JOURNAL OF BACTERIOLOGY, Feb. 1973, p. 963-968

Vol. 113, No. 2
Printed in U.S.A.

Surface Structure of Intact Cells and Spheroplasts of *Pseudomonas aeruginosa*

RICHARD L. WEISS[1] AND DEAN FRASER

Department of Microbiology, Indiana University, Bloomington, Indiana 47401

Received for publication 10 October 1972

This report describes the ultrastructural features of *Pseudomonas aeruginosa* after freeze-etching of intact cells and enzymatically prepared spheroplasts. Freeze-etching of intact cells revealed two convex layers of the cell wall and particles within the hydrophobic interior of the cell membrane. Areas of the membrane free of particles were sometimes elevated in the form of rather large dome-shaped structures. Spheroplasts were formed from intact cells by the addition of trypsin to a reaction mixture of lysozyme and ethylenediaminetetraacetic acid. Spheroplasts contained the outer lipoid layer of the cell wall. It was possible to observe this cell wall layer in freeze-etch preparations of spheroplasts. The spheroplast membrane like that of intact cells was cleaved along a central plane to expose particles and particle-free areas.

Previous studies on gram-negative bacteria (6, 24) have produced a model for the cell envelope. However, this model based on *Escherichia coli* (21) may not apply to other gram-negative bacteria with complex cell walls. Differences in the importance of metallic ions in maintaining cell wall integrity have been observed after treatment of *E. coli* and *Pseudomonas aeruginosa* with ethylenediaminetetraacetic acid (EDTA) (7), and until recently (8) it has proven difficult to produce spheroplasts of *P. aeruginosa*. These differences have been thought to imply that the structure of the envelope of *P. aeruginosa* cannot be the same as that of *E. coli*.

Trypsin has recently been used to show structural differences between rigid layers isolated from the cell wall of *P. aeruginosa* and *E. coli* (15). In the present work, trypsin was used to prepare spheroplasts of *P. aeruginosa*, and the envelope structure of intact cells and spheroplasts was examined by freeze etching.

MATERIALS AND METHODS

Bacteria and media. *P. aeruginosa* L 24 was grown aerobically in 1% tryptone (Difco), and 0.5% NaCl at 37 C for about 24 hr to the logarithmic phase (2×10^8 to 4×10^8 cells per ml). After about 28 hr cells were in the early stationary phase (8×10^8 to 9×10^8 cells per ml). The cells were harvested by centrifugation at 7,000 × *g*. In some experiments organisms were grown in 3 × "D" medium (10).

Preparation of spheroplasts. Cells in the logarithmic phase were harvested by centrifugation at 7,000 × *g* at 4 C for 10 min in the SS-34 rotor of a Sorvall RC-2B centrifuge, washed twice in 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 8.0, and suspended at 37 C in 3 ml 0.033 M Tris buffer, containing 0.25 M sucrose (*p*H 8.0). After lysozyme (10 μg/ml), EDTA (0.25 mg/ml), and trypsin (0.5 mg/ml) were added, the cells were agitated with a Vortex mixer for 10 sec and incubated at 37 C for 5 to 10 min. Spheroplasts began to form after 1 min, and 99% of the cells were converted to spherical forms after 10 min. Optical density readings were carried out with a Spectronic 20 spectrophotometer at 580 nm on cells in 0.033 M Tris buffer (*p*H 8.0) after the addition of lysozyme, EDTA, and trypsin singly or in various combinations. Control cells were incubated at 37 C in 0.033 M Tris buffer (*p*H 8.0) for 10 min.

Electron microscopy. The bacteria or spheroplasts to be freeze-etched were concentrated and frozen rapidly in Freon 22 with or without 7% dimethyl sulfoxide (DMSO) or 10% glycerol. In the absence of these cryoprotective agents the cells were prepared directly in the medium or were washed once with 1% ammonium acetate at *p*H 7.0. The frozen pellet was fractured and etched for 2 min at −98 C in a Denton freeze-etch module following the procedure of Steere (23). The replicas were cleaned with concentrated H_2SO_4 and an aqueous solution of sodium hypochlorite for 2 hr each. Washed replicas were picked up on grids and observed in the electron microscope.

The samples prepared for electron microscopy were observed in an Hitachi HU-11C electron microscope operated at 50 kV. The magnification was calibrated with a carbon replica of a diffraction grating (2,160 lines per millimeter). Measurements were carried out with a Nikon Shadowgraph projector

[1] Present address: Department of Botany, University of California, Berkeley, Calif. 94720.

directly in the center of the negative in an area of about 25 cm^2.

RESULTS

The envelope structure of chemically fixed cells of *P. aeruginosa* can be divided into a number of layers constituting either the cell wall or the cytoplasmic membrane. The surface structure of these layers is revealed in freeze-etched preparations. When cells were frozen in the presence of cryoprotective agents (Fig. 1), the outer convex layer of the cell envelope (W1) shows a rough or warty appearance similar to that described for *E. coli* frozen in glycerol (18). The appearance of this layer is influenced by the freezing medium or cryoprotective agents. Thus, when ammonium acetate was used as a freezing medium, an outer surface (W1) of intermediate roughness was obtained (Fig. 2). A second convex surface of the cell wall (W3) was usually seen (Fig. 2). The corresponding convex surface of the *E. coli* wall had numerous particles and grooves in the absence of glycerol (24), whereas with *P. aeruginosa* this face appeared less irregular with fewer particles and practically no grooves.

When the cell wall has been completely fractured away (Fig. 2), the cleavage of the underlying cytoplasmic membrane along a central plane (5, 19) exposed numerous particles (M1) within the hydrophobic membrane interior. In some areas these particles appeared in patches, whereas in other areas patches devoid of particles show up (Fig. 2). The 5.5 to 8.0-nm particles like those in the cell membrane of *E. coli* (4, 17) appear to be embedded in a smooth surface.

Particle-free areas (M2) within the interior of the membrane sometimes appeared to be elevated as dome-like structures (Fig. 1). In Fig. 2 these hemispherical elevations appear in an extensive area (M2) devoid of particles. In both Fig. 1 and 2 it appears that the individual hemispheres form part of the layer that corresponds to the smooth particle-free area of the membrane. The diameter of the freeze-etched domes was in the range from 15 to 45 nm. Elevated structures corresponding to the particle-free area have not yet been observed in the freeze-etched membrane of *E. coli*, but they have been observed in *Bacillus stearothermophilus* (22).

Lysozyme treatment. The cells were treated with lysozyme and EDTA to solubilize a large part of the cell wall including the muramic acid (9, 15). The cells were osmotically sensitive after this treatment; however, rounding up of the cells was not observed in the presence of

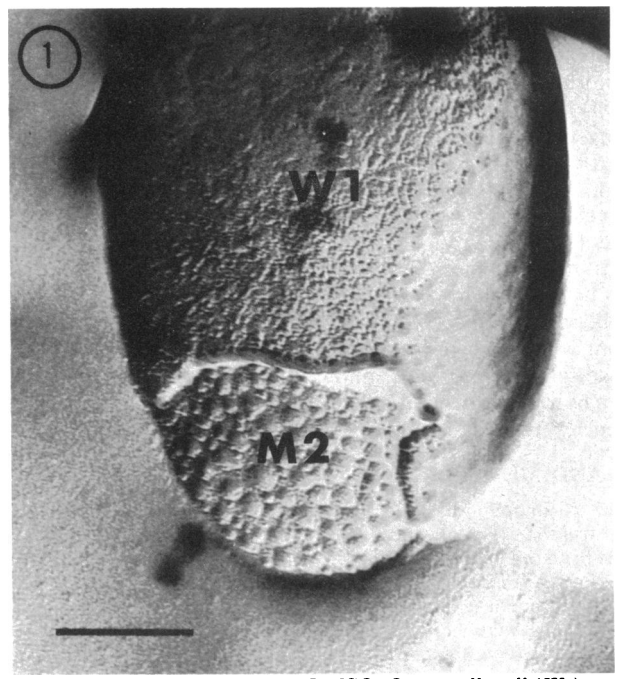

FIG. 1. *Replica of a cell frozen in 10% glycerol-7% DMSO. Outer cell wall (W1) appears rough. The metal was cast from upper left. The plasma membrane has been cleaved along a central plane exposing particle-free areas elevated as hemispherical structures (M2) within the plasma membrane. Bar = 0.25 μm.*

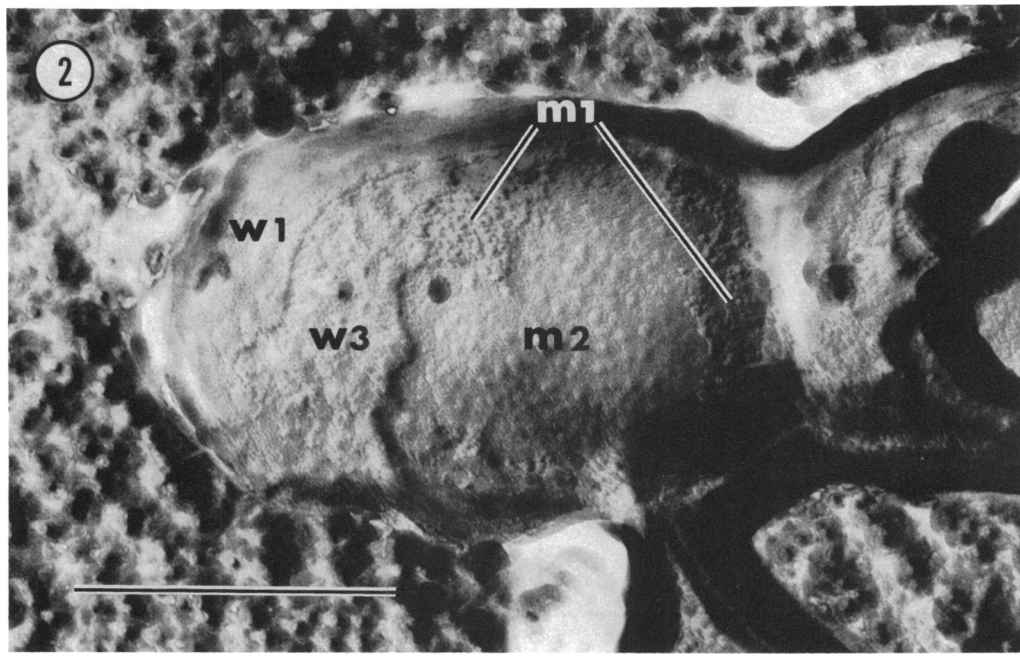

FIG. 2. *Dividing cell frozen in ammonium acetate, showing the cell wall fractured into the outer (W1) and inner (W3) convex layers. Particles (M1) and particle-free zones elevated as hemispheres (M2) appear in the hydrophobic interior of the cell membrane. Bar = 0.5 μm.*

lysozyme-EDTA and 0.25 M sucrose. Lysozyme, EDTA, or trypsin used singly or in pairs did not cause the formation of rounded spheroplasts in the presence of 0.25 M sucrose. With the addition of trypsin to the reaction mixture of lysozyme and EDTA, the rate of lysis was essentially unchanged (Fig. 3), but round spheroplasts were observed with 0.25 M sucrose in the reaction mixture. In gram-positive organisms (13) trypsin activates an autolytic enzyme, whereas in some gram-negative bacteria, it is known to hydrolyze the covalent bond between the muramic acid peptide and lipoprotein component (6). Spheroplasts of *E. coli* retain the "soft" outer layer of lipoprotein and lipopolysaccharide (9). This layer was also retained in spheroplasts of *P. aeruginosa*. The outer convex spheroplast wall (W1) was observed by freeze-etching to have some fine structure on its surface (Fig. 4), whereas a fold in this surface revealed the rather smooth concave face, W2, (Fig. 5). The hydrophobic interior of the membrane appeared as a particulate layer (Fig. 4) containing 10- to 13.0-nm globules which correspond to those observed within the cell membrane of whole cells. Cross fractures through the cytoplasm of spheroplasts (Fig. 5) revealed that the domelike elevations were directly adjacent to the cytoplasm and constituted the hydrophobic particle-free region of the plasma membrane as with whole cells. The cytoplasm did not display the fibrous structure of chemically fixed cells in either whole cells or spheroplasts.

DISCUSSION

In the present study we have shown particles (M1) in the plasma membrane of *P. aeruginosa* that correspond to those seen in the plasma membrane of *E. coli* (4, 18). These particles may well represent functional membrane components. However, their precise chemical nature has not yet been determined. Several explanations for the function of these membrane-bound particles have been offered for *E. coli* (4). It has been suggested that the particles might be multi-enzyme complexes or that they might be associated with transport functions.

The elevated hemispherical structures are an unusual feature of the cell membrane not yet observed in any other gram-negative bacterium. In a freeze-etching study of corresponding fracture moieties of gram-positive *Bacillus stearothermophilus*, Sleytr (22) found warty elevations in the particle-free "basis" layer of the membrane. These structures may be similar to the elevated hemispherical structures observed here on the particle-free area of the

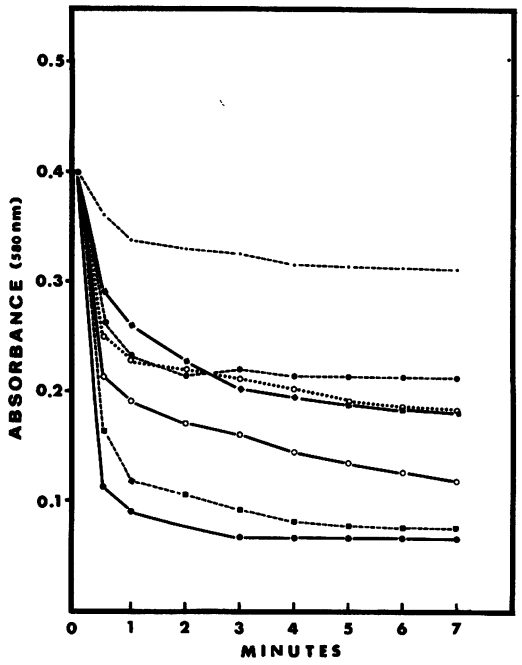

FIG. 3. *Lysis of P. aeruginosa after treatment with lysozyme-EDTA-trypsin (■-----■), lysozyme-EDTA (●——●), lysozyme-trypsin (■——■), EDTA-trypsin (O——O), lysozyme (O- - -O), EDTA (O···O), and trypsin ·---·). Cells harvested at 7,000 × g were washed twice in 0.01 M Tris buffer (pH 8.0) and suspended at 37 C in 0.033 M Tris. Lysozyme (10 μg/ml) EDTA (0.25 mg/ml), and trypsin (0.5 mg/ml) were added in various combinations to 3 ml of the cell suspension. The uncovered tubes were mixed for 3 sec and incubated at 37 C. Control cells suspended in 0.033 M Tris showed no decrease from an absorbancy of 0.4 during a 10-min incubation.*

membrane. In addition, the similarity between the hemispheres and the dome-shaped structures seen in freeze-etched preparations of the gram-positive mesosome (17, 20) suggests that such structures may be a general feature of bacterial membranes. If this is the case, these hemispheres may represent individual sites where cell wall subunits and wall material are sequestered during growth. In keeping with the idea of separate sites of growth on the cell wall, Klainer and Perkins (14) reported that treatment of *P. aeruginosa* with penicillin derivatives produced numerous saccular outpocketings. These outpocketings might be the sites where the components of the cell wall are assembled during biosynthesis. Moreover, Bayer observed multiple zones of blow-outs in *E. coli* after penicillin (2) or osmotic shock treatment (3) that were interpreted as sites of mucopolymer synthesis. It is also possible that the small hemispheres are lipid micelles. Although the hemispheres we observed in freeze-etched membranes are considerably larger than micellar subunits observed by negative staining of isolated lipids (11), it seems possible that the lipids of the membrane may be arranged in globular micelles under certain circumstances. In this connection they may be transitional components in dynamic equilibrium within the membrane sheet.

Cell wall. Two convex fracture faces appear in the freeze-etched cell wall of *P. aeruginosa*. The first convex face, the outer surface of the wall (W1), corresponds to the outer wall of *E. coli* (4, 18). The second convex face within the cell wall, W3, corresponds to a similar face located within the cell wall of *E. coli*. A more precise location of the fracture plane that exposed this face (W3) in the cell wall is possible, based on the observations of freeze-etched spheroplasts of *P. aeruginosa* which contain only the outer double-track layer of the cell wall. The outer surface (W1) of the double-track layer and a concave surface (W2) were seen in freeze-etched spheroplasts. The concave face, W2, cannot represent the underside of the outer double track, since this would imply that the plasma membrane was fractured over the outer surface, and it has been demonstrated that membranes are cleaved within the lipid bilayer (5, 19). Thus, we tend to conclude that the fracture plane exposing W2 in spheroplasts and W3 in whole cells is located within the lipoid layer as suggested for *E. coli* (4, 24).

Trypsin treatment. Treatment of the cells with lysozyme and EDTA gave results similar to those of Carson and Eagon (7). They found such treatment of *P. aeruginosa* produced osmotically sensitive rods rather than spheroplasts (9, 16) and the susceptibility of the cells to lysis seemed to depend on the selective enzymatic action of lysozyme on the peptidoglycan layer as well as the chelation of metal ions by EDTA. Cheng and co-workers (8) were able to form spheroplasts of *P. aeruginosa* by treatment with lysozyme and high concentrations of magnesium or sucrose, whereas we found that trypsin treatment produced spheroplasts from osmotically sensitive rods formed in lysozyme-EDTA (7).

Thin sections of *E. coli* spheroplasts obtained by lysozyme-EDTA treatment (9, 16) show round cells with a clear separation between the cell membrane and the remaining cell wall. Since trypsin was required in addition to lysozyme-EDTA to produce similar round spheroplasts of *P. aeruginosa*, this implies a clear

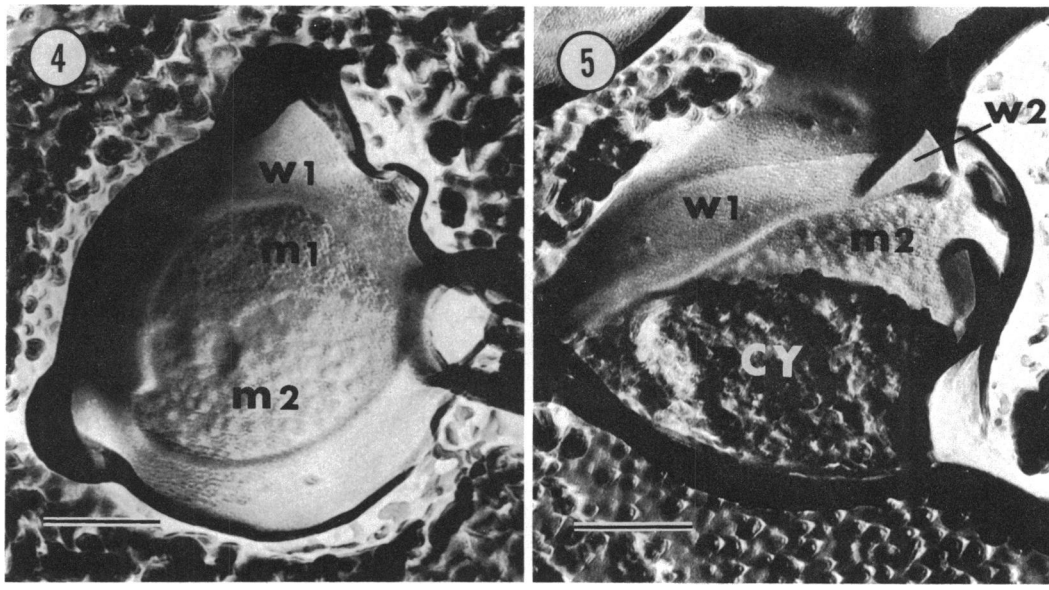

FIG. 4. *Freeze-etched spheroplast revealing the outer cell wall (W1), particles within the plasma membrane (M1), and hemispherical elevations in areas devoid of particles (M2). Cell frozen in 0.25 M sucrose. Bar = 0.25 μm.*

FIG. 5. *Portion of outer spheroplast wall (W1) folded back to expose the concave face (W2) as a smooth surface. Bare areas of the plasma membrane with elevated hemispherical structures (M2) appear next to the cytoplasm (CY). Cell frozen in 0.25 M sucrose. Bar = 0.25 μm.*

difference in the components conferring rigidity upon the cell. The role of trypsin in the production of spheroplasts from osmotically sensitive rods is not entirely clear. If trypsin increased the extraction of lipopolysaccharides from osmotically sensitive rods, as it did from isolated cell walls (12), the action of trypsin on the cell envelope could be explained, since part of the rigidity of the envelope structure of *P. aeruginosa* is associated with lipopolysaccharides (1, 12).

ACKNOWLEDGMENTS

We thank Manfred E. Bayer for critical reading of this manuscript. This work was supported by Public Health Service grant R01-CA-2772-15 from the National Cancer Institute. R. L. Weiss is the recipient of U. S. Public Health Service Microbiology training grant 5T1-GM-503 from the National Institute of General Medical Sciences. The technical assitance of J. Fisher and S. Weiss is gratefully acknowledged.

LITERATURE CITED

1. Asbell, M. A., and R. G. Eagon. 1966. Role of multivalent cations in the organization, structure and assembly of the cell wall of *Pseudomonas aeruginosa*. J. Bacteriol. **92**:380-387.
2. Bayer, M. E. 1967. The cell wall of *Escherichia coli*: Early effects of treatment and deprivation of diaminopimelic acid. J. Gen. Microbiol. **46**:237-246.
3. Bayer, M. E. 1967. Response of cell walls of *Escherichia coli* to a sudden reduction of the environmental osmotic pressure. J. Bacteriol. **93**:1104-1112.
4. Bayer, M. E., and C. C. Remsen. 1970. Structure of *Escherichia coli* after freeze-etching. J. Bacteriol. **101**:304-313.
5. Branton, D. 1966. Fracture faces of frozen membranes. Proc. Nat. Acad. Sci. U. S. A. **55**:1048-1056.
6. Braun, V., and K. Rehn. 1969. Chemical characterization, spacial distribution and function of a lipoprotein (murein-lipoprotein) of the *E. coli* cell wall. The specific effect of trypsin on the membrane structure. Eur. J. Biochem. **10**:426-438.
7. Carson, K. J., and R. G. Eagon. 1966. Lysozyme sensitivity of the cell wall of *Pseudomonas aeruginosa*: further evidence for the role of non-peptidoglycan components in cell wall rigidity. Can. J. Microbiol. **12**:105-108.
8. Cheng, K-J., J. M. Ingram, and J. W. Costerton. 1970. Alkaline phosphatase localization and spheroplast formation of *Pseudomonas aeruginosa*. Can. J. Microbiol. **16**:1319-1324.
9. De Petris, S. 1967. Ultrastructure of the cell wall of *Escherichia coli* and chemical nature of its constituent layers. J. Ultrastruct. Res. **19**:45-83.
10. Fraser, D., and E. A. Jerrel. 1953. The amino acid composition of T_3 bacteriophage. J. Biol. Chem. **205**:291-295.
11. Glauert, A. M., and J. A. Lucy. 1968. Globular micelles and the organization of membrane lipids, p. 1-30. *In* A. J. Dalton and H. Haguenau (ed.), The membranes. Academic Press Inc., New York.
12. Grey, C. W., and S. G. Wilkinson. 1965. The action of ethylenediaminetetra-acetic acid on *Pseudomonas aeruginosa*. J. Appl. Bacteriol. **28**:153-164.
13. Higgins, M. L., H. M. Pooley, and G. D. Shockman. 1970. Site of initiation of cellular autolysis of *Streptococcus faecalis* as seen by electron microscopy. J. Bacteriol. **103**:504-512.
14. Klainer, A. S., and R. L. Perkins. 1970. Antibiotic-

induced alterations in the surface morphology of bacterial cells: a scanning-beam electron microscope study. J. Infect. Dis. **122**:323-328.
15. Martin, H. H., H. D. Heilman, and H. J. Preusser. 1972. State of the rigid-layer in cell walls of some gram-negative bacteria. Arch. Mikrobiol. **83**:332-346.
16. Murray, R. G. E., P. Steed, and H. E. Elson. 1965. The location of the mucopeptide in sections of the cell wall of *Escherichia coli* and other gram-negative bacteria. Can. J. Microbiol. **11**:547-560.
17. Nanninga, N. 1968. Structural features of mesosomes (Chondrioids) of *Bacillus subtilis* after freeze etching. J. Cell Biol. **39**:251-263.
18. Nanninga, N. 1970. Ultrastructure of the cell envelope of *Escherichia coli* B after freeze-etching. J. Bacteriol. **101**:297-303.
19. Pinto da Silva, P., and D. Branton. 1970. Membrane splitting in freeze-etching. Covalently bound ferritin as a membrane marker. J. Cell Biol. **45**:598-605.
20. Remsen, C. C. 1968. Fine structure of the mesosome and nucleoid in frozen-etched *Bacillus subtilis*. Arch. Mikrobiol. **61**:40-47.
21. Schnaitman, C. A. 1971. Effect of ethylenediaminetetraacetic acid, triton X-100 and lysozyme on the morphology and chemical composition of isolated cell walls of *Escherichia coli*. J. Bacteriol. **108**:553-563.
22. Sleytr, U. B. 1970. Fracture faces in intact cells and protoplasts of *Bacillus stearothermophilus*. A study by conventional freeze-etching and freeze-etching of corresponding fracture moieties. Protoplasma **71**:295-312.
23. Steere, R. L. 1969. Freeze-etching simplified. Cryobiology **5**:306-323.
24. Van Gool, A. P., and N. Nanninga. 1971. Fracture faces in the cell envelope of *Escherichia coli*. J. Bacteriol. **108**:474-481.