A role of *Burkholderia pseudomallei* flagella as a virulent factor

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**KEYWORDS**

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**Summary** *Burkholderia pseudomallei* is an agent of melioidosis and is closely related to avirulent *B. thailandensis*. *Burkholderia thailandensis* has a 15-bp deletion within the variable region of the flagellin gene *fliC* compared with *B. pseudomallei*. The difference in the *fliC* gene might be related to virulence. In the present study, the invasion, internalization and intracellular replication of both phagocytic (mouse macrophage cell line RAW264.7) and non-phagocytic cells (human lung epithelial cell line A549) of *B. pseudomallei* *fliC* knockout mutant (MM35) complemented with its own *fliC* (Cp) or with *B. thailandensis* *fliC* (Ct) was compared with those of the wild-type strains of *B. pseudomallei* (1026b) and *B. thailandensis* (E257). In phagocytic cells, there was no significant difference in bacterial uptake between Cp and Ct, but MM35 was internalized significantly less compared with 1026b, Cp, Ct and E257. The results suggest that flagella are involved in macrophage invasion. In non-phagocytic cells, Cp and Ct showed similar invasive capacities while 1026b, Cp and Ct showed significantly higher invasiveness than MM35, suggesting that flagella facilitate the non-phagocytic cell invasion. However, the invasive capacity of MM35 was significantly higher than that of E257, suggesting that in addition to the flagella, *B. pseudomallei* may need other factor(s) to facilitate invasion in non-phagocytic cells.

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1. Introduction

*Burkholderia pseudomallei* is a Gram-negative bacterial pathogen that is a causative agent of melioidosis, a disease that is associated with a high fatality rate and is a major cause of death from community-acquired bacteraemic pneumonia in endemic regions.1,2 The organism is a facultative intracellular bacillus that can invade and multiply in various cell types.3 In contrast, *B. thailandensis* is very closely related to *B. pseudomallei* in most characteristics except for virulence in humans and animals. Both *B. pseudomallei* and *B. thailandensis* are motile by means of flagella. A comparative structural analysis of two biotypes of the flagellin gene *fliC* showed 99% amino acid homology. However, it has been reported that *B. thailandensis* has a 15-bp deletion within the variable domain of *fliC* when compared with *B. pseudomallei*.4

Motility has been implicated as one of the virulence factors in many bacterial species,5–8 and thus, in the present study, we investigated the involvement of *fliC* in *B. pseudomallei* and *B. thailandensis* in their ability to invade and replicate inside phagocytic and non-phagocytic cells.

2. Materials and methods

2.1. Bacterial strains and plasmid

The bacterial strains and plasmids used in this study are listed in Table 1. *Burkholderia pseudomallei* 1026b, MM35, and *B. thailandensis* E257 were obtained from the Wellcome-Mahidol University-Oxford Tropical Medicine Research Programme, Faculty of Tropical Medicine, Mahidol University, Thailand, and characterized previously.4,9 *Escherichia coli* DH5α, pIR and plasmid pUCP28T were obtained from the Department of Biochemistry, Faculty of Medicine, National University of Singapore, Singapore. *Burkholderia pseudomallei*, *B. thailandensis* and *E. coli* were grown at 37°C in Luria–Bertani (LB) broth or on LB agar overnight.

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When appropriate, antibiotics were added at the following concentrations: 25 µg of trimethoprim per ml for *E. coli*, and 50 µg of tetracycline and 100 µg of trimethoprim per ml for *B. pseudomallei*.

2.2. Cell lines

The mouse macrophage cell line (RAW264.7) was obtained from American Type Culture Collection (ATCC). The macrophages were cultured in DMEM (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% l-glutamine (Biological industries, Haemek, Israel). Human alveolar lung epithelial cell line (A549) was obtained from American Type Culture Collection (ATCC CCL-185). This epithelial cell line was cultured in Ham F-12 culture medium (HyClone, Logan, UT) supplemented with 1.5 g/l sodium bicarbonate (Sigma, St Louis, MO, USA), 10% fetal bovine serum (HyClone, Logan, UT), and 1% l-glutamine (Biological industries, Israel).

2.3. Construction of the *fliC* complement *Burkholderia pseudomallei* CDS and CTS

The genomic DNA of *B. pseudomallei* 1026b and *B. thailandensis* E257 were used as the templates for amplifications of the full-length *fliC* gene and its promoter by using primers Promoter1F (5' AATTCAGC CGCCCGCATCAA) and FliCSTSacI (5' GGTTGGAGCTTATTTGAGGACTTCAG). The PCR reactions were performed by using Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) in 1.5 mM MgCl2 before amplified in the PCR machine (Eppendorf: Mastercycler 1001-400). The PCR conditions were one cycle of denaturation at 99ºC for 5 min and 94ºC for 4 min, 30 cycles at 94ºC for 1 min, 56ºC for 30 s, and 72ºC for 2 min, followed by 72ºC for 15 min. The 1.5-kb PCR products were ligated into the Smal pUCP28ST, generating pUCP28STCp and pUCP28STCt, which were transformed into *E. coli* DH5αpir. The transformants were selected on LB agar plates supplemented with 25 µg/ml of trimethoprim. The *fliC* insertion was confirmed by PCR. The recombinant plasmid pUCP28STCp and pUCP28STCt were transferred into MM35 via conjugation, generating *B. pseudomallei* Cp and Ct, respectively. The *fliC* complementations were selected on LB plates supplemented with 50 µg/ml tetracycline and 100 µg/ml trimethoprim.

2.4. Motility assay

Overnight broth cultures were adjusted to the number of viable cells to 1×10⁶ cells/ml. Then 4-µl broth cultures were spotted and grown on LB plates with 0.3% agar. Motility plates were incubated at 37ºC overnight, and then the motility phenotype observed.

2.5. Bacterial uptake, intracellular survival and replication of *Burkholderia in phagocytic cells*

The macrophages (1×10⁶) were cultured in six-well plates overnight before exposure to the bacteria at a multiplicity of infection (MOI) of 2:1 (when appropriate, the coculture was immediately centrifuged at 30ºC with 170×g for 5 min to bring the bacteria directly in contact with the host cells). To investigate the internalization and intracellular replication time of the bacteria, the number of intracellular bacteria was determined at 2, 4, 6 and 8 h after incubation by standard antibiotic protection assay. The viability of the infected cells, judged by Trypan blue dye exclusion test, was greater than 90%.

2.6. Invasion, intracellular survival and replication of *Burkholderia in non-phagocytic cells*

Human lung epithelial cell line A549 (4×10⁵) was cultured in six-well plates overnight before exposure to the bacteria at a MOI of 10:1 (when appropriate, the co-culture was immediately centrifuged at 30ºC with 170×g for 5 min to bring the bacteria in direct contact with the host cells). To investigate the invasive capacity and intracellular replication time of the bacteria, the number of intracellular bacteria was determined at 3, 5, 7 and 9 h after incubation by standard antibiotic protection assay. The viability of
the infected cells, judged by the Trypan blue dye exclusion test, was greater than 90%.

2.7. Statistical analysis

One-way ANOVA test by SPSS 11.5 (SPSS Inc., Chicago, IL, USA) was used to determine if there was a statistically significant difference \( (P < 0.05) \) between the mean invasion frequencies and intracellular replication times.

3. Results

3.1. Construction and characterization of the \( fliC \) complementation of MM35

The pUCP28T derivatives, pUCP28TCp and pUCP28TCTc, were able to complement the mutation in MM35. The complemented mutants Cp and Ct restored the motility phenotype, which resulted in a larger area of growth than the non-motile MM35 (Figure 1); however, the swarm area was smaller than that of the wild type. The empty pUCP28T plasmid (no inserted fragment) did not affect the motility. It should be noted that the swarm area of \( B. \) thailandensis was larger than that of \( B. \) pseudomallei.

![Fig. 1](image)

**Fig. 1** – Motility plate assay. Complementation of MM35 by pUCP28TCp and pUCP28TCTc, resulting in motile Cp and Ct, respectively. The motility phenotype was assessed by examining the circular swarm from the site of inoculation.

3.2. Bacterial uptake and intracellular replication of \( Burkholderia \) in mouse macrophage cell line (RAW264.7)

Both Cp and Ct were able to internalize into the macrophages at nearly equal capacity (Figure 2A). In contrast, MM35 was internalized significantly less than 1026b, E257, Cp and Ct \( (P < 0.05) \). However, with an additional centrifugation step, the internalization of MM35 increased to nearly the same level as that of the other strains. By comparing 1026b and E257, the internalization of 1026b was significantly higher than that of E257 \( (P < 0.05) \).

The rate of replication among these bacteria was also determined (Figure 2B). The intracellular replication times of all bacteria calculated at 4, 6 and 8 h after infection were similar to each other. The results showed that there were no significant differences between these bacterial replication rates.

![Fig. 2](image)

**Fig. 2** – Bacterial uptake, intracellular survival, and replication of \( Burkholderia \) in mouse macrophage cells. (A) Bacterial uptake by macrophage. Internalization was determined 2 h after infection. Black bars represent the invasive capacity without centrifugation during the infection. White bars represent the invasion following an additional centrifugation step immediately after the bacteria were added to the cultured cells. Small bars represent the SE with 95% CI. (B) Intracellular survival and replication of the bacteria. Replication time was determined after a total of 4, 6 and 8 h of incubation. All experiments were performed in three independent experiments, each performed in duplicate.

3.3. Invasion and intracellular replication of \( Burkholderia \) in human lung epithelial cell line A549

As shown in Figure 3A, there was no significant difference in the invasive capacity between Cp and Ct. In contrast, 1026b, Cp and Ct showed significantly higher invasiveness than MM35 \( (P < 0.05) \). However, when bringing the bacteria into contact with the human lung epithelial cells by short centrifugation, there were no significantly different invasive capacities among all the bacteria. In addition, 1026b and MM35 exhibited an invasive capacity that was significantly higher than that of E257 \( (P < 0.05) \).

The intracellular replication times of all bacteria calculated at 5, 7 and 9 h after infection were similar to each other (Figure 3B). These rates of intracellular replication were not significantly different from their respective growth rates in the fluid broth media.
4. Discussion

Flagella are considered to be a virulence factor in many bacteria. In *Burkholderia pseudomallei*, it has been reported that adherence by flagella was required to initiate bacterial entry into the amoebic trophozoite. The importance of flagella in the pathogenesis of *B. pseudomallei* has been demonstrated in a mouse model; it was found that the flagellum was an important and necessary virulent determinant of *B. pseudomallei* during intranasal and intraperitoneal infection of mice. *Burkholderia pseudomallei* and *B. thailandensis* are very closely related, except for their capacity to cause disease. Although both bacteria contain flagella, the flagellin gene of *B. thailandensis* shows a 15-bp region (encodes for five amino acids) missing in the variable domain. Therefore, the difference in the flagellin genes should be investigated.

In the current study, the successful construction of the complemented *fliC* mutants Cp and Ct was characterized by motility assay. The results from this analysis (Figure 1) showed that the complemented mutant Cp and Ct could not fully restore the motility function of the *fliC* mutant MM35, suggesting insufficient flagellin protein production. In general, the bacteria would need approximately 20,000 copies of the flagellin protein to make one filament. This is in contrast to other bacterial proteins in which one protein unit can absolutely perform their function. In the *fliC* mutant complementation, the plasmid exists in the bacterial cell in trans, so the flagellin protein production depends on the efficiency of the plasmid. Therefore, although the vector pUCP28T is a high copy number plasmid, it may not fully restore the motility phenotype of the *fliC* mutant.

It was previously noted that the growth area of *B. thailandensis* was larger than that of *B. pseudomallei*. The fact that the bacterial growth rates in free-cell cultures of *B. thailandensis* and *B. pseudomallei* wild types are not different from each other (data not shown), indicates that the growth rate is not a factor that is involved in a larger growth area of *B. thailandensis* in the motility assay. The data from genomic analysis revealed that, in addition to *fliC*, *B. thailandensis* (strain E264) possesses another flagellin gene called flagellin D. The flagellin D gene encodes 296 amino acids in length, which is shorter than that of *fliC*, and is located in the flagellar system on chromosome II (GenBank accession no. ABC35429.1), which does not present in *B. pseudomallei* (strain K96243). Like *fliC*, the function of the flagellin D protein is primarily chemotaxis and motility (TIGR Locus: BTH_II0151). Thus, the presence of flagellin D may result in a larger growth area of *B. thailandensis*.

The results from the invasion assay of Cp and Ct in both cultured phagocytic and non-phagocytic cells indicated that the difference in the variable region of *B. pseudomallei* and *B. thailandensis* *fliC* did not involve the invasive capacity (Figures 2A and 3A). Thus, the 15-bp in the variable region of *B. pseudomallei* *fliC* is not essential for cell invasion. The brief centrifugation was performed to confirm that flagella facilitated the bacterial invasion and bacterial uptake by motility function, which may enhance the adhesion of the bacteria to the cells. Our results are consistent with those previously reported. However, the invasive capacity of *B. pseudomallei* *fliC* mutant in human lung epithelial cells was significantly higher than that of *B. thailandensis*, suggesting that in addition to flagella, *B. pseudomallei* may need other factor(s) such as type three secretion system, which present only in *B. pseudomallei* and not in *B. thailandensis* to facilitate invasion of non-phagocytic cells.

In conclusion, *B. pseudomallei* *fliC* mutant was complemented with its own *fliC* or with *B. thailandensis* *fliC*. Both complemented *fliC* mutants were equally invasive in human lung epithelial cells and were also similar to each other in the bacterial uptake by mouse macrophage cells. Thus, the 15-bp region, which is only present in the *fliC* variable region of *B. pseudomallei*, is not essential for access of *B. pseudomallei* into the phagocytic and non-phagocytic cells. However, the presence of flagella resulted in a higher invasive capacity of *B. pseudomallei* than that of the *fliC* mutant. This indicates the importance of flagella for both phagocytic and non-phagocytic cell invasion.

Authors’ contributions: PU and ST designed the study protocol; KLC provided the plasmid and supplied the materials for construction of the recombinant plasmid;
TC carried out all laboratory experiments; TC, PU, SS and ST analysed and interpreted these data; TC and PU drafted the manuscript. All authors read and approved the final manuscript. PU is guarantor of the paper.

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