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REVIEW: Surface-layer glycoproteins: an example for the diversity of bacterial glycosylation with promising impacts on nanobiotechnology

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Bacterial cell surface layers, referred to simply as S-layers, have been described for all major phylogenetic groups of bacteria, which may indicate their pivotal role for a bacterium in its natural habitat. They have the unique ability to assemble into two-dimensional crystalline arrays that completely cover the bacterial cells. Glycosylation represents the most frequent modification of S-layer proteins. S-layer glycoproteins constitute a class of glycoconjugates first isolated in the mid-1970s, but S-layer glycoprotein research is still being regarded as an "exotic field of glycobiology," possibly because of its "noneukaryotic" character. Extensive work over the past 30 years provided evidence of an enormous diversity of S-layer glycoproteins that have been created in nature over 3 billion years of prokaryotic evolution. These glycoconjugates are substantially different from eukaryotic glycoproteins, with regard to both composition and structure; nevertheless, some general structural concepts may be deduced. The awareness of the high application potential of S-layer glycoproteins, especially in combination with their intrinsic cell surface display feature, in the field of modern nanobiotechnology as a base for glycoengineering has recently led to the investigation of the S-layer protein glycosylation process at the molecular level, which has lagged behind the structural studies due to the lack of suitable molecular tools. From that work an even more interesting picture of this class of glycoconjugates is emerging. The availability of purified enzymes from S-layer glycan biosynthesis pathways exhibiting increased stabilities and/or rare sugar specificities in conjunction with preliminary genomic data on S-layer glycan biosynthesis clusters will pave the way for the rational design of S-layer *neo*glycoproteins.

Key words: bacterial glycosylation/genomic glycosylation loci/glycan diversity/glycoengineering/S-layer *nano*glycobiology

What are S-layer glycoproteins?

The progress made in prokaryotic glycoprotein research over the past 30 years has been extraordinary, providing a wealth of data on structures, biological properties, and functions of the different glycoproteins (for most recent reviews see Benz and Schmidt, 2002; Messner, 2004; Messner and Schäffer, 2003; Moens, 2000; Power and Jennings, 2003; Schäffer and Messner, 2001; Schmidt *et al*., 2003; Spiro, 2002; Szymanski *et al*., 2003; Upreti *et al*., 2003; Wacker *et al*., 2002). A major class among these glycoconjugates are bacterial cell surface layer (S-layer) glycoproteins (for review see Sleytr and Messner, 2003), which justifies the distinction between S-layer and non-S-layer glycoproteins (including intracellular, membrane-associated, and extracellular glycoproteins) as has been suggested by Sandercock *et al*. (1994). Most S-layer glycoproteins have the unique feature of assembling into two-dimensional crystalline arrays on the supporting cell envelope layer, yielding a complete coverage of the bacterial cells with the glycan moieties protruding from the cell surface. This situation compares to the polysaccharide coating of Gram-negative bacterial cells by lipopolysaccharides (Whitfield, 1995). The regular, nanometer-scale S-layer protein lattices can be best visualized by electron microscopy (Figure 1). First indications for the glycoprotein nature of a

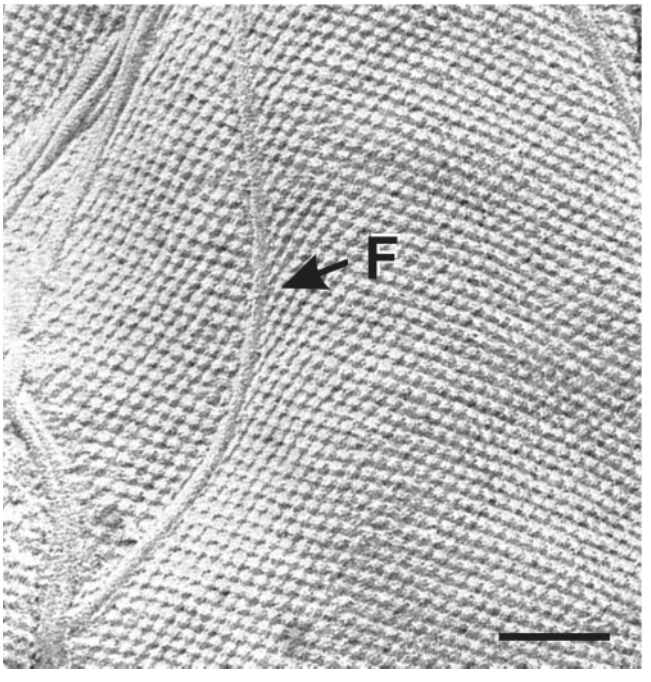

Fig. 1. Electron micrograph of the hexagonal S-layer protein lattice as observed on the cell surface of *Thermoanaerobacter thermohydrosulfuricus* L77-66 on freeze-etching and platinum-carbon shadowing. F, flagellum; bar, 100 nm.

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given S-layer protein, however, are frequently inferred from a positive carbohydrate staining reaction on an sodium dodecyl sulfate polyacrylamide (SDS-PA) gel (Schäffer *et al*., 2001).

The first reports on S-layer glycoproteins date back to the mid-1970s, when they were described simultaneously on halobacteria (Mescher and Strominger, 1976) and on thermophilic clostridia (Sleytr and Thorne, 1976). Since then a considerable body of knowledge has accumulated about archaeal (for review see Sumper and Wieland, 1995) and bacterial S-layer glycoproteins (for recent reviews see Messner and Schäffer, 2003; Schäffer and Messner, 2001), indicating that glycosylation is the major modification of S-layer proteins. (Since the introduction of archaebacteria as second prokaryotic kingdom, the living systems have been divided into three aboriginal lines of descent: the archaebacteria, the eubacteria, and the eukaryotes. According to a new nomenclature, the term *archaebacteria* was changed to *archaea* and that of *eubacteria* to *bacteria*; Woese *et al*., 1990.) It contributes to an enormous diversification potential of the bacterial cell surface, which may be advantageous for the bacterial survival in the natural, competitive habitat.

Accounting for the intrinsic, nanometer-scale cell surface display feature of bacterial S-layer glycoproteins, we have coined the neologism S-layer *nano*glycobiology, which encompasses structural, functional, and biosynthetic aspects of S-layers. Structural investigations use novel, straightforward analytical techniques (for a comprehensive survey of methods applied for S-layer glycoprotein research, the reader is referred to a recent review article by Schäffer *et al*., 2001); functional and biosynthetic studies, however, are lagging behind due to the lack of suitable molecular tools. The awareness of the promising application potential of S-layer glycoproteins as a unique matrix with inherent self-assembly properties for nanobiotechnology applications (for review see Sleytr *et al*., 1999, 2002), let us recently focus our efforts on the biosynthesis of S-layer glycoproteins at the molecular level, which represents the key to the successful modification of S-layer glycosylation and, consequently, the generation of artificial S-layer *neo*glycoconjugates with rationally designed glycosylation motifs by glycoengineering techniques.

Diversity of S-layer protein glycosylation: summary of facts

Occurrence

Glycosylated S-layer proteins are widely distributed in the major lineages of archaea (for reviews see Kandler, 1993; Sumper and Wieland, 1995) and in Gram-positive bacteria (Messner and Schäffer, 2003). In these organisms, up to 20% of the total protein synthesis effort may be devoted to the production of S-layer glycoproteins, which indicates that S-layer protein synthesis is under the control of very strong promoters (Kuen and Lubitz, 1996; Pouwels *et al*., 1997). Under laboratory cultivation conditions, the yield of S-layer glycoprotein ranges strain specifically between 0.5 and 2.0 g wet weight per L growth medium. Recently, yields up to even 5.0 g per L have been reported for some alkalitolerant and alkaliphilic methanotrophs, which dwell in specific ecological environments (Eshinimaev *et al*., 2002; Kaluzhnaya *et al*., 2001). The glycosylation degree of S-layer proteins generally varies between 2% and 10% (w/w), yielding overall apparent molecular masses of the constituting protomers between 45 and 200 kDa on SDS-PA gels.

Composition

Presently, about 40 different S-layer glycoprotein glycan structures are fully or at least partially elucidated (a summary of S-layer glycan structures is given in Table I). The observed structures and glycosidic linkage types already exceed the display found in eukaryotes. Bacterial S-layer glycan chains are linear or branched homo- or heterosaccharides, which comprise 20–50 identical repeating units, whereas in archaea, short oligosaccharides without repeats prevail. The monosaccharide constituents of the S-layer glycan chains include a wide range of neutral hexoses, 6-deoxyhexoses, and amino sugars. Among bacteria, this spectrum is further extended by rare sugars, which are otherwise typical constituents of lipopolysaccharide (LPS) O-antigens of Gram-negative bacteria (respective glycoses are marked by an asterisk in Table II). *O*-glycosidic linkage regions observed on S-layer glycoproteins occur to tyrosine, serine, and threonine; *N*-glycans have been found only in archaea. Interestingly, no antennary structures comparable to the *N*-glycans present in eukaryotes have so far been identified among S-layer glycoproteins. For those S-layer glycoproteins whose protein portion has been sequenced, the properties of the corresponding S-layer protein structural gene are given in Table III.

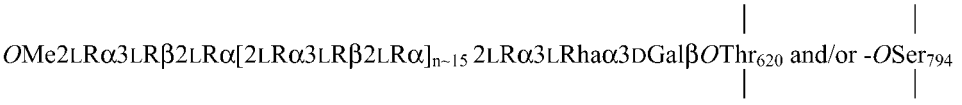
Though the information about archaeal S-layer glycoprotein glycan structures dates back several years (for review, see Sumper and Wieland, 1995), much structural data have accumulated about bacterial S-layer glycans. Despite being highly diverse on first analyses, most S-layer glycoproteins from the domain *Bacteria* follow a tripartite building plan (Figure 2). The most common scheme comprises a variable carbohydrate chain, which is linked via a core oligosaccharide to the S-layer polypeptide backbone. Capping of the terminal sugar residue at the nonreducing end of the glycan chain with an *O*-methyl-group (2-*O*-Me, 3-*O*-Me) is observed in some structures (Figure 2A). If the oligosaccharide linker is missing, the carbohydrate chain is directly bound to the glycosylated amino acid via the first repeating unit; the anomeric configuration of the linkage sugar may remain unchanged (no core) or be inverted (pseudo-core) (Figure 2B). In one organism, a truncated structure has been observed that may originate either from an enzyme defect or from mutation (Figure 2C). The current status of S-layer glycoprotein research indicates that in bacteria only one distinct scheme of S-layer glycoprotein building plan exists per individual organism, whereas in archaea, *O*- and *N*-glycans may exist simultaneously (Schäffer and Messner, 2001). Their overall structure in combination with the constituent sugars let us interpret the highly variable S-layer glycoproteins as Gram-positive equivalents of lipopolysaccharides, with the protein component replacing lipid A. All features of S-layer glycoproteins known to date, including potential modifications and glycosylation sites, are summarized in Table II.

Fresh perspectives

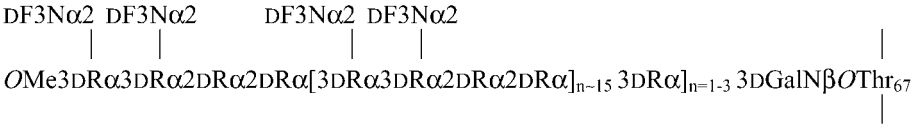
During the past few years, no new archaeal S-layer glycan structures have been reported. There was, however, considerable progress in the characterization of the respective S-layer proteins from selected organisms, such as thermophilic methanococci (Akça *et al*., 2002; Claus *et al*., 2002) or halophilic archaea (Konrad and Eichler, 2002). First crystallization experiments of the S-layer

Table I. Selected S-layer glycan structures

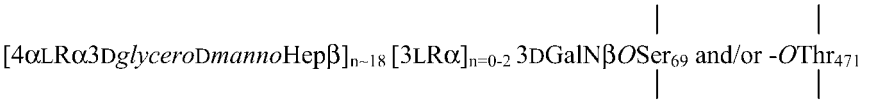
***Geobacillus stearothermophilus* NRS 2004/3a**
(Schäffer *et al*., 2002)



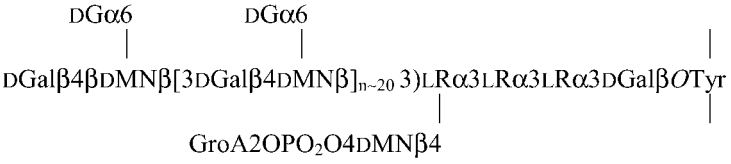
***Aneurinibacillus thermoaerophilus* L420-91^T (DSM 10154) and GS4-97**
(Kosma *et al*., 1995a; Schäffer *et al*., 1999)



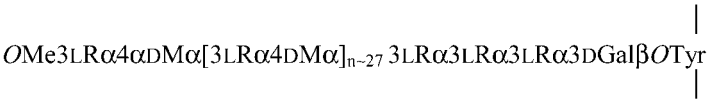
***Aneurinibacillus thermoaerophilus* (DSM 10155)**
(Kosma *et al*., 1995b; Wugeditsch *et al*., 1999)



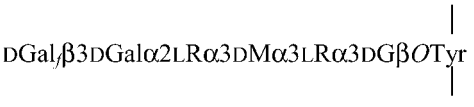
***Paenibacillus alvei* (CCM 2051)**
(Altman *et al*., 1991; Messner *et al*., 1995)



***Thermoanaerobacter thermohydrosulfuricus* L111-69 and L110-69 (DSM 568)**
(Bock *et al*., 1994)



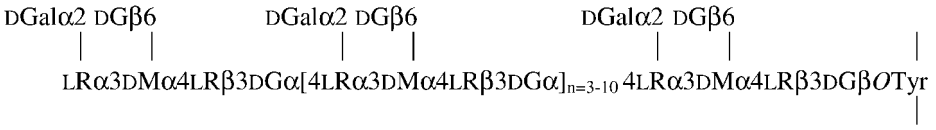
***Thermoanaerobacter thermohydrosulfuricus* S102-70**
(Messner *et al*., 1992)



***Thermoanaerobacter thermohydrosulfuricus* L77-66 (DSM 569) and L92-71**
(Altman *et al*., 1992; Messner *et al*., 1993)

Table I. continued

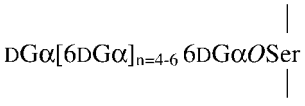
***Thermoanaerobacterium thermosaccharolyticum* D120-70**
(Schäffer *et al.*, 2000)



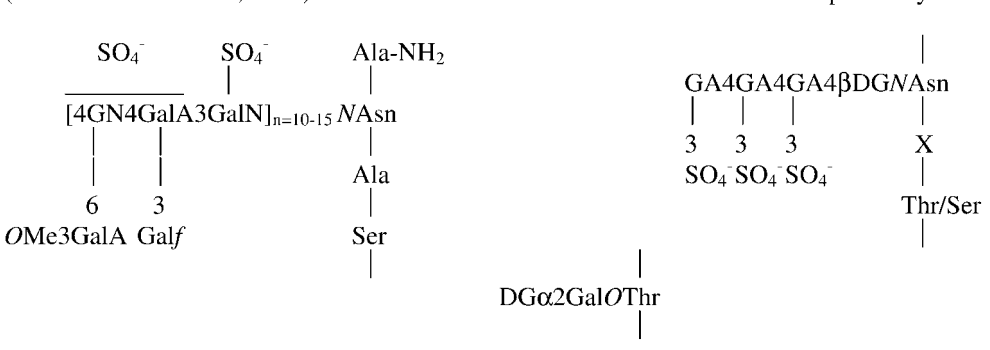
***Thermoanaerobacterium thermosaccharolyticum* E207-71**
(Altman *et al.*, 1995)

New thermophilic isolate GS5-97
(C. Schäffer, P. Messner, unpublished data)

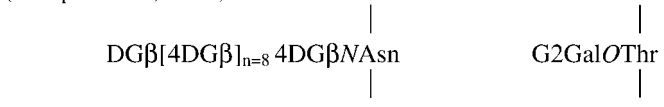
***Lactobacillus buchneri* 41021/251**
(Möschl *et al.*, 1993)



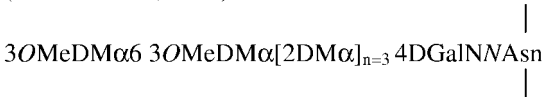
***Halobacterium halobium* R_1M_1**
(Lechner and Wieland, 1989)



***Haloferax volcanii* DS2 (NCMB 2012)**
(Sumper *et al.*, 1990)



***Methanothermus fervidus* V24S (DSM 2088)**
(Kärcher *et al.*, 1993)



G, glucose; Gal, galactose; M, mannose; LR, L-rhamnose; DR, D-rhamnose; DF, D-fucose; GN, *N*-acetylglucosamine; GalN, *N*-acetylgalactosamine; MN, *N*-acetylmannosamine; DF3N, 3-*N*-acetyl-D-fucosamine (3-acetamido-3,6-dideoxy-D-galactose); D-Q3N, 3-*N*-acetyl-D-quinovosamine (3-acetamido-3,6-dideoxy-D-glucose); D*glycero*D*manno*Hep, D-*glycero*-D-*manno*-heptose; GA, glucuronic acid; GalA, galacturonic acid; MA, mannuronic acid; IdA, iduronic acid; all sugars are in the pyranose form; Gal_f, galactofuranose; GroA2, 2-phosphoglyceric acid; PO_4, phosphate; *O*Me, O-methyl; SO_4^{2-}, sulfate; Thr, threonine; Ser, serine; Tyr, tyrosine; Ala, alanine; *X*, interchangeable amino acid; n, number of repeats.

Table II. Features of bacterial S-layer glycoproteins

Feature	Bacteria	Archaea
Type of glycan	*O*-glycan	*N*-glycan, *O*-glycan
Linkage regions	β-Glc→Tyr (Christian *et al.*, 1993; Schäffer *et al.*, 2000)	β-Glc→Asn (Mescher and Strominger, 1976; Wieland *et al.*, 1983; Paul *et al.*, 1986)
	β-Gal→Tyr (Christian *et al.*, 1988; Bock *et al.*, 1994; Messner *et al.*, 1995)	GalNAc→Asn (Mescher and Strominger, 1976; Wieland *et al.*, 1983; Kärcher *et al.*, 1993)
	β-Gal→Thr/Ser (Schäffer *et al.*, 2002)	Rha→Asn (Pellerin *et al.*, 1990)
	β-GalNAc→Thr/Ser (Schäffer *et al.*, 1999; Wugeditsch *et al.*, 1999)	Gal→Thr (Mescher and Strominger, 1976; Wieland *et al.*, 1983; Eichler, 2000)
	α-Glc→Ser (Möschl *et al.*, 1993)	
Different glycans per S-layer protomer	1	1–3
Composition of glycan	Usually heteropolysaccharides (20–50 repeats)	Short heteropolysaccharides (up to 10 sugars)
Repeating unit	Usually 2–6 sugars	Not identified
Core	Usually 1–3 sugars	Not identified
Sugar constituents	β-D-Glc*p*	α-D-Glc*p*, β-D-Glc*p*
	α-D-Gal*p*, β-D-Gal*p*, β-D-Gal*f*	Gal*p*, Gal*f*
	α-D-Man*p*	α-D-Man*p*
	α-D-Rha*p*,[a] α-L-Rha*p*	Rha*p*
	α-D-Glc*p*Nac	D-Glc*p*NAc
	α-D-Gal*p*NAc, β-D-Gal*p*NAC	D-GalNAc
	β-D-*glycero*-D-*manno*-*Hepp*[a]	GlcA
	α-D-Fuc[a]	IdA
	β-D-Man*p*Nac	
	α-D-Fuc*p*3NAc[a]	
	β-D-Qui*p*3NAc[a]	
Modifications (nonstochiometric)	2-*O*-Me (Schäffer *et al.*, 2002)	3-*O*-Me (Lechner and Wieland, 1989; SO_4^{2-} (Lechner and Wieland, 1989; Kärcher *et al.*, 1993)
	3-*O*-Me (Bock *et al.*, 1994; Kosma *et al.*, 1995a)	Kärcher *et al.*, 1993)
	GroA-PO_2^- (Messner *et al.*, 1995)	Diphytanylglycerol (Eichler, 2001)
		Mevalonic acid (Eichler, 2001)
Attachment sites on protein	1–4	3–25
Isolated glycosylation sequences	**Y**[b]T (Schäffer *et al.*, 2000) **Y**D (Schäffer *et al.*, 2000)	**N**XS/T (Lechner and Wieland, 1989; Zeitler *et al.*, 1998[c])
	YPV (Bock *et al.*, 1994)	
	YNP (Altman *et al.*, 1995)	
	YSPA (Schäffer *et al.*, 2000)	
	Q**Y** (Bock *et al.*, 1994)	
	YDGNS (Altman *et al.*, 1995)	
	S**T**GDT (Schäffer *et al.*, 1999)	
	T**T**NXK (Wugeditsch *et al.*, 1999)	
	ADLKT**T**SDNF (Schäffer *et al.*, 2002)	
	SGET (Wugeditsch *et al.*, 1999)	
	TLTSADVIRV (Schäffer *et al.*, 2002)	

[a]Also typical constituent of LPS O-antigens.
[b]Boldface indicates the glycosylated amino acid on the S-layer polypeptide.
[c]In *H. halobium*, replacement of S by V, L, or D in the sequon does not prevent S-layer *N*-glycosylation.

Table III. Amino acid sequences of S-layer glycoproteins with known glycan structures

Organism	S-layer gene	Precursor/ signal peptide	S-layer lattice	GenBank accession no.	Glycosylation sites on precursor[a]	Reference
G. stearothermophilus NRS 2004/3a	*sgsE*	903/30	Oblique	AF328862	Thr 622, Ser 794	Schäffer *et al.* (2002)
A. thermoaerophilus L420-91^T and GS4-97	*satA*	759/30	Square	AY395578	Thr 67	Pfoestl (2003)
A. thermoaerophilus DSM 10155	*satB*	738/30	Square	AY395579	Ser 69, Thr 471	Pfoestl (2003)
H. halobium R_1M_1	*csg*	852/34	Hexagonal	J02767	Asn 2, Asn 305, Asn 364, Asn 404, Asn 479, Asn 609, Asn 693, Asn 717, Asn 753, Asn 777, Asn 781; Thr 755–774	Lechner and Sumper (1987)
H. volcanii DS2	*csg*	827/34	Hexagonal	M62816	Asn 13, Asn 83, Asn 274, Asn 279, Asn 370, Asn 489, Asn 732; Thr 737–748	Sumper *et al.* (1990)
M. fervidus DSM 2088	*slgA*	539/22	Hexagonal	X58297	Not determined	Bröckl *et al.* (1991)

[a]Not all sites are necessarily glycosylated.

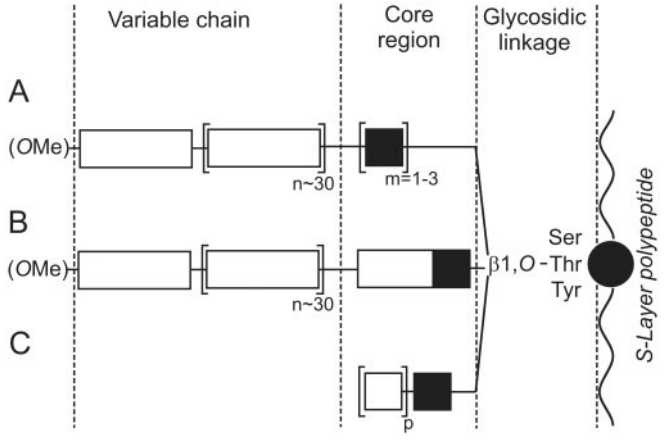


Fig. 2. Scheme of the principal building plans of S-layer glycoproteins from the domain *Bacteria*. (**A**) Extended tripartite structure; (**B**) tripartite structure with a pseudo-core; (**C**) truncated structure. Rectangles represent repeating units, the full square symbolizes the glycose attached to the glycosylation site; in bacteria from the *Bacillaceae* family this glycose is typically β-configurated. The full circle indicates the glycosylated amino acid of the S-layer protein. n, number of repeating units; m, number of core glycoses; p, number of single glycoses constituting truncated glycans.

glycoprotein of *Methanococcus fervidus* under microgravity conditions were successful in obtaining small monoclinic crystals with space group C2 (Evrard *et al.*, 1999). Currently, they are being further investigated to provide the first 3D X-ray crystal structure of an S-layer glycoprotein. With other archaeal S-layer glycoproteins there was a new understanding of both the biosynthetic stage and topological site of the S-layer protein modification. For example, like S-layer glycosylation, the lipid-modification of the S-layer glycoprotein of the halophilic archaeon *Haloferax volcanii* takes place on the external cell surface, that is, following protein translocation across the plasma membrane (Eichler, 2001; Eichler and Irihimovitch, 2003). This mevalonic acid–based maturation event of the S-layer glycoprotein becomes evident through an aberrant migration behavior of the S-layer glycoprotein monomers on SDS-PA gels, resulting in too-high molecular mass estimates and an increase in the hydrophobicity of the protein (Konrad and Eichler, 2002). Similar observations have been reported for the S-layer glycoproteins of *Halobacterium halobium* (Kikuchi *et al.*, 1999). This suggests that lipid modification of S-layer glycoproteins is a general property of halophilic archaea.

Another important question concerned the relationship between the translation and translocation event of the archaeal S-layer protein components. Using a hybrid protein approach with the signal peptide–encoding region of the S-layer glycoprotein gene of *H. volcanii* fused to either to the cellulose-binding domain of the *Clostridium thermocellum* cellulosome or a reductase of *H. volcanii*, it was demonstrated that the signal peptide-cleaved mature versions of both hybrid proteins were secreted into the growth medium, whereas the signal peptide–bearing forms remained in the cytoplasm (Irihimovitch and Eichler, 2003). The results of these experiments provide evidence that in archaea at least some of the secreted proteins are first synthesized inside the cell and only then translocated across the plasma membrane. It is assumed that the investigated archaeal preproteins rely on the Sec machinery for their secretion (Mori and Ito, 2001).

Concerning bacteria, the number of new glycan structures is growing steadily (compare with Table I). Detailed structural analyses have been performed with S-layer glycoproteins of thermophilic bacilli, such as *Geobacillus stearothermophilus* NRS 2004/3a (Schäffer *et al.*, 2002). For that organism, the S-layer protein portion SgsE has

been fully sequenced, which allowed, for the first time on a bacterial glycoprotein, the exact determination of the positions of the glycosylation sites; comparable analyses have only been performed with the thermophilic archaea *H. halobium* (Lechner and Sumper, 1987) and *H. volcanii* (Sumper *et al*., 1990). In the case of the *G. stearothermophilus* NRS 2004/3a S-layer glycoprotein, the attached poly-L-rhamnan S-layer glycan chains consist, in average, of 13–18 trisaccharide repeats that are *O*-glycosidically linked to the amino acids Thr 620 and/or Ser 794 of the precursor protein via a β-D-Gal residue (Schäffer *et al*., 2002). The C-terminal localization of the glycosylation sites on SgsE may indicate that the C-terminus of the S-layer protein is surface-exposed, which would sterically allow the attached glycan chains to protrude into the exterior environment. Furthermore, this would be in accordance with the proposed involvement of the N-terminus in anchoring the S-layer protein to the peptidoglycan network (Sára, 2001). A detailed structure of the entire S-layer glycoprotein of *G. stearothermophilus* NRS 2004/3a is given in Table I. This table also shows a novel D-fucose-containing S-layer glycan, whose complete structure is currently under investigation (Schäffer and Messner, unpublished data).

How is S-layer glycoprotein diversity created? First insights

From the analysis of several archaeal and bacterial S-layer glycoproteins (compare with Table I and Messner and Schäffer, 2000) is evident that even among phylogenetically closely related organisms, S-layer glycans can be very diverse. Consequently, the glycan modifications of the S-layer protein can be used for the recognition of Gram-positive bacteria only at the strain level. For example, *Aneurinibacillus thermoaerophilus* strain L420-91^T possesses branched hexasaccharide repeating units, whereas those of strain DSM 10155 are composed of disaccharides (see Table I). Both strains, however, show a 16S rDNA sequence similarity of 99.8% and the level of DNA relatedness is 94%, which indicates, that both organisms belong to the same species (Meier-Stauffer *et al*., 1996). Obviously, the S-layer glycan composition represents a strain-specific feature, which reflects the adaptation of the organisms to the competitive growth conditions of their natural habitat. Similar observations have been made with O-antigens of LPSs of Gram-negative bacteria (for recent reviews see Raetz and Whitfield, 2002; Valvano, 2003).

The interesting question arises as to how S-layer glycoprotein diversity is created by nature. Approaches for unravelling the details about the S-layer protein glycosylation machinery benefit form the molecular information available on the biosynthesis of LPS O-antigens, because due to structural similarities it may be speculated that both cell surface glycoconjugates are assembled via similar biosynthetic routes, involving comparable enzyme activities.

First information on bacterial S-layer glycosylation loci

Following the requirement of activated sugars for S-layer glycan biosynthesis, *A. thermoaerophilus* DSM 10155 (Graninger *et al*., 2002) and *G. stearothermophilus* NRS 2004/3a (Novotny *et al*., 2004) have been probed with *rml*-specific probes from the *Salmonella enterica* dTDP-β-L-Rha*p* biosynthesis pathway, which allowed the chromosomal localization of the biosynthesis enzymes of that nucleotide sugar. Currently, the genes encoding several other sugar processing enzymes involved in the biosynthesis of bacterial S-layer glycans are known (Table IV); these lead to the formation of GDP-α-D-Rha*p* (Kneidinger *et al*., 2001a), GDP-D-*glycero*-D-*manno*-Hep*p* (Kneidinger *et al*., 2001b), dTDP-α-D-Fuc*p*3NAc (Pfoestl *et al*., 2003), and dTDP-α-D-Qui*p*3NAc (Pfoestl *et al*., unpublished data). Sequencing of upstream and downstream regions of the respective DNA regions of *G. stearothermophilus* NRS 2004/3a, *A. thermoaerophilus* strains DSM 10155 and L420-91^T revealed the presence of S-layer glycan biosynthesis (*slg*) gene clusters (GenBank accession numbers AF328862, AF324836, and AY442352, respectively). Based on database sequence similarities, putative biological functions to most of the genes of the *slg* clusters could be assigned (Novotny *et al*., 2004). Briefly, the clusters contain components for S-layer glycan assembly, lipid activation, and export. Furthermore, there is evidence for interplay of products of glycan-specific genes and housekeeping genes as is also well known from LPS biosynthesis (Whitfield, 1995).

With the occurrence of an ABC-2 type transporter system in the investigated *slg* gene clusters, we assume that the S-glycan chains are synthesized in a process comparable to the *wzy*-independent pathway of the LPS O-polysaccharide assembly route (Raetz and Whitfield, 2002). Interestingly, the *slg* clusters of the investigated organisms are typically flanked by several transposases or remnants thereof. This, together with the observation that the G + C content (varying between 30% and 43% in individual genes) of the clusters is lower than the average of the respective organism (on average 50%), indicates that lateral transfer of the whole glycosylation machinery may have taken place. In general, lateral gene transfer under natural conditions is one of the most outstanding capacities of bacteria (Lawrence and Hendrickson, 2003; Ochman *et al*., 2000). Acquisition of the S-layer glycosylation potential of these thermophilic bacilli may be another analogy to O-polysaccharide biosynthesis of Gram-negative bacteria, because there, the major source of antigenic diversity is believed to be repeated lateral gene transfer followed by adaptation to a new niche or environment (Keenleyside and Whitfield, 1999).

Concerning the chromosomal location of the S-layer target protein in relation to the *slg* cluster, no general scheme is present in the investigated organisms. In *G. stearothermophilus* NRS 2004/3a, the S-layer structural gene *sgsE* is located immediately upstream of the *slg* gene cluster (Novotny *et al*., 2004), whereas the genes encoding the proteins SatA and SatB of *A. thermoaerophilus* L420-91^T and DSM 10155, respectively, are located elsewhere on the chromosome (Novotny *et al*., forthcoming).

In the course of investigating the glycan-based diversification potential of S-layer proteins, another interesting observation was made concerning the occurrence of specific sugar-processing enzymes in organisms whose S-layers

Table IV. Selection of genes involved in the biosynthesis of bacterial S-layer glycans

Gene designation	Enzyme name	GenBank accession no.	Reference
rmlA	Glucose 1-phosphate thymidyltransferase	AF324836, AF328862, AY278518, AY278519, AY442352	Kneidinger *et al.* (2001b, 2002); Schäffer *et al.* (2001a); Novotny *et al.* (2004)
rmlB	dTDP-glucose 4,6-dehydratase	AF324836, AF328862, AY278518, AY278519, AY442352	Kneidinger *et al.* (2001b, 2002); Schäffer *et al.* (2001a); Novotny *et al.* (2004)
rmlC	dTDP-dehydrorhamnose 3,5-epimerase	AF324836, AF328862, AY278518, AY278519	Kneidinger *et al.* (2001b); Schäffer *et al.* (2001a); Novotny *et al.* (2004)
rmlD	dTDP-dehydrorhamnose reductase	AF324836, AF328862, AY278518, AY278519	Kneidinger *et al.* (2001b); Schäffer *et al.* (2001a); Novotny *et al.* (2004)
gmd	GDP-mannose 4,6-dehydratase	AY442352	Kneidinger *et al.* (2001a)
rmd	GDP-6-deoxy-D-*lyxo*-4-hexulose reductase	AY442352	Kneidinger *et al.* (2001a)
gmhA	Sedoheptulose 7-phosphate isomerase	AF324836	Kneidinger *et al.* (2001b, 2002)
hddA	D-*glycero*-D-*manno*-heptose 7-phosphate kinase	AF324836	Kneidinger *et al.* (2001b, 2002)
gmhB	D-*glycero*-D-*manno*-heptose 1,7-bisphosphate phosphatase	AF324836	Kneidinger *et al.* (2001b, 2002)
hddC	D-*glycero*-D-*manno*-heptose 1-phosphate guanosyltransferase	AF324836	Kneidinger *et al.* (2001b, 2002)
fdtA	dTDP-6-deoxy-3,4-keto-hexulose isomerase	AY442352	Pfoestl *et al.* (2003)
fdtB	dTDP-6-deoxy-D-*xylo*-hex-3-ulose aminase	AY442352	Pfoestl *et al.* (2003)
fdtC	dTDP-D-Fuc*p*3N acetylase	AY442352	Pfoestl *et al.* (2003)
qdtA	dTDP-6-deoxy-3,4-keto-hexulose isomerase	AY422724	Pfoestl (2003)
qdtB	dTDP-6-deoxy-D-*ribo*-hex-3-ulose aminase	AY422724	Pfoestl (2003)
qdtC	dTDP-D-Qui*p*3N acetylase	AY422724	Pfoestl (2003)

are nonglycosylated (for a selection of organisms, see Messner *et al.*, 1984). Probing, for instance, the chromosomal DNA of *G. stearothermophilus* L32-65 with an *rmlA*-specific probe, coding for the glucose 1-phosphat thymidylyltransferase RmlA of the L-rhamnose pathway (Giraud and Naismith, 2000), indicated that in this organism the *rml* genes are present and, surprisingly, the enzyme proteins are also fully active (Novotny *et al.*, 2004). Because the presence of other rhamnosylated glycoconjugates in that organism could be ruled out, it is conceivable to assume that the S-layer glycosylation process must have been switched off by a yet unknown regulatory event. This finding may implicate that this particular organism and probably many others, too, have been naturally endowed with the potential to synthesize rhamnose-containing S-layer glycoproteins. It may be concluded that S-layer protein glycosylation is more widespread among bacteria than initially assumed (compare with Table I), but obviously, due to the absence of a selective pressure when grown under laboratory conditions, this ability may be subject to change or even loss.

Protein-based variations

Another reason for S-layer glycoprotein diversity was found in *Clostridium difficile*, the causative agent of antibiotic-associated diarrhea. This Gram-positive organism is unusual in expressing two S-layer proteins with molecular masses in the range of ~45 kDa and ~36 kDa, respectively (Cerquetti *et al.*, 2000; Poxton *et al.*, 1999). Both S-layer proteins have been shown to derive by processing of a larger precursor molecule, including the removal of a signal sequence and internal cleavage (Calabi *et al.*, 2001). Proteolytic cleavage takes place approximately in the middle of the precursor protein at a site that is highly conserved among different *C. difficile* strains, releasing the N-terminal portion with the signal peptide that, later on, is removed to yield the lower molecular weight S-layer protein. The C-terminal portion represents the higher-molecular-weight S-layer protein; it exhibits peptidoglycan hydrolase activity and acts as an adhesin to bind to human gut tissues (Calabi *et al.*, 2002). An unusual feature of the S-layer of *C. difficile* is that the sizes of the two mature S-layer proteins vary widely between strains. Sequence analyses of six strains of *C. difficile* showed that this variation is due to insertions and deletions of stretches of DNA within the *slpA* gene, rather than to the expression of alternate structural genes (Calabi and Fairweather, 2002). Early reports indicated that the S-layer proteins from *C. difficile* strain 253 were glycosylated (Mauri *et al.*, 1999). Recent studies on other strains showed that in these organisms only the 45-kDa protein was significantly *O*-glycosylated, whereas the 36-kDa protein is either nonglycosylated or only glycosylated to a minor extent (Calabi *et al.*, 2001). Besides the already known substantial differences in the primary amino acid sequences of the S-layer proteins of different *C. difficile* strains, analyses of their specific glycosylation patterns should show whether distinct genes are present specifying the glycosylation reactions.

Outlook: S-layer glycoproteins in applied research

Detailed structural studies present S-layer glycoproteins as highly variable bacterial cell surface glycoconjugates that show remarkable differences to the well-investigated eukaryotic glycoproteins with regard to composition and structure. Thus S-layer glycoprotein research makes valuable contributions to the field of glycobiology in general. Furthermore, bacteria are regarded ideal models for studying protein glycosylation in a relatively simple, single-celled system. Considering the constantly increasing reports of glycoproteins that are important in pathogenesis (such as pilins, adhesins, flagellae) (Power and Jennings, 2003; Schmidt *et al*., 2003; Upreti *et al*., 2003), elucidating the mechanism of bacterial protein glycosylation is of utmost importance (Messner, 2004). This will be facilitated by a detailed understanding of the involved genes.

Recent molecular investigations of the S-layer glycoprotein biosynthesis have revealed several novelties about nucleotide-sugar synthesis. The heptose genes of *A. thermoaerophilus* DSM 10155 led to the first elucidation of the overall pathway for the biosynthesis of a nucleotide-activated D-*glycero*-α-D-*manno*-heptose from D-seduheptulose 7-phosphate (Kneidinger *et al*., 2001a). Furthermore, these data allowed the assignment of the steps involved in the biosynthesis of the ADP-L-*glycero*-β-D-*manno*-heptose precursor of the inner core lipopolysaccharide of prominent laboratory organisms like *Escherichia coli* or *Salmonella enterica* (Kneidinger *et al*., 2002). During analysis of the S-layer glycosylation of *A. thermoaerophilus* L420-91^T, Gmd was identified to be a novel bifunctional enzyme displaying both dehydratase and reductase activities, which is required for GDP-D-rhamnose biosynthesis (Kneidinger *et al*., 2001b). In the course of the functional characterization of the biosynthesis of dTDP-D-Fu*cp*3NAc from the same organism, a novel type of isomerase capable of synthesizing dTDP-6-deoxy-D-*xylo*-hex-3-ulose from dTDP-6-deoxy-D-*xylo*-hex-4-ulose was described (Pfoestl *et al*., 2003). Recombinant enzymes from that pathway may be relevant for the synthesis of several antibiotics that contain C-3-aminated deoxy-sugars, such as erythromycin or tylosin. Furthermore, recombinant RmlABCD enzymes from the thermophile *A. thermoaerophilus* DSM 10155 may be beneficial for dTDP-L-rhamnose-targeted antibacterial therapy, because the instability of enzymes from mesophilic organisms limits productivity. Most Rml enzymes from the thermophilic source exhibit significantly higher stability at 37°C than the enzymes from *S. enterica* (Graninger *et al*., 2002).

The knowledge of the enzyme apparatus involved in S-layer glycoprotein glycan biosynthesis and the understanding of the underlying mechanisms, should eventually allow the alteration or the rational design of S-layer protein glycosylation patterns to obtain bioactive S-layer *neo*glycoproteins. The common trend of glycoengineering is reflected by many recent review articles on that topic (Endo and Koizumi, 2000; Mendez and Sala, 2001; Saxon and Bertozzi, 2001). The potency of glycoengineering as a strategy for increasing the *in vivo* activity and duration of action of therapeutic proteins has recently been shown by Elliott *et al*. (2003), using glycosylated analogs of recombinant human erythropoietin or leptin. The presentation of glycoengineered motifs on the cell surface of bacteria is a newly unfolding area of research. An impressing example was stated by Paton *et al*. (2000), who demonstrated that a recombinant *E. coli* that displayed a Shiga toxin receptor mimic on its cell surface was capable of adsorbing and neutralizing Shiga toxins with very high efficiency. In general, the display of heterologous (glyco)proteins on the surface of bacteria, enabled by means of recombinant DNA technology, has become an increasingly used strategy in various applications in microbiology, nanobiotechnology, and vaccinology (Samuelson *et al*., 2002). Besides outer membrane proteins, lipoproteins, autotransporters, or subunits of surface appendages that are being evaluated for that kind of applications, the use of the S-layer (glyco)protein cell surface anchor is a very attractive and promising alternative. It offers the unique advantage of providing a crystalline, regular "immobilization matrix" that should eventually allow the controlled and periodic surface display of "intelligent" glycosylation motifs. Nanobiotechnology applications of such tailored S-layer *neo*glycoproteins may include the fields of receptor mimics, vaccine design, or drug delivery using carbohydrate recognition. The possibility to incorporate additional functional domains into the S-layer protein portion may allow additional tuning of structural and functional features of these S-layer *neo*glycoproteins.

As glycosylation engineering of S-layer (glyco)proteins represents a rather new area of research, the benefits of S-layer *neo*glycoproteins for potential nanobiotechnology applications may currently be only deduced form the successful cell surface display of foreign peptide epitopes. For instance, a tetanus toxin fragment C that is cell surface–exposed via the S-layer protein system and is produced by recombinant *Bacillus anthracis* protects against tetanus toxin (Mesnage *et al*., 1999). Also the S-layers of various lactic acid bacteria have been successfully exploited for the surface display of different bioactive epitopes (Åvall-Jääskeläinen *et al*., 2003; Smit *et al*., 2001, 2002). Even though not yet cell surface-displayed *in vivo*, the high potential of chimeric S-layer proteins for nanobiotechnology applications could be unambiguously demonstrated by several other recent studies. For instance, the fusion of the major birch pollen allergen Bet v 1 to the C-terminal domain of the S-layer protein SbsC of *B. stearothermophilus* ATCC 12980 (Breitwieser *et al*., 2002) and *Bacillus sphaericus* CCM 2177, respectively (Ilk *et al*., 2002) yielded stable hybrid proteins that retained the ability to self-assemble into monomolecular lattices while presenting the allergen in its functional form. Other S-layer hybrid proteins contain the variable domain of a camel heavy chain antibody (Pleschberger *et al*., 2003). Due to the selected fusion sites, the functional epitope remains located on the outer S-layer surface, which ensures its accessibility for binding of target molecules in newly developed diagnostic devices. Other potential nanobiotechnology applications may utilize a chimeric streptavidin S-layer as a self-assembling nanopatterned molecular affinity matrix to arrange biotinylated compounds on a surface (Moll *et al*., 2002).

Consequently, the S-layer (glyco)protein-inherent biomolecular self-assembly properties, in combination with

tailored glycosylation, is recognized as a powerful tool for future nanoscale engineering.

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Abbreviations

LPS, lipopolysaccharide; SDS-PA, sodium dodecyl sulfate polyacrylamide; S-layer, cell surface layer.

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