รายงานทุนพัฒนานักวิจัย ฉบับสมบูรณ์

หน้าที่และความสำคัญของ *Burkholderia pseudomallei sctV2* และ *bipB* genes: การศึกษาหลังยุค genomic

รศ.ดร. สุนีย์ กอรปศรีเศรษฐ์

ภาควิชาวิทยาภูมิคุ้มกัน คณะแพทยศาสตร์ศิริราชพยาบาล

มหาวิทยาลัยมหิดล

สิงหาคม 2548

สัญญาเลขที่ RSA4580034

รายงานวิจัย ฉบับสมบูรณ์

หน้าที่และความสำคัญของ *Burkholderia pseudomallei sctV2* และ *bipB* genes: การศึกษาหลังยุค genomic

รศ.ดร. สุนีย์ กอรปศรีเศรษฐ์
ภาควิชาวิทยาภูมิคุ้มกัน คณะแพทยศาสตร์ศิริราชพยาบาล
มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

ผู้วิจัยขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ที่ให้การสนับสนุนทุนวิจัยโครงการนี้ ภาควิชาวิทยาภูมิคุ้มกัน คณะแพทยศาสตร์ศิริราชพยาบาล ที่ให้การสนับสนุนสถานที่และเครื่องมือการวิจัย หน่วยอณูชีววิทยาการแพทย์ สถานส่งเสริมการวิจัย คณะแพทยศาสตร์ศิริราชพยาบาลที่อนุเคราะห์ tissue culture facility

รวมทั้งเจ้าหน้าที่และนักศึกษาในภาควิชาวิทยาภูมิคุ้มกัน ที่ให้ความช่วยเหลือร่วมมือและสนับสนุนการทำวิจัยครั้งนี้อย่างดียิ่ง สุดท้ายนี้ผลงานวิจัยชิ้นนี้จะสำเร็จลุล่วงไม่ได้ถ้าขาด ศ.ดร.ศกรณ์ มงคลสุข ผศ.ดร.กาญจนา เลิศมีมงคลชัย และนพ.ชาญวิทย์ ตรีพุทธรัตน์ ที่ให้คำแนะนำและกำลังใจตลอดมา

จึงขอขอบพระคุณมาในโอกาสนี้

รศ.ดร.สุนีย์ กอรปศรีเศรษฐ์

Abstract

Project Code: RSA4580034

Project Title: Molecular and functional characterization of *Burkholderia pseudomallei sctV2* and *bipB* genes: a post-genomic study

Investigator:
Assoc. Prof. Sunee Korbsrisate
Department of Immunology, Faculty of Medicine Siriraj Hospital
Mahidol University
Bangkok 10700, Thailand
Tel : 02-419-7000 ext. 6636 Fax : 02-418-1636
E-mail address: grsks@mahidol.ac.th

Project Period: 3 years

Burkholderia pseudomallei is the etiological agent of melioidosis which is endemic in southeast Asia and tropical Australia. Uniquely among intracellular bacterial pathogens, *B. pseudomallei* induce host cell fusion leading to multinucleated giant cell (MNGC) formation in tissue culture models of infection. In addition to MNGC formation, *B. pseudomalle* is able to induce apoptotic death in infected host cells. The virulence factors contributed to these pathogenic characteristics have not been elucidated. In this study, mutagenesis of *B. pseudomallei bipB* gene was undertaken. The *bipB* mutant showed defective in MNGC formation, induction of apoptosis of J774A.1 macrophages and was also significantly attenuated following intranasal challenge of BALB/c mice. It is indicated that MNGC formation and apoptosis in infected host cells is mediated by *B. pseudomallei* BipB.

Little is known about the regulatory mechanisms that determine adaptation of *B. pseudomallei* to environmental stress. In this study, we investigated the importance of *B. pseudomallei* RpoE in survival under different stress conditions. To accomplish this, an *rpoE* operon mutant was constructed by insertion mutagenesis in order to examine its response to osmotic stress and its survival within phagocytic cells. The result demonstrated that the *rpoE* operon contributes, at least in part, to the survival of *B. pseudomallei* in stressful environments.

Keywords: *Burkholderia pseudomallei*, MNGC, *bipB, rpoE*, stress response

บทคัดย่อ

รหัสโครงการ : RSA4580034

ชื่อโครงการ : หน้าที่และความสำคัญของ *Burkholderia pseudomallei sctV2* และ *bipB* genes: การศึกษาหลังยุค genomic

ชื่อนักวิจัย :

รศ.ดร. สุนีย์ กอรปศรีเศรษฐ์

ภาควิชาวิทยาภูมิคุ้มกัน

คณะแพทยศาสตร์ศิริราชพยาบาล

มหาวิทยาลัยมหิดล

Tel : 02-419-7000 ext. 6636 Fax : 02-418-1636

E-mail address: grsks@mahidol.ac.th

ระยะเวลาโครงการ : 3 ปี

Bukholderia pseudomallei เป็นจุลชีพก่อโรค melioidosis ซึ่งพบระบาดในเอเชียตะวันออกเฉียงใต้และออสเตรเลีย จุลชีพนี้จัดเป็น intracellular bacteria โดยมีลักษณะเด่นเฉพาะคือ สามารถก่อให้เกิดการเชื่อมกันของเซลล์เนื้อเยื่อเป็น multinucleated giant cell นอกจากนั้นยังสามารถทำให้เซลล์ติดเชื้อเกิดการตายแบบ apoptosis ด้วย ในปัจจุบันยังไม่มีการศึกษา virulence factor ของ *B. pseudomallei* ที่นำไปสู่การเกิดพยาธิภาพลักษณะดังกล่าวข้างต้น ดังนั้นงานวิจัยนี้จึงมีวัตุประสงค์ที่จะศึกษาว่าโปรตีน BipB มีส่วนเกี่ยวข้องหรือไม่โดยการใช้เทคนิคทางอณูชีววิทยาผลิต *B. pseudomallei* ที่มีความผิดปกติของยีน bipB แล้วเปรียบเทียบพยาธิสภาพที่เกิดขึ้นในเซลล์ติดเชื้อว่ามีความแตกต่างจาก *B. pseudomallei* สายพันธุ์ปกติหรือไม่ ผลการทดลองพบ *B. pseudomallei bipB* mutant สูญเสียความสามารถในการก่อให้เกิด MNGC และการตายแบบ apoptosis ใน macrophage J.774A.1 และมีลักษณะอ่อนฤทธิ์ (attenuated) ต่อการก่อโรคในหนู BALB/c แสดงว่า BipB เกี่ยวข้องกับการเกิด MNGC และการตายแบบ apoptosis ในเซลล์ที่มีการติดเชื้อ

กลไกที่ทำให้ *B. pseudomallei* สามารถปรับตัวเพื่อให้มีชีวิตรอดในสภาวะแวดล้อมต่างๆ นั้นยังมีการศึกษาน้อยมาก คณะผู้วิจัยจึงได้ศึกษาความสำคัญของโปรตีน RpoE ที่มีต่อการมีชีวิตของ *B. pseudomallei* ในสิ่งแวดล้อม โดยการใช้เทคนิคทางอณูชีววิทยาผลิต *B. pseudomallei rpoE* operon mutant จากนั้นเปรียบเทียบความสามารถในการเจริญเติบโตภายใต้สภาวะ oxidative stress และภายในเซลล์ฟาโกซัยท์ ผลการทดลองพบว่าโปรตีน RpoE มีความสำคัญต่อการปรับตัวให้มีชีวิตรอดของ *B. pseudomallei* ในสภาวะแวดล้อมต่าง ๆ

Keywords: *Burkholderia pseudomalle,* MNGC, *bipB, rpoE*, stress response

Output:

1. ผลงานวิจัยตีพิมพ์ในวารสารวิชาการนานาชาติ 2 เรื่อง

- Suparak S, Kespichayawattana W, Haque A, Easton A, Damnin S, Lertmeongkolchai G, Gregory J Bancroft, **Korbsrisate S.** Multinucleated giant cell formation and apoptosis in infected host cells is mediated by *Burkholderia pseudomallei* type III secretion protein BipB. J Bacteriol 2005; **187:**6556-6560. Impact factor 4.1
- **Korbsrisate S,** Vanaporn M, Kerdsuk P, Kespichayawattana W, Vattanaviboon P, Kiatpapan P, Lertmemongkolchai G. The *Burkholderia pseudomallei* RpoE (AlgU) operon is involved in environmental stress tolerance and biofilm formation. FEMS Microbiol Letters (in press). Impact factor 1.9

2. ทุนวิจัยที่ได้รับทำให้
   - สามารถผลิตบัณฑิตระดับปริญญาโท 1 คน คือ น.ส. ผัลย์สุภา เกิดสุข
   - นักศึกษาปริญญาเอกที่คาดว่าจะจบการศึกษาในปี 2549 จำนวน 1 คนคือ น.ส. สุภาพร สุภารักษ์
   - นักศึกษาปริญญาเอกที่คาดว่าจะจบการศึกษาในปี 2551 จำนวน 1 คนคือ น.ส. มุทิตา วณาภรณ์
3. ผลงานวิจัยเรื่อง The *Burkholderia pseudomallei* RpoE (AlgU) operon is involved in environmental stress tolerance and biofilm formation ได้รับรางวัลที่ 2 จากการประกวด oral presentation ในงานประชุมวิชาการศิริราช ประจำปี 2548
4. ความร่วมมือทำวิจัยด้าน *B. pseudomallei* กับนักวิจัยต่างประเทศ เช่น Dr. R. Titball, Dr. M.P. Stevens, Dr. E.E Galyov และ Dr. G.J. Bancroft

ภาคผนวก

JOURNAL OF BACTERIOLOGY, Sept. 2005, p. 6556–6560 Vol. 187, No. 18
0021-9193/05/$08.00+0 doi:10.1128/JB.187.18.6556–6560.2005

NOTES

Multinucleated Giant Cell Formation and Apoptosis in Infected Host Cells Is Mediated by *Burkholderia pseudomallei* Type III Secretion Protein BipB

Supaporn Suparak,[1] Wannapa Kespichayawattana,[2] Ashraful Haque,[3] Anna Easton,[3] Suwat Damnin,[1] Ganjana Lertmemongkolchai,[4] Gregory J. Bancroft,[3] and Sunee Korbsrisate[1]*

Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand[1]; Laboratory of Immunology, Chulabhorn Research Institute, Laksi, Thailand[2]; Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel St., London, United Kingdom[3]; and Department of Clinical Immunology, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand[4]

Received 11 March 2005/Accepted 18 June 2005

Here we have assessed the role of a type III translocator protein, BipB, in the cell biology and virulence of *Burkholderia pseudomallei*. Genetic inactivation of *bipB* reduced multinucleated giant cell formation, cell-to-cell spreading of bacteria, and induction of apoptosis of J774A.1 macrophages. The *bipB* mutant was also significantly attenuated following intranasal challenge of BALB/c mice, whereas virulence was fully restored by complementation with a functional *bipB* gene.

Burkholderia pseudomallei, the etiological agent of melioidosis in humans and animals, is a gram-negative bacterium. Melioidosis is endemic in southeast Asia and tropical Australia and has been reported sporadically elsewhere (6). Currently, there is no vaccine against melioidosis. Uniquely among intracellular bacterial pathogens, *B. pseudomallei* induces host cell fusion leading to multinucleated giant cell (MNGC) formation in tissue culture models of infection (14). This novel phenotype may be relevant to pathogenesis, since granuloma formation and generation of MNGC are also found in tissues of humans with melioidosis (23). In addition to inducing MNGC formation, *B. pseudomallei* is able to spread from cell to cell and induce apoptotic death in infected host cells (14). The molecular mechanisms of these pathogenic characteristics have not been elucidated.

Analysis of the *B. pseudomallei* genome and several other studies have demonstrated the presence of a type III secretion system (TTSS) (for reviews, see references 3, 12, 17, 20, and 22). A knockout mutant of *B. pseudomallei* lacking a functional *bipD* gene, a homologue of *Salmonella enterica* serovar Typhimurium *sipD*, on the TTSS3/*bsa* cluster of TTSS exhibited reduced replication in murine macrophage-like cells (20), was significantly attenuated in BALB/c mice and gave partial protection against subsequent challenge with wild-type *B. pseudomallei* (19). These data correlated with the recent report that the TTSS3/*bsa* cluster is required for the pathogenicity of *B. pseudomallei* (21). In addition to BipD, *B. pseudomallei* BipB and BipC (46 and 30% amino acid identity to *Salmonella* SipB and SipC, respectively) have been identified in the TTSS3/*bsa* cluster (3). Here, we report on the role of BipB in the pathogenesis of infection with *B. pseudomallei*. With *Salmonella* organisms, purified SipB integrates into artificial membranes and induces liposome fusion (10), and it is required for inducing apoptosis in murine macrophages (11). By analogy with SipB, therefore, we investigated the role of BipB for MNGC formation, cell-to-cell spreading, and induction of apoptosis in infected host cells. We also examined the virulence of a *B. pseudomallei bipB* mutant in a murine model of melioidosis.

Construction of a *B. pseudomallei bipB* mutant. Analysis of the *B. pseudomallei* genome (http://www.sanger.ac.uk/Projects/B_pseudomallei), by use of the *sipB* sequence from *S. enterica* serovar Typhimurium as the query in a TBLASTX search, identified a coding sequence of 1,860 bp encoding the predicted BipB protein of 620 amino acids. In order to determine the function of BipB in *B. pseudomallei*, a chromosomal *bipB* mutant of *B. pseudomallei* was constructed. In brief, a 250-bp internal fragment of the *bipB* gene was amplified from *B. pseudomallei* K96243 genomic DNA by use of primers BipB-45 (5′-AACCAGGCCACGCAGCAG-3′) and BipB-46 (5′-CGT CTTCTGCATCTCCTC-3′). The amplified fragment was cloned into a suicide vector, pKNOCK-Tc (1), kindly provided by M. F. Alexeyev. This constructed plasmid was introduced from *Escherichia coli* S17-1λpir (7) into *B. pseudomallei* K96243 by conjugation. Transconjugants were selected by plating on pseudomonas agar supplemented with SR103 (Oxoid, United Kingdom) containing tetracycline. The isolated mutant, designated *B. pseudomallei* BS46 (*bipB*::pSSB-1), was verified by PCR and Southern blot hybridization to ensure insertion of the *bipB* suicide plasmid at the correct location (data not shown). For complementation analysis, the amplified *bipB*

* Corresponding author. Mailing address: Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Prannok Road, Bangkoknoi, Bangkok 10700, Thailand. Phone: 66-2-418-0569. Fax: 66-2-418-1636. E-mail: grsks@mahidol.ac.th.

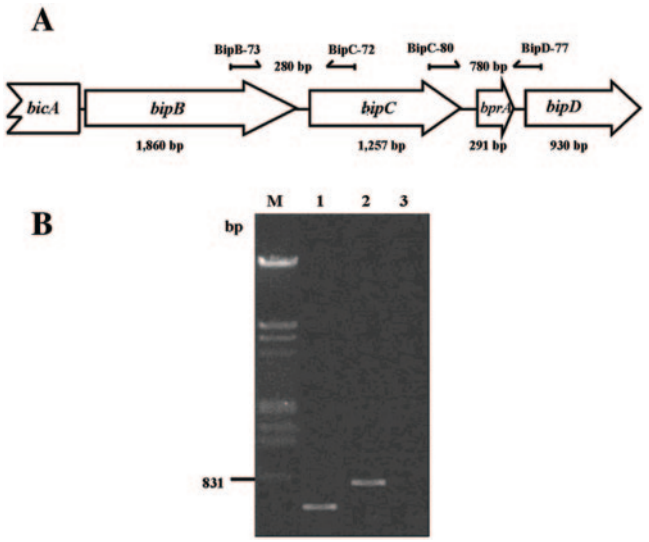

FIG. 1. The *B. pseudomallei bipB* operon. (A) Physical map of *bipB-bipC-bprA-bipD* gene organization together with locations of primer pairs BipB-73–BipC-72 and BipC-80–BipD-77 for RT-PCR analysis of *B. pseudomallei bipB* operon. (B) Ethidium bromide-stained gel showing the amplified DNA of RT-PCR products from primer pairs BipB-73–BipC-72 (lane 1) and BipC-80–BipD-77 (lane 2). Lane 3 is an RNA sample subjected to PCR to ensure no DNA contamination in the RNA preparation. Lane M shows lambda DNA markers.

gene was cloned into pBBR1MCS (15) and introduced into *B. pseudomallei* BS46. To confirm that *B. pseudomallei* BS46pBipB contained the *bipB* gene, the DNA plasmid was extracted and sequenced (data not shown).

To determine whether *bipB* was cotranscribed with the downstream genes *bipC-bprA-bipD*, reverse transcription-PCR (RT-PCR) was undertaken. Extraction of total RNA, by use of the modified hot acid phenol method, was carried out as described previously (2). In brief, mid-exponential-phase cultures were harvested and extracted with hot acid phenol. Total RNA was precipitated and resuspended with RNase-free distilled water. For RT-PCR analysis, *bipB-bipC-bprA-bipD* was reversed transcribed into cDNA (Invitrogen) and then amplified with different primers, namely, BipB-73 (5′-CTGCTCGGCG ATCTGCTCAA-3′), BipC-72 (5′-ACCGCCTTGTCGCCCT G-3′), BipC-80 (5′-GAGCAGAAAGAGGACGAGA-3′), and BipD-77 (5′-CGCAGATCGTCGTCGGTCA-3′) (Fig. 1A).

As depicted in Fig. 1B, *B. pseudomallei bipB-bipC-bprA-bipD* was transcribed in a single transcriptional unit. It is likely that *B. pseudomallei* BS46 is a polar *bipB* mutant. To investigate whether this mutation does not have effect on expression of other secreted proteins, Western blot analysis using anti-BopE (kindly provided by M. P. Stevens, United Kingdom) to detect BopE in whole-cell and secreted protein fractions of *B. pseudomallei* BS46 and wild-type strains was undertaken. BopE, homologous to the *Salmonella* SopE, was an effector protein secreted by the *B. pseudomallei* TTSS (18). BopE was detected in both whole-cell and secreted protein fractions of *B. pseudomallei* BS46 (data not shown). This suggests that the TTSS of *B. pseudomallei* BS46 is still functional to express and secrete other proteins such as BopE.

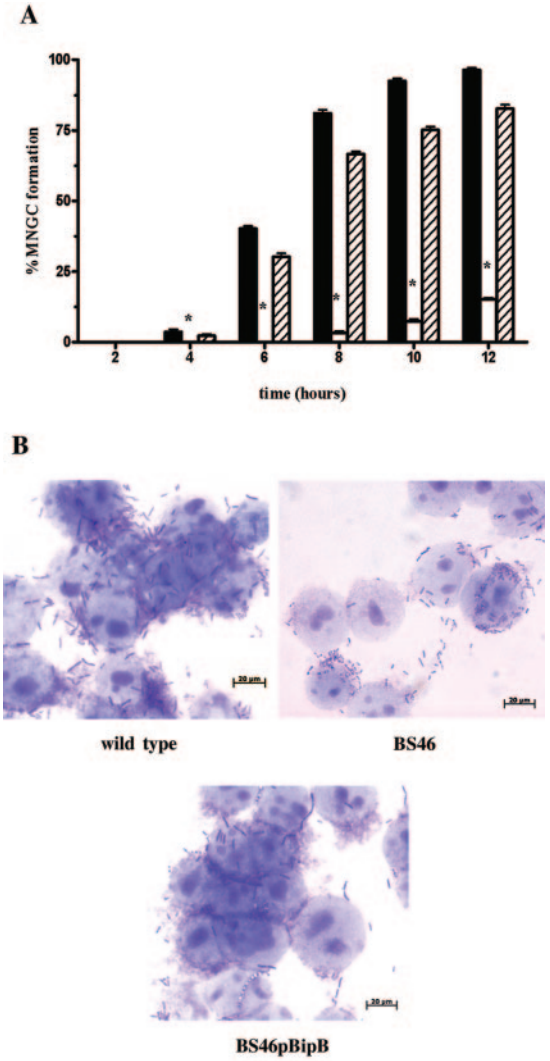

FIG. 2. MNGC formation of *B. pseudomallei*. (A) The percentages of MNGC formation of J774A.1 cells infected with *B. pseudomallei* K96243 (wild type; solid bars), BS46 (*bipB*::pSSB-1; open bars), and BS46pBipB (BS46 harboring pBipB; striped bars) were determined every 2 h. Asterisks indicate significant differences ($P < 0.05$, *t* test) between the wild type and BS46 at 4 h ($P = 0.0142$) and 6 to 12 h ($P < 0.0001$) and between BS46 and BS46pBipB at 4 h ($P = 0.0155$) and 6 to 12 h ($P < 0.0001$). Percentage of MNGC formation was determined by the following equation: MNGC formation = (number of nuclei within multinucleated giant cells/total number of nuclei counted) × 100. Error bars represent standard errors of the means for experiments performed in triplicate. (B) Giemsa staining of MNGC formation of J774A.1 cells infected with wild type, BS46, or BS46pBipB. Bars, 20 μm.

The polar *bipB* mutant is defective in MNGC formation. To investigate the potential role of BipB in MNGC formation, *B. pseudomallei* K96243 (wild type), BS46 (*bipB*::pSSB-1), and BS46pBipB (BS46 harboring pBipB) were used to infect J774A.1 murine macrophage-like cells as described previously (14). At different times after initiation of the challenge, the infected cells were fixed, Giemsa stained, and evaluated for MNGC formation. Figure 2 shows that BipB protein plays a role in *B. pseudomallei*-induced MNGC formation. At 12 h

postinfection (Fig. 2A), wild-type bacteria induced extensive MNGC formation (96.46%), while BS46 did not (15.12%). The formation of MNGC was restored in a complementation assay using strain BS46pBipB (82.7%). Figure 2B shows that MNGC loaded with numerous bacilli could be readily observed at 6 h after infection with wild-type bacteria but that this was abolished in the *bipB* mutant BS46. However, this defective phenotype was transcomplemented by reintroduction of the plasmid-born *bipB* gene. However, when the observation period was extended to 24 h, formation of MNGC in BS46-infected macrophage did occur but was still significantly less than the wild-type strain. Thus, BipB is necessary for optimal MNGC induction, but BipB-independent fusion can also occur, albeit at a reduced efficiency.

The mechanism for the MNGC formation is still unknown, and to our knowledge, this altered phenotype has not been observed in other intracellular bacteria that possess the TTSS. Based on the *Salmonella* SipB-induced fusion events in vitro (10) and those that would be transient in vivo (9), we hypothesize that BipB may have membrane fusion activity as well. It may act in concert with other proteins to induce fusion of host cell membranes. A combination of biochemistry, cell biology, and proteomics will be required to unveil the detailed pathways of MNGC formation.

The polar *bipB* mutant is defective in cell-to-cell spread and invasion into epithelial cells. The observation of MNGC led us to look closely at cell-to-cell spread of infected host cells by using a plaque assay previously described (14). HeLa cells were infected with *B. pseudomallei* and overlaid with an agarose medium containing kanamycin (250 μg/ml). To enhance visualization, plaques were overlaid with agarose containing an additional 0.01% neutral red and observed 4 h later. Figure 3A demonstrates that plaque-forming efficiencies for *B. pseudomallei* wild type (2.66) and BS46pBipB (0.68) were significantly higher than that for BS46 (0.2). It is possible that only partial complementation in BS46pBipB could have resulted from a polar effect that disrupted downstream *bipC* and *bipD* genes also participating in cell-to-cell spreading. This hypothesis is supported by a previous report, from Stevens et al. (20), that a *bipD* mutant exhibited an inability to escape from endocytic vacuoles, a requirement for cell-to-cell spread. If so, it would indicate that BipB works cooperatively with BipC and BipD in a manner similar to that of SipABCD in *Salmonella* (4).

The strategies that intracellular bacteria, i.e., *Listeria* sp. and *Shigella* sp., use to spread from cell to cell via interepithelial protrusion are quite similar (8). The process depends on the efficiency of bacterial invasion into the epithelial cytosol, protrusion formation, and the lysis of the double-membrane-bound protrusion vacuole to release bacteria into the adjacent cell. To investigate whether defective cell-to-cell spread (as detected by plaque assay) was due to an invasion defect, invasion efficiency was determined by using human respiratory epithelial cell line A549 challenged with *B. pseudomallei* as described earlier. This cell line was chosen because it is more susceptible to invasion than HeLa cells. Intracellular bacteria were counted after lysing of infected cells. Invasion efficiency of BS46 was severely restricted (0.09%) when compared to that of the wild type (0.39%), but invasion efficiency was restored to nearly normal levels in BS46pBipB (0.28%) (Fig. 3B). These data correlated with those for the *bipD* mutant that exhibited

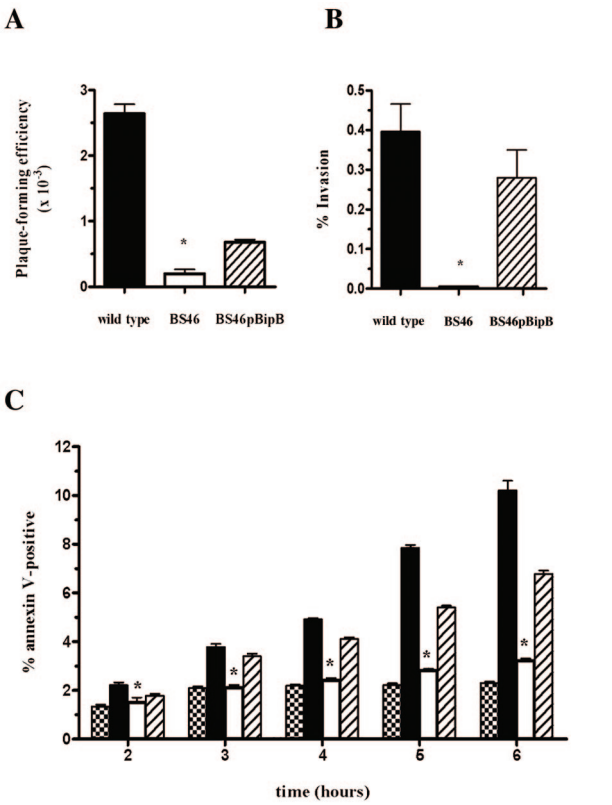

FIG. 3. Plaque formations, invasion, and apoptosis induction. (A) Plaque formations of HeLa cells by *B. pseudomallei* K96243 (wild type; solid bars), BS46 (*bipB*::pSSB-1; open bars), and BS46pBipB (BS46 harboring pBipB; striped bars). Asterisks indicate significant differences ($P < 0.05$, *t* test) between wild type and BS46 ($P = 0.0001$) and between BS46 and BS46pBipB ($P = 0.0031$). Plaque-forming efficiency was determined by the following equation: plaque-forming efficiency = number of plaques/bacterial CFU added per well. Error bars represent standard errors of the means for experiments performed in triplicate. (B) Invasion of A549 cells by *B. pseudomallei* K96243 (wild type; solid bars), BS46 (*bipB*::pSSB-1; open bars), and BS46pBipB (BS46 harboring pBipB; striped bars) strains. Asterisks indicate significant differences ($P < 0.05$, *t* test) between wild type and BS46 ($P = 0.0050$) and between BS46 and BS46pBipB ($P = 0.0173$). Percent invasion was determined by the following equation: invasion = (number of intracellular bacteria postinfection/number of CFU added) × 100. Error bars represent standard errors of the means for experiments performed in triplicate. (C) Effect of *bipB* mutation on induction of apoptosis. J774A.1 cells were infected with *B. pseudomallei* K96243 (wild type; solid bars), BS46 (*bipB*::pSSB-1; open bars), BS46pBipB (BS46 harboring pBipB; striped bars), and uninfected cells (checkered bars). The percentages of J774A.1 cells stained fluorescein isothiocyanate positive and propidium iodide negative by flow cytometry were analyzed. Asterisks indicate significant differences ($P < 0.05$, *t* test) between wild type and BS46 at 2 h ($P = 0.0123$), 3 h ($P = 0.0004$), and 4 to 6 h ($P < 0.0001$) and between BS46 and BS46pBipB at 2 h ($P = 0.1064$), 3 h ($P = 0.0006$), and 4 to 6 h ($P < 0.0001$). Error bars represent standard errors of the means for experiments performed in triplicate.

impaired entry into nonphagocytic host cells (18). In this scenario, we believe that several effector proteins, such as BopE, that contribute to invasion (18) would not be delivered into the host cell cytoplasm, even though it was expressed. This proposed mechanism is based on the study of *Salmonella* in which inactivation of *sip* genes resulted in impaired invasion effi-

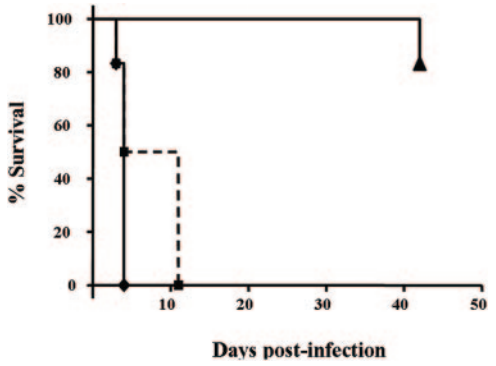

FIG. 4. Survival of BALB/c mice (six mice per group) inoculated intranasally with 10^3 CFU of *B. pseudomallei* K96243 (■) or BS46 (▲) or BS46pBipB (◆). Mice were observed daily, and percent survival was plotted against time.

ciency due to the lack of translocation of effector proteins, such as SopE, into host cells (4, 13, 24). In addition to invasion, BipB may play a role in other steps involved in cell-to-cell spreading. Further experiments are required to investigate this possibility.

The polar *bipB* mutant is defective in induction of apoptosis. *B. pseudomallei* can induce apoptotic death in infected macrophages (14). To determine the role of BipB in this process, J774A.1 cells were infected with *B. pseudomallei* strains. At different time intervals, the supernatant and cells were collected to quantify the apoptosis level by using an annexin V-fluorescein isothiocyanate detection kit (BD Biosciences, CA). At 6 h postinfection (Fig. 3C), cells infected with wild-type *B. pseudomallei* yielded significantly higher numbers of positive cells (10.20%) than those infected with BS46 (3.21%). Infection with BS46pBipB restored cytotoxicity (6.77%). These data indicated that BipB was required for efficient induction of apoptosis in host cells, although a low level of apoptosis may occur via a BipB-independent mechanism, since the level of apoptosis in uninfected cells is 2.3%. This is the first report identifying a *B. pseudomallei* virulence factor that mediates apoptosis. Interestingly, this finding joins a growing list of bacteria, including *Pseudomonas aeruginosa*, *Yersinia* sp., *Salmonella* sp., and *Shigella flexneri*, that kill host cells via apoptotic death through a type III secretion-mediated mechanism. In *Salmonella* and *Shigella*, SipB and IpaB have been shown to induce macrophage apoptotic death by activating caspase-1 (11, 25). Here, we also expect that apoptosis induced by *B. pseudomallei* will involve BipB interaction with the caspase pathway (14).

Effect of *bipB* mutation on virulence of *B. pseudomallei* in vivo. The finding that BipB is important in induction of MNGC, plaque formation, bacterial invasion, and killing of phagocytic cells in vitro led to the hypothesis that a mutant unable to produce this protein could be less virulent than the wild-type strain in vivo. We therefore assayed the virulence of the *bipB* mutant in a pulmonary model of melioidosis in BALB/c mice as previously described (19). *B. pseudomallei* strains were administered via the intranasal route. Viable counts were performed to confirm the inoculation dose, and the mice were monitored twice daily for signs of infection. There was a significant difference in percentage survival (the *P* value was <0.05, as determined by a log rank test) for mice infected with wild-type *B. pseudomallei* versus mice infected with BS46 (Fig. 4). All mice given the wild-type strain died within 5 to 11 days, whereas five of six mice infected with the *bipB* mutant survived until day 42 (termination of experiment). To confirm that attenuation resulted from the inactivation of *bipB*, we also challenged mice with strain BS46pBipB, and all died by day 4 postchallenge (Fig. 4), which was not significantly different from the wild-type strain. These observations indicated that a functional *bipB* gene was required for full virulence of *B. pseudomallei* in mice. This result is supported by previous reports (19, 21) that TTSS3/Bsa plays an important role for maximal virulence in all of its animal hosts.

Delivery of virulence-associated effector proteins into eukaryotic cells requires a set of translocator proteins. The translocons are components of oligomeric protein channels that insert themselves into the eukaryotic cell membrane to form a pore which effector proteins can pass through to gain access to the cytosolic host targets (5, 16). We have shown here that BipB translocator plays a critical role in the intracellular lifestyle of *B. pseudomallei* (i.e., MNGC formation, invasion of nonphagocytic cells, and induction of apoptotic death). We hypothesize that the *bipB* mutant is unable to deliver the effector proteins into the host cell cytoplasm and was thus impaired in invasion efficiency and ability to induce apoptosis. However, it is also possible that BipB acts as an effector protein to induce apoptotic death. Deletion of BipB clearly also reduces the efficiency of MNGC formation; however, the relationship between BipB protein and the fusion process is still under investigation. In vivo, BipB was required for full virulence of *B. pseudomallei* in mice, thus further confirming the importance of BipB for virulence in murine models of melioidosis.

This work was supported by the Thailand Research Fund (TRF) grant PHD/0093/2546 through the Royal Golden Jubilee Ph.D. program to S. Suparak and S. Korbsrisate, grant RSA4580034 from the TRF to S. Korbsrisate, and a Senior Research Scholar grant (RTA4580010) to S. Mongkolsuk.

We thank S. Lerdwana for flow cytometric analysis, P. Vattanaviboon for his suggestion, and T. W. Flegel for critical reading of the manuscript. We also acknowledge the staff from the Medical Molecular Biology Unit, Siriraj Hospital, Thailand, for assistance in cell culture techniques.

REFERENCES

1. **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.
2. **Ambulos, N. P., Jr., E. J. Duvall, and P. S. Lovett.** 1987. Method for blot-hybridization analysis of mRNA molecules from *Bacillus subtilis*. Gene **51:** 281–286.
3. **Attree, O., and I. Attree.** 2001. A second type III secretion system in *Burkholderia pseudomallei*: who is the real culprit? Microbiology **147:**3197–3199.
4. **Collazo, C. M., and J. E. Galan.** 1997. The invasion-associated type III system of *Salmonella typhimurium* directs the translocation of Sip proteins into the host cell. Mol. Microbiol. **24:**747–756.
5. **Collazo, C. M., and J. E. Galan.** 1996. Requirement for exported proteins in secretion through the invasion-associated type III system of *Salmonella typhimurium*. Infect. Immun. **64:**3524–3531.
6. **Dance, D. A.** 2000. Ecology of *Burkholderia pseudomallei* and the interactions between environmental *Burkholderia* spp. and human-animal hosts. Acta Trop. **74:**159–168.
7. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Methods Enzymol. **235:**386–405.
8. **Dramsi, S., and P. Cossart.** 1998. Intracellular pathogens and the actin cytoskeleton. Annu. Rev. Cell Dev. Biol. **14:**137–166.

9. **Finlay, B. B., and S. Falkow.** 1990. *Salmonella* interactions with polarized human intestinal Caco-2 epithelial cells. J. Infect. Dis. **162:**1096–1106.
10. **Hayward, R. D., E. J. McGhie, and V. Koronakis.** 2000. Membrane fusion activity of purified SipB, a *Salmonella* surface protein essential for mammalian cell invasion. Mol. Microbiol. **37:**727–739.
11. **Hersh, D., D. M. Monack, M. R. Smith, N. Ghori, S. Falkow, and A. Zychlinsky.** 1999. The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. Proc. Natl. Acad. Sci. USA **96:**2396–2401.
12. **Hueck, C. J.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. **62:**379–433.
13. **Kaniga, K., S. Tucker, D. Trollinger, and J. E. Galan.** 1995. Homologs of the *Shigella* IpaB and IpaC invasins are required for *Salmonella typhimurium* entry into cultured epithelial cells. J. Bacteriol. **177:**3965–3971.
14. **Kespichayawattana, W., S. Rattanachetkul, T. Wanun, P. Utaisincharoen, and S. Sirisinha.** 2000. *Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. Infect. Immun. **68:**5377–5384.
15. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
16. **Miao, E. A., C. A. Scherer, R. M. Tsolis, R. A. Kingsley, L. G. Adams, A. J. Baumler, and S. I. Miller.** 1999. *Salmonella typhimurium* leucine-rich repeat proteins are targeted to the SPI1 and SPI2 type III secretion systems. Mol. Microbiol. **34:**850–864.
17. **Rainbow, L., C. A. Hart, and C. Winstanley.** 2002. Distribution of type III secretion gene clusters in *Burkholderia pseudomallei*, *B. thailandensis* and *B. mallei*. J. Med. Microbiol. **51:**374–384.
18. **Stevens, M. P., A. Friebel, L. A. Taylor, M. W. Wood, P. J. Brown, W. D. Hardt, and E. E. Galyov.** 2003. A *Burkholderia pseudomallei* type III secreted protein, BopE, facilitates bacterial invasion of epithelial cells and exhibits guanine nucleotide exchange factor activity. J. Bacteriol. **185:**4992–4996.
19. **Stevens, M. P., A. Haque, T. Atkins, J. Hill, M. W. Wood, A. Easton, M. Nelson, C. Underwood-Fowler, R. W. Titball, G. J. Bancroft, and E. E. Galyov.** 2004. Attenuated virulence and protective efficacy of a *Burkholderia pseudomallei bsa* type III secretion mutant in murine models of melioidosis. Microbiology **150:**2669–2676.
20. **Stevens, M. P., M. W. Wood, L. A. Taylor, P. Monaghan, P. Hawes, P. W. Jones, T. S. Wallis, and E. E. Galyov.** 2002. An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen. Mol. Microbiol. **46:**649–659.
21. **Warawa, J., and D. E. Woods.** 2005. Type III secretion system cluster 3 is required for maximal virulence of *Burkholderia pseudomallei* in a hamster infection model. FEMS Microbiol. Lett. **242:**101–108.
22. **Winstanley, C., B. A. Hales, and C. A. Hart.** 1999. Evidence for the presence in *Burkholderia pseudomallei* of a type III secretion system-associated gene cluster. J. Med. Microbiol. **48:**649–656.
23. **Wong, K. T., S. D. Puthucheary, and J. Vadivelu.** 1995. The histopathology of human melioidosis. Histopathology **26:**51–55.
24. **Wood, M. W., R. Rosqvist, P. B. Mullan, M. H. Edwards, and E. E. Galyov.** 1996. SopE, a secreted protein of *Salmonella dublin*, is translocated into the target eukaryotic cell via a sip-dependent mechanism and promotes bacterial entry. Mol. Microbiol. **22:**327–338.
25. **Zychlinsky, A., B. Kenny, R. Menard, M. C. Prevost, I. B. Holland, and P. J. Sansonetti.** 1994. IpaB mediates macrophage apoptosis induced by *Shigella flexneri*. Mol. Microbiol. **11:**619–627.

ELSEVIER

FEMS Microbiology Letters xxx (2005) xxx–xxx

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

The *Burkholderia pseudomallei* RpoE (AlgU) operon is involved in environmental stress tolerance and biofilm formation

Sunee Korbsrisate [a,*], Muthita Vanaporn [a], Phansupa Kerdsuk [a], Wannapa Kespichayawattana [b], Paiboon Vattanaviboon [c], Pornpimon Kiatpapan [d], Ganjana Lertmemongkolchai [e]

[a] *Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand*
[b] *Laboratory of Immunology, Chulabhorn Research Institute, Bangkok, Thailand*
[c] *Laboratory of Biotechnology, Chulabhorn Research Institute, Bangkok, Thailand*
[d] *Biochemistry Unit, Faculty of Science, Rangsit University, Bangkok, Thailand*
[e] *Department of Clinical Immunology, Faculty of Associated Medical Sciences, Khon Kaen University, Thailand*

Received 17 February 2005; received in revised form 8 August 2005; accepted 1 September 2005

First published online

Edited by M. Schembri

Abstract

Burkholderia pseudomallei, the causative agent of melioidosis, can be isolated from soil and water. To persist, adapt and survive within and outside their human host, bacteria rely on regulatory mechanisms that allow them to respond rapidly to stressful situations. We have examined the possible role of *B. pseudomallei* alternative sigma factor σ^E (RpoE) in the stress response and found that *rpoE* and its putative regulators (*bprE-rseB-mucD*) are transcribed in a single transcriptional unit. Inactivation of the *rpoE* operon changed the *B. pseudomallei* phenotype. Changes included increased susceptibility to killing by menadione and H_2O_2, susceptibility to high osmolarity, reduced ability to form biofilms, and reduced survival in macrophage J774A.1. Therefore, we conclude that *rpoE* controls gene expression that contributes, at least in part, to *B. pseudomallei* adaptation to adverse environmental conditions.

Keywords: *Burkholderia pseudomallei*; RpoE (AlgU); Oxidative and osmotic stresses; Biofilm; Intracellular survival

1. Introduction

Burkholderia pseudomallei is a Gram-negative bacterium and is the etiological agent of melioidosis. The disease is endemic throughout south-east Asia and northern Australia [1]. The bacterium is a saprophyte found in soil and water, and transmission occurs through direct contact via cuts and abrasions or by inhalation. The incidence of clinical melioidosis is estimated to be 3.6–5.5 cases per 100,000 per annum in the north-eastern part of Thailand [2]. At present, there is no vaccine available. Although there are many studies on virulence factors [3], little is known about the genes involved in stress tolerance by this bacterial pathogen.

To persist, adapt and survive within and outside their human host, bacteria rely on regulatory mechanisms that allow them to respond rapidly to stressful situations. The responses in Gram-negative bacteria such as *Escherichia coli* can be localized in cytoplasmic and extra-cytoplasmic compartments and are controlled by distinct alternative sigma factors (RNA polymerase

* Corresponding author. Tel.: +66 2 418 0569; fax: +66 2 418 1636.
E-mail address: grsks@mahidol.ac.th (S. Korbsrisate).

0378-1097/$22.00 © 2005 Published by Elsevier B.V. on behalf of the Federation of European Microbiological Societies.
doi:10.1016/j.femsle.2005.09.002

subunits). The well-characterized cytoplasmic response is co-ordinated by σ^{32} [4]. The extracytoplasmic response, in contrast, is less well defined and is controlled by at least three partially overlapping signal transduction systems including the CpxRA and BaeSR two-component system, and the σ^E-mediated system [5]. σ^E, encoded by *rpoE*, is induced not only by heat or ethanol but also, and uniquely, by disruption of protein folding in the periplasm [6]. On activation, σ^E transcribes target genes including those encoding chaperones and proteases targeted to the cell envelope that will refold or degrade misfolded proteins [6]. In *E. coli*, σ^E activity is under the control of three genes, *rseABC* (for regulator of sigma E), which are encoded immediately downstream of the sigma factor [6,7]. This genetic organization (*rpoE rseA rseB rseC*) is conserved in the genome of other bacterial species such as *Pseudomonas aeruginosa*. In this bacterium, the organization is *algUmucABCD*. AlgU is the functional equivalent to σ^E in *E. coli* [8]. Besides contributing to the stress response, *algU* (σ^E) also controls alginate production by *P. aeruginosa*. The exopolysaccharide (EPS) alginate has traditionally been considered the major EPS of *P. aeruginosa* biofilms during cystic fibrosis pathogenesis [9]. One of the most clinically significant characteristics of biofilm communities is that they are more resistance to antibiotics and host immunity than are their free-living conditions [9].

Little is known about the adaptations of *B. pseudomallei* for survival outside and inside the human host. During infection, *B. pseudomallei* must survive major environmental changes such as the presence of reactive oxygen intermediates within phagocytic cells, changes in osmolarity and even antibiotic treatment. To characterize the cellular role of σ^E, we identified the genomic region that comprises the *rpoE* operon and investigated its importance in *B. pseudomallei* survival under stress conditions, and in biofilm formation. To accomplish this, an *rpoE* operon mutant was constructed by insertional mutagenesis in order to examine its response to osmotic stress and reactive oxygen intermediates, its ability to produce biofilms, and its survival within phagocytic cells.

2. Materials and methods

2.1. Strains, media and growth

B. pseudomallei K96243 (kindly provided by Prof. T. Dharakul) and *E. coli* S17-1*λpir* [10] were routinely maintained in Luria–Bertani (LB) agar or broth. Pseudomonas agar base supplemented with SR103 from Oxoid (UK) was used as a selective medium to inhibit growth of *E. coli* after conjugation. Antibiotics chloramphenicol (40 μg ml^{-1}), tetracycline (60 μg ml^{-1}) and ampicillin (100 μg ml^{-1}) were used as required. Where needed, M9 minimal medium (Gibco, BRL) was supplemented with 2 M NaCl. Murine macrophage (J774A.1) cell line was obtained from the American Type Culture Collection (ATCC, Manasssas, Va.). Unless indicated otherwise, cells were cultured in Dulbecco's modified Eagle's medium (Gibco Laboratory, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah) at 37 °C under a 5% CO_2 atmosphere. Biofilm, motility and cell aggregation assays were carried out as previously described [11–13].

2.2. Construction of a B. pseudomallei rpoE operon mutant

An *rpoE* operon knockout mutant was constructed as previously described [13]. Briefly, a 270-bp internal fragment of the putative *rpoE* coding sequence (CDS) was PCR amplified from *B. pseudomallei* K96243 genomic DNA using primers ALG36 (5′ CTC CAA ATA CCA CCG CAA GAT 3′) and ALG37 (5′ TAT CCC TTA GTT GGT CCG 3′) corresponding to *B. pseudomallei rpoE* nucleotide positions 78–98 and 332–349, respectively. The 270-bp PCR product was cloned into the *Eco*RV restriction site of the pKNOCK-Cm vector [14] to create pPK-1. This construct was introduced from *E. coli* S17-1*λpir* [10] into *B. pseudomallei* K96243 by conjugation. An insertion mutant was selected on Pseudomonas agar supplemented with SR103 containing chloramphenicol. Southern blot analysis and PCR assay confirmed insertion of the *rpoE* suicide plasmid at the correct location (data not shown).

2.3. RNA extraction and RT-PCR analysis

Extraction of total RNA by the modified hot acid phenol method was carried out as described previously [15]. For RT-PCR analysis, the *rpoE-bprE-rseB-mucD* operon was reverse transcribed into cDNA (Invitrogen, USA) then amplified with primers. The primers ALG58 (5′ GCG GAG ATG ATG GGT TGC C 3′) and ALG59 (5′ GCG GGG CTG GGC GGA CAA C 3′) were designed according to the *rpoE* and *rseB* genes of the *B. pseudomallei* sequences. The primers ALG70 (5′ TTG CGG CGG GCG TGG TCG T 3′) and ALG71 (5′ AGG GCT GGC AGA TCG CAC C 3′) were designed on the basis of the *rseB* and *mucD* genes of *B. pseudomallei*. PCR conditions were 35 cycles of 1-min denaturation at 95 °C, annealing at 59 °C for 50 s, and extension at 72 °C for 30 s.

2.4. Electron microscopy and stress response assays

To examine bacterial cell morphology by electron microscopy, *B. pseudomallei* was cultured on tryptic soy agar. After 48 h incubation, a small piece of agar with bacterial cells on the surface was removed, fixed

with 2% glutaraldehyde and subjected to processing for transmission and scanning electron microscopy [16].

Susceptibility of *B. pseudomallei* to oxidative stress by disc inhibition was performed as previously described [13]. 6 μl of 1 M H_2O_2 and 100 mM menadione were used and the zone of growth inhibition around the disk was measured after 24 h incubation. The measurement of sensitivity to osmotic stress was tested essentially as previously described [17].

2.5. Replication in a macrophage cell line

The ability of *B. pseudomallei* to replicate in a macrophage cell line was examined as previously described [18]. Briefly, macrophage cells were infected with wild-type and mutant *B. pseudomallei* (kanamycin-sensitive strains) at 37 °C in a 5% CO_2 atmosphere. After 2 h incubation (T2), culture medium containing kanamycin was added to completely kill residual extracellular bacteria. The infected cell monolayers were subsequently lysed at T4, T6 and T8 with 0.1% Triton X-100 (Sigma Chemical Company). The intracellular bacteria released were quantitated by dilution and plating on tryptic soy agar. The colonies were scored after 24 h incubation at 37 °C.

2.6. Statistical analysis

Data were analysed for statistical significance by the Student *t* test. Differences with *P* values of <0.05 were considered to be statistically significant.

3. Results and discussion

3.1. B. pseudomallei rpoE *gene organization*

According to the *B. pseudomallei* genome sequence [19], the *rpoE* gene was identified on chromosome 1. The predicted molecular weight of *B. pseudomallei* RpoE was approximately 33 kDa (200 amino acids), which is slightly larger than the 27.5 kDa (194 amino acids) of *P. aeruginosa* RpoE (AlgU). Fig. 1 shows the *rpoE* gene cluster in *B. pseudomallei* and its homology with the *rpoE* operons of *P. aeruginosa* and *E. coli*. The first downstream CDS, *bprE* (BPSL2435), is proposed to be a putative sigma E factor negative regulator (anti-sigma factor) of *B. pseudomallei*. The second and third downstream CDSs were proposed to be *rseB* (BPSL2434) and *mucD* (BPSL2433), respectively. We could not find any CDSs downstream from *rpoE* or other regions on the *B. pseudomallei* genome that had putative amino acid sequence homology to *E. coli* RseC and *P. aeruginosa* MucB and MucC. Based on three known types of clusters encoding RpoE (AlgU): five-gene, four-gene and three-gene clusters [20], the predicted *rpoE* gene cluster in *B. pseudomallei* correlates with the four-gene cluster type. The absence of *mucC* equivalents in some bacterial *rpoE* gene clusters has been reported [20] and this suggests that *mucC* and its homologs may have an optional or independent regulatory function.

In *E. coli*, the *rpoE* gene is located in an operon that includes three downstream genes, *rseA*, *rseB* and *rseC*. The molecular role of the *E. coli rpoE* operon has been described as the control of gene expression needed to assist cells in overcoming accumulation of unfolded and misfolded outer membrane proteins (OMP) in the periplasmic space [6,7,21]. Such OMP intermediates could be generated upon exposure to conditions such as heat, oxidative or osmotic stress. The misfolded or unfolded OMP are recognized by the protease DegS that degrades RseA, the anti-sigma factor, thus releasing RpoE to interact with the RNA polymerase core enzyme. The RNA polymerase holoenzyme directs expression of various genes of the σ^E regulon, including periplasmic proteases and genes

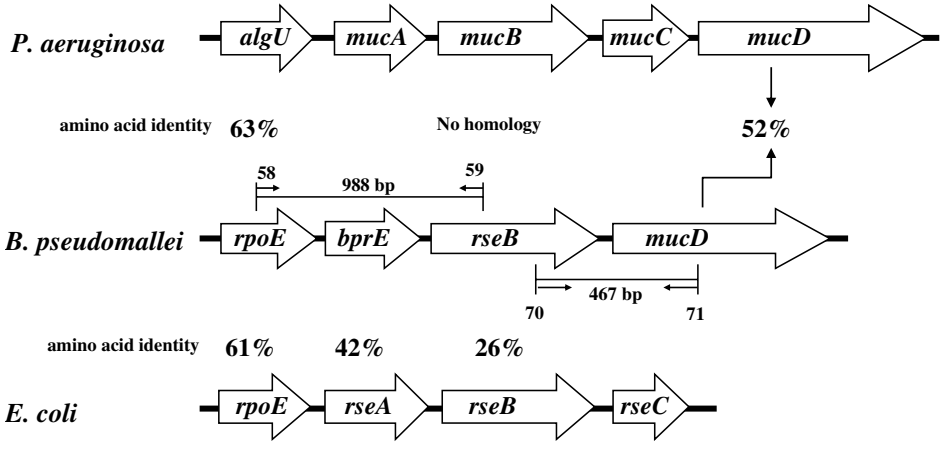

Fig. 1. Physical map of the *B. pseudomallei rpoE* operon (*rpoE-bprE-rseB-mucD*) and its relationship to the *rpoE* operons of *P. aeruginosa* and *E. coli*. Numbers between operons indicate percentages of amino acid sequence identity. Location of primers ALG 58/59 and ALG 70/71 for RT-PCR analysis of *B. pseudomallei rpoE* (*algU*) operon are indicated.

involved in lipid and lipopolysaccharide metabolism and in cell wall biogenesis [7,22]. In *P. aeruginosa*, the σ^E homolog AlgU is negatively regulated by MucA (RseA homolog) and MucB (RseB homolog) by a mechanism similar to that for *E. coli* σ^E [8,20]. Since homologues of *E. coli rseA* and *rseB* are present in *B. pseudomallei* and are organized in the same manner as in *E. coli* and *P. aeruginosa*, it is highly likely that regulation of *B. pseudomallei* RpoE employs a similar mechanism to that in *E. coli* and *P. aeruginosa*.

3.2. rpoE *transcription and mutagenesis of* rpoE *operon*

Reverse transcription-PCR (RT-PCR) with primers located within the *rpoE* gene cluster (Fig. 1) revealed that *B. pseudomallei rpoE-bprE-rseB-mucD* was transcribed in a single transcriptional unit (data not shown). This is consistent with the *P. aeruginosa algU*(*rpoE*) in that *algU*(*rpoE*), *mucA* and *mucB* genes are co-transcribed [20]. With this result, we reasoned that inactivation of *rpoE* gene in the *rpoE* knockout mutant, AL30, would have a polar effect on the expression of downstream genes (*bprE-rseB-mucD*). In *E. coli, rpoE* is essential for cell growth and its inactivation leads to cell death unless a suppressor mutation arises [23]. However, in *B. pseudomallei*, *rpoE* mutants are viable. Although the possibility of a suppressor mutation in the *rpoE* operon mutant in *B. pseudomallei* cannot be ruled out, *Salmonella enterica* serovar Typhimurium *rpoE* [24] and *P. aeruginosa algU* (an *rpoE* ortholog) mutant [25] strains have been constructed and no suppressor mutations have been reported for them. In addition, an amino acid sequence homology search could not find any *rpoE* homologues in the *B. pseudomallei* genome that might compensate for the mutation of *rpoE*.

3.3. *Morphological characteristics of the* rpoE *operon mutant*

The *B. pseudomallei* AL30 *rpoE* operon mutant had a distinct colony morphology. Wild-type K96243 colonies were uniform in size, with rounded colonies having a single defined edge and a raised center. Mutant colonies had ill-defined, double layered edges with the outer layer less dense than the inner layer (data not shown). This characteristic enabled primary differentiation of AL30 from parental K96243 and clearly showed that inactivation of *rpoE* and its regulators affected bacterial phenotype. By transmission electron microscopy, it was found that the majority of AL30 cells contained numerous vacuoles whilst the parental strain K96243 contained only a few (Fig. 2A). Scanning electron microscopy showed that AL30 cells were connected to each other in a chain-like manner while those of K96243 were aggregated in clusters (Fig. 2B). In spite of differences in colony and cell morphology,

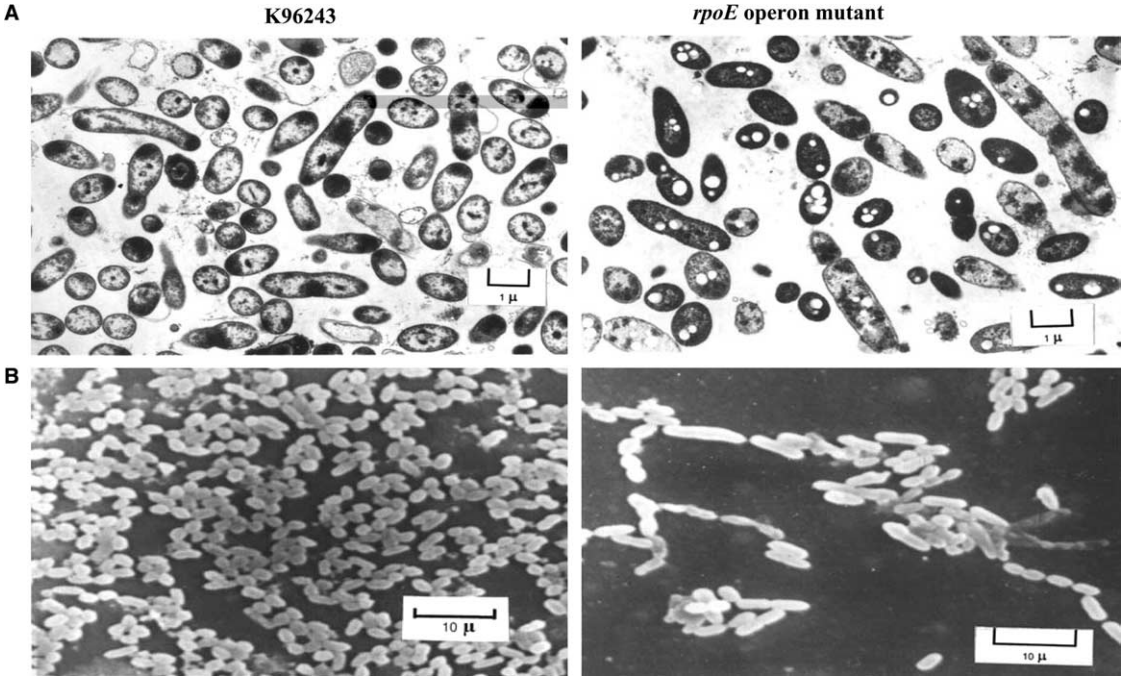

Fig. 2. Electron micrographs of *B. pseudomallei* K96243 and mutant AL30 after incubation at 37 °C for 48 h on tryptic soy agar. (A) By transmission electron microscopy, the majority of the mutant cells contained numerous vacuoles while the parental strain K96243 contained few. (B) By scanning electron microscopy, AL30 cells are connected in a chain-like manner while those of K96243 were aggregated in clusters.

we were unable to demonstrate any alteration in cell autoaggregation between the *rpoE* knockout mutant (AL30) and the wild-type strain K96243 (data not shown).

3.4. Sensitivity of rpoE knockout mutant to osmotic and oxidative stress

Since AlgU (RpoE) from *P. aeruginosa* participates in adaptation to adverse environmental conditions, we reasoned that the *rpoE* operon from*B. pseudomallei* might do the same. Thus, we compared growth characteristics of AL30 and K96243 under oxidative stress. AL30 was significantly more sensitive than K96243 to reactive oxygen intermediates on exposure to 100 mM menadione (a superoxide generator) [$P = 0.00005$] and 1 M H_2O_2 [$P = 0.004$] (Fig. 3A) and to high osmolarity using medium containing 2 M NaCl [P values after 2 and 6 h incubation were 0.013 and 0.0004, respectively] (Fig. 3B). This is correlated with the role of RpoE for tolerance towards reactive oxygen intermediates in *P. aeruginosa* [8]. The results suggest that the *rpoE* sigma factor is important in the adaptation of *B. pseudomallei* to high osmolarity and reactive oxygen intermediates. Since *B. pseudomallei*, a saprophyte found in soil and water, may be exposed to dry and harsh osmolarity conditions in soil and water, the capacity to adapt to these conditions is likely to be important for their survival. In comparison with other *B. pseudomallei* sigma factors, the role of sigma factor *rpoE* in oxidative stress is similar to *B. pseudomallei* sigma factor *rpoS* reported by Subsin et al. [26]. However, the *B. pseudomallei rpoS* knockout mutant exhibited no significant difference with the wild-type in sensitivity to osmotic stress [26].

3.5. Reduced biofilm formation in the rpoE operon knockout mutant

B. pseudomallei is an infectious pathogen able to produce biofilms that may contribute to antibiotic resistance [27] and resistance to the host immune system. They may also improve survival during latency. *P. aeruginosa algU* (*rpoE*) is known to tightly control exopolysaccharide biosynthesis that eventually affects biofilm formation. Our results revealed that the ability of *B. pseudomallei* AL30 to form biofilms was reduced by approximately 50% in comparison to K96243 (Fig. 3C). However, since AL30 retained some ability to form biofilms, there must be other factors in addition to RpoE involved in their formation.

The formation of biofilm is a multistep process that requires the participation of structural appendages such as flagella and type IV pilli [12]. Reduction in biofilm formation in *B. pseudomallei* AL30 could be due to altered activities of flagella. To test this, we compared the motility of the mutant and wild-type strains and no significant difference was observed (data not shown). The ability of bacteria to form biofilms has been associated with their capacity to cause disease in the human host [28]. By this reasoning, *B. pseudomallei* AL30 should be less virulent than the wild-type *B. pseudomallei*.

Although the AL30 mutant had a reduced ability to form biofilms like *algU* mutants in *P. aeruginosa*, no ORFs homologous for enzymes in the alginate biosynthesis pathway (i.e., *algA*, *algF*, etc.) were found in *B. pseudomallei* genomic sequences (data not shown). Thus, it is possible that *algU* (*rpoE*) in *B. pseudomallei* regulates expression of a different type of exopolysaccharide or some other factor that enhances biofilm formation.

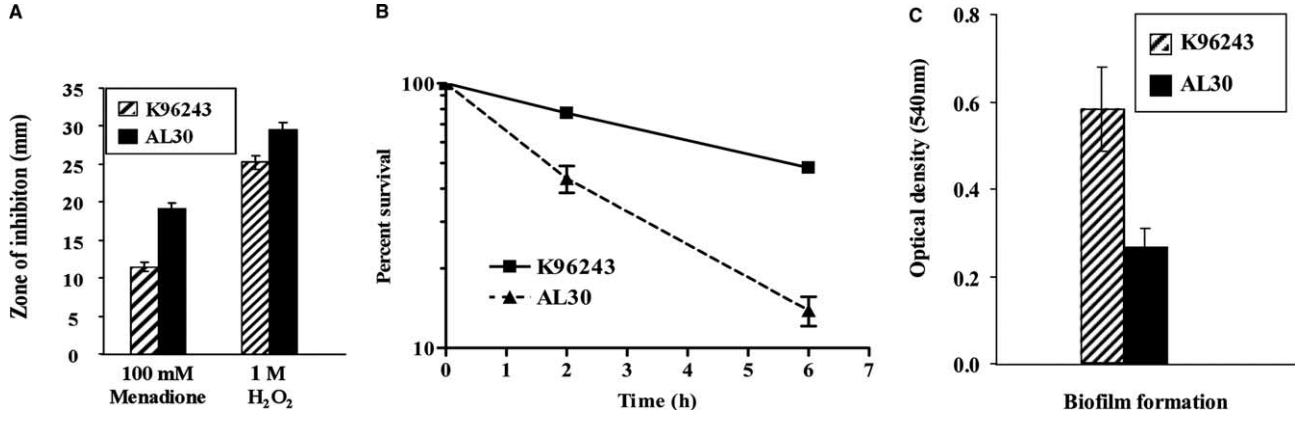

Fig. 3. Comparison of *B. pseudomallei* K96243 and its *rpoE* operon mutant (AL30) for sensitivity to stress and for biofilm formation. (A) Sensitivity to killing by H_2O_2 and menadione expressed as diameter of growth inhibition zones in mm. (B) Survival after exposure to 2 M NaCl where cell viability was determined at 2 and 6 h and where 100% viability corresponds to the CFU count immediately following resuspension in 2 M NaCl. Data shown are averages from three experiments. (C) Biofilm formation by *B. pseudomallei* K96243 and its AL30 mutant. The data are based on at least three experiments and were analysed for statistical significance using the unpaired *t* test.

Immunofluorescence staining with *B. pseudomallei* anti-exopolysaccharide (kindly provided by T. Atkins, UK) showed no significant difference between K96243 and AL30 (data not shown). Since mutagenesis of *P. aeruginosa algU (rpoE)* has been reported to affect lipopolysaccharide (LPS) biosynthesis [9,22] then LPS from AL30 and K96243 was extracted and compared but no striking differences were found (data not shown). However, our analyses cannot exclude more subtle changes or a possible expression of other factors. A comparative, two-dimensional gel electrophoresis or microarray study of AL30 and K96243 may help to identify AlgU regulated factors that play roles in biofilm formation.

3.6. *Effect of* algU *mutation on intracellular survival*

B. pseudomallei is a facultative intracellular bacterium. Once inside phagocytic cells, it has to withstand several toxic substances including reactive oxygen intermediates. Based on the finding that RpoE is involved in tolerance towards menadione and H_2O_2, known to generate reactive oxygen intermediates, it is suggested that RpoE could contribute, at least in part, to the survival of intracellular *B. pseudomallei* in oxidative stress environments such as within phagocytic cells. To investigate this hypothesis, we compared the ability of *B. pseudomallei* AL30 and K96243 to survive within macrophage J774A.1. Fig. 4 shows that the percentages of intracellular bacterial survival for AL30 at T6 and T8 were significantly lower than for parental K96243. The significantly reduced survival of AL30 cells in macrophages supports the results from the acellular assays indicating their decreased ability to withstand stress (i.e., oxidative stress) when compared to the parental cells.

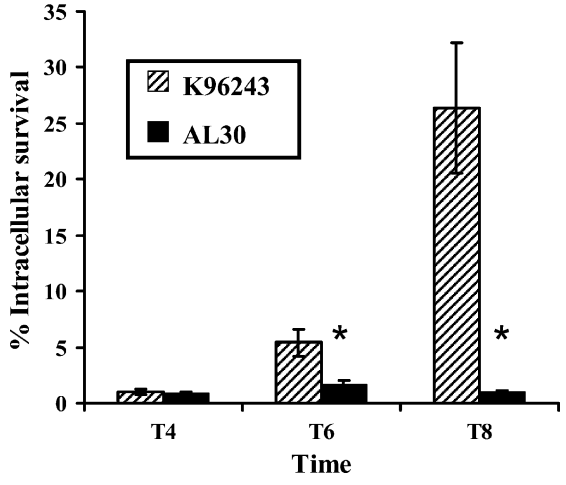

Fig. 4. Intracellular survival of *B. pseudomallei* K96243 and its *rpoE* operon mutant (AL30) in macrophage J774A.1. Intracellular survival (%) = (number of intracellular bacteria after infection/number of bacteria added per well) × 100. This graph shows the results from one of three experiments, all of which gave similar results. Data are expressed as means of intracellular survival (%) ± standard deviations of the means. Asterisks indicate significant differences between wild-type and AL30 at T6 ($P = 0.019$) and T8 ($P = 0.004$).

Many attempts have been made to complement the *B. pseudomallei rpoE* operon mutant with either the *rpoE* gene or *rpoE* operon. Unfortunately, no transformants have ever been isolated. A possible explanation is that high expression of *rpoE* may cause lethal effects on bacterial growth [29]. In addition, comparison of Southern blot and PCR analyses between the *B. pseudomallei* wild-type and *rpoE* operon mutant indicated the insertional inactivation of the *rpoE* gene.

In conclusion, little is known about the regulatory mechanisms that determine adaptation of *B. pseudomallei* to environmental stress. *rpoE* and its potential negative regulators *bprE-rseB-mucD* have been identified from the *B. pseudomallei* genome. Insertional inactivation of the *rpoE* operon affected morphology and a variety of responses including those related to osmotic stress, oxidative stress, biofilm formation, and survival within a macrophage cell line. Thus, we conclude that the *rpoE* operon contributes, at least in part, to the survival of *B. pseudomallei* in stressful environments.

Acknowledgements

We thank N. Anuntakool for performing LPS detection and S. Damnin for skillful technical assistance. R. Sallabhan and S. Rattanaprapanporn for RT-PCR analysis. We also acknowledge Prof. B. Wren, Dr. S. Loprasert and Dr. P. Utaisincharoen for their helpful suggestions. Prof. T.W. Flegel for a critical review of the manuscript. This research was supported by Grants RSA4580034, RDG4530209 and RTA4580010 from the Thailand Research Fund (TRF), and the Siriraj Grant for Research Development and Medical Education. M. Vanaporn is supported by the TRF Grant PHD/0044/2545.

References

[1] Dance, D.A.B. (2002) Melioidosis. Curr. Opin. Infect. Dis. 15, 127–132.
[2] Suputtamongkol, Y., Hall, A.J., Dance, D.A., Chaowagul, W., Rajchanuvong, A., Smith, M.D. and White, N.J. (1994) The epidemiology of melioidosis in Ubon Ratchatani, northeast Thailand. Int. J. Epidemiol. 23, 1082–1090.
[3] Cheng, A.C. and Currie, B.J. (2005) Melioidosis: epidemiology, pathophysiology, and management. Clin. Microbiol. Rev. 18, 383–416.
[4] Yura, T., Nagai, H. and Mori, H. (1993) Regulation of the heat shock response in bacteria. Annu. Rev. Microbiol. 47, 321–350.
[5] Raivio, T.L. and Silhavy, T.J. (2001) Periplasmic stress and ECF sigma factors. Annu. Rev. Microbiol. 55, 591–624.
[6] Ades, S.E. (2004) Control of the alternative sigma factor σ^E in *Escherichia coli*. Curr. Opin. Microbiol. 7, 157–162.

[7] De Las Penas, A., Connolly, L. and Gross, C.A. (1997) The σ^E-mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of σ^E. Mol. Microbiol. 24, 373–385.

[8] Yu, H., Schurr, M.J. and Deretic, V. (1995) Functional equivalence of *Escherichia coli* σ^E and *Pseudomonas aeruginosa* AlgU: *E. coli rpoE* restores mucoidy and reduces sensitivity to reactive oxygen intermediates in *algU* mutants of *P. aeruginosa*. J. Bacteriol. 177, 3259–3268.

[9] Govan, J.R.W. and Deretic, V. (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. 60, 539–574.

[10] 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.

[11] Stepanovic', S., Vukovic', D., Dakic', I., Savic', B. and Svabic-Vlahovic', M. (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. Microbiol. Methods 40, 175–179.

[12] O'Toole, G.A. and Kolter, R. (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. 30, 295–304.

[13] Loprasert, S., Sallabhan, R., Whangsuk, W. and Mongkolsuk, S. (2002) The *Burkholderia pseudomallei oxyR* gene: expression analysis and mutant characterization. Gene 296, 161–169.

[14] 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.

[15] Ambulos, N.P., Duvall, E.J. and Lovett, P.S. (1987) Method for blot-hybridization analysis of mRNA molecules from *Bacillus subtilis*. Gene 51, 281–286.

[16] Beveridge, T.J., Popkin, T.I. and Cole, R.M. (1994) Electron microscopy In: Methods for General and Molecular Bacteriology (Gerhardt, P., Ed.), pp. 42–71. American Society for Microbiology, Washington DC.

[17] Suh, S.-J., Silo-Suh, L., Woods, D.E., Hassett, D.J., West, S.E.H. and Ohman, D.E. (1999) Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. J. Bacteriol. 181, 3890–3897.

[18] Kespichayawattana, W., Rattanachetkul, S., Wanun, T., Utaisincharoen, P. and Sirisinha, S. (2000) *Burkholeria pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. Infect. Immun. 68, 5377–5384.

[19] 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., Sebaihia, M., Thomson, N.R., Bason, N., Beacham, I.R., Brooks, K., Brown, K.A., Brown, N.F., Challis, G.L., Cherevach, I., Chillingworth, T., Cronin, A., Crossett, Z., Davis, P., DeShazer, D., Feltwell, T., Fraser, A., Hance, Z., Hauser, H., Holroyd, S., Jagels, K., Keith, K.E., Maddison, M., Moule, S., Price, C., Quail, M.A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Simmonds, M., Songsivilai, S., Stevens, K., Tumapa, S., Vesaratchavest, M., Whitehead, S., Yeats, C., Barrell, B.G., Oyston, P.C. and Parkhill, J. (2004) Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. Proc. Natl. Acad. Sci. USA 101, 14240–14245.

[20] Schurr, M.J., Yu, H., Martinez-Salazar, J.M., Boucher, J.C. and Deretic, V. (1996) Control of AlgU, a member of the σ^E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. J. Bacteriol. 178, 4997–5004.

[21] Walsh, N.P., Alba, B.M., Bose, B., Gross, C.A. and Sauer, R.T. (2003) OMP peptides signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. Cell 113, 61–71.

[22] Dartigalongue, C., Missiakas, D. and Raina, S. (2001) Characterization of the *Escherichia coli* σ^E regulon. J. Biol. Chem. 276, 20866–20875.

[23] De Las Penas, A., Connolly, L. and Gross, C.A. (1997) σ^E is an essential sigma factor in *Escherichia coli*. J. Bacteriol. 179, 6862–6864.

[24] Humphreys, S., Stevenson, A., Bacon, A., Weinhardt, A.B. and Roberts, M. (1999) The alternative sigma factor, σ^E, is critically important for the virulence of *Salmonella typhimurium*. Infect. Immun. 67, 1560–1568.

[25] Martin, D.W., Holloway, B.W. and Deretic, V. (1993) Characterization of a locus determining the mucoid status of *Pseudomonas aeruginosa*: AlgU shows sequence similarities with a *Bacillus* sigma factor. J. Bacteriol. 175, 1153–1164.

[26] Subsin, S., Thomas, M.S., Katzenmeier, G., Shaw, J.G., Tungpradubkul, 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.

[27] Vorachit, M., Lam, K., Jayanetra, P. and Costerton, J.W. (1993) Resistance of *Pseudomonas pseudomallei* growing as a biofilm on sialic discs to ceftazidime and co-trimoxazole. Antimicrob. Agents Chemother. 37, 2000–2002.

[28] Costerton, J.W., Stewart, P.S. and Greenberg, E.P. (1999) Bacterial biofilm: a common cause of persistent infections. Science 284, 1318–1322.

[29] Schnider-Keel, U., Lejbolle, K.B., Baehler, E., Haas, D. and Keel, C. (2001) The sigma factor AlgU (AlgT) controls exopolysaccharide production and tolerance towards desiccation and osmotic stress in the biocontrol agent *Pseudomonas fluorescens* CHA0. Appl. Environ. Microbiol. 67, 5683–5693.