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Cell Envelope and Shape of *Escherichia coli* K12

(unit membrane/long-range order/envelope proteins/murein)

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ABSTRACT **Rod-shaped "ghosts" that are free of murein have been isolated from *E. coli*. The shape of these "ghosts" is maintained by a unit membrane soluble in sodium dodecyl sulfate. Ghosts consist of about 20–30% phospholipid (almost exclusively phosphatidylethanolamine) and 50–60% protein; a large fraction of the remaining material is lipopolysaccharide. Sodium dodecyl sulfate–gel electrophoresis reveals 4–5 different bands corresponding to molecular weights between 10,000 and 40,000. Treatment of ghosts with Pronase reduces this number to 3, and the rod shape still is not lost. Results of treatment of ghosts with a crude extract from *Dictyostelium discoideum* have supplied tentative evidence that at least one of these proteins is involved in the maintenance of rod shape. It does not appear too unlikely that these polypeptide chains are the final products of the genetic information specifying cellular shape.**

E. coli is a rod-shaped organism and, as for all other bacteria, it is not known how this shape is determined, i.e., how genetic information is translated into cellular morphology. The expression of specificity for a certain morphology must, of course, involve assembly of the cell envelope. The *E. coli* cell envelope consists of three layers: cytoplasmic membrane, outer membrane or cell wall, and in between the two, the murein or peptidoglycan (1–3). The murein can be isolated intact and the isolated layer, the "sacculus," retains the shape and dimensions of the cell from which it was made (4).

However, the specificity we are looking for cannot reside solely in the sacculus or in its precursors. This covalently closed net is not a self-assembly system. Thus, the information required to produce sacculi of a certain shape can only involve the relevant biosynthetic enzymes *in connection with a matrix* onto which murein is laid down.

In this communication, we report purification and some properties of a membrane from *E. coli* K12 that is a good candidate for such a matrix.

MATERIALS AND METHODS

Cells, Media, and Growth Conditions. Strain W945-T3282 (subsequently called 3282), which among other markers (5) carries diaminopimelate and lysine auxotrophies, was grown at 30° in Antibiotic Medium no. 3 (Difco) under aerobic conditions. The medium was supplemented with diaminopimelate (20 μg/ml) and thymine (50 μg/ml).

Isolation of Ghosts. All operations were performed at 20–25°, and all solutions contained 8 mM $MgSO_4$.

Procedure I: Cells grown to 5 to 8 × 10^8/ml were collected by centrifugation, washed once with 0.85% NaCl, and suspended, per g wet weight, in 10 ml of 20 mM Tris·HCl (pH 7.5) containing 40% sucrose. Lysozyme was added (150 μg/ml) and was allowed to act until >90% of the cells lysed upon dilution into water (about 2 hr). About 0.1 volume of chloroform was added; the suspension was shaken vigorously and then left overnight. Viscosity was broken by forcing the suspension through a hypodermic needle. The cells were centrifuged (10 min, 16,000 × *g*) and resuspended in the same volume of 20 mM Tris·HCl (pH 9). Chloroform was removed by strong aeration at 37°, and trypsin was added (500 μg/ml). After 2 hr, cells were centrifuged for 10 min at 16,000 × *g* and resuspended in 0.2 volume of 4 M urea. After 2 hr, ghosts were recovered by centrifugation at 16,000 × *g* for 10 min.

Procedure II: Cells were suspended at 100 mg wet weight/ml in 20 mM Tris·HCl (pH 7.5) containing 40% sucrose and 1% Triton X-100 or Brij 58 and left overnight. Viscosity was not a problem and the cells, recovered by centrifugation as above, were resuspended in 0.5 volume of 4 M urea. After 2 hr, they were centrifuged, washed twice with 20 mM Tris·HCl (pH 9), and resuspended in 0.25 volume of 20 mM Tris·HCl (pH 9). Trypsin was added (1 mg/ml). After 2 hr, cells were centrifuged, washed once with 8 mM $MgSO_4$, and resuspended in 0.2 volume of 50 mM ammonium acetate containing 65 mM NaCl. Lysozyme was added (200 μg/ml) and allowed to act for at least 4 hr. Ghosts were centrifuged (10 min, 16,000 × *g*), resuspended in the same volume of 4 M urea, and after about 30 min, centrifuged, washed once with 8 mM $MgSO_4$, once with water, resuspended in water, and usually lyophilized.

Subsequent Treatment of Ghosts. Pronase (200 μg/ml) digestion was performed with ghosts suspended in 25 mM Tris·HCl (pH 7.5), and incubation was for 12 hr at 22°. *Dictyostelium discoideum* treatment was done with ghosts in 50 mM sodium citrate (pH 3.5) (6) for 6 hr at 22°. A crude extract from axenically grown *D. discoideum* was adjusted to pH 3.5. Insoluble material was removed by centrifugation, and 2 mg of protein of the supernatant was added per ml of ghost suspension (about 10^9 particles per ml). *Phospholipid* was extracted (7) from dry ghosts and analyzed (8) as described (9). *Murein* content was analyzed chemically (10) and by incorporation of [^{3}H]diaminopimelate under conditions described (5).

Electrophoresis. The gel was 10% acrylamide–0.133% methylene bisacrylamide in 200 mM sodium phosphate (pH 7). Electrophoresis buffer was 20 mM sodium phosphate (pH 7). Samples were dissolved in 10 mM sodium phosphate (pH 6.9) containing 1% Na dodecyl sulfate and 100 mM mercaptoethanol. The sample was kept for 5 min at 100° (longer

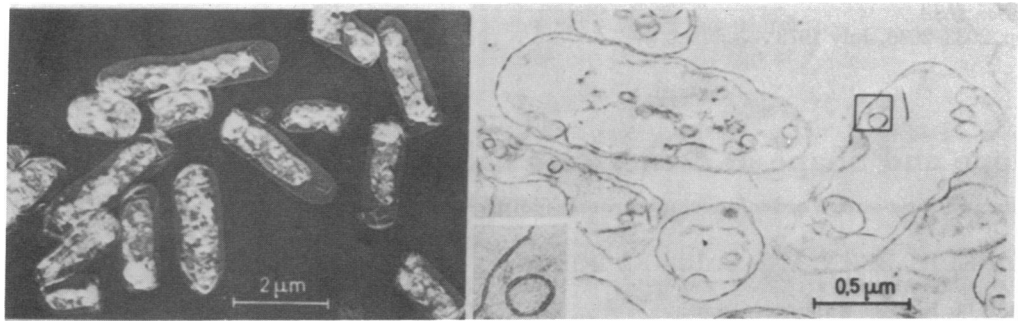

FIG. 1. *Shadowed and sectioned ghosts.* The *inset* represents a 2.5-fold further magnification.

boiling had no effect); the gel contained 0.2% and the buffer 0.08% Na dodecyl sulfate (higher Na dodecyl sulfate concentrations, up to 1% in the gel, had no effect). Staining was done with Coomassie Brilliant Blue (11).

Electron Microscopy was performed with a Philips EM 201 microscope, by described methods (9).

RESULTS

Isolation of rod-shaped membranes

Our strategy was guided by an earlier report (12) showing that treatment of plasmolyzed *E. coli* with lysozyme results in cells that, although they are osmotically sensitive, retain their rod shape. When the plasmolyzing sucrose is diluted, such cells round up into spheres. It appeared to us that sphere formation might have its basis in osmotic pressure or surface tension phenomena that could be avoided if the cytoplasmic membrane were disrupted or made leaky. The resulting "ghosts," if they remained rod-shaped, could then be investigated further to determine precisely which components were required for maintenance of the rod shape.

The first step of purification procedure I (see *Methods*) indicated that these assumptions may be correct. Plasmolyzed HfrH rods were treated with lysozyme until >90% of the cells became spherical upon dilution into 12% sucrose or lysed upon dilution into water. Shaking of this cell suspension with chloroform produced rod-shaped cells that, upon dilution into water, did not change shape. Subsequent purification steps are the results of trial and error experiments having the aim to remove as much protein as possible from the preparations without loss of rod shape. The final preparation of ghosts contains about 50% rods plus a mixture of spheres and structures of indiscernible shape.

An alternative procedure was used to obtain ghost preparations with a greater percentage of rod-shaped forms. In procedure II, lysozyme is used as the last purification step; in addition, the cytoplasmic membrane is disrupted by Triton X-100 (13) (or, equally well, Brij 58) rather than chloroform. The resulting preparations consist almost entirely of rod-shaped ghosts. Since the two procedures differ in the manner in which the cytoplasmic membrane is destroyed and in the time at which murein is removed, a comparison of ghosts prepared by the two methods should reveal whether the treatment has any effect on ghost composition.

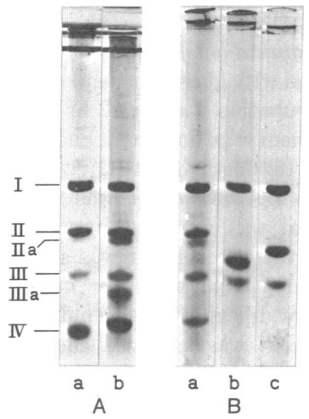

FIG. 2. *Na dodecyl sulfate–gel electrophoreses of ghosts* (*A*) Extremes of variations in band patterns obtained from different ghost preparations (*a*, purification method I; *b*, method II; the relatively small amount of band III in *a* is not typical for method I nor is the amount of band IIa typical for method II). (*B*) *a*, ghost preparation before; *b*, after treatment with Pronase; *c*, after treatment with *D. discoideum*.

General properties of ghosts and electron microscopic appearance

As observed by light microscopy, rod shape of the ghosts is resistant to trypsin, chymotrypsin, Pronase, EDTA (5 mM), Triton X-100, or Brij 58 (both 1%). Ghosts are soluble in 1% Na dodecyl sulfate and partially soluble (the suspension remains turbid but ghosts are not longer visible) in Brij 58 or Triton X-100 plus EDTA.

Fig. 1 shows that ghosts consist of an outer membrane that encloses additional, mostly vesicular, membranous material. The morphology of the inner membranous structure was found to be rather variable. In some cases it consisted only of small vesicles, while in others its appearance approached that of a continuous membrane. Obviously, this material has nothing to do with the shape of the ghosts.

Overall composition of ghosts

The only difference found between ghosts obtained by procedure I and procedure II was the amount of material extracted by chloroform–methanol. Ghosts obtained by procedure I lose 25–30% of their dry weight when extracted this way. The phospholipid is almost exclusively phosphatidylethanolamine; very little phosphatidylglycerol and only traces of cardiolipin are present. Ghosts obtained by procedure II lose 30–40% of their dry weight upon extraction. It is very likely that part of this material is Triton X-100 because the chloroform–methanol extract exhibits a strong adsorption at 280 nm and the phospholipid composition does not differ from that described for ghosts prepared by procedure I.

The residue after chloroform–methanol extraction con-

tains about 70% protein (biuret) and not precisely known quantity of lipopolysaccharide. Lipopolysaccharide was determined with Osborn's modification (14) of the ketodeoxyoctonate assay (15), and perhaps because of interference with some capsular material the values found were much too high when related to protein and phospholipid content of dry ghosts. Application of the phenol–water procedure (16) to two different phospholipid-extracted ghost preparations showed that 10 and 15% of the dry weight was recovered as lipopolysaccharide, but that separation of ketodeoxyoctonate-positive material from the protein residue was not quantitative. We estimate that at least 50% of the nonprotein and nonphospholipid material is lipopolysaccharide. We do not know whether other carbohydrate material is present.

Murein

The rod-shaped ghosts obtained by either procedure do not contain significant amounts of murein. Three different ghost preparations (procedure I) were used to measure muramic acid by aminoacid analysis of acid hydrolyzates. Muramic acid was not detectable. Experiments in which murein was labeled *in vivo* likewise indicated that very little murein is present in ghosts. Whole envelopes (17) and ghosts were prepared from strain 3282 grown in the presence of [^{3}H]diaminopimelate, and the amount of radioactivity in the two fractions was compared. Ghosts obtained by procedure I contained, per mg dry weight, 1% of the radioactivity present in the corresponding envelope; with procedure II, this value was 1.5%. In view of the fact that the *E. coli* murein most likely is a monolayer (Braun, Henning, Gnirke, & Rehn, *J. Bacteriol.*, in press), the small amount of radioactivity remaining in ghosts cannot possibly represent intact murein sacculi. Thus, it is clear that *a membrane without murein can possess rod shape.*

Protein

Preliminary Characterization. The protein was analyzed by Na dodecyl sulfate–polyacrylamide gel electrophoresis. The band pattern obtained (Fig. 2*A*) reproducibly shows four bands (apparent molecular weight): I (40,000), II (28,000), III (18,000), and IV (8000–10,000). Two more bands, IIa and IIIa, have been found in varying quantities, and in several preparations both of them were absent. It is likely that IIIa is lysozyme; it is not separable from lysozyme and is usually found in barely detectable quantities in ghost preparations obtained by procedure I, although it is always present in preparations obtained by procedure II (lysozyme used last).

It is very likely that band IV is the lipoprotein containing palmitic acid described in detail by Braun and collaborators (10, 18). In addition to exhibiting the same molecular weight as this protein, it appears to contain palmitic acid. Ghosts were prepared from cells grown in the presence of [^{14}C]-palmitate, lyophilized, extracted extensively with chloroform–methanol, and subjected to electrophoresis; upon autoradiography of the gels, radioactivity was found exclusively in band IV. In thin-layer chromatographs (19) of alkaline hydrolysates of extracted ghosts, about 70% of the radioactivity was found at the position of palmitate; thus this amount of radioactivity associated with band IV most likely represents undegraded palmitate. It may be mentioned that this experiment shows that there is no cross contamination of the other bands by the palmitic-containing protein.

Relation to Rod Shape. In experiments aimed at removal of still more material from the ghosts without destruction of rod shape, we found that Pronase destroys proteins of bands IIIa and IV, partially degrades protein(s) II, has no effect on bands I and III (Fig. 2*B*), and does not alter the shape of the ghosts. Ghosts were also exposed to a crude extract of *D. discoideum*. The extract also destroyed proteins IIIa and IV, did not alter bands I and III, and partially degraded protein(s) II; however, the fragment of protein(s) II exhibited a larger molecular weight than the Pronase fragment (Fig. 2*B*) and the ghosts became spherical.

D. discoideum contains various hydrolytic enzymes (6), and it is not clear whether loss of rod shape and fragmentation of protein(s) II are causally related. If so, one would have to assume that Pronase fragmentation is, while *D. discoideum* fragmentation is not, tolerable for the maintenance of rod shape. Therefore, Pronase-treated ghosts were subjected to digestion by the *D. discoideum* extract. Although they did not become spherical, the rods were fragmented; the pattern of protein bands was not different from that obtained after Pronase treatment alone. The experiments tend to suggest that, among other parameters, a certain conformation of protein II (or perhaps of a protein present in band II) is involved in the maintenance of rod shape while proteins in bands IIIa and IV are not.

Homogeneity. The Pronase and *D. discoideum* experiments suggest that band II is a homogeneous polypeptide chain because the relative staining intensity of the two fragments is not measurably different from band II. The following experiments tend to argue against gross heterogeneity of all other bands. Performic acid oxidation of ghosts does not change the band pattern, although this treatment—and to our experience to date only this treatment—makes the proteins of bands I, II, and III susceptible to trypsin digestion. Gel electrophoresis under conditions of method 2 of Bragg and Hou (20) also does not change the band pattern; this method is superior in resolving power to the one we have used. During the preparation of this manuscript, a paper appeared (21) describing other powerful separation procedures; we have not yet tested them.

Artifacts. Since trypsin treatment is an essential step in the purification procedure, we may be looking only at fragments of polypeptides normally found in the membrane. Such degradation products could also arise from the action of cellular proteases.

Degradation by cellular proteases cannot be excluded. The following experiment indicates that at least bands I and IV are not grossly fragmented trypsin products. Ghosts were prepared by procedure I, omitting the trypsin digestion. Although many more bands were visible in Na dodecyl sulfate gels than those shown in Fig. 2*A*, bands I and IV were clearly predominant. Unfortunately, we could not determine the effect of trypsin on bands II and III because at these positions an unresolvable number of bands was present.

Localization. Ghosts consist of an outer membrane and, to differing extents, of inner membranous material. Preliminary evidence indicates that all the proteins reside in the outer membrane. Strain 3282 was carried through the separation procedure of Osborn *et al.* (14). H-band (outer membrane) and L_1-band (cytoplasmic membrane) were then treated according to procedure II. All material from L_1 was solubi-

lized, while most of H could be recovered in insoluble form. The electrophoretic band pattern of this insoluble fraction was indistinguishable from that shown in Fig. 2*A*.

DISCUSSION

Proteins of the Outer Membrane. Chemical data are not available for most of the proteins of the *E. coli* cell envelope. It therefore does not seem worthwhile at this point to attempt a detailed comparison between our Na dodecyl sulfate–gel bands and those observed by other workers with envelope fractions of *E. coli* or *S. typhimurium* enriched for the outer membrane (13, 14, 20). In general, the patterns observed exhibit more bands than ours, but otherwise are fairly similar.

Ghosts and Membrane Structure. The observations made with the ghosts are to some extent inconsistent with at least one of the present concepts on the general structure of biological membranes (see, e.g., ref. 22). Supposedly in most membranes specific structural proteins do not exist and there is no long-range order. We have isolated a rod-shaped membrane soluble in Na dodecyl sulfate. Thus, there is noncovalent long-range order, and it is difficult to imagine that primarily anything other than the protein in the membrane would establish this order. Assuming the absence of gross heterogeneity of the polypeptides in each Na dodecyl sulfate–gel band, it would also be obvious that their arrangement would have to be one of repeating subunits. They, or at least one of them, should then be classified as structural proteins. Rejection or acceptance of these speculations awaits, of course, chemical evidence on the actual number of different polypeptide chains in ghosts.

It may be mentioned that we have purified morphologically identical ghosts from *Serratia marcescens*, *Proteus mirabilis*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*. Except for the last organism, the band patterns in Na dodecyl sulfate gels are similar or identical (*Salmonella*) to that of *E. coli*.

Ghosts and Cellular Morphology. We cannot answer our central question: Are the ghost membranes and, in particular, the polypeptide chains therein the final shape determinants of the cell? It certainly is very tempting to assume this, especially in the light of experiments of Brinton and associates with *Bacillus brevis* (23, 24). This organism has an outside protein layer (T-layer) that consists of identical polypeptide chains. Reassociation of the isolated protein can occur *in vitro* and, under certain circumstances, results in the formation of hollow cylinders (open at the ends) that can have the same diameter as the cells from which the protein was obtained. Although it is not known if this protein is involved in shape determination, it is quite clear that such "large containers" (25) can be self-assembly systems, and it appears possible that the ghost membrane (i.e., outer membrane of the cell envelope) is the product of such a shape-determining assembly system. It may be difficult to ascertain if this is so, however. Were we to find mutants defective in one of the ghost proteins and having an abnormal morphology, this still would not prove the case. Because of the (largely unknown) interdependence of many components of the cell envelope, it is extremely difficult, if not impossible, to draw direct causal conclusions. We feel that *in vitro* reconstitution studies have a greater chance to resolve the issue.

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