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Research Article

Identification of immunoreactive secretory proteins from the stationary phase culture of *Burkholderia pseudomallei*

Bacterial secreted proteins are known to be involved in virulence and may mediate important host–pathogen interactions. In this study, when the stationary phase culture supernatant of *Burkholderia pseudomallei* was subjected to 2-DE, 113 protein spots were detected. Fifty-four of the secreted proteins, which included metabolic enzymes, transcription/translation regulators, potential virulence factors, chaperones, transport regulators, and hypothetical proteins, were identified using MS and database search. Twelve of these proteins were apparently reactive to antisera of mice that were immunised with *B. pseudomallei* secreted proteins. These proteins might be excellent candidates to be used as diagnostic markers or putative candidate vaccines against *B. pseudomallei* infections.

Keywords:
Burkholderia pseudomallei / Immunoreactive / Proteomics / Secreted proteins
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1 Introduction

Burkholderia pseudomallei, a Gram-negative, motile bacillus is the causative agent of melioidosis and has been included in the category B priority agent list of the Center for Disease Control and Prevention [1]. It poses a worldwide emerging infectious disease problem and a bioterrorism threat due to its severe course of infection, aerosol infectivity, low infectious dose, intrinsic resistance to commonly used antibiotics and lack of a currently available vaccine [2]. A number of secreted products of *B. pseudomallei*, such as protease, haemolysin, lipase, catalase, superoxide dismutase, and lecithinase, have been identified as virulence factors [3–6]. However, the pathogenesis of the disease due to these virulence factors of *B. pseudomallei* still remains unclear.

Secreted proteins of pathogenic or symbiotic bacteria mediate important interactions with their eukaryotic host in the host extracellular environment [7]. These proteins are usually involved in various functions ranging from provision of nutrients, cell-to-cell communication, detoxification of the environment, and host cell toxicity to alterations of the host cell for the benefit of the invader [8]. Furthermore, secreted proteins of intracellular pathogens are known to be the primary antigen targets for host immune response [9]. Thus, these proteins may be key factors to induce immune protection as well as in the development of vaccines.

In this study, a proteome reference map of *B. pseudomallei* secreted proteins expressed at stationary phase of growth and identification of the proteins using MALDI-TOF analysis was obtained. Subsequently, Western blot analysis of these proteins were performed using mice antisera raised to *B. pseudomallei* secreted proteins in order to ascertain potential diagnostic markers or putative candidate vaccines.

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Abbreviations: BLAST, Basic Local Alignment Search Tool; **CFA,** culture filtrate antigen; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase; **ICD,** isocitrate dehydrogenase; **LB,** Luria Bertani broth; **SCOT,** succinyl-CoA:3-ketoacid-coenzyme A transferase

2 Materials and methods

2.1 Bacterial isolate and growth

B. pseudomallei CMS, a haemoculture isolate from a patient with septicemic melioidosis at the University Malaya Medical Center, was used in this study. The isolate was confirmed biochemically using the API 20NE system (Biomerieux, France). A single colony of the culture on nutrient agar was inoculated into 10 mL Luria Bertani Broth (LB) and grown aerobically at 37°C overnight with an agitation of 150 rpm. The bacteria were recovered by centrifugation with fresh LB and used to inoculate a second liquid culture to obtain an $OD_{600\,nm}$ of 0.1. Subsequently, 100 μL of the culture was inoculated into 250 mL LB broth and grown aerobically at 37°C for a further 24 h with agitation at 150 rpm. Samples (5 mL) were taken out at 4, 8,

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12, 16, 20 and 24 h time-points and the optical density of the culture samples was read at 600 nm. The remaining bacterial culture was then centrifuged at 20 000 × *g* for 40 min at 4°C and the supernatant filtered through a 0.22 μm filter (Millipore) to obtain a bacteria-free culture supernatant. Isocitrate dehydrogenase (ICD) activity was performed by measuring the reduction of $NADP^+$ at room temperature spectrophotometrically at 340 nm to determine the degree of autolysis [10].

2.2 Preparation of secreted proteins for 2-DE

Stationary phase (20 h) bacterial-free culture supernatant was prepared. Prechilled 25% (w/v) TCA was added to the culture supernatant at a ratio of 1:3 and the proteins were left to precipitate on ice for 2 h after which the precipitated proteins were collected by centrifugation at 10 000 × *g* for 20 min at 4°C. The resulting pellet was then washed three times with acetone and resuspended in lysis buffer (8 M urea, 4% CHAPS, 2% Pharmalyte 3–10) before protein concentration was determined using the Bradford method [11]. The precipitated secretory proteins were stored in aliquots at −80°C during the course of the study.

2.3 2-DE

Approximately 450 μg of proteins was resuspended in rehydration buffer (8 M urea, 2% CHAP, 2% Pharmalyte 3–10, 0.002% bromophenol blue) and DTT was added to a final concentration of 20 mM. The resuspended proteins were applied to immobilised pH gradient (IPG) strips (pH 3–10, 13 cm) and focused using an IPGphor isoelectric focusing system (GE Healthcare, Uppsala, Sweden) at 500 V for 500 Vh, 1000 V for 1000 Vh, and 8000 V for 12 500 Vh. Focused IPG strips were equilibrated in equilibrium buffer (50 mM Tris-HCL, pH 8.8; 6 M urea, 30% glycerol; 2% SDS) containing 1% DTT and 2.5% iodoacetamide followed by second-dimension separation on 12.5% SDS-PAGE gel. The separated proteins were then visualised using CBB G-250 stain [12]. The gels were scanned with an Image Scanner (GE Healthcare) and analysed using the Image Master™ 2D Platinum version 5.0 (GE Healthcare).

2.4 Antibody production

B. pseudomallei were grown in LB for 24 h at 37°C with an agitation of 150 rpm. Culture filtrate antigen (CFA) was prepared by concentration of the bacterial-free culture supernatant as described previously by Kumar et al. [13]. Antibody production was carried out according to Mariappan et al. [14] with slight modifications. Briefly, three male Balb/C mice (6–8 weeks old) were injected with 50 μg CFA in Freund's complete adjuvant via subcutaneous injection. Hundred microgram of antigen in Freund's incomplete adjuvant was injected at every two-week interval for two months. The sera was collected and evaluated for the presence of antibodies using ELISA as described by Chenthamarakshan et al. [15]. Sera that elicited high antibody titre were used in Western blot analysis. Unimmunised mice sera were used as negative control for ELISA.

2.5 Western blot of 2-DE gels

The separated proteins on replicate 2-DE gels were transferred onto nitrocellulose membranes in a semi-dry transfer apparatus (GE Healthcare) for 2 h after which the membranes were blocked with 3% gelatine in PBS for 1 h. The membranes were then washed with PBS containing (0.05%) Tween-20 and incubated with 1:1000 dilutions of mice anti-*B. pseudomallei* CFA sera for 2 h, followed by incubation with 1:5000 dilution of alkaline phosphatase conjugated rabbit anti-mouse IgG secondary antibody (CalBiochem) in PBS containing (0.05%) Tween-20 for 2 h. The membranes were developed using Western Blue Stabilising Substrate (Promega). Unimmunised mice serum was used as the negative control.

2.6 MS and protein identification

Selected protein spots were excised from the CBB G-250 stained 2-DE gels of the *B. pseudomallei* secreted proteins and the gel plugs were placed in 1.5 mL eppendorf tubes containing 200 μL of sterile distilled water in order to keep them hydrated prior to analysis. The plugs were sent to Biomolecular Research Facility, University of Newcastle, Australia for MALDI-TOF MS analysis where MALDI was performed using Ettan MALDI-TOF Pro (GE Healthcare).

Protein identification was based on peptide fingerprint map obtained from MALDI-TOF mass spectrometer. The spectra were submitted for database searching using MASCOT as the search engine (Matrix Science, London, UK). The searching criteria exploited carboxymidomethylation of cystein as fixed modification and oxidation of methionine as variable modification. One missed cleavage per peptide was allowed and an initial peptide tolerance of 50 ppm was used in all searches. All searches were performed using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/) and a non-redundant NCBI library database comprising annotated proteins of *B. pseudomallei* K96243. In silico analysis was carried out using PSORTb v.2.0 (http://www.psort.org/psortb2/index.html) to predict the cellular location of the identified proteins, SignalP v.3.0 (http://www.cbs.dtu.dk/services/SignalP/) to infer the presence of signal peptides in the proteins and TMHMM v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) to predict the transmembrane proteins topology with a hidden Markov model. Protein similarities with other closely related bacteria were also performed using BLAST analysis.

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3 Results

3.1 Visualisation of *B. pseudomallei* secreted proteins

The growth profile of *B. pseudomallei* isolate CMS was studied and the ICD activity assayed at every 4 h showed an increase in the activity throughout the growth albeit at a very low level (0–0.152 Units/mL) (Data not shown). Secreted proteins were precipitated from the stationary phase culture supernatant of *B. pseudomallei* and a total of 450 μg of the protein were profiled using linear IPG strip pH 3–10. Approximately 113 protein spots could be detected on the CBB G-250 stained 2-DE gel (Fig. 1).

3.2 Identification of *B. pseudomallei* secreted proteins

Fifty-four of the 113 distinct protein spots visualised were identified using MALDI-TOF analysis (Table 1). The remaining 59 proteins could not be identified due to insufficient protein in the spot for identification using MALDI-TOF analysis. Protein identification was based on the peptide fingerprint map obtained from MALDI-TOF mass spectrometer and the mass list generated. A PMF result of spot number B10 (identified as chaperonin GroEL) and MASCOT search engine query result is shown in Fig. 2. Three proteins including putative hydrolase (C7 and B5), hyphothetical protein BPSL1622 (C1 and D1), and hyphothetical protein BPSL2466 (A6 and F5) produced more than one spots (Table 1).

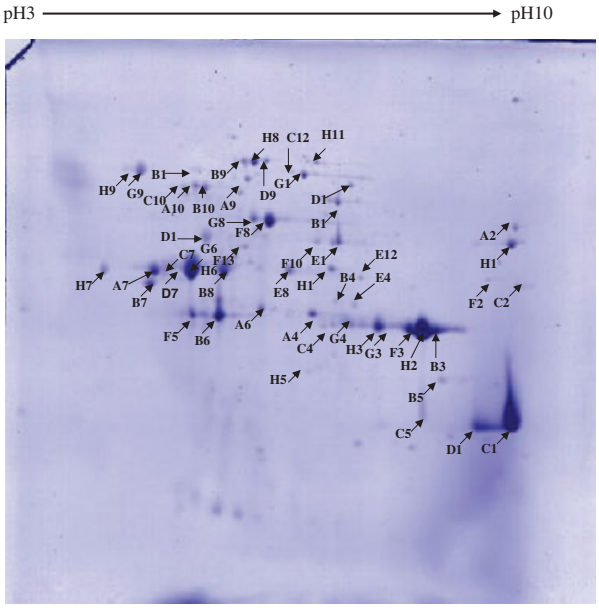

Figure 1. Analysis of *B. pseudomallei* (CMS) proteins by 2-DE. Total secreted proteins of *B. pseudomallei* grown to stationary phase in LB medium were prepared using TCA precipitation method and analysed using 2-DE. Four hundred and fifty microgram of secreted protein was separated on an IPG strip pH 3–10 in the first dimension, followed by the separation on SDS-12.5% PAGE for the second-dimension separation. The separated proteins were detected by CBB G-250 staining and picked for identification using MALDI-TOF analysis. Marked spots indicate protein spots that were able to be identified with confidence using MALDI-TOF analysis.

3.3 Identification of proteins reactive to mice antisera

Twelve proteins reactive to specific mice antisera raised to *B. pseudomallei* secreted proteins were identified by Western blot analysis of the 2-DE gel (Fig. 3). The 12 proteins identified includes flagellin, cell invasion protein (BipC), putative hydrolase, chaperonin GroEL, pyruvate dehydrogenase, cell division protein (FtsQ), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), succinyl-CoA: 3-ketoacid-coenzyme A transferase (SCOT), short-chain dehydrogenase, putative tRNA thiotransferase protein MiaB, hyphothetical protein BPSL1538, and monooxygenase. The proteins were found to be in the pH range of 4–10 and molecular weight ranging from 15 000 to 100 000. Control sera from unimmunised mice did not yield any cross-reacting signal.

3.4 In silico analysis of the proteins

The 54 identified proteins were assigned into functional classes based on Clusters of Orthologous Groups. The proteins were found to be involved in three major classes including metabolism (18 proteins), cellular processes (13 proteins), and information storage and processing (6 proteins). However, functions of 17 proteins were poorly characterised.

Metabolic proteins were mainly found to be involved in energy production and conversion (22.2%), carbohydrate transport and metabolism, lipid metabolism (22.2%), secondary metabolites biosynthesis, transport and metabolism (22.2%), and amino acid transport and metabolism (11.1%). Among the proteins involved in cellular processes, 33.3% were found to play a role in cell envelop and outer membrane biogenesis. Similarly, 33.3% were also involved in cell motility and secretion and other functions including PTM and chaperones (25.0%) and cell division and chromosome partitioning (8.3%). Among the information storage and processes proteins, 33.3% of each were found to be involved in translation, ribosomal structure and biogenesis, transcription, and DNA replication, recombination and repair.

Cellular locations prediction using PSORTb v.2.0 showed that among the 54 proteins identified, only two, *i.e.* flagellin and flagellar hook associated protein, were predicted as extracellular proteins (Table 1). The remaining 15 proteins were predicted to be cytoplasmic proteins, 10 cytoplasmic membrane-associated proteins, and one periplasmic protein. Twenty-six other proteins were from

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Table 1. Secretory protein of *B. pseudomallei* identified using MALDI-TOF analysis

Spot no.[a)]	Protein name/function	Locus tag	Sequence coverage (%)	Peptides matched	Exp/Theo (MW)	Exp/Theo (p*I*)	SignalP[b)]	TMHMM[c)]	pSORT[d)]
Information storage and processing									
Transcription									
A2	Flagellar hook associated protein	YP_110870	23	7	52.1/52.0	8.78/8.79	+	–	Extracellular
H5	GntR family transcriptional regulator	YP_108300	11	3	25.5/24.8	5.29/6.71	–	–	Unknown
Translation, ribosomal structure and biogenesis									
B7[e)]	Putative tRNA thiotransferase Protein MiaB	YP_107303	35	4	50.4/ 50.5	5.86/ 5.86	–	–	Cytoplasmic
D7[e)]	Hyphothetical protein BPSL1538	YP_108158	50	5	49.5/ 49.7	4.88/5.87	–	–	Cytoplasmic
DNA replication, recombination and repair									
B11	Transposase	YP_110723	13	4	25.5/55.9	8.63/9.69	–	–	Cytoplasmic
C8	DNA gyrase subunit B	YP_106698	33	4	15.6/91.0	8.81/5.82	–	–	Cytoplasmic membrane
Metabolism									
Energy production and conversion									
H3	Glycerophosphoryl diester phosphodiesterase family protein	YP_110428	63	4	32.4/33.4	5.91/6.44	+	1	Unknown
B17[e)]	Pyruvate dehydrogenase subunit E1	YP_108897	20	4	100.2/100.3	5.51/ 5.63	–	–	Unknown
H7	Succinate dehydrogenase flavoprotein subunit	YP_111724	57	1	42.7/64.4	4.21/6.55	–	–	Unknown
G4	NAD(P) transhydrogenase subunit alpha	YP_109481	52	4	21.6/39.1	6.02/6.33	–	–	Cytoplasmic membrane
Carbohydrate transport and metabolism									
C4	ABC transporter, periplasmic binding protein	YP_110795	39	2	4.63/35.6	9.68/8.82	+	–	Periplasmic
A7[e)]	Glyceraldehyde 3-phosphate dehydrogenase	YP_109546	34	3	35.9/36.1	4.70/ 6.37	–	–	Cytoplasmic
B9	Phosphotransferase system, II BC component	YP_107124	10	2	61.1/61.2	8.09/8.70	–	10	Cytoplasmic membrane
C12	Putative transporter protein	YP_106875	17	2	30.1/45.5	10.0/9.65	–	12	Cytoplasmic membrane
Lipid metabolism									
H9[e)]	Monooxygenase	YP_110337	34	3	43.3/43.5	10.0/ 6.81	–	–	Unknown
G9[e)]	Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A	YP_108553	32	5	25.1/ 25.2	5.85/5.56	–	–	Cytoplasmic
H10	Putative acyl-CoA dehydrogenase oxidoreductase protein	YP_107277	5	2	51.5/64.9	10.56/5.67	–	–	Unknown
B3	Phosphatidylserine decarboxylase	YP_107821	23	2	16.6/23.3	9.10/9.26	–	1	Unknown
Amino acid transport and metabolism									
C10	Peptidase	YP_109684	40	3	44.4/79.0	7.82/6.14	+	–	Unknown
C5	ABC transport system ATP-binding protein	YP_110365	19	2	10.7/38.8	8.52/6.90	–	–	Cytoplasmic
Secondary metabolites biosynthesis, transport and metabolism									
F8	Hyphothetical protein BPSS1116	YP_111129	67	3	54.6/64.4	5.97/8.94	–	–	Cytoplasmic
G11	Multidrug efflux system exported	YP_110312	12	4	41.1/42.9	7.66/9.37	+	1	Cytoplasmic membrane
F13	Cytochrome monooxygenase-related protein	YP_110047	6	2	25.4/51.5	6.21/8.97	–	–	Cytoplasmic

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Table 1. Continued

Spot no.[a)]	Protein name/function	Locus tag	Sequence coverage (%)	Peptides matched	Exp/Theo (MW)	Exp/Theo (p*I*)	SignalP[b)]	TMHMM[c)]	pSORT[d)]
B8[e)]	Short chain dehydrogenase	YP_108558	45	5	27.6/27.7	6.60/ 6.97	–	–	Cytoplasmic
Cellular processes									
Cell envelope biogenesis, outer membrane									
C2	Putative ADP-heptose-LPS Heptosyltransferase II	YP_107416	14	4	39.1/37.8	9.07/9.08	–	–	Unknown
H6[e)]	Cell division protein FtsQ	YP_109618	58	4	27.9/ 28.1	4.85/ 6.34	+	1	Cytoplasmic membrane
H11	Peptidoglycan synthetase FtsI	YP_109627	14	1	54.6/66.2	5.04/ 9.46	+	1	Cytoplasmic membrane
E10	Transferase	YP_112247	6	2	38.7/50.0	9.71/ 9.50	–	–	Cytoplasmic
PTM, protein turnover and chaperones									
F2	Hyphothetical protein BPSL0264	YP_106891	21	3	21.7/23.8	5.20/6.31	–	–	Unknown
B10[e)]	Chaperonin GroEL	YP_109293	19	10	56.5/57.0	5.18/5.17	–	–	Cytoplasmic
F10	Putative heat shock protein	YP_109423	11	3	20.5/19.6	6.60/4.78	–	–	Unknown
Cell motility and secretion									
G6[e)]	Flagellin	YP_109915	49		39.1/39.3	5.05/5.05	–	–	Extracellular
E8	Putative type II/IV secretory system ATP-binding protein	YP_108493	13	4	47.3/48.9	5.99/5.90	–	–	Cytoplasmic
D9	Chemotaxis related protein	YP_111880	30	4	42.6/60.3	4.22/5.27	–	2	Cytoplasmic membrane
D11	Chemotaxis-related methyltransferase protein	YP_111878	8	3	53.3/71.2	7.14/5.51	–	–	Cytoplasmic
Cell division and chromosome partitioning									
H8	Hyphothetical protein BPSL0566	YP_107192	17	2	42.7/71.8	8.90/ 6.00	–	–	Cytoplasmic
Poorly characterised									
C7[e)]	Putative hydrolase	YP_107999	8	2	17.3/16.2	5.88/ 8.00	–	–	Unknown
B5	Putative hydrolase	YP_107999	8	2	17.3/16.2	5.88/ 8.00	–	–	Unknown
D10	Hyphothetical protein BPSL2050	YP_108649	19	2	87.3/ 97.3	10.08/ 6.20	–	–	Unknown
E4	Undecaprenyl pyrophosphate phosphatase	YP_109239	25	2	21.4/30.2	8.74/8.65	–	5	Cytoplasmic membrane
A10	Putative lipoprotein	YP_108354	41	2	34.0/58.1	4.94/8.54	+	–	Unknown
B4	Hyphothetical protein BPSL1094	YP_107715	14	4	16.2/16.0	5.16/5.55	–	–	Unknown
A9	Hyphothetical protein BPSL2092	YP_108689	38	2	50.3/52.1	9.30/7.10	–	–	Unknown
H2	Chitin binding protein	YP_110514	24	7	39.5/39.2	8.59/8.59	+	–	Unknown
G3	Hyphothetical protein BPSS1981	YP_111987	26	3	10.4/10.1	6.96/6.50	–	–	Cytoplasmic
C1	Hyphothetical protein BPSL1622	YP_108236	36	8	26.7/26.9	9.30/9.30	+	–	Unknown
D1	Hyphothetical protein BPSL1622	YP_108236	47	10	26.7/26.9	9.30/9.30	+	–	Unknown
H1[e)]	Cell invasion protein	YP_111537	34	10	44.2/44.2	8.67/8.01	–	–	Unknown
F3	Hyphothetical protein BPSL0584	YP_107212	3	3	19.1/48.3	5.37/8.95	+	3	Cytoplasmic membrane
A6	Hyphothetical protein BPSL2466	YP_109058	100	2	9.89/15.8	6.12/9.79	+	1	Unknown
B6	Intracellular spread protein	YP_111530	19	3	13.4/56.7	9.80/ 9.85	–	–	Unknown

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Table 1. Continued

Spot no.[a)]	Protein name/function	Locus tag	Sequence coverage (%)	Peptides matched	Exp/Theo (MW)	Exp/Theo (p*I*)	SignalP[b)]	TMHMM[c)]	pSORT[d)]
F5	Hyphothetical protein BPSL2466	YP_109058	100	2	9.89/15.8	6.12/9.79	+	1	Unknown
A4	Hyphothetical protein BPSL1182	YP_107804	12	3	23.1/22.7	6.75/6.13	–	–	Unknown
D10	Hyphothetical protein BPSL0345	YP_106972	20	3	53.2/96.3	5.24/6.74	–	–	Unknown

a) Protein spot corresponding to position on gel (Fig. 1).
b) Output of computer algorithms that predict the presence (+) or absence (–) of signal peptide.
c) Output of computer algorithms that predict transmembrane helices.
d) Output of computer algorithms that predict the subcellular location of protein.
e) Reactive to mice hyperimmune sera raised against *B. pseudomallei* secreted proteins.

unknown location. Eleven proteins including eight predicted as cytoplasmic membrane protein by PSORT analysis were also predicted as cytoplasmic proteins by the TMHMM analysis.

Further characterisations of the proteins were carried out using SignalP v3.0 whereby the presence of signal peptides was predicted in 25.9% of the proteins identified. The proteins predicted as secreted via the classical sec pathway includes ABC transport system ATP-binding protein, peptidase, peptidoglycan synthetase FtsI, putative lipoprotein, ABC transporter periplasmic-binding protein, glycerophosphoryl diester phosphodiesterase family protein, cell division protein FtsQ, chitin-binding protein, multidrug efflux system transported protein and hyphothetical proteins BPSS1116, BPSS1981, BPSL2466, BPSL1622, BPSL0584.

The presence of *B. pseudomallei* secretome proteins in other closely related *Burkholderia* species, *B. mallei* strain 23344 and *B. thailandensis* strain E264, was also determined using BLAST analysis. Forty-five of 54 proteins identified in the *B. pseudomallei* secretome showed high homology ($\geq$80%) to the predicted proteins of *B. mallei* (23344). Similar homology was also demonstrated by 44 of the proteins with predicted proteins of *B. thailandensis* (E264) (Table 2). One of the proteins (hypothetical protein BPSS 1981) did not have any orthologue in *B. mallei* and one protein (hypothetical protein BPSL 1622) did not have any orthologue in *B. thailandensis*.

3.5 In silico analysis of the proteins reactive to mice antisera

Five of the 12 proteins reactive to antisera of mice following immunisation with *B. pseudomallei* secreted proteins were found to be involved in metabolic functions which include specific functions of lipid metabolism (16.7%), carbohydrate transport and metabolism (8.3%), secondary metabolites biosynthesis, transport and metabolism (8.3%), and energy production and conversion (8.3%). Three proteins were involved in cellular processes with functions including cell envelope biogenesis and outer membrane (8.3%), PTM, protein turnover and chaperones (8.3%) and also cell motility and secretion (8.3%). Two of the reactive proteins were involved in information storage and processes with translation, ribosomal structure, and biogenesis functions. However, functions of two other proteins were poorly characterised.

In silico analysis of the proteins reactive to mice antisera using PSORT predicted 50.0% cytoplasmic proteins, 8.3% extracellular proteins, and another 8.3% cytoplasmic membrane protein. The remaining 33.3% of the reactive proteins were from unknown locations. Only one protein, cell division protein FtsQ, showed the presence of signal peptide indicating that it is secreted via the classical Sec pathway. However, this protein was also predicted as cytoplasmic protein by the TMHMM algorithm with one transmembrane helix. BLAST analysis showed that all the 12 proteins reactive to antisera of mice following immuni-

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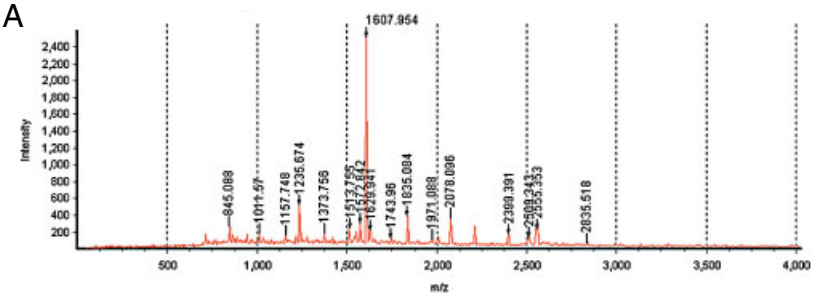
A



B

YP_109293: Chaperonin GroEL [Burkholderia pseudomallei; Score 120

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
2 - 13	1235.67	1234.67	1234.63	29	1	M.AAKDVVFGDSAR.A
14 - 28	1572.84	1571.83	1571.87	-23	1	R.AKMVEGVNILANAVK.V Oxidation (M)
16 - 28	1373.76	1372.75	1372.73	8	0	K.MVEGVNILANAVK.V Oxidation (M)
81 - 101	2078.10	2077.09	2077.03	29	0	K.TSDNAGDGTTTATVLAQSIVR.E
106 - 118	1513.76	1512.74	1512.71	27	1	K.YVASGMNPMDLKR.G 2 Oxidation (M)
396 - 404	1011.57	1010.56	1010.51	48	0	R.VEDALHATR.A
405 - 421	1607.95	1606.95	1606.90	26	0	R.AAVEEGIVPGGGVALIR.A
405 - 423	1835.08	1834.08	1834.04	19	1	R.AAVEEGIVPGGGVALIRAR.T
422 - 441	1971.09	1970.08	1970.05	13	1	R.ARTAIASLTGVNADQNAGIK.I
424 - 441	1743.96	1742.95	1742.92	21	0	R.TAIASLTGVNADQNAGIK.I

Figure 2. MALDI-TOF MS analysis for spot number B10. (A) Illustration of a representative PMF spectra typical for Chaperonin GroEL. (B) Mass list obtained from the PMF was subjected to the MASCOT search engine. Ten of the 17 queried masses were matched to the theoretical masses of with ±50 ppm tolerance and 0–1 missed cleavage. Peptide that caused oxidation at methionine residue is shown.

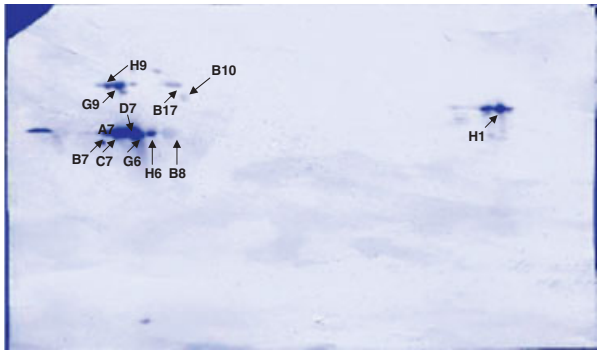


Figure 3. Western Blot analysis of *B. pseudomallei* secretome using mice anti-*B. pseudomallei* secreted protein sera. Secreted proteins from *B. pseudomallei* culture supernatant captured through TCA precipitation was separated using 2-DE, transferred onto nitrocellulose membrane and probed with sera collected from mice injected with *B. pseudomallei* culture supernatant proteins. Marked spots indicate the proteins reactive to antisera of mice following immunisation with *B. pseudomallei* secreted proteins corresponding to the spots on the 2-DE gels.

sation with *B. pseudomallei* secreted proteins had orthologues in *B. mallei* (23344) and *B. thailandensis* (E264) with more than 85% homology.

4 Discussion

Bacterial secretome, including those of *Burkholderia cepacia* [14], *Bacillus anthracis* [16, 17], *Staphylococcus aureus* [18], *Pseudomonas aeruginosa* [19], and *Helicobacter pylori* [20] has been the subject of recent proteomics and immunoproteomics studies. Interest in the secretome comes from the fact that some of these secreted proteins mediate important host–pathogen interactions when they come into direct contact with the host compartments during the course of infection. Identifying the secreted immunogenic proteins will allow the discovery of novel antigens that may be important for the development of diagnostics, vaccines, and passive immunotherapies.

In this study, we report the 2-DE separation and identification of proteins that are actively secreted by *B. pseudomallei* at the stationary phase of growth. Western blot analysis was carried out using mice hyperimmune antisera raised to *B. pseudomallei* secreted proteins, in order to identify the potential diagnostic markers and/or putative vaccine candidates. Many reports have been published on the 2-DE analysis of intracellular and surface proteins of *B. pseudomallei* [21–23], and altered secretome of *B. pseudomallei* due to salt stress [24]. However, to our knowledge, this is the first report on the proteomic mapping and identification of the whole secretome captured through TCA precipitation and also identification of the secretome proteins that are reactive to mice hyperimmune sera raised to *B. pseudomallei* secreted proteins.

Stationary phase culture was used to harvest the majority of the bacterial secreted proteins based on an earlier report by Wehmhöner *et al.* [19]. In addition, Lefebre and Valvano [25] also reported that in an in vivo condition, bacterial cells that are able to establish chronic infection might face a host physiological environment similar to the in vitro stationary phase of growth. Detection of more immunogenic proteins in the secretome of stationary-phase cells as compared with that of logarithmic-phase cells has also been reported in a similar study [26]. Therefore, proteins secreted during the stationary phase were considered most suitable to obtain a complete secretome map and identify proteins that have diagnostic or immunoprotective value.

Several measures were taken to ensure that the proteins detected in the *B. pseudomallei* secretome were purely

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Table 2. Similarity of the proteins identified compared with *B. mallei* and *B. thailandensis*

Spot number[a)]	Identity to *B. Mallei* 23344 (%)	Identity to *B. thailandensis* E264 (%)
A2	99	93
H5	26	26
B7[b)]	100	96
D7[b)]	99	96
B11	99	35
C8	100	99
H3	42	33
B17[b)]	100	96
H7	99	99
G4	100	98
C4	99	95
A7[b)]	99	98
B9	98	96
C12	99	95
H9[b)]	97	94
G9[b)]	100	97
H10	99	99
B3	99	99
C10	100	96
C5	99	97
F8	99	96
G11	100	96
F13	100	83
B8[b)]	100	98
C2	99	98
H6[b)]	99	98
H11	99	96
E10	99	28
F2	100	95
B10[b)]	99	99
F10	100	93
G6[b)]	100	90
E8	29	98
D9	100	95
D11	99	82
H8	42	42
C7[b)]	99	94
B5	99	94
D10	99	93
E4	100	94
A10	99	87
B4	97	90
A9	99	91
H2	100	95
G3	No significant similarity	92
C1	97	No significant similarity
D1	97	No significant similarity
H1[b)]	100	86
F3	34	28
A6	29	85
B6	99	90
F5	29	85
A4	99	92
D10	26	27

a) Identities of spots are as described in Table 1.

b) Reactive to mice hyperimmune sera raised against *B. pseudomallei* secreted proteins.

secreted proteins and not proteins that were medium derived or released due to bacterial cell lysis. LB broth, containing minimal protein, was used for culture in order to limit medium derived contamination. Furthermore, the ICD activity in the secretome was also monitored at different growth phases. ICD serves as an indicator of autolysis as it is an intracellular enzyme, which is not secreted by the actively dividing cells [27]. Low level of ICD activity was detected at the stationary phase of growth indicating minimal contamination due to autolysis.

The secretome map of *B. pseudomallei* yielded 113 spots using linear IPG strip pH 3–10. Andersen and colleagues [27] reported that the numbers and types of proteins released to the culture supernatant is highly dependent on the cultivation, growth time of the bacterial culture, the medium used and environmental factors such as temperature and aeration during culture. Among the 113 spots detected, only 54 were able to be identified using MALDI-TOF analysis. The low number of proteins identified using MALDI-TOF may be attributed to the problems faced with the identification of low molecular mass proteins or low abundance of certain proteins under the growth conditions used [28].

The majority of proteins identified in the *B. pseudomallei* secretome were predicted to be associated with the cell wall or the cytosol despite the low ICD activity, which suggests minimal cell lysis. Antelmann et al. [29] and Riedel et al. [30] had similarly reported that half of the identified secretome proteins were associated with intracellular or surface-related proteins. Abundant cytoplasmic proteins have also been reported in the secretome of other pathogens such as *B. cepacia* [14], *Mycobacterium tuberculosis* [31], *Listeria monocytogenes* [32] and *S. aureus* [18]. Among the detected proteins, GroEL, GAPDH, and flagellin have also been identified as natural components of the secretome in other studies [33–35]. However, Cole and colleagues [36] reported GroEL and GAPDH, as cellular, cell wall associated and also secreted. In case of the *H. pylori* ribosomal protein L11, its presence in the culture media was demonstrated to occur by active secretion and not due to non-specific cell lysis [37]. Therefore, cytoplasmic-associated proteins may in fact have dual functions and that can be targeted by the cell to different subcellular sites or secreted during certain stages of the cell growth.

Based on the genomic annotation and translation, some proteins, including those involved in secondary metabolism, drug resistance, intracellular stress, motility and chemotaxis, have been associated with the survival of *B. pseudomallei*. On the other hand, types I, II, III, and IV secretion system proteins, surface components, exoproteins, fimbriae/pili and adhesion proteins have been associated with the virulence of *B. pseudomallei* [4]. Likewise, we also identified proteins that may be associated with survival and virulence in the *B. pseudomallei* secretome including flagellar hook associated protein, flagellin, multidrug efflux system exported proteins, chaperonin GroEL, putative heat shock protein, chemotaxis-related methyltransferase protein

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and chemotaxis-related protein, cell invasion protein, intracellular spread protein, chitin-binding protein and putative lipoprotein.

BLAST analysis demonstrated that the hyphothetical protein BPSL1622 (poorly characterised protein) did not have any orthologue in *B. thailandensis*, a non-virulent counterpart of *B. pseudomallei*. This suggests that the protein may be involved in the virulence of *B. pseudomallei*. Obviously, this protein should be investigated further in order to identify its specific functions. Other proteins including the hyphothetical protein BPSL0584, glycerophosphoryl diester phosphodiesterase family protein, hyphothetical protein BPSL0345, hyphothetical protein BPSL0566, and GntR family transcriptional regulator proteins showed less than 50% homology with *B. mallei* strain 23344 and *B. thailandensis* strain E264. Therefore, these proteins or antibodies towards these proteins have the potential to be developed as diagnostic markers because they do not cross-react with the two closely related *Burkholderia* spp. However, further evaluation and characterisation of these proteins have to be carried out to confirm their potentials.

Among the secretome proteins, 12 were found to be reactive to hyperimmune mice sera raised against the *B. pseudomallei* CFA. Three of these proteins have already been described as highly immunoreactive in the secretome of other organisms including GroEL in *B. anthracis* [15], GAPDH in *Streptococcus suis* [38], and flagellin in *P. aeruginosa* [19]. Several of the reactive proteins identified are of interest due to their possible potential to be developed as diagnostic markers or putative vaccine candidates.

Flagelin, a bacterial flagellar subunit protein coded by the gene *fliC*, is known as a factor involved in the pathogenesis of *B. pseudomallei*. Brett and co-workers [39] have reported that anti-flagella (flagellin) antibody was able to reduce bacterial motility *in vitro* and provide passive protection for diabetic rats infected with *B. pseudomallei*. In addition, bacterial flagellin has also been recognised as a strong immunostimulator capable of activating NF-κβ-signalling [40] and Chen *et al.* [41] have used plasmid DNA encoding flagellin as a vaccination candidate against infection of *B. pseudomallei* in Balb/c mice.

It is surprising that cell invasion protein, BipC, a type III secretion protein that is common among bacterial pathogens and symbionts for delivery of effector proteins into eukaryotic host cells [42, 43] was present in the secretome albeit the absence of the host. However, Uchiya *et al.* [44] reported that these proteins are commonly found in the culture supernatants of bacteria grown under laboratory conditions. One of the *B. pseudomallei* Type III secretion systems, *Burkholderia* secretion apparatus, shares high homology with type III secretion system of *Salmonella typhimurium* and *Shigella flexneri* [45, 46] reported that the inactivation of *Burkholderia* secretion apparatus components resulted in impaired invasion and survival within eukaryotic cells, inability to escape from endocytic vacuoles, and failure to produce membrane protrusions and actin tails. Another potential vaccine candidate, chaperonin GroEL, is known to produce strong antigen–antibody response with melioidosis patient's sera [47]. Role of GroEL in vaccination against tuberculosis [48], brucellosis [49] and yersiniosis [50] have been studied. In addition, a virulence property of GroEL has also been suggested [47].

Table 3. Similar proteins previously identified in other *Burkholderia pseudomallei* proteome studies

Spot number	Protein name	Identified previously in other *B. pseudomallei* proteome studies. Reference(s)
G9[a)]	Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A	[20, 22]
F10	Putative heat shock protein	[20]
B10[a)]	Chaperonin GroEL	[20–23][b)]
H2	Chitin binding protein	[20, 21]
H6[a)]	Cell division protein FtsQ	[58]
G4	NAD(P) transhydrogenase subunit alpha [58]	

a) Reactive to mice hyperimmune sera raised against *B. pseudomallei* secreted proteins.
b) Immunogenic using human sera.

Four of the reactive proteins, pyruvate dehydrogenase, GAPDH, SCOT, and monooxygenase, were identified to have metabolic functions. Although metabolic proteins are known to play a major role in energy production for survival, their role in virulence has also been suggested [20]. In *P. aeruginosa* E1 and E2, components of pyruvate dehydrogenase were found to be involved in Type III secretion system-dependent cytotoxicity [51] but in *B. subtilis* they were involved in regulation of sporulation [52] and transcriptional activation of protoxin genes [53]. Meanwhile, GAPDH, a typical enzyme of the glycolysis pathway, might also play an important role in bacterial pathogenesis. In several fungi and Gram-negative bacteria, GAPDH is known to be a multifunctional protein displayed on the surface and contribute to their adhesion and virulence. It is associated with physiologic functions such as ADP-ribosylation [54], adhesion to fibronectin, myosin, and actin [55] as well as the ability to serve as a receptor for plasmin on the surface of Streptococcus [56]. However, the role for extracellular localisation of GAPDH in the pathogenesis of Gram-negative bacteria has not been described [57].

In addition, in a study on the intracellular proteins expression at stationary phase, Wongtrakoongate and colleagues [20] reported that the metabolic enzyme SCOT is highly expressed in B. *pseudomallei* but not in the non-virulent *B. thailandensis*. This suggests that SCOT might also be a potential protein marker although BLAST analysis shows a 97% homology with predicted proteins of *B. thailandensis*.

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Six of the proteins identified in this study (SCOT, chaperonin GroEL, chitin-binding protein, putative heat shock protein, Cell division protein FtsQ, and NAD(P) transhydrogenase subunit α) have been previously identified in other proteome studies of *B. pseudomallei* (Table 3). These include the proteome analysis of total cellular protein extracted from *B. pseudomallei* [21, 22, 58], surface proteins of *B. pseudomallei* [23], and also secretome [24]. Harding *et al.* [23] identified the surface located proteins of *B. pseudomallei* using biotin labelling and also found GroEL to be immunogenic when probed with convalescent human sera. Protein microarray to identify serodiagnostic and cross-reactive antigens using a large number of melioidosis and other bacterial infection patient sera also identified GroEL to be seroreactive and giving the best single antigen discrimination to accurately distinguish melioidosis cases from control [59]. This suggests the suitability of GroEL to be used in serodiagnosis. Felgner *et al.* [59] also identified several components of Type Three Secretion System (TTSS3) including BPSS1532 (BipB), BPSS1525 (BopE) to be potential for serodiagnostic. On the other hand, in our study, we identified two TTSS3 components including BPSS1531 (BipC) and BPSS1524 (BopA) in the secretome of *B. pseudomallei* and BipC was found to be reactive to hyperimmune mice sera raised to *B. pseudomallei* secreted proteins.

The BipC protein encoded by *bipC* gene has high homology with the *sipC* gene in the *S. typhimurium*, which encodes for a translocator protein involved in the type three secretion systems (T3SS). Research has shown that the mutation in the two Bip protein family, BipB and BipD, has shown attenuation of *B. pseudomallei* virulence [2, 45, 60]. BipD mutants were found to exhibit impaired invasion of HeLa cells, reduced intracellular survival in murine macrophage-like cells and a marked reduction in actin-tail formation. Similarly, BipB was found to be important in the induction of MNGC, plaque formation, bacterial invasion, and killing of phagocytic cells *in vitro*. However, up to date there are no reports regarding the role of the BipC protein on the *B. pseudomallei* virulence. Therefore, this warrants the investigation of the role of BipC in pathogenicity and virulence of *B. pseudomallei*.

In conclusion, this study highlights the mapping of the whole secretome of *B. pseudomallei* and the identification of stationary phase secretome proteins reactive to mice hyperimmune sera raised to *B. pseudomallei* secreted proteins. On the other hand, other proteome studies carried out have emphasised on the cellular proteins and surface proteins of *B. pseudomallei* [21, 22, 58]. Although Pumirat and coworkers [24] mapped the secretome of *B. pseudomallei*, they only identified the proteins that are altered under exposure to high salt-environment. Some of the identified proteins especially the proteins reactive to mice hyperimmune sera raised to *B. pseudomallei* secreted proteins are potential to be used in serodiagnosis, as protein markers or developed as vaccine candidates. However, further experiments are needed to evaluate the potentials of these proteins since this conclusion is only based on the comparison with other studies.

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The authors have declared no conflict of interest.

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