SEPARATION OF THE CYTOPLASMIC AND OUTER MEMBRANE OF PSEUDOMONAS AERUGINOSA PAO.1

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SUMMARY

A method is described for the isolation of the cytoplasmic and outer membranes of *Pseudomonas aeruginosa* PAO.1. The cytoplasmic membrane exhibits nicotinamide adenine dinucleotide oxidoreductase, lactate dehydrogenase DD-carboxypeptidase and succinate dehydrogenase activities. The outer membrane is rich in 2-keto-3-deoxyoctonate and exhibits phospholipase A and DD-carboxypeptidase activity. At least 25 protein species have been detected in the cytoplasmic membrane by polyacrylamide gel electrophoresis. Using the same technique, the outer membrane contains only five protein species of molecular weights, 56,000, 53,000, 38,000, 21,000 and 16,000.

INTRODUCTION

In general, Gram-negative bacteria, including *Pseudomonas aeruginosa*, are intrinsically more resistant to antimicrobial agents than gram-positive bacteria. There is now evidence to suggest that the outer membrane of gram-negative bacteria acts as a barrier to many antibiotics and thus prevents them reaching their targets (1,2,3). The cell envelope of *Ps. aeruginosa* has been extensively investigated in terms of protein (4,5), lipopolysaccharide (6,7), mucopeptide (8) and lipid (9) con-

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tent, but little work has been conducted on isolated envelope components. The cytoplasmic and outer membranes of several gram-negative bacteria, including S. typhimurium (10), E. coli (11,12) and P. mirabilis (13) have been separated by lysozyme and EDTA* treatment of whole cells followed by lysis of the resulting osmotically fragile spheroplasts. This has led to a greater understanding of the structure of the bacterial envelope and also the localisation of various enzyme activities such as those relating to phospholipid and lipopolysaccharide biosynthesis (10,14). However, such a separation technique did not appear to be suitable for Ps. aeruginosa due to the lability of the outer membrane in the presence of EDTA (15). However, the use of lithium chloride in place of EDTA has met with some success (4).

The method reported here shows that the cytoplasmic and outer membranes of Ps. aeruginosa can be separated using the lysozyme and EDTA technique.

MATERIALS AND METHODS

Preparation of membrane material

Pseudomonas aeruginosa PAO.1 was grown to mid log phase in nutrient broth (3 litres) harvested by centrifugation (10,000xg, 10 min) and resuspended at room temperature in 0.25M sucrose, 33mM tris (hydroxymethyl) methylamine buffer pH.8 (150 ml). Lysozyme and EDTA were added to final concentrations of 30 μg/ml and 250 μg/ml respectively and the suspension allowed to stand at room temperature for 10 min. The cells were then poured into four volumes of water at 4°C, stirred until mixing was complete and then 100 ml aliquots sonicated using a Dawe Soniprobe at 6 amps for 60 sec. Whole cells were sedimented by centrifugation (5,000xg, 30 min) and the clarified lysate centrifuged at 38,000xg for 2hr. to sediment the cell envelope material. The pellet was washed once in sterile water at 4°C, resedimented and finally resuspended in 5 ml cold water.

Separation of envelope fractions

Aliquots (1.5 ml) of the cell envelope preparation were layered onto 35-65% (w/w) sucrose gradients, and centrifuged

* EDTA ethylenediaminetetra-acetic acid

for 64 hr. at 75,000xg. Gradients were fractionated and bands of envelope material visualised by measuring the % transmission of each fraction at 280 nm. Sucrose step gradients were prepared by layering 55% (3 ml), 50% (5 ml), 45% (5 ml), 40% (5 ml) and 35% (2 ml) sucrose solutions (w/w) over a cushion of 65% sucrose. All sucrose solutions were made up in sterile distilled water.

Enzyme assays

Nicotinamide adenine dinucleotide oxidoreductase (EC. 1.6.99.22):- incubation mixtures contained 0.1M tris buffer pH 7.5 (2.8 ml), 7mM NADH* (0.1 ml), envelope material (0.01 - 0.1 ml) and water to 3 ml. Absorbance at 340 nm was measured over a ten minute period at 23°C in a Pye Unicam SP1800 recording spectrophotometer.

Succinate dehydrogenase (EC. 1.3.99.2) and lactate dehydrogenase (EC. 1.1.2.4):- incubation mixtures contained 50mM phosphate buffer pH 7 (2.5 ml), neutralised 0.1M potassium cyanide (0.1 ml), 1mg/ml solution of dichlorophenol indophenol (0.05 ml), 1mM phenazine methosulphate (0.2 ml), 4% succinate or 0.4% sodium lactate (0.1 ml) and envelope material (50-250 μg protein). Absorbance at 600 nm was measured over a ten minute period at 23°C.

Glucose-6-phosphate dehydrogenase (EC. 1.1.1.49) was assayed by the method of Malamy and Horecher (16).

DD-carboxypeptidase activity was determined as described by Pollock et al (17) Radioactive substrate (UDP-MurNAc-L.ala-D.glu-mesoDap-D.ala-D.ala, 1.2mM) labelled in the two terminal D-ala residues, was exposed to membrane preparations (24μl) for 3 hr at 37°C in a system composed of 0.06M Tris-HCl, pH 8.6, 0.05M $MgCl_2$, 0.14mM dithiothreitol and 0.5% w/v Brij 36T. The final reaction volume was 40μl. After incubation, the mixtures were boiled briefly, loaded onto Whatman 3MM chromatography paper, and developed by descending chromatography in isobutyric acid : N NH_4OH (5:3 v/v) at room temperature.

The chromatogram was fractionated into 1cm units which were digested in scintillation vials containing 12 ml. of triton-toluene-water scintillant, at 50°C overnight. The amount of D-alanine released from the substrate was determined by counting the digested fractions in a Packard 3330 scintillation spectrometer.

Protein was estimated by the method of Lowry et al (18) using bovine serum albumen as standard. 2-keto-3-deoxyoctonate was estimated by this barbituric acid method (19). DNA was estimated by the diphenylamine method of Burton (20) and RNA by the orcinol method (21).

Polyacrylamide gel electrophoresis of envelope proteins was carried out as described by Beard and Connolly (22).

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* NADH Reduced nicotinamide adenine dinucleotide

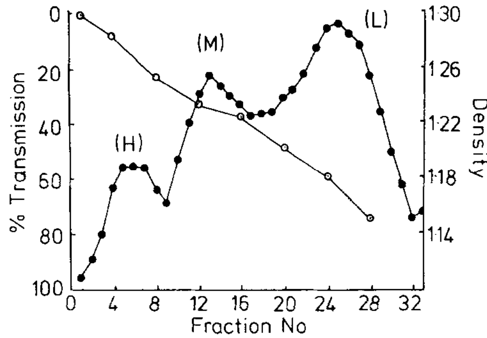

Figure 1

Separation of the inner and outer membranes of Pseudomonas aeruginosa PAO.1 (for details see materials and methods)

●—● % transmission at 280 nm
○—○ density of the gradient (g/cc).

Results and discussion

The results in Figure 1 show the separation of envelope material into three bands, H, density 1.26 g/cc, L, density 1.18 g/cc and M density 1.22 - 1.23 g/cc. Fractions of gradients containing material from each of the three bands were pooled, centrifuged at 38,000xg for 2 h, the pellet washed in cold distilled water and resedimented before finally being resuspended in 1 ml cold distilled water. The washed envelope material was then assayed for protein, KDO*, DNA, RNA, lactate dehydrogenase, succinate dehydrogenase, glucose-6-phosphate dehydrogenase and nictominamide adenine dinucleotide oxidoreductase.

The cytoplasmic membrane, but not the outer membrane of S. typhimurium, has been found to contain LDH*, SDH*, and NADH oxidising activity (10). The results presented in Table 1

* KDO 2-keto-3-deoxyoctonate
* LDH lactate dehydrogenase
* SDH succinic dehydrogenase

	Density	Protein	KDO	RNA	DNA
Inner (L)	1·18	49	0·3	1·7	0·6
Total (M)	1·22	50	1·5	1·8	nd
Outer (H)	1·26	41	2·5	1·6	nd

Table 1

The inner total and outer membranes of *Ps. aeruginosa* PAO.1 were isolated as described above. Density as g/cc : all assay measurements as % dry weight of envelope material.

nd : none detected.

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suggest such an asymmetric distribution of enzyme activities between the H and L bands isolated from *Ps. aeruginosa* PAO.1. The L band material was found to be enriched for respiratory enzyme activities while the H band was enriched for P-lipase* activity. No such asymmetric distribution of Cp-ase* activity was found.

The eight carbon sugar, KDO, is found almost exclusively in the LPS* component of the outer membrane of *E. coli* and *S. typhimurium* (11,10) and Chester *et al* (6) have found KDO to be an integral part of the LPS extracted from the cell envelope of *Ps. aeruginosa*. The results in Table 2 show that the H band material contains eight times more KDO than the L band.

Contamination of the envelope material was monitored with respect to DNA, RNA and the cytoplasmic enzyme G.6.P*

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* Cp-ase DD-carboxypeptidase * LPS lipopolysaccharide
* P-lipase phospholipase A *G.6.P glucose.6.phosphate

	LDH	SDH	NADHox	G6P-DH	P-Lipase	Cp-ase
Inner (L)	0·24	0·08	0·17	nd	0·08	30
Total (M)	0·13	0·02	0·05	0·002	not done	not done
Outer (H)	0·01	<0·01	nd	nd	0·41	47

Table 2

The inner total and outer membranes of Ps. aeruginosa PAO.1 were isolated and each assayed for the enzyme activities shown above, LDH, SDH, NADHox and G.6.P.DH activities are given as μ moles substrate/min./mg protein., carboxypeptidase as percentage of the initial substrate counts released as ^{14}C D-alanine by the membrane preparations and phospholipase as n-moles Phosphorus released per min/mg protein. For details of membrane separation and enzyme assay methods, see materials and methods.

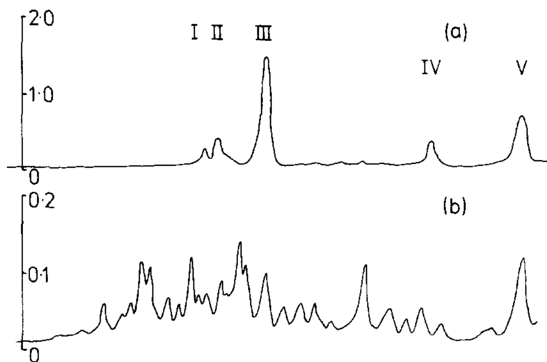

Figure 2

Inner and outer membranes of Ps. aeruginosa PAO. 1 were isolated and the membranes protein separated by polyacrylamide gel electrophoresis as described by Beard & Connolly (20). Gel proteins were stained overnight using Coomassie blue and gel scanning was carried out at 540 nm using an SP1800 recording spectrophotometer fitted with an SP1805 scanning control unit.

(a) Outer membrane proteins
(b) Inner membrane proteins

Ordinate: Absorbance at 540 nm

dehydrogenase (Tables 1 and 2). L band material contained 2.3% of its dry weight as RNA and DNA and H and M band material less than 2% RNA with no detectable DNA.

The activity of G.6.P dehydrogenase was detectable at a very low level in the M band material but was undetectable in the L and H band material.

From the data presented it was concluded that the H band material was enriched for outer membrane, the L band for cytoplasmic membrane and that the M band contained unresolved membranes but not necessarily total envelopes.

The protein complement of the cytoplasmic and outer membrane fractions was investigated by SDS-PAGE* and the results shown in Figure 2 show an asymmetric distribution of protein species between these two envelope components. The protein component of the cytoplasmic membrane was resolved into at least 25 molecular species none of which accounted for more than 12% of the total protein applied to the gel. The outer membrane proteins were resolved into five molecular species. Two of these, proteins III and V, molecular weights 38,000 and 15-16,000 respectively, are probably the proteins A and B, reported by Stinnett and Eagon (4). They suggested the two major proteins of the outer membrane, molecular weights 42,000 and 16,000 may help to maintain the structural integrity of this envelope component. The results presented here show that protein III accounts for 45% and protein V 20% of the total protein applied to the gel. Other protein species of the outer membrane each account for less than 10% of the total outer membrane protein and are of molecular weights 56,000, 53,000 and 21,000.

* SDS-PAGE Sodium dodecylsulphate polyacrylamide gel electrophoresis

Lipoprotein, covalently bound to mucopeptide, is found in the envelope of *Ps. aeruginosa* (8). However, the gel system used here would not be expected to detect a molecule with such a low molecular weight, (10,500).

The large number of protein species in the cytoplasmic membrane probably reflects the presence of a wide range of enzyme activities within this structure. On the other hand, only a few molecular species have been detected in the outer membrane. It should prove interesting to investigate whether any of these proteins possess enzyme activity, since although the outer membrane is largely devoid of known enzymes, phospholipase and DD-carboxypeptidase activities are detectable in this structure (10, present work). The finding that the outer membrane, as well as the inner membrane, exhibits DD-carboxypeptidase activity was surprising in view of the fact that in *E. coli* this enzyme was only detected in the cytoplasmic membrane fraction (17). The significance of this observation, as yet, remains unknown. It should also be of interest to investigate whether the major outer membrane proteins of *Ps. aeruginosa* are involved in phage receptor sites or conjugation as has been reported for *E. coli* (23).

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