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EXTRACELLULAR VIRULENCE FACTORS OF *STREPTOCOCCUS PNEUMONIAE*

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1. ABSTRACT

Streptococcus pneumoniae is one of the major human bacterial pathogens. Current prophylactic agents against this pathogen are limited in their protective abilities and the role of therapeutics has been inadequate as resistant strains emerge. The development of new and improved therapies to combat the pneumococcal disease is necessary. In order to accomplish this, an understanding of the interactions between this bacterium and the host tissues is essential. Such interactions largely involve extracellular virulence factors that are expressed by the pathogen to interact with the host. These virulence factors include those based on sugars (glycome-based) as their building blocks, and proteins that are built from amino acids (proteome-based). The first group includes primarily the capsule, teichoic and lipoteichoic acids. The second group is diverse and includes numerous surface proteins that are attached to the cell wall of pneumococci utilizing a variety of methods. For the purpose of this review these surface proteins were divided into three categories, proteins bound to peptidoglycan, those bound to choline residues present

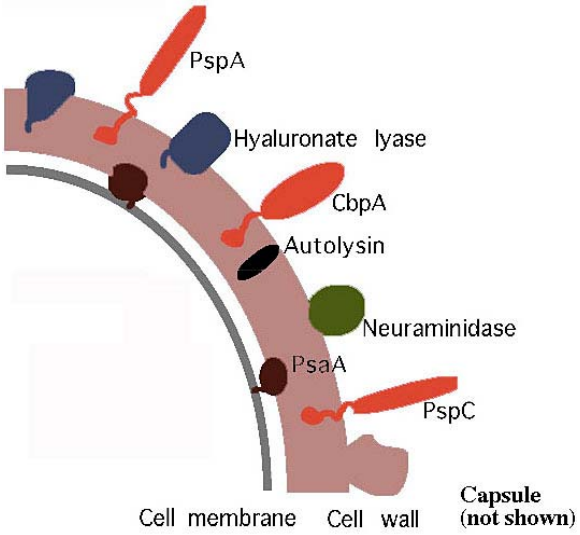

Figure 1. Diagram of the surface of the cell wall of *Streptococcus pneumoniae*. The capsule (not colored), murein layer, and cytoplasmic membrane are shown. Examples of proteins attached to the cell wall through various attachment modes are also illustrated.

on the surface of penumococci, and those bound to the lipids of the cytoplasmic membrane.

Both the glycome-based and protein-based virulence factors are described, analyzed, and represented graphically. Whenever possible, structural properties of these molecules were introduced.

2. INTRODUCTION

Streptococcus pneumoniae is a Gram-positive bacterium pathogenic to humans (1, 2). The organism colonizes the nasopharyngeal cavity, its normal environment for growth. However, under certain conditions this bacterial pathogen turns infectious and invades its human hosts usually through the lungs. Once in the lungs, these bacteria produce toxins that facilitate further spread into other tissues, primarily into the blood stream and the nervous system, including the brain (2). As a consequence, *S. pneumoniae* affects a wide variety of tissues and has the ability to cause bacteremia and meningitis, in addition to regular pneumonia (3, 4). Current prophylactic agents include two vaccines, one based solely on polysaccharides of the 23 most common disease causing strains in the United States (5-8), and the other, a conjugate vaccine of an inactivated protein toxin (diphtheria toxin) linked covalently with capsular polysaccharides. In the second case the number of polysaccharides is limited, due to the expense and the technical difficulties in producing such conjugates; to date only 7 of the main disease causing serotypes of *S. pneumoniae* have been used (9).

The cytoplasm of *S. pneumoniae* is encased by phospholipid bilayer surrounded by the outer most part of the pneumococci, a cell wall composed of peptidoglycan layers. The peptidoglycan layers are built from repeating units of N-acetyl glucosamine and N-acetyl muramic acid (10, 11). This polymeric structure is connected by cross bridges, which often contain unusual amino acid residues such as D-alanine (12-14). In addition, the surface of this bacterium contains teichoic and lipoteichoic acids protruding through the peptidoglycan layers (15, 16). The terminal parts of these (lipo)teichoic acid chains contain choline residues. The most external part of the cell is a capsule, which is built from certain glycans, often branched and with well-defined repeat units (17). The capsule is often highly electronegatively charged, however, neutral or zwitterionic capsules have also been observed. The capsule is considered the primary virulence factor of this bacterium and at least ninety different serotypes have been observed so far (18). It becomes clear that the capsule takes part in various interactions with the host cells and tissues. Host cells also express a variety of polysaccharides as well as proteins that are utilized by this bacterium for interaction within the host environment.

The cytoplasmic membrane and the cell wall, in addition to defining the boundaries of the bacterium, serve to anchor many proteins displayed on the surface (19). These proteins play a role in the elaborate interaction between bacterial cells and the host tissues. Such protein-protein or protein-polysaccharide interactions complement those of the capsule. In general, the variability of the capsule is greater than that of cell surface proteins, as antibodies against one protein of a certain serotype elicit cross protection against the majority, if not all, other serotypes. For this reason cross protectivity of such proteins might be utilized in either a new generation of vaccines that are under development or in those vaccines still to be developed (1, 19). The surface proteins expressed by the bacteria are utilized in various ways such as the degradation of host tissues, protection from recognition by the immune system, or even providing necessary nutrients in the environment of bacterial cells.

3. KNOWN PNEUMOCOCCAL VIRULENCE FACTORS

3.1. Structure of cell-wall of Gram-positive bacteria

Gram-positive bacteria have several layers of peptidoglycan, known also as murein, surrounding their cytoplasmic membrane. This moiety provides for structural rigidity of bacteria and facilitates the maintenance of elevated turgor pressure within pneumococcal cells, among other functions (Figure 1). The thickness of the murein varies from 150 to around 300 Å, with one layer of peptidoglycan being approximately 10 Å thick (10). The layer of peptidoglycan is built from repeating units of N-acetyl glucosamine and of N-acetyl muramic acid connected through a beta 1,4 glycosidic linkage (Figure 2). The individual strands/layers of such polysaccharides are crosslinked to adjacent layers or strands by covalent linkage of N-acetyl muramyl residues by amino acids, some of them in the unusual D-conformation. The individual amino acid composition of the crossbridges is diverse and strain dependent. Examples of residues commonly found in such crossbridges are L-alanine, D-isoglutamate, L-lysine (12-14). The composition of crossbridges appears to be serotype specific as well as

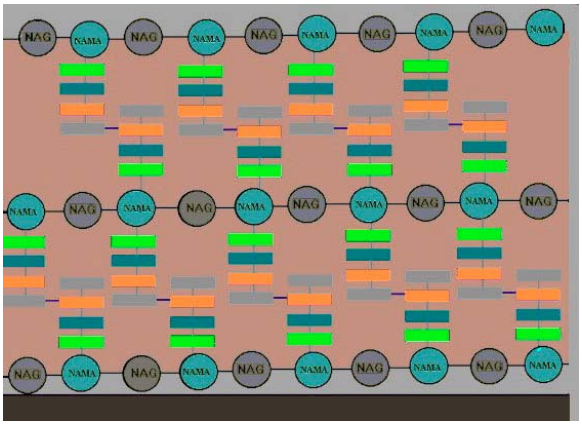

Figure 2. Schematic structure of peptidoglycan of layers of pneumococcal cell wall. The building blocks of glycan strands are N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAMA) connected by a beta 1,4 glycosidic linkage. The glycan layers are connected by linked peptide crossbridges covalently attached to the muramyl residue (shown as elongated rectangular boxes). The crossbridges vary in composition and in the number of amino acid as well as in the crosspeptide linkage.

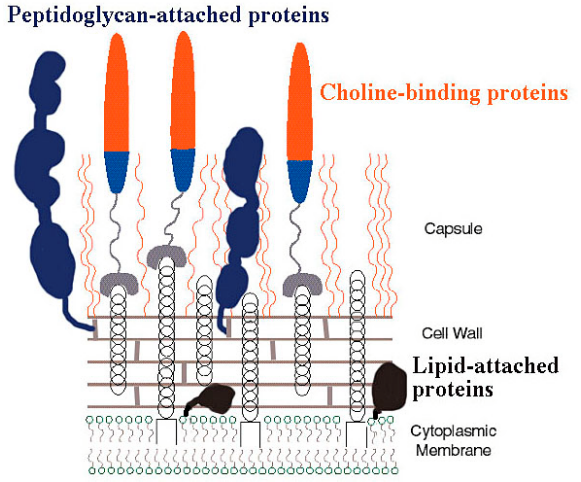

Figure 3. Modes of attachment of surface proteins to the cell wall of glycome- and protein-based virulence factors. Capsule and (lipo)teichoic acids are attached through distinct covalent linkages of unknown nature. Proteins are attached using direct peptidoglycan covalent linkage (in blue color), choline-binding domains and choline molecules of (lipo)teichoic acids (in red/blue color), and covalent lipid attachment (in brown color) as shown.

specific for the genus (20). The large number of pneumococcal serotypes identified produce unique peptidoglycan structures in addition to unique and different capsular types. The length of the crossbridging peptides varies from as short as three to significantly longer, such as a tri-tetrapeptide. The crossbridges originating from different muramic acid residues are linked usually through alanyl-serine and analyl-alanyl linkages.

The cell wall of *S. pneumoniae* can be extracted by, for example, solubilization with detergent, such as sodium dodecyl sulfate, and additional procedures yielding relatively pure preparations of pneumococcal cell wall – peptidoglycan, also known as murein sacculus. The isolated sacculus preserves the shape of pneumococcal cells (21). The murein layers are constantly being hydrolyzed by various hydrolases, which accounts for the bacteria's constantly synthesis of new layers of peptidoglycan. As a consequence, the murein layers are continuosly being renewed. This process of peptidoglycan turnover is important for cell division and growth.

Unlike their Gram-negative counterparts, Gram-positive bacteria do not have a clearly defined periplasmic space. However numerous molecules are associated with the murein layer of the cell wall, making the cell wall of these organisms an integrated periplasmic space (11, 22). There are numerous (macro)molecules covalently attached to peptidoglycan and its layers or other (macro)molecules protruding through such layers. Examples are teichoic acid (15) and capsule (23) which are directly covalently linked to the peptidoglycan (24, 25) (Figure 3). The lipotechoic acid structures (16) are anchored to the cytoplasmic lipidic membrane by covalent attachment, but still protrude thorough the cell wall (23, 24, 26). Another group are protein molecules directly attached to the peptidoglycan crossbridges via a covalent linkage that involves a signaling sequence, such as LPxTG, which guides other enzymes present in the cell wall, sortases, to form such linkages (27-29). In addition, pneumococci have proteins attached to choline resides associated with (lipo)teichoic acid structures. These proteins utilize an additional protein repeat domain, termed choline-binding domains, to attach themselves to bacterial cells (1, 19, 30, 31). Also, there are proteins directly bound covalently to lipid bilayer of the cytoplasmic membrane of *S. pneumoniae*. They are also packed inside cell wall peptidoglycan (32). There are likely other molecules attached to pneumococci by still unknown mechanisms that are located within the cell wall. However, the majority of these (macro)molecules (glycans and proteins), if not all, are virulence factors for this organism as antibodies directed towards them are often protective against pneumococcal disease. Such a role is a logical property of these (macro)molecules which are located on or close to the pneumococcal surface and can therefore interact with both host tissues and the immune system. These (macro)molecules are described in more detail below. This review largely focuses on the structural properties of such virulence factors. However, pathogenic aspects as well as public health issues are also discussed.

3.2. Glycome-based virulence factors

3.2.1. Capsule and capsular-type polysaccharide

Capsular polysaccharides (CPS) constitute the outer most part of *S. pneumoniae* and creates a hydrated shell that surrounds the spherical coccus of the non-encapsulated part of this organism. It extends approximately 200 to 400 nm from the cell wall (23). CPS is built from monosaccharides connected by glycosidic linkages (linear or branched) as well as some nonsugar components. These are predominantly anionic structures

(as is the case for the majority of pathogenic bacteria), however neutral or zwiterionic capsular polysaccharides are also observed. Functionally, CPS modulates interaction between the bacterium and the environment, including the interaction with the host and its tissues such as adherence to the host tissues, transport of molecules and ions to and from the bacterium, and the formation of biofilms and microcolonies built from individual bacteria. As a consequence of its functional properties CPS is often considered the primary virulence factor of *S. pneumoniae*. The capsule and is also known to have antiphagocytic properties. CPS is connected to the pneumococci through an unknown covalent linkage to the peptidoglycan of the bacterial cell wall. There is some doubt if the capsule of the serotype 3 is an exception to this rule and is not covalently linked to peptidoglycan (25).

As of 1998, 90 different types or strains of *S. pneumoniae* had been observed and each can have specific type of capsule (reviewed in 17). These different strains, characterized by the different capsules, were divided into 46 groups numbered 1 to 48 (26 and 30 are not used). The CPS are polymeric and are built from repeating units of oligosaccharides. They can reach a large size and high molecular weight. The molecular composition of all 90 capsular types (serotypes) of pneumococcus is shown in Table 1 (18). The most frequent monosaccharides found in CPS are: D-glucose, D-galactose, L-rhamnose, N-Acetyl-D-glucosamine, N-Acetyl-D-galactosamine, N-Acetyl-D-mannosamine, N-Acetyl-D-fucosamine, and D-glucuronate. Less frequently found sugars are: L-fucose, D-ribose, D-galacturonate, N-Acetyl-D-manosaminuronate, N-Acetyl-D-pneumosamine, and 2-acetamido-4-amino-2,6-didioxy-D-*xylo*-hexos-4-ulose. All sugars, except D-galactose and D-ribose, are based on a 6-atom pyranose ring. D-galactose can occur in both pyranose and 5-atom member furanose ring structures; D-ribose exists only based on a furanose ring form. In addition, phosphate can be present either as a phosphodiester bridge between saccharides or as a CPS backbone subsistent. All CPS polysaccharides with known structures except 7A, 7F, 14, 33F, and 37, are polyanionic due to negatively charged groups such as uronic acid, phosphate, and pyruvate. *S. pneumoniae* type 37 has the only known homoglycan-based capsule in this species. The capsules of serotypes 7A, 7F, 14, 33F, and 37 are either neutral or zwiterionic in their electrostatic properties. The detailed description of the chemical structure of all ninety capsular polysaccharides is described and reviewed by Kamerling, 2000 (17, 18). All fresh clinical isolates of pneumococci are encapsulated (smooth). Non-encapsulated bacteria (rough) are vastly less virulent than their encapsulated counterparts (33, 34).

The structural properties of capsular polysaccharides were also investigated by X-ray diffraction (35-38), nuclear magnetic resonance (NMR) spectroscopy (39-45), and three-dimensional modeling calculations (37, 39-43). As an example the X-ray fiber diffraction of type 3 CPS concluded that this polysaccharide has a structure of a 3-fold helix with a repeat of 9.2 Å. Subsequent computational conformation analysis suggested a left hand orientation of this helix (35). Also, a crystalline neutral tetrasaccharide alpha-D-Gal*p*-(1→3)-alpha-D- Glc*p*-(1→3)-alpha-L-Rha*p*-(1→4)-D-Rib-ol derived from CPS of serotype 6B was investigated by X-ray diffraction. These studies suggested a linear planar chain for this polysaccharide (36, 46). Additional hints about three-dimensional structural properties of polysaccharides, including those of bacterial capsules, are structures of protein complexes interacting with such sugar moieties. The first such enzyme complex was the structure of *S. pneumoniae* hyaluronate lyase with the disaccharide products of hyaluronan degradation (47-50). This structure was later supplemented by a complex of hexasaccharide unit of hyaluronan with the same enzyme (49, 51) (Figure 4). In this structure complex hyaluronan assumes a left-handed helix-like conformation with at least 4 disaccharides for a full turn. The hyaluronan structure is, however, distorted by the interaction with the enzyme's long cleft like binding site (49, 51-53). Even though we currently do not know any pneumococci with hyaluronan as a capsule constituent, there are other bacteria even within the *Streptococcus* genus, such as certain strains of group A streptococcus, that do have hyaluronan as the only component of their capsule (54, 55). Therefore, availability of a relatively long capsular polysaccharide structure at high resolution of X-ray diffraction is important and essential. For many of the serotypes studied, CPS is known to be attached to pneumococci through an unknown attachment to peptidoglycan (25, 56).

3.2.2. Teichoic and lipoteichoic acids

Pneumococci have two additional polysaccharide types that are antigenic: teichoic acid or C-polysaccharide (C-substance) (15) and lipoteichoic acid or F-antigen (Forssman antigen) (16). Structurally the teichoic acid part (polysaccharide part) of both entities is identical (57). The molecular composition of both molecules is shown in Table 2 (18).

The F-antigen is attached to pneumococci through a covalent bond to the cytoplasmic membrane, an ester linkage to fatty acids of the lipid bilayer (23, 24, 26). The C-antigen is not attached to lipid but to the peptidoglycan. The nature of such linkage is not known (24). However it is equally distributed on both sides of the murein layer (23, 58). The nature of this linkage is most likely different than that of capsular attachment to peptidoglycan (25, 59).

The (lipo)techoic acid is often utilized by pneumococci as one of the modes of attachment of its macromolecules such as proteins of the bacterial cell (1). Examples of such molecules attached to *S. pneumoniae* utilizing (lipo)techoic acid are pneumococcal surface protein A (PspA) (60), N-acetylmuramyl-L-alanine amidase LytA (61, 62), or choline binding protein C (CbpC) (also known as pneumococcal surface protein C (PspC)) (63-65). The choline binding module of the surface proteins consists of approximately 20 amino acid repeats, usually at the C-terminus of surface proteins of *S. pneumoniae* (1, 19, 30, 31). The number of repeats varies from as few as two, for example for *S. pneumoniae* LytA amidase, to as many as twelve. The structure of the

Table 1. Primary structures of capsular polysaccharides of Streptococcus pneumoniae

Serotype[a]				Primary structure[b]
Danish		American		
1		1		→3)-AAT-α-D-Gal*p*-(1→4)-α-D-Gal*p*A-(1→3)-α-D-Gal*p*A-(1→ +0.3 OAc
2		2		→4)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→ 2 ↑ 1 α-D-Glc*p*A-(1→6)-α-D-Glc*p*
3		3		→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→
4		4		→3)-β-D-Man*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*2,3(*S*)Pyr-(1→
5		5		→4)-β-D-Glc*p*-(1→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Sug*p*-(1→ 3 ↑ 1 α-L-Pne*p*NAc-(1→2)-β-D-Glc*p*A
6	A	6		→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-D-Rib-ol-(5→*P*→
	B	26		→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→4)-D-Rib-ol-(5→*P*→
7	F	51		→6)-α-D-Gal*p*-(1→3)-β-L-Rha*p*2Ac-(1→4)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→ 2 4 ↑ ↑ 1 1 β-D-Gal*p* α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*
	A	7		→6)-α-D-Gal*p*-(1→3)-β-L-Rha*p*2Ac-(1→4)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→ 4 ↑ 1 α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*
	B	48		→6)-α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→2)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→*P*→ 3 ↑ 1 β-D-Rib*f*-(1→4)-α-L-Rha*p*
	C	50		No information
	8	8		→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Gal*p*-(1→
9	A	33		→4)-α-D-Glc*p*A-(1→3)-α-D-Gal*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→
	L	49		→4)-α-D-Glc*p*A-(1→3)-α-D-Gal*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-(1→
	N	9		→4)-α-D-Glc*p*A-(1→3)-α-D-Glc*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-(1→
	V	68		→4)-α-D-Glc*p*A-(1→3)-α-D-Gal*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→ : : : 2Ac (17%) 4Ac (6%) 2Ac (3%) 3Ac (25%) 6Ac (55%) 3Ac (4%)
10	F	10		→6)-β-D-Gal*f*-(1→3)-α-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→3)-α-D-*Gal*p-(1→*2*)-D-Rib-ol-(5→*P*→ 6 ↑ 1 β-D-Gal*f* β-D-Gal*p* 1 ↓ 6
	A	34		→5)-β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→2)-D-Rib-ol-(5→*P*→ 3
	B			No information
	C			No information
	11	F	11	→6)-α-D-Glc*p*NAc3Ac0.5-(1→4)-α-D-Gal*p*2Ac-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 4 ↑ +0.5 OAc Rib-ol-(1→*P*
		A	43	→6)-α-D-Glc*p*2/3Ac-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 4 ↑ +OAc Gro-(1→*P*
		B	54/76	→6)-α-D-Glc*p*NAc3Ac0.5-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 4 ↑ +0.5 OAc Rib-ol-(1→*P*
		C	53	→6)-α-D-Glc*p*NAc3Ac-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 4 ↑ Gro-(1→*P*
		D		No information
	12	F	12	→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→4)-β-D-Man*p*NAcA-(1→ 3 3 ↑ ↑ 1 1 α-D-Gal*p* α-D-Glc*p*-*(1→2)*-α-D-Glc*p*
		A	83	→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→4)-β-D-Man*p*NAcA-(1→ 3 3 ↑ ↑ 1 1 α-D-Gal*pNAc* α-D-Glc*p*-(1→2)-α-D-Glc*p*

Table 1 (continued)

Serotype[a]			Primary structure[b]
Danish		American	
	B		No information
	13	13	→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*2/3Ac-(1→3)-β-D-Gal*f*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-Rib-ol-(5→*P*→
	14	14	→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 4 ↑ 1 β-D-Gal*p*
15	F	15	→3)-α-D-Gal*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 3 ↑ Cho$_{0.2}$→*P* +2 OAc
	A	30	→3)-α-D-Gal*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 3 ↑ Gro$_{0.7}$-(2→*P*
	B	54	→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 4 ↑ +0.7 OAc 1 α-D-Gal*p*-(1→2)-β-D-Gal*p* 3 ↑ Cho$_{0.2}$→*P*
	C	77	→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→ 4 ↑ 1 α-D-Gal*p*-(1→2)-β-D-Gal*p* 3 ↑ Cho$_{0.2}$→*P*
16	F	16	Constituents: Glc, Gal, Rha, GlcN, GalN, and Gro-*P*
	A	85	No information
17	F	17	→3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→3)-β-L-Rha*p*2Ac-(1→4)-α-L-Rha*p*-(1→2)-D-Ara-ol-(1→*P*→ 4 ↑ 1 α-D-Gal*p*
	A	78	→3)-β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→3)-β-L-Rha*p*2Ac-(1→4)-α-L-Rha*p*-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*f*-(1→ 4 2 ↑ ↑ 1 1 β-D-Gal*p* α-D-Glc*p* Gro-(1→*P* ↓ 3
18	F	18	→4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*2Ac-(1→ 2 ↑ 1 α-D-Glc*p*6Ac D-Gro-(1→*P* ↓ 3
	A	44	→4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*NAc-(1→3)-β-L-Rha*p*-(1→ 2 ↑ 1 α-D-Glc*p* D-Gro-(1→*P* ↓ 3
18	B	55	→4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→ 2 ↑ 1 α-D-Glc*p* Gro-(1→*P* ↓ 3
	C	56	→4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→ 2 ↑ 1 α-D-Glc*p*6Ac$_{0.3}$
19	F	19	→4)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→*P*→
	A	57	→4)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→*P*→ α-L-Fuc*p*-(1→*P* ↓ 3 →4)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→*P*→ 2 ↑ β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→*P*
	B	58	→4)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→4)-β-D-Man*p*NAc-(1→4)-α-L-Rha*p*-(1→*P*→ 3 ↑ 1 β-D-Rib*f*-(1→4)-α-L-Rha*p*

Table 1 (continued)

Serotype[a]			Primary structure[b]
Danish	American		
			β-D-Glc*p* 1 ↓ 6
	C	59	→4)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→4)-β-D-Man*p*NAc-(1→4)-α-L-Rha*p*-(1→*P*-→ 3 ↑ 1 β-D-Rib*f*-(1→4)-α-L-Rha*p*
	20	20	→6)-α-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Gal*f*-(5,6Ac$_2$)-(1→3)-β-D-Glc*p*-(1→3)-α-D-Glc*p*NAc-(1→*P*-→ 4 ↑ 1 β-D-Gal*f*
	21	21	Constituents: Glc, Gal, and GlcN
22	F	22	→4)-β-D-Glc*p*A-(1→4)-β-L-Rha*p*2Ac$_3$-(1→4)-α-D-Glc*p*-(1→3)-α-D-Gal*f*-(1→2)-α-L-Rha*p*-(1→ 3 ↑ 1 α-L-Glc*p*
	A	63	No information
			Gro-(2→*P* ↓ 3
23	F	23	→4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-β-L-Rha*p*-(1→ 2 ↑ 1 α-L-Rha*p*
	A	46	No information
	B	64	No information
24	F	24	Constituents: Glc, Rha, GlcN, Rib, and Rib-ol-*P*
	A	65	Constituents: Cho-*P*
	B	60	No information
25	F	25	Constituents: Gal, GalA, GlcN, and GalN
	A		No information
27		27	→3)-β-D-Glc*p*NAc4,6(*S*)Pyr-(1→3)-α-D-Gal*p*-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→ 2 ↑ Cho→*P*
28	F	28	Constituents: Glc, Rha, Gro, and Cho-*P*
	A	79	Constituents: Cho-*P*
29		29	→4)-β-D-Gal*p*NAc-(1→6)-β-D-Gal*f*-(1→3)-β-D-Gal*p*-(1→6)-β-D-Gal*f*-(1→1)-D-Rib-ol-(5→*P*-→
31		31	→2)-β-L-Rha*p*-(1→3)-β-D-Gal*f*-(1→3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*NA-(1→3)-β-D-Gal*f*-(1→
32	F	32	→4)-β-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-β-L-Rha*p*2Ac-(1→ 2 3 ↑ ↑ α-L-Rha*p*-(1→*P* Cho→*P*
	A	67	→4)-β-D-Glc*p*-(1→3)-α-D-Glc*p*4Ac-(1→4)-β-L-Rha*p*2Ac-(1→ 2 3 ↑ ↑ α-L-Rha*p*-(1→*P* Cho→*P*
33	F	70	→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→3)-β-D-Gal*f*(2Ac$_{0.4}$)-(1→3)-β-D-Glc*p*-(1→5)-β-D-Gal*f*-(1→ 2 ↑ 1 α-D-Gal*p*
	A	40	No information
	B	42	→6)-β-D-Glc*p*-(1→5)-β-D-Gal*f*-(1→3)-β-D-Gal*p*NAc-(1→4)-α-D-Gal*p*-(1→2)-D-Rib-ol-(5→*P*-→ +OAc 2 ↑ 1 α-D-Gal*p*
	C	39	No information
	D		No information
34		41	→3)-β-D-Gal*f*-(1→3)-α-D-Glc*p*-(1→2)-β-D-Gal*f*(6Ac$_{0.5}$)-(1→3)-α-D-Gal*p*-(1→2)-D-Rib-ol-(5→*P*-→
35	F	35	No information
	A	47/62	→3)-β-D-Gal*p*-(1→3)-β-D-Gal*f*(5,6Ac$_2$)-(1→3)-β-D-Glc*p*-(1→6)-β-D-Gal*f*(2Ac-(1→1)-Man-ol-(6→*P*-→
	B	66	→4)-β-D-Gal*p*NAc-(1→6)-β-D-Gal*f*-(1→3)-β-D-Glc*p*-(1→6)-β-D-Gal*f*(2Ac$_{0.5}$)-(1→1)-Rib-ol-(5→*P*-→
	C	61	No information
36		36	No information
37		37	→3)-β-D-Glc*p*-(1→ 2 ↑ 1 β-D-Glc*p*
38		71	No information
39		69	No information
40		45	No information
41	F	38	No information
	A	74	No information
42		80	No information
43		75	No information
44		81	No information
			Gro-(1→*P*-→6)-β-D-Glc*p*NAc 1 ↓ 4
45		72	→3)-α-D-Gal*p*-(1→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→2)-α-L-Rha*p*-(1→ 6 ↑ 1 α-D-Gal*p*
46		73	Constituents: D-Gal, D-GalNAc, D-GlcNAc, and L-FucNAc
47	F	52	No information
	A	84	No information
48		82	No information

[a] Based on Kamerling, 2000 (18), [b] Abbreviations used are: AATGal, 2-acetamido-4-amino-2,4,6-trideoxygalactose; Ara-ol, arabinitol; Fuc, fucose; FucNAc, *N*-acetylfucosamine; Gal, galactose; GalA, galacturonic acid; GalN, galactosamine; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; Gro, glycerol; ManNAc, *N*-acetylmannosamine; ManNAcA, *N*-acetylmannosaminuronic acid; Man-ol, mannitol; PneNAc, *N*-acetylpneumosamine, 2-acetamido-2,6-dideoxytalose; Rha, rhamnose; Rib, ribose; Rib-ol, ribitol; Sug, 2-acetamido-2,6-dideoxy-*xylo*-hexos-4-ulose; Ac, acetate; Cho, choline; *P*, phosphate; Pyr, pyruvate; *p*, pyranose; *f*, furanose.

Table 2. Primary structures[a] of the C-polysaccharide and F-antigen of *Streptococcus pneumoniae*

	Primary structure
C	Cho→ *P* (↓ 6) Cho→ *P* (↓ 6) H[→6)-*β*-D-Glc*p*-(1→3)-AAT-*α*-D-Gal*p*-(1→4)-*α*-D-Gal*p*NAc-(1→3)-*β*-D-Gal*p*NAc-(1→1)-D-Rib-ol-(5→P→]$_n$.....peptidoglycan
F[b]	Cho→ *P* (↓ 6) Cho→ *P* (↓ 6) H[→6)-*β*-D-Glc*p*-(1→3)-AAT-*α*-D-Gal*p*-(1→4)-*α*-D-Gal*p*NAc-(1→3)-*β*-D-Gal*p*NAc-(1→1)-D-Rib-ol-(5→P→]$_{2\text{-}8}$→6) -*β*-D-Glc*p*-(1→3)- -AAT-*β*-D-Gal*p*-(1→3)-*α*-D-Glc*p*-(1→3)-acyl$_2$Gro

[a] For abbreviations of monosaccharides see Table 1; based on Kamerling, 2000 (18). [b]I dentified fatty acids in the diacylglycerol moiety are 12:0, 14

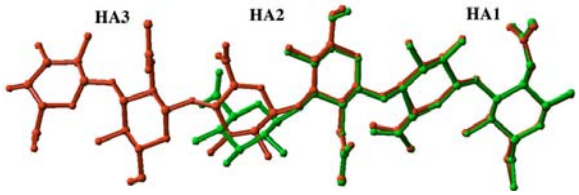

Figure 4. Structure of hyaluronan saccharide as seen in the complex with the pneumococcal hyaluronate lyase enzyme. The structure represents a bound conformation of hyaluronan to hyaluronate lyase enzyme (51; pdb codes: 1LOH and 1LXK) as tetra- and hexasacchride units (two structures overlaid together). The reducing and non-reducing ends of the hyaluronan chains and the consecutive disaccharide units numbered from the reducing end as HA1, HA2, and HA3 are labeled. The atom spheres are color-coded using standard criteria (green: carbon, blue: nitrogen, red: oxygen). The pyranose rings are in a boat conformation with the exception of the terminal sugars at the non-reducing end, which are in distorted boat conformation due to the presence of an unsaturated bond between carbons C4 and C5 (result of the production process of the tetra- and hexasaccharide). The sugar chain assumes a distorted left-handed helix like conformation with at least four disaccharide hyaluronan units per helix turn. The sugar chain is distorted, in part, due to the interactions with the enzyme especially the non-reducing end.

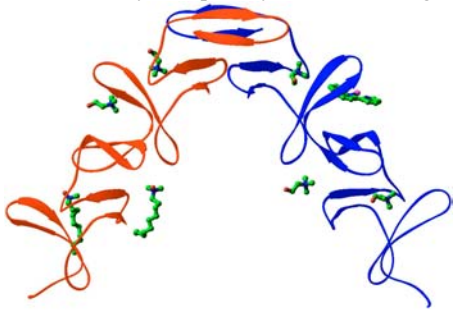

Figure 5. Structure of choline-binding domain of *S. pneumoniae* LytA amidase. The drawing is based on three-dimensional X-ray structures of LytA dimeric choline-binding domain (red and blue, respectively) (66, 67; pdb code: 1GVM). Two monomers compose one functional unit of the domain for LytA. The structure is built from □eta-sheet structures with choline-binding (or choline analogs) (shown in ball and stick fashion) the regions between the sheet structures. There are four choline-binding sites per monomer.

choline-binding part of LytA was elucidated structurally by means of X-ray diffraction (Figure 5) (66, 67). LytA is an example of the domain organization of many pneumococcal surface proteins that have usually three components or modules, a surface attachment module, a linker (usually flexible), and a functional module (1, and see below). The choline binding domain(s) is only one example of an attachment module to the surface of this bacterium.

3.3. Proteome-based virulence factors - surface proteins

3.3.1. Introduction

A variety of proteins or enzymes displayed on the surface of bacteria, including *S. pneumoniae*, contribute to their virulence and are involved in pathogenesis as well as the disease process. Examples of such protein function(s) are interactions with the host tissues or concealing the bacterium from the host's protective mechanisms. The polysaccharide capsule was considered until very recently to be the primary virulence factor of *S. pneumoniae*. One of the reasons for this was that rough pneumococci are nearly completely harmless when compared to their smooth counterparts. Identification of surface proteins, and their antigenic properties, provided an alternative for the composition of new prophylactic vaccines and therapeutics. Such surface proteins include hyaluronate lyase (Hyl) (68-70), two neuraminidases (NanA and NanB) (71), major autolysin (LytA) (72), pneumococcal surface protein A (PspA) (60, 73-75), choline binding protein A/pneumococcal surface protein C (CbpA/PspC) (63-65), and pneumococcal surface antigen A (PsaA) (76) (Figure 1 and 3). Antibodies generated to these proteins are often cross-protective against numerous serotypes of *S. pneumoniae*, and these proteins elicit a longer T-cell memory that their capsule counterparts. As such, these surface proteins are now considered virulence factors of the pneumococci along with the polysaccharide-based capsule. Some of these proteins such as PspA or PsaA have already been shown to elicit significant protection in animals and humans and show a significant promise for an alternative vaccine. These proteins are described in more detail below. In addition, a description of genome identification of essentially all such surface proteins, which are candidates for a novel pneumococcal vaccine to be developed, is provided (19). These proteins are grouped into four major groups: peptidoglycan bound proteins (Hyl, NanA), proteins attached through choline-binding domains (LytA,

PspA, CbpA/PspC), those covalently linked to cytoplasmic membrane (PsaA), and a small group of other types of proteins (*e.g.*, pneumolysin). The molecules belonging to these attachment-based groups are described in more detail below.

3.3.2. Peptidoglycan-bound molecules

Proteins attached to peptidoglycan have a distinct signature sequence usually at their C-terminus, LPxTG (x - any amino acid) or similar (*i.e.*, VPxTG, IPxTG, YPxTG) proceeded by hydrophobic residues and followed by charged residues which are recognized by an extracellular protease/transpeptidase, termed sortase (77-80). The sortase enzyme cleaves the Thr-Gly peptide bond and covalently links the remaining part of the enzyme to the crossbridges of the peptidoglycan and leaves it exposed extracellularly following the release of the ~30 amino-acid C-terminal tail. The hydrophobic and the charged residues likely help in proper orientation (81) of the protein with respect to cytoplasmic membrane of pneumococci. Below I describe two relatively well-studied examples of such pneumococcal proteins: hyaluronate lyase, Hyl, and neuraminidase, NanA (1).

3.3.2.1. Hyaluronate Lyase

3.3.2.1.1. General properties

Hyaluronate lyase (Hyl) is one of the major surface, antigenic proteins of this organism (1, 82) and is produced by most pneumococcal strains (11, 83, 84, 122). Studies on the precise role of this enzyme in pneumococcal disease are still in progress and are consistent with its major role during the progress of infection, especially in crossing barriers between tissues and spreading throughout the host (85). The enzyme, by degrading hyaluronan, a major component of extracellular matrix of tissues, is directly involved in host invasion by facilitating the organisms in their ability to penetrate the host's physical defenses and subsequently spread to its tissues (86-88). In laboratory cultures of *S. pneumoniae*, the enzyme is found in both cell-associated fractions and in culture supernatant, suggesting that part of the enzyme is released by the pathogen to surrounding host tissues. This property most likely directly facilitates infection and invasion of the host (11).

The enzyme is one of a broader group of enzymes called hyaluronidases which facilitate host tissue invasion by breaking down components of the matrix outside of cells; primarily the extracellular matrix (ECM) hyaluronan. This "spreading factor" property was first suggested by Duran-Reynals (89, 90). The increase in tissue permeability after hyaluronidase treatment appears to play a significant role in wound infections, as well as diseases such as pneumonia, bacteremia, or meningitis. During recent years the sequences of hyaluronidases of both prokaryotic and eukaryotic origin have been determined, and the functional properties were studied in detail. In general, eukaryotic hyaluronidases appear to use hydrolysis in order to degrade the extracellular barriers while the bacterial enzymes utilize an elimination mechanism. *S. pneumoniae* hyaluronate lyase, for example, cleaves the beta1,4-glycosidic linkage between N-acetyl-beta-D-glucosamine and D-glucuronic acid residues in hyaluronan (polymer of D-glucuronic acid(1-beta-3)*N*-acetyl-D-glucosamine(1-beta-4)) and also in certain chondroitins and chondroitin sulfates (polymer of D-glucuronic acid(1-beta-3)*N*-acetyl-D-galactosamine(1-beta-4) sulfated at positions 2, 4, or 6 of the galactosamine moiety). The results of this enzyme catalysis are unsaturated polysaccharides. For hyaluronan the final degradation product is a disaccharide unit, 2-acetamido-2-deoxy-3-O-(beta-D-gluco-4-enepyranosyluronic acid)-D-glucose, (91, 92).

3.3.2.1.2. Hyaluronan

Hyaluronan, the primary substrate for pneumococcal hyaluronidase (hyaluronate lyase), is a major and ubiquitous component of the ECM in essentially all vertebrates as well as being a component of capsules in some bacteria. This polymer is present in every tissue or fluid of higher animals, including humans (93). Hyaluronan has more than one function. It is a structural component of the extracellular space and, due to it ability to absorb water, has viscoelastic properties that are found to be important in, for example, the joints of animals as they protect and cushion surrounding structures. It also plays a role in restriction of free diffusion and movement of molecules in the extracellular space. However, hyaluronan, by interacting with other molecules such as CD44 or RHAMM hyaluronan receptors (94-96) has additional functions that involve the immune system, fertilization, embryonic development, cell migration and differentiation, wound healing, inflammation, and growth and metastasis of tumor cells (97, 98). For example hyaluronan levels on endothelial cells, lung fibroblasts, and other cell surfaces are controlled by various cytokines and the regulation of production and degradation of hyaluronan is crucial for life (99-101). Another known substrate for hyaluronate lyases are the unsulfated chondroitin and chondroitin sulfates, primarily sulfated in positions -4 or -6 of the galactosamine moiety. Chondroitins and chondroitin sulfates have similar viscoelastic properties to hyaluronan and, therefore, have related mechanical function(s) (91, 92, 102, 103).

3.3.2.1.3. Mechanism of attachment to pneumococci

The well studied Hyl of *S. pneumoniae* type 23 has a molecular weight of 107, and 89 kDa when expressed in *Escherichia coli* (11, 68, 69). As expected, the carboxy-terminus of the mature enzyme contains a hydrophobic tail proceeded by a signature LPqTG sequence, Leu-919 to Gly-923, and a group of charged residues responsible for anchoring the enzyme through covalent attachment to peptidoglycan crossbridges (104, 105).

3.3.2.1.4. Hyaluronate lyase structure

The truncated but functional 89 kDa form of the *S. pneumoniae* type 23 Hyl enzyme containing 724 amino acids (plus 6 His tag), of the full length enzyme (1066 amino acids including the signal peptide for the genomic TIGR4 strain) was crystallized (69, 70) and the three-dimensional crystal structure determined using X-ray diffraction (47). The crystallized enzyme lacks the first two domains at the N-terminus, namely the carbohydrate-binding domain and the spacer domain (106). The C-terminal truncation of the crystallized form lacks only

several amino-acid residues past Glu891 responsible for the anchoring to the peptidoglycan crossbridges (69, 70, 105).

The determined pneumococcal Hyl enzyme structure contains two domains: an N-terminal domain composed primarily of alpha-helices arranged in an $alpha_5$/$apha_5$-barrel structure and the C-terminal domain built from beta-sheets arranged in a four-layered beta-sandwich (Figure 6). A large, deep and long cleft spans the wider side of the barrel structure of the entire helical domain (47, 49, 53). Its size perfectly complements the size of a hyaluronan chain and was found to accommodate and bind as many as three disaccharide units of the substrate (49, 53). This cleft was determined to be responsible for substrate binding and degradation (47, 48, 51, 52). The surface of the cleft region of the protein is predominantly positively charged due to the accumulation of a large number of positively charged residues on the cleft surface. This positive charge complements the negatively charged hyaluronan and facilitates the binding, largely through charge-charge and hydrophobic interactions involving amino acid residues located in the cleft of the enzyme and the polymeric hyaluronan substrate.

3.3.2.1.5. Catalytic cleft and mechanism of action

The catalytic residues of the enzyme were identified in the central part of the cleft using structural and site-directed mutagenesis studies (47). These catalytic residues are Asn349, His399, and Tyr408. They are responsible for the substrate degradation through a proton acceptance and donation mechanism (PAD) (Figure 6b and c) (47, 49, 50, 53). In addition to these three catalytic residues identified in the cleft, additional residues were found to be involved in important aspects of the enzyme's action: a patch of hydrophobic residues, Trp291, Trp292, and Phe343, as well as a patch of residues generating negative potential and the end of the cleft, Glu388, Asp398, and Thr400 (Figure 6c). The hydrophobic patch positions the substrate for catalysis, whereas the negative patch was implicated in the release of the product from the cleft (49, 51, 53). The structure of the enzyme complex with the product of degradation, the disaccharide unit of hyaluronan, and hexa- as well as tetrasaccharides of hyaluronan revealed the exact substrate positioning with respect to the identified residues in the cleft and showed that that the enzyme degrades the substrate starting from the reducing end (47). This degradation process is progressive by endolytic degradation of one disaccharide unit at a time. The consensus of this model of action is initial exolytic binding of the enzyme to the polymeric substrate, hyaluronan or chondroitin(s), followed by an initial cut of the polymer; this 'initial bit' is followed by progressively endolytic degradation from the reduced end towards the non-reducing end of substrate until the polymeric chain is fully degraded (50, 92). The final degradation products are disaccharide units of the substrate.

The proposed PAD mechanism of hyaluronan degradation involves five steps: (i) the enzyme's binding to the substrate (ii) neutralization of the carboxyl group of glucuronic moiety of hyaluronan by Asn349 resulting ina cidification of the C5 carbon atom of the substrate; iii) extraction of the now more acidic C5 proton by His399 resulting in the formation of a double bond between carbon atoms C4 and C5 of hyaluronan, and at the same time (iv) donation of a proton by Tyr408 to the beta 1,4 glycosidic bond between building blocks of hyaluronan causing breaking of this beta 1,4 bond and the resultant (v) cleavage of the disaccharide substrate. These steps are followed by release of product from the active site and the enzyme's return to its original state by proton exchange between the two catalytic residues, His399, and Tyr408, and the water microenvironment (Figure 6c). As a final result, the enzyme is ready for the next round of catalysis of processive degradation of the substrate(s). The substrate is translocated by two disaccharide units toward the reducing end of the chain, and endolytically degraded in the processive manner using the PAD mechanism until all remaining substrate is digested (91, 92).

3.2.2.1.6. Function and movement of the beta-domain and enzyme flexibility

The role of the N-terminal alpha-helical barrel domain was clearly established as a catalytic site as it supports the substrate-binding and catalysis by the formation of the cleft where all this happens. However, the role of the beta-sheet domain of the enzyme is still not clear but it likely modulates the substrate's access to the cleft. The flexibility analysis of the enzyme suggests that the beta-domain can move sufficiently to even cover the cleft entrance. In such case the substrate cannot enter the cleft and bind (51, 52).

In addition, studies of the enzyme's flexibility identified two more types of enzyme motion. One is based on predominantly positively charged residues from the cleft being exposed in a breathing like motion towards the substrate binding area and, as such, likely facilitating the electrostatic attraction of the negatively charged substrate binding to the cleft. Another is based on the twisting motion of the cleft walls moving the hydrophobic patch residues along the sides of the cleft for translation of the substrate by exactly two disaccharide units towards the reducing end. For more details see Ponnuraj and Jedrzejas, 2000 (48), recent reviews by Jedrzejas, 2000, 2001, and 2002 (41, 9, 50, 53), and finally the details of flexibility analysis and processive mechanism of action in Jedrzejas *et al.*, 2002 and Mello *et al.*, 2002 (51, 52).

3.2.2.1.7. Carbohydrate-binding domain

The hyaluronate lyase containing the N-terminal portion was not amendable to crystallization and structure determination (70, 107). However, recent bioinformatics and modeling analysis of this part of the lyase clearly shows a carbohydrate binding ability (106). Between the alpha-domain and the most N-terminal carbohydrate binding region there is an additional linker domain that is thought to act as a spacer. Functionally, the most N-terminal segment is likely responsible for the enzyme's localization in the site of action (similar to enzyme concentration in the site of action) and for facilitation of the processive mechanism by feeding the polymeric substrate,

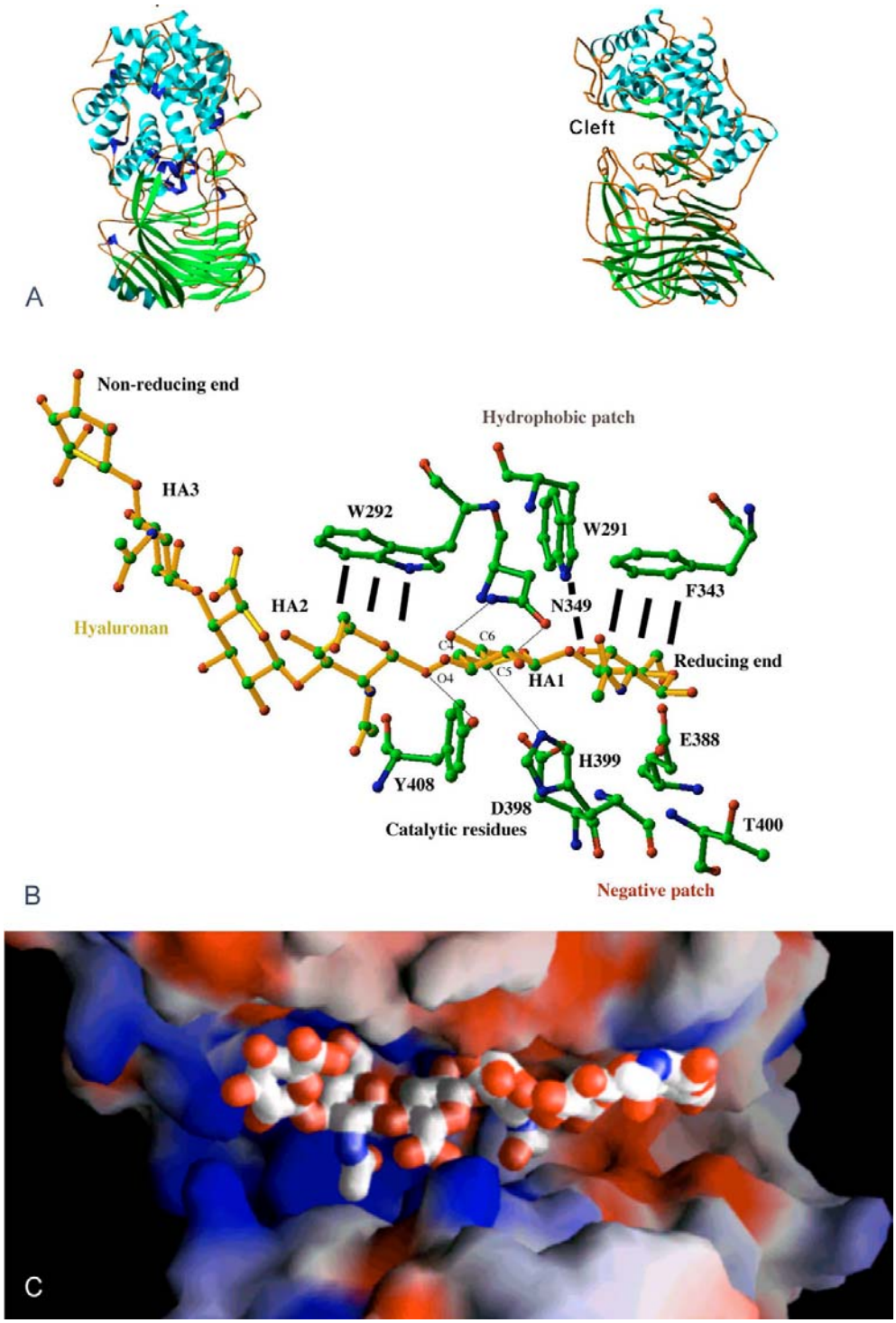

Figure 6. Hyaluronan lyase. A**:** Overall structure. The enzyme is built from an alpha-helical catalytic domain and a supportive beta-sheet domain (pdb code: 1EGU). Both domains are connected by one flexible peptide linker. Two perpendicular views of the enzyme are shown. B**:** Structure of the hexasaccharide substrate in the binding/catalytic cleft. The cleft present at one end of the alpha-domain is where the substrate binds and is being degraded. The positive nature of the cleft and negative charge on the substrate are evident through color-coding of the surfaces (positive potential – blue, negative – red). At the end of one side of the cleft a negative patch is present C**:** Catalytic residues of the enzyme. The residues directly involved in catalysis are Asn349, His399, and Tyr408; positioning of the substrate are Trp291, Trp292, and Phe343; release of the product are Glu388, Asp398, and Thr400. The most essential interactions are shown as black lines.

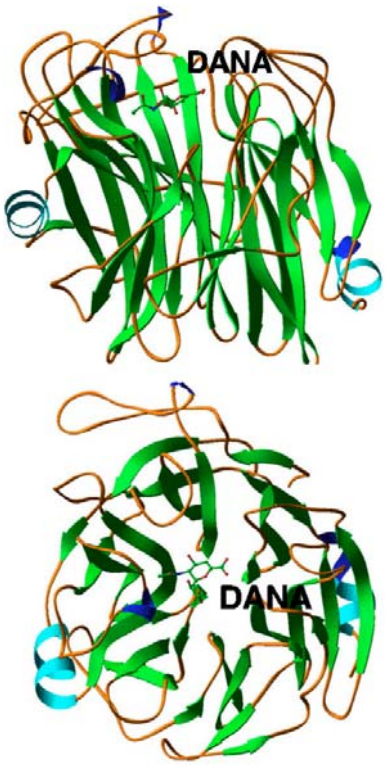

Figure 7. Structure of *Salmonella typhimurium* LT2 neuraminidase. The 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA) is bound in the active site (pdb code: 3SIM). Two perpendicular views of the enzyme are shown.

from the reducing towards the non-reducing end, into the cleft of the catalytic part. In this mechanism the weak binding of the substrate to the catalytic part of the enzyme is necessary for sliding of the enzyme along the substrate, a process in which the enzyme could get detached from the polymer. The N-terminal domain could prevent such substrate detachment. Finally, the extra domain might simply separate the substrate chains in the ECM by spreading them out for easy access of the alpha-helical catalytic domain (19, 106).

3.3.2.2. Neuraminidase

Another example of a peptidoglycan bound surface protein is neuraminidase. The enzyme was found on all examined strains of pneumococci (108-110). The function of the enzyme is based on cleaving off terminal sialic acids from the host cell surface glycans such as mucin, glycolypids, glycoproteins. This function modifies the glycosylation patterns of the host cells causing exposure of the cell surface and surface receptors for possible interaction with *S. pneumoniae* (111). Such action likely facilitates adhesion of the bacterial cell to host cell surfaces or tissues. However, the precise functionality of neuraminidase enzyme in pneumococcal pathogenesis has not been fully established (112). Based on its function, the enzyme should enhance adhesion and therefore colonization of the host tissues.

3.3.2.2.1. Types of neuraminidase enzymes in pneumococci

Two forms of the pneumococcal neuraminidases, NanA and NanB, have been discovered. The NanA enzyme has a molecular weight of ~108 kDa (113) while the NanB is smaller with a molecular weight of ~75 kDa (108). Both of these genes have been cloned and sequenced (Figure 7) (108, 113) and do not exhibit any significant sequence homology. Similarly to hyaluronate lyase, neuraminidases degrade to smaller fragments that still maintain enzymatic activity during *in vitro* growth as well as protein purification. For NanA, for example, fragments as small as 85 kDa were found to be active (71). The activity of NanB is ~100 times smaller than NanA (71, 108). In addition, NanA, but not NanB, contains a C-terminal signature sequence, LPxTG, indicative of binding to peptidoglycan crossbridges suggesting its surface character (Figure 7) (105, 113, 114,). Antibody studies have identified NanA on the surface of pneumococci (25).

Why *S. pneumoniae* has two neuraminidases is unclear, but they likely perform their function in different environments as indicated by different activities of the enzymes at different pH. For example, NanA exhibits its maximum activity at ~pH 5 whereas the maximum activity of NanB is at pH ~7 (108). Pneumococci, therefore, most likely utilize the different enzymes to degrade surface sialic acid of host tissues during various stages of infection or invasion of the host.

3.3.2.2.2. Structural homology

No three-dimensional structural or mechanistic information is available for either of the pneumococcal neuraminidases (NanA or NanB). However, intensive structural and mechanism of action studies have been performed on various forms of viral enzymes (115-118), and the structure of a *Salmonella typhimurium* neuraminidase is also available (Figure 7) (120, 121). The bacterial neuraminidases are monomers in solution whereas the viral ones act on their substrate as tetramers. The neuraminidase of *S. typhimurium* is significantly smaller than the 1035 amino acid NanA, being composed of only 379 residues. Only the three-dimensional structural information and structure-based related studies of both NanA and NanB will likely show the details of their precise function and mechanism of action. Currently the *S. typhimurium* neuraminidase structure can serve as a model that might show some insight into pneumococcal neuraminidase structure and function. The enzyme from *S. typhimurium* consists of six four-stranded antiparallel beta-sheets arranged in a propeller like fashion, which is also characteristic of the viral neuraminidase (Figure 7). The active site is located on the surface in a crevice in the central part of the enzyme. Similarly to viral enzymes, the catalytic site includes three arginine residues responsible for stabilizing the carboxyl group of the substrate, sialic acid. At the bottom of the active site crevice is a tyrosine residue interacting with the substrate pyranose-based ring structure. The N-acetyl group of sialic acid fits in and interacts with a hydrophobic pocket lining the end of the active site opposite to the Arg residues. A Glu residue, Glu231, is proposed to be a proton donor in order to release

sialic acid from the remaining part of carbohydrate chain (121). This is a well-conserved arrangement of residues in the active sites of all neuraminidases.

3.3.3. Choline-bound proteins

Choline binding proteins are attached to S. *pneumoniae* through interactions of a choline-binding domain with the choline present on (lipo)teichoic acid structures on the surface of pneumococci. These proteins have a characteristic modular design composed of a functional module connected through a usually flexible peptide linker to the anchoring module on the surface of this bacterium, a choline-binding domain (CBD). The CBD domain usually is composed of around 10 repeat segments, each around 20 amino acid long, and a hydrophobic tail (30, 31). The flexible linker is often rich in proline residues due to their flexibility as reflected by their presence in a variety of structural turns in proteins. Known examples of proteins with CBDs are PspA, CbpA/PspC, and LytA. Only recently was the structure of the first CBD, a part of LytA, obtained by X-ray crystallography (Figure 5) (66, 67). In this case the structure has a boomerang-like shape composed of two monomeric units. Each monomer is composed of six beta-hairpins and each monomer is capable of binding at least two choline residues, (four are shown in the figure), in the space between the beta-hairpins. The CBD seems to be a more general mechanism of attachment to bacterial surfaces as more bacteria were recently identified to have choline on their surface. Choline was identified among other bacteria (30) and CBDs have been found in organisms such as *Clostridium acetobutylicum*, *Clostridium difficile*, *Streptococcus mutans*, and *Streptococcus downei*.

3.3.3.1. Pneumococcal surface protein A

3.3.3.1.1. General properties

Pneumococcal surface protein A (PspA) is one of the major surface proteins and antigens of *S. pneumoniae* and is produced by all strains examined (122). Antibodies raised against PspA have confirmed its cell-wall associated character. Such antibodies were protective against disease caused by pneumococci (123). The molecule itself shows a significant degree of variability in its molecular size ranging from 67 to 99 kDa depending on the strain (124). Primary sequence analysis indicates a modular protein with four distinct domains: an N-terminal highly-charged alpha-helical, coiled-coil domain (functional module; 288 amino acids for theRx1 strain), a proline-rich, flexible peptide linker domain (83 amino acids in Rx1), ten highly conserved 20 amino acid repeat units (choline-binding domain) and a C-terminal tail of 17 hydrophobic residues (60, 74, 125). The N-terminal functional module is thought to extend out of the cell wall and even protrude through the capsule (60, 74, 125). Secondary structure predictions have shown that the N-terminal domain is highly helical and most likely a coiled-coil structure (60, 74, 125).

3.3.3.1.2. Attachment to the surface

S. pneumoniae bacteria display an unusual surface molecule, a phosphocholine residue, on their cell surface teichoic acid and the membrane bound lipoteichoic acid (126). Studies have shown that PspA attaches itself to *S. pneumoniae* through non-covalent binding to these choline residues via its C-terminal CBD domain consisting of the 10, 20 amino acid repeat region (Figure 5) (60, 74, 127).

3.3.3.1.3. Coiled-coil structure

Studies on the structural properties of PspA as well as a number of other surface exposed molecules of Gram-positive organisms (128) have shown a very high alpha-helical component in their structure. Subsequent sequence analysis revealed repeating seven residue blocks that are characteristic of proteins exhibiting coiled-coil structures. Analysis of other surface molecules of bacterial organisms exhibit predominantly beta-sheets, beta-turns, and random coils with only a small amount of helical conformation (19, 128). Therefore PspA and several other bacterial macromolecules are representative of group of non-standard proteins. The seven-residue repeat or the heptad pattern of PspA has been identified only in the N-terminal portion that is the functional module (125). The N-terminal PspA sequence, as the sequence of other coiled-coils, show this characteristic seven-residue motif (residues are labeled consecutively at these positions as *a,b,c,d,e,f,g*) with hydrophobic residues located at position *a* and *d*, and hydrophilic residues at the remaining five positions (129, 130) (Figure 8). Such sequence based conformational analysis suggests a coiled-coil structure of the N-terminal module of PspA. Investigations of the structural properties of PspA using biophysical methods such as circular dichroism spectroscopy and sedimentation velocity and equilibrium studies confirmed the sequence-based analysis and showed an elongated, rod-like, molecule built from coiled-coils (Figure 9a and b). Computer modeling led to the creation of a three-dimensional model of the molecule and the subsequent elucidation and rationalization of its role and function in the interactions with the host and the pneumococcal infection (60).

The molecule's N-terminal functional part is highly charged and polar. The C-terminal attachment module anchors PspA to the pneumococcal surface. The proline-rich region acts as a tether and allows for greater flexibility and movement of the N-terminal functional module.

3.3.3.1.4. Proposed function

Functionally PspA, a protective antigen for pneumococci, appears to play a crucial role in protecting *S. pneumoniae* from the host complement system, including phagocytosis by macrophages (38, 125, 127). This anti-complement function was attributed to the structural properties of PspA. Biophysical studies together with sensitive modeling studies showed that PspA has a highly polar electrostatic charge resulting in a dipole-like molecule with opposite charges at each end, positive at one and negative charge at the other. Such a propensity allows for the interaction of PspA with the capsular polysaccharides through its electropositive end (charge stabilization through interactions with electronegative capsule; essentially all pneumococcal capsules are negative in charge). Such an interaction of PspA with the capsular sugar points the other end of this rod like molecule away

```
    a   b   c   d   e   f   g
  1                           Met Glu Glu Ser Pro Val Ala Ser Gln Ser
 11           Lys Ala Glu Lys Asp
 16  Tyr Asp Ala Ala Lys Lys Asp
 23  Ala Lys Asn Ala Lys Lys Ala
 30  Val Glu Asp Ala Gln Lys Ala
 37  Leu Asp Asp
 40  Ala Lys Ala Ala Gln Lys Lys
 47  Tyr Asp Glu Asp Gln Lys Lys
 54                   Thr Glu Glu
 57  Lys Ala Ala Leu Glu Lys Ala
 64            Ala Ser Glu Glu
 68  Met Asp Lys Ala Val Ala Ala
 75  Val Gln Gln Ala Tyr Leu Ala
 82  Tyr Gln Gln Ala Thr Asp Lys
 89           Ala Ala Lys Asp Ala
 94  Ala Asp Lys Met
 98  Ile Asp Glu Ala Lys Lys Arg
105  Glu Glu Glu Ala Lys Thr Lys
112  Phe Asn Thr Val Arg Ala Met
119  Val
120                           Val Pro Glu Pro
124                     Glu Gln
126  Leu Ala Glu Thr Lys Lys Lys
133  Ser Glu Glu Ala Lys Gln Lys
140  Ala Pro Glu Leu Thr Lys Lys
147  Leu Glu Glu Ala Lys Ala Lys
154  Leu Glu Glu Ala Glu Lys Lys
161  Ala Thr Glu Ala Lys Gln Lys
168  Val Asp Ala Glu Glu
173                           Val Ala Pro
176                   Gln Ala Lys
179  Ile Ala Glu Leu Glu Asn Gln
186  Val His Arg Leu Glu Gln Glu
193  Leu Lys Glu
196  Ile Asp Glu Ser Glu Ser Glu
203           Asp Tyr Ala Lys Glu
208                           Gly Phe Arg Ala Pro
213               Leu Gln Ser Lys
217               Leu Asp Ala Lys
221  Lys Ala Lys Leu Ser Lys
227  Leu Glu Glu Leu Ser Asp Lys
234  Ile Asp Glu Leu Asp Ala Glu
241  Ile Ala Lys Leu Glu Asp Gln
248  Leu Lys Ala Ala Glu Glu Asn
255                     Asn Asn
257  Val Glu Asp Tyr Phe Lys Glu
264           Gly Leu Glu Lys Thr
269  Ile Ala Ala Lys Lys Ala Glu
276  Leu Glu Lys Thr Glu Ala Asp
283  Leu Lys Lys Ala Val Asn
```

Figure 8. The heptad repeat present in the N-terminal functional module of PspA. The deduced amino acid sequence of the Rx1 PspA is illustrated. The sequence of the first 288 amino acids of the Rx1 PspA are arranged in heptad repeat blocks characteristic of coiled-coil proteins. The residues in the right part of the figure 120-123, 173-175, and 208-212 do not satisfy the requirements for the coiled-coil structure.

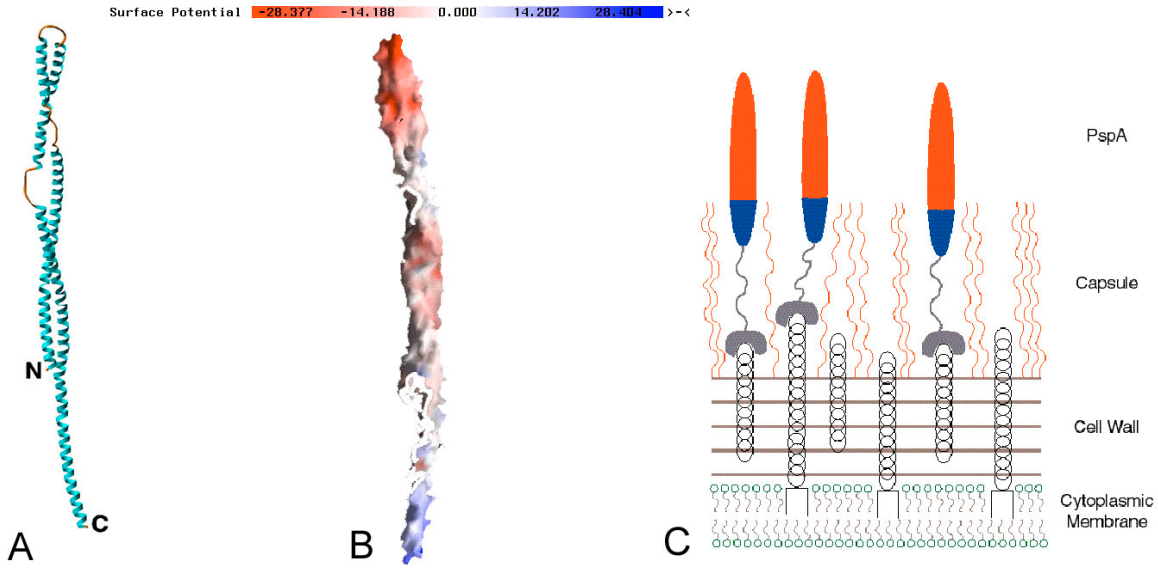

Figure 9. Structural model of PspA. A: Structure of the N-terminal functional module. The N-terminal part of PspA has an elongated rod-like shape built from antiparallel coiled-coil alpha-helices. The drawing is based on the model of a PspA molecule containing amino acids 1 to 288 (60, 74, 75). B: Electrostatic potential distribution. The color-coding of the PspA surface corresponds to the magnitude of the electrostatic potential (blue - positive, red – negative). The model also depicts the highly charged and polar character of PspA. C: Schematic arrangement of PspA molecules on the surface of *S. pneumoniae*. The character of the surface interactions of PspA with teichoic acids and the capsule exposes the highly electronegative end of the molecule outside of the bacterial cell.

from the surface of the bacterium (electrostatic repulsion of negatively charged end with negative capsule). The electronegative end of the molecule is then available for interactions with the environment, including molecules of the host complement system (Figure 9c). Receptor interactions utilizing such highly negatively charged portion of the molecule are highly unlikely. It was proposed that PspA actively repulses binding of the complement proteins and as a consequence, prevents complement activation (60). This proposed function was confirmed by biological evidence demonstrating PspA's anti-complement properties and reduced complement-mediated clearance and phagocytosis of *S. pneumoniae* (132).

Studies with an encapsulated mutant *S. pneumoniae* strain lacking PspA as well as an isogenic pneumococcal strain expressing PspA showed that the $PspA^-$ mutant cells fixed more complement than the isogenic one in which PspA was found to activate C3 but not deposit it on the pneumococci. Similarly, infections of nonimmune mice with $PspA^-$ type 3 capsular *S. pneumoniae* lead to greater early activation of serum complement (as evidenced from increased disappearance of antigenic C3 from the circulation) than infections with $PspA^+$ isogenic parent (132). All these results suggest PspA's ability to reduce complement consumption by pneumococci and thereby lead to their reduced complement-mediated clearance and phagocytosis.

More recently it has been discovered that human lactoferrin (hLfi) has receptor-like properties for the PspA molecule (133-135). Lactoferrin is an iron storage glycoprotein that is predominantly located in mucosal secretions where the level of Fe is limiting for growth. As such, the lack of Fe is considered as a host defense mechanism. Lactoferrin has been shown, on one hand, to inhibit complement activation (similarly to PspA properties) and to suppress immune activity, and on the other hand to do the opposite suggesting that Lfi behavior depends strongly on the environment (134). However, all reports available fail to demonstrate a cross-species significant binding of hLfi and PspA (133). The results indicate that hLfi and PspA-hLfi binding may not play an important role in pneumococcal pathogenesis. The PspA-hLfi binding might be coincidental and not reflective of true function of PspA (133).

All protective monoclonal antibodies (mABs) reactive to PspA on the pneumococcal cell surface were found to bind to the N-terminal functional module of the molecule (136). This part, which is mostly alpha-helical, coiled-coil has also been shown to exhibit more variability due to accumulation of mutations, reinforcing the surface exposed character of PspA and identifying the functional part of PspA as the N-terminal module of the entire molecule.

3.3.3.2. Choline binding protein A

3.3.3.2.1. Structural properties

Choline binding protein A (CbpA) was also termed Pneumococcal surface protein C (PspC) or even novel pneumococcal surface protein, SpsA, (63-65). The molecule is another example of a surface exposed protein that attaches to through a specific choline-binding motif. The choline-binding module from CbpA consists of ten 20 amino acids repeats. CbpA was identified and characterized as a major CBP with properties somewhat similar to PspA. It represents a major part of the mixtures of CBPs isolated from pneumococci (65). Antibodies to CbpA have shown that the molecule is surface exposed with a strong ability to react with both human convalescent antibody and the mouse protective anti-CBP serum. Cloning and production of CbpA showed a molecule consisting of 663 amino acids and a molecular weight of ~75 kDa. Sequence analysis showed the functional N-terminal module consists of amino acids 1-373, which is followed by a proline linker region from amino acid 374 to 403, and by the choline-binding repeat region comprising residues 404-663 (65). Even though there exist striking similarities between CbpA and PspA the primary sequence of the N-terminal module of CbpA is clearly different from that of the PspA N-terminal functional domain. Sequence analysis of the CbpA functional region has shown the existence of a high percentage of alpha-helices arranged in coiled-coils as was found for the PspA molecule. The structural parts of CbpA comprise six alpha-helices and five coiled-coils. Such secondary structure is consistent with an elongated, rod-like molecule similar to PspA (60, 74). Because the structure and biophysical properties of PspA were investigated in greater detail than those of CbpA, at least for a time being, the structural model of PspA can serve as a model for CbpA (Figure 9).

3.3.3.2.2. Functional properties

The functional properties of CbpA are related to adherence to the host tissues, making it a first known pneumococcal adhesin molecule (137). The *cbpA*-deficient mutant cells generated in these studies did not have the ability to interact with cytokine-activated host cells, or with immobilized 6'siallylactose-HSA (6'SL), or with lacto-N-neotetraose-HSA (LnNT). The adhesin properties of the molecule appear to physically bridge the pneumococcal cells and host tissue cell by utilization of the choline of (lipo)teichoic acid at one end and the host glycoconjugates on the other end. This adhesion/bridging interaction seems to be limited to cytokine-activated human cells expressing certain glycocongugates or platelet-activating factor (PAF) receptor.

It is also possible, but perhaps too simplistic, that CbpA as well as the series of other proteins containing CBPs simply block the cell wall choline residues and prevent choline from interacting with the host cells. For example, cytokine-activated human cells express the platelet-activating factor (PAF) receptor, which can bind phosphocholine of the pneumococcal cell wall and therefore act as adhesins (137). It is also obvious that carbohydrates, including the capsule of pneumococci or other glycans of the host cells, play a crucial role in the adhering of bacteria to the host cells. The interactions between bacterial and the host cells are complex, diverse,

and likely tissue specific. CbpA and carbohydrates are possibly only part of the process.

3.3.3.3. Autolysin

3.3.3.3.1. General properties

Autolysins are members of a widely distributed group of enzymes that degrade the peptidoglycan backbone of bacteria. The action of these cell wall-degrading enzymes ultimately leads to cell lysis (138). These enzymes are located in the cell envelope and are postulated to play roles in a variety of physiological cell functions associated with cell wall growth, turnover, and cell separation in microorganisms (139). One of the major functions of this group of enzymes causes significant physiological consequences which leads to cell death (139, 140). *S. pneumoniae* N-acetylmuramoyl-L-alanine amidase, also known as LytA amidase or major autolysin is a representative of this group (72, 141). Arguably, it is the best-characterized enzyme of autolysin group. LytA involvement in the pathogenesis of pneumococci is related to the release of the toxic components of cell wall that are highly inflammatory to some animals (142) as well as release of cytoplasmic proteins of the pneumococcal cell including virulence factors such as the pneumolysin toxin (143, 144). The precise role of LytA in virulence is, however, still under debate (145). Mutations of the *lytA* gene in *S. pneumoniae* chromosome lead to decreased virulence of this organism as compared to wild-type strain (85). Other studies have shown LytA to induce a protective response in mice against streptococci (68, 146, 147). Autolysin was reported to play a major role in middle ear infection (148), but others reported a lack of autolysin influence on the virulence of pneumococci (145). The contribution of LytA varies between different disease states and different animal models (149). There is still clearly some controversy regarding the role of autolysin in pathogenesis and its possible use as a component of a protein based pneumococcal vaccine (150, 151).

The gene for pneumococcal LytA amidase, *lytA*, has been cloned and the protein produced in recombinant form from *Escherichia coli* (152, 153). LytA was found to have a molecular weight of ~36 kDa and a modular organization based on two distinct domains. One domain at the C-terminus, a CBD as described earlier, is composed of six 20-21 amino acid repeats located at the C-terminus end (similar to that of PspA's repeat domain) and is responsible for the attachment to (lipo)teichoic acid residues on the surface of pneumococci. The N-terminal portion is directly responsible for the lytic activity of this enzyme against pneumococcal peptidoglycan (72, 141). However, there is an additional connection between these two domains. The interactions of the enzyme with the choline in the cell wall are essential for the lytic activity (154). It has been suggested that the carboxy-terminal attachment module influences the activity of the enzyme by stabilizing or by inducing its active conformation (155).

3.3.3.3.2. Structural aspects

The three-dimensional structure of entire LytA is unknown. However, the structure of the CBD domain was elucidated by X-ray crystallography (Figure 5) (66, 67). The structure was shown to have a boomerang-like shape and was composed of a dimer. The structure of each monomer was composed of six beta-hairpins and is capable of binding of up to four choline residues in the space between the beta-hairpins. The entire enzyme as well as its domains alone, were extensively characterized by biophysical methods including spectroscopy and analytical sedimentation analysis (71, 141). These studies indicated LytA forms primarily dimers in solution. The monomer/dimer equilibrium shifts towards the formation of more dimeric entities when the choline concentration in solution was increased. No higher order aggregates were reported (62). Analysis of the shape of the dimeric molecule from the sedimentation velocity analysis suggests an elongated, rod-like molecule with the length/width ratio of ~15/1 with dimensions 190 x 13 Å. The catalytic sites were identified to be located at both ends of the elongated rod-like molecule. The shape of LytA most likely facilitates the ease of diffusion of the enzyme in the crosslinked peptidoglycan structures, allowing for greater access to the site of action. The secondary structure of LytA was identified to be 47 % beta-sheets, 19 % alpha-helices, and 23 % turns as well as 11 % irregular structures (61). The secondary structure of the C-terminal module is very similar to the whole LytA molecule with the characteristic high beta-sheet content. The binding of LytA to choline was shown not to induce significant changes in the secondary structure, indicating lack of structural changes during the choline-binding event (61). However, two modes of choline binding to the C-terminal module have been detected; one is low affinity and the other high affinity. Saturation of choline binding to the high affinity sites induces dimerization and subsequent increase of the affinity for the substrate to be degraded (61, 62). The dimerization involves primarily the C-terminal part of the molecule with preferential binding of two choline molecules to the dimer. The presence of these two choline-binding sites together with inducible dimerization might play an essential role in LytA cellular targeting by preferential location of the enzyme at the sites of its action on the cell wall.

3.3.4. Cytoplasmic lipid bilayer attached macromolecules

Yet another group of pneumococcal surface proteins constitute proteins covalently linked to the cytoplasmic lipid bilayer. These proteins have a signature sequence Lx_1x_2C (x_1 is usually A, S, V, Q, or T; x_2 is G or A), or similar sequence at the N-terminus immediately following the signal sequence (32). During this attachment process a covalent linkage is created between the Cys residue (becoming the first N-terminal residue of the mature protein) of the signature motif and the diacylglyceryl component of the lipid bilayer. The signal peptide is then cleaved off. The N-termini of such proteins are usually highly flexible which facilitates attachment of the functional part of proteins to the bacterium's surface (156).

3.3.4.1. Pneumococcal surface antigen A

An example of a lipid-attached protein is pneumococcal surface antigen A (PsaA). PsaA is a virulence factor of pneumococci shown to elicit protection

against *S. pneumoniae* in mice (157). PsaA$^-$ mutants were avirulent in the same model (156). The protein is

Table 3. Genome identified extracellular surface proteins

Group of proteins	Number of members	Percentage with annotated function
Choline binding (PspA-like)	13	23%
Peptidoglycan-attached (Hyl-like)	20	55%
Lipid-attached (PsaA-like)	33	70%
Pht-type	4	0%
Signal sequence-peptidase I, II, type IV preplin	NA[a]	NA

[a] NA, Not Available

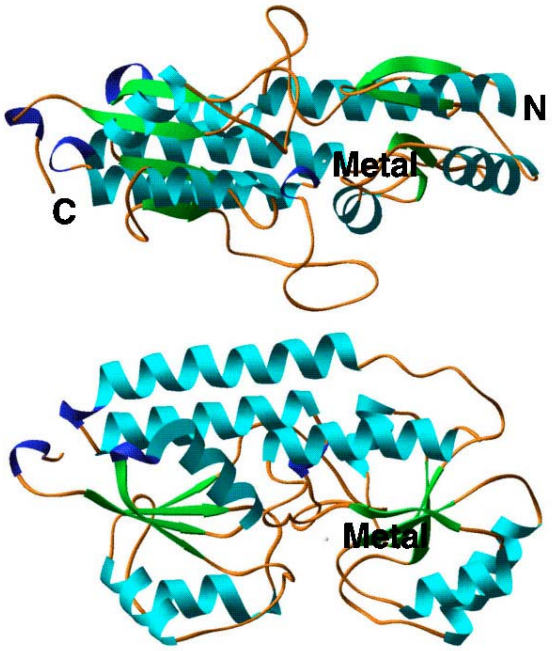

Figure 10. Structure of PsaA. The structure picture is based on the X-ray diffraction data (pdb code: 1PSZ). The metal bound in its binding site is labeled. Two perpendicular views of the protein are shown.

composed of 309 amino acid residues, having molecular weight of ~34 kDa. Functionally it is an extracellular, solute-binding part of an ABC bacterial transport system which facilitates the transport of Mn^{2+} and Zn^{2+} into the cell (158). PsaA is anchored to the cell wall of *S. pneumoniae* cytoplasmic membrane using the characteristic signature in the C-terminal part of the protein signal sequence Lx_1x_2C (32).

3.3.4.1.1. Three-dimensional structure

The three-dimensional structure of a 290 amino acid truncate consisting of residues Ala19 to Lys309, of the 309 amino acid full-length mature PsaA protein, has been elucidated by X-ray crystallography (159, 160). The structure consists of two 2-fold pseudosymmetrical $(beta/alpha)_4$ sandwich domains, together with N- and C-terminal domains (Figure 10). The beta-strands of each sandwich domain are arranged in parallel sheets (160). The N-terminal part responsible for lipid attachment is not visible in the structure. However, the whole N-terminal part of the amino-terminal domain structure clearly protrudes outside the molecule indicating its availability for flexible attachment. A sequence comparison of PsaA from different strains shows the N-terminal attachment part as being the least conserved and, therefore, likely to assume a number of different flexible structures (156).

3.3.4.1.2. Transport of metals

Analysis of the PsaA sequence shows that it is a solute(metal)-binding part of an ABC type transporter (156). The ABC transport system is characteristic of prokaryotic and eukaryotic cells (161) and has up to three components, an extra-cytoplasmic protein responsible for solute binding (such as PsaA), an integral membrane part responsible for transport of the solute through the cell membrane, and a cytoplasmic ATP binding protein. ATP hydrolysis is coupled with solute transport to provide the necessary energy. The specificity for metal ion transport, Mn^{2+} and/or Zn^{2+}, still needs to be determined. However, the growth requirements of PsaA$^-$ mutants suggest that PsaA plays an essential role in transport of Mn^{2+} (158). PsaA has dimensions of 40 x 40 x 70 Å and is likely present beneath or within the peptidoglycan and the capsule as the thickness of the pneumococcal cell wall ~0.36 micrometer (24) is to large to allow PsaA to protrude outside of the cell wall. Investigations of PsaA clearly suggest that this molecule is not an adhesin as was initially thought (76, 156). The initial adhesin features of PsaA were deduced based on analysis of PsaA$^-$ pneumococcal cells. These effects may have been due to a secondary effect related to the availability of metals such as Mn^{2+} or Zn^{2+}.

4. PUTATIVE, GENOME IDENTFIED VIRULENCE FACTORS

The availability of the sequence of the genome of *S. pneumoniae* of three strains, capsular serotype 4 or TIGR4 (162), non-encapsulated strain R32A originating from capsular type 2 clinical isolate strain D39 (163), and strain G54 of type 19 clinical isolate (partial sequence; 164) provides an unique opportunity to investigate the virulence factors of this organism on the genomic level. A recent review by Rigden *et al.*, (19) summarized such an approach for careful and reliable identification of pneumococcal proteins of the three types described in detail above, peptidoglycan bound, choline attached, and lipid bound (Table 3). Out of total identified 66 surface proteins only very limited number of them, 56 %, were characterized or even properly functionally annotated (19).

In addition to the proteins described above, new groups of virulence factors of the pneumococci were identified. This includes the Pht family of proteins with hydrophobic leader

sequences (165), and proteins that are transported outside of the cell having various signal sequences such as the peptidase I, II, or type IV preplin signal (19). More such proteins are likely to be identified in the near future.

It is likely that not all of these proteins are expressed by this pathogen at all times during the life stages of *S. pneumoniae*. However, it would be expected that they be expressed at different times, in different host tissues, or at various stages of infection. Examples of such diverse conditions related to life-cycle of pneumococci are low iron environment, high osmolarity, atmosphere containing carbon dioxide, or even a static temperature shift. Many of the genome-identified molecules were already identified experimentally (*e.g.*, 165, 166) but more studies are required to elucidate their more detailed properties.

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