

Chapter 2

S-layer Structure in Bacteria and Archaea

Chaithanya Madhurantakam, Stefan Howorka and Han Remaut

2.1 Introduction

The majority of bacteria and archaea develop multicomponent cell envelopes with layered supramolecular architectures surrounding the cytoplasmic membrane. Of the finest examples of such supramolecular cell wall components are surface or S-layers. These regular paracrystalline pericellular structures were first observed in the bacterium *Spirillum serpens* and in the archaeum *Halo-bacterium salinarum* (Houwink 1956, 1953). Following the confirmation that S-layers were of proteinaceous nature, the first S-layer protein (SLP) gene to be sequenced was that of *Brevibacillus brevis* 47 in 1986 (Tsuboi et al. 1986). It is now well established that S-layers are composed of monolayers of glycoproteins/proteins with an Mr range of 40–200 kDa, many of which have been thoroughly characterized by genetic and morphogenetic studies as well as by structural, biophysical and biochemical means (Altman et al. 1990; Fagan and Fairweather 2014; Kuen et al. 1994; Messner et al. 1986a, b; Sleytr et al. 1986, 1993). Generally, different SLPs give rise to defined monomolecular lattices, although bimolecular S-layers are known for *Clostridium difficile* (Takeoka et al. 1991) and *Bacillus anthracis* (Etienne-Toumelin et al. 1995). The two-dimensional (2D) arrays are formed through non-covalent self-assembly of the SLP subunits, though exceptionally covalent cross links between the protein subunits are found, as in *Methanosprillum hungatei* (Beveridge et al. 1985;

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L. L. Barton et al. (eds.), *Nanomicrobiology*, DOI 10.1007/978-1-4939-1667-2_2

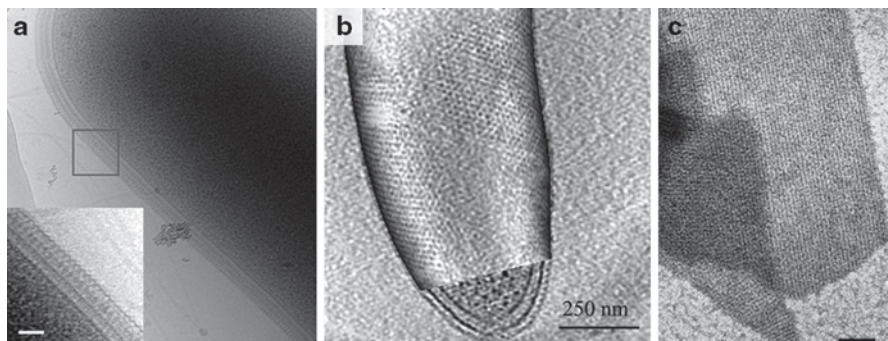


Fig. 2.1 **a** Cryo electron tomogram of the intact cell-bound S-layer (wt-SbpA) of *Lysinibacillus sphaericus* (formerly, *Bacillus sphaericus*), a Gram-positive soil bacterium that forms a 2D crystalline envelope with p4 symmetry (cell constant: ~ 13 nm). *Inset* shows the close-up view of the cell wall and the enveloping SbpA S-layer. Scale bar: 50 nm (image courtesy of Luis R. Comolli). **b** Electron tomogram of *Caulobacter crescentus* cell, overlaid with the 3D reconstruction of the RsaA S-layer, which can be seen to form hexagonal repeating units (six RsaA monomers) interconnected at their junctions by p3 symmetry. The cutaway region at the cell pole shows the S-layer as the outermost component of the cell envelope. Scale bar: 250 nm (image courtesy of Luis R. Comolli). **c** Negative stain EM micrograph of an *in vitro* self-assembly product of the *Geobacillus stearothermophilus* PV72/p2 SLP SbsB (mutant S347-HA). (Reproduced from Kinns et al. 2010). Scale bar: 100 nm

Messner et al. 2008; Sleytr and Beveridge 1999; Stewart et al. 1985). Bacterial S-layer lattices have p1, p2 (oblique), p4 (square) or p3 or p6 (hexagonal) symmetries, while archaeal S-layers are predominantly hexagonal in symmetry (Albers and Meyer 2011; Sleytr et al. 1993). Topologically, S-layers completely encapsulate the SLP-producing cell (Figure 2.1). Their mode of anchoring to the cell envelope can be broadly classified in accordance with the archaeal, Gram-positive or Gram-negative cell architecture (Pum et al. 2013). In archaea, SLPs are directly associated with the cytoplasmic membrane through a single-span transmembrane segment. The lattices form the dominant component of the cell wall and function primarily as a protective barrier, though in *Methanosarcina mazei* the SLPs were also shown to be involved in cell-cell association (Mayerhofer et al. 1992). In Gram-positive bacteria, the S-layers lie atop the peptidoglycan layer and are anchored to the peptidoglycan or the associated nonclassical secondary cell wall polymers (SCWPs; Mesnage et al. 2000; Schaffer and Messner 2005; Zhao et al. 2006). In Gram-negatives, the S-layers are associated with the lipopolysaccharides (LPS) in the outer membrane (OM; Ford et al. 2007). SLPs reach the cell surface using the general secretory pathway (SEC translocon), except in Gram-negative bacteria, where the diderm cell walls require more elaborate translocative routes such as type I and II secretion pathways (Noonan and Trust 1995; Thompson et al. 1998).

2.2 SLP Composition and Post-Translational Modifications

SLPs are distributed over a wide range of phylogenetic branches (Messner and Sleytr 1992; Sleytr et al. 1999) and generally show little (<20% identity) to no sequence similarity, even within a single species or closely related taxons. The recent advances in SLP structural biology (Baranova et al. 2012; Ethordic et al. 2012; Fagan et al. 2009; Jing et al. 2002; Kern et al. 2011; Pavkov et al. 2003, 2008; Stetefeld et al. 2000; see below) reveal a diverse domain composition. This indicates that different SLPs do not generally have a common origin, even though relatively higher similarity is observed among S-layers of Gram-positive bacteria (Engelhardt and Peters 1998). Nevertheless, SLPs show similar overall amino acid compositions that are low in methionine and negligible cysteine. For the majority of SLPs, an enrichment in aspartic and glutamic acid results in isoelectric points (pIs) that lie in the weakly acidic range (Messner et al. 1997; Sara and Sleytr 2000), with the exception of *Methanothermus fervidus* (pI: 8.4; Brockl et al. 1991) and lactobacilli (pI: 9–11; Boot et al. 1995). An exception to the low overall sequence similarity is the ~55-amino-acid S-layer homology (SLH) motif, frequently found in tandem repeats near the N-terminus of many bacterial SLPs and surface-anchored enzymes (Mesnage et al. 2000; Schaffer and Messner 2005; Zhao et al. 2006). The X-ray structure of the three tandem SLH motifs in the *B. anthracis* surface array protein (Sap) shows that these form helix-loop-helix structures that together organize into a single three-prong spindle domain (Kern et al. 2011). The SLH domains are cell-wall-anchoring domains that bind the peptidoglycan, either directly as in *Paenibacillus alvei* CCM 2051 (Janesch et al. 2013a, b) or through the associated pyruvylated carbohydrate moieties in the nonclassical SCWPs (Mesnage et al. 2000; Schaffer and Messner 2005; Zhao et al. 2006). SLH domains are not universal S-layer-anchoring modules, however. In *Geobacillus stearothermophilus* SbsC, an extended three-helix bundle is found as N-terminal glycan-binding domain (Pavkov et al. 2008), and in *Corynebacterium glutamicum* this role is taken by the C-terminal hydrophobic domain (Bahl et al. 1997; Chami et al. 1997). In Gram-negative bacteria, the S-layers attach to the OM LPS via direct or divalent cation-mediated charge interactions as in *Campylobacter fetus* or *Aeromonas salmonicida* (Garduno et al. 1992a, b), or by covalently bound fatty acids as in *Deinococcus radiodurans* Hpi (Peters et al. 1987).

Both bacterial and archaeal SLPs are frequently glycosylated, and S-layer glycoproteins (SLGPs) have provided a major impetus to the prokaryotic glycoprotein research. SLGPs have been described for halophilic archaeons such as *H. salinarum* (glycoprotein with a M_r of 200,000 and carbohydrate content of 10–12%; Mescher and Strominger 1976), *Haloferax volcanii* (presence of O-glycosidic linkages like “Thr/Ser-Gal” and 1, 2-linked glucosyl-galactose as a structural element in S-layer; Eichler and Adams 2005; Sumper et al. 1990), hyperthermophilic methanogens such as *M. fervidus* (Brockl et al. 1991), *Methanothermus sociabilis* and *Methanococcus voltae* (Konisky et al., 1994) and the thermoacidophilic crenarchaeote *Sulfolobus acidocaldarius* (oligosaccharide is a complex, branched, six-membered glycan tree; Peyfoon et al. 2010). In Gram-positive bacteria, SLP glycosylation is

found in clostridia and bacilli such as *Thermoanaerobacter*, *Geobacillus*, *Aneurinibacillus*, *Paenibacillus* and *Lactobacillus* species, whereas in Gram-negative bacteria SLP glycosylation has only been described for *Tannerella forsythia* TfsA and TfsB (Posch et al. 2011; Schaffer and Messner 2004; Sleytr and Thorne 1976). The glycan chains can be branched, though in bacteria typically contain an extended linear stretch of repeating units (10–50 units). This is frequently capped by O-methylation in the 2' or 3' position of the nonreducing glycan and anchored to the protein via a core of two to four sugar residues and an O-glycosidic linkage (galactose–tyrosine, glucose–tyrosine, N-acetyl galactosamine–serine, N-acetyl galactosamine–threonine). Archaeal glycans are shorter and generally attached by an N-glycosidic bond to asparagine, though O-linked chains have also been observed (Altman et al. 1996; Messner 1997; Wugeditsch et al. 1999). The number of glycosylation sites can vary from two to four in Gram-positive SLPs, to up to 25 in archaeal SLPs (Lechner et al. 1986). Additional post-translational modifications of SLPs include sulphated glycan chains of *Halobacter halobium* to provide stability to the S-layer or phosphorylation of tyrosine residues in *Aeromonas hydrophila* to decrease the pI (6.7–4.6).

2.3 Functional Diversity of S-Layers

The primary function of S-layers is to stabilize cells against mechanical, thermal and osmotic stress (Engelhardt 2007a). From the accumulated data it is evident that S-layers exhibit high levels of physical and chemical stability, indicating they can act as protective and/or shape-maintaining exoskeleton (Engelhardt and Peters 1998). For example, experiments with *Halobacteria* found that the rod-like appearance was lost upon S-layer lattice degradation. (Engelhardt 2007a; Mescher and Strominger 1976). Even though S-layers can maintain and modify the cell morphology, the endoskeleton is the primary factor in determining shape. (Baumeister and Lembecke 1992; Engelhardt 2007a; Messner et al. 1986a; Peters et al. 1995; Pum et al. 1991). Another role of S-layers is to shield against environmental and biological factors. S-layers in Gram-negative bacteria such as *A. salmonicida*, *C. fetus* and *Caulobacter crescentus* can form a protective covering towards bacterial parasites like *Bdellovibrio bacteriovorus* (Koval 1988; Koval and Bayer 1997; Koval and Hynes 1991), though the S-layers did not provide a barrier for protozoans. S-layers have also been observed to attenuate host immune responses in the periodontal pathogen *T. forsythia* (Sekot et al. 2011). In the opportunistic pathogen *C. fetus*, the surface expression of SLPs prevented binding of complement component C3b, and in *A. salmonicida* the S-layer (A-layer) imparts high to moderate levels of anti-bactericidal activity against complement systems (Dworkin and Blaser 1997). The A-layer also imparts an adhesive property, able to bind laminins, fibronectins (Doig et al. 1992) and basement membrane protein collagen type IV (Trust et al. 1993). In other pathogens like *T. forsythia*, *C. difficile* and *B. anthracis*, the S-layers contribute to virulence by mediating host cell adhesion (Calabi et al. 2002; Kern and Schneewind 2010; Sakakibara et al. 2007).

Besides a supporting role in maintaining cell integrity or influencing bacterial virulence, several S-layers can also function as scaffold for the adhesion of other cellular components. Archaeal S-layers are composed of long hydrophobic protrusions entering the plasma membrane, which have been proposed to provide a contact zone for macromolecules that function in folding and export of proteins and/or nutrient degradation and transport mechanisms (Lechner and Sumper 1987). Similarly, in many Bacillaceae, S-layers provide adhesion sites for cell-associated exoenzymes (Sleytr et al. 1999). Well-documented examples include the high-molecular-weight amylases in *G. stearothermophilus* strains DSM 2358 and ATCC 12980 (Egelseer et al. 1995, 1996), endo-xylanase in *Thermoanaerobacterium thermohydrosulfurigen* strain JW/SL-YS 485 (Liu et al. 1996) and endo-glucanase in *Clostridium thermocellum* (Leibovitz et al. 1997). Finally, an interesting feature observed in the unicellular cyanobacterium *Synechococcus* strain GL24 (strain isolated from a meromictic Fayetteville Green Lake, New York, USA, with high levels of calcium and sulphate ions) is that the hexagonal S-layer acts as a template for sulphate and carbonate mineral formation over its surface (Schultzelam and Beveridge 1994a, b).

Thus, although a general function of an S-layer appears to be the provision of a structurally supporting 2D array outside the cell, various genetic and functional studies found that they are phylogenetically and structurally dissimilar and attain varied roles in cellular activities, leading to a high degree of functional heterogeneity.

2.4 Structural Biology of S-Layers

Over the past two decades, there has been a steady increase in the level of molecular and structural understanding of the surface layer proteins (Table 2.1). In the initial phase, S-layer structural biology was mainly based on freeze-etching, freeze-drying, negative staining followed by 2D and 3D transmission electron microscopy (TEM) and on scanning probe microscopy (Beveridge 1993; Beveridge et al. 1993; Firtel et al. 1994b). This was followed by efforts towards the 3D prediction of the building components using the mean force approach, and by simulations of self-assembly process by Monte-Carlo simulations (Horejs et al. 2008, 2011). Cryo-electron microscopy and tomography (cryo-EM, cryo-ET) in turn provided depth to the topographical information derived from the SLPs (Baumeister and Lembecke 1992; Kinns et al. 2010; Norville et al. 2007; Rachel et al. 1986; Shin et al. 2013; Smit et al. 1992; Figure 2.1). Atomic force microscopy (AFM) has provided dynamic views of the crystallization pathways during in vitro S-layer formation (Chung et al. 2010; Shin et al. 2012). The search for high resolution structural information by 3D crystallization of SLPs has long been plagued by the inherent tendency of SLPs to form 2D lattices. Nevertheless, in recent years, X-ray crystallography has started to reveal the molecular architecture of the SLPs and/or non-assembling SLP fragments (Baranova et al. 2012; Ethordic et al. 2012; Fagan et al. 2009; Jing et al. 2002; Kern et al. 2011; Pavkov et al. 2003, 2008; Stetefeld et al. 2000; Figures 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8) and provide more molecular insights into the assembly of the supramolecular structures. In the following paragraphs, these recent advances in SLP structural biology are systematically reviewed. As common feature, bacterial SLPs

Table 2.1 Archaeal and bacterial members with characterized S-layers and methods employed to analyze the properties and structural characteristics of the S-layer lattice

Species	Methods employed	Reference
<i>Acinetobacter</i> sp. strain MJIT/F5/199A	EM	Thornley et al. (1973)
<i>Aeromonas salmonicida</i> A449-TM5	EM	Dooley et al. (1989); Garduno et al. (1992a, b); Stewart et al. (1986)
<i>Aeromonas hydrophila</i> TF7	EM	Al-Karadaghi et al. (1988); Dooley and Trust (1988); Murray et al. (1988)
<i>Aneurinibacillus thermoaerophilus</i>	Negative staining, thin-sectioning and immuno-gold labelling, EM	Kadurugamuwa et al. (1998)
<i>Aneurinibacillus thermoaerophilus</i> DSM 10155	Spectroscopy	Steindl et al. (2002)
<i>Aquaspirillum serpens</i> MW5	SAXS	Sekot et al. (2013)
<i>Bacillus anthracis</i>	EM	Etienne-Toumelin et al. (1995); Mesnage et al. (1997)
	X-ray crystallography	Kern et al. (2011)
<i>Bacillus coagulans</i> E38-66	Atomic force microscopy (AFM)	Dufrene (2001)
	EM	Sara et al. (1992)
	Freeze etching	Pum et al. (1989)
<i>Bacillus pseudofirmus</i> OF4	EM, 2D gel-electrophoresis	Gilmour et al. (2000)
<i>Bacillus subtilis</i> 168	EM	Graham and Beveridge (1994)
<i>Bacillus sphaericus</i> CCM2177	AFM	Gyorvary et al. (2003); Toca-Herrera et al. (2004)
	Scanning force microscopy (SFM)	Ohnesorge et al. (1992)
	Molecular recognition force spectroscopy (MRFS)	Tang et al. (2008)
	TEM, SAXS	Horejs et al. (2010)
	SPR	Huber et al. (2005)
	Cryo-EM, tomography, spectroscopy	Shin et al. (2013)
	Electron crystallography	Norville et al. (2007)
<i>Bacillus sphaericus</i> NCTC 9602	TEM and electron holography	Simon et al. (2004)
<i>Bacillus stearothermophilus</i> NRS2004/3a	EM	Kupcu et al. (1984); Messner et al. (1986a); Sleytr et al. (1986)
	Spectroscopy, mass spectrometry (MS)	Steiner et al. (2006)
<i>Bacillus stearothermophilus</i> PV72/p2	EM	Howorka et al. (2000); Sara et al. (1998)
	Spectroscopy	Runzler et al. (2004)
	Limited proteolysis, spectroscopy, Cryo-EM	Kinns et al. (2010)
<i>Caulobacter crescentus</i>	Negative staining, thin-section EM	Smit et al. (1992)
	Cryo-electron tomography (Cryo-ET) and statistical image processing	Amat et al. (2010)

Table 2.1 (continued)

Species	Methods employed	Reference
<i>Clostridium difficile</i>	MS and EM	Calabi et al. (2001)
<i>Clostridium difficile</i> CD630	X-ray crystallography and SAXS	Fagan et al. (2009)
<i>Clostridium thermocellum</i>	Immuno-blotting, EM	Leibovitz et al. (1997)
<i>Clostridium thermohydrosulfuricum</i> L111-69	Freeze etching, EM	Sara et al. (1988)
<i>Clostridium thermosaccharolyticum</i> D120-70		
<i>Corynebacterium glutamicum</i>	AFM imaging, single molecule force spectroscopy (SMFS)	Scheuring et al. (2002)
	Freeze-etching, EM	Peyret et al. (1993)
	Cryo-electron microscopy of vitreous sections (CEMOVIS)	Zuber et al. (2008)
<i>Deinococcus radiodurans</i>	AFM	Karrasch et al. (1994); Lister and Pinhero (2001); Muller et al. (1996, 1999); Wiegrabe et al. (1991)
	Cryo-microscopy	Baumeister et al. (1986); Karrenberg et al. (1987); Rachel et al. (1986); Sleytr et al. (1973)
<i>Desulfotomaculum nigrificans</i>	EM	Sleytr et al. (1986)
<i>Escherichia coli</i> K29	EM	Koval and Bayer (1997)
<i>Geobacillus stearothermophilus</i>	X-ray crystallography and SAXS	Pavkov et al. (2003, 2008)
<i>Geobacillus stearothermophilus</i> ATCC 12980	SAXS	Sekot et al. (2013)
<i>Geobacillus stearothermophilus</i> NRS2004/3a	Spectroscopy, MS	Schaffer et al. (2002)
<i>Geobacillus stearothermophilus</i> PV72/p2	Spectroscopy, MS, electrospray spectrometry (ES)	Petersen et al. (2008)
	EM	Kinns et al. (2010)
	X-ray crystallography, cryo-EM and SAXS	Baranova et al. (2012)
<i>Lactobacillus brevis</i> ATCC 14869	EM	Jakava-Viljanen et al. (2002)
<i>Lactobacillus buchneri</i> CD034	MS	Anzengruber et al. (2013)
<i>Lactobacillus helveticus</i> CNRZ 892	Immuno-blotting, EM	Callegari et al. (1998)
<i>Lactobacillus salivarius</i> 16	Spectroscopy and thermal denaturation	Lighezan et al. (2012)
<i>Lysinibacillus sphaericus</i>	Cryo-EM, Cryo-ET	Shin et al. (2013)
<i>Methanosarcina mazei</i>	X-ray crystallography	Jing et al. (2002)
<i>Methanosarcina acetivorans</i>	X-ray crystallography	Arbing et al. (2012)
<i>Methanospirillum hungatei</i> Gp1	Scanning-tunnelling microscopy (STM)	Blackford et al. (1994)
	Thin sectioning, negative staining, platinum shadowing and image processing, EM	Firtel et al. (1994a, b)

Table 2.1 (continued)

Species	Methods employed	Reference
<i>Staphylococcus marinus</i>	EM	Peters et al. (1955)
<i>Staphylothermus marinus</i>	X-ray crystallography	Stetefeld et al. (2000)
<i>Tannerella forsythia</i>	TEM, immune-fluorescence microscopy (IFM) and AFM	Oh et al. (2013); Sekot et al. (2011, 2012)
	Spectroscopy	Posch et al. (2011, 2013)
<i>Thermococcus litoralis</i> Z-1301	Thin-sectioning, freeze etching, EM	Kostyukova et al. (1999)
<i>Thermoproteus tenax</i> <i>Thermoproteus neutrophilus</i>	Freeze etching, freeze drying, negative staining, image enhancement, EM	Messner et al. (1986a)

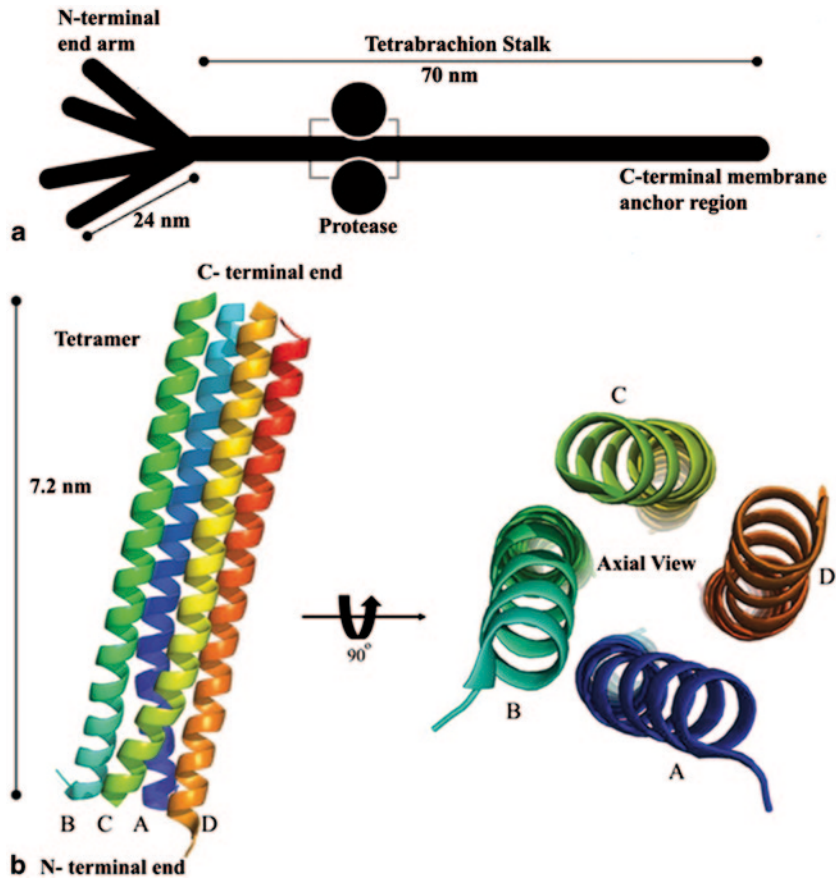


Fig. 2.2 **a** Schematic diagram of the *S. marinus* SLP which forms a filiform tetrameric unit called tetrabrachion (70 nm in length). The tetrabrachion is composed of an extended parallel-coiled coil stalk region with C-terminal membrane anchor region and four extending arms (24 nm each in length) at its N-terminal end (Peters et al. 1995). The tetrameric stalk contains a protease (represented as globular structures)-binding domain, the atomic structure of which (grey boxed area) is shown in panel **b**. **b** Crystal structure of the right-handed parallel coiled coil region (RHCC) (PDB code: 1FE6) corresponding to the protease-binding domain (grey boxed area), shown in lateral and axial view. (Stetefeld et al. 2000)

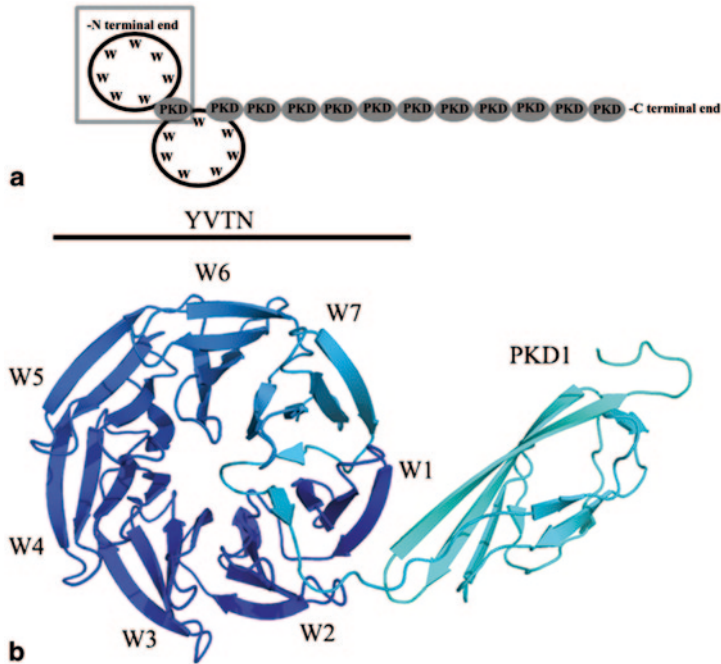


Fig. 2.3 **a** Schematic representation of the domain composition of the *M. mazei* SLP MA1904 (Jing et al. 2002). At its N-terminus, the SLP contains two “YVTN” β -propeller domains (each with seven blades composed of four-stranded β -sheets labelled *W*), followed by twelve (1+11) consecutive PKD domains. **b** Crystal structure of a 42 kDa N-terminal SLP fragment (boxed area in panel **a**) reveals the molecular structure of a “YVTN” β -propeller domain (blades labelled *W1* to *W7*) and a single-polycystic-kidney disease (*PKD1*) domain (PDB code: 1L06)

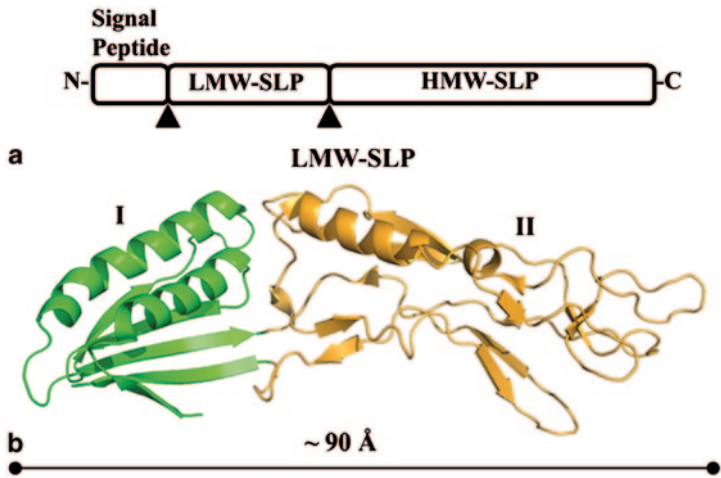


Fig. 2.4 **a** Domain architecture of the SlpA preprotein from *C. difficile* CD 630. The preprotein is proteolytically processed into a high- and low-molecular weight SLP (*HMW*- and *LMW*-SLP, resp.). Cleavage sites are represented by *small triangles*. **b** Crystal structure of LMW-SLP₁₋₂₆₂ (PDB code: 3CVZ) encompassing domains I and II, spanning ~90 Å in length

Fig. 2.5 **a** Schematic representation of the Sap protein from *B. anthracis*, indicating the N-terminal SLH domains and C-terminal crystallization domain. **b** Crystal structure of the cell-wall-anchoring domain of Sap (residues 31–210) (PDB code: 3PYW) with the SLH domains labelled as SLH₁, SLH₂ and SLH₃

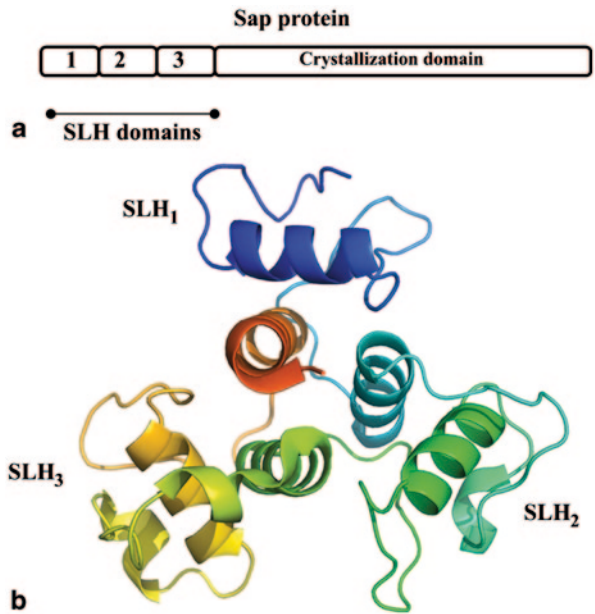
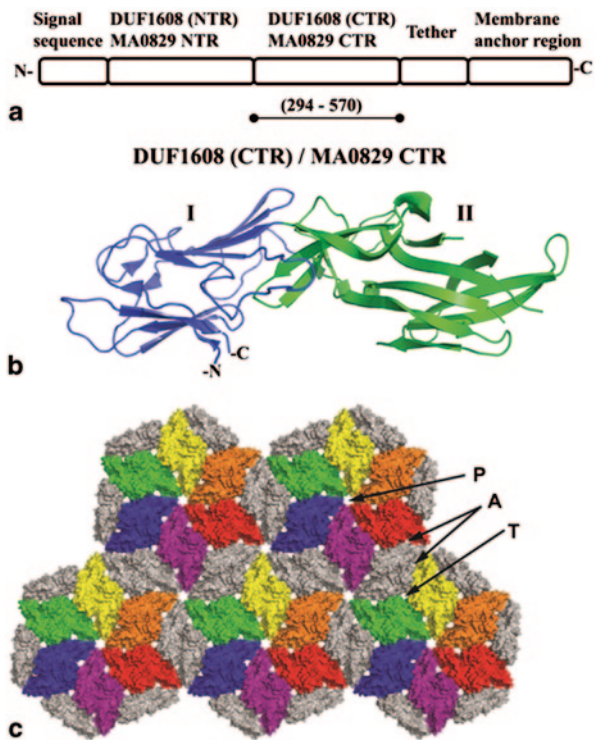


Fig. 2.6 **a** A schematic representation of the *M. acetivorans* MA0829 S-layer protein with N-terminal signal sequence, followed by a tandem repeat of DUF1608 domains (*NTR* and *CTR*), a tether sequence and finally, the C-terminal membrane-anchoring region. **b** The crystal structure of DUF1608 (CTR)/MA0829 CTR (294–570 residues) (PDB code: 3U2G) reveals two subdomains, domain I and II. **c** Putative model of the MA0829 S-layer lattice generated based on crystallographic dimers of the CTR. The model shows a semiporous lattice with three distinct pores, labelled *P* (primary), *A* (asymmetric) and *T* (trimer). (Image courtesy of Mark A. Arbing)



Nanobiotechnology

Physiological and Environmental Characteristics

Barton, L.L.; Bazylnski, D.; Xu, H. (Eds.)

2014, X, 184 p. 39 illus., 28 illus. in color., Hardcover

ISBN: 978-1-4939-1666-5