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Abstract:	*Streptococcus pneumoniae* (pneumococcus) has evolved sophisticated strategies to survive in several niches within the human body either as a harmless commensal or as a serious pathogen causing a variety of diseases. The dynamic interaction between pneumococci and resident host cells during colonization of the upper respiratory tract and at the site of infection is critical for bacterial survival and the development of disease. Pneumococcal lipoproteins are peripherally anchored membrane proteins and have pivotal roles in bacterial fitness including envelope stability, cell division, nutrient acquisition, signal transduction, transport (as substrate-binding proteins of ABC transporter systems), resistance to oxidative stress and antibiotics, and protein folding. In addition, lipoproteins are directly involved in

Pneumococcal lipoproteins involved in bacterial fitness, virulence and immune evasion

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Summary

Streptococcus pneumoniae (pneumococcus) has evolved sophisticated strategies to survive in several niches within the human body either as a harmless commensal or as a serious pathogen causing a variety of diseases. The dynamic interaction between pneumococci and resident host cells during colonization of the upper respiratory tract and at the site of infection is critical for bacterial survival and the development of disease. Pneumococcal lipoproteins are peripherally anchored membrane proteins and have pivotal roles in bacterial fitness including envelope stability, cell division, nutrient acquisition, signal transduction, transport (as substrate-binding proteins of ABC transporter systems), resistance to oxidative stress and antibiotics, and protein folding. In addition, lipoproteins are directly involved in virulence-associated processes such as adhesion, colonization, and persistence through immune evasion. Conversely, lipoproteins are also targets for the host response both as ligands for toll like receptors and as targets for acquired antibody. This review summarizes the multifaceted roles of selected pneumococcal lipoproteins and how this knowledge can be exploited to combat pneumococcal infections.

Keywords

Streptococcus pneumoniae, lipoprotein, virulence, immune response, antibacterial therapy

Introduction

Streptococcus pneumoniae (pneumococcus) is a Gram-positive human-specific commensal of the upper and lower respiratory tract. Under certain circumstances, particularly in immune-deficient individuals, young children, and elderly persons, pneumococci can also cause non-invasive infections such as otitis media and sinusitis, and life-threatening diseases such as meningitis, septicaemia, and pneumonia [1, 2]. Pneumococcal colonization of the nasopharyngeal epithelium and subsequent dissemination of pneumococci into other parts of the human body to cause disease are highly dynamic processes [3]. Multiple bacterial surface-displayed or released molecules contribute to direct and indirect interactions with the host such as adhesion and invasion. In addition, they help to maintain bacterial survival within the host by physiological adaptation and immune evasion [4-6]. The polysaccharide capsule (CPS) is the sine qua non virulence determinant of *S. pneumoniae* as it protects against mucus-mediated clearance, uptake by professional phagocytes and facilitates pneumococcal escape from neutrophil extracellular traps. The CPS has a variable structure between pneumococcal strains and is divided into more than 90 serotypes differing in their efficacy for promoting immune evasion [7-10]. The pneumococcal capsule masks other bacterial surface components such as the cell wall coated with phosphorylcholine (PCho)-containing teichoic acids (TAs) and proteins, thus preventing an intimate contact of

pneumococci with host cells or tissues. However, upon phase variation from an opaque to a transparent phenotype, less CPS is produced and higher amounts of TAs and PCho are detectable in the pneumococcal cell wall [11-13]. The increased accessibility of surface-exposed components may allow *S. pneumoniae* to survive, multiply and spread into new niches of the human host. For instance, pneumococcal surface proteins play critical roles in virulence as they maintain bacterial viability and participate in different stages of infection, including adhesion, colonization, invasion, immune evasion and dissemination into various host tissues and organs [4-6]. Four classes of proteins have been distinguished according to their anchoring mechanism: i) choline-binding proteins that are non-covalently attached to cell wall PCho, ii) proteins carrying an LPxTG motif can be covalently incorporated into the peptidoglycan polymer in a sortase-dependent manner, iii) non-classical surface proteins (NCSP), also referred to as moonlighting proteins, that are surface-associated but do not possess classical secretory and anchoring motifs, and iv) lipoproteins that are covalently bound to the cytoplasmic membrane by the action of lipoprotein diacylglyceryl transferase (Lgt) and lipoprotein signal peptidase II (Lsp). Lipoproteins are the subject of this review.

Genes encoding for putative lipoproteins are distributed throughout the pneumococcal genome and yet almost equally located on the positive or negative strand (Figure 1). It has been estimated that lipoproteins account for approximately 2-3% of the pneumococcal proteome and about 40% of the indicated and predicted pneumococcal surface proteins [14, 15]. A large proportion of pneumococcal lipoproteins are substrate-binding proteins (SBPs) of ABC transporter systems responsible for the acquisition of multiple nutrients including amino acids and short peptides, sugars, polyamines, and various metal ions. Other functions of lipoproteins in *S. pneumoniae* include folding of excreted proteins, antibiotic and oxidative stress resistance, cell shape maintenance and enzymatic activity [6, 16]. The contribution of lipoproteins to the virulence of *S. pneumoniae* has been studied extensively and they have been linked to crucial processes in the pathogenesis of pneumococcal infectious diseases such as adhesion, invasion, and immune evasion [17-21]. Moreover, lipoproteins trigger the activation of host innate immune responses via Toll-like receptor 2 (TLR2), which contributes to the establishment of cellular and humoral adaptive immune responses [22, 23]. Therefore, lipoproteins have emerged as promising candidates for the development of novel vaccines and therapeutics against severe pneumococcal infections [24, 25]. Moreover, due to their uniqueness in prokaryotes and their importance for anchoring and further processing of secreted lipoproteins, lipoprotein maturation enzymes are also potential candidates for the development of novel antimicrobials, thus providing another valuable therapeutic aspect for the treatment of pneumococcal disease.

1. Pneumococcal Lipoproteins

1.1. Lipoprotein Biosynthesis, Maturation, and Localization

Like in other bacteria, pneumococcal lipoproteins are initially translated in the cytoplasm as preprolipoproteins containing a classical N-terminal signal peptide followed by a Lipobox motif (LVI)(ASTVI)(GAS)C. After export via the sec or tat secretion machineries, lipoprotein diacylglyceryl transferase (Lgt) catalyzes the covalent attachment of the lipoprotein precursor at its indispensable cysteine residue within the Lipobox to phosphatidylglycerol of the cytoplasmic membrane. Afterwards, lipoprotein signal peptidase II (Lsp) cleaves the signal peptide of the prolipoprotein leaving the lipidated cysteine as new amino-terminal residue (Figure 2) [26]. Lgt and Lsp are conserved in all bacteria, whereas lipoprotein N-acyltransferase (Lnt), a third lipoprotein-modifying enzyme downstream of Lgt and Lsp, was thought to be present only in Gram-negative bacteria [27]. However, Lnt homologues have been identified in several high-GC bacteria and a lipoprotein of *Staphylococcus aureus* was found to be N-acylated by a so far unidentified enzyme [28].

Bacterial lipoproteins have formerly been classified into nine clusters, according to their sequence similarities [29, 30]. However, due to increasingly available structural data, a new classification based on structural similarity has been introduced, now numbering cluster A to F [31]. The *in silico* analysis predicted up to 37 lipoproteins in *S. pneumoniae* serotype 2 and 4 according to their Lipobox motif [15, 32]. Recently, a comprehensive proteomic analysis has been performed to elucidate the global surface and exoproteome of *S. pneumoniae*, including the role of lipoprotein maturation on the protein composition of the bacterial cell envelope and extracellular milieu. Optimization of a biotinylation approach allowed the identification of 95% of the 35 predicted surface-exposed lipoproteins of a nonencapsulated D39 strain, among them, two out of four hypothetical lipoproteins with unknown functions [15]. As expected, the majority of lipoproteins accumulated in the culture supernatant after deletion of Δ*lgt*, indicating improper anchoring in the cytoplasmic membrane. Deletion of Δ*lsp* resulted in higher molecular weight protein bands in immunoblots compatible to retention of N-terminal signal peptide-containing lipoproteins (Figure 3) [15, 33]. Nevertheless, the protein amount detectable on the surface and in the exoproteome of the Δ*lgt* and Δ*lsp* mutants varied substantially between the different lipoproteins due to either rapid degradation or higher expression, respectively [15]. It has yet to be determined whether unprocessed lipoproteins are still at least partially functional when retained on the pneumococcal surface. Some of the phenotypes of the *S. pneumoniae* Δ*lgt* mutant are weaker than deletion of the relevant lipoprotein suggesting some residual lipoprotein functionality even when the processing has been disrupted as recently described for the DacB lipoprotein [15, 17, 33]. Similar to previous studies, additional lower molecular

weight signals for the streptococcal lipoprotein rotamase A (SlrA) lipoprotein were detectable in the Δ*lsp* mutant suggesting another signal peptidase or membrane protease can substitute for loss of the primary signal peptidase Lsp (Figure 2) [15, 34]. Therefore, a model for maturation of lipoproteins has been proposed accounting for alternative processing in the absence of Lsp [15, 34].

2. A General view on the Role of Pneumococcal Lipoproteins for Bacterial Fitness and Virulence

Lipoproteins are involved in many important cellular processes and generally classified into different functional groups, e.g. substrate-binding for ABC transporter systems, resistance to antibiotics, cell envelope stability, and protein folding. However, several lipoproteins are multifunctional and their roles overlap these categories. In some cases redundant functions of several lipoproteins can be explained by a conserved overall three-dimensional structure, despite their variation in size and low sequence similarity [21, 30, 31]. In contrast, incomplete maturation of lipoproteins due to functional deficiency of Lgt, Lsp or both enzymes has significant but pleiotropic effects on metabolic and virulence functions modulating bacterial survival and pathogenesis in the human host [15, 33-35]. However, the impact of Δ*lgt* or Δ*lsp* mutations on growth and virulence strongly depends on the bacterial species and strain as well as on the animal infection model employed in the respective study. For example, a Δ*lgt* mutant of *Streptococcus equi* was attenuated in a mouse model of infection but not in the horse serving as natural host [36]. Likewise, deficiency of Lsp caused an impaired intracellular growth and moderate attenuation in virulence of *Listeria monocytogenes* while no effect on virulence was detectable for *S. suis* [37, 38]. In *S. pneumoniae*, functional deficiency of Lgt had no effect on bacterial growth in artificial media but resulted in a significant growth defect in blood and bronchoalveolar lavage fluids representing physiologically relevant infection conditions [15, 33]. The identified phenotypes of Δ*lgt* and Δ*lsp* mutants strongly associated with defective functions of several ABC transporters. For instance, a higher susceptibility of these mutants to oxidative stress can be attributed to impaired uptake of divalent cations and, consequently, to low intracellular concentrations of manganese ions usually imported by the lipoprotein pneumococcal surface adhesion A (PsaA) (Section 2.2.1.) [33]. Importantly, Δ*lgt* mutants of *S. pneumoniae* are avirulent in mouse models of pneumonia and septicaemia and show a decreased capacity to colonize the nasopharynx [33, 35]. Pneumococcal mutants deficient in the signal peptidase Lsp displayed only moderate defects in growth in complex media but had impaired growth in blood, which is compatible with a significantly reduced virulence in mouse infection models of pneumonia and septicaemia [15, 34].

2.1. Functions of Pneumococcal classical Non-ABC Transporter Lipoproteins

The majority of lipoproteins in *S. pneumoniae* are predicted to be part of ABC importer systems. However, the pneumococcal genome also encodes for several lipoproteins not associated with ABC transporters crucial for bacterial fitness and full virulence (Table 1) [32, 39]. For instance, six lipoproteins have been implicated in folding or activation of surface-exposed proteins, such as the peptidyl-prolyl isomerases (PPIases) putative proteinase maturation protein A (PpmA) and SlrA, though PPIase activity was only demonstrated for SlrA [19]. Both PpmA and SlrA have been shown to promote pneumococcal colonization and contribute to virulence in a mouse model of acute pneumonia [18, 19, 39]. In addition, non-ABC transporter lipoproteins exert important functions in cell wall biosynthesis and integrity, as demonstrated for the L,D-carboxypeptidase DacB (also known as LdcB), as well as stress responses of *S. pneumoniae*, as recently shown for the extracellular thioredoxin-like lipoproteins Etrx1 and Etrx2 [17, 21, 32, 40].

2.1.1. Keep the shape: Impact of cell wall hydrolases on pneumococcal pathogenesis

The cell wall of *S. pneumoniae* is a rigid layer composed of peptide-linked glycan strands (peptidoglycan, PGN) to maintain cell shape and integrity but flexible enough to ensure physiological processes such as cell division, autolysis, DNA uptake, and trafficking of nutrients and ions. In addition, the pneumococcal cell wall contains teichoic acids, either covalently attached to the PGN (wall teichoic acids, WTAs) or anchored to the cytoplasmic membrane (lipoteichoic acids, LTAs). Strikingly, in *S. pneumoniae* WTAs and LTAs share an identical chemical structure within their repeating units composed of several different sugar residues and contain up to two PCho residues per TA repeat [41]. The first steps of peptidoglycan synthesis occur intracellularly and include i) the addition of five amino acids, known as stem peptide, to UDP-N-acetylmuramic acid (MurNAc), ii) substitution of UMP in the UDP-MurNAc-pentapeptide precursor for undecaprenyl phosphate of the phospholipid bilayer (Lipid I), and iii) transfer of an UDP-N-acetylglucosamine (GlcNAc) residue yielding Lipid II [42]. Afterwards, Lipid II is translocated to the extracellular side of the membrane by the flippases FtsW and RodA [42, 43]. In the extracellular face of the cytoplasmic membrane, penicillin-binding proteins (PBPs) catalyse the incorporation of Lipid II into the existing peptidoglycan network in a cell cycle-dependent manner [42]. This also results in release of undecaprenyl pyrophosphate which is flipped back across the membrane, dephosphorylated and can serve again as lipid carrier for new rounds of peptidoglycan synthesis [44]. Within the cell wall, N-acetylamino sugars may be subjected to modification, i.e. acetylation and deacetylation. In addition, pneumococcal stem peptides can be modified by amidation, hydrolysis, and transpeptidation to form direct or dipeptide-containing cross linkages between peptide chains of neighbouring glycan strands [17, 45, 46]. Importantly, formation of

branched stem peptides and further modification of the peptidoglycan backbone are associated with pneumococcal resistance to antimicrobial agents, thus preventing bacterial cell death and release of immunostimulatory molecules, and consequently increasing the pneumococcal virulence potential [47-50].

Growth and division of *S. pneumoniae* are strictly dependent on the biosynthesis, turnover and remodelling of peptidoglycan, whose complexity is determined by the function of several different enzymes acting either sequentially or in concert [42]. In this regard, PBPs are highly conserved, extracellular membrane-bound enzymes crucial to bacterial growth and division as they catalyse various steps of the PGN biosynthetic pathway [42]. Among them, Penicillin-binding protein 3, also referred to as DacA, exhibits D,D-carboxypeptidase activity cleaving the fifth amino acid residue off the stem peptide to generate tetrapeptides [51]. Subsequently, the L,D-carboxypeptidase DacB (also referred to as LdcB) catalyses the truncation of generated tetrapeptides into tripeptides (Table 1) [52]. Importantly, the presence of tetra- and tripeptide-containing stem peptides enables transpeptidation reactions catalysed by several PBPs to generate direct cross linkages between neighbouring glycan strands [53]. DacB was first described in *E. coli* and homologues have been identified in *Lactococcus lactis* and *Bacillus* species [40, 54, 55]. Barendt et al. first reported a *dacB* gene in *S. pneumoniae* likely encoding for an extracellularly anchored 27-kDa protein of 238 amino acids acting downstream of DacA in generating PGN-tripeptides [52]. And very recently, a surface proteome analysis of *S. pneumoniae* D39 confirmed DacB as a lipoprotein processed by the lipoprotein maturation enzymes Lgt and Lsp (Table 1; Figure 3) [15]. The crystal structure of DacB lacking the membrane-anchoring domain (aa 1-55) has been solved independently by Abdullah et al. and Hoyland et al. in 2014. Both structures are almost identical comprising a small N-terminal region formed by a three-stranded, antiparallel β-sheet and a V-shaped catalytic cavity formed by a four-stranded, antiparallel β-sheet and seven α-helices [17, 40]. However, Abdullah et al. propose two 3_{10}-helices downstream of α3 and α4, respectively, instead of two extended α-helices [17]. Nonetheless, the catalytic site contains a single Zn(II) ion co-ordinated by a His-Asp-His triad and only accepts D-tetrapeptides, containing the D-isomer of alanine at the fourth position, as substrate [17, 40]. Loss of function of DacA, DacB or both enzymes resulted in severe defects in cell morphology and division as expressed by decreased pneumococcal growth and yield, rounded and less dense cells suggesting enhanced autolysis, and division asymmetry. In addition, Δ*dac* mutants showed an increased susceptibility to the antibiotic vancomycin, which binds to the terminal D-Ala-D-Ala dipeptide of Lipid II [17, 52]. Interestingly, deficiency in lipoprotein diacylglyceryl transferase Lgt did not affect L,D-carboxypeptidase activity of DacB, indicating that attachment of the lipid anchor to the DacB-preprolipoprotein is not essential for enzymatic activity. Importantly, it has been suggested that due to reduced

integrity of the peptidoglycan network in pneumococcal Δ*dac* mutants an improper sortase-dependent anchoring of surface-proteins, e.g. pneumococcal adherence and virulence factor B (PavB), might result in the diminished pneumococcal adherence to human lung epithelial cells *in vitro*. In addition, impaired cell division has been shown to enhance uptake and to reduce survival of pneumococci in professional phagocytes, negatively affecting the success of *S. pneumoniae* to cause infection. Likewise, attenuation of Δ*dac* mutants has been demonstrated *in vivo* in a mouse model of acute pneumonia. While DacA had only minor effects on sepsis development, Δ*dacB* and double Δ*dacA*Δ*dacB*-mutants were severely impaired in their ability to spread from the nasopharynx into the bloodstream (Table 1) [17]. In conclusion, the importance of DacA and DacB for peptidoglycan architecture and bacterial shape as well as the close connection between PGN metabolism and virulence of *S. pneumoniae* could provide new opportunities for the development of novel drugs and vaccines.

2.1.2. Two thiroredoxin-lipoproteins as key factors for the extracellular antioxidant defence in pneumococci

Resistance to oxidative stress is of utmost importance for *S. pneumoniae* as it ensures successful colonization and subsequent disease development. Reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radicals, are produced from oxygen and known to irreversibly damage many types of cellular macromolecules. Exogenous sources of ROS are professional phagocytes (oxidative burst) and other lactic acid bacteria in the nasopharynx. In addition, pneumococci endogenously produce up to millimolar concentrations of hydrogen peroxide by the pyruvate oxidase SpxB. High local peroxide concentrations provide a selective advantage for *S. pneumoniae* against competing organisms in the oxygen-rich upper respiratory tract and contribute to pneumococcal pathogenesis due to DNA damage-induced cell death in airway epithelial cells [56-58]. Although lacking catalase, the major peroxide-degrading enzyme in all aerobic organisms, pneumococci are able to survive in oxidative environments by both detoxifying oxygen and ROS and repairing oxidative damage. Scavenging by glutathione as well as detoxification by superoxide dismutase (SodA), NADH oxidase (Nox), alkyl hydroperoxidase (AhpD), thiol peroxidase (TpxD), and thioredoxin-methionine sulfoxide reductase (Trx-Msr) systems are known resistance mechanisms to intracellular oxidative stress in *S. pneumoniae* [59]. Extracellular protein integrity is maintained by chaperones and proteases such as heat-shock-induced serine protease A (HtrA) and Clp ATP-dependent proteases. In addition, the surface-exposed CTM system, consisting of a cytochrome c-type biogenesis protein (CcdA), a thioredoxin-like lipoprotein (TlpA), and a methionine sulfoxide reductase A/B protein (*Sp*MsrAB), has been implicated in extracellular ROS resistance. It was hypothesized that

TlpA (renamed to Etrx1 by Saleh et al.), reduced by CcdA, shuttles electrons to *Sp*MsrAB which can subsequently reduce oxidized methionine residues (MetSO) in damaged surface-exposed proteins, thereby restoring their functional capacities [21, 59, 60]. Recently, Saleh et al. expanded our knowledge on the composition of the pneumococcal CTM system as well as its importance for pneumococcal oxidative stress resistance and pathogenesis *in vivo* [21]. In addition to the previously described CTM operon, a second incomplete CTM operon has been identified to be surface-exposed in *S. pneumoniae* D39. Therefore, components of the "classical" CTM were renamed to CcdA1, Extracellular thioredoxin protein (Etrx) 1 (formerly TlpA), and *Sp*MsrAB2 (to distinguish it from the homologous intracellular *Sp*MsrAB1 protein). The incomplete CTM operon consists of a *ccdA*-like gene, designated *ccdA2*, and a paralogous gene to *etrx1*, assigned as *etrx2*, but lacks an *msrAB*-like gene. Both Etrx1 and Etrx2 are surface-exposed lipoproteins and, despite low sequence identity, share significant structural features common to members of the thioredoxin family: a three-layer α/β/α sandwich containing seven β-strands flanked by five α-helices and the invariant CXXC motif, whose two cysteine residues shuttle between the oxidized (disulfide) and reduced (dithiol) states (Table 1) [21, 61].

Several observations support the conclusion that, albeit functionally redundant, both Etrx proteins are required for efficient regeneration of oxidized *Sp*MsrAB2: i) genetic deletion of both Etrx lipoproteins renders *S. pneumoniae* susceptible to extracellular peroxide stress and enhances phagocytic killing of pneumococci *in vitro*, ii) loss of both Etrx proteins significantly reduced nasopharyngeal carriage and dissemination of pneumococci into the lower respiratory tract and bloodstream in mouse models of infection (Table 1), and iii) relevant structural differences in the active sites of Etrx1 and Etrx2, different redox states and potentials as well as varying kinetic activities of *Sp*MsrA2/B2-Etrx1/2 complexes suggest that Etrx1 preferentially reduces the *Sp*MsrA2 domain while Etrx2 can shuttle electrons to *Sp*MsrA2 and, with lower efficiency, to *Sp*MsrB2. Therefore, a model for two CcdA-Etrx-*Sp*MsrAB2 electron pathways has been proposed in which an electron transport chain initiates with the intracellular NADPH-Trx system, passes through the cytoplasmic membrane via either CcdA1 or CcdA2, which reduce either Etrx1 or Etrx2, continues with reduction of *Sp*MsrAB2 and terminates with MetSO in ROS-damaged surface-exposed proteins as final electron acceptor [21].

2.2. Functions of Pneumococcal ABC Transporter Lipoproteins

The ATP-binding cassette (ABC) transporter systems are universally distributed among eukaryotes and prokaryotes. They play critical roles in bacterial physiology as they import essential nutrients and export toxic molecules (e.g. bacteriocins and antibiotics) and pheromones. ABC transporter systems are composed of two intracellular nucleotide-binding

domains that hydrolyse ATP for energy and two transmembrane domains transporting molecules and ions across the cytoplasmic membrane, also referred to as permeases. ABC importer systems additionally possess an extracellular substrate-binding protein (SBP), which determines substrate specificity and is often a lipoprotein in both Gram-negative and Gram-positive bacteria [62]. Up to 30 ABC transporter-associated lipoproteins have been predicted in *S. pneumoniae* and for some, their surface-localisation and impact on bacterial fitness and virulence has been experimentally confirmed or implicated by large-scale genomic analyses. These include ABC transporter lipoproteins for acquisition of metal ions (e.g. PsaA; AdcA and AdcAII; PiaA, PiuA and PitA), amino acids and short peptides (MetQ, GlnH; AmiA-AliA/AliB), sugars (MalX), and inorganic compounds (i.e. PstS) (Table 2) [15, 32, 63-65]. For instance, the three iron-uptake ABC transporter systems Pia, Piu, and Pit are required for bacterial growth and virulence in both pulmonary and systemic models of infection, albeit loss of PiaA, the iron-binding SBP of Pia, has stronger effects on pneumococcal phenotype than loss of the iron-binding lipoproteins PiuA and PitA (Table 2) [65-67]. Furthermore, an additional iron-uptake ABC transporter lipoprotein (SPD_1609) is encoded in the pneumococcal genome and expressed in Δ*piaA*/Δ*piuA*/Δ*pitA* triple mutants in iron-repleted medium but has not yet been investigated in detail [15, 68, 69]. Acquisition of metal ions is essential for survival of *S. pneumoniae* in their infected host. Therefore, two important interconnected pneumococcal metal ion uptake systems are discussed in more detail below.

2.2.1. From tolerance to toxicity: Importance of manganese and zinc homeostasis on pneumococcal fitness and virulence

Manganese is one of the essential micronutrients for the growth and survival of pneumococci in the human host as it is a cofactor for pneumococcal superoxide dismutase (SodA), an enzyme which detoxifies endogenously produced hydrogen peroxide. Pneumococcal uptake of Mn(II) ions is mediated by the PsaBCA transporter consisting of PsaA as substrate-binding lipoprotein, PsaC as permease, and PsaB as ATP-binding protein (Table 2; Figure 3). Expression of the *psaBCA* operon is repressed by PsaR in presence of high Mn(II) concentrations and activated by the two-component system TCS04 [70, 71]. PsaA is an ubiquitously expressed 35-kDa lipoprotein belonging to cluster A-I of bacterial ABC transporters. In crystals, the protein folds into a two-lobed structure that consists of an N- and C-terminal domain providing the metal-binding site and linked by a rigid α-helix which has been proposed to restrict conformational flexibility [72]. Very recently, conformational states of metal-free PsaA in solution have been resolved showing that i) the C-terminal lobe is more flexible than the N-terminal lobe, providing additional proof for the importance of the C-terminal lobe as recognition site for the permease PsaC, ii) the α-helical hinge between the

two lobes allows a greater conformational flexibility important for ligand binding, and iii) the metal-binding site is larger and more solvent exposed than indicated by crystallography and *in silico* simulations [73]. PsaA was shown to bind both manganese and zinc. Although PsaA kinetically prefers Mn(II) to Zn(II), zinc bound to PsaA induces a higher thermal stability of the protein complex, therefore remains bound to the lipoprotein and is not released to the Psa permease [74, 75]. If the Zn(II) to Mn(II) ratio in the respective host niche exceeds a critical level (Zn(II):Mn(II) of 50-100:1), zinc competes with Mn(II) for binding to PsaA which leads to an intracellular depletion of Mn(II) and results in phenotypes similar to Δ*psaA* knockout mutants. Pneumococci deficient in PsaA have a significant requirement for additional Mn(II) for growth and competence and are highly susceptible to oxidative stress and greatly decreased virulence in local and systemic models of infection (Table 2) [74]. In addition, Δ*psaA* knockout strains display reduced adherence to human pneumocytes and E-cadherin has been suggested as eukaryotic cell receptor. However, a direct role for PsaA as an adhesin has not yet been conclusively clarified (Table 2) [20, 76].

2.2.2. Zinc acquisition via AdcA and AdcAII is critical for pneumococcal pathogenesis

Zinc belongs to the essential trace metals for pneumococci and is an important cofactor for basic housekeeping metalloenzymes and transcription factors. However, zinc is toxic at high concentrations, for example when released from damaged or apoptotic cells during inflammation or bacterial infection, in part due to outcompeting Mn(II) for binding to PsaA as discussed above [77]. Therefore, the balance between zinc and manganese homeostasis are crucial for survival and virulence of *S. pneumoniae*. Pneumococcal Zn(II) acquisition occurs via AdcA and AdcAII lipoproteins and is supported by Polyhistidine triad (Pht) family proteins (Table 2; Figure 3) [78, 79]. The *adcRCBA* ABC transporter operon encodes for the transcriptional repressor AdcR, the zinc-binding SBP AdcA, the AdcB permease and AdcC ATPase [80]. In contrast, AdcAII is not encoded in a classical ABC transporter operon. In fact, the *adcAII* gene is part of a long transcript together with *phtD*, encoding for the Pht protein D, downstream of a*dcAII* [81]. The pneumococcal lipoproteins AdcA and AdcAII share high structural similarities to PsaA and belong therefore also to the cluster A-I SBPs [78, 81]. In contrast to PsaA, which was shown to bind both Mn(II) and Zn(II), AdcA and AdcAII demonstrate absolute substrate-specificity for zinc [78, 82]. AdcAII transfers bound Zn(II) to the AdcB permease indicating that both AdcA and AdcAII use the same permease/ATPase-system to transport Zn(II) into the cell. AdcA and AdcAII are individually redundant in zinc acquisition, and Δ*adcA* and Δ*adcAII* single mutants show only modest attenuation in virulence in mouse models of colonisation and infection. However, combined loss of both zinc-binding proteins significantly impaired growth in artificial media, in bronchoalveolar lavage fluid, and human serum, affected pneumococcal cell division and

competence, as well as completely abolishing virulence (Table 2) [78, 82]. Interestingly, in contrast to AdcA, AdcAII does not possess structural features allowing a direct sequestration of Zn(II) indicating that accessory molecular interactions are needed for AdcAII function and zinc homeostasis. Indeed, the four Pht proteins, PhtA, PhtB, PhtD and PhtE, have been shown to collectively aid in Zn(II) acquisition via AdcAII, though the molecular mechanism, either by a direct physical interaction of AdcAII and Pht or enrichment of local zinc concentrations by Pht, is still elusive [79]. AdcR transcriptionally represses expression of the *adcRCBA* operon and the *adcAII/phtD* operon. Computational analysis revealed a variable number of AdcR-binding sites upstream of *adcRCBA*, *adcAII/phtD* and, interestingly, also upstream of *phtE*, which is encoded contiguous to *phtD*. Hence, it has been suggested that a single AdcR-binding site upstream of *adcR* permits autoregulation of the *adcRCBA* operon and provides a basal supply of Zn(II) in zinc-rich host environments, while two binding sites upstream of *adcAII/phtD* and three AdcR-binding sites upstream of *phtE* enable upregulation of AdcAII and Pht proteins to adapt to zinc limiting conditions [78]. Therefore, niche-specific adaptations to zinc and manganese availability are likely to maintain fitness and contribute to pneumococcal virulence.

2.2.3. The pneumococcal nucleoside receptor A interferes with virulence

Facing the dramatically increasing resistance of *S. pneumoniae* to antibiotics, the replacement of vaccine serotypes by non-vaccine serotypes, and the immense individual costs for each serotype to be included in a glycoconjugate vaccine, development of a serotype-independent, protein-based vaccine is an attractive alternative. Several pneumococcal surface-associated proteins, including PsaA, PiaA and PiuA, have been identified as vaccine candidates and already tested in mouse and human, though long-term effects aren´t yet known. However, combination of different pneumococcal proteins in one vaccine formulation tends to be more effective in establishment of protection against disease in mouse models of infection and ongoing research is striving to discover novel pneumococcal proteins as vaccine candidates [83, 84]. Recently, SP_0845 has been proposed as potential vaccine antigen identified by affinity and gel-free proteomic approaches [15, 85]. SP_0845 is the substrate-binding protein of the sole carbohydrate uptake ABC transporter superfamily 2 (CUT2) in *S. pneumoniae* and encoded in an eight-gene operon also containing the corresponding ATPase and two permeases predicted to function as heterodimers [86]. Since SP_0845 exhibits 70% identity to the orthologue in *S. mutans* and is similarly involved in uptake and metabolism of ribonucleosides preferentially containing cytidine, uridine, and guanosine, it has been designated as purine nucleoside receptor A (PnrA, in the literature also referred to as TmpC) (Table 2) [85, 87]. PnrA has been confirmed as conserved, surface-exposed lipoprotein accessible to (protective)

antibodies in both encapsulated and nonencapsulated pneumococci (Figure 3; see also section 3.2.) [15, 85]. Importantly, deletion of *pnrA* completely abolished virulence of *S. pneumoniae* type 2 strain D39 and type 4 strain TIGR4 [85].

2.2.4. Strain-dependent influence of MetQ on pneumococcal fitness and virulence

In all living organisms, methionine is essential for the biosynthesis of proteins, phospholipids and nucleic acids. However, this amino acid is very rare in mammalian physiological fluids and hardly available to bacteria in their natural host niche. Therefore, *S. pneumoniae* possesses a methionine ABC uptake transporter that has been recently identified as surface-exposed by proteomic analysis [15, 88]. MetQ is the substrate-binding lipoprotein of the MetQNP ABC transporter system, including the ATP-binding protein MetN and the permease MetP (Table 2; Figure 3). MetQ exhibits high affinity for L-methionine but also binds, albeit with significantly lower affinity, D-methionine and the non-proteinogenic amino acid DL-homocysteine. Loss of function of MetQ impaired growth in both chemically defined medium and human blood indicating that methionine uptake via MetQ is crucial for pneumococcal fitness [88]. In signature-tagged mutagenesis screens, MetQ has been identified as essential for pneumococcal nasopharyngeal colonisation and invasive disease [89, 90]. Indeed, MetQ has been shown to contribute to pneumococcal colonization and virulence, though in a strain-specific manner. In a mouse model of mixed infection with a pneumococcal serotype 3 strain and its respective Δ*metQ* mutant, deletion of *metQ* had no impact on nasopharyngeal colonisation and two opposing results have been reported on the competitive ability of the Δ*metQ* strain against the wild-type in pulmonary and systemic mouse infection models [88, 91]. Interestingly, when administered separately, the Δ*metQ* and wild-type strain showed a similar virulence pattern in a murine pneumonia model [88]. In contrast, deletion of *metQ* in *S. pneumoniae* serotype 2 strain D39 significantly attenuated virulence in a mouse model of acute pneumonia as monitored real time with bioluminescent imaging (Table 2; Figure 3) [92]. Moreover, vaccination with recombinant MetQ failed in a first attempt to prevent systemic pneumococcal infection after heterologous challenge [88]. Hence, extensive studies are required to obtain a holistic understanding of the impact of MetQ on pneumococcal virulence and to conclusively evaluate the applicability of MetQ in future protein-based pneumococcal vaccines.

3. Immunogenicity and Protective Potential of Pneumococcal Lipoproteins

3.1. Inflammatory Immune Responses to Pneumococcal Lipoproteins

S. pneumoniae infections are characteristically associated with inflammation [93], with a strong acute phase response, rapid leukocyte recruitment to the site of infection, and

endothelial / epithelial barrier breakdown. *S. pneumoniae* causes inflammation partly through recognition of bacterial ligands by host cell surface Toll-like receptors (TLR), with mouse and human data both demonstrating that genetic deficiencies of TLRs or their signalling proteins increases susceptibility to pneumococcal infections [94-98]. TLR2 seems to be the dominant TLR driving responses to *S. pneumoniae*, especially macrophage production of pro-inflammatory cytokines such as TNFα and IL-6 [22, 99-102]. TLR2 has been shown to be important for inflammation and control of infection in mouse models of infection [99-101], and indirectly increases inflammatory responses to the pneumococcal toxin pneumolysin [103]. In addition, TLR2 responses are required for the development of adequate cellular and humoral adaptive immune responses to *S. pneumoniae* [104, 105].

Lipoproteins are known TLR2 agonists, and recent data obtained using an Δ*lgt* mutant has demonstrated that the complete TLR2 response to *S. pneumoniae* requires bacterial lipoproteins [22]. Infection of macrophages with the pneumococcal lipoprotein deficient Δ*lgt* mutant strain resulted in reduced activation of the pro-inflammatory transcriptional factor NFκB, and lower levels of pro-inflammatory cytokine release; cytokine levels were also lower in samples recovered from a murine model of early pneumonia for animals infected with the Δ*lgt* mutant compared to those infected with the wild type strain. The differences in inflammatory responses between wild-type and the Lgt deficient strains were not due to indirect effects of loss of lipoproteins on bacterial growth or cell wall structure, and were lost when bacteria were incubated with TLR2 deficient macrophages [22]. There were striking but selective differences in human macrophage transcriptional responses between the Δ*lgt* mutant and wild-type *S. pneumoniae* strains. Although infection with *S. pneumoniae* caused up-regulation of over 900 macrophages genes, only a small proportion of these had significantly greater expression in response to the wild-type strain compared to the Δ*lgt* strain. However, the genes showing stronger responses to wild-type bacteria compared to the Δ*lgt* mutant included many important pro-inflammatory proteins, and in general were also the genes showing the greatest up-regulation in response to wild type *S. pneumoniae* or to the TLR2 agonist Pam2CSK4 [22]. Hence, the dominant macrophage transcriptional responses to *S. pneumoniae* are largely TLR2-dependent and usually require the presence of lipoproteins for maximal stimulation.

These results demonstrated that pneumococcal lipoproteins are major contributors (probably the dominant ligand) to the macrophage TLR2-mediated inflammatory response. The *S. pneumoniae* cell wall is highly pro-inflammatory, and it was previously suggested that PGN and LTA are the major pneumococcal TLR2 agonists [106-108]. However, cell-wall dependent inflammation is also mediated by nucleotide-binding oligomerization domain (NOD) recognition of PGN, and purified pneumococcal LTA does not activate a TLR2 reporter cell [41, 109, 110]. Taken together the data suggest that TLR2 dependent

inflammatory responses to the pneumococcal cell wall are largely driven by lipoprotein contamination rather than TLR2 recognition of PGN or LTA, a similar situation to that described for *S. aureus* [111, 112]. Importantly, the pattern of human inflammatory cytokine responses to the Δ*lgt* mutant strain were similar to those seen for subjects with IRAK-4 deficiency, a rare genetic defect of the TLR activation signalling pathway that causes greatly increased susceptibility to *S. pneumoniae* infection [96, 97]. This suggests that lipoproteins are important ligands driving IRAK-4 dependent inflammatory responses and therefore protective immunity in children.

Several important questions remain unanswered about lipoprotein-dependent TLR2 responses. Firstly, how do TLR2 receptors interact with *S. pneumoniae* lipoproteins when the lipoprotein moiety is at the cell membrane level and is separated by both the cell wall and the capsule from interactions with host cells? TLR2 activation occurs in response to live bacteria [22], but it is difficult to understand how lipoproteins can interact with TLR2 receptors unless they are released from the cell membrane to some extent, or TLR2 receptors penetrate through the pneumococcal capsule and cell wall. A related question is whether *S. pneumoniae* capsular serotype affects TLR2 / lipoprotein interactions, thereby causing differences in inflammatory responses between different strains and perhaps influencing the clinical phenotypes associated with capsular serotype. Another important question is which lipoproteins are the most dominant in generating the TLR2 response? TLR2 receptors could respond to all the pneumococcal lipoproteins, in which case the most common will dominate the inflammatory response (e.g. PsaA), or to a specific subset that might not necessarily include the most abundant lipoproteins. Finally, the relative roles and potentially synergistic interactions of TLR2 induced responses with other *S. pneumoniae* pro-inflammatory mechanisms such as NOD or in response to pneumolysin need further exploration [103, 109, 110]. A fuller understanding of TLR2 / lipoprotein interactions and how they affect inflammatory responses during *S. pneumoniae* infection may reveal potential novel therapeutic mechanisms for immunomodulation aiming to improve patient outcomes.

3.2. Pneumococcal Lipoproteins as Next-Generation Vaccine Candidates or drug targets

Two different types of vaccines are currently available to combat pneumococcal infections: the 23-valent polysaccharide vaccine PPV-23 and the 7-, 10- or 13-valent pneumococcal polysaccharide conjugate vaccines (PCV-7, -10, -13). Despite their efficacy and potential impact, there are some limitations to the current pneumococcal vaccines, including the need for multiple doses, the lack of protection against non-vaccine serotypes and, particularly for developing countries, the cost [113, 114]. In the last two decades, efforts have been increasingly directed to overcome these shortcomings by finding new generation

pneumococcal vaccines, which provide serotype-independent protection against invasive as well as non-invasive pneumococcal disease [83, 115-117].

Various pneumococcal proteins have been studied as potential vaccine candidates. Especially surface-exposed proteins getting in contact with the extracellular milieu in the host and are therefore likely accessible for antibodies to generate an effective immune response, are the most promising candidates for a protein-based vaccine. Importantly, these proteins have to be highly immunogenic, conserved among pneumococcal serotypes and able to cause both humoral and cellular immune responses required for protection against *S. pneumoniae* infection [118]. The best studied potential vaccine candidate among the surface-localized lipoproteins is the Pneumococcal surface adhesin A (PsaA) [119]. PsaA is a highly immunogenic protein as antibody responses to PsaA have been described in children and were related to pneumococcal exposure [120-123]. Moreover, PsaA is highly conserved among almost all pneumococcal serotypes [124, 125]. In both passive and active immunization studies, PsaA failed to confer protection against systemic infection, which was reasoned to be due to low accessibility of PsaA for circulating host antibodies [126, 127]. In contrast, in several studies the protective efficacy of PsaA against pneumococcal carriage has been demonstrated. Mice immunized intranasally with PsaA and Cholera toxin B (CTB) subunit, as mucosal adjuvant, showed significantly reduced bacterial loads in the nasopharynx. This protection was even higher when a combination of PsaA together with PspA was used [128]. The best results in protective efficacy could be achieved for using PsaA in combination with other antigens as shown in both intraperitoneal and intranasal challenge models [129-131].

Preclinical investigations demonstrated that protection against pneumococcal carriage is mediated by a T_H17-dependent immune response when immunizing mice with a heat killed whole-cell vaccine. High levels of IL-17 in the blood were correlated with non-detectable numbers of pneumococci in the nasopharynx of mice [105, 132]. A nasal formulation containing PsaA, pneumococcal surface protein C (PspC, also designated as CbpA), and detoxified pneumolysin toxoid (PdT) in the presence of cholera toxin elicited the secretion of IL-17 by $CD4^+$T cells in mice and protected against nasal colonization [131]. Lu and colleagues also tested a conjugate vaccine based on a PsaA-PdT fusion protein and the cell-wall polysaccharide by the nasal route. This vaccine could also stimulate IL-17 responses and high antibody production against the three antigens. Protection could be finally shown in the nasal colonization and the fatal aspiration pneumonia model [133]. Beside immunization with recombinant protein, a live attenuated *Salmonella* vaccine strain heterologously expressing PsaA on its surface was used to analyse its protective potential. BALB/c or C57BL/6 mice vaccinated either orally or intranasally exhibited a significant reduction in colonization of nasopharyngeal tissues after intranasal challenge with *S.*

pneumoniae serotype 6B and 23. In contrast, none of the vaccine constructs provided protection against intraperitoneal challenge with *S. pneumoniae* serotype 3 [134]. Moreover, three different lactic acid bacilli (LAB) strains expressing surface-attached PsaA were used to immunize mice intranasally with following colonization challenge with *S. pneumoniae* serotype 6B. All lactobacilli-based vaccines except the recombinant *L. lactis* strain significantly reduced pneumococcal nasal colonization [135].

Besides PsaA, other surface-localized lipoproteins of pneumococci have been investigated regarding their protective efficacy as vaccine antigens. In 2000, Overweg *et al.* isolated a pool of hydrophobic, surface-associated proteins of *S. pneumoniae* and analyzed the opsonophagocytic activity of hyperimmune serum raised against this protein fraction. The opsonophagocytic activity was serotype-independent and mainly caused by three proteins: choline-binding protein PspA, and the two lipoproteins PpmA and AmiA. Thus, they concluded that these proteins might have potential to elicit protective immune responses as opsonophagocytic activity correlated with *in vivo* protection [136]. Later, by analysis of acute and convalescent phase sera from young children (< 2 years) related to otitis media infection, it was shown that both lipoproteins PpmA and SlrA are immunogenic and elicit antibody responses early in life [137]. This was confirmed in another study where the association between specific antibodies against pneumococcal virulence proteins, colonization, and respiratory tract infections (RTI) was assessed in young children. Increased levels of IgG could be identified for e.g. PsaA, PpmA and SlrA, which were furthermore associated with a decreased number of RTIs [138]. Moreover, the Gram-positive Enhancer Matrix (GEM)-based vaccine system was used to display PpmA, SlrA and IgA1 protease (IgA1p) on the surface of a non-recombinant, killed *L. lactis*-derived strain [139]. Significant protection against fatal pneumococcal pneumonia could be shown in mice following an intranasal immunization with the SlrA-IgA1p or trivalent vaccine combinations without any additional adjuvants [140].

The two iron uptake ABC transporter substrate-binding lipoproteins PiuA and PiaA were investigated in a systemic infection model with *S. pneumoniae* D39. Recombinant PiuA and PiaA in conjunction with Alum as adjuvant were administered intraperitoneally to mice followed by pneumococcal challenge. Immunized mice showed a significant increase of survival time while this effect augmented when both antigens were used together [141]. In addition, mucosal immunization of mice with PiuA and PiaA elicited specific antibody responses in serum and respiratory secretions, and protected against intranasal challenge with *S. pneumoniae* [142]. Mice were also protected after passive immunization with antisera raised against these proteins and following systemic pneumococcal challenge leading to the assumption that protection is antibody-mediated [141]. Indeed, anti-PiaA and anti-PiuA antibodies increase complement-independent and -dependent opsonophagocytosis of

different pneumococcal serotypes. As these antibodies did not alter growth of *S. pneumoniae* in cation-depleted medium, it was suggested that vaccination with PiaA and PiuA protects against *S. pneumoniae* infection by inducing antibodies that promote bacterial opsonophagocytosis rather than inhibiting iron transport [142].

The highly conserved putative nucleoside ABC transporter binding protein PnrA (SP_0845 in *S. pneumoniae* TIGR4) was shown to be accessible to antibodies in encapsulated pneumococcal strains. In addition, subcutaneous immunization with recombinant PnrA induced high titers and functional antibodies in mice while these sera were able to promote killing of encapsulated pneumococcal strains in a blood bactericidal assay. Mice immunized with PnrA were protected against heterologous challenge with serotype 3 and serotype 14 strains in a murine sepsis model (Table 2) [85]. Thus, PnrA might me a suitable candidate for future vaccines.

As IL-17-secreting $CD4^+$ T cells (T_H17) mediate resistance to mucosal colonization by multiple pathogens including *S. pneumoniae*, an expression library containing >96% of predicted pneumococcal proteins was screened. Thereby, antigens recognized by T_H17 cells from mice immune to pneumococcal colonization were identified. Two lipoproteins, the maltose/maltodextrin ABC transporter binding protein MalX and the putative polar amino acid ABC transporter binding protein SP_0148, were among the identified antigens which provided protection from pneumococcal colonization [143]. As a lot of pneumococcal lipoproteins have already been identified to elicit protective immune responses, they are promising candidates for innovative and serotype-independent vaccines in the future.

The most prominent pneumococcal protein candidates investigated in phase I/II clinical trials include PhtD, the choline-binding protein a (PcpA) and the highly detoxified pneumolysin mutant (dPlyD1). Vaccine formulations containing these proteins were safe and immunogenic, and PhtD could also trigger production of functional post-immune human antibodies [144-146]. Another multivalent recombinant subunit protein vaccine in a phase I trial is IC47 (Intercell AG, Austria, NCT00873431), which contains the highly conserved lipoprotein pneumococcal surface adhesion A (PsaA), serine/ threonine protein kinase (StkP) and a putative murine hydrolase (PcsB). It was shown to be safe and induced protective antibodies against all three proteins in a dose-dependent manner [84]. Despite these promising outcomes, not any protein-based vaccine has reached phase III clinical trials yet, not to mention being on the way of licensing. So far, only polysaccharide-protein conjugate vaccines (PCVs) and the polysaccharide vaccine PPV23 are commercially available and recommended by the World Health Organization (WHO) [147]. However, both have major shortcomings like serotype replacement, limited serotype coverage, and high costs caused by complex manufacturing processes [84]. For this reason, development of new generation

vaccines, which can provide serotype-independent protection against pneumococcal infections and are affordable for especially developing countries, is of highest concern.

Conclusions and Perspectives

Lipoproteins represent an important class of cell envelope proteins crucial for pneumococcal fitness, colonization and pathogenesis. Loss of function of either Lgt or Lsp, essential for lipoprotein maturation, strongly affects in vitro bacterial fitness and pneumococcal virulence in experimental mouse models of infection. The detailed analysis of lipoprotein functions indicated their variety regarding their function in cellular and pathogen-host functions. Strikingly, lipoproteins, irrespective whether they are part of an ABC transporter or “classical” lipoproteins, are highly conserved among pneumococci and immunogenic. These lipoproteins affect further both the innate and acquired immune system via TLR2 signalling or by stimulating the production of protective antibodies. Thus, several pneumococcal lipoproteins have been extensively evaluated as vaccine candidates. In addition, the essentiality of lipoprotein maturation enzymes for the survival and virulence of *S. pneumoniae* supports them as attractive and broad spectrum antimicrobial targets.

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Figures

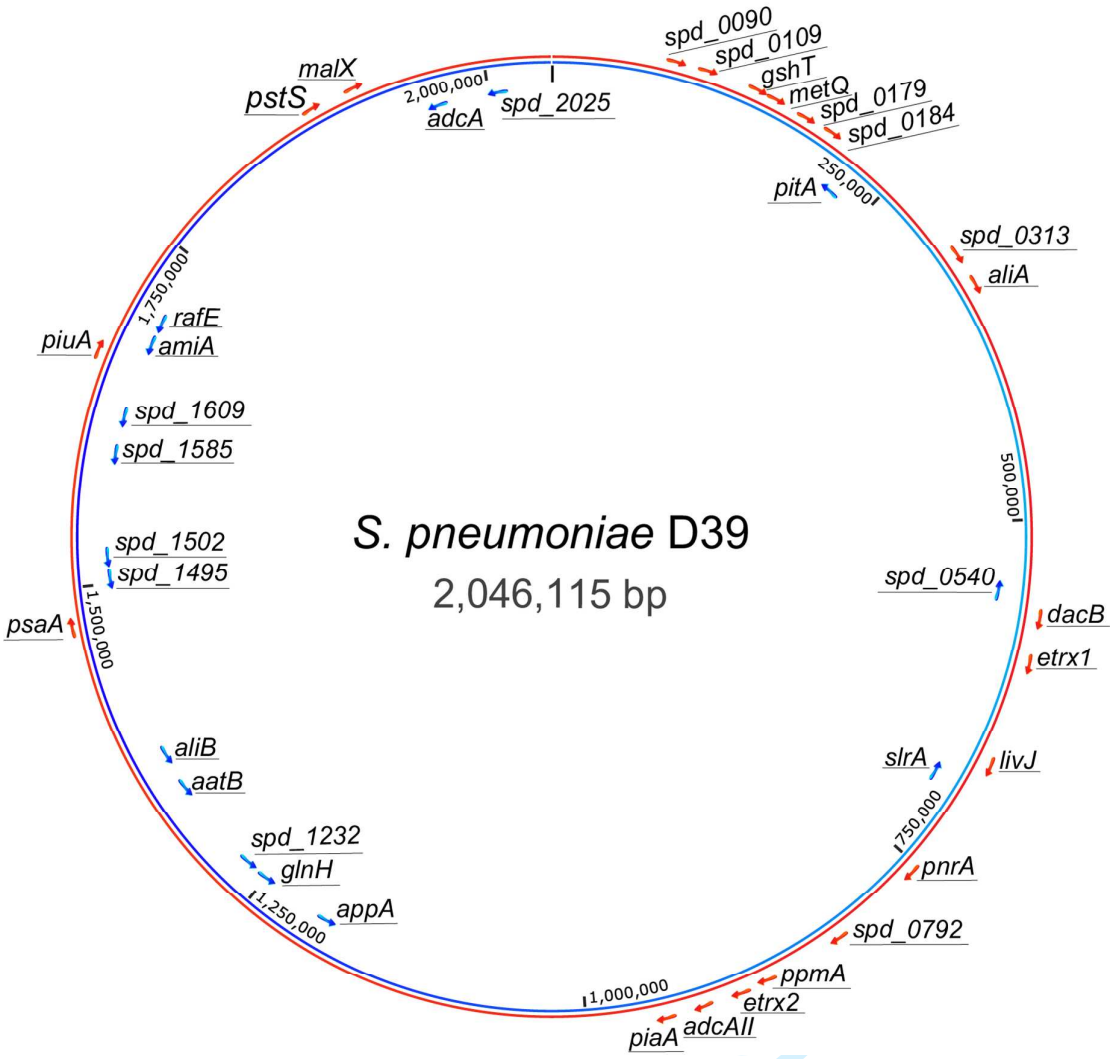


Fig. 1. Chromosomal distribution and orientation of lipoproteins in *S. pneumoniae*. Lipoproteins genes localization and orientation are depicted as arrows, not drawn at scale, in a circular illustration of the pneumococcal genome. The color code of the arrows and the circle represents the coding strand (red) and the reverse strand (blue).

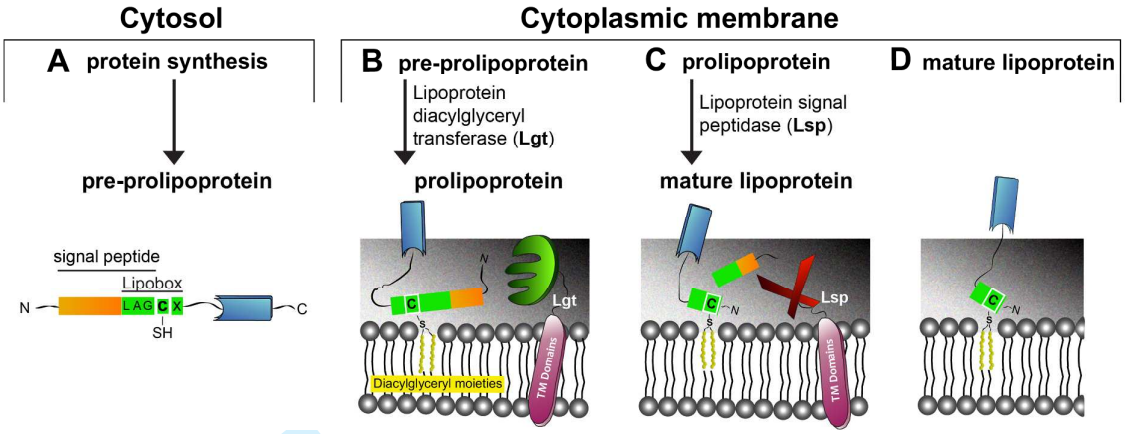


Fig. 2. Generation and processing of lipoproteins in Gram-positive bacteria.
(A) The precursor of the lipoproteins (pre-prolipoprotein) is synthesized in the cytosolic compartment of the bacterial cell with a characteristic signal peptide in the N-terminus conformed by a conserved lipobox motif. (B) The pre-prolipoprotein is translocated across the cytoplasmic membrane by either the sec or tat pathway and is anchored to the cell membrane by the addition of diacylglyceryl moieties (lipidation) to the thiol group of an invariant cysteine residue in the lipobox via the lipoprotein diacylglyceryl transferase (Lgt). (C) Following lipidation, the signal peptide is cleaved off the prolipoprotein by the lipoprotein signal peptidase II (Lsp), resulting in a final mature lipoprotein. (D) The new mature lipoprotein has now the lipidated cysteine residue as the new N-terminus.

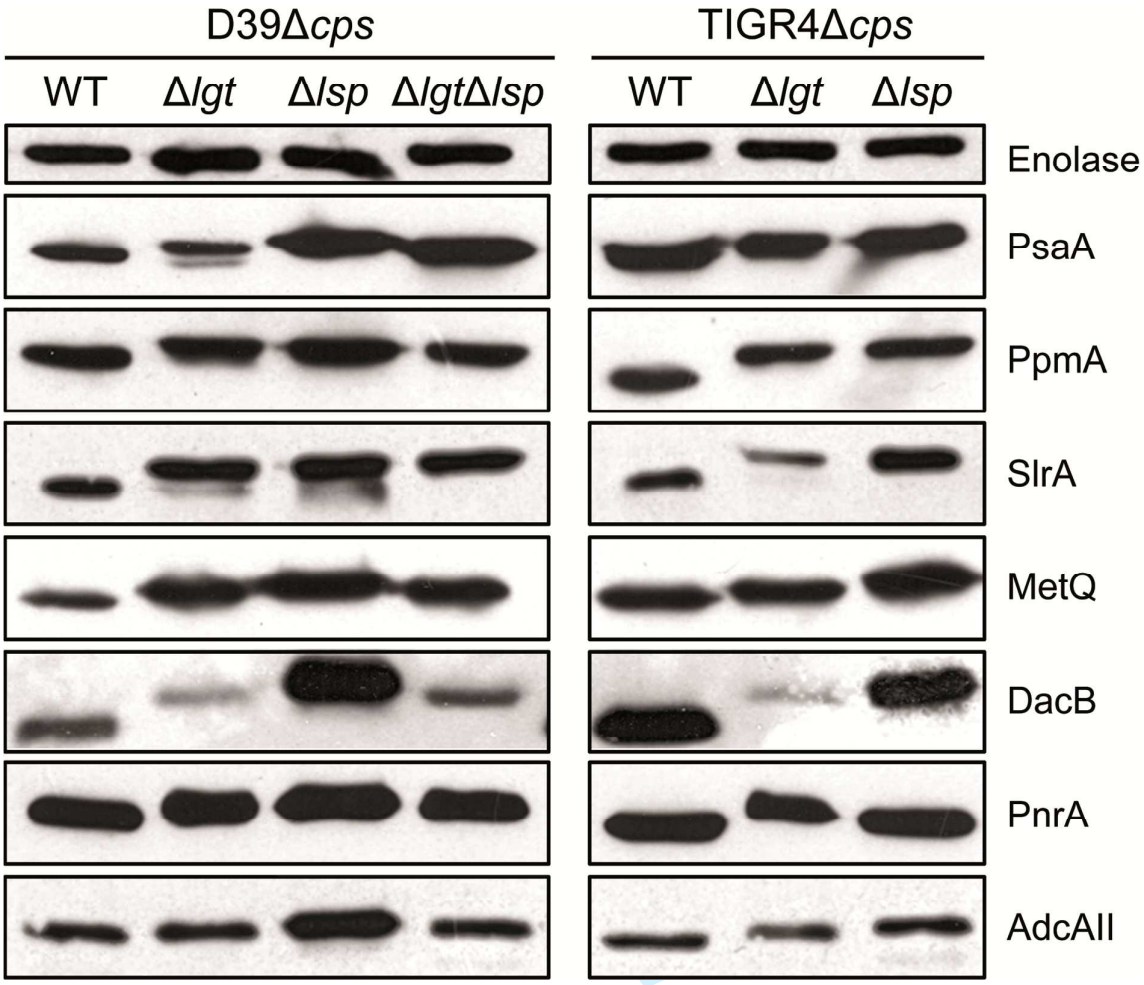


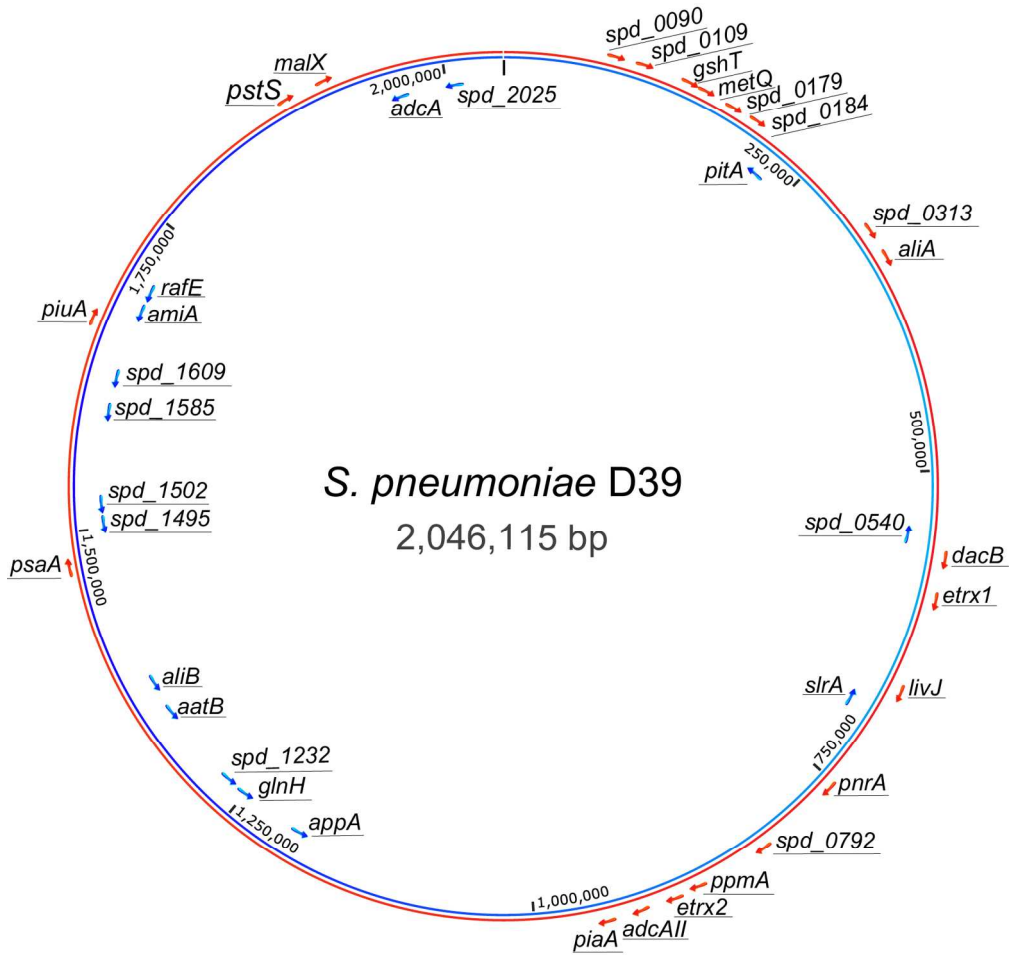
Fig. 3. Effects of mutations in the lipoprotein maturation on processing and localization of pneumococcal lipoproteins.
Immunoblot analysis of bacterial lysates from parental strains D39Δ*cps* and TIGR4Δ*cps* and their isogenic mutants D39Δ*cps*Δ*lgt*, D39Δ*cps*Δ*lsp*, D39Δ*cps*Δ*lgt*Δ*lsp* and TIGR4Δ*cps*Δ*lgt*, TIGR4Δ*cps*Δ*lsp* are shown. Specific polyclonal antibodies raised against the pneumococcal lipoproteins PsaA, PpmA, SlrA, MetQ, DacB, PnrA, and AdcAII were used to study molecular weight and localization of the protein species observed in the parental strain and its mutants. Enolase acted as a loading control and was detected with a specific rabbit anti-enolase antibody. As secondary antibody a peroxidase-coupled anti-mouse or anti-rabbit antibody was used. Bacteria were cultured in THY supplemented with appropriate antibiotics, harvested at an OD_{600} of 0.7, and $1x10^8$ cells were loaded for detection.

Table 1. Selected Non-ABC transporter lipoproteins and their impact on pneumococcal pathogenesis.

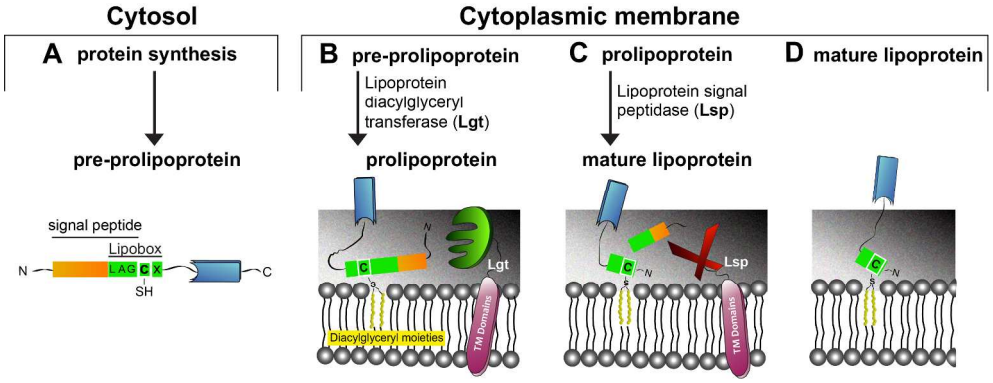
locus (TIGR4/D39)	NCBI annotation	protein	description; molecular function	pathogenic function	references
SP_0771 SPD_0672	SPD_RS03575	SlrA	streptococcal lipoprotein rotamase A; cyclophilin-type peptidyl-prolyl cis-trans isomerase	adherence, colonization; anti-phagocytosis	[15, 19, 39]
SP_0981 SPD_0868	SPD_RS04630	PpmA (PrsA)	putative proteinase maturation protein A; peptidyl-prolyl cis-trans isomerase		[15, 18, 39]
SP_0659 SPD_0572	SPD_RS03065	Etrx1 (TlpA)	extracellular thioredoxin-like protein 1; thiol-disulfide oxidoreductase	extracellular oxidative stress resistance; colonization, pulmonary & systemic infection; anti-phagocytosis	[15, 21, 60]
SP_1000 SPD_0886	SPD_RS04725	Etrx2	extracellular thioredoxin-like protein 2; thiol-disulfide oxidoreductase		
SP_0629 SPD_0549	SPD_RS02935	DacB	L,D-Carboxypeptidase; Muramoyl-tetrapeptide carboxypeptidase	antibiotic resistance; adherence, colonization, pulmonary & systemic infection; anti-phagocytosis	[15, 17, 40, 52]

Table 2. Selected ABC transporter lipoproteins and their pathogenic role.

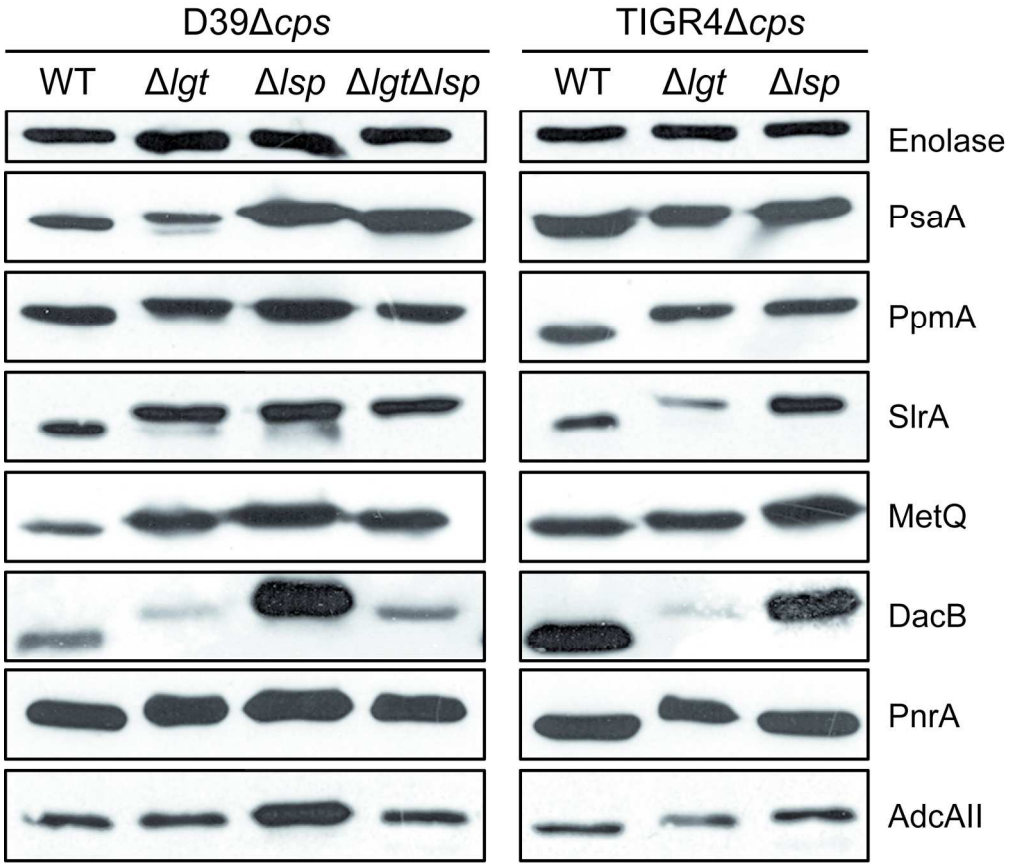
locus (TIGR4/D39)	NCBI annotation	protein	description; substrate specificity		pathogenic function	references
SP_1032 SPD_0915	SPD_RS04885	PiaA (Pit2A)	pneumococcal iron acquisition protein A; iron		pulmonary & systemic infection	[15, 32, 66]
SP_1872 SPD_1652	SPD_RS08740	PiuA (Pit1A)	pneumococcal iron uptake protein A; iron		pulmonary infection	[15, 32, 65, 66]
SP_0243 SPD_0226	SPD_RS01215	PitA	pneumococcal iron transporter protein A; iron(III)		systemic infection	[32, 67]
SP_1650 SPD_1463	SPD_RS07720	PsaA	pneumococcal surface adhesin A; manganese(II), zinc(II)		oxidative stress resistance; adherence (?), colonization; pulmonary & systemic infection	[15, 20, 32, 70-76]
SP_2169 SPD_1997	SPD_RS10490	AdcA	adhesin competence protein A; zinc(II)		pulmonary & systemic infection (single mutants: attenuated; double mutant: avirulent)	[15, 32, 78-80, 82]
SP_1002 SPD_0888	SPD_RS04735	AdcAII	adhesin competence protein AII; zinc(II)			[15, 32, 78, 79, 81, 82]
SP_1891 SPD_1671	SPD_RS08840	AmiA	aminopterin resistance locus protein A	oligopeptides	colonization; systemic infection (AliA)	[15, 32, 64, 65]
SP_0366 SPD_0334	SPD_RS01780	AliA (PlpA)	amiA-like protein A (formerly permease-like protein A)			
SP_1527 SPD_1357	SPD_RS07140	AliB	amiA-like protein B			
SP_0845 SPD_0739	SPD_RS03910	PnrA	pneumococcal nucleoside receptor A; ribonucleosides		systemic infection	[15, 32, 85, 86]
SP_0149 SPD_0151	SPD_RS00835	MetQ	methionine-binding lipoprotein Q; D-/L-methionine, DL-homocysteine		pulmonary & systemic (?) infection	[15, 32, 88-92]



Chromosomal distribution and orientation of lipoproteins in *S. pneumoniae*.
Lipoproteins genes localization and orientation are depicted as arrows, not drawn at scale, in a circular illustration of the pneumococcal genome. The color code of the arrows and the circle represents the coding strand (red) and the reverse strand (blue).
Figure 1
155x147mm (300 x 300 DPI)



Generation and processing of lipoproteins in Gram-positive bacteria.
(A) The precursor of the lipoproteins (pre-prolipoprotein) is synthesized in the cytosolic compartment of the bacterial cell with a characteristic signal peptide in the N-terminus conformed by a conserved lipobox motif. (B) The pre-prolipoprotein is translocated across the cytoplasmic membrane by either the sec or tat pathway and is anchored to the cell membrane by the addition of diacylglyceryl moieties (lipidation) to the thiol group of an invariant cysteine residue in the lipobox via the lipoprotein diacylglyceryl transferase (Lgt). (C) Following lipidation, the signal peptide is cleaved off the prolipoprotein by the lipoprotein signal peptidase II (Lsp), resulting in a final mature lipoprotein. (D) The new mature lipoprotein has now the lipidated cysteine residue as the new N-terminus.
Figure 2
255x95mm (300 x 300 DPI)



Effects of mutations in the lipoprotein maturation on processing and localization of pneumococcal lipoproteins.\r\nImmunoblot analysis of bacterial lysates from parental strains D39Δ*cps* and TIGR4Δ*cps* and their isogenic mutants D39Δ*cps*Δ*lgt*, D39Δ*cps*Δ*lsp*, D39Δ*cps*Δ*lgt*Δ*lsp* and TIGR4Δ*cps*Δ*lgt*, TIGR4Δ*cps*Δ*lsp* are shown. Specific polyclonal antibodies raised against the pneumococcal lipoproteins PsaA, PpmA, SlrA, MetQ, DacB, PnrA, and AdcAII were used to study molecular weight and localization of the protein species observed in the parental strain and its mutants. Enolase acted as a loading control and was detected with a specific rabbit anti-enolase antibody. As secondary antibody a peroxidase-coupled anti-mouse or anti-rabbit antibody was used. Bacteria were cultured in THY supplemented with appropriate antibiotics, harvested at an OD600 of 0.7, and 1x10^8 cells were loaded for detection.

Figure 3

173x149mm (300 x 300 DPI)