**REVIEW
ARTICLE****S-layers of *Bacillus* species**

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Overview

S-layers are regular crystalline surface layers in prokaryotic organisms composed of protein or glycoprotein subunits (Beveridge, 1994; Sleytr *et al.*, 1993, 1988a, 1986b; Sleytr & Messner, 1992, 1988a, b, 1983; Beveridge & Graham, 1991; Hovmöller *et al.*, 1988; Koval, 1988; Baumeister & Engelhardt, 1987). Most of the presently known S-layers are composed of identical proteins or glycoproteins of molecular mass 30–220 kDa (Sleytr *et al.*, 1994; Messner & Sleytr, 1992). Such layers have been recognized as common features of both prokaryote domains (archaeobacteria and eubacteria). S-layers can be associated with quite different cell envelope structures, such as peptidoglycan, pseudomurein or components of the outer plasma membrane (Fig. 1). In Gram-negative eubacteria (e.g. *Aeromonas salmonicida*), S-layers are associated with the outer membrane and in some archaeobacteria (e.g. *Methanococcus jannaschii*), S-layers are the sole cell wall structure and therefore associated only with the plasma membrane. The subunits are linked together and also to the underlying cell envelope layers by non-covalent forces (Messner & Sleytr, 1992; Beveridge & Graham, 1991; König, 1988). S-layers can be exclusive wall components in cell envelope structures of Gram-negative archaeobacteria (Sleytr & Messner, 1992). Most of the presently known S-layers are composed of a single (glyco)protein species endowed with the ability to assemble into two-dimensional arrays on a supporting envelope layer, and they are characterized by defined symmetry and pores of uniform size (Hovmöller *et al.*, 1988; Sleytr & Messner, 1983). When S-layers are compared with heteroporous materials, they can be considered as an ideal matrix (e.g. *Bacillus* and *Clostridium* species), because functional groups are not only aligned in high density on the S-layer surface but also show identical position and orientation on each protomer (Sára *et al.*, 1993a, b). On the outer surface of S-layers from most members of the family *Bacillaceae*, an equimolar amount of amino and carboxyl groups is present, and in the case of S-layer glycoproteins there is also a high number of surface-located hydroxyl groups

available (Sleytr *et al.*, 1994). Comparative analysis of amino acid and genetic studies on S-layers showed that crystalline arrays are usually composed of weakly acidic proteins at neutral pH. The content of hydrophobic amino acids is generally high and the cysteine or methionine content low (Sleytr *et al.*, 1993). S-layers are porous crystalline membranes 5–15 nm thick (Sleytr *et al.*, 1994), which completely cover the cell surface and can provide micro-organisms with a selective advantage by functioning as protective coats, molecular sieves, molecule and ion traps, and structures involved in cell adhesion and surface recognition (Sleytr *et al.*, 1993; Messner & Sleytr, 1992; Hovmöller *et al.*, 1988; Sleytr & Messner, 1983). S-layers were identified as contributing to virulence when present as a structural component of pathogens and possess great potential for various biotechnological, biomedical and non-biomedical applications (Sleytr *et al.*, 1993).

The genus *Bacillus* consists of facultatively anaerobic Gram-positive, spore-forming rod-shaped bacteria and is a principal member of the family *Bacillaceae*. Most *Bacillus* species are saprophytic, using a range of naturally occurring substrates (Maiden *et al.*, 1992). Some species are associated with human and animal infections and food spoilage. A number of major pathogens are encountered among the many recognized *Bacillus* species. *B. anthracis* causes anthrax in animals and humans (Thorne, 1993). *B. cereus* is a major cause of food poisoning (Drobniewski, 1993; Beecher & Macmillan, 1991). Other *Bacillus* species may cause serious infections such as bacteraemia, meningitis, endocarditis and eye infections as opportunists, often in immunocompromised patients. *Bacillus* species are also isolated sporadically from pockets associated with marginal periodontitis and implantitis, and from the root canal (unpublished observations). Some *Bacillus* species such as *B. thuringiensis* are insect pathogens and are used as 'biological insecticides' (Aronson, 1993). Only sporadically have reports on the existence of S-layers in *Bacillus* species appeared in the literature. This article gives a review of the subject, together with information on aspects such as their structure, isolation, synthesis, functions and genetics.

Fig. 1. Schematic illustration of major classes of prokaryotic cell envelopes containing crystalline surface layers (S-layers). Left, thin-section profile as revealed by electron microscopy; right, molecular architecture showing major components. (a) Gram-positive cell envelope as observed in eubacteria and archaeobacteria (containing peptidoglycan or pseudomurein, respectively); (b) Gram-negative cell envelope as observed in eubacteria. S, crystalline surface layer; SA, location of possible additional S-layer; CW, rigid cell wall layer composed primarily of peptidoglycan in eubacteria or pseudomurein in archaeobacteria; PG, peptidoglycan layer; CM, cytoplasmic membrane; OM, outer membrane. (Modified from Sleytr & Messner, 1992.)

Crystalline surface layers in *Bacillus* species

S-layers have been found in several *Bacillus* species (Doyle *et al.*, 1986; Abe & Kimoto, 1984; Messner *et al.*, 1984; Abe *et al.*, 1983; Howard & Tipper, 1973; Nermut & Murray, 1967; Goundry *et al.*, 1967) and different types are listed in Table 1. The S-layer of *B. sphaericus* NCTC 9602, which is composed of an acidic glycoprotein containing a single homogeneous peptide chain, and that of *B. brevis* 47 are the best studied in the *Bacillaceae*. *B. brevis* 47 has a three-layered wall between 27 and 29 nm thick in which the thin innermost layer of peptidoglycan and teichoic acid is covered by a hexagonal array of subunits (Udaka *et al.*, 1989; Tsuboi *et al.*, 1982). Pure S-layer preparations reveal more than one band on SDS-PAGE gels. This explains the presence of two closely associated S-layers composed of different subunit species (Abe *et al.*, 1983; Tsuboi *et al.*, 1982). *B. brevis* HPD31 contains one major protein, with an approximate molecular mass of 135 kDa, in its cell wall (Gruber *et al.*, 1988), while the two S-layer wall proteins of *B. brevis* 47 are 115 and 104 kDa in size (Daugulis *et al.*, 1993; Tsuboi *et al.*, 1982; Yamada *et al.*, 1981). A similar multilayered cell wall structure was also observed in S-layer-deficient bacteria, such as *Bacillus* sp. CIP 76-111, *B. polymyxa* and in *Clostridium nigrificans* (Leduc *et al.*, 1977; Nermut & Murray, 1967). Two S-

layer proteins are further reported in *B. macroides* strain D (Holt & Leadbetter, 1969), *B. brevis* CCM 1463 (Sára *et al.*, 1990) and *B. brevis* HPO33 (Gruber *et al.*, 1988; Yamada *et al.*, 1981). The two S-layers exhibit uniform pore morphologies, while individual lattices can display more than one type of pore (Hovmöller *et al.*, 1988; Sleytr *et al.*, 1988a). The purified S-layer from *B. stearothermophilus* NRS strain 2004/3a and *Desulfotomaculum nigrificans* strain NCIB 8395 produces more than one band on SDS-PAGE, and this can be explained by variation in the degree of glycosylation rather than by differences in the polypeptide portions of the subunits (Sleytr *et al.*, 1986b). The S-layer of *B. sphaericus* P-1 has square (p4) symmetry, a lattice constant of 13 nm, a thickness of 8 nm, and its structure is similar to that of the closely related species *Sporosarcina ureae*. Also, its rough surface layer faces towards the bacterial cell wall (Engelhardt *et al.*, 1986). According to Aebi *et al.* (1973), the T-layer (S-layer) structure can be divided into three domains: a major domain, a minor domain and an arm. The S-layer of *B. sphaericus* P-1 has minor and arm domains (Aebi *et al.*, 1973) which form a basket-like structure raised away from the cytoplasm, and such a feature seems to be characteristic of most S-layers (Baumeister *et al.*, 1986). The basket-like structure of the S-layer is highly perforated having a channel diameter less than 3 nm, thus suggesting that the S-layer

functions as a selective molecular barrier (Stewart *et al.*, 1986; Stewart & Beveridge, 1980). Gruber & Sleytr (1988) observed distinct insertion bands for the S-layer in *B. stearothermophilus*, which spirals around the cylindrical surface at a pitch angle related to the orientation of the lattice vectors of the arrays. Little or no S-layer was inserted into pre-existing S-layer at old cell poles, whereas new cell poles were covered with new proteins.

An interesting feature of many archaeobacteria and certain Gram-positive and Gram-negative eubacteria is the ability to glycosylate their S-layer proteins (Messner & Sleytr, 1991). The first glycoprotein S-layer was detected in *B. stearothermophilus* (Messner & Sleytr, 1991), containing two glycan substituents, an asparagine-linked rhamnan and an oligosaccharide having a diaminouronic acid (Messner *et al.*, 1987; Christian *et al.*, 1986). Studies of the S-layer glycoproteins from different *Bacillus* and *Clostridium* species showed that the composition of the glycans is a strain-specific feature. More than one type of carbohydrate residue may be linked to S-layer proteins and the chain length can vary from a few sugars up to approximately 150 monosaccharide residues (Messner & Sleytr, 1992, 1991). The glycan chain can be linked to the protein moiety by N- or O-glycosidic linkages (Sleytr *et al.*, 1994). Glycosylated S-layers with varying carbohydrate contents have been detected in a number of strains from the *Bacillaceae* such as *B. sphaericus* (Lewis *et al.*, 1987; Word *et al.*, 1983), *B. stearothermophilus* (Messner *et al.*, 1987; Küpcü *et al.*, 1984) and *D. nigrificans* (Sleytr *et al.*, 1986b). Glycosylation of the S-layer proteins may be of crucial importance in protecting the cells from their own (or foreign) exoproteases (Sleytr & Messner, 1983). Protein glycosylation is a costly process to the organisms in terms of energy and materials, because the carbohydrate moieties are secondary gene products, necessitating the synthesis of specific enzymes (Sleytr *et al.*, 1986b). Preliminary taxonomic characterization of *Bacillus* sp. L420-91 revealed that the S-layer glycoprotein of this organism is closely related to that of *B. aneurinolyticus*, having p4 symmetry with molecular mass 109 kDa. The total carbohydrate content of the purified glycoprotein was approximately 3.5% (Kosma *et al.*, 1995).

Preparation and isolation of S-layers

S-layers of different bacteria may vary considerably with respect to their resistance to disruption into monomeric subunits, and a wide range of methods has been applied for their isolation and purification. The subunits of most S-layers interact with each other and with the supporting envelope layer through non-covalent forces. In Gram-positive bacteria a complete disintegration of the layer into monomers can be achieved by treatment of intact cells or walls with high concentrations of H-bond-breaking agents such as urea, guanidine hydrochloride (Sára & Sleytr, 1994; Kuen *et al.*, 1994; Sleytr *et al.*, 1986a) or SDS (Kotiranta *et al.*, 1995), or by lowering or raising the pH value (Sleytr, 1976). Procedures used for

extracting S-layers from Gram-positive bacteria are summarized in Table 2. S-layers from Gram-negative bacteria frequently disrupt upon application of metal chelating agents such as EDTA, EGTA, cation substituents such as Na^+ to replace Ca^{2+} , or pH changes such as a reduction to $\text{pH} < 4.0$ (Sleytr & Messner, 1992, 1988a, 1983; Sleytr *et al.*, 1986a). In the case of *B. stearothermophilus* DSM 2358, S-layer fragments were released during preparation of spheroplasts by treatment of whole cells with lysozyme (Egelseer *et al.*, 1995). S-layers are generally isolated from cell envelope fragments prepared by mechanical cell disruption using, for example, sonication, French pressure-cells, cell homogenization with glass beads or, in special cases, freeze-thawing. Treatment with nucleases may be necessary before final isolation of the cell wall by differential centrifugation (Sprott *et al.*, 1994). The cell-wall preparation of *B. stearothermophilus* (Egelseer *et al.*, 1995; Breitwieser *et al.*, 1992) and *B. brevis* HPD31 (Ebisu *et al.*, 1990), was obtained by treatment with detergents such as Triton X-100 to selectively solubilize adherent cytoplasmic membrane fragments contaminating the cell walls (Breitwieser *et al.*, 1992; Beveridge, 1979; Sleytr & Glauert, 1976). Removal of the solubilizing agent by dialysis or dilution allowed the S-layer to be reassembled into two-dimensional crystalline lattices identical to those observed in intact cells (Jaenicke *et al.*, 1985). From extraction and disintegration experiments it can be concluded that the bonds holding the S-layer subunits together are stronger than those binding the crystalline array to the supporting envelope layer (Beveridge, 1994). Studies of S-layer-carrying organisms, particularly members of the family *Bacillaceae*, show that an inner S-layer can be formed on the peptidoglycan of intact cells when the plasma membrane becomes detached during plasmolysis or autolysis (Sleytr, 1978), for example in *B. polymyxa* (Nermut & Murray, 1967). There are some indications that S-layers of some archaeobacteria are stabilized by covalent bonds between adjacent subunits (Sleytr *et al.*, 1993).

Synthesis of S-layers

S-layer protein can constitute up to 15% of the total protein of a cell in the exponential growth phase (Sleytr *et al.*, 1993). Under artificial conditions, when bacteria are cultivated in laboratory media, S-layer-deficient mutants can outgrow the wild-type strain (Koval & Murray, 1986). S-layers are frequently lost upon prolonged cultivation under laboratory conditions indicating that they provide the organisms with a selective advantage in their natural and competitive habitats (Sleytr, 1978). Although many S-layer proteins are synthesized as secretory precursors, mature proteins are usually not liberated into the surrounding medium but are assembled into regular arrays on the cell surface (Sleytr & Messner, 1992; Sleytr *et al.*, 1988b). An exception is the case of certain strains of *B. brevis* which, dependent on the growth conditions, produce excess amounts of S-layer material. This is shed into the medium (Sleytr & Messner, 1992). Only a few organisms

Table 1. Types of S-layer lattices in *Bacillus* speciesModified from Sleytr *et al.* (1996, 1988b); Messner & Sleytr (1992); Sleytr & Messner (1983).

Species	Strain	Lattice symmetry*	Spacing (nm)	Molecular mass (kDa)	Reference
<i>B. alvei</i> †	CCM 2051	p2	10·0–7·9	127/128/147	Altman <i>et al.</i> (1991); Sára <i>et al.</i> (1990)
	183	p6	10–12	ND	Yoshii (1966)
<i>B. aneurinolyticus</i> (type I)	7 strains	p4	9·6	129	Abe & Kimoto (1984)
	13 strains	p4	9·6	114	Abe & Kimoto (1984)
<i>B. anthracis</i>	–	p6	7–10	94	Toumelin <i>et al.</i> (1995); Farchaus <i>et al.</i> (1995); Holt & Leadbetter (1969); Gerhardt (1967)
		p1			Doyle <i>et al.</i> (1986)
<i>B. brevis</i>	HPD31	p6	17·4–18·4	110–150	Ebisu <i>et al.</i> (1990); Tsuboi <i>et al.</i> (1988); Gruber <i>et al.</i> (1988)
	CCM 1089‡	p2	11·6–8·5	119/102	} Lupas <i>et al.</i> (1994); Sára <i>et al.</i> (1990)
	–		16·7	ND	
	–	p6			
	–	p4	ND	ND	Sára <i>et al.</i> (1990)
	47‡	p6	14·5	150	} Koval (1993); Tsuboi <i>et al.</i> (1986, 1982); Yamada <i>et al.</i> (1981)
	–			130	
	47-57 (phage-resistant mutant)	p6	14·5		
		p6	14·5	150	Tsuboi <i>et al.</i> (1982); Yamada <i>et al.</i> (1981)
	CCM 1463‡	p2	11·0–8·0	102	} Koval (1993); Sára <i>et al.</i> (1990)
	–	p6	17·0	118	
	–	p2	ND	ND	Sára <i>et al.</i> (1990)
	S-1	p6	17	129	Abe <i>et al.</i> (1983)
<i>B. cereus</i>	599-1-95	p6	ND	97·4	Authors' unpublished results
	600-3-95	p6	ND	97·4	Authors' unpublished results
	ATCC 4342	p4	9·0–10·0	ND	Ellar & Lundgren (1967)
<i>B. circulans</i>	CCM 1084	p2	9·6–8·0	117	Sára <i>et al.</i> (1990)
	CCM 2048	p4	8·6	120	Sára <i>et al.</i> (1990)
<i>B. coagulans</i>	E36-68	p2	9·4–7·4	100	Pum <i>et al.</i> (1993); Sára <i>et al.</i> (1992); Ohnesorge <i>et al.</i> (1992)
<i>B. fastidiosus</i>	–	p4	12·0–13·5	ND	Holt & Leadbetter (1969)
<i>B. licheniformis</i>	NM105	p4	11·6	98	Tang <i>et al.</i> (1989)
<i>B. macroides</i>	Strains A and D‡	p4	5·0–6·0	ND	Holt & Leadbetter (1969)
	–	p4	9–10	ND	Holt & Leadbetter (1969)
<i>B. megaterium</i>	–	p4	19·0	ND	Sawatake (1966)
<i>B. polymyxa</i>	CCM 1459	p4	10·0	142	Sára <i>et al.</i> (1990)
	NCIB 4747	p4	10·0	ND	Burley & Murray (1983); Finch <i>et al.</i> (1967); Goundry <i>et al.</i> (1967); Nermut & Murray (1967)
<i>B. psychrophilus</i>	W16A	p4	15–16	ND	Holt & Leadbetter (1969)

Table 1. Cont.

Species	Strain	Lattice symmetry*	Spacing (nm)	Molecular mass (kDa)	Reference
<i>B. schlegelii</i>	DSM 2000	p4	9.0	ND	Schenk & Aragno (1979)
<i>Bacillus</i> sp.	CIP 76-111	p6	11.0	255	Leduc <i>et al.</i> (1977)
	KL8	p6	9–10	ND	Kari <i>et al.</i> (1990)
	L420-91	p4	ND	109	Kosma <i>et al.</i> (1995)
	M3198	p2	9.5	ND	Haapasalo <i>et al.</i> (1988)
<i>B. sphaericus</i>	Several strains	p4	13.0	ND	Sleytr & Glauert (1975); Holt & Leadbetter (1969)
	Several strains	ND	ND	127–133	Lewis <i>et al.</i> (1987); Word <i>et al.</i> (1983)
	NCTC 9602	p4	13.0	142	Hastie & Brinton (1979a, b)
	9602 (Lmw)	p4	13.0	120	Hastie & Brinton (1979a, b)
	P-1§	p4	13.0	140	Lepault <i>et al.</i> (1986); Howard <i>et al.</i> (1982); Howard & Tipper (1973)
	CCM 2120	p4	11.2	120	Sára <i>et al.</i> (1990)
	CCM 2177	p4	12.6	122	Ohnesorge <i>et al.</i> (1992); Sára <i>et al.</i> (1990)
	2362	p2	ND	125	Bowditch <i>et al.</i> (1989)
	39 strains	p6, p4, p2	5.2–17.9	83–170	Messner <i>et al.</i> (1984); Sleytr <i>et al.</i> (1969)
	4 strains	p6, p4, p2	8.5–22.5	93–170	Sleytr <i>et al.</i> (1986b)
<i>B. stearothermophilus</i>	PV72	p6	22.5	130	Sára <i>et al.</i> (1996); Kuen <i>et al.</i> (1994); Sára & Sleytr (1994); Gruber & Sleytr (1988)
	ATCC 12980	p2	ND	ND	Gruber & Sleytr (1988)
	E4-65	p4	10.8	97	Sleytr <i>et al.</i> (1986b)
	DSM 2358	p2	ND	98	Egelseer <i>et al.</i> (1995)
	NRS 1536/3c	p4	13.8	120	Sára & Sleytr (1994); Sára <i>et al.</i> (1992); Sleytr <i>et al.</i> (1986b)
	NRS 2004/3a	p2	11.6–9.4	96; 119 , 147 ; 170	Sára & Sleytr (1994); Sleytr <i>et al.</i> (1986b)
	3a/V1	p2	9.9–7.6	97	Sára & Sleytr (1994)
	3c/p2	p2	9.9–7.6	97	Sára & Sleytr (1994)
	PV72/p2	p2	9.9–7.6	97	Sára & Sleytr (1994)
<i>B. subtilis</i>	–	p4	7.5	ND	Howatson & Russell (1964)
<i>B. thuringiensis</i> 4045 subsp. <i>galleriae</i>	–	p2	8.5–7.2	91.4	Luckevich & Beveridge (1989)
<i>B. thuringiensis</i> var. <i>berliner</i>	1715 (AH 418)	p6	ND	66.2–97.4	Authors' unpublished results

ND, Not determined.

* p6, hexagonal; p4, square; p2, oblique.

† Now *Paenibacillus alvei* (Messner & Sleytr, 1992).

‡ Two S-layers.

§ Formerly *Bacillus brevis* P-1 (Howard & Tipper, 1973).

|| According to the periodate acid–Schiff (PAS) staining procedure, bands are glycosylated.

have been reported to shed large or small quantities of excess material into the medium as assembled S-layer fragments (Yamada *et al.*, 1981; Sleytr & Glauert, 1976; Thorne *et al.*, 1976; Udaka *et al.*, 1989; Udaka, 1976). *B.*

brevis 47 is known to secrete 16 g of S-layer proteins l⁻¹ into the medium (Wight *et al.*, 1992), particularly on supplementation of the medium with amino acids (Sára *et al.*, 1996). However, according to Takagi *et al.* (1989)

Table 2. General isolation procedures for S-layers from Gram-positive bacteria

Procedure	Reference
Removal of peptidoglycan layer with lysozyme	Egelseer <i>et al.</i> (1995); Lupas <i>et al.</i> (1994); Sára <i>et al.</i> (1992); Abe <i>et al.</i> (1983); Beveridge (1979); Masuda & Kawata (1979); Nermut & Murray (1967); Sleytr (1976)
Extraction of S-layer proteins with low concentrations of chaotropic agents, e.g. 4 M/6 M urea, 1.5 M guanidinium hydrochloride, etc.	Takumi <i>et al.</i> (1991); Bowditch <i>et al.</i> (1989); Sleytr & Plohberger (1980); Nermut & Murray (1967)
Extraction of S-layer proteins with high concentrations of chaotropic agents or detergents, e.g. 8 M urea, 5 M guanidinium hydrochloride, 1% SDS, 100% formamide etc.	Sára <i>et al.</i> (1996, 1992, 1990); Sára & Sleytr (1994); Pum <i>et al.</i> (1993); Tsuboi <i>et al.</i> (1989); Hastie & Brinton (1979a); Masuda & Kawata (1979); Sleytr & Thorne (1976); Howard & Tipper (1973); Goundry <i>et al.</i> (1967); Nermut & Murray (1967)
Extraction of S-layer from whole cells by treatment with 0.2 M glycine hydrochloride at pH 3.0	Dooley <i>et al.</i> (1988)
Extraction with chaotropic agents followed by column chromatographic purification, e.g. Sepharose CL-6B in 5 M guanidinium hydrochloride	Takumi <i>et al.</i> (1991); Küpcü <i>et al.</i> (1984)

B. brevis HPD31 secretes up to 30 g l⁻¹ of a protein derived from a cell-wall component under optimal growth conditions. Characteristic of most members of the family *Bacillaceae* is the lack of S-layer proteins in the culture supernatant during exponential growth. Only during the late exponential–early stationary phase are free S-layer sheets frequently detected (Sleytr & Glauert, 1975). In the case of *B. sphaericus*, the S-layer protein is synthesized during vegetative growth (Lewis *et al.*, 1987) and upon completion of exponential growth, there is a burst in synthesis of the 122 kDa protein (Broadwell & Baumann, 1986). A pool of S-layer protein must, even in a cell with no overproduction, exist either in the periplasm of Gram-negative bacteria (Beveridge & Graham, 1991) or in the peptidoglycan network of Gram-positive bacteria.

In certain Gram-positive archaeobacteria two different types of lipid carriers are involved in the biosynthesis of cell envelope components. Undecaprenol is the common lipid carrier for pseudomurein biosynthesis, whereas dolichol is involved in the biosynthesis of the S-layer glycoproteins (Messner & Sleytr, 1992). Synthesis of the S-layer glycoprotein of *B. alvei* demonstrated that the glycan biosynthesis in eubacteria is very similar to that in archaeobacteria (Hartmann *et al.*, 1993). The rapid appearance of granular regions close to the regular S-layer lattice on the cell surface of *B. stearothermophilus* indicates that synthesis of original S-layer proteins by wild-type strains stops immediately on an increase in the oxygen supply (Sára *et al.*, 1996; Sára & Sleytr, 1994). The S-layer proteins from three different *B. stearothermophilus* strains revealing oblique, square or hexagonal lattice symmetry were preserved during growth in continuous culture on complex medium only under oxygen-limited conditions in which glucose was used as the sole carbon source (Sára & Sleytr, 1994). This shows that synchronous S-layer protein synthesis is not a stable

process in the case of *B. stearothermophilus* PV72 (Sára *et al.*, 1996).

Electron microscopy

Unlike most other surface structures, the S-layer arrays can be unequivocally identified only by electron microscopy (EM). With EM, freeze-etching (Sleytr & Messner, 1992) and negative staining (Beveridge, 1994, 1981) are the best methods for determining S-layer structures. Recently, high resolution three-dimensional structures of several S-layers have been obtained by transmission electron microscopy and scanning probe microscopy (both atomic force and scanning tunnelling microscopy) provided topographical details (Beveridge, 1994). Etching will expose the subunit profile because the grains of the shadowing metal such as platinum are relatively large (approximately 0.2–0.5 nm). Freeze-etching reveals only the general form of the alignment of the S-layer subunits around the cell. Crystal defects are often seen with this method (Sleytr & Messner, 1988a, 1983). Negative staining is a better resolving technique and relies on the fact that heavy metal salts such as phosphotungstate and uranyl acetate (Beveridge *et al.*, 1994; Pum *et al.*, 1989) can enshroud subunits in an electron-dense ‘glass’ revealing contour and linkage of proteinaceous components. On negatively stained cells an S-layer may be seen as a thin, periodic fringe at the cell periphery. It is very difficult to determine directly cell wall–S-layer interactions in cells by EM without fixation, embedding and thin sectioning. Since this technique requires dehydration with organic solvents, correct protein conformation can be altered and organic constituents extracted. According to Pum *et al.* (1993), EM studies of the crystalline S-layer of *B. coagulans* E38-66 was enhanced by negative staining of the protein and protein–lipid monolayer with uranyl acetate after

fixation with glutaraldehyde, but in the case of *B. anthracis* bacteria were adsorbed on to Formvar and carbon copper grids previously made hydrophilic by glow discharge. The thin sections were stained with uranyl acetate and lead citrate after pre- and post-fixation (Sleytr *et al.*, 1988a). Three-dimensional reconstruction of negatively stained S-layers of *B. sphaericus* P-1, displays a smooth and a rough surface on either side of the protein layer (Lepault *et al.*, 1986).

S-layer symmetries

S-layers are planar arrays of identical proteinaceous or glycoproteinaceous subunits which can be aligned in unit cells of hexagonal (p6), tetragonal (p4), trimeric (p3) or oblique (p2) symmetries (Sára *et al.*, 1996; Egelseer *et al.*, 1995; Beveridge, 1994, 1981; Beveridge & Koval, 1993; Hovmöller *et al.*, 1988; Koval, 1988; Sleytr & Messner, 1988a, b, 1983; Baumeister & Engelhardt, 1987; Koval & Murray, 1986). The morphological units of oblique, trimeric, tetragonal and hexagonal lattices consist of two, three, four and six monomers, respectively. The centre-to-centre spacing of the morphological units can range from 3 to 35 nm (Sleytr *et al.*, 1994, 1993). The regularity of their arrangement makes them convenient structures for crystallographic analysis and computer image enhancement (Stewart, 1988, 1986; Baumeister & Engelhardt, 1987). Different types of S-layer lattices in *Bacillus* species are summarized in Table 1 and Fig. 2. Holt & Leadbetter (1969) and Gerhardt (1967) described a hexagonal lattice on the surface of vegetative cells of *B. anthracis*. This structure most likely represented an S-layer with p1 symmetry and a centre-to-centre spacing of the particle of 7–10 nm (Doyle *et al.*, 1986). Strains of a single phenotypic and genetic species can show several distinctive lattice forms among their number as seen in *B. stearothermophilus* (Messner *et al.*, 1984), and the specific properties of S-layer proteins from three different *B. stearothermophilus* strains revealed oblique, square or hexagonal symmetry (Sára & Sleytr, 1994). According to Luckevich &

Beveridge (1989), the *B. thuringiensis* S-layer is composed of linear arrays of small particles arranged with p2 symmetry.

Self-assembly of S-layers

Isolated S-layer subunits of Gram-positive and Gram-negative bacteria have the ability to recrystallize on the cell envelope fragments from which they have been removed, on those of other organisms or on untextured charged or uncharged inanimate objects (Sleytr & Messner, 1992). The most detailed self-assembly and reattachment experiments have been performed with S-layers from the *Bacillaceae*. S-layers from these organisms reveal a high anisotropic charge distribution, the inner surface being net negatively charged due to an excess of carboxylic acid groups, whereas the outer surface is neutral due to an equimolar amount of carboxylic acid and amino groups (Pum & Sleytr, 1996). This appears to be essential for proper orientation during local insertion in the course of lattice growth (Sleytr & Messner, 1992). Sleytr *et al.* (1994) found that synthetic and biological lipid membranes maintained their structural and functional integrity for a much longer period of time when S-layers were used as supporting structures (Pum & Sleytr, 1994; Pum *et al.*, 1993). An identical crystalline pattern of S-layers can be observed upon reconstitution on solid supports such as carbon polymers, silica, glass, mica, metals, etc. (Pum & Sleytr, 1993; Sleytr *et al.*, 1992). Large-scale recrystallized S-layers were used as a supporting and stabilizing structure for Langmuir–Blodgett films and reconstituted biological membranes (Pum & Sleytr, 1994, 1993; Pum *et al.*, 1993). S-layer proteins isolated from *B. coagulans* E38-66 can be recrystallized into large-scale coherent monolayers at an air–water interface on phospholipid films spread on a Langmuir–Blodgett trough (Pum & Sleytr, 1996; Pum *et al.*, 1993). Structural analysis of *in vitro* self-assembly products have shown that in double S-layers, two identical layers can be linked in a minor symmetric fashion (Sleytr *et al.*, 1992). Isolated S-layer

Fig. 2. Schematic drawing of the most common types of S-layer lattices observed in *Bacillus* species. (a) Oblique (p2) lattice, (b) square (p4) lattice, (c) hexagonal (p6) lattice. (Modified from Sleytr & Messner, 1992.)

subunits from the wild-type strain of *B. stearothermophilus* reassembled into distinct types of cylindrical and sheet-like self-assembly products correlated with the presence of mono- or bivalent cations during dialysis (Pum *et al.*, 1989; Messner *et al.*, 1986). Chemical analysis of peptidoglycans containing sacculi, and extraction and recrystallization experiments revealed that the wild-type strain *B. stearothermophilus* PV72 had a cell-wall polymer consisting of *N*-acetylglucosamine and glucose, which are responsible for binding the p6 S-layer protein to the rigid cell wall layer (Sára *et al.*, 1996).

Adsorption

In comparison with S-layer-deficient strains, S-layer-carrying members of the *Bacillaceae* adsorb much more readily to positively charged or hydrophobic surfaces (Sára *et al.*, 1992; Sleytr & Messner, 1992; unpublished observations). The adsorption properties for protein molecules strongly depended on the charge and hydrophobicity of attached nucleophiles (Küpcü *et al.*, 1993, 1991). In addition, a direct relationship was observed between the molecular dimensions of attached nucleophiles and pore size reduction. This was expressed in significant changes in rejection characteristics (Küpcü *et al.*, 1993). Adsorption studies by Sára *et al.* (1992) demonstrated that free amino and carboxyl groups exposed on the outer surface of the native S-layer lattice, and in the interior of the pores, are directly neutralized by electrostatic interactions, leading to a charge-neutral surface in the pore areas. In *B. coagulans* E38-66, the S-layer lattice masks the net negative surface of the peptidoglycan and prevents nonspecific adsorption of macromolecules, which can be essential requirements to prevent pore plugging and to maintain an unhindered transport of low-molecular-mass substances (nutrients and metabolites) and secretion of exoproteins (Sára *et al.*, 1992; Pum *et al.*, 1989; Sára & Sleytr, 1987b). Egelseer *et al.* (1995) concluded that the S-layer proteins from *B. stearothermophilus* DSM 2358 function as an adhesion site for a high-molecular-mass amylase. S-layers in *Bacillaceae* act as specific sites for phage adsorption. The crystalline arrays were present on all the 24 phage-resistant mutants of *B. sphaericus* P-1, although the molecular mass of the S-layer subunits had changed in many mutants (Sleytr & Messner, 1992). A mutant strain of *B. brevis* 47-52 was isolated as a phage-resistant colony having a two-layered cell wall consisting of the middle and inner wall layers and containing only a 150 000 Da protein as the major cell-wall protein (Tsuboi *et al.*, 1982). Removal of the protease-sensitive domain from the S-layer protein of *B. sphaericus* resulted in the inability to adsorb to the cell walls but did not destroy the capacity for macromolecular assembly (Hastie & Brinton, 1979a). Adsorption, structural labelling and chemical modification studies revealed that the S-layer lattice from *B. coagulans* E38-66 is a highly specialized supramolecular structure in possessing (a) anisotropic topographical and physicochemical surface properties (Sleytr & Messner, 1992; Pum *et al.*, 1989),

(b) very precise molecular-sieving properties, with charge-neutral pores preventing unspecific adsorption (Sára & Sleytr, 1993; Sleytr & Messner, 1992; Sára *et al.*, 1992), (c) a charge-neutral characteristic of the more elevated domains on the outer surface (Sára & Sleytr, 1993; Pum *et al.*, 1989), (d) a net negatively charged inner S-layer capable of binding to the net negatively charged peptidoglycan-containing layer (Sára & Sleytr, 1993), and (e) an outer surface more hydrophobic than the inner surface (Pum *et al.*, 1993).

Specific functions of S-layers

Although there is strong evidence that crystalline arrays have the potential to function as protective coats, the S-layers from thermophilic members of the family *Bacillaceae* studied so far cannot be considered as such. Studies by Sára *et al.* (1990) showed that the S-layers of two of the eight mesophilic members of the *Bacillaceae* (*B. circulans* CCM 2048, and *B. brevis* strains CCM 1089 and CCM 1463), do not act as a protective layer. Whole cells from these three mesophilic species were rapidly lysed by the muramidase lysozyme (molecular mass 14 600 Da) and mutanolysin (molecular mass 24 000 Da). This showed that S-layers possessing pores larger than 3.5 nm in size allow free passage for both these enzymes (Sára *et al.*, 1990). It has been suggested that S-layers from some members of the *Bacillaceae* can protect the peptidoglycan from muramidases and the protoplast from proteases (Sleytr & Messner, 1983), but not in the case of *B. stearothermophilus* (Egelseer *et al.*, 1995). S-layers possess pores with sizes of 4–5 nm which allow the passage of proteins with a molecular mass of up to 45 000 Da (Sára & Sleytr, 1987a), including neuraminidases and proteases (Sleytr & Messner, 1983). S-layers with pores of this size cannot be considered effective protective barriers (Sára *et al.*, 1992). For a few *Bacillus* species it has been demonstrated that lysozyme resistance is related to a modified peptidoglycan layer, but does not result from smaller pores in their S-layer lattices (Sára *et al.*, 1994, 1990). Among 39 strains of *B. stearothermophilus* possessing S-layers with different structures, not a single strain was found to be insensitive to lysozyme, indicating that channels with diameters larger than 3 nm (Stryer, 1975) are present in the lattices (Messner *et al.*, 1984). A wild-type strain of *B. stearothermophilus*, and both variant 3a/V1 and 3a/V2 were sensitive to the chosen muramidase, indicating that in all S-layer types the pores are large enough for passage of proteins with molecular masses of at least 24 000 Da (Messner *et al.*, 1984). S-layer-carrying *B. stearothermophilus* strains can produce large amounts of exoprotein with molecular masses above the exclusion limit of their S-layer (Archibald, 1989). The involvement of lattices in exoprotein secretion has yet to be examined. It has been suggested that S-layers from members of the *Bacillaceae* can delineate a kind of periplasmic space in their cell envelopes (Sturm *et al.*, 1993; Breitwieser *et al.*, 1992). Computer image reconstruction of S-layers from several thermophilic *Bacillus* strains revealed two types of pores differing in size and morphology (Sára *et al.*, 1994; Pum

et al., 1989; Messner *et al.*, 1986). Different pores in the lattices may have different functions (Sára *et al.*, 1992). One of the pore types may guarantee unhindered passage of nutrients, whereas the other type may play an important role in exoenzyme secretion. *B. stearothermophilus* revealed sharp exclusion limits for molecules with molecular masses larger than 45 000 Da, including a limiting pore diameter of about 4.5 nm in the crystalline meshwork (Egelseer *et al.*, 1995; Sleytr & Messner, 1992). In some mesophilic *Bacillus* species, a pore size as small as 2.5 nm has been determined (Sleytr & Messner, 1992).

S-layers and virulence

S-layers have been found in pathogenic *Bacillus* species and many other pathogenic bacteria (Sleytr & Messner, 1988b). Their presence may be required for an infection to occur (Smith, 1986; Ishiguro *et al.*, 1981). It has been suggested that S-layers are important virulence factors in pathogenic bacteria, protecting against complement killing, facilitating binding of the bacterium to host molecules or enhancing its ability to associate with macrophages (Sleytr *et al.*, 1993). The S-layer may have an important function in linking the capsule to the peptidoglycan layer and controlling the exchange of molecules with the environment. *B. thuringiensis* is distinguished from *B. cereus* by the production in the cell of a protein parasporal crystal. The ability to synthesize the parasporal crystals is plasmid-encoded (Ward & Ellar, 1987). Authentic cultures of *B. cereus* can acquire the ability to produce crystals following growth in mixed culture with *B. thuringiensis* (Gonzalez *et al.*, 1982). Smith *et al.* (1993) described some immunological effects of oligosaccharide–S-layer conjugates isolated from *B. stearothermophilus* NRS 2004/3a, PV72, *B. alvei* CCM205 and *Clostridium thermohydrosulfuricum* L111-69. S-layers have the intrinsic property of assembling into large two-dimensional arrays (Messner & Sleytr, 1992; Sleytr & Messner, 1983; Sleytr, 1978) and produce effective antigenic aggregates which correspond to those produced by mixing conventional carrier protein conjugates with adjuvants such as aluminium hydroxide (Smith *et al.*, 1993). The suitability of S-layers as carriers/adjuvants for the production of conjugate vaccines has been described (Malcolm *et al.*, 1993a, b; Smith *et al.*, 1993). In conjugate vaccines, haptens are bound to a protein by covalent linkages and, due to the crystalline nature of S-layer proteins or glycoproteins, the functional groups available for hapten-binding are present on each protomer in identical positions and orientations. Hapten–S-layer conjugates function as immunogens without the need for extraneous adjuvants (Smith *et al.*, 1993; Messner *et al.*, 1992).

Genetic studies

Molecular cloning and characterization of the genes encoding S-layer proteins and glycoproteins are important for elucidation of the mechanisms involved in

their biosynthesis, translocation across membranes, assembly at the external cell surface and the precise biological properties of these macromolecules. Studies of the genetics and secretion of S-layers have been shown to be most relevant for recombinant DNA technology. Genetic studies on S-layer proteins demonstrated that most genes encoding these proteins exist as single copies on the genome (Messner & Sleytr, 1992). The DNA fragments of the protein-producing *B. brevis* 47 were first cloned into *Escherichia coli* and *B. subtilis* (Yamagata *et al.*, 1987; Tsukagoshi *et al.*, 1984). The complete nucleotide sequence of the OWP (outer-wall protein) gene of *B. brevis* 47 was determined by Tsuboi *et al.* (1986). A portion of each of the OWP and MWP (middle-wall protein) genes of *B. brevis* 47 was cloned in *E. coli* by an immunological procedure (Tsukagoshi *et al.*, 1984). Analysis of transcripts in *B. brevis* 47 revealed that the genes for the MWP and OWP constitute a cotranscriptional unit and they are transcribed from multiply and tandemly arranged promoters located upstream of the MWP gene [*cwp* (cell-wall protein gene) operon] (Yamagata *et al.*, 1987; Tsuboi *et al.*, 1986). After analysis of the DNA sequence of the promoter region (Yamagata *et al.*, 1987), the complete nucleotide sequence of the *cwp* operon was determined (Tsuboi *et al.*, 1988). Different promoters were used in *B. brevis* 47 at different stages of growth and they play distinct roles in growth-phase-specific expression of the cell-wall proteins (Adachi *et al.*, 1989). The MWP gene has two tandemly located translation initiation sites. Both of them can be utilized to start translation in *B. brevis* 47, resulting in two different leader sequences (Adachi *et al.*, 1990). Conserved structures of cell-wall protein genes in different S-layer-protein-producing *B. brevis* strains were investigated to determine the complex organization of the regulatory regions of these genes (Ebisu *et al.*, 1990). *B. brevis* HPD31 contains a hexagonally arranged S-layer protein (HWP) and the gene encoding this protein was cloned and sequenced. Analysis of the DNA sequence revealed that there is an open reading frame encoding a polypeptide of 1087 amino acid residues with a molecular mass of 123 456 Da. The deduced amino acid sequence of the protein showed a high degree of homology (78%) with that of *B. brevis* 47. For amino acid residues 1–548, the homology was 90% (Gruber *et al.*, 1988). The complete nucleotide sequence of the S-layer protein gene (*sbsA*) of *B. stearothermophilus* PV72 has been studied; after comparison with that of other species it was concluded that the amino acid similarity of S-layer proteins is weak (Kuen & Lubitz, 1996; Kuen *et al.*, 1994). A domain from residue 27 to approximately residue 200 (18 kDa) is defined by sequence homology to the OWP of *B. brevis*. A 4251 bp DNA fragment containing the gene for the S-layer glycoprotein of *B. sphaericus* 2362 was cloned into *E. coli* encoding a protein with 1176 amino acid residues (Bowditch *et al.*, 1989). Ochterlony immunodiffusion experiments, sequence identity of the N-termini of these molecules and antibody reaction suggested that the S-layer protein of *B. sphaericus* 2362 serves as a precursor of the 110 kDa larvicidal protein, which appears during

sporulation (Bowditch *et al.*, 1989). The gene encoding the non-glycosylated, hexagonally arranged S-layer of *B. stearothermophilus* PV72 was cloned and sequenced by Kuen *et al.* (1994). These authors showed sequence comparisons with other signal sequences and found a high degree of homology within signal sequences of extracellular proteins in *Bacillus* species, such as alkaline phosphatase and neutral protease from *B. amyloliquefaciens* (Vasanthan *et al.*, 1984), as well as the signal sequence for *B. sphaericus* gene 125 (Bowditch *et al.*, 1989), and the outer-wall protein gene for *B. brevis* (Tsuboi *et al.*, 1986). Peptide mapping and N-terminal sequencing results strongly indicated that the S-layer protein of *B. stearothermophilus* NRS 2004/3a and two induced variant strains are encoded by different genes and are not derived from a universal precursor form (Sára *et al.*, 1994). The results from Southern hybridization indicated that chromosomal rearrangement is responsible for S-layer protein variation in *B. stearothermophilus* PV72 (Sára *et al.*, 1996, 1994; Sára & Sleytr, 1994). The gene encoding the S-layer protein was cloned from a *B. licheniformis* NM105 gene library and was expressed in *E. coli* NM539 and JM109 and *B. subtilis* MI112 (pMK462) (Tang *et al.*, 1989). In *B. brevis*, Tsukagoshi *et al.* (1984) observed that transferred cells of *E. coli* JM539 produced only a truncated polypeptide (75 kDa), while the S-layer protein expressed in *B. subtilis* MI112 had the same molecular mass as the authentic protein purified from *B. licheniformis* NM105 (Tang *et al.*, 1989).

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