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MINIREVIEW

S-Layer Proteins

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Cell walls are an important structural component of prokaryotic organisms and essential for many aspects of their life. Particularly, the diverse structures of the outermost boundary layers strongly reflect adaptations of organisms to specific ecological and environmental conditions (6).

Over the past 3 decades of research, it has become apparent that one of the most common surface structures on archaea and bacteria are monomolecular crystalline arrays of proteinaceous subunits termed surface layers or S-layers (125, 131, 132). Since S-layer-carrying organisms are ubiquitous in the biosphere and because S-layers represent one of the most abundant cellular proteins, it is now obvious that these metabolically expensive products must provide the organisms with an advantage of selection in very different habitats (133). This minireview provides a brief survey of the current state of our knowledge about S-layers with a particular focus on molecular biological and genetic aspects. Other recent reviews (5, 7, 127, 133, 135) are recommended for a more detailed introduction to and treatises on this subject.

OCCURRENCE, STRUCTURE, AND ASSEMBLY

S-layers have now been identified in hundreds of different species belonging to all major phylogenetic groups of bacteria, and they represent a feature common to almost all archaea (for a recent compilation, see reference 133). This widespread occurrence on prokaryotic organisms has not always been appreciated, since S-layers are often lost during prolonged cultivation under laboratory conditions. Consequently, fresh isolates should be examined by electron microscopic techniques as soon as possible, preferably by freeze-etching of pellets of unwashed cells (Fig. 1) in complete medium (64, 125). For high-resolution studies on the mass distribution of S-layer lattices, negatively stained preparations of isolated or recrystallized S-layers have been used (for reviews, see references 4, 5, 50, and 130). More recently, high-resolution images of S-layers were also obtained by applying underwater atomic-force microscopy (38, 135).

The S-layer lattices can have oblique (p1, p2) square (p4), or hexagonal (p3, p6) symmetry. The data now available show that hexagonal symmetry is predominant among archaea (for a compilation, see reference 133). Depending on the lattice type, one morphological unit consists of one, two, four, three, or six identical (glyco)protein subunits, respectively, and they exhibit center-to-center spacings of approximately 2.5 to 35 nm. Most S-layers are 5 to 25 nm thick, and they reveal a rather smooth outer surface and a more corrugated inner surface. Among S-layer lattices of archaea, pillar-like extensions on the inner surface can be observed (80, 99, 100, 103). Since S-layers are monomolecular assemblies of identical subunits, they exhibit pores identical in size and morphology. In numerous S-layer lattices, two or even more distinct classes of pores (generally in the 2- to 8-nm range) could be identified, occupying up to 70% of the surface area. Since S-layers are found in gram-positive and gram-negative bacteria and archaea, they can be associated with quite different supramolecular cell envelope structures. In gram-positive bacteria and archaea, the S-layer subunits are linked to the peptidoglycan-containing layer or to the pseudomurein. In gram-negative bacteria, attachment involves components of the outer membrane (e.g., lipopolysaccharides [LPS]). In archaea lacking a rigid wall layer, S-layers are the only wall component, being closely associated with the plasma membrane (38, 59).

S-layers represent unique model systems for studying the dynamic process of assembly of a supramolecular structure during cell growth and cell division. Different methods have been developed for the detachment of S-layers and for their disintegration into protomeric units (5, 62, 90, 133). Most S-layer proteins can be solubilized with high concentrations of agents that break hydrogen bonds (e.g., guanidine hydrochloride). Particularly S-layer proteins from gram-negative bacteria may be disintegrated by applying metal-chelating agents or cation substitution. These data have shown that the individual subunits of S-layers interact with each other and with the supporting cell envelope components through noncovalent forces. Isolated S-layer subunits frequently maintain the ability to recrystallize into regular arrays in suspension or on surfaces (including that cell envelope component they were originally associated with) upon removal of the agent used for their isolation (5, 105, 115, 126, 133). Up to now, the most detailed in vivo and in vitro assembly studies were performed with S-layers from members of the family *Bacillaceae* (115, 129, 133). It was shown that differences in the surface net charge and in the hydrophobicity of the inner and outer surfaces, binding domains specific to components of the supporting rigid envelope layer and defined intersubunit binding properties, are responsible for the proper orientation and incorporation of the S-layer subunits on cell surfaces. From a more general point of view, it is now evident that S-layers are dynamic closed surface crystals with the intrinsic ability to continuously assume a structure of low free energy during cell growth and division. In most bacteria and archaea, the rate of synthesis of S-layer subunits appears to be strictly controlled, as at different growth rates only small amounts are detectable in the medium. Analysis of the lattice faults in S-layers of lobed archaea possessing hexagonal arrays as the exclusive wall component provided strong evidence that complementary pairs of pentagons and heptagons play an important role as sites for the incorporation of

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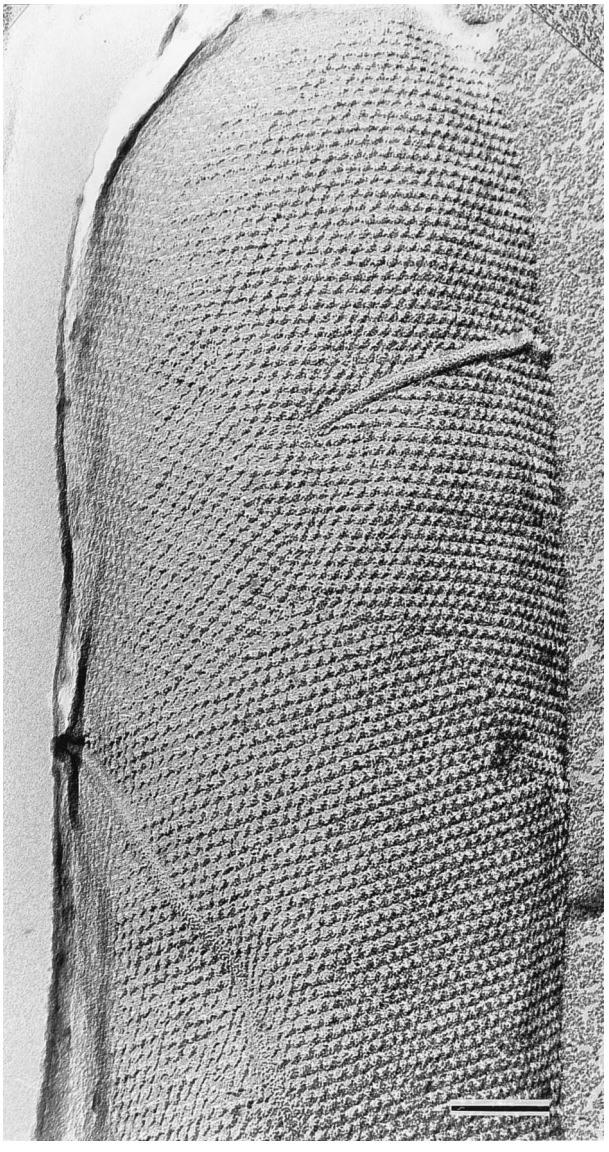

FIG. 1. Electron micrograph of a freeze-etched preparation showing a whole cell with a hexagonally ordered S-layer lattice. Bar, 100 nm.

new subunits in the formation and maintenance of the cell structure and in the fission process. The latter was assumed to be dependent on the ratio of the increase in protoplast volume to the increase in actual S-layer surface area during cell growth (106).

S-LAYER PROTEINS AND GENES

Chemical analyses of isolated S-layers showed that they are mostly composed of a single protein or glycoprotein species with apparent relative molecular weights of 40,000 to 200,000. Only the S-layers of a few organisms, such as that of *Clostridium difficile* (140) and *Bacillus anthracis* (39, 84, 85), consist of two types of S-layer subunits which together form a defined lattice type but do not cross-react with polyclonal antibodies. S-layer protein EA1 of *B. anthracis* was proposed to establish the main lattice, whereas the second S-layer protein, Sap, is most probably only incorporated into this template. Two superimposed S-layer lattices consisting of different types of S-layer subunits were identified in the cell envelopes of *Brevibacillus brevis* (formerly *Bacillus brevis*; 154) and *Aquaspirillum serpens* (58).

Amino acid analyses revealed that S-layer proteins of organisms from different phylogenetic branches are rather similar in overall composition (5, 115, 128, 133). Typically, S-layer proteins possess a high content of acidic and hydrophobic amino acids. Lysine is the predominant basic amino acid, while the arginine, histidine, and methionine content is generally low and cysteine was only detected in a few S-layer proteins (86, 115). According to sequence analyses, the isoelectric points (pIs) of most S-layer proteins are in the weakly acidic pH range. An exception was found for the S-layer proteins of *Methanothermus fervidus* (17) and for different lactobacilli (11), which possess pIs of 8.4 and 9 to 11, respectively. According to circular-dichroism measurements, approximately 20% of the amino acids are organized as α helices and about 40% occur as β sheets. Aperiodic foldings and β-turn content may vary between 5 and 45%. Secondary-structure predictions based on protein sequence data (see Table 1) revealed that most α-helical segments are arranged in the N-terminal part of the proteins. For the remaining part of the sequences, mainly short β-strands connected by loops and turns have been predicted.

S-layer proteins of many archaea and those of gram-positive bacteria can possess covalently bound carbohydrate chains (for reviews, see references 70, 86, 87, and 89). Typically, the glycan chains are composed of 20 to 50 identical repeating units containing neutral hexoses, pentoses, heptoses, 6-deoxyhexoses, and amino sugars. The glycan chains of S-layer proteins from gram-positive bacteria are more similar to the LPS occurring in the outer membranes of gram-negative bacteria than to eukaryotic glycan structures (118). Core oligosaccharides of S-layer glycoproteins consist of two to four sugar residues attaching the carbohydrate chains mostly via O-glycosidic linkages (galactose–tyrosine, glucose–tyrosine, *N*-acetylgalactosamine–serine, *N*-acetyl galactosamine–threonine) to the protein moiety (86, 87, 153). However, in a few examples, N-glycosidic linkages (asparagine–rhamnose) have been observed (86, 87). S-layer glycoproteins of gram-positive bacteria possess two to four glycosylation sites, whereas up to 25 glycosylation sites have been determined for those of archaea in which N-glycosidic linkages predominate (70). The presence of sulfate residues in the glycan chains of the extremely halophilic bacterium *Halobacterium halobium* (82) endows the S-layer lattice with a strong net negative charge. Since only neutral glycan chains were identified for the S-layer protein of the moderate halophile *Haloferax volcanii* (81), it was suggested that the negative charges are required for stabilizing of the S-layer lattice (138). A different type of posttranslational modification than glycosylation was reported for the S-layer protein of *Aeromonas hydrophila*. Phosphorylation of tyrosine residues decreases the pI of this S-layer protein from 6.7 to 4.6 (141).

During the last decade, numerous S-layer genes from organisms having quite different taxonomical positions have been cloned and sequenced (for reviews, see references 66 and 135). A survey of all of the sequenced S-layer genes is given in Table 1. Although it was proposed for several years that sequence identities among S-layer proteins are extremely rare or do not even exist, it is now evident that high sequence identities are limited to the N-terminal region, which is responsible for binding of the S-layer subunits to the underlying cell envelope layer (135). On the other hand, only low sequence identities (~20%) were found for the middle and C-terminal parts, comprising those domains that are involved in the self-assembly process and that are exposed inside the pores and on the S-layer surface. Despite these low sequence identities, conserved four-

TABLE 1. Survey of S-layer proteins whose amino acid sequences are known

Species	Strain	Gene	No. of amino acids, including N-terminal leader peptide/ N-terminal leader peptide	Lattice type[a]	GenBank accession no.	Reference
Aeromonas hydrophila	TF7	*ahs*	467/19	S	L37348	141
Aeromonas salmonicida	A450	*vapA*	502/21	S	M64655	23
Bacillus anthracis	Sterne derivative substrain 9131	*sap*	814/29	O[c]	Z36946	39
		eag	862/29	O	X99724	85
Brevibacillus brevis (*Bacillus brevis*)	47	*owp*	1,004/24		M14238	144
		mwp	1,053/23	H	M19115	145
Brevibacillus brevis (*Bacillus brevis*)	HPD31	*HWP*	1,087/23	H	D90050	33
Bacillus licheniformis	HM105	*olpA*	874/29		U38842	156
Bacillus sphaericus	P1	*sequence 8*	1,252/30	S	A45814	26
Bacillus sphaericus	2362	*gene 125*	1,176/30	S	M28361	14
		gene 80	745 (silent)			14
Bacillus stearothermophilus	PV72/p6	*sbsA*	1,228/30	H	X71092	67
	PV72/p2	*sbsB*	920/31	O	X98095	65
Bacillus stearothermophilus	ATCC 12980	*sbsC*	1,099/30	O	AF055578	53
Bacillus thuringiensis		*ctc*	842/29		AJ012290	
Campylobacter fetus subsp. *fetus*		*sapA*	933/none	H, S[b]	J05577	10
Campylobacter fetus subsp. *fetus*	23B	*sapA1*	920/none	H, S[b]	L15800	146
Campylobacter fetus subsp. *fetus*	82-40LP3	*sapA2*	1,109/none	H, S[b]	S76860	31
Campylobacter fetus subsp. *fetus*	84-91	*sapB*	936/none		U25133	31
	CIP 5396T	*sapB2*	1,112/none		AF048699	
Campylobacter rectus	314	*crs*	1,361/none		AF010143	149
Caulobacter crescentus	CB15	*rsaA*	1,026/none	H	M84760	44
Clostridium thermocellum	NCIMB 10682	*slpA*	1,036/26	O	U79117	72
Corynebacterium glutamicum	ATCC 17965	*csp2*	510/30	H	X69103	102
Deinococcus radiodurans		*HPI gene*	1,036/31	H	M17895	98
Halobacterium halobium		*csg*	852/34	H	J02767	69
Haloferax volcanii			828/34	H	M62816	139
Lactobacillus acidophilus	ATCC 4356	*slpA*	444/24	O	X89375	12
		slpB	456 (silent)		X89376	12
Lactobacillus brevis	ATCC 8287		465/30	O	Z14250	147
Lactobacillus crispatus	JCM 5810	*cbsA*	440/30	O	AF001313	
Lactobacillus helveticus	CNRZ 892	*slpH1*	440/30	O	X91199	18
Lactobacillus helveticus	CNRZ 1269	*slpH2*	440/30	O	X92752	92
Methanococcus voltae		*sla*	565/12	H	M59200	60
Methanosarcina mazei	S-6	*slgB*	652/31	H	X77929	155
Methanothermus fervidus	DSM 2088	*slgA*	593/22	H	X58297	17
Methanothermus sociabilis	DSM 3496	*slgA*	593/22	H	X58296	17
Rickettsia prowazekii	Brein 1	*spaP*	1,612/32		M37647	20
Rickettsia rickettsii	R	*p120*	1,645/32		X16353	45
Rickettsia typhii	Wilmington	*slpT*	1,645/32		L04661	48
Serratia marcescens	Isolate 8000	*slaA*	1,004/none		AB007125	56
Staphylothermus marinus	F1		1,524/putative		US7967	99
Thermoanaerobacter kivui (*Acetogenium kivui*)	DSM 2030	*slp*	762/26	H	M31069	101
Thermus thermophilus	HB8	*slpA*	917/27	H, S	X57333	40

[a] H, hexagonal; S, square; O, oblique.
[b] In *Campylobacter fetus* subsp. *fetus*, the lattice type was found to be dependent on the molecular weights of the S-layer subunits (H, 97,000; S, 127,000 and 149,000).
[c] Presumably.

to six-amino-acid sequences were observed at relatively constant distances over the whole middle and the corresponding C-terminal parts of S-layer proteins from different *Bacillus* spp. (SbsC, Sap, CTC, OlpA, and SbsB; Table 1), most probably assembling into oblique lattices (53). Considering the highly competitive situation of closely related organisms in their natural habitats, it is obvious that the S-layer surface has to contribute to diversification rather than to conservation. With respect to this, the importance of S-layer variation leading to the expression of alternative S-layer genes under different stress factors such as those imposed by the immune system of a host in response to an S-layered pathogen or drastic changes in the growth and environmental conditions for nonpathogens is conceivable (29, 112).

For S-layered bacteria with a generation time of 20 min, approximately 500 S-layer subunits have to be synthesized per second, translocated through the cell wall, and incorporated into the existing S-layer lattice (128). To keep the bacterial surface completely covered with a closed protein lattice, promoters preceding S-layer genes must be very strong (66). Actually, the promoter of the S-layer gene of *Lactobacillus acidophilus* is twice as efficient as that preceding the gene encoding lactate dehydrogenase (12, 13, 104), which is considered to be one of the strongest promoters in bacteria. Two

promoters were identified for the S-layer gene of *L. brevis* ATCC 8287 (55), *A. salmonicida* (24), and *B. stearothermophilus* ATCC 12980 (53), which are most probably used during different growth stages. The P2 promoter preceding the S-layer gene *sbsC* in *B. stearothermophilus* ATCC 12980 seems to be 2.4 times more frequently used in exponentially growing cells than the P1 promoter (53). The existence of three tandemly arranged promoters has been reported for the *cwp* operon of *B. brevis* 47 (1), an organism which concomitantly produces two different types of S-layer proteins that assemble sequentially into superimposed S-layer lattices. The promoter of the *slpA* gene encoding the S-layer protein of *L. brevis* (147) was recently exploited for heterologous expression of proteins and was recognized in *Lactococcus lactis*, *L. plantarum*, and *L. gasseri* (54).

With the exception of the S-layer proteins of the gram-negative bacteria *Caulobacter* sp. and *Campylobacter* sp., all of those sequenced to date are produced with an N-terminal secretion signal peptide which is cleaved off after translocation through the plasma membrane. Linker peptide insertions at the C-terminal end of the S-layer protein of *Caulobacter crescentus* (Table 1) indicated the existence of a type I secretion signal (8, 9). A similar observation was reported for the S-layer protein of *Campylobacter fetus*, which also reveals potential secretion signals at the C-terminal part (142). In *Serratia marcescens*, the *slaA* gene is located in the upstream region of the *lip* exporter genes and most probably encodes an S-layer protein (56). The putative S-layer protein is strictly recognized by the *lip* system, which belongs to the ATP-binding cassette exporter and which is responsible for signal peptide-independent secretion of a lipase and a metalloprotease. On the other hand, the S-layer proteins VapA and AhsA of the gram-negative bacteria *A. salmonicida* and *A. hydrophila* are produced with either a 21- or a 19-amino-acid-long signal peptide (23, 24, 141). A conserved gene (*abcA*) which is located immediately downstream of the *vapA* gene and affects its expression in *Escherichia coli* was identified. The corresponding AbcA protein possesses P-loop-containing residues at the N terminus, suggesting that this protein has ATP-binding activity and belongs to the ABC family of transport proteins (25). Further, it could be demonstrated that AbcA is a bifunctional protein in which the N-terminal part regulates the synthesis of the O-polysaccharide side chains of the LPS, functioning as a binding site for the VapA protein. The C-terminal leucine zipper increases expression of the *vapA* gene and can thus be considered as a transcriptional activator of the P2 promoter (25, 95, 96).

The existence of a translational autoregulation system was reported for the S-layer protein SlpA of *Thermus thermophilus* HB8 (41). Three genes which specifically repressed the expression of the S-layer protein promoter were cloned in *E. coli*. Sequence comparison revealed that one of these genes encodes a protein (Rep54) which corresponds to the C-terminal fragment of the S-layer protein and could bind to the 5′-untranslated region of the S-layer protein mRNA.

ATTACHMENT OF S-LAYER PROTEINS TO THE UNDERLYING CELL ENVELOPE LAYER

In gram-positive bacteria, the rigid cell envelope layer is composed of peptidoglycan and accessory (secondary) cell wall polymers which may be teichoic acids, teichuronic acids, lipoteichoic acids, or lipoglycans (2, 93). The polymer chains are either covalently linked to the peptidoglycan backbone via phosphodiester bonds or tethered to a lipid anchor moiety (2). Most of the biological functions discussed for secondary cell wall polymers, such as binding of cations, keeping of the peptidoglycan sacculus in an expanded state by charge repulsion, binding of protons to create an acidic cell wall during bacterial growth and division, or provision of a physical barrier to prevent diffusion of nutrients and metabolites, were seen in context with their net negative charge, and only for *B. subtilis* were the teichoic acids reported to serve as a binding site for a cell wall autolysin (49).

By sequence comparison, S-layer-homologous (SLH) motifs (74) have been identified at the N-terminal part of many S-layer proteins (14, 26, 33, 39, 40, 51, 65, 71, 72, 85, 97, 144) and at the C-terminal end of cell-associated exoenzymes (71, 78, 79) and other exoproteins (71, 73) of gram-positive bacteria. According to their origin (S-layer proteins, cell-associated exoproteins, porins), SLH motifs have been divided into three main groups whose specific properties have recently been reviewed by Engelhardt and Peters (38). Typically, S-layer proteins and cell-associated exoenzymes possess three repeats of SLH motifs each consisting of 50 to 60 amino acids. Due to their wide distribution among gram-positive bacteria, SLH motifs were suggested to anchor cell-associated exoproteins permanently or transiently to the cell surface (74). In contrast to the original assumption that peptidoglycan functions as a binding site (74), it is now evident that secondary cell wall polymers serve as anchoring structures for many S-layer proteins, cell-associated exoenzymes, and exoproteins (15, 22, 51, 72, 73, 83, 107).

First studies regarding the importance of secondary cell wall polymers for anchoring of S-layer proteins to the rigid cell wall layer (107) were carried out with the S-layer protein SbsB (65) from *B. stearothermophilus* PV72/p2, an oxygen-induced variant strain of *B. stearothermophilus* PV72/p6 (112). The S-layer protein SbsB carries three typical SLH motifs at the N-terminal part. Affinity studies using the whole S-layer protein (107) and an N-terminally truncated form (Δ3 SLH motifs; amino acids 208 to 920; Moll, unpublished data), as well as native peptidoglycan-containing sacculi and those extracted with hydrofluoric acid, leading to pure peptidoglycan, revealed that the N-terminal part recognizes a secondary cell wall polymer composed mainly of *N*-acetylglucosamine and *N*-acetylmannosamine and not the peptidoglycan as a binding site (107). However, affinity studies with proteolytic cleavage fragments indicated the coexistence of a binding region for peptidoglycan and the secondary cell wall polymer on the N-terminal part of this S-layer protein (111). The isolated secondary cell wall polymer had unique effects on the S-layer protein SbsB (110). Firstly, the presence of the secondary cell wall polymer inhibited the in vitro self-assembly of the guanidine hydrochloride-extracted S-layer protein and kept the S-layer protein in a water-soluble state. Under these conditions, the S-layer protein showed a significantly enhanced tendency to recrystallize into closed monolayers on those solid supports to which the subunits bind with their outer surface. Secondly, the polymer chains protected the S-layer protein against proteolytic attack and specifically masked an N-terminal endoproteinase Glu-C cleavage site. Preliminary studies indicate that a highly specific lectin-type recognition mechanism (110) exists between the S-layer protein and this type of secondary cell wall polymer.

More recently, the complete structure of the secondary cell wall polymer functioning as a binding site for the SLH motifs of the S-layer protein from *B. sphaericus* CCM 2177 was provided by nuclear magnetic resonance analysis (51). This secondary cell wall polymer is a teichuronic acid and is composed of disaccharide repeating units having the structure →3)-[4,6-*O*-(1-carboxyethylidene)]$_{\sim 0.5}$-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→. In contrast to the SLH motifs of most S-layer proteins, which reveal a positive net charge, those of *B. sphaericus*

strains (14, 26) are net negatively charged, which explains why bivalent cations are required for binding of the S-layer subunits to the rigid cell envelope layer (51).

Experimental evidence that the SLH motifs recognize a cell envelope component that could be extracted from peptidoglycan-containing sacculi with hydrofluoric acid was further provided for the S-layer proteins Sap and EA1 of *B. anthracis* (22, 85) and SlpA of *C. thermocellum* (22, 72) and the S-layer protein and cell wall-associated xylanase of *Thermoanaerobacterium thermosulfurigenes* EM1 (15). In affinity studies in which peptidoglycan-containing sacculi were used as a binding matrix, the K_d values for the polypeptides comprising either the SLH motifs of EA1 and Sap were 1.8×10^{-7} and 1.0×10^{-7} M, respectively (22). Similar values were determined for the SLH motifs and peptidoglycan-containing sacculi of *C. thermocellum* (22). In all cases, pure peptidoglycan was unable to bind the SLH motifs (22).

In contrast to most S-layer proteins of gram-positive bacteria, those of *B. stearothermophilus* wild-type strains (53, 65) and *Lactobacillus* (11, 12, 18, 92, 147) do not possess SLH motifs. Nevertheless, the N-terminal part of the S-layer proteins of *B. stearothermophilus* wild-type strains is highly conserved and recognizes a net negatively charged secondary cell wall polymer as the binding site (35, 53). This type of secondary cell wall polymer is composed of tetrasaccharide repeating units that contain glucose, *N*-acetylglucosamine, and 2,3-diacetamido-2,3-dideoxymannuronic acid in a molar ratio of 1 to 1 to 2 (117). Sequence analysis of the S-layer proteins from *B. stearothermophilus* PV72/p6 and ATCC 12980 (Table 1) revealed an accumulation of arginine, lysine, and tyrosine at the N-terminal part. Because of a high density of positively charged amino acids, the N terminus possesses a positive net charge, which is in contrast to that of the whole S-layer proteins (53). Thus, bivalent cations were not required for binding of the S-layer subunits to the rigid cell wall layer (35), indicating the existence of direct electrostatic interactions between the N-terminal region and the secondary cell wall polymer. The accumulation of tyrosine in the N-terminal part is especially interesting, since in carbohydrate-binding proteins such as lectins, tyrosine interacts with N-acetylated sugars via hydrogen bonds (108, 152). The production of truncated forms of SbsC confirmed that the N-terminal part is not involved in the self-assembly process (35, 53) and seems to fold independently of the remainder of the protein sequence. According to secondary-structure prediction, about 70% of the N-terminal amino acids are organized as α helices.

As described above, the S-layer proteins of *Lactobacillus* do not possess SLH motifs (11, 12, 18, 92, 147). In earlier studies, Masuda and Kawata (77) reported that these S-layer proteins recognized a neutral polysaccharide but not teichoic acids or the peptidoglycan as a binding site. To conclude, the S-layer proteins from most gram-positive bacteria can be considered cell surface-located carbohydrate-binding proteins with the ability to self-assemble into monomolecular crystalline arrays (110). For a better understanding of the importance of secondary cell wall polymers for S-layer protein folding, prevention of self-assembly within the peptidoglycan-containing layer (16), and anchoring of the S-layer subunits to the cell surface, more detailed studies are required. According to the suggestion that S-layers may be considered cell surface-located carbohydrate-binding proteins, the N-terminal region of the S-layer protein of the gram-negative bacterium *C. crescentus* recognizes distinct oligosaccharides of the LPS as binding sites (9, 148). Two serotypes that depend on the type of LPS present in the outer membrane are distinguished in *C. fetus*, a pathogen for humans and ungulates (29). In type A cells, the S-layer protein SapA and its homologs are bound via a conserved N-terminal region of 184 amino acids to the type A LPS. The S-layer protein SapB and its homologs possess a different N-terminal region and recognize cells with type B LPS as a binding site (31, 32).

The example of *Corynebacterium glutamicum* ATCC 17965 demonstrated that in gram-positive bacteria, interactions other than that of the S-layer protein–polysaccharide type may exist. The S-layer protein of *C. glutamicum* ATCC 17965 has a hydrophobic C-terminal end which binds to a hydrophobic cell wall layer possessing a high content of mycolic acids (21). In archaea missing a rigid cell wall layer, the S-layer subunits closely interact with the plasma membrane. The S-layer protein of *H. halobium* shows a hydrophobic stretch of 21 amino acids on its C-terminal part which integrates into the plasma membrane (69). A similar observation was reported for the S-layer protein of *Staphylothermus marinus*, which carries an extremely stable protease on its 70-nm-long stalk (80, 99).

S-LAYER-DEFICIENT PHENOTYPES AND S-LAYER VARIATION

The importance of insertion sequence (IS) elements for the generation of S-layer-deficient phenotypes has been demonstrated for at least three different organisms. In general terms, IS elements are small translocating DNA segments which occur on the host chromosome or on plasmids with the potential to move within and between bacterial genomes (76). Because of the existence of IS target sequences, transposition does not occur randomly. The integration of IS elements can either repress or activate genes located downstream. Variants of *A. salmonicida*, a fish pathogen, with S-layer-deficient phenotypes were generated by insertion of two different IS elements into different sites of the *vapA* gene and its promoter region (47). The bacteria with S-layer-deficient phenotypes were isolated after a temperature upshift from 20 to 30°C. While IS*AS1* was unique among reported IS elements, IS*AS2* showed strong similarities to transposases encoded by the IS*30* family (76). However, both IS elements were found to be restricted to *A. salmonicida*, where they occurred in a low copy number. The insertion of IS elements was linked to the attenuation or complete loss of virulence (47). Temperature-dependent transposition was also described for IS*4712* (120), an IS element which inhibits the expression of the S-layer gene *sbsA* (67) in *B. stearothermophilus* PV72/p6, leading to the S-layer-deficient phenotype strain PV72/T5 (112). Integration of IS*4712* into the upstream region of the *sbsA* gene was induced by cultivating the organism at 67°C instead of 57°C. An S-layer-deficient phenotype variant of *B. stearothermophilus* ATCC 12980 was isolated from agar plates which were stored for at least 2 years at 4°C. PCR analysis revealed that the coding region of the *sbsC* gene was interrupted by a new type of IS element designated ISBst*12*, which currently cannot be attributed to any of the known IS families. Southern hybridization showed that ISBst*12* was present in multiple copies in the S-layer-carrying strain, as well as in that with the S-layer-deficient phenotype (34). Interestingly, ISBst*12* was also detected in *B. stearothermophilus* PV72/p6, its oxygen-induced strain variant PV72/p2, and the strain with the S-layer-deficient phenotype (PV72/T5). These findings are in agreement with the observation that organisms carrying one type of IS element tend to possess other types since multiple ISs can be delivered by a single vector (76).

S-layer variation leads to the expression of different types of S-layer genes or to the recombination of partial coding sequences. S-layer variation was studied in detail for *C. fetus*, an

important pathogen for humans and ungulates (29), but was also observed for nonpathogens such as *B. stearothermophilus* (112, 121). In the case of pathogens, S-layer variation can be considered a kind of antigenic variation responding to the lytic activity of the immune system of the host cells. Both type A and B cells of *C. fetus* can produce multiple S-layer proteins with a single form predominating (31, 32). The apparent molecular weights of SapA and its homologs lie between 97,000 and 149,000, and depending on the molecular size, the S-layer subunits assemble into lattices with either hexagonal or square symmetry (42). Eight or nine S-layer gene cassettes are present in *C. fetus* wild-type cells and are tightly clustered on the genome in a region of less than 93 kb. Several studies confirmed that antigenic variation is due to the recombination of partial coding sequences (30, 31). In addition to promotor inversion between two oppositely oriented gene cassettes, one of the S-layer gene cassettes bracketing the invertible element with a nonflanking, previously silent gene cassette is exchanged. Thus, *C. fetus* uses a single promoter strictly by a single DNA inversion event at frequencies independent of DNA fragment size, permitting expression of different S-layer gene cassettes (29, 30). Although the S-layer protein SapB and its homologs possess an N-terminal region different from that of SapA, noncoding regions 300 bp upstream and 1,000 bp downstream of the *sapA* and *sapB* open reading frames, including promotor and transcriptional terminator sequences, were essentially identical for both types of cells (31).

Environmental factors inducing change in S-layer gene expression in nonpathogens were studied for *B. stearothermophilus* (112, 121). Under oxygen-limited growth conditions, the *sbsA* gene (67) encoding the S-layer protein SbsA, which assembles into a hexagonally ordered lattice type, was stably expressed during continuous cultivation of *B. stearothermophilus* PV72/p6 (112). After a change to non-oxygen-limited growth conditions (oxygen stress), expression of the second S-layer gene *sbsB* (65) was induced. The S-layer protein SbsB assembles into an oblique lattice and shows only 25% identity to SbsA. Change in S-layer gene expression was highly coordinated with the synthesis of a different type of secondary cell wall polymer (112). Interestingly, the S-layer protein SbsA recognized both types of secondary cell wall polymers as binding sites, guaranteeing complete coverage of the cell surface during the oxygen-induced switch. On the other hand, the S-layer protein SbsB showed only affinity for binding of the newly synthesized secondary cell wall polymer. Preliminary studies indicate that the *sbsB* gene is generated from partial coding sequences, while the *sbsA* gene is disrupted during the switch (121).

FUNCTIONAL ASPECTS

Although considerable knowledge has accumulated on the structure, assembly, biochemistry, and genetics of S-layers, relatively little information is available about their specific functions (5, 109, 127). Considering that S-layer-carrying organisms are ubiquitous in the biosphere, the supramolecular concept of a closed, isoporous, crystalline surface layer could have the potential to fulfill a broad spectrum of functions. In functional terms, S-layers must not be seen as isolated components since they are generally only part of more complex envelope structures.

Because S-layer lattices possess pores identical in size and morphology in the 2- to 8-nm range, they work as precise molecular sieves, providing sharp cutoff levels for the bacterial cells (113). The largest pores in the 5- to 8-nm range were found in S-layer lattices of those archaea lacking a rigid cell wall layer in which these crystalline arrays fulfill a shape-determining or shape-maintaining function (3, 4, 50, 80, 99). As a general feature, the S-layer subunits of archaea lacking a rigid cell wall layer possess long, hydrophobic protrusions which integrate into the plasma membrane. Thereby, a kind of periplasmic space is formed between the S-layer and the plasma membrane where secreted macromolecules involved in nutrient degradation, nutrient transport, and folding and export of proteins could be stored (46, 103). According to the proposed function, the S-layer glycoprotein of *S. marinus* is held at a distance of as much as 70 nm from the plasma membrane by membrane-anchored stalks. Furthermore, two copies of a hyperthermostable protease showing significant similarity to subtilisin are attached near the middle of each stalk. This enzyme is resistant to heat inactivation to 95°C in the free form and to 125°C in the stalk-bound form (80, 99).

S-layers from *Bacillaceae* were found to function as adhesion sites for cell-associated exoenzymes. The high-molecular-weight exoamylase from two *B. stearothermophilus* wild-type strains were bound to the S-layer surface in a density that did not disturb diffusion of nutrients or metabolites through the S-layer lattice (36, 37). S-layer-associated exoenzymes were also described for *T. thermohydrosulfurigenes* (71, 78, 79).

Regarding protective functions, S-layers from gram-negative bacteria such as *A. salmonicida*, *C. fetus*, *A. serpens*, and *C. crescentus* were found to protect the cells from attack by bacterial parasites such as *Bdellovibrio bacteriovorus*, but they could not shield them from other predators like protozoa (63). A quite interesting type of protective function was reported for the S-layer lattice of *Synechococcus* GL-24 (122, 123), a cyanobacterium capable of growing in lakes with exceptionally high calcium and sulfate ion concentrations. The hexagonally ordered S-layer lattice functions as a template for fine-grain mineralization and is continuously shed from the cell surface to prevent clogging of further cell envelope layers.

S-layers can contribute to virulence when they are present as a structural component of the cell envelope of pathogens. The A-layer of *A. salmonicida* endows this organism with high or intermediate resistance to the bactericidal activity of complement in immune and nonimmune sera. Furthermore, the A-layer plays an important role in uptake of porphyrins and shows unique immunoglobulin- and extracellular matrix protein-binding capabilities (28, 43). A similar observation was reported for the S-layer from *B. cereus* isolated from periodontal infections (61). Only the S-layer-carrying cells adhered to matrix proteins and were resistant to polymorphonuclear leukocytes in the absence of opsonins. The serum resistance of *C. fetus* was found to be due to the inability of complement component C3b to bind to the S-layer surface. Thus, effective opsonization occurred only after the addition of specific antibodies (29). In *Rickettsia prowazekii* and *R. typhii*, causing either epidemic or endemic typhus, the S-layer protein is responsible for humoral and cell-mediated immunity (19). In *L. acidophilus*, the S-layer is responsible for the adhesion of the bacterial cells to the intestinal epithelium (119).

APPLICATION POTENTIAL

During the last 10 years, studies on the structure, morphogenesis, genetics, and function of S-layers revealed that these isoporous monomolecular arrays have a considerable application potential in biotechnology, molecular nanotechnology, and biomimetics (for reviews, see references 105, 116, 134, and 137). Many applications depend on the ability of isolated S-layer proteins to assemble into regular arrays in suspension or on suitable surfaces (e.g., silicon wafers, metals, and polymers)

or interfaces (e.g., lipid films and liposomes) upon removal of the disrupting agent used for their isolation.

Since S-layer lattices are assemblies of identical (glyco)protein species, these crystalline arrays exhibit repetitive physicochemical properties down to the subnanometer scale and possess pores identical in size and morphology. S-layers from various *Bacillaceae* were shown to be suitable for the production of isoporous ultrafiltration membranes with well-defined molecular weight cutoffs (113, 114, 134, 136). The S-layer lattice and the pore areas of S-layers contain functional groups (carboxylic acid, amine, and hydroxyl groups) which are aligned in well-defined positions and orientations. Accordingly, highly reproducible chemical modifications can be applied for optimization of molecular sieving properties and nonspecific adsorption (antifouling) characteristics (150, 151). The repetitive features of S-layers have led to their use as immobilization matrices for binding of monolayers of functional molecules (e.g., enzymes, antibodies, and immunogens) in a geometrically well-defined way. This application potential has been exploited for the production of bioanalytical sensors, immunoassays, affinity microparticles, and affinity membranes (116, 137).

Another line of development is directed toward the use of S-layer self-assembly products in suspension as a combined carrier-adjuvant system against infection with pathogenic bacteria in the immunotherapy of cancers and in antiallergic immunotherapy (52, 91, 135). On the other hand, S-layers of pathogenic organisms were identified to be essential for virulence. Whole-cell preparations or partially purified cell products are currently used as attenuated vaccines against fish pathogens (57, 143). Another broad application for S-layers is based on the ability of subunits to recrystallize into coherent lattices on functional lipid membranes (105, 135), including liposomes (68, 75, 94). These S-layer-stabilized lipid membranes mimic the supramolecular structure of those archaeal envelopes which possess S-layers as exclusive wall components or virus envelopes. They can be used in diagnostics, as vaccines, for drug targeting or delivery, and for gene therapy (115, 116, 135, 137). Alternative or complementary to existing S-layer technologies, genetic approaches are used to incorporate functional domains (proteins and glycans) in S-layer subunits while maintaining their ability to self-assemble into regular arrays (137).

Finally, S-layer lattices recrystallized on solid supports (e.g., silicon wafers) can be patterned by microlithographic procedures as required for lab-on-a-chip technologies (105, 135). The observation that S-layers of cyanobacteria may induce precipitation of minerals (122, 123) led to the concept of their use as templates for the formation of regular arrays of metal clusters as required in molecular electronics and nonlinear optics (27, 105, 124).

CONCLUSION

It is now evident that S-layers are the most common cell surface components of prokaryotic organisms. In comparison with the considerable accumulation of data concerning the structure, chemistry, assembly, and genetics of S-layer proteins, relatively little is known about their evolutionary relationship; their biosynthesis, including glycosylation; their transfer through intermediate envelope layers to sites of lattice extension; and their functional potential. Since S-layers of gram-positive and gram-negative bacteria can be lost in the course of subculturing in the laboratory, elucidation of their functional significance for a particular organism requires ecological approaches mimicking complex natural habitats and potential hazards. Although structurally diverse S-layers with barely any sequence identities in the constituent subunits are observed even among strains of the same species, these proteins must have domains with common structural and functional significance. In this context, it is important to remember that some organisms possess multiple S-layer genes and can even assemble different superimposed S-layers on their surface. This diversity and differentiation potential is further stressed by the observation that in S-layer proteins of strains of the same species can be either glycosylated or not (87, 88).

Finally, since S-layers can be considered supramolecular structures with the intrinsic ability to assume closed structures with low free energy during cell growth, it is tempting to speculate that S-layers, like membranes, could have fulfilled barrier and supporting functions as required by self-reproducing systems during the early periods of biological evolution (132).

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