneumococcal proteins it is near the N-terminus. The N-terminal sequence may provide a mechanism for the surface location of proteases [35]. The C-terminal consensus is a recognition sequence for sortase enzymes, which recognize LPXTG and anchor the protein to the cell surface by covalent linkage of the threonine of the motif to the pentaglycine linkage of the peptidoglycan of the cell wall. The roles of several of these LPXTG proteins in virulence have been investigated. These include hyaluronidase, neuraminidase, and serine protease PrtA.

Hyaluronidase breaks down the hyaluronic acid component of mammalian connective tissue and extracellular matrix, and is secreted by 99% of clinical isolates [36]. The degradation of hyaluronic acid may aid bacterial spread and colonization, as demonstrated for other microorganisms [37]. In addition, hyaluronidase may potentiate pulmonary inflammation during pneumococcal pneumonia by complex interactions with proinflammatory cytokines and chemokines. Tumour necrosis factor-α and IL-1β are able to induce the production of hyaluronic acid by fibroblasts [38]. Hyaluronic acid can promote further cytokine secretion by binding to CD44 on host cells.

Neuraminidase cleaves *N*-acetylneuraminic acid from glycolipids, lipoproteins and oligosaccharides on cell surfaces and in body fluids [39]. This may cause direct damage to the host or it may unmask potential binding sites for the organism. Loss of sialic acid as a result of neuraminidase activity accompanies the advance of pneumococci up the Eustachian tube to the middle ear [40]. Pneumococci have genes encoding several enzymes with neuraminidase activity, and the role of neuraminidase A (NanA) in pathogenesis has been investigated. It plays a role in colonization and the development of otitis media in the chinchilla model [41], but does not play a role in meningitis-associated deafness [29]. NanA plays an important role in biofilm formation, and sialic acid released by the action of NanA may be an important signal controlling pneumococcal virulence [42,43].

The presence of the serine protease *prtA* gene was originally determined in a screen of convalescent patient sera [44], and was subsequently confirmed in all pneumococcal isolates tested [45]. This screening for *prtA*, which encodes a member of the subtilase family, also detected considerable nucleotide sequence variation resulting in amino acid changes across the central domain of the protein, but not affecting the conserved catalytic triad. In a murine model of disease, a PrtA-negative mutant of D39 was attenuated, and vaccination provided protection from intraperitoneal challenge [45]. Similar antibody levels are detected in healthy control individuals and in patients with invasive disease, although there is a trend towards increased response in early convalescence [46]. Although the exact role of PrtA is unknown, *PrtA* expression is co-regulated with a number of other virulence genes, including the pilus locus and PLY genes, by the transcription factor pneumococcal surface antigen (Psa)R [47]. Regulation of *PrtA* expression by PsaR has also been demonstrated to be oppositely repressed and stimulated by manganese or zinc [48].

The Pneumococcal Pilus

Pili were observed on the surface of pneumococci in 2006 [49]. The genes encoding pilus production, the *rlr* locus or pilus islet 1 (PI-1), code for three structural proteins and three sortase enzymes responsible for linking structural subunits. The pilus is involved in virulence, and mediates binding of pneumococci to cells. It also stimulates proinflammatory cytokine production [49]. A second pilus type has been identified in pneumococci, and is involved in adherence of pneumococci to epithelial cells [50]. Not all pneumococci have pili, but some strains are capable of expressing both types [50].

Lipoproteins

In a screen for pneumococcal adhesins, Cundell *et al.* [51] identified mutations in genes identified as those encoding peptide permeases, which reduce adherence to resting lung or endothelial cells. A homologue of these permeases, PsaA, has been ascribed a role in pneumococcal virulence [52]. PsaA is part of an ABC transporter in which PsaA is the substrate-binding lipoprotein, PsaB is the ATP-binding protein, and PsaC is the permease [53]. The complex transports manganese ions. Mutations in *psaA* cause decreased adhesion to cells, decreased virulence, and increased sensitivity to oxidative stress [54–56]. The receptor for PsaA is E-cadherin [57]. Two lipoprotein peptidyl prolyl isomerases, SlrA and PpmA, have been shown to play a role in virulence and colonization [58,59]. Pia and Piu are lipoproteins involved in iron uptake, and these are also involved in virulence [60,61].

Choline-Binding Proteins (CBPs)

CBPs are anchored to the cell surface via interaction of repeat domains with choline present in the pneumococcal cell wall. The number of CBPs present depends on the strain of pneumococcus. There are ten in strain R6 and 15 in TIGR4 [34,62]. Several CBPs have been implicated in virulence. These include four cell wall hydrolytic enzymes (LytA, LytB, LytC, and CbpE). LytA plays a role in virulence through release of highly inflammatory cell wall degradation products and also release of PLY from the cytoplasm [63,64]. LytB, LytC and CbpE are involved in nasopharyngeal colonization [65]. Pneumococcal surface protein A (PspA) is an important virulence factor, as mutants lacking this protein have reduced virulence in a sepsis model [66]. PspA interferes with the complement system [67] and also binds to lactoferrin [68]. Lactoferrin plays an important part in innate immunity. Pneumococcal surface protein C (PspC) is a multifunctional virulence factor. Its contribution to virulence varies between strains [69]. PspC acts as an adhesin, binding to the polymeric immunoglobulin receptor [70,71], and binds the complement regulatory protein factor H [72], to provide resistance to complement.

Other Surface Proteins

Several surface proteins have been identified that do not contain recognized anchor motifs (choline-binding, LPXTG, or lipoprotein). Such proteins have also been reported in other Gram-positive bacteria [73]. This family includes pneumococcal adherence and virulence factor A (PavA), two glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase and enolase, and the pneumococcal histidine triad protein (Pht) family. PavA is a crucial virulence factor, and mutants lacking PavA are attenuated in models of sepsis and meningitis [74,75]. PavA binds to fibronectin and mediates attachment to endothelial cells [75]. Glyceraldehyde-3-phosphate dehydrogenase and enolase are plasminogen-binding proteins [76,77]. Binding of plasminogen has been shown to facilitate transmigration of pneumococci through the basement membrane [78]. The Pht family was discovered through the ability of its members to provide protection against infection when used as vaccines [79,80]. The family consists of four proteins: PhtA, PhtB, PhtD, and PhtE. The proteins contain a conserved motif, HxxHHxH, that is believed to be involved in binding of zinc ions. Expression of the Pht genes is regulated by zinc [81], and crystallographic investigations have shown zinc bound in the histidine motif of PhtA [82]. Mutagenesis of all four proteins is required to attenuate the organism [81]. The Phts reduce binding of complement to pneumococci by recruiting the complement regulator factor H [81].

Gene and Genome Variation

Genes or proteins involved in the pathogenesis and interaction with the host immune system have been identified in different ways using available technologies. From signature-tagged mutagenesis (STM) screens [83–85] and through microarray analysis [86,87], various individual genes or loci with roles in virulence have been identified. STM identifies bacterial genes involved in pathogenesis. Interestingly, of the more than 300 genes identified in the three STM studies, only a small proportion are common to all three studies. This suggests that different virulence genes may be involved in different strains of the pneumococcus. The pneumococcus is naturally transformable, and can take up and incorporate DNA from closely related species [88]. For example, genetic variation resulting in resistance to penicillin is due to acquisition of fragments of the genes encoding penicillin-binding proteins from *Streptococcus mitis*, reducing the affinity of these proteins for the drug [89]. Understanding genetic diversity is important in understanding the virulence of pneumococcal strains.

The advent of whole genome sequencing has furthered the discovery of a number of pathogenicity islands, and larger-scale comparative genomics is further illuminating the complexity of this organism. The genome of the pneumococcus consists of 2–2.2 million base pairs, and contains upwards of 2000 genes, depending on the strain. Genetic heterogeneity ranges from single-nucleotide polymorphisms to the presence or absence of large genetic islands coding for virulence factors such as the capsule and pili. There are more than 90 types of capsule, and these can be exchanged by genetic recombination [90]. This type of variation has important implications for the design of vaccines based on capsular antigens. Variation in the presence of regions of diversity or pathogenicity islands across a range of isolates has been documented [87,91]. These include the pili loci for the two types of pneumocccal pilus, described above, PI-1 [49] and the related pilus islet 2 [50], the iron uptake locus *piaABCD* [92], and several other genetic loci. The PI-1 locus is detected in around 30% of isolates, segregating with clonal type but not serotype [93]. Through genetic diversity of the islet, this locus itself separates into three clades [94]. Bagnoli *et al.* have also reported the independent coexistence and

expression of both pilus islets in strains from the multilocus sequence typing clonal complex ST271. Although not as efficient in adherence to host cells, the second pilus locus is associated with emerging serotypes. Variation in pilus expression can alter the ability of pneumococci to bind to cells and induce inflammation [49].

Pneumococcal phages have also been described from comparative studies of newly sequenced genomes [95,96]. Although these appear to segregate with serotype, the role of phage genes in either the bacterial life cycle or pneumococcal disease process has yet to be determined.

Individual genes can also vary at the sequence level. For example, the known virulence factor PspC exists as a large number of allelic forms. Analysis of *pspC* sequences from 43 strains showed that the derived protein sequence had the same domain organization, but each one had a unique sequence that could be divided into subgroups [97]. The majority of PspC alleles contain a C-terminal cell wall choline-binding motif (see above), but 17 of the allelic variants contain a cell wall anchor LPTXG motif instead. The contribution of PspC to virulence is also known to differ between strains [69]. A recently identified allele, *pspC4.4*, contributes further to the role of the PspC protein in avoidance of host complement by binding the inhibitor C4-binding protein [98].

PLY is a major virulence factor of the pneumococcus (see above). The PLY gene also exists in a number of allelic forms [99]. One clinical strain was identified that was unable to produce the toxin, owing to the insertion of a transposon into the gene. Sequence variation in one of these alleles (allele 5) results in the production of a toxin with no haemolytic acivity [100]. Interestingly, strains producing this form of the toxin (serotype 1, sequence type 306) are increasing in prevalence in Europe [100]. Expression of allele 5 PLY is also associated with strains of serotype 1 and serotype 8 pneumococci that are associated with the ability to cause disease outbreaks [99].

Differences in gene expression pathways may also be involved in changes in pathogenicity. The pneumococcus has 13 two-component signal transduction systems that are involved in regulating gene expression [101]. Mutation of one of these systems was shown to attenuate the TIGR4 strain of the pneumococcus but not two other strains (D39 and 0100993) [102]. The mutation in TIGR4 was shown to alter the expression of PsaA and change resistance to oxidative stress. PsaA is a known virulence factor, and is a lipoprotein that is part of a manganese transporter as well as being an adhesin (see above). The change in PsaA expression may result in reduced ability to acquire manganese, which, in turn, affects the ability to resist oxidative stress. The change in PsaA expression was not seen in the other two strains [102].

The above examples indicate how genetic variation at different levels, from single-nucleotide polymorphism to genetic island, can contribute to the disease-causing ability of pneumococci.

Following the publication of a number of pneumococcal genomes [34,62,103,104] and comparative genomic studies [105], a core genome of 1400 genes has been calculated [105]. As a typical pneumococcal strain such as TIGR4 has approximately 2300 genes, such a huge capacity for genomic diversity allows for a high degree of flexibility. This adaptability may facilitate responses to evolutionary pressures, which might include changes in the host environment, such as in different stages of disease progression, or contribute to the development of antibiotic resistance. Integrative and conjugative elements (ICEs) constitute another mechanism by which the pneumococcus can share a larger gene pool [104]. ICE elements have been found to carry genes for antibiotic resistance, possible repair of peroxidative damage to DNA, and the SOS response. Further evidence for the role of ICEs in genome diversity comes from a study in group A streptococcus, where genomic analysis of four new strains of group A streptococcus showed that 41% of new genes identified were carried on ICEs [106].

One-third of the pneumococcal genome is composed of hypothetical proteins that have not yet been ascribed a function. The role of some of these hypothetical genes in virulence is slowly being elucidated, as is the multifunctionality of proteins not previously directly associated with virulence. However, the combinatorial and complementary nature of the assortment of genes required for virulence or colonization in any given pneumococcal isolate is proving to be highly complex. Continuation of the genome sequencing approach and comparison across larger numbers of strains from varying serotypes and lineages will be required to truly define the pneumococcal virulome.

Transparency Declaration

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References

1. Jonsson S, Musher DM, Chapman A, Goree A, Lawrence EC. Phagocytosis and killing of common bacterial pathogens of the lung by human alveolar macrophages. *J Infect Dis* 1985; 152: 4–13.
2. Musher DM. Infections caused by *Streptococcus pneumoniae*: clinical spectrum, pathogenesis, immunity, and treatment. *Clin Infect Dis* 1992; 14: 801–809.
3. Nelson AL, Roche AM, Gould JM, Chim K, Ratner AJ, Weiser JN. Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. *Infect Immun* 2007; 75: 83–90.
4. Van der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet* 2009; 374: 1543–1556.
5. Benton K, Paton J, Briles D. Differences in virulence for mice among *Streptococcus pneumoniae* strains of capsular types 2, 3, 4, 5, and 6 are not attributable to differences in pneumolysin production. *Infect Immun* 1997; 65: 1237–1244.
6. Berry AM, Lock RA, Hansman D, Paton JC. Contribution of autolysin to virulence of *Streptococcus pneumoniae*. *Infect Immun* 1989; 57: 2324–2330.
7. Balachandran P, Hollingshead SK, Paton JC, Briles DE. The autolytic enzyme LytA of *Streptococcus pneumoniae* is not responsible for releasing pneumolysin. *J Bacteriol* 2001; 183: 3108–3116.
8. Morgan PJ, Hyman SC, Rowe AJ, Mitchell TJ, Andrew PW, Saibil HR. Subunit organisation and symmetry of pore-forming oligomeric pneumolysin. *FEBS Lett* 1995; 371: 77–80.
9. Mitchell TJ, Andrew PW, Saunders FK, Smith AN, Boulnois GJ. Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein. *Mol Microbiol* 1991; 5: 1883–1888.
10. Berry AM, Yother J, Briles DE, Hansman D, Paton JC. Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae*. *Infect Immun* 1989; 57: 2037–2042.
11. Canvin JR, Marvin AP, Sivakumaran M *et al.* The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type-2 pneumococcus. *J Infect Dis* 1995; 172: 119–123.
12. Kadioglu A, Gingles NA, Grattan K, Kerr A, Mitchell TJ, Andrew PW. Host cellular immune response to pneumococcal lung infection in mice. *Infect Immun* 2000; 68: 492–501.
13. Kadioglu A, Taylor S, Iannelli F, Pozzi G, Mitchell TJ, Andrew PW. Upper and lower respiratory tract infection by *Streptococcus pneumoniae* is affected by pneumolysin deficiency and differences in capsule type. *Infect Immun* 2002; 70: 2886–2890.
14. Rubins JB, Paddock AH, Charboneau D, Berry AM, Paton JC, Janoff EN. Pneumolysin in pneumococcal adherence and colonization. *Microb Pathog* 1998; 25: 337–342.
15. Marriott HM, Mitchell TJ, Dockrell DH. Pneumolysin: a double-edged sword during the host–pathogen interaction. *Curr Mol Med* 2008; 8: 497–509.
16. Malley R, Henneke P, Morse SC *et al.* Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci USA* 2003; 100: 1966–1971.
17. Srivastava A, Henneke P, Visintin A *et al.* The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease. *Infect Immun* 2005; 73: 6479–6487.
18. Malley R, Srivastava A, Lipsitch M *et al.* Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide. *Infect Immun* 2006; 74: 2187–2195.
19. Aujla SJ, Dubin PJ, Kolls JK. Interleukin-17 in pulmonary host defense. *Exp Lung Res* 2007; 33: 507–518.
20. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity* 2004; 21: 467–476.
21. Mangan PR, Harrington LE, O'Quinn DB *et al.* Transforming growth factor-β induces development of the T_H17 lineage. *Nature* 2006; 441: 231–234.
22. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006; 24: 179–189.
23. Bettelli E, Carrier Y, Gao W *et al.* Reciprocal developmental pathways for the generation of pathogenic effector T_H17 and regulatory T cells. *Nature* 2006; 441: 235–238.
24. Basset A, Thompson CM, Hollingshead SK *et al.* Antibody-independent, $CD4^+$ T-cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins. *Infect Immun* 2007; 75: 5460–5464.
25. Higgins SC, Jarnicki AG, Lavelle EC, Mills KH. TLR4 mediates vaccine-induced protective cellular immunity to *Bordetella pertussis*: role of IL-17-producing T cells. *J Immunol* 2006; 177: 7980–7989.
26. Sato K, Quartey M, Liebeler C, Le C, Giebink G. Roles of autolysin and pneumolysin in middle ear inflammation caused by a type 3 *Streptococcus pneumoniae* strain in the chinchilla otitis media model. *Infect Immun* 1996; 64: 1140–1145.
27. Friedland IR, Paris MM, Hickey S *et al.* The limited role of pneumolysin in the pathogenesis of pneumococcal meningitis. *J Infect Dis* 1995; 172: 805–809.
28. Wellmer A, Zysk G, Gerber J *et al.* Decreased virulence of a pneumolysin-deficient strain of *Streptococcus pneumoniae* in murine meningitis. *Infect Immun* 2002; 70: 6504–6508.
29. Winter AJ, Comis SD, Osborne MP *et al.* A role for pneumolysin but not neuraminidase in the hearing loss and cochlear damage induced by experimental pneumococcal meningitis in guinea pigs. *Infect Immun* 1997; 65: 4411–4418.
30. Braun JS, Sublett JE, Freyer D *et al.* Pneumococcal pneumolysin and H_2O_2 mediate brain cell apoptosis during meningitis. *J Clin Invest* 2002; 109: 19–27.
31. Spreer A, Kerstan H, Bottcher T *et al.* Reduced release of pneumolysin by *Streptococcus pneumoniae* in vitro and in vivo after treatment with nonbacteriolytic antibiotics in comparison to ceftriaxone. *Antimicrob Agents Chemother* 2003; 47: 2649–2654.
32. Boulnois GJ, Paton JC, Mitchell TJ, Andrew PW. Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae*. *Mol Microbiol* 1991; 5: 2611–2616.
33. Benton K, Everson M, Briles D. A pneumolysin-negative mutant of *Streptococcus pneumoniae* causes chronic bacteremia rather than acute sepsis in mice. *Infect Immun* 1995; 63: 448–455.
34. Tettelin H, Nelson KE, Paulsen IT *et al.* Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. *Science* 2001; 293: 498–506.
35. Bender MH, Weiser JN. The atypical amino-terminal LPNTG-containing domain of the pneumococcal human IgA1-specific protease is required for proper enzyme localization and function. *Mol Microbiol* 2006; 61: 526–543.
36. Humphrey JH. Hyaluronidase production by pneumococci. *J Pathol Bacteriol* 1948; 55: 273–275.
37. Fitzgerald TJ, Repesh LA. The hyaluronidase associated with *Treponema pallidum* facilitates treponemal dissemination. *Infect Immun* 1987; 55: 1023–1028.

38. Irwin CR, Schor SL, Ferguson MW. Effects of cytokines on gingival fibroblasts in vitro are modulated by the extracellular matrix. *J Periodontal Res* 1994; 29: 309–317.
39. Camara M, Boulnois GJ, Andrew PW, Mitchell TJ. A neuraminidase from *Streptococcus pneumoniae* has the features of a surface protein. *Infect Immun* 1994; 62: 3688–3695.
40. Linder TE, Daniels RL, Lim DJ, DeMaria TF. Effect of intranasal inoculation of *Streptococcus pneumoniae* on the structure of the surface carbohydrates of the chinchilla eustachian tube and middle ear mucosa. *Microb Pathog* 1994; 16: 435–441.
41. Tong HH, Blue LE, James MA, DeMaria TF. Evaluation of the virulence of a *Streptococcus pneumoniae* neuraminidase-deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. *Infect Immun* 2000; 68: 921–924.
42. Trappetti C, Kadioglu A, Carter M *et al.* Sialic acid: a preventable signal for pneumococcal biofilm formation, colonization, and invasion of the host. *J Infect Dis* 2009; 199: 1497–1505.
43. Parker D, Soong G, Planet P, Brower J, Ratner AJ, Prince A. The NanA neuraminidase of *Streptococcus pneumoniae* is involved in biofilm formation. *Infect Immun* 2009; 77: 3722–3730.
44. Zysk G, Bongaerts RJ, Ten Thoren E, Bethe G, Hakenbeck R, Heinz HP. Detection of 23 immunogenic pneumococcal proteins using convalescent-phase serum. *Infect Immun* 2000; 68: 3740–3743.
45. Bethe G, Nau R, Wellmer A *et al.* The cell wall-associated serine protease PrtA: a highly conserved virulence factor of *Streptococcus pneumoniae*. *FEMS Microbiol Lett* 2001; 205: 99–104.
46. Zysk G, Bethe G, Nau R *et al.* Immune response to capsular polysaccharide and surface proteins of *Streptococcus pneumoniae* in patients with invasive pneumococcal disease. *J Infect Dis* 2003; 187: 330–333.
47. Hendriksen WT, Bootsma HJ, Van Diepen A *et al.* Strain-specific impact of psaR of *Streptococcus pneumoniae* on global gene expression and virulence. *Microbiology* 2009; 155: 1569–1579.
48. Kloosterman TG, Witwicki RM, Van der Kooi-Pol MM, Bijlsma JJ, Kuipers OP. Opposite effects of Mn^{2+} and Zn^{2+} on PsaR-mediated expression of the virulence genes *pcpA*, *prtA*, and *psaBCA* of *Streptococcus pneumoniae*. *J Bacteriol* 2008; 190: 5382–5393.
49. Barocchi MA, Ries J, Zogaj X *et al.* A pneumococcal pilus influences virulence and host inflammatory responses. *Proc Natl Acad Sci USA* 2006; 103: 2857–2862.
50. Bagnoli F, Moschioni M, Donati C *et al.* A second pilus type in *Streptococcus pneumoniae* is prevalent in emerging serotypes and mediates adhesion to host cells. *J Bacteriol* 2008; 190: 5480–5492.
51. Cundell DR, Pearce BJ, Sandros J, Naughton AM, Masure HR. Peptide permeases from *Streptococcus pneumoniae* affect adherence to eukaryotic cells. *Infect Immun* 1995; 63: 2493–2498.
52. Talkington DF, Brown BG, Tharpe JA, Koenig A, Russell H. Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA). *Microb Pathog* 1996; 21: 17–22.
53. Dintilhac A, Alloing G, Granadel C, Claverys JP. Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. *Mol Microbiol* 1997; 25: 727–739.
54. Ogunniyi AD, Folland RL, Briles DE, Hollingshead SK, Paton JC. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect Immun* 2000; 68: 3028–3033.
55. Berry AM, Paton JC. Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*. *Infect Immun* 1996; 64: 5255–5262.
56. Marra A, Lawson S, Asundi JS, Brigham D, Hromockyj AE. In vivo characterization of the *psa* genes from *Streptococcus pneumoniae* in multiple models of infection. *Microbiology* 2002; 148: 1483–1491.
57. Anderton JM, Rajam G, Romero-Steiner S *et al.* E-cadherin is a receptor for the common protein pneumococcal surface adhesin A (PsaA) of Streptococcus pneumoniae. *Microb Pathog* 2007; 42: 225–236.
58. Hermans PW, Adrian PV, Albert C *et al.* The streptococcal lipoprotein rotamase A (SlrA) is a functional peptidyl-prolyl isomerase involved in pneumococcal colonization. *J Biol Chem* 2006; 281: 968–976.
59. Cron LE, Bootsma HJ, Noske N, Burghout P, Hammerschmidt S, Hermans PW. Surface-associated lipoprotein PpmA of *Streptococcus pneumoniae* is involved in colonization in a strain-specific manner. *Microbiology* 2009; 155: 2401–2410.
60. Brown JS, Gilliland SM, Holden DW. A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. *Mol Microbiol* 2001; 40: 572–585.
61. Jomaa M, Yuste J, Paton JC, Jones C, Dougan G, Brown JS. Antibodies to the iron uptake ABC transporter lipoproteins PiaA and PiuA promote opsonophagocytosis of *Streptococcus pneumoniae*. *Infect Immun* 2005; 73: 6852–6859.
62. Hoskins J, Alborn WE, Arnold J *et al.* Genome of the bacterium *Streptococcus pneumoniae* strain R6. *J Bacteriol* 2001; 183: 5709–5717.
63. Tuomanen E. Molecular and cellular biology of pneumococcal infection. *Curr Opin Microbiol* 1999; 2: 35–39.
64. Berry AM, Paton JC. Additive attenuation of virulence of *Streptococcus pneumoniae* by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. *Infect Immun* 2000; 68: 133–140.
65. Gosink KK, Mann ER, Guglielmo C, Tuomanen EI, Masure HR. Role of novel choline binding proteins in virulence of *Streptococcus pneumoniae*. *Infect Immun* 2000; 68: 5690–5695.
66. Mcdaniel LS, Yother J, Vijayakumar M, Mcgarry L, Guild WR, Briles DE. Use of insertional inactivation to facilitate studies of biological properties of pneumococcal surface protein-A (PspA). *J Exp Med* 1987; 165: 381–394.
67. Ren B, Szalai AJ, Thomas O, Hollingshead SK, Briles DE. Both family 1 and family 2 PspA proteins can inhibit complement deposition and confer virulence to a capsular serotype 3 strain of *Streptococcus pneumoniae*. *Infect Immun* 2003; 71: 75–85.
68. Hammerschmidt S, Bethe G, Remane PH, Chhatwal GS. Identification of pneumococcal surface protein A as a lactoferrin-binding protein of *Streptococcus pneumoniae*. *Infect Immun* 1999; 67: 1683–1687.
69. Kerr AR, Paterson GK, McCluskey J *et al.* The contribution of PspC to pneumococcal virulence varies between strains and is accomplished by both complement evasion and complement-independent mechanisms. *Infect Immun* 2006; 74: 5319–5324.
70. Zhang JR, Mostov KE, Lamm ME *et al.* The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. *Cell* 2000; 102: 827–837.
71. Hammerschmidt S, Talay SR, Brandtzaeg P, Chhatwal GS. SpsA, a novel pneumococcal surface protein with specific binding to secretory immunoglobulin A and secretory component. *Mol Microbiol* 1997; 25: 1113–1124.
72. Dave S, Brooks-Walter A, Pangburn MK, McDaniel LS. PspC, a pneumococcal surface protein, binds human factor H. *Infect Immun* 2001; 69: 3435–3437.
73. Chhatwal GS. Anchorless adhesins and invasins of gram-positive bacteria: a new class of virulence factors. *Trends Microbiol* 2002; 10: 205–208.
74. Holmes AR, McNab R, Millsap KW *et al.* The *pavA* gene of *Streptococcus pneumoniae* encodes a fibronectin-binding protein that is essential for virulence. *Mol Microbiol* 2001; 41: 1395–1408.
75. Pracht D, Elm C, Gerber J *et al.* PavA of *Streptococcus pneumoniae* modulates adherence, invasion, and meningeal inflammation. *Infect Immun* 2005; 73: 2680–2689.

76. Bergmann S, Rohde M, Hammerschmidt S. Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pneumoniae* is a surface-displayed plasminogen-binding protein. *Infect Immun* 2004; 72: 2416–2419.
77. Bergmann S, Rohde M, Preissner KT, Hammerschmidt S. The nine residue plasminogen-binding motif of the pneumococcal enolase is the major cofactor of plasmin-mediated degradation of extracellular matrix, dissolution of fibrin and transmigration. *Thromb Haemost* 2005; 94: 304–311.
78. Eberhard T, Kronvall G, Ullberg M. Surface bound plasmin promotes migration of *Streptococcus pneumoniae* through reconstituted basement membranes. *Microb Pathog* 1999; 26: 175–181.
79. Adamou JE, Heinrichs JH, Erwin AL *et al.* Identification and characterization of a novel family of pneumococcal proteins that are protective against sepsis. *Infect Immun* 2001; 69: 949–958.
80. Wizemann TM, Heinrichs JH, Adamou JE *et al.* Use of a whole genome approach to identify vaccine molecules affording protection against *Streptococcus pneumoniae* infection. *Infect Immun* 2001; 69: 1593–1598.
81. Ogunniyi AD, Grabowicz M, Mahdi LK *et al.* Pneumococcal histidine triad proteins are regulated by the Zn^{2+}-dependent repressor AdcR and inhibit complement deposition through the recruitment of complement factor H. *FASEB J* 2009; 23: 731–738.
82. Riboldi-Tunnicliffe A, Isaacs NW, Mitchell TJ. 1.2 Å crystal structure of the *S. pneumoniae* PhtA histidine triad domain: a novel zinc binding fold. *FEBS Lett* 2005; 579: 5353–5360.
83. Polissi A, Pontiggia A, Feger G *et al.* Large-scale identification of virulence genes from *Streptococcus pneumoniae*. *Infect Immun* 1998; 66: 5620–5629.
84. Lau GW, Haataja S, Lonetto M *et al.* A functional genomic analysis of type 3 *Streptococcus pneumoniae* virulence. *Mol Microbiol* 2001; 40: 555–571.
85. Hava D, Camilli A. Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. *Mol Microbiol* 2002; 45: 1389–1406.
86. Orihuela CJ, Radin JN, Sublett JE, Gao G, Kaushal D, Tuomanen EI. Microarray analysis of pneumococcal gene expression during invasive disease. *Infect Immun* 2004; 72: 5582–5596.
87. Silva NA, McCluskey J, Jefferies JMC *et al.* Genomic diversity between strains of the same serotype and multilocus sequence type among pneumococcal clinical isolates. *Infect Immun* 2006; 74: 3513–3518.
88. Johnsborg O, Havarstein LS. Regulation of natural genetic transformation and acquisition of transforming DNA in *Streptococcus pneumoniae*. *FEMS Microbiol Rev* 2009; 33: 627–642.
89. Dowson CG, Hutchison A, Brannigan JA *et al.* Horizontal transfer of penicillin-binding protein genes in penicillin resistant clinical isolates of *Streptococcus pneumoniae*. *Proc Natl Acad Sci USA* 1989; 86: 8842–8846.
90. Coffey TJ, Enright MC, Daniels M *et al.* Recombinational exchanges at the capsular polysaccharide biosynthetic locus lead to frequent serotype changes among natural isolates of *Streptococcus pneumoniae*. *Mol Microbiol* 1998; 27: 73–83.
91. Obert C, Sublett J, Kaushal D *et al.* Identification of a candidate *Streptococcus pneumoniae* core genome and regions of diversity correlated with invasive pneumococcal disease. *Infect Immun* 2006; 74: 4766–4777.
92. Brown JS, Gilliland SM, Spratt BG, Holden DW. A locus contained within a variable region of pneumococcal pathogenicity island 1 contributes to virulence in mice. *Infect Immun* 2004; 72: 1587–1593.
93. Moschioni M, De Angelis G, Melchiorre S *et al.* Prevalence of pilus encoding islets among acute otitis media *Streptococcus pneumoniae* isolates from Israel. *Clin Microbiol Infect* 2009 [Epub before print].
94. Moschioni M, Donati C, Muzzi A *et al. Streptococcus pneumoniae* contains 3 *rlrA* pilus variants that are clonally related. *J Infect Dis* 2008; 197: 888–896.
95. Romero P, Croucher NJ, Hiller NL *et al.* Comparative genomic analysis of ten *Streptococcus pneumoniae* temperate bacteriophages. *J Bacteriol* 2009; 191: 4854–4862.
96. Romero P, Garcia E, Mitchell TJ. Development of a prophage typing system and analysis of prophage carriage in *Streptococcus pneumoniae*. *Appl Environ Microbiol* 2009; 75: 1642–1649.
97. Iannelli F, Oggioni MR, Pozzi G. Allelic variation in the highly polymorphic locus PspC of *Streptococcus pneumoniae*. *Gene* 2002; 284: 63–71.
98. Dieudonne-Vatran A, Krentz S, Blom AM *et al.* Clinical isolates of *Streptococcus pneumoniae* bind the complement inhibitor C4b-binding protein in a PspC allele-dependent fashion. *J Immunol* 2009; 182: 7865–7877.
99. Jefferies JM, Johnston CH, Kirkham LA *et al.* Presence of nonhemolytic pneumolysin in serotypes of *Streptococcus pneumoniae* associated with disease outbreaks. *J Infect Dis* 2007; 196: 936–944.
100. Kirkham LA, Jefferies JM, Kerr AR *et al.* Identification of invasive serotype 1 pneumococcal isolates that express nonhemolytic pneumolysin. *J Clin Microbiol* 2006; 44: 151–159.
101. Throup JP, Koretke KK, Bryant AP *et al.* A genomic analysis of two-component signal transduction in *Streptococcus pneumoniae*. *Mol Microbiol* 2000; 35: 566–576.
102. McCluskey J, Hinds J, Husain S, Witney A, Mitchell TJ. A two-component system that controls the expression of pneumococcal surface antigen A (PsaA) and regulates virulence and resistance to oxidative stress in *Streptococcus pneumoniae*. *Mol Microbiol* 2004; 51: 1661–1675.
103. Lanie JA, Ng WL, Kazmierczak KM *et al.* Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6. *J Bacteriol* 2007; 189: 38–51.
104. Croucher NJ, Walker D, Romero P *et al.* Role of conjugative elements in the evolution of the multidrug-resistant pandemic clone *Streptococcus pneumoniae*^{Spain23F} ST81. *J Bacteriol* 2009; 191: 1480–1489.
105. Hiller NL, Janto B, Hogg JS *et al.* Comparative genomic analyses of seventeen *Streptococcus pneumoniae* strains: insights into the pneumococcal supragenome. *J Bacteriol* 2007; 189: 8186–8195.
106. Beres SB, Musser JM. Contribution of exogenous genetic elements to the group A *Streptococcus* metagenome. *PLoS ONE* 2007; 2: e800.