REVIEW / SYNTHÈSE

Molecular organization of selected prokaryotic S-layer proteins

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Abstract: Regular crystalline surface layers (S-layers) are widespread among prokaryotes and probably represent the earliest cell wall structures. S-layer genes have been found in approximately 400 different species of the prokaryotic domains bacteria and archaea. S-layers usually consist of a single (glyco-)protein species with molecular masses ranging from about 40 to 200 kDa that form lattices of oblique, tetragonal, or hexagonal architecture. The primary sequences of hyperthermophilic archaeal species exhibit some characteristic signatures. Further adaptations to their specific environments occur by various post-translational modifications, such as linkage of glycans, lipids, phosphate, and sulfate groups to the protein or by proteolytic processing. Specific domains direct the anchoring of the S-layer to the underlying cell wall components and transport across the cytoplasma membrane. In addition to their presumptive original role as protective coats in archaea and bacteria, they have adapted new functions, e.g., as molecular sieves, attachment sites for extracellular enzymes, and virulence factors.

Key words: prokaryotes, cell walls, S-layer (glyco-) proteins, protein stabilization.

Résumé : Les couches de surface (couches S) cristallines régulières sont répandues parmi les procaryotes et représentent probablement les structures de parois cellulaires primitives. Des gènes de couches S ont été retrouvés chez environ 400 espèces différentes des domaines procaryotes bacteria et archaea. Les couches S sont habituellement constituées d'un seul type de glycoprotéine de masses moléculaires allant de 40 à 200 kDa qui forment des réseaux ayant une architecture oblique, tétragonale ou hexagonale. Les séquences primaires des espPces achéales hyperthermophiles présentent certaines caractéristiques distinctives. Des adaptations subséquentes à leurs environnements spécifiques furent facilitées par diverses modifications post-traductionnelles telles que des liens de la protéine à des glycanes, des lipides, des groupes phosphates et sulfates ou par une transformation protéolytique. Des domaines spécifiques sont responsables de l'ancrage de la couche S aux composantes de la paroi cellulaire inférieure et au transport à travers la membrane cytoplasmique. En plus de leur rôle original présumé comme couche protectrice des archéobactéries et des eubactéries, elles ont adopté de nouvelles fonctions, p. ex. des tamis moléculaires, des sites d'attachement pour des enzymes extracellulaires et des facteurs de virulence.

Mots clés : procaryotes, paroi cellulaire, glycoprotéines de la couches S, stabilisation protéique.

[Traduit par la Rédaction]

Introduction

During evolution, prokaryotic microorganisms adapted various cell wall structures in response to their specific and often harsh habitats. A rigid proteinaceous envelope, termed the S-layer, is regarded as the most ancient biological membrane that is ubiquitous and maintained in most species of bacteria and archaea (Baumeister et al. 1989; Beveridge and Koval 1992; Messner and Sleytr 1992; Murray 1993; Sleytr et al. 1993; Sleytr 1997; König and Messner 1997). Mono-

Received 3 September 2004. Revision received 17 February 2005. Accepted 3 March 2005. Published on the NRC Research Press Web site at http://cjm.nrc.ca on 21 October 2005.

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 doi: 10.1139/W05-093

molecular arrays of proteinaceous subunits have also been observed in archaeal sheaths (Beveridge et al. 1990) and on the surface of the cell wall of cyanobacteria (Šmarda et al. 2002). The S-layers usually consist of a single type of protein that has the intrinsic feature of being able to assemble into highly ordered two-dimensional crystalline structures. In some archaea and bacteria, two superimposed S-layer lattices, composed of different subunit species, may be present (Sleytr et al. 2001). In most archaea, the S-layer has a hexagonal lattice symmetry, whereas in bacteria, oblique and tetragonal lattices are found more frequently (König 1988; Messner and Sleytr 1992; Sleytr et al. 1996). The centre-to-centre distance of the morphological units varies between 2.5 and 35 nm and the thickness of the layer between 5.0 and 25 nm in bacteria and up to 70 nm in archaea. Owing to their crystalline nature, S-layer lattices contain pores of identical size, usually between 2 and 8 nm, and morphology (Sleytr et al. 2001). This strict modular construction of the S-layer lattice is the basis for many promising applications in nanobiotechnology (Sleytr et al. 1999, 2001; Ilk et al. 2002; Schäffer and Messner 2004).

Depending on the individual cell wall composition of the microorganism, the S-layer is noncovalently attached to the cytoplasmic membrane, outer membrane, peptidoglycan, pseudomurein, or secondary cell wall components, respectively (Sleytr et al. 2001). The S-layer proteins contain about 40–60 mol% hydrophobic residues and 25 mol% charged amino acids (Sleytr 1997; Sleytr and Beveridge 1999; Sára and Sleytr 2000). Occurrence of cysteine is typically for the S-layer proteins of hyperthermophilic archaea, which also have more charged residues compared with their mesophilic counterparts (Akça et al. 2002; Akça 2004; Claus et al. 2002). Most S-layer proteins are weakly acidic, but some are basic, e.g., those of *Methanothermus fervidus*, *Methanothermus sociabilis*, *Methanosarcina mazei*, *Methanobacterium thermoautotrophicum*, and *Lactobacilli* with isoelectric points between pH 8.0 and 10.0 (Bröckl et al. 1991; Sleytr 1997; Claus et al. 2002). The amino acids of S-layer proteins are organized such that about 40% are as β-sheets and 10%–20% are as α-helices. Their molecular masses range from 40 to 200 kDa.

Domains

Bacterial S-layer proteins are secreted by either the universally conserved general pathway (SEC) or an ATP-binding cassette transporter (Fernández and Berenguer 2000; Sára and Sleytr 2000; Kawai et al. 1998). S-layer proteins from *Methanothermus* spp. and *Methanococcus* spp. were predicted to be secreted by the SEC pathway (Bröckl et al. 1991; Akça et al. 2002), which is a common route for archaeal protein translocation (Eichler 2000). Indeed, most prokaryotic S-layer proteins exhibit a typical 30 amino acid leader peptide with a positively charged N terminus, a hydrophobic core, and a C-terminal recognition site for the cleavage by specific signal peptidases (Brendtsen et al. 2004).

The S-layer proteins from some species share homologous domains with specific functions. Most halobacterial S-layer proteins are anchored in the cytoplasmic membrane by a C-terminal transmembrane domain. The S-layer of Gram-positive bacteria is fixed by its N terminus or C terminus to the peptidogycan or by secondary cell wall polymers, e.g., teichoic acids, teichuronic acids, lipoteichoic acids, or lipoglycans (Egelseer et al. 1998; Smit et al. 2001; Antikainen et al. 2002). This attachment is mediated by so-called S-layer homologous (SLH) domains found in some *Bacillaceae*, *Deinococcus radiodurans*, *Thermotoga maritima*, and the Gram-negative bacteria *Synechocystis* sp. and *Thermus thermophilus* (Archibald et al. 1993), but not all S-layer proteins possess SLH domains (cf. *Geobacillus stearothermophilus*, see Table 3). SLH domains were identified not only at the N-terminal part of S-layer proteins but also at the C-terminal part of cell-associated exoproteins and enzymes of Gram-positive bacteria (Lupas et al. 1994; Engelhardt and Peters 1998). Typically, S-layer proteins and cell-associated exoenzymes possess three repeats of SLH motifs, each consisting of 50–70 amino acids (Engelhardt and Peters 1998; Mesnage et al. 2000; Sára and Sleytr 2000). The N-terminal sequences following the docking regions are necessary for the self-assembly process as found for the S-layer proteins from *Bacillus* and *Geobacillus*, whereas about 200 amino acids could be truncated at the C terminus without negative effect in this respect (Jarosch et al. 2001; Ilk et al. 2002; Rünzler et al. 2004).

Post-translational modifications

Proteolytic cleavage of N- and C-terminal fragments, phosphorylation, sulphurylation, glycosylation, and lipid transfer are well-documented mechanisms that alter sizes and molecular features of translated prokaryotic S-layer proteins (Boot and Pouwels 1996; Eichler 2003). By proteolytic cleavage of a proprotein, two S-layer proteins arise in *Clostridium difficile* (Calabi et al. 2001). Phosphorylation of tyrosine residues has been demonstrated for the S-layer protein of *Aeromonas hydrophila* (Thomas and Trust 1995). The S-layer proteins of the archaeal halobacteria are modified by highly sulfonated glycans and represent one of the first prokaryotic glycoproteins ever described (Mescher and Strominger 1976; Lechner and Sumper 1987; Sumper 1993). After the first description of glycosylated bacterial S-layer proteins in thermophilic *Clostridia* (Sleytr and Thorne 1976), presently about 40 different S-layer glycan structures have been fully or at least partially elucitated in the Gram-positive bacterial genera *Bacillus*, *Geobacillus*, *Lactobacillus*, and *Clostridium* as well as in archaea (Kärcher et al. 1993; Sumper and Wieland 1995; Eichler 2003; Upreti et al. 2003; Schäffer and Messner 2004). Glycosylation of S-layer proteins generally varies between 2% and 10% (*m/m*). Bacterial S-layer glycan chains are linear or branched homo- or hetero-saccharides, which comprise 20–50 identical repeating units, whereas in archaea, short oligosaccharides prevail (Kärcher et al. 1993; Schäffer and Messner 2004). The monosaccharide constituents include a wide range of neutral hexoses, 6-deoxyhexoses, and amino sugars. Whereas in archaea, *O*- and *N*-glycosidic linkages may exist simultaneously in the same strain, only one linkage scheme, predominantly O-bonds, occurs in the Gram-positive bacteria. Lipid modification of S-layer (glyco-)proteins has been demonstrated for the halophilic archaea *Haloferax volcanii* and *Halobacterium halobium* (Eichler 2003; Konrad and Eichler 2002). Like glycosylation, lipid attachment takes place on the exter-

nal cell surface, i.e., following protein translocation across the plasma membrane.

Post-translational modifications and (or) intrinsic features of the S-layer proteins sometimes provoke an unusual electrophoretic behaviour and thus confusion about the exact molecular mass of the monomer when comparing results from SDS–PAGE and gene sequences. As an example, the purified S-layer glycoprotein from *Methanothermus fervidus* exhibits two size conformations on SDS gels (Bröckl et al. 1991). The electrophoretic migration of the purified S-layer glycoprotein from *Methanocaldococcus jannaschii* is highly dependent on temperature and pH used for sample preparation (Akça 2004). Similarly, the electrophoretic mobility of the S-layer protein from the hyperthermophilic archaeon *Nanoarchaeum equitans* in SDS–PAGE depends on the temperature used for sample preparation (Schuster et al. 2004). Aberrant migration behaviour has been also observed for the lipid-modified S-layer glycoproteins from halobacteria owing to increased hydrophobicity (Konrad and Eichler 2002) or to protein phosphorylation in the case of *Aeromonas hydrophila* (Thomas and Trust 1995). A recent two-dimensional electrophoresis approach showed the prevalence of S-layer and SLH proteins in the membrane fraction of a *Bacillus anthracis* strain (Chitlaru et al. 2004). Five new SLH proteins were found, and most notably, the S-layer protein EA1 was present in a high number of isoelectric and mass variants. This situation is indicative of substantial post-translational modifications. Accordingly, the cell envelope of *Methanosarcina mazei* is composed of a mosaic of several S-layer-like proteins (Mayerhofer et al. 1998).

Functions

Although S-layers are almost ubiquitously found in bacteria and archaea, more knowledge is desirable about their physiological role. The strong resistance of these simple biological membranes to extreme environmental conditions, such as high temperatures, low pH, and high ionic strength (Engelhardt and Peters 1998; Claus et al. 2002), suggests that they contribute to the stabilization and protection of cells. This is especially true for the S-layers of extremophilic Gram-negative archaea, which are directly anchored into the cytoplasmic membrane and represent probably the most ancient prokaryotic cell wall. In all other taxa, the S-layer seems to be a more or less a remnant cell wall component and not essential for cell shape or rigidity. Owing to the high energy needed to produce and translocate large amounts of protein across the cytoplasmic membrane, other essential functions of the S-layer must have evolved.

The pores in the S-layer lattice present a barrier for macromolecules in the 30- to 40-kDa range (Breitwieser et al. 1992; Sára et al. 1992). Thus, exoenzymes may be retained within a periplasma-like space and cell-lytic enzymes may be excluded.

The S-layers of the Gram-negative species *Aeromonas salmonicida*, *Campylobacter fetus*, *Aeromonas serpens*, and *Caulobacter crescentus* shield the bacteria from predation by *Bdellovibrio bacteriovorans* (Koval 1993). Owing to their protective role against humoral and cellular immune defense, the S-layers of *Aeromonas salmonidica*, *Campylobacter fetus*, *Bacillus cereus*, *Bacillus anthracis*, and *Rickettsia* sp. contribute to the pathogenicity of these microorganisms (Carl and Dasch 1989; Etienne-Toumelin et al. 1995; Kotiranta et al. 1997; Mesnage et al. 1997). The S-layers of *Lactobacillus acidophilus* and *Lactobacillus crispatus* cells (Schneitz et al. 1993) and *Clostridium difficile* (Calabi et al. 2001) strains mediate adhesion to mammalian gut epithelial cells.

S-layers from *Bacillaceae* were found to function as adhesion sites for cell-associated exoenzymes, e.g., high molecular mass exoamylase from *Geobacillus stearothermophilus* (Sára and Sleytr 2000). Two copies of a hyperthermostable protease are attached to the S-layer glycoprotein of *Staphylothermus marinus* (Engelhardt and Peters 1998). The high molecular mass S-layer fragment from *Clostridium difficile*, obtained after specific post-translational proteolytic cleavage of a precursor protein, expresses an *N*-acetylmuramoyl-L-alanine amidase activity (Calabi et al. 2001). A metalloprotease from *Caulobacter crescentus* combines the catalytic portion of a protease located at the N terminus with an S-layer-like protein at the C terminus (Umelo-Njaka et al. 2002). Finally, a unique ecological role for the cyanobacterial S-layer was shown for *Synechococcus* sp. strain GL24. Its hexagonal S-layer units function as discrete crystallization nuclei for the biomineralization of gypsum and calcite (Schultze-Lam et al. 1992; Šmarda et al. 2002).

Gene sequences

To date, about 400 species from all important taxa of bacteria and archaea have been shown to have an S-layer (Messner and Sleytr 1992; Smit et al. 2002). Recently, a corresponding gene (*NEQ300*) was also found in the nanosized hyperthermophilic symbiont *Nanoarchaeum equitans* (Waters et al. 2003), a representative of a new archaeal phylum (Huber et al. 2002). *NEQ300* codes for a glycosylated protein with 941 amino acids and a relative mass of 103 920 Da. It possesses a 22 amino acid leader peptide and five putative N-glycosylation sites. The protein subunits occur as dimers and are arranged on a lattice with sixfold symmetry and a centre-to-centre distance of 15 nm (Schuster et al. 2004). A survey of complete or partial S-layer gene sequences of bacteria and archaea is given in Table 1.

Three-dimensional crystallization

The occurrence of a regular crystalline structure on the surfaces of a microorganism can usually be easily demonstrated by transmission electron microscopy after negative staining (Baumeister et al. 1990; Harris 1997; Sleytr et al. 2001). However, their detection is sometimes difficult because they are masked by superimposed amorphous cell materials. In our investigations, S-layer sheets were rendered visible when additional proteins were removed from, e.g., sporulating cells of *Bacillus sphaericus* and *Bacillus fusiformis* by gentle ultrasonication. Antibodies generated against the purified S-layer protein of *Bacillus fusiformis* B3 also gave strong signals with the corresponding S-layer sheet preparation obtained from spores. In agreement with this observation, the S-layer protein EA1 of *Bacillus anthracis* has recently been demonstrated to be a persistent contaminant of spore preparations (Williams and Turnbough Jr. 2004).

Table 1. S-layer-homologous protein genes.

Species	Systematic grouping (class)	Size (amino acids)	Molecular mass (Da)	Gene designation	Nucleotide accesssion No.	Protein accession No.
Halobacterium halobium	Halobacteria	852	89 814	*csg*	J02767[a]	P08198[b]
Halobacterium sp. NRC-1	Halobacteria	836	88 071	*csg*	AE005139[a]	Q9HM69[b]
Haloarcula japonica TR-1	Halobacteria	862	89 814	*csg*	D87290[a]	Q9C4B4[b]
Haloferax volcanii	Halobacteria	827	85 189	*csg*	M62816[a]	P25062[b]
Methanothermobacter thermautotrophicus Delta H	Methanobacteria	1755	191 961	*MTH716*	AE000851[c]	AAB85221[c]
Methanocaldococcus jannaschii DSM 2661	Methanobacteria	558	60 547	*slmj1*[d]	AJ311636[a]	Q58232[b]
Methanocaldococcus jannaschii DSM 2661	Methanobacteria	440	50 745	*MJ0954*	U67539[a]	Q58364[b]
Methanothermococcus thermolithotrophicus DSM 2095	Methanobacteria	559	59 225	*slmt1*[d]	AJ308554[a]	Q8X235[b]
Methanococcus vannielii DSM 1224	Methanobacteria	566	59 064	*slmv1*[d]	AJ308553[a]	Q8X234[b]
Methanococcus voltae DSM 1537	Methanobacteria	565	59 707	*sla*	M59200[a]	Q50833[b]
Methanococcus maripaludis S2	Methanobacteria	553	56 644	*slpB*	NC005791[c]	NP987995[c]
Methanococcus maripaludis S2	Methanobacteria	575	589 948	*slp*	NC005791[c]	NP987503[c]
Methanotorris igneus DSM5666	Methanobacteria	519	55 669	*slmi1*[e]	AJ564995[a]	Q6KEQ4[b]
Methanosarcinan mazei S-6	Methanobacteria	652	68 878	*slgB*	X77929[a]	Q50244[b]
Methanosarcina acetivorans C2A	Methanobacteria	557	62 086	*slg*	AE010797[c]	AAM04705[c]
Methanothermus fervidus DSM 2088	Methanobacteria	593	65 481	*slgA*	X58297[a]	P27373[b]
Methanothermus sociabilis DSM 3496	Methanobacteria	593	65 503	*slgA*	X58296[a]	P27374[b]
Nanoarchaeum equitans	Nanoarchaeota	941	103 920	*NEQ300*	AE01799[c]	AAR39148[c]
Euryarchaeta 37F11	Uncultured marine group II	564	61 747	—	AAF97179[c]	AAF97179[c]
Pyrococcus abyssi Orsay	Thermococci	604	65 985	—	AJ248285[c]	CAB49670[c]
Pyrococcus horikoshii OT3	Thermococci	236	26 128	*PH1395*	BA000001[c]	BAA30501[c]
Thermococcus kodakaraensis KOD1	Thermococci	540	57 367	—	AP006878[c]	BAD85778[c]
Thermococcus kodakaraensis KOD1	Thermococci	573	61 819	—	AP006878[c]	BAD84353[c]
Thermococcus kodakaraensis KOD1	Thermococci	612	66 631	—	AP006878[c]	BAD85778[c]
Sulfolobus acidocaldarius DSM 639	Crenarchaeota Thermoprotei Sulfolobales	1424	151 040	*slp1*	BN000824[a]	CP000077[a]
Sulfolobus solfataricus P2	Crenarchaeota Thermoprotei Sulfolobales	1231	131 868	*slp1*	BN000829[a]	CAJ31324[a]
Staphylothermus marinus F1	Crenarchaeota Thermoprotei Desulfurococcales	1524	166 287	—	U57967[c]	AAC44118[c]
Thermotoga maritima MDB8	Thermotogae	456	51 807	—	NC000853[c]	NP228650[c]
Deinococcus radiodurans Sark	Deinococci	1036	108 028	*hpi*	M17895[a]	P13126[b]
Deinococcus radiodurans	Deinococci	1167	123 729	*DR2508*	AE002080[a]	P56867[b]
Thermus thermophilus HB8	Deinococci	917	96 133	*slpA*	X57333[c]	P35830[c]
Thermus thermophilus HB27	Deinococci	469	52 219	—	NC005835[c]	YP005376[c]
Gleobacter violaceus PCC 7421	Cyanobacteria	697	72 111	*Gll4200*	AP006582[a]	BAC92141[b]
Synechococcus lividus PCC7942	Cyanobacteria	521	57 139	*somA*	D64077[a]	P7754[b]
Synechococcus sp. PCC 6301	Cyanobacteria	525	56 213	*somB*	U702564[c]	AAC33402[c]
Synechococcus sp. PCC 6301	Cyanobacteria	532	57 386	*somA*	U702564[c]	AAC33403[c]

Table 1 (*continued*).

Species	Systematic grouping (class)	Size (amino acids)	Molecular mass (Da)	Gene designation	Nucleotide accesssion No.	Protein accession No.
Synechocystis sp. PCC 6803	Cyanobacteria	630	67 600	*slr1841*	D90906[a]	BAA17449[b]
Synechocystis sp. PCC 6803	Cyanobacteria	321	34 543	*slr2000*	D90910[a]	BAA17888[b]
Caulobacter crescentus CB 15	α-Proteobacteria	1073	103 613	*rsaA*	AE005779[c]	AAK22991[c]
Caulobacter crescentus JS3001	α-Proteobacteria	1026	98 132	*rsaA*	AF193063[a]	P35828[b]
Caulobacter cresecntus JS4000	α-Proteobacteria	359	35 434	*rsaA*	AF193064[a]	Q9RMN1[b]
Caulobacter vibriodes	α-Proteobacteria	658	68 471	*sapA*	AY064211[c]	AAL47190[c]
Rickettsia japonica YH	α-Proteobacteria	1656	168 098	*rOmpB*	AB003681[a]	Q06653[b]
Rickettsia prowazekii Brein1	α-Proteobacteria	1643	169 854	*spaP*	M37647[a]	Q53020[b]
Rickettsia rickettsii R	α-Proteobacteria	1300	132 802	*p120*	X16353[a]	Q53047[b]
Rickettsia typhi Wilmington	α-Proteobacteria	1645	169 698	*slpT*	L04661[a]	P96989[b]
Desulfotalea psychrophila LSv54	δ-Proteobacteria	504	56 155	—	NC006138[c]	YP064946[c]
Aeromonas hydrophila TF7	γ-Proteobacteria	472	47 646	*ahsA*	L37348[a]	Q44072[b]
Aeromonas salmonicida A450	γ-Proteobacteria	502	52 869	*vapA*	M64655[a]	P35823[b]
Serratia marcescens Sr41	γ-Proteobacteria	1004	102 406	*slaA*	AB007125[a]	Q54455[b]
Camplobacter fetus subsp. *fetus* 23D	ε-Proteobacteria	942	96 634	*sap4*	J05577[a]	Q841Z1[b]
Camplobacter fetus subsp. *fetus* 23B	ε-Proteobacteria	922	95 109	*sapA1*	L15800[a]	Q841X3[b]
Camplobacter fetus subsp. *fetus* 82-40LP3	ε-Proteobacteria	1109	111 805	*sapA2*	S76860[a]	Q53505[b]
Camplobacter fetus subsp. *fetus* 23D	ε-Proteobacteria	811	88 153	*sapAp8*	AAO64214[c]	Q84177[b]
Camplobacter fetus subsp. *fetus* 23D	ε-Proteobacteria	1167	117 324	*sapA3*	AAO64231[c]	Q841X1[b]
Camplobacter fetus subsp. *fetus* 23D	ε-Proteobacteria	1106	111 525	*sapA5*	AAO64211[c]	Q841Z0[b]
Camplobacter fetus subsp. *fetus* 23D	ε-Proteobacteria	1257	129 306	*sapA6*	AAO64213[c]	Q841Y8[b]
Camplobacter fetus subsp. *fetus* 23D	ε-Proteobacteria	1292	130 736	*sapA7*	AAO64215[c]	Q841Y6[b]
Camplobacter fetus subsp. *fetus* 23D	ε-Proteobacteria	939	95 508	*sapA*	A37284[a]	Q841X5[b]
Camplobacter fetus subsp. *fetus* 84–91	ε-Proteobacteria	1112	112 504	*sapB*	U25133[a]	Q46037[a]
Camplobacter fetus subsp. *fetus* CIP 5396T	ε-Proteobacteria	1112	112 504	*sapB2*	AF048699[a]	Q52781[b]
Camplobacter rectus 314	ε-Proteobacteria	1361	144 386	*Crs*	AF010143[a]	Q30524[b]
Camplobacter rectus ATCC 33238	ε-Proteobacteria	1361	144 904	—	AB001876[a]	Q87083[b]
Camplobacter rectus ATCC 33238	ε-Proteobacteria	1123	118 954	*csxA*	AF035193[a]	Q9ZIB3[b]
Clostridium thermocellum NCIB 10682	Clostridia	1664	178 194	*cipA*	X67506[a]	Q06852[b]
Clostridium thermocellum NCIB 10682	Clostridia	688	74 970	—	X67506[a]	Q06853[b]
Clostridium acetobutylicum DSM 792	Clostridia	439	48 116	*CAC3558*	AE007852[c]	AAK81482[c]
Clostridium acetobutylicum DSM 792	Clostridia	1939	210 335	*CAC3389*	AE007836[c]	AAK81319[c]
Clostridium tetani E88	Clostridia	354	37 516	—	AE015937[c]	AAO35145[c]
Clostridium tetani E88	Clostridia	642	72 100	—	AE015937[c]	AAO35101[c]
Clostridium tetani E88	Clostridia	708	77 200	—	AE036592[c]	AAO36592[c]
Clostridium tetani E88	Clostridia	1334	145 565	—	AE015937[c]	AAO35096[c]
Clostridium tetani E88	Clostridia	642	72 100	—	NC004557[c]	NP781164[c]
Clostridium difficile MRY04-0409	Clostridia	335	35 623	*slpA*	AB181350[c]	BAD22753[c]

Table 1 *(continued).*

Species	Systematic grouping (class)	Size (amino acids)	Molecular mass (Da)	Gene designation	Nucleotide accesssion No.	Protein accession No.
Thermoanaerobacter kivui	Clostridia	702	76 511	—	M31069[c]	AAA21930[c]
Aneurinibacillus thermoaerophilus L420-91T	Bacilli	759	81 431	*satA*	AY395578[c]	AAS44591[c]
Aneurinibacillus thermoaerophilus DSM 10155	Bacilli	738	78 306	*satB*	AY395579[c]	AAS44592[c]
Bacillus anthracis Ames	Bacilli	862	91 362	*eag*	X99724[a]	P94217[b]
Bacillus anthracis Ames	Bacilli	814	86 620	*sap*	Z36946[a]	P49051[b]
Bacillus licheniformis NM 105	Bacilli	874	92 735	*olpA*	U38842[a]	P49052[b]
Bacillus pseudofirmus OF4	Bacilli	931	96 855	*slpA*	AF242295[a]	Q9L655[b]
Bacillus sphaericus P1	Bacilli	1252	129 935	—	A45814[a]	CAA02847[b]
Bacillus sphaericus WHO 2362	Bacilli	1176	125 226	—	M28361[a]	P38537[b]
Bacillus sphaericus CCM 2177	Bacilli	1268	132 047	*sbpA*	AF211170[a]	Q9RER7[b]
Bacillus sphaericus JG-A12	Bacilli	184[f]	19 687[f]	—	AJ292965[a]	Q9EXR5[b]
Bacillus sphaericus NCTC 9602	Bacilli	1228	129 728	*slfA*	AJ866974[c]	CA129282[c]
Bacillus cereus DSM 31	Bacilli	577	64 599	*BC0991*	AE017001[c]	AAP07978[c]
Bacillus cereus DSM 31	Bacilli	530	58 834	*BC0902*	AE017000[c]	AAP07889[c]
Bacillus cereus DSM 31	Bacilli	410	45 571	*BC3524*	AE017009[c]	AAP10458[c]
Bacillus cereus ZK	Bacilli	578	64 382	—	NC006274[c]	YP082487[c]
Bacillus cereus ATCC 10987	Bacilli	272	30 467	—	NC003909[c]	NP981245[c]
Bacillus thuringiensies subsp. *galleriae* NRRL 4045	Bacilli	821	87 123	*slpA*	AJ249446[a]	Q9RED0[b]
Bacillus thuringiensies subsp. *israelensis* 4Q2	Bacilli	393	45 437	*slp*	X62090[a]	P35826[b]
Bacillus thuringiensies subsp. *finitimus* CTC	Bacilli	816	87 293	*ctc*	AJ012290[a]	Q9ZES5[b]
Bacillus thuringiensis serovar konkudian 97-27	Bacilli	578	64 750	—	NC005957[c]	YP035240[c]
Bacillus fusiformis DSM 2898	Bacilli	576[f]	62 007[f]	*sl2*[e]	AJ781693[a,e]	Q6EV58[b,e]
Bacillus fusiformis B3	Bacilli	575[f]	61 874[f]	*sl1*[e]	AJ781692[a,e]	Q6EV59[b,e]
Brevibacillus brevis HDP31	Bacilli	1116	123 397	*hwp*	D90050[a]	P38538[b]
Brevibacillus brevis 47	Bacilli	1084	120 768	*mwp*	M19115[a]	P06546[b]
Geobacillus stearothermophilus ATCC 12980	Bacilli	1099	115 395	*sbsC*	AF055578[a]	O68840[b]
Geobacillus stearothermophilus ATCC 12980/G+	Bacilli	903	96 200	*sbsD*	AF228338[a]	Q9KIQ5[b]
Geobacillus stearothermophilus NRS 2004/3a	Bacilli	903	96 640	*sgsE*	AF328862[c]	AAL46630[c]
Geobacillus stearothermophilus DSM 2358	Bacilli	439[f]	47 082[f]	*sl1*	AJ781694[a,e]	Q6EV57[b,e]
Geobacillus stearothermophilus PV 72/p6	Bacilli	1228	131 075	*sbsA*	X71092[a]	P35825[b]
Geobacillus stearothermophilus PV 72/p2	Bacilli	920	97 916	*sbsB*	X98095[a]	Q45664[b]
Geobacillus kaustophilus HTA426	Bacilli	969	101 668	—	NC006510[c]	YP149039[c]
Lactobacillus acidophilus ATCC 4356	Bacilli	444	46 570	*slpA*	X89375[a]	P35829[b]
Lactobacillus acidophilus ATCC 4356	Bacilli	456	47 773	*slpB*	X89376[a]	Q48508[b]
Lactobacillus brevis ATCC 8287	Bacilli	465	48 159	*slpA*	Z14250[a]	Q05044[b]
Lactobacillus brevis ATCC 14869	Bacilli	483	50 924	*slpB*	AY040846[c]	AAK84948[c]
Lactobacillus brevis ATCC 14869	Bacilli	461	48 856	*slpC*	AY040847[c]	AAK84949[c]

Table 1 (*concluded*).

Species	Systematic grouping (class)	Size (amino acids)	Molecular mass (Da)	Gene designation	Nucleotide accesssion No.	Protein accession No.
Lactobacillus brevis ATCC 14869	Bacilli	413	44 572	*slpD*	AY040848[c]	AAK84950[c]
Lactobacillus crispatus JCM 5810	Bacilli	440	46 771	*cbsA*	AF001313[a]	Q07120[b]
Lactobacillus crispatus JCM 5810	Bacilli	452	47 602	*cbsB*	AF079365[a]	Q86015[b]
Lactobacillus crispatus LMG 12003	Bacilli	458	48 750	*slpNA*	AF253043[a]	Q9L5C3[b]
Lactobacillus crispatus LMG 12003	Bacilli	439	46 796	*slpNB*	AF253044[a]	Q9L5C2[b]
Lactobacillus fermentum BR11	Bacilli	264	28 627	*bspA*	U97348[a]	Q06530[b]
Lactobacillus helveticus JCM 1007	Bacilli	388	40 899	—	AB061777[c]	BAB72067[c]
Lactobacillus helveticus JCM 1008	Bacilli	391	41 199	—	AB061778[c]	BAB72068[c]
Lactobacillus helveticus ATCC 12046	Bacilli	439	46 702	—	AJ388558[a]	Q9S3A0[b]
Lactobacillus helveticus ATCC 15009	Bacilli	439	46 716	—	AJ388559[a]	Q9S399[b]
Lactobacillus helveticus CNRZ 303	Bacilli	439	46 688	—	AJ388560[a]	Q9S398[b]
Lactobacillus helveticus CNRZ 35	Bacilli	437	46 485	—	AJ388561[a]	Q9XB19[b]
Lactobacillus helveticus IMPC i60	Bacilli	439	46 704	—	AJ388562[a]	Q9S397[b]
Lactobacillus helveticus IMPC M696	Bacilli	439	46 602	—	AJ388563[a]	Q9S396[b]
Lactobacillus helveticus IMPC HLMI	Bacilli	438	46 622	—	AJ388564[a]	Q9S395[b]
Lactobacillus helveticus CNRZ 892	Bacilli	439	46 688	*slpH1*	X91199[a]	P38059[b]
Corynebacterium glutamicum ATCC 17965	Actinobacteria	510	55 425	*csp2*	X69103[a]	Q04985[b]
Propionibacterium acnes KPA171202	Actinobacteria	463	48 114	—	NC006085[c]	YP056872[c]
Pirellula sp. 1	Planctomycetacia	524	55 241	—	BX294138[c]	CAD72961[c]
Pirellula sp. 1	Planctomycetacia	818	90 680	*butB*	BX294149[c]	CAD76355[c]
Leptospira interrogans 56601	Spirochaetes	527	59 704	*slpM*	NC004342[c]	NP71086[c]
Leptospira interrogans L1-130	Spirochaetes	527	59 693	—	NC005823[c]	YP002865[c]
Bacteroides thetaiotaomicon VPI-5482	Bacteroidetes	393	44 178	—	NC004663[c]	NP809362[c]
Fusobacterium nucleatum ATCC 25586	Fusobacteria	643	71 502	*FN0694*	AE010580[c]	AAL94890[c]
Fusobacterium nucleatum subsp. *vincentii* ATCC 49256	Fusobacteria	643	71 874	*FNV1357*	AABF01000035[c]	EAA24374[c]
Fusobacterium nucleatum subsp. *vincentii* ATCC 25586	Fusobacteria	640	71 489	—	NZAABF02000035[c]	ZP00144025[c]
Cytophaga sp. Jeang 1995	Sphingobacteria	1047	108 718	—	AF068060[a]	Q9RB95[b]

Note: The systematic grouping was performed according to Garrity and Holt (2001).
[a]EMBL database.
[b]UniProt/SwissProt database.
[c]GenBank/NCBI database.
[d]Akçà et al. (2002).
[e]Akça (2004).
[f]Incomplete sequence.

Treatment with lysozyme has been described as a simple strategy for rapidly screening for the presence of S-layers on Gram-positive cells by transmission electron microscopy or scanning force microscopy (Wahl et al. 2001). Electron holography of nonstained bacterial S-layer proteins has been applied to avoid possible structural artefacts caused by the heavy metal treatment (Simon et al. 2004). Freeze-etching preparation of whole cells is another well-established method for the characterization of S-layer lattices (Sleytr and Glauert 1976; Rachel et al. 1986). Atomic force microscopy is a powerful tool for studying biological molecules in their native environment and has already been successfully applied to study the conformation and crystallization dynamics of S-layers (Müller et al. 2002; Györvary et al. 2003).

The topography of S-layers can be readily modelled by three-dimensional reconstruction from electron micrographs (Baumeister et al. 1990; Beveridge et al. 1990; Lupas et al. 1994). Nevertheless, X-ray crystal structures have therefore been obtained only for small recombinant S-layer fragments from the hyperthermophilic species *Staphylothermus marinus* (Stetefeld et al. 2000), *Methanosarcina mazei* (Jing et al. 2002), and *Geobacillus stearothermophilus* ATCC 12980 (Pavkov et al. 2003).

To obtain S-layer proteins for biochemical and biophysical studies, they are isolated after breaking whole cells by sonification, removal of the cytoplasmic membrane with Triton X-100, extraction, and dissociation into monomers by treatment with high molar concentrations of chaotropic agents (urea, guandinium chloride) or by cation substitution, e.g., Na^+ or Li^+ replacing Ca^{2+} (Sleytr et al. 2001). After removal of the chaotropic agent by dialysis at neutral pH values, the S-layer monomers reassemble into water-insoluble materials. Repeated rounds of extractions and dialysis result in highly purified S-layer proteins, which can represent up to 15% of the whole cell protein. Further purifications can be achieved for example by preparative isoelectric focusing, which has been recommended to improve protein quality for crystallization experiments (Bott et al. 1982). Using these strategies and under low-gravity conditions, we obtained protein crystals and the first X-ray data of complete archaeal and bacterial S-layer proteins (Evrard et al. 1999; Debaerdemaeker et al. 2002).

S-layer structures from selected archaea and bacteria

S-layers of methanococci

Cells of the order *Methanococcales* are covered by a single S-layer in a hexagonal arrangement that is directly exposed to the environment and cannot be stabilized by cellular components. As *Methanococcales* comprise species living at extremely different temperature conditions, their S-layer proteins represent an ideal model for studying the molecular mechanisms of protein stabilization at elevated temperatures. Molecular data of S-layer proteins from *Methanococcales* deduced from their gene sequences are compiled in Table 2.

More predicted *N*-glycan sites have been found in the S-layer primary sequences of the hyperthermophilic *Methanocaldococcus jannaschi* compared with its mesophilic relatives (Akça et al. 2002; Claus et al. 2002). The same was

Table 2. S-layer genes and proteins from selected mesophilic and (hyper)thermophilic *Methanococcales*.

Species	Growth optimum (°C)	Accession No.[a] (gene designation)	Size (amino acids)	Molecular mass (Da)	Isoelectric point	N-glycosylation sites	Cysteine (mol%)	Alanine (mol%)
Methanotorris igneus	88	AJ564995 (*slmi1*)	519	55 669	4.68	8	0.4	9.4
Methanocaldococcus jannaschii	85	AJ311636 (*slmj1*)	558	60 547	4.27	8	0.4	9.9
Methanothermococcus thermolithotrophicus	65	AJ308554 (*slmt1*)	559	59 225	4.30	5	—	12.2
Methanococcus vannielii	37	AJ308553 (*slmv1*)	566	59 064	4.29	—	—	16.3
Methanococcus voltae	37	M59200 (*sla*)	565	59 707	4.15	2	—	14.0

[a]EMBL database.

Table 3. S-layer homologous proteins of the *Bacillaceae*.

Species	Amino acid composition				Molecular mass (Da)	pI	Leader peptide (amino acids)	SLH domains	Accession No.[a]
	Nonpolar	Polar	Acidic	Basic					
Bacillus cereus ATCC 14579	39.9	37.5	10.2	12.4	64 599	8.34	29	3	AAP07978
Bacillus anthracis Ames									
Plasmid	42.5	31.2	12.9	13.5	45 044	6.3	29	3	AAD32358
Genome	47.7	27.5	12.2	12.4	91 362	5.7	29	3	CAA68063
Bacillus licheniformis NM 105	47.8	25.5	13.4	13.2	92 735	5.71	29	3	JC4930
Bacillus thuringiensies									
NRRL4045	47.4	24.1	14.7	13.7	87 123	5.43	29	3	CAB63252
Bacillus pseudofirmus OF4	47.8	34.9	10.6	6.6	96 855	4.42	31	3	AAF68436
Bacillus sphaericus									
DSM 396	48.3	32.3	9.6	9.9	85 509	5.56	31	3	AJ292964
CCM 2177	48.2	34.8	7.4	9.6	132 047	4.80	30	3	AAF22978
P1	48.6	32.9	8.3	10.5	129 935	4.85	30	3	CAA02847
WHO 2362	44.2	29.2	12.3	14.3	125 226	5.05	30	3	A33856
Bacillus fusiformis									
B3	47.1	28.2	11.3	13.4	61 874[b]	5.01	30	3	AJ781692[b]
DSM 2898T	47.4	27.7	11.4	13.2	62 007[b]	5.09	30	3	AJ781693[b]
Geobacillus stearothermophilus									
ATCC 12980	46.3	30.5	11.4	11.6	115 395	5.72	30	0	AAC12757
NRS 2004/3a	45.0	31.8	11.9	11.3	96 640	6.67	30	0	AAL46630
DSM 2358	44.9	30.5	13.9	10.7	47 082[b]	9.18	30	0	AJ781694[b]
PV 72/p6	45.2	31.4	11.3	12.0	131 076	5.40	30	0	I40468
Brevibacillus brevis									
HDP31	41.0	27.7	17.2	14.0	123 397	4.85	53	2	A35129
47	40.6	24.6	20.0	14.6	120 768	4.65	24	2	A28555

[a]EMBL database.
[b]Incomplete gene sequence (Akça 2004).

found for the S-layer protein of the hyperthermophilic *Methanotorris igneus* (Akça 2004), suggesting a role for glycosylation in the thermostabilization of these proteins. Although experimental data did not unequivocally prove S-layer glycosylation in hyperthermophilic methanococci, indirect evidence for post-translational modification is suggested by the smaller size of the S-layer protein from *Methanocaldococcus jannaschii* when heterologously expressed in *Escherichia coli* compared with the native protein (Akça 2004). On the other hand, glycans have been identified and characterized in the hyperthermophilic methanogenic species *Methanothermus fervidus* and *Methanothermus sociabilis* (Bröckl et al. 1991; Kärcher et al. 1993).

Another signature of the hyperthermophilic methanococci *Methanocaldococcus jannachii* and *Methanotorris igneus* is an increase in the number of charged residues, a decrease in alanine, and the occurrence of cysteine. Formation of disulfide bridges, decrease in hydrophobicity, and increased glycosylation may contribute to stability of hyperthermophilic archaeal S-layer proteins (Akça et al. 2002; Claus et al. 2002).

The S-layer proteins of *Methanocaldococcus jannaschii* and *Methanotorris igneus* were purified by preparative isoelectric focusing (Akça 2004). The native and the recombinant S-layer protein from *Methanocaldococcus jannaschii* exhibited an unusual electrophoretic behaviour in response to cations, pH, and temperature. Whether similar conformational adaptations of S-layer proteins may occur under the extreme living conditions of archaea has to be investigated.

The S-layer protein of *Methanotorris igneus* is 519 amino acids in length. The presumptive 28 amino acid leader peptide of the S-layer protein displays typical characteristics of a signal sequence with a positively charged N-region, a hydrophobic core, a polar C-region, and an alanine residue at the peptide cleavage site (Bendtsen et al. 2004). We found that this leader peptide sequence is highly conserved (approximately 68%) in all S-layer proteins of *Methanococcales*. Overall sequence comparisons (e.g., by BLAST 2.0) showed that the S-layer protein of *Methanotorris igneus* shares 43% identity with *Methanothermococcus thermolithotrophicus*, 39% with *Methanocaldococcus jannaschii*, 35% with *Methanococcus vannielii*, and 34% with *Methanococcus voltae*.

The S-layer gene of *Methanotorris igneus* consists of 1987 nucleotides (accession No. AJ564995) (Table 1). We identified the putative promoter region including the transcription factor B recognition element (BRE box, -GGTAA-) and TATA box (-TTTATATA-) at nucleotide positions –35 and –28 upstream from the transcription initiation site (-ATCG-). The

Table 4. Proposed regulatory gene sequences of the S-layer proteins from *Bacillaceae*.

Strain	-35- box	-10- box	Ribosome binding site
Bacillus anthracis Ames	-TTGTAT-	-TACTTT-	-GGAGGAA-
Bacillus licheniformis NM105	-TTGTAT-	-TACTTT-	-GGAGGAA-
Bacillus thuringiensis NRRL4049	-TGTATG-	-TTCTAT-	-GGAGGAA-
Bacillus sphaericus DSM 2362	-TTGAAT-	-TATAAT-	-GGAGGAA-
Bacillus sphaericus CCM 2177	-TTGTAT-	-TATAAT-	-GGAGGAA-
Bacillus sphaericus P1	Not found	-TATAAT-	-GGAGGAA-
Bacillus fusiformis B3	-TTGAAT-	-TATAAT-	GGAGGAA-
Bacillus fusiformis DSM 2898T	-TTGAAT-	-TATAAT-	-AGGGAGG-
Bacillus sphaericus DSM 396	-TTGCAT-	-TCAATT-	-TGAGGAA-
Geobacillus stearothermophilus DSM 2358	-TAGCAC-	-TAATAA-	-ATTTTAG-
Geobacillus stearothermophilus ATCC 12980	-TAGCAC-	-TAATAA-	-ATTTTAG-

Shine–Dalgarno sequence (-AGGTGAT-) is localized downstream at nucleotide +37. Translation starts (-ATG-) at nucleotide +45 and stops with -UAA- at nucleotide +1985.

S-layer of bacilli

Much sequence information is available about the S-layer genes of the family *Bacillaceae*. A comparison of the corresponding deduced primary protein sequences with respect to biochemical and phylogenetical relatedness is given in Table 3. All S-layer proteins exhibit signal peptides of 29–31 amino acids in size, except for two *Brevibacillus brevis* strains. This finding suggests a common phylogenetic development of the leader sequences for translocation across the cytoplasmic membrane. The proteins are generally weakly acidic with the exception of the more basic proteins from *Bacillus cereus* and *Geobacillus stearothermophilus* DSM 2358 (incomplete sequence). The molecular masses range from approximately 45 to 132 kDa. In contrast with most *Geobacillus* spp., one to three SLH domains have been found in all *Bacillus* spp. This is the only obvious difference in the primary peptide structure between the thermophilic and the mesophilic S-layer proteins of bacilli. When grown at 67 °C instead of 55 °C, a variant of *Geobacillus stearothermophilus* ATCC 12980 developed a glycosylated S-layer protein, whereas the wild-type protein is not glycosylated (Egelseer et al. 2001). This observation may indicate a role of glycans for thermostabilization of S-layer proteins. On the other hand, Novotny et al. (2004) observed no change of either the protein banding pattern or the glycan staining behaviour.

Overall sequence alignments generally exhibit no significant similarities among S-layer proteins of different species and even strains of bacilli. However, this is not true for the N-terminal parts harbouring the domains for the membrane transport, docking, and self-assembly processes: we found 95% homology (575 amino acids) between *Bacillus fusiformis* strains (B3, DSM 2898T), 94% (290 amino acids) between *Geobacillus stearothermophilus* strains (ATCC 12980, NRS 2004/3a, DSM 2358), 80% (192 amino acids) between *Bacillus sphaericus* strains CCM 2177, P1, WHO 2362), and 69% (201 amino acids) between *Bacillus anthracis*, *Bacillus licheniformis*, and *Bacillus thuringiensis*. On the basis of similarities in protein sequence and composition, the S-layer proteins of *Bacillaceae* can be divided into four groups: (I) *Bacillus anthracis*, *Bacillus licheniformis*, and *Bacillus thuringiensis*, (II) (*a*) *Bacillus sphaericus* (CCM 2177, P1, WHO 2362), (*b*) *Bacillus fusiformis* (DSM 2898T, B3), and (*c*) *Bacillus pseudofirmus*, *Bacillus sphaericus* DSM 396, and *Bacillus cereus* ATCC 14579, (III) *Geobacillus stearothermophilus* (ATCC 12980, NRS 2004/3a, DSM 2358), and (IV) *Brevibacillus brevis* (HDP 31, 47).

This division is also reflected by similarities within the corresponding regulatory gene sequences (Table 4). With respect to the high diversity among *Bacillus* spp. and the relative few members included in this study, the taxonomic value of this grouping is limited. However, it is remarkable that group I includes at least two phylogenetically related species (*Bacillus anthracis* and *Bacillus thuringiensis*) and group III consists exclusively of the thermophilic members. Group II comprises a genetically heterogenous group of mesophilic round-spored bacteria. On the basis of 16S rRNA analysis, Nakamura (2000) recently showed that *Bacillus sphaericus*-like organisms segregate into seven phylogenetically different clusters. Data from Hastie and Brinton (1979) and Lewis et al. (1987) revealed that the proteins composing the S-layer of *Bacillus sphaericus*-like strains have a high molecular mass (120–155 kDa), are slightly acidic (pI 4.6–4.9), and have no or only low glycosylation (0.4–1.81 mol%). The insect pathogenic strains possess a protein surface layer but lack the tetragonal arrays detected in the nonpathogenic strains by electron microscopy and negative staining (Lewis et al. 1987). A possible relatedness between the S-layer protein and the parasporal crystals of insecticide strains is still controversial (Bowditch et al. 1989).

Antibodies generated against the purified S-layer protein of *Bacillus fusiformis* strain B3 also gave strong signals with that of *Bacillus fusiformis* DSM 2898. Only a weak cross reactivity was obtained with the S-layer proteins isolated from *Bacillus sphaericus* DSM 396, *Geobacillus stearothermophilus* DSM 22, *Geobacillus stearothermophilus* DSM 458, and *Geobacillus stearothermophilus* DSM 2358 (unpublished results). The differences in morphology and the primary amino acid sequences of S-layers reflect the phylogenetical distance among the *Bacillus*-like bacteria.

Conclusions

S-layer proteins are ancient biomolecules that are almost ubiquitously distributed in all prokaryotic taxa. Their intrinsic ability to crystallize into two-dimensional lattices on the

cell surface may be one important step in the evolution of ordered structures and early life. Archaeal S-layer proteins resist high temperatures and acidic, alkaline, and high-salt environments. In bacteria that live under more moderate conditions, S-layer proteins have been maintained but with new physiological roles. In spite of the abundancy of S-layer proteins in many prokaryotic cells, these functions have been explained in only a few cases. Apart from some conserved N-terminal structures, the majority of the high molecular mass S-layer proteins show no phylogenetic clusters and heterogeneity is further enhanced by post-translational modifications. It is a challenge of future research to investigate the mechanisms and meaning of this variability. Taken together, the different S-layer proteins are exciting materials, not only for nanobiotechnological applications (Sleytr et al. 1997) but also as models to learn more about strategies for stabilization, self-organization, and functional evolution of proteins.

Acknowledgements

The authors want to thank the "Ministerium für Bildung, Wissenschaft, Forschung und Technologie (BMFT)" and the "Deutsches Zentrum für Luft - und Raumfahrt (DLR)", Bonn, Germany, for supporting these studies.

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