

Lysis of *Vibrio cholerae* Cells: Direct Isolation of the Outer Membrane from Whole Cells by Treatment with Urea

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Cells of *Vibrio cholerae* underwent rapid autolysis when suspended in media of low osmolarity under non-growing conditions. Chaotropes like urea and guanidine·HCl which are potent protein denaturants caused complete and immediate lysis of whole cells. This unique sensitivity of *V. cholerae* to protein denaturants led to the development of a rapid method for the selective isolation of the outer membrane upon treatment of whole cells with urea. The composition of the outer membrane isolated from both whole cells and crude envelopes by treatment with urea was comparable with that of the outer membrane isolated by other conventional methods.

INTRODUCTION

Lysis of a bacterial cell generally results from damage to the peptidoglycan layer (Weibull, 1953). Autolysis occurs due to an uncontrolled action of endogenous peptidoglycan hydrolases or autolysins. In Gram-negative bacteria the peptidoglycan substrate is often inaccessible to the action of exogenously added hydrolases due to the existence of the outer membrane which acts as a diffusion barrier (Repaske, 1956; Weidel *et al.*, 1960; Dirienzo *et al.*, 1978). Mutants of *Escherichia coli* and *Salmonella typhimurium* altered in the organization of the outer membrane are more susceptible to the action of detergents and chelating agents (Yem & Wu, 1978; Suzuki *et al.*, 1978; Nikaido, 1979).

Studies on the cell envelope of *Vibrio cholerae* are of special interest since its enterotoxin is membrane-bound (Fernandes & Bayer, 1977; Pearson & Mekalanos, 1982) and is also released into the surrounding medium. However, until recently, little work had been carried out on the outer membrane proteins of *V. cholerae* (Sur & Chatterjee, 1979; Kabir, 1980; Kelley & Parker, 1981). Virtually no information is available on the sensitivity of these cells to membrane-active agents except for a preliminary report by Adhikari *et al.* (1969).

The present investigation was undertaken to study the inherent lytic properties of *V. cholerae* and its susceptibility to protein denaturants and to isolate the outer membrane directly from whole cells by treatment with urea.

METHODS

Bacteria and growth conditions. The bacterial strains used were *Vibrio cholerae* 569B (Serotype Inaba; hyper-toxinogenic), *Escherichia coli* C600, *E. coli* JE5513 (Lpo), *Salmonella typhimurium* LT2 and *S. typhimurium* SL1102 (Re). *Vibrio cholerae* was grown in medium containing 1% (w/v) Lab-Lemco powder (Oxoid), 1% (w/v) peptone (Difco) (pH 8.5) with 0.18 M-NaCl. All other strains were grown in the above medium (pH 7.2) supplemented with 0.09 M-NaCl. Cells were grown at 37 °C on a gyratory shaker (180–200 r.p.m.) and bacterial growth was monitored by following the increase in culture turbidity at OD₅₅₅. Strains C600 and JE5513 of *E. coli*, and *S. typhimurium* LT2 and SL1102 were gifts from Dr J. T. Park, Tufts University, Boston, USA.

Lysis of whole cells. Routinely, a 2% inoculum of an overnight culture was added to fresh broth and cells were allowed to grow with shaking at 37 °C for 120–180 min, to yield 4–6 × 10⁸ c.f.u. ml⁻¹ (A₅₅₅ 0.6–0.7). Cells were

harvested by centrifugation at 10 °C at 6000 g for 5 min and immediately resuspended in the appropriate buffer, at either 10 °C or 37 °C. The volume was adjusted to maintain 5×10^8 c.f.u. ml⁻¹. The tubes were incubated at different temperatures and lysis was monitored by the decrease in OD₅₈₅.

Preparation of crude cell envelopes. The method of preparation of crude cell envelopes followed by separation of outer and inner membranes by sucrose density gradient centrifugation was essentially that of Schnaitman (1970), modified as noted below. Exponential phase cells (about 6×10^8 c.f.u. ml⁻¹) of *V. cholerae* were harvested (6000 g, 5 min) and resuspended in 10 mM-HEPES (Sigma, pH 7.0) containing 20% (w/v) sucrose. The cells were disrupted in an MSE ultrasonic disintegrator until the OD₅₈₅ of the suspension decreased by about 95%. The cell lysate was treated with 100 µg DNAase and RNAase ml⁻¹ each (Sigma) for 10 min. Intact cells were removed by low speed centrifugation and the cell lysate was diluted with two volumes of 10 mM-HEPES buffer and centrifuged for 60 min at 105000 g in a Sorvall AH 627 rotor. The pellet constituting the crude envelope fraction was either used as such or further purified after washing it once by centrifugation with HEPES buffer. All the above operations were carried out at 4 °C.

Isolation of outer membrane. (a) *Sucrose density gradient centrifugation.* The crude cell envelope was resuspended in 20% sucrose in 10 mM-HEPES buffer. Approximately 1 ml (about 10 mg protein) of this suspension was layered onto an 18 ml linear sucrose density gradient of 45–60% (w/v) sucrose in 10 mM-HEPES (pH 7.0) with a 1 ml cushion of 75% (w/v) sucrose, and centrifuged at 4 °C for 9 h at 105 000 g in a Sorvall AH 627. The rest of the tube (16 ml) was filled with 10 mM-HEPES buffer (pH 7.0). This modification was based on the short column gradient technique of Griffith (1978). Four distinct bands were visible which were collected by puncturing the tube at the bottom or by manual aspiration. The outer membrane was identified by its buoyant density in the gradient (1.23 g ml⁻¹) as the fourth band from the top. After collection, each of the four bands was diluted with 10 mM-HEPES (pH 7.0) and pelleted by centrifugation at 105000 g for 30 min at 4 °C. The pellets were washed once and resuspended in 10 mM-HEPES buffer and stored at –20 °C. Identical results were obtained when the more established method of sucrose density gradient centrifugation, using a linear sucrose gradient (45–60%, w/v) throughout the length of the tube and a longer running period of 24 h at 105000 g, was used. The short column gradient technique was used in this investigation because of its convenience.

(b) *Treatment with Sarkosyl NL97.* Outer membrane proteins were isolated from the crude cell envelope after treatment with 0.5% Sarkosyl NL97 (Geigy Industrial Chemicals, Ardsley, NY, USA) for 30 min at 24 °C (Filip *et al.*, 1973) followed by centrifugation at 105000 g for 60 min. The pellet containing the outer membrane proteins was washed once with cold distilled water, resuspended in distilled water and stored at –20 °C. The supernate was dialysed against distilled water for 24 h at 4 °C and centrifuged at 105 000 g for 60 min. The pellet containing the inner membrane proteins was resuspended in cold distilled water and stored at –20 °C.

Treatment with urea. Late exponential phase cells of *V. cholerae* (6×10^{10} c.f.u. ml⁻¹) were harvested and resuspended in 20 ml 4 M-urea and incubated for 10 min at 24 °C, by which time more than 90% lysis had occurred, as judged by the decrease in OD₅₈₅. The viscous lysate was thoroughly homogenized and the remaining unbroken cells were removed by two cycles of low speed centrifugation at 24 °C. The supernate was centrifuged at 105 000 g for 60 min at 4 °C. The pellet was resuspended in a small volume of 10 mM-HEPES buffer and incubated with 100 µg DNAase and RNAase ml⁻¹ each at 24 °C for 30 min. The resulting suspension was re-extracted with 4 M-urea and centrifuged at 105 000 g for 60 min at 4 °C. The pellet was finally washed in cold distilled water and stored at –20 °C until use.

Crude cell envelopes were treated with 4 M-urea at 24 °C for 10 min and centrifuged at 105 000 g for 60 min. The pellet was washed once with cold distilled water, resuspended in distilled water and stored at –20 °C. The supernate was dialysed against water (24 h, 4 °C), centrifuged at 105 000 g for 60 min at 4 °C and the resulting pellet was resuspended in distilled water and stored at –20 °C.

Analytical methods. Protein was measured by the method of Markwell *et al.* (1978) with bovine serum albumin as standard. LPS was isolated from crude cell envelope by 45% (w/v) aqueous phenol at 68 °C (Westphal & Jann, 1965). Total carbohydrate was estimated by phenol/sulphuric acid (Dubois *et al.*, 1956) and heptose by cysteine/sulphuric acid (Wright & Rebers, 1972). Phospholipids were extracted by chloroform/methanol/water (Bligh & Dyer, 1959) and quantified from the lipid phosphate. Phosphate was measured by the method of Ames & Dubin (1960). Phospholipids were identified by thin-layer chromatography on silica gel G plates (Merck) using the solvent chloroform/methanol/water (65:25:4, by vol.). The chromatogram was developed in iodine vapour.

Cytochrome *b*₁ was quantified from the oxidized – reduced difference spectra with sodium dithionite as the reducing agent and the value calculated from the ϵ_{427} (Hager & Deeb, 1967).

SDS-polyacrylamide gel electrophoresis. Proteins were analysed on a 10% SDS-polyacrylamide gel by the method of Laemmli (1970). The polysaccharide profile of purified LPS and the various outer membrane preparations was detected electrophoretically on a 5.6% SDS-polyacrylamide gel which was stained with periodic acid-Schiff's reagent (Jann *et al.*, 1975; Stewart-Tull, 1965). Approximately 20 µg total carbohydrate was loaded for each sample. A fixed current of 40 mA per slab was used for a run time of 6 h at 24 °C for a 10 cm gel length.

Electron microscopy. Outer membrane preparations were negatively stained with 2% phosphotungstate (pH 7.0) and examined under a JEOL 100C transmission electron microscope at 60 kV.

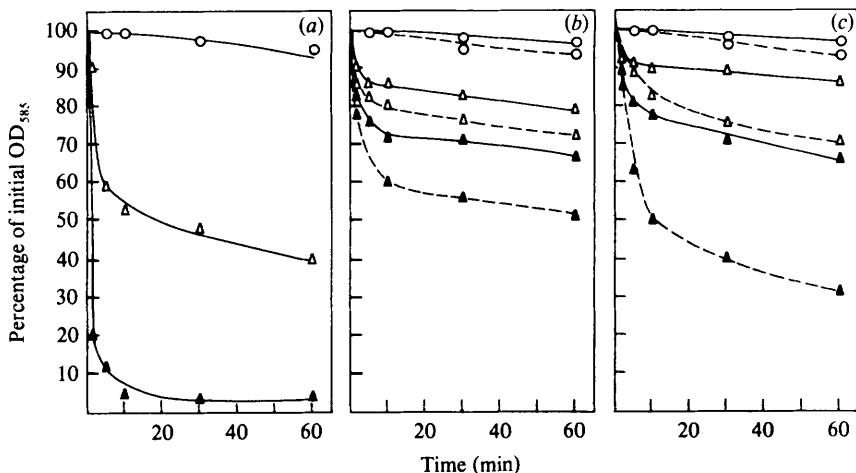


Fig. 1. Effect of urea on *V. cholerae*, *E. coli* and *S. typhimurium*. Exponential phase cells of (a) *V. cholerae* 569B, (b) *E. coli* (solid lines C600, broken lines JE5513), and (c) *S. typhimurium* (solid lines LT2, broken lines SL1102) were harvested and resuspended in: 50 mM-phosphate buffer (pH 7.0) (○); 2 M-urea (△); 4 M-urea (▲). Lysis was monitored by the decrease in OD₅₈₅.

RESULTS

Lysis of *V. cholerae* cells

V. cholerae cells harvested in the exponential phase of growth lysed rapidly when suspended in a hypotonic medium. Relatively little increment in lysis was observed with addition of EDTA and Tris/HCl. With increasing concentrations of Tris/HCl and NaCl the rate of cellular lysis decreased progressively. Stationary phase cells exhibited a similar effect except that the rate of lysis was slower.

Effect of urea and other protein denaturants

Cells of the different bacterial strains harvested in the late exponential phase of growth were suspended in water containing urea (2 M and 4 M) and incubated at 24 °C. Figure 1 shows that 4 M-urea caused instantaneous lysis of *V. cholerae* cells, the OD₅₈₅ of the suspension decreasing by more than 85% in 5 min. A further increase in the concentration of urea did not affect the degree of lysis. Similar lytic effects could be seen with other protein denaturants like 6 M-guanidine·HCl and 6 M-ammonium thiocyanate. No detectable lysis was observed if heat-treated cells of *V. cholerae* (100 °C, 5 min) were treated with urea. Different strains of *E. coli* and *S. typhimurium* were also used to compare the effect of urea on well characterized strains having defined lesions in their outer membrane. The effect of urea was significantly lower on whole cells of *E. coli* C600 and *S. typhimurium* LT2 (Fig. 1b, c), both of which are known to have a normal complement of cell envelope components. Response of the mutant strains, one deficient in the lipoprotein (*E. coli* JE5513) and the other (*S. typhimurium* SL1102) with a 'deep rough' LPS, to urea was relatively more, but still much less, than that seen with *V. cholerae* 569B (Fig. 1a). These mutant strains have been shown to be more sensitive to lysis by exogenous agents such as EDTA and detergents (Suzuki *et al.*, 1978; Nikaido, 1979).

Characterization of the insoluble residue isolated after lysis of cells by 4 M-urea

The composition of the cellular components resistant to solubilization by urea was compared with the crude cell envelope and outer membrane preparations isolated by established techniques such as sucrose density gradient centrifugation and detergent extraction of the crude envelope. The urea-insoluble material was isolated from both whole cells and crude envelope.

Proteins. The protein profiles of the urea-insoluble residue isolated from whole cells (Fig. 2, lane C) or crude envelope (lane G) were identical and matched the outer membrane protein profile isolated by Sarkosyl treatment of crude envelope (lane F). On the other hand, the protein

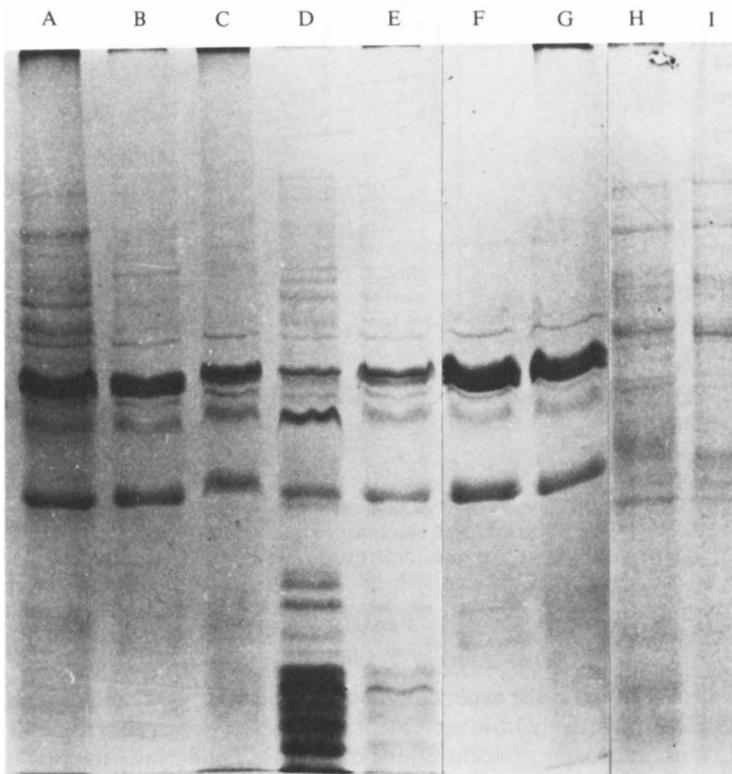


Fig. 2. SDS-polyacrylamide gel electrophoresis of *V. cholerae* 569B membrane proteins. Lane A, protein profile of *V. cholerae* 569B crude cell envelope; lane B, outer membrane isolated after density gradient centrifugation; lane C, urea-insoluble residue from whole cells; lane D, residue after lysis of cells in distilled water; lane E, residue after lysis of cells in Tris/HCl and EDTA; lane F, outer membrane proteins isolated by treatment of crude envelope with Sarkosyl; lane G, urea-insoluble residue isolated from crude envelope; lane H, Sarkosyl-extracted inner membrane proteins; lane I, urea-soluble proteins from crude envelope.

profiles of residues obtained after lysis of whole cells in distilled water alone (lane D) or by Tris/HCl and EDTA (lane E) were not comparable to either outer membrane (lane F) or crude envelope proteins (lane A). Sucrose density gradient centrifugation of the crude envelope yielded four well separated bands. The protein profile of the fourth band from the top of the gradient, whose buoyant density matches that of the outer membrane of other enteric bacteria (Lugtenberg & Van Alphen, 1983), is shown in Fig. 2, lane B. This was identical to the protein profile of the crude envelope (lane A), suggesting incomplete separation of the two membranes by this technique. The protein profiles of the other three bands were found to be similar to that of the fourth band. In contrast, treatment of crude envelopes by Sarkosyl resulted in distinct inner and outer membrane protein profiles (Fig. 2, lanes H and F, respectively).

LPS and phospholipids. Two species of phospholipids, phosphatidylethanolamine and phosphatidylglycerol, could be detected in the chloroform/methanol extract of the urea-insoluble residue. SDS-PAGE of the urea-insoluble residue revealed two periodic acid-Schiff-positive bands which had the same mobility as purified LPS (distance travelled from the origin – 6.5 and 8.5 cm). Since KDO (2-keto-3-deoxyoctonate) is not present in the LPS of *V. cholerae* (Raziuddin & Kawasaki, 1976) the ratio of heptose to total carbohydrate was measured in the urea-insoluble residue and found to be identical to that obtained with purified LPS (1:2.7). The total yield of the urea-insoluble residue isolated from whole cells or crude envelope was similar to the Sarkosyl-extracted outer membrane and was about half that of the crude envelope (Table 1). The

Table 1. *Total yield and composition of different membrane preparations*

Values given indicate the range obtained with three different sets of membrane preparations.

Membrane preparation	Percentage of cell dry wt	Protein*	Phospholipid*†	LPS*‡
Crude cell envelope	20-26	42-45	31-39	6-7
Sarkosyl-extracted outer membrane	9.6-10	44-46	5-6	16-18
Urea-insoluble residue isolated from whole cells or crude envelope	9.8-12	46-54	13-15	18-20

* Expressed as percentage dry weight of the preparation.

† Total phospholipids were quantified by multiplying the amount of extractable lipid phosphate by 700, which was taken to be the average molecular weight of phospholipids.

‡ LPS was quantified by determining the heptose content of the various membrane preparations and from the known amount of heptose present in purified LPS isolated from crude cell envelopes.

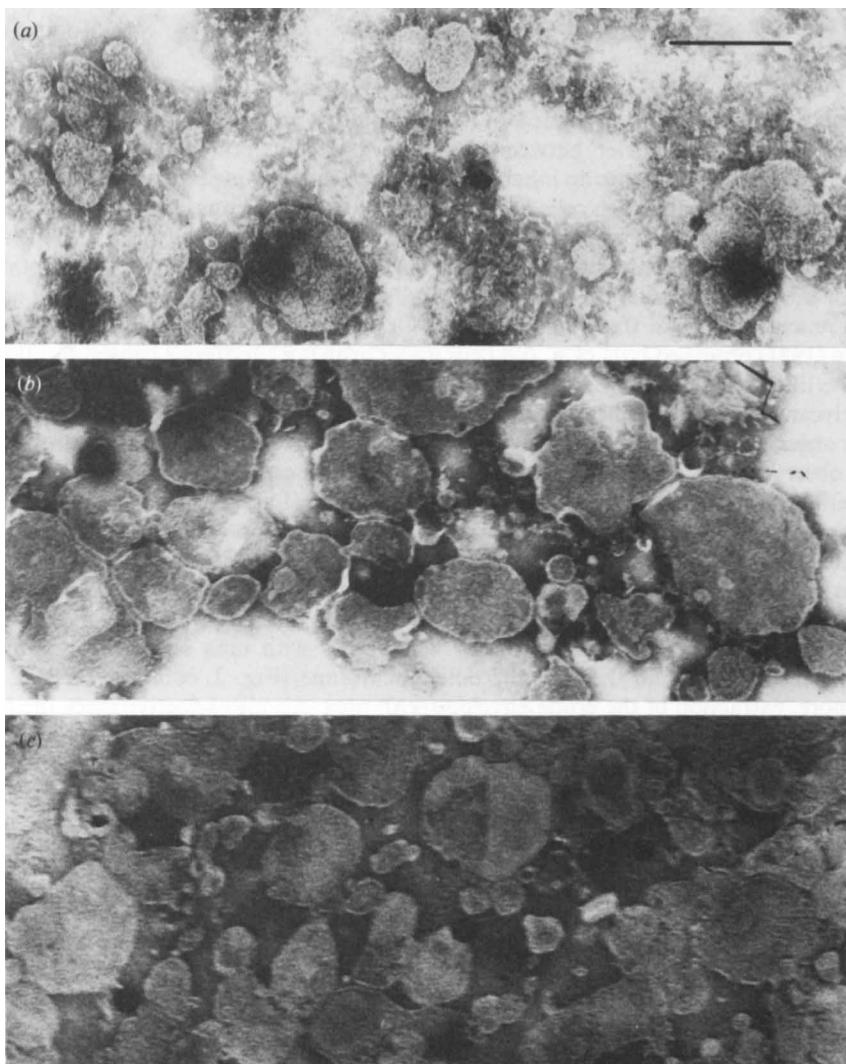


Fig. 3. Electron micrographs of negatively stained outer membrane preparations from *V. cholerae* 569B. Outer membranes isolated by (a) sucrose density gradient centrifugation, (b) treatment of crude envelope with urea, (c) treatment of crude envelope with Sarkosyl, and stained with 2% phosphotungstate. Bar represents 0.2 μm.

LPS present in the crude envelope was quantitatively recovered in the urea-insoluble residue and Sarkosyl-extracted outer membrane. About 30% of the total phospholipids present in the crude cell envelope was recovered in the urea-insoluble residue. As one would expect, the detergent-extracted outer membrane had a low content of phospholipids.

Cytochrome content. Cytochrome b_1 , which is a characteristic constituent of the inner membrane, was assayed in all the outer membrane preparations to determine the extent of contamination by inner membrane constituents. The relative distribution of cytochrome b_1 in crude cell envelope, density gradient-separated outer membrane, urea-insoluble residue and Sarkosyl-extracted outer membrane was 100 : 46 : 20 : 0. Since the protein profile of the urea-insoluble residue did not reveal any detectable contamination by inner membrane proteins, it is possible that such a presence of cytochrome b_1 may be due to a specific association which can only be removed by strong detergent treatment (Horio *et al.*, 1960).

Electron microscopy. Negatively stained electron micrographs of outer membranes prepared by sucrose density gradient centrifugation and by Sarkosyl or urea treatment of crude envelopes are shown in Fig. 3.

DISCUSSION

Lysis of *V. cholerae* cells suspended in low osmolarity medium could be due either to the absence of the putative 'barrier' between autolysins and the peptidoglycan as suggested for *E. coli* (Hartmann *et al.*, 1974) or to an inherently fragile cell supporting structure. The fact that this lysis is slower at 10 °C and with cells harvested in the stationary phase of growth suggests that autolytic enzymes may also be involved. However, it is also possible that the peptidoglycan network in *V. cholerae* is inherently fragile. It may be relevant to note that the peptidoglycan content of the related species *V. fetus* is around 0.2–0.26% (dry weight) (Winter *et al.*, 1971), which is much lower than that found in other Gram-negative bacteria (Ghysen, 1968).

Ingram (1981) observed lysis of *E. coli* cells under growing conditions caused by ethanol and chaotropes like urea and thiocyanate. He proposed an alteration in the membrane association of peptidoglycan cross-linking enzymes as the possible basis of such lysis. This is clearly different from our observation where non-growing cells were lysed by urea and other chaotropes. Earlier workers observed lysis of halobacteria by urea and hypothesized that the cell envelopes were held together by predominantly non-covalent interactions (Larsen, 1967). Lysis of *V. cholerae* cells by protein denaturants indicated the importance of non-covalent interactions in the maintenance of cellular integrity. Although extensive lysis of whole cells was observed upon resuspension in distilled water or Tris/HCl buffer (pH 8.0) containing EDTA, the protein profile of the residues isolated after lysis clearly indicated that they were associated with cytoplasmic proteins. In contrast, extraction of whole cells with urea selectively solubilized all cellular proteins other than those of the outer membrane (Fig. 2, compare lanes C and F). Furthermore, extraction of the crude envelope with urea was also selective since the insoluble residue contained the outer membrane proteins (lane G) while the soluble material contained the inner membrane proteins (Fig. 2, compare lanes H and I). A clean separation of the outer and inner membranes of *V. cholerae* was not achieved by the sucrose density gradient centrifugation. Interestingly, Kelley & Parker (1981) also failed to separate the two membrane systems cleanly by this technique and proposed that the secretory behaviour of this organism might result in linking the inner and outer membranes more tightly than in other Gram-negative bacteria. The other widely used technique for separating the two membrane systems is based on selective solubilization by detergents, but one of the principal disadvantages in the use of detergents is the loss of phospholipids from such outer membrane preparations (Lugtenberg & Van Alphen, 1983). Our method of isolation of outer membrane with its full complement of phospholipids, proteins and LPS may be applicable to other Gram-negative bacteria which display similar susceptibility to lysis.

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