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Biogenesis and functions of bacterial S-layers

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ABSTRACT

The outer surface of many Archaea and Bacteria is coated with a proteinaceous surface layer (S-layer), which is formed by the self-assembly of monomeric proteins into a regularly spaced, two-dimensional array. Bacteria possess dedicated pathways for the secretion and anchoring of the S-layer to the cell wall and some Gram-positive species have large S-layer-associated gene families. S-layers have important roles in growth and survival and their many functions include maintenance of cell integrity, enzyme display and, in pathogens and commensals, interaction with the host and its immune system. Here we review our current knowledge of S-layer and S-layer associated proteins including their structures, mechanisms of secretion and anchoring and their diverse functions.

S-layers are found on both Gram-positive and Gram-negative bacteria and are highly prevalent in the Archaea[1-3]. They are defined as a two-dimensional crystalline array that coats the entire cell and they are thought to provide important functional properties. S-layers consist of one or more (glyco)proteins, termed S-layer proteins (SLPs), that undergo self-assembly to form a regularly spaced array on the surface of the cell. As some of the most abundant proteins in the cell[2], their biogenesis must consume considerable metabolic resources, reflecting their importance to the organism. S-layers were first recognized in the 1950s and studied in numerous species during the following decades, which revealed considerable detail of their structures using techniques such as freeze-etch electron microscopy (see BOX 1). Such studies[1,2,4] showed structurally diverse S-layers, with oblique (p1, p2), square (p4) or hexagonal (p6) **lattice symmetries**. Some Gram-positive species harbour protein families containing SLPs and SLP-related proteins that share a common cell wall anchoring mechanism. However it is not yet clear if all family members contribute productively to S-layer self-assembly. These proteins, for example the *Bacillus anthracis* S-layer associated proteins (BSLs) and the *Clostridium difficile* cell wall proteins (CWPs) (see below) are included in this Review as many are functionally relevant.

The lack of S-layers in the model organisms *Escherichia coli* and *Bacillus subtilis* hindered their molecular analysis during the “molecular microbiology” era of the 1980s. Recent advances in genomics and structural biology together with the development of new molecular cloning tools for many species has facilitated structural and functional studies of SLPs. Comprehensive reviews on S-layers

were written over a decade ago[1,2] and others have emphasized the exploitation of SLPs in nanotechnology[1,2,5 6]. An excellent review of the Archaeal cell envelope was published recently[3] which included the properties of S-layers in this domain of life. Here, we review the biology of bacterial S-layer proteins and highlight recent discoveries that have shaped our understanding of these important proteins. For convenience, Table 1 outlines all the SLPs described in this review.

Diversity of S-layer genes and proteins

S-layers are usually composed of a single protein, and their structural genes can be linked to genes encoding modification or secretory pathways. Despite the apparently conserved function of providing a two-dimensional array surrounding the cell, genetic and functional studies reveal a wide diversity in both sequences and roles for S-layer proteins.

Genetic Variation

Several bacterial species show genetic variation in SLP expression, perhaps the best example is *Campylobacter fetus* in which S-layer variation is very well characterized[7]. In serotype A strains of *C. fetus,* the genome contains up to eight *sapA* homologs and one promoter element within a ~65 kb *sap* island. Usually only one *sap* homolog is expressed in culture, although bacterial sub-populations can be identified that express additional *sap* proteins. Sap homologs are expressed from a single *sap* promoter[8] and exhibit extensive sequence homology. Broad, high frequency chromosomal rearrangements involving DNA inversion and recombination lead to phenotypic switching and expression of

alternate *sap* homologs on the cell surface, resulting in an antigenically distinct S-layer[8-10].

The *Clostridium difficile* S-layer is composed of two proteins, the high-molecular weight (HMW) SLP and the low-molecular weight (LMW) SLP, derived by proteolytic cleavage of the precursor SlpA. The LMW SLP, which likely faces the environment, exhibits considerable sequence variability between strains[11,12]. This variation affects recognition by antibodies, which presumably reflects pressure from the host immune response. The genetic basis of S-layer diversity in *C. difficile* was analyzed using high-throughput genome sequencing of a panel of clinically diverse strains, which revealed the presence of a ~10 kb cassette encoding *slpA, secA2* and two CWPs (see below)[13]. 12 divergent cassettes were identified among the strains and the authors proposed that recombinational switching occurs in *C. difficile* populations to generate antigenic diversity. Interestingly, one of the cassettes is substantially larger than the others (24 kb vs 10 kb) and contains 19 extra genes encoding a putative glycosylation island (see below). Another *C. difficile* S-layer protein, CwpV, undergoes **phase-variable expression**[14] mediated by DNA inversion of an element situated between the promoter and the structural gene[15].

S-layer gene families

Some Firmicutes contain multiple S-layer gene homologs that exhibit varying degrees of sequence identity, which suggests that gene duplication has led to a family of genes with functional diversity.

The best studied examples of SLP and associated protein gene families are found in *B. anthracis* and *C. difficile.* Based on the presence of three tandem surface layer homology (SLH) motifs (see below) within predicted surface proteins, 24 putative BSLs were identified in *B. anthracis* Sterne[16], including the two major S-layer proteins, Sap and EA1 (FIG. 1). The *B. anthracis* SLPs are incorporated into the S-layer at different stages of growth; the Sap S-layer is produced during the exponential growth phase and is replaced in the stationary phase by the EA1 S-layer[17]. Three BSLs are encoded by plasmids: two by pOX1 and one by pOX2 [16]. In each BSL the three tandemly arranged SLH motifs are located either at the N- or C-terminus of the protein. In some cases the proteins are considerably larger than the ~220 residues required for the SLH domains, the Sap1 and EA1 proteins, for example, are over 800 residues in length. In some cases functional effector domains can be identified in these larger proteins, including domains encoding leucine rich repeats (LRRs), β lactamase and several involved in peptidoglycan synthesis and hydrolysis. *B. cereus*, a close relative of *B. anthracis,* lacks the *bslG*, *bslK* and *amiA* genes of *B. anthracis* yet harbors three unique *bsl* genes, *bslV*, *bslW*, and *bslX*, not found in *B. anthracis*[18]. It should be emphasized that these BSLs have not been shown to form two-dimensional arrays; this property is seen only in Sap and EA1 in *B. anthracis*.

A remarkably similar situation is found in *C. difficile.* A number of Clostridia use the Cell Wall Binding 2 (CWB2) motif in an analogous fashion to the SLH motifs to anchor the S-layer to the underlying cell wall (see below). In *C. difficile* we find 29 CWPs each with three tandem CWB2 domains, including the major SLP, SlpA[19]. Comparison of the effector domains associated with the *B. anthracis* BSL

proteins and the *C. difficile* CWPs reveal many similarities including peptidoglycan hydrolases, putative adhesins and LRR proteins (FIG. 1). Families of CWB2-containing surface proteins, some with effector domains, are also found in *C. botulinum* and *C. tetani*[20,21].

The large number of SLP paralogs in the Clostridia and Bacilli that carry an effector domain, many of which are predicted to be exposed to the environment, inspires the idea that the S-layer functions as a scaffold to display proteins or glycoproteins to the external environment. In this way, the S-layer can impart a variety of functions on its host (see below), depending on the properties of the protein or glycoprotein displayed.

From proteins to functional S-layers

SLPs are transported to the cell surface, where they assemble into the ordered structures of the S-layers. In addition, to build a fully functional S-layer, SLPs are anchored to the cell wall and in some organisms highly glycosylated.

Secretion of S-layer proteins

Translocation of proteins across the cell envelope is an essential process in all bacteria. Secretion of S-layer proteins presents a particular problem for bacteria owing to the large quantity of protein required to form a contiguous para-crystalline array; for example, we estimate that the *C. difficile* S-layer contains up to 500,000 subunits requiring the secretion of approximately 400 subunits per second per cell during exponential growth. Several distinct mechanisms have

evolved to cope with this high protein flux but now, as S-layer secretion is studied in several bacterial species, a number of trends are emerging (FIG. 2).

In many Gram-negative species, including *Caulobacter crescentus, Serratia marcescens,* and *C. fetus*, S-layer secretion relies on a specific **type I secretion system**[22-24] comprising an inner membrane ABC transporter and an outer membrane pore (FIG. 2b). In *C. fetus* a four-gene operon, adjacent to the phase-variable S-layer cassette, encodes three proteins, SapDEF, with homology to type I secretion systems, and an additional unique protein, SapC. Mutagenesis of this operon blocks S-layer secretion and the four gene operon is sufficient for the secretion of the S-layer protein SapA in a heterologous host[24].

In the fish pathogens, *Aeromonas hydrophila* and *Aeromonas salmonicida*, S-layer secretion appears to be dependent on specific **type II secretion systems** (FIG. 2b). The SLPs of both species possess an amino-terminal secretion signal and in each species additional secretion proteins with homology to components of the prototypic pullulanase secretion system, a type II secretion system found in Klebsiella, have been identified: *Aeromonas hydrophila* SpsD is homologous to PulD and *Aeromonas salmonicida* ApsE is homologous to PulE. Mutagenesis of either *spsD* or *apsE* results in periplasmic accumulation of the SLPs[25,26]. *A. salmonicida* ApsE is encoded within a complete type II secretion cluster adjacent to the S-layer gene, *vapA*. The other genes in this cluster have not been studied in depth but it seems likely that the encoded type II secretion system is responsible for the secretion of VapA.

S-layer secretion has been studied in detail in two Gram-positive bacteria, *B. anthracis* and *C. difficile* (FIG. 2a). In both cases the secretion of the S-layer precursor is dependent on the accessory Sec secretion system[27,28]. The accessory Sec secretion system was first identified in *Mycobacterium tuberculosis*[29] and has since been characterized in a small number of Gram-positive species[30]. Organisms possessing an accessory Sec system have two copies of the ATPase SecA (SecA1 and SecA2); some bacteria, such as *B. anthracis*, also have an accessory SecY (SecY2). The accessory ATPase, SecA2, is responsible for the secretion of a small subset of proteins. In *B. anthracis* efficient secretion of both major S-layer proteins, EA1 and Sap, requires SecA2 and the S-layer assembly protein, SlaP[27]. In *C. difficile* the accessory Sec system is responsible for the secretion of the S-layer precursor SlpA and the major phase-variable cell wall protein CwpV[28].

Interestingly, there is a striking degree of genetic linkage between the genes encoding S-layer proteins and their dedicated secretion systems in many of the organisms described above, including *C. difficile, B. anthracis, A. salmonicida, C. fetus* and *C. crescentus*. There is also evidence for horizontal transfer of the gene cassette containing *slpA* and *secA2* between different lineages of *C. difficile*[13]. This emphasizes the importance of the S-layer and its secretion in the life cycle of these organisms. As molecular characterization of S-layers extends to new species it will be fascinating to see if dedicated secretion systems and tight genetic linkage are common features of S-layer biogenesis.

Anchoring of S-layer proteins to the cell surface

The S-layer is anchored to the cell surface via non-covalent interactions with cell surface structures, most commonly with LPS in Gram-negative and with cell wall polysaccharides in Gram-positive bacteria. In general, S-layer anchoring in Gram-negative bacteria is less well characterized than in Gram-positive bacteria. However, the S-layers of *C. crescentus* and *C. fetus* have been studied in some detail. In *C. crescentus* the N-terminal ~225 amino acids of the 98 kDa RsaA SLP is required for binding to LPS on the cell surface[31,32]. The exact mechanism of this interaction has yet to be characterized, partly because the exact structure of the *C. crescentus* LPS is unknown, but the interaction does require an intact O-antigen[31]. The highly variable *C. fetus* S-layer also binds non-covalently to LPS. However *C. fetus* strains possess one of two distinct LPS serotypes and, consequently, two distinct S-layer anchoring modules: serotype A is exclusively associated with a *sapA*-type S-layer and serotype B with a *sapB*-type S-layer. Each *C. fetus* genome contains multiple copies of either *sapA* or *sapB*, allowing high-frequency antigenic variation (see above). All SapA-type homologues have a highly conserved N-terminal domain which is responsible for anchoring to serotype A LPS. SapB-type SLPs are similar to SapA in general but have an entirely unrelated N-terminal domain which anchors the proteins to serotype B LPS[10,33,34].

The anchoring of S-layers has been studied in many Gram-positive species. To date, two conserved Gram-positive S-layer anchoring modules have been identified, utilizing either the SLH domain or the CWB2 domain. Both modules employ three domains which are located either at the N- or C-terminal region of the protein. The SLH domain is the most widely distributed, being found in the

SLPs of many *Bacillus* species, *Thermus thermophillus, Deinococcus radiodurans* and at least one Clostridia species, *C. thermocellum*[35],[36-40]. The two major SLPs produced by *B. anthracis*, Sap and EA1, each have three tandem copies of the SLH motif [41,42]. These motifs fold as a pseudo-trimer (see BOX 1)[43] and act cooperatively to bind a pyruvylated **secondary cell wall polymer (SCWP)**. Pyruvylation of the SCWP relies on an enzyme, CsaB, which is encoded adjacent to Sap and EA1 in the *B. anthracis* genome[40]. The thermophilic bacterium *Geobacillus stearothermophilus* possesses one of the most intensively studied S-layers. *G. stearothermophilus* strains can produce five different S-layer types, encoded by *sbsA-D* and *sgsE*[44-47] with two distinct anchoring mechanisms. SbsB has three N-terminal copies of the SLH domain that anchor the protein to a pyruvylated SCWP [48]. However, no SLH domains can be identified in the remaining four *Geobacillus* SLPs. Instead, SbsC has been shown to interact with a N-acetylmannosaminuronic acid–containing SCWP[49] via the first 240 residues of the mature SLP[50] (BOX 1). As these residues are highly conserved in SbsA, SbsD and SgsE it is likely that these SLPs are anchored by the same mechanism.

The second conserved mechanism involves the CWB2 motif, which was first identified in CwlB – an autolysin that cleaves peptidoglycan in the cell wall of *B. subtilis*[51,52]. *B. subtilis* does not produce an S-layer but the CWB2 motif is necessary for retention of CwlB in the cell wall. The CWB2 motif is found in many Clostridia species, including the important human pathogens *C. difficile*, *C. tetani* and *C. botulinum* [53]. In *C. difficile* a family of 29 CWPs, including the S-layer precursor SlpA and CwpV, all utilize the CWB2 domain for anchoring to the cell wall [19]. The cell wall ligand for this domain is currently unknown but is likely to

be a cell surface polysaccharide, which is either free or linked to peptidoglycan. We know little about the specificity of CWB2-polysaccharide interactions. However, surface polysaccharides in the Firmicutes show considerable diversity[54], and it is possible that the CWB2 motif recognizes more than one chemical entity, or that in different species the motif has evolved to recognize a specific polysaccharide. Each cell wall protein has three tandem copies of the CWB2 motif, analogous to the arrangement of SLH domains seen in other S-layer proteins. As more structural information becomes available it will be interesting to see whether the pseudo-trimer binding arrangement is also shared between SLH and CWB2 domains, which would suggest a common or convergent evolutionary origin.

Formation of an ordered array on the cell surface

S-layers are by definition a two dimensional array of a single protein, but how exactly is the array formed? It is clear that SLPs that form arrays have at least two functional domains: an anchoring domain, such as the tandem SLH or CWB2 motifs, that attaches the protein to the underlying cell wall and a crystallization domain that mediates SLP-SLP interaction. Crystallization domains, which may contain several structural domains, have been identified in *G. stearothermophilus* SbsB[55] and SbsC[56] and are present in SLPs of other species including those of *B. anthracis*[57]. In a landmark publication[58], the three-dimensional crystal structure of SbsB was described, showing the atomic contacts between adjacent 718 residue SLP crystallization domains and how individual structural domains within molecules are co-ordinated by Ca^{2+}, an anion known to be essential for S-layer formation in *G. stearothermophilus* ([58] and see BOX 1). The structure also

shows pores of approximately 30 Å diameter formed at the interface between three adjacent subunits, consistent with a role in permeability (see below).

It should be noted that not all SLPs have been shown to form two-dimensional arrays and little is known about the potential for self-assembly of the associated proteins such as the *B. anthracis* BSL and *C. difficile* CWP proteins. These proteins however are found within the S-layer, and although they are likely held in place by interaction with cell wall ligands, we cannot rule out lateral interactions with the rest of the S-layer.

Glycosylation of bacterial S-layers

The first description of bacterial protein glycosylation was in the S-layer of *Halobacterium salinarium*[59], and since then a large number of glycosylated S-layer proteins have been identified in numerous species of Bacteria and Archaea. S-layer glycosylation has been reviewed in excellent detail elsewhere[60-62] and only the salient points will be discussed here. S-layer glycan modifications involve sugars commonly found in glycosylated eukaryotic proteins, together with some unusual sugars ([60] and see below). While **N- and O-linkages** have been described in Archaeal SLPs[3], to date only O-linkages have been found in Bacterial SLPs, despite other Bacterial surface proteins exhibiting N-linked glycans[63]. O-linkage in SLPs of the *Bacillaceae* can involve serine, threonine or tyrosine. The overall structure and architecture of the S-layer glycan resembles that of Gram-negative LPS, containing a linkage unit and up to 50 repeating units, each consisting of 2-6 sugars. This resemblance suggests a common evolutionary origin of LPS biosynthesis and S-layer glycosylation[64], an idea further

strengthened by recent descriptions of the S-layer glycan (*slg*) gene clusters in *G. stearothermophilus, Paenibacillus alvei, Geobacillus tepidamans, Aneurinibacillus thermoaerophilus* and *Tannerella forsythia* encoding the glycosyltransferases, glycan processing enzymes and membrane transport machinery sufficient for glycan biosynthetic pathways (for a review, see [60]). S-layer glycosylation pathways have been described in several species, including *G. stearothermophilus*[65], *P. alvei*[66] and *T. forsythia*[67], leading to the proposal of a biosynthetic route involving transfer of galactose from the nucleotide-activated sugar UDP-α-D-Gal to a lipid carrier, formation of the linkage unit by addition of glycans and assembly of the growing repetitive glycan chain onto the linkage unit. These reactions occur in the cytoplasm prior to transport of the completed glycan chain via an ABC-transporter (in the case of *G. stearothermophilus*) to the distal side of the membrane where the glycan is ligated to the S-layer protein substrate[60]. The glycan chains decorating S-layer proteins are fairly diverse: for example, in *G. stearothermophilus* the glycan chain is a simple polymer of L-rhamnose, in *P. alvei* L-rhamnose, N-acetylmannosamine, D-glucose and D-galactose are found and in *T. forsythia* the unusual sugars *N*-acetylmannosaminuronic acid, 5-acetimidol-7-*N*-glycolylpseudaminic acid and digtoxose are present[67]. Whether the glycan chain is co-transported with the S-layer protein substrate remains to be determined, but protein transport (secretion) is not dependent on glycosylation.

Recently, strains of *C. difficile* were described that contain a putative *slg* locus adjacent to *slpA*[13]. This bacterium does not normally elaborate a glycosylated S-layer[68] but these variant strains contain a distinct S-layer cassette (see above)

and produce S-layer proteins of reduced polypeptide length[12,13]. Whether these strains do indeed have a glycosylated S-layer and what, if any, phenotype that might confer is currently unknown.

Functional heterogeneity of S-layers

It is perhaps not surprising that, as the major proteinaceous surface component of the cell, a variety of functions have been described and proposed for the S-layer[1,69] (FIG. 3). However, after decades of research, no single function can be ascribed to the S-layer and in many species the S-layer has no known function. The ability to form a two-dimensional array appears to be the result of convergent evolution and is seen in proteins of quite distinct sequence. SLPs and associated proteins have further evolved to adopt a multitude of activities, some essential to the physiology of the cell and others facilitating survival in specific niches. Although we do not definitively know the function of many S-layers, it is clear from their wide occurrence in the Bacterial kingdom, apparent convergent evolution and the enormous metabolic load required to produce and maintain these structures that they fulfil some important role for the bacteria that produce them.

Roles of the S-layer in pathogenesis and immunity

In *Lactobacillus crispatus,* an indigenous member of the human and chicken gut microflora, the S-layer CsbA (SlpB) has an N-terminal domain that binds types I and IV collagen and a C–terminal domain that interacts with the bacterial cell wall [70]. This collagen binding activity is thought to mediate bacterial colonization

of the gut (FIG 3a). Interestingly, two other putative S-layer genes are present in this strain: *slpC,* located downstream of *csbA,* is transcriptionally active and *slpA* is silent[70-72].

As the major surface antigen of *C. difficile*, the S-layer has been investigated for its ability to be recognized by and activate the immune system. The SlpA proteins (HMW and LMW SLPs) induce release of pro-inflammatory cytokines from human monocytes and induce maturation of human monocyte-derived dendritic cells (MDDCs)[73]. Further work advanced these findings by showing that the SLPs induced maturation of mouse bone marrow derived dendritic cells and the production of the cytokines IL-12, TNFα and IL-10, but not of IL-1β. Importantly, activation of dendritic cells was dependent on Toll-like receptor 4 (TLR4) and subsequently induced Th responses known to be involved in clearance of bacterial pathogens[74]. Infection of TLR4 knockout mice resulted in increased severity of symptoms compared to wild-type mice, suggesting an important role of TLR4 in bacterial clearance[74]. Both the HMW and LMW SLPs of *C. difficile* were required for dendritic cell activation, suggesting either the entire complex is recognized by TLR4, or that one of the SLPs is the ligand, but requires to be seen in the context of the HMW-LMW complex (FIG. 3a). SlpA has also been shown to bind to fixed gut enterocytes[75], which could be related to an immune stimulatory role and infers that the S-layer might function as an adhesin *in vivo* (FIG 3a). To date, the role of SlpA as an adhesin has not been investigated through mutagenesis as *slpA* is an essential gene, but recent advances in genetics[76-78] should allow creation of conditional mutant strains that could be used in infection experiments to test this hypothesis. Finally the phase-variable CwpV

protein has bacterial auto-aggregation activity that might have a role during infection[15].

In *B. anthracis*, the precise role of Sap and EA1 are unknown, but activities of some BSLs have been elucidated. The BslA protein has been shown to mediate adhesion of encapsulated *B. anthracis* to HeLa cells[16]. *bslA* mutants display limited dissemination to tissues and decreased virulence in a guinea pig model of infection, suggesting BslA is a functional adhesin required for full virulence of *B. anthracis*[79]. Another S-layer protein, BslK, mediates heme uptake by utilizing a near iron transporter (NEAT) domain[80] (FIG 3c). In *B. cereus,* which has highly similar BSLs to *B. anthracis* but a very different capsule composition, a *csaB* mutant was found to be defective in retaining Sap, EA1 and BslO on the cell wall[18]. The *csaB* mutant exhibited reduced virulence in a mouse model of infection, implicating one or more BSLs in the pathogenesis of anthrax.

The Gram-negative pathogen *C. fetus* is a leading cause of abortions in sheep and cattle and can cause persistent systemic infections in humans. The S-layer, composed of the Sap protein, is essential for host colonization[81]. Sap is antigenically variable (see above) which contributes to the evasion of the host immune response[82,83]. The S-layer is crucial for pathogenesis as mutant strains do not cause disease and are sensitive to phagocytosis and killing in serum mediated by complement C3[84-86] (FIG 3a). Antigenic variation occurs during infection in humans, as shown by strains isolated at early and late stages of infection from four individuals with relapsing *C. fetus* infections[87]. In three patients, the strain had undergone a switch in the predominant S-layer

expressed.

T. forsythia (previously known as *Bacteroides forsythus)* is a Gram-negative anaerobe that is associated with severe forms of periodontal disease[88,89]. Strains of *T. forsythia* produce two high molecular weight proteins, each over 200 kDa, that are concomitantly expressed and constitute the S-layer [90-92]. The structural genes *tfsA* and *tfsB* encode proteins of 120 and 140 kDa[93], suggesting post-translational modification. This was explored in detail in a study[67] that showed a complex pattern of glycosylation on both proteins including a modified pseudaminic acid Pse5Am7Gc not previously found on bacterial proteins. This sugar, a sialic acid derivative, was suggested to participate in bacterium-host interactions[67], based on the prevalence of sialic acid-like sugars in Gram-negative structures involved in pathogenesis such as LPS, capsules, pili and flagella. In *T. forsythia* transposon mutants that exhibited altered biofilm formation (FIG 3c), an operon involved in exo-polysaccharide biosynthesis was identified. Mutation of one gene, *weeC*, which encodes a putative UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase, increased biofilm formation and altered the mobility of the two *T. forsythia* S-layer proteins on SDS-PAGE, an effect consistent with glycosylation[94]. A proteomics study also found increased quantities of S-layer proteins in *Tannerella* biofilms compared to planktonic cells[95]. The *T. forsythia* S-layer appears essential for virulence of this pathogen as adhesion to and invasion of human gingival epithelial cells (Ca9-22 cells) and mouth epidermal carcinoma cells (KB cells) were decreased or abolished in a mutant defective in *tfsA* and *tfsB*[92].

SLPs have also been demonstrated to have a role in protection against predation.

The parasitic bacterium *Bdellovibrio bacterovirus* has a wide host range and infects and replicates in the periplasm of susceptible Gram-negative species[96]. Strains of *Aquaspirillum serpens and Caulobacter cresentus* possess an S-layer and are not normally parasitized by *Bdellovibrio*, but S-layer negative variants of both species were shown to be sensitive to predation, indicating that the S-layer can provide a protective coat against infection with *Bdellovibrio*[97](FIG 3a). In addition to this, one might speculate that the S-layer would act as a receptor for bacteriophage and bacteriocins, but to our knowledge there is no evidence for such activities.

Permeability and biogenesis of the cell envelope

S-layers are often proposed to function a permeability barrier and a direct role for SLPs as barriers has been investigated in *B. coagulans* and other species[2,98]. Experimentally determined exclusion limits of isolated **sacculi** and associated proteins of 15 to 34 kDa are consistent with pore diameters of 20 – 60 Å within the S-layer lattice[2,98] and the crystal structure of SbsB reveals pores of approximately 30 Å[58]. Thus a role for the S-layer as a permeability barrier (FIG 3b) is certainly conceivable, but our knowledge of this activity would be strengthened by genetic analysis and atomic-resolution imaging of alterations in pore size *in vivo*.

In *Deinococcus radiodurans,* the S-layer, (known as hexagonally packed intermediate (HPI) layer) is composed of two S-layer proteins, SlpA and Hpi, in addition to lipids and carbohydrates. Deletion of *slpA* results in major structural alterations such as loss of the Hpi protein and surface glycans, which can be

visualized by electron microscopy as layers of material peeling off the cell wall[99]. This suggests that SlpA is involved in the attachment of Hpi and other components to the underlying cell wall, which is consistent with the presence of SLH domains within SlpA. Other examples of a role for the S-layer in cell envelope biogenesis include *C. difficile*, in which inhibition of cleavage of the S-layer precursor SlpA through inactivation of the protease Cwp84 leads to incorrect assembly of the S-layer and shedding of full-length SlpA from the cell wall[100,101] (FIG 3b). Interestingly, virulence of this *cwp84* mutant is not diminished in the hamster model of infection, presumably because gut proteases such as trypsin can cleave SlpA resulting in a fully functional S-layer[100].

In *Bacillus anthracis*, the physiological functions of the Sap, EA1 and Bsl proteins have been investigated. Sap and EA1 both have a peptidoglycan hydrolase activity[102], although they lack known functional domains that specify such an activity. BslO, which has putative N-acetylglucosaminidase activity and is localized at the cell septa, has a role in catalyzing cell division with *bslO* mutants exhibiting increased lengths of cell chains[103].(FIG 3c). Elongated chains of bacteria are also seen in a *sap* mutant, and this lack of Sap can be complemented by the addition of purified BslO protein to the culture medium, restoring cell division and reducing the chain length. In wild-type cells, Sap was visualized predominantly on the lateral cell wall, away from the septa, suggesting that *B. anthracis* controls the spatial deposition of the SLPs in order to ensure correct localization of functional entities such as BslO [104]. In the *C. difficile* family of CWPs/SLPs, several proteins potentially mediate peptidoglycan synthesis and remodeling, including Cwp22 which has L,D-transpeptidase activity[105].

Other functions of the S-layer

Another function associated with the S-layer includes swimming in the marine bacterium *Synechococcus*[106] (FIG 3c). This species is highly motile but does not possess any obvious flagellum or other organelles that might mediate swimming, and the mechanical basis for its motility is largely a mystery. In mutants defective for swimming two surface proteins essential for this activity were identified: SwmA and SwmB[107]. SwmA is a 130 kDa glycosylated S-layer protein and SwmB a large 1.12 mDa protein. Other genes essential for swimming encode an ABC transporter and several glycosyltransferases[108]. Although these results indicate another function for an S-layer and suggest glycosylation of SwmA is required for motility they do not, unfortunately, lead us much further in elucidating this highly unusual mechanism of swimming.

Perspectives

Following their discovery in the 1950s and after decades of research, our knowledge of Bacterial SLPs has increased considerably in the last few years. It is clear that S-layers do not have one single function, rather a diversity of functions is apparent and we expect to see new functions revealed as more species are studied. In some Archaea, for example *Sulfolobus,* the S-layer appears to be the sole non-lipid constituent of the envelope[3], suggesting structural integrity might be an ancestral function of SLPs. In some bacterial species, such as the *Clostridia*, the S-layer appears essential for cell viability, as unconditional deletion mutants

cannot be constructed. In these species, the SLP is the main protein component of the cell surface although a diversity of other macromolecules is found.

Increasingly sophisticated and high-resolution techniques such as AFM and electron crystallography are being applied to study S-layer morphology and symmetry[109]. Ultimately these techniques will be combined with structural information from X-ray crystallography or NMR to generate atomic resolution models of the complete S-layer. Recently, progress has been made with atomic resolution structures of several SLPs, and we look forward to a complete description of an assembled S-layer structure in complex with the ligand responsible for anchoring to the cell wall. Only then will we be able to address the crucial outstanding questions in S-layer biology: what is the biochemical basis of paracrystalline array self-assembly? What mechanisms are employed to attach the S-layer to the cell surface? And finally, what is the structural basis for the known S-layer functions?

It is clear that SLPs and their associated proteins have evolved specialized functions, and in some species of Firmicutes SLPs act as a scaffold to display enzymes on the cell surface. It is likely that many more SLPs will be identified from genome sequencing and it will be a challenge to assign meaningful functions to this diverse family of proteins without laboratory investigation. Priorities for future research include establishing the functions of S-layers present in bacterial pathogens, investigating their potential as therapeutic targets for antimicrobial or vaccine development, and in depth structural analysis of the interactions between S-layers and other surface components.

With the availability of increasingly sophisticated structural and imaging tools, we are now in a position to push forward Bacterial S-layer research and perhaps determine the full contribution of these fascinating structures to the growth and survival of Bacteria which produce them.

Table 1 | **Summary of SLPs described in text**

Organism	SLPs	Features
Campylobacter fetus	SapA SapB	High frequency antigenic variation of S-layer through recombinational switching of *sap* homologues; secreted by a specific type I secretion system
Clostridium difficile	SlpA and the CWP family	SlpA essential for cell growth; S-layer functionalised by decoration with up to 28 additional CWPs; secreted by the accessory Sec system; mediates interactions with epithelial cells and activates dendritic cells
Bacillus anthracis	Sap, EA1 and the BSL family	Sap and EA1 are alternate SLPs; S-layer functionalised by decoration with BSLs; secreted by the accessory Sec system; anchored via interaction with pyruvylated SCWP
Caulobacter crescentus	RsaA	Secreted by a specific type I secretion system; anchored via interaction with LPS
Aeromonas salmonicida	VapA	Secreted by a dedicated type II secretion system
Geobacillus stearothermophilus	SbsA SbsB SbsC SbsD SgsE	Anchored via interaction with pyruvylated SCWP (SbsB) or N-acetylmannosaminuronic acid (SbsA, C, D and SgsE); Glycosylated
Tannerella forsythia	TfsA TfsB	Glycosylated; S-layer includes both SLPs; glycosylation required for biofilm formation; S-layer essential for virulence
Lactobacillus crispatus	CsbA SlpA SlpC	Mediates binding to types I and IV collagen (CsbA)
Deinococcus radiodurans	SlpA Hpi	S-layer includes both SLPs; plays a role in maintenance of envelope integrity
Synechococcus	SwmA	Glycosylated; required for swimming motility

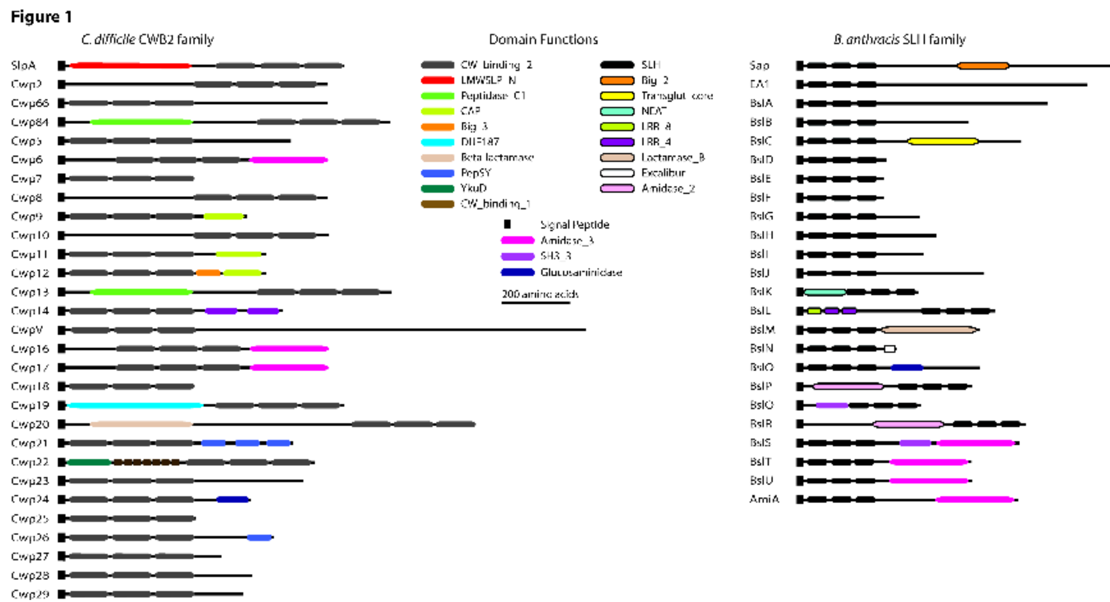


Figure 1 | ***Clostridium difficile* and *Bacillus anthracis* cell surface protein families.** Domain identification and organization among all members of the *C. difficile* CWB2 family and the *B. anthracis* SLH family was determined using the Pfam protein families database[120] and outlined in Supplementary Table 1. The N-terminal signal peptide, which is removed upon translocation through the Sec membrane channel, is shown as a black box, see also Figure 2a. In *C. difficile* the secretion of at least SlpA and CwpV is dependent on the accessory Sec secretion system[28]. In *B. anthracis* the two major S-layer proteins, Sap and EA1, also require the accessory Sec system for secretion[104]. Although limited data is available, it is possible that secretion via the accessory Sec system is a common feature of these two protein families.

Figure 2

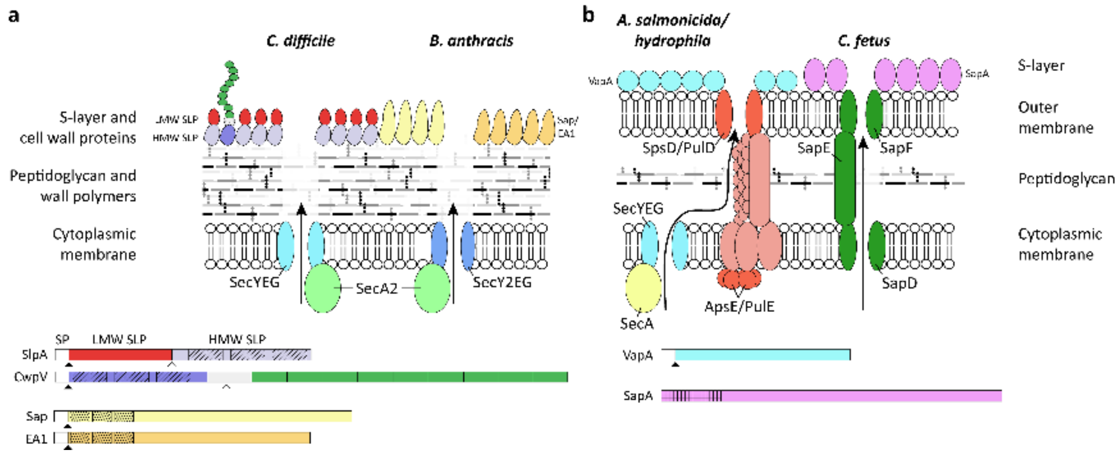


Figure 2 | **Secretion of Bacterial S-layer proteins.** To date, S-layer secretion has been studied in a small number of species, in which a number of dedicated secretion systems have been identified. **a** | In the Gram-positive bacteria *Clostridium difficile* and *Bacillus anthracis* secretion of the S-layer precursors is mediated by the accessory Sec secretion system[30]. The proteins contain an N-terminal signal sequence (white box) which directs the nascent polypeptide to the secretion apparatus and is cleaved upon membrane translocation (indicated with the black triangle). In both *C. difficile* and *B. anthracis*, translocation requires the accessory ATPase, SecA2[27,28]. Following recognition by SecA2, the nascent polypeptide is translocated across the membrane through a pore consisting of SecYEG (*C. difficile*) or SecY2EG (*B. anthracis*). **b** | Secretion of the S-layer proteins (SLPs) in *Aeromonas salmonicida* and *Aeromonas hydrophil*a requires a dedicated Type II secretion system[25,26]. Type II secretion is a two-step process: the unfolded precursor is first translocated across the cytoplasmic membrane by the canonical Sec secretion system, the protein then folds and is transported across the outer membrane by a complex multi-protein secretion apparatus which is closely related to type IV pili[121]. A complete type II secretion system is encoded alongside *vapA* in *A. salmonicida* but only one component of

this system has been directly linked to SLP secretion; *A. salmonicida* ApsE is homologous to the secretion ATPase, PulE. A PulD homologue, SpsD, likely forming an outer membrane pore, has also been identified in *A. hydrophila*. Further analysis is required to confirm whether or not SpsD and ApsE are from the same conserved secretion system. In *Campylobacter fetus* the SLPs are secreted in a single step by a Type I secretion system encoded by SapDEF[24]. Type I secretion involves an inner membrane ABC transporter, a membrane fusion protein and an outer membrane pore. Where known, the anchoring domains of the SLPs are highlighted as hatched boxes, see Figure 1.

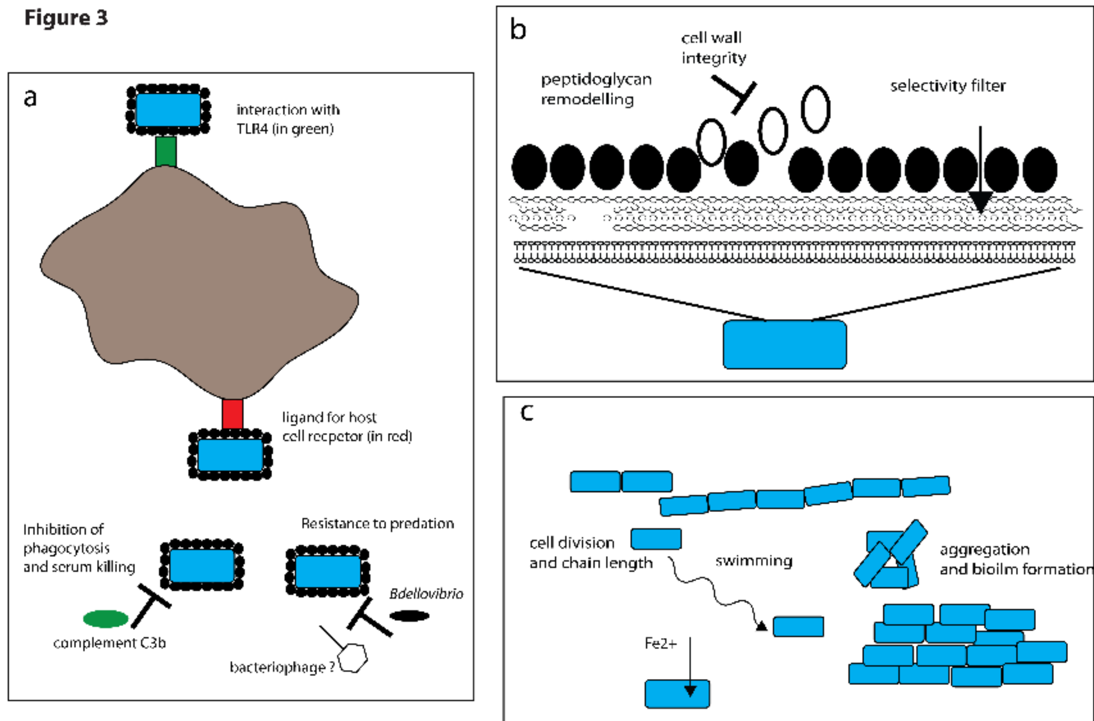
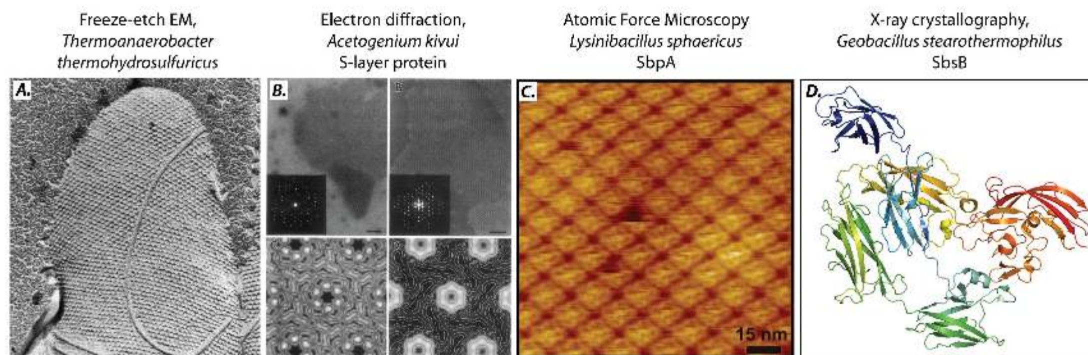


Figure 3 | **Functions of S-layer proteins.**

Probable roles in infection (**panel a**) include adhesin activity, found in several species: BslA of *B. anthracis* binds to HeLa cells and mutants are attenuated in models of infection[79], binding of SLPs to enteric cells has been observed in *C. difficile*[75] and to defined ligands (types I and IV collagen) in *Lactobacillus crispatus*[70]. In *C. difficile,* interaction of SlpA with host TLR4 receptors is linked to innate immunity[74] and in *C. fetus*, SLPs prevent binding of complement factor C3b, protecting the bacterium from host mediated phagocytosis and serum killing[86]. A role in resistance to predation has been demonstrated in *Bdellovibrio bacteriovirus*[97] and potential roles in resistance to bacteriophage and bacteriocins are possible but remain speculative (**panel a , bottom**). SLPs have roles in maintenance of cell envelope integrity (**panel b**) in *Deinococcus radiodurans* where inactivation of *slpA* causes shedding of surface molecules[99] and in *C. difficile* where loss of *cwp84* results in an abnormal S-layer and

shedding of surface proteins[101]. *B. anthracis* BslO and the *C. difficile* Cwp22 have peptidoglycan hydrolase activity that may remodel the peptidoglycan. A role for SLPs as a permeability barrier has been demonstrated in *Bacillus coagulans* [98]. A role in cell division has been found in *B. anthracis* (**panel c**) where inactivation of BslO, a putative N-acetylglucosaminidase, results in increased lengths of chains of bacteria[103]. SLPs have roles in aggregation (*C. difficile* CwpV[15]), biofilm formation (*T. forsythia*[94]) and swimming (*Synechococcus*[106]). Finally BslK from *B. anthracis* mediates iron uptake by scavenging heme, transferring it to the surface protein IsdC[80].

Box 1 Figure



Box 1

Since the first reported observation of an S-layer in 1953[110] increasingly sophisticated and high-resolution techniques have been applied to the study of their morphology, symmetry and, ultimately, atomic structure. Some of the most striking early visualizations of S-layer morphology came from electron microscopy of negatively-stained cell wall fragments and isolated S-layers and freeze-fracture microscopy of intact cells[4,111]. S-layer proteins (SLPs) spontaneously form 2-dimensional crystals *in vitro*, which can be studied using electron microscopy (**panel A** shows the regular hexagonal surface array of a *Thermoanaerobacter thermohydrosulfuricus* cell[112]). More detailed structural information can be obtained from two-dimensional crystals using electron crystallography (for example *Acetogenium kivui*, transmission electron microscopy images of negatively stained S-layer fragments (**panel B**, top), the resulting electron diffraction patterns (**panel B**, top, insets), 3D projection map shown in **panel B** (bottom)). The projection map clearly shows the p6 hexagonal symmetry of the S-layer and the 6 individual SLP monomers which form the ring-like core component of the supramolecular structure[113]. In recent years, atomic force microscopy (AFM) has been developed as a high resolution imaging

technique to visualize macromolecules and is particularly well suited to the study of bacterial surfaces [114], as it allows unprecedented resolution on intact S-layer samples under native aqueous conditions and, with functionalised tips, the ability to map individual proteins within the layer. (**Panel C,** AFM image of the re-assembled *Lysinibacillus sphaericus* SbpA S-layer showing clear tetragonal symmetry[115]). Owing to difficulties in growing 3D crystals suitable for X-ray crystallography, which is in part attributable to the propensity of SLPs to form two-dimensional crystals, there is a dearth of high resolution S-layer structures. Many groups have taken a divide and conquer approach by crystallizing except from structures of recombinant or proteolytically-derived fragments of SLPs. The first to be published was a 52 residue coiled-coil fragment of the tetrabrachion SLP from the extreme thermophile *Staphylothermus marinus*[116] followed by two partial structures of Archaeal SLPs from *Methanosarcina* spp.[117,118]. One comprises a novel seven-bladed β propeller (the YVTN domain), a polycystic kidney disease superfamily fold domain and a third, as yet unstructured, domain which is predicted to adopt a parallel right-handed β helix fold. The other SLP has two highly related DUF1608 domains, one of which has a pair of linked β sandwich folds, and a transmembrane anchor[118]. Partial structures have also been solved for two of the *Geobacillus stearothermophilus* SLPs (SbsC lacking the C-terminal crystallization domain[55], and SbsB lacking the N-terminal SLH domains [58]) and *C. difficile* LMW SLP[119]. SbsC anchors to the cell surface via non-covalent interactions between domain 1 of the protein (residues 31-270) and a negatively charged secondary cell wall polymer (SCWP) (see main text). The crystal structure of this domain revealed a series of surface exposed

positively charged residues, with spacing approximating that of the negatively charged ManNAcUA groups on the SCWP, as well as a potential carbohydrate-binding stack of aromatic side chains. Further research will hopefully determine the exact contribution of these residues to binding the SCWP. SbsB was the first SLP to be crystallized in an intact form, employing nanobodies to inhibit 2D lattice formation and allow 3D crystallization. The cell wall binding SLH domains did not resolve in the finished structure but the crystal packing, in combination with cryo-EM and crosslinking studies, allowed the proposal of a plausible model of the complete 2D paracrystalline array (see **panel D and text**)[58]. The 3D structure of the SLH domains from another SLP, the *B. anthracis* Sap1, has been determined, revealing a pseudo-trimer with each SLH domain contributing one component of a three pronged spindle, allowing modelling of binding of the SLH domains to the SCWP[43]. Panels A, B and C are reproduced with permission from [112,113] and [115] respectively. Panel D: the structural coordinates for SbsB32–920 (4AQ1) [58] were downloaded from the PDB (http://www.rcsb.org/pdb) and the image shown was generated using PyMOL.

SIDE BARS NOTES

Phase-variable expression – random variation of gene expression in a bacterial population in which expression in individual cells is either on or off leading to phenotypic heterogeneity in the population.

Secondary Cell Wall Polymers – carbohydrate based polymers other than peptidoglycan and anionic polymers present in the cell wall, for example the pyruvylated *B. anthracis* SCWP that anchors the SLPs EA1 and Sap to the cell wall.

N- and O-linked glycosylation – linkage of a sugar to the nitrogen (N) atom of asparagine or to the oxygen (O) atom of serine, threonine or tyrosine.

Type I secretion - a *sec*-independent protein secretion system in Gram-negative bacteria consisting of an inner membrane ABC transporter, a periplasmic membrane fusion protein and an outer membrane pore.

Type II secretion – a *sec*-dependent multi-protein secretion system in Gram-negative bacteria which is closely related to type II pili.

Sacculi – the sacculus (singular) is the sac of polymerised peptidoglycan surrounding the bacteria. Isolated from the bacterium, it retains the shape of the cell.

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