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Chemical Characterization of the Regularly Arranged Surface Layers of *Clostridium thermosaccharolyticum* and *Clostridium thermohydrosulfuricum*

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Clostridum thermosaccharolyticum and *Clostridium thermohydrosulfuricum* possess as outermost cell wall layer a tetragonal or hexagonal ordered array of macromolecules. The subunits of the surface layer can be detached from isolated cell walls with urea (8 M) or guanidine-HCl (4 to 5 M). Triton X-100, dithiothreitol, ethylenediaminetetraacetate, and KCl (3 M) had no visible effect on the regular arrays. Sodium dodecyl sulfate-polyacrylamide electrophroesis showed that, in both organisms, the surface layer is composed of glycoprotein of molecular weight 140,000. The glycoprotein from both microorganisms has a predominantly acidic amino acid composition and an acidic isoelectric point after isoelectric focusing on polyacrylamide gels. The glycocomponent is composed of glucose, galactose, mannose, and rhamnose.

Clostridium thermosaccharolyticum and *Clostridium thermohydrosulfuricum*, two taxonomically closely related hyperthermophilic clostridia (8), carry on their cell surfaces a layer of tetragonally or hexagonally ordered macromolecules, respectively. The appearance and arrangement of these surface (S) layers on intact cells and isolated cell walls have been studied in detail using negative staining, thin sectioning, and freeze-etching techniques (8, 17-19). Detached S-layer subunits from both clostridia were found to possess the ability to reattach either to the cell walls from which they had been removed or to the cell walls of the other strain. The resulting regular arrays are identical to the arrays found on the cell walls from which the subunits had been isolated (17). These results, together with the observation that isolated S-layer subunits have the ability to assemble in vitro to form the same pattern as that seen on intact cells, have shown that the regular arrays are determined only by the directional bonds between the S-layer subunits and not by any pattern in the binding sites of the supporting cell wall layer (17).

Although regular arrays of macromolecules have been observed on a variety of both gram-positive and gram-negative bacteria, few chemical analyses have been done. So far it has been found that the surface subunits are composed of acidic protein of molecular weight 125,000 to 150,000 in *Spirillum serpens* (3), 67,000 in *Acinetobacter* sp. strain MJT/F5/199A (22), and 86,000 to 150,000 in *Bacillus sphaericus* (9).

The present paper describes the results of a study of the detachment and chemical characterization of the S-layer subunits from *C. thermosaccharolyticum* and *C. thermohydrosulfuricum*. Evidence was obtained that the subunits are glycoproteins of molecular weight 140,000 and that they interact with each other through ionic and hydrophobic bonds and with the underlying cell wall by means of hydrogen bonds.

MATERIALS AND METHODS

Organisms and growth conditions. *C. thermohydrosulfuricum* L111-69 and *C. thermosaccharolyticum* D120-70, obtained from the culture collection of the Austrian Sugar Research Institute (8), were grown under anaerobic conditions in 500-ml flasks in TSE medium (BBL) (8, 10). Strain L111 was incubated at 70 C and strain D120-70 was incubated at 62 C. Cells in the logarithmic or early stationary phase of growth were harvested by centrifugation at 2,000 × *g* for 90 min in a cooled MSE Mistral centrifuge (Measuring & Scientific Equipment, Ltd., London) at 4 C, followed by centrifugation at 12,000 × *g* for 15 min.

Preparation of cell walls. The method of preparation of cell wall fragments consists of the breakage of the cells in a French pressure cell and treatment with deoxyribonuclease (5 μg/ml) and ribonuclease (20 μg/ml), followed by four washes in 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.4. To remove contaminating plasma membrane, the crude cell wall preparation was treated with 0.5% Triton X-100 and washed four more times in Tris-hydrochloride, pH 7.4.

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Detachment of S-layer. Different methods were tested for the removal of the S-layer subunits from isolated cell walls. A portion of cell walls was suspended in 3 to 5 ml of each of the solutions to be studied, at a concentration of 10 to 30 mg of protein per ml, and incubated under the conditions shown in Table 1. The suspensions were then chilled in an ice bath centrifuged at 25,000 × *g* for 15 to 50 min and washed twice with cold 50 mM Tris-hydrochloride buffer, pH 7.4. After treatment, preparations of cell walls, negatively stained with 0.5 to 1.0% uranyl acetate, pH 5.2, were examined in the electron microscope for evidence of loss of the S-layer. Extraction with 5 M guanidine-HCl was found to be the most effective treatment and was used routinely for the detachment of the S-layer. After treatment the supernatant was dialyzed for up to 48 h against three changes of distilled water and then used for chemical characterization.

Electrophoresis. Disc electrophoresis was performed on 10% polyacrylamide gels in 0.1% sodium dodecyl sulfate as described by Weber and Osborn (25), with lysozyme, ovalbumin, serum albumin, paramyosin, and myosin as standards for molecular weight estimations. Before electrophoresis, samples (10 to 50 μg of protein) were incubated with 0.1% sodium dodecyl sulfate in 0.1 M sodium phosphate (pH 7.2) and 5% mercaptoethanol in a total volume of 50 to 100 μl for 20 min at 70 C. After electrophoresis proteins were detected by staining with 0.01% Coomassie brilliant blue R (Gurr, High Wycombe, Bucks, U.K.). Glyco compounds were detected by the periodate-Schiff method (4).

Isoelectric focusing was done in Ampholine (pH 3.5 to 10) in polyacrylamide gels containing 1% Triton X-100. Gels were stained with Coomassie brilliant blue and by the periodate-Schiff method (4).

Chemical analysis. Protein determinations were made by the Folin method of Lowry et al. (12), with bovine serum albumin as the standard. Hexoses were determined by the improved anthrone method for determination of carbohydrates (11), with glucose as standard. Rhamnose was determined by the method of Gibbons (5), and hexosamine was determined by the method of Neuhaus and Letzring (15). For uronic acid determination the carbazole method, modified by Bitter and Muir (1), was applied, with glucuronic acid as standard. Phosphorus was determined with ammonium molybdate and ammonium vanadate after perchloric acid digestion (test combination 15920 from Boehringer [Bell Lane, Lewes, Sussex, U.K.]).

For qualitative sugar determination by paper chromatography, preparations containing S-layer subunits were hydrolyzed with H_2SO_4 (0.1-ml sample, containing 15 mg of protein per ml and 5 μl of concentrated H_2SO_4) in a sealed tube for 1 h at 120 C. The hydrolysate was neutralized with 25 mg of $BaCO_3$, diluted with distilled water to 3 ml, and separated from the precipitate by filtration. Traces of Ba^{2+} were recovered from the filtrate with Amberlite IR 120H (frequent shaking for 90 min). The sample was decanted from the residue and evaporated to dryness with nitrogen. Dry samples were redissolved in distilled water and spotted onto Whatman no. 1 filter paper for descending chromatography using *n*-butanol, pyridine, 0.1 N HCl (5:3:2, by volume), with glucose, galactose, mannose, rhamnose, glucosamine, and glucuronic acid as standards (10 μg/spot). The chromatograms were stained with alkaline $AgNO_3$ (23).

For analyses of the amino acid composition of the S-layer protein, samples were hydrolyzed with 6 N HCl in the presence of 2 mg of phenol per 100 μg of protein for 12 h at 105 C. Phenol was added to protect tryptophan during hydrolysis. Samples were analyzed with a Locarte amino acid analyzer (Locarte, London, England).

RESULTS

Isolated cell walls were treated in various ways to remove the surface subunits. The effect of the different treatments was tested by examination of negatively stained preparations in the electron microscope, and the results are listed in Table 1. Figures 1 and 2 show typical examples of cell wall preparations from *C. thermosaccharolyticum* and *C. thermohydrosulfuricum* before removal of the S-layer subunits. After removal of the S-layers (Fig. 3), the smooth structure of the supporting layer was revealed and 80 to 85% of the total cell wall protein was lost. Treatment with buffer with a pH lower than 3 causes only a disintegration (uncoiling) of the S-layer subunits, but no protein loss is detectable (17). The regular S-layer arrays were completely unaffected by treatment with the chelating agent ethylenediaminetetraacetic acid alone or in combination with urea (1 to 4 M) or guanidine-HCl (1 to 2 M). Also, no visible change in the S-layer pattern was observed after treatment with the detergent Triton X-100 (0.5%) or with dithiothreitol, which reduces disulfide bridges. The only reagents that removed the S-layer were high concentrations of urea (8 M) or guanidine-HCl (5 M).

The results of disc electrophoresis in sodium dodecyl sulfate of cell walls treated with guanidine-HCl are shown in Fig. 4. Intact cell walls gave rise to one strong band and a number of weak bands of lower molecular weight (Fig. 4a). After guanidine or urea treatment only a very weak band is seen in the region of the strong band of untreated cell walls (Fig. 4b). The guanidine extract, dialyzed against distilled water to remove guanidine, was composed predominantly of this strong band (Fig. 4d) and stained positively by the periodate-Schiff method (Fig. 4c). The same results were obtained with urea extracts. Since these treatments also lead to a complete loss of S-layer subunits, as shown in negatively stained prep-

TABLE 1. *Detachment of S-layer subunits from isolated cell walls*

Agent	Concn (M)	Time (h)	Temp (C)	Effect on S-layer[a]
Urea in 50 mM Tris-hydrochloride, pH 7.4	1-4	15	35	−
	5-7	3	35	−(+)
	8	2	60	+
	9	2	25	+
Guanidine-HCl in 50 mM Tris-hydrochloride, pH 7.4	0.1-2	15	35	−
	3	15	35	−(+)
	4	2	25	+[b]
	5	2	25	+[c]
EDTA,[d] pH 7.4	0.02	2	25	−
Urea in 10 mM EDTA, pH 7.4	1-4	2	25	−
Guanidine-HCl in 10 mM EDTA, pH 7.4	1-2	2	25	−
Triton X-100, pH 7.4	0.5 (%, wt/wt)	2	25	−
Dithiothreitol, pH 7.4	0.05	15	35 (20)	−
KCl	3	1	25	−
Glycine-HCl, pH <3	0.1	0.6	20	D
Sodium phosphate, pH <3	0.2	0.6	20	D
Sodium acetate, pH 3.6	0.2	0.6	20	D (partial)

[a] +, S-layer subunits removed; −, S-layer subunits not removed; −(+), S-layer subunits partially removed; D, S-layer subunits not removed, but uncoiled.
[b] *C. thermosaccharolyticum.*
[c] *C. thermohydrosulfuricum.*
[d] EDTA, Ethylenediaminetetraacetate.

arations (Fig. 3), the band seen in disc electrophoresis is believed to contain the S-layer material.

The molecular weight, as estimated by sodium dodecyl sulfate-polyacrylamide electrophoresis, was 140,000 for the S-layer subunits isolated from both organisms.

The amino acid composition of the S-layer subunits is shown in Table 2. There is a close similarity in the quantitative amino acid composition of the subunits derived from both organisms. The protein is predominantly acidic, as shown by its content of 20 to 25% acidic amino acid residues compared with 8 to 9% basic. No cysteine was found. Histidine is the least abundant amino acid in the subunit protein from both organisms. The minimum molecular weight for the subunit protein, based on the histidine content, would be 21,700 for *C. thermohydrosulfuricum* and 21,000 for *C. thermosaccharolyticum.*

It was shown by paper chromatography that the glyco component of the S-layer subunits is composed of glucose, galactose, mannose, and rhamnose. Quantitative measurements, by colorimetric methods, gave the values shown in Table 3. No uronic acid or hexosamine was detected. Since values of less than 1 μg of phosphorus per mg of protein were found, the protein apparently contains little or no phospholipid.

The acidity of the glycoprotein was confirmed by isoelectric focusing on polyacrylamide gels. Gels stained with Coomassie blue and by the periodate-Schiff method gave the same pattern of bands (Fig. 5), confirming that the material is indeed glycoprotein. The sample from *C. thermosaccharolyticum* gave two bands with a pK of 3 to 3.8, and these bands were particularly well separated when the samples were adjusted to pH 2 (with 0.01 N H_3PO_4) before placing on the gels. The adjustment to pH 2 was done to disintegrate S-layer aggregates, since it was shown previously (17) that during dialysis of guanidine-HCl extracts against distilled water self-assembly products were formed that could be disintegrated at acid pH. The subunits from *C. thermohydrosulfuricum* gave a number of narrow bands, but the number decreased significantly in the acidic sample. The pK of the major components, as indicated in Fig. 5, is between 5 and 5.2.

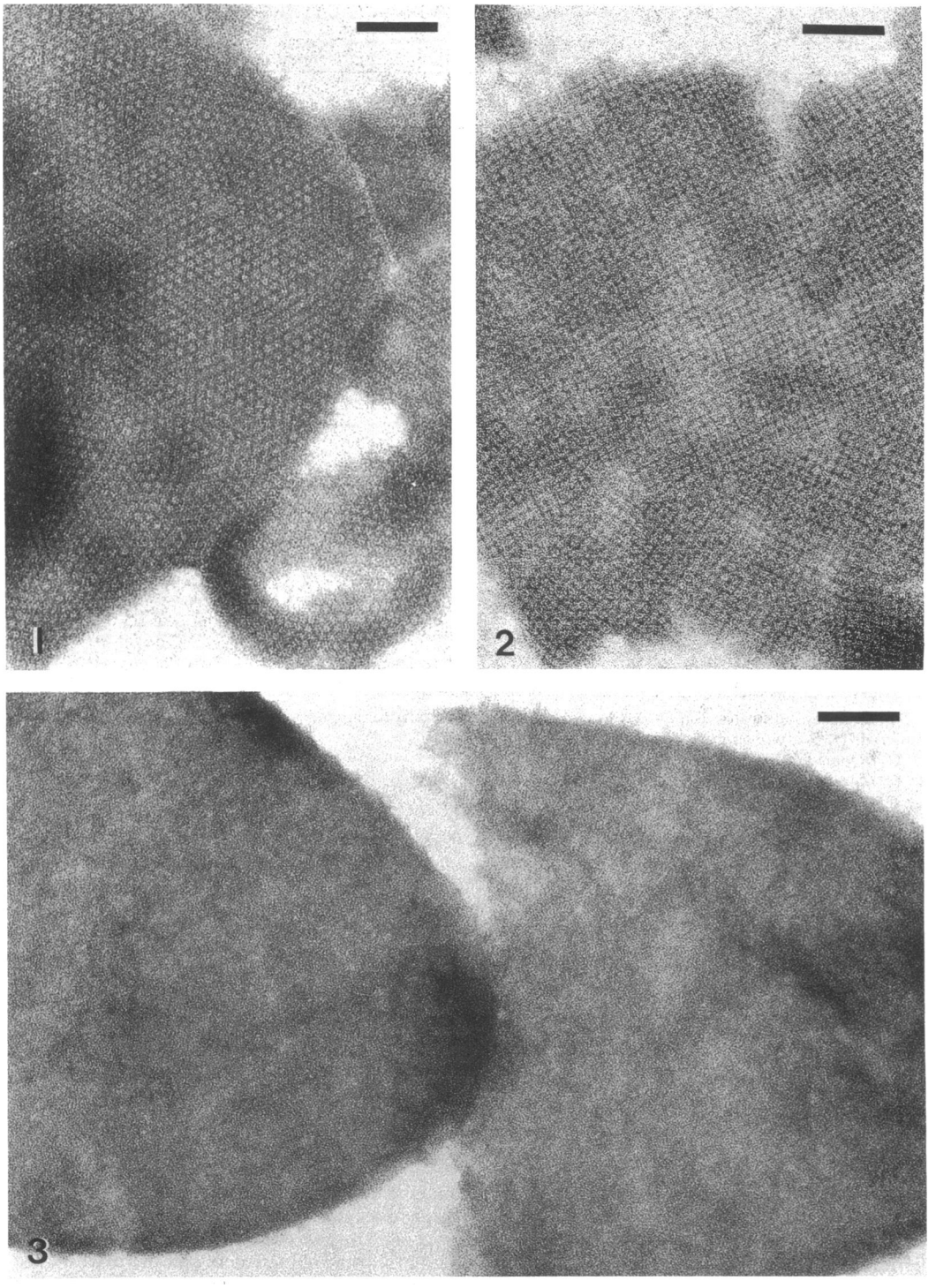

FIG. 1. *Negatively stained preparation of cell walls of C. thermohydrosulfuricum. Note the hexagonally ordered arrays of subunits. The scale mark represents 100 nm.*

FIG. 2. *Negatively stained preparation of cell walls of C. thermosaccharolyticum. Note the tetragonally ordered arrays of subunits. The scale mark represents 100 nm.*

FIG. 3. *Negatively stained preparation of cell walls of C. thermohydrosulfuricum after treatment with 5 M guanidine-HCl. The S-layer subunits have been completely removed and only the smooth supporting layer remains. The scale mark represents 100 nm.*

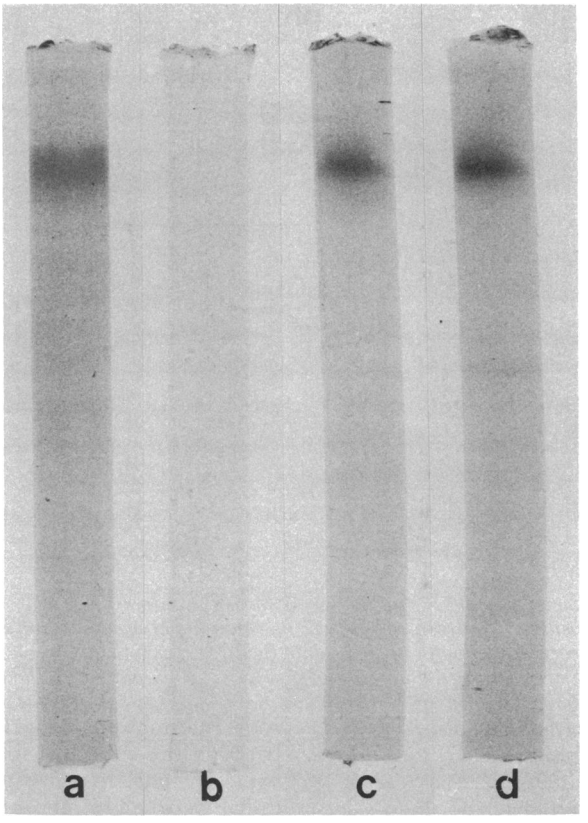

FIG. 4. *Polyacrylamide disc gel electrophoresis in sodium dodecyl sulfate of (a) intact cell walls, (b) cell walls treated with 5 M guanidine-HCl, (c) and (d) guanidine-HCl extract. (a), (b), and (d) Stained with Coomassie blue; (c) stained by the periodate-Schiff method.*

TABLE 2. *Amino acid composition of S-layer protein of C. thermohydrosulfuricum and C. thermosaccharolyticum*

Amino acid	Percentage (residues/100 residues)	
	C. thermohydrosulfuricum	*C. thermosaccharolyticum*
Aspartic acid	14.16	17.46
Threonine	10.01	9.96
Serine	6.97	8.11
Glutamic acid	6.19	7.28
Proline	4.47	3.17
Glycine	7.90	8.15
Alanine	8.71	9.32
Valine	10.71	10.55
Methionine	0.95	
Isoleucine	4.79	5.78
Leucine	6.41	5.89
Tryptophan	5.25	3.13
Phenylalanine	3.63	2.82
Histidine	0.49	0.50
Lysine	7.18	5.74
Arginine	2.15	2.13

TABLE 3. *Carbohydrate composition of S-layer subunits*

Component	μg/mg of protein	
	C. thermohydrosulfuricum	*C. thermosaccharolyticum*
Hexose	0.8	0.175
Rhamnose	0.135	0.105
Uronic acid	0	0
Hexosamine	0	0

DISCUSSION

The present data show that the S-layer subunits of *C. thermosaccharolyticum* and *C. thermohydrosulfuricum* are an acidic glycoprotein of molecular weight 140,000 and thus of the same size as the regularly arranged surface protein of the S-layer of *B. sphaericus* strain P-1 (9). The acidity of the S-layer proteins of the two clostridia is a feature common to the various regularly arranged surface proteins, which

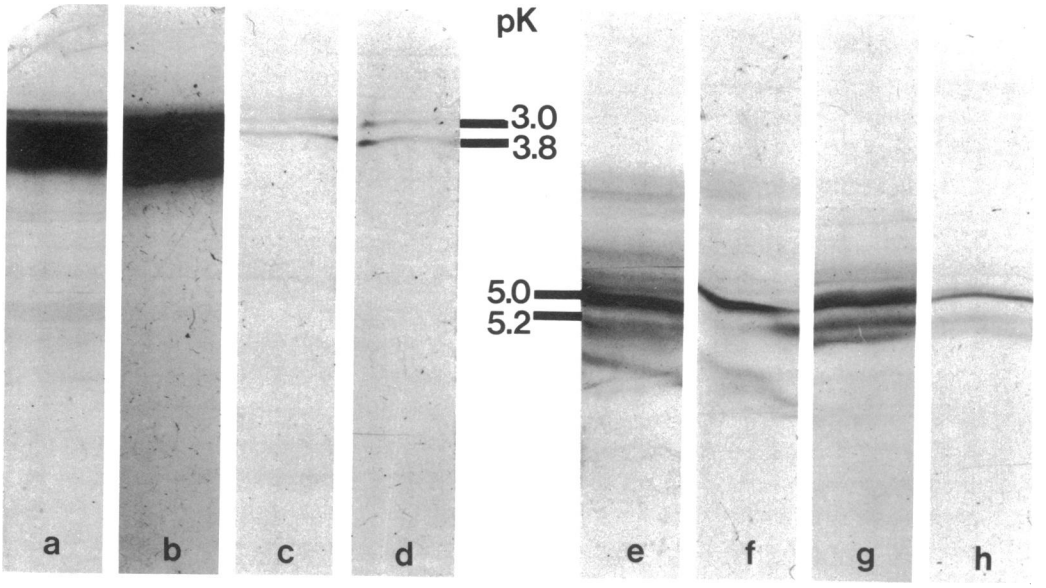

FIG. 5. *Isoelectric focusing in polyacrylamide gels. S-layer subunits from C. thermosaccharolyticum (a–d) and from C. thermohydrosulfuricum (e–h). Gels (a), (c), (e), and (g) were stained with Coomassie blue and gels (b), (d), (f), and (h) were stained with the periodate-Schiff reagent (SP). Samples (a), (b), (e), and (f) had a neutral pH, and samples (c), (d), (g), and (h) had a pH of 2 when placed on the gels.*

have been analyzed from gram-positive *B. sphaericus* (9) and *B. polymyxa* (7) and gram-negative *Acinetobacter* 199A (22) and *S. serpens* (3) bacteria. The protein from *C. thermosaccharolyticum* is more acidic than the protein from *C. thermohydrosulfuricum*. As in other gram-positive bacteria, the S-layer accounts for the majority of the cell wall protein.

The S-layer of clostridia differs significantly from the regular arrays isolated from other organisms in being composed of glycoprotein. Although glycoproteins have been found in the cell envelope of *Escherichia coli* (16) and *Halobacterium salinarium* (13), this is the first report of glycoproteins in the cell walls of gram-positive bacteria. The carbohydrate content is quite low, 3% in *C. thermosaccharolyticum* and 9% in *C. thermohydrosulfuricum*.

Isoelectric focusing revealed some heterogeneity in the S-layer preparations. All of the components stained positively for protein and carbohydrate, but they varied slightly in their apparent isoelectric points. There may be a real heterogeneity in the protein, or more likely the polysaccharide component, of the subunits, or perhaps an induced alteration during the isolation procedure. There is some evidence for changes during isolation, as not all of the subunits in a preparation will self-assemble into regular arrays.

The methods used for the detachment of the subunits from the cell wall surface provide some information about the nature of the bonds between the subunits and the underlying cell wall. The only reagents that removed the subunits were high concentrations of urea and guanidine. This suggests that hydrogen bonds are involved. It seems unlikely that the subunits are attached by covalent or ionic bonds or by disulfide or salt bridges, since they could not be removed with ethylenediaminetetraacetic acid, KCl, acid, or dithiothreitol. Similar evidence for the involvement of hydrogen bonds in the attachment of surface subunits has been described for the gram-positive *B. polymyxa* (14) and *B. sphaericus* (9) and for the gram-negative *S. serpens* (2). The attachment of *Acinetobacter* sp. 199A (20) and *Nitrosomonas* sp. (24) subunits to the cell envelope is by a different mechanism involving divalent cations.

It has been shown previously that S-layers, obtained by lysozyme treatment of intact cell walls or by an in vitro self-assembly process, disintegrate below pH 3 (17). Acid treatment does not, however, remove the subunits from cell walls. Presumably there are ionic bonds between the subunits, which contribute to the formation of the regular array, although they are not important in the binding of subunits to the cell wall.

Little is known about the biological function of the S-layer subunits (21), but they appear to have a different function in gram-positive and gram-negative organisms. In a detailed study

of *B. sphaericus* Howard and Tipper (9) demonstrated that mutants with S-layer subunits of almost half the molecular weight of the wild-type subunits can be found, but that it is not possible to select mutants completely devoid of S-layers. In contrast, in gram-negative organisms, the S-layers seem to have a less essential function, since strains with and without surface subunits attached to the outer membrane have been isolated from *Acinetobacter* (6). In addition, strains of *S. serpens* with the potential ability to form surface subunits do not develop them when grown in a calcium-free medium (2). Further studies, possibly with mutants that lack surface protein, will be necessary to elucidate its function.

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