JOURNAL OF BACTERIOLOGY, Jan. 1970, p. 304-313

Vol. 101, No. 1
Printed in U.S.A.

Structure of *Escherichia coli* After Freeze-Etching

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Received for publication 25 September 1969

Survival of *Escherichia coli*, quick-frozen under conditions similar to those employed for freeze-etching, is close to 100%. For determination of cell shrinkage, the diameters of freeze-etched *E. coli* cells (average, 0.99 μm) were compared with those of preparations after negative staining and after ultrathin sectioning. Negatively stained cells measured from 0.65 to 1.0 μm in diameter, and ultrathin sections showed average cell diameters of 0.70 μm. Freeze-etched replicas of logarithmically growing, as well as stationary, *E. coli* B cells revealed a smooth, finely pitted cell surface in contrast to cell surfaces seen with other preparative methods. The frozen cell wall may cleave in two planes, exposing (i) a smooth fracture face within the lipid layer and (ii) in rare instances an ill-defined particulate layer. Most frequently, however, cleavage of the envelope occurred between wall and protoplasmic membrane; large areas of the membrane were then exposed and showed a surface studded with predominantly spherical particles, an appearance which did not significantly change when the cells were fixed in formaldehyde and osmium tetroxide before freeze-etching. The distribution of these particles differed between logarithmically growing cells and stationary cells.

The surface of *Escherichia coli* B has been investigated by a number of different morphological methods, all of which were known or suspected to introduce a variety of structural changes. It seemed, therefore, worthwhile to compare these "conventional" methods with the newly introduced freeze-etching technique (24, 35).

When bacteria are fixed, dehydrated, and embedded for ultrathin sectioning, the production of "artifacts" such as extraction of material and shrinkage, as well as compression during sectioning, may occur (29). On the other hand, the negative-staining technique (7, 12, 18, 20) allows us to examine biological material in an unfixed state and without extensive pretreatment. Nevertheless, as with other techniques, preparative alterations of the object are also encountered, such as shrinkage during the drying procedure and interactions of the staining fluid with the specimen (33). Furthermore, the interpretation of structures after negative staining is often difficult because of the superposition of images of surface structures onto images of structures "inside" the object (11, 21). The freeze-etching method (24, 35), which represents a replica technique, shows the structure of surfaces produced by cleaving of the frozen specimen and subsequent differential sublimation of the "glass" from the exposed components of the specimen. Thus, the replica yields a one-sided view of an unfixed object. The artifacts produced by freeze-etching, however, are considered to be different from those of other methods. Two possible sources of structural distortion have previously been accounted for (22, 23): (i) effects of the freezing process, and (ii) the damage of the object in production of the replica.

In this paper, the surface of freeze-etched *E. coli* B is described. Since severe damage to the cellular structure due to freezing would be expressed by a high killing rate, the viability of the bacteria was studied after quick-freezing in liquid nitrogen as well as in the presence of Freon and two "antifreeze" compounds; thus, the conditions for freeze-etching were simulated. In addition, the amount of shrinkage of the cells associated with the various preparative methods was investigated. Furthermore, the fine structure of the freeze-etched cell envelope of *E. coli* is described and is compared with results obtained with conventional techniques.

MATERIALS AND METHODS

For all but a few experiments *E. coli* B was grown logarithmically in nutrient broth [1% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, 0.1% glucose; adjusted to *p*H 7 with 1 M NaOH] at 37 C under aeration with a generation time of between 25 and 30 min, until cell counts between 10^8 and 3×10^8 cells/ml were reached. For studies of stationary cells, the cultures were grown for 16 to 22

hr at 37 C in aerated nutrient broth. In a number of experiments cultures were grown in 3 × D medium (15).

Survival of logarithmically growing *E. coli* B after freezing was tested by centrifuging 2 ml of the cultures in nutrient medium (2,000 × *g* for 10 min), decanting the supernatant fluid, and quick-freezing the pellets by pouring the coolant into the centrifuge tube and simultaneously immersing the tube in liquid nitrogen. Liquid nitrogen or Freon MF (DuPont) at −100 C, and Freon 12 at −140 C, served as coolants. In a number of experiments, 10% (v/v) glycerol (final concentration) or 10% (w/v) sucrose was added to the culture medium before centrifugation and subsequent freezing of the pellets. The frozen pellets were quickly thawed by addition of 10 ml of nutrient medium at 37 C; when the cells were frozen in glycerol or sucrose, the medium in which the cells were thawed contained these compounds at the same concentration. The effect of Freon of 15 C on the unfrozen cells was determined by mixing 1 ml of the cells in nutrient medium with 1 ml of Freon MF; after gentle shaking, the Freon settled within a few seconds, and the viability of cells in the supernatant medium was measured. The suspended cells were plated on nutrient agar, and the number of colonies was counted after 15 hr of incubation at 37 C.

For negative staining, silicotungstic acid, brought to *p*H 7 with 1 M NaOH, was mixed with a drop of bacterial suspension on a Formvar-coated electron microscope grid, the excess fluid was removed, and the specimen was dried in the vacuum of the electron microscope. For the ultrathin sectioning, *E. coli* B was fixed for 2 hr in 1% osmium tetroxide in nutrient broth of *p*H 7. In some cases, the fixation was preceded with 2 hr of formaldehyde treatment (4 to 8% formaldehyde made from paraformaldehyde in nutrient broth, *p*H 7). The osmium fixation was followed by centrifugation at 150 × *g* for 5 min; the pellet was suspended in a mixture of 0.5% (w/v) OsO_4 and 0.5% (w/v) uranyl acetate in water and fixed overnight at 20 C. The cells were then dehydrated in acetone, embedded in Vestopal W (Jaeger, Zurich, Switzerland), and sectioned with a diamond knife (DuPont) in a Porter-Blum ultramicrotome. The frozen-state microtomy, which yields unfixed flattened walls, has been described before (5). For freeze-etching, small portions of bacterial pellets were quick-frozen in the presence of Freon 12 and freeze-etched for 3 min at −100 C in a Balzer BA360M unit (31). In a number of experiments, the cells were fixed in formaldehyde or in formaldehyde followed by osmium tetroxide, as described above; subsequently they were freeze-etched without prior removal of the fixing solutions.

Electron micrographs were taken on Ilford Contrasty Lantern Plates with a modified Siemens Elmiskop I electron microscope at magnifications of 20,000 or 40,000 times and on Kodak Plates with a Philips 300 electron microscope at magnifications of 12,000 to 16,000 times. The magnification was frequently checked with a cross-grating replica with 2,160 lines/mm. The distortion at the outer plate area was below 10%. For measurements, the central fields (≈25 cm^2) of the plates were used. Contact copies of the plates were enlarged so that the metal source appears as source of illumination on the final print, and the "shadows," containing no metal, appear dark. Thus, dark spots on the figures represent imperfections in the emulsion (clear areas in the negative copy), whereas residual, unremoved organic matter on the replica would appear bright.

RESULTS

Survival of cells. Quick-freezing for freeze-etching was always performed in the presence of Freon. After freezing and thawing in Freon, more than 85% of the cells maintained their ability to form colonies ("survived") when compared with colony counts of the unfrozen controls. Immersion of the cells in media containing glycerol or sucrose, before freezing, did not significantly increase the number of colony-forming cells. No loss in viability was observed when *E. coli* was frozen in liquid nitrogen alone, indicating that freezing in the presence of Freon and other additives is of no advantage with respect to maintaining viability of our material. It remains open to what extent the small amount of killing of *E. coli* B in the Freons is caused by the quick-freezing or the thawing procedure, or by both. Although the replica of the freeze-etched material has been subjected to the quick-freezing step only, we had to thaw the material to test for viability. Slow thawing (in 20 sec from −140 to 37 C) caused more than 50% of the cells to lose their ability to form colonies. This is probably due to ice crystal formation, since keeping cells in liquid media for a short time at temperatures close to 0 C is not accompanied by killing. Exposure of cells to Freon MF, without freezing, had no effect on the colony counts.

Freeze-etching and cell volume. The cutting or fracturing process through the frozen material cleaved the cells in all directions. During the subsequent freeze-etching procedure, the sublimation of ice from the cells was slower than from the surrounding frozen medium. Therefore, one finds in the shadow-cast replicas of such a surface that the bacteria protrude above the surface of the surrounding medium (Fig. 1c and 3–5). The dimensions of cells that were fractured normal to their long axis can be measured. Table 1 shows that the cross-sectional dimensions from ultrathin sections of fixed, dehydrated, and embedded *E. coli* cells are about 35% (i.e., 0.29 μm) smaller than those of the freeze-etched preparations, which have an average diameter of 0.99 μm. The confidence level for these data is better than 99.9%. The cross-sectional area has shrunk ~50%; calculated as the loss of volume of

TABLE 1. *Mean diameters of E. coli as observed after various preparative procedures (the symbols d_1 to d_4 are used in Fig. 2)*

Measurement	Avg diam (μm)	SD	SEσ_m
Ultrathin sections...	0.70[a]	±0.10	±0.0158
Negative stained whole cells ($d_1 \leftrightarrow (d_2)$).....	1.0 ↔ 0.65		
Freeze-etched cells..	0.99[b]	±0.09	±0.0118
Difference between d_3 and ultrathin sections.....	0.29		
Cell walls flattened after frozen-state microtomy and calculated for a cylindrical cell [$\delta = 2(d_4)/\pi$][c].....	0.94	±0.20	±0.042

[a] Average of 40 measurements.
[b] Average of 56 measurements.
[c] Average of 21 measurements of d_4 (*see* Fig. 2).

a cylinder, the shrinkage amounts to 65%. In other words, during dehydration and embedding the volume has been reduced to 35% of its former value after freeze-etching. Prefixation in formaldehyde had no significant effect on the dimensions of the sectioned material.

In comparison, the diameters of negatively stained cell populations were found to measure between 0.65 and 1.0 μm, which equals about 65 to 100% of those of freeze-etched preparations. The rather wide spread of these values might be caused by a varying area of cell wall adhering to the supporting membrane during drying (*see also* Fig. 2); if this is so, the adhering portion of the cell wall remains extended whereas the non adsorbed "free" portions of the wall can shrink and wrinkle. Thus, cells with a substantial area of their walls adhering to the supporting film (Fig. 2, d_1) will yield larger diameters than cells which shrink without being in contact with the supporting membrane (Fig. 2, d_2).

When bacterial walls were prepared by frozen-state microtomy (5), they did not reveal extensive waviness but were more or less flattened onto the grid (Fig. 2, d_4). From measurements of the distance across such walls, the diameter (δ) of an idealized cylindrical cell can be calculated since $\delta = 2d_4/\pi$. As shown in Table 1, the resulting values for δ are equal to the diameters of the freeze-etched cells. Electron micrographs of the freeze-etched bacteria indeed showed only occasionally some waviness (left portion of Fig. 1c) of an otherwise relatively smooth cell surface, thus coming close to the idealized cylindrical cell. Frequently, a dividing cell was found showing a furrow of the surface at the division site (Fig. 3) The deep grooves or valleys which are an abundant feature of the surface of negatively stained whole cells of *E. coli* (Fig. 1a) and related bacteria have not been observed in freeze-etched preparations.

Fine structure of the envelope. The cleaving plane intersects the long axes of the bacteria in the frozen pellet in all directions. However, to some extent the fracture is guided along certain cell structures, like cell wall and membrane. Therefore one finds larger areas of these components of the cell envelope exposed and etched. When in freeze-etched preparations the fracture plane lies more or less tangential to the cell surface (Fig. 1c and 3), the cleavage might have taken place along the outside surface of the cell (Fig. 1c and 4) or within the cell envelope (the "envelope" representing cell wall and protoplasmic membrane); frequently, after cleaving the envelope, the fracture plane runs through the protoplasmic contents. The cell wall usually revealed a second fracture plane beyond the surface plane [Fig. 3 (arrow) and 4], which suggests that the wall is probably cleaved within the lipoprotein-lipopolysaccharide layer; when the second plane was exposed, it always showed a smooth surface. In addition to this fracture plane, we observed, in rare cases, a layer within the wall in close proximity to the protoplasmic membrane This layer was rarely visible and rather ill-defined; it seemed to consist of particulate elements, but further investigations will be necessary to elucidate its precise ultrastructure. The wall of stationary cells did not exhibit any structural difference from logarithmic cells (Fig. 4). In addition, the underside of the wall became visible in the micrograph shown in Fig. 4 because of a folding-over of a portion of the wall (L). There was no indication of any particulate structure in or on the innermost layer of the wall except for a few particles which were probably torn from the protoplasmic membrane. However, a row of spherical elements was observed at the edge of the portion of the wall that is folded back (Fig. 4, arrow). They are covered by a fine granular layer, which would normally face the protoplasmic membrane and which might represent the peptidoglycan layer (14, 36).

The protoplasmic membrane appeared in close contact with the wall and was fused with the protoplasmic contents. The membrane was covered with particulate elements about 6 to 10 nm in diameter, most of them about 7.5 nm (Fig. 3–5). The distribution of these particles was not

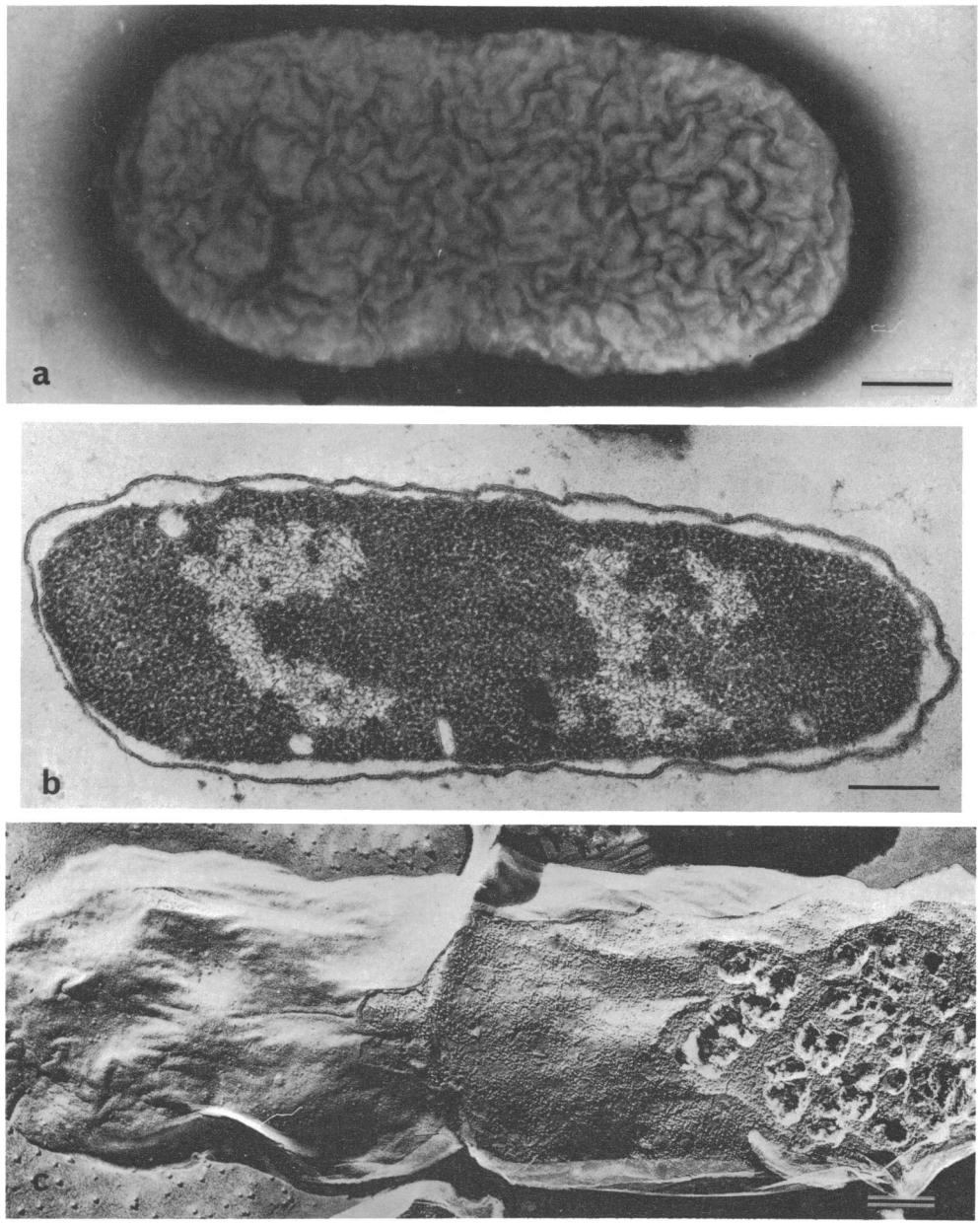

FIG. 1. *Comparison of Escherichia coli B from logarithmically growing cultures after: (a) negative staining in silicotungstate; (b) fixation in osmium tetroxide, dehydration, embedding, and ultrathin sectioning (wall and membrane have separated); (c) freeze-etching. The intact cell surface is seen at left, protoplasmic membrane is exposed in the center, the protoplasm at right. In this and all the following micrographs, the areas of metal shadow are shown dark. The bar represents 250 nm in all micrographs.*

even; instead, over the entire membrane "patchy" areas, measuring 35 to 50 nm across, were abundantly visible, and these areas were devoid of particles (Fig. 1 and 3). The distribution over the cell surface of these "patches" seemed to be random. In stationary cells, the distribution of the particulate elements was rather uniform (Fig. 4), since only occasionally did one find patches devoid of particles. These areas (A) were then of considerably larger dimensions than those in logarithmic cells; we have never seen more than two of these large patches per exposed protoplasmic membrane, i.e., a total of about four of them per stationary cell. In addition to the

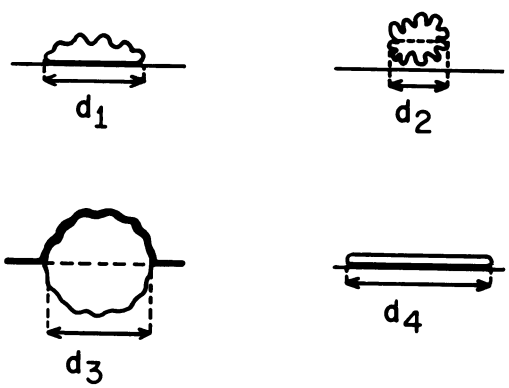

FIG. 2. *Diagram showing the influence of various preparative procedures on the dimension of bacteria; the same symbols are used in Table 1:* d_1 = *diameter of a cell, the wall of which adhered to support membrane while drying (negative staining);* d_2 = *cell diameter of a cell having dried without adhering to support membrane (negative staining);* d_3 = *cell diameter as seen with a freeze-etching replica;* d_4 = *distance across totally flattened wall prepared with frozen-state microtomy.*

more or less spherical particles, filamentous elements also became visible on the protoplasmic membrane, especially in areas where the wall had been folded (Fig. 4, double arrow). From Fig. 4, it is evident that the underside of the wall did not pick up and collect the missing particles from a "patch" on the protoplasmic membrane.

The protoplasmic contents were less well preserved in these preparations, and numerous relatively coarse "granules" became visible (Fig. 1c and 5). The presence of 10% (w/v) sucrose during freezing often diminished this granularity (Fig. 5, right side), but the protoplasm was still rather coarse. Possibly as a consequence of this, the chromosomal material was not visible in our preparations. Growth of the cultures in 3 × D medium did not seem to influence the structural composition of the freeze-etched cells.

In a separate group of experiments, we observed that fixation for 1 hr with formaldehyde or 1 hr of formaldehyde fixation followed by 1 hr of osmium tetroxide prior to freeze-etching revealed significant structural changes in neither the cell wall nor the protoplasmic membrane, nor were the size and distribution of the "patches" on the membrane affected.

DISCUSSION

Chemically, the wall of a gram-negative cell consists of lipoproteins, lipopolysaccharides, and peptidoglycan, with a particulate protein. The lipoid substances are assigned to the outer portions of the cell wall (14), and the peptidoglycan is supposed to face the protoplast (27). With the conventional electron microscopic techniques, the cell wall of *E. coli*, as well as that of other gram-negative cells, appears as a layered structure: in thin sections, electron-dense material is seen to be arranged in parallel layers (10, 27). Negative staining also shows a layering of wall substances which reflects the variation in penetrability of the wall layers by the scattering fluid (5). It is not known whether the areas providing for the "double-layered" contrasty structure in thin sections correspond to the layers in negatively stained preparations; the stringent correlation of visible contrast distribution and biochemically defined components is still not very satisfactory, although some progress has been made in this direction (10). With reference to the cleavage of the frozen wall within the lipoid layer, which would require an oriented structure, it is of interest that high-angle X-ray diffraction studies of the cell walls of *E. coli* have suggested an orientation of lipid molecules normal to the cell surface (17). A density profile derived from electron microscopy and X-ray diffraction data also lent support to the assumption of a layering of cell wall components in unfixed or fixed and dried bacterial preparations (8).

The image of the cell surface in ultrathin sections or after negative staining or after freeze-etching does not normally indicate functional areas; the surface of *E. coli* appears smooth with a fine pitting, the latter being visible only when the shadow cast occurred at small angles of incidence. One of us (2) has recently demonstrated that the cell wall exhibits some degree of a mosaicism: (i) the action of penicillin (at high concentrations) is found to cause structural alterations simultaneously at numerous zones scattered over the entire wall (4); (ii) when plasmolyzed, an average cell has 250 to 400 small areas at which wall and protoplasmic membrane do not separate; (iii) bacteriophages adsorb almost exclusively to these areas (3). The relationship of these specialized wall areas to the "islands" of wall substance which are sometimes found adhering to the cytoplasmic membrane in our freeze-etched preparations (Fig. 5), and which represent remaining patches of the former continuous cell wall, is unknown.

The rather smooth outermost surface of *E. coli* might represent the wall's lipopolysaccharide-lipoprotein layer. However, questions about the position of the "true" outer border of the cell wall were raised previously (27, 28), and some observations on noncapsulated *E. coli* strains indicate the presence of additional surface material outside the identifiable wall contour. After negative staining (Bayer, *unpublished*), as well as

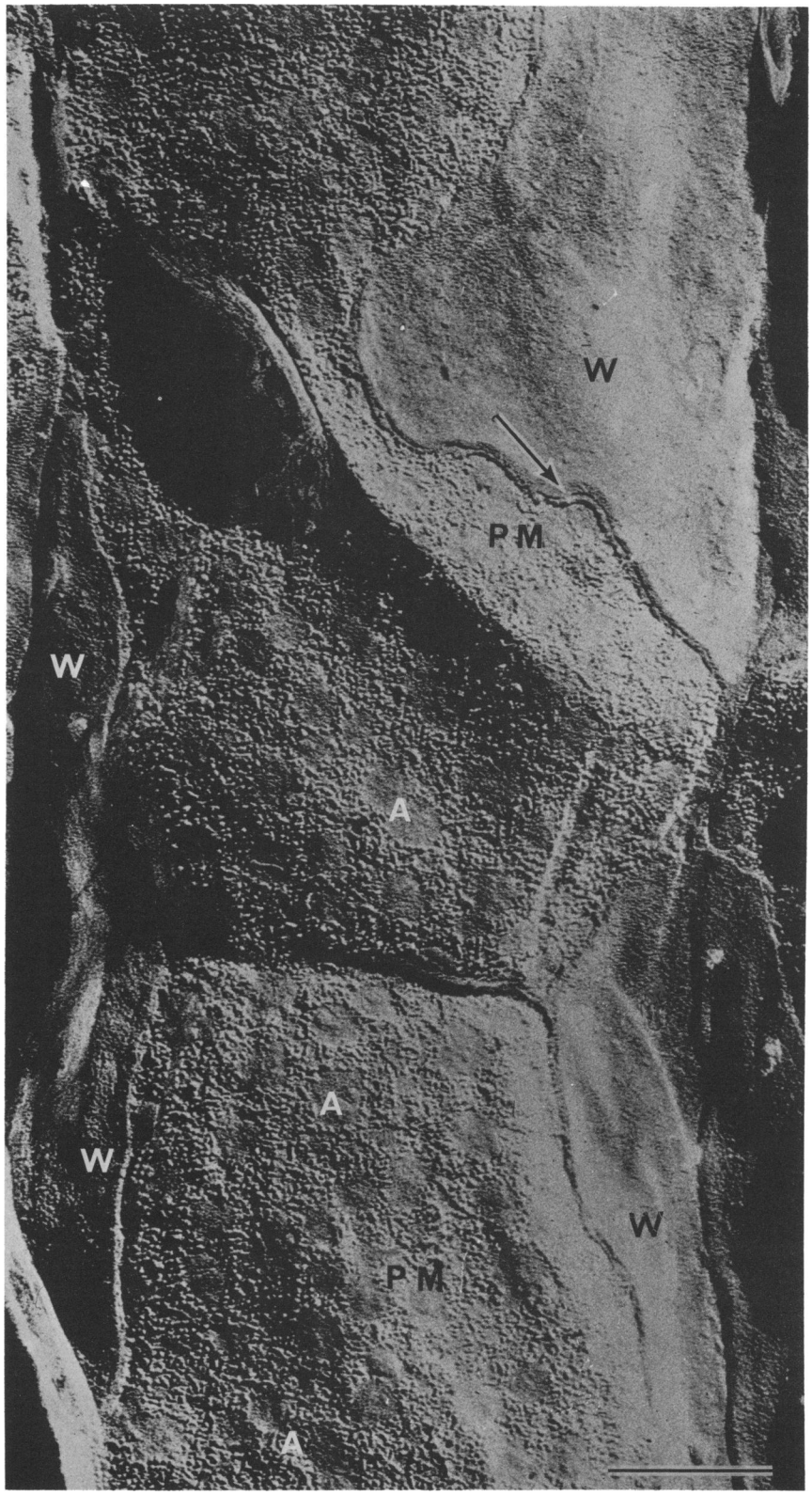

FIG. 3. *Replica of log-phase Escherichia coli B after freeze-etching. The metal was cast from upper right direction. The cell is dividing; the smooth cell wall (W) fractured into two layers (arrow). At larger areas, the wall has come off, exposing the protoplasmic membrane (PM); area (A) devoid of particles.* × *90,000.*

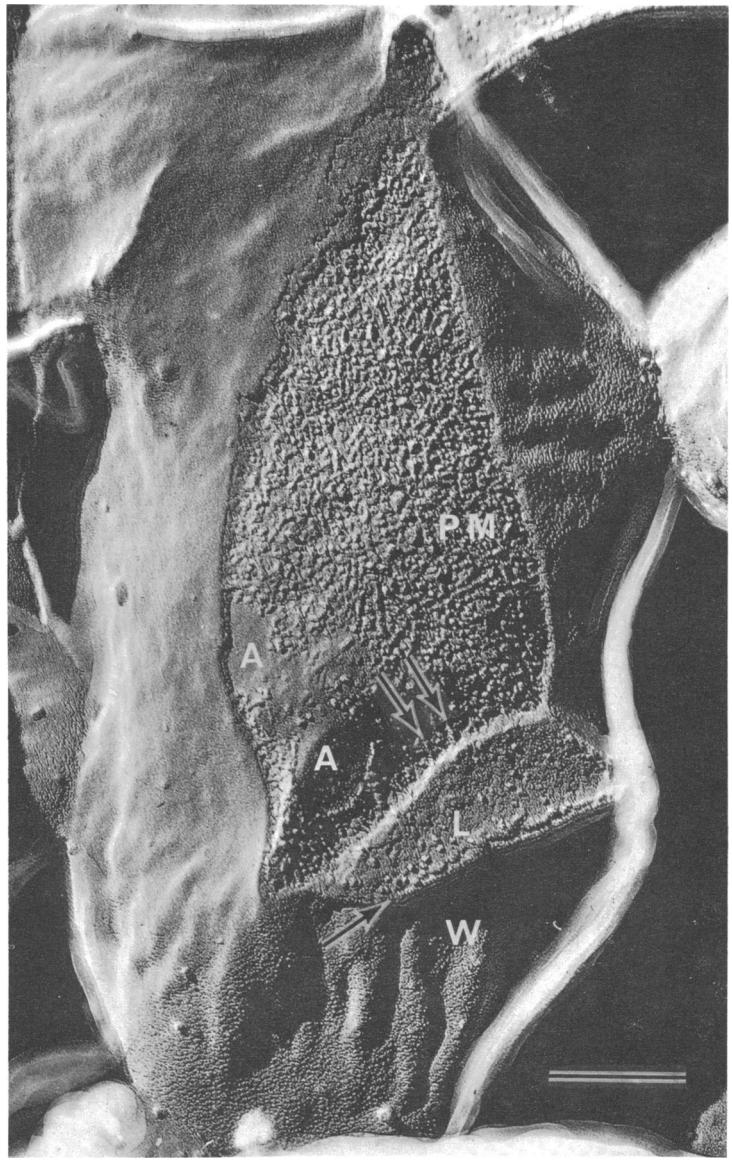

FIG. 4. *Replica of stationary cell. Symbols as in Fig. 3. Single arrow pointing at row of exposed particles, which are apparently masked by layer (L). Double arrows pointing at fibrous elements on PM.* × *70,000.*

in ultrathin sections (27), two surfaces of wall material facing each other do not seem to be able to fuse; instead, they remain separated by a gap measuring about 6 nm in negatively stained preparations and 3 to 4 nm in thin sections. This effect might be caused by the presence of material remaining invisible in the electron microscopic preparation but becoming apparent by its capability to separate the cell surfaces. Further, the somatic antigens of *E. coli* and *Salmonella* are not confined to the visible portions of the cell wall only, but have been demonstrated for longer distances beyond the layered contour of the cell surface (34). In addition, an "invisible" layer on the surface of a different microorganism was elegantly demonstrated by P. Glaister (Thesis, The Univ. of Western Ontario, London, Canada, 1967).

We observed, in freeze-etched walls, a lack of the small "finger-like protrusions" which become so clearly visible in negatively stained preparations of flattened walls (5). Only occasionally one

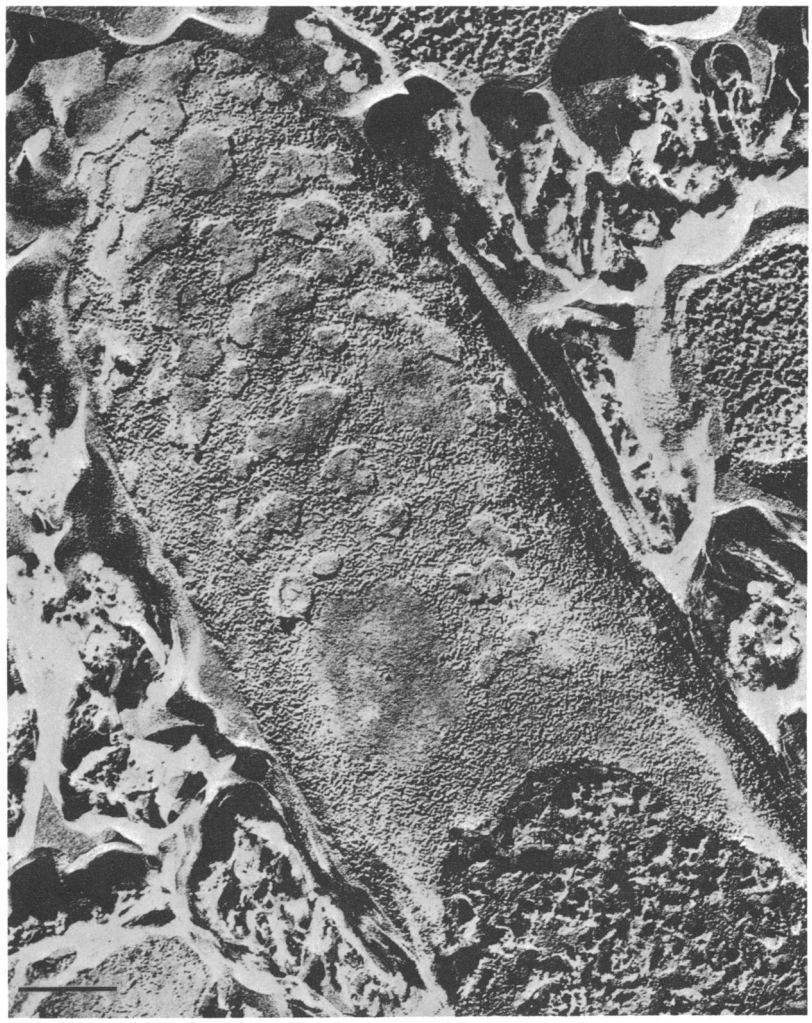

FIG. 5. *Cell wall fragments remaining attached to protoplasmic membrane. Preparation was frozen in presence of 10% sucrose.* × *52,000.*

finds a small number of knoblike globules, which are too few to account for the relatively numerous extrusions seen after negative staining. One can presently only suggest several possible causes for these differences between the methods. (i) The protrusions might be produced during negative staining. (ii) The protrusions might be embedded within some material that remains invisible in negatively stained preparations. (iii) Surface components might be released into the vacuum during freeze-etching, a phenomenon which had been proposed by Anderson (1) for freeze-dried preparations. Also, the action of "vapor-winds" as carrier for nonvolatile structures has been suggested (16). In addition, it has been shown (9) that during the freeze-etching procedures entire latex balls can be torn out from the ice surface. (iv) A flow process in the transition state between the glass phase and recrystallization of the ice at temperatures of −100 C might be taken into consideration. However, the reproducibility of the same structures with high resolution under various preparative conditions (different media, prefixation, variation in etching time, and replica formation) seems to make an extended melting or reorientation process in our preparations unlikely.

A protoplasmic membrane studded with numerous particles has been observed previously in several other freeze-etched microorganisms (19, 29–32); the particles are believed to represent multienzyme complexes (23, 25) or possibly specialized sites associated with transport functions (37). A bacterial membrane-bound adeno-

sine triphosphatase has been solubilized, and the fractions containing enzyme activity showed particles of about 10 nm diameter after negative staining (26). The presence of the particles seems to be indicative of "active membranes" (25, 37), since meylin layers are devoid of particles (6). A widening of particle-free patches has also been demonstrated by Fiil and Branton (13) in magnesium-starved *E. coli*. They relate the membrane changes primarily to metabolic "perturbances." Similar effects were also observed after calcium or carbon depletion.

In conclusion, one can state that the image of the surface of *Escherichia coli* B after freeze-etching is not inconsistent with current models derived from thin-sectioned preparations, whereas there exist some differences with regard to the surface contour in negatively stained bacteria. Freeze-etching reveals the surface views of unfixed cell envelopes with a surprising amount of detail. The method inflicts only a negligible amount of irreparable damage to the living bacterium, and shrinkage of the cells during the etching procedure, if occurring at all, appears to be of a considerably lesser consequence than with other methods. Although one has to be aware of the possible artifacts of the method, one can assume that the micrographs of the freeze-etched *E. coli* envelope impart an image with relatively close approximation to that of the living cell. However, the accurate positioning of the fracture planes within or along some of the cellular structures requires additional study.

ACKNOWLEDGMENTS

This work was supported by grant GB-4640 from the National Science Foundation, by Public Health Service grants GM-11214, GM-16754, CA-06927, and FR-05539, and by an appropriation from the Commonwealth of Pennsylvania. Contribution no. 2313 from the Woods Hole Oceanographic Institution.

We thank T. F. Anderson for his help and suggestions in the writing of this manuscript, S. W. Watson for his continued interest in the study, and G. T. Rudkin for his help in the statistical program. We also acknowledge the technical assistance of John Mackenzie, Jr., and Thomas and Eileen Starkey.

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