Are S-layers exoskeletons?
The basic function of protein surface layers revisited

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Abstract

Surface protein or glycoprotein layers (S-layers) are common structures of the prokaryotic cell envelope. They are either associated with the peptidoglycan or outer membrane of bacteria, and constitute the only cell wall component of many archaea. Despite their occurrence in most of the phylogenetic branches of microorganisms, the functional significance of S-layers is assumed to be specific for genera or groups of organisms in the same environment rather than common to all prokaryotes. Functional aspects have usually been investigated with isolated S-layer sheets or proteins, which disregards the interactions between S-layers and the underlying cell envelope components. This study discusses the synergistic effects in cell envelope assemblies, the hypothetical role of S-layers for cell shape formation, and the existence of a common function in view of new insights.

Keywords
bacterial cell wall, bacterial cell envelope, outer membrane, peptidoglycan, cell shape, osmotic pressure

1. Introduction

Protein surface layers (S-layers) were detected in 1952, when Houwink and LePoole (1952) looked at cell wall fragments of the bacterium *Spirillum serpens* in the electron microscope. Houwink (1956) was also the first to find S-layers in *Archaea*, i.e. in *Halobacterium salinarum* (formerly *H. halobium*), but of course could not be aware of the full impact of his discovery at that time. He offered an indirect explanation for the lack of interest from microbiologists in S-layers in the following years (Murray, 1988): neither *Escherichia coli* nor *Bacillus subtilis*, the two model organisms in bacteriology, possessed a regularly structured cell wall (Houwink, 1953). While it became obvious in the recent decades of S-layer research that 2-D protein arrays are common components of the prokaryotic cell wall, it proved to be astonishingly difficult to convincingly explain why they are needed. The purpose to find the function of S-layers led to a set of functional aspects being seen as useful or important for selected organisms, but a common function for all microbes was not obvious (Sára and Egelseer, 1996; Beveridge et al., 1997). This situation has been apparently (and resignedly) accepted. Nevertheless, S-layers have been attractive for biophysical studies and structural research, particularly regarding their perspectives for nanotechnological applications (for reviews see, e.g., Mertig et al., 1999; Sleytr et al., 1999, 2001; Debabov, 2004; Sára et al., 2006). The approaches to improve the structural insight comprise cryo-electron microscopy (Lembcke et al., 1993), scanning probe microscopy (Karrasch et al., 1994; Müller et al., 1996), and X-ray crystallography (Evrard et al., 1999). The hope was and still is to gain a new understanding of the functional basis of S-layer proteins at atomic resolution (Stetefeld et al., 2000; Jing et al., 2002). S-layers have been thoroughly investigated as isolated proteins and 2-D crystals but there are limited studies on S-layers in their natural environment. This approach is more complex but it accounts for the functional conditions of S-layers as opposed to purified protein sheets (Engelhardt and Peters, 1998; Sleytr et al., 2001).

The structural and functional aspects of S-layers have been discussed in a number of comprehensive articles (Sára and Egelseer, 1996; Beveridge et al., 1997; Engelhardt and Peters, 1998; Sára and Sleytr, 2000; Claus et al., 2005; König et al., 2007). This study is an attempt to reconsider assumptions on S-layer functions in the view of new insights and of mutual effects between cell envelope components. The focus is the role of S-layers in cell-envelope assemblies, for the maintenance and determination of cell shape, and common functional aspects.

## 2. Investigation and interpretation of S-layer functions

2.1. Investigating S-layer characteristics in the cell envelope

The majority of functional aspects were derived from structural investigations of purified S-layers, a strategy that is necessary and fruitful but incomplete. Natural S-layer functions also integrate interactions with partners in the cell envelope. The biophysical consequences of synergistic functions between S-layers and the cell membrane in archaea, and the outer membrane or the peptidogylcan in bacteria are still largely unexplored.

2.1.1. S-layer-peptidoglycan associations

S-layer-peptidoglycan associations are mainly mediated by specific contacts. The S-layer homology (SLH) domains found in S-layers of *Bacillus spp.*, *Clostridium spp.* and relatives (Engelhardt and Peters, 1998) either bind directly to the peptidoglycan (Zhao et al., 2006) or to a pyruvylated carbohydrate that is covalently linked to the peptidoglycan (Chauvaux et al., 1999; Ilk et al., 1999; Mesnage et al., 2000; Schäffer and Messner, 2005). The SLH domain is a modular protein component and occurs in other proteins as well. It is involved in the association of extracellular enzymes with Gram-positive bacteria (Bayer et al., 1998) and of outer membranes proteins in Gram-negative bacteria (Lupas et al., 1994; Engelhardt and Peters, 1998). Amino acid residues participating in the binding process were investigated by May et al. (2006). They identified an arginine residue in the conserved sequence motif TRAE that significantly contributes to or even determines binding and appears to interact with the pyruvylated cell wall component. The S-layers of *Lactobacillaceae* and of other Gram-positive bacteria lack SLH domains but possess alternative

binding sites for charged or neutral targets (Smit et al., 2001; Smit and Pouwels, 2002; Schäffer and Messner, 2005; Desvaux et al., 2006). SLH domains act efficiently as oligomers (Engelhardt and Peters, 1998; May et al., 2006) and it appears likely that every S-layer unit cell can bind at least one target molecule. Specific binding not only associates the S-layer with the cell wall but introduces attachment points for the peptidoglycan network in turn. This should have impact on the flexibility, elasticity, and dilation of the peptidoglycan layer, a functional impact of S-layers that still awaits experimental investigation.
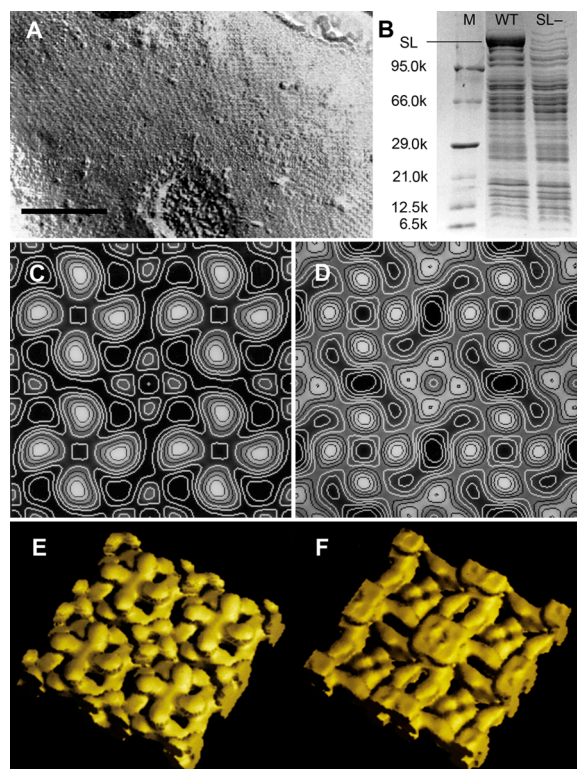
Fig. 1 Conformational changes of the S-layer from *Sporosarcina ureae*. (A) Freeze-etched and unidirectionally metal-coated (Pt) cell. Bar indicates 200 nm. (B) Polyacrylamide gel electrophoresis of total cell protein from the *S. ureae* wildtype (WT) and an S-layer-negative mutant (SL-). (M) denotes marker proteins and (SL) S-layer protein. (C) Surface-relief reconstruction from averaged unit cells of the electron micrograph depicted in A. The relief height is coded by the grey values; dark areas denote valleys. (D) Surface-relief reconstruction from the outer surface of the isolated S-layer. Note that the central and peripheral tetragonal domains are almost equal in height in contrast to the structure of the S-layer in its natural environment on the cell. (E, F) Surface-rendered model of the isolated S-layer as obtained from negatively stained preparations after 3-D reconstruction following the hybrid approach of correlation averaging and crystallographic reconstruction (Engelhardt et al., 1986), viewed from the outer (E) and the inner (F) surface. Image size 25.6 nm (C, D), lattice constant of the S-layer 12.8 nm.

The topology of S-layers unit cells in the purified and natural form, i.e. bound to the intact cell, may be different. The *Sporosarcina ureae* S-layer changes its conformation in the isolated state (Engelhardt, 1991), illustrating that the association stabilises a certain 3-D structure of the protein layer *in vivo* (Fig. 1). Since only a few S-layers have been investigated, it is difficult to assess the frequency of this phenomenon.

2.1.2. S-layer-outer membrane associations
Similar observations apply for S-layers in association with the outer membrane of Gram-negative bacteria. The protein lattices of *Aeromonas salmonicida* and *Azotobacter vinelandii* relax upon removal from the cells and by cation depletion, and change their lattice constants (Bingle et al., 1987; Dooley et al., 1989; Garduño et al., 1992). As a consequence, the porosity of the S-layers differs in the natural and isolated states.

The association of S-layers to the outer membrane is mediated by interaction via divalent cations with the charged components in the lipopolysaccharide head groups (Garduño et al., 1992; Walker et al., 1994), apparently by specific or semi-specific interactions between the N-terminal region of S-layers and particular forms of lipopolysaccharides (Dworkin et al., 1995; Nomellini et al., 1997; Ford et al., 2007), possibly by carbohydrates of glycosylated S-layer proteins (Engelhardt et al., 1990), by hydrophobic anchors, i.e. covalently bound fatty acids (Peters et al., 1987), or by the S-layer protein itself (Chami et al., 1997; Hansmeier et al., 2004). Similar to the expected situation in Gram-positive bacteria, the S-layer has an impact on the physico-chemical characteristics of the underlying cell envelope component. Although experimental data on natural systems are rare (Gerbl-Rieger et al., 1992), measurements with artificial S-layer-membrane (phospholipid) assemblies indicate possible effects (Diederich et al., 1996; Hirn et al., 1999; Küpcü et al., 1998; Mader et al., 1999; Schuster et al., 1998, 1999; Schuster and Sleytr, 2002; Sleytr et al., 2001). Here, S-layers from Gram-positive bacteria were recrystallised on homogeneous lipid monolayers or bilayers. The lipid molecules are immobilised indirectly by non-specific association to the S-layer protein whereby the membranes become less fluid, less flexible, more stable and heat-resistant, and presumably more resistant to hydrostatic pressure. The S-layer protein from

Delftia acidovorans (formerly *Comamonas acidovorans*) tightly binds LPS and can be reconstituted on dimyristoyl phosphatidylcholine (DMPC) membranes via hydrophobic anchoring (Engelhardt et al., 1991; Paul et al., 1992). Fourier-transform infrared spectroscopy revealed an altered phase transition behavior of the lipid membrane (Fig. 2), which is in qualitative accordance with the findings described for other non-specific S-layer-phospholipid assemblies (Diederich et al., 1996; Küpcü et al., 1998). The quantitative effects and functional implications for outer membranes await investigation.

Since the protein content of outer membranes can be very high (Chalcroft et al., 1987; Engelhardt et al., 1990; Kocsis et al., 1993) non-specific but also specific interactions of outer membrane and S-layer proteins are possible or even likely (Engelhardt and Peters, 1998). In contrast to small peptides, which intercalate into the head group regime of lipid membranes but do not influence the phase behavior of the lipids significantly (Weygand et al., 2002), large membrane proteins are in close contact with lipids that form a monomolecular annulus or even larger domains of immobilised lipids around the protein molecules (Jensen and Mouritsen, 2004). If outer membrane proteins were specifically immobilised by the crystalline surface protein, significant effects on the physico-chemical properties of the outer membrane could be expected in addition to possible functional interactions of the membrane and surface proteins.

2.1.3. S-layer-membrane associations
Natural association of S-layers with the cytoplasmic membrane occur in *Archaea* only. S-layers that are anchored by hydrophobic stretches of protein 'stalks' (Baumeister et al., 1989; Baumeister and Lembcke, 1992) presumably act as immobilisation structures for lipids and proteins, with currently unknown consequences for the membrane properties *in vivo* (Fig. 3). The phase characteristics of membranes containing tetraether lipids is different from that of phospholipid bilayers (Koga and Morii, 2005) but similar effects as discussed in Section 2.1.2 may be expected. Taking the dimensions of the tetrameric coiled-coil structure of the S-layer stalk from *Staphylothermus marinus* (Stetefeld et al., 2000) as a model, the range of unit cell sizes with lattice constants from 15 to 30 nm, and a projection area of ≈ 0.8 nm^2 per lipid molecule (Baba et al., 1999) suggests that up to 5% of the
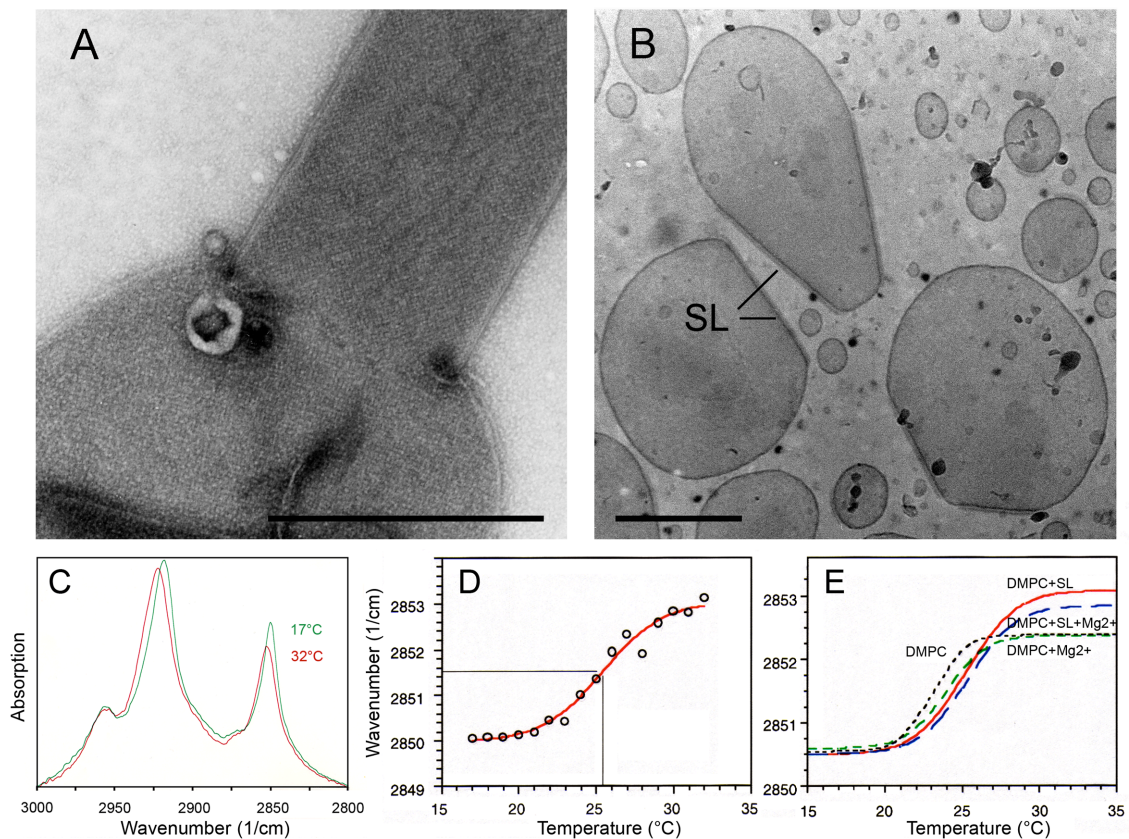
Fig. 2 Reconstituted S-layer protein from *Delftia acidovorans* on lipid vesicles. (A) Electron micrograph of a negatively stained vesicle partly shaped as a cylinder by a self-contained 2-D crystal of the S-layer. (B) Cryo-electron micrograph of frozen-hydrated vesicles partly covered with S-layer sheets. The lipid membrane made of dimyristoyl-phosphatidylcholine (DMPC) is flattened where the S-layer (SL) formed extended 2-D crystals on the surface. The reassembly products were obtained by the dialysis approach. Images are adapted from Paul et al. (1992). Scale bars indicate 0.5 μm. (C)-(E) Phase transition behavior of DMPC vesicles and S-layer-lipid assemblies. (C) Fourier-transform infrared spectra from DMPC vesicles covered by the S-layer protein from *D. acidovorans,* indicating the temperature effect on the symmetrical and asymmetrical stretching vibrations of the -CH$_2$ groups of the fatty acids. (D) Positions of the symmetrical stretching vibrations as a function of temperature. The position of the steepest slope defines the phase transition temperature. (E) Phase transition curves from DMPC vesicles (dashed line), from DMPC vesicles in the presence of Mg^{2+} that bridges the charged carboxyl groups in the DMPC head groups (dashed green curve), from vesicles with reconstituted S-layer protein (red curve), and from DMPC-S-layer assemblies in the presence of Mg^{2+} (blue curve). The S-layer is anchored by lipopolysaccharides attached to the S-layer protein. The S-layer not only shifts the phase transition temperature of DMPC but also affects the basic order of the fatty acids in the membrane as indicated by the shift to higher wavenumbers. Mg^{2+} ions only interact with the head groups and do not influence the order of fatty acid residues directly.

lipids may be immobilised by interactions with the S-layer anchor, disregarding other proteins. The important point is that the anchors, unlike common membrane proteins, do not freely float in the lipid phase.

Particularly interesting are stabilising effects of archaeal S-layers, since most of the *Archaea* and almost all of the *Crenarchaeota* possess S-layers as the sole cell wall component. It is intuitively clear that the protein layer must be the stabilising agent but experimental data are astonishingly rare (Mescher and Strominger, 1976). One obvious experimental difficulty is the lack of S-layer-negative mutants, which indicates the pivotal role of the surface protein. However, reconstituted membranes made of ether lipids and archaeal S-layers could serve as a realistic model for the cell envelope of many species. A theoretical study analyses the osmotic stability of S-layer-membrane assemblies, reveals mutual effects, and offers surprising insights into the rationale of S-layer architecture (Engelhardt, 2007). It becomes obvious that the lattice constant and symmetry of S-layers are functionally important.

2.2. Assumption: S-layers determine and maintain the cell shape

S-layers of archaea that do not possess other cell wall components are expected to maintain or even determine the cell shape (see, e.g., Sára and Egelseer, 1996). There is experimental evidence that the S-layer is shape-maintaining for halobacteria that lose their rod-like form upon removal of the surface glycoprotein or by disintegration of its lattice (Mescher and Strominger, 1976; Engelhardt, 2007), but reports on shape-determining effects are usually based on indirect conclusions.

The most impressive examples for putative shape determination are the S-layers of the thermophiles *Thermoproteus tenax* and its relatives (Messner et al., 1986a; Wildhaber and Baumeister, 1987), *Pyrobaculum* (Phipps et al., 1990), and *Thermofilum* (Stetter, 1986). *T. tenax* is rod-like up to 80 μm in length, and exhibits an apparently constant diameter ranging from 0.3 to 0.5 μm for different cells (Stetter, 1986; Zillig et al., 1981). The S-layer is very stable as attempts to dissociate isolated layers failed. The constant diameter of individual rods is consistent with the formation of self-contained 2-D lattices (Wildhaber and Baumeister, 1987). S-layers indeed have an intrinsic potential to assume a stable shape, thereby minimising the energy state, and to form cylinders upon reassembly (Messner et al., 1986b). They are even capable of reshaping lipid vesicles made of DMPC upon forming self-contained lattices (Fig. 2; Paul et al., 1992).

However, if the formation of self-contained lattices were a principle of shape determination, why then are other archaea not shaped accordingly, especially as 2-D lattices from *p*1 to *p*6 symmetries have the capacity to form self-contained cylinders (Paul et al., 1992)? The cells of *Sulfolobales* (Brock, 1981; Prüschenk et al., 1987)*, Desulfurococcales* (Wildhaber et al., 1987)*, Nanoarchaeum* (Huber et al., 2002; Briegel, 2005)*, Archaeoglobus* (Kessel et al., 1990) and of other archaea are rounded or irregularly shaped in thin-sectioned preparations. S-layers on a sphere, or on apparent half-spheres at the poles of rod-like cells, possess sites of dislocations (Harris and Scriven, 1970) and/or disclinations to cover the surface (Messner et al., 1986a; Wildhaber and Baumeister, 1987; Pum et al., 1991). S-layer proteins do have the capacity to cover both spheres and rods at the same time, as reflected by artificial reconstitution of S-layers on lipid vesicles (Paul et al., 1992; Mader et al., 1999). Fig. 2 illustrates the two modes of reassembly. Moreover, *Haloferax volcanii* and *Halobacterium salinarum* possess very similar S-layers but distinctly different cell shapes, i.e. flat to irregular and rod-like (Kessel et al., 1988; Pum et al., 1991; Trachtenberg et al., 2000). Pum et al. (1991) examined the role of S-layers with lobed cells and discussed the significance of lattice faults for growth, cell fission and shape. They observed the shape-modifying impact of lattice inclinations and suggested, in contrast to other hypotheses, a shape-determining role for S-layers of spherical, irregular and flattened cells. Taken together, it becomes evident that the inherent properties of S-layers are probably insufficient to constitute a distinct shape-determining function by themselves, beyond that of a passive shape-modifying effect. There is obviously a need for additional structural or functional ingredients.

The intriguing question is how shape-determination *in sensu stricto* is defined. Recent results from cytoskeleton research help to address the problem. The peptidoglycan has usually been assumed to determine the shape of bacteria (Beveridge, 2006). Studies with *B. subtilis* now reveal that cytoskeletal elements of the Mre system and related proteins are responsible for the definition of a typical cell form by determining the manner in which the peptidoglycan grows (Daniel and Errington, 2003; Leaver and Errington, 2005; Stewart, 2005; Carballido-López, 2006). Basically, it is the molecular machinery controlling the mode of peptidoglycan synthesis and influencing cell division that *actively* determines the shape of the murein sacculus and eventually of the cell, rather than the peptidoglycan itself that *passively* remains in the form it was given. The situation may even be more complicated with prosthecate bacteria such as *Caulobacter crescentus* (Briegel et al., 2006). Nevertheless, shape maintenance is of course an indispensible component of the mechanism of shape determination. This insight leads to a useful definition: shape determination is the result of a process, i.e. the mode of cell wall synthesis and its underlying mechanisms, while shape maintenance is an effect of the cell wall properties and depends on the stability of the respective components.

Applying this view to archaeal S-layers, we must question the location and mode of protein translocation and insertion. The fact that crystal growth takes place at crystal edges and sites of crystal faults points to the putative distribution of

the translocation machinery. Spherical archaea should therefore not exhibit distinct locations for S-layer protein transport during growth. As a consequence, the monomers are potentially available at arbitrary sites on the surface, leading to polycrystalline S-layer patterns that are not prone to reproducibly form self-contained cylindrical assemblies. A random distribution of protein translocators presumably does not enforce sophisticated mechanisms and can thus be regarded as primordial. Those cells are principally determined to be spherical but may be irregularly *shape modified* by faults in the protein lattice or become flattened by the physico-chemical properties of the S-layer-membrane system (Engelhardt, 2007).

Rod-shaped archaea, on the other hand, can be expected to organise the transport machinery in distinct regions of the cell membrane, creating ring-like growth zones of the S-layer. Now, the surface protein would assemble in a more coordinated manner, preferrably forming a cylindrical layer. The coordinated spatial distribution of membrane proteins, particularly of components of the Sec machinery, has already been observed in bacteria (Campo et a., 2004; Shiomi et al., 2006). The local organisation of protein translocation appears reasonable also from another point of view, particularly regarding the extremely long cells (80 μm) of *T. tenax* and relatives. If the S-layer protein transport occurred at any place along the cell cylinder, the molecules would either occupy most of the periplasmic space uselessly, or they could even go astray if they were not anchored to the cell membrane. The hypothesis of local (or dislocated) S-layer assembly and corresponding protein transport could be tested easily by labeling experiments (Howard et al., 1982). Cytoskeletal proteins have already been detected in *Archaea*, although their functions are still enigmatic (Löwe et al., 2004; Roeben et al., 2007). Cryo-electron tomography may help to identify corresponding cellular structures in native cells (Baumeister, 2005; Kürner et al., 2005).

The conclusion from these considerations is that S-layers may be shape-maintaining and shape-modifying but they are not *shape-determining* in a strong (process-related) sense.

2.3. Assumption: S-layers do not have a common function

None of the various functional aspects of S-layers discussed in the literature to date appear to have

Table I
Primary and secondary functions of S-layers

Primary functions	Secondary functions
Cell stabilisation: Mechanical stabilisation Thermal stabilisation Osmotic stabilisation	*Compartmentalisation:* Periplasmic space Pore-formation in conjuction with outer membrane proteins (hypothetical) *Protection against environmental factors:* Protection against particles Protection against immunological defense Protection against predators *Interaction with environment:* Ion trap, metal binding, biomineralisation matrix Protein immobilisation Phage receptor Adhesion to surfaces Specific contacts Pathogenic virulence factor

the attributes of a general function that would be important for the majority of prokaryotes (Sára and Egelseer, 1996; Beveridge et al., 1997; Sleytr et al., 2001). This arouses the suspicion that the search for a general S-layer function might end unsuccessfully. The conclusion, however, that no common functional principle exists because of missing evidence, would be illegitimate. We have certainly not judged all (known and unknown) functions in the light of all relevant criteria because we are missing a comprehensive overview of the general and specific ecological characteristics of the organisms' natural environments. This is illustrated by the fact that S-layer-negative mutants of bacteria are easily obtained in laboratory cultures and in fact outgrow the wildtype (e.g., Baldermann et al., 1998) but we have been unable to satisfactorily explain why S-layers are advantageous in the wild, with only few exceptions (Koval and Hynes, 1991). A general function, if it exists, is shared by the layers of all microorganisms. This assumption essentially considers the original functions of protein layers. However, the significance of those functions might have been modified in the course of evolution, so that they are not evident or dominating in all species today.

It is likely that protein layers stabilised early cells as primitive precursors prior to the eduction of more complex wall polymers such as peptido-glycan or pseudomurein (Dose and Rauchfuss, 1975; Sleytr and Plohberger, 1980). Since structural integrity is an absolute requirement for cells, the stabilising effect of protein layers can be regarded as more important and essentially basic compared to, e.g., protection against immunological defense that did not play a role until higher organsims came into existence. So, primary, basic, or primordial functions of S-layers can be distinguished from secondary, specific, or acquired ones. The compilation of functions in Tab. I is probably not complete, but it illustrates that the primary functions comprise features that are clearly basic to cell walls in general and originate from an inherent requirement of biological cells. Mechanical and osmotic stabilisation is a general requirement, particularly for microorganisms living in unprotected environments. A theoretical analysis of the contributions of archaeal S-layers to cell integrity and structural maintenance upon osmotic stress is given in Engelhardt (2007). Thermal stability is not a special function of the cell wall but S-layers might contribute to it mechanically. Cells experience a shift in osmotic pressure π when they are transferred from cold to boiling water ($\pi = RT \cdot \Sigma c_i$ – where c_i denotes the concentration differences of osmotically active substances). Drastic temperature changes may happen near black smokers, for instance. All basic characteristics in Tab. I refer to the compensation of forces that could jeopardise the membrane and the integrity of cells.

The specific functions reflect interactions with and adaptations to the environment. The protection against particles (e.g. macromolecules), is likely to be of importance for cells but S-layer pores are quite variable in size (Engelhardt and Peters, 1998) and do not generally protect underlying cell wall components from the attack of lytic enzymes (Sára et al., 1990). Another effect of limited porosity is the creation of a periplasmic space. The biological significance of this compartment is apparently accepted for Gram-negative bacteria but has probably been underestimated for Gram-positive bacteria and Archaea. Recent investigations by cryosectioning and cryo-electron tomography showed that B. subtilis, S. aureus, and mycobacteria, i.e. S-layer-less organisms, have evolved a periplasmic space

(Matias and Beveridge, 2005; Matias and Beveridge, 2006; Wang et al., 2000; Hoffmann et al., unpublished results). Nevertheless, the benefits of a periplasm are certainly secondary to the basic mechanical protection of cells.

Archaeal S-layers retain the primordial function in a particularly clear manner where they represent the only cell wall component. Mechanical, osmotic, and possibly thermal stabilisation of cells are indispensible achievements. It is therefore not astonishing that spontaneous S-layer-negative mutants have not been detected in Archaea (Felicitas Pfeiffer and Helmut König, personal communications). Investigations of viable S-layer-less mutant cells were especially helpful since the particular contributions of S-layers to the stability of archaeal cells are still unexplored. Despite this lack, it is apparent that S-layers possess a common function in Archaea by generally providing cell stability.

While this view is essentially not in contradiction to other discussions (Sára and Egelseer, 1996; Beveridge et al., 1997), it is not clear whether it holds true for bacterial S-layers as well. The eduction of other cell wall components such as peptidoglycan has attenuated the significance of mechanical stabilisation by S-layers but there is no obvious reason why they could not still act as structural protectants. Unfortunately, experiments addressing this question do not exist to the best of my knowlege, but indirect evidence supports a corresponding role of S-layers in Bacillus spp. at least. Beveridge et al. (1997) described the characteristics of the Gram stain and discussed the increased integrity of stained cells possessing an S-layer. Moreover, species that are devoid of an S-layer seem to have developed a particularly thick peptidoglycan. Recent investigations re-vealed a thickness of the peptidoglycan-teichoic acid network of ≈33 nm for B. subtilis (Matias and Beveridge, 2005) and 19 nm for S. aureus (Matias and Beveridge, 2006). The peptidoglycan of bacilli and clostridia possessing an S-layer is considerably thinner and measures only 3–6 nm (Beveridge and Graham, 1991). Interestingly, Bacillus anthracis and Brevibacillus brevis (formerly Bacillus brevis) even bear double S-layers (Couture-Tosi et al., 2002; Tsuboi et al., 1989), a strategy that is also observed with Aquaspirillaceae (Austin et al., 1989; Smith and Murray, 1990) and some archaea (Phipps et al., 1991; Firtel et al., 1994).
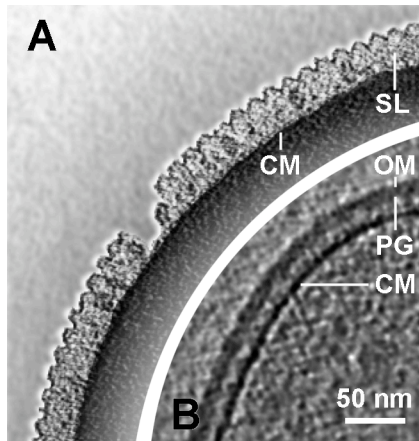
Fig. 3 Cell envelopes of an archaeon and a Gram-negative bacterium in a near-to-life state as obtained by cryo-electron tomography. (A) Section through the tomogram of an ice-embedded cell of *Pyrodictium abyssi*. The S-layer (SL) is anchored via long stalks in the cell membrane (CM) and defines the quasi-periplasmic space of the cell wall. The cytoplasmic density is removed. (B) A corresponding tomographic section through an ice-embedded cell of *Escherichia coli*, showing the cell envelope with the cell membrane (CM), the peptidoglycan (PG) and the outer membrane (OM). Note the considerable distance between the cell membrane and the peptidoglycan. The images were kindly provided by Stephan Nickell and Christian Hoffmann, Martinsried.

Gram-negative bacteria are usually characterised by a thin peptidoglycan layer (Beveridge and Graham, 1991) that compensates for the osmotic pressure since the cytoplasmic membrane is not expected to withstand higher pressure differences. The non-invasive and structure-preserving techniques of cellular cryo-electron tomography (Baumeister, 2005; Lucic et al., 2005) and cryo-electron microscopy of vitreous sections (Matias et al., 2003; Al-Amoudi et al., 2004; Zhang et al., 2004) show that the cytoplasmic membrane is clearly separated from the peptidoglycan (Fig. 3; Hoffmann et al., unpublished results). This distance and the periplasmic space between the inner and the outer membrane is exactly reflected by the ring structure of the flagellar basal body (Thomas et al., 2001) and the molecular size of the TolC-AcrB complex that bridges the periplasmic gap in *E. coli* (Murakami et al., 2002). Thus, under physiological conditions, the cell membrane is not pressed against the peptidoglycan layer and the pressure difference between cytoplasm and periplasm must be minimal. Membrane-derived charged oligosaccharides and other macromolecules are accumulated and trapped in the periplasm, account for a Donnan potential across the outer membrane (Stock et al., 1977; Miller et al., 1986; Sen et al., 1988), and serve as an osmotic buffer between the cytoplasm and the extracellular environment (Bartlett, 2000). This concept was challenged by Koch (1998), who concluded that the periplasm and the environment are almost in osmotic balance and that the cytoplasmic membrane must be pressed against the peptidoglycan under normal conditions. This assumption can now be refused by experimental evidence. Sudden hypoosmotic stress acts on the outer membrane first. Lipoproteins, OmpA-related outer membrane proteins (Baldermann et al., 1998) and periplasmic linker proteins (Engelhardt and Peters, 1998; Grizot and Buchanan, 2004) anchor the outer membrane to the peptidoglycan, which should prevent dilation in an analogous way to the S-layer-membrane assemblies in *Archaea* (Fig. 3; Engelhardt, 2007). It is highly conceivable that an S-layer sustains the outer membrane in addition (Section 2.1.2), particularly in the case of bacteria living in unprotected environments with fluctuating osmotic conditions.

Although it is less obvious, bacterial S-layers could also share a basic and common function similar to that in *Archaea,* albeit with reduced significance. There is reason to assume that S-layers contribute to or determine the mechanical and osmotic stability of prokaryotic cells.

3. Conclusion

The survey on S-layer-cell envelope interactions, the discussion on shape maintenance, and on primary and secondary functions, all share the common aspect of cell or cell wall stabilisation. This fact has escaped attention essentially because the interactions of S-layers with other components in archaeal and bacterial cell envelopes and their significance for cell stability still await more detailed investigation. Studying S-layers in association with their interacting partners promises to increase our insight into S-layer functions (Engelhardt, 2007). But is there already enough information available to answer the initial question – are S-layers exoskeletons? The answer is split. Firstly, S-layers are not exoskeletons in a sense like the cuticula for arthropodes is, giving them a characteristic and apparently invariable shape. Shape-determining elements of microorganisms appear to be of endoskeletal origin. Secondly, S-layers are exoskeletons regarding their contribution to

mechanical and osmotic cell stabilisation although, thirdly, this function is apparently not of equal importance in *Archaea* and *Bacteria* (and is still more hypothetical than proven for the latter). As a conclusion, S-layers possess characteristics of an exoskeleton but they are more like a mail shirt than a suit of armour.

Acknowledgements

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