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Outer Membrane Proteome of *Burkholderia pseudomallei* and *Burkholderia mallei* From Diverse Growth Conditions

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Abstract

Burkholderia mallei and *Burkholderia pseudomallei* are closely related, aerosol-infective human pathogens that cause life-threatening diseases. Biochemical analyses requiring large-scale growth and manipulation at biosafety level 3 under select agent regulations are cumbersome and hazardous. We developed a simple, safe, and rapid method to prepare highly purified outer membrane (OM) fragments from these pathogens. Shotgun proteomic analyses of OMs by trypsin shaving and mass spectrometry identified >155 proteins, the majority of which are clearly outer membrane proteins (OMPs). These included: 13 porins, 4 secretins for virulence factor export, 11 efflux pumps, multiple components of a Type VI secreton, metal transport receptors, polysaccharide exporters, and hypothetical OMPs of unknown function. We also identified 20 OMPs in each pathogen that are abundant under a wide variety of conditions, including in serum and with macrophages, suggesting these are fundamental for growth and survival and may represent prime drug or vaccine targets. Comparison of the OM proteomes of *B. mallei* and *B. pseudomallei* showed many similarities but also revealed a few differences, perhaps reflecting evolution of *B. mallei* away from environmental survival toward host-adaptation.

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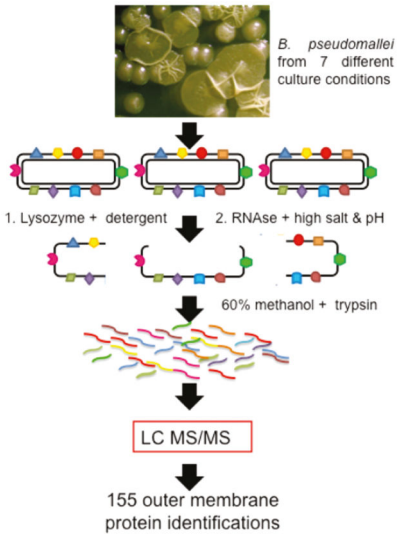
Supporting Information

Supplementary tables. This material is available free of charge via the Internet at http://pubs.acs.org.



Keywords

Burkholderia; LC–MS/MS; melioidosis; glanders; outer membrane; vaccine; OMP

INTRODUCTION

Many macromolecules in the outer membrane (OM) of bacterial pathogens, especially proteins exposed on the cell surface, are important virulence factors and targets for host immune recognition. Identification of abundant and/or novel outer membrane proteins (OMPs), and characterization of their roles in pathogen physiology, disease, and defense against the host, is an important preliminary step in development of diagnostics, vaccines, and therapeutics. Until recently, OMPs were identified individually using two-dimensional gel electrophoresis (2-DE) of solubilized OMs followed by peptide mass fingerprint (PMF) identification of individual spots.[1–4] However, recent advances in liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) technology has opened new avenues to identify en masse large numbers of proteins in whole cells or subcellular fractions.[5] The OM is an excellent subcellular fraction to target for en masse shotgun proteomics since its protein complexity is relatively low. On the other hand, its purification to homogeneity free of inner membranes (IM), cell wall components, and cytoplasmic proteins is challenging. Moreover, many OMPs are difficult to solubilize or release from the OM and often separate poorly during 2-DE.[5]

Current OM purification methods for MS analyses involve cell breakage by French pressure cell or sonication followed by differential centrifugation and carbonate or detergent extraction of crude membranes.[1] However, for potentially lethal human pathogens such as *Burkholderia pseudomallei* (Bp) and *Burkholderia mallei* (Bm) that can infect via inhalation,[6,7] sonication, or other aerosolizing cell-breakage methods must be avoided. Bp causes melioidosis, a disease endemic to Southeast Asia, while Bm causes glanders, a disease that largely affected only horses and mules until it was eradicated from most areas

by the early 1950s.[6,7] Bm is a deletion clone of Bp that has lost >1200 genes by insertion sequence-mediated deletion;[8] essentially all genes of Bm are found in Bp with >99.7% DNA sequence identity. Both are classified as biothreat agents requiring highly regulated biosafety level 3 (BSL3) and select agent containment making them difficult to work with. Thus, knowledge of their OM and surface constituents essential for development of countermeasures is much more limited than for other pathogens. Although identification of 35 surface proteins of Bp under one growth condition has been reported,[9] the majority were predicted or documented cytoplasmic proteins; only 3 were predicted OMPs. Moreover, most expected OM surface proteins (e.g., flagellar components, secretins, efflux pumps and TonB-type receptors) were not detected.

To more accurately, quantitatively, and comprehensively assess the OM proteome of Bp and Bm, we developed a safe and rapid method to purify OM fragments in BSL3-containment and then used trypsin shaving and LC–MS/MS to identify >155 OMPs from these pathogens grown under a variety of conditions.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

B. pseudomallei strain DD503 and *B. mallei* ATCC 23344 were grown in the following media: (1) M9 minimal salts (0.6% Na_2PO_4 + 0.3% KH_2PO_4 + 0.05% NaCl + 0.1% NH_4Cl + 0.02% $MgSO_4$ + 0.015% $CaCl_2$)[10] + 3% glycerol, to mimic an oligotrophic water environment; (2) M9 minimal salts + 3% glycerol+1× BME and MEM (20 amino acids; Sigma-Aldrich), to mimic a more nutrient rich water environment; (3) LB (1% tryptone + 0.5% yeast extract + 0.5% NaCl)[10] + 3% glycerol, a common media used for culturing of Bp and Bm; (4) a tissue culture medium, Dulbecco's Modified Eagle's Medium (DMEM) High Glucose (0.01% Na_2HPO_4 + 0.04%KCl + 0.6% NaCl + 0.1% NH_4Cl + 0.01% $MgSO_4$ + 0.02% $CaCl_2$ 0.1% glucose +0.01% Na-pyruvate +10^{-5} % $FeNO_3$ + 20 amino acids and vitamins (Thermo-Fisher) + 10% fetal bovine serum, to mimic a host tissue environment; (5) 1% glucose + 50% fetal bovine serum to model growth in blood or serum; (6) 3% glycerol + 3% yeast extract + 3% casamino acids, to model a nutrient-rich soil environment; (7) DMEM High Glucose + 10% fetal bovine serum with a near confluent monolayer of RAW 264.7 murine macrophages to mimic host microbe interactions involving phagocytic immune system cells. Bacteria were grown in 250-mL flasks overnight at 37 °C shaking at 200 rpm; when using DMEM, growth was in unshaken tissue culture flasks in 5% CO_2. Cells were grown for 16–24 h from initial cell densities of 0.1 $OD_{600\ nm}$ until harvest at mid- or late-log phase ($OD_{600\ nm}$ between 0.8 and 1.5 depending on media).

OM Preparation

Cells were harvested by centrifugation at 7500× *g* for 10 min, washed once with 0.1 volume of 20 mM Tris-HCl pH 7.0 + 3 mM $MgCl_2$ (TM) and frozen at −80 °C. Pellets (200 $OD_{600\ nm}$) were resuspended in 3 mL of 10 mM Tris-HCl pH 7 + 25% sucrose. Lysozyme and protease inhibitor, 4-(2-Aminoethyl) benzenesulfonyl fluoride, were added to 5 mg/mL and 0.1 mg/mL, respectively. After 20 min at 37 °C, $MgCl_2$ was added to 3 mM. After another 20 min one volume of 4% Triton X-100 in TM was added. After mixing 4 min the

lysate was frozen at −80 °C and thawed at 37 °C twice with 1 min of mixing between cycles. After centrifugation at 7500× *g* for 15 min, the supernatant was removed and recentrifuged. The second supernatant was removed, filter-sterilized by passing through a 0.2 μm polyethersulfone filter, and confirmed as sterile by plating on LBG agar. The outside of tube with the frozen sterile filtrate was decontaminated with SporKlenz, removed from BSL3 containment, thawed, and centrifuged at 110 000× *g* for 1 h at 5 °C. The supernatant was discarded and the pellet of crude OM was resuspended in 0.3 mL of TM by bath sonication for 5 min (Bransonic 1510). One volume of 4% Triton X-100 was mixed in. After 30 min on ice the sample was centrifuged at 7500× *g* for 15 min and the resultant supernatant centrifuged at 110 000× *g* for 1 h. The pelleted membranes were resuspended in 0.4 mL of TM as before and centrifuged (7500× *g*; 15 min). The OM-containing supernatant (2 mg protein) was removed and diluted 4-fold with 5 mM Tris-HCl pH8, and centrifuged at 110 000× *g* for 1 h. The pellet was resuspended by sonication in 0.6 mL 5 mM Tris-HCl and a mixture of RNase A + RNase T1 added to 0.5 mg/mL. After incubation at 37 °C for 10 min, ethylenediaminetetraacetic acid (EDTA) was added to 10 mM and incubation continued for 40 min. The sample was adjusted to 0.05 M Na_2CO_3 and 1 M NaCl. After 1 h on ice and 15 min at 37 °C, the nonvesicular membranes were pelleted at 110 000× *g* for 1.25 h. The membrane pellet was washed twice by sonication in 0.1 mL of 0.1 M Na_2CO_3 /1 M NaCl, incubation at 4 °C for 30 min, followed by 1 h centrifugation at 110 000× *g*. The final yield of purified OM was 300 μg of protein as measured with bicinchoninic reagent (Pierce). 2-keto-3-deoxyoctanoate (KDO) was measured using 50 μg of OM protein.[11] RNA content was estimated by agarose gel electrophoresis after extraction of 50 μg of OM protein with phenol followed by precipitation with 3 volumes of ethanol at −80 °C.

Shotgun Proteomics

Purified OM (75 μg protein) was resuspended in 0.2 mL of 50 mM ammonium bicarbonate pH 8.2 (ABC), adjusted to 10 mM dithiothreitol (DTT), and placed at 37 °C for 45 min; iodoacetamide (IAA) was added to 30 mM and the sample incubated at 25 °C for 30 min in the dark after which 0.2 mL of ABC was added. The reduced, alkylated membranes were centrifuged at 110 000× *g* for 1.25 h. The pellet was suspended by sonication in 0.2 mL of ABC, treated with 5 μg of modified trypsin (Promega) at 37 °C overnight and then centrifuged 110 000× *g* for 1.25 h. The pellet was sonicated for 5 min in 0.2 mL of ABC, placed at 90 °C for 15 min, then quick chilled on ice. Methanol was added to 60% followed by 5 μg of trypsin. After 18 h at 37 °C, the sample was centrifuged at 110 000× *g* for 1.25 h and the supernatant removed, vacuum-dried, dissolved in 0.1% trifluoroacetic acid (TFA), and centrifuged at 10 000× *g* for 5 min. The supernatant (5–10 μg peptides) was dried, dissolved in 0.1% formic acid in 2% acetonitrile (ACN), and nitrogenbomb loaded on a 75 μm × 105 mm C18 reverse phase column (packed in house, YMC GEL ODS-AQ120 Å, Waters). Peptides were eluted directly into the nanospray source of a linear ion trap MS instrument (Thermo Finnigan LTQ)[12] with a 160-min linear gradient of 4–80% ACN in 0.1% formic acid over 100 min at a flow rate of ~250 nL/min. Full scan MS spectra were acquired from *m/z* 300 to 2000 followed by 8 MS/MS events of the most intense ions. A dynamic exclusion window was used to prevent the same *m/z* value from being selected for 12 s after acquisition. Data were automatically acquired using Xcalibur (ver. 2.0.7, Thermo

Fisher) and subsequently analyzed using SEQUEST (Bioworks 3.3, Thermo Fisher)[13] and the sequences of all predicted proteins of Bp K96423 or Bm ATCC 23344 (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi). Data was filtered to a 1% false discovery rate on the protein level using ProteoIQ[14] and identifications based on <2 peptides discarded. The abundance of each identified protein was estimated from the normalized spectral counts, calculated as the number of spectral counts (SpC) for each protein divided by its number of amino acids (L) divided by the sum of SpC/L for all proteins in the experimental data set. Replicate analyses of membranes from two identically grown LBG cultures showed >90% coincidence in OMP identification indicating, a high level of reproducibility.

Peptide Mass Fingerprinting

Gel slices containing stained bands from sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) were twice incubated in 0.3 mL of 50% ABC in methanol at 37 °C for 30 min and the liquid discarded. Then 0.5 mL 100% ACN was added. After 15 min ACN was discarded, slices were vacuum-dried, and incubated in 10 mM DTT in ABC at 60 °C for 20 min; IAA was then added to 0.1 M. After 30 min at 25 °C, the supernatant was removed and slices extracted twice with 50% ABC in methanol for 15 min. Slices were dried and treated with 1 μg of modified trypsin in 50 μL of 10% ACN in ABC for 18 h at 37 °C. The supernatant was removed and gel slices were incubated with 60 μL of 0.1% TFA in 50% ACN at 25 °C for 40 min. Both peptide-containing supernatants were combined, dried to 10 μL, 35 μL of 0.1% TFA added, and peptides bound to an activated C-18 NuTip (Glygen) by filling and expulsion 20 times. After washing with 0.1% TFA the peptides were eluted with 2.5 μL of matrix-assisted laser desorption/ionization (MALDI) matrix (α-cyano-4-hyroxycinnamic acid; Sigma C2020) onto a MALDI plate and run on a Bruker Daltronics Autoflex time-of-flight(TOF) mass spectrometer.[15] Peptide masses (0.95 to 2.5 kDa) were analyzed with Mascot PMF (http://www.matrixscience.com/search_form_select.html) using NCBInr (no missed cleavages; ±2 Da mass tolerance). Protein identifications were based on 5 to 18 peptide masses with sequence coverage ranging between 30 and 42%. Expect values ranged from 0.05 to 10^{-8} with p-values <0.05; Z-scores ranged from 77 to 146.

Bioinformatic Methods

Using keywords such as outer membrane, lipoprotein, flagella, pili, secretin, porin, receptor, and surface at Integrated Microbial Genomes (IMG) (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi), we manually assembled a list of clusters of orthologous groups (COG) family numbers containing proteins likely to be OM-associated. Using these COG numbers we extracted a list of ~150 predicted OMPs of Bp. Similarly, we extracted a list of all Bp proteins scoring >8 in the OM localization category in PSORTb (http://www.psort.org/genomes/genomes.pl). Lists were combined and redundant entries removed resulting in a list of 201 potential membrane proteins (Table S1; Supporting Information). Approximately 145 proteins were found on both the PSORTb- and IMG-derived lists representing a catalog of highly probable OMPs of Bp.

RESULTS AND DISCUSSION

Purification and Characterization of Outer Membrane Fragments

To isolate an OM-enriched fraction, Bp or Bm cells were treated with lysozyme and then freeze–thawed in the presence of Triton X-100 and $MgCl_2$. This lysed the cells and solubilized proteins of the cytoplasm, periplasm, and inner membrane (IM). The insoluble OM fragments were recovered from this lysate by differential centrifugation. Initial LC–MS/MS analyses of tryptic peptides from these crude OM preparations identified >300 proteins. However, a significant portion of these were abundant, documented cytoplasmic proteins (e.g., TCA cycle enzymes, amino acyl-tRNA synthetases, RNA polymerase subunits). IM proteins were in relatively low abundance, but many ribosomal proteins were detected suggesting ribosomal contamination. To remove any ribosomes and proteins adsorbed to or trapped inside vesicular membranes, the OM-enriched fraction was treated with RNase and EDTA, and then subjected to extensive washing with 1 M NaCl/0.1 M Na_2CO_3 pH 11. This increased the KDO to protein ratio of the OM preparation from 0.01 μmol/mg protein to 0.03, near the value of 0.04 reported for highly purified OM of Bp;[16] rRNA was no longer detectable. Thus, ribosomes and other cytoplasmic contamination were dramatically reduced by the treatment. SDS-PAGE analysis (Figure 1) suggested this highly purified OM preparation contained 5 major protein species (bands) comprising the majority of Coomassie Blue stained material. The banding profile is similar to that of sucrose density gradient purified OM preparations from Bp.[16] Tryptic peptides from each band were analyzed by MALDI-TOF MS. Four of the five bands were identified as containing probable OMPs: BPSL0294, BPSS0879, and BPSL2522 that are predicted porins and BPSL1775, a predicted iron uptake receptor. These data indicated that the OM preparation was highly enriched for OMPs. Tryptic peptides could not be obtained from the fuzzy fifth (~100 kDa) band.

Preliminary LC–MS/MS Analyses

The highly purified OM fraction was treated with trypsin and the released peptides analyzed by LC–MS/MS. Over 225 proteins were identified at >99% confidence. Four of the most abundant proteins identified were the same OM proteins detected by SDS-PAGE and PMF; however, peptides from some ribosomal and other abundant cytoplasmic proteins were still detected at moderate to low levels. Abundant IM proteins (e.g., NADH dehydrogenase, F1–F0 ATPase, permeases[17]) were not detected. The pelleted trypsin-shaved membranes were then washed with 1 M NaCl/0.1 M Na_2CO_3, dissolved in 60% methanol, and treated with trypsin to release peptides from OMPs that were more deeply imbedded in the lipid bilayer. LC–MS/MS analyses of these peptides identified ~120 proteins. On the basis of their annotation and COG family assignments, the majority appeared to be likely OMPs, but ~20% were abundant cytoplasmic proteins (e.g., ribosomal proteins, subunits of RNA polymerase, pyruvate dehydrogenase, oxoglutarate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, translation elongation factors, amino acyl-tRNA synthetases, GroEL, glutamine/glutamate synthetases). Others have reported that ribosomal and some other highly abundant cytoplasmic proteins strongly associate with OM;[18–20] some even have suggested this is physiologically relevant.[20] More likely these proteins, or fragments

thereof, strongly adhere to the OM as an artifact of preparation and thus were not included in later analyses.

Elucidation and Analysis of the Outer Membrane Proteome of *B. pseudomallei*

We purified and analyzed OM fractions as described above from Bp cells grown under 7 different growth/media conditions. These conditions were chosen to mimic some of the diverse habitats of *B. pseudomallei*, including soil, water, and blood, such that we could attempt to identify fundamental OMPs always present in the OM. Proteins identified in these 7 OM preparations were pooled, duplicates removed, and a nonredundant list of 155 OMPs assembled and designated the OM proteome of Bp (Table S2; Supporting Information); 66% of these were detected in at least 2 preparations. Eighty-eight (57%) were in the comprehensive list of in silico predicted OMPs of Bp (Table S1, Supporting Information) and ~40% were PsortB-predicted OMPs. This proportion is similar to that reported for the OM proteomes of *Actinobacillus pleuropnemoniae*[2] and *Erwinia chrysanthemi*.[1] We obtained similar results using OMs from *B. mallei* (see below).

Of the 31 porins (COG 3203) that comprise the most populous group of predicted and observed OMPs of Bp, we detected 13, nearly twice that of previous studies.[1,2,19] Porins form water-filled channels that allow the diffusion of hydrophilic molecules across the OM. Another populous family of OMPs are OM efflux channels (COG 1538). These proteins form trimeric channels with a β-barrel that spans the OM and is coupled to a helical barrel spanning the periplasm to allow export of diverse substrates including antibiotics;[21] we detected 11 of the 21 efflux channels predicted in the Bp genome. Members of the OmpW and OmpA families are also predicted to be prominent in the OM, playing multifunctional roles that are mechanistically unclear; we detected 4 of 8 predicted OmpAs and 3 of 5 predicted OmpWs. The expected structural components of the flagella: flagellin (BPSL3319), L and P ring proteins (BPSL0276 and BPSL0277), hook and rod proteins (BPSL0273 and BPSL2750) were also detected.

Metal acquisition, especially of iron, is essential for all bacteria. In Gram-negative bacteria, it often involves OM surface receptors that bind a chelated metal complex (e.g., Fe-siderophores) that is then imported across the OM using TonB and a membrane coupling protein. These receptors are assigned to COGs 1629, 4773, and 4774. We detected all 8 Bp proteins assigned to these COGS, including those predicted to bind heme, copper, iron, and a pyochelin-like Fe-siderophore. One of these, BPSL1775, a predicted catechol-based Fe-siderophore receptor, was one of the most abundant OMPs and was detected in cells from all growth conditions (see below). Its abundance and reaction with sera from Bp-infected patients[22,23] suggest BPSL1775 is a major surface antigen targeted by the host immune system.

Extracellular polysaccharides (EPS) are essential virulence factors that probably protect Bp, Bm, and other pathogens from host recognition.[24–28] Bp has 5 proteins that are predicted to be similar to Wza, the probable translocon channel for secretion of colanic acid EPS through the OM of *E. coli*.[29] Although Bp has been reported to produce only 3 types of EPS,[28] this suggests it may have the capacity to produce two more. Three of these predicted EPS translocon channels in COG1596 were detected in the Bp OM: BPSS0417 from the

lipopolysaccharide (LPS) O-antigen biosynthesis gene cluster,[24] BPSL2807 from the capsule-associated *wcb* gene cluster,[26] and BPSS1831 from the type IV O-PS polysaccharide biosynthesis gene cluster.[27]

The OM is not only a primary barrier against host defenses, but is also the site of many offensive weapons Bp and Bm use against the host, including parts of its multiple protein secretion systems that are essential for pathogenicity.[30–34] The Bp genome encodes 4 secretin proteins in COG 1450; each is predicted to be a pore for a different secretion system used to export proteins across the OM. All of these were detected: BPSS1545 (BsaO) part of the Type III secretion system required for virulence in hamsters;[31,32] BPSL0007 (GspD) part of the general (Type II) secretion apparatus also involved in virulence;[30] BPSS1592, part of another Type III secretion system of unknown function and BPSS1600, part of a Type IV pilus assembly which could be involved in adhesion or surface motility. BPSL0007 was among the 20 most abundant proteins detected in OMs from cells grown under all conditions (see below). Interestingly, BPSL0007 and BPSL2807, one of the EPS translocon channel proteins mentioned above strongly reacted with sera from recovered melioidosis patients indicating these are prominent surface antigens expressed in vivo.[22,23]

We detected multiple components of a Type VI secretion system (T6SS).[33,35–37] Essentially all were from only one of the six distinct T6SS gene clusters in Bp. They were not from the T6SS cluster required for virulence[34] but rather from the T6SS gene cluster that is orthologous to T6SSs clusters present in dozens of genera of pathogenic and nonpathogenic proteobacteria.[37] Two of the T6SS components, BPSL3105 and BPSL3107, were among the 15 most abundant OMPs detected under all growth conditions (see below). BPSL3105 encodes the “HCP” protein that forms a ring-like structure proposed to stack into a secretion tube that passes through the OM to the cell surface.[35–37] The other abundant T6SS component detected, BPSL3107, was predicted to interact with the IcmF-like protein of the T6SS (BPSL3097) that in turn is predicted to interact with a lipoprotein similar to BPSL3108.[37] Both BPSL3097 and BPSL3108 were also detected in the OM, as was BPSS0078, a VgrG-family protein predicted to be on the end of the HCP tube where it assists in penetration of the target cell.[38] These observations support the proposed structural/topological model of the T6SS machine.[39] However, we also found 3 proteins in our OM preparations encoded by the same T6SS gene cluster that are not part of this model: BPSL3103, BPSL3106, and BPSL3110. In summary, our results suggest that proteins in COGs 3501 (Rhs/Vgr family), 3516 (ImpB/EvpA family), 3157 (HCP family), 3517 (Evp/ImpD family), 3519 (ImpG family), 3522 (ImpJ family), and 3523 (IcmF/VasK family) comprise the major OM components of the T6SS; some of these undoubtedly are surface exposed. Consistent with this BPSS0078 (COG3501) and BPSL3103 (COG3519) react with convalescent sera of Bp-infected patients.[22,23] We did not detect any predicted T6SS proteins from COG3515 (ImpA family), COG3520, COG3521 (FHA family) COG3456, COG3455 (DotU family) or COG3518. These may be located in the IM or periplasm; alternatively they may only be transiently involved in assembly of the T6SS machine.

We identified 15 “hypothetical proteins” in the Bp OM; two, BPSS1996 and BPSL0994, are antigenic and expressed in vivo since they react with sera from melioidosis patients.[22,23] Moreover, BPSL0994 has PFAM domain PF01103 found in the D15/OMA87 family of

bacterial surface antigens.[40] Six hypothetical proteins were PsortB-predicted OMPs assigned to COGs containing OMPs (e.g., COG 2558 OmpA) or proteins associated with OM functions (e.g., LPS biosynthesis, efflux). Four had no COG assignment or PFAM domain, but had predicted signal peptides. Many of these likely represent examples of new types of OMPs with functions yet to be elucidated.

Proteins Always Present in the Outer Membrane of *B. pseudomallei*

From the lists of OMPs detected under each of the 7 growth conditions used, we derived a catalog of OMPs found in every preparation, irrespective of growth conditions. The 20 most abundant of these “always-present” OMPs are listed in Table 1 and represent >50% of total protein in the Bp OM. The fact that these are abundantly present in cells growing in such a wide variety of conditions (e.g., in minimal and rich medium, in serum, in the presence of macrophages) implies they are fundamental to *Burkholderia* physiology in many environments. Orthologs of nearly all these were also detected in the Bm OM (Table 1 and below). The vast majority are porins and OmpW-like proteins. Orthologs of 3 of the 7 most abundant OMPs (BPSL2522, BPSL2989, BPSL0289) were also detected in highly purified membrane preparations of the closely related bacterium *Ralstonia solanacearum* (not shown). Thus, these 20 abundant and always-present OMPs should be good targets for multivalent vaccines, especially porins BPSS0879 and BPSS1679, as well as lipoprotein BPSL1913 and iron receptor BPSL1775 that already have been shown to react with convalescent sera from Bp-infected patients.[22,23] Two other always-present OMPs and potential vaccine targets are the Type II secretion pore GspD (BPSL0007) and BPSL3105 the tube-forming component (Hcp) of a T6SS. Two of the most abundant always-present OMPs, BPSS1356 and BPSL2003, are hypothetical proteins. The former is a 120-kDa protein with orthologs only in the closely related species *Burkholderia thailandensis* and *Burkholderia oklahomensis*. The latter is a 12-kDa protein that has orthologs in all sequenced *Burkholderia* and a few other bacteria. When purified amino acids were added to the growth media, the relative abundance of 2 porins, BPSL0289 and BPSL0294, increased >6-fold, suggesting they may be involved in amino acid uptake. Other than these we did not observe any reproducible, major differences in the abundance of major OMPs in Bp grown under the 7 different culturing regimes.

Elucidation of the OM Proteome of *B. mallei*

B. mallei is a deletion clone of Bp which has lost >1200 genes making it less metabolically capable than Bp.[8] It has been suggested that this massive genome reduction and altered regulation have made Bm better adapted to life in an animal host. We attempted to analyze the Bm OM proteome as done for Bp, but since Bm grew poorly in media 1, 5, and 7, we only could determine the OM proteome of cells grown in media 2, 3, 4, and 6. The nonredundant list of proteins detected in the Bm OM from cells grown in at least one of these conditions contained 100 proteins (Table S3, Supporting Information). Orthologs of 70 of these were also detected in the Bp OM; these 70 likely represent the most fundamental OMPs of these two pathogens. We also generated a list of the 20 most abundant OMPs detected in Bm cells regardless of growth condition (Table 2). Orthologs of 6 of these are in the analogous list of abundant, always-present OMPs of Bp (Table 1). Orthologs of the remaining 14 were detected in the Bp OM, but in lower abundance and sometimes not in

cells from all growth conditions. While 8 of the most abundant always-present OMPs of Bp were porins, only 4 porins are in the analogous Bm list. Moreover, 3 of these Bm OMPs were TonB-dependent receptor proteins involved in metal acquisition, in contrast to only one in the Bp list. One of these is predicted to be involved in heme uptake. The disparity between amounts and identities of the 20 most abundant always present OMPs of Bp and Bm can be partially explained by the fact that Bp produces OMPs encoded by genes have been deleted from Bm, thus skewing the relative abundances. For example, the T6SS genes encoding two of the most abundant OMPs of Bp (BPSL3105 and BPSSL107) are missing from Bm. Another possible reason for the disparity is that because Bm has become a host-adapted pathogen and is not nearly as proficient in environmental survival as Bp, it expresses a somewhat different spectrum of OMPs more appropriate for survival in animal hosts. For example, BMAA0749, the so-called BimA protein required for actin-based motility in macrophages, and BMA0729.1, an IcmF-like protein of the T6SS required for virulence in hamsters,[33] were detected, albeit at very low levels (~0.04% the total OMP), in preparations of the Bm OM from cells grown only in media 3 and 6 but not in any of the Bp OM preparations. Several other OMPs unique to the Bm OM are predicted surface proteins possibly related to virulence: BMAA0251, another TonB-dependent receptor, and BMAA1936 and BMA1701, lipoproteins related to a Rickettsial surface antigen.

CONCLUSION

A Triton-insoluble fraction of lysozyme-treated Bp or Bm cells was digested with RNase and repeatedly extracted with high salt at pH 11 yielding a highly purified OM preparation as evidenced by its KDO to protein ratio and SDS-PAGE analysis. The Bp preparation appeared as pure as that of Gotoh et al.,[16] who used a more hazardous and laborious method involving sonication to break the cells and sucrose density gradient centrifugation. Trypsin shaving of Bp OM preparations from 7 growth conditions followed by methanol dissolution and retreatment with trypsin coupled to LC–MS/MS identified >155 possible OMPs; of these 90 (58%) were predicted by PSORTb or IMG to be likely OMPs. Of the remaining 65 about half were proteins whose predicted functions are OM-associated (e.g., biosynthesis of EPS, LPS, and cell wall, T6SS, cell division, etc.); 7 were hypothetical proteins possibly representing new families of OMPs.

The 90 highly probable OMPs we detected is substantially more than the number of OMPs identified in most previous MALDI-TOF determinations of proteobacterial OM proteomes that relied on 2-DE fractionation of OMs purified away from IM and cytoplasmic proteins by a single detergent or pH 11 extraction.[1–4] Only one other OM proteome determination reported more OMP identifications than here; however, it employed a much more laborious combination of 1-DE and 2-DE followed by LC–MALDI-TOF-TOF analysis of dozens gel slices.[41] Because we directly analyzed total peptides from highly purified OM preparations in an LC–MS/MS ion trap instrument without prior electrophoresis and inefficient peptide extraction from gel slices, we could analyze multiple samples from cells grown under a wide variety of conditions, with a higher sensitivity, and also estimate relative abundances. This allowed us to assemble a list of 20 OMPs that are always present and abundant in Bm and Bp under several different culturing regimes. Many of these are likely to be indispensible for growth/survival and accessible on the surface of Bp and Bm cells in many environments, and

thus should be ideal targets for therapeutics and vaccines. In support of this, 20% of the identified OMPs of Bp, and 2 of the 5 most abundant ones, react with sera of recovered melioidosis patients indicating they are expressed and targeted in vivo during disease.[22,23] Surprisingly, we observed only a couple of major differences in the abundance of major OMPs in the OM proteomes of Bp or Bm in response different culturing regimes. Finally, we made several novel observations about the probable OM localization of 7 of the 15 components of a T6SS machine. Although the detailed structure and topology of these machines remains to be fully elucidated, for the most part, our findings confirmed predictions and models based on in vitro structural analyses of T6SS proteins.[36] However, several T6SS-associated proteins not accounted for in previous models were detected in the OM and hence require further study and integration into newer models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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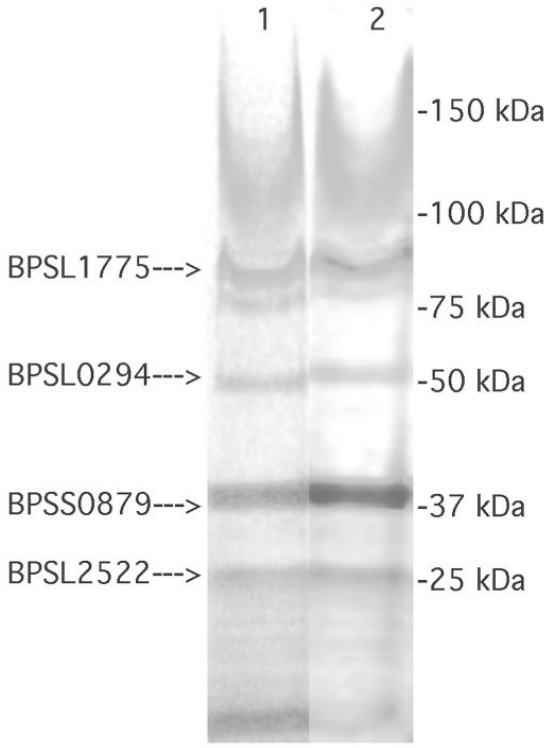

Figure 1.

SDS-PAGE and PMF analysis of purified OM from *B. pseudomallei*. Ten migrograms (lane 1) and 40 μg (lane 2) of protein from a Triton-insoluble OM pellet that had been further purified by extraction with RNase, Na_2CO_3, and NaCl were boiled for 5 min in denaturing solution (10 mM Tris-HCl pH 6.8, 2% SDS, 2% mercaptoethanol) and electrophoresed at 100 V for 3 h on a 4 to 20% polyacrylamide gradient gel (Thermo Fisher 25204). After Coomassie Blue staining, bands were excised and the protein species present in them were identified by PMF. Locus tags of PMF-identified OMPs are on the left; migration of molecular weight markers is shown on right. Cells were grown in medium 2.

Table 1

Twenty Most Abundant OM Proteins of *B. pseudomallei* Detected Under All Growth Conditions[a]

Bp locus tag	locus tag of Bm ortholog	predicted function	COG	% of total OMPs[b]	detected in Bm OM	% of total OMPs[c]
BPSL2559	BMA2089	porin	3203	18.8	yes	6.82
BPSL2989	BMA2507	lipoprotein	3133	13.0	yes	5.23
BPSL2704	BMA2010	OmpW-family protein	3407	5.88	yes	0.37
BPSS0879 [d]	BMAA1353	porin	3203	4.63	yes	11.0
BPSS1356	BMAA0912	hypothetical protein	3246	3.69	yes	1.10
BPSL2522 [d]	BMA0436	OmpA-family protein	2885	3.53	yes	7.63
BPSL2003	BMA0904	hypothetical protein	0393	1.78	yes	1.05
BPSS1679[d]	BMAA1698	porin	3203	1.67	yes	1.47
BPSS0943	BMAA1286	porin	3203	1.42	yes	3.10
BPSL1029	BMA0743	porin	3203	1.08	yes	1.47
BPSL1775 [d]	BMA1178	uptake receptor for Fe-catechol complex	4774	0.79	yes	3.89
BPSL3036	BMA2562	porin	3203	0.77	yes	1.06
BPSL3161	BMA2738	OmpW-family protein	3047	0.61	no	0.50
BPSL0816	BMA0317	OprB OM efflux protein	1538	0.58	yes	0.48
BPSL3105	None	T6SS-associated HCP protein	3157	0.30	n/a	0.48
BPSL3107	None	T6SS-associated protein	3516	0.29	n/a	0.22
BPSL0289	BMA3345	porin	3203	0.26	yes	1.87
BPSL0007[d]	BMA2786	general secretory pathway secretin GspD	1450	0.20	yes	0.22
BPSL0294	BMA3354	selective porin	3659	0.19	yes	1.25
BPSL1913[d]	BMA1056	OM lipoprotein in NodT family	1538	0.20	no	0.63

[a] Bold indicates proteins also detected by SDS-PAGE and PMF (Fig. 1). Locus tags, COG assignments, and predicted function were derived from Integrated Microbial Genomes (IMG: http://img.jgi.doe.gov/cgi-bin/pub/main.cgi).

[b] Percent of total OMPs was determined by spectral counts from cells grown in M9 minimal media with 3% glycerol.

[c] Percent of total OMPs was determined by spectral counts from cells grown in M9 media with 3% glycerol and 20 amino acids.

[d] Reported to reacted with sera from Bp infected patients.[22,23] n/a, not applicable.

Table 2

Twenty Most Abundant OM Proteins of *B. mallei* Detected Under All Growth Conditions[a]

Bm locus tag	locus tag of Bp ortholog	predicted function	COG	% of total OMPs
BMA3354	BPSL0294	selective porin in OprB family	3659	15.3
BMA0436	BPSL2522	OmpA-family protein	2885	10.6
BMA1178	BPSL1775	TonB-dependent siderophore receptor	4774	5.22
BMAA1826	BPSS0244	TonB-dependent heme/hemoglobin receptor family protein	1629	4.29
BMAA1353	BPSS0879	porin in OpcP family	3203	2.46
BMA0317	BPSL0816	RND efflux system, OM lipoprotein, NodT family	1538	2.15
BMA0465	BPSL2543	lipoprotein	None	1.49
BMAA1464	BPSS0294	RND efflux system, OM lipoprotein, NodT family	1538	1.33
BMA2307	BPSL2807	capsular polysaccharide biosynthesis/export protein	1596	1.19
BMA1547	BPSL2151	OMP85 family protein	4775	1.07
BMA0208	BPSL0658	organic solvent tolerance protein	1452	0.52
BMAA0356	BPSS2136	porin in OprD family	None	0.51
BMA0685	BPSL0976	BtuB-like vitamin B12 receptor	4206	0.50
BMAA2092	BPSS2331	lipoprotein	2853	0.47
BMA2723	BPSL3147	lipoprotein	2853	0.33
BMA2786	BPSL0007	general secretion pathway secretin GspD	1450	0.28
BMAA0427	BPSS1742	TonB-dependent copper receptor	1629	0.22
BMA0316	BPSL0815	hydrophobe/amphiphile efflux family protein	841	0.14
BMA0705	BPSL0994	OMP85 family protein	729	0.07
BMAA0486	BPSS0562	porin	3203	0.03

[a]Locus tags, COG assignments, predicted function and % of total OMPs were derived as in Table 1. Cells used were grown in M9 media with 3% glycerol and 20 amino acids.