The structure of secondary cell wall polymers: how Gram-positive bacteria stick their cell walls together

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The cell wall of Gram-positive bacteria has been a subject of detailed chemical study over the past five decades. Outside the cytoplasmic membrane of these organisms the fundamental polymer is peptidoglycan (PG), which is responsible for the maintenance of cell shape and osmotic stability. In addition, typical essential cell wall polymers such as teichoic or teichuronic acids are linked to some of the peptidoglycan chains. In this review these compounds are considered as ‘classical’ cell wall polymers. In the course of recent investigations of bacterial cell surface layers (S-layers) a different class of ‘non-classical’ secondary cell wall polymers (SCWPs) has been identified, which is involved in anchoring of S-layers to the bacterial cell surface. Comparative analyses have shown considerable differences in chemical composition, overall structure and charge behaviour of these SCWPs. This review discusses the progress that has been made in understanding the structural principles of SCWPs, which may have useful applications in S-layer-based ‘supramolecular construction kits’ in nanobiotechnology.

Overview

The cell walls of Gram-positive bacteria usually contain a variety of polysaccharides, a significant proportion of which are covalently linked to peptidoglycan (PG), the major scaffolding structure of the cell wall. As established in the 1980s, cell wall polysaccharides of Gram-positive organisms can be classified on the basis of their structural characteristics into three distinct groups: (i) teichoic acids (Archibald et al., 1968, 1993), (ii) teichuronic acids (Hancock & Baddiley, 1985; Ward, 1981), and (iii) other neutral or acidic polysaccharides which cannot be assigned to the two former groups (Araki & Ito, 1989; Naumova & Shashkov, 1997). As all these compounds have long been attributed a secondary role in cell wall function, they have been termed ‘secondary’ cell wall polymers (SCWPs). Biochemical and genetic data accumulated over the past 30 years indicate that the first two groups of these polysaccharides (‘classical’ SCWPs) play pivotal roles in normal cell function, and consequently considerable energy is expended in their biosynthesis (Archibald et al., 1993; Munson & Glaser, 1981; Pooley & Karamata, 1994).

In the course of recent investigations of bacterial cell surface layer (S-layer) proteins from Bacillaceae (Messner & Schäffer, 2003; Sleytr, 1978; Sleytr et al., 1996, 2002), novel aspects of a group of ‘non-classical’ SCWPs have emerged with regard to their structure and function. S-layers are generally composed of identical (glyco)protein species forming regular two-dimensional, lattices on bacterial cell surfaces. The high metabolic load associated with the biosynthesis of S-layer proteins, together with their prominent cellular location, indicates that they provide a positive selective advantage in the environment. It is conceivable that the stable attachment of S-layer (glyco)-proteins to the cell wall is important for the cell, and ‘non-classical’ SCWPs have been identified as mediators for non-covalent attachment of S-layers to the underlying PG meshwork (Cava et al., 2004; Mesnage et al., 2000; Sára, 2001) (Fig. 1).

We review here the current knowledge on structural features, possible linkage types to the cell wall, and interactions of ‘non-classical’ SCWPs in S-layer-carrying organisms from the family Bacillaceae. Structural comparisons have only recently started to be made, and nothing is as yet known about the biosynthesis of the ‘non-classical’ SCWPs. The structural information, however, has considerable impact on the development of S-layer-based applications in nanobiotechnology, for which these SCWPs may serve as a basic building block in a ‘supramolecular construction kit’ (Pum et al., 2004; Sampathkumar & Gilchrist, 2004; Schäffer & Messner, 2004; Sleytr et al., 2002).

SCWPs of Gram-positive bacteria – a brief retrospective

Teichoic and teichuronic acids are among the best-characterized SCWPs, with respect to both structure and function. Although they are structurally diverse (Araki & Ito, 1989; Naumova & Shashkov, 1997), the negative charge of these anionic polymers mainly originates from...
phosphate (teichoic acids) or carboxyl (teichuronic acids) groups, and they may also elaborate acidic side chains containing glycerol, phosphate, organic acids (e.g. pyruvic and succinic acid), or sulphate. While the overall structure of the different types of teichoic acids is well documented (Archibald et al., 1993; Fischer, 1988; Hancock & Baddiley, 1985; Munson & Glaser, 1981), the structural features of teichuronic acids are less well understood and only a few have been subjected to full chemical analyses (Munson & Glaser, 1981; Ward, 1981). These anionic polymers account for 10–60 % (by weight) of the bacterial cell wall, with the relative amount depending on the culture conditions (Archibald et al., 1993; Ellwood & Tempest, 1969; Rogers et al., 1980). Although the exact biological function (or functions) of these negatively charged SCWPs is not fully understood, several general functions have been attributed to them. These include (i) binding of divalent cations, (ii) role in the balance of metal ions for membrane functionality, (iii) binding of proteins, (iv) role in folding of extracellular metallo-proteins, (v) providing a source of phosphate under phosphate starvation conditions, (vi) interaction with cell wall lytic enzymes, and (vii) formation of a barrier to prevent diffusion of nutrients and metabolites (Archibald et al., 1993; Baddiley, 1972; Fischer, 1994; Hancock & Baddiley, 1985; Munson & Glaser, 1981; Navarre & Schneewind, 1999).

Besides the considerable body of knowledge that has accumulated about structural, biochemical and immunological features of teichoic and teichuronic acids from cell walls of Gram-positive bacteria, it should be kept in mind that further cell wall polysaccharides may be present in these organisms. These will be discussed in the next section.

The ‘non-classical’ group of SCWPs

The discovery of ‘non-classical’ SCWPs

Our research has focused on glycosylated S-layer proteins, which are regarded as ideal model systems for studying the glycosylation of prokaryotic proteins (for recent reviews see Messner & Schäffer, 2003; Novotny et al., 2004; Schäffer & Messner, 2004). In the course of the purification of S-layer glycoproteins, which constitute up to 15 % of the cell walls of Bacillaceae (Schäffer et al., 2001), minor amounts of a second glycoconjugate are frequently co-isolated (Altman et al., 1990, 1996; Messner et al., 1987; Schäffer et al., 1999, 2000, 2004; Steindl et al., 2002). Considering the available data about multi-glycosylated S-layer proteins from archaea (Sumper & Wieland, 1995), these ‘additional carbohydrate structures’ were originally interpreted as being a second set of S-layer glycoprotein glycan chains of low abundance. Improved purification and separation methods, however, eventually led to the clear assignment of these compounds as a class of SCWPs, which accounts for a substantial amount (7–15 % by weight) of the PG of the investigated organisms. The investigated SCWP–PG complexes comprise the intact glycan moieties and portions of PG of variable size, due to random degradation during the preparation procedure. Based on compositional and structural data, we suggest that classification of the ‘non-classical’ SCWPs from S-layer-carrying Bacillaceae into group (iii) of Araki & Ito (1989) is most appropriate.

Common and variable features of ‘non-classical’ SCWPs from Bacillaceae

Although the structural analysis of SCWPs from S-layer carrying Bacillaceae is still in its infancy, comparison of those SCWPs for which data are available has revealed a number of common features (Table 1; see also Fig. 2). These can be summarized as follows.

Group I. The structure of the glycan portion of the SCWP–PG complex of Paenibacillus alvei CCM 2051 was elucidated to be [(Pyr4,6)-β-D-ManpNAC-(1→4)-β-D-GlcpNAC-(1→3)]ₙ₋₁·[(Pyr4,6)-β-D-ManpNAC-(1→4)-α-D-GlcpNAC-(1→) (Schäffer et al., 2000). Each repeating unit disaccharide of this SCWP is substituted with 4,6-linked pyruvic acid residues, conferring the overall anionic character of the SCWP. Upon prolonged exposure of the

![Fig. 1. Schematic representation of the cell wall profile of (A) S-layer-protein- and (B) S-layer-glycoprotein-carrying Gram-positive bacilli to indicate the cellular location of SCWP. The dotted ovals serve to highlight the SCWP. The SCWP is covalently bound to muramic acid residues of PG but attached non-covalently, presumably by a lectin-type interaction (Sára, 2001), to the S-layer protein. CM, cytoplasmic membrane; MP, membrane protein; PG, peptidoglycan; SP, S-layer protein; SLG, S-layer glycoprotein glycan; SCWP, secondary cell wall polymer.](image-url)
Table 1. Features of 'non-classical' SCWPs from S-layer-carrying *Bacillaceae*

<table>
<thead>
<tr>
<th>Features</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycan composition</td>
<td>Heteropolysaccharide</td>
</tr>
<tr>
<td>Sugar constituents</td>
<td>Glc, ManpNAC, GalpNAC, Manp-2,3-diNAcA, Glcp, Ribf</td>
</tr>
<tr>
<td>Possible non-carbohydrate modifications</td>
<td>Pyruvate, phosphate, acetate</td>
</tr>
<tr>
<td>Charge</td>
<td>Negative or neutral</td>
</tr>
<tr>
<td>Repeating units</td>
<td>2–15; linear or branched (chain-length variation possible)</td>
</tr>
<tr>
<td>Number of glycoses per repeating unit</td>
<td>2–5</td>
</tr>
<tr>
<td>Average molecular mass</td>
<td>~4–6 kDa</td>
</tr>
<tr>
<td>Backbone structures</td>
<td></td>
</tr>
<tr>
<td>Group I SCWP</td>
<td>([→3]-β-D-ManpNAC-(1→)-β-D-GlcANac-(1→])</td>
</tr>
<tr>
<td>Group II SCWP</td>
<td>([→4]-β-D-Manp-2,3-diNAcA-(1→6)-α-D-Glc-(1→4)-β-D-Manp-2,3-diNAcA-(1→3)-α-D-GlcANac-(1→])</td>
</tr>
<tr>
<td>Group III SCWP</td>
<td>No common motif</td>
</tr>
<tr>
<td>Antennary structure</td>
<td>Possible</td>
</tr>
<tr>
<td>Content in PG</td>
<td>7–15 % (by weight)</td>
</tr>
<tr>
<td>Substitution of muramic acid residues</td>
<td>20–25 %</td>
</tr>
<tr>
<td>Type of linkage to muramic acid</td>
<td>Under investigation; phosphodiester or pyrophosphate linkages may exist</td>
</tr>
<tr>
<td>Number of different SCWPs per organism</td>
<td>1</td>
</tr>
</tbody>
</table>

**Fig. 2.** Structures of SCWPs of S-layer-carrying *Bacillaceae*. Open symbols, **gluco**-configuration (☐, *N*-acytlyglucosamine; ○, *D*-glucose; □, *N*-acytlymuramic acid); black symbols, **manno**-configuration (■, *N*-acytlymannosamine; ●, *D*-2,3-di-*N*-acytlymannosaminuronic acid, *R*₁ = COOH, *R*₂ = CONH₂, *R*₃ = CONHCONH₂, *R*₄ = CON(COOH)₂); light grey symbols, **galacto**-configuration (□, *N*-acytlygalactosamine); dark grey symbol, **ribo**-configuration (●, *D*-ribofuranose); ◊, phosphate-containing linker group.
polymers to acidic conditions, as obtained upon solvation in D$_2$O during NMR experiments, considerable loss of pyruvate residues can occur. In a first set of experiments, $^{31}$P-NMR spectroscopy revealed two phosphorus signals with a markedly different integral ratio, which was interpreted as a possible pyrophosphate linkage between SCWP and PG. Indirect support for this assumption came from treatment with EDTA, SDS, and sonication of the SCWP–PG complex immediately prior to the NMR measurement. The $^{31}$P spectrum then revealed two phosphorus signals with an integral ratio of almost 1:1. Thus, the presence of an $\alpha$-D-GlcPNAc-(1→O)-PO$_3^-$-O-PO$_3^-$-(O→6)-MurNAc linkage unit was inferred from that set of biochemical and NMR data.

The ManNAc-GlcNAc backbone disaccharide motif corresponds to that frequently observed in other cell wall polysaccharides, albeit with an inversion of the anomeric configuration of the D-GlcNAc residue at the reducing end (Fig. 2). It has been suggested that inversion of the anomeric linkage plays a role in the initial polymerization event of the carbohydrate chain during glycoconjugate biosynthesis, because the attachment of the first repeating unit is a critical step for the complete and correct elongation of the polysaccharide chain (Olsthoorn et al., 2000).

The ManNAc-GlcNAc backbone disaccharide motif is also reminiscent of the linkage unit of certain teichoic acids (Araki & Ito, 1989). However, in the 'non-classical' SCWPs this motif is repeated several times, thus constituting the entire glycan moiety of those SCWPs.

Pyruvic-acid-containing SCWPs have also been reported for the S-layer carrying organisms Bacillus sphaericus CCM 2177 (Iké et al., 1999) and Bacillus anthracis (Mesnage et al., 2000); however, information is not available on either their full structures or their linkage to the PG layer.

In contrast to anionic polymers, neutral polysaccharides possessing the identical backbone motif are found in the PG of other Bacillaceae. The SCWPs of Thermoanaerobacterium thermosaccharolyticum strains D120-70 (Altman et al., 1990; C. Schäffer, H. Kählig & P. Messner, unpublished results) and E207-71 (Altman et al., 1996) display the commonly encountered $\rightarrow 3$-\(\beta\)-D-ManpNAc-(1→4)-\(\beta\)-D-GlcPNAc-(1→4)-\(\beta\)-D-ManpNAc-(1→3)-\(\beta\)-D-GlcPNAc-(1→) motif. Although previously it was thought that in strain D120-70 this motif is extended by galactose residues (Altman et al., 1990), the most recent results show that alternating ManNAc residues are substituted by ribofuranose side chains (C. Schäffer, H. Kählig, R. Christian & P. Messner, unpublished results), as also demonstrated for the SCWP of strain E207-71 (Altman et al., 1996).

**Group II.** The first SCWP–PG complex of an S-layer-carrying organism for which the structure was completely elucidated was purified from Geobacillus stearothermophilus NRS 2004/3a (Messner et al., 1987; Schäffer et al., 1999). The anionic polymer, comprising on average six tetrasaccharide repeating units with the structure \(\rightarrow 4\)-\(\beta\)-D-Manp$_2$3-diNAcA-(1→6)-\(\alpha\)-D-Glc$_p$-(1→4)-\(\beta\)-D-Manp$_2$3-diNAcA-(1→3)-\(\beta\)-D-GlcPNAc(1→), represents the SCWP of many G. stearothermophilus wild-type strains. Preliminary analysis of that SCWP indicated a mass range of 4000 to 6000. To identify the linkage region between the repeating units and the PG, $^1$H spectra were recorded with simultaneous $^{31}$P decoupling to detect signals of phosphorylated sugars. Recent NMR data suggest that, instead of a phosphodiester bond which serves as linkers for teichoic and teichuronic acids, this SCWP is linked to C-6 of muramic acid of PG via a pyrophosphate bridge, with about 20–25% of muramyl residues being substituted by SCWP glycans (Schäffer et al., 1999).

Interestingly, preliminary analyses of the SCWP of Geobacillus tepidamans G55-97 indicate that its backbone structure is reminiscent of that of G. stearothermophilus NRS 2004/3a, with additional modifications of the carboxyl groups of the Manp-2,3-diNAcA residues, turning the anionic character of the glycan into a neutral one. A recent investigation has demonstrated that, in this organism, the SCWP is linked to muramic acid by a common phosphodiester linkage. The definitive structure of the SCWP of G. tepidamans G55-97 is currently being elucidated (C. Steindl, C. Schäffer, P. Messner & N. Müller, unpublished).

**Group III.** The charge-neutral SCWP isolated from Aneurinibacillus thermoauricus DSM 10155 represents a hitherto unique bacterial glycan structure (Steindl et al., 2002; Wugeditsch, 1998). Salient features of that SCWP, as deduced from NMR spectroscopy, are: (i) the biantennary oligosaccharide structure, and (ii) the chemical homogeneity of the oligosaccharide part (defined glycan chain length) (Fig. 2). It is important to note that a biantennary SCWP has, so far, not been reported for a member of the domain Bacteria. Thus, this SCWP resembles the glycoprotein glycans of eukaryotic rather than polysaccharides of prokaryotic organisms (Varki et al., 1999).

**General considerations about ‘non-classical’ SCWP structure**

Comparison of the overall composition of all ‘non-classical’ SCWPs analysed so far reveals that the more complex group II glycans exhibit the same alternating order of gluco and manno sugars as the group I glycans (Fig. 2). The glycan chain starts with a GlcNAc residue at the reducing end and ends with Manp-2,3-diNAcA. It is possible that the group II structures have evolved from the simpler group I structures by the introduction of residues such as glucose or Manp-2,3-diNAcA through the action of strain-specific enzymes. Preliminary data on the biosynthetic origin of the Manp-2,3-diNAcA residues of the lipopolysaccharide of Bordetella pertussis (Wing et al., 2004) have established that the relevant nucleotide-activated intermediate is synthesized from UDP-GlcNAc via several reaction steps including oxidation at C-6,amination, transacetylation,
and epimerization at C-3, prior to its insertion into the repeating unit of the SCWP. Likewise, deacetylation and deamination of GlcNAc to Glc is conceivable.

Concerning the linkage type between SCWPs and PG, our studies suggest that phosphodiester and pyrophosphate linkages may exist in different organisms. However, it might well be that the pyrophosphate-linked glycans identified in SCWP preparations represent biosynthetic intermediates, which under certain conditions can be detected as dominating molecular species in $^{31}P$-NMR spectra (N. Müller, C. Schäffer & P. Messner, unpublished data). It is known, for instance, from *Bacillus cereus* that sugar-pyrophosphoryllipid intermediates can be present in membrane preparations (Yamamori et al., 1978). Under *in vivo* conditions, the pyrophosphate bridge might represent a predetermined cleavage site with possible relevance for glycan biosynthesis. In general, pyrophosphate bridges are commonly formed in intermediates involved in the biosynthesis of different prokaryotic cell wall polymers. Currently the linkages between SCWPs and PG are being investigated in detail. Interestingly, for all investigated SCWPs, the glucose residue linked to the bridging phosphate residue is in the α-configuration. Whether this has any impact on the biosynthesis of the SCWPs remains to be established.

**Interactions of SCWPs and S-layers from *Bacillaceae***

In addition to the previously mentioned general features, novel properties for SCWPs have emerged from research on S-layers. S-layers are two-dimensional crystalline protein lattices on the outermost surface layer of bacteria from almost all phylogenetic branches (Sleytr, 1978; Sleytr et al., 1996). S-layer proteins are of interest because of their involvement in important physiological processes which include, in the case of pathogenic bacteria, the infection mechanism (Navarre & Schnewind, 1999). If present, S-layer (glyco)proteins are the most abundant cellular proteins. Provision of any kind of selection advantage by the S-layer to a bacterium in its natural environment requires the stable attachment of the S-layer to the cell surface *in vivo*. During evolution, Gram-positive bacteria have developed various strategies for displaying proteins on their surface. These strategies include predominantly covalent binding of LPXTG-carrying proteins to PG, but a number of non-covalent binding strategies have also been developed (Navarre & Schnewind, 1999). Reattachment experiments of isolated S-layer glycoproteins from thermophilic clostridia revealed the non-covalent character of the interaction between S-layer and PG (Sleytr, 1976). For *Lactobacillus buchneri* it was shown very early that hydroxyl groups of a neutral cell wall polysaccharide are responsible for the attachment of the S-layer protein to the cell wall (Masuda & Kawata, 1985).

Possible cell-wall-targeting mechanisms. Recently, the cell-wall-targeting mechanism of S-layer proteins has been investigated in more detail (Cava et al., 2004; Mesnage et al., 2000; Sára, 2001). These studies suggest that, in general, S-layer proteins have two functional regions: a cell-wall-targeting domain, which in most of the organisms investigated thus far is located at the N-terminus, and a C-terminal self-assembly domain. The existence of a cell-wall-targeting domain in S-layer proteins was substantiated by Fujino et al. (1993) and Lupas et al. (1994), who identified motifs of approximately 55 amino acids, containing 10–15 conserved residues, which were designated SLH (S-layer homology) domains. SLH domains, usually composed of one to three modules, are frequently used means for targeting proteins to the cell surface. They are found not only in various S-layer proteins but also in many other surface-associated proteins, e.g. the cellulosomes (Bayer et al., 2004) or other surface-associated enzymes (Liu et al., 1996). In the case of *Bacillus anthracis*, the aetiological agent of anthrax, the molecular basis of the interaction between SLH domains and SCWPs was established by elucidating their binding properties (Mesnage et al., 2000). Both S-layer proteins of *B. anthracis* (EA1 and Sap) possess SLH domains that bind the S-layer proteins directly to PG (Mesnage et al., 1997, 1999). A different binding mechanism was proposed for the S-layer protein of *Geobacillus stearothermophilus* strain PV72/p2 (Ries et al., 1997; Sára et al., 1998a). In this organism two different binding domains were identified in the N-terminal region of the S-layer protein SbsB, one for SCWP and another for PG (Sára, 2001; Sára et al., 1998b). In *Thermus thermophilus* a similar binding mechanism was identified between an S-layer–outer membrane complex and the cell wall. There is a strong interaction of the SLH domain of the S-layer protein with a pyruvylated component of a highly immunogenic SCWP (Cava et al., 2004).

Originally the involvement of pyruvyl groups in S-layer binding was inferred from observations in *B. anthracis* (Mesnage et al., 2000). It was demonstrated that CsaB is involved in the addition of pyruvate to a PG-associated polysaccharide fraction, and that this modification is necessary for binding of the S-layers via SLH domains. Interestingly, the *csaAB* operon was found to be present in several bacterial species (Mesnage et al., 2000). The amount of pyruvate in cell walls of slh<sup>+</sup> csaB<sup>+</sup> strains was in the range of ~2 µg pyruvate per mg cell wall. Pyruvate was also identified in the SCWP repeating units of *Bacillus sphaericus* CCM 2177 (Ilk et al., 1999) and *Paenibacillus alvei* CCM 2051 (Schäffer et al., 2000) (compare with Fig. 2), which may be taken as an indication that pyruvate, or more generally speaking, negative charges of SCWPs, constitute a widespread mechanism for anchoring S-layer proteins containing SLH domains to the bacterial cell wall. In this context it should be mentioned that, according to recent investigations with *B. sphaericus* CCM 2177, SCWP–S-layer protein interaction occurs via four SLH domains (Huber et al., 2005), which is different from the accepted paradigm of the involvement of one to three SLH motifs for S-layer protein binding.

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Besides the anchoring mechanism involving SLH domains, another mechanism that possibly utilizes basic amino acids, present in the cell-wall-targeting region and known for their direct interaction with carbohydrates, may apply for S-layer proteins devoid of SLH domains (Table 2). In the genus Geobacillus, SLH domains have only been identified on the S-layer protein SbsB of G. stearothermophilus PV72/p2 (Kuen et al., 1997; GenBank accession no. X98095). All other investigated strains, such as G. stearothermophilus PV72/p6 (SbsA; Kuen et al., 1994; X71092), G. stearothermophilus ATCC 12980 (SbsC; Jarosch et al., 2000; AF055578), a variant strain of G. stearothermophilus ATCC 12980 (SbsD; Egelseer et al., 2001; AF228338), and G. stearothermophilus NRS 2004/3a (SgsE; Schäffer et al., 2002; AF328862) possess S-layer proteins devoid of SLH domains. For the S-layer protein SbsC it was shown experimentally that a positively charged N-terminal fragment (amino acids 31–257) is responsible for anchoring the S-layer subunits. Using several truncated forms of SbsC it was demonstrated that this region is not required either for self-assembly or for generating the oblique lattice structure (Jarosch et al., 2001). Interestingly, none of the SCWPs of the organisms that possess S-layer proteins without SLH domains are modified with pyruvyl groups and some of them have a net-neutral charge (see Fig. 2 and Table 2). These observations support the notion that, in addition to the previously discussed involvement of pyruvate, other mechanisms can be involved in the binding of S-layer proteins to the PG (Cava et al., 2004; Mesnage et al., 2000; Sára, 2001). In addition, the non-conserved character of S-layer binding mechanisms is shown by the observation that the cell-wall-targeting domain is not necessarily located in the N-terminal region of the S-layer protein. Well-documented examples of C-terminal anchoring are the S-layer proteins of Lactobacillus acidophilus ATCC 4556 (Smít et al., 2001) and Lactobacillus crispatus (Antikainen et al., 2002). Recently, for an S-layer protein...

Table 2. ‘Non-classical’ SCWPs as linker structures for S-layer proteins to the cell wall

<table>
<thead>
<tr>
<th>Organism</th>
<th>S-layer protein</th>
<th>SCWP</th>
<th>Site of interaction between SCWP and S-layer protein</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geobacillus stearothermophilus PV72/p2</td>
<td>SbsB (X98095)</td>
<td>Negatively charged (structure under investigation)</td>
<td>N-terminus (aa 33–204), three SLH motifs</td>
<td>Kuen et al. (1997); Ries et al. (1997); Sára et al. (1998a, b)</td>
</tr>
<tr>
<td>Bacillus sphaericus CCM 2177</td>
<td>SbpA (AF211170)</td>
<td>Negatively charged</td>
<td>N-terminus (aa 33–202), four SLH motifs; involvement of pyruvyl residues of SCWP</td>
<td>Huber et al. (2005); Ilk et al. (1999)</td>
</tr>
<tr>
<td>Paenibacillus alvei CCM 2051</td>
<td>Sequence unknown</td>
<td>Negatively charged</td>
<td>Unknown</td>
<td>Schäffer et al. (2000)</td>
</tr>
<tr>
<td>Bacillus anthracis</td>
<td>Sap (P49051), EA1 (P94217)</td>
<td>Negatively charged (structure unknown)</td>
<td>N-terminus, three SLH motifs; involvement of pyruvyl residues of SCWP</td>
<td>Mesnage et al. (1999, 2000)</td>
</tr>
<tr>
<td>G. stearothermophilus NRS 2004/3a</td>
<td>SgsE (AF328864)</td>
<td>Negatively charged</td>
<td>N-terminus, no SLH motifs</td>
<td>Messner et al. (1987); Schäffer et al. (1999)</td>
</tr>
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<td>G. stearothermophilus PV72/p6</td>
<td>SbsA (X71092)</td>
<td>Negatively charged</td>
<td>N-terminus (aa 31–257), no SLH motifs</td>
<td>Kuen et al. (1994)</td>
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<td>G. stearothermophilus ATCC 12980</td>
<td>SbsC (AF055578)</td>
<td>Negatively charged</td>
<td>N-terminus (aa 31–257), no SLH motifs</td>
<td>Jarosch et al. (2000, 2001)</td>
</tr>
<tr>
<td>Geobacillus tepidamans GS5-97</td>
<td>SgTA (AY883421)</td>
<td>Neutral</td>
<td>N-terminus, no SLH motifs (proposed site of interaction)</td>
<td>C. Steindl and others, in preparation</td>
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<tr>
<td>Aneurinibacillus thermaeolerophilus DSM 10155</td>
<td>SatB (AY395579)</td>
<td>Neutral</td>
<td>C-terminus, no SLH motifs (proposed site of interaction)</td>
<td>Wugeditsch (1998); Steindl et al. (2002); S. Zayni, P. Messner &amp; C. Schäffer, unpublished</td>
</tr>
<tr>
<td>Thermoanaerobacterium thermosaccharolyticum D120-70</td>
<td>Unknown</td>
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<td>T. thermosaccharolyticum E207-71</td>
<td>Unknown</td>
<td>Neutral</td>
<td>Unknown</td>
<td>Altman et al. (1996); C. Schäffer, H. Kählig, R. Christian &amp; P. Messner, unpublished</td>
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</tbody>
</table>
protein of another member of the Bacillaceae (Aneurini-bacillus thermoaeerophilus DSM 10155), C-terminal cell wall anchoring was proposed (S. Zayni, P. Messner & C. Schäffer, unpublished data).

The current hypothesis is that the binding between S-layer proteins and SCWPs is based on a lectin-type interaction (Sára, 2001). Although, generally, lectins display low affinities for carbohydrates, their interactions should be highly specific (Lee & Lee, 2000). Indeed, high binding specificity was demonstrated for the S-layer protein SbSB of G. stearothermophilus PV72/p2 and the negatively charged SCWP of that organism by real-time surface plasmon resonance biosensor technology (Mader et al., 2004). That study revealed the presence of at least two binding sites on a single SCWP molecule with a separation of about 14 nm and an overall $K_d$ of $7 \times 10^{-7}$ M. This poses the question of which of the motifs on the structurally defined SCWPs (compare with Fig. 2) would be available for the proposed lectin-type binding. In group I SCWPs the substitution of the ManNAc residues and, in particular, the ManNAc at the non-reducing end, occurs mainly at C-4 (and C-6 in the case of pyruvate substituents). This implies that only the hydroxyl groups at C-3 of ManNAc would be available for the proposed lectin-like linkage. Consequently, for the non-covalent interaction between SCWP and S-layer protein, a specificity similar to that of a mannose/ mannosamine-specific lectin may be predicted. In the case of the group II SCWPs of G. stearothermophilus only the hydroxyl groups at C-4 would be available for lectin binding. Consequently, as with group I SCWPs, mannose-like specificity is predicted for resulting lectin-like interaction. In contrast, for the group III SCWP of A. thermoaeerophilus DSM 10155, in which the glycan chains are terminated with GlcNAc residues, a lectin-like interaction with glucose/ glucosamine-specificity is predicted with additional doubling of the binding motifs due to the presence of two antennae.

Additional properties of S-layers. In the course of the investigation of the SCWP of Aneurinibacillus thermoaeerophilus DSM 10155, an interesting observation was made, which let us propose a novel property for this SCWP: mediation of water-solubility of the organism’s S-layer glycoprotein under in vitro conditions. Indeed the S-layer glycoprotein of that organism is, so far, the only reported completely water-soluble S-layer glycoprotein (Wugeditsch, 1998). In vivo, the S-layer subunits of A. thermoaeerophilus DSM 10155 assemble into a square S-layer glycoprotein lattice, whereas isolated S-layer glycoprotein subunits do not self-assemble; this property can, however, be restored in vitro after addition of polyethylene glycols as crystallizing agents to a glycoprotein preparation. Detailed analysis of the purified S-layer self-assembly products indicated that the self-assembly process is preceded by the dissociation of the co-purified SCWP from the S-layer glycoprotein (Steindl et al., 2002). This suggests the following scenario for the in vivo biological role of the SCWP of A. thermoaeerophilus DSM 10155. On the intact bacterial cell, the SCWP holds the S-layer subunits in close proximity, enabling inter- and intermolecular interaction, and thus permitting S-layer glycoprotein lattice formation to occur. When the supporting PG layer is removed during S-layer glycoprotein preparation, the covalent bonds between SCWP and the PG backbone are cleaved, releasing the S-layer glycoprotein with the polymer attached. It is conceivable that polyethylene glycols can replace SCWP at the lectin-like binding site on the S-layer protein, thereby functioning as macromolecular crowding agents. The resulting situation might be seen as mimicry of the excluded volume effect thought to prevail on the bacterial cell surface in vivo, which enables the glycosylated S-layer subunits of A. thermoaeerophilus DSM 10155 to interact with each other through hydrophobic interactions, resulting in S-layer lattice formation (Steindl et al., 2002). This interpretation supports the assumption of a lectin-type binding between S-layer (glyco)proteins and SCWP (Sára, 2001). The biantennary structure of the SCWP obviously results in a doubling of binding motifs for the S-layer glycoprotein, allowing a biologically relevant binding strength to be attained.

Conclusions and outlook

The diversity observed among different SCWP structures of Bacillaceae follows a general theme which is well known from other cell surface structures, such as the serotypes of lipopolysaccharides (Raetz & Whitfield, 2002) and capsular polysaccharides (Sutherland, 1999). Presumably, this diversity is responsible for creating microenvironments in which different organisms can survive under unfavourable conditions. Current data indicate that ‘non-classical’ SCWPs function as mediators for anchoring S-layer (glyco)proteins from Bacillaceae to the bacterial cell wall.

One interesting aspect of future research will concern the coordination of S-layer (glyco)protein biosynthesis and SCWP biosynthesis. It is known that the biosynthesis of S-layer proteins is a very complex, finely tuned process in which the amount of the protein component, its translocation through the cell wall, and its incorporation into the existing S-layer has to be coordinated with the growth rate of the bacterium and the production of other cell wall components, such as PG and SCWP. By analogy with the biosynthesis mechanisms of other glycoconjugates, it seems reasonable to assume that the biosynthesis of SCWP and aglycone (PG) occur at different cellular locations and that a later ligation step results in formation of the covalent linkage between the SCWP and PG.

Overall, the complete elucidation of the structure and biosynthesis of several ‘non-classical’ SCWPs will contribute to our general understanding of the various mechanisms underlying the tethering of S-layer (glyco)proteins to the cell surface of Gram-positive bacteria.
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