*Molecular Biology, Vol. 38, No. 4, 2004, pp. 482–493. Translated from Molekulyarnaya Biologiya, Vol. 38, No. 4, 2004, pp. 578–591.*


**REVIEWS**

*UDC 577.1*

# Bacterial and Archaeal S-Layers as a Subject of Nanobiotechnology

**V. G. Debabov**

*State Research Center GosNIIgenetika, Moscow, 117545 Russia*
*E-mail: debabov@genetika.ru*

Received December 25, 2003

**Abstract**—Many bacteria and archaea have a crystalline surface layer (S-layer), which overlies the cell envelope. S-layers each consist of one protein or glycoprotein species. Protein subunits of the S-layer noncovalently interact with each other and with the underlying cell-envelope component. On average, the S-layer lattice has pores of 2–6 nm and is 5–10 nm high. Isolated S-layer proteins recrystallize to form two-dimensional crystalline structures in solution, on a solid support, and on planar lipid membranes. Owing to this unique property, S-layers have a broad range of applications. This review focuses on the structural features and applications of S-layers and their proteins, with special emphasis on their use in nanobiotechnology.

*Key words*: cell S-layer, nanotechnology, biosensors, vaccines

## INTRODUCTION

Nanotechnology deals with objects sized 1–1000 nm (i.e., $10^{-9}$–$10^{-6}$ m). Knowledge of how to manipulate such objects opens a way to construct fundamentally new materials and devices for molecular electronics, medicine, chemistry, and other fields of science and technology.

The importance of nanotechnology for progress in science and engineering is well understood by the people and governments of developed countries. Thus, up to 25% of the total sum assigned to science in 2003–2006 is intended for supporting nanotechnological research in the European Community.

There are two major principles of nanotechnological construction: top-down, which involves microscopic manipulation with a few atoms or molecules and generation of an ordered pattern of such structures, and bottom-up, which involves the parallel self-assembly of molecules [1, 2].

It is biological structures capable of self-assembly that are expected to play a central part in the latter case. In essence, nanobiotechnology takes advantage of the natural properties of proteins and nucleic acids, which are capable of self-organization and molecular recognition (use is apparently also made of nonnatural biopolymers such as synthetic peptides, recombinant proteins, etc.).

Biological macromolecules are several nanometers in size. The DNA double helix is about 2 nm in diameter, and its period (helix turn) is 3.4–3.6 nm; the diameter of a globular protein is 4–8 nm on average [3]. A wide-ranging branch of research, which is now developing, regards proteins and nucleic acids as subjects of materials science that are especially feasible for constructing nanostructures. This is due to the natural properties of such macromolecules. For instance, it is possible to construct linear, two-dimensional, and three-dimensional DNA structures by linking complementary sticky ends [2]. Such nanostructures may be used as a basis to obtain nanowires or ordered nanogratings [4].

Self-assembly of protein subunits is common in nature. Examples are provided by virus capsids, bacteriophages, pili, bacterial flagella, etc. Less known are surface layers (S-layers), which are ordered two-dimensional protein structures involved in the envelope of some bacteria and archaea. This review focuses on the natural distribution and properties of S-layers and on the possibility of employing S-layers or their components in nanobiotechnology.

## GENERAL CHARACTERIZATION OF S-LAYERS

The S-layer is the outermost component of the bacterial cell envelope, overlying the cell wall and completely covering the cell. Each S-layer consists of a single protein (a glycoprotein), which has a well-ordered pattern (two-dimensional crystal). This structure was first observed in bacteria of the genus *Spirillum* in 1953 [5]. In the following 20 years, similar structures were found on the surface of several other bacteria, although they were still considered to be rare and unusual for microorganisms. Extensive studies of the 1970s and 1980s showed that S-layers occur in bacteria and archaea of all genera examined and even

0026-8933/04/3804-0482 

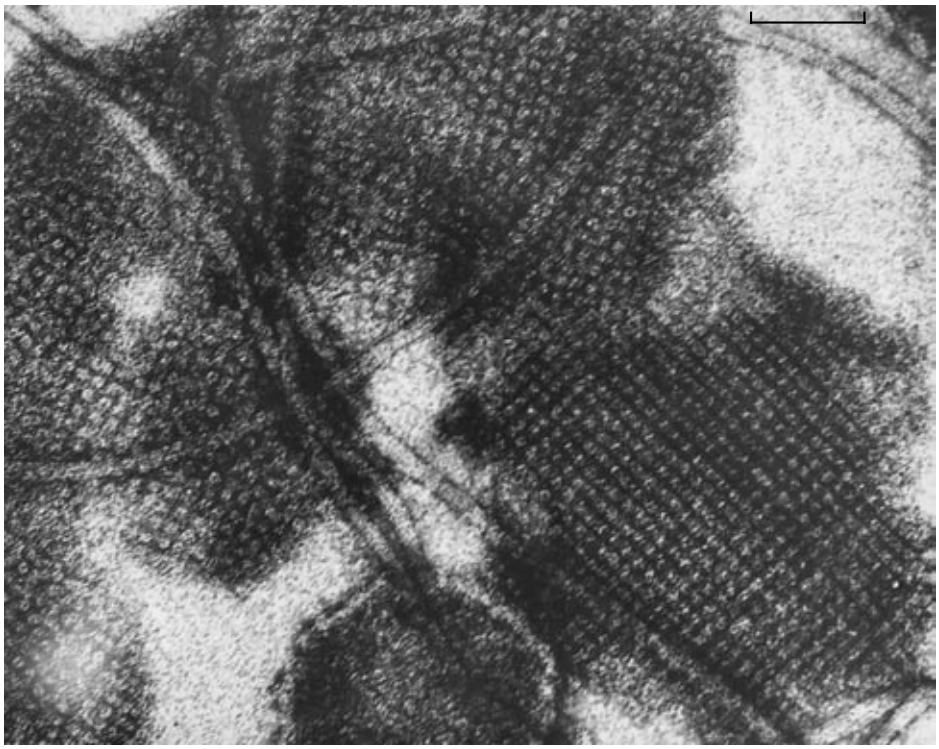

**Fig. 1.** Electron microscopic image of the *Bacillus sphearicus* S-layer. The preparation was stained with uranyl acetate. The distance between subunit centers is 8 nm; the pore size is 2 nm. Bar, 100 nm. (The photograph was kindly provided by T.A. Smirnova and R.R. Azizbekyan, State Research Center GosNIIgenetika).

represent the only rigid cover in Gram-negative archaea. By 2000, S-layers were described for 600 microbial species, 10% of all characterized bacteria and archaea. Comprehensive information on S-layers is available from reviews [5–8]. The broad distribution of S-layers among microorganisms points to their important role in the cell life. However, the S-layer is often lost when cells are cultured under laboratory conditions, indicating that the role is not vital.

Electron microscopy is the major method for detecting and studying the S-layer, making it possible to determine the arrangement of protein subunits, to analyze the general geometric pattern, to estimate the pore size, etc. (Fig. 1) [7–10]. Atomic force microscopy recently came to be employed in characterizing the S-layer [11–13]. The S-layer crystal lattice may have oblique (p1, p2), tetragonal (p4), or hexagonal (p3, p6) symmetry. Depending on the pattern, a morphological unit of the S-layer consists of one, two, three, four, or six protein subunits. The distance between subunit centers varies from 2.2 to 35 nm, and the S-layer height is 5–25 nm. Pore size varies from 2 to 8 nm, and different pores may occur in one S-layer (Fig. 2). Pores may account for up to 70% of the S-layer area. The two sides of the S-layer differ: the outward side is commonly more hydrophilic, whereas the side adjacent to the cell wall is more hydrophobic [14]. Protein subunits are noncovalently bound in the S-layer. Hydrogen bond-disrupting agents (high concentrations of acidic guanidine or urea) cause the dissociation of the S-layer into subunits [4, 6, 12–16].

A remarkable feature is that protein subunits of S-layers are capable of recrystallization when a denaturing agent is removed. It is this feature—the ability of subunits to reproduce S-layers on the cell surface, at the water–air interface, on lipid membranes, on metal or silica surfaces, etc.—that provides the basis for using S-layers in nanobiotechnology [6, 8, 14, 15, 17].

## S-LAYER PROTEINS

With a few exceptions, all bacteria and archaea have an S-layer consisting of one protein (glycoprotein) species. S-layer proteins vary in molecular weight from 40 to 200 kDa. Most S-layer proteins are weakly acidic (pI 3–5) and contain 50–60% hydrophobic residues and almost no sulfur-containing residues. In spite of these common characteristics, homology between S-layer proteins is extremely low. By now more than 40 genes for S-layer proteins have been cloned and sequenced from various microorganisms [8]. Yet X-ray quality crystals were not obtained and the exact spatial structure was not established for any of the S-layer proteins.

Many S-layer proteins are glycosylated, having a sugar residue at Tyr (O-glycosylation) or Asn (N-gly-

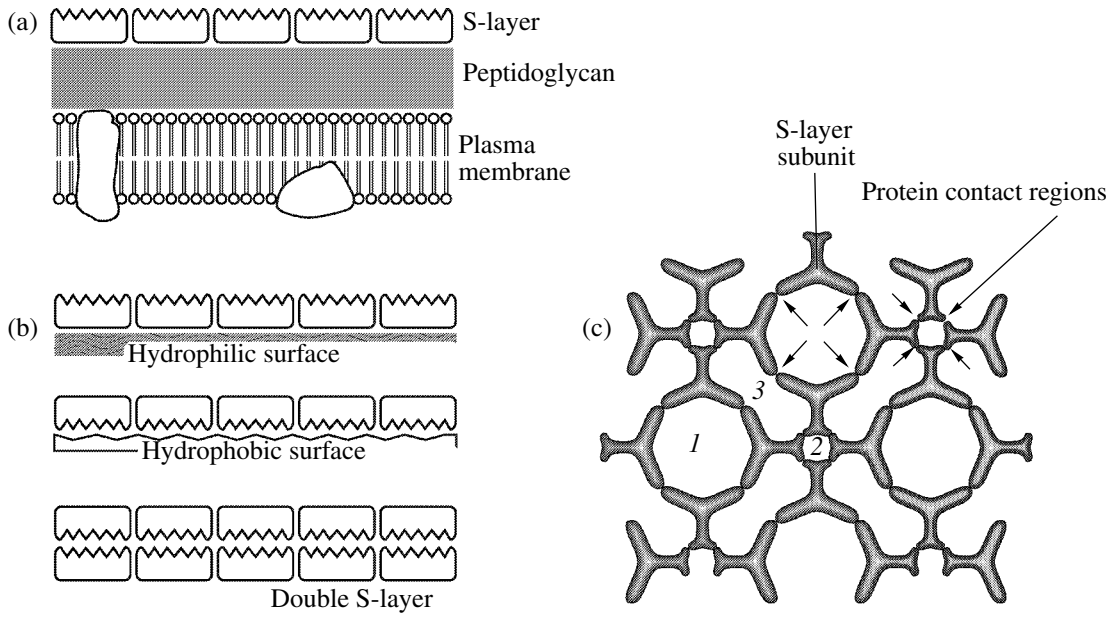


**Fig. 2.** Schemes of S-layers. (a) The S-layer on the surface of a Gram-positive bacterium overlies the peptidoglycan layer of the cell wall. (b) The two sides of the S-layer differ. Different sides of protein subunits face a hydrophilic or a hydrophobic surface after S-layer recrystallization. In solution, bilayers are often formed, with the hydrophobic surfaces of subunits facing each other. (c) The S-layer has tetragonal (p4) symmetry and contains pores of three types (*1–3*).

cosylation) [6]. S-layer proteins contain 2–20 glycosylation sites per molecule. Their oligosaccharides consist of 2–6 (rarely 10) sugar residues, including hexose, pentose, 6-deoxyhexose, and amino saccharides [6, 18, 19].

Since the S-layer overlies the cell wall, a certain mechanism must ensure secretion of S-layer proteins through the plasma membrane. S-layer proteins with known primary structures have a signal peptide (19–24 residues) at the N end. The peptide is cleaved in the course of protein translocation across the plasma membrane [8]. In some Gram-negative bacteria, such as *Caulobacter* and *Campylobacter* species, the secretion signal is at the C end of the S-layer protein and secretion follows the type I mechanism similar to that of α-hemolysin secretion in *Escherichia coli* [20, 21].

Since the S-layer completely covers the bacterial cell surface, it may be expected that S-layer proteins are intensely produced and account for an appreciable proportion of the total microbial protein. With a 20-min period between cell divisions, the production rate was estimated at about 500 S-layer protein molecules per second [22]. As shown with a few examples, such intense production is determined by potent promoters, high mRNA stability, and the use of preferential codons. For instance, the promoter of the *Lactobacillus acidophilus* S-layer protein gene is twice as efficient as the lactate dehydrogenase gene promoter, which is one of the most potent bacterial promoters [23]. The half-life of the mRNA of this S-layer protein is 15 min. A similarly high stability (half-life 14 min) is characteristic of the mRNA of the *Lactobacillus brevis* S-layer protein [24], whereas the mean half-life of bacterial mRNAs is 2–4 min.

Notwithstanding their intense synthesis, S-layer proteins are absent from the culture medium; i.e., their production is strongly regulated and correlates with cell growth. Little is still known about the molecular mechanisms regulating the activity of S-layer protein genes. The regulation was most comprehensively studied for *slp*A, which codes for the S-layer protein of thermophilic bacterium *Thermus thermophilus* HB8 [25]. It has been found that the product of another gene, *slr*A, suppresses transcription of *slp*A, but the mechanism of suppression remains obscure. Interestingly, SlpA binds to its own mRNA and thereby controls its translation [26].

The S-layer is noncovalently bound to the bacterial cell surface, since S-layer proteins dissociate from the cell and are solubilized upon cell exposure to detergents or high concentrations of hydrogen bond-disrupting agents [6]. Concerning the interaction of S-layer proteins with the bacterial cell surface, it is necessary to answer two major questions: which domains of the S-layer protein are involved in its binding to the cell surface, and which surface molecules contact the S-layer proteins. Although the total homology of S-layer proteins is surprisingly low, a certain region of the N-terminal domain displays relatively high homology among S-layer proteins of Gram-positive bacteria and is known as the SLH (S-layer homolo-

gous) motif [11, 27]. The N-terminal domain (no more than 200 residues) of an S-layer protein usually harbors three SLH motifs, each containing a conserved sequence of 10–15 residues.

The sequence was first revealed in the S-protein of *Acetogenium kivui* [28] and later was found in *Clostridium thermocellum* [29], *Thermus thermophilus* [30], *Bacillus anthracis* (a causal agent of anthrax) [31], and numerous other Gram-positive bacteria [27]. Interestingly, the SLH motif has homologs in the C-terminal region of some exoenzymes associated with cells of Gram-positive bacteria. First and foremost, these enzymes include cellulases, pullanase, and xylanase, which hydrolyze polysaccharides [32].

The anchoring of S-layer proteins on the cell wall with the SLH sequences is not universal even in Gram-positive bacteria. Thus, S-layer proteins of *Bacillus stearothermophilus* [33], *Lactobacillus* [34, 35], and *Carynebacterium glutamycum* [36] lack the SLH motif. The sequences responsible for binding to the cell wall are in the N-terminal region of the S-layer protein in *Lactobacillus* [34, 35] and in the C-terminal region in *Corynebacterium glutamicum* [36]. In Gram-negative bacteria and archaea, such sequences were found in the N- and the C-terminal regions of S-layer proteins [20, 35–38]. The diversity of S-layer protein sequences and the mechanisms of their interaction with the cell envelope is not surprising in view of a great variety of the envelopes themselves. Thus, the S-layer is the only solid cover and its proteins directly interact with the plasma membrane in some Gram-negative archaea [39, 40]. It has long been believed that S-layer proteins bind to peptidoglycan of the cell wall in Gram-positive bacteria [28], but recent experiments showed that the binding is with secondary cell-wall polymers. These are teichoic, teichuronic, and lipoteichoic acids and lipoglycans, which are bound to peptidoglycan either covalently or noncovalently, through lipid molecules [41].

As shown initially, *B. stearothermophilus* S-layer protein SbsB does not bind to the peptidoglycan cover that lacks secondary polymers as a result of extraction with hydrofluoric acid. The SLH motif is contained in SbsB, and its deletion prevents SbsB even from binding to the complete cell wall possessing secondary metabolites [42]. The SLH motifs of S-layer proteins bind with teichuronic acids on the cell wall [27, 43]. Yet it is still unclear which secondary polymers are responsible for the binding of S-layer proteins lacking SLH motifs [27].

Although S-layers are widespread among various microorganisms, their role is still incompletely understood [27, 44]. The role is probably related to the function of the whole cell envelope, which contains the S-layer as an integral part. In archaea having no other rigid cover, the S-layer apparently contributes to maintaining the cell shape [9, 45].

Yet the broad distribution and maintenance at a relatively high cost for the cell (since S-layers proteins are major) indicate that S-layers are evolutionarily conserved and are necessary for cell life beyond the lab.

Most of the functions ascribed to S-layers are hypothetical. It is possible that S-layers act as a molecular sieve allowing access to the cell only for molecules of a certain size, serve as ion traps, are responsible for cell adhesion on a solid support, protect the cell from phagolysis, etc. Experimental data on S-layer properties were obtained in only a few studies. For instance, it has been shown that the S-layer protects Gram-negative bacteria from the parasite *Bdelbovibrio bacteriovorus* [46] but not from other predators, such as protozoans [47].

In addition, S-layers are involved in the interaction of symbiotic or pathogenic bacteria with the host organism. The *Lactobacillus crispatus* S-layer binds with collagen. The binding site is within the 287 N-terminal residues of the S-layer protein. Collagen binding does not take place in the case of disturbed polymerization of the S-layer protein into a regular structure [49]. The N-terminal domain of this protein was shown to be responsible for the formation of a regular S-layer and for the binding with collagen, laminin, and secondary cell-wall polymers [50]. The *Lactobacillus brevis* S-layer is also capable of the binding with collagen and fibronectin and ensures adhesion of bacterial cells with an intact S-layer on human epithelial cells but not on erythrocytes [51]. The S-layer of *Lactobacillus acidophilus*, another lactic acid bacterium, provides for its adsorption on chicken intestinal epithelium [53]. Collagen and fibronectin form the basis of the intercellular matrix in higher animals and are a common target of pathogenic bacteria. For instance, the fish pathogen *Aeromonas salmonicida* utilizes the S-layer to enter the intercellular matrix. Thus, the S-layer is an important pathogenicity factor [54]. This is also true for the S-layer of *Campylobacter fetus*, a chicken pathogen [37].

An interesting function is performed by the S-layer of cyanobacterium *Synechococcus* (strain GL24). This bacterium inhabits a highly mineralized lake in the State of New York, and pores of its S-layer provide a matrix for gypsum or calcite crystallization [56]. A mineral layer is continuously removed from the cell surface, providing the cell with additional protection. Possibly this contributes to the formation of fine-grain minerals in nature.

## APPLICATIONS OF S-LAYERS

As already noted, S-layers are two-dimensional crystalline structures with pores. The period of the crystal pattern and the pores are several nanometers in size; i.e., these are nanostructures. This property of S-layers is of practical significance. In addition, various

fields may take advantage of the self-assembly of S-layers from purified proteins or glycoproteins on various surfaces [6, 8, 14, 15, 17].

S-layers are of potential utility for constructing ultrafiltration membranes with uniformly sized pores, nanospheres, epitope carriers to be employed in certain vaccines, highly efficient sensors with antibodies or other molecules immobilized in nodes of the crystalline matrix, or lithographic copies of S-layer lattices on a solid support.

## ULTRAFILTRATION MEMBRANES

Ultrafiltration membranes based on S-layers have an advantage over other membranes: all pores of one type are strongly uniform in size and shape in the former, whereas pore size fluctuates about a certain mean in all other cases. To date, ultrafiltration membranes have been constructed with S-layers of bacilli, mostly *Bacillus stearothermophilus* [56]. Pore size in such membranes varies from 2–8 nm with *Bacillus* strain or species [57].

Membranes are obtained with isolated fragments of S-layers of bacterial cells (about 500 nm in diameter). The fragments are layered onto a microfiltration membrane and fixed with glutaraldehyde [56–58]. Alternatively, a membrane may be obtained by linking S-layer fragments together with glutaraldehyde without a support [58]. Pores of such membranes display low nonspecific sorption of various proteins [56]. The membranes have a sharp exclusion limit, retaining proteins above a certain molecular weight. In the case of *B. stearothermophilus* membranes, the exclusion limit is between 35 and 40 kDa [56, 57].

Various protocols of membrane preparation on the basis of isolated or reconstructed S-layers were developed and patented [59, 60]. Yet in the approximately 15 years elapsed since then, ultrafiltration membranes based on S-layers have shown little promise and competed poorly with some other materials that are similar in properties, more stable, less expensive, and simpler to produce on a mass scale. Membranes based on S-layers are more promising for studying the S-layer characteristics, e.g., the pore size as dependent on the ionic strength or pH in solution [57] or the effects of chemical modification on the hydrophilic or hydrophobic properties of S-layers [61].

## S-LAYERS AS VACCINES

Although the S-layer is contained in the envelope of numerous human and animal pathogens, including anthrax-causing *B. anthracis* [31], its role as a virulence factor was demonstrated only for Gram-negative bacteria of the genus *Campylobacter* [62]. These bacteria cause acute intestinal disorders, which account for about 15% of the total intestinal disorder incidence in humans. In 1991, the genus *Helicobacter* was separated from *Campylobacter.* One of its species, *Helicobacter pylori*, is responsible for human peptic ulcer and is a subject of intensive studies [63]. In 1995, the S-layer of *Campylobacter fetus* was used as an antigen to construct a vaccine preventing abortion in chickens infected with this pathogen [64].

Given the regular arrangement of protein subunits in S-layers, haptens chemically attached to an S-layer may also have a regular pattern and thereby confer new interesting properties on the resulting combined vaccines. This expectation was realized in part. Indeed, S-layers proved to be convenient as carriers of low-molecular-weight haptens and as adjuvants [65]. Conjugate vaccines cause no visible adverse effect upon intramuscular or subcutaneous injection and are effective upon oral or nasal administration [66].

By now, combined vaccines based on S-layers were employed in three projects aimed at constructing vaccines against bacterial infection, cancer, or allergy [67–69].

A conjugate vaccine containing the *Streptococcus pneumoniae* type 8 capsular polysaccharide attached to the *Bacillus alvei* S-layer induces protective antibodies in mice, whereas the polysaccharide alone does not have this effect [65]. Moreover, the conjugate vaccine causes delayed hypersensitivity reaction, suggesting T-cell involvement. Again, this effect may be achieved with inactivated *Streptococcus pneumoniae* cells but not with the polysaccharide alone [65].

In anticancer vaccines, haptens were mucin oligosaccharides and T- and $Le^y$-antigens associated with epithelial tumors. The oligosaccharides do not cause an immune response when injected alone and are immunogenic when chemically linked to S-layers of *B. alevi*, *B. stearothermophilus*, and *Clostridium thermohygrosulfuricum* in suspension. The conjugate vaccines induce mostly the T-cell response; i.e., antibody production is low, whereas T killers and macrophages are activated to eliminate cancer cells. It should be noted that the T-antigen linked to serum albumin, which is often used as a carrier, fails to induce the T-cell response [69].

Human allergies are associated with elevated IgE production, which is regulated by $T_H2$ cells (humoral response). Normally, synthesis of allergen-specific IgG is low and the response is regulated by $T_H1$ cells (T-cell response). Switching T-cell regulation from the $T_H2$ to the $T_H1$ type is a promising strategy in preventing allergies [70]. As the above results demonstrate, vaccines with haptens conjugated to S-layers induce mostly the $T_H1$ response when employed in anticancer or antibacterial therapy. Hence, Betv1, the major birch pollen antigen, was conjugated to the S-layer. Stimulation of allergen-specific human lymphocytes with the resulting vaccine changed the

cytokine production from the $T_H2$ to the $T_H1$ type. This indicates that such conjugates are promising for immune therapy of allergies in humans [70].

## S-LAYERS AS MATRICES FOR IMMOBILIZATION OF FUNCTIONAL MOLECULES

Presumably, the regular arrangement and similar orientation of protein subunits in the S-layer lead to a high density and regular arrangement of biologically active molecules covalently bound to the S-layer surface. This assumption proved to be true within the accuracy of chemical crosslinking.

Immobilization matrices are usually the above ultrafiltration membranes or 1- to 2-μm microparticles obtained by ultrasound disruption of bacterial cell envelopes. S-layer microparticles (SMP) consist of a peptidoglycan layer covered with S-layers on both sides [72]. In some cases, an S-layer to be used for immobilization is recrystallized on a proper surface [73].

Experimental mobilization of ferritin, *Staphylococcus* protein A, and some enzymes (glucose oxidases, β-galactosidases, invertases) on such matrices has shown that protein ligands are bound at two or three molecules per S-layer subunit; this approximately corresponds to a ligand monolayer. Immobilized enzymes retain 16–60% of the initial activity. Flexible spacers (e.g., 6-aminocapronic acid) improve activity preservation [74].

To construct amperometric biosensors for quantitating glucose or sucrose in solution, an electric contact with the sensitive layer (an S-layer with immobilized glucose oxidase or invertase) is achieved with a thin gold film placed between the sensitive layer and a solid support [75, 76]. In optical biosensors, a sensitive layer is placed on the end of an optical fiber [77].

As biosensor matrices, S-layers have several advantages over standard materials. A high packing density and monolayer arrangement of an enzyme allow high sensitivity and short-distance diffusion of the substrate and the product of the reaction. In turn, this reduces the response time and improves the signal-to-noise ratio [73].

Sensors about 1 μm in size have already been constructed, yet it is technically feasible to obtain sensitive surfaces of several tens of nanometers [78]. Chemical attachment of biologically active molecules to S-layer proteins has several undesirable effects. Since chemical reactions are not absolutely specific, the binding of target molecules to S-layer subunits does not reach 100%. As a result, the density of biologically active molecules on the S-layer surface is insufficient and their arrangement irregular.

All these difficulties may be overcome with hybrid molecules containing a target protein or oligopeptide structurally incorporated in the S-layer protein so that the self-assembly of S-layer subunits is not impaired. Although the idea is self-apparent, generation and testing of such constructs started only in 2002 [79].

First and foremost, it is essential to identify the insertion sites where an insert would not interfere with the self-assembly of the S-layer and would be exposed on one of the S-layer surfaces. For instance, SbpA, a *Bacillus sphearicus* CCM2177 S-layer protein of 1268 residues, binds to the cell wall via its N-terminal region, which harbors SLH motifs [79]. Deletion of the 200 C-terminal residues does not affect the self-assembly of SbpA. Based on these observations, the full-length *sbp*A or its derivative 3'-truncated by 600 bp were each fused with a DNA fragment coding for the peptide Ala-Trp-Arg-His-Pro-Gln-Phe-Gly-Gly (Strp-tagI), which has high affinity for streptavidin. The constructs were expressed in *E. coli*. The two hybrid proteins were each capable of self-assembly into a S-layer, but the deletion derivative showed a far higher streptavidin binding. It is possible that Strp-tagI was sterically more accessible to streptavidin in the deletion derivative [79].

In dialysis, the purified hybrid proteins polymerize in solution to produce two-dimensional bilayer structures about 2 μm. In such structures, individual S-layers interact with each other via hydrophobic regions, which normally contact the cell wall. This was also observed for the wild-type SbpA [43]. Reconstructed from the hybrid proteins on the surface of the cell-wall cover of parental cells, the S-layer is similar in geometry to the native one [79].

Another study employed S-layer SbsB of *Geobacillus stearothermophilus* PV72/P2. The protein consists of 889 amino acid residues and, in S-layers reconstructed on liposomes or other solid supports, is oriented so that its inner surface faces the solvent (Fig. 2). Hence it is desirable to have hybrid proteins of two types, with a peptide insert exposed on the inner or on the outer surface of the S-layer [80]. The objective of another work was to obtain a construct exposing streptavidin on the S-layer surface in order to allow the binding of various biotinylated molecules [81]. Deletions from the N-terminal region do not hinder the self-assembly of SbsB, whereas the deletion of the 15 C-terminal amino acid residues completely prevents it. Hybrid genetic constructs contained the streptavidin-coding sequence either 5' or 3' of *sbs*B. The efficiency of construct expression in *E. coli* was about 50 mg of a hybrid protein per liter culture. Since streptavidin acts as a homotetramer, hybrid proteins were combined with streptavidin at a ratio of 1:3 before recrystallization. It was observed that N-terminal fusion proteins readily crystallized on liposomes or silica surfaces, whereas C-terminal fusion proteins formed S-layers only on the cell wall (the cover of parental bacterial cells) [81].

The above results [79, 81] demonstrate the possibility of constructing surfaces with a regular high-density arrangement of biologically active molecules. This may be taken advantage of in the construction of various biosensors.

Homologous expression of chimeric S-layer proteins may be used to obtain live vaccines. Lactic acid bacteria, which have long been employed in the food industry and are considered to be safe, are especially suitable for this purpose. In particular, these include *Lactobacillus brevis*. Owing to its properties, this bacterium is promising as a probiotic and a transport system able to deliver antigens into the human or animal organism. It is known that *L. brevis* tolerates low pH and bile acids and persists in the intestine for a long time. The S-layer makes *L. brevis* capable of adhesion on epithelial cells [51]. Hydrophilic site profiling of the *L. brevis* S-layer protein, SlpA, revealed at least four hydrophilic sites, which may be used as targets for inserting foreign peptides if exposed on the SlpA surface. On experimental evidence, at least two sites—Lys249–Ala850 and Ala313–Asn314—are suitable for insertion. Epitopes of poliovirus VPI (10 residues) and human oncoprotein c-Myc (11 residues) were inserted in these sites. The S-layer formed on the surface of *L. brevis* cells by hybrid SlpA was similar in geometry to the wild-type one and exposed the cloned epitopes [82]. The efficiency of immunization with such vaccines is yet to be determined.

## S-LAYERS AS SUPPORTS FOR LIPID MEMBRANES

Two-dimensional lipid bilayers, especially those incorporating proteins, are broadly used as a model of biological membranes. In particular, such bilayers make it possible to study the ionic channels or the behavior of receptors in ligand binding. Protein-incorporating membranes are the key components of electric and optic biosensors [83]. Low stability is a major drawback of such structures. Hence, membranes are fixed on a solid support to be of utility, although this changes the membrane properties. In particular, the mobility of lipid molecules within the bilayer decreases; i.e., membrane fluidity changes. In addition, only one surface of a fixed membrane is exposed to the aqueous environment, and the behavior of transmembrane proteins is poorly predictable in this case. To improve such a system, a water-saturated polymer layer several nanometers high (a "cushion") is placed between the membrane and the solid support [84, 85].

Liposomes represent another type of lipid bilayer structures that have found broad application. Liposomes proved to be advantageous for the addressed delivery of drugs and especially DNA into the cell. However, like planar lipid bilayers, liposomes are unstable.

As already mentioned, S-layer subunits readily crystallize on the interface of two phases, including lipid bilayers [86]. Apparently, S-layers may be used to support artificial lipid membranes, the more so as the resulting structures imitate the envelopes of archaea, which commonly live under extreme conditions [17, 68, 87]. Indeed, S-layers were reconstructed on lipid bilayers [88], phospholipid monolayers [89], and liposomes [90].

S-layers appreciably stabilize liposomes under thermal or mechanical stress [91]. This is of immense importance for medicine, as liposomes are exposed to stress when sprayed with a syringe or another device or transported through the vascular system [91].

When S-layers are used to support planar membranes, pores of less than 10 μm may be overlaid in the porous support [92]. Such structures are stable and easy to manipulate and, consequently, are convenient to use in place of free membranes [93].

As noted above, a cushion several nanometers high may be places between a lipid membrane and a solid support to improve the whole construct. Like some other materials, S-layers are efficient as such cushions [92].

Fixation on the S-layer considerably changes the properties of lipid membranes. Membrane fluidity again decreases because movements of lipid molecules within the bilayer are limited. Membranes supported by S-layers are known as semifluid [92, 93].

## S-LAYERS AS TEMPLATES FOR CONSTRUCTING REGULAR INORGANIC NANOSTRUCTURES

Another topical problem is the construction of two-dimensional ordered metal clusters of a nanometer scale. Such clusters are known as nano-dots, have unique properties, and may be employed in molecular electronics and nonlinear optics [78, 94].

Used as organic templates, S-layers make it possible to obtain planar ordered inorganic superlattices corresponding in dimensions to the S-layer lattice.

As mentioned above, calcium sulfate and calcium carbonate are crystallized on pores of cyanobacterial S-layers in nature [55]. Under laboratory conditions, S-layers were used to construct two-dimensional regular superlattices of cadmium sulfide [95] or gold [96]. In the case of CdS, two S-layers were recrystallized on carbon-covered nickel grids, which are commonly used in electron microscopy [95]. The S-layers had oblique (p1) or tetragonal (p4) symmetry. Cadmium sulfide crystals formed in S-layer pores when a $CdCl_2$ solution overlying an S-layer was gradually saturated with hydrogen sulfide. Crystals were about 5 nm with one S-layer and about 3 nm with the other. The crystal arrangement reproduced the lattice of the

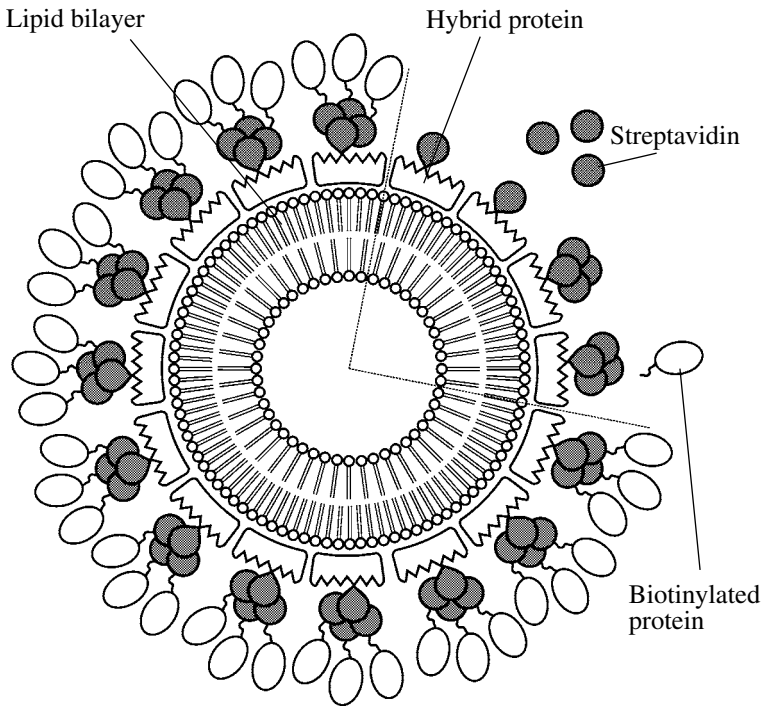


**Fig. 3.** Hybrid S-layer on the liposome surface. The hybrid protein is the *Geobacillus stearothermophilus* PV72/P2 S-layer protein with an insert of streptavidin (filled circles) [81]. Since streptavidin acts as a homotetramer, the hybrid protein was combined with free streptavidin at a ratio of 1:3 to reconstruct the S-layer on liposomes. Hybrid protein molecules each bind three molecules of a biotinylated protein on the liposome surface. The binding of the fourth molecule is sterically hindered.

corresponding S-layer, and the distance between nanocrystals was 10–13 nm [95].

Another protocol involves chemical modification of a recrystallized S-layer: protein subunits are provided with thiol groups. The S-layer is then incubated in tetrachloroauric acid and thereby covered with a thin gold film. Electron irradiation results in a two-dimensional regular superlattice of gold nanoparticles with a period of 3–5 nm. Gold particles correspond to pores in location on evidence of electron microscopy. Yet X-ray analysis showed that gold particles fail to form a perfect lattice and that particle centers deflect from the ideal positions (±0.5 nm) [96]. In addition, preliminary experiments were carried out with other metals: platinum, lead, and iron [93].

In the above works, S-layers were used as matrices for the formation of ordered arrays of nanoparticles. Another approach is to use an S-layer as a template (mask) to replicate its nanostructural pattern on a solid support. This takes advantage of the well-known ability of S-layers to recrystallize on the surface of silica, gallium arsenide, glass, carbon, and polymers, which are broadly employed in microelectronics [97]. To obtain a replica, an S-layer is placed on a silica plate, and a thin layer of a metal (titanium) is evaporated onto it at an angle of about 40°. Titanium covers the S-layer subunits with a layer of about 1.2 nm. In air, titanium is oxidized to titanium oxide. When the resulting modified (decorated with titanium oxide) S-layer is bombarded with argon ions at 2 keV, 7 $\mu A/cm^2$ for 12 min, hollows (pits) are formed in the silica plate at sites of S-layer pores [98, 99]. The pit pattern on the plate reproduces the pore pattern in the S-layer. Such experiments were performed with the *Sulfolobus acidocaldarius* S-layer, which has hexagonal symmetry, pores 5 nm in diameter, and a unit of 22 nm [100]. A drawback of this method is that exposure to a high-energy ion beam damages the silica layer. More recently, low-energy etching procedures were developed for silica [101, 102] and gallium arsenide [103] plates. Etching is performed in chlorine–hydrogen or hydrogen–helium plasma under an electron beam of 1–15 eV [103], which reduces damage to the silica surface.

After etching, the decorated S-layer is removed from the silica plate with sulfuric acid–water (1:1) at 130°C. Titanium evaporation onto a nanostructured silica template results in nanoclasters about 12 Å thick, which are formed in pits. The technique was patented in 2003 in several variants employing different metals for decorating S-layers, different materials (silica, etc.) for generating secondary masks, etc. [104].

The lithography principle is used not only in nanotechnology, but also in microtechnology. Microlithography makes it possible to apply nanostructures onto a wider surface (Fig. 4). It was shown that irradiation

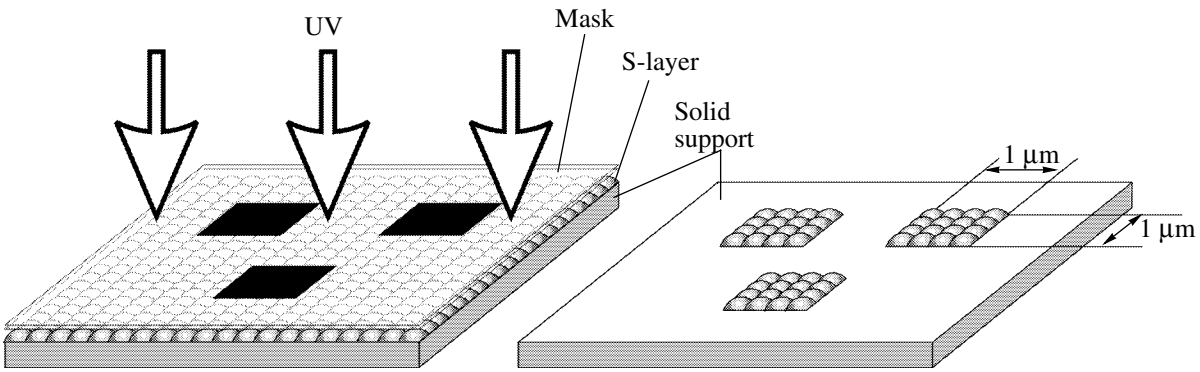


**Fig. 4.** Construction of micrometer-scaled S-layer zones on a solid support with a mask. Irradiation with short-wave UV light (λ = 193 nm) removes the S-layer from the regions that are unprotected with the mask oblique to UV light. Micrometer-sized nanostructured zones with S-layers are retained on the solid support.

with short-wave UV light (fluorine–argon laser, λ = 193 nm) destroys and completely removes S-layers from solid supports [17, 105]. When an S-layer placed on a solid support is irradiated through a mask (e.g., a quartz one) with certain sites covered with a material opaque to UV light, the resulting plate retains the S-layer in some sites and lacks it in the others. The mask must be tight against the S-layer. The sizes of such lithographic objects are restricted to about 200 nm (i.e., they are comparable with the irradiation wavelength) [17]. With short-wave radiation (e.g., an electron beam), the size may be reduced to 100 nm [78]. Long-wave UV light (fluorine–krypton laser, λ = 248 nm) only destroys (chars) S-layers and fails to remove these from a solid surface [105].

## CONCLUSIONS

It is clear now that S-layers are widespread among prokaryotes and, like other elements of the cell envelope, play an important part in interactions of the microbial cell with the environment. The functions of S-layers are far from being completely understood and await further investigation.

The attention to S-layers is due to their structural properties and the capability of self-assembly on various surfaces. S-layers are of interest for nanobiotechnology, a new field of research. In this field, classical biological macromolecules—proteins, nucleic acids, and lipids—are considered from the viewpoint of their applicability to constructing nanostructures and new materials, rather then from the functional one. The above examples illustrate the use of S-layers as ultrafiltration membranes, carriers and adjuvants in vaccines, stabilizers of planar lipid membranes or liposomes, and systems for micro- or nanolithography. The capabilities of S-layers (or their biomimetics) are further extended with the employment of gene or protein engineering in constructing hybrid S-layer subunits.

For instance, it seems possible to address the self-assembly of S-layers to surfaces of a particular type. Recently, phage display allowed selection of oligopeptides (7–21 amino acid residues each), which have specific affinity for individual inorganic materials, including silica, gallium arsenide, gold, silver, calcium carbonate, etc. [106]. It is possible that the introduction of such peptides into protein sites exposed on the S-layer surface will provide for specific sorption of the S-layer on a particular material. Rather than utilizing full-size S-layer proteins, further studies may employ their natural or changed fragments that allow directional self-assembly or have additional necessary functions.

Finally, S-layers and similar structures may be of importance for constructing extremely sensitive biosensors suitable for studying the elementary events in interactions of individual molecules.

## REFERENCES

1. Mazzola L. 2003. Commercializing nanotechnology. *Nature Biotechnol*. **21**, 1137–1143.
2. Seeman N.C. 2003. DNA in a material world. *Nature*. **421**, 427–431.
3. Cantor Ch., Shimmel P. 1984. *Biophysical Chemistry.* Moscow: Mir, vol. 1, 111–112.
4. Yan H., Ha Park S., Finkelstein G., Peif J.H., La Bean T.H. 2003. DNA-templated self-assembly of protein arrays and highly conductive nanowires. *Science*. **301**, 1882–1884.
5. Sleytr U.B. 1978. Regular arrays of macromolecules on bacterial cell walls: Structure, chemistry, assembly and function. *Int. Rev.Cytol*. **53**, 1–64.
6. Messner P., Sleytr U.B. 1992. Crystalline bacterial cell surface layers. *Adv. Microbiol. Physiol*. **33**, 213–275.
7. Beverdge T.J., Graham L. 1991. Surface layers of bacteria. *Microbiol. Rev.* **55**, 684–705.
8. Sara M., Sleytr U.B. 2000. S-layer proteins. *J. Bacteriol*. **182**, 859–868.

9. Sleytr U.B., Messner P., Pum D. 1988. Analysis of crystalline bacterial surface layers by freeze-etching, metal shadowing, negative staining and ultrathin sectioning methods. *Microbiology.* **20**, 29–60.
10. Hovmoller S., Sjogren A., Wang D.N. 1988. The structure of crystalline bacterial surface layers. *Prog. Biophys. Mol. Biol*. **51**, 131–163.
11. Engelhardt H., Peters J. 1998. Structure research on surface layers: A focus on stability, surface layer homology domains, and surface layer-cell wall interaction. *J. Struct. Biol*. **124**, 276–302.
12. Müller D.J., Baumeister W., Engel A. 1996. Conformational change of the hexagonally packed intermediate layer of *Deinococcus radiodurans* imaged by atomic force microscopy. *J. Bacteriol*. **178**, 3025–3030.
13. Muller D.J., Baumeister W., Engel A. 1999. Controlled unzipping of a bacterial surface layer with atomic force microcopy. *Proc. Natl. Acad. Sci. USA*. **96**, 13170–13174.
14. Sleyts U.B., Messner P., Pum D., Sara M. 1996. *Crystalline bacterial cell surface proteins*. Austin, TX: Academic Press.
15. Beveridge T.J. 1994. Bacterial S-layers. *Curr. Opin. Struct. Biol*. **4**, 204–212.
16. Kova S.F. 1988. Paracrystalline protein surface arrays on bacteria. *Can. J. Microbiol*. **34**, 407–414.
17. Sleytr U.B., Bayley H., Sara M., *et al*. 1997. Application of S-layers. *FEMS Microbiol. Rev.* **20**, 151–175.
18. Messner P. 1997. Bacterial glycoproteins. *Glycoconj. J*. **14**, 3–11.
19. Wugedtisch T., Zacchara N.E., Puchberger M., *et al*. 1999. Structural heterogeneity in the core oligosaccharide of the S-layer glycoprotein from *Aneurinibacillus thermoaerophylus* DSM1055. *Glycobiology.* **9**, 787–795.
20. Bingll W.H., Nomellini J.F., Smith J. 1997. Linker mutagenesis of the *Canlobacter crescentus* S-layer protein: Toward a definition of an N-terminal anchoring region and C-terminal secretion signal and the potential for heterologous protein secretion. *J. Bacteriol*. **179**, 601–611.
21. Thompson S.A., Shedd O.L., Ray K.C., *et al*. 1998. Campylobacter fetus surface layer proteins are transported by a type I secretion system. *J. Bacteriol*. **180**, 6450–6458.
22. Sleytr U.B., Messner P. 1983. Crystalline surface layers on bacteria. *Annu. Rev. Microbiol*. **37**, 311–339.
23. Boot H.J, Kolen C.P.A.M., Andreadaki F.J., *et al*. 1996. The *Lactobacillus acidophylus* S-layer protein gene expression site comprises two consensus promoter sequences, one of which directs transcription of stable mRNA. *J. Bacteriol*. **178**, 5388–5394.
24. Palva A. 1997. Molecular biology of the *Lactobacillus brevis* S-layer gene (*slpA*). *FEMS Microbiol. Rev.* **20**, 83–88.
25. Fernandez-Herrero L.A., Olabarria G., Berengner J. 1997. The S-layer of *Thermus thermophylus* HB8: structure and genetic regulation. *FEMS Microbiol. Rev.* **20**, 64–67.
26. Fernandez-Herrero L.A., Olabarria G., Berengner J. 1997. Surface proteins and a novel transcription factor regulate the expression of the S-layer gene in *Thermus thermophylus* HB8. *Mol. Microbiol*. **24**, 61–72.
27. Sara M. 2001. Conserved anchoring mechanisms between crystalline cell surface S-layer proteins and secondary cell wall polymers in Gram-positive bacteria. *Trends Microbiol*. **9**, 47–49.
28. Lupas A., Engelhardt H., Peters J., *et al*. 1994. Domain structure of the *Acetogenium kivui* surface layer revealed by electron crystallography and sequence analysis. *J. Bacteriol*. **176**, 1224–1233.
29. Lemaire M., Ohayon H., Gounon P., Fujino P., *et al*. 1995. OlpB, a new outer layer protein of *Clostridium thermocellum*, and binding of its S-layer-like domains to component of the cell envelope. *J. Bacteriol*. **177**, 2451–2459.
30. Olabarria G., Carrascosa J., De Pedro J. *et al*. 1996. A conserved motif in S-layer proteins is involved in peptidoglycan binding in *Thermus thermophilus. J. Bacteriol*. **178**, 4765–4772.
31. Fonet A., Mesange S., Tosi-Couture E., Gounon P., Mock M. 1997. *Bacillus anthracis* S-layer. *FEMS Microbiol. Rev.* **20**, 55–59.
32. Leibovitz E., Lemaire M., Miras I., *et al*. 1997. Occurrence and function of a common domain in S-layer and other exocellular proteins. *FEMS Microbiol. Rev.* **20**, 127–133.
33. Kuen B., Koch A., Asenbauer E. *et al*. 1997. Molecular characterization of the second S-layer gene *sbsB* of *Bacillus stearothermophilus* PV72 expressed by oxidative stress. *J. Bacteriol*. **179**, 1664–1670.
34. Callegari L., Riboli B., Sanders W., *et al*. 1998. The S-layer gene of *Lactobacillus helveticus* CNRZ892: Cloning, sequencing and heterologous expression. *Microbiology.* **144**, 719–726.
35. Vidgren G., Pabva I., Pakkanen R., Lounatmaa K., Palva A. 1992. S-layer protein gene of *Lactobacillus brevis* cloning by polymerase chain reaction and determination of nucleotide sequence. *J. Bacteriol*. **174**, 7419–7427.
36. Chami M., Bayan N., Peyret J.L., *et al*. 1997. The S-layer protein of *Corenebacterium glutamicum* is anchored to the cell wall by its C-terminal hydrophobic domain. *Mol. Microbiol*. **23**, 483–492.
37. Dworkin J., Blaser M.J. 1997. Molecular mechanism of *Campylobacter fetus* surface layer protein expression. *Mol. Microbiol*. **26**, 433–440.
38. Yao R., Macario A.J., Conway de Macario E. 1994. An archeal S-layer gene homolog with repetitive units. *Biochim. Biophys. Acta*. **1219**, 607–700.
39. Beveridge T.J. 1997. The response S-layered bacteria to the Gram stain. *FEMS Microbiol. Rev.* **20**, 101–114.
40. Lechner J., Sumper M. 1987. The primary structure of a prokaryotic glycoprotein. Cloning and sequencing of the cell surface gene of Halobacteria. *J. Biol. Chem*. **262**, 9724–9729.
41. Navarre W.W., Schneewind O. 1999. Surface protein of Gram-positive bacteria and mechanisms of their targeting to the cell envelope. *Microbiol. Mol. Biol. Rev.* **63**, 174–229.
42. Ries W., Hotzy C., Schoches I., Sleytr U.B., Sara M. 1997. Evidence that a secondary cell wall polymer recognizes the N-terminal part of the S-layer protein from

*Bacillus stearothermophilus* PV72/p2. *J. Bacteriol*. **179**, 3892–3898.

43. Ilk N., Kosma P., Puchberger M., *et al*. 1999. Structural and functional analyses of the secondary cell wall polymer of *Bacillus sphaericus* CCM2177 serving an S-layer-specific anchor. *J. Bacteriol*. **181**, 7643–7646.
44. Sleytr U.B. 1997. Basic and applied S-layer research: An overview. *FEMS Microbiol. Rev.* **20**, 5–12.
45. Phipps B., Huber R., Baumeister W. 1991. The cell envelope of the hyperthermophilic *Archaebacterium pyrobacillum organothrophum* consist of two regularly arrayed protein layers: Three-dimension structure of the outer layer. *Mol. Microbiol*. **5**, 523–528.
46. Koval S.F., Hynes S.H. 1991. The effect of paracrystalline protein surface layers on predation by *Bdellovibrio bacteriovorous. J. Bacteriol*. **173**, 2244–2249.
47. Koval S.F. 1997. The effect of S-layers and cell surface hydrophobicity on prey selection by bacteriovorous protozoa. *FEMS Microbiol. Rev.* **20**, 138–142.
48. Toba T., Varkola R., Westerlund B., *et al*. 1995. A collagen-binding S-layer protein in *Lactobacillus crispatus. Appl. Environ. Microbiol*. **61**, 2467–2471.
49. Sillanpää J., Martinez B., Antikainen J., *et al.* 2000. Characterization of the collagen-binding S-layer protein CbsA of *Lactobacillus crispatus. J. Bacteriol*. **182**, 6440–6450.
50. Antikainen J., Anton L., Sillanpaa J., *et al.* 2002. Domain in the S-layer protein CbsA of *Lactobacillus crispatus* involved in adherence collagens, laminin and lipotechoic acids and self assembly. *Mol. Microbiol*. **46**, 381–394.
51. Hynönen U., Westerlund-Wikstrom B., Palva A., *et al.* 2002. Identification by flagellum display of an epithelial cell and fibronectin-binding function in the SlpA surface protein of *Lactobacillus brevis. J. Bacteriol*. **184**, 3360–3367.
52. Lorea G., Torino M.J., Font de Valdez G., Ljungh A. 2002. *Lactobacillus* express cell surface proteins which mediate binding of immobilized collagen and fibronectin. *FEMS Microbiol. Lett*. **206**, 31–37.
53. Noonan B., Trust T.J. 1997. The synthesis, secretion and role in virulence of the paracrystalline surface protein layers of *Aeromonas salmonicida* and *Aeromonas hydrophila. FEMS Microbiol. Lett*. **154**, 1–7.
54. Schneitz C., Nuotioo L., Lounatmaa K. 1992. Adhesion of *Lactobacillus acidophilus* to avian intestinal epithelial cells mediated by the crystalline bacterial cell surface layer (S-layer). *J. Appl. Bacteriol*. **74**, 290–299.
55. Schultze-Lam S., Haranz G., Beveridge T.J. 1992. Participation of a cyanobacterial S-layer in fine-grain mineral formation. *J. Bacteriol*. **74**, 7971–7981.
56. Sara M., Sleytr U.B. 1987. Molecular sieving through S-layers of *Bacillus stearothermophilus* strain. *J. Bacteriol*. **169**, 4092–4098.
57. Sleytr U.B., Sara M. 1987. Ultrafiltration membranes with uniform pores from crystalline bacterial cell envelope layers. *Appl. Microbiol. Biotechnol*. **25**, 83–90.
58. Sara M., Sleytr U.B. 1987. Production and characteristics of ultrafiltration membranes with uniform pores from two-dimensional arrays of protein. *J. Membrane Sci*. **33**, 27–49.
59. Sleytr U.B., Sara M. 1988. US Patent 4.752.395.
60. Sleytr U.B., Sara M. 1989. US Patent 4.886.604.
61. Weigert S., Sara M. 1996. Ultrafiltration membranes prepared from crystalline bacterial cell surface layers as model system for studying the influence of surface properties on protein adsorption. *J. Membr. Sci*. **121**, 185–196.
62. Blaser M.J., Pai Z. 1993. Pathogenesis of *Campylobacter fetus* infection. Critical role of high molecular mass S-layer proteins in virulence. *J. Infect. Dis*. **167**, 372–377.
63. Korotyaev A.I., Babicheva S.A. 1998. In: *Meditsinskaya mikrobiologiya, immunologiya i virusologiya* (Medical Microbiology, Immunology, and Virology), St. Petersburg: Spetsial'naya Literatura, 403–405.
64. Grogono-Thomas R., Dworkin J., Blaser M.J. 1997. The role of S-layer proteins in ovine *Campylobacter* abortion. *FEMS Microbiol. Rev.* **20**, 133–135.
65. Malcolm A.J., Best M.W., Szarka R.J., *et al.* 1993. Surface layers from *Bacillus alvei* as a carrier for a *Streptococcus pneumoniae* conjugate vaccine. In: *Advances in Paracrystalline Bacterial Surface Layers*. Eds. Breveridge T.J., Roval S.F. N.Y.: Plenum Press, 219–233.
66. Smid B.J., Messner P., Unger F.M., *et al.* 1996. Toward selective elicitation of $T_n$I-controlled vaccination responses: Vaccine application of bacterial surface layer proteins. *J. Biotechnol*. **44**, 225–231.
67. Unger F.M., Messner P., Smid B.J., Sleytr U.B. 1997. Vaccine application of crystalline bacterial surface layer proteins (S-layers). *FEMS Microbiol. Rev.* **20**, 157–158.
68. Sara M., Sleytr U.B. 1996. Crystalline bacterial cell surface layers (S-layers) from cell structure to biomimetics. *Prog. Biophys. Mol. Biol*. **65**, 83–111.
69. Sleytr U.B., Mundt W., Messner P., Smith R.H., Unger F.M. 1991. Immunogenic composition containing ordered carriers. US Patent 5.043.158.
70. Holt P.G. 1994. A potential vaccine strategy for asthma and allied atopic diseases during early childhood. *Lancet*. **344**, 456–458.
71. Jahn-Schmid B., Graninger M., Glozik M., *et al.* 1996. Immunoreactivity of allergen ($Bct_v$I) conjugated to crystalline bacterial cell surface layers (S-layers). *Immunotechnology.* **2**, 103–113.
72. Sara M., Sleytr U.B. 1989. Use of regularly structured bacterial cell surface layers as matrix for immobilizing macromolecules. *Appl. Microbiol. Biotechnol*. **30**, 184–189.
73. Sara M., Sleytr U.B. 1996. Biotechnology and biomimetic with crystalline bacterial cell surface layers (S-layers). *Micron*. **27**, 141–156.
74. Kupcu S., Mader C., Sara M. 1995. The crystalline cell surface layer from *Thermoanaerobacter thermohydrosulfuricum* LIII–69 as an immobilization matrix: Influence of the morphological properties and the pore size of the matrix on loss of activity of covalently bound enzymes. *Biotechnol. Appl. Biochem*. **21**, 275–286.
75. Neubauer A., Pum D., Sleytr U.B. 1993. An amperometric glucose sensor based on isoporous crystalline protein membranes as immobilization matrix. *Anal. Lett*. **26**, 1347–1360.

76. Neubauer A., Hodl C., Pum D., Sleytr U.B. 1994. A multistep enzyme sensor for sucrose based on S-layers microparticles as immobilization matrix. *Anal. Lett*. **27**, 849–865.

77. Neubauer A., Pum D., Sleytr U.B., *et al.* 1996. Fibre-optic sensor using enzyme membrane with 2D crystalline structure. *Biosensors Bioelectronics*. **113**, 317–325.

78. Pum D., Sleytr U.B. 1999. The application of bacterial S-layers in molecular nanotechnology. *Trends Biotechnol*. **17**, 8–12.

79. Ilk N., Völlenkle C., Edelseer E.M., *et al.* 2002. Molecular characterization of the S-layer gene, sbpA, of *Bacillus sphaericus* CCM2177 and production of functional S-layer fusion protein with ability to recrystallize in a definite orientation while presented the fused allergen. *Appl. Environ. Microbiol*. **68**, 3251–3260.

80. Sara M., Kuen B., Mayer N.F., *et al.* 1996. Dynamics in oxygen-induced changes in S-layer protein synthesis from *Bacillus stearothermophilus* PV72 and the S-layer-deficient variant T5 in continuous culture, and studies of the cell wall composition. *J. Bacteriol*. **178**, 2108–2117.

81. Moll D., Huber C., Schlegel B., *et al.* 2001. S-layer-streptavidin fusion proteins as template for nanopatterned molecular arrays. *Proc. Natl. Acad. USA*. **99**, 14646–14651.

82. Avall-Jaaskelainen S., Kylä-Nikkilä., Kahala M., *et al.* 2002. Surface display of foreign epitopes on the *Lactobacillus brevis* S-layer. *Appl. Environ. Microbiol*. **68**, 5943–5951.

83. Steiner G., Zimmerer G., Friedrich S., Salzer R. 2003. Optical biosensor with Ion Channel Array. *Screening*. **4**, 32–33.

84. Sackmann E. 1996. Supported membranes: Scientific and practical application. *Science*. **271**, 43–48.

85. Schindler H. 1989. Planar lipid-protein membranes: Strategies of formation and detecting dependencies of ion transport functions on membrane conditions. *Methods Enzymol*. **171**, 225–253.

86. Pum D., Weinhand M., Hodl C., Sleytr U.B. 1993. Large-scale recrystallization of the S-layer of *Bacillus coagulans* E-3866 at the air-water interface and on lipid films. *J. Bacteriol*. **175**, 2762–2766.

87. Sleytr U.B., Sara M. 1997. Bacterial and archaeal S-layer proteins: Structure-function relationships and their biotechnological applications. *Trends Biotechnol*. **15**, 20–26.

88. Schuster B., Pun D., Braha O., *et al.* 1998. Self assembled α-hemolysin pores in an S-layer-supported lipid bilayer. *Biochim. Biophys. Acta*. **1370**, 280–288.

89. Wetzer B., Pfandler A., Gyozvary E., *et al.* 1998. S-layer reconstitution at phospholipid monolayers. *Langmuir*. **14**, 6899–6906.

90. Kupcu S., Sara M., Sleytr U.B. 1995. Liposomes coated with crystalline bacterial cell surface protein (S-layer) as immobilization structures for macromolecules. *Biochim. Biophys. Acta*. **1235**, 263–269.

91. Mader C., Kupcu S., Sara M., Sleytr U.B. 1999. Stabilizing effect of an S-layer on liposomes towards thermal or mechanical stress. *Biochim. Biophys. Acta*. **1418**, 106–116.

92. Pum D., Sleytr U.B. 1994. Large-scale reconstitution of crystalline bacterial surface layer proteins at the air-water interface and on lipid films. *Thin Solid Films*. **244**, 882–886.

93. Sleytr U.B., Messner P., Pum D., Sara M. 1999. Crystalline bacterial cell surface layers (S-layers): from supramolecular cell structure to biomimetic and nanotechnology. *Angew. Chem. Int. Ed*. **38**, 1034–1054.

94. Bohr M.T. 2002. Nanotechnology goals and challenges for electronic application. *IEEE Trans. Nanotechnol*. **1**, 56–62.

95. Shenton W., Pum D., Sleytr U.B., Mann S. 1997. Synthesis of cadmium sulphide superlattices using bacterial S-layers. *Nature*. **389**, 585–587.

96. Dielnweit S., Pum D., Sleytr U.B. 1998. Formation of a gold superlattice on an S-layer with square lattice symmetry. *Supramol. Sci*. **5**, 15–19.

97. Pum D., Sleytr U.B. 1996. Mononuclear reassembly of a crystalline bacterial cell surface (S-layer) on untreated and modified silicon surface. *Supramol. Sci*. **2**, 193–197.

98. Douglas K., Devand G., Clark N.A. 1992. Transfer of biologically derived nanometer-scale patterns to smooth substrates. *Science*. **257**, 642–644.

99. Douglas K. 1996. Biomimetic approaches to nanostructural fabrication. In: *Biomimetic Materials Chemistry.* Ed. Mann S. N.Y.: VCY, 117–142.

100. Lembcke G., Durr R., Hegerl R., *et al.* 1991. Image analysis and processing of an imperfect two dimensional crystal: The surface layer of the archaebacterium *Sulfjlobus acidocalgarius* reinvestigated. *J. Microsc*. **161**, 263–278.

101. Gillis H.P., Clemons J.L., Chamberlain J.P. 1992. Low-energy electron beam enhanced etching of Si (100) (2x1) by molecular hydrogen. *J. Vac. Sci. Technol*. **B10**, 2729–2732.

102. Gillis H.P., Chontov D.A., Steiner I.V., *et al.* 1995. Low-energy electron-enhanced etching of Si (100) in hydrogen/helium direct-current plasma. *Appl. Phys. Lett*. **66**, 2475–2477.

103. Gillis H.P., Chontov D.A., Martin K.P., *et al.* Low-energy electron-enhanced etching of GaAs (100) in a clorine/hydrogen PC plasma. *Appl. Phys. Lett*. **68**, 2255–2257.

104. Winningham T.A., Douglas K. 2002. US Patent Application 20020123227; 2003. US Patent 65.518.194.

105. Pum D., Stangl G., Sponer C., *et al.* 1997. Patterning of monolayers of crystalline S-layer proteins on a silicon surface by deep ultraviolet radiation. *Microelectron. Eng*. **35**. 297–300.

106. Sarikaya M., Tamerler C., Jen A.R.Y., *et al.* 2003. Molecular biomimetics: Nanotechnology through biology. *Nature Materials*. **2**, 577–585.