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Altered Proteome in *Burkholderia pseudomallei rpoE* Operon Knockout Mutant: Insights into Mechanisms of *rpoE* Operon in Stress Tolerance, Survival, and Virulence

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We have previously shown that the alternative sigma factor σ^E (RpoE), encoded by *rpoE*, is involved in stress tolerance and survival of *Burkholderia pseudomallei*. However, its molecular and pathogenic mechanisms remain unclear. In the present study, we applied gel-based, differential proteomics to compare the cellular proteome of an *rpoE* operon knockout mutant (RpoE Mut) to that of wild-type (K96243 WT) *B. pseudomallei*. Quantitative intensity analysis ($n = 5$ gels from 5 individual culture flasks in each group) revealed significantly differential expression of 52 proteins, which were subsequently identified by Q-TOF MS/MS. These included oxidative, osmotic, and other stress response proteins; chaperones; transcriptional/translational regulators; metabolic enzymes; proteins involved in cell wall synthesis, fatty synthesis, glycogen synthesis, and storage; exported proteins; secreted proteins; adhesion molecule; protease/peptidase; protease inhibitor; signaling proteins; and other miscellaneous proteins. The down-regulation of several stress response proteins, chaperones, transcriptional/translational regulators, and proteins involved in cell wall synthesis in RpoE Mut provided some new insights into the mechanisms of the *rpoE* operon for the stress tolerance and survival of *B. pseudomallei*. In addition, the proteomic data and *in vivo* study indicated that the *rpoE* operon is also involved in the virulence of *B. pseudomallei*. Our findings underscore the usefulness of proteomics for unraveling pathogenic mechanisms of diseases at the molecular level.

Keywords: Proteome • Proteomics • Melioidosis • Burkholderia • RpoE • Stress tolerance • Survival • Virulence

Introduction

Burkholderia pseudomallei is a Gram-negative bacillus found in soil and water and is the causative agent of melioidosis, a disease of which clinical manifestations can be acute or chronic. Organ involvement in melioidosis ranges from local to systemic, and its severity varies from mild to fatal. Almost all organs, with the exception of hair and nails, can be affected by melioidosis.[1] Mortality rates in patients with septic shock caused by melioidosis are approximately 80−95% despite adequate treatment.[1] Previous studies on melioidosis have focused not only on the improvement of therapeutic outcome, but also on the understanding of the pathogenic mechanisms of this infectious disease. Moreover, *B. pseudomallei* has been considered as a potential bioterrorism weapon.[2] Better understanding of the molecular basis and pathogenic mechanisms of this organism is, therefore, critically required for the discovery of new therapeutic targets and vaccine development for disease prevention.

As *B. pseudomallei* is a saprophyte found in soil and water, it is a difficult microorganism to kill, and it can survive in these environments for years. Additionally, this microorganism is resistant to several antibiotics, chemicals, organic compounds, and other stressful conditions.[3] Moreover, it can survive within different eukaryotic cell types, including mammalian phagocytes. Thus, stress tolerance has been thought to be one of the

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important factors for the survival of *B. pseudomallei* both inside and outside of the human body. While much progress has been made regarding its virulence factors, that is, secretory proteins and cell-associated antigens,[3] little is known about the stress tolerance of this bacterial pathogen.

In *Escherichia coli*, the alternative sigma factor σ^E (RpoE), encoded by *rpoE*, plays an important role in maintaining the integrity of the cell envelope (by controlling the transcription of several genes associated with cell envelope integrity) and is, thus, essential for viability of the bacterium.[4] During stresses (heat stress, chemical exposure, etc.), RpoE is activated and transcribes genes in its regulon, including those encoding chaperones and proteases, which subsequently refold and degrade misfolded proteins, respectively. Recently, RpoE has been shown to play a critical role in survival, stress response, and virulence of several other bacteria, that is, *Azotobacter vinelandii*,[5,6] *Bacillus subtilis*,[7,8] *Haemophilus influenzae*,[9] *Mycobacterium tuberculosis*,[10] *Pseudomonas aeruginosa*,[11,12] *Pseudomonas fluorescens*,[13] *Salmonella enterica*,[14] *Streptomyces antibioticus*,[15] and *Vibrio cholerae*.[16] More recently, we have demonstrated that the *rpoE* operon also plays a pivotal role in stress tolerance and biofilm formation in *B. pseudomallei*.[17] These data underscore the significance of RpoE in various bacteria. However, the available information is limited and does not provide sufficient insights into the mechanisms of how the *rpoE* operon controls such functions in these bacteria.

In the present study, we explored further the mechanisms of the *rpoE* operon in controlling the stress tolerance and survival of *B. pseudomallei*. Fortunately, the complete genome sequence and annotation of *B. pseudomallei* (K96243 strain) have recently been made available.[18] We, thus, performed a proteomic analysis of RpoE-associated proteins in *B. pseudomallei*. The cellular proteome of K96243 wild-type (K96243 WT) was compared with that of an *rpoE* operon knockout mutant (RpoE Mut) using a gel-based, differential proteomics strategy. The results showed that several components of stress response proteins were down-regulated in RpoE Mut. Additional findings were the down-regulation of two potential virulence factors. *In vivo* experiments using BALB/c mice showed that animals infected with RpoE Mut had a marked delay in time to death, indicating that the *rpoE* operon is also involved in the virulence of *B. pseudomallei*.

Materials and Methods

Bacterial Culture. *B. pseudomallei* K96243 WT (kindly provided by Prof. T. Dharakul) and *rpoE* operon mutant (RpoE Mut)[17] were maintained in Luria−Bertani (LB) broth at 37 °C until the stationary phase was reached. The RpoE Mut was constructed as described previously by Korbsrisate et al.[17] Briefly, a 270-bp internal fragment of the putative *rpoE* coding sequence was PCR-amplified from *B. pseudomallei* K96243 genomic DNA using the primers ALG36 (5′ CTC CAA ATA CCA CCG CAA GAT 3′) and ALG37 (5′ TAT CCC TTA GTT GGT CCG 3′), which correspond to *B. pseudomallei rpoE* nucleotides at the positions of 78−98 and 332−349, respectively. The 270-bp PCR product was cloned into the *Eco*RV restriction site of the pKNOCK-Cm vector[19] to create pPK-1. This construct was introduced from *E. coli* S17−1*λpir*[20] into *B. pseudomallei* K96243 by conjugation. An insertion mutant was selected on *Pseudomonas* agar supplemented with SR103 and 30 μg/mL chloramphenicol.

Southern Blot Analysis. Southern blot hybridization was done according to the method described by Southern.[21] Briefly, chromosomes of both strains were digested with restriction enzymes, including *Xho*I and *Xho*I/*Eco*RV, and separated by agarose gel electrophoresis. DNAs in the gel were denatured and transferred onto a nylon membrane using a capillary blotting system. The blot was fixed by baking at 80 °C for 2 h, then hybridized with a 270-bp *rpoE* homologue DNA labeled probe. After eliminating the nonspecific binding of probe, the hybridized bands were detected by radiography.

Protein Extraction for Proteomic Analysis. At the stationary phase with a comparable bacterial count, bacteria were collected using 1000*g* centrifugation for 5 min and washed three times with phosphate-buffered saline (PBS). Bacterial proteins were extracted using a buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS), 2% (v/v) ampholytes (pH 3−10), 120 mM dithiothreitol (DTT), and 40 mM Tris-base and incubated at 4 °C for 30 min. After centrifugation at 12 000*g* for 5 min, the supernatant was saved and the protein concentration was measured by spectrophotometry using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) based on Bradford's method. Because urea, thiourea, CHAPS, and other compositions in the sample/lysis buffer can interfere with the protein estimation, we generated the standard curve using bovine serum albumin at concentrations of 0, 2, 5, 7, and 10 μg/μL in the same sample/lysis buffer to ensure that the standards and the samples had the same background that might occur due to chemical interference. Proteins extracted from each cultured flask were further resolved in individual 2-D gels; $n = 5$ gels (from 5 cultured flasks) for each group; total $n = 10$ gels.

Two-Dimensional Electrophoresis (2-DE) and Staining. Immobiline DryStrip (nonlinear pH 3−10, 7 cm long; Amersham Biosciences, Uppsala, Sweden) was rehydrated overnight with 200 μg of total protein (equal loading for each sample) that was premixed with a rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 2% (v/v) ampholytes (pH 3−10), 120 mM DTT, 40 mM Tris-base, and bromophenol blue (to make the final volume of 150 μL per strip). The first dimensional separation (IEF) was performed in an Ettan IPGphor II IEF System (Amersham Biosciences) at 20 °C, using a stepwise mode to reach 9000 Vh. After completion of the IEF, proteins on the strip were equilibrated in a buffer containing 6 M urea, 130 mM DTT, 30% glycerol, 112 mM Tris-base, 4% sodium dodecyl sulfate (SDS), and 0.002% bromophenol blue, for 10 min, and then with another buffer containing 6 M urea, 135 mM iodoacetamide, 30% glycerol, 112 mM Tris-base, 4% SDS, and 0.002% bromophenol blue for 10 min. The IPG strip was then transferred onto a 12% acrylamide slab gel (8 × 9.5 cm), and the second-dimensional separation was performed in an SE260 Mini-Vertical Electrophoresis Unit (Amersham Biosciences) with the current of 20 μA/gel for 1.5 h. Separated protein spots were then visualized with Coomassie Brilliant Blue R-250 stain (Fluka Chemica AG, Buchs, Switzerland).

Spot Analysis and Matching. Image Master 2D Platinum (Amersham Biosciences) software was used for matching and analysis of protein spots on 2-D gels. 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 matching of corresponding protein spots between gels. Background subtraction was performed, and the intensity volume of each spot was normalized with total intensity volume (summation of the intensity volumes obtained from all spots

within the same 2-D gel). The variability of the 2-D spot pattern was evaluated by determining the coefficient of variation (CV) of the normalized intensity of corresponding spots across different gels using the following formula: CV = Standard deviation/Mean.

In-Gel Tryptic Digestion. Differentially expressed protein spots were excised from 2-D gels, and the gel pieces were washed with 200 μL of 50% acetonitrile (ACN)/25 mM NH_4HCO_3 buffer (pH 8.0) for 15 min twice. The gel pieces were then washed once with 200 μL of 100% ACN and dried using a Speed Vac concentrator (Savant, Holbrook, NY). Dried gel pieces were swollen with 10 μL of 1% (w/v) trypsin (Promega, Madison, WI) in 25 mM NH_4HCO_3. The gel pieces were then crushed with a 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 and dried with the Speed Vac concentrator. The peptide pellets were then resuspended in 10 μL of 0.1% TFA, and the resuspended solutions were purified using $ZipTip_{C18}$ (Millipore, Bedford, MA). Ten microliters of sample was drawn up and down in the ZipTip 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.

Protein Identification by Q-TOF MS/MS. The proteolytic samples were premixed 1:1 with the matrix solution (5 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN, 0.1% v/v TFA, and 2% w/v ammonium citrate) and spotted onto the 96-well sample stage. The samples were analyzed using the Q-TOF Ultima mass spectrometer (Micromass, Manchester, U.K.), 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 (all details are available at http://proteome.sinica.edu.tw). The MASCOT (http://www.matrixscience.com) search engine was used for obtaining protein identities and peptide sequences, based on the assumptions that peptides were monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. The search was performed using the entire protein databases of the Swiss-Prot and TrEMBL and MSDB. A mass tolerance of 50 ppm was used, and up to 1 missed trypsin cleavage was allowed.

***In Vivo* Virulence Study.** Evaluation of the virulence of RpoE Mut was performed using a pulmonary model of melioidosis in BALB/c mice as described previously.[22] In summary, 1000 CFU of either K96243 WT or RpoE Mut was administered *via* the intranasal route ($n = 6$ per group), and the mice were then monitored twice daily for signs of infection and mortality.

Statistical Analysis. Comparisons between groups were performed using unpaired t test. For the virulence study, differences in survival of the infected animals were analyzed using a log rank test. P-values less than 0.05 were considered statistically significant.

Results and Discussion

Validation of the *rpoE* Operon Knockout Mutant. Southern hybridization was performed to confirm the integration of the pKNOCK suicide vector on the *rpoE* operon knockout mutant (RpoE Mut). The genomic DNAs from the K96243 WT and RpoE Mut of *B. pseudomallei* were prepared. Both genomic DNAs were digested with restriction enzymes, including *Xho*I and *Eco*RV/*Xho*I. Southern blot of the digested genomic DNAs was hybridized with an *rpoE* DNA probe. As expected, one DNA hybridization fragment (909 bp) was detected in the *XhoI* genomic DNA of K96243 WT, whereas two DNA hybridizing fragments (2632 bp and 410 bp) were detected in *XhoI* genomic DNA of RpoE Mut (Figure 1; lanes 1−3). As also predicted, a 384 bp *Eco*RV/*Xho*I DNA fragment from K96243 WT was detected (Figure 1; lane 7), whereas 2107 bp and 410 bp *Eco*RV/*Xho*I DNA fragments from RpoE Mut were shown (Figure 1; lanes 5 and 6). These results indicated that there was an insertion of the pKNOCK vector at the *rpoE* gene on the chromosome of *B. pseudomallei* RpoE Mut.

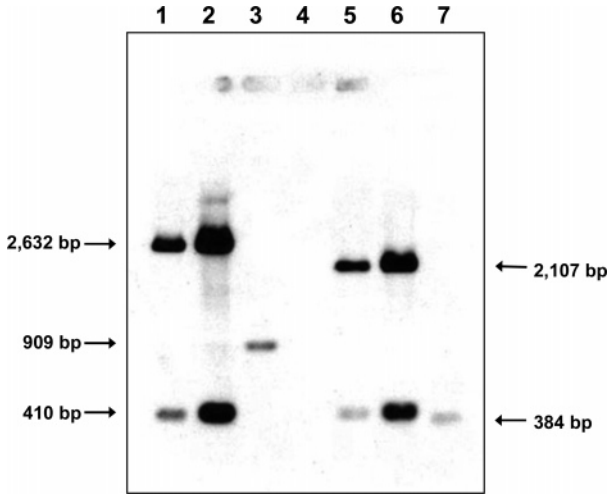


Figure 1. Southern blot analysis of digested *B. pseudomallei* genomic DNA hybridized with a 270-bp *rpoE* specific DNA probe. Lanes 1 and 2, *Xho*I-digested genomic DNA from RpoE Mut; lane 3, *Xho*I-digested genomic DNA from K96243 WT; lane 4, standard marker (1 kb DNA ladder); lanes 5 and 6, *Xho*I/*Eco*RV-digested genomic DNA from RpoE Mut; lane 7, *Xho*I/*Eco*RV-digested genomic DNA from K96243 WT.

Altered Proteome in RpoE Mut *B. pseudomallei*. As RpoE is a transcriptional factor, it is expected that production of several proteins (gene products) are controlled by RpoE. To explore such gene products and to further investigate the molecular mechanisms of RpoE, we performed a proteomic analysis of differentially expressed proteins in RpoE Mut compared to K96243 WT. Proteins extracted from bacteria in each cultured flask were resolved in individual 2-D gels; $n = 5$ gels (from 5 cultured flasks) for each group; total $n = 10$ gels. Figure 2 shows representative 2-D gels of cellular proteins extracted from K96243 WT and RpoE Mut. Up to 450 protein spots were visualized in each gel. Among these, quantitative intensity analysis and statistics revealed 52 differentially expressed protein spots (with $p < 0.05$) between the K96243 WT and RpoE Mut groups.

These differentially expressed proteins were subsequently identified by Q-TOF MS/MS, and their identities as well as quantitative data are shown in Table 1. The amino acid sequences of peptides identified are provided in Supporting Information (Table S1). These identified proteins were classified into functional groups based on their functions provided in the Swiss-Prot and TrEMBL protein databases and on literature

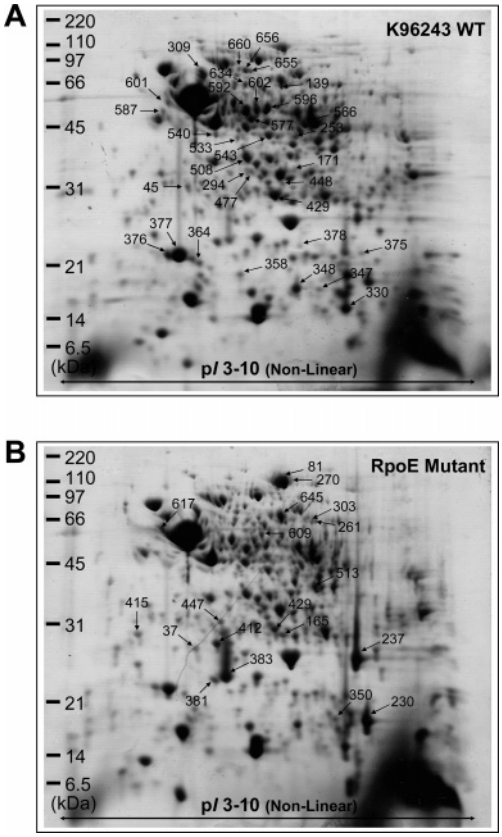


Figure 2. Proteome maps of differentially expressed proteins. (A) Representative 2-D gel for K96243 WT and (B) for RpoE Mut ($n = 5$ gels for each group; total $n = 10$). Quantitative intensity analysis revealed 52 differentially expressed protein spots between the two groups. Down-regulated proteins are labels with numbers in panel A, whereas up-regulated proteins are labeled in panel B. These differentially expressed proteins were subsequently identified by Q-TOF MS/MS (see Table 1 and Supporting Information).

search in PubMed. Some of the identified proteins were 'hypothetical proteins' of which the functions are unknown or have not previously been determined. However, sequences of some of these hypothetical proteins were identical (100% similarity) or almost identical (89−94% similarity) to the known proteins, of which functions have been clearly defined. Therefore, these hypothetical proteins were functionally classified based on their respective homologues.

Impaired Stress Tolerance and Decreased Intracellular Survival of the *rpoE* Operon Knockout Mutant. Because *B. pseudomallei* is a saprophyte found in soil and can survive in eukaryotic cells as well as in phagocytes, it is expected that this bacterium must have regulatory mechanisms for adaptation in these stressful environments, particularly the enrichment of free radicals or reactive oxygen intermediates and high osmolarity. In our previous report [see Korbsrisate et al.[17]], we had examined the effects of *rpoE* operon knockout on the susceptibility of *B. pseudomallei* to oxidative (using 100 mM menadione, 1 M H_2O_2, and 4 M H_2O_2) and osmotic stresses (using 2 M NaCl), and evaluated the viability of RpoE Mut inside mammalian (murine) macrophages. The results clearly showed that the *rpoE* operon knockout caused impaired tolerance to the oxidative stress (as the zone of growth inhibition was greater in RpoE Mut compared to K96243 WT) and to the osmotic stress.[17] For the intracellular survival, the viability of RpoE Mut in mammalian phagocytes was significantly reduced.[17] These data indicated that the *rpoE* operon is crucial for stress tolerance and intracellular survival of *B. pseudomallei*.

Interestingly, 16 out of 52 differentially expressed proteins identified in our present study were oxidative stress response proteins, osmotic stress response proteins, chaperones, and other stress response proteins (Table 1). Almost all of these stress response proteins were down-regulated (RpoE Mut/K96243 WT ratios ranged from 0.24 to 0.79; average = 0.47). The down-regulation (approximately 50% from the baseline) of these stress associated proteins, particularly the oxidative stress response group (i.e., AhpC/Tsa family antioxidant protein, ferritin-like domain protein, flavohemoprotein, and peroxidase/catalase) and chaperones (i.e., 60 kDa chaperonin, GroEL, heat shock protein HtpG, PspA/IM30 family protein, and universal stress protein family), in RpoE Mut was concordant with the phenotype of the *rpoE* operon knockout mutant, of which the stress tolerance was impaired. Osmotically inducible Y domain protein was one among the three up-regulated stress associated proteins. While it was up-regulated, the phenotype of RpoE Mut showed impaired osmotic stress tolerance. These disparate results were not surprising as osmotic stress in several other models can be regulated by various chaperones and other stress associated proteins,[23−25] some of which were down-regulated in our present study (Table 1). These data strengthened the information that the *rpoE* operon is required for stress tolerance in *B. pseudomallei* via controlling transcription/translation of various stress associated proteins.

Other groups of the differentially expressed proteins were transcriptional/translational regulators and proteins involved in cell wall synthesis. Almost all of these altered proteins were down-regulated or absent (or might be under the detection limit of our experimental procedures) in RpoE Mut. The down-regulation of these two groups of proteins together with the down-regulation of several stress response proteins, as mentioned above, most likely led to the decreased survival of RpoE Mut in mammalian (murine) phagocytes.[17]

There were a few groups of proteins (i.e., metabolic enzymes and exported proteins) in which the number of the up-regulated proteins was comparable to that of the down-regulated proteins within the same group. This pattern of changes implicates that there was a balance between the up-regulations and the down-regulations, similar to changes almost always observed in several other models of experimental interventions or gene manipulation in our previous studies.[26−30] Further examination of their roles and association with RpoE is required to better understand the functional significance of these altered proteins in *B. pseudomallei*.

Impaired Virulence of the *rpoE* Operon Knockout Mutant. Potential virulence factors of *B. pseudomallei* (which include both well-known and possible ones) are secretion type I−IV systems, surface components (i.e., lipopolysaccharide, capsular polysaccharide, and potential surface polysaccharide biosynthesis), fimbriae/pili, exoproteins (phospholipase C, metalloproteases A, collagenase, and other proteases), and adhesins or adhesive molecules that modulate host-cell interaction.[3,18] In the present study, we identified the down-regulation of a protease/peptidase (serine-type carboxypeptidase family protein; spot no. 634) and of an adhesion molecule (phospholipid-binding protein; spot no. 364) (Table 1) that may be the potential virulence factors of *B. pseudomallei*. Although there is no direct evidence demonstrating that carboxypeptidase is

Table 1. The Altered Proteins in RpoE Mut

altered proteins	spot no.	Quantity (Intensity) (Mean ± SEM) K96243 WT	RpoE Mut	*p*	ratio (Mut/WT)	change	ID	ion score	%cov	p*I*	MW
Oxidative Stress Response Proteins											
Antioxidant, AhpC/Tsa family [*Burkholderia mallei*][a]	**376**	0.5071 ± 0.0466	0.2176 ± 0.0311	0.001	0.43	Down	Q62JI8_BURMA	197	31	5.1	20.46
Antioxidant, AhpC/Tsa family [*Burkholderia mallei*][a]	**377**	2.6473 ± 0.4435	1.3248 ± 0.1059	0.020	0.50	Down	Q62JI8_BURMA	187	31	5.1	20.46
Antioxidant, AhpC/Tsa family [*Burkholderia mallei*][a]	**429**	0.8098 ± 0.0580	0.3810 ± 0.0662	0.001	0.47	Down	Q62I24_BURMA	294	30	5.8	23.90
Ferritin-like domain protein [*Burkholderia mallei*]	**348**	1.1054 ± 0.0998	0.6615 ± 0.0450	0.004	0.60	Down	Q62H71_BURMA	153	22	6.0	18.14
Flavohemoprotein [*Burkholderia pseudomallei*]	**566**	0.2350 ± 0.0156	0.1357 ± 0.0061	<0.001	0.58	Down	Q63R34_BURPS	157	16	6.1	43.64
Peroxidase/catalase (EC 1.11.1.6 (Catalase-peroxidase) [*Burkholderia mallei*]	**655**	0.0832 ± 0.0089	0.0227 ± 0.0095	0.002	0.27	Down	Q62H74_BURMA	248	6	5.7	79.45
Putative phenylacetic acid degradation oxidoreductase [*Burkholderia pseudomallei*]	**645**	0.6072 ± 0.0358	0.7921 ± 0.0618	0.032	1.30	Up	Q63QI2_BURPS	335	10	5.9	60.85
Osmotic Stress Response Proteins											
Osmotically inducible protein Y domain protein [*Burkholderia mallei*]	**412**	0.2610 ± 0.0387	0.3757 ± 0.0211	0.032	1.44	Up	Q62E87_BURMA	135	12	5.3	23.15
Other Stress Response Proteins											
60 kDa chaperonin [*Burkholderia pseudomallei* K96243]	**601**	0.3586 ± 0.0571	0.1402 ± 0.0121	0.006	0.39	Down	CAH36705	157	10	5.1	57.14
GroEL (Fragment) [*Burkholderia pseudomallei*]	**540**	0.1241 ± 0.0093	0.0572 ± 0.0049	<0.001	0.46	Down	Q83WK0_BURPS	195	10	5.2	56.49
GroEL (Fragment) [*Burkholderia pseudomallei*]	**577**	0.2034 ± 0.0154	0.0996 ± 0.0109	0.001	0.49	Down	Q83WK0_BURPS	175	10	5.2	56.49
GroEL (Fragment) [*Burkholderia pseudomallei*]	**587**	0.1323 ± 0.0202	0.0410 ± 0.0048	0.002	0.31	Down	Q83WK0_BURPS	190	10	5.2	56.49
Heat shock protein HtpG [*Burkholderia mallei*]	**309**	0.9076 ± 0.0816	0.6645 ± 0.0665	0.049	0.73	Down	Q62ID1_BURMA	274	14	5.1	71.22
PspA/IM30 family protein (phage shock protein A) (suppresses sigma54-dependent transcription) [*Burkholderia mallei*]	**45**	0.2709 ± 0.0174	0.2152 ± 0.0072	0.018	0.79	Down	Q62JH7_BURMA	100	18	5.1	24.48
Trigger factor (EC 5.2.1.8) [*Burkholderia mallei*]	**617**	0.2335 ± 0.0191	0.4046 ± 0.0418	0.006	1.73	Up	Q62JK6_BURMA	38	6	5.0	49.71
Universal stress protein family [*Burkholderia mallei*]	**340**	0.2697 ± 0.0095	0.0647 ± 0.0066	<0.001	0.24	Down	Q62EI9_BURMA	320	44	5.8	16.62
Transcriptional/Translational Regulators											
Adenosylhomocysteinase [*Burkholderia pseudomallei* K96243]	**596**	0.5861 ± 0.0413	0.3618 ± 0.0269	0.002	0.62	Down	CAH37303	140	7	5.7	52.51
Adenosylhomocysteinase [*Burkholderia pseudomallei* K96243]	**602**	0.4094 ± 0.0555	0.1955 ± 0.0383	0.013	0.48	Down	CAH37303	186	11	5.7	52.51
Arginine deiminase [*Burkholderia pseudomallei* K96243]	**592**	0.7188 ± 0.0774	0.3946 ± 0.0434	0.006	0.55	Down	CAH35742	273	16	5.6	46.42
DNA-directed RNA polymerase alpha chain [*Burkholderia pseudomallei* K96243]	**543**	0.3397 ± 0.0050	0.3037 ± 0.0064	0.002	0.89	Down	CAH37198	295	26	5.8	35.78
Endoribonuclease, L-PSP family [*Burkholderia mallei*]	**347**	0.1282 ± 0.0075	0.0850 ± 0.0055	0.002	0.66	Down	Q62HN3_BURMA	122	22	6.2	15.95
Hypothetical protein [*Burkholderia pseudomallei*][b]	**237**	1.3144 ± 0.1586	3.0324 ± 0.2261	<0.001	2.31	Up	Q63NT6_BURPS	119	16	6.7	21.65
Putative methyltransferase [*Burkholderia pseudomallei*]	**448**	0.0540 ± 0.0137	0.0000 ± 0.0000	0.004	0.00	Absent	Q63L99_BURPS	155	18	5.9	29.52
Ribonuclease PH [*Burkholderia pseudomallei*]	**171**	0.0926 ± 0.0109	0.0571 ± 0.0095	0.040	0.62	Down	CAH36573	51	4	6.0	26.21
Transcriptional regulator, AsnC family [*Burkholderia mallei*]	**350**	0.0392 ± 0.0025	0.1066 ± 0.0248	0.027	2.72	Up	Q62M96_BURMA	63	11	6.5	19.08
YbaK/prolyl-tRNA synthetases-associated domain family protein [*Burkholderia mallei*]	**358**	0.1446 ± 0.0096	0.0758 ± 0.0080	0.001	0.52	Down	Q62C75_BURMA	159	21	5.5	18.14
Metabolic Enzymes											
3-Oxoadipate CoA-succinyl transferase beta subunit [*Burkholderia mallei*]	**415**	0.0749 ± 0.0078	0.2232 ± 0.0343	0.003	2.98	Up	Q62KH2_BURMA	236	23	4.7	22.33
Aldehyde dehydrogenase family protein [*Burkholderia mallei*]	**609**	0.1036 ± 0.0128	0.2361 ± 0.0371	0.010	2.28	Up	Q62FN8_BURMA	235	16	5.7	55.72
Carbamate kinase (EC 2.7.2.2) [*Burkholderia pseudomallei*]	**508**	0.5604 ± 0.0263	0.4655 ± 0.0098	0.010	0.83	Down	Q63U71_BURPS	225	17	5.5	33.51
GMP synthase [glutamine-hydrolyzing] (EC 6.3.5.2) [*Burkholderia pseudomallei*]	**139**	0.0845 ± 0.0056	0.0063 ± 0.0063	<0.001	0.07	Down	Q63T42_BURPS	196	11	5.9	60.48
Spermidine n(1)-acetyltransferase (EC 2.3.1.57) [*Burkholderia pseudomallei*]	**378**	0.0699 ± 0.0098	0.0000 ± 0.0000	<0.001	0.00	Absent	Q63YU3_BURPS	169	14	5.9	22.22
Urocanate hydratase (EC 4.2.1.49) [*Burkholderia mallei*]	**261**	0.0717 ± 0.0042	0.1054 ± 0.0055	0.001	1.47	Up	Q62LJ4_BURMA	125	6	6.1	61.86
Urocanate hydratase (EC 4.2.1.49) [*Burkholderia mallei*]	**303**	0.0535 ± 0.0079	0.0849 ± 0.0082	0.025	1.59	Up	Q62LJ4_BURMA	297	11	6.1	61.86

Table 1 (Continued)

altered proteins	spot no.	Quantity (Intensity) (Mean ± SEM) K96243 WT	Quantity (Intensity) (Mean ± SEM) RpoE Mut	*p*	ratio (Mut/WT)	change	ID	ion score	%cov	p*I*	MW
Cell Wall Synthesis Pathway											
Glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24) (dTDP-glucose pyrophosphorylase) (dTDP-glucose synthase) [*Burkholderia mallei*]	**477**	0.1201 ± 0.0095	0.0742 ± 0.0033	0.002	0.62	Down	Q9AEV6_BURMA	51	6	5.6	33.07
Putative dTDP-D-glucose 4, 6-dehydratase (EC 4.2.1.46) [*Burkholderia pseudomallei*]	**253**	0.1977 ± 0.0178	0.1521 ± 0.0059	0.041	0.77	Down	Q9AEV7_BURPS	127	7	6.0	39.34
Glycogen Biosynthesis and Storage Pathway											
Inorganic pyrophosphatase (EC 3.6.1.1) [*Burkholderia mallei*]	**381**	0.2477 ± 0.0067	0.4036 ± 0.0300	0.001	1.63	Up	Q62LB4_BURMA	42	17	5.2	19.26
Fatty Acid Synthesis											
Putative beta-ketoacyl-ACP synthase [*Burkholderia pseudomallei*]	**533**	0.1011 ± 0.0062	0.0573 ± 0.0019	<0.001	0.57	Down	Q63N14_BURPS	96	10	5.4	40.75
Exported Proteins											
Putative exported protein [*Burkholderia pseudomallei*]	**330**	1.2433 ± 0.0680	0.9420 ± 0.0898	0.028	0.76	Down	Q63VW9_BURPS	110	59	9.2	13.75
Putative porin related exported protein [*Burkholderia pseudomallei*]	**513**	0.0619 ± 0.0099	0.3803 ± 0.0648	0.001	6.14	Up	Q63JN8_BURPS	188	15	7.8	40.14
Secreted Proteins											
Hypothetical protein [*Burkholderia pseudomallei*][c]	**447**	0.0595 ± 0.0014	0.1156 ± 0.0079	<0.001	1.94	Up	Q63IV8_BURPS	177	22	5.3	23.46
Adhesion Molecule											
Hypothetical protein [*Burkholderia mallei*][d]	**364**	0.1374 ± 0.0121	0.1078 ± 0.0043	0.050	0.78	Down	Q62FL1_BURMA	274	33	5.3	18.34
Protease/Peptidase											
Serine-type carboxypeptidase family protein [*Burkholderia mallei*]	**634**	0.1359 ± 0.0104	0.0730 ± 0.0062	0.001	0.54	Down	Q62AX8_BURMA	224	14	5.6	60.18
Protease Inhibitors											
Ecotin [*Burkholderia mallei*]	**375**	0.1133 ± 0.0081	0.0000 ± 0.0000	<0.001	0.00	Absent	Q62FK6_BURMA	33	6	8.5	19.47
Signaling Proteins											
Hypothetical protein [*Burkholderia pseudomallei*][e]	**656**	0.1637 ± 0.0064	0.0805 ± 0.0016	<0.001	0.49	Down	Q63TY9_BURPS	101	5	5.5	73.72
Hypothetical protein [*Burkholderia pseudomallei*][e]	**660**	0.0516 ± 0.0043	0.0179 ± 0.0079	0.006	0.35	Down	Q63TY9_BURPS	118	7	5.5	73.72
Miscellaneous and Unknown Function											
Chitin binding protein, putative [*Burkholderia pseudomallei*]	**165**	0.1212 ± 0.0344	0.2440 ± 0.0327	0.032	2.01	Up	Q63PN3_BURPS	66	11	6.2	25.89
Hypothetical bacteriophage protein [*Burkholderia pseudomallei*]	**230**	1.0938 ± 0.1523	2.5481 ± 0.2037	<0.001	2.33	Up	Q63LD3_BURPS	313	40	8.4	19.48
Hypothetical protein [*Burkholderia pseudomallei*][f]	**294**	0.0998 ± 0.0020	0.0796 ± 0.0067	0.021	0.80	Down	Q63KM8_BURPS	132	15	5.6	31.14
Hypothetical protein [*Burkholderia pseudomallei*]	**37**	0.1138 ± 0.0277	0.2363 ± 0.0215	0.008	2.08	Up	Q63UP7_BURPS	285	37	5.1	23.38
Hypothetical protein [*Burkholderia pseudomallei*]	**81**	0.8615 ± 0.0449	1.8358 ± 0.0917	<0.001	2.13	Up	Q63KK6_BURPS	224	6	5.9	124.63
Hypothetical protein [*Burkholderia pseudomallei*]	**270**	0.0857 ± 0.0155	0.1621 ± 0.0198	0.016	1.89	Up	Q63KK6_BURPS	279	7	5.9	124.63
Hypothetical protein [*Burkholderia pseudomallei*]	**383**	0.6012 ± 0.0935	1.7693 ± 0.2578	0.003	2.94	Up	Q63NT7_BURPS	90	16	5.3	22.45

[a] Protein is 100% identical to peroxiredoxin (gi|67763109; ZP_00501806). [b] Protein is 94% identical to methylase of polypeptide chain release factors (gi|67760879; ZP_00499595). [c] Protein is 89% identical to predicted periplasmic or secreted lipoprotein [*Burkholderia pseudomallei* S13] (gi|67762369; ZP_00501070). [d] Protein is 91% identical to phospholipid-binding protein [*Burkholderia pseudomallei* S13] (gi|67760526; ZP_00499246). [e] Protein is 100% identical to putative Ser protein kinase [*Burkholderia pseudomallei* S13] (gi|67758379; ZP_00497148). [f] Protein is 100% identical to *O*-methyltransferase involved in polyketide biosynthesis [*Burkholderia pseudomallei* Pasteur] (gi|67754005; ZP_00492924).

a virulence factor of *B. pseudomallei*, several lines of references have demonstrated its potential role in the virulence of *Naegleria fowleri*,[31] *Porphyromonas gingivalis*,[32,33] *Brucella abortus*,[34] ans so forth. Similarly, there are some indirect evidence demonstrating that proteins with a phospholipid-binding domain may play role in the virulence of bacteria.[35−38]

On the basis of these data, we therefore hypothesized that the *rpoE* operon is also involved in the virulence of *B. pseudomallei*. An *in vivo* experiment was performed to address whether *rpoE* operon knockout affects the virulence of *B. pseudomallei* (Figure 3). All mice challenged with K96243 WT ($n = 6$) succumbed within 7 days, whereas all RpoE Mut infected mice ($n = 6$) survived for the duration of the experiment (terminated at 40 days; $p < 0.002$). At this time, no bacteria were detected in the spleen or lungs of the RpoE Mut infected mice (limit of detection ≤100 CFU/organ), suggesting that this mutant is severely attenuated *in vivo*. The decreased virulence of the RpoE Mut was probably due to the down-regulation of potential virulence factors and also from the down-regulation of several various stress response proteins, as well as transcriptional/translational regulators and proteins involved in cell wall synthesis, all of which are very important for the bacteria to survive in the host. These novel findings underline the usefulness of proteomics as a screening tool to generate a new hypothesis from a set of candidate proteins that can finally be confirmed by conventional functional methods.

Technical Concerns. Some technical issues in the present study need to be discussed. First, there are four genes in the *B. pseudomallei rpoE* operon, including *rpoE*, *bprE*, *rseB*, and *mucD*, making the *rpoE* operon a four-gene cluster type. In

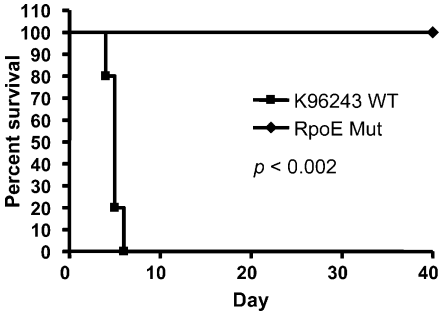


Figure 3. Impaired virulence of *rpoE* operon knockout mutant (RpoE Mut). BALB/c mice (6 per group) were infected intranasally with 1000 cfu of K96243 WT or RpoE Mut, and the survival of the infected mice was monitored.

the present study, we generated an *rpoE* operon knockout mutant. Therefore, the effects of this knockout on the other three genes could also contribute to the altered proteome observed in the present study.

Second, we examined the altered proteome of RpoE Mut only at the stationary phase. Analysis of the altered proteins at the other growth phases should provide more complete information of the molecular mechanisms of the *rpoE* operon in *B. pseudomallei*.

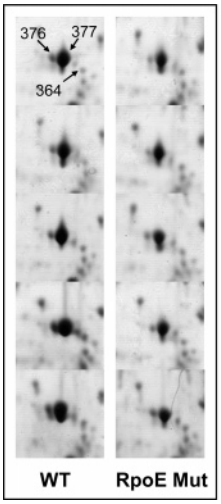
Third, we examined only the cellular proteins of *B. pseudomallei* in the present study. As we identified a periplasmic or secreted lipoprotein as one of the altered proteins, we believe that the production of other secreted proteins is also regulated by the *rpoE* operon. Hence, proteomic analysis of proteins secreted into the culture medium is of potential interest.

Fourth, the total number of protein spots visualized in each gel in the present study was relatively small for cellular proteins extracted from a bacterium. This limitation might be due to the gel-size and staining used in the present study. Many more spots should have been detected using the larger format 2-D gel and a more sensitive stain (i.e., silver or fluorescence).

Finally, we performed proteomic analysis of cellular proteins obtained from 10 different cultures to address the reproducibility and comparability of 2-D spot patterns across different gels, one of the most important issues in gel-based, differential proteomics studies. We calculated the coefficient of variation (CV) on selected spots (nos. 364, 376, and 377) across different gels. Figure 4 illustrates such selected areas and demonstrates the CVs of individual spots that ranged from 0.08 to 0.37 within the K96243 WT or RpoE Mut groups. When all these 3 spots were considered together, the CVs were reduced to 0.29 and 0.13 for K96243 WT and RpoE Mut groups, respectively. Moreover, when all spots detected in each gel were evaluated together, the CVs were only 0.0029 for K96243 WT and 0.0056 for RpoE Mut group. The CVs in our present study were in acceptable ranges of CVs previously observed in standard, gel-based, differential proteomics studies for analyzing tissues, cell lines, and body fluids.[30,39−41] In addition, the summation of normalized intensity volumes of all spots detected in each 2-D gel was comparable between the two different groups. Therefore, quantitative intensity analysis in our present study was justified.

Conclusions

We have identified a number of *B. pseudomallei* cellular proteins that were altered in an *rpoE* operon knockout mutant using proteomic technology. The down-regulation of several stress response proteins, chaperones, transcriptional/translational regulators, and proteins involved in cell wall synthesis in this mutant provided some new insights into the mechanisms of *rpoE* operon for stress tolerance and survival of *B. pseudomallei*. In addition to the stress tolerance and survival, the proteomic data and *in vivo* study indicated that the *rpoE* operon is also involved in the virulence of *B. pseudomallei* in the mammalian host.



Coefficient of Variation (CV)		
Spot No.	K96243 WT	RpoE Mut
364	0.1974	0.0889
376	0.2057	0.3196
377	0.3746	0.1787
364+376+377	0.2851	0.1294
All spots in each gel	0.0029	0.0056

Figure 4. Reproducibility and variability of gel-based proteomics approach. Coefficients of variation (CVs) of selected spots were obtained using the formula [CV = SD/Mean]. The results show that CVs in our present study were in acceptable ranges of CVs previously detected in standard, gel-based, differential proteomics studies analyzing human tissues, cell lines, and body fluids.[30,39−41]

Abbreviations: 2-DE, two-dimensional electrophoresis; ACN, acetonitrile; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate; CHCA, α-cyano-4-hydroxycinnamic acid; CV, coefficient of variation; DTT, dithiothreitol; IEF, isoelectric focusing; K96243 WT, *B. pseudomallei* wild-type; MN, menadione; Q-TOF MS/MS, quadrupole time-of-flight tandem mass spectrometry; RpoE, sigma factor σ^E; RpoE Mut, *rpoE* operon knockout mutant of *B. pseudomallei*; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid

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Supporting Information Available: Table listing the sequences of the identified peptides using Q-TOF MS/MS. This material is available free of charge via the Internet at http://pubs.acs.org.

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