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 (known as an 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 the maintenance of cell integrity, enzyme display and, in pathogens and commensals, interaction with the host and its immune system. In this Review, we discuss our current knowledge of S-layer and related proteins, including their structures, mechanisms of secretion and anchoring and their diverse functions.

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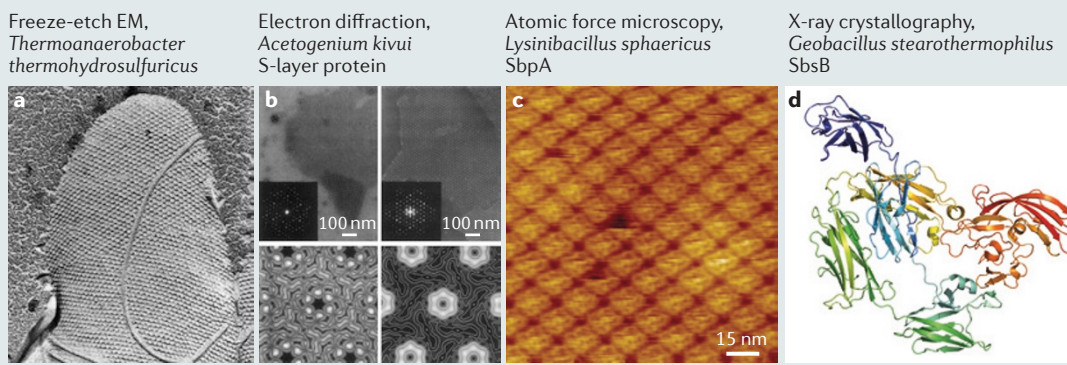*Correspondence to N.F.F.*
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S-layers are found on both Gram-positive and Gram-negative bacteria and are highly prevalent in archaea[1–3]. They are defined as two-dimensional (2D) crystalline arrays that coat the entire cell, and they are thought to provide important functional properties. S-layers consist of one or more (glyco)proteins, known as S-layer proteins (SLPs), that undergo self-assembly to form a regularly spaced array on the surface of the cell. As SLPs are some of the most abundant proteins in the cell[2], their biogenesis consumes considerable metabolic resources, which reflects their importance to the organism. S-layers were first recognized in the 1950s and were studied in several species during the following decades, which revealed considerable details of their structures using techniques such as freeze-etch electron microscopy (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. TABLE 1 outlines all the SLPs and related proteins that are described in this Review.

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, have facilitated structural and functional studies of SLPs. Comprehensive reviews about 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, which includes the properties of S-layers in this domain of life, was recently published[3]. In this Review, we discuss the biology of bacterial SLPs and highlight recent discoveries that have shaped our understanding of these important proteins.

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 that encode components of modification or secretory pathways. Despite the apparently conserved function of providing a 2D array surrounding the cell, genetic and functional studies show that there is a wide diversity in both the sequences and the roles of S-layer proteins.

Genetic variation. Several bacterial species show genetic variation in SLP expression; perhaps the best example of this 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 surface array A gene (*sapA*) homologues and one promoter element within a ~65 kb *sap* island. Usually only one *sap* homologue is expressed in culture, although bacterial subpopulations that express additional Sap proteins can be identified.

Box 1 | S-layer structures

Since the first reported observation of a surface layer (S-layer) in 1953 (REF. 110), increasingly sophisticated and high-resolution techniques have been applied to the study of S-layer 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, as well as freeze-fracture microscopy of intact cells[4,111]. S-layer proteins (SLPs) spontaneously form two-dimensional (2D) crystals *in vitro*, which can be studied using electron microscopy (EM). Part **a** of the figure shows the regular hexagonal surface array of a *Thermoanaerobacter thermohydrosulfuricus* cell[112]. More detailed structural information can be obtained from 2D crystals using electron crystallography; for example, transmission EM images of negatively stained S-layer fragments from *Acetogenium kivui*, (part **b**, upper panel), the resulting electron-diffraction patterns (part **b**, upper panel, insets), three-dimensional (3D) projection map shown in part **b** (lower panel). The projection map clearly shows the p6 hexagonal symmetry of the S-layer and the six individual SLP monomers that 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 enables unprecedented resolution of intact S-layer samples under native aqueous conditions and, with functionalized tips, the ability to map individual proteins within the layer (part **c** shows an AFM image of the re-assembled *Lysinibacillus sphaericus* SbpA S-layer, showing clear tetragonal symmetry[115]). Owing to difficulties in growing 3D crystals that are suitable for X-ray crystallography, which are, in part, attributable to the propensity of SLPs to form 2D crystals, there is a dearth of high-resolution S-layer structures. Many groups have taken a 'divide and conquer' approach by crystallizing recombinant or proteolytically derived fragments of SLPs. The first structure 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 (known as the YVTN domain), a polycystic kidney disease superfamily fold domain and a third, as yet unstructured, domain that 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 carboxy-terminal crystallization domain[55] and SbsB lacking the amino-terminal surface layer homology (SLH) domains[58]) and the *Clostridium difficile* low molecular weight (LMW) SLP[119]. SbsC anchors to the cell surface via non-covalent interactions between domain 1 of the protein (which comprises residues 31–270) and a negatively charged secondary cell wall polymer (SCWP). The crystal structure of this domain showed a series of surface-exposed positively charged residues, with spacing that approximated that of the negatively charged N-acetylmannosaminuronic acid (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, using nanobodies to inhibit 2D-lattice formation and to enable 3D crystallization. The cell wall-binding SLH domains did not resolve in the finished structure, but the crystal packing, in combination with cryo-electron microscopy and crosslinking studies, enabled the proposal of a plausible model of the complete 2D paracrystalline array[58] (see part **d**). The 3D structure of the SLH domains from another SLP, the *Bacillus anthracis* surface array protein 1 (Sap1), has been determined, revealing a pseudo-trimer in which each SLH domain contributes one component of a three-pronged spindle, enabling modelling of binding of the SLH domains to the SCWP[43]. Part **a** is reproduced, with permission, from REF. 112 © (1999) John Wiley and Sons. Part **b** is reproduced, with permission, from REF. 113 © (1994) American Society of Microbiology. Part **c** is reproduced, with permission, from REF. 115 © (2010) Proceedings of the National Academy of Sciences, USA. The structural coordinates for SbsB32–920 (REF. 58) in part **d** were obtained from from Protein Data Bank (PDB) (PDB accession: 4AQ1) and the image shown was generated using PyMOL.

Freeze-etch EM,
Thermoanaerobacter thermohydrosulfuricus

Electron diffraction,
Acetogenium kivui
S-layer protein

Atomic force microscopy,
Lysinibacillus sphaericus
SbpA

X-ray crystallography,
Geobacillus stearothermophilus
SbsB

100 nm   100 nm

15 nm

Sap homologues are expressed from a single *sap* promoter[8] and have extensive sequence homology. Broad, high-frequency chromosomal rearrangements that involve DNA inversion and recombination lead to phenotypic switching and expression of alternate *sap* homologues on the cell surface, which results in an antigenically distinct S-layer[8–10].

The *C. difficile* S-layer is composed of two proteins: the high-molecular weight (HMW) SLP and the low-molecular weight (LMW) SLP, both of which are produced by proteolytic cleavage of the precursor S-layer protein SlpA. The LMW SLP, which probably faces the environment, shows considerable sequence variability among strains[11,12]. This variation affects recognition by

Table 1 | **Summary of S-layer proteins (SLPs)**

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

BSL, Bacillus S-layer-associated protein; CsbA, collagen-binding S-layer protein A; CWP, cell wall protein; Hpi, hexagonally packed intermediate-layer surface protein; LPS, lipopolysaccharide; Sap, surface array protein; SCWP, secondary cell wall polymer; SgsE, surface layer glycoprotein E; SLP, S-layer protein; SwmA, cell surface protein required for swimming motility A; Tfs, *Tannerella forsythia* S-layer protein; VapA, tetragonal surface virulence array protein A.

antibodies, which presumably reflects pressure from the host immune response. The genetic basis of S-layer diversity in *C. difficile* was analysed using high-throughput genome sequencing of a panel of clinically diverse strains, which revealed the presence of a ~10 kb cassette that encodes SlpA, a protein translocase subunit (SecA2) and two CWPs (see below)[13]. The authors identified 12 divergent cassettes among the strains and 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 compared with 10 kb) and contains 19 extra genes that encode a putative glycosylation island (see below). Another *C. difficile* S-layer protein, cell wall protein V (CwpV), undergoes phase-variable expression[14], which is mediated by DNA inversion of an element that is situated between the promoter and the structural gene[15].

S-layer gene families. Some firmicutes contain multiple S-layer gene homologues that exhibit varying degrees of sequence identity, which suggests that gene duplication has led to a family of genes that have functional diversity.

The best studied examples of SLP and related protein gene families are found in *B. anthracis* and *C. difficile.* On the basis of 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 of these are encoded by pOX1 and one is encoded by pOX2[16]. In each BSL, the three tandemly arranged SLH motifs are located at either the amino terminus or carboxyl terminus of the protein. In some cases, the proteins are considerably larger than the ~220 residues that are required for the SLH domains; for example, the Sap1 and EA1 proteins are more than 800 residues in length. In some cases, functional effector domains can be identified in these larger proteins, including domains that encode leucine rich repeats (LRRs) and β-lactamase and several domains that are involved in peptidoglycan synthesis and hydrolysis. *Bacillus cereus*, which is a close relative of *B. anthracis,* lacks the *bslG, bslK* and *amiA* genes of *B. anthracis* but harbours three unique *bsl* genes, *bslV, bslW,* and *bslX,* which are not found in *B. anthracis*[18]. It should be emphasized that these BSLs have not been

Phase-variable expression
The random variation of gene expression in a bacterial population. Expression in individual cells is either on or off, leading to phenotypic heterogeneity in the population.
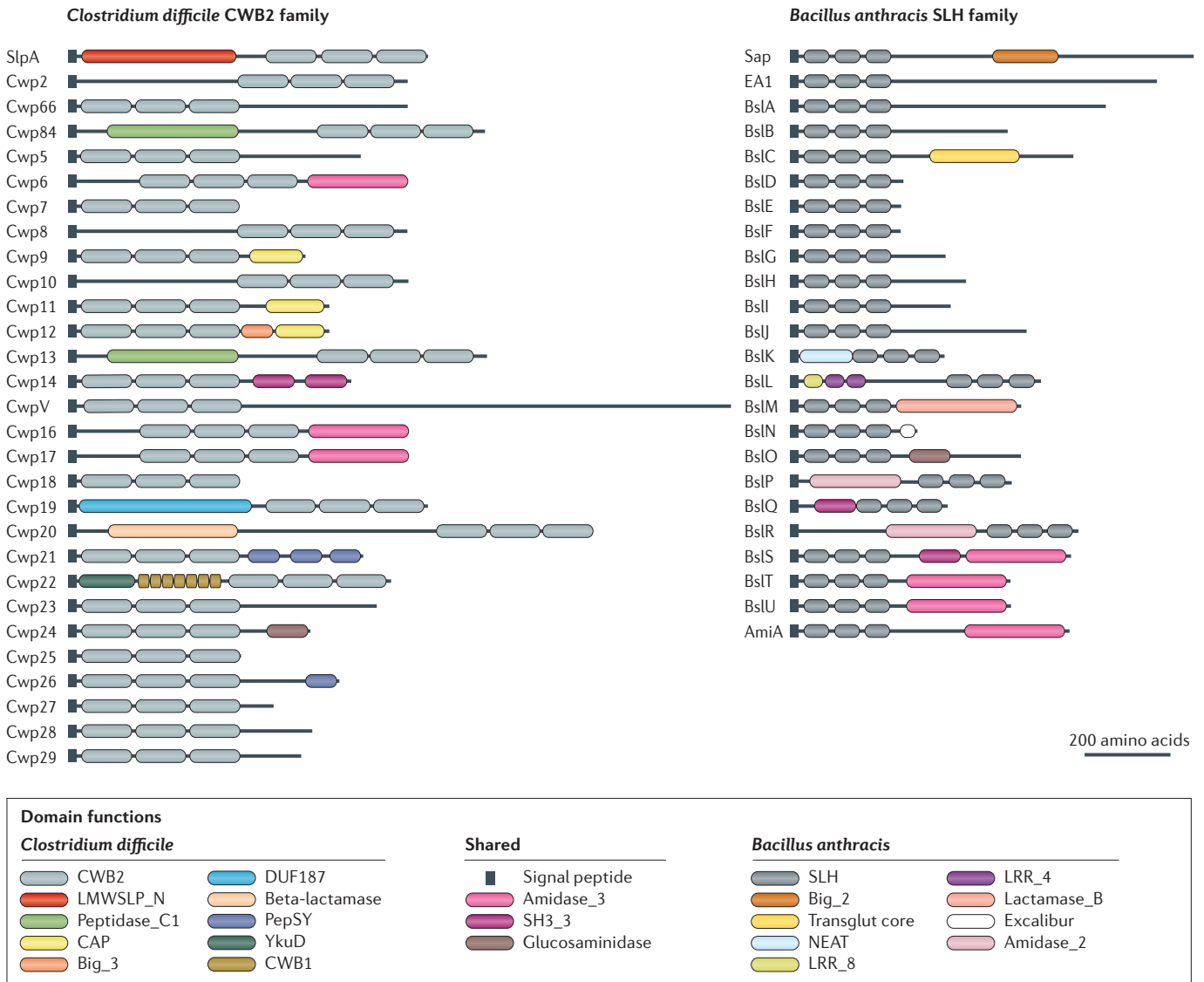
Figure 1 | *Clostridium difficile* and *Bacillus anthracis* cell surface protein families. Domain identification and organization among all members of the *Clostridium difficile* CW_binding_2 (CWB2) family and the *Bacillus anthracis* surface layer homology (SLH) family were determined using the Pfam protein families database[120] and outlined in Supplementary information S1 (table). The amino-terminal signal peptide, which is removed upon translocation through the Sec membrane channel, is shown as a black box (see also FIG. 2a). In *C. difficile*, the secretion of at least surface layer protein A (SlpA) and cell wall protein V (CwpV) is dependent on the accessory Sec secretion system[28]. In *B. anthracis*, the two major S-layer proteins, surface array protein (Sap) and EA1, also require the accessory Sec system for secretion[104]. Although limited data are available, it is possible that secretion via the accessory Sec system is a common feature of these two protein families.

shown to form 2D 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 spp. use the cell wall binding 2 (CWB2) domain in an analogous manner to the SLH motifs to anchor the S-layer to the underlying cell wall (see below). In *C. difficile*, there are 29 CWPs, each of which has three tandem CWB2 domains, including the major SLP, SlpA[19]. Comparison of the effector domains that are associated with the *B. anthracis* BSL proteins and the *C. difficile* CWPs show many similar functions, including peptidoglycan hydrolases, putative adhesins

and LRR proteins (FIG. 1). Families of CWB2-containing surface proteins, including some that have effector domains, are also found in *Clostridium botulinum* and *Clostridium tetani*[20,21].

The large number of SLP paralogues 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 various functions to its host (see below), depending on the properties of the protein or glycoprotein that is displayed.
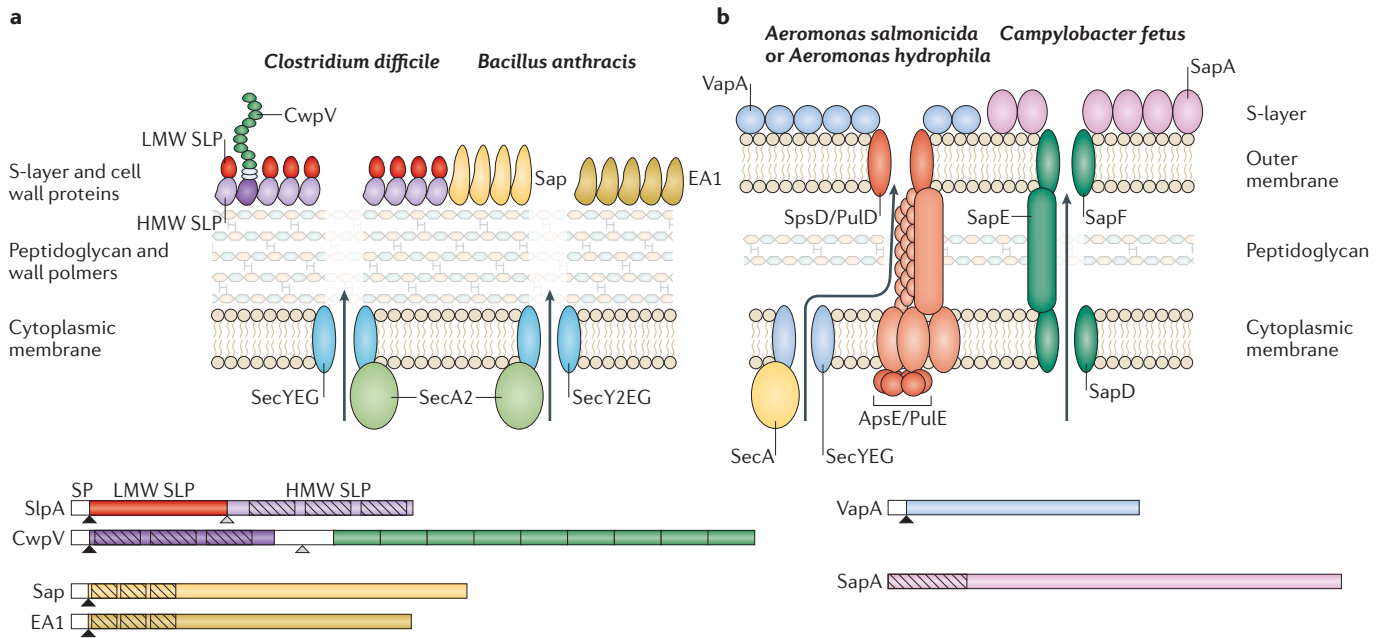
Figure 2 | **Secretion of bacterial S-layer proteins.** So far, S-layer secretion has been studied in a small number of species, in which several dedicated secretion systems have been identified. **a** | In the Gram-positive bacteria *Clostridium difficile* and *Bacillus anthracis*, secretion of the S-layer precursors is mediated by the accessory Sec secretion system[30]. The proteins contain an amino-terminal signal peptide (white box, SP) which directs the nascent polypeptide to the secretion apparatus and is cleaved upon membrane translocation (indicated by the black arrow). In both *C. difficile* and *B. anthracis*, translocation requires the accessory ATPase, SecA2 (REFS 27,28). Following recognition by SecA2, the nascent polypeptide is translocated across the membrane through a pore that consists of SecY, SecE and SecG (in *C. difficile*) or SecY2, SecE and SecG (in *B. anthracis*). **b** | Secretion of the S-layer proteins (SLPs) in *Aeromonas salmonicida* and *Aeromonas hydrophila* 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 multiprotein secretion apparatus that is closely related to type IV pili[121]. A complete type II secretion system is encoded alongside tetragonal surface virulence array A (*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, pullulanase secretion protein PulE. A PulD homologue, S-protein secretion component D (SpsD), which probably forms 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[24] that is encoded by *sapDEF*[24]. Type I secretion involves an inner membrane ATP-binding cassette (ABC) transporter, a membrane fusion protein and an outer membrane pore[168]. Where known, the anchoring domains of the SLPs are highlighted as hatched boxes (see FIG. 1). ApsE, A-protein secretion protein E; CWP, cell wall protein; HMW, high molecular weight; LMW, low molecular weight; Sap, surface array protein.

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, are 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 that is required to form a contiguous paracrystalline array; for example, we estimate that the *C. difficile* S-layer contains up to 500,000 subunits, which requires the secretion of approximately 140 subunits per second per cell during exponential growth. Several distinct mechanisms have evolved to cope with this high protein flux, but, as S-layer secretion is now being 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] that comprises an inner membrane ATP-binding cassette (ABC) transporter and an outer membrane pore (FIG. 2b). In *C. fetus*, a four-gene operon that is adjacent to the phase-variable S-layer cassette, encodes three proteins, SapD, SapE and SapF, which have 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 seems 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 that have homology to components of the prototypic pullulanase secretion system — a type II

Type I secretion system
A *sec*-independent protein secretion system in Gram-negative bacteria. It consists of an inner membrane ATP-binding cassette (ABC) transporter, a periplasmic membrane fusion protein and an outer membrane pore.

Type II secretion systems
sec-dependent multiprotein secretion systems in Gram-negative bacteria; they are closely related to type IV pili.

secretion system that is found in *Klebsiella* spp. — have been identified: *A. hydrophila* S-protein secretion component D (SpsD) is homologous to pullulanase D (PulD) and *A. salmonicida* secretion protein 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 that is adjacent to the S-layer gene *vapA*. The other genes in this cluster have not been studied in depth but it seems probable 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 that possess 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 that encode S-layer proteins and their dedicated secretion systems in many of the organisms that are 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 that contains *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, usually with lipopolysaccharides (LPSs) in Gram-negative bacteria 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 amino-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; however, 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*, enabling high-frequency antigenic variation (see above). All SapA-type homologues have a highly conserved amino-terminal domain that is responsible for anchoring to serotype A LPS. SapB-type SLPs are similar to SapA in general but have an entirely unrelated amino-terminal domain that anchors the proteins to serotype B LPS[10,33,34].

The anchoring of S-layers has been studied in many Gram-positive species. So far, two conserved Gram-positive S-layer-anchoring modules have been identified, which use either the SLH domain or the CWB2 domain. Both modules use three domains, which are located either at the amino-terminal or carboxyl-terminal region of the protein. The SLH domain is the most widely distributed and is found in the SLPs of many *Bacillus* species, *Thermus thermophillus, Deinococcus radiodurans* and at least one Clostridia species, *Clostridium thermocellum*[35–40]. The two major SLPs that are 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[43] (BOX 1) and cooperatively function to bind to a pyruvylated secondary cell wall polymer (SCWP). Pyruvylation of the SCWP relies on an enzyme, CsaB, which is encoded adjacent to genes that encode 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, which are encoded by *sbsA–sbsD* and *sgsE*[44–47], with two distinct anchoring mechanisms. SbsB has three amino-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 an *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 probable 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 the 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 CwpV and the S-layer precursor SlpA, all use 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 that 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

Secondary cell wall polymer (SCWP). A carbohydrate-based polymer, other than peptidoglycan and anionic polymers, that is present in the cell wall — for example, the pyruvylated *Bacillus anthracis* SCWP that anchors the S-layer proteins EA1 and Sap to the cell wall.

tandem copies of the CWB2 motif; this is analogous to the arrangement of SLH domains that is 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 that they have a common or convergent evolutionary origin.

Formation of an ordered array on the cell surface. An S-layer is, by definition, a 2D 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, which attaches the protein to the underlying cell wall; and a crystallization domain that mediates the 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 3D crystal structure of SbsB was described, showing the atomic contacts between adjacent 718 residue-long SLP crystallization domains, as well as the way in which individual structural domains within molecules are coordinated by Ca^{2+} — an anion that is known to be essential for S-layer formation in *G. stearothermophilus*[58] (BOX 1). The structure also shows pores of approximately 30 Å in diameter that are formed at the interface between three adjacent subunits, which is consistent with a role in permeability (see below).

It should be noted that not all SLPs have been shown to form 2D arrays, and little is known about the potential for self-assembly of the associated proteins, such as the *B. anthracis* BSL proteins and *C. difficile* CWPs. However, these proteins are found within the S-layer and, although they are probably held in place by interactions with cell wall ligands, we cannot rule out that they form 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, many glycosylated S-layer proteins have been identified in numerous bacterial and archaeal species. 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 that are often found in glycosylated eukaryotic proteins, together with some unusual sugars[60] (see below). Although N- and O-linkages have been described in archaeal SLPs[3], so far, only O-linkages have been found in bacterial SLPs, despite other bacterial surface proteins having N-linked glycans[63]. O-linkage in SLPs of the Bacillaceae family 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 of which consists of 2–6 sugars. This resemblance suggests a common evolutionary origin of LPS biosynthesis and S-layer glycosylation[64], which is an idea that is 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 that are sufficient for glycan biosynthetic pathways (reviewed in REF. 60). S-layer glycosylation pathways have been described in several species, including *G. stearothermophilus*[65], *P. alvei*[66] and *T. forsythia*[67], which has led to the proposal of a biosynthetic route that involves the transfer of galactose from the nucleotide-activated sugar uridine diphosphate (UDP)-α-D-galactose to a lipid carrier, formation of the linkage unit by the addition of glycans and assembly of the growing repetitive glycan chain onto the linkage unit. These reactions occur in the cytoplasm before 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 that decorate 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 (that is, secretion) is not dependent on glycosylation.

Strains of *C. difficile* that contain a putative *slg* locus adjacent to *slpA* were recently described[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, various 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 2D array seems to be the result of convergent evolution and is observed in proteins of quite distinct sequence. SLPs and associated proteins have further evolved to carry out a multitude of functions, some of which are essential to the physiology of the cell and some of which facilite 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 domain, apparent convergent evolution and the enormous metabolic load that is required to produce and maintain these structures, that they have an important role in the bacteria that produce them.

Roles of the S-layer in pathogenesis and immunity. In *Lactobacillus crispatus*, which is an indigenous member of the human and chicken gut microflora, the S-layer

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N- and O-linkages
Linkage of a sugar to the nitrogen (N) atom of asparagine or to the oxygen (O) atom of serine, threonine or tyrosine.
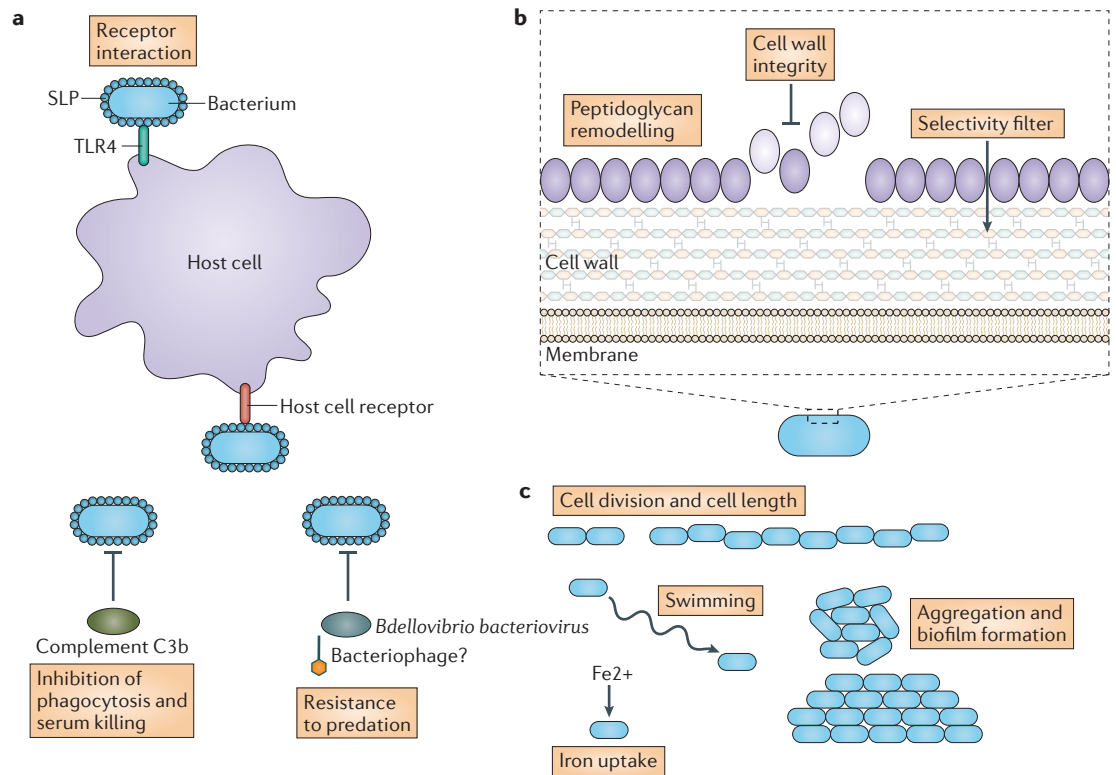
Figure 3 | Functions of S-layer proteins. Probable roles of S-layer proteins in infection (part **a**) include adhesin activity, which is found in several species: S-layer- associated protein A (BslA) of *Bacillus anthracis* binds to HeLa cells and mutants are attenuated in models of infection[79], binding of surface layer proteins (SLPs) to enteric cells has been observed in *Clostridium difficile*[75] and binding to defined ligands (such as types I and IV collagen) has been observed in *Lactobacillus crispatus*[70]. In *C. difficile*, interaction of SlpA with host toll-like receptor 4 (TLR4) receptors is linked to innate immunity[74] and, in *Campylobacter fetus*, SLPs prevent binding of complement factor C3b, which protects the bacterium from host-mediated phagocytosis and serum killing[86]. A role in resistance to predation has been shown in *Bdellovibrio bacteriovirus*[97], and potential roles in resistance to bacteriophage and bacteriocins are possible but remain speculative (part **a**; lower panel). SLPs have roles in the maintenance of cell envelope integrity (part **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 the shedding of surface proteins[101]. *B. anthracis* BslO and *C. difficile* Cwp22 have peptidoglycan hydrolase activity that may remodel the peptidoglycan. A role for SLPs as a permeability barrier has been shown in *Bacillus coagulans*[98]. A role in cell division has been found in *B. anthracis* (part **c**) where inactivation of S-layer associated protein O (BslO), which is a putative *N*-acetylglucosaminidase, results in increased bacterial chain length[103]. SLPs have roles in aggregation (such as *C. difficile* CwpV15), biofilm formation (in *Tanerella forsythia*[94]) and swimming (in *Synechococcus* spp.[106]). Finally, BslK from *B. anthracis* mediates iron uptake by scavenging haem and transferring it to iron-regulated surface determinant protein C (IsdC)[80].

CsbA (also known as SlpB) has an amino-terminal domain that binds to types I and IV collagen and a carboxyl-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*, which is 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 to activate, the immune system. The SlpA proteins (HMW and LMW SLPs) induce the release of pro-inflammatory cytokines from human monocytes and induce the maturation of human monocyte-derived dendritic cells (MDDCs)[73]. Further work advanced these findings by showing that the SLPs induced the maturation of mouse bone marrow-derived dendritic cells and

the production of the cytokines interleukin-12 (IL-12), tumour necrosis factor α (TNFα) and IL-10, but not the production of of IL-1β. Importantly, activation of dendritic cells was dependent on Toll-like receptor 4 (TLR4) and subsequently induced T helper cell responses that are known to be involved in the clearance of bacterial pathogens[74]. Infection of TLR4-knockout mice resulted in more severe symptoms than in wild-type mice, which suggests that TLR4 has an important role in bacterial clearance[74]. Both the HMW and LMW SLPs of *C. difficile* were required for the activation of dendritic cells, which suggests that either the entire complex is recognized by TLR4, or that one of the SLPs is the ligand, but that it 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 which infers that the

S-layer might function as an adhesin *in vivo* (FIG. 3a). So far, the role of SlpA as an adhesin has not been investigated using mutagenesis, as *slpA* is an essential gene, but recent advances in genetics[76–78] should enable the 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 roles of Sap and EA1 are unknown, but activities of some BSLs have been elucidated. The BslA protein has been shown to mediate the adhesion of encapsulated *B. anthracis* to HeLa cells[16]. Mutants of *bslA* show limited dissemination to tissues and decreased virulence in a guinea pig model of infection, which suggests that BslA is a functional adhesin that is required for full virulence of *B. anthracis*[79]. Another S-layer protein, BslK, mediates haem uptake by using 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, which is 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 (REFS 84–86) (FIG. 3a). Antigenic variation occurs during infection in humans, as shown by strains that were 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 protein that is expressed.

T. forsythia (which was 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 HMW proteins, each more than 200 kDa, that are concomitantly expressed and that constitute the S-layer[90–92]. The structural genes *tfsA* and *tfsB* encode proteins of 120 kDa and 140 kDa[93], which suggests that post-translational modification occurs. 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 that has not previously been found on bacterial proteins. This sugar, which is a sialic acid derivative, was suggested to participate in bacterium–host interactions[67] on the basis of the prevalence of sialic acid-like sugars in Gram-negative structures that are involved in pathogenesis, such as LPS, capsules, pili and flagella. In *T. forsythia* transposon mutants that exhibited altered biofilm formation (FIG. 3c), an operon that is involved in exopolysaccharide 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 that is consistent with glycosylation[94]. A proteomics study also found increased quantities of S-layer proteins in *Tannerella* biofilms compared with planktonic cells[95]. The *T. forsythia* S-layer seems to be essential for the 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 that was defective in *tfsA* and *tfsB*[92].

SLPs have also been shown 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 *C. cresentus* possess an S-layer and are not normally parasitized by *Bdellovibrio* spp.; however, S-layer-negative variants of both species were shown to be sensitive to predation, which indicates that the S-layer can provide a protective coat against infection with *Bdellovibrio* spp.[97] (FIG. 3a). In addition to this, one might speculate that the S-layer would function as a receptor for bacteriophage and bacteriocins but, to our knowledge, there is no evidence of such activities.

Permeability and biogenesis of the cell envelope. S-layers are often proposed to function as a permeability barrier, and a direct role for SLPs as barriers has been investigated in *Bacillus coagulans* and other species[2,98]. Experimentally determined exclusion limits of isolated sacculi and associated proteins of 15–34 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 in diameter[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 *D. radiodurans*, the S-layer (which is known as the 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 by 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 as gut proteases, such as trypsin, can cleave SlpA, resulting in a fully functional S-layer[100].

Sacculi
The sacs of polymerized peptidoglycan that surround bacteria. When they are isolated from the bacterium, they retain the shape of the cell.

In *B. 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*-acetyl-glucosaminidase activity and is localized at the cell septa, has a role in catalysing cell division; *bslO* mutants exhibit increased cell chain length[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 predominantly visualized on the lateral cell wall, away from the septa, which suggests 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 and SLPs, several proteins potentially mediate peptidoglycan synthesis and remodelling, including Cwp22, which has L,D-transpeptidase activity[105].

Other functions of the S-layer. Another function that is associated with the S-layer is swimming in marine *Synechococcus* spp.[106] (FIG 3c). This bacterium 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 mostly a mystery. In mutants that were defective for swimming, two surface proteins that are essential for this activity were identified: SwmA and SwmB[107]. SwmA is a 130 kDa glycosylated S-layer protein and SwmB is a large 1.12 MDa protein. Other genes that are essential for swimming encode an ABC transporter and several glycosyltransferases[108]. Although these results indicate another function for an S-layer and suggest that glycosylation of SwmA is required for motility, they unfortunately do not 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, such as *Sulfolobus* spp., the S-layer seems to be the sole non-lipid constituent of the envelope[3], which suggests that structural integrity might be an ancestral function of SLPs. In some bacterial species, such as the Clostridia, the S-layer seems to be 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 range of other macromolecules are found.

Increasingly sophisticated and high-resolution techniques, such as atomic force microscopy (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 nuclear magnetic resonance (NMR) spectroscopy to generate atomic-resolution models of the complete S-layer. Progress has been recently 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 that is 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 functions of S-layers?

It is clear that SLPs and their associated proteins have evolved to have specialized functions, and in some species of Firmicutes, SLPs function as a scaffold to display enzymes on the cell surface. It is probable 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 the S-layers that are present in bacterial pathogens, investigating their potential as therapeutic targets for antimicrobial or vaccine development and in-depth structural analyses of the interactions between S-layers and other surface components. As increasingly sophisticated structural and imaging tools are becoming available, 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 the bacteria that produce them.

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