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Rapid Method for Isolation of Large Quantities of Outer Membrane from *Escherichia coli* K-12 and Its Application to the Study of Envelope Mutants

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A rapid method for the isolation of large quantities of bacterial outer membrane is described. This cell envelope component was removed from plasmolyzed cells of *Escherichia coli* K-12 by lysozyme-ethylenediaminetetraacetic acid treatment, aggregated by lowering the pH to 5.0, and recovered by centrifugation. Aggregates of membrane fragments were clearly identified in an electron microscope. A criterion of homogeneity of the preparation was obtained by isopycnic sucrose gradient centrifugation. A single band appeared at a density of 1.24 g/cc. The cytoplasmic membrane marker, succinate dehydrogenase activity, was 40 times lower in the outer membrane preparation than in complete cell envelope preparations. A rich activity was, however, found for the outer membrane marker, phospholipase A. The compositions of outer membranes from a transductant pair were compared. One transductant was a chain-forming, antibiotic-supersensitive *envA* strain, whereas the other contained the *envA*$^+$ allele. The *envA* strain showed a slightly modified protein pattern and a lower relative content of phosphatidylglycerol.

The cell envelope of *Escherichia coli* is both chemically and morphologically complex (for a recent review, see Freer and Salton [6]). Electron microscopy of thin-section preparations reveals the cell envelope as a multilayered structure, in which the most peripheral component is the triple-layered, roughly 7.5 to 9.0 nm thick outer membrane. This outer membrane is separated from the cytoplasmic membrane by the thin (~1.0 nm thick) murein layer (5). The outer membrane has been separated from the cytoplasmic membrane by isopycnic sucrose gradient centrifugation of lysed spheroplasts (13, 17) or by separation of wall (outer membrane plus murein) from cytoplasmic membrane by discontinuous sucrose gradient centrifugation of cell envelope fragments (19, 20). De Pamphilis and Adler (4) utilized extraction with Triton X-100 in the presence of Mg^{2+} to solubilize the cytoplasmic membrane from spheroplasts, thereby isolating an enriched outer membrane fraction.

Little is known about the function of the outer membrane. This may be partly due to the fact that few outer membrane mutations except those affecting the biosynthesis of the lipopolysaccharide carbohydrate chains have been described. It has been suggested that the outer membrane constitutes a penetration barrier, preventing large molecules from entering or leaving the cell (6, 8, 15). Isolation of antibiotic-supersensitive mutants could be a fruitful approach for detecting outer membrane mutations. For the screening of such mutants, we have developed a rapid method by which large quantities of reasonably pure outer membrane can be isolated. As one antibiotic-permeable chain-forming mutant containing the *envA* mutation has been isolated and studied in these laboratories (14-16), it was considered of interest to apply the present technique to this strain and its wild-type analogue.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The transductant pair, strains E64-113 (*envA*$^+$) and E64-120 (*envA*), has been described previously (14, 15). The strains are isogenic except for the *envA* mutation.

The complete medium used was the LB of Bertani (2) supplemented with medium E of Vogel and Bonner (22), thiamine, and 0.2% glucose. The Casamino Acids medium contained medium E, thiamine, 0.2% casein hydrolysate supplemented with L-tryptophan (50 μg/ml), and 0.2% glucose. The experiments

were carried out at 37 C. Growth was recorded as optical density reading by use of a Klett-Summerson colorimeter with a W66 filter.

Materials. Lysozyme, trypsin, L-leucine, *meso*-α,ϵ-diaminopimelic acid, pancreatic ribonuclease, and pancreatic deoxyribonuclease were obtained from Sigma Chemical Co., St. Louis, Mo. ^{32}P (orthophosphate, 94 mCi/mg of P), ^{3}H-galactose (300 mCi/mmol), ^{14}C-leucine (55 mCi/mmol), ^{3}H-glycerol (500 mCi/mmol), and ^{3}H-2,5-diaminopimelic acid (300 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., England.

Isolation of cell envelopes. For the preparation of cell envelopes, cells grown in Casamino Acids medium were harvested by centrifugation at 4 C in late logarithmic phase (about 6×10^8 cells/ml). The cells were washed once in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.8, suspended in the same Tris buffer, and broken up in a French press (4 C) or by using glass beads in a Sorvall Omnimixer (4 C). The suspension was centrifuged at 3,000 rpm ($1,100 \times g$) in a Sorvall GSA rotor for 10 min at 4 C. The supernatant fluid was centrifuged again as above. The envelopes were recovered from the supernatant fluid by centrifugation at 17,000 rpm ($35,000 \times g$) in a Sorvall SS34 rotor for 15 min.

Electron microscopy. Outer membrane was prepared as described in Results. The outer membrane fractions were fixed in ice-cold 4% (wt/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 2 h (18). Rinsing was carried out at 4 C in the same buffer for 1 h. Thereafter, specimens were postfixed for 1 h at 4 C in 1% (wt/vol) OsO_4 dissolved in 0.1 M phosphate buffer (pH 7.3) containing 0.2 M sucrose. After rinsing in distilled water, step-wise dehydration in ethanol (30, 70, 90, and 95% and absolute alcohol) was carried out at room temperature and embedding was performed in Epon 812 (10). Polymerization was carried out at 37 C overnight and for an additional period of 24 h at 60 C. Sections were cut on an LKB ultratome with glass knives. The sections were poststained with uranyl acetate and lead citrate and were examined in a Philips EM 300 electron microscope.

Lysozyme-ethylenediaminetetraacetic acid (EDTA)-treated, plasmolyzed cells of the strain E64-113 (*envA*$^+$) were also fixed and prepared for electron microscopy according to the above technique.

Isopycnic buoyant sucrose density gradient centrifugation and enzyme measurements. Isopycnic buoyant sucrose density gradient centrifugation was carried out according to Osborn et al. (17). Their techniques for determination of succinate dehydrogenase (EC 1.3.99.1) and phospholipase A (EC 3.1.1.4) plus lysophospholipase (EC 3.1.1.5) activities were also applied.

Solubilization and gel electrophoresis of membrane proteins. The procedure followed that of Maizel (12). The envelope and outer membrane fractions were dissolved in 2% sodium dodecyl sulfate (SDS) in 0.01 M sodium phosphate buffer (pH 7.2) containing 1% 2-mercaptoethanol. The final protein concentration was 5 mg/ml. The suspension was allowed to stand overnight at 22 C. The sample was then diluted to 1 mg of protein/ml in 0.01 M sodium phosphate buffer (pH 7.2), and glycerol was added to a concentration of 25% (vol/vol). A portion (100 μg of protein) was applied to each gel (10 cm long, 6 mm in diameter, 5% polyacrylamide). The electrophoresis buffer was 0.1 M phosphate (pH 7.2) containing 0.1% SDS. Electrophoresis was run for roughly 2 h at 15 mA/gel. The gels were fixed and stained by the method of Inouye and Guthrie (7).

Quantitative measurements of macromolecular content. Protein was measured according to the method of Lowry et al. (9). Lipids were extracted by chloroform-methanol (2:1, vol/vol) from lyophilized outer membrane fragments. The organic solvents were removed by evaporation. Lipopolysaccharide was extracted from lyophilized outer membrane preparations by the phenol-water method (23), dialyzed, and freeze-dried.

Extraction and analysis of phospholipids and fatty acids. Labeling of phospholipids was obtained by growing the cells for seven generations in 1,000 ml of LB medium containing ^{32}P orthophosphate, (1 μCi/ml). The outer membrane from late log phase cells was isolated as described in Results and extracted in 10 ml of chloroform-methanol (2:1, vol/vol) overnight. The analysis of the phospholipids was carried out according to Skipski et al. (21). The fatty acid composition of outer membranes was determined as previously described (15).

RESULTS

Isolation of "outer membrane" fragments. For the isolation of outer membranes, a 1,000-ml culture of cells grown in LB or Casamino Acids was harvested at late log phase by centrifugation (4 C) and resuspended to a volume of 200 ml in 0.05 M Tris buffer (pH 7.8) containing 1 mM EDTA. This suspension was treated in a Sorvall Omnimixer for 1 min at a speed control setting of 7.5. This procedure removes flagella, pili, and capsular material, and does not result in cell breakage (19). The cells were then centrifuged at 4 C and suspended in 10 ml of 30% sucrose in 0.05 M Tris buffer (pH 7.8). Lysozyme (20 mg) and 1 ml of 0.1 M EDTA were stirred into the suspension. After 10 min of incubation at 22 C, 1.0 ml of 0.2 M $MgCl_2$ and approximately 0.1 mg each of pancreatic ribonuclease and deoxyribonuclease were added. This treatment caused a 6 to 7% decrease in the optical density. The cell suspension was then centrifuged at 20,000 rpm ($48,000 \times g$) in a Sorvall SS34 rotor for 40 min to remove spheroplasts and ghosts. The pH of the supernatant fluid was lowered to 5.0 by the addition of HCl (0.1 M), after which a visible aggregation occurred. The precipitated material was centrifuged at 12,000 rpm ($35,000 \times g$) for 15 min. The pellet was washed twice in 5 ml of ice-cold distilled water and centrifuged again. After lysozyme-EDTA treatment, 40% of re-

leased radioactivity (labeled proteins, phospholipids, lipopolysaccharides, and murein) was recovered. The particulate outer membrane fraction amounted to 6% of the total cellular dry weight.

Characterization of the "outer membrane" fraction. The effect of lysozyme-EDTA treatment of plasmolyzed E64-113 cells is depicted in Fig. 1. Fragments of the outer components of the cell envelope are peeled off from the cell surface and frequently rolled up to form circular structures. It is also evident that in certain locations outer membrane fragments are not detached but adhere closely to the inner components of the cell envelope.

Electron microscopy of the outer membrane material isolated by the method described above reveals an abundance of aggregates of short membrane fragments and occasional membrane-enclosed spherical structures (Fig. 2). The membrane fragments are triple-layered; the electron-dense layers measure 2.5 to 3.0 nm in thickness whereas the intermediate electron-lucid layer is roughly 2.0 to 2.5 nm thick.

As it has been reported (13, 17, 19) that the outer membrane exhibits a higher buoyant density than the cytoplasmic membrane, our particulate fraction was subjected to isopycnic buoyant sucrose density centrifugation (17). When the material was labeled with respect to the proteins, phospholipids, or the lipopolysaccharides (Fig. 3a–c), only one density band (outer membrane band; ρ = 1.24 g/cc) could be detected after centrifugation to equilibrium. However, when the particulate fraction was labeled with respect to the murein (^{3}H-diaminopimelic acid), about 40% remained in the top fractions (Fig. 3d). Roughly 40% of the diaminopimelic acid-labeled murein in the outer membrane fraction could be removed by trypsin digestion. For the sake of comparison, the cell envelope membranes of spheroplasts from E64-113 were separated according to the method of Osborn et al. (17). As can be seen in Fig. 3e, our outer membrane band showed approximately the same density as the corresponding so-called H band of Osborn et al. (17).

Our outer membrane fraction was assayed for succinate dehydrogenase, a known constituent of the cytoplasmic membrane, as well as for phospholipases known to be present in the outer membrane (17). Our outer membrane fraction

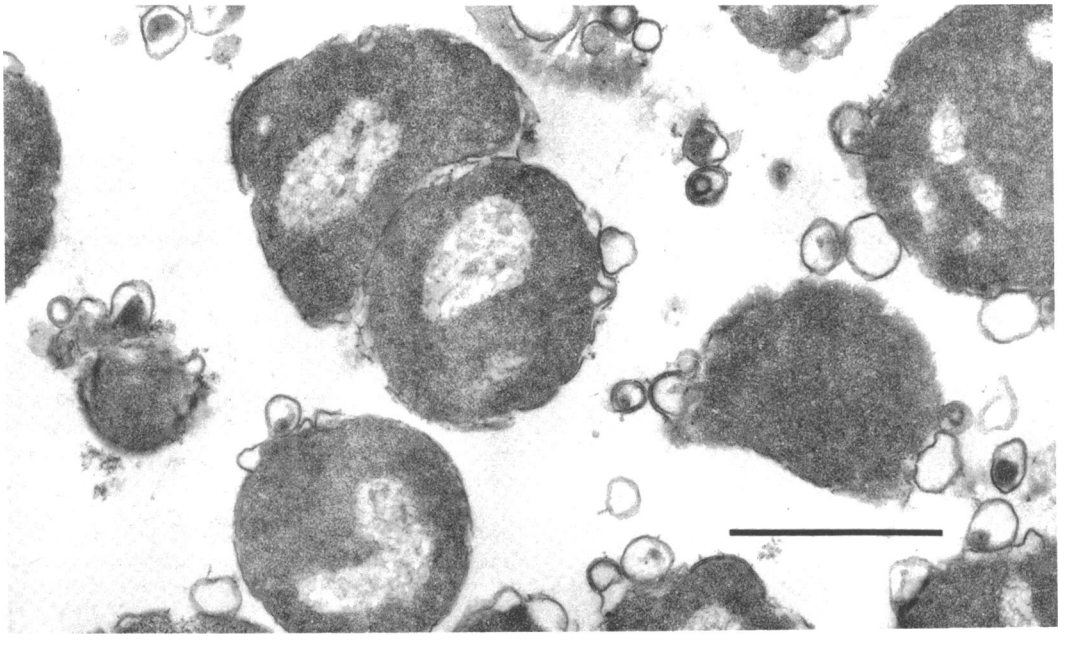

FIG. 1. *Survey electron micrograph of lysozyme-EDTA-treated plasmolyzed E64-113 cells. A 10-ml culture of cells growing in LB medium was harvested by centrifugation at late logarithmic phase. The cells were suspended in 1 ml of 30% sucrose in Tris buffer (pH 7.8). Lysozyme (final concentration, 100 μg/ml) and EDTA (final concentration, 1 mM) were added. After 10 min of incubation at 22 C, the spheroplasts were spun down at 2,000 rpm (4 C). Processing for electron microscopy was as described in Materials and Methods. Spheroplasts and cytoplasmic constituents are retained. Note the "peeling off" of outer membrane segments which frequently roll up into spherical structures. Marker bar denotes 1 μm.*

FIG. 2. *Sediment of outer membrane preparation from the E64-113 E. coli strain. Fragments of triple-layered outer membrane are clearly seen as sectioned vesicular formations surrounded by outer membrane segments. Marker bar denotes 0.1 μm.*

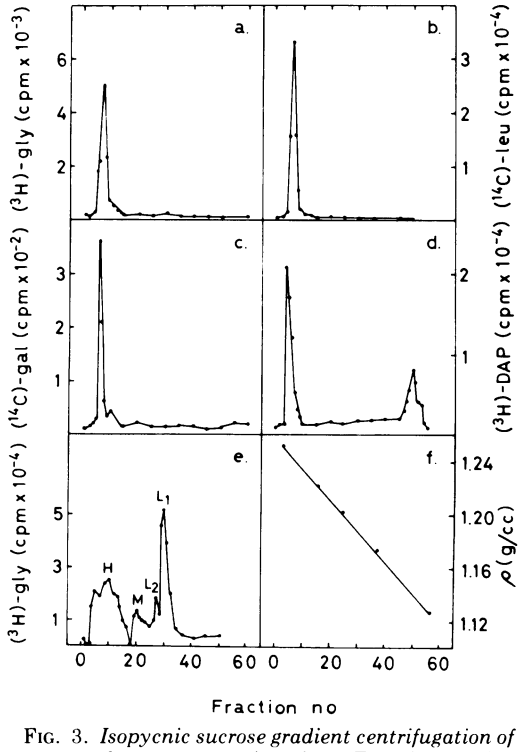

FIG. 3. *Isopycnic sucrose gradient centrifugation of outer membrane preparations from E. coli according to the method of Osborn et al. (17). (a-d) Strain E64-113 was grown in Casamino Acids medium overnight and inoculated into four flasks containing 500 ml of Casamino Acids medium plus lysine (60 μg/ml). Each flask contained one of the following isotopes: (a) 0.25 mM [2-³H]glycerol (2.0 μCi per μmol), (b) ¹⁴C-leucine (0.5 μCi per ml), (c), 0.10 mM ³H-galactose (2.0 μCi per μmol), and (d) 0.10 mM ³H-diaminopimelic acid (2.0 μCi per μmol). (e) The total membrane fraction of a ³H-glycerol-labeled E64-113 culture was prepared according to Osborn et al. (17) and subjected to the same centrifugation procedure. (f) Linearity of the sucrose gradient.*

showed 40-fold less succinate dehydrogenase activity than an acidified, complete cell envelope preparation (Table 1). On the other hand, no reduction was found in phospholipase A plus lysophospholipase activities. Furthermore, the outer membrane fraction showed approximately the same phospholipase activity as the H band prepared according to Osborn et al. (17).

Chemical composition of outer membrane isolated from a transductant pair (envA⁺ and envA). In both strains E64-113 (*envA*⁺) and E64-120 (*envA*), the recovery of outer membrane amounted to roughly 6% of the total cell dry weight. The outer membrane fraction of both the wild type and the antibiotic-susceptible strain contained total lipids, lipopolysaccharides, and proteins in the ratio 1:2:6, wt/wt (Table 2). The amount of murein was not

TABLE 1. *Enzyme activities of outer membrane and cytoplasmic membrane markers*

Material	Succinate dehydrogenase[a]	Phospholipase A plus lysophospholipase[b]
Envelope[c]		
Not acidified	0.81	0.59
Acidified	0.40	0.67
OM fraction[d]	0.01	0.56
H band[e]	0.05	0.60
$L_1 + L_2$ bands[e]		
Not acidified	1.33	0.10
Acidified	0.75	

[a] One unit is defined as the amount of enzyme which increases the absorbance at 550 nm 1 absorbance unit per min. Specific activity is expressed as units per milligram of protein.

[b] Specific activity is expressed as micromoles per minute per milligram of protein.

[c] Cell envelopes were prepared with a French press as described in Materials and Methods. The envelope fraction was divided into two parts. One was kept as a control, and the other was acidified to pH 5.0 in 30% sucrose and treated exactly as our outer membrane fraction.

[d] Outer membrane prepared as described in the text.

[e] The H band (outer membrane) and $L_1 + L_2$ bands (cytoplasmic membrane) were obtained after isopycnic sucrose density centrifugation as described by Osborn et al. (17). The $L_1 + L_2$ fractions were acidified as described in footnote *a*.

TABLE 2. *Chemical composition of the outer membrane prepared from E64-113 (envA$^+$) and E64-120 (envA).*

Strain	Envelope genotype	Content in outer membrane (percentage of dry weight)		
		Protein[a]	LPS[b]	Lipid[b]
E64-113	*envA*$^+$	50	17	8
E64-120	*envA*	50	19	6

[a] Protein was determined according to Lowry et al. (9).

[b] Lipopolysaccharide (LPS) and total lipids were recovered after phenol-water and chloroform-methanol extraction, respectively, as described in Materials and Methods.

determined. All proteins separated from the outer membrane fraction of the *envA*$^+$ strain E64-113 by SDS polyacrylamide gel electrophoresis were also present in the cell envelope of the same strain. However, certain proteins were enriched in the outer membrane fraction. Only minor relative differences in the amount of proteins were noted between the *envA* and the wild-type strain (Fig. 4). Upon comparison of the phospholipids in the two strains, it was found that the *envA* strain E64-120 showed a decrease in the relative amount of phosphatidylglycerol in outer membranes as well as in whole cell envelopes (Table 3). This difference could not be explained by a changed phospholipase activity in the mutant strain. In both strains, the outer membranes showed a decreased diphosphatidylglycerol and phosphatidylglycerol to phosphatidylethanolamine ratio when compared with whole cell envelopes. The outer membrane fraction from both strains contained β-hydroxymyristic acid amounting to 20% of the total fatty acid content. No differences were noted in the fatty acid distribution between the wild type and the permeable strain.

DISCUSSION

Our method is based on the findings by Birdsell and Cota-Robles (3) and Normark et al. (16) that lysozyme-EDTA treatment of plasmolyzed cells partly removes the outer membrane (Fig. 1).

Under physiological growth conditions, the surface of the *E. coli* cell envelope is negatively charged (11). This charge probably prevents spontaneous aggregation of the cells. Lowering the pH to 5.0 in our isolation technique decreases the net negative charge, a change which may be responsible for the marked tendency of the outer membrane fragments to aggregate.

In several experiments, the recovery of membrane amounted to roughly 6% of the total dry weight of the cells. At the pH employed (pH 5.0), about 40% of released membrane material was precipitated. Lysozyme-EDTA treatment does not completely remove the outer membrane from the spheroplasts. It is therefore reasonable to assume that the outer membrane constitutes at least 10% of the total cell dry weight.

The outer membrane and the cytoplasmic membrane have been shown to differ in their apparent buoyant density (13, 17). Our outer membrane fraction yielded no band corresponding to the density of the cytoplasmic membrane (ρ = 1.14 to 1.16 g/cc). Our material migrated to a density of 1.24 g/cc. This is slightly higher than that of the H fraction of Osborn et al. (17). Since the outer membrane material was concentrated to four fractions, as compared with 20 for the H band, it would appear that our material is more homogeneous. It has previously been shown that there are sites of adhesion between the cytoplasmic membrane and cell wall in

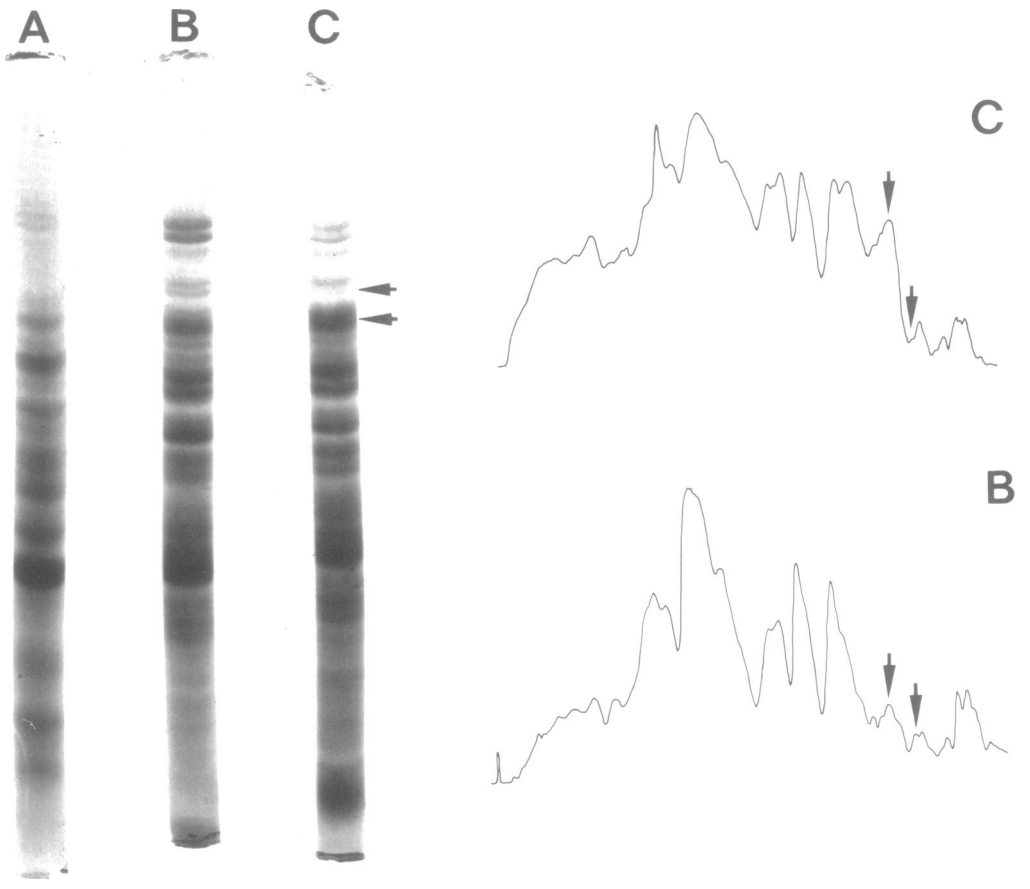

FIG. 4. *Gel electrophoresis of (A) wild-type (E64-113) cell envelope and of outer membrane preparations of (B) E64-113 (envA⁺) and (C) E64-120 (envA). Scans of the gels B and C are depicted to the right. The main differences in the protein pattern between the envA⁺ and the envA strains are marked by arrows.*

TABLE 3. *Relative amounts of phospholipids in cell envelopes and outer membrane in envA⁺ and envA strains*

Strain	Envelope genotype	Fraction	Percentage of total ^{32}P counts in phospholipids separated by thin-layer chromatography[a]		
			DPG	PG	PE
E64-113	*envA*⁺	Cell envelopes	3.5	19.8	77.1
		Outer membrane	1.8	14.8	83.3
E64-120	*envA*	Cell envelopes	4.3	17.0	78.5
		Outer membrane	1.7	10.5	87.9

[a] DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine. The diphosphatidylglycerol value is calculated on the basis of two phosphate molecules per molecule diphosphatidylglycerol.

lysozyme-treated plasmolyzed cells (1, 16). These sites may have caused some cytoplasmic membrane contamination of Osborn's H fraction.

The distribution of cytoplasmic and outer membrane markers (succinate dehydrogenase and phospholipase A plus lysophospholipase) in cell envelope and our outer membrane fraction also indicates that our preparation is virtually free from cytoplasmic membrane contamination.

When the overall compositions of outer membranes from E64-113 (*envA*⁺) and E64-120 (envA) were compared, no significant differences were found (Table 2). It is notable that both strains contained the same amount of phenol-extractable material (lipopolysaccharide). This finding, together with an earlier observation that the *envA* mutation does not affect the chemical composition of lipopolysaccharide (15), strongly suggests that the increased permeability and the cell division abnormality are not due to a mutation affecting the lipopoly-

saccharides. It has previously been shown that the cell envelopes of *envA*-containing strains show a relative decrease of phosphatidylglycerol (15). This decrease is very evident when the lipid distribution of outer membrane preparations is studied (Table 3). Other antibiotic-supersensitive mutants must be isolated and analyzed, however, before it can be concluded that the phospholipid content of the outer membrane influences the penetrability of this barrier.

A comparison of the protein profiles of outer membrane from E64-113 (*envA*$^+$) and E64-120 (*envA*) showed that all protein components were present in the mutant. However, in the chain-forming, permeable strain (*envA*), the protein pattern was slightly modified (Fig. 4). This difference may be significant for the *envA* mutation, as the parental *envA* strain D22 (14) showed the same modified protein pattern. More recently, a mutant with alteration in the outer membrane proteins of the cell envelope has been described (24). However, the phenotypic expression of this modification of the protein pattern has not been clarified.

In conclusion, a rapid method has been developed by which large quantities of reasonably pure outer membranes can be isolated. We hope this method will be useful for the characterization of envelope mutants and for the study of the physiology of the outer membrane.

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