Journal of Microbiology (2015) Vol. 53, No. 2, pp. 134–140
DOI 10.1007/s12275-015-4522-9

Transcription level analysis of intracellular *Burkholderia pseudomallei* illustrates the role of BPSL1502 during bacterial interaction with human lung epithelial cells[§]

Teerasit Techawiwattanaboon[1,2], Tanachaporn Bartpho[2], Rasana Wongratanacheewin Sermswan[2,3], and Sorujsiri Chareonsudjai[1,2*]

[1]Department of Microbiology, Faculty of Medicine, [2]Melioidosis Research Center, [3]Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

(Received Sep 22, 2014 / Revised Nov 10, 2014 / Accepted Dec 5, 2014)

Melioidosis caused by *Burkholderia pseudomallei* is a globally important disease of increasing concern according to high case-fatality rate and epidemic spreading. The ability of *B. pseudomallei* to attach and invade host cells and subsequently survive intracellularly has stimulated many questions concerning the comprehension of bacterial pathogenesis progression. Transcription levels of intracellular *B. pseudomallei* genes in human lung epithelial cells were therefore analyzed using bioinformatic tools, RT-PCR and real time RT-PCR. Here, it is reported that the identification of *bpsl1502*, encoding *B. pseudomallei* SurE (stationary phase survival protein E) located in a global transcriptional regulation operon was accomplished. The up-regulation of *B. pseudomallei* SurE was demonstrated during intracellular survival of A549 cells at 12, 18, and 24 h post-infection. To investigate the role of this protein, a *B. pseudomallei* SurE defective mutant was constructed. The invasion and initial survival of the SurE mutants within the A549 cells were impaired. There was no difference, however, between the growth of *B. pseudomallei* SurE mutant as compared to the wild type in Luria-Bertani culture. These data suggest that SurE may assist *B. pseudomallei* host cells invade and facilitate early intracellular infection but is not crucial during the stationary growth phase. The identification of *B. pseudomallei* SurE provides more information of bacterial strategy during an early step of the pathogenesis process of melioidosis.

Keywords: BPSL1502, *B. pseudomallei*, transcription level analysis

*For correspondence. E-mail: sorujsr@kku.ac.th; Tel.: +66-43-363808; Fax: +66-43-363808
[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

Introduction

Burkholderia pseudomallei, a Gram-negative soil bacillus is a cause of fatal infectious melioidosis which remains a problematic health concern worldwide especially in Southeast Asia and northern Australia (Currie *et al.*, 2008). In northeast of Thailand, melioidosis is one of three most prominent infectious diseases after HIV/AIDS and tuberculosis (Limmathurotsakul *et al.*, 2010). Most melioidosis cases occur during the rainy season and in particular during the heavy monsoonal rains with winds that may cause a shift from percutaneous inoculation to aerosol inhalation of the pathogens leading to fulminant pneumonia and septic shock, which can be rapidly fatal (Vuddhakul *et al.*, 1999; Currie, 2003; White, 2003; Cheng and Currie, 2005).

As intracellular pathogens, the ability of *B. pseudomallei* to survive and multiply in various mammalian cells reflect how the bacteria responds to the *in vivo* environments of the cells correlated with numerous features of melioidosis including latency, recrudescence and treatment difficulty (Jones *et al.*, 1996; Allwood *et al.*, 2011). A number of essential proteins involved in pathogenesis of melioidosis have been identified as key components of *B. pseudomallei* during entry, survival and replication within host cells (Lazar Adler *et al.*, 2009; Allwood *et al.*, 2011). Even if high-throughput molecular techniques have provided knowledge in intracellular survival of *B. pseudomallei*, bacterial pathogenesis is a complex procedure and a number of crucial components and mechanisms of bacterial strategies still have to be revealed.

The survival protein E (SurE) family was discovered when its existence correlated to the stationary phase survival of *E. coli* and when a defect in the *surE* gene resulted in poor survival in the stationary phase at an elevated temperature and in high salt media (Li *et al.*, 1994). Biochemical analysis suggested that SurE is an acid phosphatase and highly conserved in bacteria, archaea and plants (Zhang *et al.*, 2001). Furthermore, the predicted structures and enzymatic property of SurE as a phosphatase of *Salmonella typhimurium* and *Thermotoga maritima* were also later identified (Zhang *et al.*, 2001; Pappachan *et al.*, 2008). SurE of *B. pseudomallei* is composed of 253 amino acids with a calculated molecular weight of 27.0 kDa. The protein is annotated as a 5′ nucleotidase that shows phosphatase activity. The condition-dependent transcriptional study of *B. pseudomallei* revealed the expression of *surE* gene of *B. pseudomallei* that is located upstream of the global transcriptional regulation operon region (BPSL1502-BPSL1506) of chromosome 1 in various stress environments including inside host cells (Holden *et*

al., 2004; Ooi *et al.*, 2013). However, the role of *surE* of *B. pseudomallei* during host interaction or the stationary phase growth of the bacteria has never been identified.

This study sought to identify the response of *B. pseudomallei* genes that involved in the interaction with human lung epithelial cells, A549. The gene expression profiles from microarray data of *B. pseudomallei* 1909a infected BALB/c and C57BL/6 lungs compared to Luria-Bertani (LB) medium (Chirakul *et al.*, 2014) were analyzed. The selected genes from integrated microarray data and design criteria excluded type III secretion system (T3SS), type VI secretion system (T6SS), and some known metabolic genes were further validated whilst *B. pseudomallei* infected the A549 cells using RT-PCR and real time RT-PCR according to time of infection during melioidosis pathogenesis. Furthermore, the role of BPSL1502 was investigated to engage the ability of *B. pseudomallei* to adhere, invade and survive in human lung epithelial cells.

Materials and Methods

Gene selection and *in silico* analysis

The transcript levels from microarray data of *B. pseudomallei* 1909a infected BALB/c and C57BL/6 lungs after 4 days of infection compared to LB medium (Chirakul *et al.*, 2014) were analyzed. The CLUSTER program performed hierarchical clustering from microarray data and graphically presented dendrograms of gene expression based on statistical analysis by the TREEVIEW program (Eisen *et al.*, 1998). The results of genes that displayed up to 6 fold change or more in BALB/c lungs compared to LB but less than 2.5 fold in C57BL/6 lungs compared to LB as well as the other correlated genes from TREEVIEW were recruited that for further bioinformatics analysis whilst metabolic genes and T3SS and T6SS genes were excluded. Selected gene data were downloaded from GenBank® and UniProt (Consortium, 2013), then further processed for protein properties prediction using bioinformatic tools as follows: PSORTb 3.0, CELLO II, SOSUI-GramN and Gneg-mPLoc (Yu *et al.*, 2004, 2010; Imai *et al.*, 2008; Shen and Chou, 2010) were applied to predict the sub-cellular locations. Thereafter, signal peptide prediction was carried out using SignalP 4.0, Signal-BLAST, and PrediSi (Hiller *et al.*, 2004; Frank and Sippl, 2008; Petersen *et al.*, 2011). The predicted protein molecular weight was calculated using Compute pI/Mw (ExPASy Server).

Bacterial strains

B. pseudomallei K96243 wild type (a clinical isolated), *bpsl-1502* mutant (MT1502, this study) and *E. coli* S17-1 *λpir* were routinely grown at 37°C in LB broth or on agar plates, supplemented with the following antibiotics, when appropriate: gentamicin (8 μg/ml) and tetracycline (10 μg/ml).

Bacterial growth in LB and host cells

Single colonies of *B. pseudomallei* K96243 and MT1502 on LB agar plate were cultured in 3 ml LB broth with antibiotics at 37°C, 200 rpm overnight before being diluted by fresh media to obtain an OD_{600nm} = 0.1. One milliliter of the diluted bacterial suspension was subsequently transferred into 99 ml LB broth and then incubated for 18 h. The bacterial growth was counted at indicated times by the drop plate technique (Herigstad *et al.*, 2001).

Survival of *B. pseudomallei* K96243 and MT1502 within the human lung epithelial cells (A549) was assessed as previously described (Jones *et al.*, 1996) with some modifications. A549 cells were infected with the mid-log phase *B. pseudomallei* K96243 or MT1502 at a multiplicity of infection (MOI) 10 for 2 h. Media containing 250 μg/ml kanamycin was used to kill extracellular bacteria for 2 h followed by maintaining in media containing 20 μg/ml kanamycin. Intracellular bacteria were monitored at 4, 6, 12, 18, and 24 h post-infection (p.i.) after lysed with 0.1% v/v Triton X-100 (Merck).

Adhesion and intracellular survival of *B. pseudomallei* in A549 cells

B. pseudomallei K96243 and MT1502 adhesion and intracellular survival in A549 cells were performed according to a previously described protocol with some modifications (Jones *et al.*, 1996). After co-culture at a MOI of 10, the bacteria were enumerated at 2 h p.i. to determine the adhesion ability and 4 and 12 h p.i. to determine the invasion and multiplication inside A549. The unbound bacteria at 2 h p.i. were removed by five times washing with PBS for the adhesion assay.

Reverse transcriptase PCR for intracellular *B. pseudomallei* genes expression

B. pseudomallei RNA was extracted using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions and subsequently treated with RNase-Free DNase Set (QIAGEN). Transcriptional responses of intracellular *B. pseudomallei* K96243 in A549 cells and in LB were verified by One Step RT-PCR Kit (QIAGEN) with specific primers listed in Supplementary data Table S1. Genomic DNA and sterilized distilled water were used as positive and negative controls. The housekeeping gene, 16S rRNA was used as an internal control.

Real-time RT-PCR

The cDNA preparation for real-time RT-PCR was carried out using the SuperScript® III First-Strand Synthesis System (Invitrogen) with random hexamers according to the manufacturer's instructions. The real time RT-PCR was performed using LightCycler® FastStart DNA Master ^{PLUS} SYBR Green I (Roche), according to the manufacturer's manual.

Construction of a *B. pseudomallei* BPSL1502 mutant

The insertion mutation method was employed to construct a BPSL1502 defective mutant using pKNOCK-Tc in *B. pseudomallei* K96243 (Alexeyev, 1999). The *B. pseudomallei* K96243 *bpsl1502* nucleotide sequence (NCBI Reference Sequence: NC_006350.1; locus_tag = "BPSL1502") was retrieved from GenBank® database. An internal 153-bp of the *bpsl1502* gene was amplified using a Fw-1502-*Xba*I (5′-ACTCTCTAGAT TCGGTGCACGTTGCGCTGA-3′) and Rv-1502-*Xho*I (5′-ATTACTCGAGATTGCCGGCACGCCGAACAT-3′) corresponding to nucleotide positions 210-229 and 340-362. The 165-bp amplicon was digested and cloned into the *Xba*I and *Xho*I restriction sites of pKNOCK-Tc to yield pTT1502.

Table 1. List of 14 selected genes from microarray data and predicted protein properties

Target gene	Functional class	Predicted location[a]	Predicted signal peptide[b]	Predicted MW[c] (kDa)
BPSL0773	Transmembrane transport protein	Inner membrane	-	59.50
BPSL1151	Hypothetical protein	Cytoplasm	-	65.90
BPSL1205	Amino acid transport system, exported protein	Periplasm	+	45.45
BPSL1502	Stationary phase survival protein	Cytoplasm	-	26.99
BPSS0129	Transcriptional regulator protein	Cytoplasm	-	43.94
BPSS0525	Hypothetical protein	Cytoplasm	-	81.82
BPSS0717	Hypothetical protein	Inner membrane	-	45.76
BPSS0873	Porin protein	Outer membrane	+	41.66
BPSS0919	Hypothetical protein	Inner membrane	-	31.40
BPSS1228	Cation transporter protein	Inner membrane	-	38.58
BPSS1774	Hypothetical protein	Extracellular	+	43.03
BPSS1929	Outer membrane lipoprotein	Outer membrane	+	51.64
BPSS2179	Hypothetical protein	Cytoplasm	-	17.31
BPSS2330	Hypothetical protein	Inner membrane	-	92.51

[a] Protein location prediction using PSORTb 3.0, CELLO II, SOSUI-GramN and Gneg-mPLoc
[b] Signal peptide prediction using SignalP 4.0, Signal-BLAST and PrediSi
[c] Theoretical MW using Compute pI/Mw (ExPASy Server)
+ indicate signal peptide is contained while - indicate not contain signal peptide

The pTT1502 was then transformed into *E. coli* S17-1 *λpir* (de Lorenzo and Timmis, 1994) and delivered into *B. pseudomallei* K96243 by conjugation. The transconjugants were selected on LB agar containing 50 μg/ml tetracycline and 8 μg/ml gentamicin. The *bpsl1502* mutants were subsequently verified by PCR and designated as *B. pseudomallei* MT1502.

Statistical analysis

Statistical analysis was performed using SPSS version 13.0 statistical package (SPSS Inc.). Data were analyzed for statistical significance by the independent Student's *t*-test. Differences with *P* values of <0.05 were designated to be statistically significant.

Results

Bioinformatic analysis of *B. pseudomallei* genes up-regulated during intracellular survival inside A549 cells

Bioinformatic analysis from microarray data revealed 14 genes likely to be essential for intracellular *B. pseudomallei* to survive in host cells according to the design criteria. Meanwhile, some known genes including T3SS and T6SS genes and common metabolic genes were excluded. Of these, 4 genes were located in large chromosomes whereas the other 10 genes were located in small chromosomes. Seven of the 14 genes were already annotated for their functions including BPSL0773 (transmembrane transport protein), BPSL1205 (amino acid transport system, exported protein), BPSL1502 (stationary

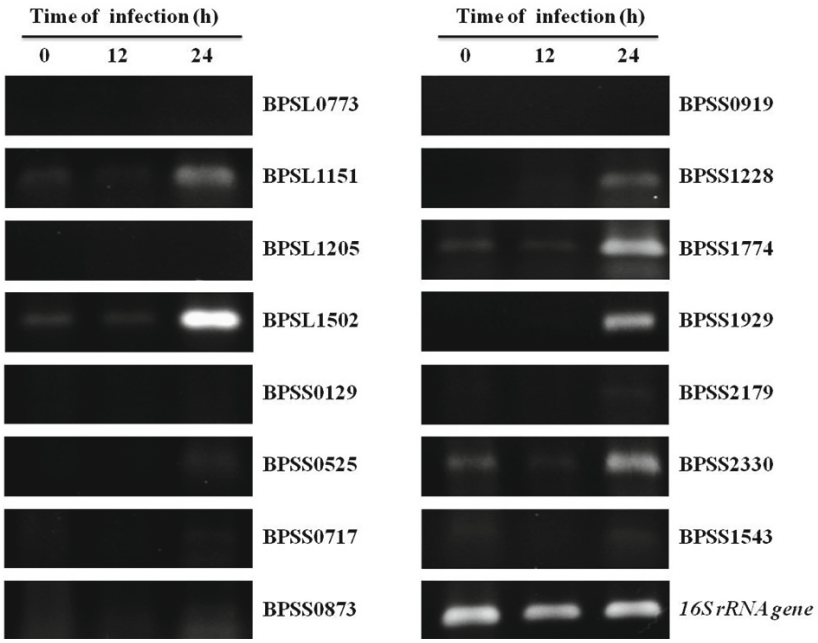

Fig. 1. Transcription analysis by RT-PCR of 14 selected genes of *B. pseudomallei* K96243 while growing inside A549 cells at 0, 12, and 24 h post-infection. Each row represents an individual gene, a house-keeping gene 16S rRNA and *bpss1543*, T3SS gene that also performed as internal and positive controls.

phase survival protein, SurE), BPSS0129 (transcriptional regulator protein), BPSS0873 (porin protein), BPSS1228 (cation transporter protein), and BPSS1929 (outer membrane lipoprotein) while the other 7 genes were marked as hypothetical proteins.

Prediction of sub-cellular localization, signal peptide, and molecular weight of proteins using bioinformatic tools were demonstrated in Table 1.

Transcription level profiles of the selected *B. pseudomallei* genes during intracellular survival in A549 cells

The validation confirmation of essential intracellular *B. pseudomallei* gene expressions using conventional one-step RT-PCR from two independent experiments revealed that, 7 of the 14 selected genes increased in band intensity whilst staying alive in A549 at 12 or 24 h p.i. compared to the extracellular growth condition at 0 h; these were BPSL1151, hypothetical protein; BPSL1502, stationary phase survival protein (SurE); BPSS1228, cation transporter protein; BPSS1774, hypothetical protein: BPSS1929, outer membrane lipoprotein; BPSS2179, hypothetical protein and BPSS2330 hypothetical protein (Fig. 1). Three other genes, however: BPSS0525, hypothetical protein; BPSS0717, hypothetical protein and BPSS0873, and porin protein were just slightly expressed at 24 h p.i.. Meanwhile, the BPSS1543, the *bsaQ* gene, which encoded structural components of the T3SS and the 16S rRNA gene were used as controls. The microarray processed procedure to identify the function of BPSL1502 was demonstrated in Supplementary data Fig. S1.

B. pseudomallei K96243 similar growth in LB and A549 cells

The growth curve of *B. pseudomallei* K96243 at 37°C in A549 cells (MOI 10) was parallel but slightly delayed compared to that in LB medium. The generation time of intracellular *B. pseudomallei* in A549 cells was 61.99±4.67 min whereas it was only 44.22±0.50 min in LB (Fig. 2A). The growth information first disclosed the growth of intracellular *B. pseudomallei* in A549 at 12 h as the mid-log phase and 24 h as the early stationary phase.

BPSL1502 was highly up-regulated during *B. pseudomallei* survival in A549 cells but only slightly up-regulated in LB medium growth

The RT-PCR data was convincing that BPSL1502 may associate with *B. pseudomallei* adaptation during intracellular survival from the transcript levels of *bpsl1502* of *B. pseudomallei* that were re-performed in comparison to the expression of intracellular survival in A549 cells (MOI 10) and in LB culture. The results demonstrated remarkable transcript levels of *bpsl1502* associated with intracellular residence of the pathogens in A549 cells at 12, 18, and 24 h p.i. (Fig. 2B). In contrast, only slight expression of *bpsl1502* in LB culture during stationary growth was observed at 12-24 h of incubation (Fig. 2C).

Furthermore, real-time RT-PCR of *B. pseudomallei bpsl1502* expression of the mid-log phase in A549 cells (12 h p.i.) was compared to the mid-log in LB (7 h of incubation). The relative transcription level of *bpsl1502* was normalized with the internal control, 16S rRNA, from the 2 independent attempts

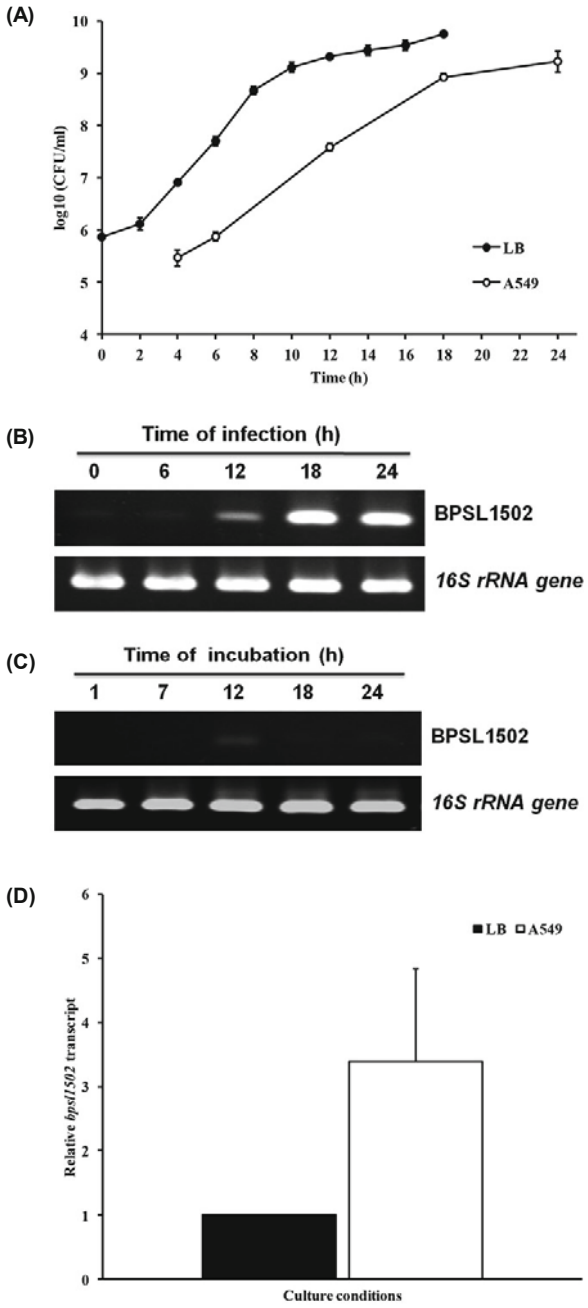

Fig. 2. (A) Growth of *B. pseudomallei* K96243 in LB medium and A549 cells. *B. pseudomallei* (1% innoculum) in LB broth were incubated at 37°C with shaking at 200 rpm. Bacterial cell counts were enumerated every 2 h for up to 18 h of incubation by the drop plate technique. *B. pseudomallei* intracellular growth in A549 cells (MOI 10) was also investigated at 4, 6, 12, 18, and 24 h post-infection. Intracellular bacteria were lysed and enumerated by a drop plate technique. The data shown represent mean ± SD from 3 time independent tests. Transcription profile of *bpsl1502* during *B. pseudomallei* K96243 growth by RT-PCR. (B) *B. pseudomallei* growth inside A549 cells at 6, 12, 18, and 24 h post-infection. (C) *B. pseudomallei* growth in LB medium at 1 (lag phase), 7 (mid-log phase), 12 (early-stationary phase), 18, and 24 h (late-stationary phase). (D) Transcription levels of *B. pseudomallei bpsl1502* compared between mid-log phase point of extracellular (grown in LB for 7 h) and intracellular A549 cells (12 h post-infection) by real time PCR. The relative gene transcript was calculated by ratio of Ct values of target genes normalized with an internal house-keeping gene control, 16S rRNA. The data shown represent mean ± SD from 2 time independent tests.

and revealed consistency with the RT-PCR. The results indicated that *bpsl1502* was remarkably higher up-regulated during *B. pseudomallei* survival in A549 cells than in LB, suggesting that this gene may be essential for *B. pseudomallei* intracellular survival in A549 cells (Fig. 2D).

SurE mutant does not affect *B. pseudomallei* growth in LB

To analyze the crucial role of BPSL1502 in *B. pseudomallei* stationary-phase survival, the *bpsl1502* mutant strain using insertion mutagenesis and designated as *B. pseudomallei* MT1502 was constructed. Afterward, the growth of the *B. pseudomallei* wild type and the *surE* mutant in LB were determined (Fig. 3). The results from two independent experiments demonstrated that the SurE mutation did not affect *B. pseudomallei* growth in LB medium.

SurE plays a role in bacterial invasion but not adhesion of *B. pseudomallei* in A549 cells

To confirm the crucial role of SurE in *B. pseudomallei* host cell interactions, the *B. pseudomallei* MT1502 was examined its adhesion, invasion and multiplication ability in human lung epithelial cells compared to the wild type strain. The *surE* mutant was similar in ability to adhere to A549 cells but was significantly less invasive than the wild type strain at 4 h p.i. ($P < 0.05$) (Fig. 4A). The percentage of bacterial invasion of K96243 and MT1502 were 1.7397±0.22 and 0.9882±0.09, respectively, indicating that the *B. pseudomallei* that lacked BPSL1502 had significantly decreased bacterial invasion ability (Fig. 4B). Nevertheless, there were no significant differences, however, in survivals between the *B. pseudomallei* wild type and the SurE mutant strains at 12 h p.i. (Fig. 4A).

Discussion

As an intracellular pathogen, *B. pseudomallei* could manipulate the regulation of gene expression to attach and survive inside host cells including under overwhelming stress conditions as well as avoiding or subverting the host immune system (Lazar Adler *et al.*, 2009). Pathogenesis mechanisms of *B. pseudomallei* have been intensively studied to uncover molecular mechanisms of the pathogens and provide information that is essential to manipulate the bacterial number leading to control of fatal infectious melioidosis. The strategies of *B. pseudomallei* to survive intracellularly in host cells may facilitate the bacteria adaptation to host environments, avoid host immune responses and induce melioidosis progress. This study presents the parallel growth curves of *B. pseudomallei* K96243 in A549 and in LB media (Fig. 2) consistent with the efficient invasive and intracellular survival of the pathogen inside A549 cells as previously reported (Jones *et al.*, 1996; Harley *et al.*, 1998; Kespichayawattana *et al.*, 2004). The ability of survival within host cells with a decrease in generation time compared with its extracellular

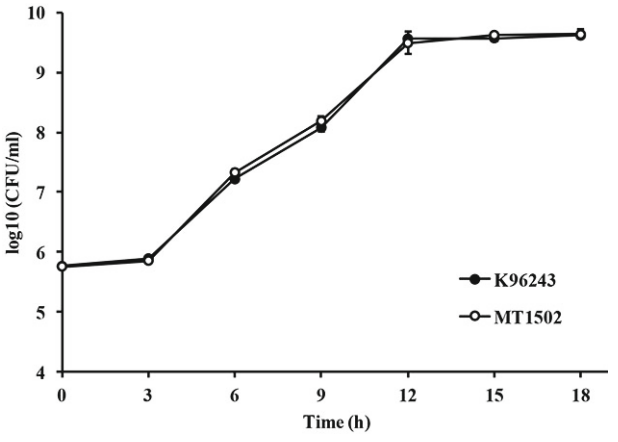

Fig. 3. Growth of *B. pseudomallei* K96243 (wild type) and MT1502 (*bpsl1502* mutant) in LB broth with appropriate antibiotics at 37°C at 200 rpm. Bacterial cells counts were examined at every 3 h for up to 18 h by the drop plate technique. The data shown represent mean ± SD from 2 time independent tests.

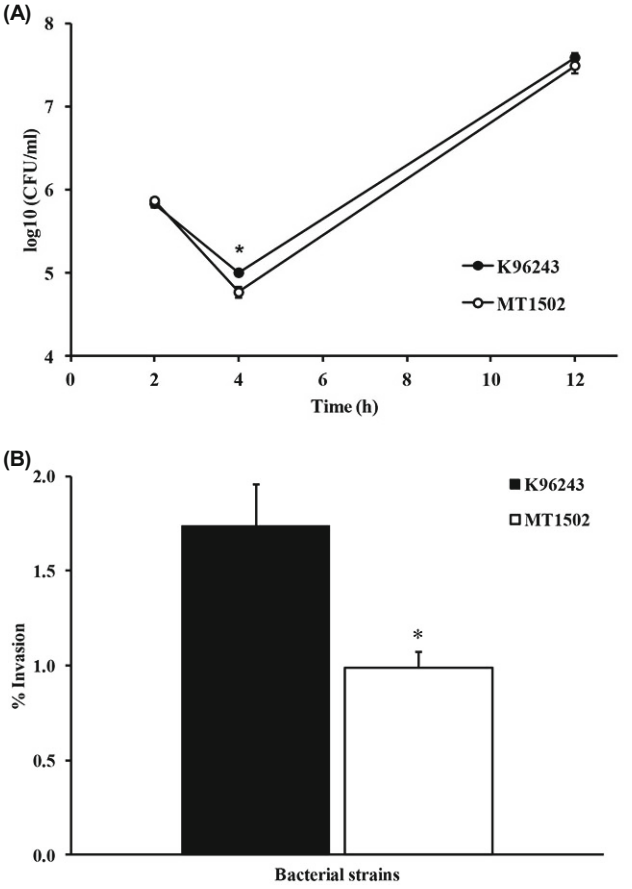

Fig. 4. (A) Adhesion and survival of *B. pseudomallei* strains in A549 cells. K96243 (wild type) and MT1502 (*bpsl1502* mutant) pathogenesis were investigated in A549 cells (MOI 10). The number of bacterial cells was counted at 2, 4, and 12 h post-infection represent adhesion, invasion and intracellular multiplication. The data shown represent mean ± SD from 3 time independent tests. (B) Invasion of A549 cells by *B. pseudomallei* strains. K96243 (wild type) and MT1502 (*bpsl1502* mutant) were used to infect A549 cells at MOI 10. The numbers of intracellular bacteria were calculated at 4 h post-infection after an antibiotic protection assay. The percentage of invasion represented the number of intracellular bacteria relative to the inoculums. The data shown represent mean ± SD from 3 independent tests.

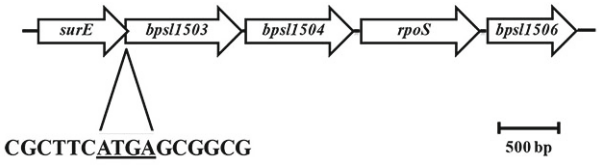

Fig. 5. Illustration of the *B. pseudomallei rpoS* operon (*surE-bpsl1503-bpsl1504-rpoS-bpsl1506*). The gene nucleotide sequences were retrieved from *B. pseudomallei* K96243 genome sequence analysis (NCBI Reference Sequence: NC_006350.1) (Holden *et al.*, 2004). Open reading frames are indicated as open arrows. The overlap 4 bases of *surE* and *bpsl1503* are underlined.

existence may demonstrate that the pathogen can overcome some stress conditions, acquire some micronutrients inside host cells as well as adapt bacterial behavior to influence host cell performance to provide for their intracellular growth.

In this study, the transcription level investigation using bioinformatics and molecular approaches has demonstrated a difference of expression of intracellular *B. pseudomallei* from extracellular expression. The remarkable up-regulation of the *bpsl1502* gene encoding the SurE protein of *B. pseudomallei* existing inside A549 cells at 12-24 h p.i. than while growing in LB broth was revealed. Thereafter, the SurE mutant was constructed to uncover the association of SurE *B. pseudomallei* survivals. The growth of *B. pseudomallei* SurE mutant in LB demonstrated similar growth kinetics between the mutant and the wild type. Notably, the *B. pseudomallei* SurE mutant had significantly diminished bacterial invasion ability to A549 cells at 4 h p.i. compared to the wild type strain. The results indicated that interrupted SurE had no effect on *B. pseudomallei* growth in the rich culture medium but disturbed the pathogen early step of infection and survival while exposed to a very stressful milieu during the host cells approach.

The conserved bacterial survival protein E (SurE) is known as a stationary phase survival protein that improves bacterial viability during stressful conditions previously identified in *Escherichia coli.* The *surE* gene is located upstream of *pcm* (an *L*-isoaspartate, *O*-methyltransferase), *nlpD* (a gene for lipoprotein) and *rpoS* genes, and hence it belongs to the *rpoS* operon (Li *et al.*, 1994). The *surE* gene was demonstrated to be co-transcribed with the *pcm* gene. Moreover, the operon organization of *surE* and *pcm* with their proximities to *rpoS* was evidenced. Therefore, SurE may be defined as key a factor in the stationary growth phase and again in the stress environments of *E. coli* (Visick *et al.*, 1998).

The *B. pseudomallei bpsl1502* (*surE)* gene lies at the upstream and overlap 4 bases of *bpsl1503* (L-isoaspartate O-methyltransferase) follow by *bpsl1504* (peptidase) and *rpoS* gene (*bpsl1505*) which is involved in the global transcriptional regulation operon (Fig. 5) (Holden *et al.*, 2004; Nandi *et al.*, 2010). The expression of this operon was marked as constitutively expressed and associated with core cellular functions and pathogenesis (Ooi *et al.*, 2013). Furthermore, *B. pseudomallei rpoS* gene was up-regulated according to the growth phase particularly reaching a peak during the stationary phase but the possibility that *rpoS* subjected to cell density-dependent regulation has not been ruled out. The *rpoS* mutant was more sensitive to peroxide and acid stress conditions but this was not crucial for the bacterial intracellular survival (Subsin *et al.*, 2003). Moreover, the *B. pseudomallei* RpoS was determined upon the functions of invasion and intracellular survival in the mouse macrophage (Utaisincharoen *et al.*, 2006). Based on current knowledge, this study reveals for the first time that *surE* is not required for the stationary phase growth of *B. pseudomallei* in LB medium since the growth curves of wild type and the *surE* mutant are identical, consistent with no transcription level of *surE* in LB. In contrast, the data represent an essential role for *surE* during the second step for bacterial interaction with host cells in the co-cultured assay. Therefore, it is possible that *surE* gene expression may be positioned to introduce the bacteria with an approach to host cells and may facilitate the invasion into host cells. However, according to the location of the *bpsl1502* gene, the possible polar effect on downstream genes, the deletion mutation or complement strain may confirm the function of the survival protein E (SurE) of *B. pseudomallei*.

These findings firstly displayed the role of *B. pseudomallei* SurE based on transcription levels of the bacteria residing in epithelial cells. The bacterial growth between the *B. pseudomallei* SurE mutant and the wild type in LB did not show any differences in bacterial plate count. This indicates that SurE might not be essential for *B. pseudomallei* to survive in a rich culture medium even through the stationary phase of growth. Nevertheless, the *B. pseudomallei* mutant defect SurE had reduced abilities in host epithelial cell invasion and initial intracellular survival. These finding points to the possible role of *B. pseudomallei* SurE that may be crucial for the pathogen to initially invade and protect against stressful surroundings during pathogenesis.

Acknowledgements

We gratefully acknowledge a financial support by Khon Kaen University under incubation researcher project and Faculty of Medicine, Khon Kaen University. We thank M.F. Alexyev for the kindly provided pKNOCK-Tc vector. We would like to acknowledge Emeritus Prof. James A. Will, University of Wisconsin-Madison, USA, for editing the manuscript via the Faculty of Medicine Publication Clinic, Khon Kaen University, Thailand.

References

Alexeyev, M.F. 1999. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of Gram-negative bacteria. *Biotechniques* **26**, 824–826, 828.

Allwood, E.M., Devenish, R.J., Prescott, M., Adler, B., and Boyce, J.D. 2011. Strategies for intracellular survival of *Burkholderia pseudomallei*. *Front Microbiol* **2**, 170.

Cheng, A.C. and Currie, B.J. 2005. Melioidosis: epidemiology, pathophysiology, and management. *Clin. Microbiol. Rev.* **18**, 383–416.

Chirakul, S., Bartpho, T., Wongsurawat, T., Taweechaisupapong, S., Karoonutaisiri, N., Talaat, A.M., Wongratanacheewin, S., Ernst, R.K., and Sermswan, R.W. 2014. Characterization of BPSS1521 (*bprD*), a regulator of *Burkholderia pseudomallei* virulence gene expression in the mouse model. *PLoS One* **9**, e104313.

Consortium, T.U. 2013. Update on activities at the Universal Protein Resource (UniProt) in 2013. *Nucleic Acids Res.* **41**, D43–47.

Currie, B.J. 2003. Melioidosis: an important cause of pneumonia in residents of and travellers returned from endemic regions. *Eur. Respir. J.* **22**, 542–550.

Currie, B.J., Dance, D.A., and Cheng, A.C. 2008. The global distribution of *Burkholderia pseudomallei* and melioidosis: an update. *Trans. R. Soc. Trop. Med. Hyg.* **102 Suppl 1**, S1–4.

de Lorenzo, V. and Timmis, K.N. 1994. Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Methods Enzymol.* **235**, 386–405.

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. 1998. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* **95**, 14863–14868.

Frank, K. and Sippl, M.J. 2008. High-performance signal peptide prediction based on sequence alignment techniques. *Bioinformatics* **24**, 2172–2176.

Harley, V.S., Dance, D.A., Drasar, B.S., and Tovey, G. 1998. Effects of *Burkholderia pseudomallei* and other *Burkholderia* species on eukaryotic cells in tissue culture. *Microbios* **96**, 71–93.

Herigstad, B., Hamilton, M., and Heersink, J. 2001. How to optimize the drop plate method for enumerating bacteria. *J. Microbiol. Methods* **44**, 121–129.

Hiller, K., Grote, A., Scheer, M., Munch, R., and Jahn, D. 2004. PrediSi: prediction of signal peptides and their cleavage positions. *Nucleic Acids Res.* **32**, W375–379.

Holden, M.T., Titball, R.W., Peacock, S.J., Cerdeno-Tarraga, A.M., Atkins, T., Crossman, L.C., Pitt, T., Churcher, C., Mungall, K., Bentley, S.D., *et al.* 2004. Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. *Proc. Natl. Acad. Sci. USA* **101**, 14240–14245.

Imai, K., Asakawa, N., Tsuji, T., Akazawa, F., Ino, A., Sonoyama, M., and Mitaku, S. 2008. SOSUI-GramN: high performance prediction for sub-cellular localization of proteins in Gram-negative bacteria. *Bioinformation* **2**, 417–421.

Jones, A.L., Beveridge, T.J., and Woods, D.E. 1996. Intracellular survival of *Burkholderia pseudomallei*. *Infect. Immun.* **64**, 782–790.

Kespichayawattana, W., Intachote, P., Utaisincharoen, P., and Sirisinha, S. 2004. Virulent *Burkholderia pseudomallei* is more efficient than avirulent *Burkholderia thailandensis* in invasion of and adherence to cultured human epithelial cells. *Microb. Pathog.* **36**, 287–292.

Lazar Adler, N.R., Govan, B., Cullinane, M., Harper, M., Adler, B., and Boyce, J.D. 2009. The molecular and cellular basis of pathogenesis in melioidosis: how does *Burkholderia pseudomallei* cause disease? *FEMS Microbiol. Rev.* **33**, 1079–1099.

Li, C., Ichikawa, J.K., Ravetto, J.J., Kuo, H.C., Fu, J.C., and Clarke, S. 1994. A new gene involved in stationary-phase survival located at 59 minutes on the *Escherichia coli* chromosome. *J. Bacteriol.* **176**, 6015–6022.

Limmathurotsakul, D., Wongratanacheewin, S., Teerawattanasook, N., Wongsuvan, G., Chaisuksant, S., Chetchotisakd, P., Chaowagul, W., Day, N.P., and Peacock, S.J. 2010. Increasing incidence of human melioidosis in Northeast Thailand. *Am. J. Trop. Med. Hyg.* **82**, 1113–1117.

Nandi, T., Ong, C., Singh, A.P., Boddey, J., Atkins, T., Sarkar-Tyson, M., Essex-Lopresti, A.E., Chua, H.H., Pearson, T., Kreisberg, J.F., *et al.* 2010. A genomic survey of positive selection in *Burkholderia pseudomallei* provides insights into the evolution of accidental virulence. *PLoS Pathog.* **6**, e1000845.

Ooi, W.F., Ong, C., Nandi, T., Kreisberg, J.F., Chua, H.H., Sun, G., Chen, Y., Mueller, C., Conejero, L., Eshaghi, M., *et al.* 2013. The condition-dependent transcriptional landscape of *Burkholderia pseudomallei*. *PLoS Genet.* **9**, e1003795.

Pappachan, A., Savithri, H.S., and Murthy, M.R. 2008. Structural and functional studies on a mesophilic stationary phase survival protein (Sur E) from *Salmonella typhimurium*. *FEBS J.* **275**, 5855–5864.

Petersen, T.N., Brunak, S., von Heijne, G., and Nielsen, H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* **8**, 785–786.

Shen, H.B. and Chou, K.C. 2010. Gneg-mPLoc: a top-down strategy to enhance the quality of predicting subcellular localization of Gram-negative bacterial proteins. *J. Theor. Biol.* **264**, 326–333.

Subsin, B., Thomas, M.S., Katzenmeier, G., Shaw, J.G., Tungpradabkul, S., and Kunakorn, M. 2003. Role of the stationary growth phase sigma factor RpoS of *Burkholderia pseudomallei* in response to physiological stress conditions. *J. Bacteriol.* **185**, 7008–7014.

Utaisincharoen, P., Arjcharoen, S., Limposuwan, K., Tungpradabkul, S., and Sirisinha, S. 2006. *Burkholderia pseudomallei* RpoS regulates multinucleated giant cell formation and inducible nitric oxide synthase expression in mouse macrophage cell line (RAW 264.7). *Microb. Pathog.* **40**, 184–189.

Visick, J.E., Ichikawa, J.K., and Clarke, S. 1998. Mutations in the *Escherichia coli surE* gene increase isoaspartyl accumulation in a strain lacking the pcm repair methyltransferase but suppress stress-survival phenotypes. *FEMS Microbiol. Lett.* **167**, 19–25.

Vuddhakul, V., Tharavichitkul, P., Na-Ngam, N., Jitsurong, S., Kunthawa, B., Noimay, P., Noimay, P., Binla, A., and Thamlikitkul, V. 1999. Epidemiology of *Burkholderia pseudomallei* in Thailand. *Am. J. Trop. Med. Hyg.* **60**, 458–461.

White, N.J. 2003. Melioidosis. *Lancet* **361**, 1715–1722.

Yu, C.S., Lin, C.J., and Hwang, J.K. 2004. Predicting subcellular localization of proteins for Gram-negative bacteria by support vector machines based on n-peptide compositions. *Protein Sci.* **13**, 1402–1406.

Yu, N.Y., Wagner, J.R., Laird, M.R., Melli, G., Rey, S., Lo, R., Dao, P., Sahinalp, S.C., Ester, M., Foster, L.J., and Brinkman, F.S. 2010. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* **26**, 1608–1615.

Zhang, R.G., Skarina, T., Katz, J.E., Beasley, S., Khachatryan, A., Vyas, S., Arrowsmith, C.H., Clarke, S., Edwards, A., Joachimiak, A., *et al.* 2001. Structure of *Thermotoga maritima* stationary phase survival protein SurE: a novel acid phosphatase. *Structure* **9**, 1095–1106.