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Isolation and characterization of the outer-membrane proteins of *Burkholderia (Pseudomonas) pseudomallei*

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Membranes obtained from whole-cell lysates of *Burkholderia (Pseudomonas) pseudomallei* (strain 319a) were separated into four fractions by sucrose density gradient centrifugation. Membranes were characterized by enzymic and chemical analyses, and by SDS-PAGE. Cytoplasmic membranes and two forms of outer membranes (OM-1, OM-2) were detected. The major outer-membrane proteins had M_r values of 70000, 38000, 31000, 24000 and 17000. To determine which outer-membrane proteins were common to *B. pseudomallei* strains, OM-1 fractions from 12 different strains were prepared. SDS-PAGE analysis of these fractions demonstrated that the five major outer-membrane proteins were common to the strains tested. Further studies have shown that an M_r 110000 protein, which is oligomeric in that it migrates as an M_r 38000 protein upon heating at 95 °C and which is peptidoglycan-associated, serves as a porin in *B. pseudomallei*. Using proteoliposomes reconstituted from this protein and phospholipid, it was demonstrated by the liposome-swelling assay that this protein acts as a porin through which small saccharides may diffuse. Further characterization of this M_r 38000 protein will be important in delineating the role of this molecule in the permeability of the *B. pseudomallei* outer membrane.

Keywords: *Burkholderia (Pseudomonas) pseudomallei*, outer-membrane proteins, porin

INTRODUCTION

Burkholderia pseudomallei (formerly *Pseudomonas pseudomallei*: see Yabuuchi *et al.*, 1992) is the causative agent of melioidosis, cases of which have been documented in Korea, the Philippines, Central and Southern America, the West Indies, Turkey, Malaysia, Madagascar, Guam and Australia (Beamer *et al.*, 1954; Dance, 1991). Most cases occur in the 20° N to 20° S latitudes. The disease is rare in the Western Hemisphere, but has been identified in Panama, Ecuador, Haiti, Brazil, Peru and Guyana. Some investigators feel that the organism will cause disease only in endemic areas; whereas others (Bremmelgaard, 1975; Thomas *et al.*, 1979), feel that because of its nutritional versatility, the organism and the disease due to it should be found in many other areas of the world.

Melioidosis can be manifested in several ways. It can be seen as an inapparent infection, asymptomatic pulmonary infiltration, acute localized suppurative infection, acute pulmonary infection (most common), acute septicaemic infection or chronic suppurative infection (Sanford, 1985, 1987). With better diagnosis and more prolonged appropriate therapy, mortality in forms except the septicaemic should be low (Chaowagul *et al.*, 1989; Eickhoff *et al.*, 1970).

B. pseudomallei is a motile Gram-negative rod and grows on most standard media (Wetmore & Gochenour, 1956). It can grow at temperatures of 18–42 °C. Routine laboratory identification of the organism is difficult due principally to problems in differentiating this organism from *Burkholderia (Pseudomonas) cepacia* (Howe *et al.*, 1971). A low but significant level of DNA homology between *B. cepacia* and *B. pseudomallei* has been reported (Ballard *et al.*, 1970; Rogul *et al.*, 1970). Various biochemical testing procedures have been employed for identification of *B. pseudomallei*; however, these have not proved to be totally satisfactory (Ashdown, 1979; Thomas, 1983).

The pathogenic determinants of *B. pseudomallei* have not

Abbreviations: DOC, sodium deoxycholate; KDO, 2-keto-3-deoxyoctonic acid (3-deoxy-D-*mano*-2-octulosonic acid); 2-ME, 2-mercaptoethanol; β-OG, n-octyl β-D-glucopyranoside; SDH, succinate dehydrogenase.

been well studied. A thermolabile toxin and a protease have been described, though not well characterized (Heckley, 1964; Heckley & Nigg, 1958; Rapaport *et al.*, 1961). Endotoxin has also been considered in the pathogenesis of the disease; however, the toxicity of *B. pseudomallei* endotoxin is reportedly low (Rapaport *et al.*, 1961). A competitive ELISA for detection of the thermolabile toxin has been described and should prove useful in defining the role of this toxin in *B. pseudomallei* pathogenesis (Ismail *et al.*, 1987). Intracellular survival of *B. pseudomallei* has also been reported (Kishimoto & Eveland, 1975, 1976a, b) but not confirmed, as it has been shown that immune macrophages will ingest and kill these organisms (Pruksachartvuthi *et al.*, 1990; Tanphaichitra & Srimuang, 1984). In regard to antibody responses to the organism, the haemagglutination test, agglutination test and complement fixation test are an aid in diagnosis, if a fourfold or greater rise in titre is demonstrated in paired sera (Strauss *et al.*, 1969). Distinguishing between IgM and IgG antibody responses has not been helpful in distinguishing active from inactive disease (Ashdown, 1979; Khupulsup & Petchclai, 1986).

Much remains to be discovered regarding the epidemiology, diagnosis, pathogenesis and treatment of disease due to *B. pseudomallei*. Studies of cell structure and function, including the structure and function of outer-membrane proteins, may be important in the understanding of the pathogenesis of disease due to *B. pseudomallei*. In the studies described here we have isolated and characterized the outer membranes of *B. pseudomallei*.

METHODS

Bacteria and growth conditions. Thirteen strains of *B. pseudomallei* were used in this study. These included NCTC 8708, ATCC 23343 and eleven clinical isolates obtained from patients with melioidosis. Cells were grown aerobically with vigorous shaking at 37 °C in Brain Heart Infusion (BHI) broth (Difco). Late-exponential-phase cells were harvested by centrifugation at 10000 ***g*** for 15 min at 4 °C. The cell pellet was immediately frozen in a dry ice/acetone bath and stored at −70 °C.

Outer-membrane preparations by sucrose density gradient centrifugation. Outer membranes were prepared by a modification of the method of Hancock & Nikaido (1978). All of the following manipulations were performed at 4 °C. The cell pellet (approx. 6 g) was thawed and suspended in 60 ml 20% (w/v) sucrose in 30 mM Tris/HCl, pH 8·0. Cells were broken by oscillation with a Branson sonicator equipped with a medium tip (50% duty cycle; power, 9; total time, 30 min). Unbroken cells and large cell debris were removed by centrifugation at 10000 ***g*** for 30 min. The supernatant was decanted and diluted with 20 ml 30 mM Tris/HCl, pH 8·0. For concentration of membranes and removal of cytoplasmic materials such as ribosomes, DNA and RNA, 25 ml supernatant was layered onto a two-step sucrose density gradient consisting of 5 ml 70% (w/v) sucrose and 10 ml 15% (w/v) sucrose in 30 mM Tris/HCl, pH 8·0. The suspension was centrifuged at 26000 r.p.m. (approx. 100000 ***g***) in a Beckman SW28 rotor for 3 h. Both top and bottom layers were removed and diluted with 30 mM Tris/HCl, pH 8·0. Two millilitres of the suspension were layered onto a seven-step sucrose density gradient consisting of 5 ml 70% (w/v) sucrose, 7 ml 58% (w/v) sucrose, 7 ml 52% (w/v) sucrose, 7 ml 48% (w/v) sucrose, 3 ml 40% (w/v) sucrose, and 3 ml 30% (w/v) sucrose. All sucrose solutions were made up in 30 mM Tris/HCl, pH 8·0. The gradients were centrifuged at 26000 r.p.m. for 15 h. Bands were collected from the centrifuge tube with a 12 gauge needle attached to a 10 ml syringe, diluted in cold 30 mM Tris/HCl, pH 8·0, and centrifuged at 170000 ***g*** for 30 min. Pellets were washed three times by resuspension in 30 mM Tris/HCl, pH 8·0, and centrifuged at 170000 ***g*** for 30 min. Membrane pellets were resuspended in a minimum volume of 30 mM Tris/HCl, pH 8·0, and the suspension was used immediately for enzyme analysis or stored at −70 °C.

Nonionic detergents, Triton X-100 (Sigma), CHAPS (Sigma) and n-octyl β-D-glucopyranoside (β-OG; Sigma), and an ionic detergent, sodium deoxycholate (DOC; Sigma), were used for detergent-insoluble preparations. Total membranes (10 mg) described above were suspended in 10 ml 10 mM Tris/HCl, pH 8·0, containing 1% (w/v) detergent, and incubated at 30 °C for 1 h. After centrifugation at 200000 ***g*** for 1 h, pellets were suspended in 10 mM Tris/HCl, pH 8·0, and centrifuged under the same conditions. The pellets obtained were resuspended in 10 mM Tris/HCl, pH 8·0, and were subjected to SDS-PAGE analysis on a 12·5% (w/v) separating gel.

Enzyme assay and chemical analyses. Membranes were assayed for succinate dehydrogenase (SDH) activity by the method of Mizuno & Kageyama (1978). Protein content was determined with a Bio-Rad protein assay kit using bovine serum albumin as the standard. 3-Deoxy-D-*manno*-2-octulosonic acid (2-keto-3-deoxyoctonic acid, KDO) content was estimated by the method of Karkhanis *et al.* (1978) after samples had been hydrolysed in 0·05 M H_2SO_4 at 100 °C for 30 min. Pure KDO (Sigma) was used as the standard.

SDS-PAGE. The protein profiles of membranes were examined by SDS-PAGE by the method of Laemmli (1970). The separating gel consisted of 8, 10 or 12·5% (w/v) acrylamide (monoacrylamide: *N*,*N*′-methylenebisacrylamide, 30:0·8%). Membranes were solubilized in the sample buffer of Hancock & Carey (1979) at various temperatures for 5 min in the presence or absence of 5% (v/v) 2-mercaptoethanol (2-ME).

Protein M_r standards were myosin (200000), *Escherichia coli* β-galactosidase (116250), rabbit muscle phosphorylase *b* (97400), bovine serum albumin (66200), hen egg white ovalbumin (45000), bovine carbonic anhydrase (31000), soybean trypsin inhibitor (21500), and hen egg white lysozyme (14400).

Characterization of outer-membrane proteins. Oligomeric proteins were identified by a modification of the two-dimensional SDS-PAGE system of Hindahl & Iglewski (1984). Membranes were solubilized in the sample buffer in the absence of 2-ME at 30 °C and run on an 8% (w/v) separating gel. A 5 mm wide strip of the gel was cut out, placed in a sealed tube containing the sample buffer in the presence of 2-ME, and heated for 5 min at 95 °C. The gel strip was placed in a horizontal position on top of a second 12·5% (w/v) separating gel, and fixed in position with 1% (w/v) agarose in 0·125 M Tris/HCl pH 6·8, 0·2% SDS. Proteins from both one- and two-dimensional gels were stained with Coomassie brilliant blue R-250.

Peptidoglycan-associated proteins from three *B. pseudomallei* strains (319a, NCTC 8708, ATCC 23343) were identified based on a modification of the method of Hindahl & Iglewski (1984). Briefly, total membranes (envelopes) recovered from whole-cell lysates by centrifugation at 200000 ***g*** for 1 h were suspended in 10 mM Tris/HCl, pH 8·0, containing 2% (w/v) SDS, and individual samples were heated at 37 °C for 15 min. Crude

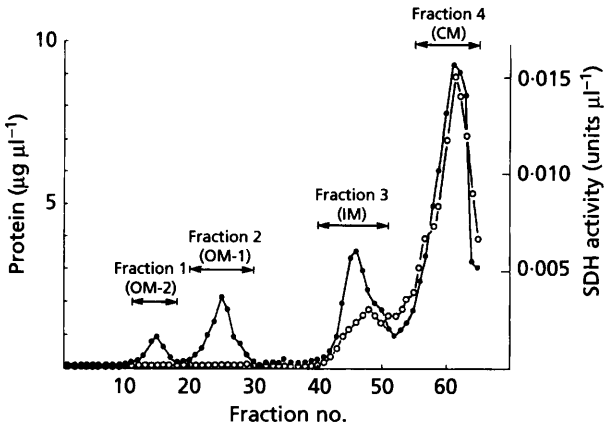

Fig. 1. Profile of *B. pseudomallei* 319a membranes fractionated by sucrose density gradient centrifugation. The direction of sedimentation is from right to left. Succinate dehydrogenase (SDH) activity (○) was determined on a 50 ml sample of each fraction and is expressed as nmol cytochrome *c* reduced min^{-1} (μl fraction)$^{-1}$. Protein concentration (●) was measured by a Bio-Rad protein assay kit.

peptidoglycan was pelleted by centrifugation at 200000 ***g*** and resuspended in 10 mM Tris/HCl, pH 8·0, containing 2% (w/v) SDS and 0·5 M NaCl. This material was incubated at 37 °C for 30 min. Released proteins and peptidoglycan were separated by centrifugation at 200000 ***g*** for 1 h. The pellets obtained were resuspended in Tris/HCl, pH 8·0. After solubilization in sample buffer in the presence of 2-ME at either 37 or 95 °C for 5 min, samples from each step were subjected to SDS-PAGE analysis.

Peptidoglycan-associated proteins were subjected to gel-filtration chromatography (Sephacryl S-200, Pharmacia). Proteins released from crude peptidoglycan in Tris/HCl, pH 8·0, containing 2% (w/v) SDS and 0·5 M NaCl were applied to a column (2·6 × 95 cm) equilibrated in 10 mM Tris/HCl, pH 8·0, containing 1% (w/v) SDS and 0·5 M NaCl. Fractionation was performed at a flow rate of 1 ml min^{-1}, and 2 ml fractions were collected. Eluted protein was measured by A_{280}. Protein peaks were pooled, dialysed against 50 mM sodium phosphate buffer, pH 7·5, and concentrated using Centriprep 10 concentrators (Amicon). SDS was removed by application of the sample to a Sephadex G25 column (Pharmacia, 2·0 × 40 cm) equilibrated in MEGA-9 buffer (50 mM sodium phosphate, pH 7·5, containing 2%, w/v, nonanoyl-*N*-methylglucamide) and eluted with the same buffer. The eluted protein was subjected to ion-exchange chromatography (CM-Sepharose, Pharmacia) using a 1·0 × 10 cm column equilibrated in MEGA-9 buffer. Proteins were fractionated by elution with a 0–0·5 M linear gradient of NaCl in MEGA-9 buffer. Eluted protein was detected by A_{280} and analysed by SDS-PAGE.

Table 1. Physical, enzymic and chemical characteristics of membrane fractions

Membrane fraction	Buoyant density (g ml^{-1})	SDH*	KDO†
CM	1·12	142	0·002
IM	1·21	78	0·016
OM-1	1·28	8	0·038
OM-2	1·33	5	0·042

* Succinate dehydrogenase activity, expressed as nmol cytochrome *c* reduced min^{-1} (mg protein)$^{-1}$.

† Expressed as μmol (mg protein)$^{-1}$.

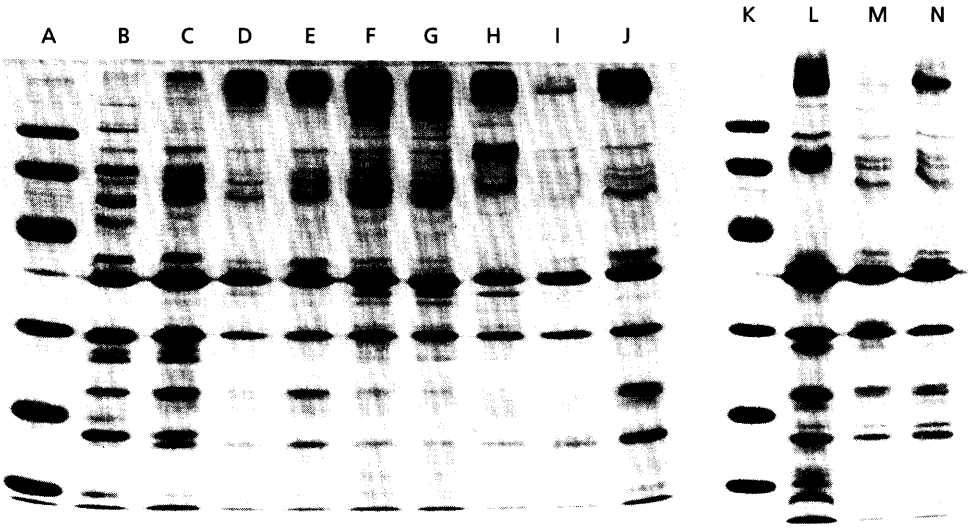

Fig. 2. SDS-PAGE of outer-membrane (OM-1) proteins of 12 strains of *B. pseudomallei*. Each membrane preparation was isolated by sucrose density gradient centrifugation and solubilized in the sample buffer containing 2-ME at 95 °C for 10 min. Lanes: A, M_r standards (top to bottom: phosphorylase *b*, 97400; bovine serum albumin, 66200; hen ovalbumin, 42700; bovine carbonic anhydrase, 31000; soybean trypsin inhibitor, 21500; hen egg white lysozyme, 14400); B, NCTC 8708; C, 199a; D, 230; E, 231a; F, 244a; G, 264a; H, 319a; I, U4; J, U14; K, M_r standards as above; L, U28; M, U31; N, U50.

Proteoliposome membrane studies. Relative diffusion rates for neutral saccharides were determined by the liposome-swelling assay using proteoliposome membranes reconstituted from various amounts of an M_r 110000 protein purified from peptidoglycan as described above and phospholipids (phosphatidylcholine:dicetyl phosphate, 97:3). Diffusion rates were calculated from dA^{-1}/dt as described by Yoshimura *et al.* (1983). The preparation of proteoliposomes and determination of diffusion rates were as described by Gotoh *et al.* (1989).

RESULTS

Membrane fractionation

Lysis by sonication of *B. pseudomallei* strain 319a cells grown in BHI broth, followed by sucrose density gradient centrifugation, produced four distinct bands. The location of each band in the gradient corresponded to the interface of the sucrose step. Bands were designated in order of increasing density: cytoplasmic membrane (CM; interface between 30% and 40%), intermediate band (IM; interface between 40% and 48%), outer membrane-1 (OM-1; interface between 52% and 58%), and outer membrane-2 (OM-2; interface between 58% and 70%). The CM band was a clear red colour; the IM and OM-1 were yellowish-white, and OM-2 was a white band.

Protein contents of membrane fractions are shown in Fig. 1. Assessment of SDH activity and KDO content (Table 1) was used to determine the efficiency of separation of cytoplasmic membranes from outer membranes. Although SDH activity as a marker of cytoplasmic membrane was highest in the CM fraction, this enzyme was also detected in the IM, OM-1 and OM-2 fractions. Specific activity of this enzyme in the CM fraction was approximately 18-fold and 28-fold greater than in OM-1 and OM-2, respectively. The CM fraction and the OM-1 fraction had protein contents of 0·1 and 0·3 mg (mg dry wt)$^{-1}$, respectively. Assay for KDO was used as an indicator of LPS content in the membrane fractions. These materials gave a positive thiobarbituric acid (TBA) reaction product which absorbed strongly at 549 nm, and appeared to be spectrally identical to that produced by the purified KDO (the standard). We observed little difference in the amount of KDO between OM-1 and OM-2, and a higher amount of KDO (approximately 20-fold) was found in these fractions than in the IM fraction (Table 1).

To determine which major proteins were common among various strains of *B. pseudomallei*, we isolated outer membranes from 12 different strains by sucrose density gradient centrifugation and examined the preparations using the conditions described above (Fig. 2). The outer-membrane protein profiles were strikingly similar among the 12 strains; however, slight variability among strains was noted. Two outer-membrane proteins, M_r 38000 and M_r 31000, were consistently noted as major outer-membrane proteins in all strains examined. Additionally, proteins of M_r 70000, 24000 and 17000 were consistently seen in outer-membrane preparations of all strains examined.

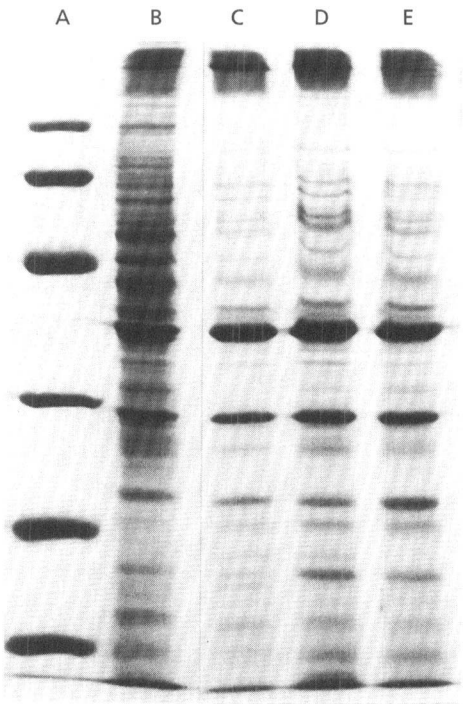

Fig. 3. Protein profiles of outer membranes of *B. pseudomallei* strain 319a isolated by detergent extraction. Membranes were extracted as described in the text and solubilized in sample buffer containing 2-ME at 95 °C for 10 min. Lanes: A, M_r standards as described in the legend to Fig. 2; B, 1% β-OG insoluble fraction; C, 1% DOC insoluble fraction; D, 1% Triton X-100 insoluble fraction; E, 1% CHAPS insoluble fraction.

Comparison of techniques for isolation of the outer membrane

For isolation of outer membranes from other Gram-negative bacteria, sucrose density gradient centrifugation is the most widely used technique and is based on the different density between the outer and inner membranes (Osborne *et al.*, 1972). Alternative techniques for membrane isolation have been developed based on the observation that the outer membranes are relatively insoluble in some detergents (Murphy & Loeb, 1989). Therefore, preparation of detergent-insoluble vesicles was employed in an attempt to identify a more convenient way for the isolation of *B. pseudomallei* outer membranes using Triton X-100, β-OG, CHAPS and DOC. Fig. 3 shows SDS-PAGE protein profiles of these vesicle preparations from strain 319a. Outer-membrane protein profiles virtually identical to those observed in the OM-1 fraction obtained by the sucrose density centrifugation procedure were seen in the detergent-insoluble vesicle preparations. Thus, the vesicle technique may be suitable for the detection of the major outer-membrane proteins of *B. pseudomallei*. Extraction with Sarkosyl, which is a frequently used detergent for outer membrane isolation, produced inconsistent results in our attempts to isolate OM from *B. pseudomallei*, and may not be suitable for use with this organism.

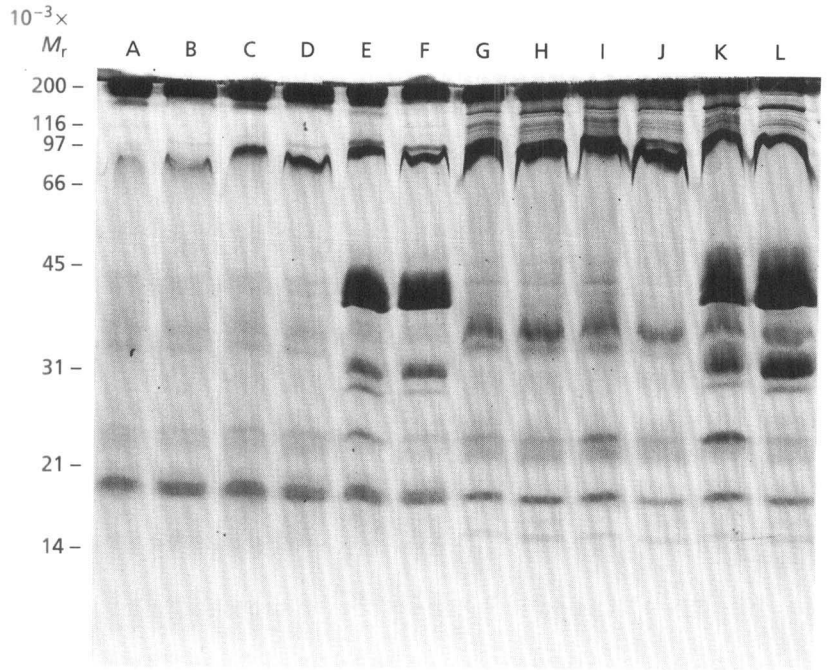

Fig. 4. Effects of solubilization temperature and 2-ME on the electrophoretic mobilities of the OM-1 proteins of *B. pseudomallei* strains 319a (lanes A–F) and NCTC 8708 (lanes G–L). Each membrane was solubilized in the sample buffer with 2-ME (lanes B, D, F, H, J and L) or without 2-ME (lanes A, C, E, G, I and K) at 30 °C (lanes A, B, G and H), 60 °C (lanes C, D, I and J) or 95 °C (lanes E, F, K and L).

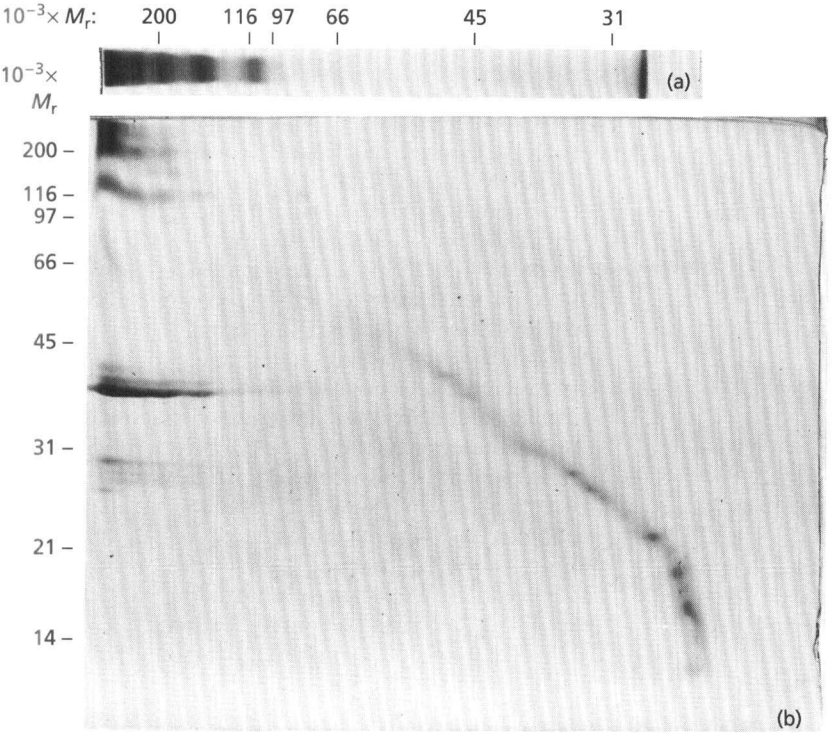

Fig. 5. Two-dimensional SDS-PAGE of heat-modifiable proteins. Fraction OM-1 was solubilized in the sample buffer containing 2-ME at 37 °C for 30 min before running in the first dimension (a). A gel strip cut from the first dimension was placed in sample buffer containing 2-ME and heated at 95 °C for 10 min before running in the second dimension (b).

Depolymerizable proteins

To determine the effect of solubilization temperature and 2-ME on the electrophoretic mobility of the outer-membrane proteins, the outer membranes (OM-1 fractions) of *B. pseudomallei* strains 319a and NTCC 8708 were solubilized in sample buffer in the presence or absence of 2-ME at 30, 60 or 95 °C, and subjected to SDS-PAGE. Few differences were evident between the strains (Fig. 4). Mobility of outer-membrane proteins was unaffected by the presence or absence of 2-ME in sample buffer at all temperatures tested. The outer membrane proteins of M_r 37 000, 36 000 and 27 000 are commonly heat-modifiable in both strains. These protein bands moved as large aggre-

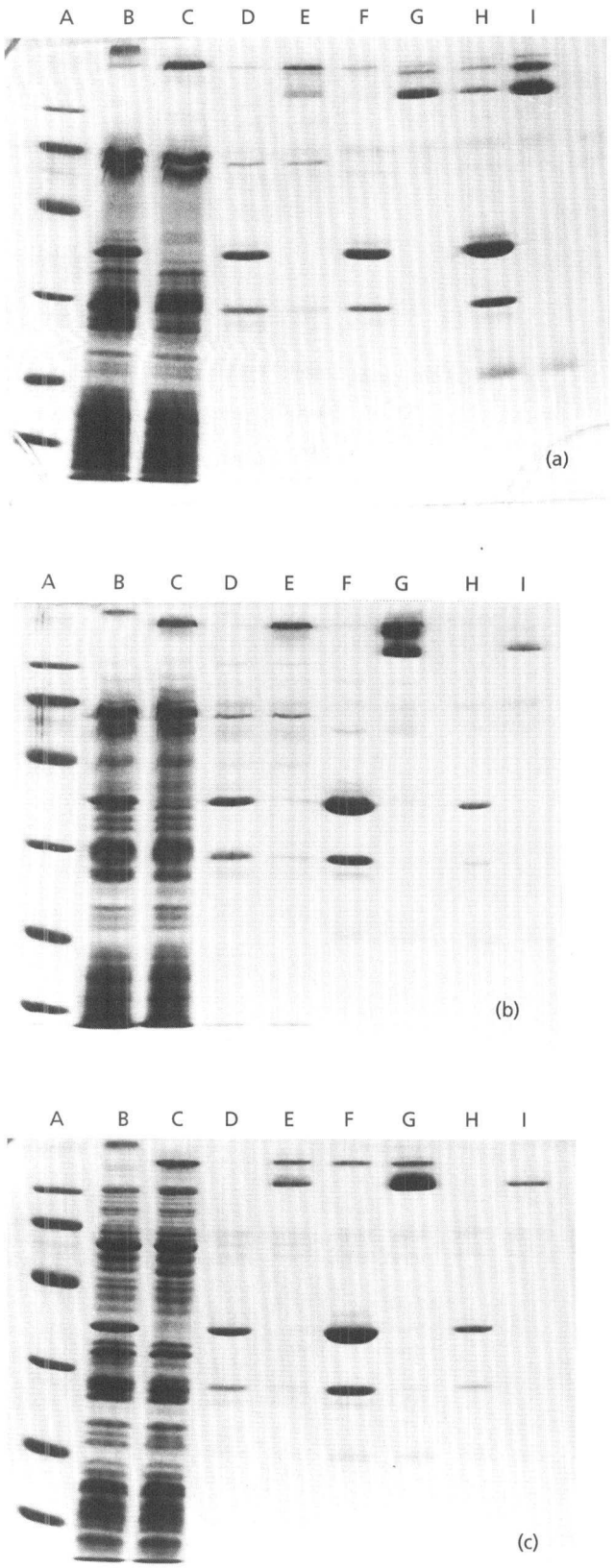

Fig. 6. SDS-PAGE of peptidoglycan-associated proteins from *B. pseudomallei* strains 319a (a), NCTC 8708 (b) and ATCC 23343 (c). SDS-soluble materials from cell envelopes were extracted with 10 mM Tris/HCl, pH 8·0, containing 2% SDS, at 37 °C (lanes B and C). Insoluble material (crude peptidoglycan) was pelleted by centrifugation at 200000 *g* (lanes D and E). This material was resuspended in 10 mM Tris/HCl, pH 8·0, containing 2% SDS and 0·5 M NaCl. Proteins released into the buffer containing NaCl are displayed in lanes F and G. Proteins bound to peptidoglycan are displayed in lanes H and I. Samples were solubilized in sample buffer either at 95 °C (lanes B, D, F and H) or at 37 °C (lanes C, E, G and I). Lane A contains M_r standards as described in the legend to Fig. 2.

gates at the top of the 12·5% separating gel. The M_r 37000 and 36000 proteins were prominent bands in samples heated to 95 °C (Fig. 4), but could not be clearly identified in samples incubated at 30 or 60 °C in the 12·5% gel (Fig. 4). To further characterize the heat-modifiable proteins, two-dimensional SDS-PAGE analysis was performed with the OM-1 fraction. The first-dimensional fractionation of the sample incubated at 30 °C in the absence of 2-ME by 8% gel electrophoresis yielded at least four bands, of apparent M_r 110000, 160000, 200000 and 222000 (Fig. 5a). The gel slice containing these proteins was heated at 95 °C for 5 min in sample buffer in the presence of 2-ME and subjected to second-dimensional electrophoresis. All four bands observed after first-dimensional electrophoresis disassociated to M_r 37000, 36000 and 27000 bands, and some other membrane proteins were seen on the diagonal and extensional lines of the gel (Fig. 5b).

Peptidoglycan-associated proteins

Outer-membrane proteins of M_r 120000, 110000, 38000 and 31000 were found to be associated with SDS-insoluble material, i.e. crude peptidoglycan, in preparations from *B. pseudomallei* strain 319a (Fig. 6a). This was in contrast to the results obtained for strains NCTC 8708 (Fig. 6b) and ATCC 23343 (Fig. 6c), where outer-membrane proteins of M_r 110000 and 38000 were found to be peptidoglycan-associated.

Proteins released from crude peptidoglycan in Tris/HCl, pH 8·0, containing 2% SDS and 0·5 M NaCl, were used as the starting material in a protocol designed to purify peptidoglycan-associated proteins. Fig. 7 illustrates the elution profile of this starting material following chromatographic separation on a Sephacryl S-200 column. The protein which eluted as a single major peak was pooled, dialysed against starting buffer, and protein separated by ion-exchange chromatography. The elution profile of protein released by NaCl gradient elution is shown in Fig. 8 and indicates that the majority of the protein eluted as a single peak at a NaCl concentration of 0·4–0·5 M. When this material was examined by SDS-PAGE, the protein migrated as a single major band of M_r 110000 when solubilized at 37 °C and as major bands of M_r 110000, 38000 and 30000 when solubilized at 95 °C (Fig. 9). As may also be seen in Fig. 9, no differences were noted in the SDS-PAGE profiles of protein obtained from *B. pseudomallei* strain 319a versus strain ATCC 23343.

This material which migrated as a single major protein

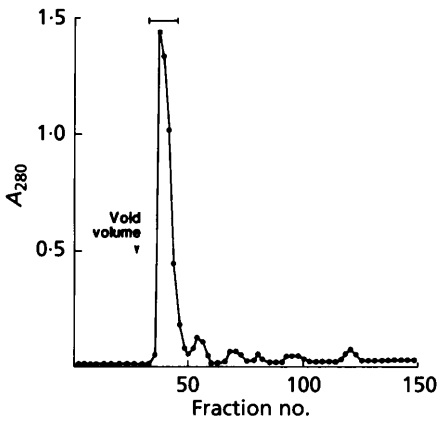

Fig. 7. Elution profile of peptidoglycan-associated proteins subjected to gel-filtration chromatography (Sephacryl S-200, Pharmacia). Proteins released from crude peptidoglycan in Tris/HCl, pH 8·0, containing 2% SDS and 0·5 M NaCl, were applied to a column (2·6 × 95 cm) equilibrated in 10 mM Tris/HCl, pH 8·0, containing 1% SDS and 0·5 M NaCl. Fractionation was performed at a flow rate of 1 ml min^{-1}, and 2 ml fractions were collected. Eluted protein was measured by A_{280}.

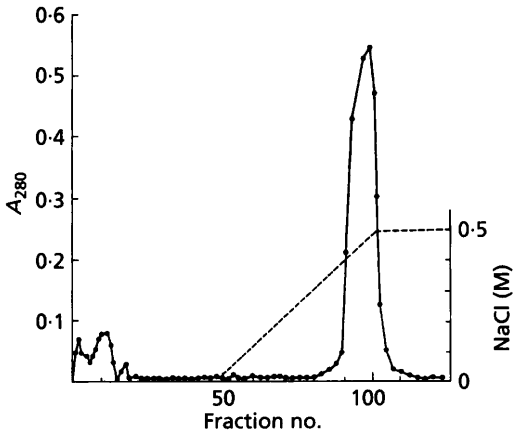

Fig. 8. Protein peaks eluted from the Sephacryl S-200 column were pooled, dialysed against 50 mM sodium phosphate buffer, pH 7·5, and concentrated using Centriprep 10 concentrators (Amicon). SDS was removed by application of the sample to a Sephadex G25 column as described in Methods. The eluted protein was subjected to ion-exchange chromatography (CM-Sepharose, Pharmacia) using a 1·0 × 10 cm column equilibrated in MEGA-9 buffer. Proteins were fractionated by elution with a 0–0·5 M linear gradient of NaCl in MEGA-9 buffer. Eluted protein was detected by A_{280} and analysed by SDS-PAGE.

band of M_r 110000 at 30 °C and as a band of M_r 38000 at 100 °C was used to reconstitute proteoliposomes for the purposes of measuring relative diffusion rates of saccharides through liposome membranes in order to determine if this protein could function as a porin. It can be seen in Fig. 10 that this is indeed the case, in that

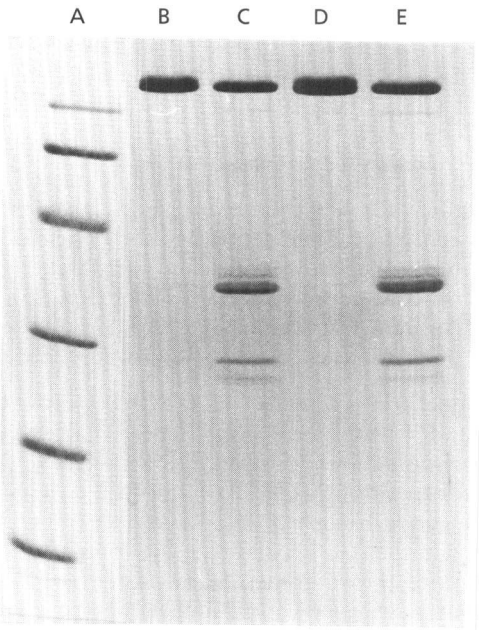

Fig. 9. SDS-PAGE of peptidoglycan-associated protein purified from *B. pseudomallei* strains 319a (lanes B and C) and ATCC 23343 (lanes D and E) by gel-filtration and ion-exchange chromatography. Samples were solubilized in sample buffer either at 37 °C (lanes B and D) or at 95 °C (lanes C and E). Lane A contains M_r standards as described in the legend to Fig. 2.

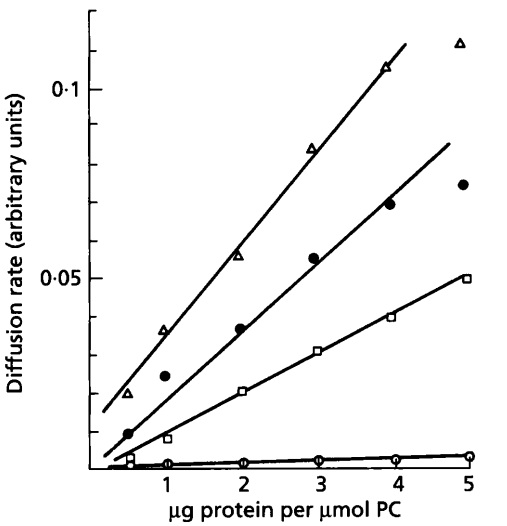

Fig. 10. Relative diffusion rates for neutral saccharides were determined by the liposome-swelling assay using proteoliposome membranes reconstituted from various amounts of an M_r 115000 protein purified from peptidoglycan and phospholipids (phosphatidylcholine:dicetyl phosphate, 97:3). The preparation of proteoliposomes and determination of diffusion rates were as described by Gotoh *et al.* (1989). △, Arabinose; ●, glucose; □, mannose; ○, sucrose. PC, phosphatidylcholine.

increasing the ratio of the M_r 110000 protein to phospholipid resulted in a dose-dependent increase in the diffusion rates of small saccharides such as arabinose (M_r 150),

glucose (M_r 150) and mannose (M_r 180), but not sucrose (M_r 342).

DISCUSSION

We have described the isolation and characterization of cytoplasmic and outer membranes from *B. pseudomallei* by sucrose density gradient centrifugation. The SDS-PAGE protein profiles of the cytoplasmic membrane (CM) and the inner membrane (IM) were distinct from those of the outer-membrane fractions, OM-1 and OM-2, whereas the profiles of OM-1 and OM-2 were virtually identical.

Five major protein bands, of M_r 70000, 38000, 31000, 24000 and 17000, were consistently detectable in the outer-membrane fractions by SDS-PAGE; the M_r range of these proteins is typical of the outer membrane of Gram-negative bacteria. These five proteins were found to be common to 12 different *B. pseudomallei* strains. Furthermore, the outer membrane protein profiles among these strains were strikingly similar. Among these common major proteins, the M_r 38000 protein was the most abundant in the OM-1 and OM-2 fractions, and the M_r 38000, protein entered SDS-PAGE gels only after solubilization at elevated temperature, suggesting that this protein exists as large aggregates. Similar conditions are required to resolve the major outer-membrane proteins from other Gram-negative bacteria (Hindahl & Iglewski, 1984).

No major proteins in the *B. pseudomallei* outer membrane were affected by the presence of the reducing agent, 2-ME, in SDS-PAGE sample buffer. The M_r 38000 protein from *B. pseudomallei* was recovered from SDS-insoluble material (i.e. crude peptidoglycan) and was not liberated by treatment at temperatures exceeding 60 °C. This characteristic is similar to some major outer-membrane proteins of other Gram-negative bacteria such as porins in *Escherichia coli* (Lugtenberg *et al.*, 1977), *Legionella pneumophila* (Hindahl & Iglewski, 1984) and *Alcaligenes faecalis* (Ishii & Nakae, 1988), and the OprF protein in *Pseudomonas aeruginosa* (Hancock *et al.*, 1990). This M_r 38000 protein was present as aggregates of apparent M_r 110000 when outer-membrane proteins were solubilized in SDS-PAGE sample buffer at 30 °C.

Mutharia & Hancock (1985) demonstrated that one class of monoclonal antibodies directed against OprF of *P. aeruginosa* recognized an outer membrane protein of *P. syringae*. Furthermore, Ullstrom *et al.* (1991) have shown that only the nine members of rRNA homology group I of *Pseudomonadaceae*, including *P. syringae*, hybridized with the cloned *oprF* gene. These results suggested that some homologous outer membrane proteins exist in rRNA homology group I of the genus.

B. pseudomallei is a member of rRNA homology group II, which includes *B. cepacia* and *B. mallei* (Palleroni, 1977). We compared the protein profile of *B. pseudomallei* to that of *B. cepacia* (unpublished observations). Both protein profiles were very similar, the M_r of the most abundant proteins of the two species being 38000. Anwar *et al.* (1983) have reported that this protein is an oligomeric protein. Furthermore, Parr *et al.* (1987) observed that the oligomeric composition of this protein and the minor protein of M_r 27000 forms a diffusion pore for permeation of small hydrophilic solutes through the *B. cepacia* outer membrane. These observations support the theory that the M_r 38000 protein forms aggregates of M_r 110000. In the present studies, we have demonstrated that the purified M_r 110000 protein in the *B. pseudomallei* outer membrane dissociates into an M_r 38000 protein. We have also shown that the M_r 38000 protein serves as a porin in *B. pseudomallei*. Further studies to elucidate the role of this molecule in antibiotic permeability and the relationship of this molecule to porins in other *Burkholderia* and *Pseudomonas* spp. are in progress.

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