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Sylvie S. Lortal, J. van Heijenoort, K. Gruber, U.B. Sleytr

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S-layer of *Lactobacillus helveticus* ATCC 12046: isolation, chemical characterization and re-formation after extraction with lithium chloride

SYLVIE LORTAL,[1]* JEAN VAN HEIJENOORT,[2] KARIN GRUBER[3] and UWE B. SLEYTR[3]

[1]*INRA, Laboratoire de Recherches de Technologie Laitière, 65 rue de St-Brieuc, 35042 Rennes cedex, France*
[2]*CNRS, Centre National de la Recherche Scientifique, Unité de Recherche Associée 1131, Université Paris Sud, 91405 Orsay, France*
[3]*Zentrum für Ultrastrukturforschung und Ludwig Boltzmann Institut für Molekulare Nanotechnologie, Universität für Bodenkultur, 1180 Vienna, Austria*

In a previous study, electron microscopic examinations of thin sections of *Lactobacillus helveticus* ATCC 12046 revealed a three-layered structure of the cell wall. The outermost component was identified as a layer of a non-glycosylated 52 kDa protein. Freeze-etched preparations of intact cells have now demonstrated that this protein layer is an oblique surface layer (S-layer) lattice ($a = 4.5$ nm, $b = 9.6$ nm, $\gamma = 77$ °) which completely covers the cell surface. Treatment with 5 M-LiCl extracted the S-layer protein from intact cells efficiently and selectively. Viability did not decrease significantly. Moreover, the S-layer reappeared when treated cells were allowed to grow again. *In vitro* self-assembly products obtained upon aggregation of isolated S-layer subunits exhibited the same oblique S-layer symmetry as observed on intact cells *in vivo*. The purified S-layer protein had a high content (44%) of hydrophobic amino acids. The N-terminal sequence was mainly composed of alanine, threonine, asparagine and aspartic acid.

Introduction

Crystalline surface layers (S-layers) are a common feature among eubacteria and archaeobacteria (Kandler & König, 1985; Sleytr & Messner, 1988; Messner & Sleytr, 1992). They are composed of identical subunits consisting of a single protein species, which may be glycosylated, and completely cover the cell surface (Beveridge, 1981; Sleytr & Messner, 1983; Koval, 1988). The biological functions of S-layers in eubacteria include protection, cell adhesion and surface recognition (Beveridge, 1981; Smit, 1986; Gruber & Sleytr, 1991).

Lactobacillus helveticus has great technological significance, particularly in cheese making (Accolas *et al.*, 1980; Turner *et al.*, 1983). In previous work (Lortal *et al.*, 1991) the cell wall of *L. helveticus* ATCC 12046 was found to have a three-layered structure. The external layer was shown to be composed of a 52 kDa protein, non-covalently linked to the internal layers. These results strongly suggested the presence of an S-layer on *L.*

helveticus ATCC 12046, especially since an S-layer has been detected on other strains of *L. helveticus* by Masuda & Kawata (1983).

In this paper the presence of an S-layer on *L. helveticus* ATCC 12046 was confirmed by analysis of the cell surface topography using freeze-etching techniques. Isolation procedures and reassembly experiments *in vitro* are described as well as re-formation of the S-layer on intact cells after extraction. The amino acid composition and the N-terminal sequence of the purified S-layer protein were determined.

Methods

Bacterial strain and growth conditions. *Lactobacillus helveticus* ATCC 12046 was obtained from the Pasteur Institute, Paris, France, and stored at -30 °C in MRS broth (De Man *et al.*, 1960) containing 15% (v/v) glycerol. The strain was grown unstirred in MRS medium at 43 °C after inoculation with 1% (v/v) overnight culture.

Determination of the effect of LiCl and guanidium hydrochloride (GHCl) treatment on cell viability. Viability was determined as c.f.u. on MRS agar plates after incubation for 48 h at 43 °C under anaerobic conditions. Exponential-phase cells (1.6 mg dry mass ml⁻¹; 6.5×10^9 c.f.u. ml⁻¹) were incubated for 15 min at several temperatures with LiCl or guanidinium hydrochloride (GHCl) to extract the S-layer

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protein. The treated bacteria were recovered by centrifugation, washed once in sterile distilled water and resuspended in the initial volume of MRS broth for the determination of viable bacteria by plate counting.

Extraction of the S-layer protein with LiCl and S-layer re-formation on growing cells. Exponential-phase cells were harvested and washed once with distilled water. Ten–fifteen mg of the moist pellet were suspended per ml of 5 M-LiCl and kept at 0 °C for 15 min, followed by centrifugation (30000 g, 15 min). The supernatant was designated the crude extract (CE). The pellet of LiCl-extracted cells was washed once in distilled water, resuspended in prewarmed (43 °C) MRS broth to an OD_{650} of 0·5 (corresponding to 0·2 mg dry wt ml^{-1}) and incubated at 43 °C. An aliquot was kept at 4 °C.

Purification of the S-layer protein. The CE was dialysed against distilled water overnight at 4 °C. For analysis of the amino acid composition, a more extensive purification of the S-layer protein was carried out by gel permeation chromatography: the CE was applied to a column (2·5 × 50 cm) of Sephacryl S200 (Pharmacia) equilibrated in 5 M-LiCl. Upward elution was done at a flow rate of 50 ml h^{-1} and fractions of 5 ml were collected. Protein was determined by the Lowry method using BSA as standard. Amino acid analysis was performed with a Pharmacia LKB Alpha Plus instrument as described by Lortal *et al.* (1991).

Preparation of cell walls. Cell walls were prepared by mechanical disruption of whole cells using a French press as described by Lortal *et al.* (1991).

Electron microscopy and labelling with polycationized ferritin. Negative staining was obtained by treating the grids for 2 min with a drop of uranyl acetate (2%, w/v, in distilled water). Thin sectioning was performed as described by Lortal *et al.* (1991). Freeze-fracturing, freeze-etching and optical diffraction procedures for determining lattice constants are described by Messner *et al.* (1984) and Pum *et al.* (1989). For freeze-drying preparations, the pioloform-coated gold grids (400 mesh) were glow-discharged (CTA 010, Balzers) and treated with poly-L-lysine (M_r 56000, Sigma, 0·1 % in distilled water) for 10 min to produce a positively charged surface. After floating upside down on the bacterial suspension for 10 min, the grids were washed three times in distilled water and moved onto a drop of polycationic ferritin (PCF, Sigma), incubated for 10 min and washed three times in 0·05 M-Tris/HCl buffer, pH 7·2. The grids were blotted onto wet filter paper for 45 s (Kistler *et al.*, 1977) after washing in distilled water to remove residual buffer. The grids were immediately frozen by plunging into liquid nitrogen and were mounted onto a precooled freeze-drying table of a Balzers BAF 400 freeze-etching machine. After freeze-drying at − 80 °C for 1 h the samples were shadowed unidirectionally with 2 nm platinum at an angle of 45°. The heavy metal shadow was reinforced with a 15 nm carbon layer deposited at an angle of 90 ° (Pum *et al.*, 1989). The preparations were first rinsed in acetone and then incubated in chloroform (both from Merck) at room temperature for 3 h followed by an overnight treatment with 30% (v/v) chromic acid at 37 °C. After four washes in distilled water the grids were air-dried. Electron microscopy was performed with a Philips EM 301 microscope using an acceleration voltage of 80 kV.

SDS-PAGE. (i) For determination of the apparent molecular mass of the S-layer protein and for electroblotting experiments, SDS-PAGE was performed using a 15% (w/v) acrylamide separating gel (15 × 15 cm) as described by Lortal *et al.* (1991). Molecular mass standard proteins were obtained from Pharmacia.

(ii) For all other experiments, SDS-PAGE was performed using a 10% acrylamide separating gel (8 × 8 cm) and a 4% acrylamide stacking gel. Samples of whole cells (equal weights when comparison between lanes was required), cell walls or isolated S-layer protein were suspended in a 3% (w/v) SDS solution in 0·05 M-Tris/HCl buffer,

pH 7·2, and treated for 10 min in a boiling water bath; then the samples were centrifuged and 25 µl of the supernatant was mixed with 40 µl of the following solution: 0·1% SDS, 0·1% 2-mercaptoethanol, 30% (w/v) glycerol, 0·2% Orange G in 0·01 M-Tris/HCl buffer, pH 7·2. Gels were stained with Coomassie blue G250.

Electroblotting and N-terminal sequence. Immediately following SDS-PAGE, the proteins were electrohoretically (50 V, 1·25 h) transferred to an Immobilon sheet (PVDF 0·45 µm, Millipore). The membrane was stained for 30 s with a solution composed of 0·1% (w/v) amido black, 45% (v/v) methanol and 7% (v/v) ethanol in distilled water, and washed once in distilled water. The area of the membrane containing the 52 kDa protein was cut out with a sterile scalpel and kept at − 20 °C until microsequencing was performed with a gas-phase analyser at the Institute of Neurophysiology at the CNRS, Gif sur Yvette, France.

Treatment of intact cells with lytic enzymes. Lysozyme and mutanolysin (both from Sigma) were dissolved before use in 0·05 M-Tris/HCl buffer (pH 7·2) and kept at 4 °C. The effect on the bacteria was followed by the decrease in OD_{650} of a cell suspension in Tris/HCl buffer, pH 7·2, at 37 °C. The concentration of the buffer was 0·05 M for mutanolysin and 0·1 M for lysozyme; concentrations of the enzymes are indicated in the Figure legends or in the text.

Results

Morphological characterization of the S-layer

Freeze-etched preparations of whole cells of *L. helveticus* ATCC 12046 (Fig. 1*a*, *b*) revealed the presence of a crystalline array which completely covers the cell surface. A clear fracture plane between the peptidoglycan-containing layer and the S-layer could frequently be observed in oblique fracture faces along the cell envelope (Fig. 1*b*). In such areas [indicated by the arrowheads in Fig. 1(*b*)] the thin outermost S-layer can clearly be recognized. In thin sections (Fig. 1*c*) the S-layer appeared only approximately 9 nm thick, whereas the underlying layer was three- to four-times as thick. Optical diffraction experiments (Fig. 4*b*) with self-assembly products of the 52 kDa S-layer protein showed that the length of the lattice base vectors were 4·5 and 9·6 nm, respectively, with an interaxial angle γ of 77 °.

Isolation, purification and chemical characterization of the S-layer protein

Previous work (Lortal *et al.*, 1991) showed that the outermost layer of the cell wall was composed of a non-glycosylated 52 kDa protein. In this investigation, several procedures for extracting the S-layer protein from whole cells and isolated cell walls were tested (Table 1). Since the S-layer protein represented approximately 14% of the total protein content of the bacteria and gave a dominant band on SDS electrophoretograms (Fig. 2, lane A), the efficiency of the various extraction procedures could be followed by SDS-PAGE (data not shown).
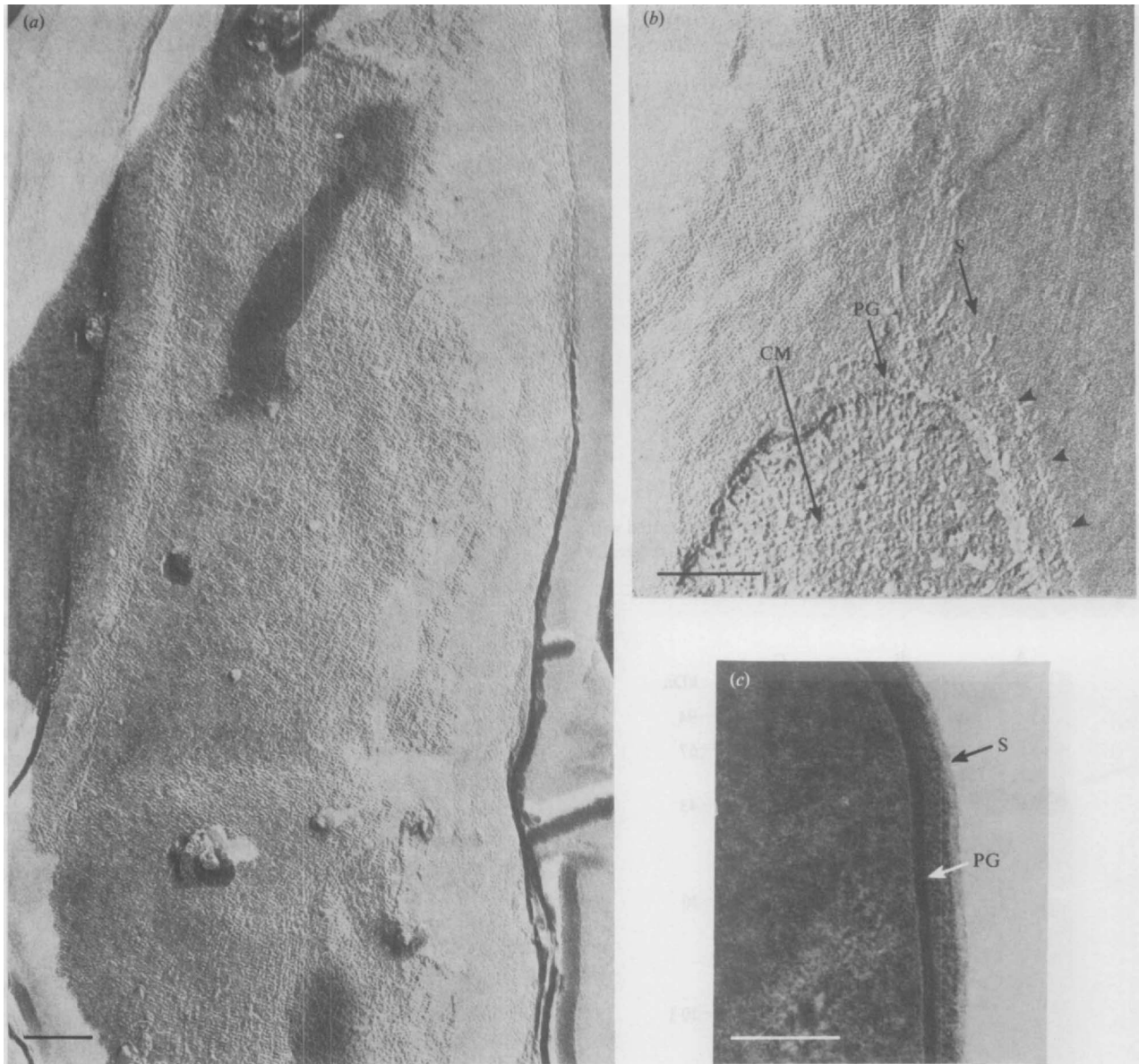
Fig. 1. (a) Freeze-etched preparations of whole cells of *L. helveticus* ATCC 12046 exhibiting an oblique (p2) S-layer lattice which completely covers the cell surface. (b) Oblique fracture faces of the cell envelope revealing a thick peptidoglycan-containing layer (PG) and a thin S-layer (S); CM, concave fracture face of the cytoplasmic membrane. (c) Thin section of whole cells of *L. helveticus* ATCC 12046; note the thin S-layer [abbreviations as for (b)]. Bars, 0·1 μm.

Tween 80, varying concentrations of urea and heating for 15 min in 0·1 M-sodium phosphate buffers at 50 °C (pH 5, 6, 7·5, 9) were inefficient. HCl (0·1 M) had an extractive effect but also led to hydrolytic damage of the S-layer protein. SDS, GHCl and LiCl were found to be efficient extraction agents. Among them, LiCl (5 M, 15 min, 0 °C) was the most selective (Fig. 2, lane B) and was therefore chosen for further assays. Lower concentrations of LiCl (1 and 2·5 M) extracted less protein. A linear relationship was observed between the concentration of LiCl and the quantity of protein solubilized. Repeated LiCl treatment had an insignificant effect on the increase in efficiency of extraction (Fig. 2, lane C).

During dialysis of the LiCl CE against distilled water at 4 °C, a white precipitate appeared which could be recovered by centrifugation. The supernatant (Fig. 3, lane A) revealed some contaminant proteins whereas the white pellet (Fig. 3, lane B) contained mainly the 52 kDa
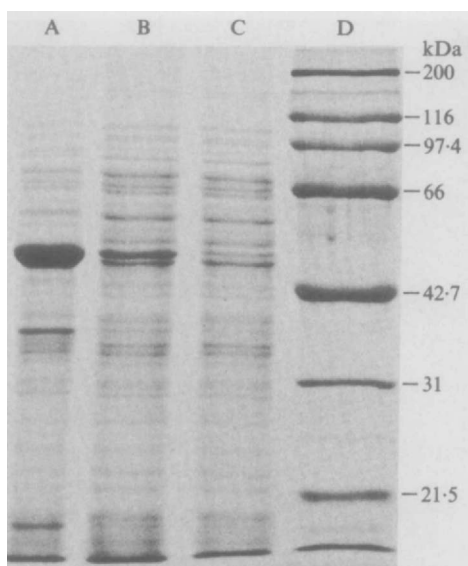
Fig. 2. SDS-PAGE. Lanes: A, whole cells; B, whole cells treated with 5 M-LiCl once; C, whole cells treated with 5 M-LiCl twice; D, molecular mass standards.
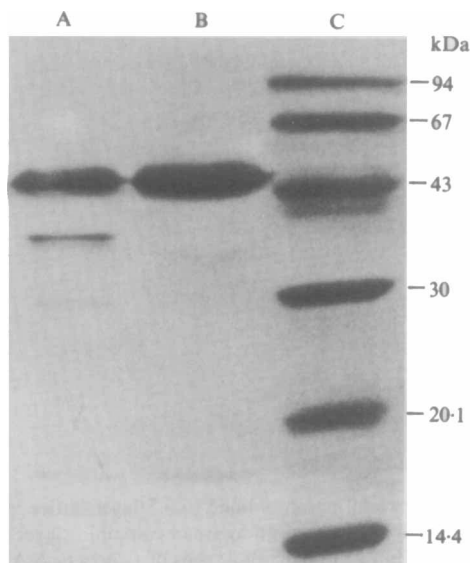

Fig. 3. SDS-PAGE of (A) the supernatant and (B) the pellet obtained upon centrifugation of the dialysed LiCl crude extract. Lane C, molecular mass standards.

protein and was therefore regarded as pure enough for analysis of its N-terminal sequence. In the sequence obtained – ATTINADSAINANTNAKYDVDVT – alanine, threonine, asparagine and aspartic acid accounted for 70% of the amino acids. Negative staining revealed that the precipitated material obtained during dialysis of the LiCl extract was reassembled S-layer

Table 1. *Effect of various chemical treatments on the S-layer of L. helveticus ATCC 12046*

Each treatment was applied at 0 °C for 10 min, except where otherwise indicated. r.t., Room temperature.

Treatment	Whole cells*	Cell walls*
Tween 80 (5%)	−	−
Urea (1 M)	−	+/−
Urea (3 M)	−	+/−
HCl (0·1 M)†	+	+
LiCl (5 M, 15 min)	+	+
GHCl (5 M, 15 min)	+	+
SDS (1%, r.t., 30 min)	+	+
SDS (1%, 100 °C, 5 min)	+	+

* +, Solubilization of S-layer protein; −, no effect.
† The S-layer protein was clearly degraded by this treatment.

protein (Fig. 4a). Optical diffraction analysis of electron micrographs (Fig. 4b) revealed that the sheet-like self-assembly products had the same oblique lattice as observed in freeze-etched preparations of intact cells (Fig. 1a). Purification of the S-layer protein was carried out on a Sephacryl S-200 column. The first peak eluted consisted of the 52 kDa protein, as verified by SDS-PAGE (data not shown). The combined fractions were dialysed against distilled water and the precipitated protein was dried overnight using a speed vacuum centrifuge (Savant Instruments). After hydrolysis, the amino acid composition of the protein was determined. As summarized in Table 2, the hydrophobic amino acids accounted for 44% of the amino acid residues present.

Effect of LiCl and GHCl treatment on viability

Treatment of bacteria with 5 M-LiCl led to a limited loss of viability (80%, i.e. <1 log unit) whereas the extraction with 5 M-GHCl led to a drastic loss of viability (6·2 log units). On repeated treatment with 5 M-LiCl, viability decreased significantly (3·2 log units). For all treatments, results were the same whether the procedures were performed at 0 °C or at room temperature.

Reformation of the S-layer after extraction with LiCl

LiCl-treated cells (sample B, Fig. 5a) incubated at 43 °C in MRS broth, grew again as shown in Fig. 5(a). Sample A (untreated cells) and samples B–K harvested at different times, as shown in Fig. 5(a), were analysed by SDS-PAGE (Fig. 5b), and labelled with PCF and freeze-dried for electron microscopical observations (Fig. 6). Hardly any PCF absorbed to S-layer-carrying cells (Fig. 6a). This implies that the surface of the S-layer is not negatively charged. However, PCF formed a dense layer
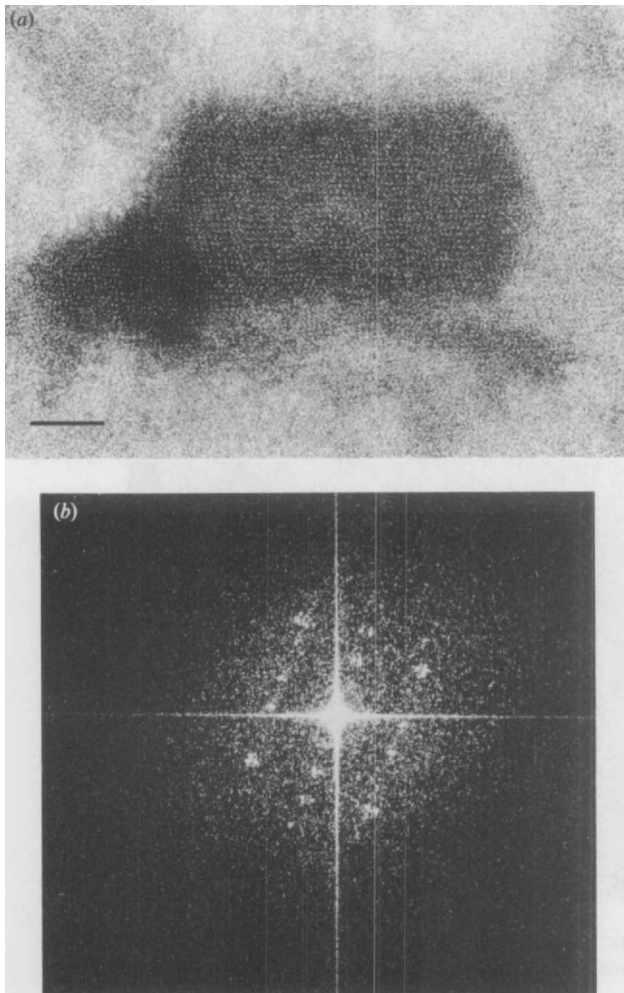
Fig. 4. (a) Negative staining of the S-layer self-assembly product obtained upon dialysis of the LiCl cell extract (bar, 0·05 μm). (b) Diffraction pattern from S-layer self-assembly product reflecting the oblique symmetry of the crystalline lattice.

Fig. 5. (a) Growth (OD$_{650}$) of LiCl-treated whole cells (B) after suspension in MRS broth at 43 °C; sample X was kept at 4 °C and did not grow. (b) SDS-PAGE of whole cells harvested at different times (B–K) indicated on the growth curve in (a), showing the reappearance of the 52 kDa S-layer protein. Lane A corresponds to untreated whole cells; lane X corresponds to sample X.

on the surface of cells from which the S-layer had been removed (Fig. 6b). This confirmed the complete extraction of the S-layer by LiCl. As PCF does not absorb onto the S-layer, Fig. 6(c) and (d) suggested that the binding of PCF molecules was prevented by the progressive reformation of the S-layer on LiCl-treated cells of *L. helveticus* ATCC 12046.

Sensitivity of cells to lytic enzymes before and after removal of the S-layer

To investigate a potential protective role for the S-layer against muramidases, whole cells before and after LiCl treatment were exposed to lysozyme and mutanolysin. LiCl treatment did not increase sensitivity to lysozyme,
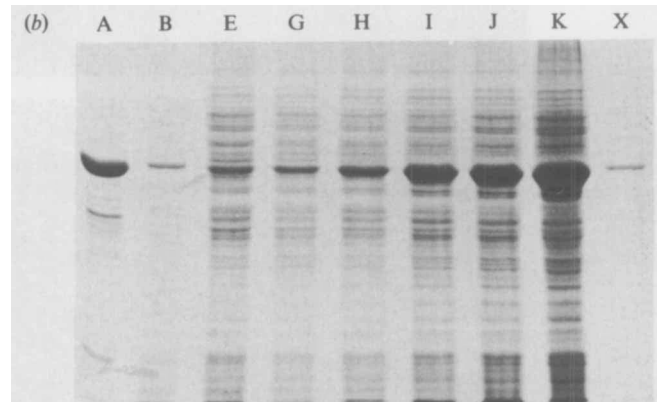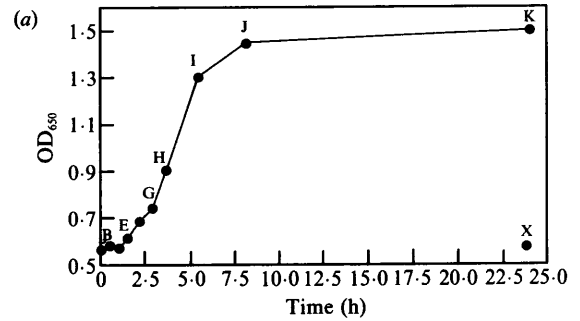
Table 2. *Amino acid composition of the S-layer protein*

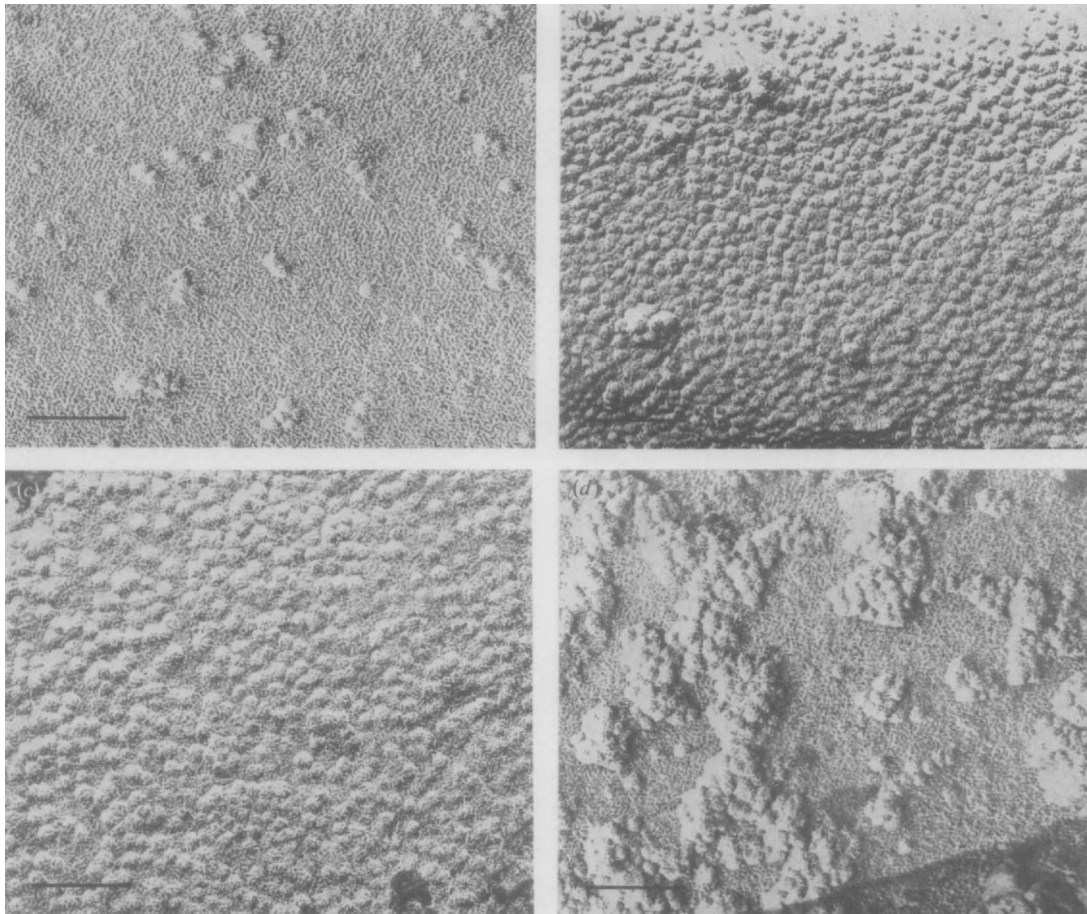| Amino acid	Molar ratio (%)
Asx	14·15
Ala	11·4
Thr	11·1
Lys	10·9
Ser	9·5
Gly	8·75
Val	7·9
Glx	7·35
Ile	4
Pro	3·8
Leu	3
Tyr	3
Phe	2·4
Arg	1·45
His	1·1
Met	0·2
Cys	0
Try	ND

ND, Not determined.

Fig. 6. Freeze-dried preparations of whole cells labelled with polycationic ferritin (PCF). (a) Untreated cells, (b) LiCl-treated cells, and (c, d) stationary phase cells J and K (Fig. 5a), respectively. The reappearance of the S-layer is shown by the concomitant decrease of the number of bound PCF molecules. Bars, 0·1 μm.

whereas mutanolysin had a more rapid lytic effect on cells devoid of an S-layer (Fig. 7).

Discussion

The presence of an oblique S-layer on the external surface of *L. helveticus* ATCC 12046 was clearly established by freeze-etching and negative staining. This has extended our previous work (Lortal *et al.*, 1991) where the more external layer of the cell wall was shown to consist of a 52 kDa non-glycosylated protein, non-covalently bound to other cell wall constituents. Other strains of *L. helveticus* have also been found to contain an S-layer (M. Sàra, personal communication). Masuda & Kawata (1983) found an S-layer in other *L. helveticus* strains.

Although several studies of the structure and chemical composition of the cell walls of lactobacilli have been performed (Baddiley & Davison, 1961; Ikawa & Snell,
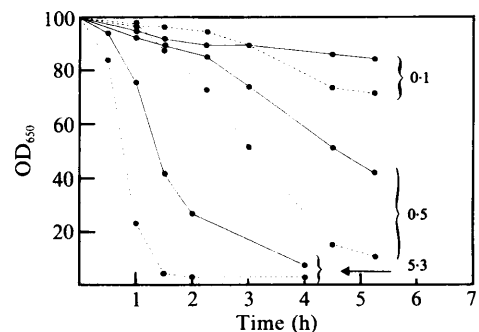

Fig. 7. *L. helveticus* ATCC 12046 cells, untreated (———) or LiCl-extracted (------), were tested for their susceptibility to the lytic action of mutanolysin at the final concentrations indicated (μg ml⁻¹) in 0·05 M-Tris/HCl buffer, pH 7·2.

1960; Williams, 1971), only two strains have been investigated using freeze-etching techniques: *Lactobacillus fermenti* (Kawata *et al.*, 1974) and *Lactobacillus brevis* (Masuda & Kawata, 1979). These displayed

regular arrays with oblique symmetry like *L. helveticus*. Release of the S-layer protein from isolated cell walls of *L. fermenti* was achieved by enzymic hydrolysis of the peptidoglycan and from *L. brevis* by extraction with GHCl, urea and SDS. Negative staining and electrophoresis also revealed S-layers on *L. casei* (Barker & Thorne, 1970), *L. brevis* (Masuda & Kawata, 1980, 1983), *L. buchneri*, *L. acidophilus* and *L. bulgaricus* (Masuda & Kawata, 1981, 1985). For all these lactobacilli the apparent molecular mass of the S-layer protein was in the range 41–52 kDa.

We found that it was possible to extract the S-layer protein from whole cells of *L. helveticus* directly at a remarkably high concentration compared to other proteins using 5 M-LiCl. During the removal of the LiCl by dialysis, the S-layer subunits reassembled spontaneously into sheets with the same p2 symmetry and lattice constants as observed *in vivo*, showing that they were not substantially altered by the LiCl treatment. Reattachment experiments with isolated S-layer subunits on naked peptidoglycan-containing layers of *L. helveticus* ATCC 12046 have not been performed although studies on homologous or heterologous reattachment with S-layers from other lactobacilli have provided interesting data on the reattachment of S-layers amongst different organisms (Masuda & Kawata, 1985). In agreement with data on S-layers from other bacterial species (Masuda & Kawata, 1983; Sleytr & Messner, 1988), the S-layer protein of *L. helveticus* had a high content of hydrophobic amino acids and no or very little sulphur-containing amino acids. As observed by Luckevitch & Beveridge (1989), no homology between the N-terminal sequence determined here and that from other S-layer proteins was evident.

The LiCl procedure used in this work for extracting the S-layer protein of *L. helveticus* is a new and interesting alternative to the classical methods with GHCl or SDS. Electron microscopy, electrophoresis and chemical analysis demonstrated the efficiency and selectivity of this method. Moreover, LiCl was less lethal than GHCl to the bacteria. One of the most interesting features was the reformation of the S-layer after its removal. The new S-layer material appeared during the exponential-phase on the cell surface in a random distribution. The change in the mode of distribution of the PCF molecules from Fig. 6(*c*) to 6(*d*) could be interpreted as a 'charge drift'. Pushed or partially masked by growing and fusing crystallites of the newly synthesized S-layer protein, the long teichoic acid molecules protruding from the peptidoglycan layer could form areas of high negative charge in the closing S-layer. For two *Bacillus* species (Gruber & Sleytr, 1988; Howard *et al.*, 1982), the incorporation of newly synthesized S-layer protein into a coherent S-layer lattice has been

shown to be non-random. However, the S-layer reformation described here was a repair process of cells stripped of the S-layer and, thus, probably reflected conditions quite different from the incorporation of S-layer domains in an existing lattice during cell growth.

No specific biological significance of the S-layer lattice from lactobacilli has been detected so far. In *L. helveticus* ATCC 12046 the S-layer appeared as a thin layer completely covering the thick peptidoglycan-containing layer. Since no morphological changes of the cells could be detected after removal of the S-layer with LiCl and during subsequent cell growth and S-layer reformation, a cell-shape-determining function of the crystalline array (Beveridge, 1988; Sleytr & Messner, 1988) can be excluded. Previous studies on the permeability properties of S-layers from mesophilic and thermophilic Bacillaceae (Sàra & Sleytr, 1987; Sàra *et al.* 1990) have shown that S-layers have the potential to function as selective molecular sieves. S-layers preventing the entry of molecules with molecular masses exceeding 10000–15000 kDa could provide the organism with a selective advantage, since most lytic enzymes present in natural habitats have larger molecular masses (Stewart *et al.*, 1986; Glenn, 1976). Since the S-layer of *L. helveticus* could not protect the peptidoglycan from lysozyme (M_r 14600) and mutanolysin (M_r 24000) it can be concluded from the molecular dimensions of these enzymes that the pores in the oblique S-layer lattice must be larger than 3·5 nm (Sàra *et al.*, 1990). Further studies will show if the S-layer can protect the cells from proteases which generally have molecular masses larger than common muramidases. Only a few molecules of polycationized ferritin were adsorbed on whole cells of *L. helveticus* showing that the S-layer surface did not exhibit a net negative charge. The same observations were made with different *Clostridium* (Sàra *et al.*, 1988) and *Bacillus* (Pum *et al.*, 1989) species. Therefore, the functional role of the S-layer of *L. helveticus* could be to mask the underlying strongly negatively charged peptidoglycan-containing layer, as has been proposed previously for mesophilic and thermophilic *Bacillaceae* (Sàra *et al.*, 1988; Sàra *et al.*, 1990; Sleytr & Messner, 1988). Further study on the interaction of *L. helveticus* ATCC 12046 with environmental components such as milk proteins will help to elucidate this functional aspect.

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