REVIEW ARTICLE

S-layers: principles and applications

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Abstract

Monomolecular arrays of protein or glycoprotein subunits forming surface layers (S-layers) are one of the most commonly observed prokaryotic cell envelope components. S-layers are generally the most abundantly expressed proteins, have been observed in species of nearly every taxonomical group of walled bacteria, and represent an almost universal feature of archaeal envelopes. The isoporous lattices completely covering the cell surface provide organisms with various selection advantages including functioning as protective coats, molecular sieves and ion traps, as structures involved in surface recognition and cell adhesion, and as antifouling layers. S-layers are also identified to contribute to virulence when present as a structural component of pathogens. In *Archaea*, most of which possess S-layers as exclusive wall component, they are involved in determining cell shape and cell division. Studies on structure, chemistry, genetics, assembly, function, and evolutionary relationship of S-layers revealed considerable application potential in (nano)biotechnology, biomimetics, biomedicine, and synthetic biology.

Introduction

With the exception of those prokaryotic organisms which have developed strategies to live under very specialized and frequently extreme environmental conditions in which monocultures are feasible, most organisms have to survive in highly competitive habitats in very complex microbiomes. Consequently, the diversity observed in the molecular architecture of bacterial and archaeal cell envelopes, particularly the structure of the outermost boundary layers, reflects evolutionary adaptations of the organism to specific environmental and ecological conditions.

Among the most commonly observed prokaryotic cell surface structures are two-dimensional arrays of proteinaceous subunits forming surface layers (termed S-layers) on prokaryotic cells (Sleytr, 1976; Sleytr *et al.*, 1988b; Table 1). Since the first 'macromolecular monolayer' described by Houwink and Le Poole (1952; Houwink, 1953) in the cell wall of a *Spirillum sp.*, S-layers have now been identified in hundreds of different species of almost every taxonomic group of walled *Bacteria* and are an almost universal feature of *Archaea* (Fig. 1; for compilation see, Messner & Sleytr, 1992; Sleytr *et al.*, 1996a, 1999, 2002; Claus *et al.*, 2005; König *et al.*, 2010; Messner *et al.*, 2010; Albers & Meyer, 2011; Hynonen & Palva, 2013).

Because S-layer proteins account for approximately ten percent of cellular proteins in *Archaea* and *Bacteria*, they represent interesting model systems for studying the processes involved in the synthesis, secretion, and assembly of extracellular proteins. Moreover, as the biomass of prokaryotic organisms surpasses the biomass of eukaryotic organism (Whitman *et al.*, 1998), S-layer proteins can be considered as one of the most abundant biopolymers on our planet. S-layers also represent the simplest biological protein or glycoprotein membranes developed during evolution (Sleytr, 1975). They are generally composed of a single molecular species endowed with the ability to assemble on the cell surface into closed regular arrays occupying a low free-energy arrangement. Studies on the *in vivo* morphogenesis of S-layers demonstrated that at high growth rates, approximately 500 subunits per second must be synthesized, translocated to the cell surface, and incorporated into the existing S-layer lattice (Sleytr & Messner, 1983; Sleytr & Beveridge, 1999). It is now evident that

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Table 1. Selected milestones in basic and applied S-layer research

Year	Milestone	Reference
1953	First evidence of a monomolecular array in a bacterial cell wall fragment	Houwink (1953)
1968	Evidence that coherent monomolecular arrays are located on the surface of the cell envelope of intact Gram-positive and Gram-negative bacteria using freeze-etching techniques	Remsen *et al.* (1968) and Sleytr *et al.* (1968)
1969	Description of *in vitro* assembly of S-layer proteins	Brinton *et al.* (1969)
1971	First evidence for a function: S-layer as protective coat against the bacterial parasite *Bdellovibrio bacteriovorus*	Buckmire (1971)
1975	Studies on the self-assembly and homologous and heterologous reattachment of S-layer proteins on cell envelopes of Gram-positive bacteria	Sleytr (1975)
1976	Evidence for glycosylation of archaeal S-layer proteins	Mescher & Strominger (1976)
1976	Evidence for glycosylation of bacterial S-layer proteins	Sleytr & Thorne (1976)
1986/1987	First nanobiotechnological application of S-layer proteins: Use of S-layer lattices for the production of ultrafiltration membranes with defined molecular sieving properties	Sleytr & Sára (1986), Sára & Sleytr (1987c) and Sleytr & Sára (1988)
1986/1991	S-layers involved in morphogenesis and cell division in archaea	Messner *et al.* (1986b) and Pum *et al.* (1991)
1986	First sequenced S-layer protein gen	Tsuboi *et al.* (1986)
1989/1991	S-layers as combined carrier/adjuvants for conjugated vaccines	Sleytr *et al.* (1989, 1991)
1994	Proposing S-layer-induced nanopatterned fluidity in lipid films (termed *semi-fluid lipid membrane model*)	Pum & Sleytr (1994)
1997/1998	First biomimetic approach copying the supramolecular building principle of archaeal cell envelopes to generate (functional) phospho- and ether lipid membranes	Schuster *et al.* (1997, 1998a, b)
2002	First monomeric and oligomeric functional S-layer fusion proteins capable to assemble into ordered arrays	Breitwieser *et al.* (2002) and Moll *et al.* (2002)
1999/2002	Surface display of foreign epitopes of SLH domain and whole S-layer protein	Mesnage *et al.* (1999a) and Avall-Jääskeläinen *et al.* (2002)
2002/2008	First atomistic structures of archaeal and bacterial S-layer protein domains obtained from X-ray studies (2.4 Å resolution)	Jing *et al.* (2002) and Pavkov *et al.* (2008)

The terminology 'S-layer' (surface layer) was introduced 1976 (Sleytr, 1976) and generally accepted at the 'First International Workshop on Crystalline Bacterial Cell Surface Layers' in Vienna, Austria (August 1984). At the 'EMBO Workshop on Crystalline Bacterial Cell Surface Layers (S-layers)' (August 31 to September 2, 1987, Vienna, Austria), S-layers were defined as: 'Two-dimensional arrays of proteinaceous subunits forming surface layers on prokaryotic cells' (Sleytr *et al.*, 1988b).

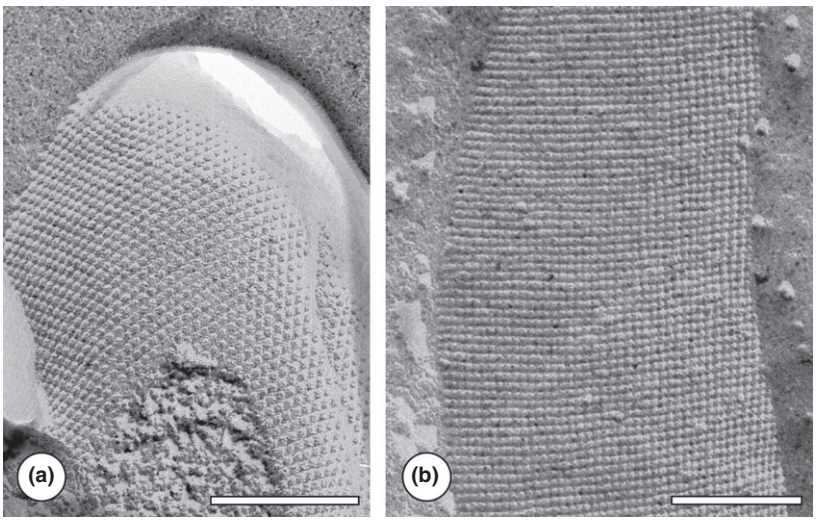

Fig. 1. TEM image of a freeze-etched and metal shadowed preparation of (a) an archaeal cell (from *Methanocorpusuculum sinense*), and (b) a bacterial cell (from *Desulfotomaculum nigrificans*). Bars, 200 nm.

S-layers as metabolic expensive products can provide organisms with an advantage of selection in quite diverse habitats. Although a considerable amount of knowledge has accumulated on the structure, assembly, chemistry, and genetics of S-layers, relatively little firm data are available about their specific biological functions (Sleytr *et al.*, 2002, 2007b; Hynonen & Palva, 2013). It is now recognized that they can function as protective coats, molecular sieves, molecule and ion traps, promoters for cell adhesion, immunomodulators, surface recognition, antifouling coat-

ings, and virulence factors in pathogenic organisms. In those *Archaea* that possess S-layers as the exclusive envelope component external to the cytoplasmic membrane, the lattice is involved in the determination of cell shape and as a structure aiding in the cell division process.

The wealth of information accumulated on the general principles of S-layers led to a broad spectrum of applications. Most relevant for applied S-layer research is the capability of isolated S-layer (glyco)proteins to assemble in defined orientations into crystalline arrays in suspension or on suitable surfaces or interfaces (Sleytr *et al.*, 1999, 2005; Pum *et al.*, 2006, 2010). As S-layers are periodic structures, they exhibit repetitive identical physicochemical properties down to the subnanometer scale and possess pores identical in size and morphology. Most important, properties of S-layer proteins can be changed by chemical modifications and genetic engineering. It is now evident that S-layers also represent a unique structural basis and pattering element for generating complex supramolecular assemblies involving all relevant 'building blocks' such as proteins, lipids, glycans, and nucleic acids (Egelseer *et al.*, 2008; Schuster & Sleytr, 2009b; Egelseer *et al.*, 2010; Sleytr *et al.*, 2010; Ilk *et al.*, 2011a; Sleytr *et al.*, 2011, 2013).

Occurrence, location, and structure

The location and ultrastructure of S-layers of a great number of *Bacteria* and *Archaea* have been studied by electron microscopy of thin-sectioned, freeze-etched, freeze-dried and shadowed, negatively stained or frozen hydrated preparations (Thornley *et al.*, 1974; Sleytr & Glauert, 1975; Sleytr, 1978; Sleytr & Messner, 1983; Baumeister & Engelhardt, 1987; Sleytr *et al.*, 1988a; Beveridge, 1994; Sleytr *et al.*, 1996a; Pavkov-Keller *et al.*, 2011). More recently, atomic force microscopy (AFM) has become an important method for characterizing S-layer lattices (Müller *et al.*, 1996, 1999; Ebner *et al.*, 2006; Tang *et al.*, 2007; Moreno-Flores *et al.*, 2008; Chung *et al.*, 2010; López *et al.*, 2011). In most *Archaea*, S-layers represent the only wall component outside the plasma membrane (Fig. 2a and b). Only a few *Archaea* possess a rigid wall layer (e.g. pseudomurein in methanogenic *Archaea*) as intermediate layer between the cytoplasmic membrane and the S-layer (Fig. 2c; Claus & König, 2010; Albers & Meyer, 2011). In Gram-positive *Bacteria*, S-layers are attached to the rigid peptidoglycan-containing layer (Fig. 2d), while in the more complex Gram-negative bacterial cell envelope, the S-layer adheres to the lipopolysaccharide of the outer membrane (Fig. 2e).

The most useful electron microscopy preparation technique for identifying S-layers on a particular organism is freeze-etching of intact cells (Fig. 1). S-layers completely cover the cell surface during all stages of cell growth and division (Sleytr & Glauert, 1975; Sleytr, 1978; Sleytr & Messner, 1989; Messner & Sleytr, 1991b; Pum *et al.*, 1991; Sleytr *et al.*, 1999; Rachel, 2010). For some organisms, two superimposed S-layer lattices composed of different proteins have been observed (Sleytr & Messner, 1983; Sleytr *et al.*, 1996b). Mono-molecular arrays of proteinaceous subunits have also been observed in prokaryotic sheaths (Beveridge & Graham, 1991; Albers & Meyer,

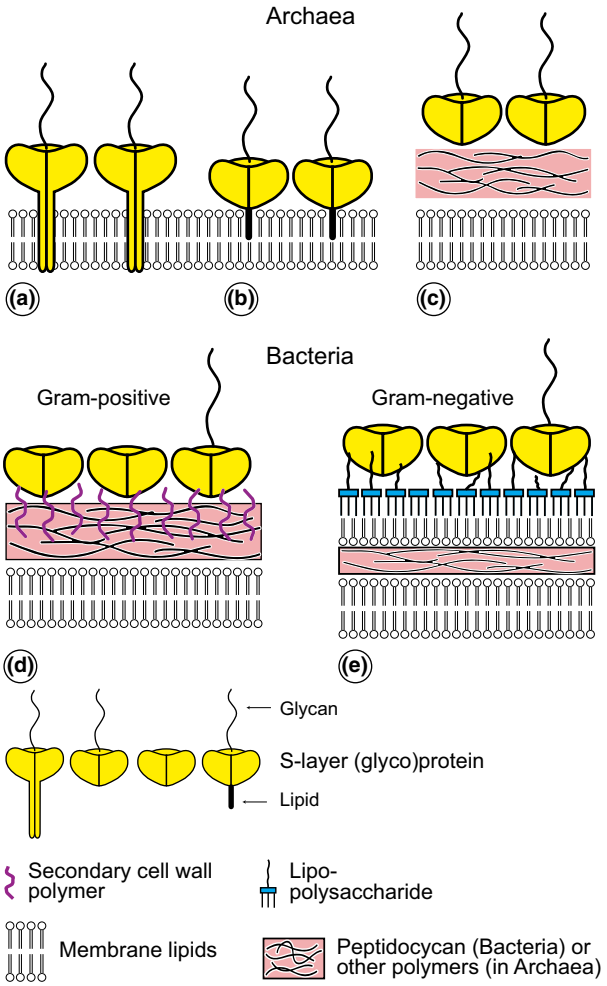

Fig. 2. Schematic illustration of the supramolecular architecture of the major classes of prokaryotic cell envelopes containing surface (S) layers. S-layers in archaea with glycoprotein lattices as exclusive wall component are composed either of mushroom-like subunits with pillar-like, hydrophobic trans-membrane domains (a), or lipid-modified glycoprotein subunits (b). Individual S-layers can be composed of glycoproteins possessing both types of membrane anchoring mechanisms. Few archaea possess a rigid wall layer (e.g. pseudomurein in methanogenic organisms) as intermediate layer between the plasma membrane and the S-layer (c). In Gram-positive bacteria, (d) the S-layer (glyco)proteins are bound to the rigid peptidoglycan-containing layer via secondary cell wall polymers. In Gram-negative bacteria, (e) the S-layer is closely associated with the lipopolysaccharide of the outer membrane.

2011), spore coats (Holt & Leadbetter, 1969), on the surface of the cell wall of eukaryotic algae (Roberts *et al.*, 1985), fungal spores (Sleytr *et al.*, 1969; Linder, 2009; Penfold *et al.*, 2012), and gas vacuoles of prokaryotic organism (Cohen-Bazire *et al.*, 1969). The two-dimensional spatial organization of S-layers has been obtained primarily by electron crystallography (Baumeister & Engelhardt, 1987; Hovmöller *et al.*, 1988; Pavkov-Keller *et al.*, 2011) and more recently through scanning probe microscopy (Müller *et al.*, 1996, 1999; Ebner *et al.*, 2006; Tang *et al.*, 2007; Moreno-Flores *et al.*, 2008; Chung *et al.*, 2010; López *et al.*, 2011), and X-ray and neutron scattering techniques (Weygand *et al.*, 1999, 2000, 2002; Horejs *et al.*, 2010; Pavkov-Keller *et al.*, 2011; Baranova *et al.*, 2012).

S-layer lattices generally exhibit oblique (p1, p2), square (p4), or hexagonal (p3, p6) space group symmetry (Fig. 3) with center-to-center spacings of the morphological units of 4–35 nm (Beveridge, 1994; Sleytr & Beveridge, 1999; Sleytr *et al.*, 1999, 2002; Albers & Meyer, 2011; Pavkov-Keller *et al.*, 2011). Hexagonal symmetry is predominant among *Archaea* (Messner & Sleytr, 1992; Messner *et al.*, 2010; Albers & Meyer, 2011). Depending on the lattice type, the morphological units consist of one, two, three, four, or six monomers, respectively (Fig. 3). Bacterial S-layers are generally 5–10 nm thick, whereas archaeal S-layers frequently exhibit a much thicker 'mushroom-like structure' with pillar-like domains anchored to the plasma membrane (Baumeister & Engelhardt, 1987; Albers & Meyer, 2011). Bacterial S-layers reveal a rather smooth outer and a more corrugated inner surface. S-layers represent highly porous protein lattices (30–70% porosity) with pores of uniform size and morphology in the 2–8 nm range. Many S-layers possess two or even more distinct classes of pores (Sleytr & Beveridge, 1999; Sleytr *et al.*, 1999, 2002; Albers & Meyer, 2011; Pavkov-Keller *et al.*, 2011).

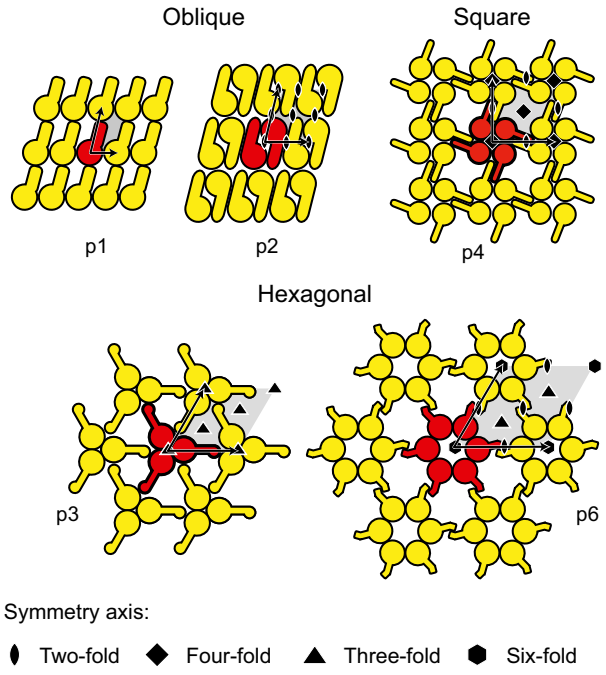

Fig. 3. Schematic drawing of the different S-layer lattice types, their base vectors, the unit cell (shaded in gray), and the corresponding symmetry axis. The proteins at one morphological unit are shown in red.

Isolation and chemistry

In both *Archaea* and *Bacteria*, S-layer lattices differ considerably in their susceptibility to isolation from the supporting envelope structure and disruption into monomeric subunits. Generally, S-layers are isolated from cell wall fragments which were obtained by breaking up the cells and removing the content including the cytoplasmic membrane by addition of hydrogen bond-breaking agents [e.g. guanidine hydrochloride (GHCl) or urea] (Sleytr & Messner, 1983; Schuster *et al.*, 2005; Schuster & Sleytr, 2013), trichloroacetic acid (Nußer *et al.*, 1988), detergents, or cation substitution (e.g. Na^+ or Li^+, replacing Ca^{2+}; Koval & Murray, 1984; Lortal *et al.*, 1992, 1993), and ethylene diamine tetraacetic acid (EDTA; Cline *et al.*, 1989). In certain cases, even washing cells with deionized water can lead to a dissociation of the S-layer lattice (Kosma *et al.*, 1995). The various extraction and disintegration experiments revealed that the intersubunit bonds in the S-layer lattices are stronger than those binding the crystalline arrays to the supporting envelope layer (Sleytr & Glauert, 1976; Sleytr & Beveridge, 1999). This characteristic property is seen as a major requirement for continuous recrystallization of the lattice into a low free-energy arrangement during cell growth and division. Some archaeal S-layers have shown to be highly resistant to common denaturizing agents (Beveridge, 1994). Special isolation procedures are required for S-layers in *Archaea* where they are associated with the cytoplasmic membrane (Nußer *et al.*, 1988; König *et al.*, 2004; Rachel, 2010). With many solubilized S-layers, it has been demonstrated that isolated subunits reassemble into lattices identical to those observed on intact cells upon removal of the disrupting agent (see also section 'Assembly and morphogenesis').

Chemical and genetical analysis of many S-layers has revealed a similar overall composition. They are generally composed of a single protein or glycoprotein species with molecular masses ranging from 40 to 170 kDa (Sleytr *et al.*, 1993a, 2002; Avall-Jääskeläinen & Palva, 2005; Claus *et al.*, 2005; Messner *et al.*, 2010). Most S-layers of *Bacteria* are composed of weakly acidic proteins or glyco-

proteins, contain 40–60% hydrophobic amino acids, and possess few or no sulfur-containing amino acids. The pI values of the proteins range from 4 to 6. For some *Archaea* (e.g. *Methanothermus fervidus*) and in *Lactobacilli*, however, pIs of the S-layer proteins between 8 and 10 have been determined. Comparative studies on S-layer genes of organisms from different taxonomic affiliations revealed that homologies between nonrelated organisms are low despite the fact that their amino acid composition shows no significant difference. Nevertheless, it is quite obvious that common structural principles must exist in S-layer proteins (e.g. the ability to form intersubunit bonds and to self-assemble into monomolecular arrays, the formation of hydrophilic pores with low unspecific adsorption, and the interaction with underlying cell envelope components).

A few post-translational modifications are known to occur in S-layer proteins, including cleavage of carboxy- or amino terminal fragments, protein phosphorylation, and protein glycosylation of amino acid residues. The latter is a remarkable characteristic of many archaeal and some bacterial S-layer proteins. In fact, S-layer proteins were the first glycoproteins detected in prokaryotes (Mescher & Strominger, 1976; Sleytr & Thorne, 1976) and still are among the best-studied examples of glycosylated prokaryotic proteins (Sára *et al.*, 1989; Sumper *et al.*, 1990; Konrad & Eichler, 2002; Sleytr *et al.*, 2002; Eichler & Adams, 2005; Messner *et al.*, 2008, 2009; Albers & Meyer, 2011; Eichler & Maupin-Furlow, 2013). The glycan chain and linkages of bacterial and archaeal glycoproteins are significantly different from those of eukaryotes (Sleytr *et al.*, 2002; Schäffer & Messner, 2004; Messner *et al.*, 2009). The *Halobacterium salinarum* S-layer glycoprotein was the first noneukaryotic protein shown to be N-glycosylated (Mescher & Strominger, 1976). Most archaeal S-layer glycoprotein glycans consist of only short heteropolysaccharides, usually not built of repeating units. Moreover, the predominant linkage types are N-glycosidic bonds where the glycan moieties are covalently linked to select asparagine residues of the target protein. Although most S-layer proteins are either N- or O-glycosylated, in few cases both modifications can be present on one protein at the same time (Albers & Meyer, 2011; Eichler & Maupin-Furlow, 2013). An example constitutes the well-characterized *Halobacterium volcanii* S-layer glycoprotein contains both N- and O-linked glycans (Sumper *et al.*, 1990).

As mentioned before, at the same time when glycosylation on haloarchaea was reported for the first time, glycosylation of S-layer proteins from the *Bacteria Thermoanaerobacter thermohydrosulfuricus* and *Thermoanaerobacterium thermosaccharolyticum* was discovered (Sleytr & Thorne, 1976; Table 1). Since then, S-layer glycoproteins from several other bacteria have been extensively studied, leading to the awareness of the wide distribution of S-layer glycoproteins among *Bacteria*.

In *Bacteria*, N-glycosylation is considered to be a rare event and is represented mainly in *Campylobacter* spp., *Helicobacter* spp., and *Desulphovibrio* spp. (Stimson *et al.*, 1995).Thus, N-glycosylation apparently is far more common in archaea than in bacteria. The degree of glycosylation of bacterial S-layer proteins, that is, the covalent O-glycosidic linkage of glycan moieties to select serine, threonine, and tyrosine residues, varies generally between 2% and 10% (w/w), and the S-layer proteins are typically multiple glycosylated (Messner *et al.*, 2009, 2013).

Moreover, the lipid carrier molecules also differ between *Archaea* and *Bacteria*. In *Archaea,* isoprene-based lipids such as dolichol phosphate and dolichol pyrophosphate play essential roles in the N-glycosylation process by delivering their bound glycan cargo to selected protein targets. In *Bacteria*, however, undecaprenol pyrophosphate is recognized to play an essential role in S-layer protein O-glycosylation. To sum up, S-layer glycoproteins are among the best-studied examples of glycosylated prokaryotic proteins (Eichler & Adams, 2005; Messner *et al.*, 2008, 2009; Albers & Meyer, 2011). Hence, a more detailed and comprehensive summary cannot be given and is out of the scope of this review. Nevertheless, at this point, we would like to refer to several recent reviews providing deeper information on the glycosylation process in general (Albers & Meyer, 2011; Eichler & Maupin-Furlow, 2013; Messner *et al.*, 2013).

Recently, yet another post-translational modification of S-layer glycoproteins was reported. It could be demonstrated that a subset of secreted euryarchaeal proteins, including the S-layer glycoprotein, is processed and covalently linked to membrane-embedded lipids involving membrane-spanning enzymes referred to as archaeosortases (Szabo & Pohlschroder, 2012; Eichler & Maupin-Furlow, 2013). A distinctive subfamily of the archaeosortase/exosortase superfamily is designated archaeosortase A (ArtA) because of its restriction to the *Archaea* and its remote homology to exosortase. To examine the role of ArtA from *Haloferax volcanii* for the first time *in vivo*, homologous recombination to construct deletion strains that lack the *artA* gene was performed (Abdul Halim et al., 2013). Comparison of wild-type and the *ΔartA* mutant strains resulted in multiple biological phenotypes including alteration in cell shape and the S-layer. These results clearly demonstrated *in vivo* that, as predicted by *in silico* work (Haft *et al.*, 2012), the C-terminal hydrophobic transmembrane segment of *H. volcanii* S-layer glycoprotein is processed by the ArtA. Because the C-terminal tripartite structure consisting of a signature motif, a transmembrane alpha helix domain, and a cluster

of basic residues, which is recognized by archaeosortases, is highly conserved in a large number of euryarchaeal S-layer glycoproteins (all of which possess an archaeosortase), it is very likely that this proposed lipid-anchoring mechanism is a broadly conserved euryarchaeal surface-anchoring mechanism. Most relevant for *in vivo* membrane function and for nanobiotechnological applications (see also section 'S-layer supported functional lipid membranes'), this mechanism provides a membrane anchor for S-layer glycoproteins without stuffing the lipid membrane with hydrophobic C-terminal transmembrane domains. Most recent studies even indicated that the S-layer lattice of *H. volcanii* is composed of two S-layer glycoprotein populations. One type of glycoprotein population is anchored to the membrane via the C-terminal hydrophobic transmembrane domain (Fig 2a), while the other one is lipid-modified for enabling membrane association in an EDTA-sensitive manner (Fig 2b; Kandiba *et al.*, 2013). Presently, however, little is known of how these protein-processing events affect S-layer behavior or architecture. Nevertheless, considering that S-layer lattices assembled on lipid membranes (see section 'S-layer protein–lipid interaction') induce a nanopatterned fluidity of constituent membrane lipids, the supramolecular concept involving two types of S-layer membrane anchoring mechanisms should provide organisms with more flexibility to adapt to changes in environmental conditions (e.g. temperature dependent membrane properties).

Assembly and morphogenesis

Assembly *in vivo*

Numerous *in vitro* and *in vivo* studies have been performed to elucidate the dynamic process of the incorporation and reassembly of new subunits into (closed) S-layer lattices during cell growth. The only requirement for maintaining highly ordered monomolecular arrays with no gaps on a growing cell surface is a continuous synthesis of a surplus of subunits and their translocation to sites of lattice growth. In most organisms, the rate of synthesis of S-layer protein appears to be strictly controlled because only small amounts are detectable in the growth medium. On the other hand, studies on a variety of *Bacillaceae* have demonstrated that a pool of S-layer subunits, at least sufficient for generating one complete S-layer on the cell surface, may be present in the peptidoglycan-containing cell wall matrix (Sleytr & Glauert, 1975, 1976; Breitwieser *et al.*, 1992).

Labeling experiments with fluorescent antibodies and colloidal gold/antibody marker methods showed that different strategies of S-layer lattice extensions exist for Gram-positive and Gram-negative *Bacteria* (Howard *et al.*, 1982; Smit & Todd, 1986; Gruber & Sleytr, 1988). In Gram-positive *Bacteria*, lattice growth occurs primarily by insertion of multiple bands of S-layers on the cylindrical part of the cell and at new cell poles. But the major area of S-layer incorporation is a band at the sites of an incipient cell division (Sleytr & Glauert, 1976; Gruber & Sleytr, 1988; Sleytr & Messner, 1989). This particular band splits and covers the new poles of each progeny cell. These pole areas resemble static lattice domains because little or no S-layer material is inserted at pre-existing S-layers at the poles. Further on, protein A/colloidal gold labeling showed for the hexagonal lattice on the cell surface of *Geobacillus stearothermophilus* PV72 that the new bands were helically arranged over the cylindrical surface of the cell at a pitch angle related to the orientation of the hexagonal lattice. In Gram-negative bacteria, however, S-layer lattices grow by insertion of new subunits at diffuse sites over the main cell body (Smit & Agabian, 1982). No indications of growth bands or zones were found. From the limited data available, it appears that at least in Gram-positive *Bacteria,* areas of lattice extensions superimpose areas of newly synthesized peptidoglycan.

Information concerning the development of coherent S-layer lattices on growing cell surfaces was gained by reconstitution experiments with isolated S-layer subunits on cell surfaces from which they had been removed (homologous reattachment) or on those of other organisms (heterologous reattachment; Sleytr, 1975, 1976; Sleytr & Glauert, 1976). For this purpose, two strains of taxonomical closely related hyperthermophilic *Bacteria* were used (*Thermoanaerobacter thermosaccarolyticum* and *Thermoanaerobacter thermohydrosulfurium*) showing square and hexagonal lattice symmetries, respectively. Upon dialysis of the disrupting agent (urea or GHCl, respectively), the isolated S-layer proteins of both organisms reassembled into regular arrays on the cell walls from which they had been removed. Contrary to the large regular arrays on intact cells, the crystalline patches were much smaller. An unexpected finding was that the S-layer proteins from one organism could attach to cell walls of the other one and form their patterns again. In addition, when a mixture of both S-layer protein species (with square and hexagonal lattice symmetry, respectively) was supplied, small arrays of both types were formed. These observations clearly demonstrated that the information for the dynamic lattice formation and orientation resides in the proteins themselves and is not affected by the support (Sleytr, 1975, 1976).

With the exception of selected methanogenic *Archaea* (e.g. *M. fervidus*) where the S-layer is located on the surface of a rigid pseudomurein sacculus (Nußer *et al.*, 1988; Fig. 2c) in most *Archaea*, S-layers assemble as exclusive

wall component in close association with the plasma membrane and consequently have been connected with a cell shape maintaining role (Mescher & Strominger, 1976; Messner *et al.*, 1986b; Pum *et al.*, 1991; Claus & König, 2010; Albers & Meyer, 2011). Analysis of cell morphology and lattice fault distribution provided strong evidence that the S-layers lattice is not only involved in cell shape maintenance but must also be involved in cell fission (Harris & Scriven, 1970, 1971; Nabarro & Harris, 1971; Harris, 1975, 1978). *Thermoproteus tenax*, an extremely thermophilic archaeon, has a cylindrical shape with constant diameter, but is variable in length (Messner *et al.*, 1986b; Wildhaber & Baumeister, 1987). While no dislocations could be observed on the hexagonal array covering the cylindrical part, six wedge disclinations could be visualized on each hemispherical cap. Thus, it was concluded that the elongation of the cylindrical part of the cell only requires insertion of S-layer subunits at these distinct lattice faults (Messner *et al.*, 1986b). More detailed studies on the involvement of an S-layer in cell morphology and division has been reported for *Methanocorpusculum sinense* (Pum *et al.*, 1991). The hexagonal S-layer of this highly lobed organism forms a porous but strongly interconnected network. In freeze-etched preparations of intact cells, numerous pentagons and heptagons could be detected in the hexagonal array. Complementary pairs of pentagons and heptagons were identified as the termination points of edge dislocations acting as sites for the incorporation of new morphological units into the lattice and as initiation points for the cell division process. In addition, the analysis of the number and distribution of lattice faults confirmed that the S-layer continuously recrystallizes during cell growth, maintaining an equilibrium of lowest free energy (Pum *et al.*, 1991; Sleytr *et al.*, 2005). The tension within the S-layer lattice is generated by the growth of the underlying protoplast and plasma membrane.

Assembly *in vitro*

The capability of isolated S-layer proteins to assemble into two-dimensional arrays *in vivo* and *in vitro* is one of their key properties exploited in basic and application-oriented research. It occurs upon dialysis of the disrupting agents as described before (Fig. 4). The formation of the self-assembled arrays is only determined by the amino acid sequence of the polypeptide chains, and consequently the tertiary structure of the S-layer protein species (Sleytr, 1975). As S-layer proteins have a high proportion of nonpolar amino acids, most likely, hydrophobic interactions are involved in the initial stage of the assembly process. Some S-layers are stabilized by divalent cations, such as Ca^{2+} (Pum & Sleytr, 1994, 1995b; Norville *et al.*, 2007; Baranova *et al.*, 2012) and in the case of extremely halophiles by Mg^{2+} (Mescher & Strominger, 1976; Cline *et al.*, 1989; Eichler *et al.*, 2010) interacting with acidic amino acids. Studies on the distribution of functional groups on the surface have shown that free carboxylic acid groups and amino groups are arranged in close proximity and thus contribute to the cohesion of the proteins by electrostatic interactions (Sára & Sleytr, 1987a; Sára *et al.*, 1988a; Pum *et al.*, 1989; Györvary *et al.*, 2004). S-layer proteins are noncovalently linked to each other and, in the case of their adhesion to supporting structures (e.g. silicon, metal or polymeric solid surfaces, or lipid membranes) differing combinations of weak bonds (hydrophobic bonds, ionic bonds involving divalent cations or direct interaction of polar groups, and hydrogen bonds), are responsible for the structural integrity as well. Nevertheless, disintegration and reassembly experiments led to the conclusion that the bonds holding the S-layer proteins together must be much stronger than those binding them to the support (Sleytr, 1975, 1976, 1978). Once formed, S-layer proteins were never observed to leave the lattice, and thus, it was concluded that lattice growth is irreversible and no S-layer protein turnover occurs. The reason for this irreversibility may be that after the addition of the 'last' protein monomer to the (incomplete) morphological unit, this monomer is locked into place and now has a low probability of leaving (Chung *et al.*, 2010; Comolli *et al.*, 2013) because this final conformational arrangement in 'confinement' constitutes the state of lowest free energy (Chung *et al.*, 2010).

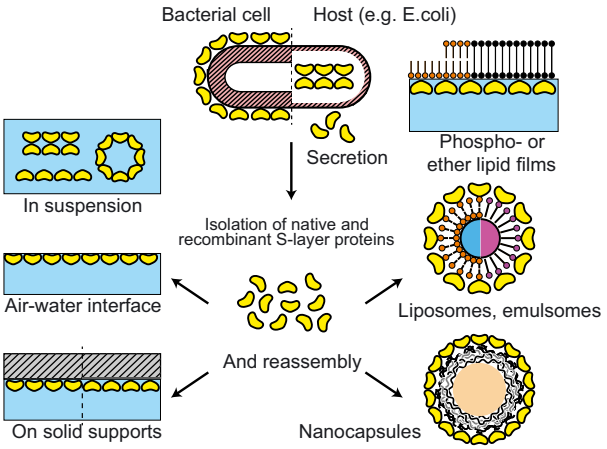

Fig. 4. Schematic drawing of the reassembly of isolated S-layer (glyco)proteins in suspension, at the air–water interface, on solid supports, on lipid films, on liposomes, emulsomes, polyelectrolyte nanocapsules, and (magnetic) beads.

Reassembly in solution

Self-assembly products are formed in solution during the dialysis of the disrupting agent against selected buffer solutions (ionic strength and pH). Monitoring the time course of self-assembly by light scattering yielded multiphasic kinetics with a rapid initial phase and slow consecutive processes of higher than second order (Jaenicke *et al.*, 1985). The rapid phase may be attributed to the formation of oligomeric precursors. Concentration-dependent light scattering measurements gave evidence for a 'critical concentration' of association, suggesting that patches of 12–16 proteins are formed and recrystallize into the final (native) S-layer structure.

Depending on the morphology and bonding properties of the S-layer proteins, either flat sheets or open-ended cylinders are formed (Messner *et al.*, 1986a; Sleytr *et al.*, 1999, 2005; Bobeth *et al.*, 2011; Sleytr *et al.*, 2011; Shin *et al.*, 2013). The self-assembly products may be composed of mono- or double layers. In addition, it was also observed that closed vesicles may be formed by S-layer proteins recrystallizing in hexagonal lattice symmetry (Sleytr, 1976; Sleytr *et al.*, 2005). In some cases, it was possible to control the self-assembly routes by changing the environmental parameters such as pH, or ionic content and strength of the subphase. In this context, one of the most detailed studied S-layer self-assembly systems is the one of *G. stearothermophilus* strain NRS 2004/3a (Messner *et al.*, 1986a). This S-layer composed of glycoproteins exhibits oblique (p2) lattice symmetry and can be extracted from the peptidoglycan by high molar GHCl. Upon dialysis, the isolated proteins assembled into both flat and cylindrical mono- and double-layer self-assembly products. Depending on the salt concentration during dialysis and dialysis duration, different self-assembly structures were formed. Generally, the presence of low concentration of bivalent cations (e.g. Ca^{2+}) led to the formation of a mixture of highly defined sheets and cylindrical self-assembly products.

Reassembly at interfaces

Crystal growth at interfaces (e.g. solid supports, air–water interface or lipid membranes) is initiated simultaneously at many randomly distributed nucleation points and proceeds in plane until the crystalline domains meet, thus leading to a closed, coherent mosaic of individual, several micro meters large S-layer domains (Pum & Sleytr, 1995a, b; Györvary *et al.*, 2003; Sleytr *et al.*, 2005). The growth of extended S-layers domains is favored at low monomer concentrations due to the corresponding low number of nucleation sites. The individual domains are mono crystalline and separated by grain boundaries.

In a recently carried out detailed study using *in situ* AFM, it was shown that the reassembly of SbpA S-layer proteins from *Lysinibacillus sphaericus* CCM2177 on mica does not necessarily follow the classical pathway of crystal growth. Instead, a kinetic trap keeping the system at a higher-energy, long-lived transient state may hinder the reassembly into extended matrices (Shin *et al.*, 2012). Over time, finally the trapped state transforms into a stable, low energy state. Careful analysis of the time and temperature dependence of formation and transformation yielded an energy difference by only 1.6 kJ mol^{-1} (or 0.7 kT). But the energy barrier to transform into the final low energy state is 38 times higher (61 kJ mol^{-1}).

The formation of coherent crystalline domains depends on the S-layer protein species used, the environmental conditions of the subphase, such as ionic content and strength, but, in particular, on the surface properties of the interface. While the reassembly of S-layer proteins at the air–water interface and at planar lipid films is well defined (Pum *et al.*, 1993; Pum & Sleytr, 1994; Weygand *et al.*, 1999, 2000, 2002), the deliberate modification of the surface properties of a solid support allows to specifically control the reassembly process (Pum & Sleytr, 1995a; Sleytr *et al.*, 1999; Györvary *et al.*, 2003; Comolli *et al.*, 2013). For example, the S-layer protein SbpA, which is currently one of the most detailed studied S-layer proteins for functionalizing solid supports, forms monolayers on hydrophobic and double layers on hydrophilic silicon supports (Györvary *et al.*, 2003; Moreno-Flores *et al.*, 2008). In addition, in comparison with hydrophilic surfaces, the layer formation is much faster on hydrophobic supports starting from many different nucleation sites and thus leading to a mosaic of small crystalline domains (2D powder). Along this line, the importance of the interplay between hydrophobic and hydrophilic regions was studied in detail by reassembling the S-layer protein SbpA on self-assembled monolayers (SAMs) on gold composed of disulfides with different end groups [hydroxyl (OH) *vs.* methyl (CH_3) groups] and lengths of the individual methylene chains (Moreno-Flores *et al.*, 2008). The formation of monolayers was observed when the hydrophobic end groups (CH_3) surmounted the hydrophilic (OH) ones. On the contrary, double S-layers were formed when hydrophilic (OH) groups superseded the hydrophobic (CH_3) end groups. The threshold for the transition between native and non-native S-layer parameters was four methylene groups. Finally, it must be noted that different lattice constants were observed on the two surfaces.

SAMs were also used to study the influence of the introduced surface chemistry (López *et al.*, 2011). The SAMs

carried CH_3, OH, carboxylic acid (COOH), or mannose ($C_6H_{12}O_6$), respectively, as terminating functional groups. It was confirmed that electrostatic interactions (COOH functional groups) induce a faster adsorption than hydrophobic (CH_3 groups) or hydrophilic (OH groups) interactions – as already shown for the reattachment on the bacterial cell (Sleytr, 1975; Mader *et al.*, 2004) and at liposomes (Küpcü *et al.*, 1995b; Mader *et al.*, 1999, 2000), polyelectrolyte nanocapsules (Toca-Herrera *et al.*, 2005), and emulsomes (Ücisik *et al.*, 2013b).

As required by technological demands, a great variety of supports, differing in their physico-chemical properties, are currently investigated. Silicon and metal surfaces are exploited for applications in nano-electronics, glasses in nano-optics, and polymeric surfaces, such as epoxy-based negative photoresists (e.g. SU-8), in microfluidics (Picher *et al.*, 2013). For example, silanization with either aminopropyltriethoxysilane or octadecyltrichlorosilane is often used in such applications to render the properties of a glass surface hydrophobic (Lopez *et al.*, 2010). AFM and Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) studies demonstrated that the S-layer protein SbpA adsorbs on aminopropyltriethoxysilane- and octadecyltrichlorosilane-modified surfaces much faster than on the native silicon dioxide rendered hydrophilic by plasma treatment (Lopez *et al.*, 2010). AFM measurements showed that the crystalline domains were much smaller on silanized substrates compared with hydrophilic silicon dioxide ones. The protein adsorption was diffusion controlled up to a threshold concentration of 0.05 mg mL^{-1} SbpA for silanized substrates and 0.07 mg mL^{-1} SbpA for silicon dioxide.

Finally, the reassembly of S-layer proteins at the air/water interface and on lipid films, and the handling of such layers by standard Langmuir Blodgett (LB) techniques, opened a broad spectrum of applications in basic and applied membrane research (Schuster & Sleytr, 2000, 2009b) (see section 'S-layer supported functional lipid membranes'). However, with respect to the reassembly of S-layer proteins *per se* at such interfaces, a detailed study using the S-layer protein SbpA as model system had been carried out on solid-supported lipid bilayers (Chung *et al.*, 2010). The reassembly of the square lattice followed a multistage, nonclassical pathway in which monomers, with extended conformation, first formed a mobile adsorbed phase from which they condensed into amorphous clusters. In a subsequent phase transition, the S-layer proteins folded into clusters of compact tetramers. In the following, crystal growth proceeded by the formation of new tetramers exclusively at cluster edges. Further studies will show how this information on S-layer recrystallization on 'model' supports will help to understand the *in vivo* assembly process in detail.

Genetics, domains, and biosynthesis

In the mid-eighties, first reports on cloning and sequencing of S-layer genes were published. The first complete S-layer gene sequence was that for the outer wall protein (OWP) from *Bacillus brevis* 47 (Tsuboi *et al.*, 1986). To date, the search term 'S-layer protein' yields more than 4000 hits in the nucleotide database of NCBI because numerous S-layer genes from *Archaea* and *Bacteria* have been sequenced and cloned (Sleytr *et al.*, 1999; Akca *et al.*, 2002; Sleytr *et al.*, 2002; Avall-Jääskeläinen & Palva, 2005; Messner *et al.*, 2010).

With the accumulation of S-layer gene sequences, screening for putative sequence identities became possible. Although S-layer proteins show low homology on the sequence level, common structural organization principles have been identified. The elucidation of the structure–function relationship of distinct segments of S-layer proteins started with the production of N- and C-terminally truncated forms which were used for recrystallization and binding studies (Jarosch *et al.*, 2001; Ilk *et al.*, 2002; Huber *et al.*, 2005). Thereby, it turned out that S-layer proteins exhibit mostly two separated morphological regions: one responsible for cell wall binding and the other required for self-assembly. The position of the cell wall-anchoring region within the protein can vary between bacterial species. Studies on a great variety of S-layer proteins from *Bacillaceae* revealed the existence of specific binding domains on the N-terminal part for sugar polymers, so-called secondary cell wall polymers (SCWPs), which are covalently linked to the peptidoglycan of the cell wall (Egelseer *et al.*, 2010).

This specific molecular interaction is often mediated by a recurring structural motif of approximately 55 amino acids, which is mostly found in triplicate at the N-terminus of S-layer proteins. These so-called S-layer homology (SLH) motifs are involved in cell wall anchoring of S-layer proteins by recognizing a distinct type of SCWP, which carries pyruvic acid residues (Ries *et al.*, 1997; Lemaire *et al.*, 1998; Chauvaux *et al.*, 1999; Ilk *et al.*, 1999; Mesnage *et al.*, 1999b, 2000; Cava *et al.*, 2004; Mader *et al.*, 2004; Rünzler *et al.*, 2004; Huber *et al.*, 2005). The need for pyruvylation was confirmed by the construction of knock-out mutants in *Bacillus anthracis* and *Thermus thermophilus* (Mesnage *et al.*, 2000; Cava *et al.*, 2004) as well as by surface plasmon resonance (SPR) spectroscopy using the S-layer protein rSbsB of *G. stearothermophilus* PV72/p2 and the corresponding SCWP (Petersen *et al.*, 2008) as binding partners (Mader *et al.*, 2004). For SbsB, the exclusive and complete

responsibility of a functional domain formed by the three SLH motifs for SCWP recognition could be confirmed, whereas for SbpA, the S-layer protein of *L. sphaericus* CCM 2177, an additional 58-amino acid-long SLH-like motif is required (Mader *et al.*, 2004; Huber *et al.*, 2005). The strong correlation between the existence of SLH motifs and the presence of the gene for the pyruvyltransferase CsaB was demonstrated once again in a very recent study (Pleschberger *et al.*, 2013). Sequencing of 8004 bp in the 5′-upstream region of the S-layer gene *sbpA* led to the identification of a novel gene cluster comprising five open reading frames (ORFs) which encode proteins involved in cell wall metabolism of *L. sphaericus* CCM 2177. The two ORFs encoding the autolysin rAbpA and the pyruvyl transferase rCsaB were cloned and expressed in *Escherichia coli*, and the recombinantly produced proteins were characterized regarding their secondary structure and their enzymatic activity (Pleschberger *et al.*, 2013).

Recently, the role of the three SLH motifs in the glycosylated S-layer protein SpaA of *Paenibacillus alvei* CCM 2051T was analyzed by site-directed mutagenesis and visualization by *in vivo* studies using homologous expression as well as *in vitro* binding assays (Janesch *et al.*, 2013). It was demonstrated that the SLH motifs of SpaA are sufficient for *in vivo* cell surface display of foreign proteins at the cell surface of *P. alvei*. Furthermore, it was shown that in *P. alvei*, SLH domains have a dual-recognition function, one for the SCWP and one for the peptidoglycan, and that cell wall anchoring of SpaA is not a prerequisite for glycosylation. The coexistence of two N-terminally located binding domains (for SCWP and peptidoglycan) was already described many years ago for the SLH domain carrying S-layer protein SbsB of *G. stearothermophilus* PV72/p2 (Sára *et al.*, 1998).

In contrast, S-layer proteins devoid of SLH motifs are anchored to different types of SCWP via their N- or C-terminal regions. Using affinity studies and SPR spectroscopy, a further main type of binding mechanism was described for *G. stearothermophilus* wild-type strains which involves a nonpyruvylated SCWP containing 2,3-diacetamido-2,3-dideoxymannuronic acid as the negatively charged component and a highly conserved N-terminal region lacking an SLH domain (Egelseer *et al.*, 1998; Schäffer *et al.*, 1999; Jarosch *et al.*, 2000, 2001; Ferner-Ortner *et al.*, 2007).

Among the S-layer proteins from Lactobacilli, which are devoid of SLH motifs, the regions important for cell wall binding and self-assembly are quite different. In the S-layer proteins SlpA of *Lactobacillus acidophilus* and CbsA *of Lactobacillus crispatus*, a putative carbohydrate-binding repeat comprising approximately the last 130 C-terminal amino acids has been identified. This is one-third of this S-layer protein and was suggested to be involved in cell wall binding (Smit *et al.*, 2001; Antikainen *et al.*, 2002). The thus far characterized cell wall ligands to which the *Lactobacillus* S-layer attaches include teichoic acids, lipoteichoic acids, and neutral polysaccharides (Avall-Jääskeläinen & Palva, 2005). In contrast, in SlpA of *Lactobacillus brevis* ATCC 8287, the domains responsible for self-assembly (C-terminal) and cell wall binding (N-terminal) are located in a reverse order compared to those in all other *Lactobacillus* S-layer proteins characterized so far (Avall-Jääskeläinen *et al.*, 2008). However, contrary to the *L. acidophilus*-group organisms, the specific cell wall component interacting with the S-layer protein in *L. brevis* ATCC 8287 was shown to be different than (lipo)teichoic acid (Avall-Jääskeläinen *et al.*, 2008).

In Gram-negative bacteria, no general S-layer-anchoring motif has been identified and the S-layer is attached with its N- or C-terminus to the lipopolysaccharide component of the outer membrane (Thomas *et al.*, 1992; Doig *et al.*, 1993; Bingle *et al.*, 1997b). For the *Caulobacter crescentus* S-layer protein RsaA, recrystallization on lipid vesicles was obtained only when the vesicles contained the specific species of *Caulobacter* smooth lipopolysaccharide that previous studies implicated as a requirement for attaching the S-layer to the cell surface (Nomellini *et al.*, 1997). The specific type of phospholipids did not appear critical; phospholipids rather different from those present in *Caulobacter* membranes or archaeal ether lipids worked equally well. However, the source of lipopolysaccharide was critical. Furthermore, efficient recrystallization and long range order could not be obtained with pure protein, although it was apparent that calcium was required for crystallization (Nomellini *et al.*, 1997).

Using selected N- or C-terminally truncated S-layer protein forms as fusion partners for foreign proteins or domains, it turned out that S-layer proteins are able to assemble into geometrically highly defined layers while incorporating a segment that has never participated in lattice formation. To date, a great variety of functional, chimeric S-layer proteins is available (Fig. 5, Table 2 and section ‘S-layers as matrix for functional molecules and nanoparticles’).

Although considerable knowledge has already been experimentally accumulated on the structure, biochemistry, assembly characteristics, and genetics of S-layer proteins, no structural model at atomic resolution was available for quite a while. Therefore, neither their tertiary structure nor exact amino acid or domain allocations in the lattices were known. A first tertiary structure prediction for an S-layer protein (SbsB from *G. stearothermophilus* PV72/p2; Sára *et al.*, 1996b) was obtained by molecular

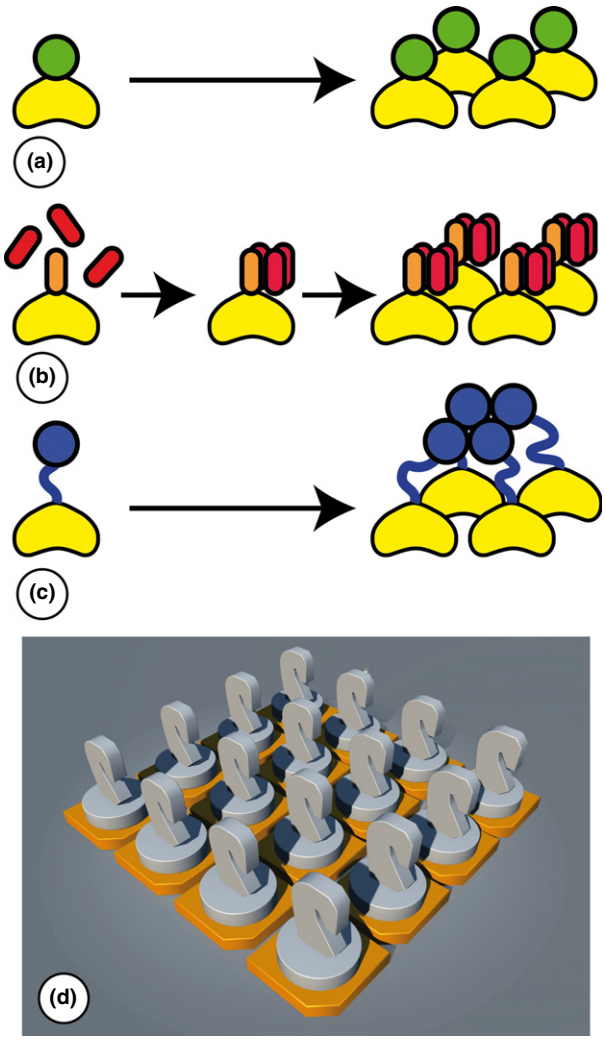

Fig. 5. Schematic drawings of mono- and oligomeric S-layer fusion proteins: (a) fusion protein with single function, (b) fusion protein acting as template for oligomeric assemblies, (c) functional domains bound via flexible linkers to S-layer proteins assemble on the surface of the S-layer lattice. (d) Cartoon illustrating self-assembled S-layer fusion proteins (see Table 2) carrying functional domains (represented as knights) in defined position and orientation (Sleytr *et al.*, 2007a, b).

dynamic simulations using the mean force method (Horejs *et al.*, 2008). For the simulation of the folding, SbsB was divided into eight structurally independent domains: three domains at the N-terminus and five domains at the C-terminus. The N-terminal domains consisted mainly of α-helices and the C-terminal ones of β-sheets. The obtained tertiary structure of SbsB showed that the N-terminus of $SbsB_{1–207}$ consists of six α-helices that are linked by turns and coils. According to secondary structure predictions and sequence similarity searches, SbsB has three SLH domains with every domain made up of two α-helices, respectively. The C-terminus of SbsB accounts for the main part of the protein. Molecular dynamics (MD) simulations performed for 30 ns (in vacuum) finally led to three main domains at the C-terminal end. The first domain is linear (aa_{208}–aa_{486}) and connects the N-terminus with the

L-shaped part of the C-terminus, which is made up of the other two domains (aa_{487}–aa_{755} and aa_{756}–aa_{920}) which are fibronectin type III and Ig-like group 2 domains, respectively. Later on, the same theoretical approach was used to predict the 3D structure of SbpA from *L. sphaericus* CCM2177 (Horejs *et al.*, 2011b).

The first high-resolution structure of a domain of a bacterial S-layer protein was obtained from X-ray studies with an assembly-negative, water-soluble, truncated form of the S-layer protein SbsC of *G. stearothermophilus* ATCC 12980 (2.4 Å resolution; Pavkov *et al.*, 2008). Despite the intrinsic property of S-layer proteins to reassemble solely in two dimensions, it turned out that this truncated form is well suited for 3D crystallization studies. The crystal structure of $rSbsC_{31–844}$ ($P2_1$ space group symmetry) revealed a novel fold, consisting of six separate domains, which are connected by short flexible linkers. Furthermore, SCWP binding induced considerable stabilization of the N-terminal domain (Pavkov *et al.*, 2008) what was later on accordingly confirmed for SbsB by AFM-based single-molecule spectroscopy (Horejs *et al.*, 2011a). To complete the structure of the full-length protein, additional soluble constructs containing the crucial domains for self-assembly were cloned, expressed, and purified (Dordic *et al.*, 2012). Currently, rebuilding and refinement of the structure is in progress and upon completion will yield the complete structure of the full-length SbsC protein.

Most recently, the full-length atomistic SbsB structure was solved by the use of nanobody-aided crystallization (Baranova *et al.*, 2012). According to this investigation, SbsB consists of seven domains formed by an amino-terminated cell wall attachment domain (SLH domain) and six consecutive immunoglobulin-like domains organized into a ϕ-shaped disk-like monomeric unit stabilized by an interdomain Ca^{2+} ion coordination. It is interesting to see that the choice of structurally meaningful parts and domain predictions used in the theoretical approach [described before (Horejs *et al.*, 2008)] are in very good agreement for the first four domains identified in this high-resolution X-ray work.

However, the first reported crystal structure of an S-layer protein from a bacterial pathogen was described for *Clostridium difficile* (Fagan *et al.*, 2009). This S-layer protein contains a low molecular weight protein (LMW) and a high molecular weight (HMW) partner. Both proteins form a tightly associated noncovalent complex, the H/L complex. The crystal structure of a

Table 2. Functional recombinant S-layer fusion proteins and their applications (modified after; Sleytr *et al.*, 2011)

Recombinant S-layer protein	Functionality	Length of function	Application	References
SbpA SbsB	Core streptavidin	118 aa	Binding of biotinylated ligands (DNA, protein), Biochip development	Moll *et al.* (2002) and Huber *et al.* (2006b)
SbpA, SbsC	Major birch pollen allergen (Bet v1)	116 aa	Vaccine development, treatment for type 1 allergy	Breitwieser *et al.* (2002) and Ilk *et al.* (2002)
SbpA	*Strep*-tag II, Affinity tag for streptavidin	9 aa	Biochip development	Ilk *et al.* (2002)
SbpA	ZZ, IgG-binding domain of Protein A	116 aa	Extracorporeal blood purification	Völlenkle *et al.* (2004)
SbpA	Enhanced green fluorescent protein (EGFP)	238 aa	Coating of liposomes, Development of drug and delivery systems	Ilk *et al.* (2004)
SbpA	cAb, Heavy chain camel antibody	117 aa	Diagnostic systems and sensing layer for label-free detection systems	Pleschberger *et al.* (2004)
SbpA	Hyperthermophilic enzyme laminarinase (LamA)	263 aa	Immobilized biocatalysts	Tschiggerl *et al.* (2008b)
SbpA	Cysteine mutants	3 aa	Building of nanoparticle arrays	Badelt-Lichtblau *et al.* (2009)
SbpA, SbsB	Mimotope of an Epstein–Barr virus (EBV) epitope (F1)	20 aa	Vaccine development	Tschiggerl *et al.* (2008a)
SbpA, SbsB	*Mycoplasma tuberculosis* antigen (mpt64)	204 aa	Vaccine development	H. Tschiggerl (pers. commun.)
SbpA	IgG-Binding domain of Protein G	110 aa	Downstream processing	Nano-S Inc. (pers. commun.)
SgsE	Glucose-1-phosphate thymidylyltransferase (RmlA)	299 aa	Immobilized biocatalysts	Schäffer *et al.* (2007)
SgsE	Enhanced cyan fluorescent protein (ECFP) Enhanced green fluorescent protein (EGFP) Yellow fluorescent protein (YFP) Monomeric red fluorescent protein (RFP1)	240 aa 240 aa 240 aa 225 aa	pH biosensors *in vivo* or *in vitro*, fluorescent markers for drug delivery systems	Kainz *et al.* (2010a, b)
SbsA	*Haemophilus influenzae* antigen (*Omp*26)	200 aa	Vaccine development	Riedmann *et al.* (2003)
SlpA	Antigenic poliovirus epitope (VP1) Human c-myc proto-oncogene	11 aa 10 aa	Development of mucosal vaccines	Avall-Jääskeläinen *et al.* (2002)
SLH-EA1, SLH-Sap	Levansucrase of *B. subtilis*	473 aa	Vaccine development	Mesnage *et al.* (1999a)
SLH-EA1	Tetanus toxin fragment C of *C. tetani* (ToxC)	451 aa	Development of live veterinary vaccines	Mesnage *et al.* (1999c)
RsaA	*Pseudomonas aeruginosa* strain K pilin	12 aa	Vaccine development	Bingle *et al.* (1997a)
RsaA	IHNV glycoprotein	184 aa	Development of vaccines against hematopoietic virus infection	Simon *et al.* (2001)
RsaA	Beta-1,4-glycanase (Cex)	485 aa	Immobilized biocatalysts	Duncan *et al.* (2005)
RsaA	IgG-binding domain of Protein G	$GB1_{xs}$	Development of immunoactive reagent	Nomellini *et al.* (2007)
RsaA	Domain 1 of HIV receptor CD4 MIP1α ligand for HIV coreceptor CCR5	81 aa 70 aa	Anti-HIV microbicide development	Nomellini *et al.* (2010)
RsaA	His-tag, Affinity tag	6 aa	Bioremediation of heavy metals (Cd) from aqueous systems, bioreactor	Patel *et al.* (2010)
RsaA	Protective coat	6 aa	Protection against antimicrobial peptide in *Caulobacter crescentus*	Patel *et al.* (2010) and de la Fuente-Núñez *et al.* (2012)

S-layer proteins: SbsB of *Geobacillus stearothermophilus* PV72/p2, SbpA of *Lysinibacillus sphaericus* CCM 2177, SbsC of *Geobacillus stearothermophilus* ATCC 12980, SgsE of *Geobacillus stearothermophilus* NRS 2004/3a, SbsA of *Bacillus stearothermophilus* PV72/p6, SlpA of *Lactobacillus brevis* ATCC 8287, SLH (SLH domain of EA1 or Sap) of *Bacillus anthracis*, RsaA of *Caulobacter crescentus* CB15A.

truncated derivative of the LMW protein was resolved down to 2.4 Å resolution and showed two domains (Fagan *et al.*, 2009).

Furthermore, using X-ray crystallography, it was shown that the three SLH domains from *B. anthracis* SAP assume the shape of a three-prong spindle (Kern *et al.*, 2011).

The first high-resolution crystal structure of an archaeal S-layer protein (2.3 Å resolution) was obtained for *Methanosarcina* species (Jing *et al.*, 2002; Arbing *et al.*, 2012) being representative for the structure of a large family of homologous archaeal *Methanosarcinaceae* proteins. While the S-layer structure reveals a protective, porous barrier, it is interesting to see that β-sandwich folds are structurally homologous to eukaryotic virus envelope proteins, suggesting that *Archaea* and viruses have found a common solution for protective envelope structures.

S-layer glycoproteins, first described in the 1970s (Table 1), were found in both domains, *Archaea* and *Bacteria* (see section 'Isolation and chemistry'). Typically, bacterial S-layer glycans are *O*-glycosidically linked to serine, threonine, or tyrosine residues, and they rely on a much wider variety of constituents, linkage types, and structures than their eukaryotic counterparts (Schäffer & Messner, 2004; Eichler & Adams, 2005; Messner *et al.*, 2008, 2009, 2010; Ristl *et al.*, 2011; Eichler & Maupin-Furlow, 2013). In the past few years, substantial progress has been made in describing the archaeal *N*-glycosylation pathway, where the glycan is linked to asparagine. Interestingly, although *Eukarya*, *Bacteria,* and *Archaea* seem to share certain features in their *N*-glycosylation pathways, the archaeal pathway is a mosaic of the eukaryal and bacterial systems (Albers & Meyer, 2011). In *Archaea* and *Bacteria*, only a single gene product (AglB and PglB, respectively) is needed for the oligosaccharyltransferase reaction, whereas in Eukarya, the oligosaccharyltransferase complex is composed of nine membrane-bound protein subunits (Albers & Meyer, 2011; Eichler & Maupin-Furlow, 2013).

For many years, due to the lack of suitable molecular tools, the understanding of the genetic basis for S-layer protein glycosylation was lagging behind the structural analyses. An important milestone was reached with the identification of the first S-layer glycosylation (*slg*) gene cluster in the bacterium *G. stearothermophilus* NRS 2004/3a (Novotny *et al.*, 2004). Until now, about 15 different S-layer glycoprotein glycan structures have been fully or partially elucidated, and several *slg* gene clusters have been identified, sequenced, and characterized (Ristl *et al.*, 2011).

In a proof-of-concept study, the transfer of the *Campylobacter jejuni* heptasaccharide and the *E. coli* O7 polysaccharide onto the SgsE S-layer protein of *G. stearothermophilus* NRS 2004/3a as well as the successful expression of the S-layer neoglycoproteins in *E. coli* could be demonstrated. The degree of glycosylation of the S-layer neoglycoproteins after purification from the periplasmic fraction reached completeness and electron microscopical investigations revealed that recombinant glycosylation is fully compatible with the S-layer protein self-assembly system (Steiner *et al.*, 2008).

Based on the fact that the two *Bacteroidales* species *Bacteroides fragilis* and *Tannerella forsythia* both have general *O*-glycosylation systems and share a common glycosylation sequon, a very recent study reports on the successful transfer of the *B. fragilis O*-glycan onto heterologously expressed *T. forsythia* proteins and vice versa (Posch *et al.*, 2013). The authors showed that 'cross-glycosylation' of proteins in *Bacteroidales* are feasible, allowing the design of novel glycoproteins. To conclude, the S-layer system is a promising strategy for multivalent glycan display approaches where strict nanometer-scale control over position and orientation of the glycan epitopes is desired.

Functional aspects

When other cell surface components (e.g. capsules, glycocalyces) are absent, S-layers as the outermost cell envelope component represent an important interface between the cell and its environment. As S-layer carrying *Bacteria* and *Archaea* are ubiquitous in the biosphere, the supramolecular concept of a closed, isoporous protein lattice represent specific adaptations to diverse environmental and ecological conditions. Most important, S-layers are generally part of complex envelope structures (Fig. 2) and consequently should not be considered as isolated layers. Several of the functions assigned to S-layers are still hypothetical and not based on firm experimental data.

Cell shape determination

Based on the fact that in most *Archaea*, the S-layer is the exclusive cell envelope component outside the cytoplasmic membrane (Albers & Meyer, 2011), it was concluded that S-layers must have a shape determining and maintaining function. This assumption was addressed in detail by studying the role of the S-layer in morphogenesis and cell division of the rod shaped *T. tenax* and *Thermoproteus neutrophilus* (Messner *et al.*, 1986b; Wildhaber & Baumeister, 1987) and the lobed archaeon *M. sinense* as described in section 'Assembly *in vivo*'; Pum *et al.*, 1991).

Surface properties and protective coats

Labeling with charged topographical markers and affinity studies revealed that S-layers from several *Bacillus* strains do not possess a net negative charge as demonstrated for the underlying peptidoglycan-containing layer or other bacterial surface structures (Sára & Sleytr, 1987a; Gruber & Sleytr, 1991; Sára *et al.*, 1992). In native S-layers, carboxyl groups are neutralized by an equal number of amino groups, leading to a charge neutral outer surface (Weigert & Sára, 1995).

For glycosylated S-layer proteins, the long carbohydrate chains were found to be exposed to the ambient environment (Sára *et al.*, 1988a, 1989; Messner & Sleytr, 1991a). Adsorption studies using whole cells which are completely covered with glycosylated S-layers revealed that they can bind to hydrophilic, hydrophobic, positively, and negatively charged materials to a comparable extent (Sára *et al.*, 1988a). In this context, data obtained on cell surface hydrophobicity of a collection of different Lactobacillus strains with and without S-layers have to be taken into account. Contact angle measurements and AFM revealed that cell surface hydrophobicity changed in response to changes in ionic strenghth offering this strains a versatile mechanism to adhere to hydrophobic and hydrophilic surfaces (Vadillo-Rodríguez *et al.*, 2004). As *Lactobacilli* can protect the host against infection by invading pathogens in the upper gastrointestinal tract and vagina, this surface properties are of great interest (Hynonen & Palva, 2013).

Interestingly, cell adhesion of the S-layer carrying strain *G. stearothermophilus* PV72 was less influenced by the environmental conditions than that of the S-layer-deficient variant T5 (Gruber & Sleytr, 1991). For the latter, hydrophobic interaction chromatography revealed a more pronounced hydrophilic surface. In the case of the pathogenic organism *Aeromonas salmonicida*, it could be demonstrated that the presence of the S-layer makes the cell surface much more hydrophobic (Trust *et al.*, 1983). S-layers are capable of interacting with particles and materials of different physiochemical properties, thereby favoring adherence of whole cells to solid surfaces. In contrast, S-layers from thermophilic Bacillaceae did not adsorb charged macromolecules on their surface or inside the pores because this would hinder the transport of nutrients and metabolites (Sára & Sleytr, 1987a; Weigert & Sára, 1995). Based on these results, S-layers can be considered as structures with excellent 'antifouling' properties.

Furthermore, S-layers have been suggested to fulfill a protective function for the living cells by excluding hostile lytic enzymes such as muramidases and proteases. However, this could only be confirmed for a few examples. The S-layer from *Sporosarcina urea* was found to protect the murein sacculus from lysozyme attack, possibly due to the presence of pores smaller than the enzyme molecules (Beveridge, 1979). This is in contrast to S-layer carrying muramidase-resistant strains of mesophilic Bacillus species which were found to have a muramidase-resistant chemically modified peptidoglycan but did not possess pores significantly smaller than strains of thermophilic *Bacillaceae* which allowed free passage of differently sized muramidases (Sára *et al.*, 1990).

S-layers from Gram-negative *Bacteria* such as *A. salmonicida*, *Campylobacter fetus*, *Aquaspirillum serpens*, and *C. crescentus* protect the cells from attack by the bacterial parasite *Bdellovibrio bacteriovorus* by masking the outer membrane components and receptors, but do not show a protective function against other predators, such as protozoa (Koval, 1993; Beveridge *et al.*, 1997).

More recently experimental data were presented which indicate that the S-layer that covers the outer membrane of *C. crescentus* is involved in protection against antimicrobial peptides present in the environment (de la Fuente-Núñez *et al.*, 2012).

A quite interesting type of protective function was reported for the S-layer lattice of *Synechococcus* (Schultze-Lam *et al.*, 1992; Schultze-Lam & Beveridge, 1994b), a cyanobacterium capable of growing in lakes with exceptionally high calcium and sulfate ion concentrations. The hexagonally ordered S-layer lattice functions as a template for fine-grain mineralization and is continuously shed from the cell surface to get rid of mineral depositions thereby maintaining basic vital processes such as growth and division as well as nutrient transport.

S-Layers related to pathogenicity

S-layers can contribute to virulence when they are present as a structural component of the cell envelope of pathogens. *Bacillus anthracis*, the etiological agent of anthrax, is capable of lethality in both animals and humans and is a biothreat of great concern (Blendon *et al.*, 2002). The surface of *B. anthracis*, the causative agent of anthrax, is unusually complex: an S-layer is present underneath a poly-γ-D-glutamic acid capsule, and the two structures are independent (Mesnage *et al.*, 1998). Two proteins, Sap (Surface array protein) and EA1 (Extractable Antigen 1), encoded by the clustered chromosomal genes *sap* and *eag*, are the S-layer components (Etienne-Toumelin *et al.*, 1995; Mesnage *et al.*, 1997). Both proteins have an N-terminal cell wall-anchoring domain consisting of three SLH motifs followed by a putative crystallization domain comprising 604 C-terminal amino acids (Mesnage *et al.*, 1999b; Candela *et al.*, 2005). During the exponential growth phase, *B. anthracis* cells are surrounded by the

Sap S-layer protein, which is replaced by the EA1 S-layer protein when the cells enter the stationary phase (Mignot *et al.*, 2002). *In vitro* translation of selected ORFs on the virulence plasmid pXO1, followed by analysis of the reactivity of the ORF products with hyperimmune anti-*B. anthracis* antisera, led to the identification of the two S-layer proteins, both carrying three SLH motifs (Ariel *et al.*, 2002). Immunoreactivity studies using a truncated S-layer protein form devoid of the SLH moiety indicated that the C-terminal segment contributes significantly to S-layer immunogenicity (Ariel *et al.*, 2002).

In a recent study, single domain antibodies (sdAbs) were isolated using a phage display library prepared from immunized llamas (Walper *et al.*, 2012). Interestingly, the protein target for all six sdAb families was determined to be the S-layer protein EA1, which is present in both vegetative cells and bacterial spores. This research demonstrates the capabilities of these sdAbs and their potential for integration into current and developing assays and biosensors (Walper *et al.*, 2012).

Also recently, evidence was provided that *B. anthracis* S-layer protein K (BslK), an SLH, and near iron transporter (NEAT) protein are surface localized and bind and transfer heme to iron-regulated surface determinant (Isd) proteins in a rapid, contact-dependent manner (Tarlovsky *et al.*, 2013). This finding suggests that the Isd system can receive heme from multiple inputs and may reflect an adaptation of *B. anthracis* to changing iron reservoirs during an infection. Understanding the mechanism of heme uptake in pathogenic *Bacteria* is important for the development of novel therapeutics to prevent and treat bacterial infections (Tarlovsky *et al.*, 2013).

Bacillus cereus G9241 is the causative agent of respiratory anthrax-like disease in humans, which is most frequently observed in welders (Callahan *et al.*, 2008). In a very recent study, Wang *et al.* (2013) showed that *B. cereus* G9241 elaborates two S-layer proteins, Sap and EA1, which are conserved relative to the S-layer proteins of *B. anthracis* but not identical to them. The S-layer and S-layer-associated proteins (BslA and BslO) of *B. cereus* G9241 are retained in the bacterial envelope in a manner requiring *csaB*, a gene responsible for adding pyruvic acid residues to the SCWP, whose sequence is virtually identical to that of *B. anthracis*. The finding that *B. cereus* G9241 *csaB* mutants cannot retain S-layer proteins and display a concomitant decrease in virulence suggests that S-layer assembly is important for the pathogenesis of this anthrax-like disease and that S-layers and S-layer-associated proteins have many different functions during infection. One of these functions is the control of the chain length of vegetative forms which represents a mechanism for bacterial escape from opsonophagocytic killing. If bacillus chain length exceeds the size of macrophages or granulocytes, *Bacteria* cannot be engulfed (Wang *et al.*, 2013).

Clostridium difficile is a frequent cause of severe, recurrent postantibiotic diarrhea and pseudomembranous colitis (Kelly & LaMont, 1998). The *C. difficile* S-layer is the predominant outer surface component which is involved in pathogen–host interactions critical to pathogenesis. S-layer proteins could mediate the binding to both the intestinal epithelial cells and some components of their extracellular matrix fibers, contributing to further tissue damage (Calabi *et al.*, 2002; Cerquetti *et al.*, 2002). Evidence was provided that the HMW subunit functions as an adhesin which mediates adherence of *C. difficile* to host cells (Calabi *et al.*, 2002). Ausiello and coworkers demonstrated the ability of *C. difficile* S-layer proteins to modulate the function of human monocytes and dendritic cells (DC) and to induce inflammatory and regulatory cytokines (Ausiello *et al.*, 2006). Thus, S-layer proteins may fine-tune the equilibrium of Th1/Th2 response and affect antibody responses. Host antibody response plays an important role in protection, in particular IgM anti-S-layer proteins have been associated with a reduced risk of recurrent *C. difficile*-associated diarrhea in humans (Drudy *et al.*, 2004). In this context, a protective effect of anti-S-layer protein serum has also been observed in a lethal hamster challenge model. The potential mechanism of action of the antiserum was shown to be through enhancement of *C. difficile* phagocytosis (O'Brien *et al.*, 2005). Therefore, the possible use of S-layer proteins in a multicomponent vaccine against *C. difficile* infections for high-risk patients can be envisaged.

It is now evident that *Lactobacilli* over a long evolutionary period have colonized the mucosa of the upper gastrointestinal tract and the vagina coexisting in mutualistic relationship with the host. With *L. acidophilus* NCFM, it was shown that the S-layer protein interacts with a major receptor on DC and that the bacterial cells regulate dendritic- and T-cell immune functions, suggesting that this probiotic bacterium could directly or indirectly interfere with pathogen-induced effects on the host immune system (Konstantinov *et al.*, 2008). As individual strains of immunomodulatory probiotic *Bacteria* (e.g. dairy Propionibacteria) possess S-layers (Lortal *et al.*, 1993), it will be interesting to study their importance in more detail (Foligné *et al.*, 2010).

Campylobacter fetus, a spiral Gram-negative bacterium, is a recognized pathogen of cattle and sheep that can also infect humans (Guerrant *et al.*, 1978; Smibert, 1978; Blaser, 1998; Thompson & Blaser, 2000). *Campylobacter fetus* may be either type A or type B based on serotype, lipopolysaccharide structure, and S-layer protein type (Dubreuil *et al.*, 1990; Blaser *et al.*, 1994; Dworkin *et al.*, 1995). The S-layer

proteins have been shown to play a critical role in *C. fetus* virulence by protecting the bacterium from phagocytosis and serum killing (Blaser *et al.*, 1988). Graham and coworkers showed that attachment to extracellular matrix components (EMC) was neither correlated with S-layer expression nor with cell surface hydrophobicity (Graham *et al.*, 2008). However, ligand immunoblots, identified the S-layer protein as a major site of fibronectin binding, and modified ECM binding assays revealed that soluble fibronectin significantly enhanced the attachment of S-layer-expressing *C. fetus* strains to other ECM components (Graham *et al.*, 2008). For further information on S-layers related to pathogenicity, see section 'S-layers for vaccine development'.

S-Layers as molecular sieves and antifouling coating

To determine the size of pores in S-layer lattices of different Bacillaceae, permeability studies were performed according to the space technique (Scherrer & Gerhard, 1971). For this purpose, native and glutaraldehyde-treated S-layer containers were prepared that resembled the shape of whole bacterial cells (Sára & Sleytr, 1987b). Native S-layer containers were composed of three adjacent layers namely the S-layer, the peptidoglycan-containing layer, and an inner S-layer attached to the inner face of the peptidoglycan layer. The latter was formed upon removal of the plasma membrane out of the pool of S-layer subunits originally entrapped in the peptidoglycan layer (Breitwieser *et al.*, 1992). To distinguish between the molecular sieving properties between the S-layer and the peptidoglycan layer, the peptidoglycan layer was digested with lysozyme. The solutions selected for the molecular sieving measurements were sugars, proteins, and dextrans of increasing molecular weights. It was clearly demonstrated that the S-layer lattices allow free passage for molecules with a molecular weight of up to 30 kD and showed sharp exclusion limits between molecular weights of 30 and 45 kD, suggesting a limiting pore diameter in the range of 3–4.5 nm which resembles the pore dimensions determined by high-resolution transmission electron microscopy (TEM) and AFM (Messner *et al.*, 2010; Pavkov-Keller *et al.*, 2011). Moreover, of great relevance in these studies was the observation that the peptidoglycan layer does not limit the passage of molecules capable of penetrating the S-layer. This information on the structure and function of different S-layers of *Bacillaceae* makes it unlikely that their S-layers have the potential to function as an effective barrier against lysogenic enzymes (Sára & Sleytr, 1987b). Most important a great variety of permeability studies on S-layers from *Bacillaceae* demonstrated that the surface and pore areas of the protein meshwork have a very low tendency for unspecific adsorption of (macro)molecules (Sleytr *et al.*, 1986; Sára & Sleytr, 1987c, 1988; Sára & Sleytr, 1993; Sára *et al.*, 1993; Sára *et al.*, 1996a). This characteristic of S-layers is seen essential for maintaining an unhindered exchange of nutrients and metabolites between the cell and its environment. In this context, it is also important to note that the S-layer in Gram-positive *Bacteria* masks the net negative charge of the peptidoglycan layer which significantly determines interactions between living cells and its environment. The information available even allows the assumption that the S-layer surface and the pore areas have excellent antifouling properties (Sára & Sleytr, 1987b; Picher *et al.*, 2013). With S-layer carrying *Lactobacillus* strains, it could be demonstrated that variations in cell surface hydrophobicity and cell adhesion to surfaces may vary upon changes in pH and ionic strength of the environment (Vadillo-Rodríguez *et al.*, 2004).

S-Layers as adhesion zone for exoenzymes

The bacterial cell wall plays a key role in the exchange of substrates between the bacterium and its surrounding environment. Because S-layer-carrying *G. stearothermophilus* strains produce large amounts of exoproteins with molecular weights above the exclusion limit of their S-layers, the role of these porous lattices with regard to exoprotein secretion and adhesion came under careful scrutiny. It was also suggested that S-layers from members of the family *Bacillaceae* could delineate a kind of periplasmic space in cell envelopes of Gram-positive organisms and consequently delay or control the release of exoenzymes (Graham *et al.*, 1991; Breitwieser *et al.*, 1992; Sturm *et al.*, 1993). Binding to the cell surface has also been reported for exoenzymes and exoproteins including the outer layer proteins OlpA and OlpB of *Clostridium thermocellum* (Fujino *et al.*, 1993; Salamitou *et al.*, 1994a, b; Lemaire *et al.*, 1995) and the extracellular enzymes xylanase XynA, pullulanase AmyB and polygalacturonate hydrolase PglA of *Thermoanaerobacterium thermosulfurigenes* EM1 (Brechtel & Bahl, 1999; May *et al.*, 2006). For the xylanase XynA, evidence could be provided that the C-terminally located SLH motifs (Engelhardt & Peters, 1998) are necessary to anchor the extracellular enzyme to the cell surface and that accessory cell wall polymers and not peptidoglycan functions as the adhesion component in the cell wall (Brechtel & Bahl, 1999).

First studies concerning the importance of the S-layer lattice with regard to exoprotein secretion were carried

out with the exoamylase-producing strain *G. stearothermophilus* DSM 2358 which indicated the putative role of the S-layer as an adhesion site for a high molecular mass amylase (HMMA; Egelseer *et al.*, 1995). Affinity experiments strongly suggested the presence of a specific recognition mechanism between the amylase molecules and S-layer protein domains either exposed on the outermost surface or inside the pores (Egelseer *et al.*, 1995). For further comparative studies, the closely related S-layer-carrying (S^+) strain *G. stearothermophilus* ATCC 12980 which is completely covered by the S-layer protein SbsC and the S-layer-deficient (S^-) variant thereof was selected as model system (Egelseer *et al.*, 1996). On the genetic level, the S^+ and the S^- strain showed similarity values of 100%, except that in the S^- variant, expression of the *sbsC* gene was found to be inhibited by the insertion of the bacterial insertion sequence (IS) element ISBst*12* (Egelseer *et al.*, 2000). On starch medium, both strains of *G. stearothermophilus* ATCC 12980 secreted two smaller amylases and one HMMA into the culture fluid (Egelseer *et al.*, 1996), but only the latter also remained cell-associated. Using heterologously produced N- or C-terminally truncated SbsC forms and the native HMMA for affinity studies, it turned out that the N-terminal part of SbsC must comprise the binding region for the exoenzyme (Jarosch *et al.*, 2001). After elucidation of the *hmma* gene sequence, the full-length rHMMA, N- or C-terminal rHMMA truncations, as well as C-terminal rHMMA fragments were heterologously produced (Ferner-Ortner-Bleckmann *et al.*, 2009). The different rHMMA forms were used either for affinity studies with rSbsC, peptidoglycan-containing sacculi, and pure peptidoglycan devoid of SCWP, or for SPR studies using $rSbsC_{31\text{-}443}$ (a truncated rSbsC form comprising the N-terminus) and isolated SCWP. On the basis of all available data, a specific binding region for each of the three cell wall components (rSbsC, SCWP, and peptidoglycan) could be identified in the C-terminal part of the rHMMA, representing the smallest regions necessary for interaction (Ferner-Ortner-Bleckmann *et al.*, 2009).

For *G. stearothermophilus* wild-type strains, changing environmental conditions led to S-layer variant formation (Sára & Sleytr, 1994; Sára *et al.*, 1996b; Egelseer *et al.*, 2000; Scholz *et al.*, 2000, 2001). During the oxygen-induced switch from the wild-type strain *G. stearothermophilus* PV72/p6 to the variant PV72/p2, not only the S-layer protein but also the type of SCWP was altered (Sára *et al.*, 1996b). However, in all variants investigated so far, the peptidoglycan-chemotype remained constant. In order to adapt to any change in the composition of the cell wall induced by altered environmental conditions and variant formation, the HMMA evolved a multifunctional binding mechanism that provides the enzyme with a great flexibility.

S-Layers as template for fine-grain mineralization and bioremediation

S-layers are a very common surface structure in *Bacteria* including *Cyanobacteria* (Smarda *et al.*, 2002). A unique ecological role could be demonstrated for the cyanobacterial S-layer of *Synechococcus* strain GL24. This bacterium was found to induce mineralization of fine-grain gypsum ($CaSO_4{\cdot}2H_2O$) and calcite ($CaCO_3$) in a fresh water lake (Thompson & Ferris, 1990). The S-layer has a hexagonal monomer arrangement and provides regularly spaced, chemically identical nucleation sites for mineral growth (Schultze-Lam *et al.*, 1992; Schultze-Lam & Beveridge, 1994a; Smarda *et al.*, 2002). Mineral formation begins within the large holes of the array when Ca^{2+} binds to negatively charged sites on the S-layer protein and is joined by SO_4^{2-}, initiating the formation of a mineral aggregate. Eventually, the S-layer becomes encrusted with mineral and is shed so that cells have a patchy appearance with respect to the location of mineralized portions of their surface. Shedding of S-layer material could be a common process of *Bacteria* to get rid of mineral depositions on their cell surface thereby maintaining basic vital processes such as growth and division as well as nutrient transport. The natural pH value of the lake (*c.* pH 7.9) promotes the formation of gypsum, but in the course of seasonal warming, further alkalization in the close microenvironment of each photosynthesizing cell pushes the solid mineral field toward the formation of stable calcite crystals in which the sulfate is replaced by carbonate. Although the involvement of cyanobacteria in the formation of calcium carbonate has been well established, microbial involvement in the formation of other carbonate minerals has not been extensively studied. In an experimental system, it could be demonstrated that *Synechococcus* mediates a similar sulfate-to-carbonate transformation when Sr^{2+} is the major divalent cation present, forming celestite and strontianite which were considered to be formed by abiogenic mechanisms such as evaporation (Schultze-Lam & Beveridge, 1994b). Due to the difficulty of examining the process of calcite nucleation on natural matrices such as *Synechococcus* S-layers, a very recent review focused on studies of nucleation at carboxyl-terminated alkane thiol SAM surfaces on noble metal substrates. To some extent, these films provide a mimic of two key features of the *Synechococcus* S-layer because they are rich in carboxyl groups that can bind Ca^{2+}, and they present an ordered array of such functional groups (De Yoreo *et al.*, 2013). The ability to form fine-grain mineral sediments may be much more common among planktonic prokaryotes endowed with S-layers than can be imagined currently (Klingl *et al.*, 2011). Because prokaryotes exist since approximately 3.5 billion

years, they could have had a major impact on global crust development.

Currently, there is much interest in the synthesis of inorganic materials using biomimetic approaches. Inspired by the process of biomineralization, the potential of S-layer proteins and their self-assembly products as catalysts, templates, and scaffolds for the generation of novel silica architectures was investigated (Göbel *et al.*, 2010). For that purpose, the S-layer protein SbpA of *L. sphaericus* CCM 2177 was used as organic template for the generation of nanostructured silica. Using tetramethoxysilane (TMOS), TEM investigations showed the formation of a nanostructured silica network resembling the S-layer lattice. QCM-D measurements of silica adsorption demonstrated that a certain amount of negatively charged sites, such as phosphate molecules or activated carboxyl groups, significantly promote the deposition of silica on the S-layer (Göbel *et al.*, 2010). Studying the formation of silicified S-layers may help to develop novel silicon-based materials with enhanced mechanical stability and optical properties (Schuster *et al.*, 2013).

Another current approach considers the use of bacterial S-layers as a potential alternative for bioremediation processes of heavy metals in field. The S-layer of *Bacillus sphaericus* JG-A12, an isolate from a uranium mining waste pile in Germany, was shown to bind high amounts of toxic metals such as U, Cu, Pd(II), Pt(II), and Au(III) (Pollmann *et al.*, 2006). Furthermore, Velásquez and coworkers determined the tolerance of different Colombian *B. sphaericus* native strains to different heavy metals and came to the conclusion that their S-layer proteins might have the ability to entrap metallic ions, either on living or dead cells (Velásquez & Dussan, 2009). In 2010, a recombinant bioremediation agent of high efficiency and low cost was developed by inserting a hexa-histidine peptide into a permissive site of the S-layer protein RsaA of the harmless, Gram-negative bacterium *C. crescentus* in order to remove cadmium from contaminated water samples (Patel *et al.*, 2010). To summarize, these special capabilities of the bacterial cells and their S-layers are highly interesting for the clean-up of contaminated waste waters, for the recovery of precious metals from wastes of the electronic industry, as well as for the production of metal nanoclusters.

Applications

Isoporous ultrafiltration membranes

Information on either the mass distribution in S-layer lattices obtained by high-resolution electron microscopy or the 'functional pore' size derived from permeability studies led to the use of isoporous protein lattices for the production of ultrafiltration membranes with very accurate molecular weight cutoffs (Sleytr & Sára, 1986; Sára & Sleytr, 1987b, c; Sára *et al.*, 1988b). S-layer ultrafiltration membranes (SUMs) were produced by depositing S-layer fragments as a coherent layer on microfiltration membranes. The mechanical and chemical stability of their composite structure is subsequently obtained by inter- and intramolecular cross-linking. The chemical and thermal resistance of these membranes was shown to be comparable to polyamide membranes. The uniformity of functional groups on both the surface and within the pore area of the S-layer lattice could be used for very accurate chemical modifications in the subnanometer range.

SUMs produced with S-layers from *Bacillus* or *Geobacillus* strains showed a molecular weight cutoff in the range of 30 to 40 kDa (Sára *et al.*, 1996a; Sleytr *et al.*, 2001). The flux of SUMs ranges from 150 to 250 L m^{-2} h^{-1} when measured at 0.2 MPa with water (Sára & Sleytr, 1988). Surface properties and molecular sieving as well as antifouling characteristics of SUMs were tuned by chemical modifications involving activation of carboxyl groups with carbodiimides and subsequently converting them with differently sized and/or charged nucleophiles (Küpcü *et al.*, 1993; Weigert & Sára, 1995; Sleytr *et al.*, 2001). In this way, depending on the specific separation processes, SUMs can be prepared with different net charges, hydrophilic or hydrophobic surface properties and separation characteristics. Most important for separation processes, in comparison with conventional ultrafiltration membranes produced by amorphous polymers, SUMs revealed an extremely low unspecific protein adsorption (membrane fouling) in buffer solutions. Because of their high stability under shear forces, SUMs have also a broad application potential as a matrix for immobilizing functional molecules (e.g. ligands, enzymes, antibodies and antigens; Weiner *et al.*, 1994a, b; Sleytr *et al.*, 2001, 2002; Sára *et al.*, 2006a, b; Egelseer *et al.*, 2010; Sleytr *et al.*, 2011). More recently, SUMs have been used as supporting and stabilizing structures for functional lipid membranes (see section 'S-layer supported functional lipid membranes').

S-Layers as matrix for functional molecules and nanoparticles

Because S-layer lattices are composed of identical protein or glycoprotein species, functional sequences introduced either by chemical modification or genetic engineering must be aligned in exact positions and orientation down to the subnanometer scale (Sleytr *et al.*, 1999, 2001, 2005; Sára *et al.*, 2006a, b; Sleytr *et al.*, 2007a; Egelseer *et al.*, 2010; Sleytr *et al.*, 2011, 2013).

Chemical modification and labeling experiments revealed that S-layer lattices possess a high density of functional

groups on the outermost surface. As in bacterial S-layers, the subunits are linked to each other and to the underlying cell envelope layer by noncovalent interactions, a stable immobilization matrix can only be obtained by cross-linking with glutardialdehyde or other intra- and intermolecular cross-linkers (Sára & Sleytr, 1989; Sára *et al.*, 1993, 1996a). For immobilization of foreign, functional molecules such as enzymes, ligands, antigens, or antibodies, free carboxylic acid groups in the S-layer protein were activated with water-soluble carbodiimide which could then react with free amino groups of the macromolecules leading to stable peptide bonds between the S-layer matrix and the immobilized protein (Sára & Sleytr, 1989; Sleytr *et al.*, 1993b; Weiner *et al.*, 1994a; Küpcü *et al.*, 1995b; Breitwieser *et al.*, 1996; Küpcü *et al.*, 1996). Independent of the type of S-layer protein originating from different Bacillaceae, large enzymes such as invertase, glucose oxidase, glucuronidase, or β-galactosidase formed a monolayer on the outer surface of the S-layer lattice (Neubauer *et al.*, 1993, 1994, 1996; Sára *et al.*, 1996a; Sleytr *et al.*, 2001). The activity of smaller enzymes retained upon immobilization strongly depended on the molecular size of the enzyme, the morphological properties of the S-layer lattice as well as the applied immobilization procedure (Küpcü *et al.*, 1995a). For enzymes such as β-glucosidase with a molecular size slightly above the pore size in the S-layer lattice, an activity loss could be prevented by introducing spacer molecules which increased the distance between the immobilized enzyme and the S-layer lattice (Küpcü *et al.*, 1995b).

Furthermore, a universal biospecific matrix for immunoassays and dipsticks could be generated by immobilizing monolayers of either protein A or streptavidin onto SUMs (Breitwieser *et al.*, 1996). SUM-based dipsticks were used for diagnosis of type I allergies, as well as for quantification of tissue type plasminogen activator (t-PA) and interleukin 8 (IL8; Sleytr & Sára, 1997; Breitwieser *et al.*, 1998; Sleytr *et al.*, 2004). Furthermore, the S-layer lattice was exploited as an immobilization matrix for a dipstick assay developed for prion diagnosis (Völkel *et al.*, 2003). SUMs were also chosen as matrix for an amperometric glucose sensor using glucose oxidase as the biologically active component (Neubauer *et al.*, 1993), and S-layer microparticles obtained by mechanical disruption of whole cells were used for the fabrication of a multienzyme biosensor for sucrose (Neubauer *et al.*, 1994).

Just recently, a lab-on-a-chip containing embedded amperometric sensors that are coated with crystalline monolayers formed by the S-layer protein SbpA of *L. sphaericus* CCM 2177 was developed to provide a continuous, stable, reliable, and accurate detection of blood glucose (Picher *et al.*, 2013). The key feature of this novel concept is the integration of a uniform bioactive S-layer with improved antifouling properties over conventional antifouling strategies that are capable of preventing blood coagulation along 'foreign' lab-on-a-chip surfaces such as glass, polydimethylsiloxane, and metal electrodes. This novel combination of biologically derived nanostructured surfaces with microchip technology constitutes a powerful new tool for multiplexed analysis of complex samples.

Several studies already demonstrated that preformed nanoparticles can be bound in regular distribution on native S-layers (Hall *et al.*, 2001; Bergkvist *et al.*, 2004; Györvary *et al.*, 2004). The pattern of bound molecules and nanoparticles reflected the size of the morphological units, the lattice symmetry, and the physicochemical properties of the array. Using electron microscopical methods, the distribution of net negatively charged domains on S-layers could be visualized after labeling with the positively charged topographical marker polycationic ferritin (Messner *et al.*, 1986b; Sleytr *et al.*, 2001).

Due to the promising results obtained with native S-layers as immobilization matrix, genetic engineering of S-layer proteins was envisaged. On the basis of recrystallization studies and surface accessibility screens with various genetically produced N- and/or C-terminally truncated forms, several bacterial S-layer proteins were selected as fusion partner for the construction of chimeric S-layer proteins. It turned out that S-layer proteins are capable of tolerating fusions with foreign proteins or domains that have never participated in lattice formation while retaining the ability to assemble into geometrically highly defined layers. The general applicability of the 'S-layer tag' to any fusion partner led to a high flexibility for variation of the functional groups. To date, a great variety of functional S-layer fusion proteins was cloned and heterologously expressed in *E. coli* or used for surface display after homologous expression (Egelseer *et al.*, 2010; Ilk *et al.*, 2011a; Sleytr *et al.*, 2011; Table 2). Using TEM and AFM as well as functional tests, it could be demonstrated that the recrystallization properties conferred by the S-layer protein moiety as well as the functionalities of the fused peptide sequences were retained in all S-layer fusion proteins. Moreover, functional proteins maintain their functionality much better on the S-layer protein matrix in comparison when being directly attached (immobilized) to solid supports.

S-layer fusion proteins incorporating either the sequence of the hypervariable region of heavy chain camel antibodies recognizing lysozyme or the prostate-specific antigen (PSA; Pleschberger *et al.*, 2003, 2004), two copies of the Fc-binding Z-domain, a synthetic analogue of the B-domain of Protein A (Völlenkle *et al.*, 2004), the major birch pollen allergen (Bet v1; Breitwieser *et al.*, 2002; Ilk *et al.*, 2002), fluorescent proteins (Ilk *et al.*, 2004; Kainz *et al.*, 2010a, b), core streptavidin (Moll *et al.*, 2002; Huber *et al.*, 2006a, b), a C-terminally fused cysteine residue for patterning of nanoparticles (Badelt-Lichtblau *et al.*, 2009), or

monomeric and multimeric enzymes from extremophiles (Schäffer *et al.*, 2007; Tschiggerl *et al.*, 2008b; Ferner-Ortner-Bleckmann *et al.*, 2013) were successfully recrystallized on various solid supports (e.g. gold chips, silicon wafers, polystyrene or magnetic beads) or on liposomes (Table 2 and Figs 4 and 5).

Based on the demonstrated suitability of the S-layer protein self-assembly system for covalent enzyme immobilization, genetic approaches were pursued to construct fusion proteins comprising S-layer proteins of Bacillaceae and enzymes from extremophiles for the development of novel biocatalysts (Schäffer *et al.*, 2007; Tschiggerl *et al.*, 2008b; Ferner-Ortner-Bleckmann *et al.*, 2011). Significant advantages for enzyme immobilization by the S-layer self-assembly system over processes based on random immobilization of sole enzymes include the requirement of only a simple, one-step incubation process for site-directed immobilization without preceding surface activation of the support. Moreover, the provision of a cushion for the enzyme through the S-layer moiety of the fusion protein prevents denaturation and consequently loss of enzyme activity upon immobilization. In a recent study, for the first time, self-assembling biocatalysts, consisting of S-layer lattices exhibiting surface exposed active multimeric extremozymes, were produced (Fig. 5; Ferner-Ortner-Bleckmann *et al.*, 2013). The challenging step forward was to use enzymes of extremophiles which are active only in the multimeric state. For proof of concept, the tetrameric enzyme xylose isomerase and the trimeric enzyme carbonic anhydrase were selected and fused via a peptide linker to the C-terminal end of the S-layer protein SbpA of *L. sphaericus* CCM 2177. The study demonstrated that the outstanding robustness and high stability of multimeric extremozymes could be combined with the unique lattice forming capability and periodicity of bacterial S-layers, thereby providing a matrix for a most accurate spatial presentation of the multimeric enzymes (Ferner-Ortner-Bleckmann *et al.*, 2013).

The design of S-layer/extremozyme fusion proteins was based on a rather similar approach chosen for the construction of S-layer/streptavidin fusion proteins allowing to arrange any biotinylated target into the regular arrays formed by the S-layer (Moll *et al.*, 2002; Huber *et al.*, 2006a, b). Core streptavidin was either fused to N- or C-terminal positions of S-layer proteins. As biologically active streptavidin occurs as tetramer, functional heterotetramers consisting of one chain fusion protein and three chains of core streptavidin were prepared by applying a special refolding procedure (Moll *et al.*, 2002). The lattice formed by the S-layer/streptavidin fusion proteins displayed streptavidin in defined repetitive spacing, capable of binding biotinylated proteins, in particular ferritin (Fig. 5b; Moll *et al.*, 2002).

In a different approach, functionalized monomolecular S-layer lattices formed by the S-layer fusion protein rSbpA/STII/Cys exhibiting highly accessible cysteine residues in a well-defined arrangement on the surface were utilized for the template-assisted patterning of gold nanoparticles (Badelt-Lichtblau *et al.*, 2009).

S-Layers for vaccine development

As surface components frequently mediate specific interactions of a pathogen with its host organism, especially S-layers of pathogenic strains are expected to have an important role in virulence (see also section 'S-layers related to pathogenicity'). For that purpose, S-layer proteins are prime candidates for vaccine development. To date, current experiments focus on the use of S-layer proteins as attenuated pathogens, as antigen/hapten carrier, as adjuvants, or as part of vaccination vesicles (Sleytr *et al.*, 1991; Messner *et al.*, 1996; Sleytr *et al.*, 2002).

Because a reproducible immobilization of peptide epitopes to common carriers which were used as monomers in solution or as dispersions of unstructured aggregates on aluminum salts could not be achieved (Brown *et al.*, 1993; Powell & Newman, 1995), the use of regularly structured S-layer self-assembly products as immobilization matrices was envisaged. Therefore, several conjugate vaccines with S-layer (glyco)proteins and carbohydrate antigens (poly- and oligosaccharides), haptens or the recombinant birch pollen allergen Bet v1 were produced which showed promising results in vaccination trials (Sleytr *et al.*, 1989; Messner *et al.*, 1992; Malcolm *et al.*, 1993a, b; Smith *et al.*, 1993; Jahn-Schmid *et al.*, 1996a, b; Messner *et al.*, 1996; Jahn-Schmid *et al.*, 1997).

In earlier studies, the crystalline surface-layer glycoproteins of *T. thermohydrosulfuricus* L111–69, *G. stearothermophilus* NRS 2004/3a and *P. alvei* CCM 2051 were used for immobilization of spacer-linked blood group A-trisaccharide and of the spacer-linked, tumor-associated T-disaccharide (Messner *et al.*, 1992). The haptens were immobilized to either the protein moiety or the glycan chains of the respective S-layer glycoproteins. The resulting conjugates were useful for assessing the application potential of haptenated surface-layer preparations as carrier/adjuvants for the induction of immunity to poorly immunogenic molecules (Messner *et al.*, 1992, 1996).

Immunization of mice with conjugates of oligosaccharide haptens and crystalline S-layers primed the animals for a strong, hapten-specific, delayed-type hypersensitivity (DTH) response (Smith *et al.*, 1993). Most important, S-layer conjugates also elicited strong antihapten DTH responses when administered by an oral/nasal route. Apparently, the natural assembly of S-layer proteins into

large, two-dimensional arrays endows them with intrinsic adjuvant properties (Smith *et al.*, 1993).

First studies concerning the applicability of S-layers as vaccine carrier for treatment of type I allergy were carried out using native or cross-linked S-layer self-assembly products and cell wall preparations from *L. sphaericus* CCM 2177 as well as *T. thermohydrosulfuricus* L111–69 and L110–69 for immobilization of recombinant major birch pollen allergen Bet v 1 (Jahn-Schmid *et al.*, 1996b). Stimulation of human allergen-specific Th2 lymphocytes with S-layer-conjugated Bet v 1 led to a modulation of the cytokine production pattern from Th2 to Th0/Th1, indicating that S-layers may be suitable carriers for immunotherapeutical vaccines for type 1 hypersensitivity. In a subsequent study, the adjuvant effect of S-layer proteins mediated by IL-12 was demonstrated (Jahn-Schmid *et al.*, 1997). In cultures of peripheral blood mononuclear cells, both S-layer protein and S-layer/Bet v 1 conjugate (but not rBet v 1) stimulated the production of high levels of IL-12, a pivotal mediator of Th1 responses (Jahn-Schmid *et al.*, 1997).

In the following years, chemical coupling procedures were replaced by genetic fusion of the major birch pollen allergen Bet v1 to bacterial S-layer proteins resulting in recombinant fusion proteins exhibiting reduced allergenicity as well as immunomodulatory capacity (Breitwieser *et al.*, 2002; Ilk *et al.*, 2002; Bohle *et al.*, 2004). This was exemplified by two S-layer/allergen fusion proteins, rSbpA/Bet v1 and rSbsC/Bet v1, carrying Bet v 1 at the C-terminus (Breitwieser *et al.*, 2002; Ilk *et al.*, 2002). Immunological studies showed that both fusion proteins displayed strongly reduced IgE binding capacity compared with free rBet v 1 and promoted the induction of allergen-specific Th0/1 cells and regulatory T cells (Bohle *et al.*, 2004; Gerstmayr *et al.*, 2007, 2009). For first studies, expression of the S-layer/allergen fusion proteins was carried out in the Gram-negative expression host *E. coli* which had to be followed by a very material and time consuming purification procedure to remove the associated endotoxin. In a more recent study, *Bacillus subtilis* 1012, a Gram-positive, nonpathogenic organism with naturally high secretory capacity, was chosen as host for expression of the pyrogen-free recombinant S-layer/allergen fusion protein rSbpA/Bet v 1 (Ilk *et al.*, 2011b). Structural and immunological investigations of the obtained fusion protein revealed that rSbpA/Bet v 1 was endotoxin-free and showed excellent recrystallization properties and immune reactivity. To conclude, for the first time, a pyrogen-free recombinant S-layer/allergen fusion protein required for vaccine development was produced using a Gram-positive expression system based on *B. subtilis* 1012 (Ilk *et al.*, 2011b).

To summarize, S-layer carrier conjugates are superior vaccine carriers because (1) they elicit DTH and immunoprotective antibody responses without the use of extraneous adjuvants, (2) they can be administered by several different immunization routes (intramuscular, subcutaneous, nasal/oral), and they are immunological unique, which means that antibody and delayed-type hypersensitivity responses to each S-layer are specific and not cross-reactive (Malcolm *et al.*, 1993b).

The mechanical and thermal stability of S-layer-coated liposomes (Küpcü *et al.*, 1998; Hianik *et al.*, 1999; Mader *et al.*, 1999) and the possibility for immobilization or entrapping biologically active molecules (Küpcü *et al.*, 1995a; Mader *et al.*, 2000; Krivanek *et al.*, 2002) introduced a broad application potential, particularly as carrier and/or drug delivery and drug-targeting systems or in gene therapy. A very recent study described a novel nanocarrier system comprising lipidic emulsomes and S-layer (fusion) proteins as functionalizing tools coating the surface (Ücisik *et al.*, 2013b). *In vitro* cell culture studies showed that S-layer coated emulsomes can be taken up by human liver carcinoma cells (HepG2) without any significant cytotoxicity. S-layer coating led to a change in the zeta potential of the emulsomes from positive to negative, thus protecting the cell from oxidative stress and cell membrane damage. By combining the high drug loading capacity of emulsomes with recombinant S-layer technology, new applications for these emulsomes in nanomedicine, especially for drug delivery and targeting, can be envisaged (Ücisik *et al.*, 2013a, b).

Bacillus anthracis spores germinate to vegetative forms in host cells and produce fatal toxins. As previously updated (see section 'S-layers related to pathogenicity'), the S-layer of *B. anthracis* is composed of two proteins, EA1 and Sap which comprise 5–10% of total cellular protein (Etienne-Toumelin *et al.*, 1995). In a former study, a recombinant *B. anthracis* strain was constructed by integrating into the chromosome a translational fusion harboring the DNA fragments encoding the SLH domain of EA1 and tetanus toxin fragment C (ToxC) of *Clostridium tetani*. The immune response to ToxC was sufficient to protect mice against tetanus toxin challenge and could be tested for the development of new live veterinary vaccines (Mesnage *et al.*, 1999c). In a very recent study, the protective effect of EA1 against anthrax was investigated (Uchida *et al.*, 2012). For that purpose, mice were intranasally immunized with recombinant EA1, followed by a lethal challenge of *B. anthracis* spores. It could be demonstrated that immunization with EA1 greatly reduced the number of bacteria in infected organs and protected the mice from lethal infection, thus suggesting that EA1 is a novel candidate for an anthrax vaccine.

The display of heterologous proteins on the cell surface of lactic acid bacteria (LAB) is an exciting and emerging research area that holds great promise for the

developments of live vaccine delivery system. In this context, the development of live mucosal vaccines using *Lactobacillus* strains carrying S-layers composed of hybrid proteins on their surface is of great interest (Hynonen & Palva, 2013). Small model peptides have already been displayed in each monomer of the S-layer of *L. brevis* ATCC 8287 and *L. acidophilus* ATCC 4356 by chromosomal integration based on homologous recombination (Avall-Jääskeläinen *et al.*, 2002; Smit *et al.*, 2002). A recent study describes a novel characteristic of the S-layer of *L. acidophilus* ATCC 4365, a GRAS status protein, because it contributes to the pathogen exclusion reported for this probiotic strain (Martinez *et al.*, 2012). The S-layer protein of this strain was shown to bind electrostatically to dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN), a cell surface adhesion factor that enhances viral entry of several virus families including HIV type 1, hepatitis C virus, Ebola virus, cytomegalovirus, Dengue virus, and SARS coronavirus and may therefore be applied as novel antiviral agent (Martinez *et al.*, 2012).

One of the greatest obstacles in developing an effective vaccine against *Aeromonas hydrophila*, an important fish pathogen in aquaculture systems, is its high heterogeneity in nature (Poobalane *et al.*, 2010). The results of the study suggested that the recombinant S-layer protein of *A. hydrophila* could be useful as a vaccine antigen to protect fish against different isolates of this pathogenic bacterium.

The S-layer proteins of *C. difficile* have been shown to be involved in gut colonization and in the adhesion process to the intestinal mucosa. Some years ago, O'Brien and coworkers showed that a passive immunization using anti-S-layer protein antibodies significantly delayed the progress of *C. difficile* infection in a lethal hamster challenge model (O'Brien *et al.*, 2005). In a subsequent study, *C. difficile* S-layer proteins were tested as a vaccine component in a series of immunization and challenge experiments with hamsters. However, none of the regimes tested conferred complete protection of animals and antibody stimulation was variable and generally modest or poor (Ni Eidhin *et al.*, 2008). In a recent, novel approach, the *C. difficile* protease Cwp84, found to be associated with the S-layer proteins, was evaluated as a vaccine antigen. Hamster immunization studies demonstrated that Cwp84 is an attractive component for inclusion in a vaccine to reduce *C. difficile* intestinal colonization in humans, which in turn may diminish the risk of *C. difficile* infection (Pechine *et al.*, 2011).

S-layer supported functional lipid membranes

The building principle of S-layer supported lipid membranes (SsLMs) is copied from the supramolecular cell envelope structure of *Archaea* (Fig. 2a and b). It is assumed that the cell envelope structure of *Archaea* is a key prerequisite for these organisms to be able to dwell under extreme environmental conditions such as temperatures up to 120 °C, pH down to 0, high hydrostatic pressure, or high salt concentrations (De Rosa, 1996; Stetter, 1999; Hanford & Peeples, 2002; Albers & Meyer, 2011). Hence, S-layers must therefore integrate the basic functions of mechanical and osmotic cell stabilization (Engelhardt, 2007). As suitable methods for disintegration of archaeal S-layer protein lattices and their reassembly into monomolecular arrays on lipid films are not yet available, S-layer proteins from Gram-positive *Bacteria* are used for the generation of SsLMs (Schuster *et al.*, 2008; Schuster & Sleytr, 2009b; Schuster *et al.*, 2010; Sleytr *et al.*, 2011). Moreover, S-layer proteins or glycoproteins can be utilized as biofunctional surfaces (Küpcü *et al.*, 1995a; Schuster *et al.*, 2008; Schuster & Sleytr, 2009b; Sleytr & Messner, 2009; Sleytr *et al.*, 2010, 2011; Ücisik *et al.*, 2013b). Disregarding emulsomes, these model lipid membranes consist either of an artificial phospholipid bilayer or a tetraetherlipid monolayer which replaces the cytoplasmic membrane and a closely associated bacterial S-layer lattice (Fig. 6). In addition, a second S-layer acting as protective molecular sieve and further stabilizing scaffold and antifouling layer can be recrystallized on the top of the previously generated SsLM (Fig. 6). These features make S-layer lattices to unique supporting architectures resulting in lipid membranes with nanopatterned fluidity and considerably extended longevity (Schuster & Sleytr, 2000; Gufler *et al.*, 2004; Schuster *et al.*, 2004; Schuster & Sleytr, 2006;

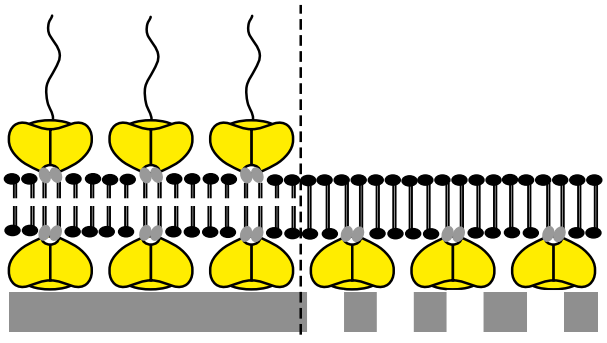

Fig. 6. Schematic drawing of a lipid membrane on S-layer (yellow) covered solid (black; left) or porous (black/white; right) supports. Some head groups of the lipid molecules within the membrane (gray) interact electrostatically with certain domains on the S-layer lattice. A further (glyco)protein S-layer lattice can be recrystallized on the outer leaflet of the lipid membrane (left). In analogy, some head groups of the lipid molecules within the membrane (gray) interact electrostatically with certain domains of the S-layer proteins. The lipid molecules on the left side depict schematically phospholipids, whereas the lipid molecules on the right side indicate ether lipids.

Schuster *et al.*, 2008; Schuster & Sleytr, 2009b; Schuster *et al.*, 2010).

SsLMs have attracted lively interest because of three main reasons: First, they constitute a versatile biomimetic model to study the characteristics of the archaeal cell envelope by a broad arsenal of surface-sensitive techniques and sophisticated microscopical methods. Second, with SsLMs, surfaces with new properties such as an antifouling characteristics for application in material science and nanomedicine can be generated. Third, SsLMs provide an amphiphilic matrix for reconstitution of (trans) membrane proteins (MPs). Hence, SsLMs may be utilized in basic research to characterize MPs. This is of paramount importance as the results of genome mapping showed that approximately one-third of all genes of an organism encode for MPs (Gerstein & Hegyi, 1998; Galdiero *et al.*, 2007, 2010) which are key factors in cell's metabolism and thus, in health and disease (Viviani *et al.*, 2007). Moreover, MPs constitute preferred targets for pharmaceuticals (at present more than 60% of consumed drugs; Ellis & Smith, 2004). Thus, SsLMs received widespread recognition in drug discovery and protein–ligand screening. In the future, the increased knowledge on MPs might allow to rebuild sensory organs, for example an artificial nose, and are of high interest for the development of biosensors based on MPs (Reimhult & Kumar, 2008; Demarche *et al.*, 2011; Srinivasan & Kumar, 2012; Tiefenauer & Demarche, 2012).

S-layer protein–lipid interaction

Formation of S-layer lattices covering the entire area of lipid films has been observed on zwitterionic phospholipids such as phosphatidyl cholines and in particular phosphatidyl ethanolamines, but not on negatively charged phospholipids (Fig. 4; Pum *et al.*, 1993; Pum & Sleytr, 1994; Diederich *et al.*, 1996; Wetzer *et al.*, 1998). Electrostatic interaction has been figured out to exist between exposed carboxyl groups on the S-layer lattice and zwitterionic or positively charged lipid head groups (Küpcü *et al.*, 1995a; Hirn *et al.*, 1999; Schuster *et al.*, 1999) At least two to three contact points between the S-layer protein and the attached lipid film have been identified (Fig. 6; Wetzer *et al.*, 1998). Hence, $< 5\%$ of the lipid molecules of the adjacent monolayer are anchored to these contact points (protein domains) on the S-layer protein. The remaining $\geq 95\%$ lipid molecules may diffuse freely within the membrane between the pillars consisting of anchored lipid molecules (Schuster, 2005; Schuster & Sleytr, 2005, 2006, 2009b). These nanopatterned lipid membranes are also referred to as 'semi-fluid membranes' (Pum & Sleytr, 1994) because of its widely retained fluid behavior (Györvary *et al.*, 1999; Hirn *et al.*, 1999). Most important, although peptide side groups of the S-layer protein interpenetrate the phospholipid head group regions almost in its entire depth, no impact on the hydrophobic lipid alkyl chains has been observed (Schuster *et al.*, 1998a, b; Weygand *et al.*, 1999, 2000, 2002; Schuster *et al.*, 2003a). To enhance the stability of the composite SsLMs, head groups of phospholipids have been covalently linked to the S-layer lattice (Schrems *et al.*, 2011a, b). Interestingly, it became evident that in nature, archaeal S-layer proteins are targeted for post-translational modifications such as the addition of a lipid (Kikuchi *et al.*, 1999; Konrad & Eichler, 2002; Szabo & Pohlschroder, 2012; Abdul Halim *et al.*, 2013). Hence, our approach to link lipids covalently to S-layer proteins is a biomimetic one as lipid modifications of S-layer glycoproteins are a general property of, for example, halophilic *Archaea*. Lipid modification of the S-layer glycoproteins takes place on the external cell surface that is following protein translocation across the membrane (Konrad & Eichler, 2002; Abdul Halim *et al.*, 2013; Kandiba *et al.*, 2013).

Planar lipid membranes

The mechanical properties of free-standing SsLMs were investigated by applying a hydrostatic pressure (Schuster & Sleytr, 2002a). SsLMs revealed a higher structural integrity when the pressure was applied from the S-layer faced side compared with plain bilayer lipid membranes (BLMs). This result supports the 'osmoprotecting effect', one putative biological function of S-layer lattices in *Archaea* (Engelhardt, 2007).

To increase the mechanical stability and longevity in particular with reconstituted peptides or MPs, the BLMs were attached to porous or solid supports to improve their practical applicability (Castellana & Cremer, 2006; Chan & Boxer, 2007; Knoll *et al.*, 2008; Reimhult & Kumar, 2008; Steinem & Janshoff, 2010).

At solid-supported lipid membranes, the task of the S-layer lattice is, beside to act as stabilizing scaffold, to provide a defined tether layer to decouple the BLM from the (inorganic) support and to generate an ionic reservoir necessary for electrochemical measurements (Schuster & Sleytr, 2000, 2009b). Moreover, the reservoir may be tailored using a mixture from full-length and truncated S-layer proteins (Schuster & Sleytr, 2009a) or by the self-assembly of a thiolated SCWP layer on the gold electrode prior S-layer protein recrystallization (Sleytr *et al.*, 2000, 2006; Schuster & Sleytr, 2009b). A very important feature of supported lipid membranes is to preserve a high degree of mobility of the lipid molecules within the membrane (fluidity) and at the same time exhibiting sound condition of the overall membrane structure (longevity).

Györvary and coworkers compared the mobility of lipid molecules of SsLMs to silane- and dextran-supported phospholipid mono- and bilayers, respectively. Most probably due to the repetitive, nanopatterned local interactions of the S-layer lattice with the lipid head groups, the fluidity of lipids was highest in SsLMs (Györvary *et al.*, 1999). Moreover, the longevity of a tetraetherlipid monolayer sandwiched by an S-layer lattice on each side revealed in comparison with other approaches (e.g. tethered membranes, polymer cushion), an exceptional long-term robustness of approximately 1 week (Schuster, 2005; Schuster & Sleytr, 2005, 2006, 2009b). This finding reflects also the optimization of the archaeal cell envelope structure by nature over billions of years.

Lipid membranes generated on a porous support combine the advantage of easy manual handling, individual excess to both membrane surfaces, and possessing an essentially unlimited ionic reservoir on each side of the BLM. The surface properties of porous supports, such as roughness or great variations in pore size, have significantly impaired the stability of attached BLMs (Nikolelis *et al.*, 1999). A straightforward approach is the use of SUMs (see section 'Isoporous ultrafiltration membranes') with the S-layer as stabilizing and smoothening biomimetic layer between the lipid membrane and the porous support (Schuster *et al.*, 2001, 2003b; Gufler *et al.*, 2004). Composite SUM-supported BLMs were found to be highly isolating structures with a life time of up to 17 h. The life time could be even significantly increased to approximately 1 day forming an S-layer–lipid membrane–S-layer sandwich-like structure on SUMs (Fig. 6; Schuster *et al.*, 2001, 2003b; Gufler *et al.*, 2004). Hence, the nanopatterned anchoring of lipids is a promising strategy for generating stable and fluid supported lipid membranes.

The most challenging property of model lipid membranes is the feasibility to incorporate membrane-active (antimicrobial) peptides (AMPs; Hancock & Chapple, 1999; Wimley & Hristova, 2011) and more important, the reconstitution of (complex) integral MPs in a functional state (Demarche *et al.*, 2011; Tiefenauer & Demarche, 2012). Table 3 summarizes the functional incorporated AMPs in SsLMs resting on gold electrodes or SUMs (Schuster *et al.*, 1998a, 2003b; Gufler *et al.*, 2004; Schrems *et al.*, 2013).

A recent study showed that the S-layer lattice of *C. crescentus* hindered positively charged AMPs in reaching its outer membrane (de la Fuente-Núñez *et al.*, 2012). Thus, the protection against APMs was proposed to be one biological function of S-layer lattices. The staphylococcal proteinaceous α-hemolysin (αHL; M_w = 33 kDa) formed lytic pores when added to the lipid-exposed side of an SsLM. However, no pore formation was detected upon addition of αHL monomers to the S-layer-faced side of this SsLM. Therefore, the intrinsic molecular sieving properties of the S-layer lattice did not allow passage of αHL monomers through the S-layer pores toward the lipid membrane which is of biological significance in competitive habitats (Schuster *et al.*, 1998b). In addition, this result confirmed the existence of a closed S-layer lattice without any defects tightly attached to the BLM. Notably, even single pore recordings have been performed

Table 3. Summary of membrane-active peptides and transmembrane proteins reconstituted in S-layer supported lipid membranes

Membrane-active peptide	Source	Remarks/References	Transmembrane protein	Source	Remarks/References
Gramicidin A (gA)	*Bacillus brevis*	Linear pentadeca peptide (Schuster *et al.*, 2003b)	α-Hemolysin (αHL)	Exotoxin from *Staphylococcus aureus*	Pore-forming; homoheptamer (Schuster *et al.*, 1998a, 2001; Schuster & Sleytr, 2002b)
Alamethicin (Ala)	*Trichoderma viride*	Linear, 20 amino acids (Gufler *et al.*, 2004)	Ryanodine receptor 1 (RyR1)	Skeletal muscle cells	Ca^{2+}-release channel; homotetramer (Larisch, 2012)
Valinomycin (Val)	Several Streptomyces strains, for example *S. tsusimaensis* and *S. fulvissimus*	Cyclic dodecadepsi peptide (Schuster *et al.*, 1998b; Gufler *et al.*, 2004)	Nicotinic acetylcholine receptor (nAChR)	Plasma membranes of neurons; on postsynaptic side of the neuromuscular junction	Ligand gated ion channel; 5 subunits (Kepplinger, 2007; Kiene, 2011)
Peptidyl-glycine-leucine-carboxyamide (PGLa) analogue	Synthesized via protein chemistry	20 amino acid; analogue negatively charged (Schrems *et al.*, 2013)	Voltage-dependent anion channel (VDAC)	Located on the outer mitochondrial membrane; produced by cell-free expression	Voltage gated; porin ion channel monomeric but can cluster (S. Damiati, pers. commun.)
			M2 segment from nAChR	Segment forms ion-conducting channel; see nAChR	Ion-conducting channel (Keizer *et al.*, 2007, 2008)

with αHL-reconstituted SsLMs (Schuster *et al.*, 2001; Schuster & Sleytr, 2002b).

Sulfolobicins, produced by several Sulfolobus strains, are an example for the interaction of proteinaceous toxins with S-layer proteins in nature. These toxins were found to be associated with cell-derived S-layer-coated vesicles resulting in no release of sulfolobicins in soluble form into the environment. These enzyme-containing vesicles of *Sulfolobus islandicus,* for example, were shown to kill cells of other strains of the same species (Prangishvili *et al.*, 2000; O'Connor & Shand, 2002).

Table 3 summarizes the pore-forming αHL (Schuster *et al.*, 1998b, 2001; Schuster & Sleytr, 2002b) and channel-forming MPs (Keizer *et al.*, 2007; Kepplinger, 2007; Keizer *et al.*, 2008; Kiene, 2011; Larisch, 2012) which have been functionally reconstituted in SsLMs resting on gold electrodes or SUMs. To sum up, the ability to act as biomimetic spacer and scaffold for composite lipid membranes with nonintrusive character on ion channel activity make S-layer proteins attractive for biosensor applications, especially those that enhance the stability of BLMs beyond the use of tethers or polymer supports (Bayley & Cremer, 2001; Sugawara & Hirano, 2005). In future, the ability to reconstitute integral membrane proteins in defined structures on, for example, sensor surfaces is one of the most important concerns in designing biomimetic sensing devices (Nikolelis *et al.*, 1999; Trojanowicz, 2001; Demarche *et al.*, 2011; Jackman *et al.*, 2012; Tiefenauer & Demarche, 2012; Schuster & Sleytr, 2013; Sleytr *et al.*, 2013).

S-layer coated liposomes and emulsomes

Unilamellar liposomes are artificially prepared spherical containers comprising of a phospholipid bilayer shell and an aqueous core (Bangham *et al.*, 1965; Tien & Ottova-Leitmannova, 2000; Cui *et al.*, 2006). In the latter, biologically active molecules such as hydrophilic drugs can be stored and transported, whereas the lipidic shell can be loaded with hydrophobic drugs. Emulsomes, however, are spherical systems with a solid fat core surrounded by phospholipid mono- and bilayer(s) (Fig. 4; Amselem *et al.*, 1994; Vyas *et al.*, 2006). Hence, emulsomes show a much higher loading capacity for lipophilic drug molecules such as curcumin for targeted drug delivery to fight against cancer and other diseases (Andresen *et al.*, 2005; Vyas *et al.*, 2006; Ücisik *et al.*, 2013a, b). Furthermore, S-layer lattices as envelope structure covering the spherical containers (Fig. 4) constitute biomimetic 'artificial virus-like particles' enabling both stabilization of the nanocarriers and presenting addressor molecules in a highly defined orientation and special distribution (Sleytr *et al.*, 2010, 2013).

When recrystallizing isolated S-layer subunits of Bacillaceae such as *G. stearothermophilus* PV72/p2 on positively charged liposomes, the S-layer is attached by its inner face (bearing a net negative charge) in an orientation identical to the lattice on intact cells (Küpcü *et al.*, 1995a; Mader *et al.*, 1999). Coating of positively charged liposomes or emulsomes with bacterial S-layer (fusion) proteins resulted in inversion of the zeta potential from an initially positive value to a negative one (Mader *et al.*, 1999; Ücisik *et al.*, 2013b). A similar behavior was observed for liposomes coated with S-layer proteins from Lactobacilli (Hollmann *et al.*, 2007).

Mader and coworkers demonstrated a much higher mechanical (shear forces, ultrasonication) and thermal stability for S-layer-coated liposomes compared with plain ones (Küpcü *et al.*, 1998; Mader *et al.*, 1999). This finding supports the notion of the high stability of archaeal cell envelope structures. Moreover, to enhance the stability, the S-layer protein on the liposome can be cross-linked (Schuster *et al.*, 2006). In addition, cross-linking can also be utilized for covalent attachment of biologically relevant macromolecules (Sleytr *et al.*, 2005, 2007a, 2010, 2013). In turn, a layer of intact liposomes can also be reversibly tethered via the specific nickel–His-tag linkage on an S-layer lattice (Kepplinger *et al.*, 2009).

S-layer-coated liposomes constitute a versatile matrix for the covalent binding of macromolecules (Küpcü *et al.*, 1995a). Biotinylation of S-layer-coated liposomes resulted in two accessible biotin residues per S-layer subunit for subsequent streptavidin binding (Mader *et al.*, 2000). By this approach, biotinylated ferritin and biotinylated anti-human IgG were attached via streptavidin to S-layer-coated liposomes. The biological activity of bound anti-human IgG was confirmed by ELISA (Mader *et al.*, 2000) and by measuring changes in ultrasound velocity (Krivanek *et al.*, 2002). Moreover, S-layer/streptavidin fusion proteins have been constructed in order to bind up to three biotinylated biomolecules per S-layer subunit in a highly defined orientation and position (Moll *et al.*, 2002).

An interesting approach is the recrystallization of the S-layer-enhanced green fluorescent protein (EGFP) fusion protein on liposomes (Ilk *et al.*, 2004). By this means, the uptake via endocytosis of S-layer/EGFP fusion protein coated liposomes into eukaryotic cells such as HeLa cells could be visualized by the intrinsic EGFP fluorescence. The most interesting advantage can be seen in co-recrystallization of, for example, S-layer/EGFP and S-layer/streptavidin fusion proteins on the same liposome. The uptake of these specially coated liposomes by target cells and the functionality of transported drugs could be investigated simultaneously without the need of any additional labels.

Likewise on liposomes, several wild-type, recombinant, and S-layer fusion proteins formed a closed S-layer lattice covering the entire surface of emulsomes composed of a solid tripalmitin core and a phospholipid shell (Ücisik *et al.*, 2013b). *In vitro* cell culture studies revealed that S-layer coated emulsomes can be up taken by HepG2 without showing any significant cytotoxicity. The utilization of S-layer fusion proteins equipped in a nanopatterned fashion by identical or diverse functions may lead to attractive nanobiotechnological and nanomedicinal applications, particularly as drug-targeting and delivery systems, as artificial virus envelopes in, for example, medicinal applications and in gene therapy (Mader *et al.*, 2000; Pum *et al.*, 2006; Schuster & Sleytr, 2009b; Ücisik *et al.*, 2013b). Finally, these biomimetic approaches are exciting examples for synthetic biology mimicking structural and functional aspects of many bacterial and archaeal cell envelopes having an S-layer lattice as outermost cell wall component (Sleytr & Beveridge, 1999; Sleytr *et al.*, 2002, 2013).

Conclusions and perspectives

Regular arrays of macromolecules were first observed about 60 years ago in electron micrographs of prokaryotic cell wall fragments and were viewed originally as a curiosity. S-layers are now recognized as one of the most common envelope surface structures in *Archaea* and *Bacteria*. The widespread occurrence and the high physiological expense of S-layers raise the question of what selection advantage S-layer carrying organisms would have in their natural and frequently highly competitive habitats. In this context, it is interesting to remember that under optimal growth conditions for *Bacteria* in continuous laboratory cultures, S-layer-deficient mutants, or variants possessing S-layers composed of (glyco)protein subunits with lower molecular mass, frequently outgrow wild-type strains (Gruber & Sleytr, 1991; Messner & Sleytr, 1992; Sára *et al.*, 1996b; Sleytr & Beveridge, 1999; Egelseer *et al.*, 2000). Moreover, if present, S-layers are also part of a more complex supramolecular envelope structure and consequently in functional terms must not be considered as isolated protein lattice. Defined domains of S-layer proteins have been identified as being involved in specific interactions with supporting cell envelope components. As S-layers are highly porous structures, some components of the supporting envelope layers such as side chains of lipopolysaccharides (in Gram-negative bacteria; Chart *et al.*, 1984), or SCWPs (in Gram-positive bacteria), may protrude through the protein meshwork. The latter may explain the phenomenon that in Bacillaceae, the expression of a different S-layer protein on the cell surface is accompanied by a change in the chemical composition of the S-layer-anchoring SCWP (Sára *et al.*, 1996b). In functional terms, such complete cell surface modifications could prevent either attachment of specific phages or delay host immune reactions in case of pathogenic organisms.

So far no general biological function has been found, and many of the functions assigned to S-layers still remain hypothetical. As S-layers cover the surface of the whole cell as coherent layers, it has been inferred that many biological functions for the layer may depend on both the completeness of the covering and the structural and physicochemical repetitive uniformity down to the subnanometer scale (Sleytr *et al.*, 2002).

A striking feature of many S-layers of *Bacteria* and *Archaea* is their excellent antifouling property. This unique characteristic was first observed in electron micrographs of freeze-etched preparations (Sleytr & Glauert, 1975; Sleytr, 1978; Sleytr & Messner, 1983; Pum *et al.*, 1991) involving ultrafast (30 000 K s^{-1}) vitrification of intact cells (Robards & Sleytr, 1985). Even when cells were harvested from complex environments or growth media containing a great variety of macromolecular components, the S-layer lattices were never masked by adsorbed molecules (Fig. 1). In this context, it has to be remembered that not only in fast frozen preparations but also in electron micrographs of negatively stained preparations the individual S-layer proteins show deviations from precise lattice positions so that high-resolution studies require digital image processing to correct the spatial distortions (Crowther & Sleytr, 1977; Saxton & Baumeister, 1982; Saxton *et al.*, 1984; Henderson *et al.*, 1986; Gil *et al.*, 2006; Pavkov-Keller *et al.*, 2011). Obviously, the different electron microscopical preparation techniques retain deviations from the ideal crystal structure created by thermal lattice vibrations. These specific S-layer properties may additionally influence interactions with molecules in close proximity and consequently the observed antifouling properties. More detailed studies on molecular interactions and permeability using isolated S-layers or S-layer ultrafiltration membranes (see section 'S-layers as molecular sieves and antifouling coatings' and 'Isoporous ultrafiltration membranes') confirmed that the surface of the lattice in *Bacteria* is charge neutral, preventing nonspecific binding of molecules and pore blocking. Moreover, in Bacillacaea, it was shown that S-layer lattices mask the net negative charge of the peptidoglycan-containing layer (Sára & Sleytr, 1987a; Gruber & Sleytr, 1991; Weigert & Sára, 1995). Data derived from antifouling zwitterionic polymer coatings on composite nanofiltration membranes can lead to the conclusion that likewise the ultra-low fouling properties of S-layers may be affected by their zwitterionic surface properties (Ji *et al.*, 2012). In zwitterionic coatings developed for many applications

that require biofouling resistance, ions alternate perfectly in the subnanometer scale between positive and negative charges preventing adsorption of naturally occurring molecules, particularly proteins. Final proof for this hypothesis will depend on structural information of S-layer lattices at atomic resolution and detailed information on the impact of an S-layer surface on the molecular organization of the adjacent water boundary structure. Most recently, the unique antifouling properties of S-layers were successfully exploited for coating microfluidic channels in lab-on-a-chip devices (Picher *et al.*, 2013; Rothbauer *et al.*, 2013).

In addition to the unique physicochemical surface properties, the repetitive topographical characteristics of S-layers should be considered as relevant features affecting hydrodynamic surface properties of cells. It is tempting to speculate that the defined roughness of S-layer surfaces determines the flow resistance of cells in natural environments. Studies on friction flows of liquids at nanopatterned interfaces have shown that the slippage of fluids at channel boundaries is greatly increased using surfaces that are patterned on the nanometer scale (Cottin-Bizonne *et al.*, 2003). With prokaryotic organisms characterized by a large surface to volume ratio, such effects should be of particular importance.

Considering the combination of antifouling properties and increased slippage, the presence of S-layers may facilitate flagella-driven cell locomotion in natural habitats (e.g. soil, mud, body fluids), thus justifying the energy expense of S-layer protein synthesis. It will be interesting to see whether S-layer glycosylation even amplifies these important boundary conditions and surface properties. We presume that besides the more specific functions identified for S-layers of different organisms (e.g. specific interactions with molecules and cell surfaces as observed in pathogens), these features may turn out to be one of the most general ones among bacterial and archaeal S-layers. Thus, antifouling properties and reduced resistance in cell locomotion might be summed up in an S-layer specific topographical 'Nano-Lotus-effect', and we should be encouraged to use different methods in surface sciences for scrutinizing this hypothesis. Experimental approaches may imply established techniques for reassembly of S-layer proteins on solid supports at macroscopic dimensions (see section 'Reassembly at interfaces'). Biomimetic approaches copying these unique S-layer surface properties could be of great technological relevance.

In those *Archaea* possessing S-layers as exclusive wall component external to the plasma membrane, there is now strong evidence that the crystalline arrays have general potential as a membrane stabilizing framework determining a nanopatterned fluidity of the lipid components (see section 'S-layer protein–lipid interactions') and consequently enabling many species of *Archaea* to dwell under most extreme environmental conditions including temperatures up to 120 °C. In this context, it is of particular relevance to remember that in *H. volcanii* S-layer lattices, two glycoprotein populations coexist regarding their association with the plasma membrane. The first presumably corresponds to S-layer glycoprotein anchored to the membrane via the C-terminal transmembrane domain, whereas the other glycoprotein population is lipid-modified (most presumably by archaetidic acid) and associated with the membrane (Kandiba *et al.*, 2013). Variations in the relative distribution of both types of populations should enable rapid changes in the membrane 'nanopatterned fluidity' and consequently facilitate adaptation of the organism to environmental changes (see also section 'Isolation and chemistry'). Furthermore, S-layers as exclusive wall component in *Archaea* appear to be involved in maintaining of cell shape and in fission processes, but more detailed studies will be required to support this notion. Presumably, many recognized and predicted functions of bacterial S-layers, such as forming a barrier against predators, as molecular sieves that exclude hazardous components and retain useful molecules in the periplasmic space and as a promoter of very specific cell adhesion to surfaces and cells co-exist in different prokaryotic organisms. An important area of future S-layers research will concern their relevance in terms of pathogenicity, immunomodulatory capacity, health beneficial (probiotic) properties (Hynonen & Palva, 2013), and virulence of organisms.

Accumulated data on the structure, chemical composition, assembly, surface, and permeability properties have clearly shown that S-layers are the simplest biological (glyco)protein membranes which have developed during evolution. Most important, S-layer morphogenesis follows the theoretically simplest mechanism for a dynamic process of assembly of a closed container composed of monomolecular arrays of identical macromolecules. As there is no theoretical possibility of forming a closed protein membrane with less redundancy of information, it is also tempting to speculate that a simple protein membrane capable of dynamic growth could have initiated a barrier membrane in an early stage of biological evolution (Sleytr & Plohberger, 1980; Pum *et al.*, 1991; Sleytr & Beveridge, 1999; Sleytr *et al.*, 2002).

Moreover, S-layers have been shown to interact specifically with a great variety of amphiphilic molecules (e.g. phospho- and ether lipids) generating more complex supramolecular membrane structures with potential for high transmembrane selectivity. It is even probable that structure–function relationships between S-layer lattices and virus capsids or animal and human virus envelopes exist (Arbing *et al.*, 2012), and we cannot rule out the

possibility that horizontal gene transfer was relevant during co-evolution of different systems. On the other hand, structures that look alike not necessarily have similar functions.

The characteristic properties of S-layers, particularly their structural and physicochemical uniformity and the spontaneous association of constituent subunits under equilibrium conditions, have led to an astonishing spectrum of applications in nano(bio)technology, synthetic biology, and biomimetics (Sleytr *et al.*, 2001; Sára *et al.*, 2006b; Egelseer *et al.*, 2008; Pum & Sleytr, 2009; Schuster & Sleytr, 2009b; Egelseer *et al.*, 2010; Göbel *et al.*, 2010; Sleytr *et al.*, 2010; Ilk *et al.*, 2011a; Sleytr *et al.*, 2011, 2013). In this context, S-layer-carrying Lactobacilli as food grade and potentially probiotic organisms will gain importance for health-related applications such as live oral vaccines. Furthermore, S-layer proteins of Lactobacilli are excellent candidates as carriers of antigens or other medically important molecules relevant for specific adhesion and immunomodulation (Hynonen & Palva, 2013).

S-layers are now recognized as versatile patterning elements for the generation of complex supramolecular structures involving other molecules such as lipids, proteins, glycans, and nucleic acids as well as inorganic materials (e.g. nanoparticles). An important line of future development concerns the combination of S-layers with planar lipid membranes, liposomes, and emulsomes (Schuster & Sleytr, 2009b; Ferner-Ortner-Bleckmann *et al.*, 2011; Ücisik *et al.*, 2013b). This biomimetic approach, copying the supramolecular principle of cell envelopes of *Archaea* or envelopes of a great variety of viruses, allows stabilizing functional lipid membranes at the macroscopic scale. Most recently, it could be demonstrated that S-layer stabilized lipid membranes can be functionalized by incorporating membrane proteins exploiting cell-free protein synthesis regimes (E.K. Sinner, B. Schuster, S. Damiati, pers. commun.). This unique possibility of significantly improving stability and life time of functional lipid membranes can also be exploited for a broad spectrum of liposome and emulsome technologies as required for drug-targeting and delivery systems, immunotherapy, and gene therapy (see section 'S-layer coated liposomes and emulsomes') and eventually may even serve in the long term as supramolecular concept for generating 'artificial life' following bottom-up strategies in synthetic biology.

Many areas of applied S-layer research will particularly be promoted by the construction of S-layer fusion proteins comprising the intrinsic self-assembly domain and a fused functional sequence (Table 2). Another interesting application for S-layers concerns tailored S-layer neoglycoproteins utilizing the recrystallization capability of the S-layer protein for the controlled and periodic surface display of 'functional' glycosylation motifs. Applications for S-layer neoglycoproteins concern receptor mimics, vaccine design, diagnostics, and drug delivery exploiting specific carbohydrate recognition (Sleytr *et al.*, 2010). Although up to now the development of applied S-layer research has focused on life sciences, in future non-life science applications (e.g. molecular electronics, nonlinear optics) will gain importance (Shenton *et al.*, 1997; Mertig *et al.*, 1999; Vyalikh *et al.*, 2004; Maslyuk *et al.*, 2008; Pum & Sleytr, 2009; Queitsch *et al.*, 2009; Sleytr *et al.*, 2013).

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