Functional reconstitution, gene isolation and topology modelling of porins from *Burkholderia pseudomallei* and *Burkholderia thailandensis*

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The intracellular pathogen *Burkholderia pseudomallei* is the causative agent of tropical melioidosis, and *Burkholderia thailandensis* is a closely-related Gram-negative bacterium that does not cause serious disease. Like other bacteria, the major outer membrane (OM) porins of *Burkholderia* strains, *BpsOmp38* and *BthOmp38* may have roles in antibiotic resistance and immunity. We purified both proteins and found them to be immunologically related, SDS-resistant, heat-sensitive trimers with M_r of approx. 110 000. In functional liposome-swelling assays, both proteins showed similar permeabilities for small sugar molecules, compatible with a pore diameter of between 1.2 and 1.6 nm. Secondary structure analysis by FTIR (Fourier-transform infrared) spectroscopy revealed almost identical spectra with predominantly β -sheet structures, typical of bacterial porins. MALDI-TOF (matrix-assisted laser-desorption ionization-time

of flight) MS and ESI/MS (electrospray ionization MS) analysis of each protein showed extensive sequence similarities to the OpcP1 porin from *Burkholderia cepacia* (later found to be 76.5 % identical). Based on information from the incomplete *B. pseudomallei* genome-sequencing project, the genes encoding Omp38 were identified and amplified by PCR from *B. pseudomallei* and *B. thailandensis* genomic DNA. The nucleotide sequences are 99.7 % identical, and the predicted processed proteins are 100 % identical. Topology prediction and molecular modelling suggest that this newly-isolated and cloned porin is a 16-stranded β -barrel and the external loops of the protein could be important determinants of the immune response to infection.

Key words: *Burkholderia*, cloning, diffusion pore, Omp38, topology.

INTRODUCTION

Burkholderia pseudomallei (formerly *Pseudomonas pseudomallei*), the causative agent of tropical melioidosis, is a motile Gram-negative bacterium mainly endemic to South-East Asia [1]. In addition to occurring in people who come into contact with contaminated soil or water in endemic areas, the infection is more common in immunosuppressed patients and must be recognized early and treated with appropriate antibiotics to ensure survival. Although the bacterium can cause acute or sub-acute symptoms, most cases are asymptomatic and it can activate many years after the initial exposure, causing chronic illness [2]. However, little is known about the climatic, physical, chemical and biological factors, which control the proliferation and survival of *Burkholderia* spp.

Burkholderia thailandensis is a closely related non-pathogenic soil organism originally isolated in Central and North-East Thailand [3]. Although the two organisms are very similar ([4,5], but see [5a]), they are different in their genetic and biochemical properties. The rRNA sequence of *B. thailandensis* differs from that of *B. pseudomallei* by 15 nucleotides, and there are significant differences in genomic macrorestriction patterns between the organisms [6]. The biochemical profiles of these two species differ in that *B. thailandensis* utilizes L-arabinose, whereas *B. pseudomallei* does not [7]. *B. thailandensis* is also substantially less virulent in Syrian golden hamster models of infection [4]. It has been reported that more than 99 % of cases of melioidosis

are caused by *B. pseudomallei*, whereas *B. thailandensis* is only responsible for less than 1 % [8]. Therefore efficient identification of *B. pseudomallei* and *B. thailandensis* is crucial in guiding clinical management of patients with suspected melioidosis.

Treatment of tropical melioidosis has proven to be extremely difficult. *B. pseudomallei* secretes potent bacteriocins that block protein synthesis in macrophages, as well as in phagocytes. These bacteriocins may be responsible for the extensive abscess formation associated with the disease [9]. There is no published evidence to relate non-pathogenicity of *B. thailandensis* with high susceptibility to antibiotics. However, it has been well established that *B. pseudomallei* is resistant to many antibiotics including β -lactams, aminoglycosides, macrolides and polymyxins [1,10]. In some cases, this is because of low permeability of these compounds through the outer membrane of the bacteria [11]. Given that these hydrophilic molecules often diffuse through water-filled porins [12], the characteristics of specific porin channels may be significant determinants of antibiotic sensitivity. In conjunction with peptidoglycan and LPS (lipopolysaccharide), porins are receptors for bacteriophages and bacteriocidins, and they also have a significant structural role in maintaining the integrity of the cells [13]. In addition, porins may play an important role in the immune response to infection. For example, an unrelated porin in another intracellular pathogen, the major outer membrane (OM) protein (MOMP) of *Chlamydia trachomatis*, is an immunodominant surface antigen that elicits both antibody production and T-cell response [14].

Abbreviations used: ESI/MS, electrospray ionization MS; FTIR, Fourier-transform infrared; L1 (etc.), Loop 1 (etc.); LPS, lipopolysaccharide; MALDI-TOF, matrix-assisted laser-desorption ionization-time-of-flight; MS/MS, tandem MS; OM, outer membrane.

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The sequences for Omp38 from *Burkholderia pseudomallei* and *Burkholderia thailandensis* have been deposited in the DDBJ, EMBL, GenBank[®] and GSDB Nucleotide Sequence Databases under the accession numbers AY312416 and AY312417 respectively.

Porins have been identified in many Gram-negative bacteria, and several porins from *Escherichia coli* (e.g. OmpF, OmpC, OmpG and PhoE) have been well-characterized in terms of their biochemical, functional, genetic, immunochemical and structural properties [15]. In particular, all porins appear to contain abundant β -sheet structures [16]. The OM of the related organism *Burkholderia cepacia* (responsible for fatal lung infections in cystic fibrosis) contains an 81 kDa porin designated OpcP0, which also shows low permeability to β -lactams [17]. This protein contains two subunits: a major 36 kDa protein designated OpcP1, and a minor 27 kDa protein, designated OpcP2. OpcP1 has been cloned and expressed in *E. coli* [18]. The predicted OpcP1 protein precursor contains 361 residues, including a 20-residue signal sequence. The calculated M_r of 35 696 for the mature protein is in agreement with the OpcP1 protein band of 36 kDa by SDS/PAGE. Similarity of the amino acid sequences between OpcP1 and several other Gram-negative bacterial porins strongly suggests that OpcP1 is the pore-forming component of OpcP0. This notion is also supported by the similarity of the hydropathy plots of OpcP1 and the major *E. coli* porin, OmpF [17].

In the present study, we describe the isolation of *BpsOmp38* from *B. pseudomallei* and *BthOmp38* from *B. thailandensis*, and characterize them using FTIR (Fourier-transform infrared) spectroscopy, MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS and nanoESI/MS. Peptide mass fingerprinting showed the amino acid sequences of the proteins to be identical, and enabled us to identify and isolate the gene encoding *BpsOmp38* from the partially sequenced *B. pseudomallei* genome. We have also cloned its homologue from *B. thailandensis* genomic DNA, and the predicted proteins have been subjected to topology prediction and structural modelling.

EXPERIMENTAL

Materials

Bacterial culture medium and bacteriological agar were purchased from Scharlau Chemie (Barcelona, Spain). Cetyltrimethylammonium bromide, dithiothreitol, iodoacetamide, ammonium hydrogen carbonate, D-stachyose, D-melezitose, D-sucrose, L-arabinose, D-glucose and D-mannose were from Acros Organics (Morris Plains, NJ, U.S.A.). Proteinase K and trypsin (sequencing grade) were from Promega. Sephacryl S-200[®] HR resin and Dextran T-40 were from Amersham Biosciences. Detergents used for protein preparation were purchased from Carlo Erba Reagenti (Milan, Italy). All other reagents for general laboratory use were from Sigma-Aldrich and Carlo Erba Reagenti. *Taq* DNA polymerase was purchased from Promega. Primers for PCR amplification were synthesized by Prologo Singapore (Singapore Science Park II, Singapore).

Preparation of OM proteins

B. pseudomallei (ATCC 23 343) and *B. thailandensis* (ATCC 700 388) were grown in LB (Luria-Bertani) broth at 37 °C with vigorous shaking. Cells were harvested at late exponential phase by centrifugation at 10 000 *g* at 4 °C for 20 min. Crude peptidoglycan was isolated using a modification of the method of Gotoh et al. [19]. Briefly, cells from 2 litres of late-exponential culture were washed once and resuspended in 10 ml of 10 mM Tris/HCl, pH 8.0, containing 1 mM PMSF and 2 mg of hen's-egg lysozyme. The suspension was sonicated using a Sonopuls Ultrasonic homogenizer with a 6 mm diameter probe (50 % duty cycle; amplitude setting, 20%; total time, 5 min), and large cellular debris and unbroken cells were removed by centrifuga-

tion at 10 000 *g* for 30 min. Cell membranes were recovered from the lysates by microcentrifugation at 12 000 *g* for 1 h, suspended in 10 mM Tris/HCl, pH 8.0, containing 0.5 % (w/v) SDS, and heated at 30 °C for 1 h to solubilize most of the remaining cytoplasmic and membrane proteins. A complex of non-solubilized peptidoglycan sheets (crude peptidoglycan) was then pelleted by microcentrifugation at 12 000 *g* for 1 h and heated in 4 ml of 10 mM Tris/HCl, pH 8.0, containing 2 % (w/v) SDS and 0.5 M NaCl at 37 °C for 1 h. Solubilized peptidoglycan-associated proteins were then separated from insoluble peptidoglycan by microcentrifugation at 12 000 *g* for 1 h.

Proteoliposome-swelling assay

The preparation of proteoliposomes and the determination of diffusion rates were based on the detailed methods described previously [19–21]. Briefly, mixtures of 2.4 μ mol of phosphatidylcholine and 0.2 μ mol of diacetyl phosphate in chloroform were dried by vacuum-centrifugation and the phospholipid films were resuspended in 0.2 ml of distilled water and mixed with 50 μ g of purified Omp (outer membrane protein). The mixtures were sonicated for 7 min in a water bath at 20 °C, dried overnight by vacuum-centrifugation, and resuspended in 0.2 ml of 10 mM Tris/HCl, pH 8.0, containing 15 % (w/v) Dextran T-40, to form proteoliposomes. The proteoliposomes were diluted into 0.6 ml of an iso-osmotic sugar solution (prepared as described in the Results section), and changes in absorbance were measured at 400 nm for 60 s. The relative permeabilities of the pore-forming proteins were assumed to be proportional to the initial swelling rates [22], and each experiment was repeated at least three times using liposomes without any protein, or liposomes containing BSA, as negative controls.

SDS/PAGE and immunological analysis

Purified *B. pseudomallei* Omp38 (2 μ g) was separated by SDS/PAGE as a monomer using 12 % (v/v) acrylamide and 8 M urea in a Laemmli buffer system [23]. Following electrophoresis, the protein was stained with Coomassie Blue. After destaining, the protein band was excised from the gel, emulsified with 50 μ l of Freund's complete adjuvant (Pierce), and used to raise rabbit polyclonal antiserum [24]. Antisera titres and cross-reactivity between *B. pseudomallei* and *B. thailandensis* proteins (5 μ g each) were analysed by Western blotting with enhanced chemiluminescence (ECL[®]; Amersham Biosciences) detection, using 1:10 000 dilutions of the anti-Omp38 antiserum in PBS, pH 7.4, in the presence of 0.2 % (v/v) Tween 20 and 5 % (w/v) non-fat dried milk. Protein concentrations were determined using a BCA (bicinchoninic acid) kit (Pierce).

Protein identification and peptide mass analysis by MALDI-TOF MS and ESI/MS

Protein identification was performed in two steps: for the initial analysis, the protein bands from SDS gels (see above) were excised, destained, reduced, alkylated with iodoacetamide and digested with sequencing-grade trypsin (Promega) following a standard protocol [25]. After overnight digestion at 37 °C, the peptides were extracted and dried in a SpeedVac vacuum centrifuge. A small fraction of these tryptic peptides was analysed by MALDI-TOF MS (Voyager-DE Pro in reflective mode) in an α -cyano-4-hydroxycinnamic acid matrix for the peptide 'mass fingerprinting'. The remaining peptides were separated by capillary HPLC using a 0–40 % linear gradient of acetonitrile containing 0.1 % acetic acid, then detected directly by ESI/MS

(Thermo Finnigan LCQ Deca) using the proprietary 'triple play' mode for obtaining MS/MS (tandem MS) sequence information for the relevant peptides. Databank searching was performed with the MS-Fit program (<http://prospector.ucsf.edu/>) for MALDI mass fingerprint data, and with the Sequest search program (<http://fields.scripps.edu/sequest/index.html>) for MS/MS data from the HPLC-MS run. In some experiments, the proteins (not gel pieces) were completely digested with sequencing-grade Trypsin in a buffer containing 5 mM CaCl_2 and 100 mM NH_4HCO_3 , and the resulting peptides were applied to an Agilent 1100 HPLC connected to the LCQ ESI/MS. Mass spectra of whole proteins were obtained with linear MALDI-TOF (Voyager-DE Pro) using sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) as the matrix.

FTIR spectroscopy measurements

FTIR spectroscopy measurements were carried out using a Bruker IFS-66 FTIR spectrometer equipped with a DTGS (deuterated triglycine sulphate) detector. The porins were measured in home-made demountable equipped cells with CaF_2 windows. Aliquots of the protein solutions (5 μl , 10 mg/ml) were first dried under vacuum for 5 min with $^2\text{H}_2\text{O}$ (5 μl). To compensate for $^2\text{H}_2\text{O}$ absorption, buffer solutions were subtracted using the same cells. The path length was about 50 μm . FTIR spectra of the protein solutions were recorded using identical scanning parameters. Typically, the nominal physical resolution was 4 cm^{-1} using a Happ-Genzel apodization function for Fourier-transformation and a zero-filling factor of 4 to yield a data encoding interval of one data point per wavenumber and 32 scans were accumulated before performing the Fourier-transform analysis to optimize the signal-to-noise ratio. The protein spectrum was obtained after subtracting the buffer spectrum from the spectrum of the protein solution measured under the same conditions. To eliminate spectral contributions due to atmospheric water vapour, the instrument was continuously purged with dry air. Spectral contributions from residual water vapour were eliminated using water vapour spectra measured under identical conditions. The absorption region 1000–4000 cm^{-1} was primarily monitored. However, to obtain secondary structure information on the proteins, the amide I and II absorption regions between 1500 and 1800 cm^{-1} were specifically acquired for second derivative plots.

Preparation of genomic DNA and cDNA cloning and sequencing

Genomic DNA was prepared using the protocol of Ausubel et al. [26]. PCR primers were synthesized by Prologo. Amplification reactions were carried out using *Taq* polymerase and the products were cloned into pGEM[®]-T vector and sequenced in both directions using an automated sequencer [Biotechnology Sequencing Unit (BSU), Thailand].

Structural prediction and molecular modelling

Protein sequences were aligned using ClustalW (<http://www.ebi.ac.uk/clustalw/>) and displayed using GeneDoc (<http://www.psc.edu/biomed/genedoc/>). Signal peptide cleavage sites were predicted using SignalP V1.1 (<http://www.cbs.dtu.dk/services/SignalP/>) and transmembrane domains obtained using prediction of the protein localization sites (<http://www.psorth.org/psorth/index.html>). RPS-BLAST (<http://www.ncbi.nih.gov/BLAST>) was used to identify conserved domains, and protein structures were modelled and displayed using 3D-PSSM (<http://www.sbg.bio.ic.ac.uk/~3dpssm>), 123D+ search (<http://123d.ncifcrf.gov>), and Swiss-Model (<http://www.expasy.org/swissmod>).

Figure 1 Elution profile and urea SDS/PAGE of *BpsOmp38* and *BthOmp38*

(A) Gel-filtration chromatography (Sephacryl S-200[®] HR, Amersham Biosciences). Proteins released from *B. pseudomallei* crude peptidoglycan in 10 mM Tris/HCl, pH 8.0, containing 2% (w/v) SDS and 0.5 M NaCl, were applied to a column (1.5 cm $\times$ 95 cm) equilibrated in 10 mM Tris/HCl, pH 8.0, containing 1% (w/v) SDS and 0.5 M NaCl. Fractionation was carried out at a flow rate of 1 ml $\cdot$ min⁻¹, and 2 ml fractions were collected. The A_{280} of the eluted protein was measured. The profile for *B. thailandensis* protein was identical. The inset displays the obtained calibration curve determining the M_r of *BpsOmp38* and *BthOmp38*. (B) SDS/PAGE analysis of *BpsOmp38* and *BthOmp38* proteins purified from *B. pseudomallei* under heated (H) or unheated (UH) conditions. Samples were prepared in the sample buffer containing 8 M urea at 25 $^{\circ}\text{C}$ for UH *BpsOmp38* and UH *BthOmp38* or at 95 $^{\circ}\text{C}$, 5 min for H *BpsOmp38* and H *BthOmp38*.

RESULTS

Purification of *BpsOmp38* and *BthOmp38*

Crude peptidoglycan isolated from *B. pseudomallei* and *B. thailandensis* (see the Experimental section) was used as the starting material to purify trimeric OM porins. Following SDS-extraction, the peptidoglycan fraction was purified further by size-exclusion chromatography (200 ml Sephacryl S-200[®] HR column) in the presence of 1% (w/v) SDS and 0.5 M NaCl. Eluted protein was detected by measuring the absorbance of the eluate at 280 nm, and peak fractions were analysed by SDS/PAGE (12.5%) in the presence of urea [23].

For each organism, the major protein peak obtained after size-exclusion chromatography (Figure 1A) corresponded to an M_r of approx. 100 000. As shown in Figure 1(B), this relatively high molecular mass band shifted to an M_r of approx. 38 000 after heating at 95 $^{\circ}\text{C}$ for 5 min. Taken together, the results from size-exclusion chromatography and SDS/PAGE suggested that the isolated proteins were SDS-resistant homotrimers comprising monomers of an M_r of approx. 38 000, consistent with trimeric

Table 1 Omp38 purification from both *B. pseudomallei* and *B. thailandensis*

The results are the means of two separate experiments for each protein.

Purification step	<i>B. pseudomallei</i> (mg)	Yield (%)	<i>B. thailandensis</i> (mg)	Yield (%)
1. Crude peptidoglycan	492.0	100	296.0	100
2. OM fraction	175.4	36	107.4	36
3. Peptidoglycan-associated fraction				
0.5 % (w/v) SDS extraction	5.4	1.1	3.4	1.1
2 % (w/v) SDS and 0.5 M NaCl extraction	5.0	1.0	3.0	1.0
4. Sephacryl S200 filtration and dialysis	0.8	0.2	0.6	0.2

Figure 2 Immunoblots of *BpsOmp38*, *BthOmp38* and *E. coli* BL21 (DE3) porin

(A) Non-denatured (unheated) SDS/PAGE analysis and (B) corresponding immunoblot using anti-*BpsOmp38* polyclonal antibodies. (C) Denatured (heated at 95 °C for 5 min) SDS/PAGE analysis and (D) corresponding immunoblot. Note that because of differences in exposure times, it was not possible to carry out immunoblots of both heated and unheated samples on the same gel.

OM porins. Similar results were seen in ten independent preparations. The proteins isolated from *B. pseudomallei* and *B. thailandensis* were therefore designated *BpsOmp38* and *BthOmp38* respectively. The final yield of each protein obtained from a 2 litre culture was approx. 0.2% (800 µg and 600 µg for *B. pseudomallei* and *B. thailandensis* respectively) (Table 1).

Antibody production and immunoblotting analysis

The protein band corresponding to the *B. pseudomallei* Omp38 monomer was excised from a gel and used to raise a rabbit polyclonal antiserum as described in the Experimental section. The antibodies cross-reacted with both proteins on immunoblots, and recognized both the high molecular mass, presumed trimeric, protein band in non-denatured samples (Figures 2A and 2B) and a 39 kDa protein from *E. coli* BL21(DE3) OM fraction (Figures 2C and 2D). In addition, the antibodies also strongly recognized the protein band corresponding to OpcP0 (81 kDa), the monomeric protein bands corresponding to OpcP1 (36 kDa) and OpcP2 (27 kDa), as well as a non-identified 18 kDa of *B. cepacia* OM crude fraction (results not shown) [17]. None of these proteins was recognized by rabbit pre-immune serum, suggesting that the *Burkholderia* proteins may be structurally related to at least one porin from *E. coli* and *B. cepacia*.

Secondary-structure analysis using FTIR

The second derivative spectra obtained from FTIR measurements revealed extensive secondary structure similarity between

Figure 3 FTIR analysis of *BpsOmp38* and *BthOmp38*

Secondary derivative spectra of *BpsOmp38* (—) and *BthOmp38* (···) between 1500 and 1800 cm⁻¹ were normalized using the IR band of Tyr (1514 cm⁻¹) and superimposed. Signals at amide I region: 1626 cm⁻¹ represents β -sheets; 1652 cm⁻¹ and 1674 cm⁻¹ represent turns and loops; 1696 cm⁻¹ represents antiparallel β -pleated sheets; 1727 cm⁻¹ and 1740 cm⁻¹ represent LPS [27].

Figure 4 Liposome-swelling assay

(A) Relative diffusion rates for neutral saccharides were determined by liposome-swelling assays using proteoliposomes containing various amounts of *BpsOmp38* and *BthOmp38*, together with 2.4 µmol of phosphatidylcholine and 0.2 µmol of diacetyl phosphate (final volume, 600 µl). The following symbols represent, in order, L-arabinose (○); D-glucose (□); D-mannose (△); D-galactose (◇); N-acetylglucosamine (●); D-sucrose (■); D-melezitose (▲); stachyose (◆). (B) Relative permeabilities. Initial relative swelling rates for liposomes containing purified *B. pseudomallei* (△) and *B. thailandensis* (○) porins plotted on a logarithmic scale against the *M_r* of the permeating solute (abscissa). Results are means ± S.E.M. (*n* = 3 experiments for each protein; rate for arabinose = 100%). The size-dependent swelling rates for the two porins are indistinguishable, and the straight line is a regression fit to all the data. The broken lines indicate the 95% confidence limits of the fit. The near-limiting *M_r* defined as 5% relative permeability through both *Burkholderia* porins is 650 (cf. 450 for *E. coli* porin reconstituted under the same conditions; results not shown).

Figure 5 Alignment of *BpsOmp38* and *BthOmp38* with other OM porins

Porins were selected on the basis of a high degree of amino acid identity with Omp38. The secondary structures of *BpsOmp38* and *BthOmp38* were predicted by comparison with porins of known structure (OmpF and Omp 32). *Ecoli2OmpF*, OmpF porin from *E. coli*; *CacOmp32*, anion-selective porin from *C. acidovorans*; *BceOPCP1*, OpcP1 porin from *B. cepacia*; *BpsOmp38*, Omp38 porin from *B. pseudomallei* A. T. C. C. 23343; *BthOmp38*, Omp38 porin from *B. thailandensis* A. T. C. C. 700388. Open box, β strand; closed box, loop; hatched box, α helix; circle, turn.

BpsOmp38 and *BthOmp38*, and their spectra could almost be directly superimposed, apart from differences in the ester carbonyl stretching regions (signals at 1727 cm^{-1} and 1740 cm^{-1}) (Figure 3) [27], which most probably originate from residual LPS tightly bound to the porin. It was noticeable that *BpsOmp38*

contained more associated LPS than *BthOmp38*. The secondary derivative plots in the amide I ($1600\text{--}1700\text{ cm}^{-1}$) region demonstrated that both proteins contained predominantly β -sheet, as indicated by the two distinct peaks at 1626 cm^{-1} and 1696 cm^{-1} , typical for the antiparallel β -pleated sheets of porins [27,28].

Minor contributions at 1652 cm^{-1} and 1674 cm^{-1} were tentatively assigned to be loop and turn structures.

Liposome swelling assay

Our data strongly suggested that *BpsOmp38* and *BthOmp38* were likely to be porins, and that they had been isolated in a non-denatured, possibly native, conformation. In particular, both membrane proteins retained significant secondary and quaternary structures (comprising mainly β -sheet, and SDS-resistant, heat-sensitive trimers respectively). The apparent retention of normal folding suggested that they might form functional channels, and we tested this idea by assaying solute transport in liposome-swelling assays.

Following reconstitution, the relative diffusion rates of L-arabinose, D-glucose, D-mannose, D-galactose, *N*-acetylglucosamine, D-sucrose and D-melezitose into multilamellar liposomes were determined, as described in the Experimental section, after establishing iso-osmotic buffer conditions in the presence of the large impermeable sugar, stachyose (667 Da). Absorbance changes for each sample were positive, and increased linearly with time from the outset of each recording (results not shown), confirming that the starting conditions were iso-osmotic and the proteoliposomes were properly equilibrated. The diffusion rates of all the sugars that were tested increased linearly with protein concentration (Figure 4A), and the rates clearly depended on the M_r of the solute.

As shown in Figure 4(B), the relative diffusion rates through *BpsOmp38* and *BthOmp38* porins were indistinguishable, with a 'limiting' M_r of approx. 650 (defined as the value corresponding to 5% relative permeability). The rates were clearly different from the rates through *E. coli* OM porin (mainly OmpF, under the growth conditions used), which, when reconstituted under exactly the same conditions, had a limiting M_r of approx. 450 (results not shown).

Gene isolation, cloning and sequencing

We analysed the primary structures of *BpsOmp38* and *BthOmp38* in order to identify the corresponding genes from genome sequences. As identified by MALDI-TOF MS, the masses of *BpsOmp38* and *BthOmp38* were 37 159 and 37 108 respectively. This is identical within the mass-dependent peak broadening of MALDI-TOF protein measurements. MALDI mass fingerprints of tryptic fragments obtained from in-gel digests revealed almost identical peptide ensembles. When these peptides were separated on a capillary HPLC and subjected to partial fragmentation (ESI/MS and MS/MS sequence analysis), eight *BpsOmp38* peptides (TDVYAQAVYQR, GSEDLGGGLK, SLWSVGAGVDQSR, LNTNGDVAVNNTVK, AYSAGASYQFQGLK, QAFVGLSSNYGTVTLGR, AIFTLESGFNIGNGR and NANASIYNGDLS-TPFSTSINQTAATVGLR) and four identical *BthOmp38* peptides (GSEDLGGGLK, SLWSVGAGVDQSR, QAFVGLSSNYGTVTLGR and AIFTLESGFNIGNGR) gave an unambiguous match to the OM porin OmpP1 of *B. cepacia*. Each of these peptides could also be aligned with a single predicted open reading frame in contig 836 of the *B. pseudomallei* genome (<http://www.ncbi.nlm.nih.gov/sutils/genomtable.cgi>).

Accordingly, we amplified full-length *BpsOmp38* and *BthOmp38* cDNAs, each containing 1112 nucleotides, from appropriate genomic DNA using the following sense primer: 3'-ATGAACAAGACTCTGATTGTTGCA-5', and antisense primer: 3'-GAAGCGGTGACGCAGACCAA-5' using *Taq* DNA polymerase. The cDNAs were cloned into pGEM[®]-T vector and were

Figure 6 Predicted transmembrane topologies of *BpsOmp38* and *BthOmp38*

Trajectories of successive C_{α} carbons plotted against residue number for the identical predicted *B. pseudomallei* and *B. thailandensis* porins (see the Experimental section). The Z value (scaled from 0–1) corresponds to the predicted position of each C_{α} carbon relative to the plane of the membrane. Values < 0.4 represent positions in periplasmic turns, values between 0.4 and 0.6 are predicted to be in transmembrane crossings, and values > 0.6 represent extracellular domains. Sixteen such membrane crossings (often with lengths compatible with a tilted β -strand) can be identified, and the corresponding external loops are labelled L1–L8.

transformed into *E. coli* host strain DH5 α . The sequences of both inserts were determined [29] and have been deposited in the GenBank[®] database under the accession numbers AY312416 and AY312417 for *BpsOmp38* and *BthOmp38* respectively. The DNA sequences were >98% identical, giving almost 100% identity of the putative amino acid sequences translated from the corresponding nucleotide sequences with the exception that the second *N*-terminal residue, asparagine, in the *BpsOmp38* sequence was replaced by aspartic acid in the *BthOmp38* sequence.

When these sequences were used to calculate the masses of our tryptic peptides, we could identify almost every peptide from the prior HPLC–MS analysis. As expected, the exceptions were peptides containing fewer than four amino acid residues. The first amino acid detected by HPLC–MS was Ala⁴¹ and the last was (as expected) Arg³⁷¹. The peptide composition as analysed by HPLC–MS was identical for both proteins.

Protein structure prediction

SignalP was used to predict Omp38 signal sequences, and when these were removed, the calculated M_r of the identical, processed, leaderless proteins was 37 163.5. This value agrees well with the estimate from MALDI–TOF analysis of the purified proteins.

The predicted amino acid sequences of the pre-proteins were next analysed for similarity with *Burkholderia* and other Gram-negative bacterial porins (Figure 5). The membrane topology of Omp38 was predicted by the method of Diederichs et al. [30], and the three-dimensional structure of the protein was analysed using 123D+ and Swiss-Model. Omp38 had most similarity to the anion-selective porin Omp32 [Protein DataBank (PDB) code 1e54a], (35.8% identity when using Swiss-Model). The predicted topology of 16 transmembrane domains, eight loops and eight periplasmic turns is compatible with a 16-stranded porin

Figure 7 Predicted three-dimensional structures of *BpsOmp38* and *BthOmp38* using Swiss-Model

The representation was drawn using Swiss-pdb Viewer version 3.7b2 and was built by alignment with the closest homologue (Omp32 porin from *C. acidovorans*). This figure shows 16 strands and eight loops. The longest loop (L3) (Ser¹²⁷–Asp¹⁵⁴) folds inside the barrel. (A) Stereo image (top view) of Omp38. (B) Side view of Omp38.

β -barrel in which the N-terminus forms a salt bridge with the C-terminus immediately after a periplasmic turn, and based on both transmembrane topology prediction and three-dimensional structure modelling, Omp38 has a β -barrel structure with 16 transmembrane strands, eight short periplasmic turns and eight external loops (Figures 6 and 7).

The longest loop, Loop 3 (L3; Ser¹²⁷ → Asp¹⁵⁴), contains two short, antiparallel β -strands at the very beginning and the end of the loop, and a short right-handed α -helix (Tyr¹¹⁹ → Leu¹²⁶) was found to precede the long L3. This loop is characteristic of many porins as a pore-confined loop responsible for the size-selectivity of the channel [31,32].

DISCUSSION

Porins play crucial roles in the interactions between Gram-negative bacteria and their environment. In the present study, we purified and characterized the major OM porin of the pathogenic bacterium, *B. pseudomallei*, and the equivalent porin from the closely-related non-pathogenic bacterium, *B. thailandensis*. When the oligomeric proteins (M_r 110 000) were heated to 95 °C, they disassociated into M_r 38 000 monomers. This process was irreversible, and it has been suggested that upon heating, porin β -sheets are converted into α -helices, preventing reassembling into trimers [33,34].

When reconstituted in liposomes for functional analysis, the results were consistent with those expected for 'general diffusion porins' [15,35]. Although the highest diffusion rate corresponded to the smallest sugar, L-arabinose (150 Da), D-glucose (180 Da) permeated measurably faster than other sugars with the same molecular mass (D-mannose and D-galactose). *B. pseudomallei* and *B. thailandensis*, like other Gram-negative bacteria, utilize glucose as a primary source of carbon for cell growth, and enhanced glucose permeation may be functionally significant. *B. thailandensis* can utilize L-arabinose, whereas *B. pseudomallei* cannot [4]. However, the two porins displayed a similar relative diffusion rate for L-arabinose (Figure 4), suggesting that Omp38 is not a sugar-specific channel for L-arabinose. On the basis of very similar sugar permeability through the unusually large channels of *E. coli* OmpG and the 42 kDa *Serratia liquefaciens* porin [22,36], the diameter of the 38 kDa porin protein can be estimated to be in the range 1.2–2.0 nm. The size-exclusion limit of Omp38 was larger than that of the *E. coli* porin when various sugars were subjected to permeability measurements under the same conditions (results not shown). Hence, *Bps*Omp38 and *Bth*Omp38 porins represent large channels, which allow a greater variety of nutrients to be taken up.

FTIR analysis showed that Omp38 contained predominantly β -strands and was likely to form the antiparallel β -barrel characteristic of bacterial OM porins [37–39]. The IR patterns of both proteins were almost identical with slight differences found in the turns and loops, and LPS regions. It is not known whether this may be reflected in genuine differences in the final conformations of the otherwise identical proteins. The analysis also showed that Omp38 preparations always contained varying amounts of LPS. Although both were isolated and purified by the same procedure, *Bps*Omp38 contained a greater amount of LPS than *Bth*Omp38 (Figure 3). The data in Figure 1(B) also suggest the presence of LPS in the purified proteins, with a 'ladder' of several bands in the unheated samples (cf. Eisele and Rosenbusch [40]). The idea of a strong porin–LPS interaction is supported by the fact that solubilization of Omp38 by a strong detergent (SDS) and gel-filtration failed to remove the LPS completely. However, small amounts of LPS remaining in the *Bps*Omp38 sample helped to improve solubility of the protein, and did not show much effect on the immunoblots, MS analysis and liposome-swelling assays carried out in this study.

We isolated the gene encoding Omp38 from *B. pseudomallei* and *B. thailandensis* genomic DNA after identifying the genes following protein MS analysis. We found that almost all the back-translated amino acid sequences of both organisms could be matched to peptide fragment data from capillary HPLC–ESI/MS of the native porins. Some amino acid sequences in the mature proteins could not be identified using HPLC–ESI/MS. At high concentrations, membrane proteins tend to aggregate, and this might hinder analysis by HPLC–ESI/MS and MALDI–MS [41].

Omp38 is one of a large number of porins that usually form trimers and have individual subunits that fold into 16- or 20-stranded barrels [16]. Topology and three-dimensional prediction programs strongly suggest that Omp38 folds as a 16-stranded barrel with eight loops and eight turns, with the closest similarity to the anion-selective porin from *Comamonas acidovorans* [31]. The longest loop (L3) folds into the barrel. This loop, which contains a short α -helix, has been suggested to constrict the size of the pore [32,39]. L8, which is the second longest loop, also folds into the barrel interior and contributes to the formation of the particularly narrow external channel opening.

Based on the three-dimensional structure of *E. coli* OmpF and PhoE [39], L3 was found to fold into the channel and to define a constriction zone, which governs pore activity. The

cluster of acid residues in L3, located in front of a positively charged cluster of basic amino acid residues (anti-L3), creates an intense electrostatic field in the pore. The resulting charge distribution regulates diffusion of solutes through the pore [32,39,42]. Several residues of L3 have been suggested to be involved in channel activity [32,43], especially Asp¹¹³ and Glu¹¹⁷. These residues participate in the architecture of the negative cluster. In addition, site-directed mutagenesis data revealed that Gly¹¹⁹ was associated with colicin resistance, drastic alterations in diffusion and antibiotic susceptibility, and local structural alterations focused inside the lumen [44,45].

In the present study, we have cloned and sequenced the genes encoding Omp38 porins from both *B. thailandensis* and *B. pseudomallei*, and have shown the proteins to be identical. Further work is underway to determine the importance of Omp38 in antibiotic resistance and immunity, and to determine its three-dimensional structure by protein crystallization and X-ray diffraction.

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