Chapter 2
Occurrence, Structure, Chemistry, Genetics, Morphogenesis, and Functions of S-Layers

Paul Messner, Christina Schäffer, Eva-Maria Egelseer, and Uwe B. Sleytr

2.1 Introduction

S-layers, as probably the most abundant bacterial cellular proteins, are being studied with regard to structure, synthesis, assembly, and function and provide excellent models for studying a proteinaceous cell component and its evolutionary relationships within the prokaryotic world. Recently, S-layers have also shown a considerable application potential in biotechnology, biomimetics, biomedicine, and molecular nanotechnology. In the past few years, several reports have been published on these application aspects, demonstrating the change of perception that has been taken place in this field of research (see Chaps. 16 and 17).

In this chapter, we give a general overview of occurrence, location, and structure of S-layers and then focus particularly on those S-layer-carrying organisms that have attracted special attention within the past decade because of their scientific, medical, or public implications (e.g. Bacillus anthracis S-layer proteins EA1 and Sap).

2.2 Occurrence, Location, and Structure

In the course of evolution, prokaryotic organisms have developed a considerable diversity in their supramolecular architectures. Although not a universal feature, many prokaryotic organisms possess a monomolecular array of proteinaceous subunits as the outermost component of the cell envelope (Sleytr 1978; Sleytr et al. 1988; Sleytr and Messner 2009). S-layers represent an almost universal...
feature of archaeal cell envelopes (see Chaps. 7–9) and have been detected in hundreds of different species of nearly every phylogenetic group of bacteria (Sleytr et al. 1996b, 2002; Åvall-Jääskeläinen and Palva 2005; Claus et al. 2005). Despite the fact that considerable variations exist in the structure and chemistry of prokaryotic envelopes (Fig. 2.1), S-layers have apparently coevolved with these diverse structures. In most archaea, S-layers are attached or inserted to the plasma membrane. In Gram-positive bacteria and Gram-positive archaea, the regular array assembles on the surface of the rigid wall matrix which is mainly composed of peptidoglycan. SCWPs are only present in bacteria. In archaea, other wall polymers (e.g., pseudomurein or methanochondroitin) are found. For some organisms, two superimposed S-layer lattices composed of different S-layer proteins have been described. S-layer-like monomolecular arrays of proteins have also been observed in bacterial sheaths (Beveridge and Graham 1991), spore coats (Holt and Leadbetter 1969), and on the surface of the cell wall of eukaryotic algae (Roberts et al. 1985).

The location and ultrastructure of S-layers of a great variety of organisms have been investigated by electron microscopy (EM) (Sleytr and Messner 1983) and atomic force microscopy (AFM) (Müller et al. 1996). The most suitable EM procedure for identifying S-layers on intact cells is freeze-etching (Fig. 2.2) (Sleytr and Glauert 1975; Sleytr 1978; Sleytr and Messner 1989). High resolution studies on the mass distribution of the lattices were performed on negatively stained preparations or unstained, ice-embedded samples. Two- and three-dimensional image analysis involving computer-image reconstruction revealed structural information down to approximately 1 nm (for reviews see Baumeister et al. 1989; Hovmöller 1993;
Beveridge 1994; Sleytr et al. 1996a). More recently, high-resolution studies on the mass distribution of S-layers were also obtained using AFM under aqueous conditions (Müller et al. 1999; Sleytr et al. 1999) and tertiary structure prediction, based on amino acid sequences (Horejs et al. 2008). A common feature of bacterial S-layers is their smooth outer and more corrugated inner surface. Archaeal S-layers frequently reveal pillar-like domains on the inner surface (see Chap. 9).

S-layer subunits can be aligned in lattices with oblique (p1, p2), square (p4), or hexagonal (p3, p6) symmetry (Fig. 2.2). Hexagonal symmetry is predominant among archaea (for compilation see Sleytr et al. 1996b, 2002). The morphological units generally have center-to-center spacings of approximately 3–30 nm. In S-layers, one or even more distinct classes of pores could be observed. Pore sizes were determined to be in the range of approximately 2–8 nm and pores can occupy 30–70% of the surface area.

2.3 Isolation, Chemistry, and Domains

Because of the diversity in the supramolecular structure of prokaryotic cell envelopes, different disruption and isolation procedures for S-layers have been developed. Usually, they are isolated from purified cell-wall fragments by the addition of hydrogen-bond breaking agents (e.g. guanidine hydrochloride or urea) (for review see Messner and Sleytr 1988; Schuster et al. 2005) or detergents or by...
cation substitution (e.g. Na\(^+\) or Li\(^+\) replacing Ca\(^{2+}\)) (Koval and Murray 1984). Extraction and disintegration experiments revealed that the inter-subunit bonds in the S-layer are stronger than those binding the subunits to the supporting envelope (Sleytr 1975). Special isolation procedures are required for S-layers in those archaea where they are associated with the plasma membrane (see Chap. 9).

Chemical and genetic analyses on many S-layers revealed that they are generally composed of a single protein or glycoprotein species with molecular masses ranging from 40 to 170 kD (for reviews see Sleytr et al. 1993, 1999, 2001a, 2002; Sumper and Wieland 1995; Messner and Schäffer 2003; Åvall-Jääskeläinen and Palva 2005; Claus et al. 2005).

S-layers from bacteria are often weakly acidic proteins, typically containing 40–60% hydrophobic amino acids, and possess few or no sulphur-containing amino acids. The pIs of these proteins range from 4 to 6 although the S-layer proteins from lactobacilli and some archaea (e.g. *Methanothermus fervidus*) have pIs ranging from 8 to 10.

Comparative studies on S-layer genes of organisms from different taxonomic affiliations have shown that homologies between non-related organisms are low, although their amino acid compositions show no significant difference. High homologies are commonly explained by evolutionary relationships but other factors such as growth conditions and environmental stress may also be responsible for structural homologies of S-layer genes. For example, if present in Bacillaceae, high sequence identities are found at the N-terminus. In some strains, S-layer homology (SLH) motives (Lupas et al. 1994) are involved in the attachment of S-layer proteins to polysaccharides (secondary cell wall polymers) that are linked to the underlying peptidoglycan layer (Sára 2001; Mader et al. 2004; Huber et al. 2005). Among the S-layer proteins of *Geobacillus stearothermophilus* strains investigated so far, strain PV72/p2 possesses three N-terminal SLH domains that have been shown to be involved in cell wall attachment of the S-layer protein (compare with Sect. 2.6.4, Fig. 2.3a). In S-layer proteins without SLH domains, positively charged amino acids contained at either the conserved N-terminal region (Fig. 2.3b) or at the C-terminal region (Fig. 2.3c) interact with a peptidoglycan-associated secondary cell wall polymer via direct electrostatic interactions or hydrogen bonds, thereby mediating attachment to the cell wall (Sára 2001; Schäffer and Messner 2005; Egelseer et al. 2008; see also Sect. 2.6.4). As recently evaluated by surface plasmon resonance studies for the S-layer protein SbsC of *G. stearothermophilus* ATCC12980\(^T\), this interaction is highly specific (Ferner-Ortner et al. 2007; compare with Sect. 2.6.4).

Traditionally, S-layers have been studied by TEM techniques and, as a result, much is known about their ultrastructure. Little is known, however, about S-layer protein structure–function relationships. In recent years, with the advent of recombinant DNA technologies, valuable new clues to the structural organization of S-layer proteins have been obtained. Methods such as deletion analysis, cloning of domains, linker mutagenesis, and cysteine scanning mutagenesis have been used (Mesnage et al. 1999; Howorka et al. 2000; Jarosch et al. 2001; Smit et al. 2001).
A few post-translational modifications are known to occur in S-layer proteins, including protein phosphorylation and protein glycosylation. S-layer glycoproteins are among the best studied examples of glycosylated prokaryotic proteins (Eichler and Adams 2005; Logan 2006; Messner et al. 2008). They are widely distributed in the major lineages of Archaea, as well as among Bacteria; in the latter lineage, they have been demonstrated mainly within Gram-positive taxa (e.g., Aneurinibacillus, Geobacillus, Clostridium, Desulfotomaculum, Paenibacillus, Thermoanaerobacter, Thermoanaerobacterium, Lactobacillus) (Messner 1996; Messner and Schäffer 2000). Complete structural analyses of S-layer glycoprotein glycans from these Gram-positive organisms have been elaborated exclusively by our group. Only recently, glycosylated S-layer proteins have also been convincingly described in the Gram-negative species Tannerella forsythia (Lee et al. 2006b). The glycan chains and linkages of bacterial and archaeal glycoproteins are significantly different from those of eukaryotes (Sleytr et al. 2002; Messner and Schäffer 2003; Schäffer and Messner 2004). Most archaeal S-layer glycoprotein glycans consist of only short heterosaccharides, usually not built of repeating units. The predominant linkage types are N-glycosidic bonds. The opposite situation is found with bacteria where up to now only O-glycosidic linkages have been found. From comparative chemical and structural studies it appears that, at least in most bacteria, S-layers have little or no taxonomic value and may merely be considered as strain-specific characteristics. Moreover, individual strains revealed the capability to synthesize and assemble more than one type of S-layer (glyco)protein array.

Fig. 2.3 Schematic drawing of the principal organization of S-layer protein domains. (a) S-layer protein without SLH domains and N-terminal cell wall anchoring (e.g., G. stearothermophilus wild-type strains ATCC 12980 and NRS 2004/a); (b) S-layer protein with SLH domains at the N-terminus and N-terminal cell wall anchoring (e.g., G. stearothermophilus PV72/p2 and Lysinibacillus sphaericus CCM 2177); (c) S-layer proteins without SLH domains and C-terminal cell wall anchoring (e.g., Aneurinibacillus thermoautotrophicus L420-91 and Lactobacillus acidophilus ATCC 4356). On the proteins, the signal peptide (closed square), the S-layer cell wall binding domain (open rectangle) and the crystallization domain (closed rectangle) are indicated. Domains as indicated are not to scale in relation to the S-layer protein. N, N-terminus; C, C-terminus.
2.4 Genetics, Biosynthesis, and Assembly

If present, S-layer proteins are among the most abundant cellular proteins, with a total protein biosynthesis effort of up to 20% being devoted to S-layer protein biosynthesis. The high amount of S-layer protein subunits is required for a complete coverage with a closed S-layer lattice during all stages of the bacterial growth cycle. This is ensured at the molecular level by a combination of strong S-layer gene promoters and high mRNA stability. More recently, the increased knowledge on diverse glycan structures of bacterial S-layer glycoproteins has led to detailed studies of the biosynthesis of these prokaryotic glycoproteins (see Sect. 2.6.4; Eichler and Adams 2005; Messner et al. 2008).

In the context of gene regulation of S-layer biosynthesis, it was interesting to observe that some bacteria can express different S-layer proteins. For example, in the pathogen *Campylobacter fetus*, synthesis of different S-layers enables the organism to circumvent the host’s immune response (for a summary see Blaser 1998). S-layer variation might have evolved as an important strategy of the respective bacteria to respond to changing environmental conditions. S-layer variation leads to the synthesis of alternate S-layer proteins, either by the expression of different S-layer genes or by recombination of partial coding sequences, and has been described in both pathogens and non-pathogens (Sára et al. 1996; Dworkin and Blaser 1997; Scholz et al. 2001; Jakava-Viljanen et al. 2002).

S-layers represent a fascinating system for studying the dynamic process of self-assembly of a supramolecular biological structure (Sleytr et al. 2001b, 2005). Detailed studies on selected S-layers from bacteria have shown that isolated subunits assemble spontaneously into regular arrays after removal of the disrupting agent used for their isolation (see Chap. 16). Studies on the in vivo morphogenesis of S-layers demonstrated that at high growth rates approximately 500 subunits per second must be synthesized, translocated to the cell surface, and incorporated into the pre-existing S-layer lattice. Differences in the net surface charge and specific (e.g. lectin-type) interactions between the inner and outer surface of the S-layer proteins to the supporting layer have been shown to be essential for proper orientation (Sleytr et al. 2002). Ultrastructural data indicate that during growth S-layer subunits (proto-mers) must have the ability to recrystallize on the supporting envelope layer, assuming a low free energy arrangement (Sleytr and Plohberger 1980; Sleytr and Messner 1989) with the intrinsic tendency to assume a continuously regular lattice during cell growth (Sleytr 1975, 1981; Sleytr and Glauert 1975).

2.5 Function

Considering that S-layer-carrying organisms are ubiquitous in the biosphere, the supramolecular concept of a closed, isoporous, protein meshwork has the potential to fulfil a broad spectrum of functions. When bacteria are no longer exposed to
natural environmental selection pressures, S-layers can be lost, indicating that the considerable biosynthesis effort is only required in natural habitats. In functional terms, S-layers are generally part of complex envelope structures (Fig. 2.1) and consequently should not be considered as isolated layers. Many of the functions assigned to S-layers are still hypothetical and not based on firm experimental data (for compilation see Table 2.1) (for reviews see Sleytr 1997; Sleytr and Beveridge 1999; Sára and Sleytr 2000; Sleytr et al. 2002; Engelhardt 2007).

### 2.6 Specific S-Layer-Carrying Organisms

Since the last comprehensive review on S-layer proteins in 1996 (Sleytr et al. 1996b), a large number of reports have appeared in the literature, documenting the universal occurrence of S-layers in the prokaryotic world. These informations are summarized in Table 2.2 including a complete coverage of GenBank accession numbers of S-layer structural genes and presently known data on surface layer glycosylation (slg) gene clusters.
Table 2.2 Bacterial S-layers (new entries are in alphabetical order, since 1995; for older references see Sleytr et al. (1996b) and Messner and Sleytr (1992)). In addition, a complete list of GenBank accession numbers of bacterial S-layer genes (see also Sleytr et al. 2002) and glycosylation-related genes is given.

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*Functions of S-Layers* (continued)
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<td>Khmelenina et al. (1999)</td>
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<td>microbes from siliceous sediments (clostridia or Desulfotomaculum sp., strains)</td>
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<td>Iino et al. (2007)</td>
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<td>Glycosylation/GenBank accession no.</td>
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<td>Higuchi et al. (2000); Sabet et al. (2003); Lee et al. (2006b)</td>
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<td>T. subterraneus DSM 13054T</td>
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<td>Liu et al. (1996)</td>
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</table>

aBacterial names in parentheses (e.g. Bacillus sp.) refer to former designations of the organisms
bAbbreviations: H, hexagonal (p6); T, trimeric, (p3); S, square (p4); O, oblique (p1, p2); P, periodic
cDeglycosylated S-layer protein
In the following subsections, information has been summarized on those organisms that were the prime research targets within the last 5–6 years.

### 2.6.1 Bacillus anthracis

*B. anthracis*, a Gram-positive, spore-forming bacterium is the etiological agent of anthrax, a disease involving toxemia and septicemia. In 2001, its potential use in biological warfare was demonstrated in the US postal system.

In addition to virulence factors (Mock and Fouet 2001), *B. anthracis* synthesizes two S-layer proteins, termed Sap and EA1 (Etienne-Toumelin et al. 1995; Mesnage et al. 1997). Both proteins have the same modular organization, an N-terminal cell wall anchoring domain consisting of three S-layer homology (SLH) motifs followed by a putative crystallization domain (Mesnage et al. 1999). Electron microscopy and genetic analyses of Sap using a bacterial two-hybrid system confirmed that the region comprising 604 C-terminal amino acids represents the crystallization domain (Candela et al. 2005).

During the exponential growth phase, *B. anthracis* cells are surrounded by the Sap S-layer protein, which is replaced by the EA1 S-layer protein when the cells enter the stationary phase (Mignot et al. 2002). By using mutant strains for electron microscopical studies and image processing, the S-layer structural organization was elucidated (Couture-Tosi et al. 2002). The projection map of EA1 revealed that the protein consists of one very large domain forming the central body around which the three smaller domains are surrounded. EA1 subunits crystallize into a lattice with p1 symmetry. For the Sap S-layer protein, six or seven domains that repeat themselves along the two axes of the crystal were identified. The Sap protein is homogeneously distributed on the cell surface, whereas the EA1 protein displays a crazy-paving aspect (Couture-Tosi et al. 2002). For both S-layer proteins, a previously unrecognized ability to function as murein hydrolases has been demonstrated (Ahn et al. 2006).

The S-layer components are encoded by the contiguous chromosomal S-layer genes *sap* and *eag*, and the S-layer switch is controlled at the transcriptional level, with *sap* being transcribed during the exponential growth phase, and *eag* being only transcribed during the stationary phase. Transcription of both genes is ensured by distinct sigma factors and the phase-specific expression of *eag* is strictly dependent on Sap, because this protein can act as a direct repressor of *eag* (Mignot et al. 2002). Mignot et al. (2003, 2004) provided evidence that expression of the chromosomally located S-layer genes is also influenced by two genes located on the virulence plasmid pXO1 encoding the two transcriptional regulators PagR and AtxA (Mignot et al. 2003, 2004). The latter was found to be a master regulator that also controls the transcription of S-layer genes by controlling the synthesis of PagR.

In vitro translation of selected open reading frames (ORFs) on the virulence plasmid pXO1, followed by analysis of the reactivity of the ORF products with hyperimmune anti-*B. anthracis* antisera, led to the identification of two S-layer
proteins, both carrying three SLH-motifs (Ariel et al. 2002). Immunoreactivity studies using a truncated S-layer protein form devoid of the SLH moiety indicated that the C-terminal segment contributes significantly to S-layer immunogenicity (Ariel et al. 2002).

Cloning and sequencing of the DNA region upstream of the S-layer gene sap led to the identification of the genes csaA and csaB (cell surface anchoring), that are organized as an operon. CsaA revealed similarity to oligosaccharide transporters of Gram-negative bacteria, whereas CsaB was identified as pyruvyl transferase, which is supposed to be involved in pyruvlation of the peptidoglycan-associated cell wall polymer (Mesnage et al. 2000). Recently, Choudhury and coworkers (2006) described the structure of the polysaccharide released from B. anthracis vegetative cell walls by hydrogen fluoride (HF). It consists of Gal, ManNAc, and GlcNAc in a molar ratio of 3:1:2. Since it was speculated that the ability of neutrophils to kill vegetative B. anthracis cells depends on the binding of α-defensins to this carbohydrate by a lectin-like binding, the major cell wall polysaccharide could be considered as a target for development of specific antimicrobials against anthrax.

Chitlaru and coworkers identified a group of B. anthracis membrane proteins which could be useful for eliciting protective immune responses and serve as potential candidates for the development of an improved anthrax vaccine (Chitlaru et al. 2004, 2007). Among these, the S-layer proteins Sap and EA1 were found to represent more than 75% of the B. anthracis membrane fraction and undergo post-translational modifications. It was postulated that subpopulations of differently glycosylated Sap and EA1 may exist (Chitlaru et al. 2004). A comparative proteomic approach was employed to elucidate the differences among the extracellular proteomes (secretomes) of three isogenic strains under host-simulated conditions (Lamonica et al. 2005). In the fully virulent strain (pXO1+ pXO2+), while the partially cured derivative (pXO1+ pXO2−), the two chromosomally encoded S-layer proteins Sap and EA1 were found to be up-regulated whereas the pXO1-encoded S-layer protein A represented a unique protein in the derivative strain devoid of plasmid pXO2 (Chitlaru et al. 2004).

Proteome analysis was used to define the exact protein composition of the current UK anthrax vaccine (Whiting et al. 2004). In addition to the protective antigen PA, the key immunogen of the vaccine, the presence of the two other toxin components, as well as the S-layer proteins, EA1 and SAP, could be established. The S-layer proteins turned out to be also immunogenic in man but their presence did not adversely affect the protective immune response induced by PA (Baillie et al. 2003). Specific identification of B. anthracis is vital for the accurate treatment of afflicted personnel during biological warfare situations and civilian terrorist attacks. Unfortunately, this pathogenic Bacillus species shares so much genetic material with B. cereus and B. thuringiensis that its discrimination from the other species can be problematic. However, unique genomic differences could be identified and the S-layer gene sequence was found to be among these B. anthracis-specific “DNA signatures” which are capable of quickly detecting all six genetically
distinct groups of *B. anthracis* with any rapid DNA-based detection platform (Radnedge et al. 2003).

It was demonstrated that EA1, the abundant, highly antigenic S-layer protein of vegetative *B. anthracis* cells, is not a spore component as previously stated but a persistent contaminant in spore preparations. Thus, EA1 is not a suitable marker for spore detection and could result in failure to detect highly purified spores or to accurately estimate spore number, which would have catastrophic consequences (Redmond et al. 2004; Williams and Turnbough 2004). On the other hand, affinity purification of antigenic protein and tandem mass spectrometry revealed that EA1 represents a highly specific biomarker that enables simultaneous identification and verification of vegetative *B. anthracis* cells (Krishnamurthy et al. 2006).

### 2.6.2 *Clostridium difficile*

*Clostridium difficile* is a Gram-positive, spore-forming anaerobic bacterium, which is recognized as the etiological agent of antibiotic-associated diarrhea and pseudomembraneous colitis in humans and which can cause significant morbidity in hospitalized patients (Kelly and LaMont 1998). Its pathogenicity is mediated by two toxins, A and B, both of which damage the human colonic mucosa and are potent tissue-damaging enzymes (Borriello et al. 1990).

S-layer proteins are the most abundant extracellular *C. difficile* proteins found during both high and low toxin production (Mukherjee et al. 2002). This organism is unusual in expressing two S-layer proteins which associate to form the S-layer, one of high molecular weight (HMW) and the other of low molecular weight (LMW). Both subunits are encoded by the *slpA* gene and are produced from post-translational cleavage of a single precursor (Calabi et al. 2001; Karjalainen et al. 2001). The HMW peptide, derived from the C-terminal portion of the precursor, is highly conserved between strains, while the LMW peptide derived from the N-terminal portion of the precursor demonstrates considerable sequence diversity and appears to be the main serotyping antigen (Calabi and Fairweather 2002; Karjalainen et al. 2002). The HMW peptide has sequence similarity to the *N*-acetylmuramoyl-α-alanine amidase from *Bacillus subtilis*, and was shown to possess amidase activity (Calabi et al. 2001). By investigating the pattern of sequence conservation among different *C. difficile* isolates, a number of other genes encoding putative amidases, known as *slpA* paralogs, were found in the vicinity of *slpA* (Calabi and Fairweather 2002).

The *slpA* gene was identified in a genetic locus carrying 17 ORFs, 11 of which encode putative surface-anchored proteins with adhesive properties (Calabi et al. 2001; Karjalainen et al. 2001). Transcriptional analysis of this putative virulence cluster revealed that the *slpA* gene is strongly transcribed during the entire growth phase as a bicistronic transcript (Savariau-Lacomme et al. 2003). The isolation and investigation of this cluster is an important step in the characterization of the process of colonization by *C. difficile*.  


McCoubrey and coworkers used a novel phenotypic typing method to determine the level of *Clostridium difficile* colonization and disease in a population of elderly patients (McCoubrey et al. 2003). This so-called “S-typing” is a simple method which utilizes the high degree in variation of the molecular masses of the two *C. difficile* S-layer proteins to compare the strains in the patients and in the ward environment. With the aid of this typing technique, the endemic nature of *C. difficile* in a geriatric population and the degree to which their environment is contaminated could be demonstrated (McCoubrey et al. 2003).

Karjalainen and coworkers found that the sequence of the variable regions of slpA were strictly identical in a given serogroup of *C. difficile* but divergent between serogroups (Karjalainen et al. 2002). Based on the strong correlation to serogroup designation, slpA sequence typing could constitute a reproducible and reliable alternative to *C. difficile* serotyping and seems to be especially useful when specimens contain only small numbers of *C. difficile* cells or are inappropriate for culturing (Kato et al. 2005). Sequence and phylogenetic analysis of the complete slpA gene sequence from 14 PCR-ribotypes of *C. difficile* also confirmed that the slpA sequence is strongly related to serogroup designation whereas PCR ribotyping that is based on the polymorphism of the intergenic rRNA region represents a more discriminatory typing method (Ní Eidhin et al. 2006). The results of a recent study suggest that the combination of PCR-ribotyping with PCR-RFLP analysis of slpA could be more suitable for studying *C. difficile* epidemiology (Poilane et al. 2007).

*C. difficile* S-layer proteins were shown to be involved in the mechanism of gut colonization and in the process of adhesion to the intestinal mucosa (Calabi et al. 2002; Cerquetti et al. 2002). It has been proposed that S-layer proteins could mediate the binding to both the intestinal epithelial cells and some components of their extracellular matrix fibers, contributing to further tissue damage (Calabi et al. 2002; Cerquetti et al. 2002). Microscopic and biochemical approaches provided evidence that the HMW peptide functions as an adhesin which mediates adherence of *C. difficile* to host cells (Calabi et al. 2002).

In a recent study, the ability of *C. difficile* S-layer proteins to modulate the function of human monocytes and dendritic cells (DC) and to induce inflammatory and regulatory cytokines was demonstrated (Ausiello et al. 2006). S-layer proteins may thus fine-tune the equilibrium of Th1/Th2 response and affect antibody responses. The fact that S-layer proteins are a target of antibody response in patients with *C. difficile*-associated diarrhea has already been demonstrated (Drudy et al. 2004). Host antibody response plays an important role in protection, in particular IgM anti S-layer proteins have been associated with a reduced risk of recurrent *C. difficile*-associated diarrhea in humans. In this context, a protective effect of anti-S-layer protein serum has also been observed in a lethal hamster challenge model (O’Brien et al. 2005). The potential mechanism of action of the antiserum was shown to be through enhancement of *C. difficile* phagocytosis. Taken together, these data are of interest in the light of the possible use of S-layer proteins in a multicomponent vaccine against *C. difficile* infections for high-risk patients.
2.6.3 *Lactobacillus* sp.

The lactobacilli are one of the industrially most important group of bacteria; this is reflected by the accumulation of genomic data on these bacteria (for review see Pfeiler and Klaenhammer 2007). Due to their GRAS (generally regarded as safe) status, these organisms are used in a variety of ways, including food production, health improvement, and production of macromolecules, enzymes, and metabolites. Lactobacilli are Gram-positive, non-sporulating rod-shaped bacteria, with many of them being confined by an S-layer. Several strains colonize important ecological niches, such as the oral cavity or the intestine of humans and higher animals. The application potential of lactobacilli is based on a profound characterization of these organisms; in particular, a detailed understanding of the properties linked to their S-layer proteins is necessary to improve the knowledge of the interactions between the bacterial cells and the surrounding environments. This is reflected by the fact that, besides a few surface enzymes, the S-layer proteins are the most frequently described components of the otherwise rather poorly understood cell wall of the lactobacilli. While the past five to six years have seen only a few reports on new lactobacterial isolates that possess an S-layer or on reclassifications, research has been focusing on the development of improved methods for identification: e.g., real-time PCR in combination with nested reverse transcription PCR for monitoring of the viable cell number of *L. helveticus* in human feces (Saito et al. 2004) and a simple and rapid antibody-based method for detection of *L. kefir* in fermented milk (Garrote et al. 2005), and for analysis of S-layer proteins, on functional aspects of S-layers, as well as on the application potential of *Lactobacillus* S-layers.

Lactobacilli with a confirmed S-layer include *L. acidophilus*, *L. crispatus*, *L. casei*, *L. plantarum*, *L. brevis*, *L. buchneri*, *L. fermentum*, *L. bulgaricus*, *L. amylovorus*, *L. gallinarum* (Sleytr et al. 1996b), and *L. helveticus*, which has been reclassified as *L. suntoryeus* (Cachat and Priest 2005; Naser et al. 2006a), as well as several strains of enteric lactobacilli (Reniero et al. 1990). Recently, S-layer encoding genes of 21 *L. helveticus* strains were characterized; phylogenetic analyses based on the identified S-layer genes revealed two main clusters, one of which includes a sequence similar to the *slpH1* gene of *L. helveticus* CNRZ 892 and a second cluster including genes with similarity to *prtY* (Gatti et al. 2005). Within *L. helveticus* species, there is a high degree of variability in relation to the presence of plasmid molecules, possibly representing different evolutionary lineages (Ricci et al. 2006). Among recent S-layer-carrying isolates of lactobacilli are *L. kefir* and *L. parakefir* (Garrote et al. 2004), the 1,2-propanediol-degrading bacterium *L. diolivorans* sp. nov. (Krooneman et al. 2002) and *L. kefiranofaciens*, which is a later synonym for *L. kefirgranum* (Vancanneyt et al. 2004), as well as *L. amylo-trophicus* sp. nov. (formerly *L. amylophilus*) (Naser et al. 2006b). On *L. johnsonii* and *L. gasseri* cells, the presence of two surface proteins with compositional characteristics similar to S-layer proteins and functioning as aggregation promoting factors have been visualized by transmission electron microscopy (TEM) and SDS-PAGE (Ventura et al. 2002). Another S-layer-related protein according to in silico
analyses is the cell division protein CdpA from *L. acidophilus* NCFM; however, due to the low amount of this protein it is unlikely that it is involved in S-layer formation (Altermann et al. 2004).

So far, S-layer structural genes of many lactobacilli have been sequenced (see Table 2.2). The primary structure of the encoded S-layers predict proteins of 25–71 kDa with basic isoelectric points (pI > 9) due essentially to lysine-rich terminal regions that confer a positive charge to various S-layer proteins with rather conserved C-terminal amino acid sequences but otherwise limited amino acid sequence similarity. With regard to gene regulatory aspects, *L. acidophilus* ATCC 4356 is best investigated. To account for the high number of S-layer protein subunits that are required to ensure a complete coverage of the bacterial cell with a closed S-layer lattice during all stages of the growth cycle, the S-layer gene *slpA* was shown to be transcribed from a very strong promoter (with twice the strength of the lactate dehydrogenase promoter, which is considered one of the strongest bacterial promoters) (Pouwels et al. 1997). Additionally, the 5′-untranslated leader sequence (UTLS) of the *slpA* gene contributes to mRNA stabilization by producing a 5′ stem and loop structure (Narita et al. 2006). *L. acidophilus* ATCC 4356, which possesses two spontaneously interchangeable S-layer genes, *slpA* and *slpB*, was also one of the first organisms to study S-layer protein variation at the molecular level (Boot et al. 1996). Change of S-layer expression in response to environmental conditions has been found in *L. brevis* ATCC 14869 (Jakava-Vilijanen et al. 2002). For that organism, three *slp* genes (*slpB*, *slpC*, and *slpD*) were identified, whose differential expression could be linked to a reversible alteration of colony morphology under different growth conditions. Under aerobic conditions, R-colony type cells produce both SlpB and SlpD proteins, whereas under anaerobic conditions, S-colony-type cells synthesize essentially only SlpB. Northern blot analysis demonstrated that *slpB* and *slpD* form a monocistronic transcription unit and are effectively expressed, but *slpD* expression is induced under aerated conditions; *slpC* was silent under the tested conditions. Promoter analysis suggests that the variation of S-layer protein content involves activation of transcription by a soluble factor rather than DNA rearrangements, which are typical for most of the known S-layer phase variation mechanisms. The presence of silent S-layer genes, termed *lgsA* and *lgsB*, respectively, was also reported for different isolates of *Lactobacillus gallinarum* cultured from the crops of broiler chickens. In these organisms, only a second, strain-specific, S-layer gene is expressed both in vivo and in vitro (Hagen et al. 2005). It is hypothesized that gene duplication and S-layer sequence variation is a means for coexistence of the bacteria in the same habitat. It is important to note that in lactobacilli which contain more than one S-layer gene, the construction of S-layer knockout mutants has so far been unsuccessful. On the other hand, S-layer gene variability is not necessarily present in lactobacilli. The molecular background of strong *Lactobacillus* S-layer gene expression constituted the basis for the development of a high-level protein expression system in lactic acid bacteria by combining the UTLS of the *slpA* gene form *L. acidophilus* with the core promoter sequence of the heterologous protein to be produced (Narita et al. 2006). Another line of development utilized a cryptic plasmid pKC5b from *L. fermentum* for the
construction of a *Lactobacillus* — *E. coli* shuttle vector that was shown to be suitable for *slpA* gene expression in a heterologous *Lactobacillus* strain, which additionally demonstrated surface-bound expression of the S-layer protein (Pavlova et al. 2002). Lindholm and coworkers (2004) used the signal sequence of the *L. brevis* S-layer protein for heterologous protein secretion in *Lactococcus lactis*, which was shown to yield a significantly higher secretion rate than the signal peptide of *L. lactis* Usp45.

S-layer self-assembly is a key characteristic of *Lactobacillus* S-layer proteins that is also pivotal to many of the applications envisaged. Interestingly, all lactobacillar S-layer arrays that have been analyzed for their lattice properties exhibit a morphologically similar, oblique (p2) lattice structure (see Table 2.2). Based on the knowledge of the primary sequence, detailed structure–function analyses of *Lactobacillus* S-layer proteins have been performed. Sequence comparison of the 43 kDa *SlpA* protein of *L. acidophilus* ATCC 4356 with S-layer proteins from *L. helveticus*, *L. crispatus*, and the S-layer proteins encoded by silent genes suggested the presence of two domains, one comprising the N-terminal two-thirds (SAN), and another making up the C-terminal one-third (SAC) of the protein. While the N-terminal sequence is variable, the C-terminal domain is highly conserved in the S-layer proteins of these organisms and contains a tandem repeat (Smit et al. 2001). Analysis of the SAN domain by insertion and deletion mutagenesis in combination with proteolytic treatment identified this protein region as a crystallization domain, consisting of a ~12 kDa and a ~18 kDa C-terminal subdomain linked by a surface-exposed loop. Mutant *SlpA* protein synthesized in *E. coli* with 7–13 amino acid insertions of the c-myec epitope indicated that insertions in conserved regions or in regions with predicted secondary structure elements (positions 30, 67, 88 and 156) destroy the crystallization capacity (Smit et al. 2002). Thus, it is proposed that the regions of higher conservation are responsible for either intra- or intermolecular subunit interaction and, hence, are important for domain structure–function. SAC was identified as the cell wall binding domain of *SlpA*, with an acid-labile peptidoglycan-associated compound serving as a mediator for binding (Smit and Pouwels 2002). It consists of a tandemly repeated ~65 amino acid sequence with a conserved tyrosine doublet. The two repeats (the N-terminal repeat SAC1 and the C-terminal repeat SAC2) share 26% identical amino acids, most of which are basic and aromatic. SAC shows homology to carbohydrate-binding regions of *Clostridium difficile* toxins and cell wall-associated proteinases of lactic acid bacteria. Although the SAC repeats and SLH domains have similar sizes, they do not show amino acid sequence similarity. In addition, secondary structure determinations predict a β-stranded structure for SAC1 and SAC2 but a helix–loop–helix structure for SLH. Interestingly, it was shown that SAC2 can be deleted without compromising the cell wall binding capacity or proteolytic cleavage. This indicates that SAC1 is both a structural and a functional unit, whereas SAC might provide strength to the cell wall interaction of SAC without possessing direct binding capacity. The second functionally characterized *Lactobacillus* S-layer protein is the 410-amino-acid protein CbsA from *Lactobacillus crispatus* (Antikainen et al. 2002). Stepwise truncation of this S-layer protein from both
termini revealed that the region comprising amino acids 32–271 carries the information for self-assembly of CbsA into a periodic structure. Short deletions or substitutions in the border regions 30–34 and 269–274, which are conserved in valine-rich short sequences, affect the morphology of self-assembly products, which vary from sheet-like to tubular appearance. This observation is interpreted by increasing destabilization of the formed self-assembly structure through loss of intermolecular interactions. The basic C-terminal part of CbsA binds to lipoteichoic acid and teichoic acids and functions to anchor the S-layer to the lactobacillar cell wall. Summarizing, these studies confirmed that *Lactobacillus* S-layer proteins can be seen as composite molecules with two structurally and functionally independent domains (see Fig. 2.3c), whose additional adhesive properties are located in the N-terminal region. Recently, single molecule atomic force microscopy (AFM) was used to gain insight into the molecular forces driving the folding and assembly of the S-layer protein CbsA (Verbelen et al. 2007). To address the N-terminal and C-terminal regions of the protein, genetically engineered His-tagged entire mature CbsA as well as several peptides were coupled onto AFM tips, supports were modified with mixed self-assembled monolayers, and the surface morphology of the modified surfaces was characterized. This study revealed that secondary structures of the entire CbsA protein and of its N-terminal region can be unfolded using relatively small forces, suggesting that they consist of α-helices rather than β-sheets. By contrast, the C-terminal region cannot be unfolded but shows large, single adhesion events attributed to electrostatic intermolecular bridges involving cationic lysine residues.

The probiotic properties of lactobacilli have stimulated various types of research on the possible roles of S-layer proteins in adherence to specific host tissues. In this context, the specific surface properties mediated through the S-layer proteins are of major importance. A variety of strains from the genus *Lactobacillus* was investigated with respect to structure, softness, and interactions of their S-layers in order to construct structure–property relations (Schär-Zammaretti and Ubbink 2003). In this context it is important to note that for *L. acidophilus* it was demonstrated that its physicochemical surface properties are influenced by the composition of the fermentation medium (Schär-Zammaretti et al. 2005). In particular, in the absence of peptones, the expression of the S-layer protein is strongly enhanced, which suggests that the S-layer protein is preferentially expressed under conditions which are not optimal for bacterial growth; this is in line with a postulated protective effect whereby the S-layer is expressed in response to a stress factor. Many efforts were put into the improvement of microscopic and biophysical techniques for probing cell surface hydrophobicity and bacterial interactions with a host or a material surface (Ubbink and Schär-Zammaretti 2005). In particular, the flexibility of AFM in probing various types of physical interactions provides prospects for the elucidation of adhesion maps and their relationship to biological and structural data. Imaging of different *Lactobacillus* strains with AFM revealed major differences in the surface topography depending on the presence or absence of an S-layer. Force volume images calculated into elasticity and adhesion force maps showed that
L. crispatus and L. helveticus have a surface with a homogeneous stiffness without adhesion events, most likely caused by the S-layer. In contrast, for the S-layer-deficient strains L. johnsonii DSM 20533 and ATCC 33200 high adhesion forces were observed, which can be related to a surface rich in polysaccharides (Schär-Zammaretti and Ubbink 2003). While the S-layer has been known to convey hydrophobicity to the lactobacillar cell surface (van der Mei et al. 2003), it was interesting to find by AFM studies that S-layer-carrying strains do not necessarily adhere better to hydrophobic substrates than strains without an S-layer (Vadillo-Rodríguez et al. 2004, 2005). Moreover, the tested strains exhibited a dynamic cell surface hydrophobicity in dependent on the ionic strength of the medium. Lactobacillus strains with an S-layer (e.g. L. acidophilus ATCC 4356) were found to be hydrophobic in 10 mmol/L KCl solution and became more hydrophilic in 100 mmol/L, while it was the opposite case for strains without an S-layer (e.g. L. crispatus). This observation suggests that cell surfaces of lactobacilli may adapt their hydrophobicity in response to environmental changes, like ionic strength or pH. The ability to adhere is thought to be important to lactobacilli in establishing or maintaining selective colonization. In general, S-layers have been shown to function as adhesins mediating the adherence of Lactobacillus cells to host epithelial cells and/or matrix proteins (Lorca et al. 2002; Åvall-Jääskeläinen and Palva 2005; Buck et al. 2005). In particular, the involvement of the S-layer in adhesion to erythrocytes has been demonstrated for many Lactobacillus species (Toba et al. 1995; Boris et al. 1997). The S-layer of an L. acidophilus isolate has been reported to act as an afimbrial adhesin in vitro, as it is involved in the interaction with avian epithelial cells (Schneitz et al. 1993; Edelman et al. 2002). The S-layer SlpA of L. brevis ATCC 8287 was identified as an adhesion with affinity for human epithelial cells and fibronectin. Using a flagellum display model of SlpA fragments on E. coli cells, the receptor-binding region was located within a fragment of 81 amino acids in the N-terminal part of the S-layer (Hynönen et al. 2002). Binding characteristics of this S-layer protein to extracellular matrix proteins were refined through surface plasmon resonance studies. SlpA was found to interact with high affinity with fibronectin and laminin with respective binding constants of 90 mM and 27 mM, while the interaction with collagen and fibrinogen was much lower with respective binding constants of 32 mM and 26 mM (de Leeuw et al. 2006). For L. acidophilus M92, which manifests a high degree of hydrophobicity, the 45 kDa S-layer protein was shown to be responsible for autoaggregation and adhesion to mouse ileal epithelial cells (Kos et al. 2003; Frece et al. 2005). Very recent studies have given further insight into the probiotic properties of S-layer-carrying lactobacilli against pathogens. For coaggregating L. kefir strains, it was demonstrated that the S-layer protein antagonizes the interaction of Salmonella enterica serovar Enteritidis with epithelial cells (Gołowczyz et al. 2007). Similarly, the S-layer protein of L. crispatus ZJ001 is involved in the adhesion and competitive exclusion of pathogenic Salmonella typhimurium and E. coli O157:H7 to HeLa cells (Chen et al. 2007; Johnson-Henry et al. 2007). Decrease of pathogen adherence after pre-treatment of host epithelial cells with S-layer protein extracts
indicates that a non-viable constituent from a probiotic strain may prove effective in interrupting the infectious process of a pathogen. A similar effect has already been described for *L. crispatus* JCM 5810, which inhibited adhesion of diarrheagenic *E. coli* strains to reconstituted basement membrane preparations (Horie et al. 2002). In addition to the functions mentioned that are clearly attributable to the respective S-layer proteins, there were several reports on newly identified surface proteins that may or may not be associated with the S-layer. Among them is an aggregation-promoting factor for maintenance of cell shape in *L. gasseri* 4B2 (Jankovic et al. 2003), a surface protein Cpf of *L. coryniformis* DSM 20001 transmediating coaggregation with and aggregation among pathogens (Schachtsiek et al. 2004), and a surface protein from *L. fermentum* 104R that binds to porcine small intestinal mucus and gastric mucin (Rojas et al. 2002). Furthermore, a cell envelope protease was extracted from S-layer-carrying *L. helveticus* Zuc2 (Scolari et al. 2006).

The utilization of lactobacilli as in vivo delivery vectors for biologically active molecules has become increasingly attractive due to their non-pathogenicity and their ability to survive the gastrointestinal tract. In particular, strategies for obtaining periodic cell surface display of epitopes are being intensively investigated. It has already been demonstrated that S-layer protein subunits can be modified to carry foreign epitopes as a uniform recombinant S-layer on the *Lactobacillus* cell surface. The capacity of the SlpA S-layer protein of *L. acidophilus* ATCC 4356 to present epitopes, up to 19 amino acids residues in length, upon recrystallization of recombinant S-layer fusion protein on the surface of *Lactobacillus* cells that have been stripped off from their S-layer upon chemical treatment, makes this system suitable in principle as an oral vaccine vector (Smit et al. 2002). In vivo surface display of foreign epitopes was for the first time obtained via the *L. brevis* S-layer protein subunits and exemplified with the human 11-amino-acid c-\textit{myc} proto-oncogene. For this purpose, a gene replacement system was optimized for replacement of the wild-type *slpA* gene with the *slpA-c-\textit{myc}* construct (Åvall-Jääskeläinen et al. 2002). According to TEM evidence, a uniform S-layer was obtained displaying on its surface the desired antigen in all of the S-layer subunits. Based on the theoretically calculated presence of approximately $5 \times 10^5$ S-layer subunits on a *L. brevis* cell, with this cell surface display system it is possible to present at least such a large number of antigen epitope molecules on each cell. Surface display of vaccines as part of an S-layer would thus be a very effective way to present antigens to the mucosa-associated lymphoreticular tissue. In the context of vaccine development based on lactobacillar S-layer, in a recent study porcine-specific S-layer-carrying strains were analyzed for later use as vaccine vectors and/or probiotics (Jakava-Viljanen and Palva 2007). However, the amount of such organisms was rather low, and their adhesion affinities to various host tissues differed considerably. As a previous stage to the development of a vaccine vehicle for oral administration, positively charged liposomes were coated with the S-layer proteins from *L. brevis* and *L. kefir* (Hollmann et al. 2007). As already demonstrated with other S-layer proteins (Mader et al. 1999), the lactobacillar S-layers increased the stability of the liposomes when resuspended in bile salts and pancreatic extract and under thermal shock.
2.6.4 Geobacillus stearothermophilus strains

The genus *Geobacillus stearothermophilus* comprises Gram-positive, strictly aerobic species of endospore-forming bacteria. Many of them are covered with crystalline S-layers of different lattice geometry (Sleytr 1978; Messner et al. 1984; Sleytr et al. 2002). Among them are also strains with a glycosylated S-layer protein. Since prokaryotic protein glycosylation in general is now a rapidly expanding field and the most frequently observed modification of S-layer proteins (for reviews see Messner and Sleytr 1991, 1992; Sumper and Wieland 1995; Eichler and Adams 2005; Szymanski and Wren 2005; Messner et al. 2008), we have focused considerable efforts on the elucidation of this important post-translational protein modification. Currently, the best investigated bacterial S-layer glycoprotein is that of the Gram-positive, moderately thermophilic organism *G. stearothermophilus* NRS 2004/3a (Sleytr and Plohberger 1980; Küpcü et al. 1984; Messner et al. 1984; Messner et al. 1986b; Schäffer et al. 2002; Novotny et al. 2004a, b; Steiner et al. 2006, 2007; Bindila et al. 2007; Messner et al. 2008). The surface of this strain is covered by an oblique S-layer lattice composed of identical glycoprotein subunits. The S-layer glycan chains protrude from the cell surface and may create a hydrophilic coat around the bacterial cell comparable to LPS O-antigens of Gram-negative bacteria (Messner et al. 1986b; Raetz and Whitfield 2002; see also Chap. 4). After isolation and purification of the glycosylated S-layer protein SgsE of *G. stearothermophilus* NRS 2004/3a, the glycan chain structure was determined by straightforward one- and two-dimensional $^1$H and $^{13}$C nuclear magnetic resonance spectroscopy. To the S-layer protein subunits are attached elongated glycan chains that are composed of, on average, 15 trisaccharide repeats with the structure $[-2]-\alpha-L-Rhap-(1\rightarrow3)-\beta-L-Rhap-(1\rightarrow2)-\alpha-L-Rhap-(1\rightarrow3)\eta$ and a 2-$O$-methyl modification of the terminal repeat at the non-reducing end of the glycan chain (Christian et al. 1986; Schäffer et al. 2002). The glycan chains are bound via core saccharides of, on average, two $\alpha$1,3-linked $\alpha-L-Rhap$ residues to carbon-3 of $\beta$-D-galactose residues. The entire 903-amino-acid-residues-long S-layer glycans are then attached via $O$-glycosidic linkages to different serine and threonine residues of the S-layer protein subunits (Schäffer et al. 2002). On SDS-PAGE gels four bands appear, three of which represent glycosylated S-layer proteins. Due to the lack of adequate analytical techniques, a conclusive interpretation of the multiple banding pattern of this S-layer glycoprotein as observed by SDS-PAGE has been hampered in the past. Recently, straightforward mass spectrometry methods have allowed the accurate determination of the average masses of the three inherently heterogenic glycoprotein species of SgsE to be 101.66 kDa, 108.68 kDa, and 115.73 kDa, corresponding to SgsE with different numbers of attached glycan chains (Steiner et al. 2006). Each of the glycoforms revealed nanoheterogeneity with variation between 12 and 18 trisaccharide repeats and the possibility of extension of the already known di-rhamnose core region by one additional rhamnose residue (Steiner et al. 2006). On the 93-kDa SgsE S-layer protein, three glycosylation sites could be unequivocally identified, namely at positions threonine-590, threonine-620, and...
serine-794. These data led to the logical interpretation that in the 101.66-kDa glycoform only one glycosylation site is occupied, in the 108.68-kDa glycoform two glycosylation sites are occupied, and in the 115.73-kDa glycoform all three glycosylation sites are occupied (Steiner et al. 2006). However, it is not yet known which of the sites are actually occupied and how long the attached glycans are in the in vivo situation. This task could only be approached by single molecule analysis of SgsE subunits, which is far beyond the resolution power of today’s analytical techniques.

Recently, the structural gene sgsE encoding the S-layer protein of G. stearothermophilus NRS 2004/3a was identified by polymerase chain reaction-based techniques (Schäffer et al. 2002). The ORF codes for a protein of 903 amino acids, including a leader sequence of 30 amino acids. The mature S-layer protein has a calculated molecular mass of 93,684 Da and a pI of 6.1. In addition to sgsE, the structural genes coding for S-layer proteins of four other strains of G. stearothermophilus strains, termed sbsA–sbsD, have now been characterized (Egelseer et al. 2001; Schäffer et al. 2002; Novotny et al. 2004b). Comparison of the amino acid sequences of the G. stearothermophilus S-layer proteins SbsA–D (protein identification CAA50409, CAA66724, AAC12757, AAF34763, respectively) with SgsE (protein identification AAL46630) showed that they share similarities with respect to the following features: (1) they are synthesized with a typical N-terminal signal sequence consisting of 30 (SbsA, SbsC, SbsD, and SgsE) and 31 (SbsB) amino acids, respectively; (2) the signal peptide cleavage site is between the Ala–Ala motif, consistent with the proposed recognition sequence for signal peptidases; (3) all G. stearothermophilus S-layer proteins exhibit a weakly acidic isoelectric point. The highest sequence identities are found within the N-terminal regions of the S-layer proteins (Schäffer et al. 2002). In contrast, the N-terminus of SbsB does not reveal significant sequence homology; instead, it is the only one among the G. stearothermophilus S-layer proteins compared to possess an S-layer homology (SLH) domain (Lupas et al. 1994) between amino acids 31 and 168 (Sára et al. 1998).

Glycosylation of SgsE in the C-terminal region of the bacterial cell wall (see also Sect. 2.5). Recently, a diacetamidodideoxyuronic acid-containing glycan of G. stearothermophilus NRS 2004/3a with the repeating unit structure \( [\rightarrow4]-\beta-D-ManpA2,3(NAc)2-(1\rightarrow6)-\alpha-D-Glcp-(1\rightarrow4)-\beta-D-ManpA2,3(NAc)2-(1\rightarrow3)-\alpha-D-GlcpNAc-(1\rightarrow)_{n-5} \) was examined to identify its linkage to the bacterial cell wall. This glycan represents the secondary cell wall polymer (see Sect. 2.5) of G. stearothermophilus NRS 2004/3a (Schäffer et al. 1999). In the meantime, chemical characterization of secondary cell wall polymers from all G. stearothermophilus strains analysed so far that do not contain SLH domains in their S-layer protein, showed that they possess diacetamidodideoxyuronic acid-containing secondary cell wall polymers of identical structure (Schäffer and Messner 2005).

In the course of the genetic characterization of the G. stearothermophilus NRS 2004/3a S-layer glycoprotein glycosylation, the ~16.5 kb surface layer glycan biosynthesis (slg) gene cluster has been sequenced (GenBank AF328862) (Novotny et al. 2004a, b). The cluster is located immediately downstream of the S-layer
structural gene sgsE and consists of 13 ORFs that have been identified by database sequence comparisons. The cluster comprises genes encoding enzymes for dTDP-\(L\)-rhamnose biosynthesis (\(rml\) operon), required for building up the polyrhamnan S-layer glycan, as well as those for assembly and export of the elongated glycan chain, and its transfer to the S-layer protein. There is evidence that this cluster is transcribed as a polycistronic unit, whereas sgsE is transcribed monocistronically (Novotny et al. 2004b). Chromosomal DNA preparations of several \(G.\) \(stearothermophilus\) strains with glycosylated (NRS 2004/3a, GenBank AF328862) and without glycosylated S-layer proteins (L32-65, GenBank AY278518; ATCC 12980\(^T\), GenBank AY278519) were screened for the presence of the \(rml\) operon, because \(L\)-rhamnose is a frequent constituent of S-layer glycans. The flanking regions of the operon were sequenced from \(rml\)-positive strains. Comparison with the \(slg\) gene cluster of \(G.\) \(stearothermophilus\) NRS 2004/3a (GenBank AF328862) revealed sequence homologies between adjacent genes. Cell-free extracts of the strains were capable of converting dTDP-\(D\)-glucose to dTDP-\(L\)-rhamnose. These results indicate that the \(rml\) locus is highly conserved among \(G.\) \(stearothermophilus\) strains, and that in the \(rml\)-containing strains investigated, dTDP-\(L\)-rhamnose is actively synthesized in vitro (Novotny et al. 2004b), even if the respective S-layer proteins are non-glycosylated.

The sequence determination of the genes of the \(slg\) gene cluster of \(G.\) \(stearothermophilus\) NRS 2004/3a was the basis for the functional characterization of the different enzyme proteins encoded. The biochemical characterization of WsaP showed this gene as a UDP-Gal:phosphoryl-polyprenol Gal-1-phosphate transferase that primes the S-layer glycoprotein glycan biosynthesis of \(G.\) \(stearothermophilus\) NRS 2004/3a and is therefore the initiation enzyme of the glycosylation reaction (Steiner et al. 2007). It was demonstrated that the enzyme transfers in vitro a galactose-1-phosphate from UDP-galactose to endogenous phosphoryl-polyprenol and that the C-terminal half of WsaP carries the galactosyltransferase function, capable of reconstituting polysaccharide biosynthesis in WbaP-deficient strains of \(Escherichia\) \(coli\) and \(Salmonella\) \(enterica\) serovar Typhimurium (Steiner et al. 2007). For a better understanding of the general glycosylation process of bacterial S-layer glycoproteins, further studies of genes involved in S-layer protein glycosylation of different organisms are required.

Other important \(G.\) \(stearothermophilus\) species include \(G.\) \(stearothermophilus\) PV72 and \(G.\) \(stearothermophilus\) ATCC 12980\(^T\). The first S-layer gene of this species which was sequenced and cloned was the S-layer gene \(sbsA\) (Kuen et al. 1994) of the wild-type strain \(G.\) \(stearothermophilus\) PV72/p6 exhibiting a hexagonally ordered S-layer lattice (Sára et al. 1996). Upon cultivation under unphysiologically high oxygen concentrations, the hexagonal S-layer lattice of the wild-type strain formed by SbsA was replaced by an oblique lattice type consisting of the S-layer protein SbsB of the variant designated PV72/p2 (Sára et al. 1996). Sequencing of the \(sbsB\) gene (Kuen et al. 1997) revealed that the S-layer protein SbsB carries three SLH motifs at the N-terminal part and showed only 25% identity to SbsA. On the molecular biological level, variant formation was found to depend on recombination events between a megaplasmid and the chromosome (Scholz et al.
In addition to the change of the S-layer protein, the synthesis of a different type of SCWP was observed (Sára et al. 1996). In the variant strain, the non-pyruvylated, 2,3-diacetamido-2,3-dideoxymannuronic acid-containing SCWP of the wild-type strain was replaced by another type of SCWP which contained GlcNAc and ManNAc as the major components and pyruvate ketals as net negatively charged groups (Ries et al. 1997; Sára et al. 1998; Sára 2001). In a very recent study, the structure of this SCWP has been resolved by NMR (Petersen et al. 2008). Interestingly, the S-layer protein SbsA recognized both types of SCWPs as binding sites, guaranteeing complete coverage of the cell surface during the oxygen-induced switch. On the other hand, the S-layer protein SbsB showed only affinity for binding of the newly synthesized SCWP.

For biophysical characterization, SbsB was cloned and expressed as two separate but complementary parts, namely, the N-terminal part defined by the three consecutive S-layer homologous motifs and the remaining large C-terminal part (Rünzler et al. 2004). By applying this dissection approach, the SLH-domain of SbsB (rSbsB32–208) was found to be exclusively responsible for SCWP binding whereas the larger C-terminal part represents the self-assembly domain. Furthermore, circular dichroism spectroscopy studies confirmed that most α-helical segments are arranged in the N-terminal SLH domain, whereas the middle and C-terminal part could be characterized as a β-sheet protein (Rünzler et al. 2004). The C-terminal part of SbsB was found to be highly sensitive against deletions since the removal of even less than 15 amino acids led to water-soluble S-layer protein forms (Howorka et al. 2000; Moll et al. 2002).

The interaction of the S-layer protein SbsB of *G. stearothermophilus* PV72/p2 and the corresponding SCWP was assessed by surface plasmon resonance (SPR) biosensor technology using native and chemically modified SCWPs devoid of pyruvic acid residues. The interaction proved to be highly specific for the carbohydrate component and glycan pyruvylation was found to be an essential requirement (Mader et al. 2004).

The cell surface of the type strain of that species, *G. stearothermophilus* ATCC12980\(^T\), is completely covered with an oblique S-layer lattice formed by the S-layer protein SbsC, which was also found to be an adhesion site for a high-molecular-mass exoamylase (HMMA) (Egelseer et al. 1996). After elucidation of the *G. stearothermophilus* S-layer genes *sbsA* and *sbsB* of PV72/p6 and PV72/p2, respectively, *sbsC* was the third S-layer gene of this species to be sequenced and cloned (Jarosch et al. 2000). The entire *sbsC* sequence showed an ORF of 3,297 bp predicted to encode a protein of 1,099 amino acids with a theoretical molecular mass of 115,409 Da and an isoelectric point of 5.73. The elucidation of the S-layer gene sequence opened the possibility of investigating whether sequence identities and a common structure–function relationship exist in S-layer proteins of *G. stearothermophilus* wild-type strains.

Thus, in a first approach, different N- or C-terminally truncated S-layer proteins were produced heterologously and their self-assembly and recrystallization properties were investigated (Jarosch et al. 2001). Based on these results, the S-layer proteins could be characterized by two functionally and structurally separated parts, namely a
highly conserved N-terminal region which interacts with an SCWP composed of N-acetylglucosamine (GlcNAc), glucose, and 2,3-diacetamido-2,3-dideoxymannuronic acid, and the larger C-terminal part responsible for formation of the crystalline array (Jarosch et al. 2001). Interestingly, SbsC turned out to be highly tolerant against deletions, since significant portions at the N- or C-terminal part could be deleted without losing the capability of the subunits for lattice formation (Jarosch et al. 2001).

On the other hand, water-soluble N- or C-terminally truncated forms of SbsC were found to be well suited for first three-dimensional (3D) crystallization studies, thereby circumventing the intrinsic property of S-layer proteins to form two-dimensional lattices which prevent the formation of isotropic 3D crystals. For the C-terminally truncated form rSbsC31–844, crystals which diffracted to a resolution of 3 Å using synchrotron radiation could be obtained (Pavkov et al. 2003). 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 (Pavkov et al. 2003). In a very recent study, refinement of preliminary data led to the first high-resolution structure of the soluble N-terminal form rSbsC31–844, showing a very elongated and flexible molecule, with strong and specific binding to the secondary cell wall polymer (SCWP) (Pavkov et al. 2008). The crystal structure of rSbsC31–844 revealed a novel fold, consisting of six separate domains which are connected by short flexible linkers. Furthermore, SCWP binding induces considerable stabilization of the N-terminal domain.

Also recently, the basic interaction in the mechanism anchoring an S-layer protein devoid of S-layer-homologous (SLH) motifs to the rigid cell wall layer was systematically investigated by SPR biosensor technology, using the S-layer protein SbsC and the corresponding non-pyruvylated SCWP of *G. stearothermophilus* ATCC 12980T as the model system (Ferner-Ortner et al. 2007). Two C-terminal truncations of SbsC (rSbsC31–270 and rSbsC31–443) carrying the SCWP binding domain as well as one N-terminal truncation (rSbsC638–1099) comprising the residual part of SbsC were produced heterologously and used for affinity and SPR studies. The SPR data from both complementary experimental setups in which either the truncated rSbsC forms or the SCWP were immobilized on the sensor surface confirmed that the N-terminal region comprising the amino acid residues 31 to 270 was exclusively responsible for SCWP binding. Analysis of data from the setup in which SCWP was immobilized on a sensor chip and rSbsC31–270 or rSbsC31–443 represented the soluble analytes indicated a binding behaviour with low ($K_d = 9.32 \times 10^{-5}$ M and $2.95 \times 10^{-6}$ M), medium ($K_d = 4.8 \times 10^{-9}$ M and $1.22 \times 10^{-8}$ M), and high ($K_d = 1.94 \times 10^{-12}$ M and $2.05 \times 10^{-12}$ M) affinity (Ferner-Ortner et al. 2007).

### 2.6.5 *Lysinibacillus sphaericus*

*Lysinibacillus sphaericus* (formerly *Bacillus sphaericus*) represents a strictly aerobic group of mesophilic endospore-forming bacteria. The S-layer lattice of
*L. sphaericus* CCM 2177 shows square symmetry and is composed of identical subunits with an estimated relative molecular weight of 127,000. The in vitro self-assembly of this S-layer protein was found to be dependent on the presence of bivalent cations (Pum and Sleytr 1995). This S-layer protein recognizes a negatively charged SCWP as the binding site in the rigid cell wall layer. The SCWP of *L. sphaericus* CCM 2177 is composed of disaccharide repeating units that consist of \( N\)-acetylg glucosamine (GlcNAc) and \( N\)-acetylmannosamine (ManNAc). Each second ManNAc residue carries a pyruvate ketal, which endows the polymer chains with a negative net charge (Ilk et al. 1999).

The gene encoding the S-layer protein of *L. sphaericus* CCM 2177 was sequenced, cloned, and expressed in *E. coli* (Ilk et al. 2002). The entire *sbpA* sequence indicated one ORF of 3,804 bp encoding a protein of 1,268 amino acids with a theoretical molecular mass of 132,062 Da and a calculated isoelectric point of 4.69. Prediction of the secondary structure from sequence data as well as by far-UV circular dichroism spectroscopy indicated the concentration of \( \alpha\)-helices in the N-terminal part, whereas the middle and C-terminal parts are dominated by loops and short \( \beta\)-strands (Huber et al. 2005).

Comparative studies of the structure–function relationship as well as of the accessibility of the C-terminus in full-length rSbpA (rSbpA\(_{31-1268}\)) and the truncated form rSbpA\(_{31-1068}\) revealed that the deletion of 200 C-terminal amino acids did not interfere with the self-assembly properties of the S-layer protein but significantly increased the accessibility of the C-terminal end (Ilk et al. 2002).

To identify the SCWP-binding domain of SbpA, seven C-terminal truncations and one N- and C-terminal truncation of this S-layer protein, as well as two chimeric proteins comprising either the SLH-domain of the S-layer protein SbsB of *G. stearothermophilus* PV72/p2 and the residual sequence of SbpA, and vice versa, were produced recombinantly and used for SPR studies (Huber et al. 2005). Surprisingly, rSbpA\(_{31-202}\) solely comprising the three SLH motifs did not bind at all. The shortest C-terminal truncation with specific affinity to SCWP was rSbpA\(_{31-318}\). Contrary to the SLH-domain-carrying S-layer protein SbsB (Mader et al. 2004), in SbpA the 3 SLH motifs and an additional 58 amino acids long SLH-like motif located behind the third SLH motif were required for reconstituting the functional SCWP domain. In the C-terminal part of this S-layer protein, up to 237 amino acids could be deleted without interfering with the square lattice structure. Most important, it was demonstrated that the deletion of a further 113 C-terminal amino acids led to a change from square (p4) to oblique (p1) lattice structure (Huber et al. 2005).

### 2.6.6 Tannerella and Bacteroides

*Tannerella forsythia* (formerly *Bacteroides forsythus*) is a filamentous, Gram-negative, oral anaerobe, which has been implicated in the development of periodontitis (Tran et al. 2001). *T. forsythia* possesses a distinctive cell envelope
ultrastructure, with an S-layer appearing as a regularly arrayed lattice formed of serrated structural subunits (Kerosuo 1988) and lipopolysaccharide but no capsule located outside of the outer membrane. Sabet and coworkers (2003) have reported on the small-scale isolation and purification of the *T. forsythia* S-layer using sodium deoxycholate followed by caesium chloride isopycnic gradient centrifugation. SDS-PAGE analysis of the S-layer preparation revealed two major protein bands, named TfsA and TfsB, exhibiting molecular masses of 200/230 kDa and 210/270 kDa, respectively (values for molecular masses vary in literature depending on the percentage of the PA gel), both of which were reactive with the periodic acid–Schiff reagent, which is indicative of glycosylation (Higuchi et al. 2000; Sabet et al. 2003). For identification of the *tfsA* and *tfsB* genes, partial amino acid sequences from electrophoretically purified S-layer proteins were compared with the genome of *T. forsythia* (Lee et al. 2006b; Sakakibara et al. 2007). Two genes, *tfsA*, encoding a ~135-kDa protein with an isoelectric point of 7.85, and *tfsB*, encoding a rather basic ~152-kDa S-layer protein with an isoelectric point of 9.1, are located in contigs TF2661–TF2662, and TF2663, respectively. The differences between the calculated molecular masses of the S-layer proteins and those estimated from the PA gels indicate a high degree of glycosylation of TfsA and TfsB. Interestingly, *tfsA* and *tfsB* are located in tandem and are transcribed as an operon (Lee et al. 2006b).

There are several indications that the S-layer functions as a virulence factor. The S-layer proteins were clearly recognized by sera from patients with adult and early-onset periodontitis, whereas the IgG response against the antigen was low in healthy control individuals (Yoneda et al. 2003). Functional tests have furthermore revealed that the S-layer is highly responsible for adherence/invasion of *T. forsythia* to human gingival epithelial cells, mediates hemagglutination, and is involved in coaggregation with *Porphyromonas gingivalis*, another crucial oral pathogen (Sabet et al. 2003; Sakakibara et al. 2007).

Comparison of the amino acid sequences of TfsA and TfsB with the protein database indicated that these S-layer proteins share no overall homology to any other deposited S-layer protein sequence. There is, however, profound similarity (between 63% and 75%) of the C-terminal domains of the *T. forsythia* S-layer proteins and nine recently described proteins of the related organism *Bacteroides distasonis*, named DgpA–I (Fletcher et al. 2007). Interestingly, there are strong indications that the Dgp proteins are also glycosylated S-layer proteins. They are highly abundant in the intestinal symbiont *B. distasonis* and their expression was shown to be phase-variable, which is taken as an indication for the importance of these S-layer glycoproteins to the bacterial–host symbiosis (Fletcher et al. 2007).

### 2.6.7 *Caulobacter vibrioides* (formerly *Caulobacter crescentus*)

*Caulobacter vibrioides* is a Gram-negative, non-pathogenic aquatic bacterium that is covered by a hexagonal S-layer lattice composed of a single 98 kDa
protein species termed RsaA (Smit et al. 1981, 1992). This S-layer protein is non-covalently bound to the cell surface via a smooth lipopolysaccharide (LPS) species whose O-side chain is composed, at least in part, of N-acetylperosamine. This LPS species is at least involved in S-layer anchoring, since strains deficient in O-side chain biogenesis were found to shed the S-layer (Walker et al. 1994; Awram and Smit 2001).

In contrast to Gram-positive bacteria, no general S-layer anchoring motifs have been identified in Gram-negative organisms. In a recent study using reattachment assays, the RsaA anchoring region was found to lie in the N-terminal region comprising approximately the first 225 amino acids (Ford et al. 2007). Assembly into the hexagonal array was revealed to require calcium, which may mediate RsaA crystallization via calcium bridging between RsaA monomers (Smit et al. 1992; Walker et al. 1992). A calcium-binding motif located near the C-terminus of RsaA, the so-called RTX (repeats in toxin) motif, is likely to mediate this process (Gilchrist et al. 1992).

The rsaA gene (Gilchrist et al. 1992), which encodes the RsaA protein comprising 1,026 amino acids with a predicted molecular weight of 98,132 Da, was exploited to display foreign peptides on the cell surface in a dense, highly ordered manner (Bingle et al. 1997). While most of the characterized S-layer transport systems from Gram-negative bacteria involve type II secretion, the S-layer protein of *C. vibrioides* is secreted by a type I mechanism which requires two outer membrane proteins, RsaFa and RsaFb (Toporowski et al. 2004). Interestingly, the S-layer-associated metalloprotease Sap (Umelo-Njaka et al. 2002) which primarily uses the S-layer type I secretion apparatus recognized a cleavage site in truncated RsaA mutants but not in full-length RsaA (Ford et al. 2007). This could be explained by inappropriate or slower folding of the RsaA mutants, thus exposing this weak cleavage site to the Sap protease. It can be speculated that this extracellular membrane-bound protease could be part of a “remove and replace” strategy for repairing S-layer damaged by various environmental causes (Ford et al. 2007).

Comparative studies between different display systems to identify peptides mimicking bovine herpes virus 1 (BoHV-1) envelope glycoprotein gE proved the feasibility of the commercially available PurePro™ *Caulobacter* expression system (Invitrogen) also as a display system (Lehmann et al. 2004). In the latter, the S-layer protein RsaA is used as a scaffold displaying the peptide of interest.

In the course of efforts to use *C. vibrioides* as a vector for immunotherapy by expressing cancer-associated peptides from genetic insertions in the S-layer gene, live and unmodified cells of wild-type *C. vibrioides* revealed unexpected tumor suppressive effects in mice (Bhatnagar et al. 2006). These results suggest that *C. vibrioides* may be a safe, bacterial immunomodulator for the treatment of tumors.

### 2.6.8 Campylobacter fetus

*Campylobacter fetus*, a microaerophilic spiral Gram-negative bacterium, has been recognized as an important pathogen in animals important for food production and
humans (Blaser 1998; Thompson and Blaser 2000). *C. fetus* has been isolated from numerous hosts including mammals, birds, and reptiles. *C. fetus* may be either type A or type B based on serotype, lipopolysaccharide structure, and S-layer protein type (Dubreuil et al. 1990; Blaser et al. 1994; Dworkin et al. 1995). *C. fetus* is covered by an S-layer composed of high-molecular-weight protein subunits (Dubreuil et al. 1988; Pei et al. 1988; Fujimoto et al. 1991). The S-layer proteins have been shown to play a critical role in *C. fetus* virulence (Blaser et al. 1987; Pei and Blaser 1990; Blaser and Pei 1993; Blaser et al. 1994; Grogono-Thomas et al. 2000, 2003) by protecting the bacterium from phagocytosis and serum killing (Blaser et al. 1988).

The *C. fetus* S-layer proteins are encoded by multiple sap genes and vary in size. Identification and characterization of the 53.8-kb chromosomal region containing the entire sap locus (termed the “sap island”) in a wild-type strain showed that all eight complete sapA homologues share conserved regions at their 5’ regions, encode S-layer proteins from 96 kDa to 131 kDa that share similar characteristics, and can be divided into three phylogenetic groups based on their 3’ sequences (Tu et al. 2003). The extensive homologies in the sap island include both direct and inverted repeats, which allow DNA rearrangements, deletion, or duplication (Tu et al. 2004a). Thereby, each sapA homologue can reciprocally recombine with the others, with rearrangements permitting the creation of new homologs and their placement downstream of the unique sapA promoter. Genetic analyses of the sap islands and their boundaries in 18 different *C. fetus* strains suggest that the sap island was not acquired by recent horizontal gene transfer but is an ancient genomic constituent that has evolved differing genotypes that are plastic, perhaps enabling colonization of varied niches, in addition to antigenic variation (Tu et al. 2004a).

Genetic analyses of the sap locus were also found to be useful tools to determine the phylogenetic relationship of *C. fetus* strains of mammal or reptile origin (Tu et al. 2001). Although phenotypic testing did not definitively identify the organism in a patient suffering recurrent bacteremia, genotypic approaches utilizing PCRs specific for sapA, sapB, and reptile sap island insertion, as well as sequence analysis of sapD and 16 S rRNA in combination with RAPD, allowed identification of a *C. fetus* strain of reptile origin as a human pathogen (Tu et al. 2004b).

Data from challenge experiments using vaccinated ewes suggested that S-layer protein-expressing vaccines could protect animals from abortion and that this effect was independent of the S-layer protein expressed, indicating involvement of conserved epitopes in the S-layer protein. By epitope mapping of the conserved 184-amino-acid N-terminal region with rabbit anti-S-layer protein antisera by using overlapping synthetic 20-mer peptides, two putative epitopes were identified between amino acids 81 to 110 and 141 to 160. Conserved antigenic regions of the S-layer protein that induce protective immune responses may enable development of synthetic vaccine candidates for *C. fetus* ssp. *fetus*-associated ovine abortion (Grogono-Thomas et al. 2003).

Interestingly, the investigation of the antigenic property of the GroEL-like protein in *C. fetus* belonging to the heat shock protein (HSP) family HSP60 revealed the presence of a common immunodominant carbohydrate epitope between the S-layer
protein and the bacterial HSP (Hinode et al. 2002). Analyses of genotypic and phenotypic variation of \(C. \text{fetus}\) isolates from human patients with relapsing infections by comparing their \(sap\) type, \(sap\) island repertoire and organization, as well as the expressed S-layer proteins, point to at least three different mechanisms underlying recurrent \(C. \text{fetus}\) infections: (1) genetic rearrangement to up-regulate virulence, (2) rearrangement for antigenic variation, and (3) true latency of a fully virulent strain with later reactivation due to a change in the clinical status (Tu et al. 2005).

2.7 Conclusions

More than 50 years ago Houwink described the presence of a “macromolecular monolayer” in the cell wall of a \(\text{Spirillum}\) sp. (Houwink and Le Poole 1952; Houwink 1953). Soon after, similar arrays were reported in \(\text{Acinetobacter}\) and \(\text{Aquaspirillum}\) strains. Some 40 years ago for the first time “regular arrays of macromolecules” were identified on the cell surface of living \(\text{Bacillus}\) and \(\text{Clostridium}\) cells (Sleytr et al. 1967, 1968). Nevertheless, since the classical working strains in microbiology, molecular biology, and genetics, \(\text{Escherichia coli}\) and \(\text{Bacillus subtilis}\), did not reveal such structures, it took a long time before the broad distribution and relevance of this cell wall structure was appreciated. In a previous review (Sleytr 1978) 80 species were already listed and currently about 800 bacterial and archaeal organisms are known to possess S-layers. In other words, S-layers can now be considered as one of the most commonly observed prokaryotic cell envelope structures. Moreover, composed of single subunits endowed with the ability to assemble into monomolecular arrays, they represent the simplest type of protein membrane developed during evolution (Sleytr and Plohberger 1980). Since S-layer-carrying organisms were demonstrated to be ubiquitous in the biosphere and S-layers are integral components of a variety of cell envelopes, it became obvious that they must provide the organisms with an advantage of selection in very difficult habitats.

Despite the significant increase in knowledge of the structure, chemistry, assembly, and genetics of S-layers over recent decades, relatively few firm data are available on the biological significance and selection advantage to organisms of producing such metabolically expensive layers (Murray 1993). Finally, it should be taken into consideration that a simple S-layer-type protein membrane capable of dynamic growth could have initiated a barrier membrane in the early stage of biological evolution (Sleytr and Plohberger 1980). The cell division process observed in lobed (e.g. \(\text{Methanocorpusculum sinense}\)) (Pum et al. 1991) or rod-shaped (e.g. \(\text{Thermoproteus tenax}\)) (Messner et al. 1986a) archaea clearly demonstrated such morphogenetic potentials of S-layers.

Finally, the increasing wealth of information on the general principles of S-layers will promote their use in life and non-life science applications (see Chap. 16).

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