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# Freeze-Etching and X-Ray Diffraction of the Isolated Double-Track Layer from the Cell Wall of a Gram-Negative Marine Pseudomonad

A. FORGE,[1] J. W. COSTERTON, AND K. ANN KERR

*Department of Biology and Departments of Chemistry and Physics, University of Calgary, Calgary, Alberta, Canada*

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The isolated double-track layer of the cell wall of the gram-negative marine pseudomonad studied here contains a cleavage plane. This finding localizes the single cleavage plane of the cell wall and shows that the molecular architecture of this layer provides the lipid-enriched layer which cleaves preferentially in the frozen cell. The observation that the isolated double-track layer of the cell wall is sufficiently ordered at the molecular level to yield a well-defined X-ray diffraction pattern with a d-spacing of 0.44 nm shows that its molecular architecture is very similar to that of true membranes. This specific d-spacing is produced by the highly ordered packing of the hydrophobic portions of phospholipid molecules. Therefore, the double-track layer of the cell wall has been shown, by these two biophysical means, to have a molecular architecture which would allow it to function as the membrane-like "molecular sieve" layer, whose presence has been deduced from physiological data. This layer is important in the retention of cell wall-associated enzymes and in the control of the movement of large molecules through the cell wall.

Physiological studies of a number of gram-negative bacteria have shown that some part of the cell wall is capable of acting as a barrier to the passage of certain molecules into the cell (10). This "molecular sieve" layer also functions as the outer boundary of the "periplasmic" space (28) in which many of the cell wall-associated enzymes are retained. These physiological data cannot, however, indicate which of the several layers of the cell wall (15, 23) perform this selective barrier function.

Recently, a method has been developed for the nondegradative chemical dissection of the cell wall of the gram-negative bacterium used in this study (11, 23), and it was determined that the only cell wall layer which contains large amounts of phospholipids and proteins is the double-track layer (24). Schnaitman (35) obtained membrane-free preparations of the double-track layer of the cell wall of *Escherichia coli* and found that they are primarily composed of phospholipids and proteins. This membrane-like chemical composition, plus the fact that this layer forms a double track when fixed for electron microscopy by the usual methods, suggests that it has the chemical potential to form the barrier layer which has been shown to be present in the cell wall.

Freeze-etching studies have shown that cleavage planes follow the organized lipids in the median area of membranes (3, 30, 37), and studies of whole cells of the organism used here have shown that there is a single cleavage plane in the cell wall and that it lies in either the underlying soluble layer or the double-track layer (17). X-ray diffraction studies also indicated that a layer composed of highly ordered lipid molecules was present in the cell wall of gram-negative bacteria (6), and the suggestion was made that these lipids are in the double-track layer (7). However, these studies used cell wall preparations which were contaminated with cytoplasmic membrane; thus, the localization of the layer of ordered lipids within the cell envelope remains undetermined.

The development of a method for the preparation of isolated double-track layer which was free of contaminating cytoplasmic membrane and contained only small amounts of material from adjoining cell wall layers (24) provided us

[1] Present address: Astbury Department of Biophysics, University of Leeds, Leeds, Yorkshire, U.K.

with the opportunity to study this layer in isolation. An examination of this material was undertaken to determine whether this layer constituted a cleavage plane in frozen preparations, whether it yielded a membrane-like X-ray diffraction pattern, and thus whether it possessed a membrane-like molecular architecture.

## MATERIALS AND METHODS

**Organism.** The organism used, designated marine pseudomonad B-16, has been classified as a *Pseudomonas* species type IV. It was deposited in the American Type Culture Collection as ATCC 19855 and with the National Collection of Marine Bacteria, Aberdeen, Scotland, as NCMB19. Studies on the ultrastructure and metabolism of this organism have been reported in several previous communications, e.g., Forsberg et al. (23, 24) and DeVoe et al. (16).

**Medium and growth conditions.** The medium used in these studies was detailed by Forsberg et al. (23).

A 10-ml volume of the liquid medium in a 50-ml Erlenmeyer flask was inoculated from a slant and incubated for 8 hr. The entire contents of this flask was used to inoculate 250 ml of the liquid medium in a 2-liter Erlenmeyer flask, and this was incubated for 5 hr. Portions (40 ml) of this culture were each added to 260-ml volumes of the liquid medium in 2-liter Erlenmeyer flasks, and incubation was continued for a further 5 hr. Incubation of all cultures was at 25 C on a rotary shaker. This procedure provided large quantities of cells in the early logarithmic phase of growth ("5 hour cells," reference 23).

**Washing of cells.** Cells were harvested by centrifugation at 16,000 × *g* at 4 C and washed three times by successive suspension in and centrifugation from volumes of 0.5 M NaCl equal to the volume of the growth medium. This removed the loosely bound outer layer of the cell wall (23).

**Isolation of the double-track layer of the cell wall.** Cells washed three times in 0.5 M NaCl were suspended in a volume of 0.5 M sucrose equal to one-quarter the initial volume of growth medium. This suspension was incubated for 30 min at 25 C on a rotary shaker and then centrifuged at 35,000 × *g* for 20 min. The cloudy supernatant fluid was retained, the cells were suspended in the same volume of fresh 0.5 M sucrose, and the washing procedure was repeated. The supernatant fractions from these two washings were pooled, and the double-track layer was collected from them by centrifugation at 73,000 × *g* for 2 hr. The double-track layer sedimented as a green gelatinous pellet. This material was washed three times in glass-distilled water, by successive suspensions and centrifugation at 73,000 × *g* for 2 hr each time, to free it of the underlying soluble layer material (23).

**Freeze-etching.** Specimens were prepared for freeze-etching by affixing drops of pelleted material to ridged gold discs, 3 mm in diameter, without cryoprotective agents, and immersing the discs in liquid Freon 22 maintained around its melting point. Freeze-etching was performed in a Balzers BA360M apparatus, and for each sample at least two replicas were prepared: one in which cleaving was performed at −135 C with no subsequent controlled sublimation (freeze-cleaved preparations), and one in which cleaving was performed at −100 C followed by etching of the surface by sublimation for 5 sec (freeze-etched preparations). The platinum coating was applied from an angle of approximately 25° above the surface in freeze-cleaved preparations and from an angle of approximately 45° in freeze-etched replicas.

**Negative staining.** A small volume of a thick suspension of the material was mixed with an equal volume of 1% zirconium oxide (24), *p*H 7.0, with 0.2% sucrose. Drops of this suspension were dried down on Formvar-coated grids.

**Thin sectioning.** The pelleted material was fixed in 5% glutaraldehyde in 0.4 M phosphate buffer, *p*H 6.8, for 1 to 2 hr at 22 C. It was then enrobed in 4% agar, and the agar cores were washed five times in the phosphate buffer. Postfixation was in 1.8% osmium tetroxide in the phosphate buffer (with 0.2% tryptone and a few drops of 1 M $MgCl_2$ added), for 1.5 to 2 hr at 22 C, followed by five washes in the phosphate buffer and dehydration in an acetone series. The cores were embedded in Vestopal W (Martin Jaeger Co., Geneva, Switzerland). Thin sections (silver) were cut on a Porter-Blum MT.2 ultramicrotome by using glass knives and poststained in 1% uranyl acetate and Reynolds' lead citrate (34).

**Electron microscopy.** Specimens were examined in an A.E.I. EM801 electron microscope by using 60 kv accelerating voltage.

**X-ray diffraction.** A concentrated suspension of the double-track material was drawn up into thin-walled glass capillary tubes, and this material was further concentrated by allowing the evaporation of the free water. The tubes were sealed and the material was examined in a Siemens flat camera using pinhole collimation. Copper k alpha radiations were provided by a Siemens Crystalloflex IV generator using 40 kv accelerating voltage and 18 ma filament current. Specimen-film distances, which were between 5 and 6 cm, were accurately determined by reference to the diffraction pattern of NaCl which was lightly dusted over the surface of the specimen tubes. Exposure times were usually 8 to 10 hr, depending on the concentration of the sample, and patterns were recorded on Ilford Industrial-G X-ray film.

## RESULTS

**Morphological studies.** The double-track layer of the cell wall of the organism used in these studies forms spherical vesicles on isolation. Because these vesicles are flattened by drying, they appear as circular structures with thickened rims (Fig. 1) in negatively stained preparations. This appearance in these preparations is very similar to that of membrane vesicles (27) and artificial liposomes (25). In thin-sectioned preparations, the vesicles are seen to be roughly round, and the double track

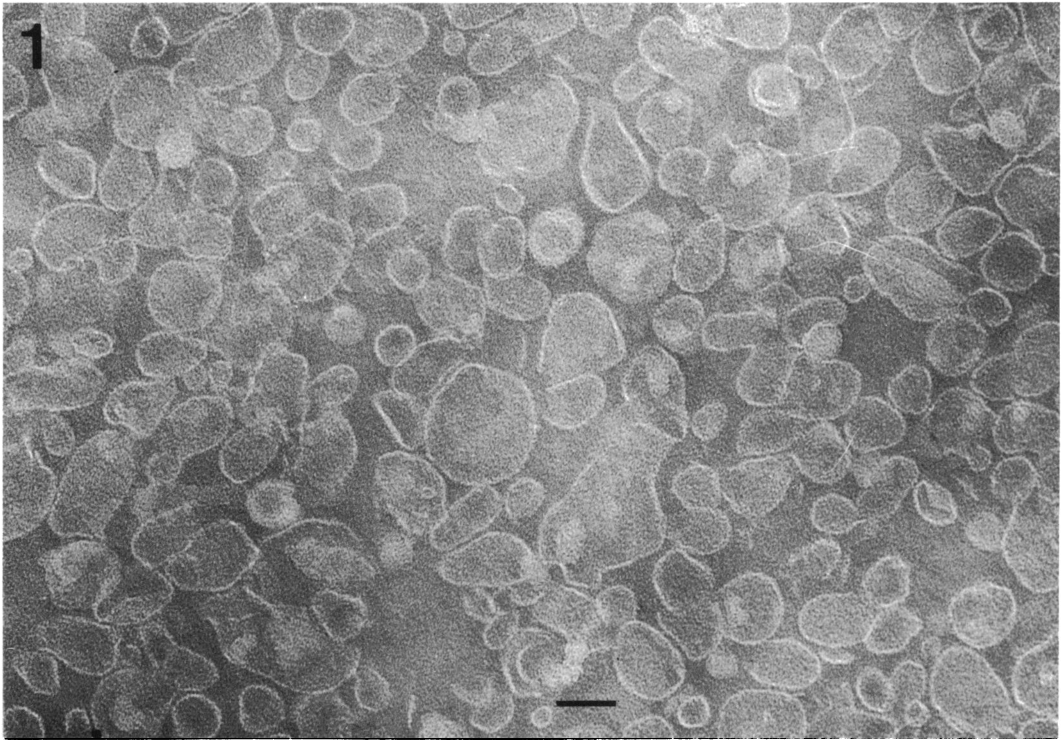

FIG. 1. *Electron micrograph of a negatively stained preparation of the isolated double-track layer of the cell wall of the marine pseudomonad (B-16). Stained with 1% zirconium oxide (pH 7.1) with 0.2% sucrose. The bar in this and subsequent figures represents 0.1 μm.*

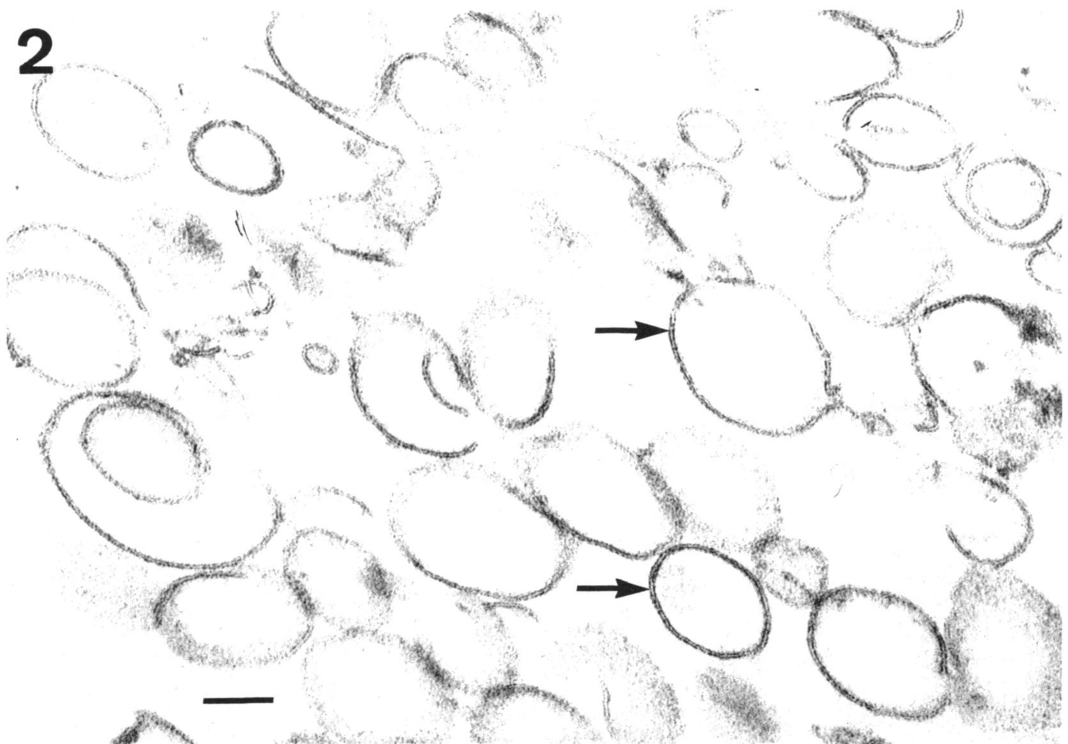

FIG. 2. *Electron micrograph of an embedded preparation of the isolation double-track layer. Note the clear double-track profile where the layer is "in phase" (arrows).*

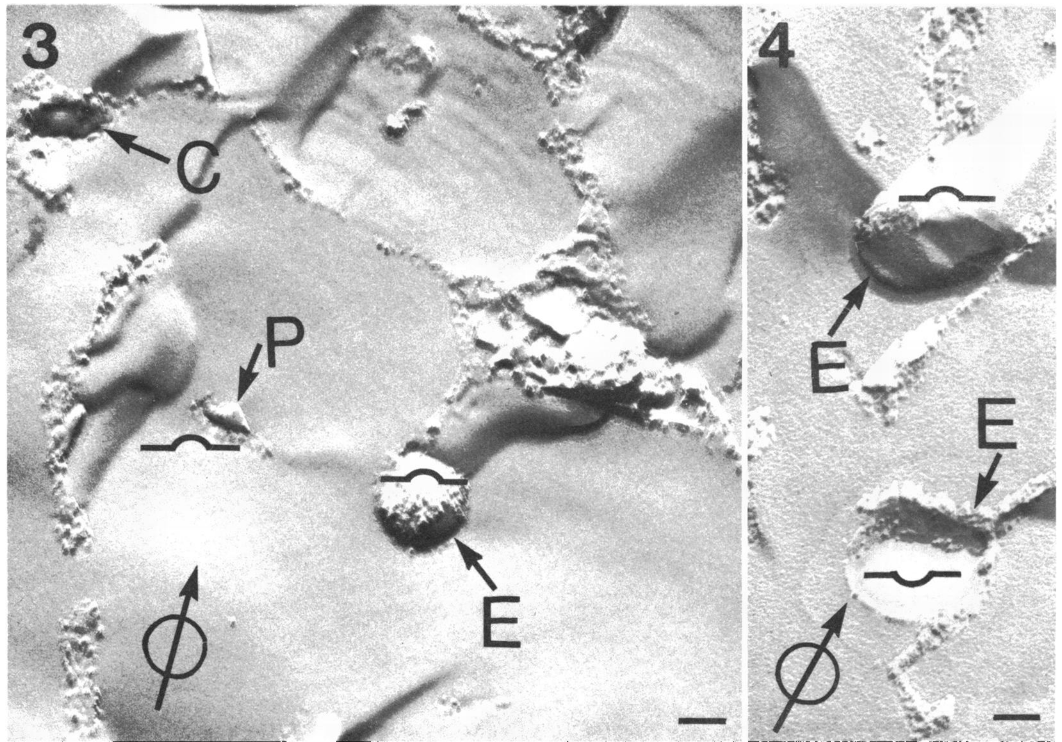


FIG. 3, 4. *Electron micrographs of freeze-cleaved preparations (no sublimation) of the isolated double-track layer. The cleavage plane in this material is weak and occasionally follows the contour of the vesicles for a great distance (E), but more often for a short distance (P), and cross-cleaved vesicles are often seen (C). Symbols:* ⌀, *direction of shadowing;* ‿, *concave cleavage;* ⁀, *convex cleavage.*

is clearly seen when it is "in phase" (Fig. 2, arrows). The curvature seen in non-equatorially sectioned vesicles indicates that most of the vesicles are spherical.

**Freeze-cleaving.** Replicas of freeze-cleaved (no sublimation) preparations of the isolated double-track layer vesicles reveal generally smooth, convex and concave fracture faces which result from a cleavage plane within this layer (Fig. 3, 4). This plane is usually weak, and cleavage may be deflected for only a short distance (Fig. 3, P) or may follow the contour of the double-track layer only briefly and then cross-cleave the vesicle to reveal its hollow center (Fig. 3, C). Occasionally, the cleavage plane is relatively strong and the whole contour of the vesicle is revealed (Fig. 3 and 4, E).

**Freeze-etching.** Replicas of freeze-etched preparations (5 sec sublimation) show both convex and concave fracture faces (Fig. 5, C) in the cleaved area which establishes that the material exposed at this level by the cleavage plane is non-etchable. Nearly all of the structures in the areas of the preparations which were exposed by etching (sublimation) are directly related to the vesicles because they were frozen in distilled water and eutectic formation (17) is minimal. Careful examination of the ridgelike aggregates of material in the areas of these specimens which were revealed by etching shows the outlines of numerous vesicles (Fig. 5, S), and these structures can be related to profiles of vesicles in the cleaved areas of the specimens. Those areas in which the surface of the ice has been lowered by sublimation are indicated (*). Thus, the spherical vesicles of the isolated double-track layer have been seen when the ice around them is sublimed away, and they have been shown to constitute a definite, if somewhat weak, cleavage plane in frozen preparations.

**X-ray diffraction.** The wide-angle X-ray diffraction pattern obtained from concentrated preparations of the isolated double-track material is a broad diffuse ring (Fig. 6). The pattern and the d-spacing (0.44 nm) are very similar to those obtained in studies of cytoplasmic membrane (19, 21) and to the structures formed when phospholipids are dispersed in water (33).

## DISCUSSION

DeVoe et al. (17) established that there is

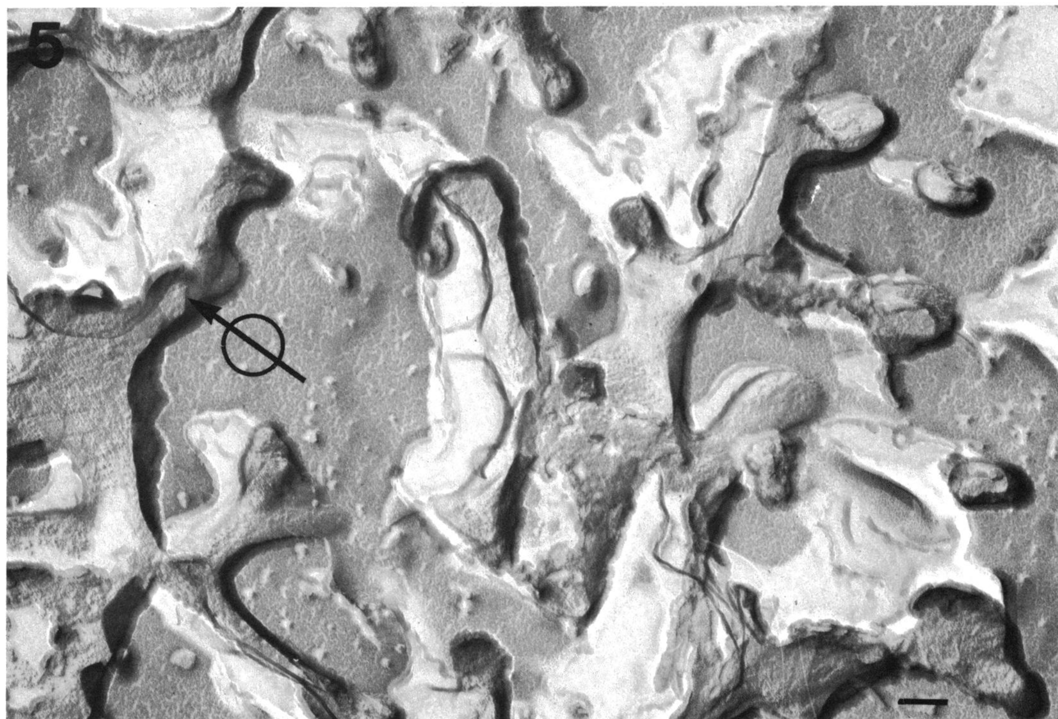


FIG. 5. *Electron micrograph of a freeze-etched preparation of the isolated double-track layer. The outlines of vesicles can be seen in this preparation, in the higher cleaved areas (C) and where their surfaces have been exposed by sublimation (S). Areas where the ice level has been lowered by sublimation are indicated (*).*

a single cleavage plane in the cell wall of frozen cells of the organism used in this study. They noted that this cleavage plane is considerably weaker than that of the cytoplasmic membrane, and its relative weakness, in this organism and in a number of gram-negative bacteria (1, 20, 37), corresponds well with the weak but definite cleavage plane found in the isolated double-track material studied here. DeVoe et al. (17) established that the single cell wall cleavage plane lies in either the underlying soluble layer of the cell wall (23) or in the double-track layer, and this present study established that it is in the latter. In both whole cells (17) and these isolated double-track layer vesicles, the cleavage plane exposes a smooth surface which is distinctly different from the particle-studded surfaces exposed by cytoplasmic membrane cleavage planes (3, 17).

The demonstration that the cleavage plane follows the median area of biological membranes (13, 30) and of artificial lipid bilayers (14) indicates that the low resistance to cleavage in this area results from its content of the hydrophobic portions of the phospholipid molecules. Since the only cleavage planes within cells are associated with membranes, the deduction can be reversed and we can state that the presence of a cleavage plane at the level of the double-track layer of the cell wall indicates that this layer has a median hydrophobic area. This conclusion is supported by the fact that this layer forms a "double-track" on fixation, because this tripartite pattern results from the action of osmium on organized layers of phospholipids (18). Recent studies have shown that this layer is composed of proteins and phospholipids (24, 35), and Costerton and Thompson (12) have shown that it has a membrane-like plasticity in response to deforming stresses. None of the other layers of the cell wall of this organism has a chemical composition (24) or a molecular arrangement (17) which would enable it to form a hydrophobic barrier zone.

The X-ray diffraction patterns produced by the isolated double-track layer vesicles indicate that there is a high degree of order in the arrangement of one of the components of this layer. The d-spacing of this pattern is the same as that obtained from membranes (19, 21), where it has been attributed to the predominantly side-by-side packing of the hydrophobic hydrocarbon chains of the phospholipids to form liquid crystalline array of roughly parallel

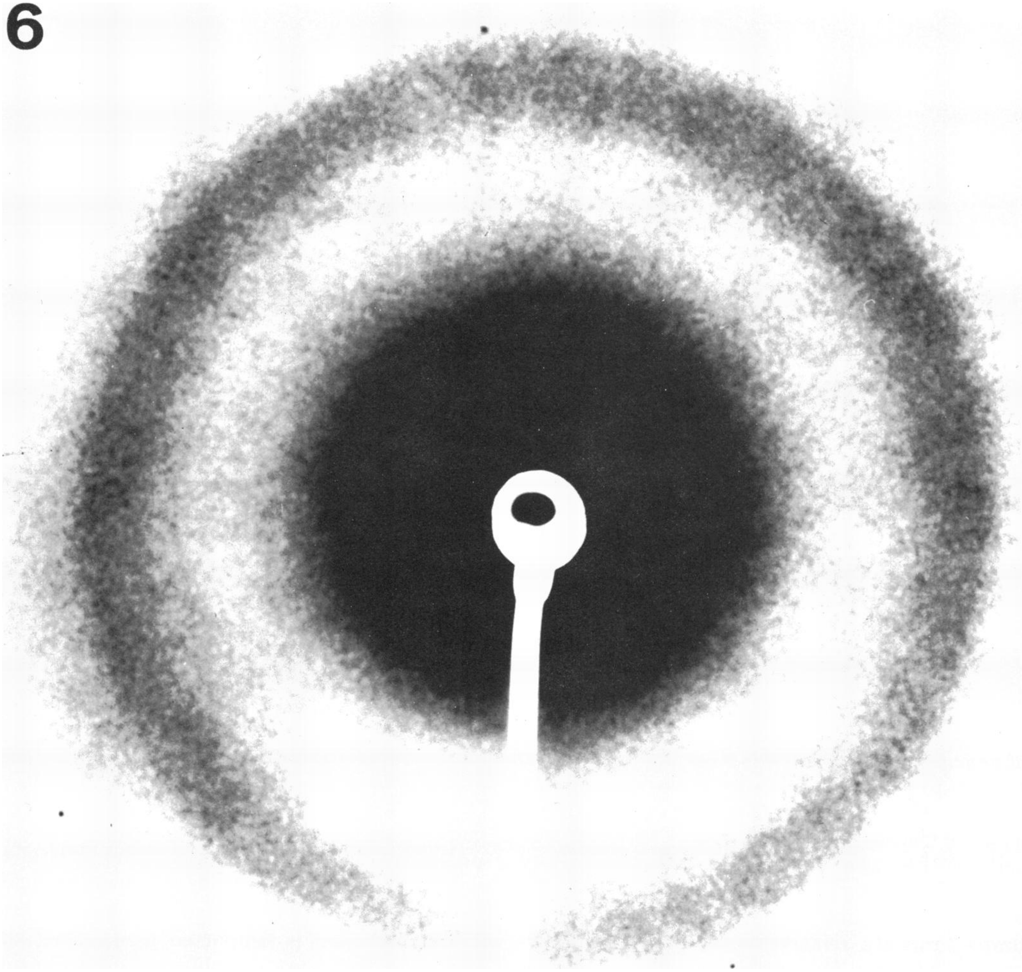


FIG. 6. *X-ray diffraction pattern produced by the isolated double-track layer of the cell wall of the marine pseudomonad (B-16).*

molecules in the median area of the membrane 21, 33).

The fact that the double-track layer has a basically membrane-like molecular architecture accounts for its efficacy as a barrier to actinomycin (36) and as a sieve (28) which limits the penetration of peptides above a definite molecular size (32). This layer also limits the outward movement of periplasmic enzymes (10), and its derangement by a variety of agents (8, 10) causes the release of these enzymes.

Cheng et al. pointed out that periplasmic enzymes are also retained by their association with the lipopolysaccharide of the cell wall (9), and Schnaitman (35) suggested that both the lipopolysaccharide and the lipoprotein described by Braun and Sieglin (4) are anchored to the double-track layer by hydrophobic associations. Thus, the membrane-like double-track layer plays an important structural role in cell wall, and is involved in the retention of wall-associated enzymes both in the periplasmic area (10) and at the cell surface (31).

It has been suggested (5) that the gram-negative bacterial cell is surrounded by two functional membranes; but, while the double-track layer is distinctly membrane-like, it is much more porous than the cytoplasmic membrane (32), and it does not appear to play a significant part in active transport (16). Thus, this double-track layer of the gram-negative bacterial cell resembles the porous and relatively metabolically inert outer mitochondrial membrane (29).

Both structures provide only weak cleavage planes in the frozen state (3), and both are profoundly affected by lipid depletion (22). This similarity of the outer membrane-like layers may then be added to the present evidence concerning equivalence of deoxyribonucleic acid and ribosomal structure to support the suggestion that mitochondria evolved from aerobic gram-negative bacteria (26).

## ACKNOWLEDGMENTS

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