Cell. Mol. Life Sci. 64 (2007) 2799 – 2822
1420-682X/07/212799-24
DOI 10.1007/s00018-007-7125-8

Cellular and Molecular Life Sciences

Review

Unveiling molecular mechanisms of bacterial surface proteins: *Streptococcus pneumoniae* as a model organism for structural studies

M. J. Jedrzejas

Center for Immunobiology and Vaccine Development, Children's Hospital Oakland Research Institute, 5700 Martin Luther King, Jr. Way, Oakland, CA 94609 (USA), Fax: +1 510 450 7914, e-mail: mjedrzejas@chori.org

Received 12 March 2007; received after revision 28 June 2007; accepted 18 July 2007
Online First 11 August 2007

Abstract. Bacteria present a variety of molecules either on their surface or in a cell-free form. These molecules take part in numerous processes in the interactions with their host, with its tissues and other molecules. These molecules are essential to bacterial pathogenesis either during colonization or the spread/invasion stages, and most are virulence factors. This review is focused on such molecules using *Streptococcus pneumoniae*, a Gram-positive bacterium, as an example. Selected surface proteins are introduced, their structure described, and, whenever available, their mechanisms of function on an atomic level are explained. Such mechanisms for hyaluronate lyase, pneumococcal surface protein A, pneumolysin, histidine-triad and fibronectin-binding proteins are discussed. Elucidation of molecular mechanisms of virulence factors is essential for the understanding of bacteria and their functional properties. Structural biology appears pivotal for these studies, as structural and mechanistic insights facilitate rational approach to the development of new treatments.

Keywords. Mechanism of action, pathogenesis, structure/function, virulence.

Introduction

Cell wall of Gram-positive bacteria

The focus of this review is the molecular analyses of extracellular proteins of Gram-positive bacterial organisms, using *Streptococcus pneumoniae* as a model system. Gram-positive bacteria have characteristic thick layers of peptidoglycan structures, known also as murein, that surround their cytoplasmic membrane. Peptidoglycan provides the structural rigidity of bacterial cells, for their cell wall, and helps maintain elevated turgor pressure within these cells. In addition, it provides space for other extracytoplasmic macromolecules and for their associated functions. The thickness of this layer varies from ~150 to 300 Å, with one layer of peptidoglycan being approximately 10 Å thick [1]. A layer of peptidoglycan is built from repeating units of *N*-acetyl glucosamine and of *N*-acetyl muramic acid, all connected through β1,4-glycosidic linkages. These individual layers are cross-linked by covalent linkages attached to individual *N*-acetyl muramyl residues. The cross-linking bridges are built from amino acids (aa), some of them with the unusual D-conformations. The aa composition of these cross-bridges is diverse, and is strongly strain-dependent [2]. For *S. pneumoniae*, for example, the length of the peptide crossbridges varies from three to four aa residues. The cross-bridges originating from different muramic acid residues are linked usually through alanyl-serine

and alanyl-alanyl covalent linkages. The peptidoglycan layers are constantly being degraded (hydrolyzed) by various hydrolases, and replaced by newly synthesized ones resulting in continuous renewal of peptidoglycan, and ultimately leading to bacterial cell wall growth. This process is essential to cell division, a process that is central to the survival of all prokaryotic lineages.

Periplasmic space and differences from Gram-negative organisms

Unlike Gram-negative organisms, Gram-positive bacteria do not have a clearly defined periplasmic space. However, numerous molecules are associated with or within the peptidoglycan layers, making it essentially an integrated periplasmic space [3, 4]. Examples of such molecules are proteins, glycans such as teichoic acid (present in pneumococci, for example) [5] and capsule [6] covalently attached to peptidoglycan [7], and other molecules protruding through such layers. In addition, protruding through murein are lipotechoic acids [8] that are covalently anchored to the cytoplasmic membrane [6]. Specifically, pneumococci have choline resides associated with (lipo)teichoic acid structures that are used as anchors for additional proteins [9–12]. Finally, other groups of proteins are covalently bound to the lipid bilayer of the cytoplasmic membrane and they, too, utilize the semi-periplasmic space [13]. The majority of molecules occupying the same region of the cell as peptidoglycan, *i.e.*, sugars or proteins, are virulence factors for some of these Gram-positive organisms that are pathogenic in nature [14, 15]. Such molecules play a role in the diseases these bacterial organisms cause. In addition, for most, if not all of these molecules, antibodies directed towards them are protective against disease. This review focuses on the molecular mechanisms underlying functional and structural features of surface proteins that are known to be virulence factors. In one way or another, these proteins are associated with the cell wall and its major structural component peptidoglycan.

The pre-genomic era

These pre-genomic era-identified molecules were discovered by relatively laborious laboratory biochemistry methods. A number of them have been characterized. Examples of such proteins for pneumococci are hyaluronate lyase (Hyal) [16, 17] and a neuraminidase enzyme (NanA) [18] and they both are linked to the prokaryotic cell by a direct covalent linkage to the peptidoglycan. Pneumococcal surface protein A (PspA) [19, 20], choline-binding protein A [CbpA; also known as pneumococcal surface protein C (PspC), and *S. pneumoniae* surface protein A (SpsA)] [11, 21], and major autolysin (LytA amidase) [22] are examples of pneumococcal proteins that utilize choline residues on bacterial surface for their attachment and binding. Finally, the pneumococcal surface antigen A (PsaA) [23] is an example of surface molecule connected to bacterial cell through a covalent linkage to the cytoplasmic bilayer lipids. In addition, other molecules such as a cytoplasmic protein pneumolysin (Ply; cholesterol–dependent cytolysin), which has the ability to be released out of the bacteria, have been studied in significant detail [24, 25]. Similarly, a second neuraminidase, NanB, which does not belong to any of the above groups, has been investigated [18, 26]. Significant advancement has clearly been made for Hyal, PspA, and Ply. This is, however, not the case for all other molecules listed above, for which only incremental knowledge has been gained.

The post-genomic era

The availability of genomic sequence data for numerous Gram-positive bacteria, including three pneumococcal strains, has facilitated the identification of additional pneumococcal surface molecules (*e.g.*, [15, 27, 28]). These analyses have led to the identification of many new surface molecules compared to the pre-genomic era, and have often relied on searches for specific signatures in sequences of open reading frames (ORF) coding for such proteins.

For example, the initial analysis of the genomic sequence of *S. pneumoniae*, reported by Tettelin et al. [29], identified 69 genes coding for proteins predicted to be exposed on the surface of the pneumococcal cell. This protein set included 19 predicted proteins with the cell wall surface anchor family sequence LPxTG-like motif responsible for covalent attachment to peptidoglycan, and are likely substrates of sortase or sortase-like proteins (*e.g.*, [30, 31]). Sortase enzymes are responsible for the creation of covalent attachment of specific proteins, LPxTG-like, to the cell wall. In addition, 15 predicted proteins with putative choline-binding motifs, 36 proteins with putative lipid-attachment motifs (predicted lipoproteins), and 60 proteins with predicted N-terminal signal peptides were found. In 62 cases, there were two or more independent sequence-based indications of the surface localization of the predicted protein [28]. The analysis of the putative surface proteins of *S. pneumoniae* performed in this laboratory identified significantly more of such proteins. These include 117 candidate secreted and membrane proteins [28]. Similarly, we identified several additional proteins with LPxTG-like motifs. In addition, 567 predicted proteins were found to contain from 3 to 12 transmembrane segments; 20 more proteins with two

Table 1. Predicted sets of pneumococcal surface proteins. The data is based on Rigden et al. [28].

Protein class	No. identified	No. with previously annotated function (%)	Largest (residues)	Smallest (residues)	Mean size (residues)
Choline binding	13	3 (23%)	744	211	458
Peptidoglycan attached	20	11 (55%)	4776	202	1350
Lipid attached	33	23 (70%)	661	236	370

predicted transmembrane segments are also likely to be membrane-anchored (Table 1).

Examples of such newly identified pneumococcal proteins that have subsequently been characterized experimentally are novel fibronectin-binding proteins, and pneumococcal adhesion and virulence A (PavA) and B (PavB) proteins [32, 33], as well as in part new discoveries related to the pneumococcal histidine triad (Pht) family proteins [15, 34–36]. In the coming years, a surge of new data is anticipated, generated by such genomic sequence-driven studies. The new data should include significant new results on the molecular mechanisms that underlie their functions in the bacterial life. Such studies will likely result from bioinformatic analyses and fold-recognition methods of the genome-identified sequences, which have the ability to suggest functions, structures, and even specific aa residues involved in functional properties. Extensive and comprehensive genome-based identification and analysis of virulence factors has been reported and reviewed, by this laboratory [28] as well as others [15, 37].

Interactions with host and host tissues during the bacterial life

Due to their spatial location, bacterial surface proteins are naturally involved in complex interactions of bacterial cells with host tissues. Some of these interactions, and proteins or other molecules involved in these interactions, might be essential only during the colonization stage (*e.g.,* in nasopharynx for pneumococci), during invasion (*e.g.*, crossing epithelial layers of mucosal surfaces), or spread to other host tissues (*e.g.*, crossing into the blood stream or nervous system). Yet others might be essential during all stages of the bacterial life. It is obvious that the capsule with its wide variety of capsular polysaccharide types and other surface glycans such as teichoic acid (C-polysaccharide, C-substance) and lipoteichoic acid (F-antigen, Forssman antigen) for *S. pneumoniae*, or other bacterial harboring such structures, are also involved in major interactions with the host [38–40]. In addition to surface glycans, a variety of surface protein molecules are involved in specific aspects of host interactions. Many details of such interactions have been discovered recently and elucidated in reasonable detail. These newly discovered means of bacterial protein-based interactions are the main focus of the present work, particularly directed towards providing details of molecular mechanisms.

Virulence factors

Pathogenic bacteria are distinguished from their non-pathogenic counterparts by their ability to cause disease. One feature of pathogenic bacteria is that they contain virulence genes that can be transcribed into protein products, virulence factors. In general, virulence factors are molecules produced by bacterial organisms, including *S. pneumoniae*, that are essential for causing disease. Some of these factors are only part of the "life style" virulence and they promote colonization. Others, "true" virulence factors, are directly involved in promoting invasion.

Most, if not all, proteins displayed on the surface of bacteria contribute significantly to the pathogenesis (*e.g.*, [12, 41]). As discussed earlier, these proteins are often involved in direct interactions with the host tissues or in concealing the bacterial surface from the host's defense mechanisms. Such proteins are, therefore, virulence factors, and they are reasonable candidates for generating immunogenicity that can be protective, for example, when utilized in vaccine compositions.

Protein virulence factors of bacteria

Hyaluronan lyase

Hyal is one of the major surface bacterial proteins with antigenetically variable properties that are essential for full virulence. Most strains of *S. pneumoniae*, as well as other Gram-positive bacterial pathogens such as *Streptococcus agalactiae*, *Streptococcus pyogenes*, or *Staphylococcus aureus*, produce Hyal. The enzyme degrades major components of the extracellular matrix (ECM) of tissues, hyaluronan (HA) and certain chondroitins (CHs) (Fig. 1) [42, 43]. By breaking down HA, an important constituent of connective tissues, Hyal appears to be directly involved in host invasion by bacteria that produce this enzyme, such as *S. pneumoniae* [44, 45], although how the enzyme facilitates bacterial penetration of the host's physical

defenses and subsequent spread to its tissues is poorly understood. Hyal involvement in pneumococcal meningitis has been studied in mice [44] and humans [45]. In *S. pneumoniae* cultures, Hyal is found in both the culture fluid and in the cell-associated fractions, suggesting that at least part of the enzyme is released by pneumococci in the process of invasion.

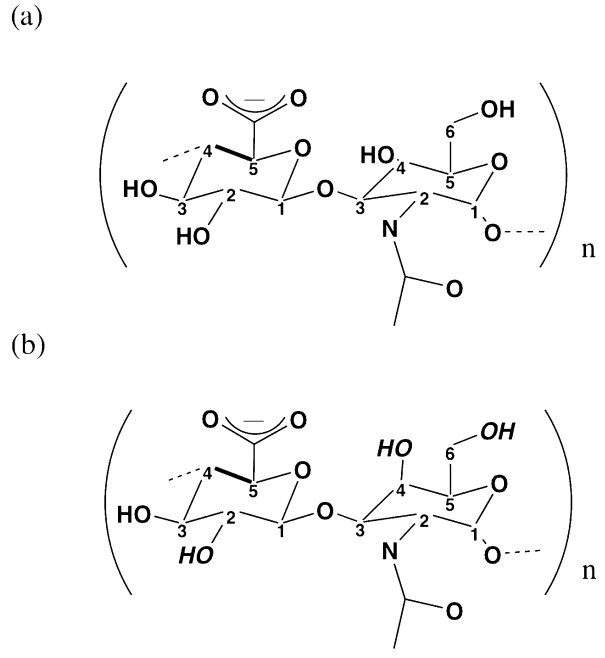

Figure 1. Chemical structures of (*a*) hyaluronan (HA), and (*b*) unsulfated chondroitin (CH) and CH sulfates. Alternating units of D-glucuronic acid and *N*-acetyl-D-glucosamine are the building blocks of the HA polymer. CH structures differ only in the anomericity at the C4 position of the *N*-acetyl-D-glycosamine, *N*-acetyl-D-glucosamine in HA and *N*-acetyl-D-galactosamine in CH. Potential sulfation sites for CH structure are marked by italicized hydroxyl groups. The main digestion product is a disaccharide unit of either HA or CH, which differs from the substrate by the introduction of a double bond between carbon atoms C4 and C5 of the D-glucuronate (marked by thicker bond). This also results in a modified puckering of the D-glucuronate pyranose ring.

HA and CH substrates

HA is composed of repeating units of [D-glucuronic acid(1-β-3)*N*-acetyl-D-glucosamine(1-β-4)] (Fig. 1) and is detectable in every tissue and body fluid of higher animals, including humans. Bacterial Hyals cleave the β1,4-glycosidic linkage between *N*-acetyl-β-D-glucosamine and D-glucuronic acid residues in HA, not by hydrolysis but by β-elimination, and catalyze the release of unsaturated polysaccharides, with the disaccharide unit 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose being the main end product [46] (Fig. 1). HA is commonly associated with its role as a structural component, allowing for protection and cushioning of surrounding structures such as in joints due to its tensile properties that result from its ability to absorb large amounts of water. In addition, HA and its interacting molecules have many other biological functions including fertilization, embryonic development, cell migration and differentiation, wound healing, inflammation, and growth and metastasis of tumor cells [47, 48]. This polymer also interacts with receptors and binding proteins on cell surfaces like CD44 and RHAMM [49, 50]. CD44, the HA receptor, is present on many different cell types and is important in different steps in the normal immune response [51]. The CD44 receptor, a member of a family of cell surface adhesion molecules [52], is present on the surfaces of macrophages [53], neutrophils, T and B cells, and various epithelial cells. There is also mounting evidence that cytokines are involved in *S. pneumoniae* sepsis and in HA metabolism [54]. HA levels on endothelial cells, lung fibroblasts, and other cell surfaces are finely controlled by various cytokines that regulate the rates of HA biosynthesis and degradation [55, 56].

The secondary substrate for Hyals are CHs. CHs differ in their structures from HA only in the anomericity at the C4 position of the *N*-acetyl-D-glycosamine, *N*-acetyl-D-glucosamine in HA and *N*-acetyl-D-galactosamine in CHs. In addition, CHs, unlike HA, can be sulfated at selected positions and are primarily associated with glycoproteins (Fig. 1b). Both polymers are members of a larger group of polymeric glycans termed glycosaminoglycans (GAGs). As HA, CHs also bind significant amounts of water, which allows protection and cushioning of surrounding structures, and they also limit the freedom of diffusion of other macromolecules. CHs that are cleaved by bacterial Hyals have very specific sulfation patterns [57], and these sulfation patterns appear to be critical for their biological function.

Hyal as a prototype for HA-protein interactions and processivity in nature

The Hyal from *S. pneumoniae* was the first protein elucidated structurally in complex with HA. As such it provided the first glimpse into HA-protein interactions on a molecular level [58, 59]. Soon afterwards bee venom hyaluronidase [60], human link protein [61, 62], and Hyal from *S. agalactiae* [63] followed. In addition, the streptococcal Hyals seem to degrade HA, but not CHs, in a processive manner [64, 65], and as such present a unique opportunity to look at the details of not only the catalytic degradation of Hyal's substrates but also the mechanism of processivity by itself. Two bacterial Hyals were elucidated at the structural level, one from *S. pneumoniae* [16] and the other from *S. agalactiae*

[63]. They clearly reveal this enzyme's common mechanistic properties.

Three-dimensional structures of Hyal

The truncated but fully active 89-kDa pneumococcal Hyal was crystallized and the structure solved in 2000 [16] (see below for the discussion of the full-length enzyme). This enzyme's three-dimensional (3-D) crystal structure reveals an enzyme that is composed of two structural domains connected by one short aa linker peptide with presumably flexibility properties (Fig. 2a). The structural domains are the N-terminal α-helical domain (termed here the α-domain) and the C-terminal β-sheet domain (termed the β-domain). The α-domain is arranged in a α_5/α_5-barrel structure (Fig. 2a) and the larger end of this barrel forms a deep and elongated cleft. The β-domain is arranged primarily in a β-sandwich. A portion of this domain is in close proximity to the cleft present in the α-domain. This cleft in the α-domain is where the HA and CHs substrates bind and are degraded by a group of catalytic residues (Fig. 2c) [16].

The substrate-binding cleft is elongated and relatively wide, which allows for easy access of the polymeric HA or CHs substrates. The surface of the cleft is highly electropositive due to the abundance of basic residues at its surface (termed a positive patch). HA and CHs are electronegative due to the negative charges of the carboxylate groups along their chains, and also have a significant hydrophobic character due to their backbone's sugar rings [64]. The charge complementarity between the cleft and HA allows for electrostatic interactions between enzyme and substrate, resulting in substrate binding (Fig. 2b). In summary, predominant features of the cleft are: (i) multiple basic residues lining the cleft surface making it highly positively charged and thereby facilitating substrate binding (positive patch); (ii) a cluster of three residues Glu388, Asp398, and Thr400 creating negative patch, located at the terminal part of the cleft, which is implicated in product release (reducing end of the bound substrate); (iii) three closely placed hydrophobic residues, Trp291, Trp292, and Phe343, located next to the catalytic residues and forming a hydrophobic patch that is implicated in precise positioning of the substrate for catalysis; and (iv) three residues, Asn349, His399, and Tyr408, proposed to be directly involved in catalysis (Fig. 2b, c). These aa residues were identified based on the analysis of the structural information of Hyals and their complexes with HA and CHs, modeling, mutagenesis, and activity studies [59, 63], and with HA tetra-, and hexasaccharides [64, 65]. The site-directed mutant enzymes were characterized kinetically, and the correlation between kinetic properties and structure formulated (Fig. 2c) [16, 46, 66].

Mechanism of action of Hyal and its processivity

The degradation of HA by the streptococcal Hyals is primarily processive and is thought to start with a initial random endolytic cuts of a long, aggregated HA chains. Once the HA chain becomes smaller and non-aggregated, its degradation is followed by exolytic, processive degradation of the substrate chain towards its non-reducing end until the entire substrate is degraded (Fig. 2c) [63, 64, 66]. The exolytic step leads to a burst of production of unsaturated HA disaccharides [66, 67]. The mechanism of such a process (action pattern) is proposed to include five steps (Fig. 2c): (i) the negatively charged HA substrate binds in the positively charged Hyal cleft, and the hydrophobic patch resides, Trp291, Trp292, and Phe343 position the substrate precisely for catalysis; (ii) a catalytic acid-base type step of HA degradation occurs with His399 acting as a base, Tyr408 as an acid, and Asn349 as a weak electron sink, followed by (iii) hydrogen exchange with the water microenvironment, and (iv) irreversible product release by the negative patch. Finally, (v) translocation of the remaining HA in the cleft in the reducing end direction takes place and the process is repeated until the entire HA chain is degraded (for the processive part) (Fig. 2c) [16]. Due to the processive nature of degradation, HA binding must be well balanced to be strong enough to allow for HA to remain in the cleft after catalysis and weak enough to allow for sliding of the HA in the cleft for the next round of the reaction.

The mechanistic details of the catalytic step (ii) above for all currently known bacterial Hyal enzymes are based on the β-elimination reaction, and this process is termed proton acceptance and donation (PAD) [16]. This catalytic step/mechanism also involves distinct steps (Figs 1a, 2c): (i) the acidification of C5 carbon atom of HA's glucuronate residue by a Hyal's Asn349 aa acting as an electron sink (Figs 1, 2c); (ii) extraction of the proton of the C5 carbon by the enzyme's His399 residue, followed by the formation of an unsaturated bond between C4 and C5 of the glucuronate on the reducing side of the glycosidic bond; and finally (iii) cleavage of the glycosidic bond after a proton is donated from the enzyme's Tyr408 residue. During the process, the C4 and C5 carbon atoms change their hybridization from sp^3 to sp^2 with respective changes in the product conformation of the sugar ring, involving a puckering of the glucuronate sugar ring, leading to a distorted half chair conformation (data not shown).

Degradation of CHs is similar; however, it always is a non-processive, endolytic process. *S. pneumoniae*

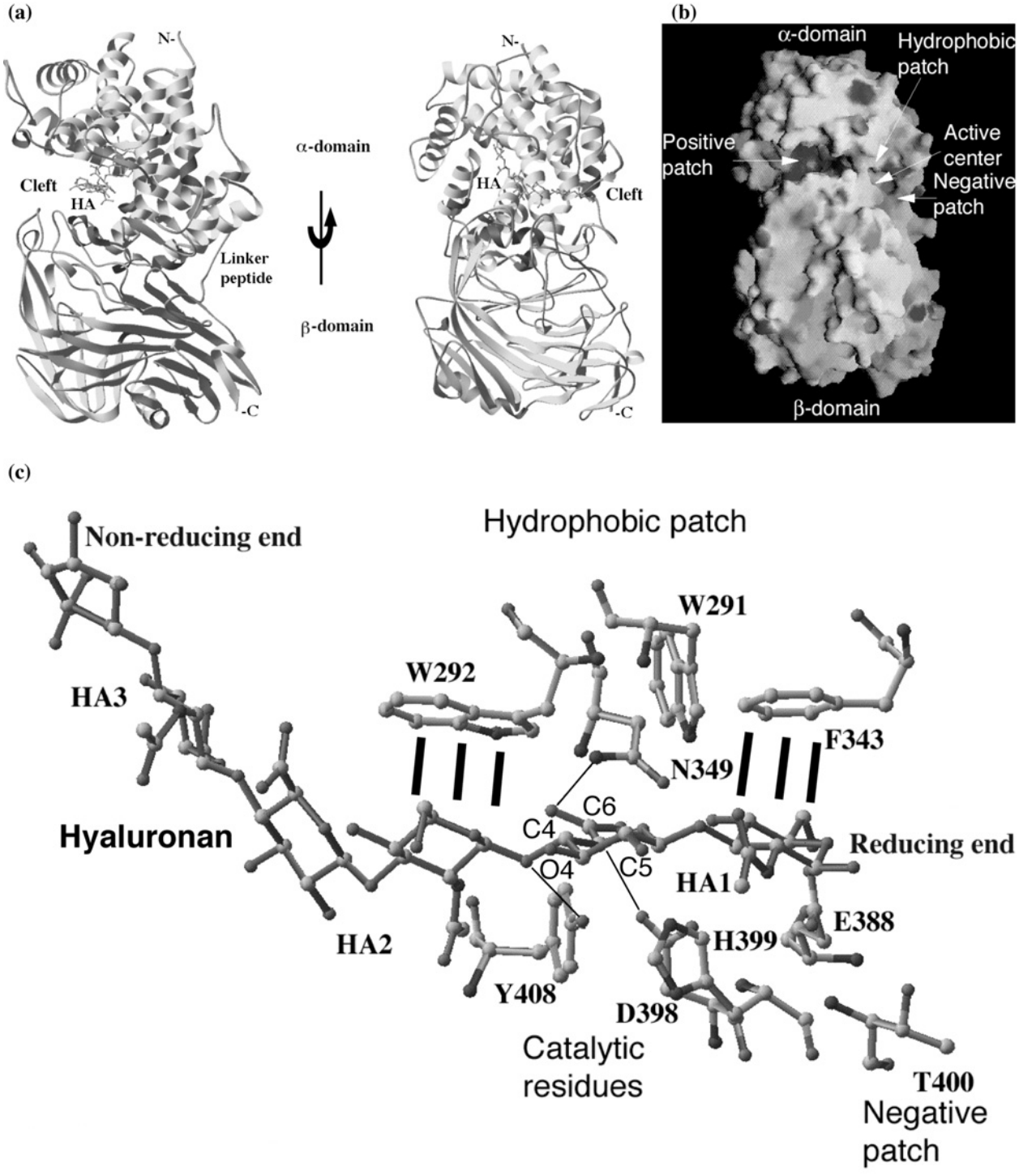

Figure 2. Structural properties of *S. pneumoniae* hyaluronate lyase (Hyal). (*a*) Three-dimensional structure of *S. pneumoniae* Hyal in perpendicular views. The domains, the substrate-binding and catalytic cleft, and the peptide linker are labeled. This structure figure is based on protein database (pdb) coordinates 1egu [16]. (*b*) Surface of Hyal enzyme. The surface of Hyal is shown with labeled locations of the positively charged cleft (positive patch), the negative patch, the hydrophobic patch, and the catalytic residues. (*c*) The catalytic part of the cleft with the bound HA and residues important for catalysis. HA1, HA2, and HA3 are HA disaccharides (HA1 and HA2 are on both sides of the glycosidic bond cleaved between them). The catalytic residues and HA are shown to illustrate the mechanism, and are based on the structure of the complex with HA hexasaccharide (based on pdb coordinates 1loh) [16, 64].

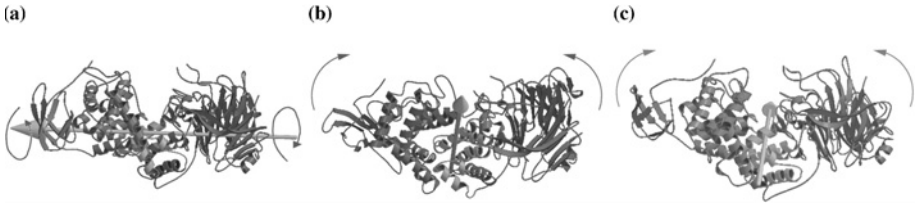

Figure 3. Flexibility of Hyal. The motions of the enzyme are described by thin drawn arrows that are purely descriptive. The thick arrow in the middle of structure corresponds to the rotation axis for the motion described by the thin arrows. The flexibility domains differ from the structural α– and β-domains and their linker (Fig. 2) [65, 71]. (*a*) Rotation/twisting motion. (*b*) Opening/closing of the cleft. (*c*) Opening/closing of the access to the cleft.

Hyal can only degrade CHs at the β1,4 linkage only when the disaccharide on the non-reducing side of the cleaved bond is unsulfated or 6-sulfated. Sulfation at the 4 position is not tolerated on the non-reducing side, although it is accepted on the reducing side of the bond to be cleaved. CHs sulfated at the 2 position are also not cleaved [57] (Figs 1, 2c). Sulfation in these unallowed positions around the β1,4 linkage causes steric clashes with the enzyme and, therefore, CHs cannot assume favorable positions for degradation. Even unsulfated CH cannot be degraded processively due to the altered steric conformation at the C4 carbon of *N*-acetyl-D-glycosamine, preventing the sliding capability for this substrate.

Exolytic processive *vs.* endolytic 'random bite' mechanism of HA degradation is dependent on the size and aggregation state of the substrate

As a consequence of HA aggregation into a large molecular mass, the initial degradation of high molecular mass HA proceeds through a random endolytic cleavage, but only at sites where the β1,4 linkage in the chain is exposed and available for Hyal binding. As the size of HA decreases, HA's ability to aggregate also decreases. At molecular masses below 300 kDa, the ability of HA to aggregate is indeed shown by electron microscopy-rotary shadowing to decrease [68]. At the same time HA chains below ~100 disaccharides in length (~40 kDa) as shown by light scattering evidence, do not aggregate in salt solutions [69]. For the review of the 3-D properties and aggregation of HA as well as CHs see [70]. These properties indicate that somewhere below 300 kDa, the molecule can be degraded by bacterial Hyals using a purely processive mechanism due to smaller degree of aggregation and the availability of all β1,4 linkages to the enzyme's action. As the average HA size decreases, the processive mechanism takes over from the random cleavage, leading to faster exponential degradation at the termination of the process with generation of vast amount of unsaturated HA disaccharides.

Hyal is highly flexible: Functional implications

The Hyal enzyme exhibits a high flexibility that facilitates the mechanism of action of this enzyme. There are three major types of motions of the enzyme [64, 65, 71]. The largest is a rotation/twisting motion of the whole α-domain relative to the top half of the β-domain (Fig. 3a). In this case, one structural domain has two flexibility parts/domains. Hence this twisting motion (movement by ~10 Å along the cleft axis) likely facilitates the shift of the ligand along the cleft to translate it by one disaccharide (also ~10 Å long) in the catalytic site, for further cleavage and processive action (sliding).

Another major motion is an opening/closing of the width of HA-binding cleft within the α-domain itself (Fig. 3b). The residues of the catalytic group and from the negative patch exhibit significant relative positional shifts in this motion. This relates to the obvious need of the enzyme to move the catalytic residues into catalytic positions and to release the electronegative disaccharide product of degradation from the active site to enable translocation (sliding) and further processing of the remaining polysaccharide.

Finally, there is evidence for the mobility of loops from the β-domain, relative to the α-domain, that are involved in the formation of the side of the cleft, resulting in opening/closing of the access/entrance to the cleft (Fig. 3c). The flexibility analysis suggests that this opening/closing of the cleft access by covering the cleft entrance indicates a role for the β-domain as the modulator of enzyme's action, or a gate, of substrate access to the cleft.

Domain structure of the bacterial Hyals

Studies of *S. pneumoniae* Hyal yield a detailed picture of its structure and the catalytic process. However, these studies were carried out with a truncated form of the enzyme, lacking a segment of approximately 285 aa residues at the N terminus (Fig. 4b, domains 3 and 4 are represented in the pneumococcal Hyal crystal structure) [16]. The structure of *S. agalactiae* Hyal [63] reveals the presence of an additional small, 74 residue domain N-terminal to the *S. pneumoniae* protein's

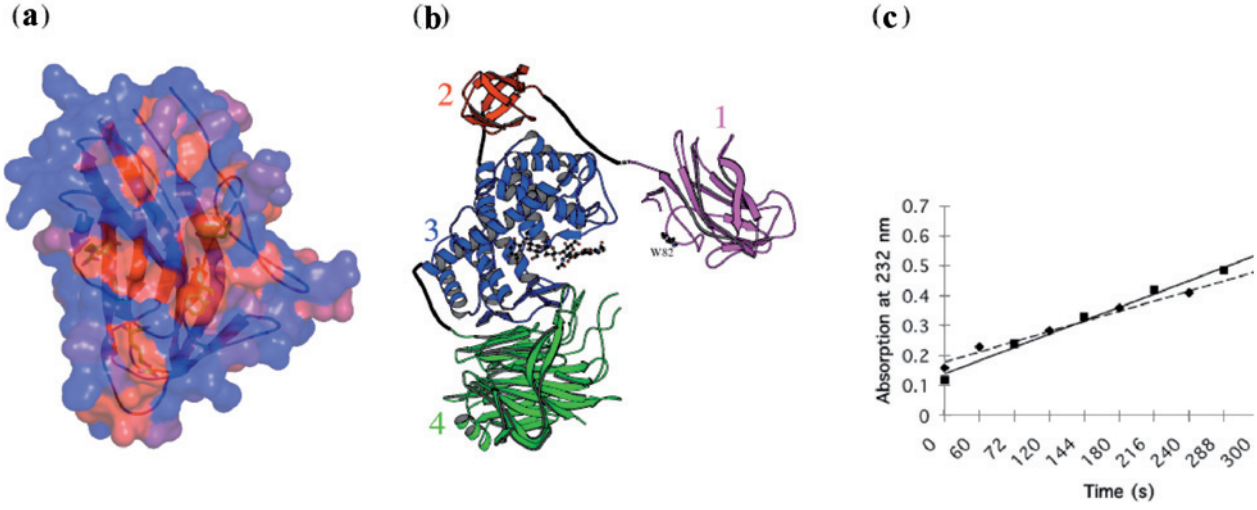

Figure 4. Multidomain, modular architecture of Hyal. The figure is adopted from Rigden and Jedrzejas, 2003 [73]. (*a*) A 3-D model of the N-terminal HA-binding domain. Sequence conservation within streptococcal Hyal on a molecular surface (red conserved, blue not conserved) reveals a HA-binding area. (*b*) Domain organization of entire Hyal. Hyal in complex with HA (domains 2–4) is shown with manually positioned HA-binding model (domain 1). HA and the carbohydrate-binding residue are drawn as balls and sticks. (*c*) Activity of the Hyal versions. The activity of the full-length molecule (■, domains 1–4) is higher than the crystallized, truncated form (♦, domains 2–4). The higher the absorption at 232 nm (absorption of the unsaturated bond created by Hyal action in HA), the higher is the activity of the enzyme.

crystal structure, but around 200 residues past the signal peptide still remain to be structurally accounted for (Fig. 4b, domains 4, 3, and 2). In both cases, full-length enzymes are not stable as they undergo (auto)degradation to the smaller forms. The presence of elongated peptide linkers between domains 1 and 2, and 2 and 3 of the full-length Hyals are presumably the reason for the instability of the protein. Such peptide linkers are easily accessible to (auto)protease degradation. These smaller and stable forms of both streptococcal enzymes, not the full-length molecules, were, therefore, investigated by structural methods. The 3-D structural information of the extreme N terminus was achieved by utilization of fold-recognition methods that clearly revealed a structural correspondence of this domain (Fig. 4a, b, domain 1) to the cellulose binding domains of cellulase of known structure (β1,4-glucanase from *Cellulomonas fimi* protein databank, pdb, codes: 1cx1, 1ulo and 1ulp; [72]). Further analysis reveals a "conserved" cleft and a potential carbohydrate-binding surface, lined up with completely conserved aromatic and positively charged residues suitable for interacting with the hydrophobic sugar faces and negative side chains of HA (Fig. 4a). Such a domain was modeled and its 3-D structure produced [73]. The function of this domain remains speculative but is implicated in any or all of the following roles: (i) co-localization of the enzyme to the sites where substrate is present; (ii) disruption of non-covalent interactions between HA chains or between HA and other polysaccharides; and (iii) orientation of the substrate with respect to the catalytic domain to facilitate substrate degradation in a processive and unidirectional manner (from reducing to non-reducing end). The comparison of activity on HA of the full-length Hyal and the truncated crystallized form demonstrates significant differences (Fig. 4b, c). Such differences under physiological conditions might be more pronounced due to the high molecular mass of HA, HA aggregation and interactions with the ECM, and the points i–iii described above.

Physiological significance of final digestion products of substrates by bacterial Hyals

The bacterial Hyal enzymes generate unsaturated disaccharides as the predominant products following exhaustive digestion. These have no direct biological function other than to serve perhaps as an energy supply for the bacterial organism. These fragments are not known to participate in the pathogenesis of pneumococcal infection.

Bacterial pathogens such as *S. pneumoniae*, can utilize Hyals to overcome human host defense mechanisms, to facilitate invasion, and to reach sites essential for invasion of the host. These exogenous pressures force the host to diversify its glycan structures as part of their defense and survival strategies. All bacterial Hyals studied to date are lyases, also known as eliminases, and may have evolved such catalytic mechanisms precisely for overcoming host defense, as part of their counter-strategy [42, 43, 74]. Bacterial

Hyals may have evolved two separate functions in their survival counter-strategy. First, bacterial spread is facilitated by degradation of the main components of the host tissue ECM, HA and CHs. The second reason is to provide their own carbon and energy source. The identity of such a disaccharide transport system for HA products essential in this case, presumably of the ATP-binding cassette (ABC) type, has yet not been determined. Other enzymes, of either bacterial or host origin, may, however, cleave the disaccharides to monosaccharides. Further degradation of HA disaccharides to individual sugars, such as glucose, glucose-P, or similar sugars, would allow pneumococci to use standard glucose transport mechanisms to gain access to this rich energy and carbon source. Alternatively, the degraded unsaturated HA product in the form of *N*-acetylglucosamine and 4-deoxy-5-dehydroglucuronate could later be cleaved to pyruvate and glycerol dialdehyde, which can be reduced to glyceraldehyde and be subsequently easily transported inside bacterial cells.

This source of carbon and energy might be the reason why bacteria degrade HA to small unsaturated disaccharide molecules, as (i) they are easier to transport or to degrade further, and (ii) they introduce an unsaturated bond in the glucuronic part of HA between carbon atoms C4 and C5. Such unsaturation allows for additional chemistry to take place, facilitating degradation of these disaccharides, compared to the presence of the saturated bonds that occur in vertebrate Hyal digestion products. The HA disaccharides with C4-C5 unsaturation are distinct in their structural properties, compared to conventional HA disaccharides. They have different 3-D structures, compared to regular HA disaccharides, and this difference is exhibited by a different puckering of the ring of the glucuronate moiety that assumes a distorted half-chair conformation [59, 63]. For normal HA, this sugar is clearly in a chair conformation [64, 65]. The puckered ring structure is more amenable to further metabolic reactions and the entire molecule might be recognized as a discrete chemical entity different from the regular HA disaccharide.

Vitamin C and its derivatives are inhibitors of bacterial Hyals

Vitamin C, salicylate, and flavonoids inhibit the Hyal enzyme activity [75]. The effects of these compounds on the activity of pneumococcal Hyal were, therefore, studied in more detail. Only vitamin C, however, demonstrates some competitive inhibitory potency. The vitamin C concentration giving 50% inhibition (IC_{50}) is ~5.8 mM [76]. This behavior is perhaps related to D-glucuronic acid being a precursor in vitamin C biosynthesis. These biochemical studies were followed by determining the crystal structure of the complex between Hyal and vitamin C (Fig. 5b) [16]. Vitamin C binds in the catalytic cleft of the enzyme. Among numerous interactions between the enzyme and vitamin C, the most significant are those with the residues of the hydrophobic patch, mainly Trp292 and catalytic Tyr408. These residues are directly implicated in positioning of substrate for catalysis [46]. Especially, the indole group of Trp292 exhibits strong interactions with the five-member ring of vitamin C (Fig. 2c, b). This structural arrangement provides a major hydrophobic interaction that stabilizes vitamin C binding within the cleft. The structure of the Hyal-vitamin C complex can provide some clues to the design of more efficient Hyal inhibitors. Based on the vitamin C-Hyal interface characteristics, a stronger Hyal inhibitor should have a larger hydrophobic surface available for binding to increase the hydrophobic interactions with the Trp292, other residues in hydrophobic patch Trp291 and Phe343, and possibly with catalytic Tyr408. Also, additional negatively charged groups available for salt bridges, such as carboxylates, would be beneficial for providing the negative charges to lead the inhibitor into the cleft region of the enzyme, and to increase its total binding capacity by creation of additional bonds.

To test the first hypothesis, that increased hydrophobicity of vitamin C derivatives might lead to better binding and to higher inhibitory activity with Hyal, L-ascorbic acid-6-hexadecanoate (L-ascorbic acid-6-palmitate; Vcpal) was designed and synthesized as a vitamin C derivative and examined as a potential hyaluronidase inhibitor. Vcpal is known to be a highly effective antioxidant [77] and an inhibitor of glutathione-S-transferase [78]. Vcpal inhibited pneumococcal Hyal with IC_{50} values of 100 μM [79]. The details of Vcpal binding to Hyal were also examined by structural methods. The enzyme co-crystal structure showed that Vcpal binds in a similar location to vitamin C, and indicated the reason for the markedly stronger binding and inhibition of Vcpal compared to vitamin C. This stronger binding is due to the long alkyl chain that interacts with all three hydrophobic patch residues Trp291, trp291, and Phe343. This chain increases the affinity of Hyal for this molecule,primarily by additional hydrophobic interactions (Fig. 5c).

Pneumococcal surface protein A

Pneumococcal surface protein A, PspA, is one of the choline-binding proteins, and appears to be specific to pneumococci or at least bacteria producing choline residues on their surface. Antibody studies have

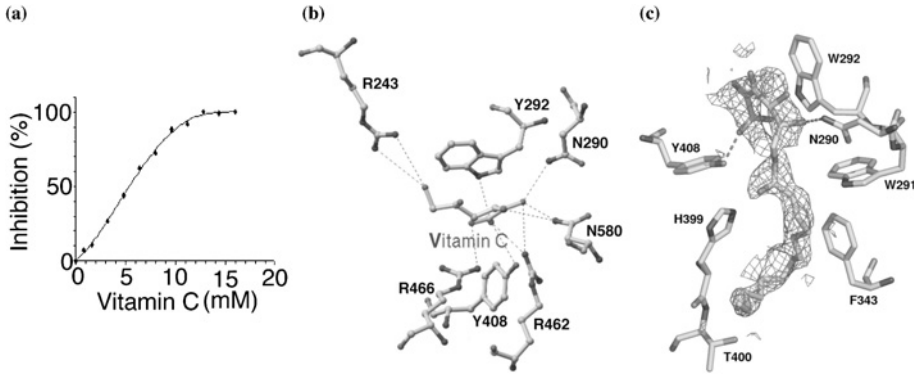

Figure 5. Vitamin C (ascorbic acid) inhibits activity of pneumococcal Hyal. (*a*) and (*b*) are adopted from Li et al. [76] and (*c*) from Botzki et al. [79]. (*a*) Vitamin C inhibits Hyal with IC_{50} ~5.8 mM. Inhibition of the enzyme as the function of vitamin C concentration is depicted [76]. (*b*) Binding and interactions of vitamin C relative to the active site in the cleft. The graph is based on the structure of the complex between Hyal and vitamin C (based on pdb coordinates 1f9 g). Interactions with Trp292 are among major interactions of this inhibitor with the enzyme. (*c*) Binding and interactions of Vcpal. The vitamin C derivative, Vcpal, shows markedly stronger binding in a similar position in the cleft to vitamin C, but it utilizes extended hydrophobic interactions not only with Trp292 but also with all three residues of hydrophobic patch Trp291, Trp291, and Phe343. The figure is based on the structure of the complex with Vcpal.

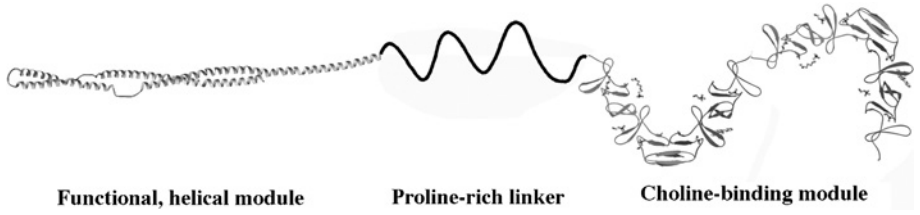

Figure 6. Schematic domain architecture of pneumococcal surface protein A (PspA). The first 288 aa residues (Rx1 strain) are believed to be in the charged/helical conformation followed by the presumably flexible peptide linker termed proline region (aa 289–370), the choline-binding domain (CBD) repeats (aa 371–571), and finally the hydrophobic 17 aa C-terminal part (not depicted) [20].

shown that PspA is located on the cell surface of pneumococci [10, 80] and is found in every *S. pneumoniae* strain discovered to date [81]. PspA is a surface protein with variable molecular mass from 67 to 99 kDa. Based on sequence, the protein has four distinct regions: an N-terminal highly charged α-helical region (288 aa in Rx1 strain), a proline-rich domain (83 aa), a stretch of ten highly conserved 20-aa repeats, and a tail of 17 slightly hydrophobic residues at the C terminus [20, 82] (Fig. 6). The N-terminal end extends through the cell wall and possibly protrudes outside the capsule. Secondary structure predictions methods predict that the N-terminal domain is highly helical and most likely a coiled-coil structure [20, 82]. Such helical coils are relatively common among bacterial surface proteins, including pneumococcal CbpA or *S. pyogenes* M proteins.

All protective monoclonal antibodies (mAbs) reactive to PspA on the pneumococcal cell surface bind to the N-terminal half, suggesting that this part of the molecule is surface exposed [83]. This is also the functional module of PspA. This functional α-helical domain exhibits more variability due to accumulation of mutations than the C-terminal half. Recent evidence indicates that PspA has at lest two virulence functions. One function is inhibition of complement C3 activation and deposition of C3 fragments [84, 85]. The other function is inhibition of killing of pneumococci by host lactoferrin, specifically its iron-deprived apo form (see below for detailed discussion) [86].

Coiled-coil structure of the functional module of PspA

A number of surface molecules of Gram-positive organisms have been discovered, cloned, and sequenced [17, 18, 20, 26, 74, 87, 88]. A conformational analysis of these sequences demonstrates that many of these molecules have a very high α-helical component with characteristic seven-residue-repeat blocks that are characteristic only of proteins exhibiting a coiled-coil structural conformation. The remaining parts of these known surface molecules exhibit predominant β-sheet, β-turn, and random coil structure with only a small amount of helical conformation [89].

The seven-residue repeat or the heptad pattern has been identified in the N-terminal part of the PspA molecule [20, 90, 91]. PspA's was subsequently shown to be an anti-parallel coiled-coil α-helical protein with

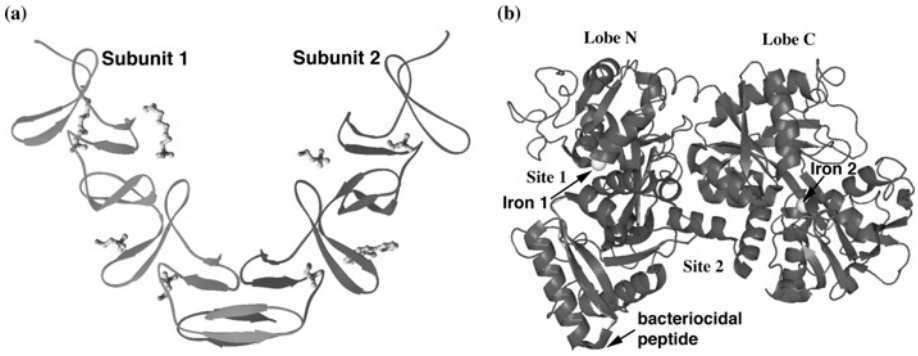

Figure 7. Structures of PspA-related molecules. (*a*) Structure of the CBD of LytA amidase from *S. pneumoniae.* The choline-binding protein (CBP) of LytA amidase is shown (based on pdb coordinates 1h8 g) [94]. The two subunits (left and right side of the figure) form a boomerang-shaped dimer. The choline (larger molecules) and other bound ligands (smaller molecules) are shown in space-filling representation (both represented as balls and sticks). These ligands occupy the choline-binding sites, four per monomer. (*b*) The 3-D structure of human iron-free (apo) lactoferrin. The location of lactoferricin bactericidal peptide is labeled, and the location of the two putative iron ions binding sites, 1 and 2, is shown as balls and labeled (based on pdb coordinates 1cb6) [157].

the characteristic seven-residue motif. In such structures the hydrophobic residues are placed inside the coiled-coils, whereas the hydrophilic residues are located on the outside, solvent-accessible surfaces [92].

PspA attachment to the surface of *S. pneumoniae*

Unlike many other bacteria, pneumococci display a specific surface molecule, phosphocholine, on the cell wall teichoic acid and the membrane-bound lipoteichoic acid [39]. Studies have shown that PspA attaches itself to *S. pneumoniae* by a non-covalent binding to this choline residue of both lipoteichoic or teichoic acids via its C-terminal end. This also comprises the repeat region also called the choline-binding domain/region (CBD) (Fig. 7a) [93, 94]. PspA is not the only choline-binding protein (CBP) [10] present on the surface of this pathogen. Examples of other pneumococcal CBPs are the major cell wall hydrolase LytA [95] and an adhesin CbpA [11]. LytA is an amidase functioning in the separation of daughter cells during cell division [96] and is required for cell lysis [97], whereas CbpA is a protein adhesin on the pneumococcal surface [11, 98]. The CBD motif has also been found among the surface proteins of other bacteria [89] like *Clostridium acetobutylicum*, *Clostridium difficile*, *Streptococcus mutans*, and *Streptococcus downei.* All these CBPs have the characteristic feature of the repeat sequences in the C-terminal region. This family of ligand-binding proteins seems to have a modular structure with the CBD region being responsible for the attachment to the pathogen and the other module responsible for the function of the molecule.

Functional properties of the N-terminal module of PspA

The modeled structures obtained in our laboratory provide new insight into the structure and function of this molecule and support the presence of a highly charged and polar surface of the N-terminal fragment of PspA (Fig. 8a, b). Such a charged molecule may interact well with the negatively charged capsule of *S. pneumoniae* through its positively charged portion close to the proline region. This interaction possibly stabilizes the capsular structure and also directs the negatively charged region of the PspA molecule at the N-terminal part of the functional module away from the *S. pneumoniae* surface. Capsules of virtually all virulent pneumococci are negatively charged [38]. The proline-rich region may act then as a tether, allowing flexible attachment to the bacteria through the CBD (Figs 6, 7a, 8c) [20].

Interactions with the host defense molecules. The PspA molecule has been shown to have anti-complementary properties [84, 85, 99, 100], and an increase of bacterial surface charge has been correlated to a decrease in antibacterial phagocytic activity [101]. The negatively charged PspA could simply repel complement molecules and prevent their interaction with pneumococci, a process necessary for proper function of complement system leading to pneumococcal cell death. PspA has been found to reduce complement-mediated clearance and phagocytosis of *S. pneumoniae* [84, 102].

The protective epitopes of PspA interactions with IgG human antibodies have been recently investigated on the molecular and immunological level [103]. Such epitopes clearly group in selected parts of PspA. Based on these studies, a 3-D model of the PspA-Fab part of IgG antibody was proposed (Fig. 9a, b). The

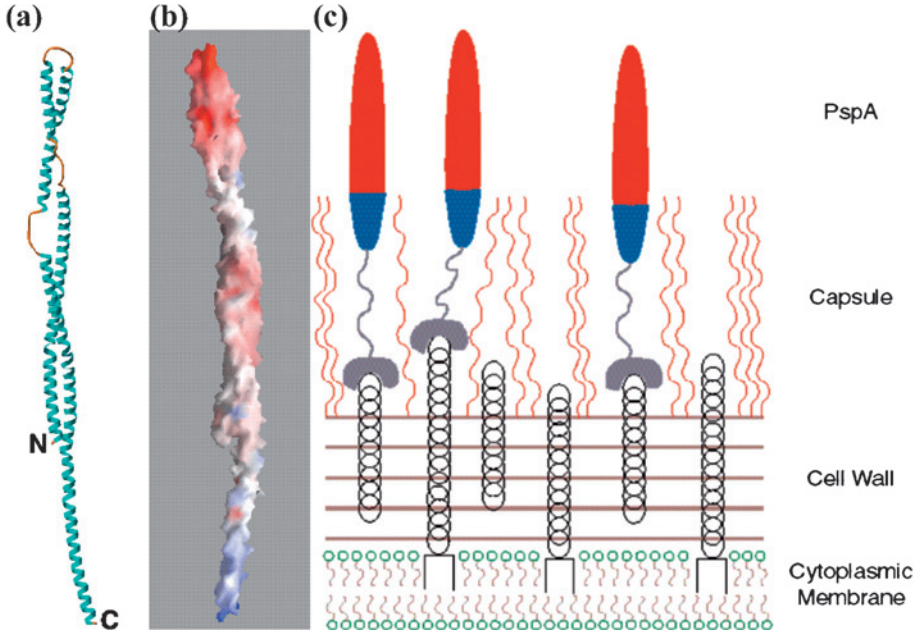

Figure 8. The 3-D modeled of PspA molecule. Figure adopted from Jedrzejas et al. [20]. (*a*) Model structure of N-terminal PspA. This highly elongated molecule assumes an anti-parallel coiled-coil fold. The breaks in the coiled-coil structure are shown as loops. (*b*) The surface potential of the model. PspA is highly charged and polar molecule with the hydrophobic parts being mainly on the interface between coiled helices (blue, positive; red, negative; gray, neutral potential). (*c*) A schematic diagram of the proposed hypothetical structure of PspA on the surface of *S. pneumoniae.* Proposed view of PspA on the surface of *S. pneumoniae* depicts its interactions with the capsule. The red- and blue-colored PspA highlight its polar charges, negatively charged distant part from the surface and positive C terminus. The positively charged C terminus may interact with the electronegative capsule. Nearly all pneumococcal capsules are negative in charge.

intermolecular interactions involve antibodies with deep groves in the complementarity determining regions (CDRs) in which an elongated coiled-coil of the PspA functional module fits and makes intermolecular binding contacts.

Human lactoferrin as a ligand for PspA antigen. One of additional functions of PspA appears to only recently been discovered and it involves a human lactoferrin (hLf) molecule, which is a ligand for PspA antigen molecule [85, 86, 104, 105]. hLf is found mostly in high concentrations in milk, saliva, and tears. hLf is ~80-kDa protein built from two distinct parts, termed lobe C and N (Fig. 7b) [106]. Each lobe has one iron-binding site and is glycosylated. In its apo form, the iron-binging sites are empty and in the holo form it binds one iron molecule per lobe, usually Fe^{3+} [107, 108]. Lf is an iron storage glycoprotein that is predominantly located on mucosal secretions where the level of iron regulates growth. As such the lack of Fe is considered as a host defense mechanism against bacteria [109]. Lf has been shown, on one hand, to inhibit complement activation (similarly to PspA properties) and on the other, to suppress immune activity [110].

The recent results indicate that the apo form of hLf (*apo*hLf) is the form involved in PspA-apohLf binding [86]. PspA, by binding to *apo*hLf, inhibits killing of pneumococci by hLf. The *apo*hLf form, but not the holo one, is bactericidal due, in part, to the Fe chelation effect, so that depletion of Fe is necessary for growth of many bacteria, including *S. pneumoniae.* The main bactericidal activity is narrowed down to a ~47-aa peptide of hLf (red ribbon in Fig. 7b) termed lactoferricin (Lfn). Lfn is a charged peptide and significantly hydrophobic. This and similar peptides (often as short as 11 aa) presumably interact with the negatively charged bacterial membranes, enter them, and then destabilize them [111]. The lack of charge, and the presence of cholesterol in human cell membranes are thought to protect humans from Lfn action. *apo*hLf was also found to have serine protease activity [112] and it might degrade itself autocatalytically to release Lfn. Alternatively, other proteolytic proteins of pneumococcal or host origin might facilitate the process of Lfn release, leading to killing of this bacterium. Due to high concentrations of *apo*hLf in secretions as compared to serum, the process presumably takes place at the mucosal surfaces and thus facilitating carriage of *S. pneumoniae.*

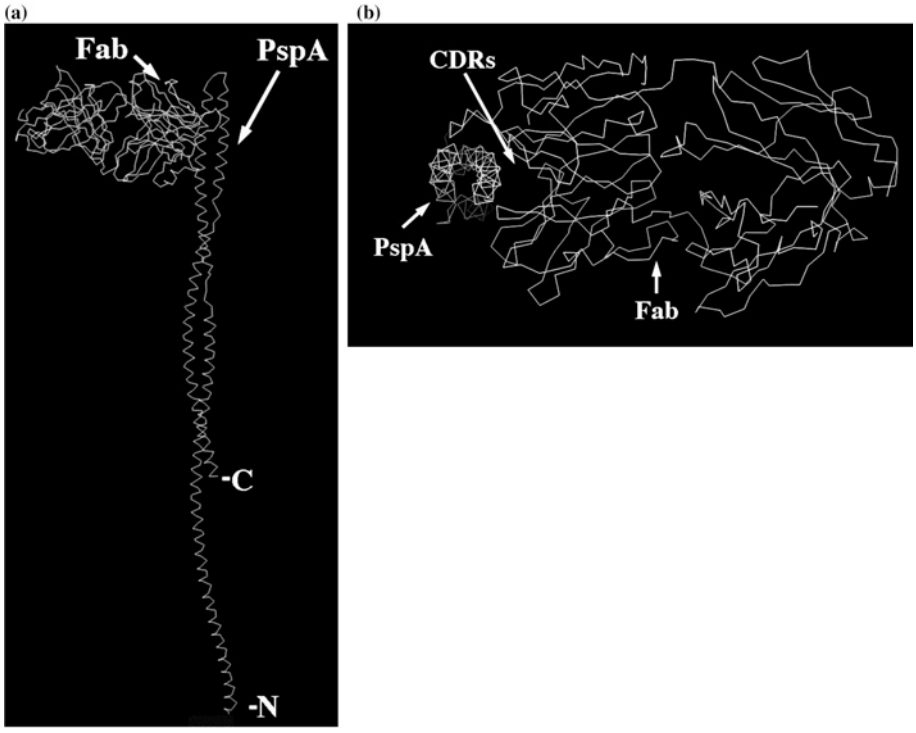

Figure 9. Model of the interactions of PspA with antibodies. The putative structure of a model PspA-Fab complex is shown. The figure drawings are based on Kolberg et al. [103]. (*a*) The 3-D model of a complex structure between PspA and the Fab part of anti-PspA. The interactions of PspA (long anti-parallel helix) and Fab (globular structure) (labeled) are depicted. The Fab molecule has a deep groove allowing for interactions and easy fit of PspA within the groove. (*b*) Conformation of epitopes available for intermolecular interactions. The CDRs region of Fab (labeled) are involved in molecular contact with PspA residues (view down the helical axis; labeled).

Putative molecular details of PspA-hLf interactions. The epitope for *apo*hLf was identified using the truncated forms of PspA. *apo*hLf binding involves aa 193–288 of PspA [104]. This epitope is exactly at the C-terminal part of the helical/functional region implicated earlier in interactions with hLf [104]. As seen in Figure 8a and b, PspA in this region assumes a positive potential. It is, therefore, conceivable that this relatively positively charged part of the helical module of PspA has the ability to interact with hLf, which binds the positively charged ions Fe^{2+} or Fe^{3+}. These positive ions must bind to negatively charged area of hLf, and the positively charged PspA segment at the C-terminal part of the helical module might to some extent mimic this property. The biochemical and structural information support one another, and can explain the binding effect on the molecular level. In addition, based on the molecular characterization of PspA-Fab interactions, *apo*hLf clearly needs to have a deep groove into which PspA might fit, and its residues interact with this human ligand. The 3-D structure of *apo*hLf in Fig. 7b shows the presence of large deep groves around areas where iron ions bind, and where PspA could clearly fit and undergo binding [113]. Upon such PspA antigen-*apo*hLf ligand interaction, the ligand might change shape and become a more compact molecule and, therefore, resistant to (auto)proteolytic degradation, by hiding the vulnerable proteolytic degradation sites inside the complex molecule. The hLf molecule has the ability to modulate its shape after binding, as demonstrated by similar behavior after binding iron ions. The lack of degradation by proteases or hLf itself might be a basis of the lack of bactericidal action of hLf-generated Lfn, due to the lack of its (auto)proteolytic release from a compact molecular antigen-ligand complex.

Pneumolysin

Pneumolysin, Ply, is a protein with a molecular mass of ~53 kDa. It is produced by all clinical isolates of *S. pneumoniae* [114]. It belongs to cholesterol-dependent cytolysin (CDC) family and it forms oligomers that embed themselves in the lipid-bilayer of target cells membranes containing a cholesterol receptor for Ply [115–117]. Such action leads to creation of pores in target cell membranes that subsequently leads to cell lysis and contributes to the invasive capability of this bacterium [118, 119]. Similar CDCs are present in number of other bacteria such as *Bacillus anthracis* (anthrolysin O), *Clostridium perfringens* (perfringolysin O, Pfo), *Clostridium sordellii* (sordellilysin), *Listeria monocytogenes* (listeriolysin O), *Listeria seeligeri* (seeligeriolysin O), or *Streptococcus mitis* (mitilysin).

In *S. pneumoniae* this CDC has several distinct functions in the early pathogenesis of infection [117, 120]. Ply has been shown to be cytotoxic to ciliated bronchial epithelial cells. In organ culture, it slows

ciliary beating, and disrupts tight junctions and the integrity of the bronchial epithelial monolayer [121]. As a result of this disruption, the ability of ciliated bronchial cells to clear mucus from the lower respiratory tract is reduced, facilitating propagation of *S. pneumoniae.* Ply also acts on alveolar epithelial cells and pulmonary endothelial cells. This action may account for the characteristic alveolar edema and hemorrhage seen in pneumococcal pneumonia. By disrupting the alveolar-capillary boundary, Ply produces an alveolar flooding which provides nutrients for bacterial growth and allows penetration through the epithelium into the pulmonary interstitium, and ultimately into the bloodstream [117]. Finally, Ply also has the ability to suppress the host inflammatory and immune responses. Ply directly inhibits phagocyte and immune cell function through its direct cytotoxic effects. But in addition to this, low concentrations of Ply are able to inhibit human neutrophil and monocyte respiratory bursts, chemotaxis, bactericidal activity, and production of lymphokines and immunoglobulins [117].

Ply release from pneumococci

Ply is not actively secreted during bacterial growth because it lacks an N-terminal secretion signal sequence. Also, its release used to be considered obligatorily coupled to *S. pneumoniae* cell lysis, as a self-sacrificial event of a portion of pneumococcal cells to release the content of their cytoplasm. Historically this process is considered to be coupled to the enzyme autolysin (LytA amidase), which is thought to degrade the bacterial cell wall during mitosis, and to disrupt the cell wall during spontaneous, antibiotic or detergent-induced autolysis [117]. This process is questioned and remains unclear. In addition to the data that LytA is not involved in Ply release in the WU2 pneumococcal strain [122], in serotype 2 of the D39 strain such release was shown to be coupled to the action of the ATP-dependent caseinolytic protease ClpCP [123]. Hopefully, future studies will delineate the mechanisms of Ply release from the cytoplasm of pneumococci.

Molecular mechanisms underlying Ply function

Ply belongs to a family of bacterial proteins known as CDCs [115]. Molecules belonging to this group of toxins share many similarities in primary structure and mode of action. The soluble CDCs usually exist in solution as monomers and dimers, but go on to form large oligomers, which become embedded in the lipid-bilayer of target cell membranes. In the case of Ply, the membrane-bound pore assembly consists of 30–50 subunits with a diameter of 350–450 Å, which can be visualized using electron microscopy [117]. These pores are the causative agents that disrupt integrity of host cell membranes leading quickly to cell lysis. The presence of cholesterol in the target membrane is required for the cytotoxicity of each member of the CDC family, and it also serves as a receptor/activator [124]. Due to the lethal effect of pore formation, Ply is also a virulence factor from pathogenic Gram-positive bacteria [115].

The 11 aa residue region referred to as the Trp-rich region is highly conserved among Ply and Ply-like proteins (Fig. 10). In Ply, this region contains a single cysteine and three tryptophan residues implicated in the mechanism of membrane binding, and pore formation [116]. However, this mechanism of interaction with the membrane is currently under debate. Recent studies document that certain regions of the monomer structure adopt a change in conformation from α-helix to β-hairpin during the transition to the oligomeric form of the toxin [119, 125, 126]. The binding of Ply to the host cell membrane is believed to be responsible for this conformational shift in the Trp-rich region [127, 128], presumably through an interaction with membrane cholesterol. The cholesterol binding and pore formation is thought to be a sequential process [129]. There appear to be several distinct steps involved, starting with (i) Ply binding to membrane inserted cholesterol, followed by (ii) membrane insertion, and finally (iii) aggregation and pore formation leading to target cell lysis. Any of these three steps might occur at the same or at a similar time. Ply binding to cholesterol is sufficient to induce structural changes, leading to decrease in β-sheet and increase in random coil structures [129]. The content of α-helices does not change. Also, upon cholesterol binding to Ply, this molecule no longer aggregates to form dimers, or any higher oligomers, as is the case for an uncomplexed molecule. The cholesterol-bound and uncomplexed forms exhibit changes of the molecule consistent with a more compact, less elongated shape for the cholesterol-bound molecule. This behavior is probably due to the rearrangement of the Ply domains with respect to one another and changes in secondary structures (Fig. 10). In addition, fluorescence studies show major changes in the environments of certain tryptophan residues into more hydrophobic ones, as supported by the major increase of quantum yield upon cholesterol binding [129]. The biophysical analyses suggest a 1:1 molar ratio between Ply and cholesterol. The affinity for cholesterol, K_d, is estimated to be ~400 nM [124]. In general, the proposed mechanism of Ply involves a structural shift within a portion of the protein in response direct binding to the cholesterol, which is immediately followed by self-association. There is a conformational change, which then exposes a hydro-

phobic domain that can insert into the host cell membranes. As the protein structure shifts, oligomers are formed and aggregate in large ring/pore structures. The study of Tilley et al. [130] demonstrates the aspects of assembly of the toxin in the membrane. The resulting pores within the cell membrane upset the delicate osmotic balance between the cell and its environment, allowing materials to leak in and out freely, quickly leading to lysis.

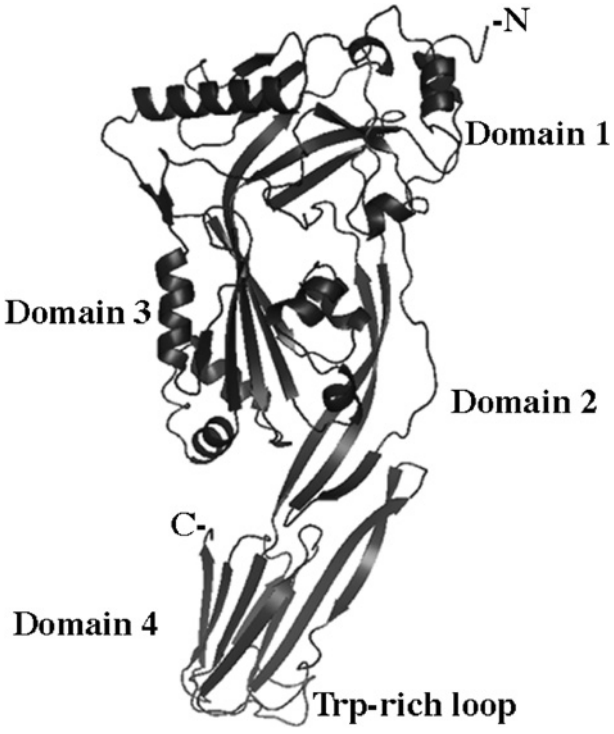

Figure 10. Homology based structural model for pneumolysin (Ply). The domains are labeled 1–4, and the Trp-rich loop in domain 4 is indicated. Domains 3 and 4 are implicated in membrane insertion accompanied by conformational changes leading to secondary structure shift. The rendition is based on Ply model coordinates (M.J. Jedrzejas, unpublished data).

Structural properties

Ply shares functional and to some extend structural similarities to other microbial membrane-inserting toxins. Some of such toxins have been elucidated structurally [127] (Fig. 10). One of these molecules is perfringolysin, whose structure is most similar, mainly due to its highest sequence identity of 60%. These proteins share gross similarity in their structure, mainly a domain-based, rod-like elongated shape and high content of β-sheets. However, a detailed comparison indicates significant differences between these structures [131]. Structural insights into the Ply molecule based on its putative structural homology to the structure of Pfo are limited, due to differences in properties of these two proteins. However, the Ply molecule likely has a similar four-domain arrangement, with the forth domain being implicated in cholesterol binding and membrane insertion through its Trp-rich region/loop [127] (Fig. 10). Recent biophysical studies, including those by small angle X-ray scattering (SAXS), suggest significant structural, biochemical, and solution property differences between Ply and Pfo [131]. A more elongated structure of Ply than previously thought is consistent with the SAXS data, based on the homology to Ply. The structure of Ply in solution appears to be more elongated (axial ratio of 1:5) than that of its Pfo-based homology model [131].

Histidine triad proteins

Recently four new pneumococcal surface-exposed proteins containing putative hydrophobic leader sequences and several histidine triad repeats (Pht family) were identified in the pneumococcal genome, and described, and characterized immunologically in mice [15, 34–36]. Each of these Pht family member molecules, termed PhtA, -B, -D, and -E, have four or five copies of the novel signature motif, HxxHxH (where 'x' is any aa residue), called "the histidine triad". Similar repeats (and homologous proteins) have been observed in other streptococci such as *S. pyogenes* or *S. agalactiae.* The Pht-type hydrophobic leader sequences found at their N-termini are of the Lx1x2C type (see below), characteristic of lipid-anchored proteins that are exported across the cytoplasmic membrane and anchored in such membranes [132, 133]. Normally, the proteins anchored to a lipid of the cytoplasmic membrane have the above signature sequence at their N terminus Lx1x2C (or similar; where x1 is usually A, S, V, Q or T whereas x2 is G or A). This protein segment is utilized to form a covalent linkage during the transport of protein out of the cell. In this attachment type, proteins form a covalent linkage between the Cys residue and the diacylglyceryl of the cytoplasmic lipid bilayer. After the attachment, cleavage of the protein's signal peptide results in a modified Cys residue as the first aa at the true N terminus of these proteins. However, the residues in the vicinity of the Lx1x2C motif of Pht proteins are unlike those expected of classical lipid-attached proteins, placing lipid anchoring of Pht proteins in question. An example of a more studied classical lipid-attachment signature protein is the PsaA molecule [23]. Although the biological functions of the Pht proteins are still unknown, they proved to be immunogenic, and elicited protection against pneumococcal infection in mice, specifically protective against sepsis and death [34].

Sequence and bioinformatics analysis

It is clear that relating the function of Pht protein family to their structure would be facilitated significantly with clues to the protein's domain composition and the location of key aa residues involved in such function. Broad comparative sequence analysis using bioinformatics-driven methods targeted to domain identification and fold recognition based on the Structure Prediction Meta Server analysis [134] were used in an attempt to predict Pht proteins domains and their probable structures. All Pht proteins are specific to streptococci and are clearly related to one another in sequence and in structure. Unfortunately, no molecules homologous to these proteins are currently known. Based on the significant number of His and Tyr residues present in the sequence of these proteins, it is attractive to speculate putative metal, nucleoside, or sugar binding. Furthermore, the *phtD* gene is located just downstream from a gene of the metal-binding cluster 9 of metal/solute binding proteins [34], confirming earlier suggestion of putative metal binding ability.

Pht proteins as zinc-binding molecules involved in regulation of adhesion to host

Recent comparative analysis of bacterial zinc regulons were identified just upstream from genes encoding the Pht proteins [135]. This initial observation provides significant insight into this family of proteins. The histidine-triad motifs are suggested to be involved in zinc binding. The aa residue environment around the His repeats is rich in other hydrophobic residue rather than negatively charged ones. Panina et al. [135] conclude that due to the conserved sequence of these repeats, they play a important functional or structural role in the Pht family of proteins. The zinc binding of this family is directly related to invasive properties of pneumococci and the regulation of their adhesion. As suggested, during colonization, pneumococci restrict their location to mucosal surfaces, and only a small fraction invades epithelial surfaces. In the invasion process pneumococci reach other tissues, including blood stream. The concentration of zinc in bronchoalveolar space is several times lower than that in the bloodstream. As suggested by Panina et al. [135], during colonization bacteria face zinc deprivation and induce expression of selected genes coding for proteins, including the Pht family. These proteins scavenge and bind available zinc ions using their histidine-triad motifs for Fe binding. Zinc binding, in turn, stimulates protein-independent streptococcal adhesion by still unknown molecular mechanisms [135]. Once invading bacterial cells reach the bloodstream, however, zinc ions are relatively abundant in their new environment, which leads to blocking the production of the Pht proteins, and allows bacterial cells to avoid adhesion, including adhesion to macrophages, leading to killing by the host immune system.

The functional model presented above for the Pht proteins is plausible. Zn^{2+} has been found in many complexes with proteins and the presence of His among the ligands with which it coordinates is relatively common. However, Zn^{2+} does not strongly discriminate in choices for ligands in its primarily (distorted)tetrahedral coordination sphere when bound to proteins [136]. Other metals are far more discriminating in this respect. Mn^{2+}, for example, is more discriminating as it accepts only carboxylates of Asp or Glu, carboxyamides of Asn or Gln, and nitrogen atom of His. As such, close association of binding Zn^{2+} ions with the His-triad repeat sequence is risky. Strongest evidence of support of such Zn^{2+} ion binding possibility comes from structural studies of a short fragment of PhtA protein containing aa residues 166–220 of this 816 residue long molecule (in the R6 *S. pneumoniae* strain) [137, 138]. This short fragment contains a stable Zn^{2+} binding motif with a novel structure. The residues coordinating to this metal are His194, His197, His199, and Asp173 in a distorted tetrahedral coordination geometry. The Zn^{2+} ions appear to originate from the production of recombinant protein in *Escherichia coli* due to the natural affinity of PhtA protein for these ions, and not from chemical reagents used in purification or crystallization [138]. This structure, however, fails to provide more hints to the functional properties of the PhtA molecule, as well as other Pht proteins, outside of the possibility outlined above.

Therefore, even though the functional outline for the Pht proteins in the earlier paragraph is logical, and certainly plausible, more studies and more experimental evidence are clearly needed to delineate functional properties and their underlying molecular mechanism of these proteins. The PsaA molecule mentioned above as an example of classical lipid-bound proteins, is also implicated in binding metal ions, specifically Mn^{2+} or Zn^{2+} [139]. The sequence of this protein, however, does not contain the histidine-triad repeats, suggesting again that more studies are needed to characterize the function, structure, and the underlying molecular mechanisms of Pht family of proteins.

Fibronectin-binding proteins PavA and PavB

Investigations of bacterial cells interactions with their host tissues suggests that adhesion and interactions with host fibronectin molecules play an important role for *S. pyogenes*, *Streptococcus gordonii*, *S. pneumo-*

niae, as well as many others. Fibronectin, a large multidomain mammalian ECM glycoprotein, is present in basement membranes, as well as in plasma, cerebrospinal, and amniotic fluids [140]. Ability of bacteria to bind to fibronectin is associated with their ability to colonize and invade. The sequence of fibronectin is highly conserved among vertebrates. It is present in solution in various states of aggregation, such as dimers and oligomers [140]. It is a versatile molecule involved in many basic biological processes including metastasis, wound healing, embryogenesis, and blood clotting. Fibronectin has specific binding epitopes for other proteins (collagen, integrins), and carbohydrates (heparin). Bacteria appear to have several types of adhesins to fibronectin, and not all classes of streptococcal fibronectin-binding protein are shared by all streptococcus species. Examples are the lack of *S. pyogenes* fibronectin-binding protein (PFBP) type in *S. pneumoniae* that, on the other hand, is found in *S. pyogenes* and *S. dysgalactiae* [141]. *S. pneumoniae* adheres to immobilized fibronectin but not to its soluble form [142, 143]. The natural conclusion would be to assign adhesive properties to either surface glycans, such as found in the capsule, or to specific surface-localized proteins.

PavA is essential for virulence

The first pneumococcal protein with fibronectin binding properties was identified through homology to *S. pyogenes* 54-kDa fibronectin-binding protein (Fbp54). This fibronectin-binding protein was termed PavA (pneumococcal adhesion and virulence A protein) [33]. PavA protein encodes a 551-aa residue protein with 74% identity to *S. gordonii* fibronectin-binding protein A (FbpA) and 67% to that of *S. pyogenes* Fbp54. The molecule is present in all 64 isolates of *S. pneumoniae* and is attached to bacterial cells by a still unknown mechanism. The possibility of binding to peptidoglycan structures through LPxTG-like motifs or to surface choline residues *via* a CBD attachment mechanism has, however, been eliminated. Immunoelectron microscopy localizes this molecule to the surface of bacterial cells. The mature, full-length recombinant version of this protein binds preferentially to immobilized fibronectin as compared to binding to its soluble counterpart. Such binding is heparin sensitive, suggesting that PavA binds to the heparin-binding domain of fibronectin. This binding is attributed to the C-terminal part of the protein. More precise details of binding and interactions between these two molecules, as well as the underlying molecular mechanism await investigation.

Pneumococci without PavA protein, however, retain about half of their fibronectin-binding capacity [33, 143], suggesting the presence of additional fibronectin-like surface binding molecules, presumably protein(s).

Characterization of properties of PavB/SP0082

An additional fibronectin-binding protein, product of SP0082 ORF from the TIGR4 genomic sequence has been identified [32]. Similarly to PavA, Sp0082 is highly immunogenic. Unlike PavA, SP0082 is a multidomain molecule having four copies of the fibronectin-binding domain. This domain has no recognizable homology with any other protein [32].

Using the PavA nomenclature as an example, the protein coded by SP0082 ORF in TIGR4 genome is termed here pneumococcal adhesion and virulence B protein (PavB). PavB consists of four repeat domains termed here streptococcal surface repeat domains (SSURE) that are located at its N terminus. The SSURE domains are followed by a proline-rich, presumably flexible peptide linker, and a segment responsible for the attachment to the peptidoglycan cross-bridges of the LPxTG type (the LPNTG motif preceded by charged residues and followed by hydrophobic ones). One of the SSURE domains has been cloned, produced in *E. coli*, and the recombinant form analyzed. Binding to immobilized fibronectin was demonstrated by enzyme linked immunosorbent assays (ELISA) methods. In addition, production of the entire PavB protein in *S. pneumoniae* and its localization at the cell surface have been demonstrated by ELISA and flow cytometry for TIGR4 and R6 strains. The SSURE domain alone was demonstrated to be highly immunogenic in mice [32].

Other streptococcal organisms display similar proteins to PavB with the number of SSURE domains varying from two to six. Such bacteria include *S. agalactiae*, *S. mitis*, and *S. gordonii.* It is tempting to speculate that repetitive repeat domain attach to repetitive host fibronectin motifs. The modular structure of fibronectin clearly facilitates such a possibility [144]. For example, two to six SSURE domains could interact with consecutive fibronectin repeats, such as 6 type I repeats or 14 type II repeats. As PavA binding is localized partially in the C-terminal part of fibronectin, the interactions with type III repeats are more feasible, as they are in similar locations. Type I repeats, on the other hand, are localized in the N-terminal portion of fibronectin. Structural studies of fibronectin demonstrate that the type III repeats are spaced by approximately 45 Å [145], whereas type I fibronectin repeat are spaced by ~22 Å [146]. The 3-D modeling of PavB SSURE domain suggests a putative fibronectin-binding site and spacing of consecutive domains by ~40 Å, surprisingly similar to the spacing of type III repeats on fibronectin molecule [32]. Closer analysis of the SSURE domain model suggests a two-domain

molecule (Fig. 11). This model also allows for the prediction of a fibronectin-binding site that is localized to the C-terminal domain. This putative binding site is lined by hydrophobic residues that are available for binding interactions with fibronectin.

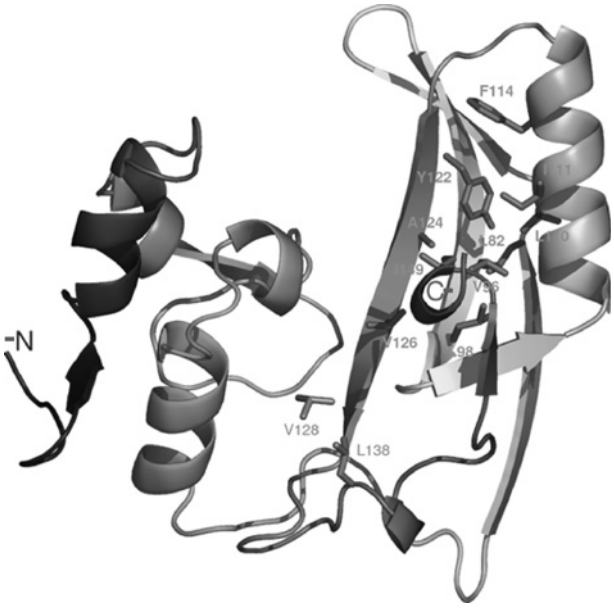

Figure 11. The 3-D model of the streptococcal surface repeat (SSURE) domain of pneumococcal adhesion and virulence B (PavB) fibronectin-binding protein. The residues comprising the putative fibronectin-binding site are shown as sticks. The two domains (left and right) at the N- and C-terminal parts of this protein are depicted. The figure is based in part on Bumbaca et al. [32].

PavA and PavB utilize a novel mode of fibronectin binding

Investigations of fibronectin-binding proteins from other organisms identified specific sequence signatures responsible for fibronectin binding. One such signature is a $GGx_{3-4}I/VDF$ repeated (where x is any aa residue) [147, 148], and the second is a VETEDT motif [149]. Neither of these two signature motifs has been identified in PavA or PavB proteins. They presumably evolved, therefore, to utilize another mode(s) of binding to ECM and fibronectin.

Fibronectin-binding proteins from other bacterial [33, 141] seem to group in homologous families such as that related to *S. pyogenes* PFBP. Different streptococcal bacteria have a different repertoire of fibronectin binders. The differences between such binders perhaps relate to differences in the specific types of fibronectin present in their respective targeted host tissues. For example, PavA and -B proteins may play a role in pneumococcal colonization and spread from nasopharynx and lungs to the blood stream and the nervous system [143]. Moreover, the listerial homologue of PavA has been shown to be essential for functional activity of the *Listeria monocytogenes* protein InlB and listeriolysin O [150, 151]. Every tissue environment in pneumococcal life is rich in fibronectin. The likely importance of this fibronectin-binding protein in aspects of the pneumococcal life cycle, the immunogenicity of the PavA [33, 143] and -B proteins in mice [32], together with their surface localization and PavA function in adherence make it an important virulence factor of pneumococci. More studies are needed, however, on the molecular mechanisms, underlying their functional properties, especially for PavB.

Other pneumococcal surface proteins with known structural properties

Examples of other major proteins from the surface of *S. pneumoniae* with known structures are CbpA [152] and CbpE [153, 154], and lipoprotein SlrA [155]. CbpA is a major adhesin; CbpE is a phosphorylcholine esterase, whereas SlrA is a peptidyl-prolyl isomerase. An insight into PsaA [23], an Mn^{2+} and/or Zn^{2+} metal transporter of ABC type, and a novel mucin-binding protein has also been provided [156]. Others can sometimes be modeled in three dimensions by computations methods, and those could presumably include neuraminidases NanA and B. In addition, the 3-D structures of numerous surface proteins from other bacterial organisms have been elucidated. New bacterial surface proteins will be structurally characterized as time progresses, and such studies will contribute to our understanding of molecular mechanisms underlying their functions and role in bacterial life, as well as in the disease process. Due to space constrains they are not discussed here in more detail.

Summary

Conclusions

Experimental identification and characterization of properties of extracellular bacterial virulence factors is time consuming and laborious. This process is made easier and faster with the availability of the sequences of chromosomal DNA, especially in cases where sequences of genomes of several strains of the same organism are available. Such sequences allow the identification of a number of these proteins directly from the genomic data. The identification and analyses of relevant ORFs coding for such proteins is not trivial, but is becoming easier and simpler to use, and is more robust as time goes on. Also, the vast advancements in the bioinformatics methods for the analysis

of such ORFs allow for broad utilization of such approaches. The outcome often consists of predictions of (i) reliable secondary structure and domain boundaries of proteins, (ii) their putative function, and often (iii) even their predicted structures. The success rates in functional and structural predictions are not great and often require a high level of expertise in these computational methods. Such analyses can lead to the discovery of new protein virulence factors and to a drastically shortening in the time required for their characterization. A list of residues for mutational analysis that are involved in function and mechanisms is not an uncommon result of detailed bioinformatics studies. Occasionally, function can be reliably predicted, even in the absence of experimental evidence. Even though these new approaches are very helpful, it is nearly impossible to avoid experimental verification of sequence analysis-driven predictions. However, once a new protein has been characterized for one bacterial organism, such data can be extended in a relatively straightforward manner to other organisms carrying the same or similar genes, especially within the major groups of bacteria within the Gram-positive or Gram-negative class. Due to marked differences in the structure of their cell walls, the surface proteins of Gram-positive and -negative bacteria are often different. For example, in contrast with Gram-positives, Gram-negative bacteria have double membranes, well-defined periplasmic spaces, lipopolysaccharides, and their secretion systems I, II, III, and IV. Two of the last secretion systems are currently being extensively studied, as their molecular mechanisms of function are relatively little known. On the other hand, Gram-positive bacteria do not have a well-defined periplasmic space, and they have different anchoring and release mechanisms for their surface proteins, such as peptidoglycan- or (lipo)teichoic acid-bound ones. As a consequence of these significant differences, the virulence factors, surface proteins, and the mechanisms of their interactions with the host of these two major groups of bacteria are certainly destined to have significant differences.

Future directions

There seems to be an increase in application of biophysical methods, especially structural ones, to characterize molecular processes of bacterial proteins involved in interaction with their hosts. Examples of major methods are X-ray crystallography, nuclear magnetic resonance (NMR), and SAXS. As these methods are becoming more easily available, their importance in characterizing these proteins is increasing. There are numerous examples of studies of entire complexes between protein virulence factors and their ligands or other proteins (macromolecular assembles), for example, that provide direct and reliable evidence not only related to structure but also upon function, and their underlying molecular mechanisms. Utilization of such approaches will continue for some time, and should yield valuable new information.

Further utilization of advanced, state-of-the-art sequence and bioinformatics analyses of proteins involved in the interactions with the host should greatly enhance advances in this field. As a consequence, for the first time, these studies can follow a designed rational path of hypothesis and experimental investigations, instead of less empirical approaches.

In addition, methods and strategies developed for pneumococcal factors, their characterization, and deconvolution of their functional, and other interactions with additional molecules, such as antibodies or complement molecules, will be invaluable in characterizing virulence factors of other bacterial organisms.

Application of molecular mechanisms in the development of cures

The information regarding the functions of virulence factors, by delineating the mechanism(s) of how these tasks occur, and by detailed mapping of the essential functional/mechanistic parts of these molecules are important. These parts of antigens are the least likely to change (in different newly emerging bacterial strains). Therefore, they are likely to elicit immunity that is effective against most, if not all, strains of a specific bacterium or group of bacteria. Such selected immunogens or portions thereof are reasonable candidates for inclusion in new vaccine compositions. These should yield prophylactics that are more effective in the long run. However, changes in these portions of the antigens would normally cause emergence of resistance, but as they would also compromise the function of the entire molecule/virulence factor, they are unlikely to emerge.

At the same time, delineated molecular mechanisms can be used to design small molecular inhibitors, to prevent or to modulate the destructive functions of bacteria. Such molecules could be inhibitors of enzymes or inhibitors of protein-protein interactions, resulting in prevention of active molecular assembly. Once inhibitory compounds are known, similar therapeutic agents could be developed for use as new antibiotics. The mechanisms of the virulence factors' action and interactions with the host are, therefore, very important, especially for the understanding of bacterial life and their pathogenesis, as well as for the generation of new drugs.

Acknowledgements. The author would like to acknowledge assistance and helpful discussions with present and past members of his laboratory and with collaborators.

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