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# Effect of Acidic pH on the Invasion Efficiency and the Type III Secretion System of *Burkholderia thailandensis*

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***Burkholderia thailandensis* is a close relative of *Burkholderia pseudomallei*. These organisms are very similar, but *B. thailandensis* is far less virulent than *B. pseudomallei*. Nucleotide sequencing and analysis of 14 *B. thailandensis* isolates revealed variation in the regions coding for the type III secreted BipD protein. The degree of *B. thailandensis* BipD sequence variation was greater than that found in *B. pseudomallei*. Western blot analysis indicated that, unlike *B. pseudomallei*, *B. thailandensis* type III secreted proteins including BipD and BopE could not be detected in the supernatant of culture medium unless induced by acidic conditions. In addition, culturing *B. thailandensis* under acidic growth conditions (pH 4.5) can induce the ability of this bacterium to invade human respiratory epithelial cells A549. The identification of an environmental stimulus that increases the invasion capability of *B. thailandensis* invasion is of value for those who would like to use this bacterium as a model to study *B. pseudomallei* virulence.**

***Keywords*:** *B. thailandensis*, acidic pH, type III secretion system, invasion

*Burkholderia thailandensis* is a Gram-negative bacterium closely related to *Burkholderia pseudomallei*. It was first isolated in Thailand and was mistakenly identified as *B. pseudomallei* because of its similar biochemical and morphological characteristics (Wuthiekanun *et al*., 1996). It has been suggested that *B. thailandensis* diverged from *B. pseudomallei* approximately 47 million years ago (Yu *et al*., 2006). The most remarkable differences between the two organisms are their virulence capacity in humans and animals and the ability to assimilate arabinose (Smith *et al*., 1997). Only two human infections with *B. thailandensis* have been recorded in Southeast Asia (Dharakul *et al*., 1999; Lertpatanasuwan *et al*., 1999). The $LD_{50}$ of *B. thailandensis* in the hamster infection model is $10^5$-fold higher than *B. pseudomallei* (Brett *et al*., 1997). *B. pseudomallei* contains a large gene cluster involved in the synthesis and export of capsular polysaccharides and this cluster is absent in *B. thailandensis* (DeShazer *et al*., 2001). In addition, *B. thailandensis* is less efficient than *B. pseudomallei* in adhering to and invading host cells (Kespichayawattana *et al*., 2004). There have been many attempts to identify the factors that are associated with virulence in *B. pseudomallei* and mechanisms of pathogenesis by comparative analysis between *B. pseudomallei* and *B. thailandensis* (Yu *et al.,* 2006; Haraga *et al*., 2008; Charoensap *et al*., 2009).

The type III secretion system (T3SS) is a toxic delivery mechanism that allows bacteria to inject substances into the host cell cytoplasm to subvert the normal cellular functions of host cells in order to benefit the invading bacterium. There are at least three loci encoding putative T3SS on the genome of *B. pseudomallei*, one of which has been designated the T3SS *Burkholderia* secretion apparatus (Bsa). The *B. pseudomallei* BipD protein was reported as a component of the Bsa T3SS, and showed similarity in sequence and is functionally analogous to IpaD and SipD of *Shigella* and *Salmonella*, respectively (Kaniga *et al*., 1995; Picking *et al*., 2005). Serum from patients infected with *B. pseudomallei* was found to contain antibodies to BipD (Stevens *et al*., 2002). *B. pseudomallei bipD* mutants show reduced virulence but are still able to cause disease (Stevens *et al*., 2004). Functional characterization has revealed that the *B. pseudomallei* BipD protein is involved in the invasion and intracellular spread of *B. pseudomallei* (Stevens *et al*., 2002, 2004). The crystal structure of the *B. pseudomallei* BipDprotein has been proposed (Erskine *et al*., 2006).

There are many studies of the *B. pseudomallei* Bsa T3SS, but far fewer of the *B. thailandensis* T3SS. Rainbow *et al*. (2002) reported that *B. thailandensis* also possesses Bsa T3SS, homologue and this was subsequently confirmed by genome sequencing. Comparative genomic analysis of *B. pseudomallei* K96243 and *B. thailandensis* E264 revealed that virulence-related genes, in particular those within the T3SS, exhibited the highest level of divergence between the two species (Yu *et al*., 2006). It was suggested that subtle functional alterations in the T3SS could have contributed to the ability of *B. pseudomallei* to infect the human host successfully. Little is known about divergence of BipD sequences among *B. thailandensis* isolates and the effect of environmental stimuli on BipD secretion and *B. thailandensis* virulence.

In this study, *B. thailandensis* BipD sequences were analyzed

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and compared with homologues from *B. pseudomallei* to gain insight into the extent of BipD sequences variation. In addition, the effect of acidic conditions on the secretion of *B. thailandensis* Bsa type III secreted proteins including BipD and BopE, together with the effect on the ability of this bacterium to invade respiratory epithelial cells were investigated.

## Materials and Methods

### Bacterial strains, cell line, and growth conditions

Eleven *B. thailandensis* isolates were collected from the environment in Southern and Northeastern Thailand. They were identified by conventional biochemical methods and confirmed by API 20NE. Both *B. pseudomallei* K96243 and *B. thailandensis* isolates were grown in Luria-Bertani (LB) medium (Miller, USA) at 37°C. The human respiratory epithelial cell line (A549) was obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA) at 37°C under a 5% $CO_2$ atmosphere.

### *B. thailandensis bipD* DNA amplification and sequencing

Genomic DNA was extracted from *B. thailandensis* with phenol-chloroform-isoamyl alcohol and precipitated with ethanol (Ausubel *et al*., 1995). PCR primers BD-F1 (5′-TGTAAAACGACGGCCAGTC CTTGGCGAA-3′) and BD-R4 (5′-CAGGAAACAGCTATGACCTT GCTTCGGA-3′) were used to amplify *B. thailandensis bipD* DNA with the following cycling conditions: 94°C (for 1 min), 60°C (for 1 min), and 72°C (for 1 min) for 30 cycles. The amplified DNA fragment from each isolate was sequenced in both directions at the Bioservice Unit (BSU) of the National Science and Technology Development Agency (NSTDA), Thailand, using an ABI PRISM 3100 automated DNA sequencer.

### Precipitation of bacterial culture supernatant and Western blot analysis

*B. thailandensis* and *B. pseudomallei* isolates were subculture in LB broth adjusted to pH 7.0 and pH 4.5 at 37°C until mid-logarithmic phase (approximately 6 h). Bacterial cells and culture supernatants were separated by centrifugation at 9,600×g for 15 min. Bacterial cells were lysed by B-PER®II (Pierce, USA). Culture supernatants were harvested and filtered (0.22 μm filter Millipore, USA) before precipitating with absolute ethanol to 50% final concentration. Proteins in samples were resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were subjected to Western blot analysis using 1:500 rabbit anti-BipD or 1:200 anti-BopE antibodies (kindly provided by Professor M.P. Stevens) and 1:1,000 diluted mouse anti-rabbit IgG antibody conjugated with horseradish peroxidase (DAKO, USA). The immunoreactive protein band was then detected using 3,3-diaminobenzidine (DAB) (Sigma-Aldrich, USA).

### Invasion assay

An invasion assay in the human respiratory epithelial cell line A549 was performed as described previously (Muangsombut *et al*., 2008) with some modifications. Briefly, *B. pseudomallei* or *B. thailandensis* strains were cultured in LB broth (pH 7.0 and pH 4.5). After overnight cultivation, the bacterial cells were washed with 1× phosphate-buffered saline (PBS) before adding to A549 cell line at a multiplicity of infection (MOI) of 50:1 for 3 h to bring bacteria in contact with the cells and allow bacterial entry. The monolayers were overlaid with a medium containing 250 μg/ml of kanamycin to kill extracellular bacteria for 1 h. The viable intracellular bacteria were released from the infected cells at 4 h post-infection (p.i.) by lysis with 0.1% Triton X-100 (Sigma-Aldrich) and plated on trypticase soy agar (TSA). Colony forming units (CFU) were measured after 36-48 h of incubation at 37°C. The invasion efficiency is calculated as: % invasion efficiency = [number (CFU) of intracellular bacteria at 4 h p.i./ CFU added] × 100.

### BipD sequences analysis

The BipD amino acid sequences from *B. thailandensis* strains were compared with those deposited in databases using BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments were performed by Jalview (Waterhouse *et al*., 2009).

### Statistical analysis

Average and standard errors of the mean (SEM) were calculated from at least three independent determinations. All tests for significance were performed using the Student's *t*-test. A *P*-value $<0.05$ was considered statistically significant.

## Results

### Variation of *B. thailandensis* BipD sequence

The *B. thailandensis* BipD protein is composed of 310 amino acids. To gain insight into the extent of variation of BipD sequences, *bipD* genes from 11 *B. thailandensis* isolates were sequenced and analyzed together with three *B. thailandensis bipD* sequences (strains E264, Bt4, and MSMB43) already available in the GenBank database (www.ncbi.nlm.nih.gov/Genbank). The 11 *B. thailandensis* isolates tested in our laboratory had been collected in Thailand (Table 1). The *B. thailandensis* E264 genome sequence strain was also isolated in Thailand; strains Bt4 and MSMB43 were isolated from Australia. The results revealed that the amino acid identity of BipD sequences among 14 *B. thailandensis* strains ranged from 89 to 99%. Of 14 strains, 11 showed 99% with-in group amino acid identity, this variant therefore can be classified as the major type of *B. thailandensis* BipD protein; this group has 93% amino acid sequence identity to *B. pseudomallei* K96243

**Table 1.** *B. thailandensis* isolated from this study

| Strain | Accession number | Source | Year | Area of isolation in Thailand |
|---|---|---|---|---|
| DV1 | GQ149082 | Soil | 1998 | Northeastern |
| D1 | HM439346 | Soil | 1998 | Southern |
| D3 | HM439347 | Soil | 1997 | Northeastern |
| D4 | HM439348 | Soil | 1997 | Southern |
| D6 | HM439349 | Soil | 1997 | Northeastern |
| D17 | HM439342 | Soil | 1997 | Northeastern |
| D31 | HM439343 | Soil | 1998 | Southern |
| D354 | HM439350 | Soil | 1998 | Northeastern |
| D335 | HM439344 | Soil | 1998 | Northeastern |
| D413 | HM439345 | Soil | 1998 | Northeastern |
| D488 | HM439341 | Soil | 1998 | Northeastern |

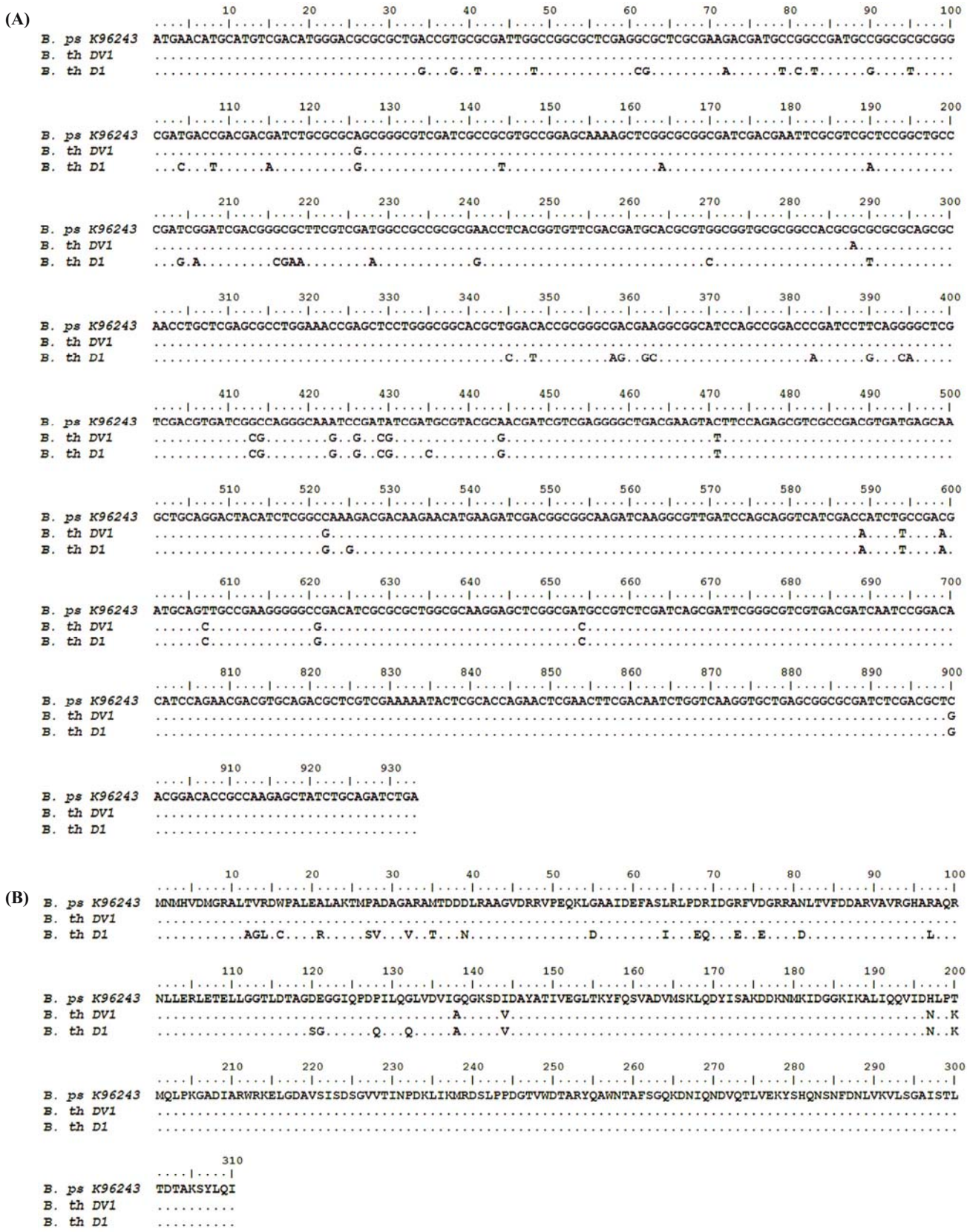


**Fig. 1.** Alignment of nucleotide (A) and amino acid (B) sequences of BipD from *B. thailandensis* (*B. th*) strains DV1, D1 and *B. pseudomallei* (*B. ps*) K96243. Identical sequences are indicated with asterisks. Alignment was derived by the CLUSTAL W alignment program (http://www.ch.embnet.org/software/ClustalW.html).

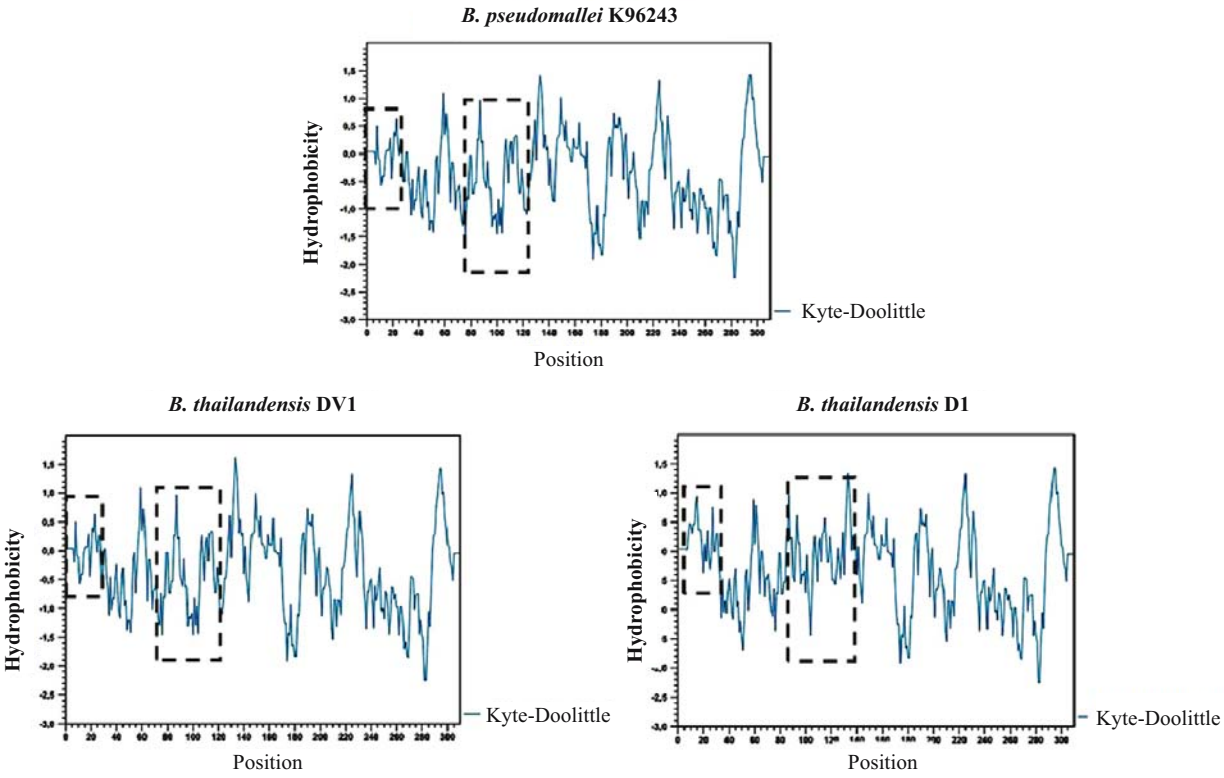


**Fig. 2.** Hydrophobicity profiles of *B. thailandensis* strains DV1, D1 and *B. pseudomallei* strain K96243. The hydrophobicity profile of *B. thailandensis* DV1 is similar to *B. pseudomallei* strain K96243 but different from *B. thailandensis* D1. Dash-Boxes indicate the amino acid positions where differences in predicted hydrophobicity profiles occurred.

BipD. A representative isolate of this group was *B. thailandensis* strain D1 (accession no. HM439346). Nucleotide and amino acid sequence comparison between *bipD* gene and BipD protein from *B. pseudomallei* K96243 and *B. thailandensis* D1 is shown in Figs. 1A and B.

Interestingly, a *B. thailandensis* strain DV1 (accession no. GQ149082) isolated during this study had 98% BipD sequence identity to that from *B. pseudomallei* K96243 (accession no. YP111535) but 93% identity to strain D1 which was chosen to be the representative of the major of *B. thailandensis* BipD proteins (Fig. 1B). The BipD sequence of the strains DV1 is closer to *B. pseudomallei* than other *B. thailandensis*: however taxonomically DV1 is still *B. thaliandensis*. This species identification was confirmed by biochemical test (kindly performed by V. Wuthiekanun) and our established PCR method based on the amplification of a central acidic (CA) domain of *B. thailandensis* BimA protein (unpublished data). When the hydrophobicity profile of BipD protein sequences were analyzed, it was found that the profile of *B. thailandensis* strain DV1 was different from *B. thailandensis* strain D1 but similar to that from *B. pseudomallei* K96243 (Fig. 2).

Notably, an Australian *B. thailandenis* strain MSMB43 (accession no. ZP02466843) had 89% identity to the major type of *B. thailandensis* BipD protein. We noticed that the low percentage of identity in strain MSMB43 was due to the deletion of 84 amino acids long at the N-terminus of the BipD protein (data not shown). Furthermore, analysis of 10 *B. pseudomallei* genome sequences available including strains 576, 668, BCC215, DM98, Pakistan9, 1106a, 1106b, 1710a, 1710b, and K96243 indicated that *B. pseudomallei* BipD amino acid sequences showed less variation than *B. thailandenis* (99-100% identity).

## Acidic pH increases *B. thailandensis* BipD and BopE proteins secretion

Since there is a high degree of homology in BipD sequences between *B. thailandensis* DV1 and *B. pseudomallei* K96243, we decided to investigate the secretion of BipD proteins from these two strains together with *B. thailandensis* D1 and E264. We found that in standard LB medium (pH 7.0), BipD proteins in cell lysates of *B. thailandensis* strains DV1, D1, and E264 are detected at almost the same level as is seen in *B. pseudomallei* (Fig. 3A). However, BipD protein could not be detected from the culture supernatant of any of the *B. thailandensis* strains tested (Fig. 3B) indicating an apparent inability or low efficiency of *B. thailandensis* secretion of BipD protein.

Previous studies reported that the T3SS activity in Gram-negative bacteria was increased under environmental stimuli (Beuzón *et al*., 1999; Mildiner-Earley *et al*., 2007). To clarify whether the inability to detect BipD from culture supernatant was due to the low secretion efficiency, we cultured *B. thailandensis* in LB medium at pH 4.5 for 6 h before detecting protein expression and secretion compared with LB medium (pH 7.0). *B. thailandensis* BipD protein expressed in whole cell lysates under acidic culture condition was significantly

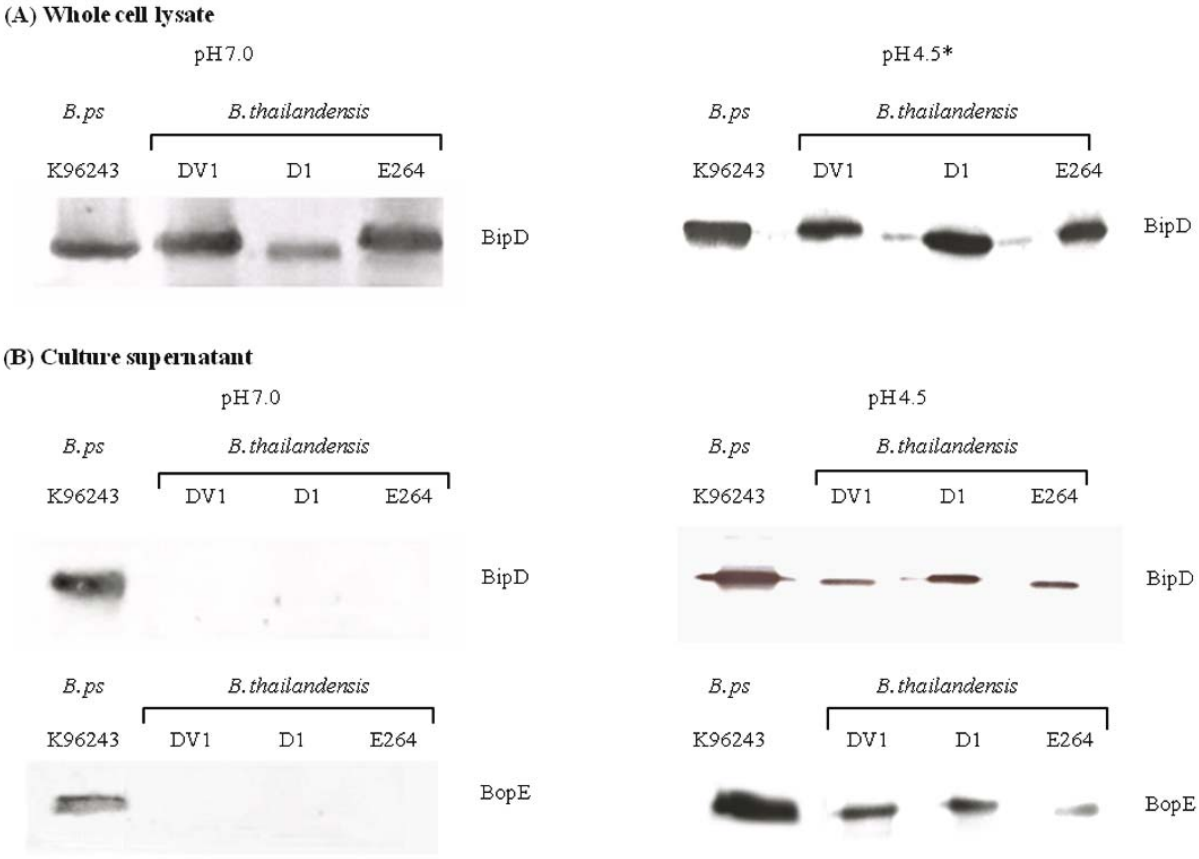


**Fig. 3.** Effect of acidic culture condition on *B. thailandensis* BopE and BipD protein secretions. Whole cell lysates (A) and culture supernatants (B) of *B. thailandensis* and *B. pseudomallei* (*B. ps*) K96243 cultured in standard LB medium pH 7.0 or 4.5 were precipitated and separated by 12% SDS-PAGE. The blotted proteins were detected by anti-BipD and anti-BopE antibodies. * The whole cell lysates from *B. thailandensis* and *B. pseudomallei* cultured in LB medium pH 4.5 were diluted 1:3 before loading.

increased. As shown in Fig. 3A, whole cell lysates from *B. thailandensis* cultured in LB medium (pH. 4.5) had to be diluted 1:3 before loading indicating that acidic condition could induce bacterial BipD protein production. Moreover, culture in LB medium at pH 4.5 could induce BipD secretion in all *B. thailandensis* strains including DV1, D1, and E264 (Fig. 3B). Therefore, the inability to detect BipD from culture supernatant may result from low secretion efficiency at pH 7.0. It is not due to a defect or inability of *B. thailandensis* T3SS to secrete BipD protein. *B. thailandensis* strain E264 was included in this assay because it has been proposed as a model for the study of virulence associated T3SS of *B. pseudomallei* (Haraga *et al*., 2008). This strain also showed increased BipD protein secretion after acid treatment.

BopE protein is the best characterized type III secreted protein from the *B. pseudomallei* Bsa T3SS. Therefore, in addition to BipD, we also determined BopE protein secretion from culture supernatants of *B. thailandensis* strains D1, DV1, and E264. The Western blot analysis of BopE secretion correlated with that seen for BipD protein (Fig. 3B), suggesting that secretion of *B. thailandensis* T3SS proteins (at least BopE and BipD) required induction. In contrast, secretion of *B. pseudomallei* BopE and BipD proteins could be detected even under LB medium (pH 7.0). In addition, the amount of *B. pseudomallei* BipD and BopE proteins secretion after acidic induction is increased and significantly higher than for the three *B. thailandensis* strains (Fig. 3B).

### Effect of acidic pH on *B. thailandensis* invasion efficiency

Previous studies indicated that the *B. pseudomallei* BipD protein is involved in invasion of this bacterial pathogen into host cells (Stevens *et al*., 2002, 2004) and *B. pseudomallei* is more efficient than *B. thailandensis* in invasion of epithelial cells (Kespichayawattana *et al*., 2004). Since Western blot analysis revealed that *B. thailandensis* BipD and BopE proteins could be induced in acidic culture conditions, we compared the ability of *B. thailandensis* to invade A549 cells after culturing in standard and acidic LB medium. Fig. 4 demonstrates that the invasion efficiencies of A549 respiratory epithelial cells by *B. thailandensis* DV1 and E264 grown in LB medium pH 4.5 were significantly increased compared to bacteria grown in standard LB medium ($p<0.024$ and $<0.005$, respectively). The increase in invasion efficiency was almost to the same level as *B. pseudomallei* cultured in standard LB medium (Fig. 4). Surprisingly the invasion efficiency of *B. thailandensis* D1 did not increase after culturing in acidic medium but decreased. When we measured the actual growth rate of *B. thailandensis* D1 in LB medium (pH 4.5), we

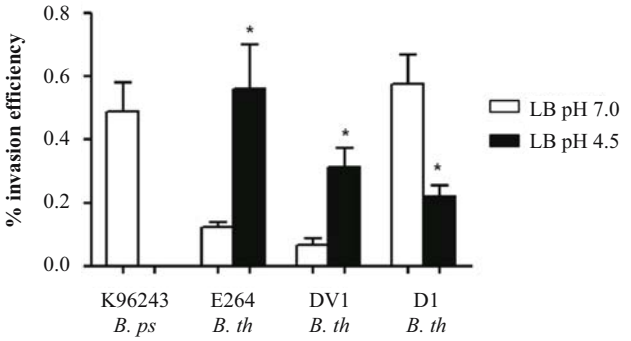


**Fig. 4.** Effect of acidic culture condition on *B. thailandensis* invasion efficiency. *B. thailandensis* (*B. th*) strains D1, DV1, E264 and *B. pseudomallei* (*B. ps*) strain K96243 were cultured in standard LB medium pH 7.0 or 4.5 before infection into A549 lung epithelial cells. * indicate significant difference at $p<0.05$ (Student's *t* test).

observed that it was slower to *B. thailandensis* DV1 and E264 despite our efforts to equalize the number of starting cells in all cases. In addition, we noticed that the invasion efficiency of *B. thailandensis* D1 when cultured in standard LB medium was almost at the same level as *B. pseudomallei* and much higher than the other *B. thailandensis* strains tested. These results suggest that there is significant biological variation among *B. thailandensis* strains.

## Discussion

*B. thailandensis* is a saprophyte found in the environment in Southeast Asia, including Thailand. It is a less virulent relative of *B. pseudomallei* and *Burkholderia mallei. B. thailandensis* has attracted increasing interest because of the potential to use it as a model to study certain aspects of *B. pseudomallei* biology (Haraga *et al*., 2008). This is partly because, unlike *B. pseudomallei*, biosafety level 3 laboratories are not required for working with *B. thailandensis.* In this study, analysis of *B. thailandensis* BipD amino acid sequences found variation among *B. thailandensis* isolates (89 to 99%), which was greater than that found among *B. pseudomallei* strains (99%). The greater heterogeneity of *B. thailandensis* BipD sequences compared with *B. pseudomallei* may suggest the "differentially evolved" state of *B. thailandensis* and confirmed previous reports that virulence related genes; in particular members of the T3SS, were more divergent between *B. thailandensis* and *B. pseudomallei* compared to the other genes in the bacterial genome (Yu *et al*., 2006). The emergence of a *B. thailandensis* strain DV1 with BipD sequence closer to *B. pseudomallei* than the most of *B. thailandensis* may be because these bacteria are closely related species that often live in the same environment, so there is a possibility of horizontal gene exchange between these two species.

Although the *B. thailandensis* genome carries a T3SS homologue to *B. pseudomallei* Bsa T3SS (Rainbow *et al*., 2002), little is known about the conditions that trigger protein secretion in the less virulent organism. The inability to detect BipD and BopE proteins secretions from *B. thailandensis* cultured in LB medium pH 7.0 indicated that although LB broth was suitable for synthesis of *B. thailandensis* BipD and BopE proteins, conditions were not optimal for its secretion. In addition, although *B. thailandensis* strain DV1 showed 98% BipD amino acid sequence identity to *B. pseudomallei* K96243, it still could not secrete BipD and BopE to the level detected by Western blot analysis suggesting that other factors may play a role in secretion e.g. chaperones are required for the effective type III secretion. Indeed, culturing *B. thailandensis* strains DV1 and the other tested strains in acidic pH of 4.5 for 6 h could induce the secretion of BipD and BopE proteins. Our data are in good agreement with a previous report in which BopE secretion in *B. thailandensis* 700388 harboring a plasmid for the expression of hemagglutinin-tagged BopE was detected only in acidic culture condition (Haraga *et al*., 2008).

Several strains of *B. thailandensis* have been shown to invade A549 respiratory epithelial cells less efficiently than *B. pseudomallei* (Kespichayawattana *et al*., 2004). To our knowledge this is the first report revealing that low pH could increase the ability of *B. thailandensis* to invade A549 cells. The mechanism behind this observation might be related to the induction of the T3SS but we cannot rule out other bacterial factors. Further studies are necessary for clarification. In addition, we also showed that there is significant biological variation among *B. thailandensis* strains. Bacterial invasion is an important step allowing bacteria to enter into host cells prior to intracellular multiplication. The phenomenon of pH induced invasion efficiency can be used by those who would like to use this bacterium to infect cell cultures to the same extent as *B. pseudomallei* for *in vitro* studies of the mechanism of intracellular survival.

In conclusion, this study demonstrated that heterogeneity of BipD sequences among *B. thailandensis* isolates was greater than in *B. pseudomallei*. Western blot analysis indicated that *B. thailandensis* isolates could be induced to secrete type III secretion proteins i.e. BipD and BopE, by culturing in acidic pH cultured medium and this process correlated with the increased invasion efficiency. However, the possibility that acidic pH can induce other bacterial factors leading to the observed increased invasion could not be ruled out.

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