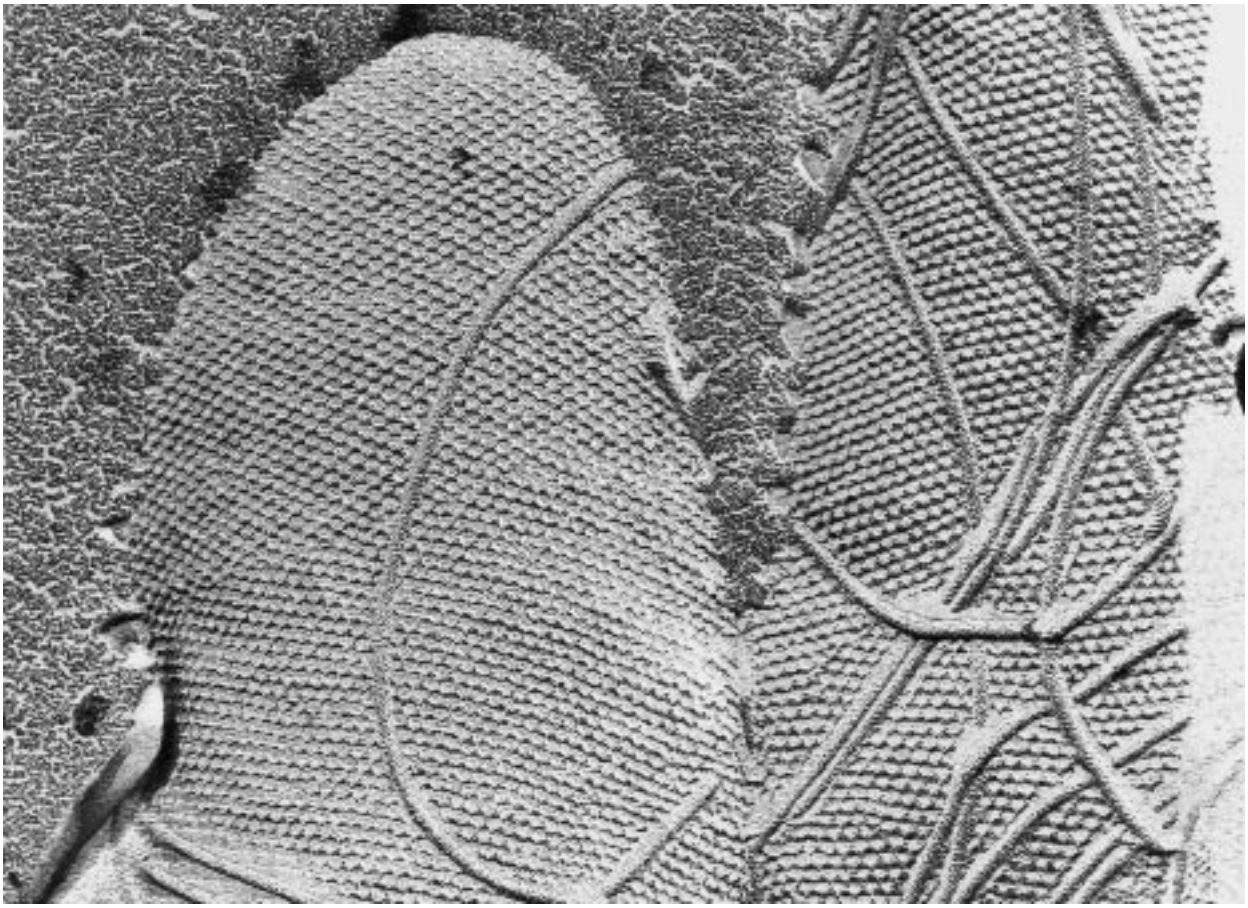
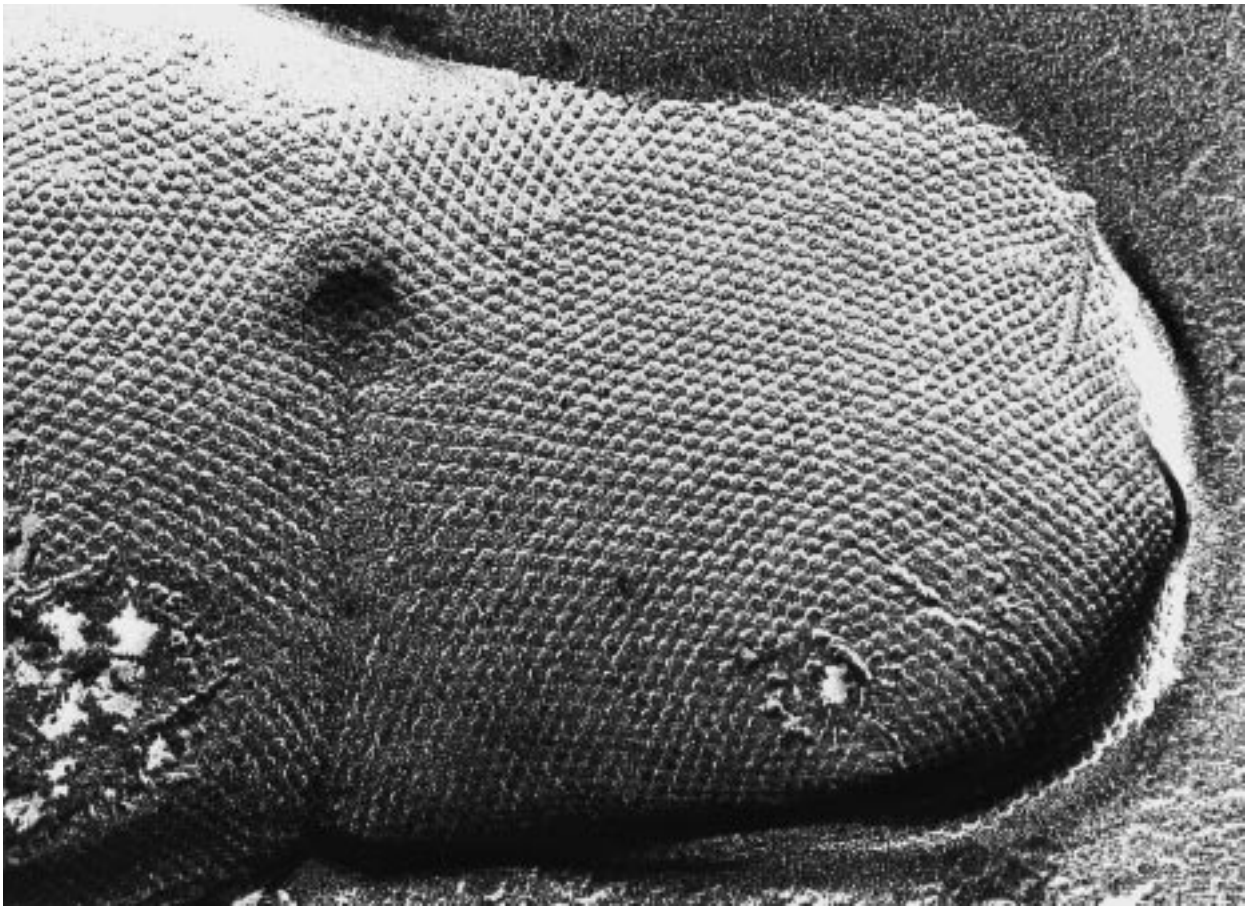


Electron micrographs of two S layer lattices on the surface of bacteria cells.
Top: *Thermoanaerobacter thermohydrosulfuricus* L111-69 (magnification 241000 times);
bottom: *Methanocorpusculum sinense* DSM 4274 (magnification 343000 times)



Crystalline Bacterial Cell Surface Layers (S Layers): From Supramolecular Cell Structure to Biomimetics and Nanotechnology

Uwe B. Sleytr,* Paul Messner, Dietmar Pum, and Margit Sára

One of the most remarkable features of many prokaryotic organisms (archaea and bacteria) is the presence of a regularly ordered surface layer (S layer) as the outermost component of the cell envelope. Surface layers are composed of a single protein or glycoprotein species and represent the simplest biological membrane developed during evolution. Owing to the fact that S layers possess a high degree of structural regularity they represent unique systems for studying the structure, synthesis, genetics, the dynamic process of assembly, and the function of a supramolecular structure. Isolated S layer subunits of numerous organisms are able to assemble into monomolecular arrays either in suspension, at liquid–surface interfaces, on lipid films, on liposomes, and on solid supports (for example, silicon wafers). Pores in S layers are of regular size and morphology, and functional groups on the protein lattice are aligned in well-defined positions and orientations. These unique features of S layers have led to a broad spectrum of applications in biotechnology, diagnostics, vaccine development, biomimetics, molecular nanotechnology, and controlled biomineralization.

Keywords: biomimetics · immobilization · membranes · nanotechnology · S layers

1. Introduction

It is now generally accepted that in the course of evolution life on this planet has divided into three domains, bacteria, archaea, and eucarya.[1, 2] Both bacteria (formerly eubacteria) and archaea (formerly archaebacteria) are prokaryotes, resembling extremely small unicellular life forms. With the exception of those prokaryotes which dwell as naked protoplasts in very specialized ecological niches, most bacteria and archaea possess a supramolecular layered cell-wall structure outside the cytoplasmic membrane. The cell wall and the cytoplasmic membrane constitute the prokaryotic cell envelope complex which regulates the molecular exchange between the cell and its environment. In most organisms the cell wall has to be stable enough to withstand the turgor pressure of the protoplast and is also involved in the determination of cell shape. Since most prokaryotic cells have to survive in highly competitive habitats the diversity observed in the molecular architecture of cell walls, particularly the structure of the outermost boundary layer, reflects specific adaptations to environmental and ecological conditions.[3]

One of the most commonly observed surface structures on archaea and bacteria are crystalline arrays of proteinaceous subunits, termed surface layers (S layers).[4, 5] Structural, chemical, genetic, and morphogenetic studies revealed that S layers represent the simplest type of biological membrane developed during evolution.[6–8] They are generally composed of a single protein or glycoprotein species endowed with the ability to assemble into closed, highly porous lattices on the cell surface during all stages of cell growth and division.

The wealth of information accumulated on the general principles of S layers led to a broad spectrum of applications.[9–11] The most relevant features exploited in applied S layer research are:

1) Isolated S layer subunits from many organisms are capable of recrystallizing as closed monolayers onto solid supports, at the air/water interface, on Langmuir lipid films, and on liposomes.
2) Pores passing through S layers show identical size and morphology and are in the range of pores in ultrafiltration membranes.
3) Functional groups on the surface and in the pores are aligned in well-defined positions and orientations and are accessible for binding functional molecules in a very precise fashion.

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These repetitive physicochemical properties down to the subnanometer scale make S layer lattices unique structures for functionalizing surfaces and interfaces close to the ultimate resolution limit. The remarkable supramolecular principles of S layers are presently exploited in the fields of biotechnology, biomimetics, biomedicine, and molecular nanotechnology.

2. Occurrence and Ultrastructure

Despite considerable variations in the supramolecular structure and complexity of prokaryotic cell envelopes, most envelope profiles can be classified into three main groups, which also reflect the phylogenetic position of the organism (Figure 1). Surface layers have now been identified on organisms of nearly every taxonomic group of walled bacteria and appear to be an almost universal feature of archaea. Unlike most other prokaryotic cell surface structures, S layers can be unequivocally identified only by electron microscopy, in particular by freeze-etching (Figure 2). In both bacteria and archaea the regular arrays completely cover the cells at all stages of cell growth and division.[4, 12–15]

The three-dimensional spatial organization of S layers has been obtained primarily by electron crystallography and more recently through scanning force microsopy. Mass distribution analysis by computer image enhancement procedures revealed structural information down to a range of 0.5 to 1.5 nm.[14, 16–19] Surface layers are planar assemblies of identical protein or glycoprotein subunits which can be aligned in lattices with oblique (p1, p2), square (p4), or hexagonal (p3, p6) symmetry with center-to-center spacings of the morphological units of approximately 2.5 – 35 nm (Figure 3). Hexagonal lattice symmetry is predominant among archaea.[20] Surface layers of bacteria are generally 5 – 20 nm thick and

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Margit Sára, born in 1957 in Vienna, studied food and biotechnology at the University of Agricultural Sciences, Vienna. After receiving her PhD in 1985 she continued her research work in the field of microbiology, biochemistry, and biotechnology, focussing on biotechnological applications of S layers. In 1989 she was promoted to Associated Professor of General Microbiology. Since then she is head of the research group "Molecular Biotechnology and Biomimetic Membranes" at the Center for Ultrastructure Research.

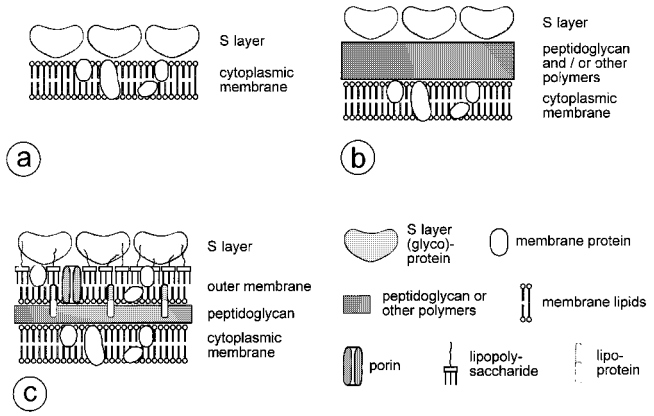


Figure 1. Schematic illustration of the supramolecular architecture of the three major classes of prokaryotic cell envelopes containing crystalline bacterial cell surface layers (S layers). a) Cell envelope structure of Gram-negative archaea with S layers as the only cell-wall component external to the cytoplasmic membrane. b) Cell envelope as observed in Gram-positive archaea and bacteria. In bacteria the rigid wall component is primarily composed of peptidoglycan. In archaea other wall polymers (e.g. pseudomurein or methanochondroitin) are found. c) Cell envelope profile of Gram-negative bacteria composed of a thin peptidoglycan layer and an outer membrane. If present the S layer is closely associated with the lipopolysaccharide of the outer membrane (modified based on reference [13]).

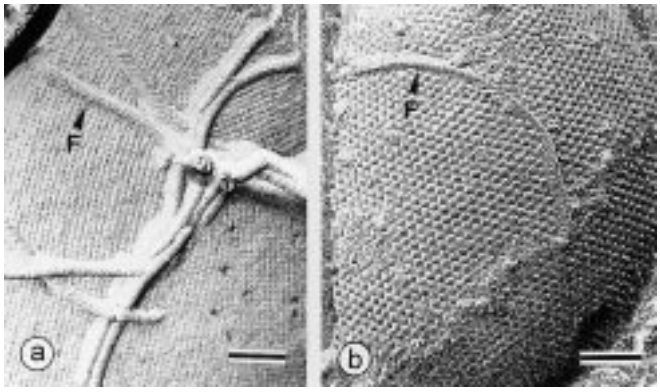


Figure 2. Electron micrograph of freeze-etched preparations of intact cells from a) *Aneurinibacillus thermoaerophilus* DSM 10155 showing a square (p4) S layer lattice and b) archaeon *Methanomicrobium mobile* DSM 1539 covered with a hexagonal (p6) array. F: flagella. The bar corresponds to 100 nm.

reveal a rather smooth outer and a more corrugated inner surface. Amongst archaeal S layers pillarlike domains associated with the plasma membrane have been described.[17, 21–23] These characteristic structural features may lead to S layer lattices with their heavy domains located at a distance of up to 70 nm from the surface of the cytoplasmic membrane.

High-resolution electron microscopical and scanning force microscopy studies have shown that S layers are very porous membranes with pores occupying up to 70% of their surface area (Figure 4). Since S layers are assemblies of identical subunits they exhibit pores of identical size and morphology. In many protein lattices two or more distinct classes of pores in the range of approximately 2–8 nm have been identified.[13–15, 18, 22]

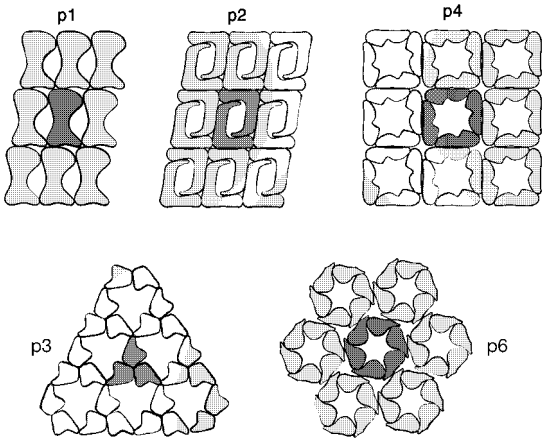


Figure 3. Schematic drawing of different S layer lattice types. The regular arrays exhibit either oblique (p1, p2), square (p4), or hexagonal lattice symmetry (p3, p6). The morphological units are composed of one, two, three, four, or six identical subunits.

3. Assembly and Morphogenesis

Surface layers represent a fascinating model system for studying the dynamic process of assembly of a supramolecular structure during cell growth. An intact closed S layer on an average-sized, rod-shaped prokaryotic cell consists of approximately 5×10^5 monomers. Thus, for maintaining a complete S layer on the surface of a cell growing with a generation time of 20 to 30 min at least 500 copies of a single polypeptide species have to be synthesized, translocated to the cell surface, and incorporated into the existing lattice per second. Detailed electron microscopical examinations of S layers on growing cells and in vitro self-assembly studies of isolated S layer subunits have been performed to elucidate the dynamic process of assembly in the course of S layer morphogenesis.

3.1. Self-Assembly In Vitro

A great variety of methods has been developed for the detachment of S layers and their disintegration into protomeric units. Generally a complete disintegration of S layer lattices can be achieved using high concentrations of chaotropic agents (for example, guanidine hydrochloride, urea), or by lowering or raising the pH value. Particularly S layers from Gram-negative bacteria may also be disintegrated by applying metal-chelating agents (for example, ethylenediaminetetraacetic acid (EDTA), ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA)) or cation substitution.[4, 12–14, 24–28] These data have confirmed that S layers are held together and onto the supporting envelope component by noncovalent forces, including hydrophobic interactions and hydrogen bonds as well as ionic bonds involving divalent cations or direct interactions of polar groups. The extraction experiments have also shown that the bonds holding the subunits together are stronger than those involved in the association of the S layers to the underlying layer.

Surface layers from some archaea are very resistant to extraction and desintegration, suggesting even covalent

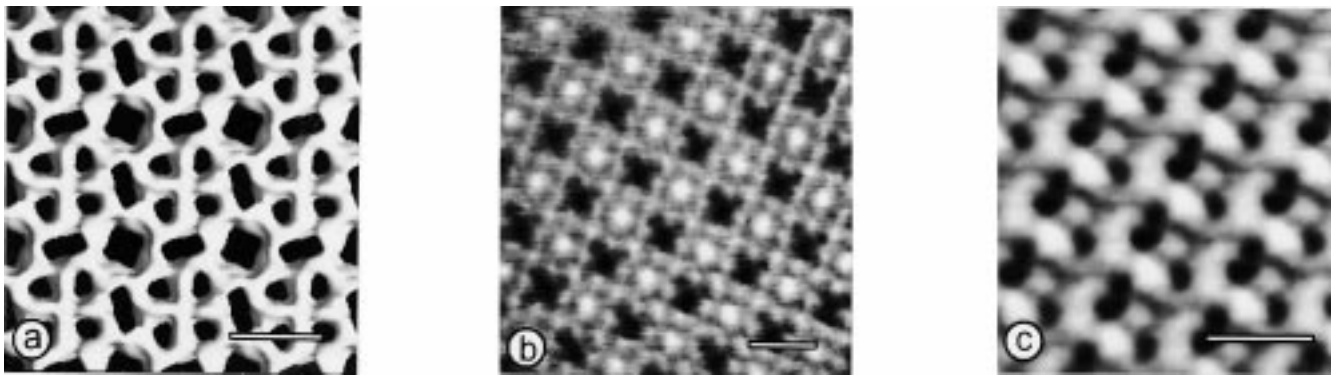


Figure 4. a) Three-dimensional model of the protein mass distribution of the S layer of *Bacillus stearothermophilus* NRS 2004/3a/V2 (outer face). The S layer is about 8 nm thick and exhibits a center-to-center spacing of the morphological units of 13.5 nm. The protein meshwork shows one square-shaped, two elongated, and four small pores per morphological unit. The model was obtained after recording several tilt series of a negatively stained preparation in a transmission electron microscope, performing Fourier-domain computer image reconstructions over each individual tilted view, and combining all processed views to a three-dimensional data volume. b), c) Computer image reconstruction of the scanning force microscopic images of the topography of the inner face of the S layer lattice from *B. sphaericus* CCM 2177 (b) and *Bacillus coagulans* E38–66/V1 (c). The images were taken under water. The surface corrugation corresponding to a gray scale from black to white is 1.8 nm. The center-to-center spacing of the morphological unit of the square lattice (b) is 14.5 nm. The S layer lattice in (c) shows an oblique unit cell ($a = 9.4$ nm, $b = 7.5$ nm, base angle $= 80^\circ$). The bars correspond to 10 nm.

intersubunit bonds.[14, 29a] Isolated S layer subunits from many prokaryotic organisms have shown the ability to assemble into regular lattices identical to those observed on intact cells upon removal of the disrupting agent used for their isolation (for example, upon dialysis).[29b] These S layer self-assembly processes may lead to the formation of flat sheets, open-ended cylinders, or spheres (Figure 5).[12, 15, 27, 30] Ionic strength,

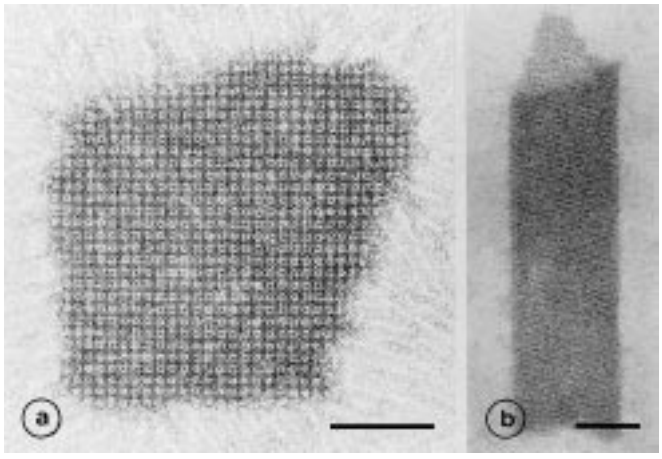


Figure 5. Electron micrographs of negatively stained preparations of a) sheet-like S layer self-assembly products of *Desulfotomaculum nigrificans* NCIB 8706 showing a p4 lattice and b) cylindrical S layer self-assembly products of *Bacillus stearothermophilus* NRS 2004/3a showing a p2 lattice. The bars correspond to 100 nm.

temperature, protein concentration, and polymers associated with S layers can determine both the rate and extent of association. With S layers from Bacillaceae it was shown that the assembly kinetics are multiphasic, with a rapid initial phase leading to oligomeric precursors and slow consecutive rearrangement steps, which finally lead to extended lattices.[31] The capability of some S layer proteins to reassemble into large coherent monomolecular arrays on solid supports, at the air/water interface, and on Langmuir lipid films (Figure 6) has led to numerous applications (see Section 7).

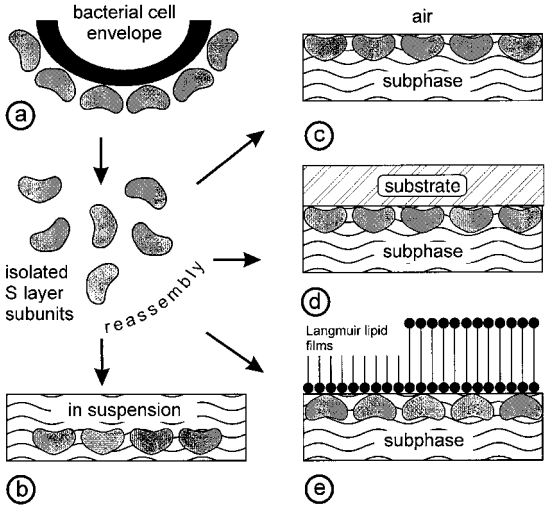


Figure 6. Schematic illustration of the recrystallization of isolated S layer subunits into crystalline arrays (a). The self-assembly process can occur in suspension (b), at the air/water interface (c), on solid supports (d), and on Langmuir lipid films (e).

3.2. Self-Assembly In Vivo

With S layers from various bacteria it was demonstrated that distinct surface properties of the subunits (charge distribution, hydrophobicity, specific interactions with components of the supporting envelope layer) are essential for the proper orientation of the S layer subunits during local insertion in the course of lattice growth. Labeling experiments (for example, with colloidal gold or fluorescent markers) provided information about the extension of the S layer lattices and incorporation sites of subunits during cell growth. In Gram-positive and Gram-negative bacteria S layer lattice growth is dependent on the extension of the supporting rigid envelope component (Figure 1) and may occur in defined domains[32, 33] or at random sites.[34, 35]

In high-resolution freeze-etching preparations dislocations and disclinations have been identified (Figure 7). Topologi-

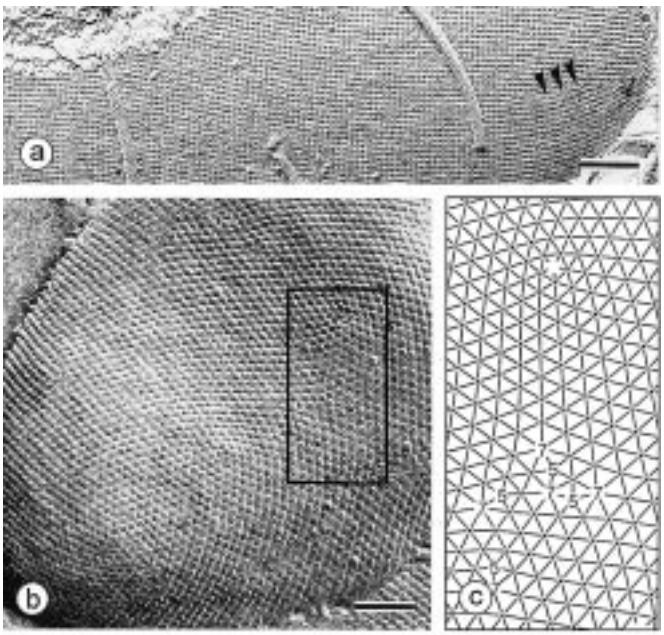


Figure 7. Freeze-etched preparations of intact bacteria showing common lattice faults of S layers. a) The arrows indicate dislocations in the square lattice on the rounded surface of the cell pole of *Aneurinibacillus thermoaerophilus* DSM 10155. b) Wedge disclinations and edge dislocations are seen as point imperfections in the crystalline array of *Methanocorpusculum sinense* DSM 4274. c) Representation of the neighboring S layer units from the boxed region in (b). The central unit cells of the $+60^\circ$ and -60° wedge disclinations are pentagons (labeled 5) and heptagons (labeled 7), respectively. The representation is modified based on reference [39]. The bars correspond to 100 nm.

cally, such lattice faults are required for covering the curved surface of the hemispherical cell poles of rod-shaped cells or the spherical surface of a coccus. On the other hand, as theoretically predicted by Harris and co-workers,[36–38] dislocations and disclinations can serve as preferential sites for incorporation of new subunits in crystalline arrays which grow by "intussusception".[36] Further, as a geometrical necessity, closed surface crystals must contain local wedge (screw) disclinations which themselves can act as source of edge disclinations.[38]

Analysis of the distribution of lattice faults in the hexagonal S layer of archaea which possess an S layer as its sole cell-wall constituent (Figure 1a) provided strong evidence that complementary pairs of pentagons and heptagons play an important role as sites for the incorporation of new subunits in the formation and maintenance of the lobed cell structure and in the fission process.[27, 39] The latter was suggested to be determined by the ratio between the increase in protoplast volume and the increase in actual S layer surface area during cell growth. In rod-shaped archaea with S layers as exclusive cell-wall component, wedge disclinations at both hemispherical poles resembling the theoretical required numbers could be visualized. Elongation of the cylindrical part of the cell most probably involves incorporation of S layer subunits at sites of sliding dislocations at the poles.[40, 41]

It is now evident that S layers are "dynamic closed surface crystals" with the intrinsic capability to assume continuously a structure of low free energy during cell growth. Further, the morphogenetic potential of S layer lattices is determined by the molecular structure of its constituent subunits.[4, 12, 14, 27, 42]

4. Chemical Composition and Biosynthesis

Chemical characterization of highly purified S layers from bacteria as well as archaea revealed that most of these cell surface macromolecules possess a rather similar overall composition, regardless of the phylogenetic origin of the respective bacteria. Extensive reviews of the relevant work have been published recently.[26, 43–53] Additionally, S layers of various nonpathogenic[45–48] and pathogenic[49–51] bacteria and archaea[52, 53] have been characterized biochemically since then. Typically they are composed of 40 to 60% of hydrophobic amino acids and possess a rather low portion, if any, of sulfur-containing amino acids. From circular dichroism measurements and protein sequence data secondary structure predictions have been performed (Table 1). In general, S layers are weakly acidic proteins or glycoproteins with isoelectric points in the range of 4–6.[5, 7, 26, 43, 44, 54, 55] For *Methanothermus fervidus*, however, a pI value for the S layer protein of 8.4[56] and for lactobacilli pI values in the range of 9–10 have been determined.[57]

Table 1. Properties of S layers.[5, 7, 14, 26, 43, 44, 54, 55]

The S layer lattices can have oblique (p1, p2), square (p4), or hexagonal (p3, p6) symmetry.
The center-to-center spacing of the morphological unit can be between 3 and 35 nm.
The lattices are generally 5 to 20 nm thick (in archaea up to approximately 70 nm).
The outer surface is generally less corrugated than the inner surface.
The S layer lattices exhibit pores of identical size and morphology.
In many S layers two or even more distinct classes of pores are present.
The pore sizes range from approximately 2 to 8 nm.
The pores can occupy 30 to 70% of the surface area.
The relative molecular mass of constituent subunits is in the range of 40000 to 200000.
These are weakly acidic proteins (pI $\approx$ 4–6), except for *Methanothermus fervidus* (pI = 8.4) and lactobacilli (pI > 9.5).
Large amounts of glutamic and aspartic acid (about 15 mol%) are present.
There is a high lysine content (about 10 mol%).
There are large amounts of hydrophobic amino acids (about 40–60 mol%).
Hydrophilic and hydrophobic amino acids do not form extended clusters.
In most S layer proteins about 20% of the amino acids are organized as α helices and about 40% occur as β sheets.
Aperiodic foldings and β-turn content may vary between 5 and 45%.
Posttranslational modifications of S layer proteins include cleavage of N- or C-terminal fragments, glycosylation, and phosphorylation of amino acid residues.

General protein secretion pathways are known in a number of bacteria. For secretion of S layers a specific pathway has been described for the first time in *Aeromonas salmonicida*.[58] A few posttranslational modifications are known to occur in S layer proteins, including protein phosphorylation and protein glycosylation (Table 1). The modification of the S layer of *Aeromonas hydrophila* (termed A layer) has been detected by a comparison of the molecular masses obtained from SDS PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) and the DNA sequence. The molecular mass of the A layer (45 000 Da) was increased by 7000 Da. Western blot analysis using a monoclonal antibody showed the presence of phosphotyrosine residues.[59]

A more frequently observed modification of S layer proteins is their glycosylation. Many archaea—including halophilic, methanogenic, thermophilic, acidophilic, and alkalophilic strains—possess S layer proteins with covalently linked glycan chains.[43, 60, 61] The hyperthermostable S layer glycoprotein complex of *Staphylothermus marinus*[62] and the S layer glycoprotein of *Haloarcula japonica*[63] were only recently characterized. In the domain bacteria, however, glycosylated S layers have been demonstrated unambiguously only in the Bacillaceae family.[43, 64, 65]

The purification regimes are identical for nonglycosylated and glycosylated S layers and have been summarized in recent reviews.[26, 43, 66, 67] In the case of glycosylated S layers, usually complete degradation to S layer glycopeptides by proteolytic enzymes is required for further analysis. Determination of the S layer glycan structure includes chemical degradation experiments of the glycan chains, ^{1}H and ^{13}C NMR spectroscopy, and mass spectrometry.[43, 68] Glycan structures of selected strains of bacteria and archaea are summarized in Table 2. From the data available the following conclusions can be drawn: Most bacterial S layer glycan chains are composed of 20 to 50 identical repeating units made of neutral hexoses and rarely pentoses, 6-deoxy hexoses, and amino sugars. In terms of structure they are comparable to lipopolysaccharide (LPS) O-antigens of Gram-negative bacteria (Figure 8).[86]

Between the O-antigen-like glycan chain and the S layer polypeptide there is usually a core oligosaccharide of two to four sugar residues which connects the proximal portions of this macromolecule. Per S layer protein monomer two to six glycosylation sites have been determined,[68, 73a, 77] where S layer glycans are attached predominantly by O-glycosidic linkages such as galactose →tyrosine,[68] glucose →tyrosine,[77] or *N*-acetylgalactosamine →threonine/serine.[73a] The reexamination[87] of an S layer glycoprotein preparation from *Bacillus stearothermophilus* of NRS 2004/3a[88a] has demonstrated that in this S layer there is only one type of glycan with a certain variation in chain length. A concomitantly isolated oligosaccharide[88a] is not covalently linked to the S layer polypeptide,[87] and therefore has to be considered as an associated secondary cell-wall polymer like in other bacilli.[88b]

Those archaeal S layer glycoproteins whose glycan structures and linkage regions have been investigated (Table 2) consist almost exclusively of short heterosaccharides with up to ten sugar residues and no repeating units.[43, 61, 85] In halobacteria up to three different glycan species with up to 25 glycosylation sites per S layer monomer can be present.[61, 84] The dominating linkage types are N-glycosidic linkages such as glucose →asparagine[61] or *N*-acetylgalactosamine →asparagine.[61, 85] However, short O-glycans, which occur in clusters, have also been found.

The remarkable diversity in glycan structures raised interesting questions about the biosynthesis of prokaryotic glycoproteins. Recently Sumper and Wieland have proposed a model for how the different N-linked glycans are synthesized in *Halobacterium halobium*.[61] It includes transfer of dolichol-linked saccharides to consensus sequences for N-glycosylation on the S layer polypeptide. Similar lipid-activated oligosaccharides with short-chain ($C_{55}-C_{60}$) dolichol species rather than undecaprenol for S layer glycosylation were also observed in *Haloferax volcanii*[89] and *M. fervidus*.[90] In comparison to archaeal S layer glycoproteins, less detailed information is available about the biosynthesis of glycosylated S layers of bacteria. In *Paenibacillus* (formerly *Bacillus*) *alvei* similar dolichol (C_{55}) carrier lipids are involved in glycan biosynthesis,[91] whereas for *Thermoanaerobacterium* (formerly *Clostridium*) *thermosaccharolyticum* only nucleotide-activated sugars have been characterized so far.[92] Of particular interest is the observation that in all investigated systems beside nucleotide-activated monosaccharides also nucleotide-activated oligosaccharides play an important role in synthesis.[90–92] Such activated metabolic intermediates are not known for eukaryotic glycoproteins. The biological relevance of this characteristic difference is currently being investigated.

Elucidation of biosynthetic pathways for prokaryotic glycoprotein glycans should enable the production of tailored S layer glycoproteins as required for different biotechnological applications (for example, S layer vaccines; see Section 7.1.3).

5. Molecular Biology and Genetics

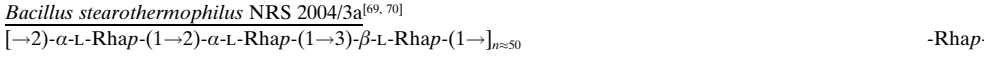
Although it is quite evident that common structural principles must exist in S layer proteins (for example, the ability to form intersubunit bonds and to self-assemble, the formation of hydrophilic pores with low unspecific adsorption, the interaction with the underlying cell envelope layer), sequencing of S layer genes from organisms of all phylogenetic branches led to the conclusion that sequence identities are rare.[93] Sequencing of S layer genes from closely related organisms (Table 3)[94–127] revealed that sequence identities depend on evolutionary relationship. For example, the nucleotide sequence of the S layer gene *slpA* from *Lactobacillus acidophilus* ATCC 4356[115] showed 80% similarity to that of the S layer gene from the closely related *L. helveticus*,[118] but almost no similarity to that from *L. brevis*.[116]

For some species the N-terminal part of the S layer proteins was found to represent the conserved structural element which is responsible for anchoring the S layer subunits to the underlying cell envelope layer. The S layer proteins SbsA[103] and SbsC[105] from two *B. stearothermophilus* wild-type strains (PV72/p6 and ATCC 12980) showed high identity for the 270 N-terminal amino acids, but only low identity (< 25%) for the

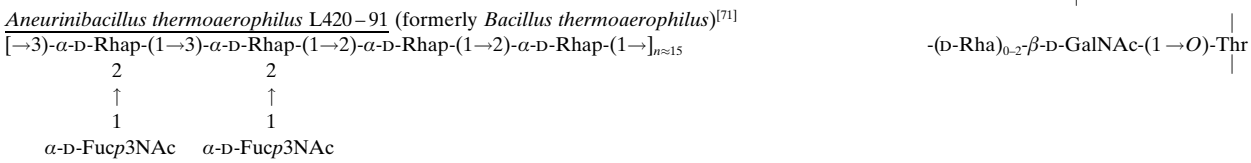
Table 2. Glycan structures of selected bacterial and archaeal S layer glycoproteins.[a][43, 61]

Bacteria

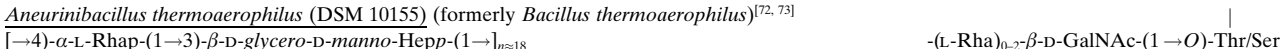
Bacillus stearothermophilus NRS 2004/3a[69, 70]



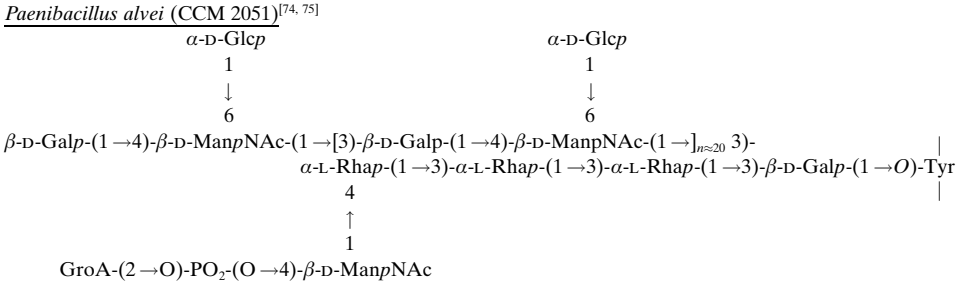
Aneurinibacillus thermoaerophilus L420–91 (formerly *Bacillus thermoaerophilus*)[71]



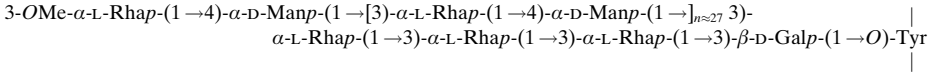
Aneurinibacillus thermoaerophilus (DSM 10155) (formerly *Bacillus thermoaerophilus*)[72, 73]



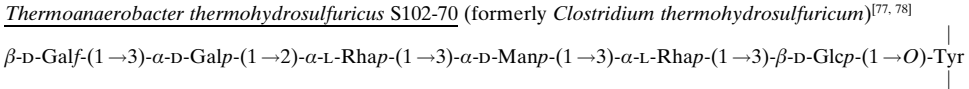
Paenibacillus alvei (CCM 2051)[74, 75]



Thermoanaerobacter thermohydrosulfuricus L111-69 and L110-69 (DSM 568) (formerly *Clostridium thermohydrosulfuricum*)[68, 76]

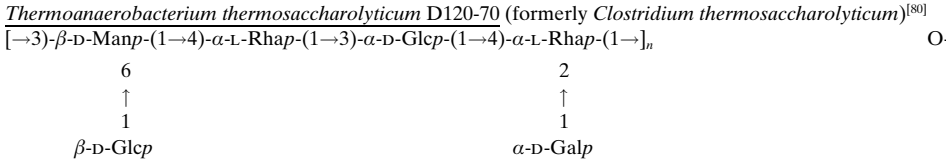


Thermoanaerobacter thermohydrosulfuricus S102-70 (formerly *Clostridium thermohydrosulfuricum*)[77, 78]

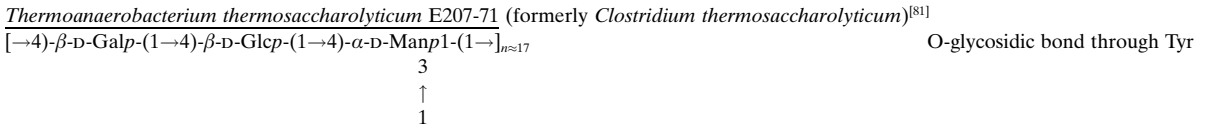


Thermoanaerobacter thermohydrosulfuricus L77-66 (DSM 569) and L92-71 (formerly *Clostridium thermohydrosulfuricum*)[79]

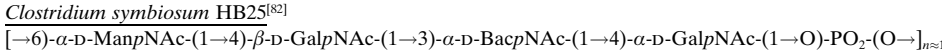
Thermoanaerobacterium thermosaccharolyticum D120-70 (formerly *Clostridium thermosaccharolyticum*)[80]



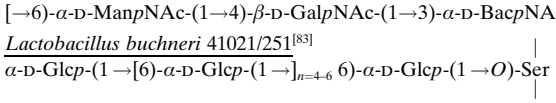
Thermoanaerobacterium thermosaccharolyticum E207-71 (formerly *Clostridium thermosaccharolyticum*)[81]



Clostridium symbiosum HB25[82]



Lactobacillus buchneri 41021/251[83]

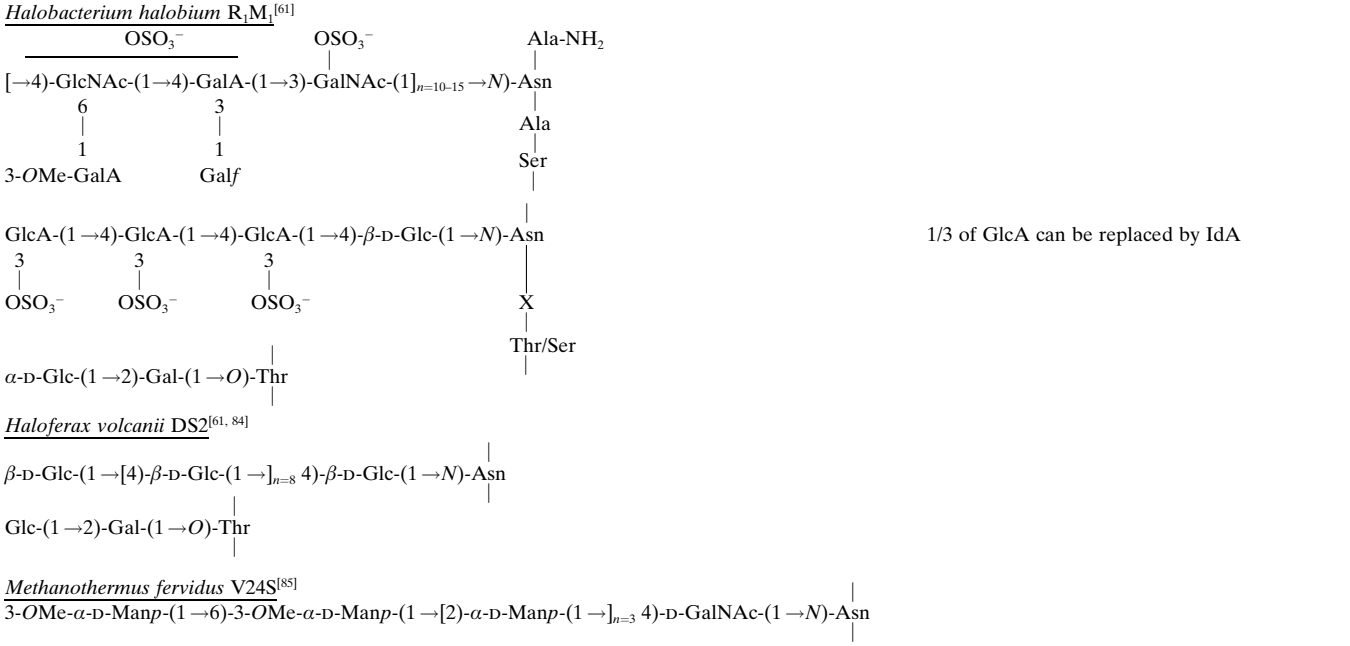


larger part of the sequences. Actually, the N-terminal part of SbsA and SbsC was found to anchor the S layer subunits through a secondary cell-wall polymer of identical chemical composition and structure to the rigid cell-wall layer,[128] while the C-terminal part most probably leads to quite diverse cell surface properties even amongst closely related strains within the same species.

Contrary to the hypothesis that evolutionary relationship correlates with sequence identities for structurally homologous domains was the observation that the S layer protein SbsB showed only low overall identity ($< 25\,\%$) to SbsA and that both S layer proteins do not even share an identical N-terminal region.[104] The S layer protein SbsB is synthesized by *B. stearothermophilus* PV72/p2, an oxygen-induced strain variant from *B. stearothermophilus* PV72/p6. Interestingly, the oxygen-induced strain variant produced a different secondary cell-wall polymer which was recognized by the SbsB as binding site, indicating that a highly coordinated switch in synthesis of cell-envelope components had occurred during oxygen-induced variant formation. Sequencing of 450 bases in

Table 2. (Continued)

Archaea



[a] The formulas represent either the repeating unit structure or the saccharide structure of S layer glycoprotein glycans. Additionally, as far as already determined, the structure of the carbohydrate-protein linkage region is presented. Abbreviations: Glc*p*: glucose (pyranose form), Gal*f*: galactose (furanose form), Man: mannose, Rha: rhamnose, GlcNAc: *N*-acetylglucosamine, GalNAc: *N*-acetylgalactosamine, ManNAc: *N*-acetylmannosamine, GlcA: glucuronic acid, GalA: galacturonic acid, ManA: mannuronic acids, IdA: iduronic acid, 3-*O*Me-GalA: 3-*O*-methylgalacturonic acid, BacNAc: *N*-acetylbacillosamine (2-*N*-acetyl-4-amino-2,4,6-trideoxyglucose), Fuc3NAc: 3-*N*-acetylfucosamine, Qui3NAc: 3-*N*-acetylquinovosamine (3-acetamido-3,6-dideoxyglucose), BacNAc: *N*-acetylbacillosamine (2-acetamido-4-amino-2,4,6-trideoxyglucose), GroA: phosphoglyceric acid, Asn: asparagine, Tyr: tyrosine, Thr: threonine, Ser: serine, Ala: alanine, X: interchangeable amino acid.

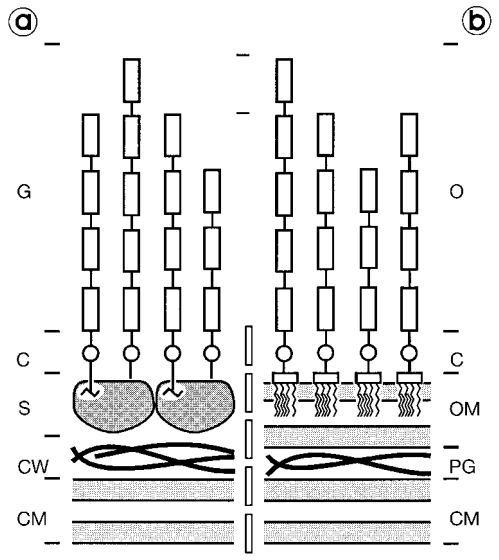


Figure 8. Schematic drawing of the cell envelope profile of Gram-positive (a) and Gram-negative bacteria (b) underlining the structural similarities between S layer glycoproteins and lipopolysaccharides. Abbrevations: C: core region; CM: cytoplasmic membrane; CW: cell wall; G: glycan chain; O: O-antigen; OM: outer membrane; PG: peptidoglycan (taken from reference [43] with permission of Landes/Academic Press).

the variable region of the 16S rDNA confirmed that the similarity values for the wild-type strain and the variant are 100%. These findings indicated that despite evolutionary relationship, other factors such as growth conditions or environmental stress are determinative for sequence identities of structurally homologous domains of S layer proteins.

Since at a generation time of 20 minutes about 500 S layer subunits have to be produced per second to keep the bacterial cell surface completely covered with S layer protein,[26] promoters preceeding S layer genes must be very strong.[93] Indeed, the promoter of the S layer gene from *L. acidophilus* is two times more efficient than that of the gene encoding lactate dehydrogenase, which is considered to be one of the strongest promoters in bacteria.[129] In *L. brevis* two promoters (P1 and P2) have been identified for S layer gene expression. The P2 promoter is located closer to the start codon and is efficiently transcribed during the exponential and early stationary growth phase.[130] The use of three promoters has been reported for the expression of the *cwp* operon of *B. brevis*.[99, 131] With exception of those from *Campylobacter* and *Caulobacter,* all S layer proteins are produced with a signal peptide, suggesting the classical route of secretion.[57]

Important for understanding S layer gene regulation has been the observation that single bacterial strains can express different (silent) genes. The most detailed studies regarding S layer variation were carried out with *Campylobacter fetus* subsp. *fetus*, a pathogen that interferes with reproductive function in ungulates.[132] In case of pathogens S layer variation can be considered as antigenic variation which is

Table 3. Survey of S layer proteins with known amino acid sequence.

Species	Strain	Gene	Number of amino acids including the N-terminal leader peptide/N-terminal leader peptide	Lattice type[a]	Gene bank accession number	Ref.
Aeromonas hydrophila	TF7	*ahs*	467/19	S	L37348	[59]
Aeromonas salmonicida	A 450	*vapA*	502/21	S	M64655	[94]
Bacillus anthracis	Sterne derivative sub-strain 9131	*sap*	814/29	O	Z36946	[95]
Bacillus anthracis	Sterne substrain 9131	*eag*	862/29	O	X99724	[96]
Bacillus brevis	47	*owp*	1004/24	–	M14238	[97]
Bacillus brevis	47	*mwp*	1053/23	H	M19115	[98]
Bacillus brevis	HPD 31	*HWP*	1087/23 or 53	H	D90050	[99]
Bacillus licheniformis	HM 105		874/29	–	U38842	[100]
Bacillus sphaericus	P1	sequence *8*	1252/30	S	A45814	[101]
Bacillus sphaericus	2362	Gen *125*	1176/30	S	M28361	[102]
Bacillus sphaericus	2362	Gen *80*	745 (silent)	–	–	[102]
Bacillus stearothermophilus	PV72/p6	*sbsA*	1228/30	H	X71092	[103]
Bacillus stearothermophilus	PV72/p2	*sbsB*	920/31	O	X98095	[104]
Bacillus stearothermophilus	ATCC 12980	*sbsC*	1099/30	O	AF055578	[105]
Bacillus thuringiensis	ssp. *israelensis* 4Q2	*slp*	393/putative	–	X62090	–
Campylobacter fetus, ssp. *fetus*	–	*sapA*	933/No	H, S[b]	J05577	[106]
Campylobacter fetus, ssp. *fetus*	23B	*sapA1*	920/No	H, S[b]	L15800	[107]
Campylobacter fetus, ssp. *fetus*	82-40LP3	*sapA2*	1109/No	H, S[b]	S76860	[108]
Campylobacter fetus, ssp. *fetus*	84-91	*sapB*	936/No	–	U25133	[108]
Campylobacter fetus, ssp. *fetus*	CIP 5396T	*sapB2*	1112/No	–	AF048699	–
Campylobacter rectus	314	*crs*	1361/No		AF010143	[109]
Caulobacter crescentus	CB 15	*rsaA*	1026/No	H	M84760	[110]
Corynebacterium glutamicum	ATCC 17965	*csp2*	510/30	H	X69103	[111]
Deinococcus radiodurans	–	*HPI*	1036/31	H	M17895	[112]
Halobacterium halobium	–	*csg*	852/34	H	J02767	[113]
Haloferax volcanii	–	–	828/34	H	M62816	[114]
Lactobacillus acidophilus	ATCC 4356	*slpA*	444/24	O	X89375	[115]
Lactobacillus acidophilus	ATCC 4356	*slpB*	456 (silent)	–	X89376	[115]
Lactobacillus brevis	ATCC 8287		465/30	O	Z14250	[116]
Lactobacillus crispatus	JCM 5810	*cbsA*	440/30	O	AF001313	–
Lactobacillus fermentum	BR 11	*bspA*	265/putative	O	U97348	[117]
Lactobacillus helveticus	CNRZ 892	*slpH1*	440/30	O	X91199	[118]
Lactobacillus helveticus	CNRZ 1269	*slpH2*	440/30	O	X92752	[119]
Methanococcus voltae	–	*sla*	565/12	H	M59200	[120]
Methanosarcina mazei	S-6	*slgB*	652/31	H	X77929	[121]
Methanothermus fervidus	DSM 2088	*slgA*	593/22	H	X58297	[56]
Methanothermus sociabilis	DSM 3496	*slgA*	593/22	H	X58296	[56]
Rickettsia prowazekii	Brein 1	*spaP*	1612/32	–	M37647	[122]
Rickettsia rickettsii	R	*p120*	1645/32	–	X16353	[123]
Rickettsia typhii	Wilmington	*slpT*	1645/32	–	L04661	[124]
Serratia marcescens	isolate 8000	*slaA*	1004	–	AB007125	–
Staphylothermus marinus	F1	–	1524/putative	–	US7967	[125]
Thermoanaerobacter kivui	DSM 2030	*slp*	762/26	H	M31069	[126]
Thermus thermophilus	HB-8	*slpA*	917/27	H, S	X57333	[127]

[a] H: hexagonal, S: square, O: oblique. [b] In *Campylobacter fetus*, ssp. *fetus*, the lattice type depends on the molecular weight of the S layer subunits (H: 97000; S: 127000 and 149000).

induced in response to the lytic activity of the immune system and leads to modified cell-surface properties. Two serotypes (type A and type B) are known which depend on the type of lipopolysaccharides in the outer membrane.[133] In both serotypes the N-terminal part of the S layer proteins recognizes a distinct type of lipopolysaccharide. Surface layer proteins from type A cells are encoded by genes designated *sapA* and $sapA_{1-n}$, while S layer proteins from type B cells represent a parallel family. Comparison of *sapA* and $sapA_1$ showed that two regions of identity exist: The first (5′-conserved region) begins 74 bp "upstream" of the open reading frame (ORF), proceeding 552 bp into the ORF to encode the 184 N-terminal amino acids, whereas the second (3′-conserved region) begins "downstream" of the ORF.[107, 134]

Eight to nine S layer gene cassettes are present in *C. fetus* wild-type strains,[135, 136] which are tightly clustered in a region less than 93 kb, representing less than 8% of the genome.[134] Several studies confirmed that only a single promoter exists[137] and that antigenic variation is due to recombination events. In addition to promoter inversion between two oppositely oriented gene cassettes, one of the S layer gene cassettes bracketing the invertible element with a nonflanking, previously silent gene cassette is exchanged.[132, 138] Thus, *C. fetus* reassembles a single promoter strictly by a single DNA inversion event, and at frequencies independent of the

size of the DNA fragment (6 – 16 kb), permitting expression of different S layer gene cassettes.

L. acidophilus ATCC 4356 was isolated from human pharynx and possesses two S layer genes, termed *slpA* and *slpB*.[115] The *slpA* gene is actively transcribed, whereas the *slpB* represents the silent gene. Interestingly, the 5′ and 3′ ends of both S layer genes are very similar, while considerable differences could be observed for the middle region.[115] The two S layer genes are located at a distance of 3 kb on the chromosome in reverse orientation relative to each other.[57]

Environmental factors inducing change in S layer gene expression in nonpathogens have been studied for *B. stearothermophilus* strains. The S layer gene *sbsA* was stably expressed by *B. stearothermophilus* PV72/p6 in continuous culture under oxygen limitation.[139–141] After relieving oxygen limitation, variant formation and expression of the second S layer gene *sbsB* was induced. The decrease in *SbsA* revealed that change in S layer gene expression is a synchronous process in the whole culture. Although the mechanism leading to change in S layer gene expression is still unclear, PCR and hybridization studies indicated that *sbsB* is generated from partial coding sequences and that *sbsA* is disrupted during the switch.[142]

6. Functional Aspects

The biosynthetic effort to keep the cell surface completely covered with S layer protein raises the question of what advantage of selection S layer carrying organisms have in their natural habitats in comparison to their unlayered counterparts. Although many of the functions assigned to S layers are still hypothetical, it is now recognized that they can act as 1) frameworks to determine cell shape and to aid in the cell division process in those archaea that possess S layers as an exclusive wall component (see Section 3.2), 2) structures involved in cell adhesion and surface recognition, and 3) protective coats, molecular sieves, and molecule and ion traps.[7, 11, 14, 20]

Because S layers possess pores identical in size and morphology in the 2 to 8 nm range, they work as precise molecular sieves with sharp cut-off levels for the cells. Interestingly, the smallest pores were reported for the S-layer-like sheaths of *Methanospirillum hungatei*,[143, 144] which uses only small molecules such as gases and salts. In contrast, S layers from Bacillaceae, which are Gram-positive bacteria producing large amounts of exoenzymes, possess pores with a size of 4 to 5 nm.[145] Since S layers from Bacillaceae reject macromolecules with a molecular weight of 30000 to 40000, which is just within the dimension of the pores and of the exoenzymes produced, S layers from these organisms were suggested to delineate a kind of periplasmic space and control the speed of exoenzyme release into the environment.[146–148] The largest pores in the 5 to 8 nm range were reported for S layers of archaea lacking a rigid cell-wall layer, in which the S layer fulfills shape-determining or shape-maintaining function.[16–18, 21–23, 39–41] In these organisms, the S layer subunits generally possess long hydrophobic protrusions which integrate into the plasma membrane. Thereby, a kind of periplasmic space is formed between the S layer and the plasma membrane in which secreted macromolecules involved in nutrient degradation and transport as well as folding and export of proteins could be stored.[113, 149–151]

Regarding protective functions, S layers from Gram-negative bacteria such as *A. salmonicida, C. fetus, A. serpens*, and *C. crescentus* were found to prevent the cells from attack by bacteria parasites such as *Bdellovibrio bacteriovorus*, but they could not shield the cells from other predators like protozoa.[152, 153] As described for *L. crispatus* and *L. acidophilus*, S layers mediate the adhesion of these bacteria to intestinal epithelial cells.[154, 155] In comparison to variants lacking S layers, strains carrying S layers have a more hydrophobic surface.[156, 157]

Surface layers from Bacillaceae were found to function as adhesion sites for cell-associated exoenzymes. The high molecular weight exoamylases from two *B. stearothermophilus* wild-type strains were bound to the S layer surface in a density that did not disturb diffusion of nutrients or metabolites through the S layer lattice.[158, 159] Exoenzymes associated with S layers were also described for *Thermoanaerobacterium thermohydrosulfurigenes*[160, 161] and *Clostridium thermocellum*.[162] A quite different function was reported for the S layer lattice from *Synechococcus* GL-24,[29a, 163] a cyanobacterium capable of growing in lakes with exceptionally high calcium and sulfate ion concentrations. The hexagonally ordered S layer lattice functioned as template for fine grain mineralization and was continuously shed from the cell surface.

Surface layers can contribute to virulence when they are present as structural components of the cell envelope of pathogens. Detailed studies have been performed with the fish pathogen *Aeromonas salmonicida*. The S layer (termed A layer) endows this organism with high or intermediate resistance towards the bactericidal activity of complement systems, in both immune and nonimmune serum. Moreover, the A layer plays an important role in uptake of porphyrins and shows unique binding capabilities for immunoglobulins and extracellular matrix proteins.[164–166] Similar observation was reported for the S layer from *B. cereus* which was recently isolated from peridontal infections.[156] The cells carrying S layers adhered to various matrix proteins and were resistant against polymorphonuclear leucocytes in absence of opsonins. Serum resistance of *C. fetus* was found to be due to the inability of complement component C3b to bind to the S layer surface, and effective opsonization occurred only after addition of specific antibodies.[132] In *Rickettsia prowazekii* and *R. typhii*, which cause either epidemic or endemic typhus, the S layer protein is responsible for humoral and cell-mediated immunity.[167] *B. anthracis*, the causative agent of anthrax, possesses two S layer proteins: Sap and EA1.[95, 96] Synthesis of EA1 in *sap*-disrupted mutants revealed that EA1 is the major cell surface antigen which is exclusively cell-associated, while Sap is also shed into the culture fluid.

7. Applications of Surface Layers

7.1. Biotechnological and Biomimetic Applications

The specific structural and self-assembly properties of S layers have led to a broad spectrum of applications in biotechnology and biomimetics.[10, 11, 67]

For obtaining S layer proteins with defined properties for bio- and nanotechnological applications, organisms carrying S layers are grown in continuous culture at a constant specific growth rate.[140, 168] However, future development is directed to the production of recombinant S layer proteins[11, 169] with integrated functional domains. Moreover, the use of truncated forms or recombinant S layer proteins with exchanged amino acids will enable the definition of those domains that are responsible for intersubunit bonding and for interactions between the S layer subunits and natural or artificial supports. These data are also required for the construction of recombinant S layer fusion proteins capable of self-assembly.

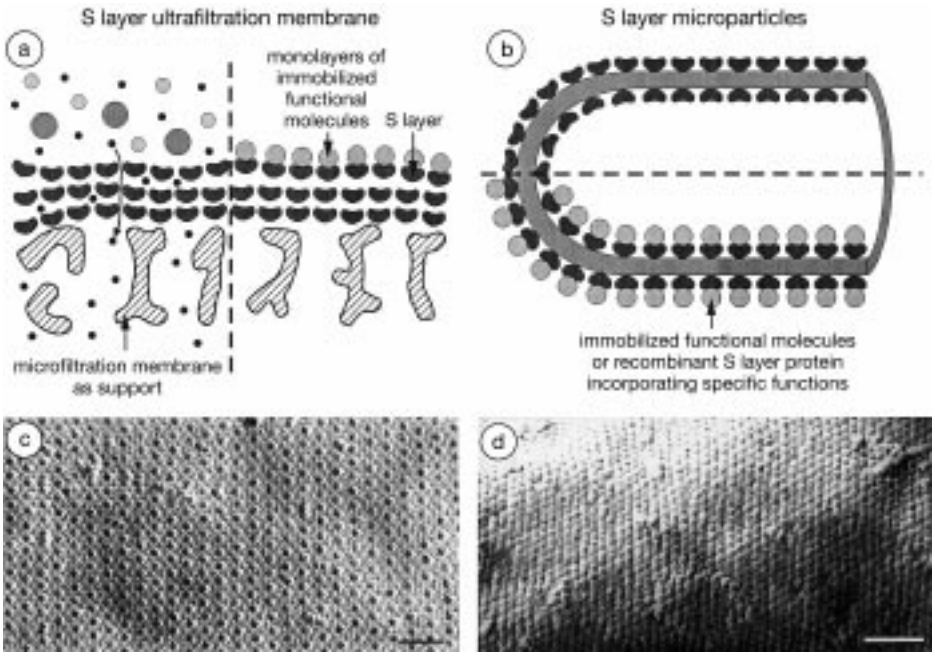


Figure 9. Schematic drawing showing a) S layer ultrafiltration membranes (SUMs) and b) S layer microparticles (SMPs) as matrix for the immobilization of functional macromolecules. c, d) Electron micrographs of freeze-etched preparations of S layers with hexagonal lattice symmetry. In (c) polycationic ferritin (PCF) was used for labeling negatively charged domains on the surface of the archaeon *Thermoproteus tenax*. In (d) ferritin was covalently linked to the carbodiimide-activated carboxy groups of the S layer lattice of *Thermoanaerobacter thermohydrosulfuricus*. The bars correspond to 100 nm.

7.1.1. Surface Layers as Isoporous Ultrafiltration Membranes

Owing to the presence of pores identical in size and morphology in the 2 to 8 nm range, S layers can be considered as isoporous ultrafiltration membranes[10, 11] with a porosity in the range of 30 to 70%. Permeability studies using proteins with defined molecular size according to the space technique were performed with S layers from *B. stearothermophilus* strains.[145] The nominal molecular weight cut-off determined by this method was in good agreement with the pore size estimated from negatively stained preparations of isolated S layers or S layer self-assembly products.[148, 170] Because of the sharp molecular weight cut-off, S layers from various Bacillaceae could be exploited for the production of ultrafiltration membranes (SUMs).[171, 172] SUMs are produced by depositing S layer self-assembly products or cell-wall fragments carrying S layers on microfiltration membranes, cross-linking the S layer protein with glutaraldeyhde, and reducing Schiff bases with sodium borohydride (Figure 9a).

Chemical modification of the carboxy groups from the acidic amino acids exposed on the SUM surface and in the pore areas allowed the preparation of neutral, or positively or negatively charged SUMs of different surface hydrophobicity.[173, 174] The isoporous structure and the defined surface properties of (chemically modified) SUMs enabled detailed studies regarding the flux losses of ultrafiltration membranes caused by adsorbed monolayers of protein molecules.[173, 174]

7.1.2. Surface Layers as Matrix for the Immobilization of Functional Molecules

The high density and defined position of carboxy groups located on the surface of S layer lattices was exploited for the immobilization (covalent attachment) of different (macro)-molecules (Figure 9). Amongst different immobilization procedures, carbodiimide activation of carboxy groups was found to be optimal with respect to the binding density and the reproducibility.[175–178] For quantification of the amount of foreign (macro)molecules which could covalently be bound to the S layer lattice, S layer microparticles (SMP; cell-wall fragments carrying S layers that show a complete outer and inner S layer and are cross-linked with glutaraldehyde) were used (Figure 9b).[175, 177] Most enzymes—for example, β-glucosidase, glucose oxidase, invertase, or naringinase—could be immobilized on S layer lattices as densely packed monolayers.[176, 178] Immobilization through spacers frequently improved the retained enzymic activity, but had no influence on the binding capacity.[176] After covalent attachment of protein A, which is a ligand that specifically recognizes the Fc part of most mammalian antibodies, SMPs were applicable as affinity particles for the isolation and purification of (monoclonal) antibodies from serum or hybridoma culture supernatants.[179, 180] Because of the high stability towards pressure and shear forces, SMPs with immobilized protein A could also be used as "escort particles" in affinity cross-flow filtration processes.[180]

Ultrafiltration membranes with immobilized IgG were used as novel reaction zone for dipstick-style solid-phase immunoassays or SUM-based dipsticks.[175, 181] Dipsticks based on SUMs are easy to handle and allow rapid determination of various analytes. For preparing the reaction zone, monoclonal antibodies were linked to the carbodiimide-activated carboxy groups of the S layer protein. The binding capacity of the square S layer lattice from *B. sphaericus* for IgG was 375 ng cm^{-2}. As derived from the saturation capacity of a planar surface and the molecular dimension of IgG, this corresponds to a monolayer of randomly oriented antibody molecules.[175] So far, different types of SUM-based dipsticks have been developed for the rapid determination of the concentration of tissue-type plasminogen activator (t-PA) in whole blood or plasma, IgE as a marker for type I allergies in serum, and interleukins to differentiate between septic and traumatic shock. The advantage of SUMs as reaction zone for dipsticks in comparison to amorphous polymers can be summed up as follows:

1) Surface layers have well-defined surface properties.
2) Immobilization of the catching antibody can only occur on the outermost surface of the crystal lattice, preventing diffusion-controlled reactions.
3) Since the catching antibody is covalently linked to the S layer protein, no leakage occurs during the test procedure.
4) SUMs do not unspecifically adsorb erythrocytes or other serum or blood components.
5) Stable, concentration-dependent precipitates are formed by peroxidase or alkaline phosphatase on the SUM surface, from which the intensities can be measured with a reflectometer.[181]

7.1.3. Surface Layers for Vaccine Development

In the past, crystalline bacterial cell S layers have been utilized in different ways for vaccine development. *Aeromonas salmonicida* and *A. hydrophila* can cause disease in salmonids in fresh-water and marine environments. Since the S layers of those organisms are essential for virulence, whole cell preparations, cell sonicates, or partially purified cell products have been applied as attenuated vaccines.[44, 182]

On the other hand, native and cross-linked S layers were used as combined carrier/adjuvants system either against infection with pathogenic bacteria, in the immunotherapy of cancers, and in the antiallergic immunotherapy.[183, 184] Owing to their crystalline nature the functional groups available for antigen/hapten binding occur on each protomer in identical position and orientation.[185, 186] Thus, ligands can be immobilized onto these protein lattices of S layer self-assembly products in a precisely defined manner (Figures 5 and 9).

The suitability of S layers as combined carrier/adjuvants for therapeutic cancer vaccines was first suggested by Smith et al.[187] Tumor-associated glycans such as T or Lewis Y antigen, chemically coupled to glutaraldehyde-cross-linked S layer preparations of different Gram-positive bacteria elicited without the use of extraneous adjuvant strong, caused hapten-specific delayed-type hypersensitivity (DTH) responses. In contrast, only low titers of antibody responses were obtained with those S layer–hapten conjugates. Adoptive transfer experiments indicated that the observed DTH responses are mediated by T-helper cells. Administration of the S layer–hapten conjugates by the oral/nasal route resulted for tumor-associated glycans in similar specific DTH responses.[187]

Surface layer–antigen conjugates were also prepared by coupling oligosaccharides derived from capsules of different *Streptococcus pneumoniae* serotypes to native, non-cross-linked S layers. Sera from mice immunized with these conjugates were found to be immunoprotective, and they reduced the colony counts of *S. pneumoniae* by 99 % on blood agar plates. Surface layer–polysaccharide conjugates of the same serotype revealed no protective antibodies. The oligosaccharide conjugates could also elicit a cellular (DTH) response on challenge with heat-killed *S. pneumoniae* bacteria. Therefore with these S layer conjugates the thymus-independent response against a bacterial polysaccharide was changed into an immunoprotective thymus-dependent response.[188]

In type I allergies the production of IgE antibodies is mediated by the Th2 helper lymphocyte subset. Nonallergic individuals, however, produce low levels of specific IgG to allergens, a response mediated by Th0/Th1 cells.[189] The modulation of regulatory T cell subsets by adjuvant effects of S layers is a promising concept for immunotherapy and perhaps also for prophylaxis of type I allergies. In the experimental S layer vaccine recombinant birch pollen allergen Bet v 1 (rBet v 1) was conjugated to cross-linked S layers. The T cell lines (TCL) and T cell clones (TCC) were established from peripheral blood from patients allergic to birch pollen using either rBet v 1 alone or rBet V 1–S layer conjugates as initial antigen stimulus. For assessment of the respective immune reaction the corresponding cytokine production was investigated (Figure 10).[190] Surface layers and rBet v 1–S layer conjugates stimulated the production of high levels of interleukin 12 (IL-12). Stimulation of rBet v 1-induced TCC with Bet v 1–S layer conjugates led to an increased production of interferone γ, which could be

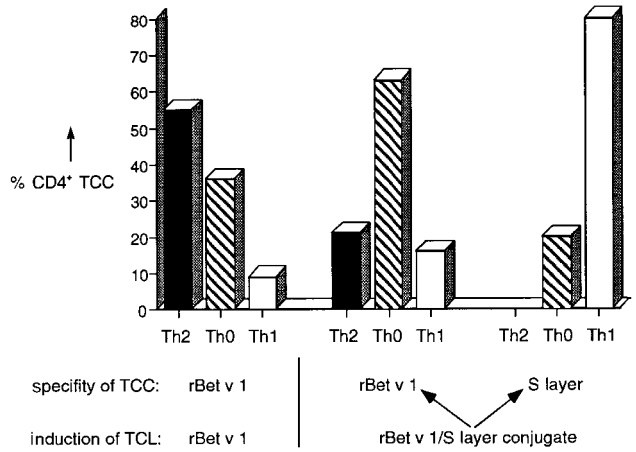


Figure 10. Distribution of T helper cell (Th) subsets within CD4$^+$ T cell clones (TCC) induced by recombinant major birch pollen allergen rBet v 1 or rBet v 1–S layer conjugates and stimulated by rBet v 1. Th subsets were classified according to the ratio of interferon γ to interleukine 4 (taken from reference [190] with permission of Oxford University Press).

reversed by neutralizing anti-IL-12 monoclonal antibodies. Together these results indicate an adjuvant effect of S layers mediated by IL-12.[190]

The experiments conducted so far have indicated that the S layer vaccine technology is suitable for a broad spectrum of applications. A number of immunologically non-cross-reactive S layers, which do not show any measurable toxicity, can already be used as carrier/adjuvants systems. A furthur strategy for the development of new model vaccines is based on liposomes stabilized by S layers, and a wide range of combinations is possible with suitable protein-, lipid-, or glycolipid-immune modulators. In all applications, the S layer vaccine technology appears to offer the versatility needed to direct vaccination responses toward predominant control by Th1 or Th2 lymphocytes to meet the different therapeutic or prophylactic requirements.

7.1.4. Surface Layers as Supporting Structure for Functional Lipid Membranes

Since a great variety of biological processes are membrane-mediated, there has always been great interest in the meso- and macroscopic reconstitution of biological membranes. Particularly functional transmembrane proteins have a broad potential for bioanalytical, biotechnological, and biomimetic applications. On the other hand, investigations are primarily impeded by a low stability of artificial planar lipid bilayer systems and liposomes.[191, 192] Consequently, there is a strong demand to develop systems that reinforce such fragile structures without interfering with their function.

The stability of lipid membranes can be increased significantly by recrystallization of isolated S layer (glyco)proteins as coherent monomolecular lattices.[193, 194] Composite S layer lipid films (Figure 11) are biomimetic structures resembling those archaeal envelope structures which are exclusively composed of monomolecular arrays of (glyco)proteins and a closely associated plasma membrane (Figure 1a). Phospholipid or tetraetherlipid films stabilized by S layers may span apertures in a solid support that are some 10 μm in diameter.[194] The possibility for handling such layers by standard Langmuir–Blodgett (LB) techniques opens a broad spectrum of applications in basic and applied membrane research, including physiology, diagnostics, and biosensor development. Composite S layer lipid films are particularly interesting supramolecular structures, since the crystallization does not involve specific key–lock mechanisms between proteins and (modified) lipids, as is the case for antigen–antibody, enzyme–substrate, or biotin–avidin systems.[195–198]

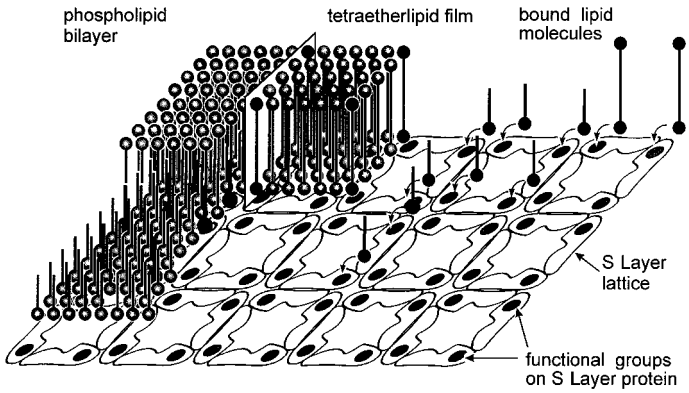


Figure 11. Schematic illustration of the "semifluid" membrane model composed of a phospholipid bilayer or tetraetherlipid monolayer Langmuir film supported by an S layer. The proportion of lipid molecules which can covalently be linked to the porous S layer lattice or which interact by noncovalent forces with domains of the S layer protein subunits significantly modulates the lateral diffusion of the free lipid molecules and consequently the fluidity of the membrane (modified based on reference [200]).

The recrystallization of S layer proteins into coherent monolayer or phospholipid films depends on 1) the nature of the lipid headgroup, 2) the phase state of the lipid film, and 3) the ionic content and pH value of the subphase.[198–201]

To obtain more information about the interaction between the S layer protein lattice and lipid membranes and to study how the interaction points influence fluidity in a lipid layer, different biophysical methods including X-ray reflectivity and grazing incidence diffraction,[202] fluorescence recovery after photobleaching,[203] atomic force microscopy,[199] sound velocity and density measurements,[204] and microcalorimetric studies[205] have been performed. As the interaction of the lipid head groups in such composite membranes with the repetitive domains of the associated S layer lattice (Figure 11) can significantly modulate the characteristics of the lipid film (particularly its fluidity and local order on the nanometer scale), the terminology "semifluid membranes" has been used to describe membranes supported by S layers.[194, 200]

Voltage clamp studies at Langmuir films[206] and black lipid membranes[207] confirmed that the associated S layer did not impede the function of the lipid membrane or incorporated molecules. Particularly stable composite structures could be obtained after intra- and intermolecular cross-linking of the S layer proteins alone or with molecules from the lipid layer.

The "semifluid membrane" concept was also exploited for the design of different types of solid-supported lipid bilayers and tetraether monolayers (Figure 12),[199, 203] as required for (highly selective) receptor surfaces of biosensors on electro-optical devices or the biofunctionalization of inorganic surfaces.[208, 209]

Surface layer subunits can also recrystallize on liposomes[205, 210, 211] composed of phospholipids and tetraetherlipids (Figure 13). Such liposomes coated with S layers are closed biomimetic structures that resemble archaeal cells (Figure 1a) or virus envelopes. The S layer proteins, once recrystallized on liposomes, can be cross-linked and exploited as a matrix for the covalent attachment of molecules. The high mechanical and thermal stability of liposomes coated with S layers and the possibility for immobilizing or entrapping biologically active molecules reveal a broad application potential, particularly as carrier and/or drug-delivery and drug-targeting systems or in gene therapy (for example, artificial viruses).

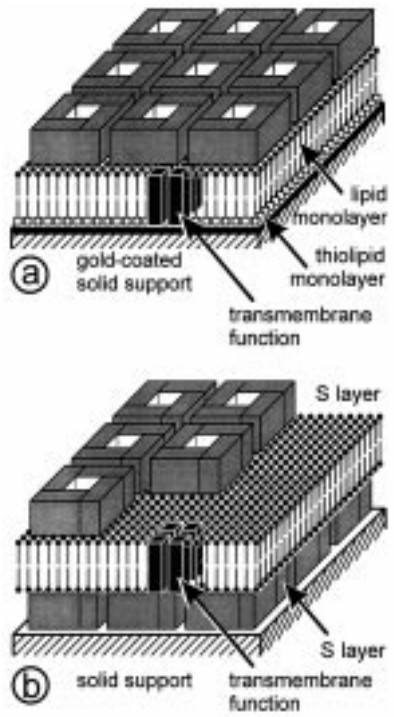


Figure 12. Schematic drawing illustrating the concept of solid-supported lipid membranes stabilized by S layers. In (a) the generation of the lipid bilayer makes use of the strong chemisorption of thiolipids to gold. The second leaflet and the S layer which had recrystallized at the lipid film before in a Langmuir trough were transferred onto the thiolipid-coated solid support by the Langmuir–Schaefer technique. Integrated functional molecules allow the investigation of transmembrane functions. b) As an alternative to soft polymer cushions an S layer is located between the solid support and the lipid layer. Optionally, the external leaflet of the lipid bilayer can be stabilized by the attachment of an S layer (modified based on reference [212]).

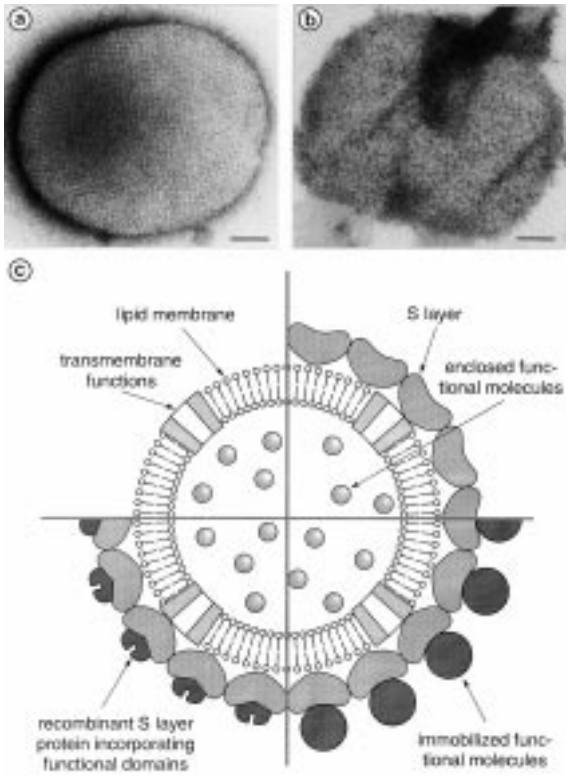


Figure 13. Electron micrographs of negatively stained liposomes coated with an oblique S layer lattice a) before and b) after covalent binding of electron-dense ferritin molecules. c) Schematic drawing of a liposome coated with an S layer lattice which is used as matrix for the immobilization of functional molecules. Alternatively, recombinant S layer proteins incorporating functional domains may be used. The bars correspond to 80 nm.

7.2. Nanotechnological Applications

7.2.1. Surface Layers Recrystallized on Solid Supports

The intrinsic property of S layer proteins to form extended crystalline arrays on solid supports is one of the most important features for functionalizing surfaces.[200, 212] For many bio- and nanotechnological applications a defined orientation of the lattice with respect to the substrate is required.[213] A common feature of most technologically relevant S layers is their asymmetrical topography, surface charge, and hydrophobicity between the outer and inner faces. High-resolution electron microscopy and scanning force microscopy have demonstrated a moderate surface roughness for the outer face and a more pronounced one for the inner face. Chemical modifications of the surfaces as well as adsorption and labeling experiments with differently charged marker molecules demonstrated a charge neutral, more hydrophobic outer face and a net negatively charged, more hydrophilic inner face for S layers of many Bacillaceae.

High-resolution electron microscopical and scanning force microscopical studies have shown that the protein monolayers are not monocrystalline over the entire surface, but consist of a mosaic of randomly aligned crystalline domains.[29b, 193, 213] Analysis of the crystallization process revealed that crystal growth of S layers at interfaces is initiated at randomly distributed nucleation sites composed of proteins or small protein assemblies from the bulk solution. Subsequently, the crystalline domains grow laterally in all directions until neighboring areas meet and a closed coherent monolayer is formed.

Although the S layer lattice exhibits a polycrystalline character, the individual crystalline domains have the same orientation with respect to the interface. This orientation is unambigously determined by the reciprocal influence of the surface properties of the S layer and support (for example, hydrophobicity, surface charge). The patch size of the crystalline areas depends on the density and lateral mobility of those protein subunits or protein assemblies which have adsorbed in a very early stage of crystal growth at the surface. Beside the surface properties of the substrate, the formation of coherent crystalline arrays depends strongly on the particular S layer used and on the environmental conditions of the bulk phase (for example, temperature, pH value, ion composition, and ionic strength). The average diameter of the crystalline patches is in the range of 1 to 20 μm, while the total covered area is only limited by the size of the substrate. Table 4 summarizes all those technologically important substrates for which the formation of S layer lattices has already been optimized.

Table 4. Technologically important substrates on which S layers recrystallize.[200]

Silicon and gallium arsenide wafers for novel applications in microelectronics and for miniaturized integrated biosensors
Glass for all developments of optical and electrooptical sensor systems
Noble metals (e.g. gold or platinum) for the development of ultrathin working electrodes for amperometric biosensors

7.2.2. Patterning of Recrystallized Surface Layers

Almost all technological applications for surfaces functionalized with S layers require patterning of the protein lattice in the (sub)micrometer range. For this purpose, S-protein monolayers recrystallized on silicon wafers are irradiated by deep ultraviolet (DUV) laser radiation through a microlithographic mask possessing feature in the range of 1 μm to 200 nm (square and line patterns, Figure 14).[214] Drying of the S layer was necessary to avoid formation of interference fringes in the patterned S layer by a thin water layer between mask and substrate. The S layer could be completely removed by two pulses of ArF radiation ($\lambda = 193$ nm; dose: approximately 100 mJ cm^{-2} per pulse) in the exposed areas, while in the unexposed regions the structural and functional integrity was completely retained. The height difference between exposed and unexposed areas was determined by scanning force microscopy, and found to be in perfect agreement with the thickness of the S layer as determined at fringes of individual monolayers.

Surface protein monolayers recrystallized on silicon wafers are currently being tested for their potential application as novel ultrathin high-resolution resists. In this application the patterned S layers are reinforced by a thin refractory layer (for example, Zr, deposited by reaction with $ZrOCl_2$), silylation,[215] or electroless metallization[216] in order to yield different etching rates between exposed and unexposed regions for subsequent dry (reactive-ion) etching. Advantages against conventional polymeric resists are expected due to the extreme thinness of S layers (approximately 10 nm).

In comparison to ArF radiation, the S layer could not be ablated when exposed to KrF radiation ($\lambda = 248$ nm).[214] Investigation of the irradiated S layer lattices showed that even a dosage of about 3500 mJ cm^{-2} (supplied in 10 shots at 350 mJ cm^{-2} each) led only to a carbonization and not to an ablation of the protein.[214] As determined by scanning force microscopy the step height between exposed and unexposed areas did not reach the thickness of the intact S layers. Since the S layer lattice could not be identified in the unexposed regions after exposure, it was concluded that during exposure heat dissipated into the unexposed areas and destroyed the molecular structure of the S layer lattice there. Nevertheless, this remarkably different sensitivity of the S layer against ArF and KrF radiation could already be utilized for transferring a submicron pattern into a conventional polymeric resist on a silicon wafer.[217] In this approach an S layers recrystallized on top of a spin-coated polymeric resist (thickness approximately 500 nm), sensitive towards KrF but not towards ArF radiation, was first patterned by ArF radiation as decribed above. Subsequently, this patterned S layer structure served as mask for a blank exposure of the resist by irradiation with KrF. In this approach very steep side walls in the developed polymeric resist could be obtained.

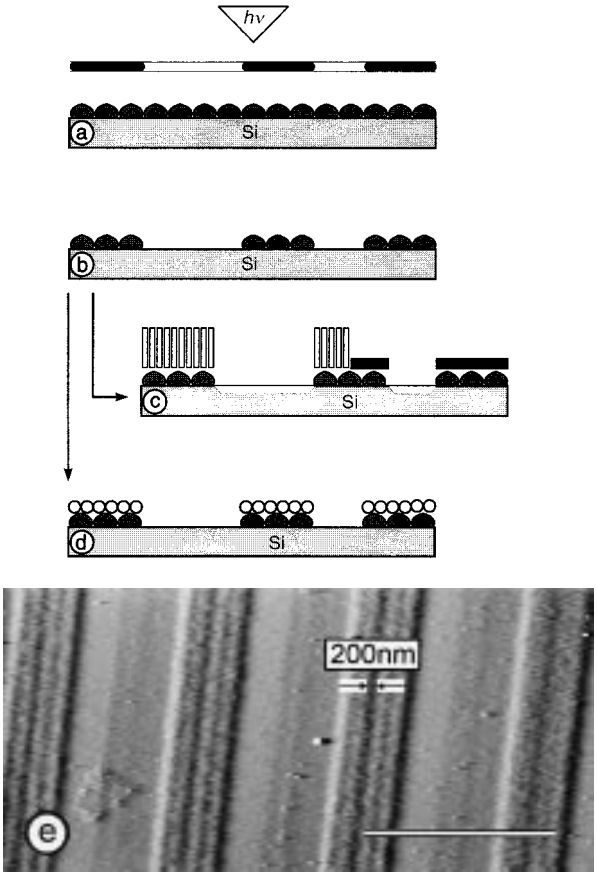


Figure 14. Schematic drawing of the patterning of S layers by exposure to deep ultraviolet radiation. a) A pattern is transferred onto the S layer by exposure to ArF excimer laser radiation ($\lambda = 193$ nm) through a microlithographic mask. b) The S layer is specifically removed from the silicon surface in the exposed regions, but retains its crystalline and functional integrity in the unexposed areas. c) Unexposed S layer areas can be used either to bind enhancing ligands or to enable electroless metallization. In both cases a layer is formed which allows a patterning process by reactive ion etching. d) Alternatively unexposed S layers may also be used for selectively binding biologically active molecules which would be necessary for the fabrication of miniaturized biosensors or biocompatible surfaces. e) Scanning force microscopical image of a patterned S layer on a silicon wafer. The bar corresponds to 3 μm. The ultimate resolution is determined by the wavelength of the excimer laser radiation (modified based on reference [212]).

Current research is concentrated on an improvement of the resolution limit of 100 nm, which is caused by the wavelength of the ultraviolet radiation. Preliminary experiments with electron and ion beams have already demonstrated that feature sizes well below the 100-nm limit can be generated in S layer lattices (unpublished results of our research group).

7.2.3. Surface Layers as Templates in the Formation of Regularly Arranged Nanoparticles

The formation of arrays of metal clusters with novel physical properties by colloidal crystallization or monolayer deposition is currently under extensive investigation in the

field of molecular electronics and nonlinear optics.[218–221] Although, in recent years, these methods have advanced tremendously it is still not possible to fabricate extended arrays on the micrometer scale. As an alternative approach to such methods S layers have already demonstrated their application potential as templates in the formation of regularly arranged nanometric metallic or semiconducting point patterns. Surface protein monolayers on substrates and sheet-like self-assembly products in suspension promote the precipitation of monodisperse cadmium sulfide[222] and gold nanoparticles (Figure 15).[223] This observation is not surprising since recent studies on a cyanobacterial S layer clearly demonstrated that a crystalline surface layer can function as template for fine grain mineralization.[163]

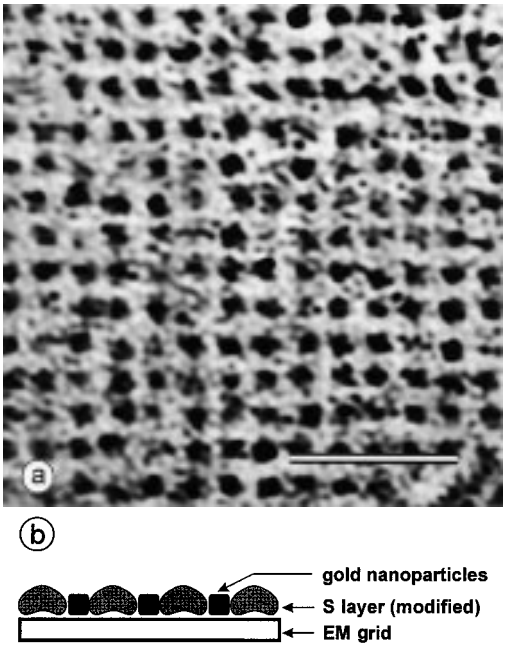


Figure 15. a) Electron micrograph of a gold "superlattice" consisting of monodisperse gold nanoparticles with mean diameters of 4 to 5 nm. b) Schematic drawing of a cross-section through a support coated with S layers, where the gold nanoparticles had formed in the pore region of the protein layer. The bar corresponds to 50 nm.

For the formation of gold nanoparticle arrays thiol groups had to be introduced in the S layer before incubation with a tetrachloroauric(III) acid solution. The gold particles were formed upon electron irradiation of an S layer covered with a thin layer of gold (gold staining of the S layer). High-resolution electron microscopical studies revealed that the particles were formed in the pore region of the S layer lattice and that their shape resembled the morphology of the pore (Figure 15). The interparticle spacing of the gold dots resembled the S layer lattice. Electron diffraction patterns and energy-dispersive X-ray analysis confirmed that the gold particles were crystalline (pure) gold, but not crystallographically aligned in terms of long-range order. Digital image processing also revealed that the mean deviation of the individual particles from their ideal position in the two-dimensional lattice was less than ± 0.5 nm.

Most recently the spectrum of available materials was extended towards the precipitation of platinum and palladium, but also magnetic materials such as iron or nickel (unpublished observations of our research group). Currently the electrical, magnetic, and optical properties of these nanoparticle arrays are being investigated.

Surface layers have also been used as nanometric etching masks in the fabrication of ordered arrays of titanium nanoparticles.[224–226] In this approach an hexagonal S layer structure was transferred into a silicon surface by (low energy enhanced) electron etching. Subsequently, the S layer mask was removed, and the patterned surface oxidized in an oxygen plasma. After deposition of a thin layer (about 1.2 nm) of titanium on the oxidized surface, an ordered array of nanometric titanium clusters was formed in the etched holes.

In contrast to other current approaches, only S layers appear to allow the synthesis of extended inorganic nanocrystal superlattices with a broad range of particle sizes (3 to 15 nm in diameter), interparticle spacings (up to 30 nm), and lattice symmetries (oblique, square, or hexagonal).

8. Conclusions and Perspectives

Surface layers are the most common envelope structures in prokaryotic organisms. As highly porous protein lattices that completely cover the cell surface, they reflect specific adaptations to ecological conditions and selection criteria. Being composed of a single protein species, S layers represent the most simple self-assembling membranes that developed during the evolution of life. The spontaneous associations of identical subunits under equilibrium conditions result in stable, structurally well defined monomolecular aggregates joined by noncovalent bonds. On growing bacterial cells S layers represent "dynamic closed surface crystals" with the intrinsic capability to assume a structure of low free energy continuously during cell growth.

The study of self-assembly is a new and rapidly growing scientific and engineering field that crosses the boundaries of several existing disciplines.[29b, 212, 227, 228] Molecular self-assembly processes are ubiquitous in biological systems and are essential for the morphogenesis of complex biological structures. The attractiveness of self-assembly for practitioners of material science lies in its capabilities to form various ultrasmall, uniform, or highly complex meso- or macroscopic area structures spontaneously. Such "bottom up" processes are based on intermolecular forces which determine, in a highly predictable way, the form or pattern of the final structure.

Learning from nature how to create supramolecular units and the elucidation of rules mediating their organization into functional materials is leading to fascinating new technologies. Our goal in applied research on S layers and prokaryotic cell envelopes has been to harness for technical applications the unique self-assembly characteristics and the repetitive structural properties of these supramolecular systems. Especially the possibility to immobilize or grow other materials on top of S layers with an accurately spatially controlled archi-

tecture opens up many new possibilities in supramolecular engineering and nanofabrication.

Similarly, the biomimetic approaches copying the supramolecular principle of plasma membranes associated with S layers (developed by archaea in the most extreme and hostile ecosystems, for example, at pH values below 1, at temperatures up to 110 °C, or in concentrated salt solutions) is expected to lead to new technologies for stabilizing functional lipid membranes and their use at the macroscopic scale.

Along the same line, liposomes stabilized by S layers resemble archaeal and viral envelopes and represent unique supramolecular systems that are suitable as carriers for drug delivery and drug targeting or as vehicles for gene therapy.

An important line of development is directed towards the genetic manipulation of S layer proteins. The possibility to change the natural properties of S layer proteins by site-directed mutagenesis opens a new horizon for the tuning of their structural and functional features by genetic engineering techniques. Incorporation of peptide segments corresponding to specific functional domains of other proteins while maintaining the self-assembly capability should lead to completely new affinity structures and enzyme membranes, ion-selective binding matrices, microcarriers, biosensors, diagnostics, vaccines, biocompatible surfaces, or bioabsorbable systems for tissue regeneration.[11] Finally, S layer technologies provide new approaches for nanopatterning of surfaces, biological templating, and formation of ordered arrays of metal clusters as required for nonlinear optics and nanoelectronics.[212]

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