Biochimica et Biophysica Acta 1794 (2009) 898–904

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Altered secretome of *Burkholderia pseudomallei* induced by salt stress

Pornpan Pumirat [a], Putita Saetun [b], Supachok Sinchaikul [c], Shui-Tein Chen [c,d], Sunee Korbsrisate [a,*], Visith Thongboonkerd [b,*]

[a] *Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, 11th Floor Adulyadej Vikrom Building, 2 Prannok Road, Siriraj Hospital, Bangkoknoi, Bangkok 10700, Thailand*
[b] *Medical Proteomics Unit, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*
[c] *Institute of Biological Chemistry and Genomic Research Center, Academia Sinica, Taipei, Taiwan*
[d] *Institute of Biochemical Sciences, College of Life Science, National Taiwan University, Taipei, Taiwan*

ARTICLE INFO

Article history:
Received 9 September 2008
Received in revised form 2 December 2008
Accepted 29 January 2009
Available online 11 February 2009

Keywords:
Adaptive response
Burkholderia pseudomallei
Melioidosis
Proteome
Proteomics
Salt stress
Secretome

ABSTRACT

Burkholderia pseudomallei is a saprophyte found in soil and water. It is a difficult microorganism to kill and can survive in these environments for many years. Mechanisms for its adaptive response to environmental changes remain largely unknown. We performed a proteomics study to examine alterations in secreted proteins (secretome) under a salt stress (with 150 mM NaCl) compared to the normal cultured condition in LB broth. The culture supernatants were filtrated and precipitated with 50% ethanol. The isolated proteins were recovered, separated with 2-D PAGE, and visualized with SYPRO Ruby stain ($n=5$ gels for each group). Differentially expressed protein spots were identified by Q-TOF MS and/or MS/MS analyses. A total of 42 protein spots representing 37 unique proteins were identified as the altered proteins during the salt stress, including metabolic enzymes, transcription/translation regulators, potential virulence factors, chaperones, phage capsid proteins, drug resistance protein, solute transport regulator, and hypothetical proteins. The presence of secreted GroEL only after NaCl exposure was confirmed by Western blot analysis. The increased level (19-fold) of a beta-lactamase-like protein suggested that the NaCl-exposed bacterium might resist to beta-lactam antibiotics. Functional analysis revealed that the NaCl-exposed bacterium had significantly greater survival rate after a treatment with ceftazidime. Our study provided the first dataset of the secretome of *B. pseudomallei* and its alterations, which may lead to novel insights into adaptive response of *B. pseudomallei* during the salt stress.

1. Introduction

Burkholderia pseudomallei is a Gram-negative saprophytic bacterium that causes melioidosis, which remains an important and common tropical disease, particularly in southeast Asia and northern Australia [1]. Melioidosis is most prevalent during rainy season in endemic areas [2,3] and mainly affects individuals who have a direct contact with wet soil (especially those with an underlying disease, which predisposes to infection, such as diabetes, chronic renal failure, chronic lung disease, etc.) [4,5]. Clinical features of melioidosis vary largely and the disease course ranges from acute to subacute and chronic infection [1,5]. In the northeastern part of Thailand, the incidence of melioidosis is approximately 3.6–5.5 cases per 100,000 annually, with a high mortality rate for primary disease (up to 50% in adults and 35% in children) [1,4]. Unfortunately, there is no effective vaccine available for the prevention of melioidosis [6].

Melioidosis research during the past few decades has thus focused on exploring pathogenic and molecular mechanisms of this bacterial infection [7]. In addition, better understanding of the bacterial survival and virulence is also crucial for successful prevention and/or treatment of this infectious disease. Regarding its survival, *B. pseudomallei*, as a saprophyte, is a difficult microorganism to kill and can survive in soil and water for many years [4]. Therefore, it must have adaptive mechanisms to survive in these environments even with various stresses due to environmental changes (e.g., alterations in salt contents, osmolarity, pH, etc.). However, these mechanisms of its adaptive response to environmental changes remain largely unknown.

The present study was therefore conducted to explore adaptive response in *B. pseudomallei* during a salt stress. After the stationary phase was reached, the bacteria were grown further with or without an addition of NaCl (at a final concentration of 150 mM) to the fresh culture medium. The secreted proteins were then isolated and analyzed by a gel-based proteomics method. Using this approach, a number of secreted proteins with altered levels were identified. Potential roles for the altered secretome in adaptive response during the salt stress are discussed.

* Corresponding authors. V. Thongboonkerd is to be contacted at Medical Proteomics Unit, Office for Research and Development, 12th Floor Adulyadej Vikrom Building, 2 Prannok Road, Siriraj Hospital, Bangkoknoi, Bangkok 10700, Thailand. Tel./fax: +66 2 4184793. S. Korbsrisate, Tel.: +66 2 418 0569; fax: +66 2 418 1636.
E-mail addresses: grsks@mahidol.ac.th (S. Korbsrisate), thongboonkerd@dr.com, vthongbo@yahoo.com (V. Thongboonkerd).

1570-9639/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbapap.2009.01.011

2. Materials and methods

2.1. Bacterial culture and sample preparation

B. pseudomallei (10276 strain) was maintained in Luria-Bertani (LB) broth at 37 °C. The growth phase was determined by measuring optical density (absorbance) at a wavelength of 600 nm for every 2 h using a UV-visible spectroscopy. After the stationary phase was reached (approximately 18–24 h), the bacteria were subcultured (with 1/10 dilution) in other sets of the fresh media and divided into two groups; with and without an addition of NaCl (to make a final concentration of 150 mM) ($n = 5$ replicates for each group). After 7 h of the growth in these two differential media, the bacteria were removed and the supernatants were saved by a centrifugation at 8000 *g* for 15 min. The supernatants were then filtrated using Millex-GP filter (Millipore; Bedford, MA) with a 0.22-μm pore size. Secreted proteins in culture supernatants were precipitated using 50% ethanol. After another centrifugation at 13,000 *g* for 10 min, the protein pellets were saved and the supernatants were discarded. The proteins in the pellets were then resuspended with a lysis buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS), 120 mM dithiothreitol (DTT), 2% ampholytes (pH 3–10), and 40 mM Tris–HCl. Protein concentrations in individual samples were then measured using the Bradford method.

2.2. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

A total of 10 gels derived from 10 individual culture flasks (5 from each group) were analyzed in the present study. Totally 100 μg proteins derived from each culture flask were premixed with a rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 120 mM DTT, 40 mM Tris–base, 2% (v/v) ampholytes (pH 3–10) and bromophenol blue) to make a final volume of 150 μl per sample. The protein mixture was then rehydrated on immobilized pH gradient (IPG) strip, linear pH 3–10, 7-cm-long (GE Healthcare; Uppsala, Sweden) at room temperature for 16 h. Subsequently, the first dimensional separation or isoelectric focusing (IEF) was performed using Ettan IPGphor II IEF System (GE Healthcare) with a step-and-hold mode until a total of 9083 Vh was achieved. The IPG strip was then equilibrated with equilibration Buffer I (6 M urea, 130 mM DTT, 112 mM Tris–base, 4% SDS, 30% glycerol and 0.002% bromophenol blue) for 15 min, followed by another equilibration step in Buffer II (6 M urea, 135 mM iodoacetamide, 112 mM Tris–base, 4% SDS, 30% glycerol and 0.002% bromophenol blue) for 15 min. Thereafter, the strip was placed onto a 12% polyacrylamide slab gel (8×9.5 cm), and the second dimensional separation was performed in SE260 mini-Vertical Electrophoresis Unit (GE Healthcare) at 150 V for approximately 2 h. Separated proteins were then visualized using SYPRO Ruby fluorescence dye (Invitrogen — Molecular Probes; Eugene, OR), and 2-D gel images were obtained by Typhoon 9200 laser scanner (GE Healthcare).

2.3. Matching and analysis of protein spots

Matching and analysis of protein spots were performed using Image Master 2D Platinum software (GE Healthcare). Parameters used for spot detection were (i) minimal area = 10 pixels; (ii) smooth factor = 2.0; and (iii) saliency = 2.0. A reference gel was created from an artificial gel combining all of the spots presenting in different gels into one image. The reference gel was then used for determination of existence and difference of protein expression among gels. Intensity volumes of individual spots were obtained and subjected to statistical analysis. Differentially expressed protein spots were subjected to in-gel tryptic digestion and identification by mass spectrometry.

2.4. Statistical analysis

To define differentially expressed protein spots, unpaired Student's *t*-test was performed to compare intensity levels of corresponding spots between control and NaCl groups using SPSS software package for Windows (SPSS; Chicago, IL). The criteria for defining spots with significant differences (either increase or decrease) included (i) p-values < 0.05, and (ii) the differentially expressed spots must be consistently present or absent in all five gels of each group.

2.5. In-gel tryptic digestion

The differentially expressed protein spots were excised from 2-D gels, washed twice with 200 μl of 50% acetonitrile (ACN)/25 mM NH_4HCO_3 buffer (pH 8.0) at room temperature for 15 min, and then washed once with 200 μl of 100% ACN. After washing, the solvent was removed and the gel pieces were dried by a SpeedVac concentrator

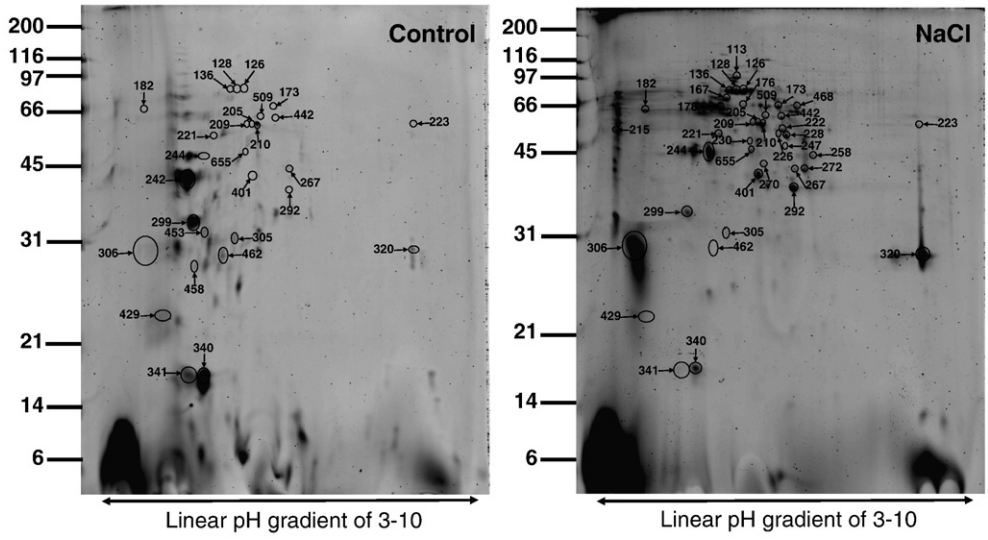


Fig. 1. Proteome map of the altered *B. pseudomallei* secreted proteins under a high-salt stress. After the stationary phase was reached, the bacteria were subcultured and divided into two groups: without (left panel) and with (right panel) an addition of NaCl (with a final concentration of 150 mM) to LB broth. The secreted proteins were then isolated, resolved by 2-D PAGE, and visualized with SYPRO Ruby stain. Proteins with significantly differential levels between the two groups (labeled with numbers, which correspond to those reported in Table 1) were excised and subjected to in-gel tryptic digestion and identification by Q-TOF MS and/or MS/MS analyses. Identities, quantitative data, degrees of changes, and other details of all these altered proteins are summarized in Table 1.

Table 1
Altered *B. pseudomallei* secreted proteins induced by salt stress

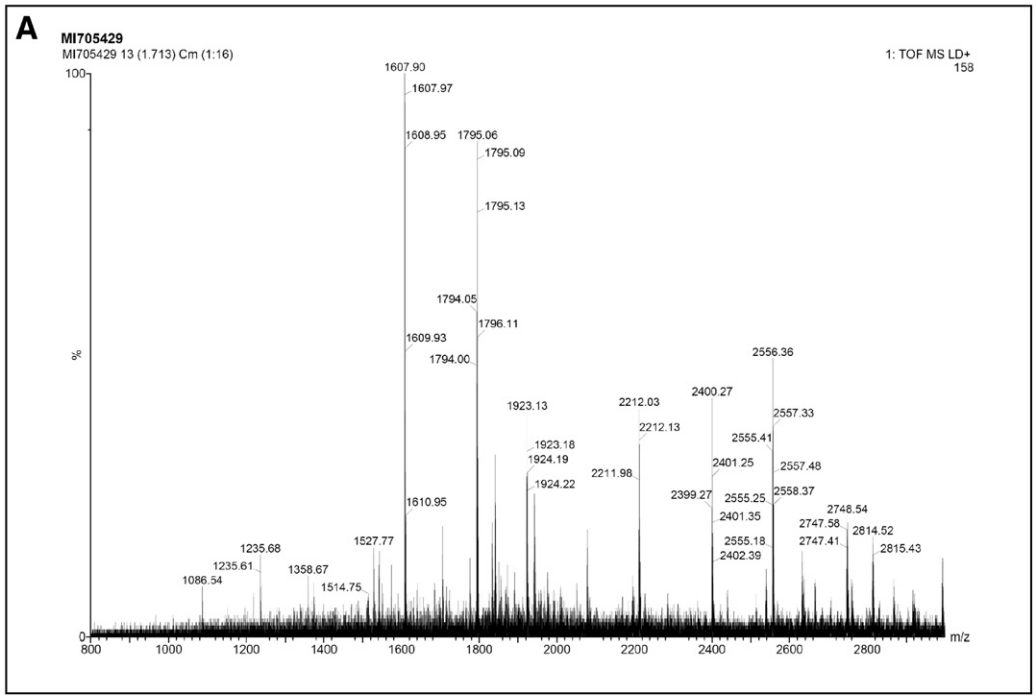
Protein name	Spot no.	NCBI ID	Identified by	Identification scores (MS, MS/MS)	%Cov (MS, MS/MS)	No. of matched peptides (MS, MS/MS)	p*I*	MW (kDa)	Intensity (mean ± SEM)		Ratio (NaCl/ control)	*p* values
									Control	NaCl		
Metabolic enzymes												
3-oxoacyl-(acyl-carrier-protein) synthase II	655	gi|86151671	MS	81, NA	35, NA	9, NA	5.65	43.10	0.0128 ± 0.0079	0.2455 ± 0.0558	19.15	0.003
Acetyl-CoA acetyltransferase	258	gi|53719169	MS	95, NA	39, NA	12, NA	6.62	40.75	0.0000 ± 0.0000	0.1077 ± 0.0445	DIV/0	0.042
AMP-dependent synthetase and ligase	509	gi|170744090	MS	83, NA	34, NA	10, NA	6.06	54.33	0.0059 ± 0.0059	0.1612 ± 0.0594	27.41	0.032
Arylsulfatase regulator (Fe–S oxidoreductase)	176	gi|90408763	MS	77, NA	28, NA	18, NA	7.16	51.35	0.0000 ± 0.0000	0.2603 ± 0.0823	DIV/0	0.013
ATP phosphoribosyl transferase regulatory subunit	299	gi|110835059	MS	77, NA	24, NA	8, NA	5.02	42.71	8.5468 ± 2.9556	1.0521 ± 0.3639	0.12	0.036
ATPase	458	gi|56551020	MS	82, NA	29, NA	11, NA	5.30	58.69	0.3773 ± 0.1316	0.0000 ± 0.0000	0.00	0.021
ATPase, AAA family protein	267	gi|157165173	MS	85, NA	31, NA	10, NA	6.30	44.36	0.0096 ± 0.0096	0.2411 ± 0.0723	25.20	0.013
Dehydratase protein	221	gi|21243028	MS	90, NA	26, NA	12, NA	8.89	70.63	2.1656 ± 0.2239	0.4120 ± 0.1029	0.19	<0.001
D-isomer specific 2-hydroxyacid dehydrogenase, NAD-binding	306	gi|148544838	MS	59, NA	32, NA	7, NA	4.80	36.60	0.0000 ± 0.0000	16.0833 ± 0.8781	DIV/0	<0.001
Glycosidase	228	gi|27367964	MS	70, NA	27, NA	10, NA	5.72	52.57	0.0000 ± 0.0000	0.3829 ± 0.0604	DIV/0	<0.001
Isopentenyl-diphosphate delta-isomerase	341	gi|149202049	MS	71, NA	56, NA	8, NA	6.54	19.49	2.8479 ± 1.0513	0.0495 ± 0.0495	0.02	0.029
Putative hydroxylase	429	gi|103487394	MS	63, NA	45, NA	6, NA	6.23	24.40	2.2644 ± 0.7858	0.0791 ± 0.0791	0.03	0.024
Putative hydroxylase	462	gi|103487394	MS	73, NA	45, NA	8, NA	6.23	24.40	0.2034 ± 0.0523	0.0063 ± 0.0063	0.03	0.006
Putative methyltransferase	242	gi|84388938	MS	84, NA	35, NA	10, NA	6.01	43.91	10.1691 ± 1.8233	0.0000 ± 0.0000	0.00	0.003
Putative UDP-galactose 4-epimerase	292	gi|68553183	MS	77, NA	30, NA	8, NA	6.47	37.05	0.1251 ± 0.0656	0.8530 ± 0.1982	6.82	0.008
Pyruvate dehydrogenase complex dihydrolipoamide acetyltransferase	244	gi|157372244	MS	75, NA	30, NA	10, NA	5.18	65.72	0.5910 ± 0.3598	4.1944 ± 0.9829	7.10	0.009
Response regulator receiver modulated metal dependent phosphohydrolase	453	gi|118743860	MS	78, NA	36, NA	9, NA	4.95	41.49	0.4248 ± 0.1363	0.0000 ± 0.0000	0.00	0.014
S-adenosyl-L-homocysteine hydrolase	205	gi|53720900	MS, MS/MS	85, 7	26, 2	10, 1	5.73	52.51	0.0106 ± 0.0106	0.2600 ± 0.0728	24.44	0.010
S-adenosyl-L-homocysteine hydrolase	210	gi|53720900	MS	64, NA	28, NA	10, NA	5.73	52.51	0.0921 ± 0.0921	0.7126 ± 0.1624	7.74	0.010
Transcription and translation regulators												
ATP-dependent RNA helicase	209	gi|53713588	MS	61, NA	31, NA	10, NA	5.84	46.93	0.0315 ± 0.0253	0.1890 ± 0.0443	6.00	0.015
Elongation factor EF-2	113	gi|53720824	MS	85, NA	27, NA	13, NA	5.33	77.74	0.0000 ± 0.0000	0.4641 ± 0.1753	DIV/0	0.029
Glutaminyl-tRNA synthetase	226	gi|66044979	MS	76, NA	25, NA	10, NA	5.73	65.24	0.0000 ± 0.0000	0.0555 ± 0.0173	DIV/0	0.012
Glutamyl-tRNA(Gln) amidotransferase, B subunit	222	gi|83319771	MS	87, NA	23, NA	10, NA	6.45	54.97	0.0000 ± 0.0000	0.1819 ± 0.0621	DIV/0	0.019
GTP-binding protein LepA	215	gi|24379799	MS	74, NA	27, NA	10, NA	5.16	68.54	0.0000 ± 0.0000	0.1532 ± 0.0430	DIV/0	0.007
Transcriptional regulator	320	gi|84357351	MS	55, NA	25, NA	4, NA	6.19	30.44	0.5721 ± 0.4213	7.4625 ± 1.8963	13.04	0.008
Transposase	305	gi|58701083	MS	88, NA	43, NA	8, NA	8.40	23.21	0.8037 ± 0.1795	0.0767 ± 0.0400	0.10	0.004
tRNA (uracil-5-)-methyltransferase Gid	182	gi|86749845	MS	70, NA	29, NA	9, NA	6.68	51.43	0.0091 ± 0.0091	0.3487 ± 0.0916	38.36	0.006
tRNA nucleotidyl transferase	247	gi|134096102	MS	64, NA	27, NA	8, NA	6.41	46.43	0.0000 ± 0.0000	0.0249 ± 0.0102	DIV/0	0.040
Potential virulence factors												
Peptidase, M1 family	126	gi|134283293	MS, MS/MS	116, 32	28, 3	15, 2	6.74	82.14	0.1035 ± 0.0935	1.1483 ± 0.3801	11.09	0.028
Peptidase, M1 family	128	gi|134283293	MS, MS/MS	99, 23	21, 1	14, 1	6.74	82.14	0.1379 ± 0.1086	1.3742 ± 0.4828	9.97	0.037
Peptidase, M1 family	136	gi|134283293	MS, MS/MS	94, 24	24, 1	14, 1	6.74	82.14	0.0116 ± 0.0116	1.6693 ± 0.7036	143.85	0.046
Peptidase, M1 family	173	gi|134283293	MS	84, NA	21, NA	12, NA	6.74	82.14	0.0058 ± 0.0058	0.1253 ± 0.0504	21.79	0.046
Chaperones												
GroEL	178	gi|28630950	MS, MS/MS	197, 100	60, 6	20, 2	5.18	56.49	0.0000 ± 0.0000	1.2901 ± 0.2463	DIV/0	0.012
Heat shock protein transcription repressor	272	gi|157693049	MS	62, NA	18, NA	5, NA	5.96	38.90	0.0000 ± 0.0000	0.3754 ± 0.1150	DIV/0	0.011
Phage capsid proteins												
Phage major capsid protein precursor	270	gi|53717812	MS, MS/MS	85, 35	33, 3	11, 1	6.78	38.18	0.0000 ± 0.0000	0.1176 ± 0.0329	DIV/0	0.007
Major capsid protein precursor	401	gi|53722091	MS, MS/MS	141, 54	37, 9	15, 2	6.78	38.22	0.0286 ± 0.0286	0.6991 ± 0.1473	24.42	0.002
Drug resistance protein												
Beta-lactamase-like protein	442	gi|149914600	MS	76, NA	27, NA	11, NA	5.50	60.76	0.0076 ± 0.0076	0.1464 ± 0.0689	19.29	0.008
Solute transport regulatory protein												
Bacterial extracellular solute-binding protein	223	gi|33599920	MS	59, NA	24, NA	10, NA	6.33	58.85	0.0112 ± 0.0112	0.0968 ± 0.0288	8.61	0.025

Table 1 (*continued*)

Protein name	Spot no.	NCBI ID	Identified by	Identification scores (MS, MS/MS)	%Cov (MS, MS/MS)	No. of matched peptides (MS, MS/MS)	pI	MW (kDa)	Intensity (mean ± SEM)		Ratio (NaCl/ control)	p values
									Control	NaCl		
Hypothetical protein												
Hypothetical protein Bpse110_02001906	340	gi|82536900	MS	72, NA	48, NA	7, NA	4.92	16.08	15.4537 ± 3.8195	2.5137 ± 0.3937	0.16	0.010
Hypothetical protein BURPSPAST_T0419	468	gi|157934517	MS	86, NA	33, NA	13, NA	6.39	56.53	0.0000 ± 0.0000	0.3126 ± 0.1011	DIV/0	0.015
Hypothetical protein mlr0590	230	gi|13470794	MS	58, NA	14, NA	6, NA	5.77	60.10	0.0000 ± 0.0000	0.0294 ± 0.0127	DIV/0	0.049
Hypothetical protein XOO4849	167	gi|116247043	MS	72, NA	23, NA	8, NA	8.48	60.57	0.0000 ± 0.0000	0.6520 ± 0.2517	DIV/0	0.032

DIV/0, Divided by zero; MS, mass spectrometry; MS/MS, tandem MS; MW, molecular weight; NA, not applicable; NCBI, National Center for Biotechnology Information; pI, Isoelectric point.

(Savant; Holbrook, NY). The dried gel plugs were then rehydrated with 10 µl of 1% (w/v) trypsin (Promega; Madison, WI) in 25 mM NH_4HCO_3. After rehydration, the gel pieces were crushed with siliconized blue stick and incubated at 37 °C for at least 16 h. Peptides were subsequently extracted twice with 50 µl of 50% ACN/5% trifluoroacetic acid (TFA); the extracted solutions were then combined



B

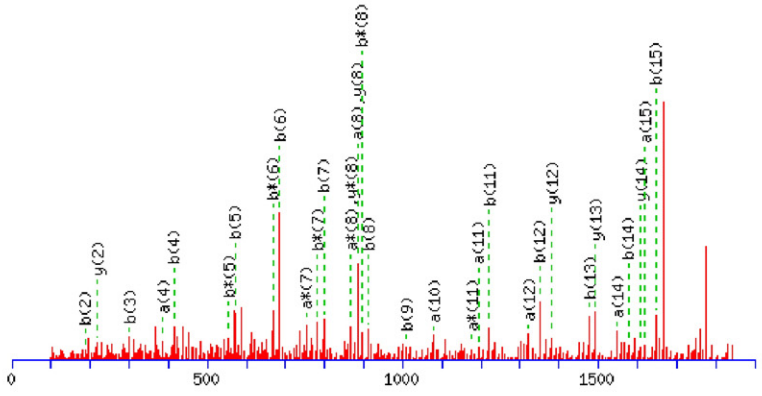
Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
2 - 13	1235.6333	1234.6260	1234.6306	-4	1	M.AAKDVVFGDSAR.A
14 - 28	1572.8392	1571.8319	1571.8705	-25	1	R.AKMVEGVNILANAVK.V*
16 - 34	1953.0045	1951.9972	1952.1129	-59	1	K.MVEGVNILANAVKVTLGPK.G
43 - 58	1549.7974	1548.7901	1548.8148	-16	1	R.SFGGPTVTKDGVSVAK.E
81 - 101	2078.0254	2077.0181	2077.0288	-5	0	K.TSDNAGDGTTTATVLAQSIVR.E
143 - 161	1889.9163	1888.9090	1888.9126	-2	0	K.EIAQVGAISANSDSSIGDR.I
172 - 197	2867.3647	2866.3574	2866.3542	1	1	K.EGVITVEDGKSLADELDVVEGMQFDR.G*
182 - 197	1839.8452	1838.8379	1838.8356	1	0	K.SLADELDVVEGMQFDR.G*
198 - 210	1527.7499	1526.7426	1526.7405	1	0	R.GYLSPYFINNPDK.Q
227 - 242	1794.0376	1793.0303	1793.0411	-6	1	K.VSNIRDLLPVLEQVAK.A
243 - 268	2746.5140	2745.5067	2745.5388	-12	0	K.AGRPLLIIAEDVEGEALATLVVNNIR.G
286 - 311	2759.4595	2758.4522	2758.4674	-5	1	R.KAMLEDIAILTGGQVIAEETGLTLEK.A*
287 - 311	2631.3484	2630.3411	2630.3724	-12	0	K.AMLEDIAILTGGQVIAEETGLTLEK.A*
323 - 345	2399.2305	2398.2232	2398.2339	-4	1	R.IEVGKENTTIIDGAGEAVNIEAR.V
348 - 362	1824.8748	1823.8675	1823.8649	1	1	K.QIRTQIEEATSDYDR.E
351 - 364	1684.7893	1683.7820	1683.7587	14	1	R.TQIEEATSDYDREK.L
405 - 421	1607.9108	1606.9035	1606.9043	-0	0	R.AAVEEGIVPGGGVALIR.A
424 - 445	2225.0896	2224.0823	2224.2539	-77	1	R.TAIASLTGVNADQNAGIKIVLR.A
474 - 501	2992.3872	2991.3799	2991.3920	-4	1	K.GNYGYNAATGEYVDMVEAGVVDPTKVTR.T*
502 - 526	2438.3027	2437.2954	2437.3428	-19	0	R.TALQNAASVAGLLLTTDAAVAELPK.E

Fig. 2. An illustration of MS and MS/MS data of protein spot #178, which was identified as GroEL (gi|28630950). (A) MS spectra. (B) Peptide mass fingerprinting (PMF) of the MS data successfully identified this protein as GroEL with sequence coverage of 60% and PMF score of 197. The identified residues are underlined in (C). (D) MS/MS spectra of the parent ion with *m/z* of 1794.0376, of which the sequence was successfully identified as VSNIRDLLPVLEQVAK (residues 227–242) by MS/MS matching (MS/MS ions score of 100 and sequence coverage of 6%) as demonstrated in (E). * = Oxidation at methionine residue.

C

```
  1 MAAKDVVFGD SARAKMVEGV NILANAVKVT LGPKGRNVVL ERSFGGPTVT
 51 KDGVSVAKEI ELKDKLQNMG AQMVKEVASK TSDNAGDGTT TATVLAQSIV
101 REGMKYVASG MNPMDLKRGI DKAVAAAVEE LKKISKPCTT NKEIAQVGAI
151 SANSDSSIGD RIAEAMDKVG KEGVITVEDG KSLADELDVV EGMQFDRGYL
201 SPYFINNPDK QVAVLENPFV LLHDKKVSNI RDLLPVLEQV AKAGRPLLII
251 AEDVEGEALA TLVVNNIRGI LKTVAVKAPG FGDRRKAMLE DIAILTGGQV
301 IAEETGLTLE KATLAELGQA KRIEVGKENT TIIDGAGEAV NIEARVKQIR
351 TQIEEATSDY DREKLQERVA KLAGGVAVIK VGAATEVEMK EKKARVEDAL
401 HATRAAVEEG IVPGGGVALI RARTAIASLT GVNADQNAGI KIVLRAMEEP
451 LRQIVTNGGE EASVVVAAVA AGKGNYGYNA ATGEYVDMVE AGVVDPTKVT
501 RTALQNAASV AGLLLTTDAA VAELPKEDAP MPGGMPGGM
```

D



E

#	a	a*	b	b*	Seq.	y	y*	#
1	72.0808		100.0757		V			16
2	159.1128		**187.1077**		S	1694.9799	1677.9534	15
3	273.1557	256.1292	**301.1506**	284.1241	N	**1607.9479**	1590.9214	14
4	**386.2398**	369.2132	**414.2347**	397.2082	I	**1493.9050**	1476.8784	13
5	542.3409	525.3144	**570.3358**	**553.3093**	R	**1380.8209**	1363.7944	12
6	657.3678	640.3413	**685.3628**	**668.3362**	D	1224.7198	1207.6933	11
7	770.4519	**753.4254**	**798.4468**	**781.4203**	L	1109.6929	1092.6663	10
8	**883.5360**	**866.5094**	**911.5309**	**894.5043**	L	996.6088	979.5823	9
9	980.5887	963.5622	**1008.5837**	991.5571	P	**883.5247**	**866.4982**	8
10	**1079.6572**	1062.6306	1107.6521	1090.6255	V	786.4720	769.4454	7
11	**1192.7412**	**1175.7147**	**1220.7361**	1203.7096	L	687.4036	670.3770	6
12	**1321.7838**	1304.7573	**1349.7787**	1332.7522	E	574.3195	557.2930	5
13	1449.8424	1432.8158	**1477.8373**	1460.8108	Q	445.2769	428.2504	4
14	**1548.9108**	1531.8843	**1576.9057**	1559.8792	V	317.2183	300.1918	3
15	**1619.9479**	1602.9214	**1647.9428**	1630.9163	A	**218.1499**	201.1234	2
16					K	147.1128	130.0863	1

Fig. 2 (*continued*).

and dried with the SpeedVac concentrator. The peptide pellets were resuspended with 10 μl of 0.1% TFA and purified using $ZipTip_{C18}$ (Millipore). The peptide solution was drawn up and down in the $ZipTip_{C18}$ 10 times and then washed with 10 μl of 0.1% formic acid by drawing up and expelling the washing solution three times. The peptides were finally eluted with 5 μl of 75% ACN/0.1% formic acid.

2.6. MS and/or MS/MS analyses

The trypsinized samples were premixed 1:1 with the matrix solution containing 5 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN, 0.1% (v/v) TFA and 2% (w/v) ammonium citrate, and deposited onto the 96-well MALDI target plate. The samples were analyzed by Q-

TOF Ultima™ mass spectrometer (Micromass; Manchester, UK), which was fully automated with predefined probe motion pattern and the peak intensity threshold for switching over from MS survey scanning to MS/MS, and from one MS/MS to another. Within each sample well, parent ions that met the predefined criteria (any peak within the m/z 800–3000 range with intensity above 10 count ± include/exclude list) were selected for CID MS/MS using argon as the collision gas and a mass dependent ± 5 V rolling collision energy until the end of the probe pattern was reached. The MS and MS/MS data were extracted and outputted as the searchable *.txt* and *.pkl* files, respectively, for independent searches using the MASCOT search engine (http://www.matrixscience.com), assuming that peptides were monoisotopic. Fixed modification was carbamidomethylation at cysteine residues, whereas variable modification was oxidation at methionine residues. Only one missed trypsin cleavage was allowed, and peptide mass tolerances of 100 and 50 ppm were allowed for peptide mass fingerprinting and MS/MS ions search, respectively.

2.7. Western blot analysis

Totally 20 μg proteins extracted from each sample were resolved with SDS-PAGE at 150 V for approximately 2 h using SE260 mini-Vertical Electrophoresis Unit (GE Healthcare). After the completion of SDS-PAGE, proteins were transferred onto a nitrocellulose membrane and non-specific bindings were blocked with 5% milk in PBS for 1 h. The membrane was then incubated with mouse monoclonal anti-GroEL (60 kDa chaperonin or HSP60) antibody (1:500 in 5% milk/PBS) (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) at 4 °C overnight. After washing, the membrane was further incubated with rabbit anti-mouse IgG conjugated with horseradish peroxidase (1:1000 in 5% milk/PBS) (Dako; Glostrup, Denmark) at room temperature for 1 h. Reactive protein bands were then visualized with SuperSignal® West Pico chemiluminescence substrate (Pierce Biotechnology, Inc.; Rockford, IL).

2.8. Evaluation of bacterial survival after treatment with a beta-lactam antibiotic

Assay to evaluate beta-lactamase activity was performed by measuring bacterial survival after an exposure to ceftazidime, which is a beta-lactam antibiotic. *B. pseudomallei* 10276 strain was maintained in LB broth as aforementioned. After 7 h of culture in two differential media (with or without NaCl), both groups were treated with ceftazidime (Millimed; Samut Prakarn, Thailand) at the final concentration of 1000 μg/ml. Their viable colony forming units (CFU) were counted before and after 2-h treatment with ceftazidime. Percentage of bacterial survival was determined using the formula: $\%\text{Survival} = [\text{CFU}_{(\text{after ceftazidime treatment})} / \text{CFU}_{(\text{before ceftazidime treatment})}] \times 100\%$.

3. Results and discussion

We evaluated changes in the secretome of *B. pseudomallei* under an exposure to the high-salt environment (by an addition of NaCl into the LB broth at a final concentration of 150 mM). To the best of our knowledge, this is the first study characterizing the secretome of *B. pseudomallei*. After 7 h of the exposure to salt stress, the 2-D proteome profile of the NaCl-exposed bacterium was compared to that of the control ($n = 5$ replicates for each group). Using 2-D PAGE and SYPRO Ruby fluorescence stain, 93 ± 21 spots were visualized in the NaCl-exposed group, whereas 148 ± 14 spots were detected in the controls. Image Master 2D Platinum software was employed to compare the protein spot pattern in all 2-D gels (5 gels in each group; totally 10 gels were analyzed). Quantitative intensity analysis and statistics revealed significant differences in spot intensity levels of totally 42 protein spots between the two groups (Fig. 1). All these significant differences had p values < 0.05, and were consistently present or absent in all five gels of each group.

Among these, 17 spots had increased levels (NaCl/control relative abundance ratios were ranged from 6.00 to 143.85 fold), 7 spots had decreased levels (NaCl/control relative abundance ratios were ranged from 0.02 to 0.19 fold), 3 spots were absent, and 15 spots were present only after an exposure to NaCl (Table 1 and Fig. 1). These data indicate the high degree of alterations in secretome of *B. pseudomallei* under a salt stress. These altered proteins were then successfully identified by Q-TOF MS and/or MS/MS analyses. Most of the altered proteins identified in this study were metabolic enzymes, transcription/translation regulators, and potential virulence factors. Other altered proteins included chaperones, drug resistance protein, solute transport regulator, and hypothetical proteins. Fig. 2 illustrates MS and MS/MS analyses of spot #178, which was identified as GroEL (gi|28630950). Fig. 2A illustrates MS spectra of this spot, whereas Fig. 2B and C demonstrates peptide mass fingerprinting (PMF) of this spot with a matching score of 197 and sequence coverage of 60% (from totally 20 significantly matched peptides). Fig. 2D and E shows the MS/MS matching of GroEL with a MS/MS ion score of 100 and sequence coverage of 6% (from 2 significantly matched peptides). Fig. 3 shows Western blot data, which confirmed the presence of GroEL in the culture supernatant only after an exposure to NaCl.

Recently, the complete genome sequence and annotation of *B. pseudomallei* has been made available [8,9], allowing the post-genomic characterizations of *B. pseudomallei* feasible and leading to a wider biological view of this microorganism. Based on the genomic annotation and translation, proteins that are associated with the survival of *B. pseudomallei* include those involving in secondary metabolism (i.e., antibiotic, surfactant, and siderophore biosynthetic pathways); drug resistance (i.e., beta-lactamases, multidrug efflux pumps, and amino-glycoside acetyltransferase); intracellular stress (i.e., superoxide and nitric oxide detoxification enzymes); and motility and chemotaxis (i.e., flagella proteins and chemotaxis-associated proteins) [4,7,8]. We identified a 19-fold increase of beta-lactamase-like protein during an exposure to the high-salt environment. The increase in secreted beta-

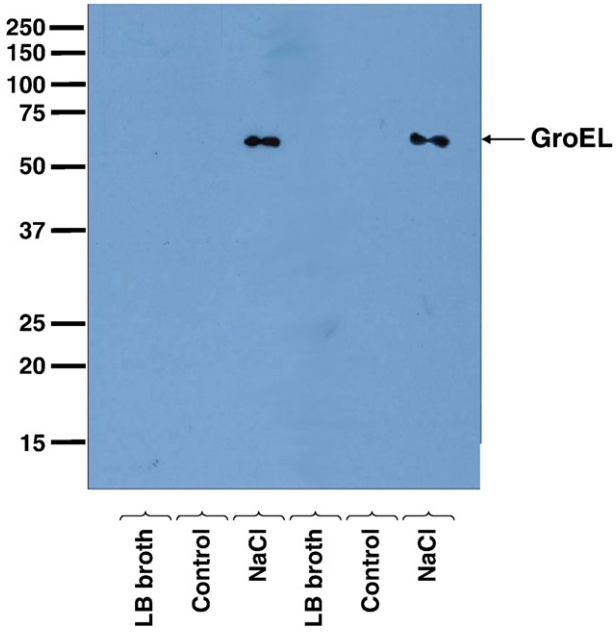


Fig. 3. Western blot analysis for GroEL. Totally 20 μg proteins extracted from each sample were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Basal LB broth (prior to bacterial culture) also served as another control. After blocking, the membrane was incubated with mouse monoclonal anti-GroEL (60 kDa chaperonin or HSP60) antibody (1:500 in 5% milk in PBS) at 4 °C overnight and then with rabbit anti-mouse IgG conjugated with horseradish peroxidase (1:1000 in 5% milk in PBS) at room temperature for 1 h. Reactive protein bands were then visualized with SuperSignal® West Pico chemiluminescence substrate.

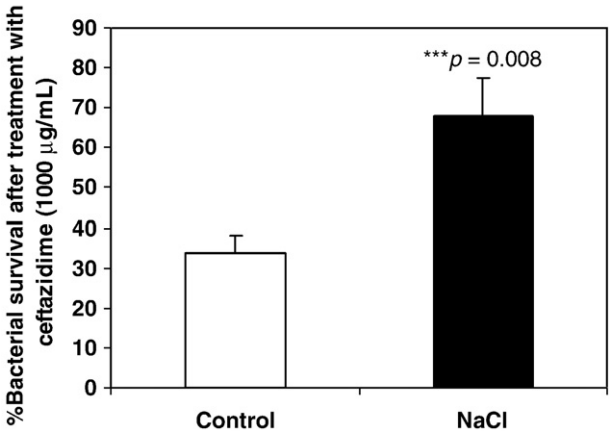


Fig. 4. Evaluation of bacterial survival after treatment with ceftazidime. *B. pseudomallei* 10276 strain was maintained in LB broth as detailed in "Materials and methods". After 7 h of culture in two differential media (with or without NaCl), both groups were treated with 1000 μg/ml ceftazidime. Their viable colony forming units (CFU) were counted before and after 2-h treatment with ceftazidime. Percentage of bacterial survival was determined using the formula: %Survival = [$CFU_{(after\ ceftazidime\ treatment)}$ / $CFU_{(before\ ceftazidime\ treatment)}$] × 100%.

lactamases or beta-lactamase-like proteins in culture supernatant might reflect the adaptive response of *B. pseudomallei* under the salt stress to resist to beta-lactam antibiotics [10,11]. We therefore performed a functional analysis to compare the bacterial survival after treatment with 1000 μg/ml ceftazidime, a beta-lactam antibiotic, in NaCl-exposed bacterium versus control. Fig. 4 demonstrates that the NaCl-exposed bacterium had significantly greater survival rate after ceftazidime treatment, indicating its resistance to ceftazidime (beta-lactam antibiotic) induced by the salt stress. Indeed, most of the identified proteins that were altered during an exposure to NaCl included metabolic enzymes and transcription/translation regulators, which are very important for bacterial growth and survival. Most of them were either increased or newly present after an exposure to NaCl, implicating their important roles in adaptive response.

During the environmental stresses, several of partly folded cellular proteins tended to aggregate with others via their exposed hydrophobic regions [12]. To combat or prevent this stress-induced effect, the chaperonin GroEL (60 kDa chaperonin or HSP60) together with GroES form a folding cage to encapsulate the individual polypeptide chains to allow these polypeptide chains to fold until the hydrophobic regions are buried within the completely folded structure [12–14]. We identified an increased level of secreted GroEL during the salt stress. This increase might reflect adaptive response of *B. pseudomallei* to prevent protein aggregation induced by salt stress. While GroEL level was increased, we observed the increased level of a protein that prevents the transcription of heat shock proteins in our model. The mechanism underlying this disparate result remains unknown and should be further elucidated.

Apart from the proteins that are involved in the survival as aforementioned, proteins that are associated with the virulence of *B. pseudomallei* include type I, II, III and IV secretion systems; surface components (i.e., lipopolysaccharide, capsular polysaccharide and potential surface polysaccharide biosynthesis); exoproteins (i.e., phospholipase C, metalloproteases A, collagenase and other proteases); fimbriae/pili; and adhesins or adhesive molecules that modulate host-cell interaction [4,7,8]. We identified 4 forms of peptidase in M1 family, which is a membrane protease, with increased levels (ranged from 9.97 to 143.85 fold that is the maximal degree of changes identified in this study). These findings provided the first evidence demonstrating that salt stress can enhance the secretion of these potential virulence factors of *B. pseudomallei*.

In summary, we report herein the first dataset of the secretome of *B. pseudomallei* and its alterations during salt stress. These findings may provide some novel insights into the adaptive response of this microorganism to survive during the salt stress. Further characterizations and functional analyses of these altered proteins may lead to identification of new therapeutic targets or vaccine development for melioidosis.

Acknowledgements

We are grateful to Napat Songtawee, Theptida Semangoen, and Veerachat Muangsombut for their technical assistance. We also thank the Core Facilities for Proteomics and Structural Biology Research, Institute of Biological Chemistry, Academia Sinica, Taiwan. This study was supported by Siriraj Grant for Research and Development, Commission on Higher Education, and The Thailand Research Fund (Grant RMU 5080015) (to S. Korbsrisate and V. Thongboonkerd). P. Pumirat was supported by Siriraj Graduate Scholarship and by the Royal Golden Jubilee Ph.D. Program.

References

[1] N.J. White, Melioidosis, Lancet 361 (2003) 1715–1722.
[2] B.J. Currie, S.P. Jacups, Intensity of rainfall and severity of melioidosis, Australia, Emerg. Infect. Dis. 9 (2003) 1538–1542.
[3] B.J. Currie, S.P. Jacups, A.C. Cheng, D.A. Fisher, N.M. Anstey, S.E. Huffam, V.L. Krause, Melioidosis epidemiology and risk factors from a prospective whole-population study in northern Australia, Trop. Med. Int. Health 9 (2004) 1167–1174.
[4] A.C. Cheng, B.J. Currie, Melioidosis: epidemiology, pathophysiology, and management, Clin. Microbiol. Rev. 18 (2005) 383–416.
[5] A. Leelarasamee, Recent development in melioidosis, Curr. Opin. Infect. Dis. 17 (2004) 131–136.
[6] S.J. Peacock, Melioidosis, Curr. Opin. Infect. Dis. 19 (2006) 421–428.
[7] W.J. Wiersinga, P.T. van der, N.J. White, N.P. Day, S.J. Peacock, Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*, Nat. Rev. Microbiol. 4 (2006) 272–282.
[8] M.T. Holden, R.W. Titball, S.J. Peacock, A.M. Cerdeno-Tarraga, T. Atkins, L.C. Crossman, T. Pitt, C. Churcher, K. Mungall, S.D. Bentley, M. Sebaihia, N.R. Thomson, N. Bason, I.R. Beacham, K. Brooks, K.A. Brown, N.F. Brown, G.L. Challis, I. Cherevach, T. Chillingworth, A. Cronin, B. Crossett, P. Davis, D. DeShazer, T. Feltwell, A. Fraser, Z. Hance, H. Hauser, S. Holroyd, K. Jagels, K.E. Keith, M. Maddison, S. Moule, C. Price, M.A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, M. Simmonds, S. Songsivilai, K. Stevens, S. Tumapa, M. Vesaratchavest, S. Whitehead, C. Yeats, B.G. Barrell, P.C. Oyston, J. Parkhill, Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 14240–14245.
[9] S. Tumapa, M.T. Holden, M. Vesaratchavest, V. Wuthiekanun, D. Limmathurotsakul, W. Chierakul, E.J. Feil, B.J. Currie, N.P. Day, W.C. Nierman, S.J. Peacock, *Burkholderia pseudomallei* genome plasticity associated with genomic island variation, BMC Genomics 9 (2008) 190.
[10] K.E. Keith, P.C. Oyston, B. Crossett, N.F. Fairweather, R.W. Titball, T.R. Walsh, K.A. Brown, Functional characterization of OXA-57, a class D beta-lactamase from *Burkholderia pseudomallei*, Antimicrob. Agents Chemother. 49 (2005) 1639–1641.
[11] C. Tribuddharat, R.A. Moore, P. Baker, D.E. Woods, *Burkholderia pseudomallei* class a beta-lactamase mutations that confer selective resistance against ceftazidime or clavulanic acid inhibition, Antimicrob. Agents Chemother. 47 (2003) 2082–2087.
[12] R.J. Ellis, Chaperomics: in vivo GroEL function defined, Curr. Biol. 15 (2005) R661–R663.
[13] Y.C. Tang, H.C. Chang, A. Roeben, D. Wischnewski, N. Wischnewski, M.J. Kerner, F.U. Hartl, M. Hayer-Hartl, Structural features of the GroEL-GroES nano-cage required for rapid folding of encapsulated protein, Cell 125 (2006) 903–914.
[14] S.E. Radford, GroEL: more than Just a folding cage, Cell 125 (2006) 831–833.