V. Functions of S-layers 1

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Abstract

Although S-layers are being increasingly identified on Bacteria and Archaea, it is enigmatic that in most cases S-layer function continues to elude us. In a few instances, S-layers have been shown to be virulence factors on pathogens (e.g. Campylobacter fetus ssp. fetus and Aeromonas salmonicida), protective against Bdellovibrio, a depository for surface-exposed enzymes (e.g. Bacillus stearothermophilus), shape-determining agents (e.g. Thermoproteus tenax) and nuclease factors for fine-grain mineral development (e.g. Synechococcus GL 24). Yet, for the vast majority of S-layered bacteria, the natural function of these crystalline arrays continues to be evasive. The following review up-dates the functional basis of S-layers and describes such diverse topics as the effect of S-layers on the Gram stain, bacteriophage adsorption in lactobacilli, phagocytosis by human polymorphonuclear leukocytes, the adhesion of a high-molecular-mass amylase, outer membrane porosity, and the secretion of extracellular enzymes of Thermoanaerobacterium. In addition, the functional aspect of calcium on the Caulobacter S-layer is explained.

Keywords: Gram stain; Cell wall; S-layer; Archaea; Protozoa; Bacteriovory; Crystalline outer membrane protein; Porin; Campylobacter fetus; Ovine abortion; Virulence factor; Glycosyl hydrolase; Cellulose; Cell wall anchoring; Caulobacter crescentus; Secretion; Adhesion; Amylase; Bacillus stearothermophilus; Lactobacillus; Bacillus; Phagocytosis; Polymerphagocytic leukocyte; Bacteria

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1. Introduction

Many bacteria and some eukaryotic algae possess surface layers (S-layers) composed of either proteins or glycoproteins. The protein subunits form porous lattices completely covering the cell. Since they are envelope structures exterior to the cell wall proper or even replace typical other cell wall structures, they fulfill a variety of biological functions and roles. Some of these roles are common to surface structures in general whereas others are specific for S-layers. Examples of the different roles are cell adhesion, protection from feeding by protozoa or phagocytes, virulence factor, antigenic properties, anchoring sites for hydrolytic exoenzymes, receptors for phages, porin function and others. In addition, these layers can affect Gram staining. Finally, there are other aspects (like assembly of the structures, modulation of their properties, excretion and regulation of their synthesis) which are interconnected with the biological functions of this cell structure. The various functional aspects will be discussed in this review.

2. The response of S-layered bacteria to the Gram stain

Terry J. Beveridge³

Microbiologists still rely on the Gram stain for the
initial screening of new isolates so that the isolates can be classified as being Gram-positive or Gram-negative. Although there have been many studies to determine the mechanism of the stain (for a review of these studies see [1,2]), most authorities have shown that the underlying staining principle relies on the type of cell wall a bacterium possesses [2–4]. All of these early studies used bacteria that possessed ‘classic’ Gram-positive (e.g. Bacillus subtilis or B. megaterium) or Gram-negative (e.g. Escherichia coli) cell walls without regard to extra superficial layers such as capsules, sheaths or S-layers. Also, at that time, prokaryotes had not been separated into Archaea (archaeobacteria) or Bacteria (eubacteria). It was therefore important to re-examine the Gram stain and its mechanism on a larger variety of microorganisms which included both of the archaean and bacterial taxonomic domains, especially representatives from each which possessed external superficial layers such as S-layers.

In 1983, Davies et al. were able to chemically define the mechanism of the Gram stain using nuclear magnetic resonance to follow the reactive species of the staining reagents [1]. Crystal violet (CV) is a chloride salt which dissociates into CV⁺ and Cl⁻ when dissolved in water. Both of these ions penetrate into bacteria (which are heat-fixed onto microscopical glass slides) during the initial step of the Gram stain. The bacteria are then immersed in Gram’s iodine (as a mordant solution) which contains highly reactive iodide (I⁻ or I₃⁻). When these ions enter the cells a metathetical anion exchange occurs whereby chloride is replaced by the bulkier iodide to form an insoluble CV-I complex which precipitates from solution. This occurs in the cytoplasm of both Gram-negative and Gram-positive cells at this stage of the staining regimen and it is this precipitate which forms the bulk of the purple coloration. It is the ethanol decolorization step which discriminates between Gram-negative and Gram-positive bacteria. Lipid bilayers are particularly sensitive to ethanol and dissolve. For Gram-negative cells, both the plasma (cytoplasmic) membrane and the outer membrane are disrupted and small punctures occur in the thin peptidoglycan layer through which the ethanol solubilized CV-I complex escapes [2]. These bacteria are, then, colorless and become red when counter-stained with carbol fuchsin or safranin. In this case, the counter-stain forms an ionic union with the remaining cell substance which retains its cellular shape.

Gram-positive bacteria do not possess outer membranes and are encased in a much thicker layer of peptidoglycan [5,6]; although the plasma membrane is disrupted by the decolorization step. Gram-positive walls are too thick and impermeable to allow the CV-I complex to escape [2]. Gram-positive cells are also dyed by the counter-stain but this only heightens the contrast of the CV-I (purple) color.

Recognizing the chemical mechanism of the Gram stain, one of the Gram reagents could be replaced with a newly synthesized electron opaque compound (potassium trichloro-(η²-ethylene)platinum II; TPt) that could react as effectively as iodide with CV; in this case, a purple CV-TPt precipitate is formed within cells [1]. In this way, using TPt, the Gram stain can be studied by both light and electron microscopy [2]. This technique has been especially advantageous since, for the first time, it has allowed minute structural differences within cells to be ascertained during staining, allowing subtle different staining responses to be seen [7–9]. Also, because the platinum in TPt produces a strong X-ray signal when illuminated by high energy electrons in a transmission electron microscope, the CV-TPt precipitates within cells can be easily identified and quantitated by energy dispersive X-ray spectroscopy (EDS); for more information on this technique see [10]. TPt can now be commercially obtained through the Aldrich Chemical Company, Milwaukee, WI, USA.

2.1. Gram stain, light microscopy (LM), transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDS)

Once cells had achieved a mid-exponential growth phase (usually an OD₆₀₀ = 0.3–0.5), they were harvested by centrifugation and processed by both the conventional and modified Gram stain regimens as outlined in Davies et al. [1]. TPt was either chemically synthesized according to the method of Chock et al. [11] or obtained from Aldrich Chem. Co.; both reagents gave identical results.

LM was used on all samples to ensure that the staining response was accurate. As eubacterial controls, Streptococcus pyogenes (Gram-positive) and

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E. coli (Gram-negative) were also processed for comparison with the S-layered bacteria. Stains for LM were done on heat-fixed smears of bacteria according to the following procedure. CV was used to flood the smear for 60 s, followed by flooding with an equal volume of Gram's iodine solution (conventional Gram procedure) or 50 mM TPt-modified procedure for an additional 60 s. The mordant stain was gently washed under running tap water (≈4°C) and blotted dry with absorbent filter paper. The smears were decolorized in a slow, steady stream of hydrous 95% ethanol for 30 s and were then washed with water, blotted dry, and counter-stained with carbol fuchsin for 60 s. The smears were then washed with water, blotted dry, and observed by light microscopy.

For electron microscopy and EDS, cells were harvested by centrifugation and washed three times in 25 ml of 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 6.8) containing 1 mM MgCl2. They were then put through either the conventional or TPt-modified Gram procedure as outlined above. More detail can be found in Fig. 1 of Davies et al. [1]. The cells were then reequilibrated to 50 mM HEPES buffer, fixed for 1 h in 5% (v/v) glutaraldehyde in buffer, washed (without heavy-metal staining), and processed into Epon 812 (CanEM, Guelph, Canada). For added contrast to decipher cellular structure after EDS analysis, some sections were stained with uranyl acetate and lead citrate as outlined in Beveridge and Davies [2].

EDS was performed on unstained thin sections, using the spectrum from the embedding resin as an index of specimen background radiation. Pt (Mα,βand Lα,β) lines were monitored for the Gram precipitate (the TPt-crystal violet complex). Spectral lines slightly upstream and downstream in the X-ray spectrum were used to establish readings of the continuum to ensure the validity of the Pt readings. Point analyses were performed using a Philips EM400T® equipped with a Link LZ-5 detector and a Link exl processor for EDS at 100 kV, using a spot size of 2.0–20.0 nm and an emission current of 80 μA. Counting times of 100–200 s (live time) were typically used.

2.2. S-layered bacteria

The B. brevis and Clostridium species were chosen because they represent both aerobic and anaerobic members of the eubacteria which possess S-layers and these bacteria are notorious for having a variable Gram staining response depending on their growth phase [7]. These bacteria do not have a thick peptidoglycan layer during their exponential growth phase (Fig. 1), have a variable response to the Gram stain and are, therefore, called Gram-variable [7], i.e. some bacteria from a single culture stain Gram-positive whereas others are Gram-negative. The variability is highest during the exponential growth phase. Presumably, during this point of active cell elongation and division in a culture, the cell's peptidoglycan metabolism is lagging behind cell growth which results in a thinner than normal peptidoglycan layer. It is possible that one of the reasons these bacteria possess an S-layer is because, at this point in time, their cell walls require the re-enforcement of such an extra layer. The B. brevis strain possessed a hexagonal array of center-to-center spacing equal to 17 nm, C. tetani a 15 nm hexagonal lattice, C. thermosaccharolyticum a 10 nm square lattice, and Butyrivibrio fibrisolvens a 18 nm hexagonal lattice (T. Beveridge, unpublished). These lattice formats and spacings were similar to those of other strains of each species [12].

B. thuringiensis was chosen because it also possesses an S-layer and, unlike those mentioned previ-
ously, it continually manufactures a thick robust cell wall similar to that of *B. subtilis* [13]. Like *B. subtilis*, *B. thuringiensis* stained Gram-positive throughout its vegetative growth phase.

When non-S-layered control bacteria, such as *E. coli* (Gram-negative) and *S. pyogenes* (Gram-positive), were processed by the modified TPt Gram stain they stained as expected by LM. In thin sections by TEM, very little cytoplasm remained in the cells of *E. coli*, few electron-dense (TPt) precipitates were seen, and little to no platinum signal could be detected by EDS [1,2]. The exact opposite was seen with *S. pyogenes*; the cells remained relatively intact, and contained cytoplasm and electron precipitates which gave a strong Pt signal by EDS (Fig. 2). *B. thuringiensis* produced exactly the same results as seen for *S. pyogenes*; the thick cell wall remained and entrapped CV-TPt precipitates within the cell. Very little of the S-layer remained after the ethanol decolorization procedure.

The results of the TPt-Gram stain by TEM confirmed the LM results for the *Bacillus-Clostridium-Butyribrio* group [7]. Lag and stationary growth phase cultures had a predominance of Gram-positive cells, whereas mid-exponential cells were frequently Gram-negative. Thin sections of the former revealed intact cells with relatively thick peptidoglycan layers (usually ~8–12 nm thick) with electron-dense cytoplasms which gave high Pt signals by EDS. On the other hand, mid-exponential cells were often undergoing lysis (Fig. 3) and possessed few electron-dense TPt precipitates in their cytoplasmic spaces (Fig. 4). It was apparent that, although the S-layers of these bacteria might be adding strength to the cell walls during exponential growth, they were not adding enough re-enforcement to resist the trauma of the

![Fig. 2. Thin section of *Streptococcus pyogenes* after using the TPt-Gram stain. The electron-dense precipitates of TPt and crystal violet are seen scattered throughout the cytoplasm. This thin section has been contrasted with uranyl acetate and lead citrate so that the cellular substance can also be seen. Bar = 100 nm. An energy dispersive X-ray spectrum of an uncontrasted thin section is shown in the inset and the TPt precipitates are identified by the Pt peaks (arrows). (This figure previously appeared in Microbiology [9] in an article by the author.)](image-url)
Gram stain. It was also difficult to ascertain whether or not the S-layer was still intact on the cell surface (Fig. 4).

*Aeromonas salmonicida* is a Gram-negative fish pathogen which possesses a tetragonal S-layer above its outer membrane [14]. Like *E. coli*, this bacterium stained Gram-negative with the TpT stain. During ethanolic decolorization the S-layer, the outer membrane and the plasma membrane were disrupted and small punctures were seen in the peptidoglycan layer through which the cytoplasmic contents and the CV-TpT precipitates escaped. Because there is a strong union between the S-layer and the lipopolysaccharide of the outer membrane [15], as ethanol dissolved the lipid constituent, the S-layer protein was also (presumably) liberated. In this way, the S-layer did not confer protection to the cells against the decolorization step of the Gram stain. Other S-layered Gram-negative bacteria (e.g. *Aquaspirillum serpens* VHA, *Caulobacter crescentus*, *Campylobacter fetus*

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Fig. 3. Thin section of *Butyribrio fibrisolvens* which is lying after the ethanol decolorization step of the TpT-Gram stain. Note the cytoplasm and electron-dense TpT precipitates which are escaping from the cell. The S-layer appears to have disrupted. Bar = 100 nm. (This figure previously appeared in J. Bacteriol. [7] in an article by the author.)

Fig. 4. Thin section of *B. brevis* after the TpT-Gram stain. Note that there are few cytoplasmic remnants left nor electron-dense TpT precipitates and that the S-layer is no longer apparent. Bar = 100 nm. (This figure previously appeared in J. Bacteriol. [7] in an article by the author.)
ssp. fetus, and Synechococcus GL 24) react in the same way (T. Beveridge, unpublished).

2.3. S-layered archaea

The archaea that were used for this part of the study were carefully chosen so that they represented the diverse number of S-layered cell envelopes that exist in this domain [12,16,17]. These can be very simple (i.e. the cell wall can consist of a single S-layer as in Sulfolobus acidocaldarius [18] and Methanococcus jannaschii [19–21]) or they can be amongst the most complex (i.e. the multiple layers of Methanospiroplum hungatei [8,22]).

*M. jannaschii* and *S. acidocaldarius* had the simplest envelopes consisting of a plasma membrane and a single proteinaceous (*M. jannaschii*) or glycoproteinaceous (*S. acidocaldarius*) S-layer (Fig. 5 is representative). *M. jannaschii* can alter the lipid composition of its plasma membrane depending on its growth temperature; at 70°C it has an extremely high proportion of tetraether lipids (~45% dry weight) in the bilayer and this is reduced to ~20% at 50°C [21]. Since a high proportion of tetraether lipids physically strengthens the membrane against freeze-fracture [23], it was important to see if these lipids could also alter the Gram stainability.

Both of these simple S-layered bacteria stained Gram-negative and this was independent of growth temperature or growth phase [9]. Thin sections of them showed little cell substance to be left after the TPT-Gram stain (Fig. 6) and uncontrasted sections (i.e. those not stained by uranyl acetate and lead citrate) showed very little electron density (Fig. 7). Pt was undetectable by EDS in these same uncontrasted sections. Like the previous results with the S-layered eubacteria (in mid-exponential growth phase; Fig. 4), these archaeal S-layers were not able to stand up to the rigors of the Gram stain. Even when there was a substantial increase of tetraether lipid in the plasma membrane, the cells still lysed and stained Gram-negative [9].

*Methanosarcina mazei* possesses a thick cell wall that consists of methanochondroitin [24,25], a substance which typically cements the cells together in large, irregular clusters of cells [26]. The clusters can be enzymatically broken apart by an inherent periplasmic ‘disaggregatase’ which hydrolyzes the cell wall under certain environmental conditions [27]. In such cases, cells of *M. mazei* are usually seen in sets of two or fours. Whether or not the cells are disaggregated, it has been reported that a particulate array exists between the methanochondroitin and

![Image](https://academic.oup.com/femsre/article-abstract/20/1-2/99/512691/1048168)
the plasma membrane [28]. For this reason, *M. mazei* was included in our study.

This archaean stained Gram-negative by LM. This was surprising because, with its thick methanochondriotic cell wall and S-layer, we would have expected it to be Gram-positive. In thin section the cells had little cytoplasm left in them after the TPt-Gram stain and few TPt precipitates. Interestingly, the cell wall was thinner than expected (∼12 nm versus ∼35-40 nm) and it was possible that the cell walls were subjected to the action of the disaggregatase under our growth conditions or during the staining regimen. In thin section we were unable to identify the S-layer after the Gram stain. Clearly, growth phase and disaggregatase activity must be taken into account when Gram staining this archaean.

Of all the archaen, the staining response of *M. hungatei* proved the most interesting; it also confirmed earlier work [8]. When cultures were Gram stained thin filaments with a shallow helix and containing ∼9 cells per filament were seen. The terminal cells were Gram-positive whereas the more internal cells were Gram-negative. Thin sections confirmed this staining response; although all cells within a

Fig. 8. Thin section of a *Methanospirillum hungatei* cell before Gram staining which shows the sheath (S), S-layered wall (W), plasma membrane (M) and spacer plug (P). Bar=100 nm.
Fig. 9. Thin section of the terminal end of a *M. hungatei* filament which has been contrasted after the TPt-Gram stain and which shows copious amounts of TPt precipitates in this terminal cell. Presumably, the staining reagents of the Gram stain have entered through the terminal plug at the end of the filament, but the precipitates, once formed, were too large to be removed during decolorization. Bar = 100 nm.

Fig. 10. Thin section of a more internal cell along the filament to that seen in Fig. 9. This cell has also been contrasted by uranyl acetate and lead citrate and can be directly compared to the cell in Fig. 9. Note that the cell is intact and there are no electron dense TPt precipitates. Bar = 100 nm.

filament possessed cytoplasm only the terminal cells contained TPt precipitates. Somehow, it appeared that the CV and TPt were only penetrating into the end cells of each filament where they formed precipitates and that these complexes could not be removed by the decolorizing step. Carbol fuchsins, as
a counter-stain, interacted with the surface of the remaining unstained filament making these cells appear to be Gram-negative.

The cellular filaments of *M. hungatei* are very complex structures and contain a number of layered structures which encompass each cell (Fig. 8 [29]). The plasma membrane (which contains tetraether lipids [23]) has a single hexagonal S-layer with a 15 nm periodicity on top of it as the sole cell wall constituent [30]. At the terminus of each cell, there resides a multilayered spacer (or terminal) plug that is composed of both periodic (hexagonal lattice of 18 nm) and amorphous layers [31]. All of these structures and the cells are packed into a hollow proteinaceous tube that possesses an S-layer with the smallest periodicity yet discovered (a p2 lattice with a 2.8 nm frequency [32]). This is called the sheath [29] and it is amongst the most resilient biological structures known, being able to resist harsh acids at 100°C and up to 400 atm of pressure [33,34].

The extremely small periodicity of the sheath makes it impermeable to all but small solutes; for example, H₂ and CO₂ are nutrients which (through metabolism) yield CH₄ and it is apparent that these substances must be able to get into and out of cells. The 2.8 nm periodicity of the sheath can only be slightly increased as gas pressure builds within cells because of the poor elasticity properties of this S-layer [34]. Accordingly, it is very possible that the chemical reagents of the Gram stain cannot penetrate through the sheath but only through the terminal (spacer) plug at the end of each cell filament [8,31]. This would mean that only the terminal cells of each filament could be subjected to CV and TPT so that they become Gram-positive. The other more internal cells of the filament would remain colorless. This was confirmed by the TPT-Gram stain (Figs. 9 and 10). When the entire filament is counter-stained, carbol fuchsine would also not be able to penetrate the sheath but it would ionically complex to the sheath surface; this would give the appearance to the internal cells as being Gram-negative. The same would happen to the terminal cells but, because they contain CV-TPT precipitates, they would still appear as being Gram-positive [8]. The sheath's minute pore size and its strong physical nature are unaffected by the Gram stain and these properties ensure this archaean's unique shape and staining response.

### 2.4. Concluding remarks

Prokaryotes possess a remarkable variety of cell envelope structural formats and chemical compositions [5,6], and those with S-layers, whether they be archaea or bacteria, are particularly intriguing since so little is known about the functionality of these outer layers [20]. S-layers are self-assembly systems and (usually) the bonding forces are between adjacent subunits and between each subunit and the underlying wall. These interactions are through charge-charge, salt-linkage, or hydrophobic interactions [5] which are not as strong as covalent bonding. But, in concert, these interactions can be sound and will re-enforce the underlying structures. Certainly, for those archaea with only single S-layers for cell walls (e.g. *M. jannaschii* and *S. acidocaldarius*) this must be intrinsically true. It is also possible that those bacteria which have a thinner peptidoglycan than normal for Gram-positives, especially during their exponential growth (e.g. *B. brevis, B. fibrisolvens, C. tetani* and *Thermoaerobacterium thermosaccharolyticum*), would utilize S-layers to buoy up their cell walls during this sensitive period. For Gram-negative S-layered bacteria such as *A. salmonicida* which have a thin peptidoglycan layer during all growth phases, this could also be true.

Because S-layers should act to strengthen the walls of both archaea and bacteria, it was possible that they could also contribute to the Gram staining response. This present study has reconfirmed that the Gram response depends on the structure and chemistry of the cell wall [1–4]. It also suggests that an S-layer rarely adds enough strength to walls to affect the staining response. All S-layered bacteria with thin walls, no matter their domain, proved to be Gram-negative at some point in their cell cycle. *B. thuringiensis* which has a thick wall plus an S-layer always stained Gram-positive. The most unique staining response was exhibited by *M. hungatei*. The majority of the cells of this archaean were Gram-negative, not because they had become disrupted, but because the staining reagents could not penetrate into their cellular substance. Only the terminal cells were penetrable and these stained Gram-positive (Figs. 9 and 10). Here we have an S-layer (the sheath) that can have a profound effect on the Gram stain because of its minute porosity. An ar-
chaeon with a similar S-layered sheath, Methanosaceta (Methanothrix) concilii [35,36], stains in a similar manner to M. hungatei because of the impermeability of its outer most lattice (T. Beveridge, unpublished). The Gram stain seems to still depend on the cell wall in archaea, but the exceptional variability of these structures between genera ensure that the essential mechanism of Gram reaction can be quite different from that seen in bacteria.

3. Function of the S-layer of some Gram-positive bacteria in phagocytosis

A. Kotiranta, Kari Lounatmaa, K. Kari, Eero Kerosuo, Markus Haapasalo

Phagocytosis is the most important defense mechanism in tissues. It is divided into three phases, attachment, ingestion, and killing. Human polymorphonuclear leukocytes (PMNs) can phagocytose bacteria without opsonizing proteins but, in the presence of serum, opsonization by complement and antibodies enhances phagocytic ingestion. Infection, however, a shortage of opsonins may arise due to proteases produced by several bacteria that can destroy these defense proteins [37,38]. Production of a thick capsule can also render bacteria resistant to phagocytosis by preventing effective opsonization [39]. Many oral anaerobic bacteria can also affect the bactericidal activity of the PMNs; for example, by modulating the generation of reactive oxygen species [40,41].

In the absence of opsonins, PMNs may recognize cell surface molecules that can mediate binding of the leukocyte to the microbial cell, for example lectin-like surface components are involved in PMN binding and phagocytic ingestion [42-44]. Several oral bacteria have fimbrial lectins [45-47], but the role of these different structures as hydrophins and hydrophobins is not established [48-50]. Nevertheless, hydrophobicity has been shown to play an important role in phagocytosis without opsonins [51-56]. The ultrastructure and surface properties of Eubacterium yurii, a Gram-positive, anaerobic rod isolated from oral infections, has been studied and it has been shown that its S-layer is possibly the major contributory factor for the high hydrophobicity of the cells [57]. These studies have now been extended to include binding of E. yurii subspecies to different host matrix molecules. In addition, experiments measuring phagocytosis of clinical isolates of Bacillus cereus, an aerobic species with an S-layer which has been isolated from oral infections, have been initiated.

3.1. Experimental conditions

Most of the bacterial strains studied were clinical isolates from endodontic and periodontal infections.

<p>| Table 1 |
| Eubacterium yurii* and Bacillus cereus strains and their sources |</p>
<table>
<thead>
<tr>
<th>Subspecies/strain</th>
<th>Source</th>
<th>Site of isolation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. yurii ssp. yurii</td>
<td>ATCC43714T</td>
<td>ATCC⁴</td>
<td>Periodontal pocket</td>
</tr>
<tr>
<td></td>
<td>ATCC⁴</td>
<td>Own isolate</td>
<td></td>
</tr>
<tr>
<td>E. yurii ssp. margaretae</td>
<td>ATCC43715T</td>
<td>ATCC⁴</td>
<td>Periodontal pocket</td>
</tr>
<tr>
<td></td>
<td>ES4C</td>
<td>Own isolate</td>
<td>Necrotic root canal</td>
</tr>
<tr>
<td></td>
<td>ES14B-8E</td>
<td>Own isolate</td>
<td>Necrotic root canal</td>
</tr>
<tr>
<td>E. yurii ssp. schleifka</td>
<td>ATCC43716T</td>
<td>ATCC⁴</td>
<td>Periodontal pocket</td>
</tr>
<tr>
<td>Bacillus cereus⁵</td>
<td>OH599</td>
<td>Clinical isolate</td>
<td>Necrotic root canal</td>
</tr>
<tr>
<td></td>
<td>OH600</td>
<td>Clinical isolate</td>
<td>Periodontal pocket</td>
</tr>
</tbody>
</table>

*Data from E. yurii have been published previously [57].
⁴ATCC, American type culture collection.
⁵Kindly provided by Dr. Ingar Olsen.
Table 2
Phagocytic ingestion (opsonic and non-opsonic) and percentage of hydrophobicity (% HP ± S.E.) of *E. yurii* strains

<table>
<thead>
<tr>
<th>Subspecies/strain</th>
<th>Non-opsonic</th>
<th>Opsonic (NHS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% PMN</td>
<td>B/PMN (± S.E.)</td>
</tr>
<tr>
<td>PMNs</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>E. yurii</em> ssp. <em>yurii</em></td>
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<td></td>
</tr>
<tr>
<td>ATCC43714T</td>
<td>93.0</td>
<td>5.0 ± 0.4</td>
</tr>
<tr>
<td>ES21-4</td>
<td>88.8</td>
<td>4.5 ± 1.0</td>
</tr>
<tr>
<td><em>E. yurii</em> ssp. <em>margaretiae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC43715T</td>
<td>3.6</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>ES4C</td>
<td>0.6</td>
<td>0.0 ± 0.1</td>
</tr>
<tr>
<td>ES14B-8E</td>
<td>1.6</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td><em>E. yurii</em> ssp. <em>schitika</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC43716T</td>
<td>93.2</td>
<td>4.8 ± 0.5</td>
</tr>
</tbody>
</table>

Bacteria were incubated for 30 min with human PMN leukocytes in the presence or absence of normal human serum (NHS; final concentration 10%) [57].

% PMN, percentage of PMNs having ingested at least one bacterium; B/PMN, average number of bacteria ingested per PMN (± S.E.); % HP, hydrophobicity percentage (± S.E.).

In addition, reference strains from culture collections were also included (Table 1). Antiserum against the denatured S-layer protein of *E. yurii* ssp. *margaretiae* ATCC 43715 was prepared from rabbits [57]. The hydrophobicity of the bacteria was measured using the hexadecane method [51,52]. Phagocytic ingestion of bacteria was measured by fluorescence microscopy and acridine orange staining and by electron microscopy [55,57]. Bacterial adherence to host matrix molecules was measured as follows: glass slides were coated with 0.1 mg ml⁻¹ of collagen type I, fibronectin, fibrinogen and laminin, blocked with 3% bovine serum albumin (BSA), and incubated with the bacteria (10⁷ CFU ml⁻¹) for 2 h at 37°C. After washing the cells were fixed with 2.5% glutaraldehyde and stained with toluidine blue and attached bacteria were counted by light microscopy.

3.2. PMN phagocytosis of *E. yurii*

All three *E. yurii* subspecies were clearly more hydrophobic than PMN cells (% HP, 38–53 vs. 16). However, experiments measuring non-opsonophagocytosis showed that, while two *E. yurii* subspecies (ssp. *schitika* and ssp. *yurii*) were rapidly ingested by the PMNs, *E. yurii* ssp. *margaretiae* was totally resistant to phagocytosis when opsonins were not available (Table 2, Fig. 11). When the bacteria were treated with normal human serum (NHS), all *E. yurii* subspecies were ingested by PMNs and the ingestions of the sensitive ssp. *yurii* and ssp. *schitika*,

Table 3
Effect of opsonization with normal human serum (NHS) and immune serum (KH4007) on phagocytic ingestion (% PMN) of *E. yurii* ssp. *margaretiae* strains

<table>
<thead>
<tr>
<th>Opsonin</th>
<th>Strain</th>
<th>ES4C</th>
<th>ATCC43715T</th>
<th>ES14B-8E</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
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<td>0.0</td>
<td>0.5</td>
<td>4.5</td>
</tr>
<tr>
<td>NHS</td>
<td>Untreated</td>
<td>93.0</td>
<td>91.5</td>
<td>94.0</td>
</tr>
<tr>
<td></td>
<td>Inactivated</td>
<td>3.5</td>
<td>7.5</td>
<td>13.8</td>
</tr>
<tr>
<td>KH4007</td>
<td>Untreated</td>
<td>97.5</td>
<td>96.5</td>
<td>99.5</td>
</tr>
<tr>
<td></td>
<td>Inactivated (56°C)</td>
<td>87.0</td>
<td>92.5</td>
<td>95.0</td>
</tr>
</tbody>
</table>

Mean of three or more experiments is given. % PMN, percentage of PMNs having ingested at least one bacterium; NHS, normal human serum in PBS (final concentration 2%); KH4007, rabbit immune serum in PBS (final concentration 2%) [57].
was enhanced as compared to phagocytosis without serum (Table 2, Fig. 12). When non-immune NHS was heat-treated to inactivate the complement, phagocytosis of *E. yurii* ssp. *margaretiae* was abolished. However, both the untreated and heat-inactivated antiserum prepared against the S-layer protein of *E. yurii* ssp. *margaretiae* ATCC 43715T allowed efficient phagocytosis of the bacterial cells (Table 3). When a mixture of a resistant strain (*E. yurii* ssp. *margaretiae* ATCC 43715T) and a sensitive strain (*E. yurii* ssp. *schititka* ATCC 43516T) was incubated with PMNs and assessed for phagocytosis, the presence of the resistant strain did not seem to affect the rate of bacterial ingestion by PMNs.

### 3.3. Binding of *E. yurii* to Human Matrix Proteins

As one of three *E. yurii* subspecies was resistant to non-opsonophagocytosis, the adhesion of the three subspecies to the host proteins fibronectin, collagen type I, fibrinogen and laminin was tested. Subspecies *yurii* bound well to collagen type I, fibronectin and laminin, binding to fibronogen was lower but still 10 times higher than to BSA which was used as a control. *E. yurii* ssp. *schititka* bound well to fibronectin while only moderate binding was measured to collagen I, laminin and fibrinogen. However, the ATCC strain of *E. yurii* ssp. *margaretiae* did not bind at all to the proteins tested (Table 4). Two other strains of *E. yurii* ssp. *margaretiae*, that were also resistant to phagocytosis were then tested for binding to the host proteins. Surprisingly, the clinical strains of *E. yurii* ssp. *margaretiae* showed good binding to fibronectin, while moderate to good binding was measured to collagen I, laminin and fibrinogen (Table 4).

### Table 4

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
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<tbody>
<tr>
<td></td>
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<td>S.E.</td>
<td>mean</td>
<td>S.E.</td>
<td>mean</td>
<td>S.E.</td>
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<td>BSA</td>
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<td>5.1</td>
<td>15</td>
<td>2.5</td>
<td>18</td>
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<tr>
<td>Fibrinogen</td>
<td>372</td>
<td>27.6</td>
<td>157</td>
<td>5.3</td>
<td>21</td>
<td>1.7</td>
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<tr>
<td>Fibronectin</td>
<td>826</td>
<td>24.4</td>
<td>824</td>
<td>39.2</td>
<td>71</td>
<td>2.7</td>
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<tr>
<td>Laminin</td>
<td>574</td>
<td>19.6</td>
<td>310</td>
<td>21.3</td>
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<td>Collagen</td>
<td>941</td>
<td>51.0</td>
<td>367</td>
<td>18.5</td>
<td>53</td>
<td>1.9</td>
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<td></td>
<td>778</td>
<td>59.4</td>
<td>330</td>
<td>24.8</td>
<td>241</td>
<td>8.6</td>
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</table>

3.4. PMN phagocytosis of B. cereus

PMN phagocytosis of two *B. cereus* strains isolated from oral infections was also studied. SDS-PAGE from detergent extracts of the cells showed a major 97,000 molecular mass band apparently representing the S-layer protein in both young (1 day old) and old (6 day old) cells of *B. cereus*. However, preliminary observations indicate that cells from young cultures are readily ingested by PMNs while cells from older cultures are not (Fig. 13).

3.5. Discussion

The amount of information available about the possible role of S-layers for the ecology, bacteria-host interaction and virulence of the cells is relatively small. Phagocytosis is regarded as the most important defense mechanism of the host. Earlier studies have implicated a role in resistance to phagocytosis for the S-layer of *Campylobacter fetus* [58]. Phagocytosis is most efficient when bacteria are opsonized by specific antibodies and complement 3b. However, PMNs are able to phagocytose bacteria also in the absence of opsonizing proteins. In lectinophagocytosis, cell surface lectins mediate the binding of PMNs to bacteria, whereas in surface phagocytosis, hydrophobic forces have been suggested to be involved [44,54,59]. Earlier studies have indicated that bacteria which are more hydrophobic than the PMNs can be ingested by surface phagocytosis, while the hydrophilic strains are more resistant [54,56,59]. Results with *E. yurii* ssp. *margaretiae* strongly indicate, however, that high hydrophobicity by itself may not be the only requirement for surface phagocytosis to occur (Table 2). Resistance to non-opsonophagocytosis by *E. yurii* ssp. *margaretiae* could have been due to two different mechanisms: (i) the bacteria could have an inhibitory effect on the PMN function by means of enzymes or toxic metabolic products or (ii) PMN cells cannot find receptors to bind to on the bacterial cell surface despite the high hydrophobicity of the cells. The latter possibility (lack of receptors) is more likely, as the resistant strain had no effect on the PMN ingestion of the sensitive strain [57]. It may, therefore, be that high hydrophobicity promotes non-opsonophagocytosis by making it easier for the two partners to get into close contact with each other. However, if receptor-mediated binding does not occur after this, ingestion fails to take place [44].

It is possible that despite possessing seemingly similar S-layer structures, seen in electron microscopy, there are differences in the molecular composition of the surface of the *E. yurii* ssp. For example, it is possible that some material (visualized by negative staining, but not by thin sectioning) is covering the S-layer protein in *E. yurii* ssp. *margaretiae* and thus masking the receptors in the S-layer protein needed for PMN binding. Differences in the chemical composition between S-layer proteins from different subspecies are also indicated by SDS-PAGE gels of extracted protein which show constant differences in molecular mass between subspecies. Similarly, isoelectric focusing of S-layer proteins revealed different pl value for *E. yurii* ssp. *margaretiae* as compared to the other two subspecies (Fig. 14).

Fig. 13. Cells of *Bacillus cereus* strain OH-600 incubated 30 min with human PMNs. No ingestion occurred. Bar = 1 μm.

Fig. 14. Two-dimensional gel electrophoresis of S-layer proteins extracted from *Eubacterium yurii* ssp. *yurii* ATCC 43714, ssp. *margaretiae* ATCC 43715 and ssp. *schmitka* ATCC 43716.
Strain specificity of the antiserum against the S-layer protein of *E. yurii* ssp. *margaritae* ATCC 43715\(^\text{F}\) also indicated inter- and even intra-subspecies variation in the antigenic composition of the cell surface [57]. Heat treatment of non-immune NHS and immunoserum against the S-layer protein of *E. yurii* ssp. *margaritae* ATCC 43715 showed that activation of the complement system is needed for the phagocytosis of this subspecies when specific immunoglobulins are not available. However, specific antibodies also strongly facilitated PMN ingestion when the complement was inactivated (Table 3).

Difference in binding pattern to host molecules between the three subspecies of *E. yurii* are evident. *E. yurii* showed good binding to fibronectin and type I collagen and binding to laminin and fibrinogen was also clearly (10–20 \(\times\)) above binding to BSA. Interestingly, one strain of *E. yurii* ssp. *margaritae*, strain ATCC 43715 did not bind at all to the proteins tested, while the two clinical strains of *E. yurii* subsp. *margaritae* showed strong binding comparable to that of other subspecies. Additional experiments including inhibition experiments must be performed using specific antibodies against the S-layer protein. Nevertheless, the binding of the S-layer protein particularly to type I collagen, fibrinogen and also laminin would be important factors contributing to the ecological establishment of these bacteria in the human oral cavity.

*B. cereus* is occasionally found in oral infections. A crystalline surface protein layer has been earlier described on this species [60]. There are differences in susceptibility to non-opsonophagocytosis between cells from young and old cultures. SDS-PAGE from detergent extracts from both cell populations revealed an equally strong major band with a molecular mass of 97000 probably representing the S-layer protein. It is possible that ecological changes in the growth condition during ageing of the culture are followed by changes in the surface properties of the cell which are again reflected in the increasing resistance to phagocytosis.

4. Evidence that the S-layer of *Bacillus stearothermophilus* strains functions as an adhesion site for a high-molecular-mass amylase

Eva M. Egelseer\(^3\), Ingrid Schocher, Uwe B. Sleytr, Margit Sára

Although S-layers can now be considered a most common surface structure in prokaryotic cells, little information is available about the specific biological function of these crystalline arrays (for reviews see [6,16,61–64]). Oblique (p1, p2), square (p4), and hexagonal (p3, p6) lattices are composed of assemblies of identical protein or glycoprotein subunits with \(M_s\) ranging from 30000 to 200000.

*Bacillus stearothermophilus* strains are aerobic, thermophilic organisms which can produce large amounts of exo-enzymes, such as proteases and amylases with molecular masses above the exclusion limit of their S-layer lattices [65–67]. In this context, the involvement of these crystalline arrays in exo-protein secretion has to be addressed. It has been suggested that S-layers in the Bacillaceae could delineate a kind of periplasmic space between themselves and the plasma membrane of these Gram-positive organisms and consequently delay or control the release of exo-enzymes [68–70]. More recently, S-layers have been reported to function as an adhesion sites for bacterial exo-enzymes [71,72].

In many S-layer proteins, three repeats of about 50 amino acids have been identified as S-layer homologous or SLH domains at the N-terminus and these same sequences can also be detected at the C-terminal region of several exo-enzymes or exo-proteins [72–77]. Recent studies indicate that the outer layer protein (OlpB) of the cellulose complex of *Clostridium thermocellum* might be able to bind non-covalently to the peptidoglycan by means of its SLH domain [74]. This observation is consistent with a putative role of SLH domains in the binding of S-layer proteins or cell-associated exo-proteins to the peptidoglycan [74,78–80]. Contrary to the hypothesis that SLH domains are responsible for binding of S-layer proteins to the peptidoglycan, studies with *B. stearothermophilus* strains strongly indicate that the N-terminal region recognizes a secondary cell wall polymer which is covalently bound to the peptidoglycan [81]. However, the question of which
domains are involved in S-layer protein-exo-enzyme association has still to be clarified.

4.1. S-layer and amylase interaction of B. stearothermophilus DSM 2358

The S-layer of B. stearothermophilus DSM 2358 completely covers the cell surface and exhibits oblique lattice symmetry. As determined by SDS-PAGE, the $M_r$ of the S-layer protein is 98,000. During growth on starch medium, three amylases with $M_r$s of 58,000, 98,000 and 184,000 are secreted into the culture fluid, but only the highest-molecular-mass amylase was found to be cell-associated [71] (Fig. 15).

The highest-molecular-mass amylase remained associated with S-layered cell wall fragments and S-layer self-assembly products (Fig. 16). During degradation of the peptidoglycan with lysozyme, no release of the highest-molecular-mass amylase into the supernatant was observed. Amylase activity was entirely associated with the pellet, which consisted of spheroplasts and S-layer fragments.

Studies of interactions between the highest-molecular-mass amylase and isolated cell wall components revealed that the enzyme had high affinity to bind to S-layered cell wall fragments and S-layer self-assembly products but none to isolated peptidoglycan-containing sacculi. Because extraction of the major portion of the highest-molecular-mass amylase did not require disintegration of the S-layer lattice, it is evident that the amylase does not participate in the self-assembly process. The ratio of S-layer subunits to the bound amylase is in the range of 8:1 which means that, on the assumption that there is a uniform distribution of the enzyme molecules on the S-layer surface, every fourth morphological unit of the S-layer lattice is covered with an amylase molecule.

As observed by immunoblotting, polyclonal antibodies raised against the highest-molecular-mass...
amylose did not cross react with the S-layer protein or the other two amylases. These results suggest that the highest-molecular-mass amylase and the S-layer protein as well as the two other amylases do not possess pronounced structurally homologous domains. The surface location of the highest-molecular-mass amylase was confirmed by immunogold labeling of whole cells and electron microscopy [71,82].

4.2. S-layer and amylase interaction of
B. stearothermophilus ATCC 12980

In a recent study, the role of the S-layer lattice with regard to exo-enzyme adhesion was investigated by using the closely related B. stearothermophilus ATCC 12980 and its S-layer-deficient variant ATCC 12980-9/1 as a model system [82]. B. stearothermophilus ATCC 12980 exhibited an oblique S-layer lattice composed of a 122 kDa protein. During growth on starch-containing medium, the S-layered strain and the deficient variant each secreted three amylases with identical $M_r$s of 58000, 122000 and 184000, but only the highest-molecular-mass amylase remained cell-associated (Fig. 17). The S-layered strain and the variant revealed remarkable differences regarding cell association of the highest-molecular-mass amylase. For the S-layer-deficient variant, no changes in the amount of cell-associated and free highest-molecular-mass amylase could be observed during growth on starch medium, while for the S-layered strain, cell association of the highest-molecular-mass amylase strongly depended on the growth phase of the cells [82]. The maximum amount of cell-associated amylase (which was comparable to that of the S-layer-deficient variant) was observed in the early exponential growth phase. The steady decrease in cell-associated amylase during continued growth correlated with the appearance and the increasing intensity of a protein band with a $M_r$ of 60000 ($P_{60}$) (Fig. 17). The highest-molecular-mass amylase could bind to S-layer self-assembly products and to the peptidoglycan-containing layer of both S-layered and deficient strains at comparable extents, indicating that the enzyme had affinity to both cell envelope components. This was in contrast to the results obtained for B. stearothermophilus DSM 2358 in which the highest-molecular-mass

Fig. 17. SDS-PAGE patterns of whole cell extracts from the S-layer-carrying B. stearothermophilus ATCC 12980 strain cultivated on complex medium. Samples were taken 1-8 h after inoculation. Protein bands were visualized by silver staining; bands with amylolytic activity could be detected with the iodine-starch reagent. In the S-layered strain, high expression of a protein with a $M_r$ of 60,000 ($P_{60}$) was accompanied by a reduced cell association and release of the highest-molecular-mass amylase into the culture fluid.

Fig. 18. SDS-PAGE patterns of (a) sedimented S-layer self-assembly products and (b) centrifugation supernatants of dialyzed GHI extracts. In the supernatants only small amounts of S-layer protein and at least 50% of $P_{60}$ could be detected, while the residual 50% of $P_{60}$ was incorporated into self-assembly products.
Amylase possessed affinity to only the S-layer surface [71]. Bonding interactions between the highest-molecular-mass amylase and the peptidoglycan-containing layer were stronger than those between the enzyme and the S-layer surface [82].

The 122 kDa S-layer protein as well as P60 could be extracted completely from cell wall fragments with 5 M guanidinium hydrochloride (GHCl). When GHCl was removed by dialysis, both the S-layer protein and P60 were detected in the suspension containing S-layer self-assembly products and soluble S-layer protein. After sedimentation of self-assembly products by centrifugation, only <10% of total S-layer protein but at least 50% of P60 were detected in this fraction (Fig. 18). Reassociation of P60 to S-layered cell wall fragments was possible only after disintegration of the S-layer lattice with 5 M GHCl and dialysis, indicating that P60 was integrated into the S-layer lattice. Furthermore, P60 retained the ability to bind to the peptidoglycan-containing layer. High-level expression of P60 in stationary growth phase cells cultivated on starch-containing medium led to the loss of the regular structure of the S-layer lattice over large areas [82].

By immunoblotting, peptide mapping, and N-terminal sequencing, P60 was identified as the N-terminal part of the whole S-layer protein. Thus, P60 and the S-layer protein showed a strong cross-reaction on immunoblots and yielded very similar, but not identical, cleavage patterns after peptide mapping with endoproteinase-Glu C (Staphylococcus aureus V8 protease). As shown in Fig. 19, three cleavage products of the S-layer protein with Mₙs of 29000, 30000 and 31000 could not be detected for the P60.

Both, this S-layer protein and P60 showed an identical N-terminal region typical of S-layer proteins of B. stearothermophilus wild-type strains [83]. From experiments with different protease inhibitors, it was apparent that P60 was not a proteolytic cleavage product of the whole S-layer protein. These results strongly indicate that under certain culture conditions such as growth on starch-containing medium, P60 is co-expressed with the whole S-layer protein and the amylases.

4.3. Comparative studies between
B. stearothermophilus DSM 2358 and
B. stearothermophilus ATCC 12980

The S-layer proteins of B. stearothermophilus ATCC 12980 and DSM 2358 showed no cross-reaction on immunoblots and yielded different patterns of cleavage products upon peptide mapping with endoproteinase-Glu C. Hence, despite an identical N-terminal region [71,82], the S-layer proteins of the wild-type strains possess only low structural homology. Because the high-molecular-mass amylases from B. stearothermophilus DSM 2358 and ATCC 12980 showed an identical Mₙ of 184000 on SDS-PAGE gels and revealed a strong cross-reaction on immunoblots, they are structurally related. As demonstrated for both strains, the highest-molecular-mass amylases did not cross-react with antibodies raised against the S-layer protein, indicating the absence of extended structurally homologous domains. Thus, the highest-molecular-mass amylases were the only cell surface components which were structurally related.

4.4. Conclusions and perspectives

For B. stearothermophilus, affinity studies and immunolabeling techniques confirmed that the S-layer lattice functions as an adhesion site for the highest-molecular-mass amylase secreted into the culture fluid. Cell association of this enzyme seems to be advantageous since it could function as a mediator.
between the living cell and high-molecular-mass substrates [82]. Because the S-layer lattice is the first contact region between the cell and its outer environment, other biological functions of these crystal lattices cannot be excluded. B. stearothermophilus wild-type strains possess identical peptidoglycan types, comparable amounts of compositionally similar secondary wall polymers, and identical N-terminal regions of their S-layer proteins [81]. However, comparative studies with the closely related B. stearothermophilus strain ATCC 12980 and its S-layer-deficient variant 12980-9/1 revealed a different type of exo-protein adhesion. In contrast to the cell-associated amylase produced by B. stearothermophilus DSM 2358, the exo-enzyme from B. stearothermophilus ATCC 12980 had affinity not only for the S-layer surface but also for the peptidoglycan-containing layer, suggesting that the enzyme carries binding domains for both cell envelope components. Furthermore, the N-terminal part of the S-layer protein anchors the subunits to the rigid cell wall, whereas the C-terminal region of the S-layer protein could function as a binding site for the cell-associated amylase.

5. Surface layer of Lactobacillus helveticus CNRZ 892

Lorenzo Morelli³, Maria-Luisa Callegari

Thermophilic species of Lactobacillus helveticus play a key role in the production of Swiss and Parmesan cheese.

Since the first paper reporting the presence of a regular, crystalline surface structure in L. helveticus, a number of studies have been published, dealing with the biochemical and the genetic characterization of L. helveticus S-layer [84–89]. The biological roles played by this S-layer are not known; it appears that resistance to lysozyme treatment could be related to its presence as the outermost envelope layer of L. helveticus ATCC 12046 [86], whereas a role of the S-layer of L. helveticus CNRZ 892 as receptor for the virulent bacteriophage CNRZ 832-B1 was suggested [86]. Few data are available about the relationships among S-layers isolated from various lactobacilli. Antiserum raised against the L. buchneri ATCC 4005 S-layer protein was used to show that S-layer monomers isolated from lactobacilli belonging to different species are not immunologically related [84]. Nevertheless, the N-terminal amino acid sequence of L. helveticus ATCC 12046 S-layer protein [88] and the deduced N-terminal amino acid sequence of L. acidophilus ATCC 4356 are nearly identical [90]. These two species belong to the obligatory homofermentative group of Lactobacillus and are phylogenetically related by DNA-DNA hybridization and rRNA sequence analyses [91]. This taxonomic observation was confirmed by means of immunological cross reaction using antisera raised against S-layer monomers isolated from different strains of these species ([92]; unpublished data); the same antisera did not react with S-layer monomers extracted from other species of the genus Lactobacillus.

Here data on the genetic characterization, the functional properties and the taxonomic significance of the S-layer protein (SlpH) of L. helveticus CNRZ 892 are presented, a strain used in the manufacture of hard cheese.

5.1. Isolation and characterization of SlpH

Electron microscopy of cells of L. helveticus CNRZ 892 has clearly shown that the wall of this strain has an outer protein array [84]. Protein extracts obtained by treating the walls of CNRZ 892 with 6 M LiCl revealed only one protein (SlpH) with an Mr close to 43 000; monomers in SDS-PAGE gels were not stained by Alcian blue, suggesting the absence of sugar residues in the protein structure. During reassembly of the SlpH, which was achieved by removal of LiCl by means of extensive dialysis, flat sheets were formed which showed the native lattice parameters.

5.2. SlpH contains the receptor for a lytic phage

The first step in phage infection involves the attachment of the phage particles to specific receptors located on cell envelopes. In the case of the lactic acid bacterial phages, the phage receptors have been poorly studied, except for phage PL-1 and J-1 of Lactococcus casei S-1 (ATCC 27092). Phage receptors have never been characterized in L. helveti-
cus, despite the commercial relevance of this species for dairy products. *L. helveticus* CNRZ 892 is the bacterial host of the lytic phage CNRZ 832-B1 [93, 94]. Preliminary evidence of the role played by StpH in phage binding was obtained by using purified and re-assembled StpH to bind phage particles (Fig. 20). Also, plaquing efficiency was reduced when the S-layer was added to a mixture of bacterial cells and phage particles. Yet, S-layer proteins extracted from several phage-resistant mutants, spontaneously arising from the sensitive bacterial population when challenged with the phage, were unable to adsorb 832-B1 and did not show any influence on the level of phage attachment to bacterial cells. StpH isolated from these mutants had an $M_1$ identical to the surface protein of the wild-type strain and the same capacity to reassociate in vitro.

5.3. Genetics of *L. helveticus* CNRZ 892 S-layer

The gene encoding the S-layer protein of strain CNRZ 892 has been cloned and sequenced in *E. coli* (EMBL bank accession number X91199); the deduced mature protein turned out to be composed of 440 amino acids, which is in good agreement with the $M_1$ estimated by SDS-PAGE. The deduced amino acid sequence shows a leader peptide of 30 amino acid residues with positively charged n- and h-regions. Computer analysis indicated that the amino acid sequence of StpH was very similar to

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*Fig. 20. Particles of the lytic phage CNRZ 832-B1 attached to re-associated S-layer isolated from the wild-type, phage-sensitive strain *Lactobacillus helveticus* CNRZ 892.*

*Fig. 21. Comparison of amino acid compositions, deduced from the nucleotide sequences of S-layers of *Lactobacillus helveticus* CNRZ 892 (upper sequence) and its phage-resistant derivative CNRZ 1269 (lower sequence). Position of the point mutation is indicated by arrows.*
that determined for the SlpA of *L. acidophilus* ATCC 4356 [88]. Homology with *L. acidophilus* SlpA was particularly evident in the C-terminal region (amino acids 193–438) while the middle region showed a lower degree of homology. Moreover, the N-terminal sequence deduced from the nucleotide sequence of *slpH* (ATTINADSAINANTNAKYDVT) was identical to that of *L. helveticus* ATCC 12046.

Two primers designed from the nucleotide sequence were used to amplify *slpH* by means of PCR from the chromosomal DNA of resistant mutants, and fragments, including a region immediately upstream of the RBS sequence and ending with the stop codon, were obtained. Comparison of all nucleotide sequences revealed that the 5'-end and the 3'-end of the amplified sequences were identical in all the studied mutants. In the central part (a 200 bp region located at 591 bp downstream of the ATG codon) were concentrated all mutations capable of affecting the phage-binding ability of the protein. Comparison of the *slpH* sequence obtained from the phage-sensitive wild-type strain and from one of the phage-resistant mutants (named CNRZ 1269) is shown in Fig. 21. In this case a glutamine in position 242 was substituted by a proline; this change was due to a point mutation of the codon AAC as it became an ACC triplet. Mutations detected in four additional mutants also consisted of single amino acid substitutions (two isolates), but one deletion and one insertion mutant were also found. Computer analysis suggested that this area had a high probability of occurring at the surface of the cell. These results confirm that SlpH does contain a receptor region for CNRZ 832-B1 phage and strongly suggest that the phage-binding site is located in this region.

5.4. Localization of *S*-layer encoding gene in *L. helveticus*

Chromosomal analysis by means of pulsed field gel electrophoresis (PFGE) revealed that in all ana-

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![Fig. 22. PFGE analysis (a) of *SmaI* digestion of five different *Lactobacillus helveticus* strains. The previous gels after Southern hybridization (b) using as probe the PCR amplified *slpH*. All examined strains harbored an S-layer gene localized in the same *SmaI* fragment.](image-url)
lyzed *L. helveticus* strains, the gene encoding S-layer monomers is localized on the same *SmaI* fragment (Fig. 22); a similar location was found in the *L. acidophilus* strains [95]. These data suggest that S-layer-encoding genes are located in a conserved position within the chromosomal structure.

5.5. Conclusions and perspectives

*Lactobacillus helveticus* is widely used as a starter culture in dairy productions, mainly in the manufacturing of hard cheese, such as Swiss and Parmesan types of cheese. In Italy, this *Lactobacillus* species (together with other thermophilic lactobacilli such as *L. bulgaricus*) is reproduced day-to-day in the so-called natural whey culture method. A rapid screening procedure to discriminate between these two species could be useful for practical applications. More than 100 isolates of thermophilic lactobacilli used for hard cheese production, previously designated *L. helveticus* species by means of phenotypic tests and by hybridization with a species-specific DNA probe designed from 23S RNA, have been characterized (unpublished results). Oligonucleotides designed on the basis of the slpH sequence were used to probe these strains for the presence of the S-layer-encoding gene. Results obtained using stringent conditions confirmed that an S-layer gene, highly homologous to *slpH*, is present in all *L. helveticus* isolates. SDS-PAGE analysis also showed the presence of monomers reacting in Western blot experiments with SlpH-specific antiserum.

Contrary to this, 68 strains isolated from the same environment and classified by means of the 23S RNA-directed probe as *L. bulgaricus* did not hybridize, even in low-stringency experiments, with *slpH* probes. Moreover, SlpH-specific antiserum did not recognize protein extracted from the surface of these strains. These results suggest that it could be possible to exploit the presence of the S-layer on *L. helveticus* strains to design an immuno-enzymatic assay in order to quantitate the presence of this species in natural microflora.

6. Form, function and utility studies with the *Caulobacter crescentus* S-layer protein

John F. Nomellini, Wade H. Bingle, John Smit

While it is becoming well known that paracrystalline S-layers are a common feature throughout the eubacterial and archaeal kingdoms [16,61,96], few have been the subject of in-depth analysis of all phases of biogenesis of such a surface structure. The analysis of the S-layer of *C. crescentus* seems to be rewarding because there has been a significant investment in gene analysis in this bacterium, driven by the adoption of this organism as a model system for understanding fundamental aspects of cell differentiation, including temporal and spatial control of the cell cycle [97,98]. Caulobacters differ from most bacteria in that they exhibit a biphasic life cycle: they have a stalked, sessile stage that adheres firmly to surfaces via an adhesion structure (the holdfast). During growth the sessile cell produces a motile stalkless cell (the swarmer) which eventually converts to the stalked form to complete the life cycle. A predominant reason to study the S-layer is that this class of bacteria is common and perhaps even prolific in many aquatic and terrestrial environments [99–101]. As a biofilm-forming bacterium, *C. crescentus* is perhaps representative of many oligotrophic bacteria in the environment whose existence depends on survival in the competitive microbial milieu that develops on inert surfaces [102]. As a Gram-negative bacterium, assessing how the S-layer is attached to the outer membrane is interesting as compared to what is known about this attachment in other Gram-negative genera [5,16,64].

Furthermore, this bacterium is a harmless, non-pathogenic organism producing a large amount of a single protein as a secreted surface-attached product that is not essential for viability. The bacterium grows rapidly to high density in chemically defined media and produces no other secreted protein. In pure culture it will spontaneously form a single cell layer as a biofilm on nearly any surface (Fig. 23). These characteristics can be considered highly appropriate for development of a bacterium that can be used to present useful activities on the cell surface or to secrete commercially interesting proteins, either in a fixed cell bioreactor or suspended culture. Adaptations.
tion of the S-layer protein to this task is considered fundamental to the success of this approach, and an understanding of the entire S-layer biogenesis process is essential for the pursuit of that goal.

6.1. General features of the *C. crescentus* S-layer

The S-layer of *C. crescentus* was first characterized as an integral part of isolated fragments of outer membrane [103]. The possibility that this S-layer was composed of more than one protein species was considered on the basis of the apparently fixed ratio between RsaA (the S-layer protein monomer) and a protein called 74K. More recently, isolated RsaA was recrystallized [104], making it clear that only it was responsible for the structure. The *rsaA* gene was cloned and sequenced [105–107]; it specifies a protein with a molecular mass of about 98 000 (1025 amino acids) which has no signal leader peptide and, beyond the removal of the initial methionine, is not processed in any way. Like many S-layer proteins, RsaA has an acidic pI, lacks cysteines and, as a consequence of covering the entire cell, is produced in large quantity (10–12% of cell protein). It is dissimilar to some other S-layer proteins by lacking a hydrophobic character; indeed fully 25% of the amino acids are serines or threonines. The level of protein production is not regulated during the cell cycle, but there is a coordination of S-layer assembly with the growth of the underlying cell surface [108]. Indeed, antibodies to the S-layer can be used to identify particular growth zones (the division septum and the stalk) on the cell surface.

The structural organization of the S-layer was first demonstrated at higher resolution than unprocessed images by two-dimensional image analysis [103] and later by a 3-D reconstruction [109]. The monomer appears to be a U-shaped molecule with one end joining five other monomers to produce a salient six-membered ring, while the other apparently contacts two other monomers to form the axis of three-fold symmetry, resulting in a hexagonally packed array.

Image analysis suggested that the *Caulobacter*
S-layer could well serve as a filter, exhibiting an apparent porosity that would exclude most proteins. It is clear, based on experimentation by Koval [110], using isogenic C. crescentus strains, with and without an S-layer, that a parasitic, Bdellovibrio-like bacterium was incapable of attacking C. crescentus when the S-layer was present. It is probable that a major role for the Caulobacter S-layer is protection by selective porosity from a variety of predatorial assaults that are likely to occur in complex bacterial biofilm communities; the inhibition to electroporation of plasmids may be a reflection of this role [111]. It has, for example, been demonstrated that a bacteriophage uses the S-layer as a necessary attachment site [112].

6.2. Biogenesis of the S-layer

At a minimum, it seems apparent that to form an S-layer three tasks must be accomplished by the monomeric RsaA protein: it must be secreted, it must crystallize into the 2-D array and it must be attached to the underlying surface, presumably the outer membrane. These same factors must be understood if one is to adapt this S-layer system to be a surface presentation system or to use it to serve as a means of producing heterologous cloned proteins. Although it has become apparent that all these attributes are inter-related and are, in some ways, difficult to separate functionally, it is useful to describe progress in each area as a separate topic.

6.2.1. Surface attachment

Poindexter first noticed that C. crescentus required calcium for growth, but when the bacterium was cultured in the absence of calcium, strains arose lacking an S-layer [113]. This observation was extended, noting that 'calcium-independent' strains appeared at mutant frequency; not all lacked an S-layer but many had lost the ability to attach the S-layer [114]. When colonies were examined, extended sheets of crystallized S-layer were seen adjacent to the cells (Fig. 24). These sheets were precisely superimposed double layers, composed of mirror imaged S-layer.

Fig. 24. Electron microscopy of negatively stained cells from a colony of a calcium-independent, S-layer-shedding strain. The S-layer sheet is actually a mirror double layer and the cells are completely devoid of attached S-layer. Bar = 0.2 \( \mu \)m.
This phenomenon could not be reproduced in recrystallization experiments with purified protein; it does not occur in liquid cultures of these strains and it is unknown how the mirror images adhere to each other.

The means of attachment to the cell surface is clearer. The primary LPS of *C. crescentus* is a ‘rough’ species, containing only a core oligosaccharide [115]. But a smooth LPS species (SLPS) is also present; it has an extended ‘O-antigen’ of nearly uniform length which is unusual for a smooth LPS species [114]. This SLPS species is missing in all calcium-independent, S-layer-shedding strains. Recrystallization studies (see below) also implicate SLPS in the attachment. The O-antigen contained two types of dideoxyaminohexose and glycerol in equal proportions [114]. Calcium ions seem to be responsible for bridging the S-layer to the SLPS. Recent studies, however, indicate that the glycerol, although tightly associated, is not covalently bound to SLPS and may be derived from the gel electrophoresis sample loading buffer. In addition, calcium bound to SLPS was undetectable (Walker, Awram and Smit, unpublished), suggesting that calcium is not mediating S-layer attachment. It may be that glycerol is mimicking the nature of association between SLPS and S-layer due to the interaction between key hydroxyl-containing amino acid residues (i.e. in fact, serine and threonine are abundant in RsaA).

The region of RsaA that is involved with the attachment has not been precisely located. It does, however, seem to be in the N-terminal region [116] (Fig. 25). Interruptions of the protein sequence by deletion or insertion of short peptides within approximately the first 100 amino acids most often has the effect of producing a version of RsaA that is shed. Of course, if such a disruption actually affected crystallization, shedding might also be expected as discussed in the next section. In contrast, numerous insertions in the central regions of RsaA (ranging from ca. 300 to 700 amino acids in length) had no effect on crystallization or attachment.

### 6.2.2. Crystallization

Although purified RsaA can be recrystallized [104], highly ordered, long-range recrystallization has not been possible using only divalent cations and purified RsaA. Recently, using low-pH extracted RsaA and lipid vesicles or a monolayer of lipids at an air-water interface in a Langmuir-Blodgett trough, rapid and highly ordered recrystallization was achieved (Nomellini, Küpcü, Sleytr and Smit, unpublished). This occurred, however, only if SLPS and certain divalent cations (calcium being the most effective) were included into the lipid mixture. The other lipids were not critical and other types of LPS were ineffective. Because long-range crystallization could be achieved with small amounts of SLPS, it is possible that SLPS-mediated surface attachment is needed only to initiate crystallization, which can then
proceed without additional surface lipids. Probably calcium ions are needed for interactions between RsaA monomers, since calcium does not appear to bind to SLPS (see previous section). This emphasizes that crystallization and surface attachment are entwined; if the native cell surface has limited amounts of SLPS, anything that disrupts crystallization will likely release considerable RsaA from pre-existing S-layer or prevent further crystallization from occurring. For example, EGTA treatment, which effectively disrupts crystallized S-layer by chelating calcium ions, effectively removes the S-layer from the cell surface [104], likely because only a small fraction of RsaA monomers are directly attached to the surface. As discussed previously, lack of surface attachment strongly impacts the ability of RsaA to crystallize. Mutations in RsaA that specifically affect crystallization are expected to result in RsaA ‘shedders’, where most monomers will be unable to find an unoccupied SLPS molecule for attachment.

6.2.3. Secretion

Initial DNA sequence information gave few clues about the mechanism of RsaA secretion. The N-terminus of the protein was relatively hydrophobic [106] and was perhaps comparable to a signal leader peptide, suggesting the potential of the N-terminal regions to facilitate secretion of reporter molecules. This hypothesis was tested using an endoglucanase (‘cellulase’) from Cellulomonas fimii and the alkaline phosphatase from E. coli. Both reporters gave similar results: when very small portions (≤20 residues) of the N-terminus were fused to the reporter, no secretion was detectable; when 21–52 N-terminal residues were used, as much as 10% of the fusion product was found in the periplasm (but not beyond); when more than 52 residues were fused, nearly all constructs were toxic to caulobacters, suggesting that the cell dies in an attempt to secrete the fusion protein [117–119]. These data probably do not indicate what the true secretion signal is. Large in-frame internal deletions of RsaA and subsequently deleting all but the C-terminal region, did not abolish secretion of the recombinant product [116]. Conversely, if the last few residues of RsaA were removed, secretion was abolished; it became clear that a C-terminal signal was necessary and sufficient for secretion.

The rsaA gene sequence was inspected in light of other so-called type I protein secretion systems [120,121] (e.g. the hemolysin of E. coli and the alkaline protease of Pseudomonas aeruginosa). The lack of an N-terminal signal coupled with the presence of a glycine-rich, calcium-binding consensus sequence [107] suggested a type I mechanism. 16 000 independent Tn5 mutants were screened for loss of RsaA secretion; nearly all insertions outside rsaA mapped to a region immediately downstream of the rsaA gene. Sequence analysis has identified the key secretion genes, including that of an ABC-transporter protein; these have a high degree of homology to cognate genes in the alkaline protease and hemolysin secretion apparatus (Awram and Smit, unpublished). To date, the Caulobacter S-layer protein is the most abundant protein produced by this class of secretion apparatus. Apparently, the S-layer proteins of Campylobacter fetus (another example of a hexagonally packed surface array in a Gram-negative bacterium) are also secreted by a type I mechanism (M. Blaser, personal communication).

One feature of the type I secretion proteins is the presence of calcium-binding motifs which are found upstream of the C-terminal secretion signal. Evidence is accumulating that this region may have a role in protein folding and thus indirectly affect secretion efficiency. Preliminary evidence with RsaA deletions has indicated that removal of this region also has the effect of reducing the quantity of protein secreted. Recalling that calcium ions are needed for monomer crystallization, it is conceivable that these calcium-binding regions serve two functions (protein folding and level of secretion); if true, this is another indication of the coordinated inter-relationships between all three aspects of S-layer biogenesis.

Many aspects of the biogenesis remain to be clarified. Despite uncertainties about the exact phenotypes of various gene constructions, the overall pattern developing thus far suggests that RsaA has relegated secretory functions to the C-terminus and the attachment function to the N-terminus. It remains to be learned where regions specifically involved with monomer-monomer interactions (crystallization) will be found. The role of calcium on biogenesis is not yet completely understood. It is unknown why calcium-independent mutants do not produce the SLPS. In addition, when calcium-independent mutants are grown without calcium, they no
longer produce RsaA (Walker, Awram and Smit, unpublished). \textit{rsaA} mRNA levels are not reduced, indicating that control is not at the level of transcription. Even so, it seems appropriate for \textit{C. crescentus} to limit RsaA production if insufficient calcium ions are available for subsequent crystallization. Also, some gene insertions (see below) at some positions in \textit{rsaA} result in a sensitivity to site-specific cleavage at or near the site of insertion. This suggests that a protease activity may be part of the secretion process, poised to disrupt defective copies of RsaA before assembly is attempted.

6.3. Comparison of the \textit{C. crescentus} S-layer with those of other Caulobacter strains

Caulobacters are readily found in many soil and aquatic environments. Caulobacter strains were isolated from waste water treatment facilities and other aquatic habitats, where these bacteria present in apparent profusion [99], attaching to surfaces via their adhesive holdfasts [122–124]. At least in western North America, the most common strain found was phenotypically similar to the laboratory \textit{C. crescentus} strains and, via 16S rRNA sequence comparisons, appears closely related [125]. One phenotypic trait, in particular, was the presence of an S-layer that was similar to the well characterized S-layer [104]. For example, they appeared similar in negative stain by electron microscopy, the methods used to extract a single RsaA protein were equally effective with these strains, Southern blot hybridization using the \textit{rsaA} gene as a probe readily identified a hybridizing DNA segment [99]. Western immunoblot analysis (using antisera raised against RsaA) was reactive against these proteins, a rapid method to identify SLPS also identified comparable polysaccharides, and these polysaccharides were reactive with antisera against SLPS. The one difference noted so far is variation in the size of the S-layer proteins. In short, it appears that not only are S-layers common in Caulobacters but, in contrast to \textit{Bacillus} species [16], they are likely to be similar to one another, perhaps even evolving from a common ancestor.

6.4. Modifying the S-layer for biotechnology applications

Efforts to define functional regions of RsaA have the additional utility of assessing whether the S-layer can be adapted for surface presentation of heterologous peptides or proteins, even when using only RsaA secretion signals, or the secretion of large proteins. Potential applications include the development of inexpensive vaccines, using the S-layer protein as a carrier vehicle or low cost production of proteins. Caulobacters are readily cultivated to high density in inexpensive mineral salt-based media. Moreover, if S-layer shedding strains are used (lacking SLPS), the recombinant RsaA proteins collect in the culture medium as a macroscopic precipitate that is readily separated from cells with a simple mesh filter, greatly simplifying purification. Also, the lipid A of Caulobacter LPS has low endotoxicity, a key consideration for the production of recombinant protein from any Gram-negative bacterium.

The development of epitope, or more generally, peptide presentation within RsaA was done by preparing numerous versions of \textit{rsaA}, each with a 12 bp insertion (specifying a unique \textit{Bam}II restriction site) at a different site [126,127]. Versions of \textit{rsaA} have been collected, each containing such sites, throughout the gene that have no effect on S-layer biogenesis [128]. These sites can then be investigated for their ability to accept additional insertions and multiple sites have been identified as such. Gene segment-carrier cassettes have been developed, which are short segments of DNA that are designed to insert at the unique \textit{Bam}II sites and create in-frame insertions with a passenger segment (Nomellini, Bingle, Rebstein and Smit, unpublished). The cassettes are also designed for ready multimerization, allowing multiple repeats of the same foreign segment or mixtures of various foreign epitopes or peptides. Thus, for example, in the development of subunit vaccines, a variety of configurations can readily be produced, combining multiple key epitopes or other factors that encourage an effective immune response. Insertions of a key epitope from a \textit{Pseudomonas aeruginosa} pilus subunit were prepared which enable the bacteria to adhere specifically to certain glycolipids present on mucosal surfaces (Nomellini, Bingle, Rebstein and Smit, unpublished). Nearly all insertions
produced crystallized S-layer that could bind an epitope-specific monoclonal antibody and the recombinant RsaAs specifically bound to the key glycolipid [129]. Insertions of up to 50 amino acids therefore usually result in retention of crystallization and surface attachment capabilities, quite large insertions (> 150 amino acids) are still efficiently secreted in many cases, but crystallization and surface attachment capabilities are lost, and multiple cysteine residues are not tolerated well, leading to loss of secretion or outright toxicity of the constructions. This is probably due to inappropriate disulfide bond formation at some point during the secretion process.

Direct fusion of heterologous proteins to segments of the C-terminal, while retaining secretion capability is also possible. As part of a project to develop a vaccine for infectious hematopoietic necrosis virus (IHNV), a salmonid fish pathogen [130], a 184 amino acid segment of the virus surface glycoprotein was fused to various RsaA C-terminal segments, ranging from 150 to 250 amino acids in length, and high-level secretion was achieved. The maximum size and the range of protein types are being explored but, since the native protein is a large (> 1000 amino acids) hydrophilic molecule and since type I secretion systems generally have considerable flexibility in the type of protein secreted, secretion of a variety of proteins will be possible using the *Caulobacter* S-layer biogenesis apparatus.

7. Occurrence and function of a common domain in S-layer and other exocellular proteins

Emmanuelle Leibovitz, Marc Lemaire, Isabelle Miras, Sylvie Salamitou, Pierre Béguin, Hélène Ohayon, Pierre Gounon, Markus Matuschek, Kerstin Sahm, Hubert Bahl

Surface (S) layer proteins of several bacteria con-

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### Table 5

Proteins containing SLH domains

<table>
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<th>Gene symbol</th>
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<th>Accession number</th>
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tain a conserved sequence at their N-terminus which is composed of up to a three-fold reiteration of a motif consisting of about 50–60 amino acids. Similar S-layer-like segments have been identified in numerous exocellular proteins (Table 5) including several exo-enzymes involved in the hydrolysis of extracellular polysaccharides. They have been termed SLH domains [80].

Here, the current knowledge about the occurrence and function of SLH domains in bacterial exocellular proteins, especially of *C. thermocellum* and *T. thermosulfurigenes* EM1 is summarized. In each of these two organisms several exocellular proteins with SLH domains were identified.

7.1. Cell surface proteins of Clostridium thermocellum

7.1.1. The cellulosome of *C. thermocellum*: an exocellular multienzyme complex bound to the cell surface

*Clostridium thermocellum*, a Gram-positive, anaerobic and moderately thermophilic bacterium, produces a highly active cellulase complex termed cellulosome [131]. The cellulosome contains a set of hydrolytic enzymes [132,133], which are anchored to a scaffolding protein termed CipA [134,135]. Each hydrolytic component comprises a catalytic domain and a separate docking domain, termed dockerin domain, which includes about 65 residues and mediates binding to CipA [135,136]. In contrast to catalytic domains, dockerin domains are highly conserved between different enzymes. CipA comprises a cellulose-binding domain and a set of nine highly conserved modules of 145–150 residues [137]. These modules, termed cohesin domains, act as receptor domains which bind the dockerin domains borne by the catalytic subunits [76,138]. In addition, the C-terminal region of CipA consists of a dockerin domain whose sequence is less conserved than those of the dockerin domains borne by the hydrolytic components.

In growing cells, a significant fraction of the cellulases is cell-bound [139]. The polypeptide sequences of cellulase subunits, including CipA,
start with signal peptides. Furthermore, CipA never appears as an non-complexed molecule in the culture medium. Thus, cellulosome subunits are probably secreted via the general secretion pathway and assembled into cellulosomes while bound to the cell surface.

7.1.2. Proteins of C. thermocellum mediating attachment of cellulases, hemicellulases and cellulosomes to the cell surface

Upon sequencing the region extending downstream from cipA, Fujino et al. [73] identified three open reading frames encoding polypeptides which appeared to contain various sequence modules (Figs. 26 and 27). In particular, the C-terminal region of the three polypeptides consisted of a three-fold reiteration of a 60-residue motif. Comparison with sequences then available in the data bases revealed similarities with segments present in the S-layer proteins of Bacillus brevis and Thermoanaerobacter kivui. This suggested that the three polypeptides were components of the cell envelope [73]. The hypothesis was verified for OlpA and OlpB by cell fractionation and by immunolabeling and electron microscopy [74,75]. The N-terminal region of the third polypeptide (OlpA) was clearly similar to the cohesin domains present in CipA, and was shown to bind a hybrid protein harboring the dockern domain of endoglucanase CelD. The affinity constant of the complex was close to that measured for the complex between the dockern domain of CelD and the seventh cohesin domain of CipA. However, the cohesin domain of OlpA was unable to bind the C-terminal dockern domain of CipA [76]. Thus, OlpA probably acts as an anchoring factor for individual cellulases, rather than for the cellulosome.

OlpB and ORF2p displayed N-terminal reiterations for which no similarity could be found in the data bases. These segments correspond to a new type of cohesin domain, which specifically binds the dockern domain of CipA. Indeed, a fourth, unlinked gene, termed sdbA, was isolated after screening a gene bank for clones expressing polypeptides that would bind the dockern domain of CipA. The SdbA polypeptide contains an N-terminal domain which is similar to the N-terminal repeats of OlpB and ORF2p, and which specifically binds the dockern domain of CipA. The same property was demonstrated for the N-terminal repeat of OlpB [140]. The new domains are termed type II cohesin domain, in contrast to the cohesin domains of type I present in CipA and OlpA, which are specific for the dockern domains of the catalytic subunits of the cellulosome. The C-terminal region of SdbA consists

![Fig. 27. Domain structures of proteins from C. thermocellum that contain SLH domains. S, signal peptide; CDI, cohesin domain type I; CDII, cohesin domain type II; P, Pro/Thr/Ser-rich region; TPSDEP, Thr/Pro/Ser/Asp/Glu/Pro repeats; K, Lys-rich region; SL, SLH domain. CBD is predicted to be a cellulose-binding domain and XDA (xylanase domain A) is a duplicated sequence with unknown function. The latter two domains were described by Winterhalter et al. [141]. GHF10 and GHF16 are catalytic domains of glycosyl hydrolase families 10 and 16, respectively [142].](https://academic.oup.com/femsre/article-abstract/20/1-2/99/512691)
of SLH repeats and, like OlpA and OlpB, the protein was shown to be located on the cell surface of the bacterium (unpublished data). Thus, the three polypeptides OlpB, ORF2p, and SdbA, probably serve as cell surface anchoring sites for the cellulosome.

7.1.3. The S-layer of C. thermocellum
This S-layer was first observed in thin sections as an electron-dense layer surrounded by an electron-transparent outer layer. The latter is a thick, irregular layer forming numerous protuberances [143], which can be visualized after staining with ruthenium red or cationized ferritin (Fig. 28). The regular structure of the S-layer was identified on freeze-fractured cells [144]. The S-layer can also be visualized after rotatory shadowing of preparations of cell envelopes washed with 1% Triton X-100 (unpublished results). Such preparations contain a 130,000 molecular mass polypeptide as the major protein component [145]. The latter appears to correspond to the exo-cellular, non-cellulosomal glycoprotein described by Lamed and Bayer [143], which is present on the cell surface and released in large amounts into the culture medium. The polypeptide is non-covalently bound to the peptidoglycan and can be released from cell envelopes by treatment with guanidine hydrochloride or lysozyme. The corresponding gene, termed slpA, was recently cloned using hybridization with a degenerate oligonucleotide derived from the sequence of an internal peptide of the S-layer protein. Sequence analysis of the slpA gene shows that the encoded polypeptide contains reiterated motifs that are also present in the S-layer protein of Bacillus sphaericus and in the outer wall protein of B. brevis (unpublished data). The N-terminus comprises two sequence elements that are reminiscent of part of the SLH consensus. Work is in progress to determine whether these sequence elements can promote the attachment of fusion proteins to the peptidoglycan of C. thermocellum.

7.2. SLH domains in exocellular proteins of T. thermosulfurigenes EM1

7.2.1. The pullulanase of T. thermosulfurigenes EM1
T. thermosulfurigenes EM1, a Gram-positive thermophilic anaerobic bacterium is capable of utilizing the carbohydrate polymers starch, xylan and pectin as carbon and energy sources [146]. For the breakdown of these substrates the organism produces exocellular enzymes. The best characterized of these enzymes, the pullulanase of T. thermosulfurigenes EM1 hydrolyzes α-1,6 as well as α-1,4 linkages in various sugar polymers, resulting in their complete breakdown and the formation of maltotriose and maltose. Immunogold labeling of cells by the low-temperature embedding technique demonstrated the localization of the pullulanase at the cell periphery of T. thermosulfurigenes EM1. However, it was not known to which component of the cell envelope the enzyme
was attached. The amount of cell-bound enzyme appeared to be dependent on the growth conditions. During growth on starch in batch culture, up to 90% of the total enzyme activity was found to be cell-associated. In contrast, almost all enzyme activity was found in the culture fluid when cells were grown in continuous culture. Under these growth conditions, degradation of the S-layer and the peptidoglycan layer of *T. thermosulfurigenes* EM1 was observed [77,147].

Sequence analysis of the pullulanase-encoding gene (*amyB*) supported by biochemical investigations revealed that the enzyme (AmyB) is an exceptionally large polypeptide (1861 aa) consisting of a single catalytic and several non-catalytic domains (Fig. 29). The C-terminus of AmyB consists of three SLH domains. Based on the available data a model was proposed in which the pullulanase is anchored to the cell envelope of *T. thermosulfurigenes* EM1 by these C-terminal domains [72].

### 7.2.2. Three extracellular enzymes of *T. thermosulfurigenes* EM1 that contain SLH domains

Southern blot analysis with a DNA probe encoding domain SLH1 of the pullulanase revealed that the genome of *T. thermosulfurigenes* EM1 contains two additional DNA regions with significant similarity to the SLH domain-encoding fragment. Cloning and sequencing of the corresponding restriction fragments led to the identification of two genes which are predicted to encode a xylanase (*XynA*) and a polygalacturonate hydrolase (*PglA*) [148]. Like the pullulanase, XynA and PglA showed a multidomain architecture. Each protein consisted of a catalytic and several non-catalytic domains including three C-terminal SLH domains (Fig. 29). In summary, sequence analysis indicated that three glycosyl hydrolases from *T. thermosulfurigenes* EM1 (AmyB, XynA and PglA which act on different carbohydrate polymers) contain strongly conserved SLH domains (95% identical residues). Less similarity was observed when the SLH sequences within the proteins were compared to each other (35–46% identical residues). Zymogram analysis of subcellular fractions from *T. thermosulfurigenes* EM1 supported the notion that the xylanase is a cell wall-associated protein. Activity staining revealed a xylanolytic enzyme with a similar molecular mass in cell wall fragments consisting of peptidoglycan and S-layer. In contrast, no enzyme activity was detected in fractions containing the cytoplasm or the cytoplasmic (plasma) membrane [149].

### 7.2.3. The S-layer of *T. thermosulfurigenes* EM1

*T. thermosulfurigenes* EM1 is covered by an S-layer that exhibits hexagonal (p6) lattice symmetry [147]. The protein subunits forming this S-layer were purified according to the procedure of Messner and Sleytr [150] and characterized. The S-layer subunits of *T. thermosulfurigenes* EM1 could be dissolved in 5 M guanidine hydrochloride. After dialysis against

![Fig. 29. Domain structures of AmyB, PglA and XynA from *Thermoanaerobacter thermosulfurigenes* EM1. S, signal peptide. PDH and PDG (for pullulanase domain H and G respectively) are domains with unknown function that were described in [161]. According to Jespersen et al. [161], only starch hydrolases acting in endo-fashion on pullulan and the α-1,6 linkages of branched substrates are predicted to possess domains that are located N-terminal to the catalytic domain. FN, fibronectin type III domain [162,163]; L, Gly/Thr/Ser/Asn-rich segment; SL, SLH domain; CBD, predicted cellulose-binding domain; XDA, xylanase domain A (see Fig. 26); GHF 10, GHF 13, and GHF 28, catalytic domain of glycosyl hydrolase families 10, 13, and 28, respectively [142].](image-url)
distilled water, sheets of S-layer self-assembly products were obtained that showed the same lattice symmetry as the S-layer in vivo. Protein chemical studies indicated that the S-layer of *T. thermosulfurigenes* EM1 is composed of a single protein with an approximate molecular mass of 83 000 which is heterogeneously glycosylated. The N-terminal sequence of the S-layer protein exhibited strongest similarity to the N-terminus of the *T. kivui* S-layer protein. Therefore, it can be concluded that the S-layer protein of *T. thermosulfurigenes* EM1 also contains an SLH domain. In contrast, the N-terminus of the S-layer protein of *T. thermosulfurigenes* EM1 showed only weak similarity to the conserved triplicated sequences of the three exo-cellular enzymes AmyB, XynA, and PglA of strain EM1 [149]. Sequence analysis of the gene encoding the S-layer protein of *T. thermosulfurigenes* EM1 and the functional analysis of the SLH domain of the exo-cellular enzymes of *T. thermosulfurigenes* EM1 are awaited.

7.3. Discussion

Bacteria have evolved various modes to display polypeptides on their cell surface, e.g. incorporation as lipoproteins into the outer membrane [151–153], covalent attachment to the peptidoglycan [154–156], non-covalent binding to the choline moiety of lipoteichoic acids [157,158] or to S-layer proteins [71] (see also contribution by Egelseer et al., this volume). As a mode of cell surface display, SLH domains have been found so far in the largest variety of bacteria. Besides in S-layer proteins, SLH domains are found in several polysaccharidases, like the pullulanase, the polygalacturonate hydrolase, and the xylanase of *T. thermosulfurigenes* EM1 discussed previously. In *C. thermocellum*, two polysaccharidases, XynX or LicA, also contain SLH domains (Table 5 and Fig. 27) and are presumably linked directly to the cell surface. However, at least four SLH-harbouring polypeptides appear to act as carrier proteins, which bind cellulases or cellulosomes by means of cohesin-dockerin interactions.

The correlation between the presence in various polypeptides of SLH domains and their location on the cell surface is now quite compelling, and their role in anchoring polypeptides to the cell surface was demonstrated in vivo for the S-layer protein of *Thermus thermophilus*. A mutant of *T. thermophilus* in which the SLH repeats were deleted from the S-layer protein could still form an S-layer, but the latter was no longer attached to the cell surface [159]. However, the interaction of SLH domains with other cell envelope components is not fully defined. On the basis of ultrastructural observations, Lupas et al. [80] proposed that SLH domains bind to peptidoglycan. Furthermore, sequence analysis concluded that a segment of each repeat should have a β-strand conformation. In the case of triplicated repeats, this leaves a single β-strand, which must interact with a β-strand from another molecule. Thus, it was also predicted that SLH domains should self-associate [80]. Both predictions were borne out by experimental evidence. The SLH domains of OlpB promote in vitro binding of the *E. coli* maltose-binding protein MalE to the peptidoglycan sacculus of *C. thermocellum* [74] and the SLH domains of the *T. thermophilus* S-layer protein bind fragments of *T. thermophilus* peptidoglycan [159]. SLH domains also mediate association of SLH domain-bearing polypeptides in vitro [74]. However, it is not clear which of the two interactions is relevant in vivo. A related point is whether enzymes carrying SLH domains are indeed enmeshed within S-layers. In the case of *T. thermosulfurigenes* EM1, the outermost cell wall layer appears to be the S-layer, which lies on the outside of the peptidoglycan [77,147]. Thus, the fact that the pullulanase, the xylanase and the polygalacturonate hydrolase are located on the cell surface makes it plausible that they are associated with the S-layer. Anchoring of the SLH-bearing carbohydrates may be mediated either by SLH-SLH interactions with the SLH domain of the S-layer protomer or by direct binding of the SLH domains to the underlying peptidoglycan. However, in the case of *C. thermocellum*, association of SLH-bearing polypeptides with the S-layer or with the peptidoglycan, if it does occur, is probably not permanent. Electron micrographs of *C. thermocellum* cells labeled with antibodies directed against OlpA and OlpB show that the two proteins are located in the thick, irregular outer layer surrounding the S-layer [74,75]. In addition, no binding of the SLH domains of OlpB to the S-layer protomer of *C. thermocellum* was detected, as would be expected if they were to be co-incorporated within the lattice of the S-layer. Two hypotheses
have been proposed to reconcile these observations with the properties of SLH domains observed in vitro [74]. Attachment to specific elements of the cell surface may not necessarily be a permanent feature of polypeptides carrying SLH domains. These may bind transiently to the peptidoglycan prior to being released and trapped in the outer layer. Alternatively, the self-interacting properties of SLH domains may contribute to the formation of a protein network within the outer layer, which might also involve a 28 000 molecular mass component that was found to bind to SLH domains [74]. Clearly, more work is required to define the composition and organization of the outer layer. The latter is heavily stained by cationized ferritin (Fig. 28), and it has been proposed that it may contain acidic polysaccharides [160]. Electron micrographs of C. thermocellum cells grown in the presence of cellulose show that the outer layer forms filamentous protractions between the cells and the cellulose fibers. It has been surmised that the protractions may act as channeling corridors facilitating the diffusion of soluble hydrolysis products towards the cells [160].

8. The role of S-layer proteins in ovine Campylobacter abortion

Rosemary Grogono-Thomas, Joel Dworkin, Martin J. Blaser, Ralph M. Woodland, Diane G. Newell

8.1. The role of the S-layer protein in the pathogenesis of ovine abortion

In 1995, a vaccine was produced from C. fetus ssp. fetus which protected against ovine abortion. This vaccine comprised a bacterial extract containing a “rather loosely attached capsular envelope protein” [164]. This material was later characterized as a surface microcapsule which mediated protection against phagocytosis [165]. The antigen was purified and characterized as a protein with an apparent molecular mass of 98 000 existing as a complex with lipopolysaccharide. This material has all the characteristics of a surface array (i.e. an S-layer) with protein subunits arranged regularly to form a two-dimensional paracrystalline surface structure. This protein was initially reported to be a glycoprotein but this is not confirmed and now seems unlikely [166].

It is now established that C. fetus ssp. fetus expresses a number of S-layer proteins encoded by up to eight genes (homologs of sapA). Recombination events at the genomic level, apparently mediated at least in part by recA, enable the organism to variably express these proteins. The molecular basis of these events is reviewed elsewhere (Blaser, this volume).

There is currently no direct evidence that the S-layer protein has a role in the pathogenesis of ovine abortion. However, recent evidence from experiments using bovine and human C. fetus isolates suggests that this is the predominant virulence factor for this organism [167]. The fact that S-layer-negative variants are never isolated from ovine clinical material and that vaccines containing the S-layer protein are protective suggest that this protein displays similar virulence properties during those ovine infections leading to abortion.

8.2. Immunity to ovine Campylobacter abortion

Immunity after abortion is protective and many animals that are in contact with aborting dams also develop a protective immune response and a lamb normally during the subsequent lambing season. Protective immunity, once acquired, appears to be long-lived. Given the high levels of protective immunity induced by natural ovine infection, vaccination is an obvious solution to Campylobacter abortion.

Experimental abortion, following challenge with a homologous strain of C. fetus ssp. fetus, can be successfully prevented by vaccination with formalin-killed, but not heat-treated vaccines [168]. Moreover protection can be conferred on susceptible ewes by vaccinating animals once the first abortion has occurred in a flock [169]. Such use of immunotherapy is preferential to antibiotic therapy because vaccination stimulates a long-term protective response and negates the need for repeated treatment. However, as campylobacter abortion is sporadic in nature the routine vaccination of a flock is seldom justifiable. Nevertheless, the production of a modern vaccine containing protective antigens from all major sheep-abortion agents would be economically beneficial. A few such commercial vaccines are currently
available. One example is Ovine Vibshield (Grand Labs, USA) which contains killed antigens of Chlamydia psittaci, C. jejuni, C. fetus and E. coli K99. The efficacy of such vaccines, containing complex antigenic components from a range of organisms is not known. Modern vaccine development requires the identification of defined subunit vaccine candidates of proven protective antigenicity in the absence of immunosuppressive effects. The later is particularly important in the development of polyvalent vaccines. The value of the S-layer protein of C. fetus ssp. fetus as a suitable antigenic candidate in vaccines against ovine abortion has yet to be established.

8.3. Establishment of a model of ovine abortion

An experimental model of ovine abortion has been developed by administration of 10⁸ CFU of C. fetus ssp. fetus strain S47 orally or subcutaneously to Welsh Mountain sheep on day 105 of pregnancy. Abortion occurred 10–25 days post-infection. Approximately 20% of sheep which were orally infected and 60% of sheep subcutaneously infected aborted. The aborted fetuses showed gross abnormal pathology of the liver with necrotic lesions in approximately 20% of the fetal livers examined. The abomasum, and occasionally the jejunum, was red and inflamed. The gross pathology is consistent with that of naturally occurring infections. The bacteria were recoverable from liver, jejunum, abomasum and placenta of aborted fetuses and aborting dams.

8.4. The use of a model of ovine abortion to investigate the role of the S-layer protein

8.4.1. The activity of an S-layer protein-deficient variant

The Campylobacter fetus 23D wild-type strain [167] usually expresses a 97000 molecular mass S-layer protein. Strain 23B is a spontaneous mutant of 23D which has a 9 kb genomic deletion, which includes the promoter region of sapA; it does not express an S-layer protein. When the aborting activity of 23B was compared to that of 23D in the ovine model using the subcutaneous route of infection, the non-S-layered mutant was unable to cause abortion.

8.4.2. The activity of S-layer protein expression-defined mutants of strain 23D

Spontaneous variants, such as 23B, may have other mutations involving genotypic and phenotypic changes than just those in the expression of the S-layer protein. In fact, preliminary data suggest that 23D and 23B also differ in the expression of additional antigens including the flagellins. Investigation of defined isogenic mutants was therefore necessary in order to clearly establish the role of the S-layer protein in this abortion model. A series of S-layer protein expression mutants with deletions of the sapA and/or recA genes have been tested in the ovine abortion model. The genotypes and phenotypes of these mutants are shown in Table 6.

The results to date clearly show that the expression of an S-layer protein is essential for ovine abortion. Mutants unable to express an S-layer protein were unable to cause abortion. In contrast, mutants which had a deleted sapA gene but which were able

Table 6
Bacterial strains used in ovine investigations

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolation site</th>
<th>LPS type</th>
<th>Major S-layer protein (molecular weight × 10³)</th>
<th>Antibiotic resistance cassette</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>S47</td>
<td>Ovine fetus</td>
<td>unknown</td>
<td>97</td>
<td>None</td>
<td>ND</td>
</tr>
<tr>
<td>23D</td>
<td>Bovine fetus</td>
<td>A</td>
<td>97</td>
<td>None</td>
<td>sapA⁺ recA⁺</td>
</tr>
<tr>
<td>23B</td>
<td>Spontaneous variant</td>
<td>A</td>
<td>None present</td>
<td>Promoter of sapA is absent</td>
<td></td>
</tr>
<tr>
<td>23D 600(2)</td>
<td>Deletion mutant of 23D</td>
<td>A</td>
<td>97</td>
<td>Kanamycin</td>
<td>sapA⁺ recA⁻</td>
</tr>
<tr>
<td>23D 600(4)</td>
<td>Deletion mutant of 23D</td>
<td>A</td>
<td>127</td>
<td>Kanamycin</td>
<td>sapA⁺ recA⁻</td>
</tr>
<tr>
<td>23D 501</td>
<td>Deletion mutant of 23D</td>
<td>A</td>
<td>None present</td>
<td>Chloramphenicol</td>
<td>sapA⁺ recA⁺</td>
</tr>
<tr>
<td>23D 502</td>
<td>Deletion mutant of 23D</td>
<td>A</td>
<td>None present</td>
<td>Kanamycin, chloramphenicol</td>
<td>sapA⁻ recA⁻</td>
</tr>
</tbody>
</table>

ND, not determined.
to switch on an alternative *sapA* gene were still capable of causing abortion. Moreover, expression of one S-layer protein phenotype (a 97,000 molecular mass protein) may confer a virulence advantage to the organism, mediating greater aborting activity, than strains expressing other S-layer phenotypes.

### 8.4.3. Antigenic variation in S-layer proteins during ovine abortion

Variations in the molecular masses of the S-layer proteins are well documented [170,171]. Antigenic variation may occur concomitant with changes in molecular mass [172]. The N-termini of these proteins are antigenically highly conserved [173] but conservation declines towards the C-terminus and non-cross-reactive epitopes are detectable using monoclonal antibodies [170]. Such antigenic variation may provide a bacterial mechanism for survival in an immunologically hostile host and enable chronic infection. The role of such antigenic variation in ovine infections is unknown.

To detect variations in antigenicity during infection, sera were collected from sheep inoculated with the S-layered 23D strain and used in Western blots against several isolates recovered from faeces over a 6-week period. The results demonstrate that the molecular mass and, presumably, the antigenicity of S-layer proteins expressed by strain 23D varies significantly over time during colonization of the gut. This variation occurs rapidly but evidently common epitopes (detectable by induction of cross-reacting antibodies) are consistently expressed.

The deletion of *recA* appears to prevent the genomic recombination enabling the expression of alternative *sapA* gene products. The deletion of *recA* should then prevent antigenic and molecular mass variations during infection. With a single exception, deletion of the *recA* gene prevented the occurrence of antigenic variation during ovine infection and abortion. The relevance of the one exception has yet to be established.

### 8.5. Conclusions

To date, evidence suggests that *C. fetus* ssp. *fetus* is an important, though probably underestimated, cause of ovine abortion with major economic consequences to farming worldwide. Current control strategies include naturally acquired immunity and antibiotics but vaccine development would be a good long-term alternative. The exact mechanism by which this pathogen causes abortion is unknown but previous work with similar infections suggest that the S-layer is a major virulence factor. To investigate this, an ovine abortion model has been developed using the subcutaneous route of inoculation. This model has been used to test a number of S-layer protein expression variants and defined mutants. The results indicate that the expression of the S-layer is essential for the abortion. Moreover, the phenotype of the expressed S-layer proteins may influence the outcome of the infection in pregnant sheep. The antigenic variation in the S-layer proteins observed in vivo appears to be dependant on *recA* gene function, although preliminary data suggest that other mechanisms also may be involved. Our results indicate that the presence of and antigenic variation of S-layer proteins have a potentially important role in ovine colonization and abortion. This role requires further investigation, if S-layer proteins are to be considered as vaccine candidates for *C. fetus* ssp. *fetus* infections.

### 9. Naturally crystalline porins or specialized S-layers

Martin Kessel

S-layers have traditionally been associated with the outermost layers of bacterial envelopes but in 1985 a specialized group of S-layers was first recognized [174] which have become known as ‘regular outer membrane proteins’ (rOMPs, [175]) or ‘crystalline outer membrane proteins’ (COMPs, [176]). As their name implies, these are proteins present in the outer membrane of Gram-negative bacteria where they have naturally assumed a crystalline two-dimensional organization.

This brief review will describe our current knowledge of some representatives of these rOMPs and attempt to draw comparisons between the different structures presented.

#### 9.1. Porins as rOMPs

The most dominant group of rOMPs are those
constituted by bacterial porins. In experiments with *E. coli* envelopes treated with SDS and heated to 60°C it was observed that a crystalline structure developed within the plane of the membrane [177]. Image analysis of micrographs of these treated cell envelopes showed that this crystalline structure was made up of trimers of stain-filled depressions organized on a hexagonal lattice. This was the first evidence of the structural organization of 'porin', the major outer membrane protein. These experiments, however, raised questions due to the harsh conditions imposed by SDS and heating. It therefore remained to isolate organisms which naturally exhibited these two-dimensional crystalline arrays in their cell membranes to prove that such a membrane organization was physiologically viable.

9.2. Naturally occurring rOMPs

The first examples of a naturally occurring porin-like rOMP were reported by Kessel et al. [174] and Chalcroft et al. [178] when both groups described a similar unusual bacterium even though they were isolated from two different habitats, a hypersaline pond at the southern tip of the Sinai Peninsula and a hot spring in the South Island of New Zealand. This bacterium was characterized by a rod-shaped body from which exceedingly long stalks (as long as 50 μm) emanate from either end (Fig. 30a,b). When examined by negative staining these stalks were seen to be covered with a periodic array which gives a strong impression of a moiré pattern (Fig. 30c). Analysis of the optical diffraction pattern from such an array (Fig. 30d) shows a very characteristic hexagonal order of two superimposed but slightly rotated lattices, each separate pattern arising from one of the two sides of the flattened cylindrical stalk. This flattening occurs when the stalk dries down on the specimen grid. By the application of a digital filter to the Fourier transform of the image, it is possible to reconstruct the lattice of one side of the cylinder and the result shows that the unit cell con-

![Fig. 30. Long-stalked bacterium from a hypersaline pond in Southern Sinai. (a) The bacterium as viewed by Nomarski interference optics. Long stalks are seen emanating from both ends of the cell body. (b) The bacterium seen at low magnification by electron microscopy after negative staining with 1% uranyl acetate. (c) Higher magnification of a short region of the stalk. Bar=0.5 μm. Inset shows the Fourier-filtered image of one side of the flattened stalk, for detail see (e). (d) Diffraction pattern from the area marked in (c). Note the double sets of intensities, each arising from one of the two sides of the flattened stalk. The single ring of intensities at high resolution indicates that this information is preserved from one of the two sides of the stalk only. (e) Fourier filtration of a region from a single side of the stalk showing the close-packed trimeric organization of the 'porin-like' layer. The thick white borders demarcating the trimers are the end-on projections through the membrane-spanning β-sheet strands normal to the plane of the membrane.](https://academic.oup.com/femsre/article-abstract/20/1-2/99/512691)
sists of a trimer of stain-filled depressions arranged hexagonally with respect to the surrounding trimers. The center-to-center spacing of the trimers is approximately 9 nm which is very typical for that found in synthetic two-dimensional sheets of various porins such as OmpF. Chalcraft et al. [178] calculated a 3-D reconstruction of the stalk protein and these authors showed that the trimeric stain-filled depressions were in fact pores.

What could be the reason for these unusually long stalks? It seems plausible to propose, since these organisms are found in extreme environments, that they have evolved an apparatus for either the efficient uptake of nutrients or the efflux of metabolites and toxins. In this case the lattice would be a densely packed array of non-selective hydrophilic channels.

Another unusual Gram-negative eubacterium, *Thermatoga maritima*, is described by Rachel et al. [179], and it is also found in a thermophilic environment. When observed in the light microscope the bacterium appears to have ‘wings’ attached to both poles of the rod-shaped organism. When thin sections of the organism were examined in the electron microscope, these ‘wings’ were seen to be balloon-like extensions of the outer membrane. Negative stains, freeze fractures and shadows of freeze-dried preparations of the membrane showed it to be composed of a 2-D array of repeating morphological units. These, too, were shown by digital filtration to be trimers but with a unit cell size of approximately 11 nm. A 3-D reconstruction of the negatively stained layer showed the three channels coalescing into a single channel as had been shown earlier by Engel et al. [180] for porin reconstituted into lipid bilayers. However, by use of the information from the freeze-fractured replicas, Rachel et al. [179] could make a definitive assignment of the topology of the trimer showing that the common opening of the trimer faced the outside of the cell while the three channels opened into the periplasm [179]. This was the first report of the correct orientation of porin trimers in the outer membrane. This incorporation of the outer membrane with a 2-D crystalline array of trimeric porins has been shown to exist in other members of the Thermotoga order, e.g. *T. thermarum* and related genera such as *Fervido* (R. Rachel, personal communication). *Thermotoga* is thus a further striking example of a bacterium which uses a crystalline porin to increase the conduction of the periplasm with the external milieu.

An observation by A. Lawn at the Poultry Research Station, Houghton, Cambridgeshire, published only as a figure in a medical textbook, showed that the outer surface of an avirulent strain of the Gram-negative bacterium causing whooping cough, *Bordetella pertussis*, appeared to be covered with periodic array. Independently, our group at the National Institute of Health had characterized the outer surface of the avirulent strain of *Bordetella pertussis* Tohama III [181] and showed this to be periodic as well. Further image analysis of images of negatively stained envelopes of the avirulent strain (Fig. 31a) showed this to be an array of porin-like stain-filled trimers arranged in an oblique (p2) lattice with two oppositely oriented trimers per unit cell and with a characteristic center-to-center spacing of 9 nm (Fig. 31a, inset). A 3-D reconstruction from a tilt series of negatively stained envelopes revealed that the three porin channels traversed the membrane, eventually coalescing to a common vestibule (Fig. 31b). The correct orientation of the trimers in *B. pertussis*, however, remained unresolved until a combination of results from unidirectional shadowing of cell envelopes and the 3-D reconstruction unequivocally showed that the correct orientation was with the common vestibule facing the outside of the cell and the three channels facing the periplasm. This orientation was also confirmed by X-ray crystallography of a porin from *Rhodobacter capsulatus* [183] which showed the rim of the vestibule (from which the three channels diverged) to consist of a series of polypeptide loops on which external epitopes could be mapped.

A comparison of the outer membrane proteins of the avirulent and virulent strains of *B. pertussis* showed that all of the virulence factors present in the virulent strain were completely absent in the non-virulent strain. It is therefore plausible that when the porin is present as the major protein in the outer membrane, it exists in high enough concentrations to allow the formation of a 2-D crystal within the plane of the membrane.

Parallel studies by Hoenger et al. [184] on overproducing OmpF mutant strains of *E. coli* showed that the major porin in these bacteria also formed 2-D crystalline arrays. These assemblies were amena-
Fig. 31. Porin in the outer membrane of *Bordetella pertussis* strain Tohama III. (a) Typical cell envelope after freezing and thawing to remove the bacterial cytoplasm, negatively stained with 1% uranyl acetate. Clear areas are covered with a periodic array. Inset: Fourier filtration of a selected area from an envelope showing the arrangement of the porin trimers seen as stain-filled depressions. There are 2 trimers per unit cell arranged in p2 symmetry. Bar = 10 nm. (b) Surface-shaded three-dimensional reconstruction of the porin trimers in which the negative stain penetrating the pores is emphasized. The three channels open into the periplasm whereas the single vestibule (v) opens to the outside of the outer membrane. Trimers are presented in both orientations to help interpretation.

...ble to image analysis and showed the typical trimeric arrangement of the porin pores with the single opening of the tri-channel on the outer face of the membrane. Other crystalline organizations in the outer membrane of bacteria have been found in *Thermus thermophilus* [185] and various species of *Bacteroides* [176].

9.3. Conclusions and perspectives

Because of the presented examples it is clear that there exist a small number of bacteria exhibiting naturally crystalline outer membrane proteins, usually porins. It should be emphasized that in all the referred cases of naturally crystalline porins, this type of organization of the outer membrane does not appear to prevent the organism from growing normally and implies that the cells are physiologically active in the presence of the crystalline arrangement of porins.

It remains for further studies of organisms from extreme environments or studies on those bacteria with a biphasic pathogenic nature for more examples of this specialized class of S-layers to be discovered and their function to be elucidated.

10. The effect of S-layers and cell surface hydrophobicity on prey selection by bacterivorous protozoa

Susan F. Koval

In nature bacteria can be at risk from starvation or from any number of other environmental stresses such as temperature, pH, salinity, and pressure. But
aquatic and terrestrial prokaryotes in the phylogenetic domain. Bacteria also face another risk: that of sudden ingestion by and demise within free-living phagotrophic protozoa. Protozoa are widely distributed in nature and found in many of the diverse habitats, both aerobic and anaerobic, populated by S-layered bacteria. Thus the diversification of S-layers could be driven by a need for a survival strategy to avoid predation by bacterivorous protozoa. This survival strategy has clearly been shown to be true for the interaction between a prokaryotic predator, *Bdellovibrio bacteriovorus*, and Gram-negative bacteria [110,186]. Prey cells with S-layers (*Aeromonas salmonicida, Aquaspirillum serpens, A. sinusus, Campylobacter fetus* and *Caulobacter crescentus*) were resistant to predation by *B. bacteriovorus*, but not isogenic S-layer-deficient variants (where available). These studies were relatively straightforward in that they involved a predator which attaches to the surface of potential Gram-negative prey cells and then proceeds with its (usually) periplasmic life cycle [110]. The interaction of protozoa and prey cells is opposite to that of prokaryotic predation in that bacteria are ingested by protozoa rather than penetrated by the predator. The feeding mechanisms of bacterivorous protozoa are considerably more varied and complex than the interaction between *B. bacteriovorus* and prey cells. To put the question of S-layers as an anti-protozoal predation device into perspective, the feeding mechanisms of protozoa must first be considered.

### 10.1. Feeding mechanisms of protozoa

Free-living protozoa can informally be divided into three groups based on their mechanism of motility: ciliates, flagellates and sarcodina (which includes ameboid forms). These unicellular eukaryotic microorganisms are exceedingly diverse morphologically, but possess one phenotypic characteristic, phagocytosis, that is still useful in defining protozoa. Phagocytosis is by definition the formation of food vacuoles around particulate matter. Most studies on feeding (grazing) have been done with ciliates and flagellates. The uptake of nutrients by phagocytosis involves a wide diversity of mechanisms, although a cytostome or fixed point of intake is not an essential requirement.

There are three principal categories of feeding mechanisms [187,188]. Filter feeding requires the presence of a filter and a means of propelling water through the filter. If the filter is a purely mechanical sieve, its mesh size will limit the minimum size of particles that can be captured. Purely mechanical filters exhibit no selectivity of food capture. A filter composed of sticky bars may intercept small particles that adhere to the filter as well as larger particles retained by the mesh. Since the mesh of filters of ciliates varies widely, different ciliates can co-exist in the same habitat and use different-size bacteria as a nutritional source. Cilia or flagella may be used to create water flows through the filtering structures. Filter feeders have a constant feeding rate and are more efficient when utilizing food particles that are small relative to the grazer. Thus many filter feeders are bacterivorous.

Direct interception is used by flagellates which either swim freely or create currents while attached to a surface. The flagellates intercept food particles either on their body or on body projections. This method of prey capture is only nutritionally adequate where the size of the prey is a reasonable proportion (at least 10%) of the predator size. Therefore it is not suitable for bacterivory by any protists except for the smallest flagellates.

For diffusion feeding, protozoa that actively search for prey use a range of mechanisms for food capture and intake: e.g. toxic and harpoon-like trichocysts which immobilize the prey. Both ciliates and flagellates can have microtubular feeding organelles which assist in the capture of other protozoa or filamentous cyanobacteria. However, this is not a common feeding mechanism for bacterivorous protozoa.

Simple models of suspension feeding have shown that, in general, small organisms are more effective in extracting small suspended particles than larger organisms [188]. Therefore, studies on the effect of S-layers on protozoan bacterivory will be restricted to small bacterivorous ciliates and flagellates, which feed mainly by filter feeding and direct interception.

### 10.2. Food selection by bacterivorous protozoa

The question of whether bacterivorous protozoa exhibit selectivity of food particles during grazing is
still open to discussion. In vitro, the feeding of ciliated and flagellated protozoa is characterized by the lack of ability to discriminate between different kinds of particles, except for their mechanical properties [189,190]. Particle size discrimination is probably the most important factor in selection. Inert dye particles and fluorescently labelled bacterial-sized latex microspheres have been used to quantitate ingestion rates by protozoa. However, Scherr et al. [191] showed that some marine ciliates and flagellates actively selected against microspheres compared to fluorescently labelled bacteria and Landry et al. [192] showed that a marine flagellate could discriminate between living and heat-killed bacterial prey. These results suggest that factors other than size selection can influence ingestion of particles. Certainly the latex microspheres do not in general mimic the shapes of bacteria.

It is not known to what extent in situ protozoa discriminate between different strains or sizes of bacteria [191]. It is a well-established fact that growth rates of different species of protozoa are strongly affected by the strains of bacterial prey available as food [189]. But this, however, does not imply any selectivity with respect to the type of particles which are ingested. In nature, the selection of food particles can be non-specific and a proportion of ingested material may be of little or no nutritional significance.

The effect of food particle surface properties on protozoan feeding has rarely been studied. Sanders [193] examined the uptake by the ciliate <i>Cyclidium</i> of fluorescent microspheres of different sizes which had been protein-coated, carboxylated or amide modified. <i>Cyclidium</i> ingested 0.6 μm particles at high rates relative to the larger 0.93 μm particles. Surface properties of the microspheres appeared to affect <i>Cyclidium</i> feeding, but discrimination between protein-coated and carboxylated spheres was only observed for 0.93 μm beads. There was no difference in clearance of amide-modified or carboxylated 0.93 μm beads. These data suggest that an interaction of size and surface properties affected feeding only on larger, less-efficiently captured spheres. Interestingly, the clearance rate by <i>Cyclidium</i> of the unicellular cyanobacterium <i>Microcystis</i> (diameter between 0.8 and 1.1 μm) was much higher than on any microsphere tested. One suggestion was that the surface chemistry of <i>Microcystis</i> may have affected ingestion. This leads to a consideration of S-layers, because many unicellular cyanobacteria, including <i>Microcystis</i> spp. [194] and <i>Synechococcus</i> [195] have S-layers.

### 10.3. Ingestion of S-layered bacteria by <i>Tetrahymena thermophila</i> and <i>Paraphysomonas vestita</i>

The only studies to address the effect of S-layers on protozoan grazing have been those of Koval [186]. Bacteria with S-layers that have been well-characterized structurally and biochemically (Table 7) have been used as potential prey cells. The availability of variants without S-layers of some of these bacteria provides a unique opportunity to assess the effect of S-layers on ingestion independently of cell size and shape. Two freshwater bacterivorous protozoa were used in these studies: the filter-feeding ciliate <i>Tetrahymena thermophila</i> (body length less than 50 μm) and the flagellate <i>Paraphysomonas vestita</i> (body length 5–20 μm) which captures prey by direct interception [192].

Bacterivory was estimated by the use of prey cells labelled with the yellow-green fluorescent dye 5-(4,6-dichlorotiazin-2-yl)aminofluorescein (DTAF). The method of Scherr et al. [191] was used to prepare fluorescently labelled bacteria (FLB) with the modification that cell suspensions were incubated with the dye at 30°C for 2 h, instead of at 60°C for 2 h [186]. Heat treatment at 60°C was not compatible with the structural integrity of S-layers and probably affects other properties of the cell wall and surface. Even brief heat treatments can diminish the palatability of food for some protozoans [191]. Short-term uptake experiments were done according to the method of Scherr [192,201]. Under the conditions used a linear uptake of FLB with time was found.

All the bacterial prey listed in Table 7 were ingested by <i>T. thermophila</i> and <i>P. vestita</i>. A comparison of uptake rates of FLB of S-layered bacteria with that of their corresponding S-layer-negative variants (where available) showed that in all cases the S-layered strains were ingested at the same or higher rates (S.F. Koval, unpublished data). The growth rates of the protozoa on these bacteria were not measured, but electron microscopy of thin sections of <i>T. thermophila</i> showed that ingested bacteria were in various stages of digestion within the food vac-
Table 7
Bacterial prey ingested by *Tetrahymena thermophila* and *Paraphysomonas vestita*

<table>
<thead>
<tr>
<th>Organism</th>
<th>S-layer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aeromonas salmonicida</em> A449</td>
<td>1</td>
<td>[196]</td>
</tr>
<tr>
<td><em>Aeromonas salmonicida</em> A449-3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Aquaspirillum serpens</em> MWS</td>
<td>2</td>
<td>[197]</td>
</tr>
<tr>
<td><em>Aquaspirillum serpens</em> VHA</td>
<td>1</td>
<td>[198]</td>
</tr>
<tr>
<td><em>Aquaspirillum serpens</em> VHL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Aquaspirillum sinusum</em></td>
<td>2</td>
<td>[199]</td>
</tr>
<tr>
<td><em>Bacillus brevis</em> 47</td>
<td>2</td>
<td>[200]</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> 168</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Caulobacter crescentus</em> CB2NY66R</td>
<td>1</td>
<td>[105]</td>
</tr>
<tr>
<td><em>Caulobacter crescentus</em> CB2A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Synechococcus</em> GL24</td>
<td>1</td>
<td>[195]</td>
</tr>
</tbody>
</table>

uoles [186]. When digestion begins, the vacuole contents become compact and dense. One exception was *Synechococcus* strain GL 24 where electron-dense irregularly shaped masses were not formed in the same manner as for the other bacteria (S.F. Koval, unpublished data).

One property that S-layers can confer on bacterial cell surfaces is increased hydrophobicity, which may affect the susceptibility of bacteria to grazing. The bacterial adherence to hydrocarbons (BATH) method [51] with hexadecane was used to assess the relative hydrophobicity of bacterial prey. All S-layered bacteria were more hydrophobic than *B. subtilis* 168 and less hydrophobic than *Staphylococcus aureus* Oxford strain, which exhibited a high affinity for hexadecane (Table 8, unlabelled cells). In addition, all strains with S-layers, except *C. crescentus* CB2NY66R, were more hydrophobic than their S-layer-negative variants. These results confirm the work of Trust et al. [202] who reported that the presence of an S-layer increases the surface hydrophobicity of *A. salmonicida* species. The relative hydrophobicities of the two *C. crescentus* strains have not previously been reported (J. Smit, personal communication), but since the S-layer is assembled via a type I secretion pathway [116], the S-layer protein need not be hydrophobic. This S-layer protein contains 25% serine and threonine residues, which would tend to make the surface more hydrophilic than hydrophobic. Schultz-Lam and Beveridge [203] also used the BATH test to show that *Synechococcus* GL 24 was considerably more hydrophobic than *B. subtilis* 168 and *Escherichia coli* AB264 which is a K12 strain.

The relative hydrophobicities of fluorescently labelled cells were then measured to determine the ef-

Table 8
Relative hydrophobicities assessed by adherence of cells to n-hexadecane

<table>
<thead>
<tr>
<th>Organism</th>
<th>S-layer</th>
<th>% Adherence of cells</th>
<th>Unlabelled</th>
<th>FLB*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aeromonas salmonicida</em> A449</td>
<td>+</td>
<td>71.5</td>
<td>47.2</td>
<td></td>
</tr>
<tr>
<td><em>Aeromonas salmonicida</em> A449-3</td>
<td>-</td>
<td>20.4</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td><em>Aquaspirillum serpens</em> MWS</td>
<td>+</td>
<td>52.0</td>
<td>37.3</td>
<td></td>
</tr>
<tr>
<td><em>Aquaspirillum serpens</em> VHA</td>
<td>+</td>
<td>78.6</td>
<td>59.6</td>
<td></td>
</tr>
<tr>
<td><em>Aquaspirillum serpens</em> VHL</td>
<td>-</td>
<td>21.9</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td><em>Caulobacter crescentus</em> CB2NY66R</td>
<td>+</td>
<td>34.8</td>
<td>14.7</td>
<td></td>
</tr>
<tr>
<td><em>Caulobacter crescentus</em> CB2A</td>
<td>-</td>
<td>69.8</td>
<td>62.7</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus brevis</em> 47</td>
<td>+</td>
<td>16.8</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> 168</td>
<td>-</td>
<td>1.3</td>
<td>17.0</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> Oxford</td>
<td>-</td>
<td>90.5</td>
<td>92.6</td>
<td></td>
</tr>
</tbody>
</table>

* FLB, fluorescently labelled bacteria.
fect of the fluorescent dye on cell surface hydrophobicity. For all Gram-negative bacteria the hydrophobicity of cells decreased when labelled with DTAF, but the hydrophobicity of S-layered cells still remained higher than that of their respective S-layer-negative variants (except for *C. crescentus*) (Table 8, FLB). Thus the uptake data of FLB could be related to cell surface hydrophobicity. The effect of DTAF on cell surface hydrophobicity varied for the three Gram-positive bacteria. An isogenic S-layer-negative strain of *B. brevis* strain 47 was not available, and thus the hydrophobicity data for this species cannot be compared directly to those of *B. subtilis* strain 168.

Three lines of evidence (S.F. Koval, unpublished data) support the conclusion that cell surface hydrophobicity does not affect prey selection. (1) S-layered bacteria labelled with DTAF were ingested at the same or higher rates than their isogenic S-layer-negative variants. (2) A hydrophobic, autofluorescent cyanobacterium, *Synechococcus* GL24, was ingested by *T. thermophila* at a rate equal to or higher than other S-layered bacteria. (3) The very hydrophobic Gram-positive bacterium *S. aureus* Oxford was also ingested at a high rate.

In another study, Gurijala and Alexander [204] tested whether bacterial growth rate and cell surface hydrophobicity determined the size of bacterial populations surviving protozoan predation. In their long-term studies, heat-killed *E. coli* cells served as an alternative prey. None of the viable bacterial prey tested had S-layers, but the three species (*Klebsiella pneumoniae*, *Flavobacterium* sp. and *Streptococcus faecalis*) that survived at highest densities when actively grazed by *T. thermophila* possessed more hydrophobic cell surfaces than the other species tested. The survival of these species was also related to the capacity of the bacterium to grow and the population density of the species before the onset of intense grazing. These studies more clearly reflect in vivo grazing conditions and population dynamics.

10.4. Conclusions

Cell surface hydrophobicity per se, and S-layers specifically, do not affect prey selection by a filter-feeding ciliate and a small heterotrophic flagellate which captures prey by direct interception. Previous studies have addressed one possible mechanism of bacterial resistance to protozoan predation: avoidance of ingestion. It is becoming increasingly evident that some Gram-negative bacteria, including pathogens, can resist predation by surviving killing and digestion within food vacuoles [205]. *Legionella pneumophila* and *Listeria monocytogenes* infect and multiply within free-living amebas [205] and a strain of *A. salmonicida* has been reported to multiply within *Tetrahymena pyriformis* [206]. The presence of an S-layer on this strain of *A. salmonicida* was not noted, but, if present, it did not hinder ingestion, as in our studies. Examination of thin sections of monocultures of *T. thermophila* and *A. salmonicida* strains A450 and A450-3 has shown that the bacteria were digested within food vacuoles (S.F. Koval, unpublished data). Viable counts of intracellular bacteria were not determined. It has been suggested, however, that resistance to digestion by predatory protozoa may have been an evolutionary prerequisite of bacterial pathogens and a survival mechanism for bacteria in aquatic environments [205]. Clearly the next step for S-layer research is to examine survival of cells within bacterivorous protozoa.

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