Effects of Lipid Phase Transition on the Freeze-Cleaved Envelope of Escherichia coli

MANFRED E. BAYER,* MARY DOLACK, AND ENOCH HOUSER

The Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Received for publication 29 November 1976

We studied the fine structure of the envelope of Escherichia coli auxotroph K1060 after the cells were grown in the presence of one of the following fatty acids: oleic, palmitelaidic, or elaic acid. The cells were freeze-fractured after exposure to temperatures above and below the lipid phase transition range. As judged by freeze-etching methods, we observed that below the transition range the fracture plane of the inner membrane showed the typical aggregation of intramembranous particles (IMP) and concomitant development of areas devoid of IMP. In these areas we found a regular arrangement of equally spaced ridges, often intersected at 90° by arrays of similar ridges. The ridges were composed of spherical particles measuring 4 to 5 nm in diameter. Formation and melting of these arrays took place within 15 to 30 s after temperature shift-down or shift-up, respectively. Fixation in glutaraldehyde prevented these changes. The outer-membrane fracture plane revealed ordered areas to a lesser degree; these were discernible only by the regular arrangement of the IMP of the concave fracture plane. We interpret the data by suggesting that the pattern of ridges in E. coli K1060 is analogous to the band patterns described for artificial liposomes, and that the particles, possibly proteins, are lined up or extruded along the ridges during membrane lipid crystallization.

The cell envelope of gram-negative bacteria, including Escherichia coli, has been shown to be composed of at least three chemically distinct macromolecular structures: an inner membrane with its phospholipids and proteins, a peptidoglycan layer external to the inner membrane, and the outer membrane, which contains in addition to phospholipids and proteins the lipopolysaccharides of the organism (12, 14, 15, 23).

The composition of the outer and inner membranes reflects their functional difference. The functions are greatly influenced by the fluidity of the membrane and respond especially to a shift in temperature at the range of lipid phase transitions (11, 19, 21), during which the membrane lipids crystallize in increasing amounts. The phase transition within a cell membrane takes place over temperature ranges reaching below the crystallization temperature of the fatty acid incorporated (18). The functional changes of these membranes during the liquid/gel transitions are correlated to structural changes, measurable with X-ray scattering, differential scanning calorimetry (DSC), fluorescence, and freeze-etching (7, 8). We used E. coli K1060, a derivative of K-12 and K1001. This strain is unable to synthesize or degrade unsaturated fatty acids, requires the acids for growth, and incorporates them into the phospholipids of the cell envelope (17, 25).

We studied structural aspects of the membranes of the K1060 envelope after culturing the cells in the presence of the following unsaturated fatty acids: oleic, elaic, and palmitelaidic. After incorporation of a fatty acid at temperatures above the particular transition temperature, a subsequent shifting to temperatures below the melting point will cause cell growth to cease, the membrane to crystallize, and the cells eventually to lyse. We present here evidence of a complex crystallization of the inner and outer membrane: (i) the appearance of particles aligned in ordered arrays in the inner membrane, and (ii) particles of the outer membrane packed in a fourfold pattern.

MATERIALS AND METHODS

Chemicals. The following mono-unsaturated fatty acids were used: (i) oleic acid = cis-C\(_{18}\), melting point (MP) = 13.4°C; (ii) palmitelaidic acid = trans-C\(_{16}\); MP = 38°C; (iii) elaic acid = trans-C\(_{18}\); MP = 44°C, all from Analab, Inc., North Haven, Conn.

Triton X-100 and thiamine-hydrochloride were obtained from Eastman Kodak, Rochester, N.Y.

Preparation of chemicals. Oleic, palmitelaidic, and elaic acids were prepared separately as a 5% solution in 95% ethanol and stored at 4°C; Triton X-
1564 BAYER, DOLACK, AND HOUSER

solution; basic (F.W. glucose (Baker); (Difco); 100 and stock ml

FIG. 1. E. coli K1060 freeze-etched after growth at
42°C in palmitelaidic acid (PLM). The inner (proto-
plasmic) membrane fracture plane (IMF) shows IMF
randomly distributed and a few areas lacking the
IMP. Convex aspect (~). White arrow at corner of
each micrograph shows direction of metal evapora-
tion. Bar in each micrograph represents 0.2 μm.

100 was stored as a 20% solution in 95% ethanol, and
thiamine-hydrochloride (1 μg/ml) was stored as an
aqueous solution; both were sterilized by filtration
and refrigerated until used.

Growth media. Nutrient medium with the follow-
ing composition (grams/liter) was prepared: 10.0 g of
tryptone (Difco); 5.0 g of yeast extract (Difco); 1.0 g
of glucose (Baker); 5.42 g of sodium phosphate mono-
basic (F.W. 138); and 21.86 g of sodium phosphate
dibasic (F.W. 358.1). The medium was autoclaved
and then supplemented with 0.1 g of fatty acid (2.0
ml of stock solution), 2.0 g of Triton X-100 (10.0 ml of
stock solution), and 1.0 mg of thiamine-hydrochlo-
ride (1.0 ml of stock solution). For plate cultures,
1.5% agar was added to the medium.

Cultures. E. coli K1060 was obtained from P.
Overath, Max Planck Institute for Biology, Tübingen,
West Germany, and from H. Goldfine, University
of Pennsylvania Medical School, Philadelphia,
and was grown in 25 ml of nutrient media supple-
mented with a particular fatty acid, inoculated from
an overnight plate culture, and incubated in a preci-
sion water bath for 4 to 5 h in culture tubes aerated
with prewarmed air at temperatures of either 41 or
42°C (for palmitelaidic or elaidic acid) and 37°C (for
oleic acid). During this period, cell densities of ap-
proximately 2.0 × 10^8 colony-forming units/ml were
obtained.

The average generation time for E. coli K1060 at
the elevated temperatures was about 30 min.

Lipid phase transition experiments. Tempera-
ture-shift experiments in the range of the lipid
phase transition temperatures (Tc) were divided
into four groups as follows:

Group I (control). Cells were grown at 37, 41, or
42°C until a turbidity (Klett-Summerson) corre-
responding to 2 × 10^6/ml was reached. The culture was
sedimented without change in temperature at 4,000
× g for 15 min. Again, without changes in tempera-
ture, the pellet was prepared on a gold carrier and
then quickly frozen in liquid Freon 22. The transfer
of the carrier from the elevated temperature to the
Freon took less than 1 s.

Group II. Cultures were grown as in group I, but
shifted down to either room temperature (25°C) or
30°C, kept at that temperature for 30 min or less,
and then sedimented, placed on gold carriers, and
quick-frozen in Freon.

Group III. Cultures were grown as for group I, treat-
as for group II (shifted down to room tempera-
ture for 30 min, sedimented, placed on grids), and
then shifted up to 40°C and frozen. In this group of
experiments we used a brass rod (2.0-cm diameter)
immersed into a precision water bath and protrud-
ing approximately 1.0 cm above the water level. For
the temperature shift-up, the gold carrier was
quickly placed on the flat surface of the rod; after
0.5, 1, 2, 4, or 8 min, the specimen was removed with
prewarmed forceps and immediately frozen in liquid
nitrogen.

The speed by which melting or crystallization of a
substance occurs after transfer of the gold carrier
was tested by observing (with a ×10 telescope) the
crystals of ethyl-m-nitrobenzoate (Eastman), a sub-
stance with a melting point of 40 to 41°C. Melting of
crystals in the hollow gold carrier took 10 to 15 s
after transfer from 22 to 42°C. Crystallization
started (at the rims of the hollow gold carrier) 11 s
after shift-down from 50 to 22°C.

Group IV. Cells were grown as for group I, fixed
at the same temperature in 1% buffered glutaralde-
hyde of pH 6.8 for 15 min, pelleted, placed on gold
carriers, and frozen.

Group V. Cells of group IV (after fixation in glu-
taraldehyde) were shifted down for 30 min to room
temperature, sedimented, put on gold carriers, and
frozen.

Group VI. Cells of group II (grown at elevated
Fig. 2. *E. coli* K1060 freeze-etched after growth at 42°C in PLM and shifted down to 22°C for 30 s. Note the aggregation of IMP and the abundance of particle-free areas. In one of these, regularly spaced ridges have developed (black arrow).

Fig. 3. Cell grown in presence of elaidic acid at 42°C; 30 min after shift-down to 22°C, the IMF (convex) shows aggregated IMP and two sets of parallel ridges, intercepting each other at about 90°. OMF, Outer-membrane fracture plane. Here the OMF plane is visible in very small segments only (see arrow).

The frozen specimens were freeze-cleaved at -100°C, etched for 1 to 3 min, and shadowed with platinum/carbon in a Balzers 360 M (Balzers, Fürstentum Liechtenstein). The replicas were obtained by standard procedures. The replicas were studied in a Siemens 101 electron microscope at ×40,000 to ×80,000 magnification. The micrographs were printed from contact...
transparencies, so that in the final prints the areas of metal shadow appear black.

RESULTS

Structure of the inner membrane. E. coli K1060 grown at high temperatures and quickly frozen at −180°C (group I) revealed the typical intramembranous particles (IMP) in the convex freeze-fracture face of the inner membrane. These particles were mostly randomly distributed. However, in this cell strain we were unable to entirely prevent the formation of a few large particle-free patches (Fig. 1), which indicate localized phase separation. This occurred especially in cells supplemented with elaidic acid, despite the short time of <1 s needed for transfer of the specimen from 42°C to liquid nitrogen. The patches devoid of IMP increased in size and number after shift-down of the cultures below the transition temperature (T1) (group II). The inner-membrane fracture plane of a cell after shift-down is shown in Fig. 2 at 30 s and in Fig. 3 and 4 at 30 min. Concomitant with the growth of the particle-free patches, the typical IMP were increasingly condensed and aggregated into small domains (compare Fig. 2 with Fig. 3 and 4).

Glutaraldehyde fixation at 42°C for 15 to 60 min (group IV) showed a random arrangement of IMP, which remained unchanged after shift-down below the transition temperature (group V) (Fig. 5). When the cells were fixed at temperatures below T1 and subsequently shifted up to temperatures above T1 (group VI), the IMP clustering was irreversible. In contrast, in unfixed preparations a dispersal of the particles became obvious within 3 min at elevated temperatures (group III) (Fig. 6).

Close inspection of the particle-free areas in K1060 cells of group II revealed a series of repeating structures consisting of equally spaced parallel ridges (Fig. 7). The distance between the crests of the ridges was 11.8 ± 1.2 nm. Each ridge seemed to be composed of spherical particles of 4-nm diameter which protruded from the convex fracture plane of the inner membrane. The parallel arrays of ridges were often seen to be intercepted at about 90° by another set of ridges (Fig. 3 and 4), giving the membrane a crystalline aspect with fourfold symmetry. In contrast, at the cell poles, we observed that in general the relative orientation of the arrays was no longer normal to each other, but showed a wide variation in the angles of interception. In addition, there were frequently smooth zones scattered between the regular arrays (Fig. 4).

The shortest time required for crystalline zones to become obvious in the group II experiments was 30 s after shift-down (Fig. 2, arrow).
On the other hand, raising of the temperature above $T_c$ for 15 s (group III) caused almost all of the regular arrays to disappear; occasionally a few linear aggregates were visible even 3 min after shift-up. The ordered distribution of ridges remained in group VI, in which the cells were fixed at 22°C in glutaraldehyde and the temperature was subsequently raised above $T_c$; however, in some cells it appeared as if only smaller ordered domains were preserved (Fig. 8, arrows).

The concave fracture plane of the inner membrane, complementary to the convex plane, also showed the regular arrays of lines, with a repeat spacing of 12.0 ± 0.4 nm (Fig. 9). The pattern was indistinguishable from that of the arrays at the convex fracture plane; the lines appeared to be smoother, but their particulate composition was still discernible.

**Outer membrane and its fracture plane.** The cell surface of freeze-etched *E. coli* K1060 appeared to be indistinguishable from that of other *E. coli* and Salmonella strains. Glutaraldehyde fixation caused an increased waviness of the cell contour (Fig. 5). The fracture plane of the outer membrane with its convex and concave aspect revealed the following structures (Fig. 10). The convex fracture formed large plateaus. Their smooth surface was studded with spherical and filamentous particles, arranged in

![Image](http://jb.asm.org)
random distribution and with relatively low density per unit area. These plateaus were preferentially located over those areas at which the IMP of the inner membrane had aggregated; as shown in Fig. 10, the areas of IMP aggregates extended beyond the borders of the plateaus of the outer-membrane material.

At temperatures above $T_c$, the concave face of the outer-membrane fracture plane was observed as densely studded with particles in random arrangement; this result is in good agreement with previous work on *E. coli* and other gram-negative organisms (12, 29). In the cells cooled below the transition point, small particle-free domains in random distribution were observed in the concave fracture plane (Fig. 11). They appeared to be without any obvious substructure or symmetrical pattern. In some areas of this cleavage plane, an ordered ("crystalline") arrangement of the outer-membrane particles was detected (Fig. 12; the thin white arrows indicate one orientation axis). The rows formed by the particles in these areas seemed to intercept each other at approximately right angles, suggesting a fourfold symmetry of packing.

**DISCUSSION**

Cooling of the fatty-acid auxotroph *E. coli* K1060 below the fatty-acid transition temperature caused a number of changes in both membranes of the cell envelope to occur.

(i) IMP of the inner membrane aggregate while large particle-free patches are formed. This structural change was reported previously by a number of laboratories in a variety of microorganisms (2, 8, 10, 12, 24, 26, 28, 30) and

---

**Fig. 6.** Cell grown in PLM at 42°C, shifted to 31°C for 30 min, and shifted up to 41°C for 3 min. A loosening or disaggregation of IMP areas is seen: small knob-like structures are visible in random orientation, and a regular array of ridges is missing.

**Fig. 7.** Inner-membrane fracture plane (convex) of an unfixed cell after growth in PLM and 30 min after shift-down to 31°C. Note the repeating pattern of ridges. The ridges seem to be composed of spherical elements.
was interpreted as being caused by a migration of lipids into domains (crystals) and a concomitant movement of membrane proteins during the formation of the gel state within the membrane (11, 30, 31). Provided that the particles which aggregate extend into both lipid monolayers of the bilayered lipid membrane, the phenomenon of particle movement could occur in asymmetric membranes as well (9), in which only one of the bilayers responds to the liquid/gel transition. Thus, although the particles are moved by the crystallization process in one membrane monolayer, their distribution can be visualized in the freeze-fracture of the whole membrane. Particle clustering takes place rather rapidly; it can be demonstrated within 15 s after shift-down. With increasing time at temperatures below the transition range, the particle-free areas increase in size. It has been shown by Haest et al. (8) that particle aggregation is enhanced with lowering of the temperature rather far beyond the point of lipid phase transition, as determined by optical techniques and electron spin resonance methods (22). Our freeze-etch data confirm this result and suggest that over a relatively wide cooling range increased effects on particle aggregation can be observed. Since in most of our experiments the pH was kept at 6.8 and the tonicity as well as the concentration of divalent cations and proteins remained unchanged, additional effects of these parameters on particle distribution and transition temperature (4–6) could be avoided. The recognizable redistribution of the aggregated particles after temperature shift-up requires the comparatively long time span of 3 min, whereas other membrane structures seem to respond to the elevated temperature considerably faster. This slow dispersal might be caused by attracting forces between the clustered particles. Fixation by glutaraldehyde at either above or below the phase transition range prevented the particles from responding to subsequent temperature changes. The random distribution of IMP observed in cells fixed above the phase transition range seems to represent the particle distribution within the living membrane of K1060 (8, 12); in unfixed E. coli strain K1060, substituted with elaidic acid, we were unable to observe an inner membrane entirely devoid of particle-free patches, in spite of the use of fast cooling methods such as spray guns (1). We attribute this to an extremely fast initiation of phase transition processes; on the other hand, one might consider the possibility that a small number of the particle-free areas occur normally in cells. If this is so, fixation might cross-link otherwise mobile IMP to form an apparently even distribution of particles. The occurrence of filamentous IMP in the fracture plane of inner and outer membranes has been discussed before (3, 13). These filamentous shapes have been attributed to plastic deformation; also, charge effects have been considered responsible for the filamentous shapes (7, 33).

(iii) The broad areas from which the typical IMP have been “pushed aside” by cooling of the cell are considered to have a more or less smooth surface (8, 10, 18). However, we observed regularly spaced lines in these areas that were often intercepted at angles of approximately 90° by similar sets of lines. These lines were composed of particulate elements. The nature and composition of these particles is unknown. They might be proteins, as is assumed for the typical IMP (4, 20). The presence of proteins in the “particle-free” patches of the

![Image](http://jb.asm.org/)

**FIG. 8.** IMF of a cell grown at 42°C in presence of elaidic acid, shifted down to 22°C, fixed in glutaraldehyde for 30 min at 22°C, shifted up to 42°C for 2 min, and freeze-etched. Note the localized "crystalline" arrays (black arrows).
inner membrane has been shown by Heerikhuizen et al. (9), who also suggest that some of the proteins become concentrated within this low density "crystalline" subfraction of the membrane. Although the particles of the ridges might be of proteinaceous origin, the repeat structure or crystalline array is reminiscent of patterns observed in liposomes made with known lipid species (31). Liposomes below the transition temperature revealed characteristic band patterns after freeze-fracturing. Their corresponding solid phase has been detected by X-ray diffraction and was described as pβ'. (27, 32). Intermolecularly mixed liposomes showed co-crystallization of their component molecules when measured by DSC. In freeze-etchings they exhibited a band pattern different from that of their pure lipid components (32). In contrast, the mixture of two different phosphatidylcholine species showed two thermotrophic peaks in DSC, with the typical band patterns of the two components becoming visible below each of the melting points. At present, we are unable to discern with freeze-etching between the two possibilities in the K1060 membrane. It should also be noted that the band patterns of fractured vesicle membranes showed no sign of a particulate composition. We tentatively interpret the nature of the crystalline areas in the K1060 cells as a composite of lipid band patterns, with proteins as particulate elements extruded within the ridges of the individual lines.

The reason for the general occurrence of the interception angles of 90° between the ridge patterns remains undetermined. This type of interception is surprising in view of hexagonal phases found in freeze-fractured lipid-water systems (7). It is interesting that the angles of interception may vary widely, mostly toward the poles of the cells, and that in such zones, domains of ordered structures change with those of smooth areas. This again reminds one of the crystallization of mixed lipid vesicles frozen from temperatures between the transition range of their two components (7). In this connection we would also like to report the observation that along the cylindrical parts of the cell, the direction of one set of the ridges was usually similar between cells and was slightly tilted relative to the long axis of the cells. We have seen this apparent directional preference of the "crystal" ridges in the cells of numerous different experiments. The reason for this might be "fixed" zones in the envelope from which crystallization starts.

(iii) Regular arrays of particles were observed in the fracture plane of the outer membrane. The obvious lack of a morphologically well-expressed crystallinity in this membrane might be a reflection of a diminished ability for crystallization of its components (16). We found a multitude of small, particle-free areas in the outer membrane after cooling, in good agreement with the results of other laboratories (12,
The ordered arrangement of the particles of the outer-membrane fracture face, however, seems to suggest either a crystallization or a particle aggregation, similar to that of the inner-membrane particles, with a preference for a fourfold symmetry. We did not find an unambiguous hexagonal pattern arrangement in any of the fracture planes of the K1060 envelope.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant AI-10414-03 from the National Institute of Allergy and Infectious Diseases and National Science Foundation grant PCM73-01121 to M.E.B., National Institutes of Health grants CA-06927-13S2 (from the National Cancer Institute) and RR-05539-14 (from the Division of Research Resources) to The Institute for Cancer Research, and also by an Appropriation from the Commonwealth of Pennsylvania.

We wish to express our gratitude to P. Overath and H. Goldfine for supplying us with the cell strains. We also...
1572 BAYER, DOLACK, AND Houser

acknowledge the reliable work of Barbara Hulme and Roberta Ridley.

LITERATURE CITED


Fig. 12. OMF, concave face, of cells grown in PLM, shifted from 42 to 25°C for 30 min. The IMP are arranged along the two axes, as pointed out by the thin double arrows.
LIPID PHASE TRANSITION IN E. COLI


