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BACTERIAL CELL-ENVELOPE GLYCOCONJUGATES

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I. Introduction

1. Outline

Nature has equipped prokaryotes (namely, bacteria and archaea) from almost all phylogenetic branches with a considerable repertoire of components from its “glycodiversification toolbox.” Especially, bacterial-cell envelopes present an amazing repertoire of glycoconjugates, including (i) surface-located compounds, such as lipopolysaccharides, capsular polysaccharides, lipooligosaccharides, and glycoproteins; (ii) cell-wall-associated compounds categorized as “classical” and “nonclassical” secondary cell-wall polymers (SCWP); as well as (iii) secreted exopolysaccharides.

This article focuses on a special class of bacterial cell-surface glycoproteins, namely, S (surface)-layer glycoproteins and their inherent SCWP cell-envelope anchor. S-Layer glycoproteins have the unique feature of self-assembling into 2-D crystalline lattices, providing a display matrix for glycans having periodicity at the nanometer scale. This feature, in combination with the high degree of glycan variability which exceeds by far that of their eukaryotic counterparts, makes them attractive targets for glycoengineering aimed at (nano)biotechnology and biomedical applications.

2. Background—Bacterial Protein Glycosylation

Glycosylation of proteins is a modification ubiquitous in the natural world; it involves the co- or postsecretional addition of sugar residues to the respective polypeptides and serves to expand the diversity of the proteome.^{1–3} A glycoprotein is a glycoconjugate in which a protein carries one or more glycans covalently attached to the polypeptide backbone, usually via an N- or O-glycosylic linkage. Many different carbohydrate components, together with a variety of covalent glycan–protein linkages, have been identified in glycoproteins originating from organisms of all three domains of life, namely, the Eukaryotes,⁴ the *Bacteria*,^{5–13} and the *Archaea*.^{14–17} S-Layer glycoproteins are the focus of this article and are described in detail herein. A detailed list of other prokaryotic glycoproteins, including glycosylated enzymes, toxins, plasma and outer membrane proteins, antigens, aggregation factors, and other cellular glycoproteins, has been given by Messner and Schäffer.¹⁸

To understand fully the potential of bacterial protein glycosylation, it is reasonable to recapitulate details about the eukaryotic glycoproteins, which have been well investigated. Eukaryotic glycoproteins have been implicated in a multitude of cellular processes, including immune response, intracellular targeting, intercellular recognition, and protein folding and stability.^{4,19–22} The common classes of glycans found in or on eukaryotic cells are primarily defined according to the nature of the linkage to the aglycone (protein or lipid). In N-linked glycans, a sugar chain is linked covalently to an asparagine residue of a polypeptide chain, commonly involving a GlcNAc residue and the consensus peptide sequence Asn-X-Ser/Thr.³ Eukaryotic N-glycans share a common pentasaccharide-core region and can be generally divided into three main classes: oligomannosidic (or high-mannose) type, complex type, and hybrid type. In O-linked glycans, an *N*-acetylgalactosamine (GalNAc) residue is usually linked to the hydroxyl group of a serine or threonine residue, and it can be extended into a variety of different structural-core classes. In addition, several other types of O-linkages have been identified in a variety of eukaryotic glycoproteins (such as mannose–serine/threonine, xylose–serine, glucose–tyrosine, galactose–hydroxylysine, and L-arabinose–hydroxyproline).^{4,21}

For a long time, reports on prokaryotic glycoproteins were restricted to a few archaeal and bacterial examples,^{23,24} and thus, initially, the presence of glycoproteins in prokaryotes was considered a controversial matter. With the investigations of halobacterial S-layer glycoproteins conducted during the late 1970s and 1980s, the occurrence of glycosylated proteins in prokaryotes became generally accepted.^{15,25,26} These first prokaryotic glycoproteins were shown to have similar types of glycosidic linkages as those found in eukaryotes. However, as the S-layer glycoproteome of more bacterial species was unraveled, it became evident that this class of bacterial cell-surface glycoconjugates displays a high structural variability which exceeds that of eukaryotic glycoproteins.¹⁸ Recent work on bacterial and archaeal S-layer glycoproteins is discussed in greater detail in Section II.

Even though S-layer glycoproteins are a major class of prokaryotic glycoproteins, glycosylation of prokaryotic proteins is not limited to the S-layers. About 15 years ago, Sandercock and colleagues coined the term “non-S-layer glycoproteins” to subsume all other representatives of this heterogeneous group of bacterial glycoproteins.²⁷ One of the very first reports in this context was the partial description of an enterococcal (formerly streptococcal) *N*-acetylmuramoylhydrolase²⁸ and also the characterization of the TibA glycoprotein from an enterotoxigenic (ETEC) *Escherichia coli* that acts as an adhesin and plays an important role in the virulence of this organism.²⁹ Other important members of “non-S-layer glycoproteins” are such cell-surface appendages as flagella and pili.³⁰ Among the first fully described species were the flagella of the archaeon *Halobacterium salinarum* (formerly *Hbt. halobium*)³¹ and the flagellin of the nitrogen-fixing bacterium *Azospirillum brasilense*.³²

During the past decade, high-resolution mass-spectrometric investigations revealed the existence of uncommon sialic acid-like sugars (5,7-diacetamido-3,5,7,9-tetra-deoxy-nonulosonate derivatives), such as pseudaminic acid or legionaminic acid, as major components of flagella^{33–37} and pili³⁸ of different pathogenic bacteria. These can also incorporate the previously mentioned sialic acid-like sugars into other virulence-associated

cell-surface glycoconjugates such as lipopolysaccharides (LPS)^{39,40} and capsular polysaccharides.⁴¹ The sialic acid-like sugars are unique to microorganisms and may exhibit configurational differences as compared to sialic acid. Legionaminic acid (5,7-diacetamido-3,5,7,9-tetradeoxy- β -glycero- β -galacto-nonulosonic acid), for instance, has the same absolute configuration as sialic acid. In this context, the properties of the sialic acid-like glycoconjugates mimicking host structures are of utmost importance for the host immune defense.

The seminal observation of the late 1990s that *Campylobacter jejuni* is able to synthesize a large number of periplasmic glycoproteins strongly supported the already proposed concept concerning the existence of prokaryotic glycoproteins.⁴² *C. jejuni* is a pathogen of the human gut mucosa that is one of the main causes of bacterial gastroenteritis worldwide. A striking finding was the identification of two glycosylation loci, which encode, respectively, both the O-linked and N-linked protein glycosylation pathways. The O-linked flagellin glycosylation system comprises a glycosylation cluster of approximately 50 genes, including genes encoding the flagellin structural proteins FlaA and FlaB. Although multiple orthologues of the genes for sialic acid biosynthesis were identified, recent structural analysis of the flagellins from *C. jejuni* and the related species *Campylobacter coli* has shown that flagellins are not modified by sialic acid, but by several monosaccharide analogues of the related sugar, pseudaminic acid.⁴³ The *Campylobacter* flagella are extensively modified, with approximately 10% of the total FlaA glycoprotein mass consisting of O-linked glycans, and each filament containing approximately 20,000 protein subunits.⁴⁴

The glycosylation system for the N-linked protein was originally thought to be involved in the biosynthesis of lipopolysaccharide (LPS), but later it was shown to be involved in the glycosylation of multiple *C. jejuni* proteins.⁴² Structural investigations revealed that the N-linked glycan consists of the heptasaccharide α -GalNAc-(1 $\rightarrow$ 4)- α -GalNAc-(1 $\rightarrow$ 4)- β -Glc-(1 $\rightarrow$ 3)- α -GalNAc-(1 $\rightarrow$ 4)- α -GalNAc-(1 $\rightarrow$ 4)- α -GalNAc-(1 $\rightarrow$ 3)- β -Bac-(1 $\rightarrow$ N)-Asn, where Bac is bacillosamine (2,4-diacetamido-2,4,6-trideoxy- β -glucose).⁴⁵ The key enzyme for protein glycosylation is the N-oligosaccharyltransferase (OTase) PglB, which has significant amino acid similarity to the eukaryotic oligosaccharyltransferase complex STT3.⁴⁶ Glycosylation in *C. jejuni* involves the transfer of the heptasaccharide from an undecaprenyl pyrophosphate donor to the asparagine side chain of proteins at the bacterial periplasmic membrane to form N-linked glycoproteins.⁹ The *in vitro* biosynthesis machinery responsible for this elaborate protein modification follows a similar overall progression, whereby an oligosaccharide is assembled in a stepwise fashion on a polyisoprenyl pyrophosphate carrier and is then ultimately transferred to a protein.²² A major scientific and biotechnological breakthrough could be achieved by the transfer of the complete *C. jejuni* N-glycosylation machinery into *E. coli*.⁴⁷ This allowed for the first time the recombinant production of glycoproteins in prokaryotes. In a series of excellent papers, the optimization of both the glycosylation process and the production conditions for biosynthesis of “humanized glycoproteins” in bacteria has been worked out.^{48–53}

Similar considerations were also applied for the characterization of other prokaryotic glycoproteins, such as type IV pili.⁵⁴ The constituting pilins are O-glycosylated in such

pathogenic bacteria as *Neisseria meningitidis* and *N. gonorrhoeae*, where they are decorated with short glycans, up to three sugars in length. The final demonstration that PglL is the O-OTase in *N. meningitidis*, responsible for the transfer of the glycan to pilin, was provided by Faridmoayer and colleagues, who showed the PglL-dependent glycosylation of pilin by reconstituting the process in *E. coli*.⁵⁵ In *N. gonorrhoeae*, the glycosylation pathway to O-linked protein is responsible for the synthesis of the trisaccharide on undecaprenyl diphosphate and subsequent *en bloc* transfer of the glycan by PglO, the respective O-OTase, to serine residues of select periplasmic proteins. The linkage sugar nucleotide UDP-2,4-diacetamido-2,4,6-trideoxy- α -D-glucose, which is the first sugar in glycan biosynthesis, was produced enzymatically, and the stereochemistry as uridine diphosphate N-diacetylglucosamine (UDP-Bac-2,4-diNAc) was assigned by NMR.⁵⁶

The biological role of many prokaryotic glycoproteins requires further exploration. During the past decade, it became evident that glycosylation plays a vital role in pathogenicity and host invasion. In addition, roles in adhesion, protection against proteolytic cleavage, protein assembly, solubility, antigenic variation, and protective immunity have been suggested.^{8,57–62}

a. General Architecture of Bacterial Cell Envelopes—In the course of evolution, prokaryotic organisms have developed a considerable diversity in their supramolecular architectures. The different cell-wall structures, particularly those facing the surrounding environment, obviously reflect evolutionary adaptations of the organisms to a broad spectrum of selection criteria in the natural habitat.⁶³ Among them are S-layers which, although not a universal feature, have been identified as common features of many organisms belonging to the prokaryotic domains *Bacteria* (Section II.1) and *Archaea* (Section II.2). These monomolecular arrays of (glyco) proteinaceous subunits present as the outermost component of the cell envelope were detected in a large number of different species of nearly every phylogenetic group of bacteria^{64–74} and represent an almost universal feature of archaeal cell envelopes.^{17,75–77} Despite the fact that considerable variations exist in the structure and chemistry of prokaryotic envelopes (Fig. 1), S-layers have apparently coevolved with these diverse structures. In Gram-positive bacteria and Gram-positive archaea (Fig. 1, left panel), the regular arrays assemble on the surface of the rigid wall matrix, which is mainly composed of peptidoglycan or pseudomurein, respectively.⁶³ In Gram-negative bacteria (Fig. 1, middle panel), the S-layer is presumably attached to the LPS of the outer membrane, and in most archaea (Fig. 1, right panel), S-layers are attached or inserted to the plasma membrane (for review, see Ref. 74).

The location and ultrastructure of S-layers of a great variety of organisms have been investigated by electron microscopy (EM) (for reviews, see Refs. 67,78–80) and atomic force microscopy (AFM) (for reviews, see Refs. 81–84). On the prokaryotic cell surface, the S-layer subunits can be aligned in lattices with oblique (p1, p2), square (p4), or hexagonal (p3, p6) symmetry. While hexagonal symmetry is predominant among archaea, in bacterial S-layers, any type of lattice has been observed.^{69,74}

It is an intrinsic property to rapidly self-assemble into two-dimensional (2-D), regularly ordered protein crystals, thus preventing the formation of 3-D crystals suitable for X-ray

diffraction analysis.^{85,86} Although S-layer proteins are usually insoluble in aqueous systems, we observed that this was not the case with the glycosylated S-layer protein of *Aneurinibacillus thermoaerophilus* (*Ane. thermoaerophilus*, formerly *Bacillus thermoaerophilus*).⁸⁷ This S-layer glycoprotein was completely water-soluble and was thus potentially suited for 3-D crystallization experiments. Later on, however, the S-layer protein SbsC from *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) ATCC 12980^T became the first one for which different N- or C-terminally truncated S-layer protein forms were recombinantly produced⁸⁸ and systematically surveyed for their self-assembling and recrystallization properties by a dissection approach.⁸⁹ Native and heavy-atom derivative data confirmed the results of the secondary-structure prediction, which indicated that the N-terminal region comprising the first 257 amino acids is mainly organized as α -helices, whereas the middle and C-terminal parts of SbsC consist of loops and β -sheets.^{90,91} The crystal-structure model of truncated rSbsC revealed a novel fold, consisting of six separate domains, which are connected by short flexible linkers. Instead of a dissection approach, Baranova et al. used a nanobody-aided crystallization system and provided the first 3-D crystal structure of the nontruncated S-layer protein SbsB from *Lysinibacillus sphaericus* (formerly *Bacillus sphaericus*) CCM 2177 with a resolution of 2.4 Å.⁹² SbsB consists of a seven-domain protein, formed by an amino-terminal cell-wall attachment domain and six consecutive immunoglobulin-like domains, that organize into a χ -shaped disk-like monomeric crystallization unit stabilized by interdomain Ca^{2+} ion coordination.

Independent of the 3-D crystallization efforts, secondary-structure predictions have been made for different S-layer proteins (as from *Aeromonas salmonicida*, *Campylobacter fetus*, and *G. stearothermophilus* PV72/p2; for review, see Ref. 93) by comparison of circular dichroism (CD) spectra under native and denaturing conditions. Furthermore, equilibrium unfolding profiles induced thermally or by guanidinium hydrochloride, monitored by intrinsic fluorescence and CD spectroscopy, revealed that the N-terminal portion of truncated rSbsB from *G. stearothermophilus* PV72/p2 is an α -helical protein. The C-terminal form was characterized as a β -sheet protein with typical multidomain unfolding.⁸⁹ The first tertiary-structure prediction for SbsB, based on molecular dynamics simulations using the mean force method, has been published.⁹⁴ By using a combination of the structural information and a Monte Carlo method with a coarse-grained model, the functional protein self-assembly of SbsB from *G. stearothermophilus* PV72/p2 was studied.⁹⁵ It was found that only a few amino acids and mainly hydrophobic ones, located on the surface of the monomer, are responsible for the formation of a highly ordered, anisotropic protein lattice. The broad potential for application of S-layers in nanobiotechnology is based on the specific intrinsic features of the monomolecular arrays composed of identical protein or glycoprotein subunits. Many applications depend on the capability of isolated subunits to recrystallize into monomolecular arrays in suspension or on suitable surfaces (as on polymers, metals, or silicon wafers) or interfaces (such as lipid films, liposomes, or emulsomes).⁹⁶

b. Biological Functions—Only little is known about S-layer glycoprotein structure–function relationships. Most of the presumptive functions of S-layer glycoprotein glycans relate to the functions attributed to the 2-D crystalline S-layer protein portions. Considering that S-layer-carrying organisms are ubiquitous in the biosphere, the supramolecular concept

of a closed, isoporous, protein meshwork has the potential to fulfill a broad spectrum of functions. When bacteria are no longer exposed to selection pressures in the natural environment, S-layers can be lost, indicating that the considerable biosynthetic effort is only required in natural habitats. In functional terms, (glycosylated) S-layers are generally part of complex envelope structures (Fig. 1) and consequently should not be considered as isolated layers. S-Layer functions are generally enabled through the prominent location at the cell surface and include, for instance, isoporous and/or protective coating of the cell, surface recognition and cell adhesion to substrates, receptor–substrate interactions, templated fine-grain mineralization, as well as mediation of pathogenicity-related phenomena.^{74,97–100} Many of these assigned functions are still hypothetical and are not based on firm experimental data.

Concerning the latter function, S-layer variation might have evolved as an important strategy of pathogens—but also of nonpathogens—to respond to changing environmental conditions.^{101–105} With regard to an interplay of glycosylation and pathogenicity, specific data have recently been accumulated for the periodontal pathogen *Tannerella forsythia*. For this bacterium, S-layer glycosylation is an important aspect affecting its cellular integrity, lifestyle, and virulence potential.¹⁰⁰ The bacterium possesses a unique Gram-negative cell envelope with a glycosylated S-layer as outermost decoration that is formed by coassembly of the two S-layer glycoproteins TfsA and TfsB.⁸⁴ The O-glycosidically linked *T. forsythia* S-layer deca-saccharide (see Section II for details) is overall a highly diverse structure containing several rare sugar residues.¹⁰⁰ This S-layer glycan directly impacts the lifestyle of *T. forsythia* because increased biofilm formation of a UDP-*N*-acetylmannosaminuronic acid dehydrogenase mutant could be correlated with the presence of truncated S-layer glycans in which the “acidic” branch of the deca-saccharide is missing.¹⁰⁶ It is also tempting to speculate that the terminal 5-acetimido-7-glycolyl pseudaminic acid (Pse5Am7Gc) residue of the S-layer glycans participates in the bacterium–host cross talk, although the relevance of the modification of the pseudaminic acid remains yet unclear. This notion is supported by the fact that members of this class of sialic acid-like sugars have been found in many Gram-negative bacterial species as constituents of important cell-surface glycoconjugates, such as LPS,⁴⁰ capsules,⁴¹ pili,³⁸ and flagella,^{33,36} all of which are important mediators of pathogenicity and possibly influence bacterial adhesion, invasion, and immune evasion.¹⁰⁷

While the S-layer has been shown to be a virulence factor and to delay the bacterium’s recognition by the innate immune system of the host,^{84,108} the contribution of glycosylation to modulating the host immunity is currently unraveling. It was already shown that surface glycosylation of *Tannerella* has a role in restraining the Th17-mediated neutrophil infiltration in the gingival tissues.¹⁰⁹

II. Surface-Layer Glycoproteins

During the past 35 years, considerable progress has been made in research on prokaryotic glycoproteins, providing a wealth of data on structures, biological properties, and functions of the different cellular and external prokaryotic glycoproteins (compare with Section I). The first data on prokaryotic S-layer glycoproteins date back to the mid-1970s, when reports

on halobacteria²³ and on thermophilic clostridia²⁴ were published. Since then a considerable body of knowledge has accumulated about bacterial (for recent reviews, see Refs. 18,110,111) and archaeal S-layer glycoproteins (for reviews, see Refs. 16,17,72,112–114), indicating that glycosylation is the major modification of S-layer proteins; however, prokaryotic S-layer proteins are not necessarily glycosylated. Glycosylation generally contributes to an enormous diversification potential of the prokaryotic cell surface, which may be advantageous for the survival of the organisms in their natural, competitive habitats.

1. Bacterial S-Layer Glycoproteins

A major class among prokaryotic glycoconjugates are the bacterial S-layer glycoproteins from members of the *Bacillaceae* (for reviews, see Refs. 5,11,18,58,74,111, 115,116). Among the *Bacillaceae* species investigated are highly thermophilic *G. stearothermophilus* strains¹¹⁷ and thermophilic clostridia,²⁴ as well as the less-thermophilic *Ane. thermoaerophilus*¹¹⁸ and *Anoxybacillus tepidamans* (*Ano. tepidamans*, formerly *Geobacillus tepidamans*) strains^{119,119a}; all of these bacteria are isolates from extraction plants of Austrian beet-sugar factories. The mesophilic Gram-positive bacteria investigated include *Paenibacillus alvei* (formerly *Bacillus alvei*), a type culture organism originally isolated from honeybee hives, and different *Lactobacillus buchneri* strains, including a dairy factory isolate¹²⁰ and a silage isolate.¹²¹

In these organisms, up to 20% of the total protein-synthesis effort may be devoted to the production of S-layer glycoproteins, and this indicates that S-layer protein synthesis is under the control of very strong promoters.¹²² The degree of glycosylation 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, according to SDS-PA gels. Most of these S-layer glycoproteins have the unique property of assembling into two-dimensional crystalline arrays on the supporting peptidoglycan layer, yielding a complete coverage of the bacterial cells during all stages of the bacterial growth cycle, with the glycan moieties protruding from the cell surface into the exterior environment (Fig. 2). This feature resembles the coating of Gram-negative bacterial cells by lipopolysaccharides.^{123,124} While in the past most of the structural investigations on S-layer glycoproteins have been performed on selected Gram-positive bacilli, clostridia and lactobacilli,^{18,111,116} investigations on Gram-negative organisms were only recently initiated. Glycosylated S-layer proteins have been unambiguously identified on the human oral pathogen *T. forsythia*,^{106,125} and comparable glycosylated proteins, though not yet explicitly identified as S-layer proteins, on the closely related human commensal *Bacteroides fragilis*.^{126,127} Thus far, only the structure of the *T. forsythia* S-layer oligosaccharide has been completely elucidated,¹⁰⁶ but preliminary analyses revealed that the *B. fragilis* glycan is structurally comparable.¹²⁷

a. Core Structures—Despite a highly diverse glycan composition, most S-layer glycoproteins of the Gram-positive bacteria thus far analyzed follow a general tripartite building plan.^{11,112,117,118} This plan comprises a short oligosaccharide, termed “adapter region,” linking the entire S-layer glycan to the S-layer polypeptide backbone. To date, in bacterial S-layer glycoproteins from Gram-positive as well as Gram-negative species, only

O-glycosidic linkages have been identified.^{111,128} This includes linkages to serine, threonine,⁴ and tyrosine residues.¹³⁰ Typically, S-layer proteins are multiply glycosylated. It is noteworthy that in all structures investigated the linkage sugar (namely, Gal, GalNAc, or Glc) is in the β -anomeric configuration.

(i) Core Oligosaccharides of Elongated S-Layer Glycans: In the long-chain S-layer glycans of *Bacillaceae*, some variation in the composition of the adapter region has been observed. The most common structure, which is present in *G. stearothermophilus* NRS 2004/3a,¹³¹ *Ano. tepidamans* GS5-97,¹³² *Ane. thermoaerophilus* (DSM 10155),¹³³ *P. alvei* (CCM 2051^T),^{134,135} *Thermoanaerobacter thermohydrosulfuricus* L111-69, and L110-69 (DSM 568) (formerly *Clostridium thermohydrosulfuricum*),¹³⁶ comprises one to three α -(1 $\rightarrow$ 3)-linked L-rhamnopyranose residues α -(1 $\rightarrow$ 3)-linked to the linkage sugars β -D-Galp or β -D-GalpNAc, respectively (Fig. 3).

In the *P. alvei* CCM 2051 S-layer glycan core, the distal L-Rhap residue is additionally decorated with a β -(1 $\rightarrow$ 4)-linked GroA-(2 $\rightarrow$ O)-P(=O)OH-(O $\rightarrow$ 4)- β -D-ManpNAc side chain (Fig. 4).¹³⁵

A slightly different adapter region oligosaccharide has been found in the S-layer glycan of *Ane. thermoaerophilus* L420-91^T (DSM 10154), where instead of α -(1 $\rightarrow$ 3)-linked L-Rhap residues, the adapter glycan is made of one to three α -(1 $\rightarrow$ 3)-linked D-Rhap residues that are α -(1 $\rightarrow$ 3)-linked to a β -D-GalpNAc residue (Fig. 5).¹³⁷

If the oligosaccharide linker is missing, the carbohydrate chain can be bound directly to the glycosylated amino acid via the first repeating unit, as in the S-layer glycan of *Thermoanaerobacterium thermosaccharolyticum* D120-70 (formerly *Clostridium thermosaccharolyticum*).^{138,139} The anomeric configuration of this linkage sugar may remain unchanged (no core) or be inverted (pseudo-core).¹¹⁵ In *Thb. thermohydrosulfuricus* S102-70, a short glycan consisting only of a tyrosine-linked hexasaccharide with β -D-Glcp as the linkage sugar has been observed.¹⁴⁰

(ii) S-Layer Glycans Without Core Oligosaccharides: A divergence from the tripartite building plan of the long-chain S-layer glycans from the *Bacillaceae* previously described was observed for the S-layer glycoproteins of Gram-positive *Lb. buchneri* strains. In *Lb. buchneri* 41021/251 and CD034, the S-layer glycans are short D-glucO homooligomers that are O-glycosidically linked to distinct serine residues within the specific amino acid sequence SSASSASSA (glycosylation sites are underlined) for glycosylation of the respective S-layer proteins (Fig. 6).^{120,141}

Interestingly, the same O-glycosylation motif has also been found on other lactobacillar proteins of the strains investigated, which led to the first proposal of a general protein O-glycosylation system in lactobacilli.¹⁴¹

Also for the *Bacteroidales*, to which the Gram-negative species *B. fragilis* and *T. forsythia* are affiliated, the glycoprotein glycans so far known are short oligosaccharides without repeats.^{106,127} For these glycans, a phylum-wide protein O-glycosylation system targeting the amino acid motif D-(S/T)-(A/I/L/V/M/T) has been proposed.^{106,127}

b. S-Layer Glycans—At present, about 40 different S-layer glycoprotein glycan structures have been fully or at least partially elucidated. The structures observed and glycosidic linkage types exceed by far the display found in eukaryotes and broaden the spectrum of components found in bacterial polysaccharides, as previously reviewed in this series.¹⁴² Most of the S-layer glycan chains of Gram-positive bacteria so far known are linear or branched homo- or heterosaccharides, which comprise 20–50 identical repeating units. In selected lactic acid bacteria (such as *Lb. buchneri* strains) as well as in the Gram-negative bacterium *T. forsythia* currently analyzed, S-layer glycans are short oligosaccharides without repeats comparable to archaeal glycans. The monosaccharide constituents of the S-layer glycan chains include a wide range of neutral hexoses, 6-deoxyhexoses, amino sugars, and uronic acids.⁷ This spectrum of carbohydrates is further extended by rare sugars, including α -D-Rhap, α -D-Fucp, α -D-FucpNAc, β -D-QuipNAc, D-glycero- β -D-manno-Hepp, β -D-ManpNAcA, or Pse5Am7Gc. Some of them are otherwise typical constituents of LPS O-antigens of Gram-negative bacteria.¹²³ O-Glycosidic linkage regions observed on S-layer glycoproteins involve tyrosine, serine, and threonine residues (for reviews, see Refs. 74,116). As is known from eukaryotic glycoproteins,⁴ there is no specific glycosylation sequence present for O-glycosylation of proteins.¹⁴³

Despite being highly diverse on first analyses, most S-layer glycoproteins from the Gram-positive bacteria analyzed possess the tripartite structure, comprising the elongated glycan chain that is made of a distinct number of repeats, an adapter region, and the glycosidic linkage to the S-layer proteins (see Section II.1.a).

In the majority of glycans, L-rhamnose is the prevailing carbohydrate residue, as in the L-rhamnan found in the S-layer glycan of *G. stearotheophilus* NRS 2004/3a (Fig. 7).¹³¹

L-Rhamnose linked to mannopyranosyl residues has also been detected frequently as a component of linear and branched repeating units in glycans of *Thb. thermohydrosulfuricus* strains L111-69, L110-69 (DSM 568)¹³⁶ and S120-70 (Fig. 8) as well as *Thm. thermosaccharolyticum* D120-70 (Fig. 9),¹³⁹ respectively.

The backbone of the *Thm. thermosaccharolyticum* D120-70 glycan comprises a $\rightarrow 4$)- α -L-Rhap-(1 $\rightarrow$ 3)- β -D-Manp-(1 $\rightarrow$ 4)- β -L-Rhap-(1 $\rightarrow$ 3)- α -D-Glcp-(1 $\rightarrow$ tetrasaccharide with branching α -D-Galp-(1 $\rightarrow$ 2)- and β -D-Glcp-(1 $\rightarrow$ 6)-side-chain residues.¹³⁹

The disaccharide L-rhamnose α -(1 $\rightarrow$ 2)-linked to D-fucose constitutes the repeating unit in the glycan of *Ano. tepidamans* GS5-97^T (Fig. 10),¹³² whereas *Ane. thermoaerophilus* DSM 10155 harbors L-rhamnose α -(1 $\rightarrow$ 3)-linked to D-glycero- β -D-manno-heptose—otherwise a common constituent of bacterial LPS in Gram-negative bacteria (Fig. 11).¹³³

The glycan of *Ane. thermoaerophilus* strains L420-91^T and GS4-97, respectively, contains a branched hexasaccharide repeating unit of α -(1 $\rightarrow$ 2)-linked 3-acetamido-3-deoxy-D-fucose residues connected to a D-rhamnosyl tetrasaccharide backbone (Fig. 12).^{137,144}

N-Acetyl- β -D-mannosamine, N-acetyl-D-glucosamine, and N-acetyl-D-galactosamine residues are present in S-layer glycans from *P. alvei* CCM 2051¹³⁴ (Fig. 13) and *Thb. thermohydrosulfuricus* strains L77-66 (DSM 569) and L92-71, respectively (Fig. 14).¹⁴⁵

A complex branched hexasaccharide repeating-unit containing the trisaccharide backbone $\rightarrow 4)\text{-}\beta\text{-D-Galp-(1}\rightarrow 4)\text{-}\beta\text{-D-Glcp-(1}\rightarrow 4)\text{-}\beta\text{-D-Manp-(1}\rightarrow$ has been identified in *Thm. thermosaccharolyticum* E207-71 (Fig. 15).¹⁴⁶ The repeating unit is extended at position 3 of the mannose residues by a branching trisaccharide side-chain $\beta\text{-D-Quip3NAc-(1}\rightarrow 6)\text{-}\beta\text{-D-Galf-(1}\rightarrow 4)\text{-}\alpha\text{-D-Rhap}$.

The overall structure of these glycans, in combination with the broad spectrum of constituent sugars, lets us interpret the highly variable S-layer glycoproteins as the Gram-positive equivalents of lipopolysaccharides, with the protein component replacing lipid A of Gram-negative bacteria.¹²³

As already mentioned, an exception among the *Bacillaceae* is the S-layer glycan of *Thb. thermohydrosulfuricus* S102-70, which consists only of a hexasaccharide $\beta\text{-D-Galf-(1}\rightarrow 3)\text{-}\alpha\text{-D-Galp-(1}\rightarrow 2)\text{-}\alpha\text{-L-Rhap-(1}\rightarrow 3)\text{-}\alpha\text{-D-Manp-(1}\rightarrow 3)\text{-}\alpha\text{-L-Rhap-(1}\rightarrow 3)\text{-}\beta\text{-D-Glc}$ linked to specific tyrosine residues of the S-layer proteins. The nonreducing end of the chain possesses a (less common) $\beta\text{-D-galactofuranose}$ residue as the terminating sugar (Fig. 16).¹⁴⁰ The reason for this truncation is not known.

The other short-chain S-layer glycans from Gram-positive strains are those of *Lb. buchneri* strains.^{120,141} In contrast to the previously mentioned S-layer glycans in the other bacilli, the $\alpha\text{-D-glucose}$ residue linking the S-layer glycan to the S-layer protein is in the α configuration (Figure 6). This observation might reflect a different biosynthetic pathway (see Section V).

For *T. forsythia*, S-layer glycosylation is an important factor in defining the bacterium's lifestyle. This S-layer glycan constitutes a complex and so far unique oligosaccharide, having the structure $4\text{-O-Me-}\beta\text{-ManpNAc6CONH}_2\text{-(1}\rightarrow 3)\text{-[Pse5Am7Gc-(2}\rightarrow 4)]\text{-}\beta\text{-ManpNAcA-(1}\rightarrow 4)\text{-[4-O-Me-}\alpha\text{-Galp-(1}\rightarrow 2)]\text{-}\alpha\text{-Fucp-(1}\rightarrow 4)\text{-[}\alpha\text{-Xylp-(1}\rightarrow 3)]\text{-}\beta\text{-GlcpA-(1}\rightarrow 3)\text{-[}\beta\text{-Digp-(1}\rightarrow 2)]\text{-}\alpha\text{-Galp}$, which is O-glycosidically linked to multiple serine and threonine residues within the *Bacteroidales* protein glycosylation motif of the two S-layer proteins constituting the S-layer (Fig. 17).¹⁰⁶ As with the anomeric configuration of the linkage sugar in S-layer glycans of lactobacilli, the linkage sugar galactose of the *T. forsythia* glycoproteins is also in the α configuration.

Mass-spectrometric analysis of native *B. fragilis* glycoproteins after subjection to β -elimination revealed the glycan to be an oligosaccharide consisting of nine sugar residues (Fig. 18).¹²⁷ Notably, the proposed structure resembles that of the *T. forsythia* O-glycan in several aspects. In both glycans, attachment to the protein occurs via a hexose residue that is succeeded by a hexuronic acid. Additionally, a nonpolar constituent branches from the first sugar of the *B. fragilis* O-glycan. Also, both glycans contain a deoxyhexose residue in their linear structure. As in the *T. forsythia* glycan, the deoxyhexose of the *B. fragilis* glycan most likely is a fucose residue, as glycoproteins of *B. fragilis* are readily detected with *Aleuria aurantia* lectin and depend on genes for GDP-fucose biosynthesis.¹²⁶

c. Terminal Groups and Noncarbohydrate Substituents—Frequently, the terminal sugar residue at the nonreducing end of long-chain S-layer glycans is capped with noncarbohydrate substituents (see section II.1.b.). Capping with 2-O-methyl groups is

observed on the S-layer glycans of *G. stearothermophilus* NRS 2004/3a¹³¹ (Fig. 7) or *Thb. thermohydrosulfuricus* L111-69¹³⁶ (Fig. 8), while 3-*O*-methylation has been found in the distal units of the *Ane. thermoaerophilus* L420-91^T (DSM 10154) and GS4-97¹³⁷ glycans (Fig. 12), respectively. On the S-layer glycan of *Ano. tepidamans* GS5-97, an unusual capping structure was observed, namely, an α -(1 $\rightarrow$ 3)-linked *N*-acetylmuramic acid and a β -(1 $\rightarrow$ 2)-linked GlcNAc residue (Fig. 19).¹³²

By analogy to pathways for LPS biosynthesis, these capping structures are assumed to be involved in chain-length determination of the polysaccharides during biosynthesis; however, no conclusive data are yet available.¹¹¹ Since not all S-layer glycans are terminated by such structures, alternative mechanisms may exist. In the case of LPS, the O-PS chain length is strictly regulated, since it plays a pivotal role for the protection of the bacterium from defense mechanisms in the host. In the biosynthesis pathway for the Wzy-dependent O-antigen, the integral inner-membrane protein Wzz determines the O-antigen chain length.¹⁴⁷

2. Archaeal S-Layer Glycoproteins

During the past few years, only a few new structures of archaeal S-layer glycans have been reported, and the initial work on S-layer glycosylation of haloarchaea and methanogens is summarized in several excellent reviews (see Refs. 16,17,113,148,149,149a). There has been, however, considerable progress made in the functional characterization of the carbohydrate-active proteins involved in archaeal S-layer-protein glycosylation. In an impressive series of research papers, Eichler and coworkers reinvestigated the structure of the S-layer glycan of *Haloferax volcanii* and established the biosynthesis pathway for an S-layer pentasaccharide.^{150–152} They further discovered that lipid modification resulted in the formation of two distinct populations of S-layer glycoproteins.¹⁵³ These observations are consistent with the S-layer glycoprotein being synthesized initially as an integral membrane protein and subsequently undergoing a processing event in which the extracellular portion of the protein is separated from the membrane-spanning domain and transferred to a “waiting” lipid moiety. In a comparative approach, protein N-glycosylation in *Haloarcula marismortui*, a second haloarchaeon also originating from the Dead Sea, was investigated.¹⁵⁴ While both species decorate the respective S-layer glycoprotein with the same N-linked pentasaccharide and employ dolichyl phosphate as the lipid glycan carrier, species-specific differences in the two N-glycosylation pathways exist. *Har. marismortui* first assembles the complete pentasaccharide on dolichyl phosphate and only then transfers the glycan to the target protein. In contrast, *Hfx. volcanii* initially transfers the first four pentasaccharide subunits from a common dolichyl phosphate carrier to the target protein and subsequently delivers the final pentasaccharide from a distinct dolichyl phosphate to the N-linked tetrasaccharide.¹⁵⁴ To cope with life in changing hypersaline environments, *Hfx. volcanii* is able to modulate not only the N-linked glycan structures decorating the S-layer glycoprotein but also the sites of posttranslational modification.¹¹⁴ This high degree of inherent structural flexibility makes this organism also a potent candidate for glycoengineering.¹⁵⁴

In the course of structural investigations of glycosylated flagellins from methanogenic archaea, Jarrell and coworkers made the interesting observation that not only the flagellin

but also the glycosylated S-layer protein of *Methanococcus voltae* were decorated with the same complex N-linked glycan, where one of the constituting glycan components (β -ManNAcA) having a carbonyl group at C-6 forms an amide bond with the amino group of a threonine residue.^{155,156} In other strains, such as *Mco. maripaludis*, glycan structures of even greater complexity, containing also a novel terminal sugar residue (5S)-2-acetamido-2,4-dideoxy-5-*O*-methyl- α -L-erythro-hexos-5-ulo-1,5-pyranose, have been identified in flagellins and potentially in S-layer glycoproteins.^{112,157–159} This common usage of identical glycan structures on S-layers and flagella was first reported with the haloarchaeon *Hbt. salinarum*.³¹

Subsequently, it was shown that the oligosaccharide of the S-layer of the thermoacidophilic crenarchaeote *Sulfolobus acidocaldarius* consists of a tri-branched hexasaccharide that is *N*-glycosylically linked via a chitobiose core to several sites at the S-layer protein.¹⁶⁰ Furthermore, this glycan contains a 6-sulfoquinovose residue, which was first described in the cytochrome *b*_{558/566} of *Sl. acidocaldarius* (Fig. 20).¹⁶¹

A gene cluster involved in the biosynthesis of sulfoquinovose and the assembly of the S-layer N-glycans was identified.¹⁶² In archaea, *N*-glycosylation relies also on phosphorylated dolichol. The organism *Sl. acidocaldarius*, however, contains an unusually short, highly reduced dolichyl phosphate, which presents a degree of saturation thus far not reported in any other organism.¹⁶³

Regarding anchoring of the archaeal S-layer proteins to the cell envelope, in many strains the S-layer entirely penetrates the cytoplasmic membrane, allowing for a stable positioning of the S-layer protomers in the membrane.¹¹³ This principle is completely different from bacterial S-layer proteins, where carbohydrate interactions obviously play a dominant role in S-layer anchoring, either via secondary cell-wall polymers (SCWPs) as in Gram-positive bacteria^{165–167} or via rough lipopolysaccharide, as suggested for Gram-negative bacteria.¹⁶⁸

III. Nonclassical Secondary Cell-Envelope Polysaccharides

1. Background

As established about three decades ago, cell-wall polysaccharides of Gram-positive organisms can be classified on the basis of their structural characteristics into three distinct groups: (i) teichoic acids,¹⁶⁹ (ii) teichuronic acids,¹⁷⁰ and (iii) other neutral or acidic polysaccharides that cannot be assigned to the two former groups.^{171,172} Since all these compounds play a secondary role in cell-wall function, they have been termed “secondary” cell-wall polymers (SCWPs). Biochemical and genetic data accumulated over the past 30 years indicate that the first two groups of these polysaccharides (classical SCWPs) play important roles in normal cell function.^{169,173}

In the course of later investigations of bacterial S-layer proteins (see Section II), novel aspects of a group of nonclassical SCWPs have emerged with regard to their structure and function. S-Layers are generally composed of identical (glyco)protein species, forming regular two-dimensional, lattices on the surfaces of bacterial cells. It can be assumed that the stable attachment of S-layer (glyco)proteins to the cell wall is important for the cell, and in

this context, nonclassical SCWPs have been identified as mediators for noncovalent attachment of S-layers to the underlying PG meshwork (Fig. 1).^{163,165,174,175} Herein we summarize the current knowledge on structural features, possible linkage types to the cell wall, and interactions of nonclassical SCWPs in S-layer-carrying organisms.

2. The Nonclassical Group of SCWPs

Besides the considerable body of knowledge that has accumulated concerning structural, biochemical, and immunological features of teichoic and teichuronic acids from cell walls of Gram-positive bacteria,^{169,170} it should be kept in mind that additional cell-wall polysaccharides may be present in these organisms. Our research has focused on glycosylated S-layer proteins (see Section II), which are regarded as ideal model systems for studying the glycosylation of prokaryotic proteins. Improved purification and separation methods, however, eventually led to the clear assignment of a distinct proportion of compounds (originally copurified) as a separate class of SCWPs, and this accounts for a substantial proportion (7–15% by weight) of the PG of the organisms investigated. The SCWP–PG complexes investigated comprise the intact glycan moieties, together usually with proportions of PG of variable size, arising because of random degradation during the preparation procedure (without any acid treatment to prevent undesired removal of acid-labile components).

a. Common and Variable Features of Nonclassical SCWPs from *Bacillaceae*—

Based on compositional and structural data, we suggest that classification of the nonclassical SCWPs from S-layer-carrying *Bacillaceae* into group (iii) as defined by Araki and Ito is the most appropriate.¹⁷¹ Recent investigations have shown that, in S-layer-carrying organisms, teichoic and teichuronic acids as typical representatives of classical SCWPs are not present. Instead, the (to some extent, structurally comparable) nonclassical SCWPs act as linkers for the random noncovalent attachment of the S-layers to carbon 6 of muramic acid residues of the PG (compare with Fig. 1, left panel). This information can now be summarized as follows:

First group: The structure of the glycan portion of the SCWP–PG complex of *P. alvei* CCM 2051^T was elucidated to be [(Pyr4,6)- β -D-ManpNAc-(1 $\rightarrow$ 4)- β -D-GlcpNAc-(1 $\rightarrow$ 3)]_{n-11}-(Pyr4,6)- β -D-ManpNAc-(1 $\rightarrow$ 4)- α -D-GlcpNAc-(1 $\rightarrow$).¹⁷⁶ Each repeating-unit disaccharide of this SCWP is substituted with 4,6-linked pyruvic acid residues, conferring the overall anionic character of the SCWP (Fig. 21).

Upon prolonged exposure of the polymer sample to acidic conditions, as occurs upon solvation in D₂O during NMR experiments, considerable loss of pyruvate residues can occur. The ManNAc–GlcNAc backbone disaccharide motif corresponds to that frequently observed in certain teichoic acids of other bacilli.¹⁷¹ However, in the nonclassical SCWPs, this motif is repeated several times, thus constituting the entire glycan moiety of those SCWPs. Pyruvic acid-containing SCWPs have also been reported for the S-layer-carrying organisms *L. sphaericus* CCM 2177¹⁷⁷ and *B. anthracis*.¹⁶⁴ So far, however, no information is available on either their full structures or their linkage to the PG layer.

In contrast to anionic polymers, neutral polysaccharides possessing the identical backbone motif are found in the PG of other *Bacillaceae*. The SCWPs of *Thm. thermosaccharolyticum* strains D120-70¹³⁸ and E207-71¹⁷⁸ display the commonly encountered $\rightarrow 3$)- β -D-ManpNAc-(1 $\rightarrow$ 4)- β -D-GlcpNAc-(1 $\rightarrow$ 4)- β -D-ManpNAc-(1 $\rightarrow$ 3)- β -D-GlcpNAc-(1 $\rightarrow$ motif.¹⁷⁸

Although previously it had been thought that in strain D120-70 this motif is extended by galactose residues,¹³⁸ later results show that alternating ManNAc residues are substituted by ribofuranose side chains (C. Schäffer, H. Kählig, R. Christian, and P. Messner, unpublished results), as has also been demonstrated for the SCWP of strain E207-71 (Fig. 22).¹⁷⁸

Second group: The first SCWP-PG complex of an S-layer-carrying organism for which the structure was completely elucidated was purified from *G. stearotheophilus* NRS 2004/3a.^{179,180} The anionic polymer, comprising on average six tetrasaccharide repeating units having the structure $\rightarrow 4$)- β -D-Manp-2,3-diNAcA-(1 $\rightarrow$ 6)- α -D-Glcp-(1 $\rightarrow$ 4)- β -D-Manp-2,3-diNAcA-(1 $\rightarrow$ 3)- α -D-GlcpNAc(1 $\rightarrow$, constitutes the SCWP of many *G. stearotheophilus* wild-type strains (Fig. 23). Preliminary analysis of that SCWP indicated a mass range of 4000–6000. About 20–25% of the muramyl residues are substituted by SCWP glycans.^{179,180} Interestingly, analyses of the SCWP of *Ano. tepidamans* GS5-97^T indicate that its backbone structure is reminiscent of that of *G. stearotheophilus* NRS 2004/3a, but with additional modifications of the carboxyl groups by amide formation at the Manp-2,3-diNAcA residues, turning the anionic character of the glycan into a neutral one. ³¹P NMR analysis has demonstrated that the SCWP of *Ano. tepidamans* GS5-97^T is linked to muramic acid by a common phosphodiester linkage.¹⁸¹

Third group: In this group, the linkage-region disaccharide (compare with the *first group*) of teichoic acids is much further extended. In addition to linear chains, branched repeating units containing neutral sugars and/or amino sugars have also been observed. The charge-neutral SCWP isolated from *Ane. thermoaerophilus* DSM 10155 represents a unique bacterial glycan structure containing repeats of the sequence $\rightarrow 3$)- α -D-GlcpNAc-(1 $\rightarrow$ 3)- β -D-ManpNAc-(1 $\rightarrow$ 4)- β -D-GalpNAc-(1 $\rightarrow$ (Fig. 24).⁸⁷ The interpretation of the observed NMR data supporting a single branched GalNAc unit, however, should be treated with caution, since the ¹³C NMR chemical shifts of the carbon atoms in the branched residue are very similar to those located in the linear chain.

A branched structure was found in the SCWP from *G. stearotheophilus* PV72/p2, which, however, had undergone treatment with HF for cleavage of the linkage to peptidoglycan. Hence information on the linkage region as well as the extent of pyruvate substitution and proof for *N*-deacetylated glycoses is compromised (Fig. 25).¹⁸²

The SCWP contains a pentasaccharide repeating unit of the sequence $\rightarrow 4$)-[β -D-GlcpNAc-(1 $\rightarrow$ 3)]- α -D-GlcpNAc-(1 $\rightarrow$ 4)- β -D-ManpNAc-(1 $\rightarrow$ 4)- β -D-GlcpN/NAc-(1 $\rightarrow$ 6)-[4,6-(*S*)-Pyr- α -D-ManpNAc-(1 $\rightarrow$ 4)]- α -D-GlcpNAc-(1 $\rightarrow$. Similar to the SCWPs of the *first group*, the (*S*)-configured pyruvate residues are present on the D-ManpNAc residues, albeit not within the linear backbone chain, but extending from the branching 4,6-disubstituted α -D-GlcpNAc residue.

Secondary cell-wall polymers of *Bacillus anthracis* and *Bacillus cereus* may also be classified into the third group. *B. anthracis*, the causative agent of anthrax disease, harbors

antigenic cell-surface carbohydrate determinants which may be utilized for diagnostic purposes and vaccine development, respectively. Nonclassical SCWPs of *B. anthracis* having approximate molecular masses in the range of 12–22 kDa mediate binding to the surface-layer homology (SLH) domain of the paracrystalline surface-array protein Sap and the extractable antigen 1, respectively.^{183,184} The SCWP of several *B. anthracis* strains was released from its covalent linkage to peptidoglycan by treatment with HF, and the resulting purified fractions were analyzed by high-field NMR spectroscopy and by mass spectrometry techniques.¹⁸⁵ The repeating unit of the SCWP comprises the backbone sequence $\rightarrow 6)-\alpha\text{-D-Glc}p\text{NAc}-(1 \rightarrow 4)-\beta\text{-D-Man}p\text{NAc}-(1 \rightarrow 4)-\beta\text{-D-Glc}p\text{NAc}-(1 \rightarrow$ with α -galactopyranosyl side-chain units linked to O-3 of the $\alpha\text{-D-Glc}p\text{NAc}$ and $\beta\text{-D-Glc}p\text{NAc}$ units, respectively, and a $\beta\text{-D-Galp}$ residue linked to O-4 of the $\alpha\text{-D-Glc}p\text{NAc}$ residue (Fig. 26). The trisaccharide backbone forms a consensus motif shared by many *B. anthracis* and *B. cereus* strains, respectively. Cross-reactive epitopes are present in these SCWPs as well as epitopes being specific for *B. anthracis* and also *B. cereus*, respectively.^{185,186}

The $\beta\text{-D-galactosyl}$ moiety attached to the nonterminal GlcNAc residue constitutes a unique binding epitope to endolysins from *B. anthracis* bacteriophages.^{187,188} Galactosylation, however, is completely absent in the SCWP of the avirulent strain *B. anthracis* CDC 648 (Ba684).¹⁸⁹ Additional modifications observed in that SCWP pertain to acetylation at O-3 and unsubstituted amino groups of the β - and α -configured glucosamine residues, respectively, at the terminal, nonrepeating sequence.

A structure similar to the *B. anthracis* SCWP has been reported to constitute the SCWP of pathogenic *B. cereus* strains G9242, 03BB87, and 03BB102, which features an additional substitution at O-3 of the β -ManNAc unit by an α -galactosyl residue in the former two strains (Fig. 27).¹⁹⁰

Unique structural features have been found for *B. cereus* strains ATCC 10987 and ATCC 14579.¹⁹¹ The trisaccharide backbone is composed of the repeating unit $\rightarrow 6)-\alpha\text{-D-GalpNAc}-(1 \rightarrow 4)-\beta\text{-D-Man}p\text{NAc}-(1 \rightarrow 4)-\beta\text{-D-Glc}p\text{NAc}-(1 \rightarrow$ substituted by a group located single $\beta\text{-D-Galp}$ residue at O-3 of the GalpNAc unit and an *O*-acetyl at O-3 of the central $\beta\text{-D-ManNAc}$ moiety (Fig. 28).

B. cereus strain ATCC 14579 produces two structurally different SCWPs, depending on environmental conditions.¹⁹² The structure of the first SCWP, which is constantly being expressed, is closely related to the canonical sequence motif comprising the sequence of $\beta\text{-D-Glc}p\text{NAc}-\alpha\text{-D-HexpNAc}-\beta\text{-D-Man}p\text{NAc}$ (as in *B. cereus* strain 10987), but it contains a highly branched $\alpha\text{-D-GalpNAc}$ residue (Fig. 29).

The second, completely unrelated, SCWP was identified as being present in a phase-specific manner, both in the planktonic phase and in late biofilm formation. The presence of both SCWPs, as well as the enrichment of the latter form in biofilm, was established by high-resolution (HR)-MAS NMR spectroscopy. The charged second SCWP could be separated from the neutral SCWP by anion-exchange chromatography as well as by mild acid hydrolysis. The SCWP contains a 4-amino-4,6-dideoxy- $\beta\text{-D-GalpNAc}$ residue extended by an amide-linked $\alpha\text{-2-(R)-hydroxyglutaric}$ acid residue (Fig. 30).

Pyruvate substitution has been detected in the HF-treated SCWPs from *B. anthracis* (Sterne 34F₂, 7702) and *B. cereus* strain G9241, 03BB87, 03BB102.¹⁹⁰ The extent of pyruvate substitution remains ambiguous, since the cleavage of the SCWP from peptidoglycan with 48% HF also leads to the liberation of the acid-sensitive ketal groups, thus preventing any quantification and even detection of pyruvate. For the *B. anthracis* SCWP CDC 684, the location of a terminal (*S*)-pyruvate substituent at O-4 and O-6 of the β -D-ManpNAc residue has been identified by NMR spectroscopy.¹⁸⁹

b. General Considerations About Nonclassical SCWP Structure—Comparison of the overall composition of all nonclassical SCWPs analyzed so far reveals that the more-complex glycans of the *second group* exhibit the same alternating order of *gluco* and *manno* sugars as glycans of the *first group*. The glycan chain starts with a GlcNAc residue at the reducing end and ends with Manp-2,3-diNAcA. It is possible that glycan structures of the *second group* have evolved from the simpler *first-group* structures by the introduction of such residues as glucose or Manp-2,3-diNAcA through the action of strain-specific enzymes. The same scenario could be imagined for glycans of the *third group*. However, biosynthesis pathways of greater complexity have to be envisaged and, so far, no experimental evidence is available to support this notion.

The linkages between SCWPs and PG are currently being investigated in more detail. Interestingly, for all SCWPs investigated, the glucose residue linked to the bridging phosphate residue is in the α configuration. Whether this has any impact on the biosynthesis of the SCWPs remains to be established.

c. Interactions of SCWPs and S-Layers from *Bacillaceae*—In addition to the general features previously mentioned, novel properties for SCWPs have emerged from research on S-layers.^{67,69} S-Layers are two-dimensional crystalline protein lattices on the outermost surface layer of bacteria from almost all phylogenetic branches.^{67,69} The S-layer proteins are of interest because of their involvement in important physiological processes, which include, in the case of pathogenic bacteria, the infection mechanism.¹⁹³ If present, S-layer (glyco)proteins are the most abundant cellular proteins. Provision of any kind of selection advantage by the S-layer to a bacterium in its natural environment requires the stable attachment of the S-layer to the cell surface *in vivo*. During evolution, Gram-positive bacteria have developed various strategies for displaying proteins on their surface. These strategies include predominantly covalent binding of LPXTG-carrying proteins to PG, but a number of noncovalent binding strategies have also been developed.¹⁹³ Reattachment experiments of isolated S-layer glycoproteins from thermophilic clostridia revealed the noncovalent character of the interaction between S-layer and PG.¹⁹⁴ For *Lb. buchneri*, it was shown very early that hydroxyl groups of a neutral cell-wall polysaccharide are responsible for the attachment of the S-layer protein to the cell wall.¹⁹⁵

d. Possible Cell-Wall-Targeting Mechanisms—Recently, the cell-wall-targeting mechanism of S-layer proteins has been investigated in more detail.^{164,165,167,174} These studies suggest that, in general, S-layer proteins have two functional regions: a cell-wall-targeting domain, which in most of the organisms thus far investigated is located at the N-terminus, and a C-terminal self-assembly domain. The existence of a cell-wall-targeting

domain in S-layer proteins was substantiated by Fujino *et al.*¹⁹⁶ and Lupas *et al.*,¹⁹⁷ who identified motifs of approximately 55 amino acids, containing 10–15 conserved residues, which were designated SLH (S-layer homology) domains. These SLH domains, usually composed of one to three modules, are the means frequently used for targeting proteins to the cell surface. They are found not only in various S-layer proteins but also in many other surface-associated proteins, such as the cellulosomes¹⁹⁸ or other surface-associated enzymes.¹⁹⁹ In the case of *B. anthracis*, the etiological agent of anthrax, the molecular basis of the interaction between SLH domains and SCWPs was established by elucidating their binding properties.¹⁶⁴ Both S-layer proteins of *B. anthracis* (EA1 and Sap) possess SLH domains that bind the S-layer proteins directly to PG.^{200,201} While the overall sequence similarity of SLH domains is rather low, the highly conserved four amino acid motif TRAE has been found to play a key role for the binding function to SCWP.^{167,202,203} The crystal structure recently elucidated of one S-layer protein from *B. anthracis* shows the SLH domains arranged in threefold pseudo-symmetry with the TRAE (or similar) motifs arranged so that they would be accessible to the SCWP.²⁰³

A different binding mechanism was proposed for the S-layer proteins of *G. stearothermophilus* PV72/p2^{204,205} and *P. alvei* CCM 2051^T.¹⁶⁷ In these organisms, two different binding domains were identified in the N-terminal region of the S-layer proteins, one for SCWP and another for PG. In *Thermus thermophilus*, a similar binding mechanism was identified between an S-layer–outer membrane complex and the cell wall. There is a strong interaction of the SLH domain of the S-layer protein with a pyruvated component of a highly immunogenic SCWP.¹⁷⁴

Originally, the involvement of pyruvate groups in S-layer binding was inferred from observations in *B. anthracis*.¹⁶⁴ It was demonstrated that the pyruvate transferase CsaB is involved in the addition of pyruvate to a PG-associated polysaccharide fraction and that this modification is necessary for the binding of the S-layers via SLH domains. Interestingly, the *csaAB* operon was found to be present in several bacterial species.¹⁶⁴ Pyruvate was also identified in the SCWP repeating units of *L. sphaericus* CCM 2177¹⁷⁷ and *P. alvei* CCM 2051,¹⁷⁶ which may be taken as an indication that pyruvate, or more generally speaking, negative charges of SCWPs, constitutes a widespread mechanism for the anchoring of S-layer proteins containing SLH domains to the bacterial cell wall. Besides the anchoring mechanism involving SLH domains, another mechanism that possibly utilizes basic amino acids, present in the cell-wall-targeting region and known for their direct interaction with carbohydrates, may apply for S-layer proteins devoid of SLH domains. In the genus *Geobacillus*, SLH domains have only been identified on the S-layer protein SbsB of *G. stearothermophilus* PV72/p2.²⁰⁶ All other investigated *G. stearothermophilus* strains possess S-layer proteins devoid of SLH domains.⁷⁴

Interestingly, none of the SCWPs of the organisms that possess S-layer proteins lacking SLH domains are modified with pyruvate groups, and some of them have a net-neutral charge.¹⁶⁶ These observations support the notion that, in addition to the previously discussed involvement of pyruvate, other mechanisms can be involved in the binding of S-layer proteins to the PG.^{164,165,167,174} In addition, the nonconserved character of S-layer-binding mechanisms is shown by the observation that the cell-wall-targeting domain is not

necessarily located in the N-terminal region of the S-layer protein. Well-documented examples of C-terminal anchoring are the S-layer proteins of *Lactobacillus acidophilus* ATCC 4556²⁰⁷ and *Lactobacillus crispatus*.²⁰⁸

The diversity observed among different SCWP structures of *Bacillaceae* follows a general theme that is well known from other cell-surface structures, such as the serotypes of lipopolysaccharides¹²³ and capsular polysaccharides.²⁰⁹ Presumably, this diversity is responsible for creating microenvironments in which different organisms can survive under unfavorable conditions. Current data indicate that nonclassical SCWPs function as mediators for anchoring S-layer (glyco)proteins from *Bacillaceae* to the bacterial cell wall. The complete elucidation of both the structure and biosynthesis of several nonclassical SCWPs would contribute to our general understanding of the various mechanisms underlying the tethering of S-layer (glyco)proteins to the cell surface of Gram-positive bacteria.

IV. Structural Analysis

1. Isolation of Cell-Envelope Polysaccharides and Glycopeptides

Cell-envelope polysaccharides and glycopeptides are characterized by a high degree of structural variability regarding their carbohydrate constituents (resembling the broad spectrum of sugars found in bacterial lipopolysaccharides), interunit linkages, and glycosylation sites of the protein, which are distributed over the whole S-layer protein but are mainly present within the proposed self-assembly domains.^{74,117} While O-linked polysaccharides seem to be limited only to S-layer glycoprotein glycans, N-glycans have also frequently been found in archaea. Usually, the polysaccharides harbor approximately 15–50 repeating units as linear or branched homo- and heteroglycans, respectively. Structural analysis has also to take into account the microheterogeneity of both the glycan and the peptide domain. In general, the isolation of inherently water-insoluble S-layer glycoproteins has been achieved by extraction from purified cell-wall preparations, employing such chaotropic agents, as 5 M LiCl, 5 M guanidine hydrochloride, or 6 M urea, followed by pronase or tryptic digestion and removal of the peptide fragments by passage over a cation-exchange resin. Subsequent final purification is usually accomplished by size-exclusion chromatography and reverse-HPLC separation, resulting in glycopeptide fractions amenable to structural analysis. Release of the O-glycans can eventually be induced by β -elimination. Hydrolysis to give the individual monosaccharide constituents has typically been performed by treatment with 4 M HCl or 2 M trifluoroacetic acid at 110 °C for several hours. The quantitation of the glycan components was achieved by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC PAD) or by gas chromatography–mass-spectrometric analysis of the trimethylsilylated methyl glycosides. The absolute configuration of the monosaccharides was based on GC–MS data of the TMS 2-butyl derivatives (for a review, see Ref. 7).

SCWPs usually exhibit an overall molecular mass in the range of 4–6 kDa.¹⁷⁵ The polymers were mostly released from the murein-containing sacculus by treatment with 48% hydrofluoric acid at low temperature, which cleaves the phosphodiester link to C-6 of the peptidoglycan.²¹⁰ The acidic conditions, however, also lead to cleavage of the acetal linkages of pyruvate substituents (see sections III.2.a. and IV.2.a.). Alternatively, the

phosphodiester bridge to the peptidoglycan may be preserved, allowing the determination of the linkage and substitution pattern of the SCWP to the murein backbone.⁸⁷ Separation of SCWPs from S-layer glycoprotein glycans can be effected by size-exclusion chromatography with 0.1 M NaCl as eluent or by RP-4 HPLC chromatography. Following degradation of proteins by trypsin digestion and re-*N*-acetylation, the material is subjected to digestion by lysozyme, dialysis, size-exclusion chromatography on Sephadex G-50, and final HPLC purification on RP-18 to give SCWP samples amenable to NMR analysis.¹⁸⁰

2. Degradation Reactions

a. HF Treatment of SCWPs—Cleavage of the phosphate linkages of SCWPs from the murein by the action of HF ($pK_a=3.2$) leads to hydrolytic degradation of the polymer under the acidic conditions. Degradation may affect the glycosidic linkages, *O*- and *N*-acetyl groups, and also pyruvate groups. The latter group has been suggested to act as a noncovalent anchor of the SCWP to confer binding to the S-layer homology (SLH) domain.^{164–166,174} Thus, in several structural reports on SCWPs, the actual amount given for the pyruvate substituent may be too low, or the native pyruvate groups may have not been detected at all in the acid-degraded material. The difference in pyruvate contents depending on the isolation protocol used may be demonstrated by comparing two SCWPs from *L. sphaericus* CCM 2177, isolated by HF treatment,¹⁷⁷ and *P. alvei* CCM 2051, treated with lysozyme, respectively.¹⁷⁶ In the latter case, full substitution of the β -D-ManpNAc residues by 4,6-pyruvate acetal groups in the intact SCWP was found (Fig. 20), but this was decreased by up to 50% upon mild acid treatment. An approximately 50% degree of substitution was also reported for the related SCWP from *P. alvei* CCM 2051 as extracted by HF treatment.¹⁷⁷

b. Smith Degradation—Smith degradation^{211,212} has frequently been employed as a common tool to detect protein glycosylation using the periodic acid–Schiff reagent, but has also been proved to be of considerable value in establishing the structure of several SCWPs and S-layer glycoprotein glycans. Smith degradation, employing periodate oxidation followed by sodium borohydride reduction and acid hydrolysis of the acetal linkages, was applied for the removal of distal side-chain units in the HF-degraded SCWP from *G. stearothermophilus* PV72/p2¹⁸² and S-layer glycoprotein glycans from *Ane. thermoaerophilus* L420-91,^{133,137} *Thb. thermohydrosulfuricus* L77-66,¹⁴⁵ *P. alvei* CCM 2051,¹³⁸ and *G. stearothermophilus* NRS 2004/3a.¹³¹ Smith degradation has also been used for in-chain cleavage to release oligosaccharide fragments, fragments originating from the core portion of S-layer glycopeptides,^{136,139,178,213} and to confirm the 3-*O*-methyl substitution of a terminal rhamnopyranosyl unit in *Thb. thermohydrosulfuricus* L111-69.¹³⁶

3. Structure Elucidation by Nuclear Magnetic Resonance Spectroscopy

The tools of modern NMR spectroscopy have been instrumental in the detailed structure elucidation of S-layer glycans and SCWPs. NMR-based findings include the identification of distal end-group modifications, substoichiometric substitution by *O*- and *N*-acetyl groups, the detailed structure of core oligosaccharides at the reducing end of the polysaccharides and the binding regions of the peptide and murein fragments, respectively. Information on the individual glycosyl constituents is now routinely derived from one- and two-dimensional

NMR spectroscopic techniques employing ^1H – ^1H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), ^1H – ^{13}C heteronuclear single quantum coherence spectroscopy (HSQC), and also ^1H – ^{13}C HSQC-TOCSY experiments, which additionally help in identifying the glycosylation sites by virtue of the observed ^{13}C NMR glycosylation shifts.^{214,215} Assignment of the anomeric configuration is based on the values of the homonuclear coupling constants $J_{\text{H1,H2}}$ as well as being confirmed by the value of the one-bond heteronuclear coupling constant $J_{\text{C1,H1}}$. Information on glycosyl sequences is mainly achieved from nuclear Overhauser effect spectroscopy (NOESY) and ^1H – ^{13}C heteronuclear multiple-bond coherence spectroscopy (HMBC) data, which additionally also support the complete assignment of the individual glycosyl spin systems. Whereas HMBC records three-bond connectivities, the H2BC technique monitors two-bond correlations only, thereby clarifying ambiguous assignments made from HMBC or HSQC-TOCSY data.^{182,216} Comparison of the integral values arising from nondegenerate signals at the termini of the polysaccharide chain with the degenerate signals of the repeat units provides an estimate of the chain length of the cell-envelope polymers.

Specific structural features of SCWPs relate to the presence of pyruvate groups and the linkage region to the peptidoglycan. The presence of pyruvate acetals is readily identified via HMBC correlations from the high-field-shifted signal of the methyl group to carbon 2 at ~102–103 ppm and carbon 1 of the carboxyl group. The (*S*)-configuration is deduced from the low-field-shifted ^{13}C NMR signal of the equatorially arranged methyl group.^{176,182,189,190,217} The linkage of SCWPs to muramic acid residues of PG has been determined for only a very limited number of examples because of the heterogeneity of the peptidoglycan backbone and substantial signal broadening. Both phosphodiester^{181,181} and putative diphosphate linkages from a GlcpNAc residue have been suggested, based on ^{31}P NMR data.^{176,181} A connectivity of the anomeric proton to the adjacent phosphorus atom could be established by ^1H { ^{31}P } decoupling difference experiments as well as by using ^1H – ^{31}P HMBC experiments.¹⁸⁰ Notably, in the case of the diphosphate unit that is suggested to occur in the SCWP of *P. alvei* CCM 2051, a reversible change in the integral values of two phosphorus signals was observed, depending on the presence or absence of micelle-breaking agents.¹⁷⁶ Low-field-shifted signals of CH_2 groups correlated to ^{31}P NMR signals agree with the existence of an attachment site at carbon 6 of the muramyl units in the PG backbone. Additional evidence is required to substantiate the presence of these diphosphate entities.

The structural elucidation of end groups occurring in S-layer glycoprotein glycans has to cover the capping components—assumed to be involved in chain termination of the polymer during biosynthesis—as well as the structurally diverse core units and their linkage to the peptide portion of the S-layer glycoprotein. Methylation at O-2 and O-3 of terminal α -L-rhamnopyranosyl residues has been found in the S-layer glycoprotein glycans from *G. stearothermophilus* NRS 2004/3a¹³¹ and *Ane. thermoaerophilus* L420-91^T and GS4-97,¹³⁷ as well as *Thb. thermohydrosulfuricus* L111-69 and L110-69,¹³⁶ respectively. The location of the methylation site was established through HMBC correlation of the methyl carbon atom to the respective proton of the rhamnose residue.¹²⁰ A unique capping component was identified in *Ano. tepidamans* GS5-97^T harboring (*R*)-*N*-acetylmuramic acid and GlcpNAc

as distal sugar units¹³²; diffusion difference experiments were used to rule out any accompanying contamination by low-molecular-weight components.¹³⁹

S-Layer glycoprotein glycans frequently contain the unusual O-glycosidic linkage to tyrosine residues, which was readily identified by the two doublet proton signals in the aromatic region (albeit occurring in low intensity). The tyrosine-connected sugars found thus far are glucopyranosyl and galactopyranosyl groups in a β -glycosidic linkage.^{135,136,139,140,218} Other glycosyl amino acid units harbor O-linked threonine/serine as well as N-linked asparagine residues. Analysis of the glycopeptide part is complicated by the inherent microheterogeneity and low intensity of the respective NMR signals. Elucidation of these domains has been accomplished by NMR analysis of fragments obtained by acid hydrolysis as well as by measurements performed in 90% H₂O/10% D₂O solution with efficient suppression of the water signal using a WATERGATE pulse-sequence. In that way, correlations arising from the NH amide signals were utilized to elucidate the amino acid sequence of the glycopeptide domain. Because of heterogeneity present in a number of core structures and the peptide part, however, a full structural elucidation has usually to be based on a combination of NMR data and mass-spectrometric evidence.

4. Mass Spectrometry

Matrix-assisted laser-desorption ionization time-of-flight (MALDI) MS has proved to be a valuable tool for the study of S-layer glycans as well as SCWPs. MALDI-TOF mass spectra of glycopeptide pools have been recorded in both negative- and positive-ion mode, giving insight into structural heterogeneities of the sample, chain-length distribution, and also the molecular mass of the repeat units.^{131,136,139} Electrospray-ionization time-of-flight (ESI Q-TOF) mass spectrometry provides enhanced sensitivity and, by analysis of multiply charged species and fragmentation patterns, allows sequencing of core units and glycopeptide fragments.^{132,219–222}

For analyzing patterns of protein glycosylation at the nanoscale, LC-ESI-MS/MS can be employed, as has been recently exemplified with *T. forsythia*.¹⁰⁶ Here, the TfsA and TfsB S-layer glycoproteins, as well as four additional carbohydrate-positive protein bands, were excised from SDS–polyacrylamide gels and the O-glycans were isolated by applying in-gel reductive β -elimination with 1 M NaBH₄ in 0.5 M NaOH at 50 °C followed by removal of excess salt and subsequent LC-ESI-MS/MS analysis. In different bacteria,¹⁸¹ such as in the *T. forsythia* S-layer glycan, mannosaminuron-amides are present which are hydrolyzed to mannosaminuronic acids under these conditions. This change is evidenced by a mass difference of 1 Da between released and bound O-glycan. Alternatively, ammonia-based nonreductive β -elimination can be applied,²²³ followed by prolonged exposure to sodium hydroxide at 50 °C, which leads to a deamidation reaction that can be readily monitored by MS.

V. Cell-Envelope Glycan Biosynthesis

1. Genetic Basis for S-Layer Glycoprotein Biosynthesis

If present, S-layer proteins are among the most abundant cellular proteins, with up to 20% of the total protein biosynthetic effort of a bacterium being devoted to production of S-layer protein. This large amount of S-layer protein is required for a complete coverage of the bacterium by a closed S-layer lattice during all stages of its growth cycle. At high growth rates of an average-sized cell, approximately 500 S-layer subunits must be synthesized per second, translocated to the cell surface, and incorporated into the preexisting S-layer lattice.²²⁴ All of these steps have to be strictly coordinated with the glycosylation process to afford a glycosylated S-layer lattice. According to recent data, S-layer glycosylation lags behind biosynthesis of S-layer protein and is likely to proceed cosecretionally, with the underlying regulatory mechanism being currently unknown.

The provision of this extraordinary high amount of S-layer protein is ensured at the molecular level by a combination of strong S-layer gene promoters and high mRNA stability. In the context of gene regulation of S-layer protein biosynthesis, it should be mentioned that some bacteria (such as *C. fetus* or lactobacilli) can express different S-layer proteins.^{103,105} S-Layer variation might have evolved as a pivotal strategy of bacteria to respond to changing environmental conditions.^{101,102,104,105}

The genetic information for S-layer protein glycosylation is encoded by S-layer glycosylation (*slg*) gene clusters (polycistronic units) or *slg* gene loci (comprising several transcriptional units), working in concert with housekeeping genes. Housekeeping genes map outside the *slg* gene regions, and the encoded gene products are required for the biosynthesis of the reducing-end glycoses of the S-layer glycans. The gene that encodes the respective S-layer target protein is transcribed independently of the genes in the *slg* gene region, and its chromosomal location is not necessarily in close vicinity. Currently, most data are available from the Gram-positive bacteria *G. stearothermophilus* NRS 2004/3a (GenBank AF328862),²²⁵ *Ano. tepidamans* GS5-97^T (GenBank AY883421),²²⁶ and *Ane. thermoaerophilus* strains L420-91^T (GenBank AY442352) and DSM 10155/G⁺ (GenBank AF324836)²²⁷ as well as from the Gram-negative *T. forsythia* ATCC 43037.¹⁰⁶ Depending on the complexity of the encoded S-layer glycan, the *slg* gene regions are ~16 to ~25 kb in size. They include genes of the nucleotide-sugar pathway that are arranged consecutively, glycosyltransferase genes, glycan-processing genes, and transporter genes. The presence of insertion sequences and the decrease of the G + C content at these gene regions in comparison to the respective bacterial genome suggest that lateral gene transfer is the source of S-layer glycan variation (for review, see Ref. 116).

The comparison of the *slg* gene clusters encoding the extended tripartite S-layer glycans of *G. stearothermophilus* NRS 2004/3a and *Ano. tepidamans* GS5-97^T¹¹⁵ revealed that the clusters are organized in a similar way.²²⁶ The central, variable part of the *slg* gene clusters is responsible for the biosynthesis of the individual repeating units and terminating elements, whereas the region having higher homology codes for proteins involved in assembling the core region, transport of the glycan to the cell surface, and its ligation to the S-layer protein. This behavior resembles the organization of O-antigen gene clusters in Gram-negative

organisms, where the variability of O-antigens is considered to be a result of recombination events in the central region of the O-antigen gene clusters.^{228,229}

A special situation exists with *T. forsythia*, where theoretical analysis of the S-layer O-glycosylation indicated the involvement of a 6.8-kb gene locus that is conserved among different bacteria from the *Bacteroidetes* phylum.¹⁰⁶

2. Nucleotide Sugar Biosynthesis

Based on the identification of the S-layer glycan-specific nucleotide-sugar genes in the *slg* gene regions, the encoded proteins were cloned and overexpressed in *E. coli*. This procedure, together with the establishment of functional assays for the recombinant enzymes, led to the characterization of the biosynthesis pathways for dTDP- β -L-Rhap,²³⁰ dTDP- α -D-Fucp3NAc,²³¹ dTDP- α -D-Quip3NAc,²³² GDP-D-glycero- α -D-manno-heptose,²³³ GDP- α -D-Rhap,²³⁴ and dTDP- α -D-Fucp²²⁶ in Gram-positive bacteria. In all these pathways, dTDP-“4-dehydro-6-deoxyglucose” (6-deoxy-D-xylo-hexos-4-ulose) plays a central role (Fig. 31).

Consequently, pathways for S-layer protein glycosylation provide a spectrum of rare enzymes that may be used for glycoengineering purposes in heterologous hosts (see Section VI). Furthermore, some of these enzymes, for example, most of the Rml enzymes from *Ane. thermoaerophilus* DSM 10155/G⁺, because of their thermophilic origin, exhibit significantly higher stability at 37 °C than the enzymes from the mesophilic strain *Salmonella enterica*. This advantage could lead to the development of improved high-throughput screening systems for specific sugars.²³⁵

3. Multispecific Glycosyltransferases

As already outlined, *G. stearothermophilus* NRS 2004/3a possesses S-layer O-glycans having the repeating-unit structure $[\rightarrow 2)\text{-}\alpha\text{-L-Rhap-(1} \rightarrow 3)\text{-}\beta\text{-L-Rhap-(1} \rightarrow 2)\text{-}\alpha\text{-L-Rhap-(1} \rightarrow]_{n=13-18}$, including a 2-O-methyl group capping the terminal repeating unit at the nonreducing end and a $\rightarrow 2)\text{-}\alpha\text{-L-Rhap-}[(1 \rightarrow 3)\text{-}\alpha\text{-L-Rhap}]_{n=1-2} (1 \rightarrow 3)\text{-adaptor}$ linked via a $\beta\text{-D-Galp}$ residue to distinct threonine and serine sites of the S-layer protein SgsE (for structure, see Fig. 7).¹³¹ During *in vitro* analysis of the biosynthesis of this S-layer glycan, using purified enzyme preparations and synthetic substrates,²³⁶ an unusual multifunctional methylrhamnosyltransferase (WsaE) was identified. The N-terminal portion of WsaE is responsible for the S-adenosylmethionine-dependent methylation reaction of the terminal $\alpha\text{-(1} \rightarrow 3)\text{-linked L-rhamnose}$ residue, and the central and C-terminal portions are involved in the transfer of L-rhamnose from dTDP- $\beta\text{-L-rhamnose}$ to the adaptor saccharide to form the $\alpha\text{-(1} \rightarrow 2)\text{-}$ and $\alpha\text{-(1} \rightarrow 3)\text{-linkages}$ during elongation of the S-layer glycan chain, with the methylation and the glycosylation reactions occurring independently.

In *P. alvei* CCM 2051^T, the S-layer polysaccharide consisting of $\rightarrow 3)\text{-}\beta\text{-D-Galp-(1}[\alpha\text{-D-Glcp-(1} \rightarrow 6)]\rightarrow 4)\text{-}\beta\text{-D-ManpNAc-(1} \rightarrow$ repeating units is O-glycosidically linked via an adaptor having the structure $\text{-(GroA-2} \rightarrow \text{O)-P(=O)OH-(O} \rightarrow 4)\text{-}\beta\text{-D-ManpNAc-(1} \rightarrow 4)]\rightarrow 3)\text{-}\alpha\text{-L-Rhap-(1} \rightarrow 3)\text{-}\alpha\text{-L-Rhap-(1} \rightarrow 3)\text{-}\alpha\text{-L-Rhap-(1} \rightarrow 3)\text{-}\beta\text{-D-Galp-(1} \rightarrow$ to specific tyrosine residues of the S-layer protein SpaA (for structure, see Fig. 4). A 24.3-kb S-layer *slg* gene

locus comprising 18 open reading frames encodes the information necessary for the biosynthesis of this glycan. The protein encoded by *wsfC* is the largest found in this gene locus, coding for a tripartite transferase of 147.3 kDa. Two glycosyltransferase family 2 motifs are predicted in the central and C-terminal part, while a single CDP-glycerol:poly(glycerophosphate) glycerophosphotransferase motif is present at the N-terminal part of the protein. It is conceivable that the two former motifs synthesize the repeating-unit backbone $\rightarrow 3)\text{-}\beta\text{-D-Galp-(1}\rightarrow 4)\text{-}\beta\text{-D-ManpNAc-(1}\rightarrow$ of the S-layer glycan,¹³⁴ and we speculate that the latter motif might be involved in chain-length termination by catalyzing the transfer of a phosphoglyceric acid. This conclusion strongly suggests a multifunctional role of this enzyme in the biosynthesis of the S-layer O-glycan of *P. alvei*.¹³⁰

The involvement of multifunctional enzymes in the biosynthesis of polysaccharides is not unusual. In polysaccharides of *E. coli* O8 and O9a, for instance, chain extension is performed by a multidomain serotype-specific mannosyltransferase WbdA.²³⁸ In contrast to WsaE of *G. stearotheophilus* NRS 2004/3a, methylation or phosphomethylation of the nonreducing end occurs in separate steps by the action of WdbD. Although it is reported that methylation plays a critical role in chain-length determination, and that overexpression of WdbD reduces O-PS chain length in serotypes O8 and O9a, it is unclear how WdbD activity is regulated.²³⁹

4. Proposed Pathway for S-Layer Glycoprotein Biosynthesis

Based on sound structural data and knowledge of the underlying *slg* gene regions, a picture of how S-layer glycoprotein diversity is created by nature is unraveling. Biosynthesis of S-layer glycan glycoprotein can be viewed as a sequence of distinct stages: initiation, assembly, and transfer to the protein. Initial approaches for investigating the details concerning the machinery of S-layer protein glycosylation benefited from the molecular information available on the biosynthesis of LPS O-antigens, because due to the structural similarities (see Section II) it was reasonable to assume that both cell-surface glycoconjugates are assembled via similar biosynthetic routes, involving comparable enzyme activities.

a. Initiation—Synthesis of the glycan chain is initiated by transfer of the first sugar residue from its nucleotide-activated form onto the membrane-bound undecaprenylphosphate (undP) lipid carrier. The initiation enzymes of S-layer-glycan biosynthesis in *G. stearotheophilus* NRS 2004/3a and *P. alvei* CCM 2051^T are the UDP-Gal:phosphoryl-polyprenol Gal-1-phosphate-transferases WsaP and WsfP, respectively. WsaP was shown to transfer Gal-1-P from UDP-Gal to phosphoryl-polyprenol *in vitro* and to reconstitute the function of WbaP in WbaP-deficient strains of *E. coli* and *S. enterica*.²²¹ Deletion of *wsfP* in *P. alvei* CCM 2051^T resulted in loss of S-layer glycosylation in this strain, while the original phenotype could be restored by expression of either WsfP or WsaP.²⁴⁰ WsaP and WsfP share 60% identity and 75% similarity, and both enzymes show high sequence homology to members of the poly-isoprenyl-phosphate hexose-1-phosphate transferase (PHPT) family such as WbaP.²⁴¹ The involvement of WbaP homologues in the S-layer glycosylation pathway of *G. stearotheophilus* NRS 2004/3a and *P. alvei* CCM 2051^T is rather unexpected, since in the

corresponding *slg* gene regions, the presence of the ABC-transporter constituents Wzm and Wzt is predicted. Consequently, by analogy to what is known from the well-investigated routes for biosynthesis of LPS O-antigen, the initiation of S-layer glycan assembly should be catalyzed by a WecA homologue (a GlcNAc-1-phosphate transferase), which is the usual initiation enzyme in the ABC-transporter-dependent pathway of O-polysaccharide biosynthesis.¹²³ Thus, despite many similarities between the biosynthesis of S-layer glycans and that of O-polysaccharides, the S-layer-specific pathways utilize a new combination of modules that has not been observed before.

b. Assembly of the S-Layer Glycan—S-Layer glycans in *G. stearotheophilus* NRS 2004/3a and *P. alvei* CCM 2051^T are assembled stepwise by the consecutive transfer of glucose residues from the nucleotide-activated forms to the lipid-bound growing glycan chain, with the individual transfer reactions being catalyzed by specific glycosyltransferases.¹³⁰ This scheme implies the growth of the glycan chain from the nonreducing end and, thus, resembles the ABC-transporter-dependent pathway of O-polysaccharide biosynthesis.¹²³

Assembly of the poly- α -rhamnan S-layer glycan of *G. stearotheophilus* NRS 2004/3a has been studied in different *in vitro* setups. The *slg* gene cluster of this bacterium encodes four rhamnosyltransferases, named WsaC through WsaF, which are involved in the biosynthesis of the S-layer glycan. The individual reactions catalyzed by each of the four enzymes have been elucidated in detail by their respective ability to transfer rhamnose from dTDP- β - α -Rha to different synthetic substrates, thus resembling the different stages of the lipid-bound growing glycan chain. Membrane-anchored WsaD was found to catalyze the initial rhamnosylation reaction, which is the transfer of activated β - α -Rha to the undP-bound galactose primer. Subsequently, WsaC adds one or two additional α -Rha residues, thereby completing the adaptor saccharide. The repeating units are synthesized in a concerted action of the enzyme proteins WsaE and WsaF. WsaF catalyzes the formation of the β -(1 $\rightarrow$ 2)-linkage,²⁴² while WsaE is a trifunctional enzyme with direct involvement in chain-length determination through its methyltransferase activity.

Insight into the assembly of the S-layer glycan of *P. alvei* CCM 2051^T was gained through a systematic gene-deletion approach of the individual genes encoded by the *slg* gene locus of this bacterium.¹³⁰ The biosynthesis pathway was proposed based on detailed analysis of the glycosylation pattern of the individual mutants. The first building block of the *P. alvei* CCM 2051^T S-layer glycan is the adaptor saccharide having the structure -[GroA-2 $\rightarrow$ O)-P(=O)OH-(O $\rightarrow$ 4)- β -D-ManpNAc-(1 $\rightarrow$ 4)] $\rightarrow$ 3)- α -L-Rhap-(1 $\rightarrow$ 3)- α -L-Rhap-(1 $\rightarrow$ 3)- α -L-Rhap-(1 $\rightarrow$ 3)- β -D-Galp-(1 $\rightarrow$ (for structure, see Fig. 4)).¹³⁵ WsfG transfers the first α -Rha residue onto the initial lipid-linked Gal obtained upon catalysis of WsfP. Subsequently, WsfF adds two more α -Rha residues. The branching ManNAc residue is predicted to be transferred by WsfE, and the GroA-2-phosphate is likely to be added by the CDP-glycerol:poly (glycerophosphate) glycerophosphotransferase domain of WsfC. WsfC is furthermore involved in biosynthesis of the repeating-unit backbone. A branching glucose residue would be α -(1 $\rightarrow$ 6)-linked to the repeating-unit ManNAc residue via a separate mechanism that was shown to depend on the activity of WsfH and WsfD. According to the current interpretation, WsfH would transfer a single glucose residue from UDP-Glc to a

separate lipid carrier, from which it would be further transferred to the S-layer glycan by the activity of WsfD.

c. Transfer of the S-Layer Glycan onto the Protein—The *slg* gene regions from both *G. stearothermophilus* NRS 2004/3a and *P. alvei* CCM 2051^T contain the integral membrane protein Wzm and the nucleotide-binding protein Wzt constituting an ABC transporter.²⁴³⁻²⁴⁵ By analogy to the ABC-dependent pathway of O-polysaccharide synthesis, the ABC transporter would be responsible for the transport of complete lipid-bound S-layer glycans across the cytoplasmic membrane. Transfer of the elongated glycan chain onto the S-layer protein most likely occurs cosecretionally. This final step of transferring the complete glycan to the O-glycosylation sites of the S-layer protein requires the action of a dedicated oligosaccharyl:protein transferase,²⁴⁶ which would act on the S-layer protein upon cleavage of the signal peptide and transfer across the cytoplasmic membrane. Eventually, the S-layer protein would undergo 2-D crystallization on the surface of the bacterial cell and form a closed S-layer lattice.

5. SCWP Glycosylation Gene Clusters

Upstream and downstream of the S-layer gene *spaA* of *P. alvei* CCM 2051^T several genes were identified, which are predicted to be involved in the biosynthesis of the bacterium's SCWP with the structure [(Pyr4,6)- β -D-ManpNAc-(1 $\rightarrow$ 4)- β -D-GlcpNAc-(1 $\rightarrow$ 3)_{n-11}-(Pyr4,6)- β -D-ManpNAc-(1 $\rightarrow$ 4)- α -D-GlcpNAc-(1 $\rightarrow$) (for structure, see Fig. 21).²⁴⁷ The prediction of transcription units for the complete ~14-kb DNA region resulted in a polycistronic RNA-containing *orf1*, *csaB*, and *tagA* in addition to four separate monocistronic RNAs for *tagO*, *slhA*, *spaA*, and *orf7*. The putative gene products of the SCWP biosynthesis locus were analyzed by database comparison.²⁴⁷ Orf1 might act as an exporter for the SCWP-like CsaA in the biosynthesis of other pyruvated SCWPs.^{164,174} Both *tagA* and *tagO* reveal high similarity to the glycosyl-transferases TagA and TagO of different *Bacillaceae*. In *B. anthracis*, these enzyme proteins mediate assembly of SCWP linkage units and tether pyruvated SCWP to the *B. anthracis* envelope. In *B. subtilis*, both enzymes are involved in the biosynthesis of teichoic acids, with TagO coupling GlcNAc to the membrane-embedded lipid undP and TagA catalyzing the addition of ManNAc to produce the undP-linked GlcNAc-ManNAc disaccharide.^{248,249} Since a ManNAc-GlcNAc backbone disaccharide motif is found in the SCWP of *P. alvei* CCM 2051^T, it is conceivable that both enzymes are involved in the biosynthesis of this SCWP.^{135,248}

In the context of the anchoring function of SCWP for the S-layer protein SpaA of *P. alvei* CCM 2051^T, CsaB is of special interest, because the *csaB* gene is highly homologous to genes coding for pyruvate transferases (CsaB) in various *Bacillus* strains. In *B. anthracis* and *Ths. thermophilus*, CsaB is involved in the addition of pyruvate groups to the respective SCWPs, a prerequisite for anchoring of cell-wall-associated proteins containing SLH domains.^{164-166,174} The presence of a pyruvate-containing SCWP in *P. alvei* CCM 2051^T suggests that CsaB is responsible for SCWP pyruvation also in this organism.¹⁷⁶ To analyze directly the contribution of the pyruvate residues for the interaction with the SLH domains of the *P. alvei* CCM 2051^T S-layer protein, an attempt was made to inactivate *csaB*; this, however, generated a lethal phenotype.¹⁶⁷ This is in line with the impossibility of

inactivating *spaA*,¹⁶⁷ which indicates that cell-envelope integrity is essential for the viability of *P. alvei* CCM 2051^T cells. In this context, it is noteworthy that also for *B. anthracis*, no null mutations of either *tagO* or *tagA* could be obtained.²⁵⁰

For *B. anthracis*, comparably little is known about SCWP biosynthesis, even though for this bacterium the important role of CsaB for proper SCWP function *in vivo* was reported for the first time.¹⁶⁴ Previous work described the SCWP of *B. anthracis* as a polysaccharide having the repeating structure $[\rightarrow 4)\text{-}\beta\text{-ManNAc}-(1 \rightarrow 4)\text{-}\beta\text{-GlcNAc}-(1 \rightarrow 6)\text{-}\alpha\text{-GlcNAc}-(1 \rightarrow)]_n$, where $\alpha\text{-GlcNAc}$ is substituted with $\alpha\text{-Gal}$ and $\beta\text{-Gal}$ at O-3 and O-4, respectively, and the $\beta\text{-GlcNAc}$ is substituted with $\alpha\text{-Gal}$ at O-3 (for structure, see Fig. 26).¹⁸⁵ A SCWP strand is tethered via a murein linkage unit (GlcNAc–ManNAc) and a phosphodiester bond to the C-6 hydroxyl group of *N*-acetylmuramic acid (MurNAc) within the peptidoglycan.^{184,251} Mesnage and coworkers¹⁶⁴ identified the two-gene operon *csaAB* for cell-surface anchoring of the SLH domain-containing S-layer proteins EA1 and Sap in *B. anthracis*. Biochemical analysis of cell-wall components showed that CsaB was involved in the addition of a pyruvate group to a peptidoglycan-associated polysaccharide fraction and that this modification was necessary for binding of the SLH domain. The *csaAB* operon was found to be present in several bacterial species that synthesize SLH-containing proteins. This observation, and the presence of pyruvate in the cell wall of the corresponding bacteria, suggested that this mechanism is widespread among bacteria. In contrast to *P. alvei* CCM 2051^T, deletion of *csaAB* affected only attachment of the *B. anthracis* S-layer proteins EA1 and Sap, but did not lead to a lethal phenotype.¹⁶⁴

Very recent studies revealed that the SCWP of *B. anthracis* is modified with acetal-pyruvate and acetyl groups.^{184,189} Acetylation of SCWP appears to be a universal feature of *B. anthracis* and *B. cereus*, and the sites of acetylation and pyruvation on the polysaccharide appear similar between *B. anthracis* CDC 684 and *B. anthracis* Sterne.^{186,189,252} Nevertheless, the genetic basis for such modification is not yet known in detail. Recently, two putative acyl-transferase systems, designated PatA1 and PatA2, were identified in *B. anthracis* and were proposed to function as peptidoglycan acetyl-transferases.²⁵³ Investigation of the contributions of PatA1 and PatA2 on SCWP acetylation and S-layer assembly revealed that mutations in *patA1* and *patA2* affect the chain lengths of *B. anthracis* vegetative forms and perturb the deposition of the BslO murein hydrolase at cell-division septa.²⁵⁴ Furthermore, *patA1* and *patA2* mutants are defective for the assembly of EA1 in the envelope, but retain the ability of S-layer formation with Sap. The SCWP isolated from the *patA1/patA2* mutant lacked acetyl moieties identified in the wild-type SCWP and failed to associate with the SLH domains of EA1. However, it still remains to be investigated experimentally whether PatA1 and PatA2 also act to acetylate the peptidoglycan of *B. anthracis*.

VI. Glycan Engineering and Applications

1. The S-Layer Glycobiology Toolbox

Glycosylation of proteins accounts for much of the biological diversity generated at the proteome level. S-Layer glycoproteins as a special class of glycoproteins are present on a wide variety of prokaryotic cell surfaces. They offer, on account of their high degree of

variability with regard to glycoses, glycosidic linkages, and structures involving many rare glycoses, an unsurpassed glycobiology toolbox (compare with Section II).^{11,111} As outlined in detail in Section II, S-layer glycoproteins additionally possess an intrinsic self-assembly feature into 2-D crystalline structures, which cover entire bacterial cells as a closed monolayer with lattice-like appearance (for reviews, see Refs. 67,74). Accounting for this intrinsic nanometer-scale cell-surface display feature of bacterial S-layer glycans via the S-layer protein matrix, we have coined the neologism *S-layer nanoglycobiology*, which comprises structural, functional, and biosynthetic aspects of S-layers.^{111,115,116} The awareness of the promising application potential of S-layer glycoproteins as a unique matrix with inherent self-assembly properties (for reviews, see Refs. 80,115,122) is the driving force behind the efforts for structural elucidation of novel S-layer glycans of different bacteria; recent focus is on medically relevant bacteria and the characterization of S-layer glycoprotein biosynthesis at the molecular level (compare with Section V).

Carbohydrate-active enzymes from pathways for S-layer protein glycosylation, especially those from nucleotide-sugar biosynthesis, offer interesting options for therapeutic intervention. For instance, the immediate source for α -rhamnose, a common component of the cell wall and the capsule of many pathogenic bacteria as well as of many S-layer glycans, is dTDP- α -Rha. Since to date neither rhamnose nor the RmlABCD genes responsible for dTDP- α -Rha biosynthesis have been identified in humans, these enzymes are ideal targets for inhibiting rhamnose-mediated bacterial pathogenicity.²⁵⁵ Other enzymes from the S-layer glycome can be beneficial for synthetic purposes because of their unusual specificities or thermostability. Thermostable Rml enzymes from *Ane. thermoaerophilus* DSM 10155 allow higher productivity of dTDP- α -Rha (compare with Section V).²³⁰ Other enzymes, such as an isomerase derived from *Ane. thermoaerophilus* DSM 420-91^T capable of synthesizing DTDP-6-deoxy-D-xylo-hexos-3-ulose (“dTDP-3-keto-6-deoxygalactose”) from dTDP-6-deoxy-D-xylo-hexos-4-ulose (“dTDP-4-keto-6-deoxyglucose”)²³⁰ (Fig. 31), may be relevant for the synthesis of antibiotics that contain C-3-aminated deoxy sugars, such as erythromycin or tylosin.

Eventually, detailed insights into the structures of S-layer glycans and the molecular understanding of the governing biosynthetic machinery will increase the possibilities offered by the glycobiology toolbox aiming at the rational design of S-layer glycans and, consequently, the generation of artificial S-layer *neoglycoconjugates* having rationally designed and biofunctional glycosylation motifs.^{247,256,257} Since the S-layer glycome is connected with a molecular self-assembly system, our approach has a strong link to the field of nanobiotechnology, because the means for organizing materials, such as biologically functional glycans, at the nanometer level are prime candidates for the fabrication of supramolecular structures and devices.⁹³

2. Glycosylation Engineering

Considering the constantly increasing number of reports about prokaryotic glycoproteins that are important in pathogenesis,^{8,9,57,60,126} the molecular understanding of S-layer protein glycosylation (see Sections V. and VI.1.) should eventually allow the rational design of glycosylation patterns for S-layer proteins to obtain bioactive S-layer neoglycoproteins.¹¹

The common trend of glycoengineering is reflected by many recent articles on that topic.^{47,53,127,258-260} In general, the display of heterologous (glyco)proteins on the surface of bacteria, enabled by means of recombinant DNA technology, has become increasingly used as a strategy in various applications in microbiology, nanobiotechnology, and vaccinology.^{247,261,262} Besides outer membrane proteins, lipoproteins, autotransporters, or subunits of surface appendages, the use of the glycosylated S-layer proteins as a surface-anchoring structure 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 functional glycosylation motifs.^{122,247} Nanobiotechnology applications of such tailored S-layer neoglycoproteins may include the fields of receptor mimics, vaccine design, or drug delivery using carbohydrate recognition. The possibility for incorporating additional functional domains into the S-layer protein portion may permit additional tuning of structural and functional features of these S-layer neoglycoproteins.

Glycosylation engineering of S-layer glycoproteins constitutes a rather new area of research, and only a few examples have been published so far. Previous studies have demonstrated that several functional domains introduced into S-layer proteins at distinct positions by genetic engineering do not interfere with the intrinsic self-assembly property of the S-layer system, and simultaneously they fully retain their specific biological properties.²⁵⁶ SgsE is a naturally *O*-glycosylated protein of *G. stearothermophilus* NRS 2004/3a which has been used in a combined carbohydrate/protein engineering approach for the production of two types of S-layer neoglycoproteins in *E. coli*. Based on the identification of a suitable periplasmic targeting system for the SgsE self-assembly protein as a cellular prerequisite for protein glycosylation, and on engineering of one of the natural protein *O*-glycosylation sites into a target for *N*-glycosylation, the heptasaccharide from the AcrA protein of *C. jejuni* and the O7 polysaccharide of *E. coli* were transferred to the S-layer protein by the action of the oligosaccharyltransferase PglB. The degree of glycosylation of the S-layer neoglycoproteins after purification from the periplasmic fraction reached completeness.²⁵⁷

Considering the manifold properties glycans provide to proteins, including stabilizing functions,²⁶³ enhanced thermal stability,²⁶⁴ and protection from proteases,²⁶⁵ glycosylation engineering seems to be promising for the future generation of glycoproteins having improved characteristics. Recently, “cross-glycosylation” of nonnative proteins was successfully performed in the closely related organisms *T. forsythia* and *B. fragilis*.¹²⁷ Both organisms are known to possess general *O*-glycosylation systems sharing a conserved amino acid motif that is targeted for glycosylation. Since the glycan structure of *T. forsythia* is already known (Fig. 17),¹⁰⁶ we next partially characterized the native *B. fragilis* O-glycan in order to determine whether that glycan would be added to *T. forsythia* proteins expressed in *B. fragilis*. Mass-spectrometric analysis of native *B. fragilis* glycoproteins subjected to β -elimination revealed the glycan to be an oligosaccharide consisting of nine sugar residues (Fig. 18).¹²⁷ Notably, the proposed structure based on MS data resembles that of the *T. forsythia* O-glycan in several aspects (compare with Section II). As in the *T. forsythia* glycan, the deoxyhexose of the *B. fragilis* glycan most likely is a fucose residue, as glycoproteins of *B. fragilis* are readily detected with *A. aurantia* lectin and depend on GDP-

fucose biosynthesis genes¹²⁶; however, there was no indication for the presence of a sialic acid-like sugar as in the *T. forsythia* S-layer glycan.¹⁰⁶ Knowing the size and approximate composition of the *B. fragilis* glycan, the respective glycans were transferred onto nonnative proteins. The known S-layer proteins TfsA and TfsB from *T. forsythia* were chosen for heterologous glycosylation in *B. fragilis*.^{84,125} Conversely, two model glycoproteins of *B. fragilis*, BF2494 and BF3567, both of unknown function,¹²⁶ were selected for heterologous expression in *T. forsythia*. The successful transfer of the O-glycans was confirmed by the appearance of novel glycoproteins with the respective O-glycans linked via serine/threonine residues to the *Bacteroides* glycosylation sequon D(S/T)(A/I/L/V/M/T).¹²⁶ Moreover, glycan transfer appeared to be highly efficient, as none of the peptides spanning a putative glycosylation site were observed as unmodified.¹²⁷ Considering the vast amount of glycoproteins synthesized by *B. fragilis*²⁶⁰—and most likely also by *T. forsythia*—it is likely that O-glycosylation of proteins has a more general function, for instance, in conferring protein stability. Analyzing the influence of (heterologous) glycosylation on protein stability in *T. forsythia* and *B. fragilis* will contribute to a better understanding of protein glycosylation in general, as well as trigger efforts specifically to improve protein stability through glycoengineering.

VII. Concluding Remarks

The remarkable diversity observed among the S-layer glycan and SCWP structures presented here reflects the high level of adaptation developed by bacteria and archaea in the course of evolution as a consequence of ongoing changes in their respective microenvironments and habitats. The important function of secondary cell-wall polysaccharides in anchoring the S-layer remains to be studied in more detail in order to unravel the mechanisms leading to the regularly patterned display of S-layers on the surface of the bacterial cell. In addition, elucidation of the underlying genetic basis and biosynthetic pathways has already opened a fertile playground in the forthcoming years for numerous applications in the fields of nanotechnology and biomedicine, using rationally designed glycoengineering tools.

Abbreviations

AFM	atomic force microscopy
Bac	“bacillosamine,” 2,4-diacetamido-2,4,6-trideoxy-D-glucose
CD	circular dichroism
COSY	correlated spectroscopy
CP	capsular polysaccharide
EM	electron microscopy
ESI	electrospray ionization
GC	gas chromatography
HF	hydrofluoric acid

HMBC	heteronuclear multiple-bond correlation
HPAEC	high-performance anion-exchange chromatography
HR MAS	high-resolution magic-angle spinning
Legionaminic acid	5,7-diacetamido-3,5,7,9-tetradecoxy- <i>D</i> -glycero- <i>D</i> -galacto-nonulosonic acid
LPS	lipopolysaccharide
MALDI	matrix-assisted laser-desorption ionization
MS	mass spectrometry
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
PA	polyacrylamide
Pse	pseudaminic acid 5,7-diacetamido-3,5,7,9-tetradecoxy- <i>L</i> -glycero- <i>L</i> -manno-nonulosonic acid
PAD	pulsed amperometric detection
PG	peptidoglycan
Quinovose	6-deoxy- <i>D</i> -glucose
RP	reverse phase
S	S-layer (surface layer)
SCWP	secondary cell-wall polymer
SDS	sodium dodecyl sulfate
SLH	S-layer homology domain
TMS	trimethylsilyl
TOCSY	total correlated spectroscopy
TOF	time of flight

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Abbreviations: CM, cytoplasmic membrane; PG, peptidoglycan; OM, outer membrane; SLG, glycosylated S-layer protein. , different S-layer glycans; , S-layer proteins; , secondary cell-wall glycan (SCWP); , peptidoglycan strand; , bacterial membrane phospholipid; , archaeal membrane tetraetherlipid; , outer membrane lipid A.

Fig. 1.

Scheme of cell envelopes of prokaryotic organisms, showing representative cellular glycan structures

Fig. 2.

Electron micrograph of a freeze-substituted and cross-sectioned cell of *Ane. thermoaerophilus* DSM 10155. With permission from Springer Science + Business Media, compare with Ref. 128, Fig. 1b.

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Fig. 4.
Core structure of *P. alvei* CCM 2051 S-layer glycan.¹³⁵

Fig. 5.
Core structure of *Ane. thermoaerophilus* L420-91^{T,137}

Fig. 6.
S-Layer glycan of *Lb. buchneri*.¹²⁰

Fig. 7.
S-Layer glycan structure of *G. stearotheophilus* NRS 2004/3a.¹³¹

Fig. 8.
S-Layer glycan structure of *Thb. thermohydrosulfuricus* strains L111-69 and L110-69.¹³⁶

Fig. 9.
S-Layer glycan structure of *Thm. thermosaccharolyticum* strain D120-70.¹³⁹

Fig. 10.
S-Layer glycan structure of *Ano. tepidamans* GS5-97^T.¹³²

Fig. 11.
S-Layer glycan structure of *Ane. thermoaerophilus* DSM 10155.¹³³

Fig. 12.
S-Layer glycan structure of *Ane. thermoaerophilus* strains L420-91^T and GS4-97.^{137,144}

Fig. 13.
S-Layer glycan structure of *P. alvei* CCM 2051^T.¹³⁴

Fig. 14.
S-Layer glycan structure of *Thb. thermohydrosulfuricus* L77-66 (DSM 569).¹⁴⁵

Fig. 15.
S-Layer glycan structure of *Thm. thermosaccharolyticum* E207-71.¹⁴⁶

Fig. 16.
S-Layer glycan structure of *Thb. thermohydrosulfuricus* S102-70.¹⁴⁰

Fig. 17.

Schematic structure of the *T. forsythia* S-layer glycan.^{100,106} Adapted from the open access journal *Biomolecules*; © 2012 by the authors, licensee MDPI, Basel, Switzerland; <http://creativecommons.org/licenses/by/3.0>.

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Fig. 19.
Terminal end of the S-layer glycan of *Ano. tepidamans* GS5-97^T.¹³²

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Fig. 21.
Repeating unit of the SCWP from *P. alvei* CCM 2051^T.¹⁷⁶

Fig. 22.
Structure of the SCWP from *Thm. thermosaccharolyticum* strain E207-71.¹⁷⁸

Fig. 23.
Structure of the SCWP from *G. stearothermophilus* NRS 2004/3a.^{179,180}

Fig. 24.
Structure of the repeating unit of the SCWP from *Ane. thermoaerophilus* DSM 10155.⁸⁷

Fig. 25.

Structure of the repeating unit of the acid-degraded SCWP from *G. stearothermophilus* PV72/p2.¹⁸² Reprinted from Ref. 182. Copyright (2008), with permission from Elsevier.

Fig. 26.
Structure of HF-treated *B. anthracis* SCWP.¹⁸⁵

Fig. 27.
Structure of the HF-treated SCWP from *B. cereus* strains G92141 and 03BB87.¹⁹⁰

Fig. 28.

Structure of the HF-treated SCWP from *B. cereus* ATCC 10987.¹⁹¹ Adapted from Ref. 191.

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Fig. 29.
Structure of the HF-treated SCWP from *B. cereus* ATCC 14579.¹⁹²

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Fig. 31.

Biosynthesis pathways for nucleotide sugars as required for S-layer protein glycosylation.^{226,230-232}