Ultrastructure of the Cell Envelope of *Escherichia coli* B After Freeze-Etching

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The cell envelope of *Escherichia coli* B was investigated with the freeze-etching technique. A considerable gain in visible structural detail over more conventional electron microscopic techniques was obtained. The inner surface of the plasma membrane revealed a smooth surface sparsely studded with particles measuring from 5 to 10 nm in diameter, whereas the outer surface of the plasma membrane showed many more particles of corresponding diameter. The freeze-etched cell wall appeared to be a multilayered structure. The innermost layer could be observed as a profile studded with closely packed elements of about 10 nm in diameter. External to this layer was a smooth surface bordering the outermost cell wall layer. When frozen in the absence of glycerol the outermost surface observed in the cell wall was smooth, but when grown in the presence of glycerol it had a "wavy" appearance with small particles attached to it. The observations support current concepts on the ultrastructure of the enterobacterial cell envelope.

The enterobacterial cell envelope (cell wall and plasma membrane) has been studied extensively with chemical and electron microscopic techniques (15, 16). In investigations of *Escherichia coli*, more emphasis has been placed on the cell wall than on the plasma membrane. Chemical characterization of the alternating electron-dense and electron-translucent lines in the cell envelope as observed in electron micrographs of thin sections has been pursued (3, 9, 17). Further, attempts have been made to remove cell wall components selectively and to follow this process with an electron microscope by making shadowed preparations (6, 7, 18). As a result of these approaches (see reference 6 for a recent discussion), the commonly accepted cell wall model of *E. coli* is that of a mucopeptide substrate to which all other cell wall components are attached. The mucopeptide layer is, on its outer surface, covered by covalently linked protein; together, these constitute the so-called "rigid layer" (18) which is believed to determine the ultimate shape of the bacterium. In addition, recent experiments (21) suggest that a phospholipid structure also contributes to the cell wall rigidity. Lipoproteins and lipopolysaccharides are thought to be situated at the outer surface of the "rigid layer," not necessarily forming separate strata, since both components can act as phage receptor sites (19, 20).

It should, however, be recognized that thin-sectioning gives limited information on the actual ultrastructure of the electron-dense or electron-translucent material in the cell envelope. Furthermore, the structures observed in shadowed specimens of chemically treated cell walls may deviate from their situation in the intact cell. Therefore, it was of interest to study the cell envelope in the intact cell as well as in isolated form by means of freeze-etching, because, unlike any other method known, this technique permits the demonstration of spatial structural organization.

**MATERIALS AND METHODS**

**Culture and medium.** *E. coli* B was grown aerobically in Heart Infusion (Difco) for approximately 4 hr at about 30 C. For some freeze-etching experiments, the organisms were grown in the above medium supplemented with 20% (v/v) glycerol (final concentration). Cells were collected by centrifugation at 7,000 × g for 15 min at room temperature.

**Isolation of the cell wall.** Cell wall preparations were made according to the method of Weidel et al. (18), which includes extraction of the cells in sodium hydroxide and shaking with glass beads in sodium dodecyl sulfate, followed by several washes in water. In the present study, instead of shaking with a Mickle disintegrator for 60 min (18), a Braun homogenizer was used for 1 min. Prolonged shaking in the Braun apparatus resulted in fragmented cell walls, visible by electron microscopy of whole mount preparations stained with uranyl acetate.

**Electron microscopic techniques.** Freeze-etching of
intact bacteria was performed on thick suspensions of cells both in the absence and in the presence of 20% (v/v) glycerol. Fracturing and etching were done for 1 min at -100 C (8). Deep-etching (10 min at -80 C) was performed on isolated cell walls in the presence of 35% (v/v) glycerol with the use of the freeze-etching apparatus of Balzers (Liechtenstein). For thin-sectioning, intact cells and isolated cell walls were fixed and embedded according to the Rytel-Kellenberger procedure (14). Sections were cut with glass knives on an LKB ultratome. No poststaining of sections was applied. Isolated cell walls have been shadowed with platinum-carbon. Electron micrographs were taken with either a Philips EM 200 or EM 300 electron microscope operating at 80 kv. The electron micrographs of freeze-etched specimens are printed in reverse to facilitate their interpretation (cf. 10).

RESULTS
The multilayered appearance of the cell envelope as observed in thin sections (2, 9, 17, and Fig. 1) was likewise found in cross-fractured cells (Fig. 2). The plasma membrane shows a single profile, whereas the cell wall exhibits a layered structure. More structural detail was, however, detected in freeze-etched cells showing the surfaces of these layers. For convenience, the order of description of the observed surface features in intact E. coli B is from the outside inwards, and subsequently from the inside outwards, dependent on the way in which the cells have been fractured, i.e., exposing either convex or concave surfaces.

Cell envelope from the outside inwards. Figures 3 and 4 show surfaces that become visible after fracturing along a convex surface. The outer surface of the cell wall was most readily observed in cells that were frozen in the absence of glycerol (Fig. 3). This surface then appeared smooth, with areas revealing a possible finer substructure (arrow, Fig. 3). On the other hand, in cells grown in the presence of glycerol, the outer surface of the cell wall had a "wavy" appearance, and was seen to carry a few small particles and fibrils measuring about 7 nm (Fig. 4). The next surface generally encountered was the outer surface of the plasma membrane (OPM in Fig. 3 and 4). It was characterized by the presence of numerous particles and strands about 7 nm in diameter. The surface morphology of the plasma membrane was not significantly influenced by the presence of glycerol. In a few cases, smooth fields revealing blunt protrusions of variable size were observed in these plasma membranes (Fig. 4). When present, the protrusions varied in diameter, as did the distances between them.

Cell envelope from the inside outwards. When the cell envelope was fractured along a concave surface, the first surface encountered appeared in close contact with the cell content (CC in Fig. 5). It is therefore believed to represent the inner surface of the plasma membrane (IPM in Fig. 5–7). This surface was characterized by a sparse distribution of granules of variable size (about 5 to 10 nm), which sometimes were turned out (Fig. 7). The next layer (CW1, see also Discussion) could only be observed in profile (Fig. 5 and 7). The innermost layer of the cell wall (CW1) was in its turn succeeded by a layer consisting of closely packed elements (CW2 in Fig. 5–7). The elements measured approximately 10 nm. By proceeding further to the outside, a smooth surface was encountered (CW3 in Fig. 6). A further subdivision in layers could not be detected.

Isolated cell wall. As an aid in evaluating the results obtained with freeze-etching, a shadowed (Fig. 8) and a sectioned specimen (Fig. 9) are shown for comparison. Thin-sectioning reveals a succession of three electron-dense lines (Fig. 9, and reference 3) resembling the profile observed in the intact cell. Since "normal" freeze-etching (fracturing at -100 C, and etching for 1 min at the same temperature) of bacterial fragments produced only cross-fractured specimens, deep-etching was applied in an effort to expose surface structures. After deep-etching, one observes a cell wall profile composed of two layers (Fig. 10 and 11). Only one type of surface structure was encountered in this preparation: the presumed inner and outer surfaces are indistinguishable from each other (Fig. 10–12; see also Discussion). Both surfaces resemble a loose network of thick strands. Closer examination indicated that the networks on either side are attached to a substrate. A smooth surface seems to border the outer surface network (Fig. 11, arrow), and a rougher surface (Fig. 12, arrow) might be identified as the basis of the inner cell wall network.

DISCUSSION
The observations made by application of the freeze-etching technique in the present study confirm the concept of the multilayered structure of the enterobacterial cell envelope (3, 6, 7, 9, 18). The special characteristics of the freeze-etching technique allowed observation of a good deal more structural detail compared with more conventional techniques.

The plasma membrane of E. coli B resembles that of other freeze-etched bacteria (e.g., see 4, 10, 12). It may well be a general feature of the bacterial plasma membrane that its inner and outer surfaces differ in structure. The chemical constitution of the particles at either side of
FIG. 1. Thin section showing the multilayered cell wall (CW) and the profile of the plasma membrane (PM). (Bar equals 0.1 μm in Fig. 1-12.)

FIG. 2. Cross-fractured cell envelope in which the main layers can be discerned. CW, cell wall; PM, plasma membrane; CC, cell content.

FIG. 3. Cell envelope fractured along a convex surface. No glycerol has been used here as a freeze-protecting agent. CW, cell wall; OPM, outer surface of the plasma membrane. Arrow points at a possible substructure in the cell wall.

FIG. 4. Cell envelope fractured along a convex surface. Cells have been grown in the medium supplemented with glycerol. The cell wall (CW) has a wavy appearance, and some small particles and fibrils are attached to it. The underlying surface represents the outer surface of the plasma membrane (OPM). In the latter surface appears a smooth field bearing blunt protrusions.
FIG. 5. Cell envelope fractured along a concave surface. The inner surface of the plasma membrane is revealed (IPM). Note the close association of the latter with the cell content (CC). CW1, innermost layer of the cell wall; CW2, next visible layer in the cell wall showing closely packed elements.

FIG. 6. Cell envelope fractured along a concave surface. CW2 has been partly removed, showing a smooth surface (CW3). IPM, inner surface of the plasma membrane.

FIG. 7. Cell envelope fractured along a concave surface. The separation of the plasma membrane (IPM) and the innermost layer of the cell wall (CW1) are shown at the left (arrows). CW2, cell wall layer bordering CW1 (cf. Fig. 5); CC, cell content.
Fig. 8. Platinum-carbon shadowed cell wall preparation.
Fig. 9. Thin section of a closely packed cell wall preparation.
Fig. 10. Freeze-etched cell wall preparation showing a double-layered cell wall (cf. Fig. 9). The inner surface of the cell wall (IS) seems to be composed of a network of thick strands.
Fig. 11. Cell wall fragment with partly removed inner cell wall layer (IS). A smooth surface is revealed (arrow).
Fig. 12. Fragment with the outer surface of the cell wall (OS) exposed. Note the similarity in surface texture of inner (IS, Fig. 10 and 11) and outer surface (OS). The underlying surface has a rough appearance (arrow).
the membrane is so far unknown. In Bacillus subtilis, the particles appear preserved when, prior to freeze-etching, a chemical fixation with osmium tetroxide is applied (11). The particles are likewise present on the membrane of B. subtilis protoplasts (unpublished). A proper interpretation of the significance of the smooth fields with blunt protrusions, sometimes observed in the outer surface of the plasma membrane, is as yet difficult to give. Fii and Branton (4) noted an increase in the size of the smooth fields in E. coli after magnesium starvation.

The most conspicuous component of the cell wall is the array of closely packed elements measuring about 10 nm in diameter (CW2 in Fig. 5-7). A layer of globular elements has been observed by De Petris (2) in thin sections, bordering what was later interpreted as the mucopeptide layer (3). In shadowed cell wall preparations (18), protein particles were found to be attached to the mucopeptide layer, but these were not so closely packed as the elements in CW2 (Fig. 5–7). In accordance with these observations (2, 3, 18), a further profile (CW1, in Fig. 5 and 7) could be discerned underneath the layer of closely packed elements (CW2).

In partly cross-fractured cells, the profile CW1 was seen to follow the course of the cell wall and not that of the plasma membrane (Fig. 7, arrows). Therefore, I like to suggest that CW2 represents the freeze-etched equivalent of the globular protein layer (2, 3, 18), and CW1, that of the mucopeptide layer. The hexagonal pattern that has been observed in negatively stained preparations of preheated cell walls (5) was not found in our freeze-etched specimens. Possibly CW2 can assume a hexagonal pattern upon heating.

No other structures were seen between the innermost profile CW1 and the plasma membrane.

Overlaying the layer of closely packed elements (CW2) a smooth surface was encountered (CW3 in Fig. 6). This surface most probably corresponds to the inner surface of the outermost cell wall layer, which is reported to be composed of lipoproteins and lipopolysaccharides (cf. 6).

The outermost surface observed in cells frozen in the absence of glycerol appears smooth (Fig. 3), whereas in cells grown in the presence of glycerol a rougher surface was seen (Fig. 4). In cells treated with glycerol just prior to freezing, no outer surface of the cell wall has been observed thus far. The rough surface could underlie the smooth, or, alternatively, the rough surface may be the result of an apposition of material against the smooth surface. The role of glycerol in this connection is not quite clear.

The finger-shaped protrusions visible on the bacterial surface after staining with silicotungstate (1) were not observed in our preparations.

Some of the structures described here for the cell envelope of freeze-etched E. coli closely resemble those found in Ferrobacillus ferrooxidans by Rensens and Lundgren (13), but the interpretation offered in the present report differs in part in the light of more recent literature (cf. 6).

As far as the isolated cell wall preparation is concerned, the possibility that the cell wall material has been rearranged during the isolation procedure or that the inside of the cell wall has been turned out during the various treatments cannot be ruled out. Therefore, tentatively, the following interpretation is offered. Freeze-etching of the isolated cell wall reveals the apposition of two layers (Fig. 10 and 11), suggesting that the interface is related to the middle electron-dense line of the sectioned profile (Fig. 9).

The smooth surface in the cell wall preparation (Fig. 11, arrow) is presumably the same as that seen in the intact cell (see Fig. 6). The rough surface (Fig. 12, arrow) could represent what remains of the layer of closely packed elements (CW2 in Fig. 5–7). No morphological trace of the mucopeptide layer could be detected in this isolated cell wall preparation. Nevertheless, it is expected to be present: first, because of the rigidity of the cell wall (see Fig. 8), and, second, because of the presence of three electron-dense lines in the sectioned profile (Fig. 9). A striking feature of the cell wall preparation is that only one type of external surface structure was observed. As this preparation has been deep-etched (see also Results), any external surface of the wall should have been exposed. It could be that the external appearance observed in these preparations (Fig. 10–12) has been influenced by the presence of glycerol in the freezing-medium. To study cell fragments, however, it is essential to avoid any ice crystal formation. In my experience the chance of obtaining preparations undisturbed by ice crystals can be enhanced by increasing the glycerol concentration (from 20 to 35%, v/v). Therefore, some caution is needed in drawing conclusions from isolated cell wall preparations with respect to cell wall structure in the intact cell.

By virtue of the fact that the freeze-etching technique allows the demonstration of large extensions of cell envelope layers and their attached particles, a three-dimensional image can be derived in which data obtained from sectioned material and shadowed cell wall fragments can be tentatively integrated. The spatial relationships of the cell envelope structures observed by freeze-etching in the intact cell are summarized schematically in Fig. 13.
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LITERATURE CITED

20. Weidel, W., G. Koch, and F. Lohss. 1954. Über die Zellmembran von E. coli B II. Der Rezeptorkomplex für die Bakteriophagen Ts, Tt und Tr. Z. Naturforsch. 9B:398-402.