

Ultrastructure of the Cell Walls of Two Closely Related Clostridia that Possess Different Regular Arrays of Surface Subunits

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Cell walls of *Clostridium thermohydrosulfuricum* and *C. thermosaccharolyticum* have a two-layered structure, consisting of a thin, lysozyme-sensitive murein layer and a surface (S) layer composed of hexagonally or tetragonally arranged subunits. The subunits can be removed from the murein layer by treatment with guanidine hydrochloride and urea. Intact S layers, obtained by lysozyme treatment of cell wall preparations, are composed of a fragile, pH-sensitive monolayer of macromolecular subunits. In both organisms the first stage of the cell division process involves only the plasma membrane and the murein layer. During the subsequent cell separation, a surplus of S-layer subunits appears at the site of division, and consequently the newly formed cell poles remain completely covered by the S layer throughout the separation process. In autolyzed cells an additional layer of subunits assembles on extended areas of the inside of the mucopeptide layer. These observations indicate that the biological function of the S layer depends on its ability to maintain a complete covering of the cell surface at all stages of cell growth and division.

Studies by electron microscopy have shown that regular arrays of subunits are present on the surfaces of a variety of bacteria, and the patterns seen on gram-positive bacteria were listed in a table of a review paper by Glauert and Thornley (11). Additional studies have been made on strains of *Desulfotomaculum* (26), *Clostridium* (25, 27), and *Bacillus* (1, 14, 15, 23, 27). Apart from the work of Howard and Tipper (15), who examined the surface subunits of *B. sphaericus* and showed that they act as receptors for bacteriophages, and an analysis (27) of the regular arrays on the surfaces of the two strains of clostridia studied in the present paper, little attention has been paid to the relationship of the patterned surface layers to other components of the cell wall or to the formation and maintenance of the patterned layer during normal growth. The aim of the present study was to investigate in detail the structure of the walls of two organisms that possess regular arrays of surface subunits, with particular emphasis on the changes occurring during cell division. The organisms studied, *C. thermohydrosulfuricum* and *C. thermosaccharolyticum*, were chosen because they had already been the subject of a detailed taxonomic study (12) which showed that, although they are very closely

related, the regular surface arrays of subunits have hexagonal symmetry in one organism and tetragonal symmetry in the other. Preliminary experiments have shown that the subunits from both organisms are glycoproteins with a molecular weight of 140,000 (24).

MATERIALS AND METHODS

Organisms and growth conditions. The two strains of clostridia used in this study, *C. thermohydrosulfuricum* L111-69 and *C. thermosaccharolyticum* D120-70, are both hyperthermophilic. They were originally isolated from extraction juice from sugar beet factories (16) and were obtained from the culture collection of the Austrian Sugar Research Institute. The taxonomic positions of the two strains were described in detail by Hollaus and Sleytr (12). They are very similar in their biochemistry and morphology, the main differences being that *C. thermohydrosulfuricum* produces much larger amounts of hydrogen sulfide and is capable of multiplying at a temperature of 75 C, which is 10 C higher than the maximum temperature for *C. thermosaccharolyticum* (5, 13, 19, 20).

Stock cultures were maintained on BBL TSE medium (16), consisting of 1% tryptone (Oxoid), 0.1% sucrose, 0.02% FeSO₄·7H₂O, 0.04% (0.02%) Na₂SO₃, 0.008% Na₂S₂O₃·5H₂O, and 0.7% agar (Oxoid). The higher concentration (0.04%) of Na₂SO₃ was used for the cultivation of strain L111-69, and the lower concentration (0.02%) was used for strain D120-70, which grew better at low sulfite concentration. Special precautions to eliminate atmospheric oxygen

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were not necessary since this semisolid medium, containing 0.6 to 0.7% agar, proved suitable for the growth of these obligate anaerobic strains when freshly prepared. For ultrastructural studies, the cells were grown in 500-ml flasks (filled to the top and carefully closed) in BBL TSE medium without FeSO_4 or agar. Strain D120-70 was incubated at 62 C, and strain L111-69 was incubated at 70 C. Cells in the logarithmic or early stationary phase of growth were harvested by centrifugation at $2,000 \times g$ for 90 min in a cooled MSE Mistral centrifuge at 4 C, followed by $12,000 \times g$ for 15 min.

Preparation of cell walls. After harvesting, the cells were washed twice in cold 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.4), centrifuged, and suspended in 5 volumes of the same buffer. The cells were broken by passing the suspension twice through an Aminoc French pressure cell at a pressure of 120 to 125 kg/cm². The broken cells were treated with deoxyribonuclease (5 $\mu\text{g}/\text{ml}$) and ribonuclease (20 $\mu\text{g}/\text{ml}$) in the presence of 5 mM MgCl_2 and then centrifuged at $80,000 \times g$ for 1 h. The pellet consisted of a few intact cells, broken cell walls, and plasma membranes. The pellet, excluding a small fraction of unbroken cells at the bottom of the centrifuge tube, was suspended in 50 mM Tris-hydrochloride buffer (pH 7.4) and washed three times in the same buffer. Because additional washes with buffer did not decrease the amount of contamination with fragments of plasma membrane, the crude cell wall preparation was treated with 0.5% Triton X-100 for 10 min at 20 C with frequent shaking and then washed four more times in 50 mM Tris-hydrochloride buffer (pH 7.4). Examination by electron microscopy of the resulting preparations of negatively stained specimens showed that only traces of plasma membrane fragments remained.

Chemical and enzymatic methods. The peptidoglycan layer was removed from the cell walls by treatment with lysozyme (Armour Pharmaceutical) (150 $\mu\text{g}/\text{ml}$) in 50 mM Tris-hydrochloride buffer (pH 7.4) for 2 h at 25 C with frequent stirring. The suspension was then centrifuged at $25,000 \times g$ for 30 min, and the pellet was washed twice with the Tris buffer. The final pellet consisted of two-dimensional arrays of surface subunits, which will be referred to as the S (surface) layer.

Isolated cell walls were treated with different concentrations of guanidine hydrochloride and urea (both in 50 mM Tris-hydrochloride, pH 7.4) at 25 C to remove the subunits of the S layer from the underlying supporting layer of the cell wall. In early experiments, tests were carried out by floating cell walls mounted on Formvar-coated electron microscope grids on the different reagents, but the results were not reproducible. Consequently the tests were done by suspending a measured amount of the cell wall preparation in the reagent and then incubating the suspension for the required time. The effects of the proteolytic enzymes papain (pH 5.8; 25 C; 2 h), trypsin (pH 7.4; 35 C; 2 h), Pronase (pH 7.4; 35 C; 2 h), and thermolysin (pH 7.4; 50 C; 3 h) at concentrations of 150 $\mu\text{g}/\text{ml}$ were tested. Papain, Pronase, and thermolysin were obtained from Sigma Chemical

Co., and trypsin was from British Drug Houses Ltd., England. After treatment, the cell wall suspension was chilled in an ice bath and centrifuged at $25,000 \times g$ for 15 to 60 min. The pellet was washed twice with cold 50 mM Tris-hydrochloride buffer (pH 7.4) to remove the reagent. Protein was determined by the method of Lowry et al. (18) as modified by Campbell and Sargent (6), with bovine serum albumin as the standard.

Electron microscopy. Preparations to be examined by thin-section electron microscopy were fixed with 2.5% glutaraldehyde in 0.09 M cacodylate buffer (pH 7.2) containing 3 mM calcium chloride for 1 h at 4 C. If the pellet dispersed during fixation, the preparation was centrifuged and the resulting pellets were found to be stable. The pellets were washed overnight in cacodylate buffer at 4 C, post-fixed with Zetterqvist Veronal-acetate-buffered osmium tetroxide (10) (pH 7.2) for 1 h at 20 C, stained with 0.5% uranyl acetate in veronal-acetate buffer for 1 h at 20 C, dehydrated in ethanol, and embedded in Araldite. Thin sections were cut with glass knives on an LKB Ultratome III or a Cambridge Huxley Mark 2 ultramicrotome and stained with lead citrate. Freeze-etching of unfixed, intact cells or cell wall preparations was carried out in a Balzers BAF 300 freeze-etching unit (Balzers A. G., Principality of Liechtenstein), using an electron gun for platinum-carbon shadowing. Replicas were cleaned with 35% chromic acid (CrO_3) and finally washed with distilled water. Cleaned replicas were picked up on Formvar-coated copper grids.

For negative staining, grids coated with Formvar-carbon or collodion-carbon films were floated for 10 s, facedown, on the surface of the suspension of cells walls or S layers and then on the surface of the negative-staining solution. In the early experiments, 1% ammonium molybdate (pH 7.2) was used as the negative stain, but subsequently it was found that 0.5 to 1.0% uranyl acetate (pH 5.2) gave better contrast. The grids were floated on the negative-staining solution for 30 s and were frequently moved about so that the surface of the grid was washed in the negative-staining solution. The grids were finally dried by touching their edges to filter paper.

Electron micrographs were taken in an AEI EM6B electron microscope operating at 60 kV with a 50- μm objective aperture. The magnification was calibrated with a replica of a cross-grating and with negatively stained catalase crystals.

RESULTS

Observations on intact cells. The cell walls of both strains of clostridia have a two-layered structure as seen in electron micrographs of thin sections. The innermost layer, adjacent to the cytoplasmic membrane, is densely stained (Fig. 1A and 2, d) and is 5 to 8 nm thick, whereas the outer layer (Fig. 1A and 2, s) is less dense and approximately 15 nm thick. In sections perpendicular to the plane of the cell wall, the outer layer appears to be composed of regularly spaced globular units, with a center-to-

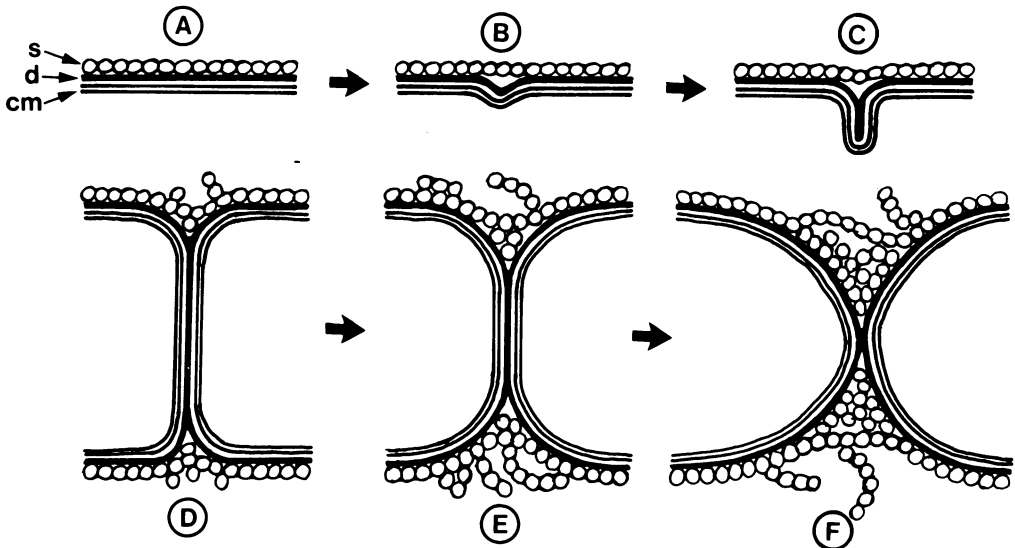


FIG. 1. Diagram illustrating the cell division process in *C. thermohydrosulfuricum* and *C. thermosaccharolyticum*. s, Surface layer of the cell wall (for the purposes of illustration, the subunits of this layer are drawn three times larger than observed by electron microscopy; compare with Fig. 2 to 7). d, Dense layer of the cell wall. cm, Cytoplasmic membrane. (A) Layers of the cell envelope in a nondividing cell. (B to C) Early stages of septum formation. The cytoplasmic membrane and the dense layer of the cell wall grow inward to form the septum by a mechanism similar to the closing of an iris diaphragm. The surface layer of the cell wall is excluded from the septum. (D) Completed septum. Excess S-layer material is present at the septum. (E to F) Cell separation. The excess of S-layer material ensures that the newly formed dense layers of the cell walls remain completely covered during the separation of the cells. Even at late stages of separation the S layer bridges the gap between the cell poles. Some of the excess S-layer material forms curved sheets, which separate from the cell surface.

center spacing between the units of approximately 14 nm in *C. thermohydrosulfuricum* and of 11 nm in *C. thermosaccharolyticum* (Fig. 2, s). The cytoplasmic membrane (Fig. 1, cm) is not always resolved as a unit membrane as a consequence of the density of the adjacent cytoplasm but is very clearly seen in partially autolysed cells (Fig. 14 and 15, cm).

The process of cell division (Fig. 1 to 7) can be divided into two stages: cell septation, followed by cell separation. During the first stage, the cytoplasmic membrane and the inner dense layer of the cell wall grow inwards symmetrically around the circumference of the cell in a manner similar to the closing of an iris diaphragm. During these early stages, the outer S layer is excluded from the septum (Fig. 1B and C, and Fig. 3 and 4). During the subsequent separation of the cells, an excess of S-layer material is observed at the site of division (Fig. 1D, E, and F, and Fig. 5 to 7) so that the newly formed dense layers of the cross wall, which eventually become the cell poles, remain completely covered throughout the separation process. Some of the excess S-layer material forms curved sheets, which can become detached from

the cell (Fig. 6 and 7, arrows), while the remainder forms dense aggregates between the separating cells (Fig. 7). Even in the latest stages of cell separation the S layer frequently bridges across between the cell poles (Fig. 7). Mesosomes have been observed to be associated with the ingrowing cytoplasmic membrane at all stages of septation (Fig. 5, m). The hexagonally and tetragonally arranged subunits of the S layer are clearly visible in replicas of freeze-etched intact cells (27). The center-to-center spacing of the subunits in the hexagonal lattice of *C. thermohydrosulfuricum* is 14 nm (Fig. 8 and 11) and in the tetragonal lattice of *C. thermosaccharolyticum* is 11 nm (Fig. 9 and 12). No internal fracture planes are seen in the cell walls of either organism. In general, the surfaces of the cells are completely covered with the regular arrays of subunits, but in cells of *C. thermohydrosulfuricum* some smooth areas, lacking the S layer, are observed (Fig. 10) in the late stationary phase of growth when some of the cells are dead.

Acid-treated cells. Intact cells were treated with acid in an attempt to remove the subunits of the S layer. The regular surface pattern is no

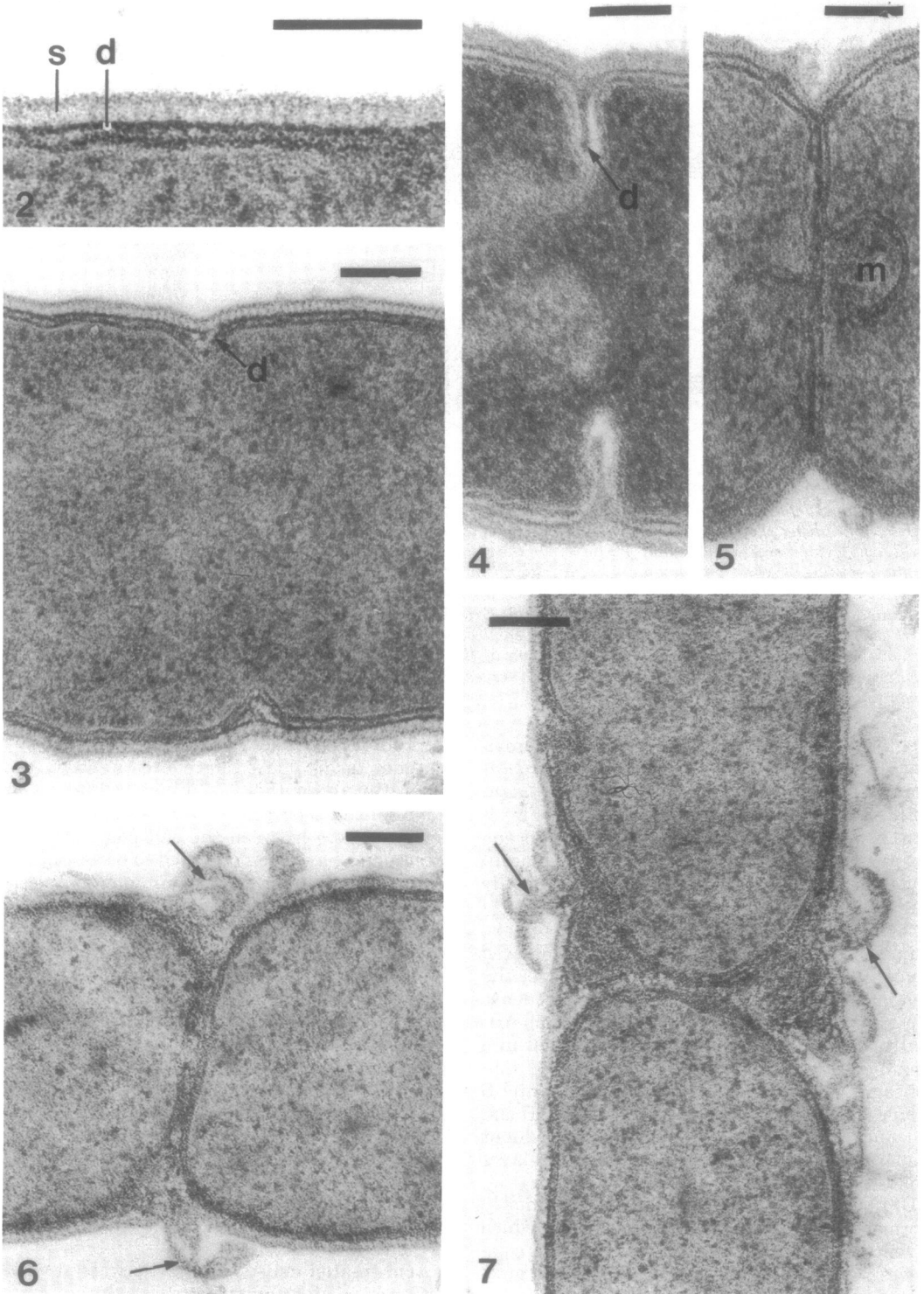


FIG. 2-27. Bar represents 100 nm.

FIG. 2-7. Electron micrographs of thin sections of *C. thermosaccharolyticum*, illustrating the stages in cell division.

longer visible in freeze-etched preparations after treatment at a pH lower than 3, and the surface has a fine granular structure (Fig. 13). However, this structure is clearly different from that of the naturally occurring smooth areas on untreated cells (cf. Fig. 10 and 13). No periodicity of the surface structure is detectable by optical diffraction analysis of electron micrographs of acid-treated cells (R. A. Crowther and U. B. Sleytr, unpublished observations).

Autolyzed cells. In some regions of partially autolyzed cells, the cytoplasmic membrane is widely separated from the inner layer of the cell wall, and in these areas an additional layer is observed on the inner surface of the dense layer (d) of the cell wall (Fig. 14 and 15). This additional layer has the same density and regular globular structure as the S layer. It is not seen in regions where the cytoplasmic membrane is still in close proximity to the cell wall (Fig. 14).

Isolated cell walls. Electron micrographs of thin sections and negatively stained preparations of cell walls treated with 0.5% Triton X-100 show that all but traces of the cytoplasmic membrane have been removed. The cell wall structure of both organisms, as seen in thin sections, is similar to that observed in intact cells, consisting of an inner dense layer and an outer layer with a globular appearance, but an additional S layer is frequently observed on the inner surface of the dense layer, particularly in *C. thermosaccharolyticum* (Fig. 16, arrows). This additional layer appears as a mirror image of the S layer and has the same globular repeating structure. However, it does not cover the entire surface area of the cell wall fragments. Tangential sections of cell walls also show that the additional layer has the same regular patterned structure as the S layer. The hexagonal array of subunits with a center-to-center spacing of 14 nm on the surface of cell walls of *C. thermohydrosulfuricum* is clearly visible in negatively stained preparations. At high magnification (Fig. 17), each morphological subunit appears to possess sixfold symmetry, and there is some indication of a central dark area in each unit. The tetragonal array of subunits with a center-to-center spacing of 11 nm on cell walls

of *C. thermosaccharolyticum* is also visible in negatively stained preparations and is particularly clear in regions where two cell walls are lying on top of one another in accurate register. In these areas there is an indication that each morphological subunit appears to consist of four smaller units in a square array (Fig. 18). When the additional layer of subunits is present on the inner surface of the cell wall, the regular patterns are not so clearly seen, since in two overlying cell walls there are four S layers and these are rarely in accurate alignment. In freeze-etched preparations, the subunits of the S layer appear globular with very little indication of the details seen by negative staining, although the hexagonally arranged subunits of *C. thermohydrosulfuricum* sometimes show armlike extensions identical to those seen on intact cells (Fig. 11). A comparison of the two types of preparation suggests that the central dark areas seen in the subunits in negatively stained preparations probably represent central cores that have been penetrated by the negative-staining solution, rather than pits that are open to the surfaces of the subunits. If surface pits are present, they are too small to be detected in shadowed replicas in which the resolution of fine detail is less than in negatively stained preparations (as a result of the filling in of surface depressions by the shadowing material). It seems likely, however, that freeze-etched preparations give the most accurate values for the center-to-center distances between the subunits in the regular arrays. Direct comparison is possible between the dimensions of the arrays on the surfaces of intact cells and on isolated cell walls, and shows that there are no detectable changes in dimensions during the isolation procedure. In addition, the dimensions of 14 nm for the hexagonal arrays and 11 nm for the tetragonal arrays are in good agreement with the values (14.2 and 11.5 nm, respectively) obtained from hydrated preparations of cell walls maintained at pH 7.4 and analyzed by low-angle X-ray diffraction (R. Henderson and U. B. Sleytr, unpublished observations). The dimensions of the patterns seen in thin sections are always smaller and less accurately

FIG. 2. Cell wall consists of an inner dense layer (d) and an outer less-dense layer (s), which has a regular globular structure.

FIG. 3 and 4. At early stages of septum formation, the cytoplasmic membrane and the dense layer of the cell wall (d) have begun to form the septum. The S layer remains as a continuous layer at the surface and is excluded from the septum.

FIG. 5. The septum is complete. A mesosome (m) is associated with the cytoplasmic membrane at the septum.

FIG. 6 and 7. Stages of cell separation. Excess S-layer material is present between the separating cells and completely covers the newly formed dense layers at the new cell poles. Some of the S-layer material has formed curved sheets (arrows).

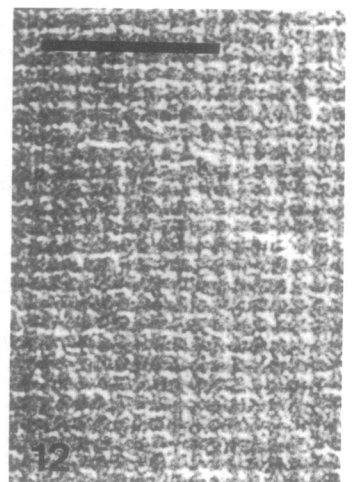
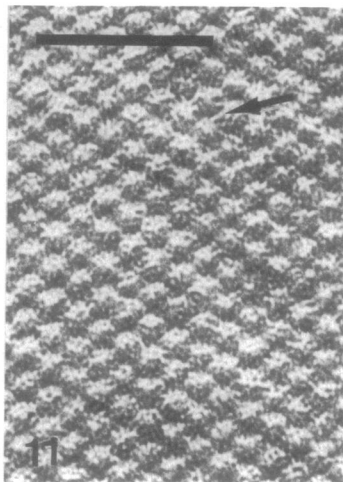
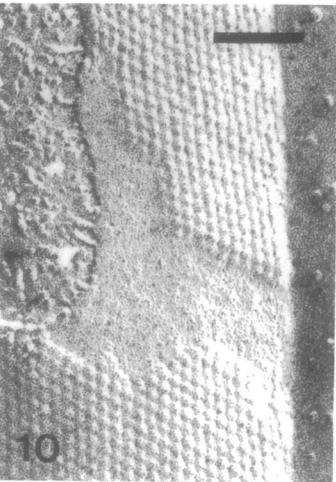
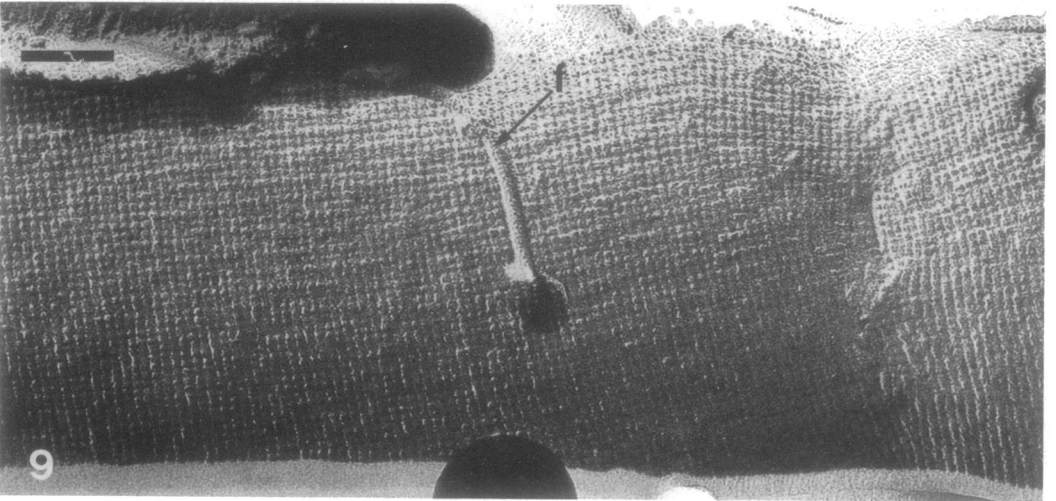
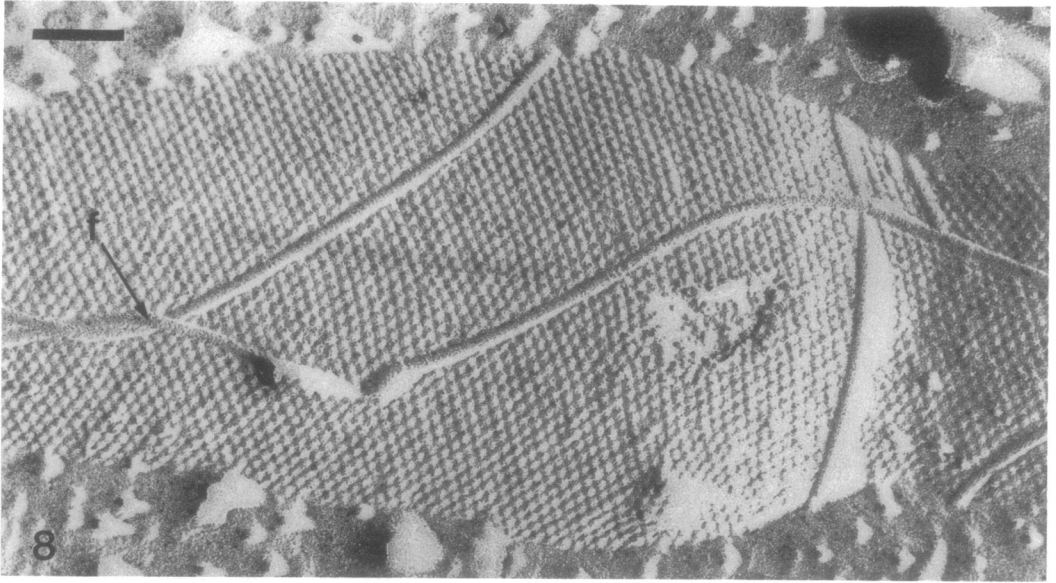


FIG. 8-12. *Electron micrographs of freeze-etched preparations after deep etching.*

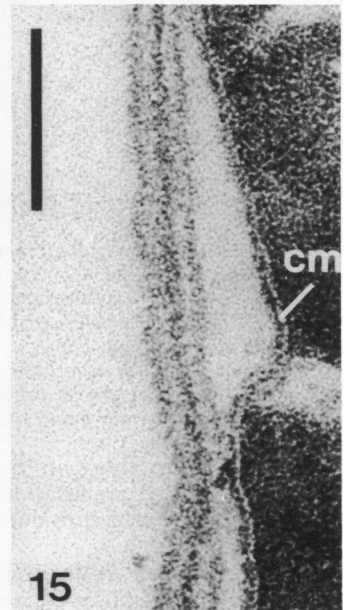
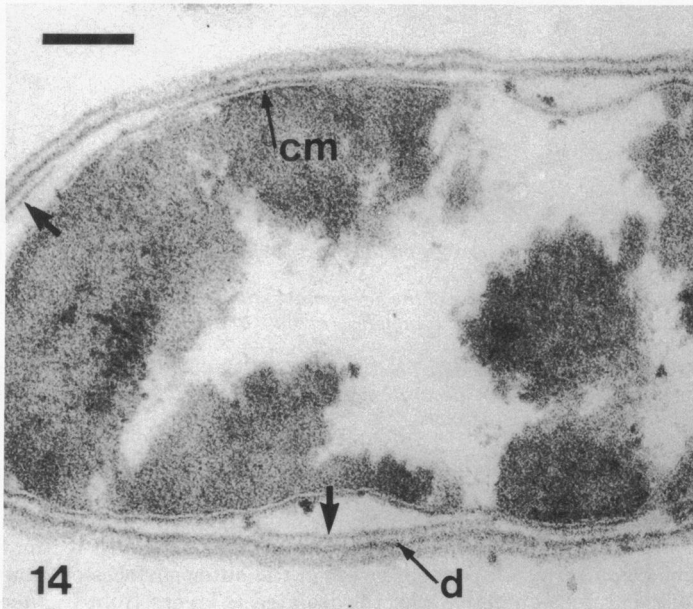


FIG. 13. No periodic structure is visible on the surface of an intact cell of *C. thermohydrosulfuricum* after acid treatment. Freeze-etched preparation.

FIG. 14. An additional layer (arrows), similar in structure to the S layer, is visible on the inner surface of the dense layer (d) of the cell wall in a thin section of a partially autolyzed cell of *C. thermosaccharolyticum*, except for a region where the cytoplasmic membrane (cm) is still in close proximity to the cell wall.

FIG. 15. Thin section of the surface of a partially autolyzed cell of *C. thermosaccharolyticum* at higher magnification. cm, Cytoplasmic membrane.

FIG. 8. A large area of the surface of a cell of *C. thermohydrosulfuricum* is covered with a regular hexagonal array of subunits with a center-to-center spacing of about 14 nm. f, Flagella.

FIG. 9. The subunits of the tetragonal arrays on the surface of *C. thermosaccharolyticum* have a spacing of about 11 nm. f, Flagellum.

FIG. 10. The array of subunits is lacking from an area of the surface of a cell of *C. thermohydrosulfuricum* that is probably dead. The exposed underlying surface appears smooth.

FIG. 11. Some of the subunits show armlike extensions (arrow) is a micrograph of the hexagonal array on *C. thermohydrosulfuricum* at higher magnification.

FIG. 12. Tetragonal array of subunits on the surface of *C. thermosaccharolyticum* at higher magnification.

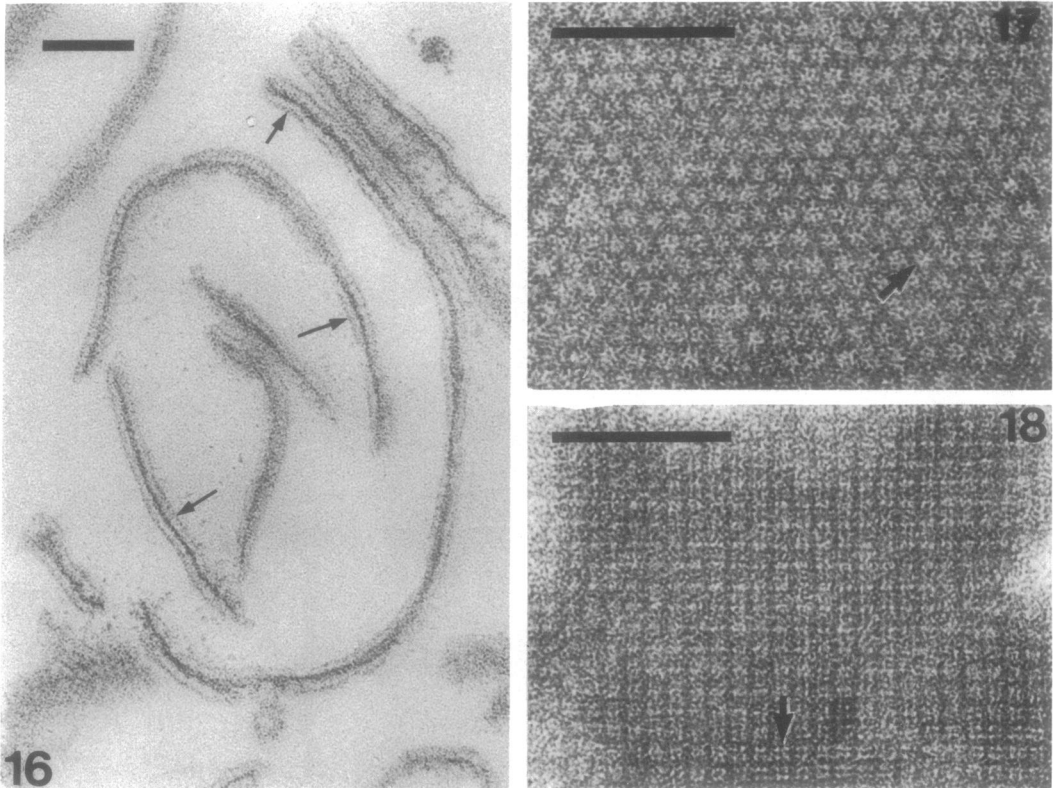


FIG. 16. An additional S layer (arrows) is frequently observed on the inner surface of isolated cell walls of *C. thermosaccharolyticum* in thin sections.

FIG. 17. At high magnification each morphological subunit of the hexagonal array on the surface of the cell wall of *C. thermohydrosulfuricum* appears to have sixfold symmetry (arrow) and a dark central core. Negatively stained preparation.

FIG. 18. Region of a negatively stained preparation of isolated cell walls of *C. thermosaccharolyticum* in which two tetragonal arrays are superimposed locally in accurate register. Each morphological subunit appears to consist of four small units in a square array (arrow).

measurable than those in freeze-etched and negatively stained preparations as a result of the shrinkage that occurs during dehydration and embedding.

Acid-treated cell walls. The surface pattern is no longer detectable in freeze-etched or negatively stained preparations of walls isolated from both organisms when the pH is lowered to less than 3, and the walls have the same granular appearance as the surfaces of acid-treated intact cells (Fig. 19 and 20). The acid treatment does not cause any loss of protein from the cell walls, and the pattern becomes clearly visible again when the pH is raised to 7 (Fig. 21). These observations suggest that the subunits of the S layer are not removed by acid treatment but that they uncoil to form a layer with a random granular substructure.

Lysozyme-treated cell walls. After treatment with lysozyme, the dense layer is no longer visible in thin sections of cell walls from

both organisms (Fig. 22 and 23), indicating that the dense layer is the peptidoglycan-containing, murein layer of the cell wall. The remaining layers are identical in structure to the S layers observed on the outer surfaces of intact cell walls; the isolated S layers from *C. thermohydrosulfuricum* tend to form curved sheets (Fig. 22), whereas those from *C. thermosaccharolyticum* form mostly flatter sheets (Fig. 23). The characteristic hexagonal or tetragonal patterns are very clearly seen in negatively stained preparations of the S layers (Fig. 24 and 25). Frequent washing in Tris-hydrochloride buffer (pH 7) causes some disruption of the S layers, but they disintegrate completely when the pH is lowered to 3. Thus, it appears that the binding sites between the subunits within the S layer are affected by acid treatment, although the subunits are not detached from the underlying dense layer in intact cell walls.

Chemically and enzymatically treated cell

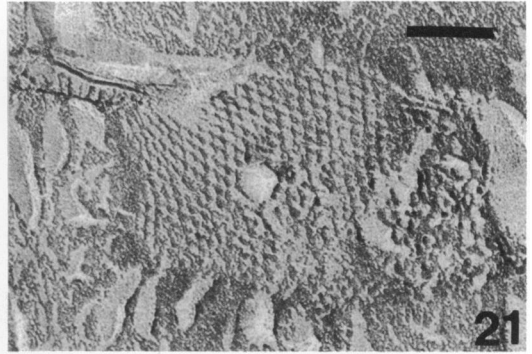
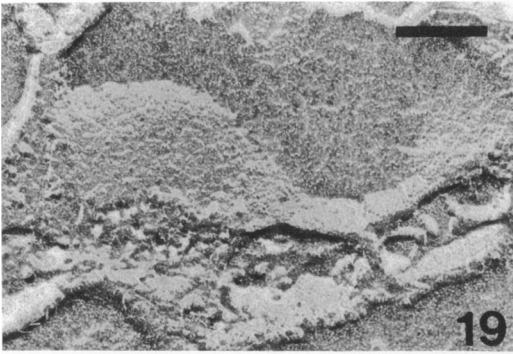


FIG. 19. The surface of an acid-treated cell wall of *C. thermohydrosulfuricum* appears smooth in a freeze-etched preparation.

FIG. 20. Negatively stained preparation of an acid-treated cell wall of *C. thermohydrosulfuricum*.

FIG. 21. When the pH is raised to 7, the hexagonal pattern becomes visible again on the surface of an acid-treated cell wall of *C. thermohydrosulfuricum*. Freeze-etched preparation.

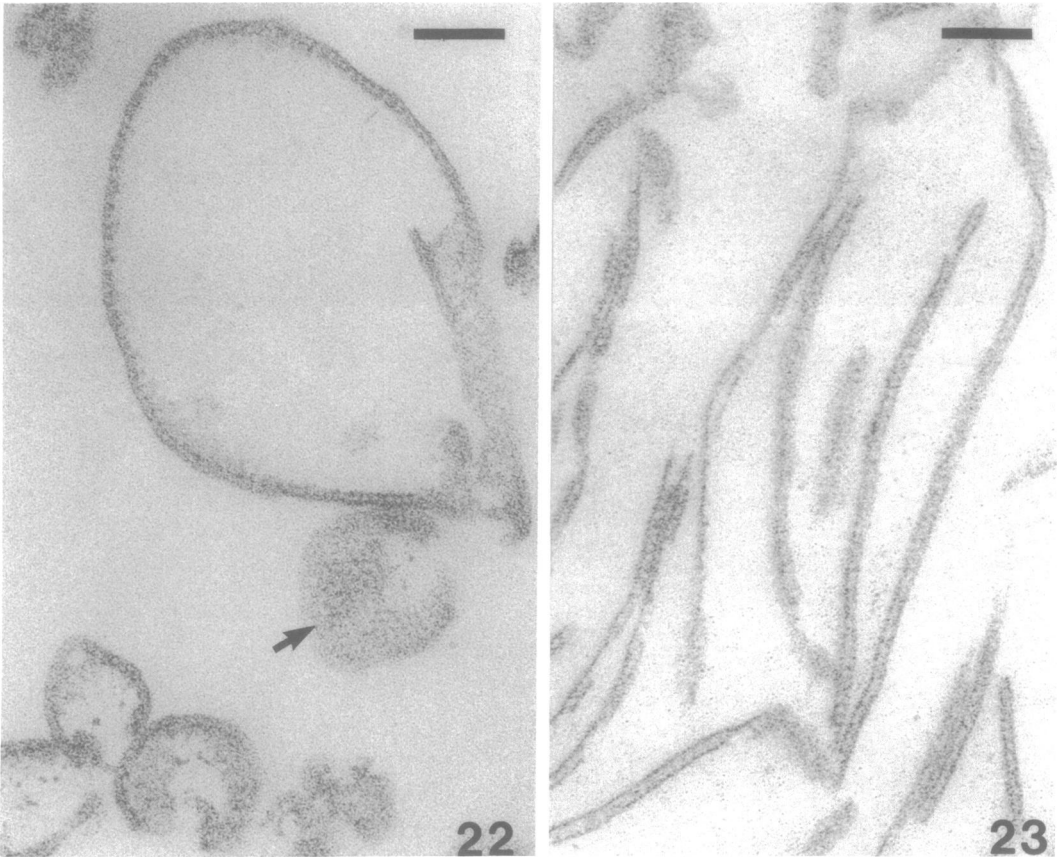


FIG. 22. Thin section of lysozyme-treated cell walls of *C. thermohydrosulfuricum*. The dense layer of the cell wall has been removed, and only curved S layers remain. The regular array of subunits is visible in a tangential section of an S layer (arrow).

FIG. 23. Thin section of the S layers remaining after lysozyme treatment of cell walls of *C. thermosaccharolyticum*. The S layers are in the form of flat sheets.

walls. Different treatments were tested in attempts to remove the S layer from isolated cell walls. At concentrations higher than 4 M, guanidine hydrochloride causes a complete disintegration and removal of the S layers from the cell walls of *C. thermosaccharolyticum*, whereas a 5 M solution is necessary to produce the same effect on the walls of *C. thermohydrosulfuricum* (Fig. 26). Complete removal of the S layers from both organisms was also achieved by treatment of the cell walls with 8 M urea. The proteolytic enzymes papain, trypsin, Pronase, and thermolysin had no visible effect on the S layers. Examination of thin sections of cell walls treated with guanidine hydrochloride or urea at sufficient concentration to cause complete removal of the S layer shows that the resulting cell walls consist of the dense layer only (Fig. 27). In both organisms the removal of the S layer is accompanied by loss of 80 to 85% of the total cell wall protein.

DISCUSSION

The two-layered structure of the cell walls of *C. thermohydrosulfuricum* and *C. thermosaccharolyticum* is unusual for gram-positive bacteria. The inner layer is the denser of the two and has been shown in the present study to be sensitive to digestion with lysozyme. It thus appears to correspond to the rigid, peptidoglycan-containing layer of the cell wall. It is unusually thin and may account for the fact that both organisms were found to be gram variable in the taxonomic study by Hollaus and Sleytr (12). The outer layer, which can be isolated by treating cell walls with lysozyme, consists of a fragile, pH-sensitive monolayer of subunits that are arranged in strikingly regular arrays on the bacterial surface. These arrays have hexagonal symmetry in *C. thermohydrosulfuricum* and tetragonal symmetry in *C. thermosaccharolyticum* and enable the two organisms to be distinguished taxonomically (12).

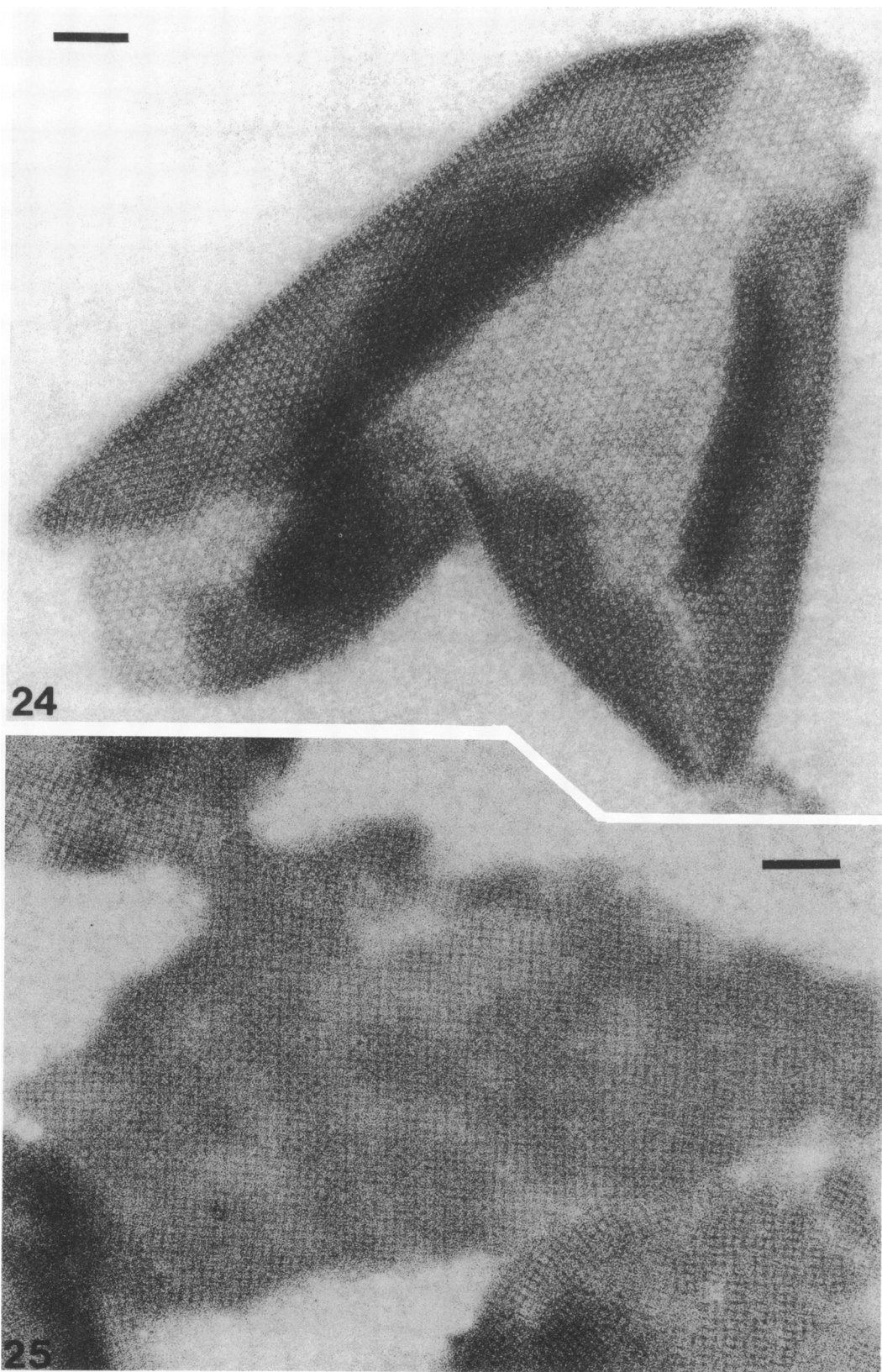


FIG. 24. The hexagonal pattern is clearly visible in a negatively stained preparation on an S layer isolated from *C. thermohydrosulfuricum*. The layer has a tendency to roll up at the edges.

FIG. 25. An S layer isolated from *C. thermosaccharolyticum* is in the form of a flat sheet, and the tetragonal pattern of subunits is clearly visible. Negatively stained preparation.

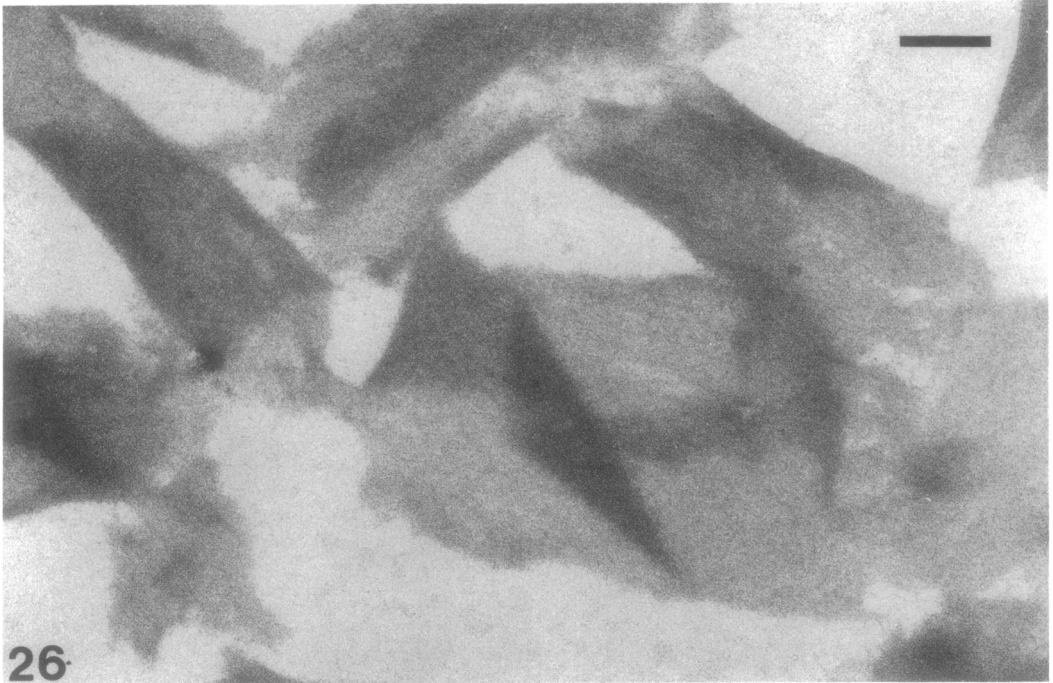


FIG. 26. Treatment of isolated cell walls of *C. thermohydrosulfuricum* with 5 M guanidine hydrochloride for 2 h has removed the S layer, and the remaining dense, peptidoglycan-containing layers have a smooth appearance in a negatively stained preparation.

FIG. 27. A thin section of the dense layers of the cell wall of *C. thermohydrosulfuricum*, remaining after guanidine hydrochloride treatment, shows that these peptidoglycan-containing murein layers are unusually thin.

The hexagonal array of subunits on the surface of *C. thermohydrosulfuricum* is somewhat similar to the patterns found on *Micrococcus radiodurans* (9, 32, 33) and *Spirillum serpens* (3, 21). The main difference is in the size of the central dark region within each subunit which appears to be penetrated by negative-staining solutions. This region is considerably larger in *M. radiodurans* and *S. serpens* and is clearly visible in freeze-etched preparations of *M. radiodurans* (27), suggesting that each subunit has a hollow core or a deep pit in its outer surface.

Tetragonal arrays of subunits similar to those on the surface of the cell wall of *C. thermosaccharolyticum* have been observed on a range of gram-positive bacteria, including *Bacillus polymyxa* (2, 8, 22), *B. cereus* (7), *B. fastidiosus* (17), *B. sphaericus* (1, 15, 23), *Clostridium tetani* and *C. botulinum* (30), *C. (Desulfotomaculum) nigrificans* (26), and *C. tartarivorum* (12, 27). In the majority of these arrays, the center-to-center distance between the subunits is in the range 9 to 12 nm (11, 31). Analysis of images of negatively stained preparations of *B. polymyxa* (8) by optical diffraction and optical filtering techniques showed that each of the morphological subunits appears to be composed of four smaller subunits, whereas a detailed analysis of images of the tetragonal arrays in *B. sphaericus* (previously named *B. brevis*) (1) by a variety of techniques, including computer filtering, suggested that the protein of these arrays has a very complex mass distribution. Previous analyses of the arrays of subunits on the surfaces of a variety of hyperthermophilic aerobic and anaerobic bacteria, including the two strains examined in the present study, using the freeze-etching technique have shown that the pattern is very regular over large areas of the cylindrical parts of the rod-shaped cells. In contrast, the orientation of the pattern frequently changes at the poles of the cells, and at the division sites the surface is covered with a mosaic of small crystallites (27, 31). These observations suggested that newly formed subunits appear primarily at the division sites and then subsequently rearrange themselves into the large regular arrays. Accumulations of granular material are sometimes present at sites of division and were interpreted as representing an excess of newly formed subunits that had not yet found a place in the regular arrays. This suggestion is strongly supported by the observations on thin sections in the present study, in which an excess of S-layer material was seen at sites of cell septation in logarithmically growing cells. After the completion of cell separation,

this surplus S-layer material is no longer present and has presumably been shed into the medium. A similar process in some gram-negative bacteria involves the formation of blebs on the outer membrane at division sites, as observed in *Acinetobacter* sp. (29), *Escherichia coli* (4), and *Micrococcus radioproteolyticus* (U. B. Sleytr and M. Kocur, unpublished observations). The observation that an additional layer of S-layer subunits is attached to the inner surface of the peptidoglycan-containing layer in regions where the cytoplasmic membrane has separated from the cell wall in partially autolyzed cells suggests that a surplus of subunits is also present in the region between the cytoplasmic membrane and the cell wall. A similar additional layer was also seen in thin sections of partially plasmolyzed or autolyzed cells of *B. polymyxa* (22) and *C. nigrificans* (26), both of which possess surface layers composed of regularly arranged subunits, but the significance of these observations was not appreciated at the time. The fact that the subunits can form a layer on the inner as well as the outer surface of the peptidoglycan-containing layer suggests that the two surfaces have considerable similarity and are both able to act as a supporting substratum. An additional layer of S-layer material is also observed on the inner surface of isolated cell walls of *C. thermohydrosulfuricum* and *C. thermosaccharolyticum* and is presumably formed from subunits released during the preparation of the cell walls.

The biological function of these S layers composed of regular arrays of subunits is still a matter for speculation, although the resistance of the S layers of the two strains of clostridia examined in the present study to digestion by proteolytic enzymes suggests that they may have a protective role. Such a function is also indicated by the most striking property of the subunit—their strong tendency to cover the bacterial surface completely, leaving no gaps (27), even at the expense of having to produce an excess of subunits which are shed into the medium.

The murein layers of these two clostridia are thinner than is usual in gram-positive bacteria and thus have a greater similarity to the murein layers of gram-negative than of gram-positive bacteria. It seems possible, therefore, that the S layers fulfil the same role as the outer membranes of gram-negative bacteria, a role that is adequately performed by the thicker, teichoic acid-containing murein layers of gram-positive bacteria. This role may be complex. For example, a protective function has been ascribed to the S layer of the gram-negative

bacterium *Spirillum serpens*, which appears to prevent invasion by the bacterial endoparasite *Bdellovibrio bacteriovorus* (F.L.A. Buckmire, Bacteriol. Proc., p. 43, 1971). In contrast, Howard and Tipper (15) have shown that the S layer of *B. sphaericus* can act as a specific receptor for bacteriophages. A greater understanding of the role of S layers in bacterial physiology awaits the results of comparative studies on intact bacteria, with and without regularly patterned surface layers, and further characterization of the properties and chemistry of the constituent subunits.

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