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Alterations in envelope structure of heptose-deficient mutants of *Escherichia coli* as revealed by freeze-etching

(membrane fracture/lipopolysaccharide/protein)

M. E. BAYER, J. KOPLOW, AND H. GOLDFINE

The Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111 and The Department of Microbiology, The University of Pennsylvania, Medical School, Philadelphia, Pa. 19104

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ABSTRACT **The surface of freeze-etched *E. coli* strain GR467, a heptose-deficient ("deep rough") mutant derived from CR34, was studied by electron microscopy. The outer membrane of GR467 has an increased ratio of phospholipid to protein, mainly due to a decreased protein content. Freeze-etched CR34 showed structural features indistinguishable from wild-type *E. coli*, i.e., the primary cleavage occurring in the inner membrane with only minor appearances of cleavage within the outer membrane. In contrast to this, in mutant GR467 most of the freeze-cleavages had taken place along a new plane, presumably in a hydrophobic region of the outer membrane. In this cleavage plane numerous particles were seen. Often the cleavage extended over the entire exposed cell surface; occasionally only a few large plateaus were visible, around which the next deeper cleavage plane, that of the protoplasmic or inner membrane, was discernible. Two spontaneous revertants (R11 and R16) with protein and lipid A levels similar to wild-type cells showed mostly freeze fractures with wild-type characteristics, and only a few cells had retained fracturing properties of GR467. A partial revertant revealed intermediate characteristics. Thus, there appears to be a morphological correlation with the chemical data relating the amount of outer membrane protein with the heptose content of the lipopolysaccharide.**

The components of the cell envelope of Gram negative bacteria, such as *Escherichia coli*, are arranged in two composite layers: (*i*) the outer membrane (OM) to which is attached the "rigid" peptidoglycan, and, still deeper in the envelope, (*ii*) the inner membrane (IM), surrounding the protoplasmic contents of the cell. In *E. coli* the OM is composed of lipopolysaccharide (LPS), proteins, and phospholipid, whereas the IM is largely composed of phospholipid and protein.

The chemical structure of the component molecules of the envelope has been extensively investigated, with special emphasis on the LPS of the outer membrane. Correlation of the chemical structure of LPS with such functions as antigenicity (1–3), virus receptor activity (4, 5), and permeability to antibiotics (6–9), mutagens, dyes (10, 11), and proteins (12) has led to an increasing understanding of the complexity of the OM as a functional cell surface layer. On the structural level, however, knowledge is still lacking with regard to the physical parameters of distribution of the phospholipid, LPS, and protein components of the outer membrane. As opposed to the rather uniform "triple layered" contour seen in electron micrographs of ultrathin cross sections, freeze-fracturing has revealed an unexpected aspect of this membrane: a "membrane" that only fractures for very short distances within its hydrophobic portions (13). This suggests that the OM is not a typical lipid bilayer which would normally be expected to fracture in its inner, hydrophobic region (14). There are only limited data on the arrangement of the proteins in the outer membrane, and none on the possibilities for membrane cleavage after introduction of known changes of the lipid/protein ratio. Such a study became possible with the description of heptose-deficient LPS mutants of *E. coli* in which concomitant changes in the protein content of the OM were observed (15). The mechanism linking heptose deficiency of the OM to diminution of the protein content is not known. We have studied the effect that alterations of LPS and of the lipid/protein ratio had on the morphology of freeze-fractured envelopes of *E. coli*.

Abbreviations: OM, outer membrane; IM, inner membrane; LPS, lipopolysaccharide.

MATERIALS AND METHODS

The parent strain CR34 is a derivative of *E. coli* K12. Strain GR467 is a temperature sensitive, deep rough mutant of CR34, deficient in the heptose content of its LPS. Strains R11 and R16 are spontaneous revertants of GR467 with heptose levels comparable to CR34, whereas R2 is a revertant of intermediate characteristics. For isolation procedures and further characteristics of these strains, see ref. 15. The medium used for growth was Trypticase soy broth (BBL) supplemented with 10 μg of thymine per ml. The cells were grown at 30° in tubes with aerators and harvested in the logarithmic state of growth at densities of 2 to 4 $\times$ 10^8 cells per ml. The cells were sedimented by spinning for 10 min at 6000 $\times$ *g*, and portions of the pellets were quickly frozen on gold grids in Freon at liquid nitrogen temperature. Care was taken that all preparations were handled at the same temperatures. The specimens were subsequently freeze-etched at −100° in a Balzer BM 360 unit and shadowed with platinum-carbon before carbon replication (see ref. 13). Micrographs of the replicas were taken in a Siemens 101 electron microscope using Kodak electron microscope film. Negative copies were printed, so that in the final micrograph the shadows are black and the source of platinum appears as a source of illumination.

RESULTS

The results of this study can best be followed by referring to the diagrammatic representation of the *E. coli* cleavage planes (Fig. 1). After freeze-fracture, cells of strains CR34 are structurally indistinguishable from other wild-type *E. coli* strains, such as *E. coli* B or Cla. The contour of the outer membrane is slightly wavy; its surface is rather smooth (Fig. 2); a detailed substructure (for example, a pittedness) is not recognizable. A fracture plane, probably within the outer membrane, becomes visible in relatively narrow areas (Fig. 2, arrows; see also diagram of Fig. 1, fracture plane OMF). From this plateau, the next deeper fracture plane is positioned in the inner membrane (in diagram, Fig. 1: IMF) and

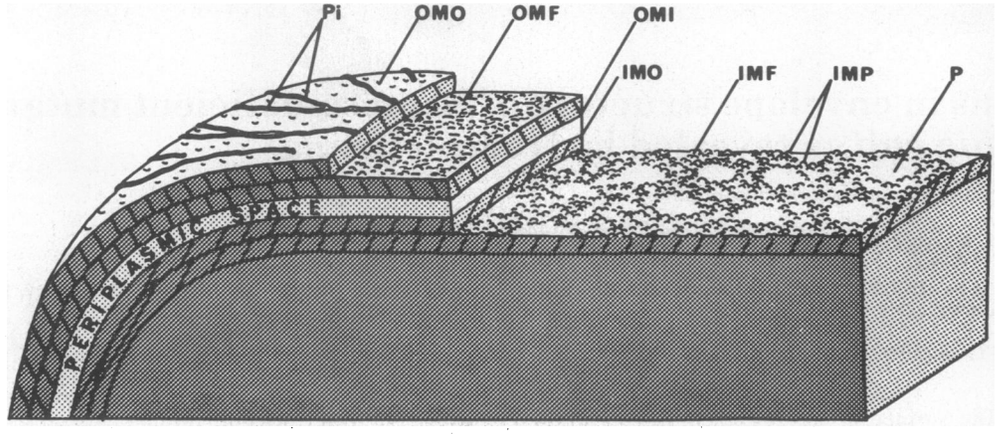

FIG. 1. Diagrammatic representation of freeze-cleavage planes of *E. coli*. Abbreviations used in all figures: OMO, outer membrane (cell wall) surface; OMF, outer membrane fracture plane; IMF, inner membrane fracture plane with intramembranous membrane particles (IMP); P, patches devoid of inner membrane particles; OMI, outer membrane innermost surface; Pi, pili; IMO, inner membrane outer surface. The peptidoglycan layer has not been included, since its exact position relative to OMI cannot be deduced from our preparations.

is characterized by the presence of numerous particles of 8.0–10.0 nm in diameter. This plane is most likely located within the hydrophobic region of the lipid molecules of the inner membrane. The distribution of particles in this membrane is more or less random. However, differences in the cooling rate of the specimens shortly before quick freezing, may introduce the appearance of patchy areas of various extent lacking the intramembranous particles (16). A few such particle-free patches can be seen in Fig. 2 (see also Fig. 1). Throughout this investigation, however, we have attempted to subject the various specimens to identical procedures within each experiment, avoiding differences in cooling or major changes in the environmental temperatures before the quick freezing step. The heptose-deficient strain GR467 reveals an entirely different aspect; the major fracture plane is now located in the outer membrane (Fig. 3a and b, OMF). The fracture plane OMF is often the only cell surface visible in these preparations. Most strikingly, this plane shows a fine structure consisting of abundant particulate elements scattered over the fracture plane. These structural elements protrude from the cleavage plane OMF and are either of spherical shape, measuring about 10 nm in diameter, or of filamentous shape of 30–40 nm in length and about 10 nm in width; at smaller regions of the fracture plane they seem to be oriented somewhat parallel to each other (Fig. 3a). However, this orientation may be related to the direction of cleavage and not to a characteristic feature of the cell envelope. Furthermore, a filamentous appearance can be introduced by freeze-cleavage of spherical elements such as latex particles. In such preparations, many spherical latex particles assume cone-shaped structures (Bayer, unpublished). The number of particles (spherical plus filamentous) per μm^2 has been estimated to be about 1000 to 1100 in strain GR467.

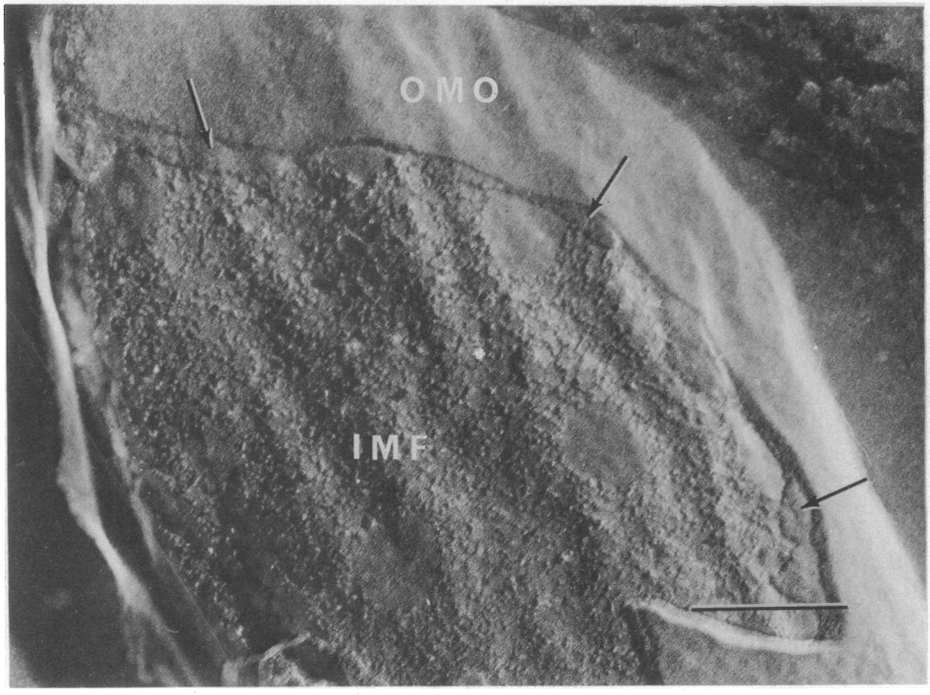

FIG. 2. Freeze-etching of *E. coli* CR34. The cleavage within the outer membrane (arrows) occurs in only relatively small marginal areas. Most of the cleavage occurs within the inner membrane (IMF). The bar represents 0.2 μm in this and in subsequent figures.

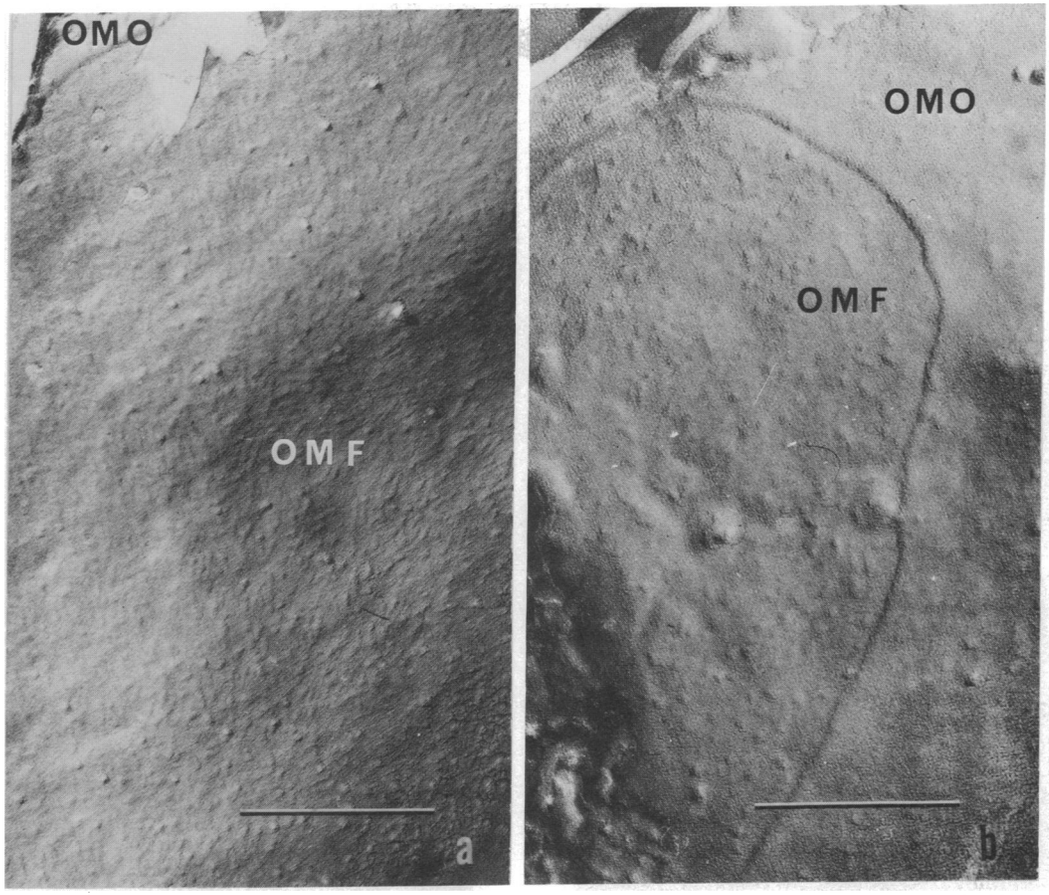

FIG. 3. (a and b) The heptose-deficient mutant GR467 shows cleavage entirely within the outer membrane (OMF). Note the filamentous and spherical particles exposed in this cleavage plane.

A relatively small number of strain GR467 cells also reveal a cleavage plane within the protoplasmic membrane. The cleavage may then alternate between outer membrane (OMF) and inner membrane (IMF), generating a multi-level appearance (Fig. 4).

The freeze-etched "full" revertant (15) strain R11, shows a fracture profile mostly indistinguishable from that of strain CR34: the cleavage takes place in the hydrophobic region of the inner (protoplasmic) membrane (Fig. 5, IMF), showing narrow areas of cleavage in the outer membrane (arrow), and only rarely are wider areas of that cleavage plane visible. In the second "full" revertant, R16, on the other hand, a few small island-like plateaus with cleavage in OMF are occasionally visible as they protrude over the fracture plane of the inner membrane (IMF) (not shown).

The spontaneous revertant R2 with partial reversion to wild-type chemical characteristics showed features in freeze-etchings resembling, at one extreme, those of GR467, but mostly features of an intermediate degree, with exposure of the cleavage planes in both the outer as well as the inner membranes (Fig. 6).

DISCUSSION

The protein content of the outer membrane of strain GR467 is reduced about 60% relative to the content of phospholipid or lipid A, whereas the ratio of phospholipid to lipid A is similar to that found in the parental strain CR34 (15). When the proteins of the outer membrane of the mutant were solubilized in sodium dodecyl sulfate, a diminution of major polypeptides was observed by means of polyacrylamide gel electrophoresis. The phenomenon that links heptose deficiency in LPS to depletion of outer membrane proteins can, at present, not be explained satisfactorily. It has also been observed by Ferro-Luzzi Ames *et al.* (17) in heptose-deficient mutants of *Salmonella typhimurium*. The changes in composition of the outer membrane of *E. coli* GR467 did not seem to be accompanied by major structural changes in the profile of ultrathin sections of fixed and Epon-embedded cells. However, the properties of membrane cleavage in the frozen state appear to be drastically altered, which suggests considerable changes in the physical state of the outer membrane.

One can speculate that several major aspects might work separately or together to cause the structural changes that we have observed in the heptose-deficient freeze-etched envelopes of the *E. coli* mutants: (*i*) a lesser degree of crosslinkage on noncovalent binding of the LPS molecules; (*ii*) a decrease in the amount of protein in the membrane which leads to a higher relative lipid content with an increased tendency to cleave within the hydrophobic region of the membrane; and (*iii*) a decreased content of a specific "linker protein" that usually holds the lipid bilayer together.

In the last respect, it is of interest that Henning and collaborators (18, 19) and also Rosenbusch (20) have proposed a model for the outer membrane in which the membrane proteins form a tight meshwork. Such an arrangement has some resemblance to the membrane model of Capaldi and Green (21). In addition, it should be noted that Nakamura *et al.*

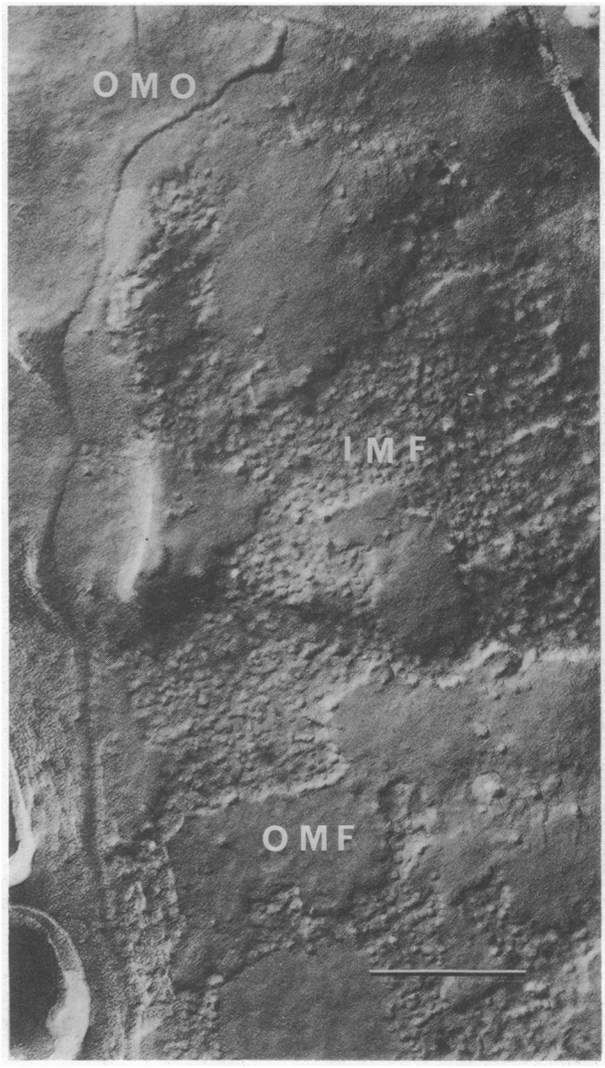

FIG. 4. A less common aspect of the cell surface of GR467. Patchy areas occur where the fracture cleaved in the OMF and also in deeper areas of cleavage in IMF.

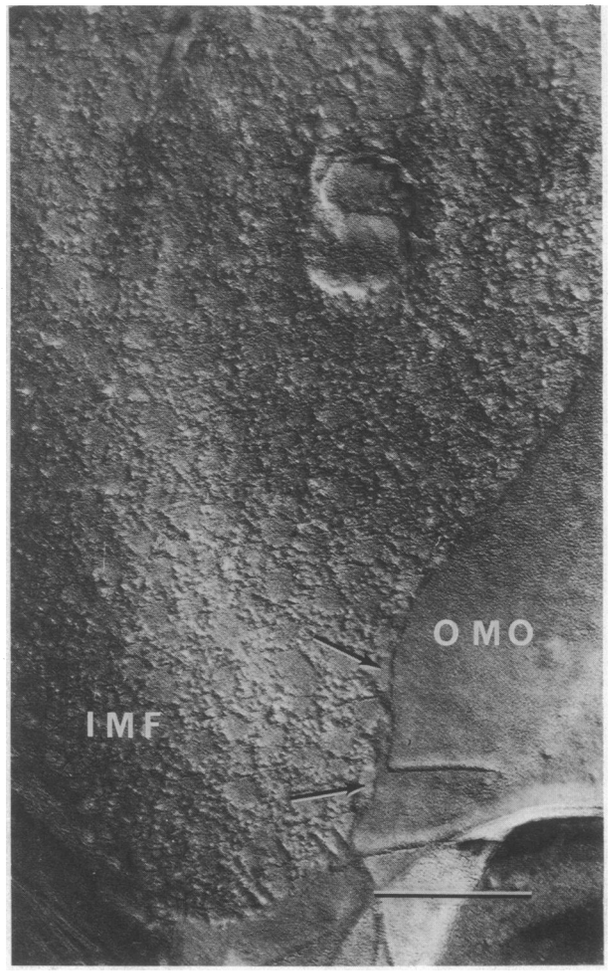

FIG. 5. The full revertant R11 shows a cleavage, almost entirely in the IMF, comparable to that seen in CR34 (Fig. 2).

(22) and Rosenbusch (20) found by infrared analysis that a large portion of the proteins in the OM of *E. coli* spheroplasts consists of beta-structured polypeptides. Although a typical triple-layered contrast distribution is observed in ultrathin cross sections through the outer membrane of wild-type *E. coli*, the architectural design of the unfixed, nondehydrated outer membrane of *E. coli* cannot easily be described as a lipid bilayer-globular protein mosaic model (23) in view of the freeze-etching data: wild-type *E. coli* B or CR34 only very rarely exhibit a tendency to cleave within the outer membrane (13, 24). The preference for cleavage in the OM of strain GR467 suggests to us that a membrane with bilayer characteristics can be generated upon withdrawal or diminution of membrane protein.

According to Schnaitman (25) and other investigators, approximately 60% of the envelope protein in *E. coli* is present in the outer membrane. Attempts to visualize the particles in the OMF of CR34 or wild-type *E. coli* B have failed due to the small area of OMF exposed in these cells. If glycerol is present during freezing, then larger areas of OMF, containing particles, can be seen in *E. coli* B as well as in CR 34. The relatively large amount of particles visible in the fracture plane of the OM of GR467 would not be in disagreement with the tentative assumption that these particles represent some aspects of the OM proteins. The size of the spherical and elongated elements in our preparations is such that it would correspond to molecular weights far in excess of those reported for the peptide fractions seen after sodium dodecyl sulfate-polyacrylamide analysis (15, 18, 25). The possibility exists that the particles seen are composed of aggregates of proteins depicted in their "normal state" in the membrane. It was thought that the extremely rapid cooling rate during freezing of the specimen would not provide sufficient time for an "artificial" aggregation; however, the possibility seems to exist (26). We found no indication for a regular "crystalline" arrangement of the particles, as has been reported for the major protein of the *E. coli* envelope (20). Under the circumstances of diminished protein content in strain GR467, the cleavage may be guided with increasing probability within the hydrophobic region of the lipid bilayer.

The "patchiness" of the cleavage observed in the full, and especially in the partial, revertants could be indicative of unevenly distributed phospholipid, LPS, and protein. Thus, there may be portions of the OM of these strains that contain sufficient amounts of protein, possibly in a meshwork as suggested by Henning *et al.* (18, 19) or a lattice structure (20), while other portions have little protein, thus allowing cleav-

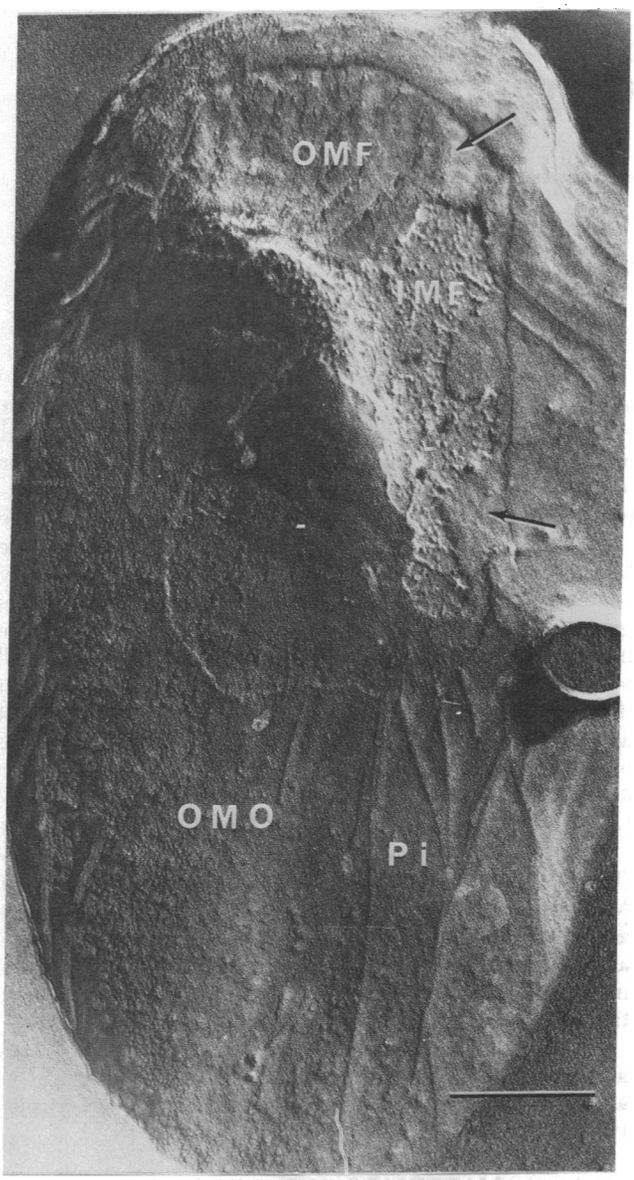

FIG. 6. Partial revertant R2 shows fracture planes in the OMF as well as in the IMF. Note the pili (Pi) that indicate the outer surface (OMO) of the cell.

age in typical bilayer-like regions. Some support for this interpretation can be drawn from the work of Leive (27). Leive found that after EDTA treatment, about 50% of the LPS of *E. coli* is released into the medium. Newly synthesized LPS is nonreleasable and possibly contains less protein than those portions from which LPS is released by EDTA (28). We recently found (Bayer and Leive, submitted) that EDTA-treated *E. coli* showed patches in freeze-etchings, in which some of the outer membrane was cleaved in a manner similar to that seen in Fig. 4 of this paper, whereas most of the cleavage had occurred in the inner membrane.

Since the chemical data of the mutant GR467 indicate considerable changes in the direction of a relative excess of lipid over protein in the outer membrane, we suggest that for the same reason the morphological/physical characteristics of this organelle have shifted away from a tightly complexed lipid/protein arrangement toward a membrane exhibiting properties more like those of a lipid bilayer.

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