A ROLE OF *BURKHOLDERIA PSEUDOMALLEI* FLAGELLA AS A VIRULENT FACTOR

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Thesis
Entitled

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A ROLE OF *BURKHOLDERIA PSEUDOMALLEI* FLAGELLA AS A VIRULENT FACTOR

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ABSTRACT

Burkholderia pseudomallei is an agent of melioidosis that is closely related to avirulent *Burkholderia thailandensis*. *B. thailandensis* has a 15-base pair deletion within the variable region of the flagellin gene (*fliC*) compared with *B. pseudomallei*. This difference in *fliC* might be related to virulence. In this study, the invasion, intracellular replication, and NF-κB activation in both phagocytic (mouse macrophage cell line RAW 264.7) and nonphagocytic 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 wild type *B. pseudomallei* (1026b) and *B. thailandensis* (E257). In both phagocytic and nonphagocytic cells, there was no difference in bacterial invasion between Cp and Ct, suggesting that the difference in *B. pseudomallei fliC* is not essential for cell invasion. However, MM35 showed significantly less invasiveness than other strains, indicating the role of flagella in macrophage and epithelial cell invasion. In nonphagocytic cells, the invasive capacity of MM35 was significantly higher than E257, indicating that in addition to the flagella, *B. pseudomallei* may need other factors to facilitate invasion. IκBα, an inhibitor of NFκB, showed a similar pattern of degradation in the wild type 1026b, E257, Cp and Ct. However, in MM35, the level of IκBα was slightly less than that of the wild type in both phagocytic and nonphagocytic cells. This suggests that flagella may also participate in NF-κB activation. It was only in nonphagocytic cells, that NF-κB activation induced by all *B. pseudomallei* strains was more rapidly inhibited compared to *B. thailandensis*.

KEY WORDS: *BURKHOLDERIA PSEUDOMALLEI*/ *BURKHOLDERIA THAILANDENSIS*/ FLAGELLA/ INVASION/ NF-κB ACTIVATION

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บทบาทของเฟลคเจลลาในการก่อความรุนแรงของเชื้อเบอร์โคลเดอเรีย สูโดมาลลิไอ
(A ROLE OF *BURKHOLDERIA PSEUDOMALLEI* FLAGELLA AS A VIRULENT FACTOR)

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บทคัดย่อ

เชื้อเบอร์โคลเดอเรีย สูโดมาลลิไอ (*Burkholderia pseudomallei*) เป็นเชื้อที่ก่อให้เกิดโรคเมลิออยโดซิส เชื้อชนิดนี้มีความคล้ายคลึงกับเชื้อเบอร์โคลเดอเรีย ไทยแลนเด็นซิส (*B. thailandensis*) ซึ่งเป็นสายพันธุ์ที่ไม่ก่อโรค จากการศึกษาก่อนหน้านี้พบว่าภายในบริเวณที่ผันแปรได้ของยีนเฟลคเจลลาของเชื้อ *B. thailandensis* มีส่วนที่ขาดหายไปสิบห้าคู่เบสเมื่อเปรียบเทียบกับเชื้อ *B. pseudomallei* ซึ่งส่วนที่ขาดหายไปนี้อาจเกี่ยวข้องกับการก่อความรุนแรงของโรค การศึกษาในครั้งนี้ได้ทำการเปรียบเทียบความสามารถในการรุกรานและการกระตุ้น NF-κB ต่อเซลล์ phagocyte และเซลล์ nonphagocyte โดยเชื้อห้าสายพันธุ์ ได้แก่ เชื้อ *B. pseudomallei*, เชื้อ*B. thailandensis*, เชื้อ *B. pseudomallei* MM35 ที่ทำการทำลายส่วนเฟลคเจลลา, เชื้อ *B. pseudomallei* Cp ที่ทำการต่อเติมส่วนเฟลคเจลลากลับเข้าไปให้สมบูรณ์โดยใช้เฟลคเจลลาของเชื้อ *B. pseudomallei*เอง และเชื้อ *B. pseudomallei* Ct ที่ต่อเติมโดยใช้เฟลคเจลลาของเชื้อ *B. thailandensis* จากการศึกษาพบว่า เฟลคเจลลามีส่วนช่วยในการรุกรานของเชื้อต่อทั้งเซลล์ phagocyte และเซลล์ nonphagocyte โดยช่วยนำพาเชื้อไปสู่เซลล์เจ้าบ้าน แต่ส่วนต่างภายในเฟลคเจลลาระหว่างเชื้อ *B. pseudomallei* และ *B. thailandensis* นั้น ไม่ได้มีส่วนช่วยในการรุกรานของเชื้อ *B. pseudomallei* นอกจากนี้ยังพบว่าเฟลคเจลลายังช่วยในการกระตุ้น NF-κB ทั้งในเซลล์ phagocyte และเซลล์ nonphagocyte อย่างไรก็ตามภายในเซลล์ nonphagocyteนั้น การกระตุ้นของ NF-κB โดยเชื้อ *B. pseudomallei* สิ้นสุดลงเร็วกว่าการกระตุ้นด้วยเชื้อ *B. thailandensis*

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LIST OF ABBREVIATIONS

/	per
%	percent
Ara	arabinose sugar
bp	base pair
BSA	Bovine serum albumin
°C	degree Celsius
CCW	counterclockwise
CW	clockwise
cm	centimeter
CFU	colony forming unit
CO_2	carbon dioxide
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
et al	et. All (Latin), and others
FBS	fetal bovine serum
fliC	flagellin gene
FliC	flagellin protein
G	gram
G+C	Guanine+cytosine
gDNA	genomic DNA
h	hour (s)
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IκB	ikappa-B
IκBα	ikappa-B alpha
IL	interleukin

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(Continued)

iNOS	inducible nitric oxide
IRF	interferon regulatory factor
kb	kilo base
kDa	kilodalton
LB	Luria-Bertani
LD_{50}	lethal dose
LPS	lipoolysaccharide
M	Molar
Mbp	Mega bp
min	minute (s)
ml	milliliter
mM	milimolar
MNGC	multinucleated giant cell
MOI	multiplicity of infection
mRNA	messenger RNA
M.Sc	Master of Science
NF-κB	Nuclear factor-kappa B
ng	nanogram
NO	nitric oxide
OD	optical density
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pH	per Hydrogen
RNA	ribonucleic acid
ROI	reactive oxygen intermediates
RNI	reactive nitrogen intermediates
rRNA	ribosomal RNA
rpm	revolutions per minute

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(Continued)

SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel Electrophoresis
sec	second
TBE	tris-borate EDTA buffer
TEMED	N, N, N', N'-tetramethylethylene diamine
TLR	Toll-like receptor
TSA	Tryptic soy agar
TTSS	Type three secretion system
U	unit
µg	microgram
µl	microliter
µm	micrometer
V	volt
w/v	weight per volume

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CHAPTER I
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 bacteremic pneumonia in Southeast Asia and Northern Australia (1, 2). The organism is a facultative intracellular bacillus that can invade and multiply in a variety of cell types (3). In contrast, *B. thailandensis* is very closely related to *B. pseudomallei* in most characteristics except virulence for humans and animals. Both *B. pseudomallei* and *B. thailandensis* are motile by mean of flagella. A comparative analysis of the *fliC* structural gene of two species showed 99% amino acid homology. However, it has been reported that *B. thailandensis* has a 15-base pair deletion within the variable domain of *fliC* when compared with *B. pseudomallei* (4). Since flagellin protein has been implicated as one of the virulence factors (the factor that supports the pathogenicity of the pathogen) in many bacterial pathogens, including *B. pseudomallei* (5-8), therefore the difference between *B. pseudomallei* and *B. thailandensis* flagellin gene should be investigated. The objective of this study is to investigate the involvement of the difference in the *fliC* gene between *B. pseudomallei* and *B. thailandensis* in their ability to invade, replicate, and activate NF-κB. *B. pseudomallei fliC* knock mutant MM35 was complemented the ability to motile by its own flagellin (Cp) or by the flagellin of *B. thailandensis* (Ct), before comparing the invasiveness capacity, internalized ability, intracellular replication, and NF-κB activation with wild type *B. pseudomallei* (1026b) and *B. thailandensis* (E257) in both phagocytic (mouse macrophage cell line, RAW264.7) and nonphagocytic (human lung epithelial cell line A549) cells. The information obtained from this study will provide a better understanding of how *B. pseudomallei* flagella contribute to the pathogenesis of melioidosis.

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CHAPTER II
LITERATURE REVIEW

1. Melioidosis

1.1 History and epidemiology

Melioidosis is a life-threatening disease caused by *Burkholderia pseudomallei* (*B. pseudomallei*). It was first described by Alfred Whitmore and C.S. Krishnaswami as a glander-like disease among morphia addicts in Rangoon, Burma, in 1912 (10). In 1932, melioidosis was named from the Greek word "melis" means distemper of asses and "eidos" means resemblance by Stanton and Fletcher (11). Melioidosis is an important infectious disease of humans and animals known to be endemic in the tropics, especially in the countries of Southeast Asia such as Thailand, Malaysia, Vietnam, Cambodia, Laos and Myanmar, and Northern Australia. In Northeast Thailand, *B. pseudomallei* accounts for up to approximately 20% of all community-acquired septicaemia and 50% of sepsis related mortality (12, 13). In Australia, it has been the most common cause of fatal community-acquired bacteremic pneumonia (14, 15). There are some reports of melioidosis cases in other parts of the world such as Central America, the Caribbean, China, Taiwan, Africa, the Middle East.

1.2 Transmission

In infected animal, *B. pseudomallei* was found in excreted sputum, pus, urine and faeces, which may cause contamination of the environment (16). In humans, melioidosis is acquired mainly by contact with contaminated soil and water through skin abrasions or wounds, by ingestion of contaminated water or by inhalation of infectious dust particle or close contact with melioidosis patient (17). *B. pseudomallei* is ubiquitous in the rice-farming areas. It is also present in rubber plantations, cleared fields, cultivated and irrigated agricultural sites as well as drains and ditches. Since *B.*

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pseudomallei is found mostly in rice paddy field and the farmers are always not wear protective footwear, therefore, rice farmers and their families are a major risk group of infection. The highest incidence of infection occurs during the rainy season. It is believed that during these periods, rising water tables percolate the organisms to expose to humans and animals (18). Sporadic cases have resulted from laboratory accidents and person-to-person or animal-to-person spread. Direct human to human infection by sexual transmission was reported in 1957 (19). Transmission by insect bites (mosquito; *Aedes agypti* and rat flea; *Xenopsylla cheopsis*) has been reported in experimental animal (20) but this infection route has not yet been proved in humans. The infection will result in disease, probably depending on the virulence of the strain, the size of the inoculum, congenital status and physiological of the host. The disease can occur in all age groups of both genders but there was a preponderance of disease among males with the ratio of male:female of 1.4:1. The range of ages with high incident of infection was 40-60 years (21).

1.3 Disease

Melioidosis is the most important cause of bacteremia and pneumonia that may be symptomatically confused with typhoid fever, malaria, leptospirosis, tuberculosis, mycotic infections and septicemia caused by other gram negative bacteria (22, 23). The clinical manifestation of melioidosis is extremely variable ranging from acute to chronic form. Acute septicemia is the most severe form of melioidosis and is responsible for much morbidity and mortality within a few days of exposure. The mortality occurs in patient with the septicemic form of melioidosis, which is characterized by dissemination of the bacteria in circulation and isolation of the bacteria from blood and from various organs. Severe septicemia is significantly associated with underlying diseases, such as diabetes mellitus, renal failure, immunodeficient conditions, chronic alcoholism and chronic infections (24). Infection occurring is probably determined by the dose of inoculum, the virulence of the infected strain, and the susceptibility of the host. Melioidosis can become chronic with formation of abscesses or can remain subclinical for many years. Relapses occur if the organism is not completely cleared after the primary infection as it has been reported that *B. pseudomallei* can survive and multiply in host cells in vitro (3). Recrudescence

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from subclinical infection was found in both humans and animals. There were cases reported that the patients infected with *B. pseudomallei* who had been treated with long-term antimicrobial drugs occurred the recrudescence of the disease after cessation of therapy. The recrudescence of the infection is more frequent in patient who survived after acute melioidosis. There are several factors that influence the relapse such as clinical severity at original presentation and type of drug treatment.

1.4 Treatment

Treatment is divided into 2 phases; intravenous and oral phase. Initial intravenous therapy is given for at least 2 weeks before switching over to oral maintenance therapy for 12 to 20 weeks. For severe melioidosis, ceftazidime combination with cotrimoxazole or with ciprofloxacin is the drug of choice. Ceftazidime monotherapy can be used in systemic melioidosis (25, 26). However, there are some resistant strains (27). Imipenem may also be considered an alternative to ceftaxidime for acute severe melioidosis treatment (28, 29). Amoxicillin clavulanate or doxycycline in combination with cotrimoxazole is a choice in localized infections (30). For maintenance therapy, Co-amoxyclav is a safe and well-tolerated antimicrobial agent (31, 32).

1.5 Diagnosis

1.5.1 Identification of *B. pseudomallei*

Culture-based methods

Ashdown's selective agar is the most common selective medium that has long been used for the isolation of *B. pseudomallei* from clinical specimens in countries where the disease is endemic. It consists of trypticase soy agar with glycerol, crystal violet, neutral red and gentamicin. The presumptive identification of colonies of *B. pseudomallei* can be made from their characteristic purple color with dry and wrinkled appearance, which becomes evident after 24 to 48 h of incubation. A second medium, *B. pseudomallei* selective agar (BPSA), which may enhance the growth of mucoid colonies is also used in *B. pseudomallei* culture. However, neither medium is commercially available, they are not rapidly accessible to laboratories in areas of nonendemicity. A third medium, *B. cepacia* medium, is widely available. It is an agar

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most commonly used to identify *B. cepacia* complex from the sputum of individuals with cystic fibrosis. These three media were compared for their ability to grow *B. pseudomallei*. The sensitivity of each medium was equivalent, but the selectivity of BPSA was lower than that of *B. cepacia* medium or Ashdown's medium (33).

Antigen detection

Antigen tests have been developed for used on direct specimens or in blood culture supernatant. A monoclonal antibody latex agglutination test against a 200-kDa protein of *B. pseudomallei* was shown to agglutinate blood culture fluid positive for *B. pseudomallei*, including strains with atypical lipopolysaccharide (LPS) patterns but not *B. thailandensis* strains (34). Immunofluoresence from direct specimen is the way to reduce the time to diagnosis but only where specialized microscopy facilities are available.

1.5.2 Serological tests

Indirect haemagglutination assay (IHA) is commonly used to assess exposure to *B. pseudomallei* despite its poor sensitivity and specificity. It is evident that the sensitivity of IHA is limited in patients with acute septic illnesses (35). ELISA based on LPS and 30- and 200-kDa proteins have been validated. It was more sensitive and specific than the IHA but still lack the performance necessary for clinical use (36). Further research is needed to develop and evaluate improved serological tests.

1.5.3 Molecular identification techniques

Several studies have been made to develop new molecular procedures to identify *B. pseudomallei* from various specimens but few have been field testsed. Many ribotyping experiments using the restriction enzymes had been studied but none of these could be assigned to a single animal species, a geographic or clinical course (37). However, ribotyping proved its value in outbreak investigations in animals and humans by identifying the source of contamination and showing that melioidosis is saprozoonotic and animal to human transmission occurs only occasionally. 16S RNA sequencing has long been used for the identification of bacterial species. This method has been used for phylogeny (38, 39). More recent studies examined the role of a PCR for the type III secretion system (TTSS) in clinical and environmental specimens (40). Real-time PCR is a method that has been developed to distinguish *B. pseudomallei*

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from other *Burkholderia* strains. For example, the primers that target gene *orf11* and *orf13* of TTSS1 and *BpSCU2* of TTSS2 were used to distinguish between *B. pseudomallei, B. mallei* and *B. thailandensis* with 100% specificity (41).

1.6 Prevention

There is currently no licensed vaccine available for protection against melioidosis. However, there are some reported data on the possible utility for vaccine development. Antibodies against *B. pseudomallei* flagellin reduce the motility of the bacterium and provide passive protection against melioidosis in animal model (42). LPS and capsular polysaccharide as subunit vaccines also showed partial protection in mouse model (43). Because of *B. pseudomallei* induced a rapid and potent IFN-γ response from natural killer T cells and conventional T cells, it has been suggested that an effective vaccine against *B. pseudomallei* should target the generation of IFN-γ-secreting T cells (44).

Since a licensed animal or human vaccine does not exist, therefore, the best way for protection against melioidosis is to avoid *B. pseudomallei* infection. Using the protective clothing such as rubber boots and gloves during agricultural work can prevent infection through the feet and hands. Person with skin lesions should avoid contact with soil and standing-water in the endemic areas. As milk from goats and dairy cows can contain *B. pseudomallei*, therefore pasteurization is essential before consumption (45). The proper disposal of sewage wastes in the endemic areas is important because wastes can attract insects and rats that encourage the spread of the disease.

2. Bacterial aspects

2.1 *B. pseudomallei*

B. pseudomallei, the agent of melioidosis, was first named *Bacillus pseudomallei* by Whitemore and Krishnaswami in 1912 (10). Thereafter, it was reported under a variety of names regarded its nomenclature such as *Bacillus whitmori, Pfeifferella whitmori, Pfeifferella pseudomallei, Flavobacterium pseudomallei, Actinobacillus pseudomallei, Loefferella whitmori, Malleomyces*

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pseudomallei and *Pseudomonas pseudomallei* (46, 47). Nucleic acid similarities among the *Pseudomonas* species have long been discussed. Palleroni divided the *Pseudomonas* genus to five RNA homology groups (48). Seven species including *P. pseudomallei* in homology group II were transferred to the new genus, *Burkholderia*, in 1992, by using 16s rRNA sequence, DNA-DNA homology value, cellular lipid and fatty acid composition, and phenotypic characteristics (49).

B. pseudomallei is a facultative intracellular, gram negative, non spore forming, and oxidase-positive bacillus which grows on ordinary media at 37 °C under aerobic conditions or under anaerobic condition when nitrate or arginine are present (49). The energy for growth is acquired by respiration but not by fermentation. Glucose and galactose are oxidized to gluconic acid and galactonic acid, respectively. *B. pseudomallei* can also multiply in soil at pH from between 4 and 8, at a minimal humidity of 10-15%, at temperatures from 4 to 42 °C but not in salt water (50). It is able to survive in the absence of nutrients in distilled water at room temperature for several years, in muddy water for up to 7 months and in soil in the laboratory for up to 30 months (51, 52). The existence of viable but not cultivable forms supports its survival in environmental stress such as heat or dry climatic conditions. *B. pseudomallei* shows characteristic bipolar staining because of intracellular deposits of β-hydroxy butyric acid. The organism is motile by means of polar tuft of flagella. Soma size is 0.8×1.5 μm. Colonies are mostly smooth initially but become dry or wrinkled on further incubation and have a strong musty, earthy odor which was used at one time as the hallmark for preliminary identification. On *B. pseudomallei*-positive broth, a tough, dry pellicle with a matte surface may be due to a quorum-sensing system reflecting aerotaxis and assumed that biofilms are produced at the air-fluid interface (53, 54).

The prevalence of *B. pseudomallei* infection increases with rainfall in the endemic areas. The agent can be found in soil and clay layers from the surface but more often and regularly in deeper layers from 25 to 120 cm (55-57). During the rainy season, the animals and humans can come into contact with muddy water and soil particles carrying bacteria from deeper layers to the surface. The strong winds associated with monsoon rainfalls are believed to increase the inhalation of the bacteria which leads to a more severe course of infection (pneumonia and

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septicaemia), resulting in an increased fatality rate. *B. pseudomallei* can survive in cells of *Acanthamoeba* or the dinoflagellate *Alexandrium minutum* or in the spores of the mycorrhizal fungus *Gigaspora decipiens*. This property may contribute to an increased ability of the bacterium to survive in the environmental stress (58-60).

B. pseudomallei is intrinsically resistant to many antibiotics including penicillin, aminoglycosides, rifamycins macrolides, colistin, and first- and second-generation of cephalosporins but is usually susceptible to amoxicillin-clavulanate, doxycyclin, trimethoprim-sulphamethoxazole, ureido-penicillin, ceftazidime and carbapenems (61). *B. pseudomallei* is resistant to macrolide and aminoglycoside antibiotics via a multidrug efflux pump (62). Mutations within the conserved motifs of the beta-lactamase enzyme (enzyme that hydrolyses the cyclic amide bond of beta-lactam antibiotics) also account for the resistance patterns (63). One mechanism of antibiotic resistance is the ability to form biofilm. Resistance of *B. pseudomallei* biofilm to conventional antibiotics explains the lack of success in the treatment of the chronic manifestations of this bacterial infection (64).

The genome of *B. pseudomallei* is composed of two chromosomes, 4.07 and 3.17 Mbp in size with a G+C content of 68% (65). The large chromosome carries many genes associated with core functions such as cell growth and metabolism. The smaller chromosome carries more genes encoding accessory functions that could be associated with adaptation and survival in different environments. Approximately 6% of the genome is made up of putative genomic islands that have probably been acquired through horizontal gene transfer. The molecular epidemiology of *B. pseudomallei* has been investigated using multilocus sequence typing (MLST) and the findings suggest a high rate of genetic recombination (66).

B. pseudomallei had been differentiated into two distinct biotypes by the ability to utilize the pentose sugar L-arabinose as a sole energy source for growth. Almost all clinical isolates of *B. pseudomallei* are unable to assimilate arabinose(Ara^-), while the soil isolated included both Ara^+ and Ara^- biotypes (67). The biotype differences are associated with the disease producing potential of the bacteria. The Ara^+ isolates are much less virulent than the Ara^- isolates in animal models (68). Differences have also been found in 16s rRNA gene sequence from virulent (Ara^-) and avirulent (Ara^+) strains. The characterization by *Bam*HI enzyme showed the different

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ribotypes. Group I ribotype, DNA is digested by *Bam*HI and was found in all clinical isolates and half of the environmental isolates. Group II ribotype, DNA is not digested, found only in the environment (69). Based on the differences in 16s rRNA-encoding gene and the less virulence, the Ara^+ biotype had been classified as a new species called *Burkholderia thailandensis* (38).

2.2 *B. thailandensis*

B. thailandensis is a facultative intracellular, gram-negative bacillus that is closely related to *B. pseudomallei*. It grows on diverse nutrient at temperatures ranging from 25 to 42 °C. The organism is motile by a polar tuft of two of four flagella (38).

The genome of *B. thailandensis* is composed of two chromosomes, 3.8 and 2.7 Mbp in size with a G+C content of 67.6% (65). *B. pseudomallei* and *B. thailandensis* are broadly similar in many genomic levels such as both species display highly conserved genomic synteny, and appear to share an extensive repertoire of genes involved in core metabolism, accessory pathways, structure-based superfamilies and bacterial virulence factors. *B. thailandensis* also produces siderophore, lipase, lecithinase and protease. It is resistant to aminoglycoside but sensitive to tetracycline, ceftazidime and trimethoprim (38). *B. thailandensis* is a soil saprophyte, and until its classification as a distinct species in 1998 was considered to represent a subtype of *B. pseudomallei*.

The significant differences between *B. thailandensis* and *B. pseudomallei*

1) Metabolism

B. thailandensis but not *B. pseudomallei* can utilize L-arabinose (Ara^+) and xylose as the carbon sources (68). This phenotype is consistent with the genomic analysis that *B. thailandensis* contains an eight-gene arabinose assimilation operon on chromosome 2, and a 64 kb region on chromosome 1 encoding several genes involved in xylose metabolism that are absent in *B. pseudomallei* (65).

2) Virulence

B. thailandensis is substantially less virulent. In Syrian hamster, the 50% lethal dose (LD_{50}) for *B. thailandensis* was approximately 10^6 bacteria whereas that for *B. pseudomallei* was only 10 bacteria (38). It has been reported that more than 99%

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of melioidosis cases are caused by *B. pseudomallei*, whereas *B. thailandensis* is only responsible for less than 1% (70).

The important surface-related proteins that could contribute to virulence are fimbriae and surface polysaccharides. Fimbriea are the short pilus structures that allow bacteria to adhere to environmental surfaces and host cells. *B. pseudomallei* has been shown to be more efficient than *B. thailandensis* in adhering to and invading host cells (71). It is consistent with the genomic analysis that *B. pseudomallei* contains twice as many fimbrial gene clusters as *B. thailandensis* (65). One cluster on Chromosome 2 is of particular interest as it contains a fimbrial usher protein, a fimbrial chaperone and two exported proteins with similarity to fimbriae proteins of *Yersinia* spp, whose family member *Y. pestis* is the causative agent of plague. Moreover, it has been reported that *B. pseudomallei* contains a large gene cluster involved in the synthesis and export of capsular polysaccharides and this cluster is absent in *B. thailandensis* (72). In addition to surface molecules, another molecule that might contribute to virulence is secondary metabolites with toxic activities (73). There are four polyketide synthase (PKS) and nonribosomal peptide synthase (NRPS) clusters involved in the production and regulation of secondary metabolites that are present in *B. pseudomallei* but absent in *B. thailandensis*. These clusters contain genes encoding putative resistance proteins, efflux proteins and lipopeptide antibiotic proteins, which could be involved in toxin production. Other difference that may contribute to its low virulence is the absence of a type III secretion system I (TTSS1) in *B. thailandensis* (74).

3) Colonial morphology

On Ashdown's selective agar, *B. thailandensis* colonies are smooth and glossy with pink pigmentations while *B. pseudomallei* colonies are rough and wrinkled with dark purple pigmentations (67).

3. Flagella

3.1 Function and structure

Flagella are long thin appendages free at one end and attached to the cell at the other end that provide the most common mechanism of motility. Flagella are so

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thin (about 20 nm in diameter) that a single flagellum can only be seen with the light microscope after staining with special flagella stain such as Leifson that allows the staining agent to adhere to and coat the thin flagella to increase their diameter. However, the staining procedure is difficult and requires patience and expertise. The other choice is the electron microscope, flagella are easily seen with the electron microscope**.** Based on the location and number of flagella, the bacteria can be divided into three groups; polar, lophotrichous, and peritrichous (75, Figure 1). In polar flagellation, single flagellum is located on one end (monotrichous) or both ends (amphitrichous) of the bacterium. Occasionally a tuft (group) of flagella may arise at one or both ends of the cell, an arrangement called lophotrichous. In peritrichous flagellation, the flagella are distributed all over the surface of the bacterium.

The basic structure of a bacterial flagellum consists of three parts; a basal body, a hook, and a filament (76, Figure 2). The basal body is a motor, anchored in the cytoplasmic membrane and the cell wall, and consists of a central rod and a series of rings surrounding the rod. In gram-negative bacteria, an outer ring, called the L ring, is embedded in the lipopolysaccharide layer. A second ring, called the P ring, is anchored in the peptidoglycan layer. A third set of rings, called the MS and C rings, are located in the cytoplasmic membrane and cytoplasm, respectively. For gram-positive bacteria which lack an outer membrane, only the inner pair of rings is present. Surrounding the inner ring and anchored in the cytoplasmic membrane are a series of motor proteins, called Mot protein. A final set of proteins, called the Fli proteins, function as the motor switch. The hook is located external to the cell and provides the link between the basal body and the filament. The filament is the portion extending into the exterior environment. It is made of protein subunits called flagellin. Flagellin protein folds into a hairpin-like conformation, with the conserved N- and C- terminal domains are inside and responsible for defining the basic filament structure, whist the central variable domain is exposed on the surface. A flagellin subunit can be divided

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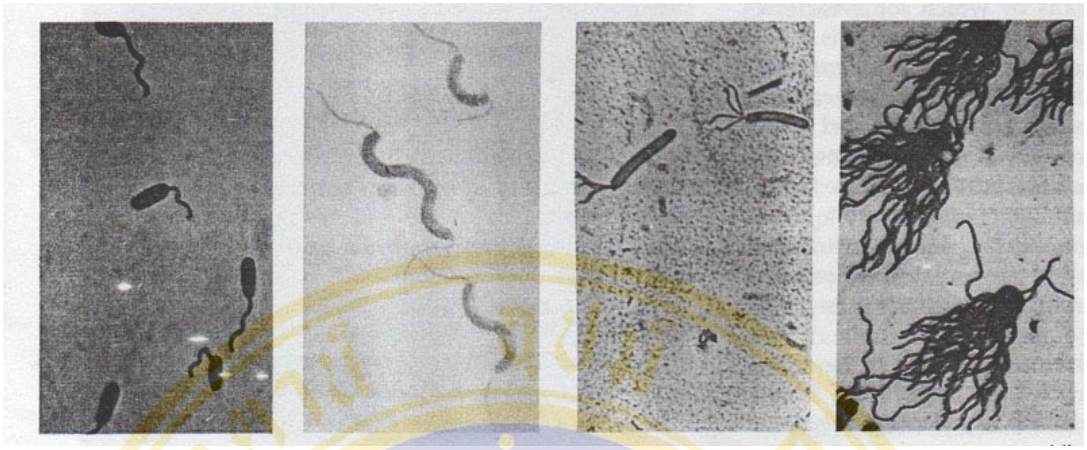

A. **B.** **C.** **D.**

Figure 1. Arrangements of bacterial flagella (75)

The arrangements of bacterial flagella can be divided into 4 groups.

A. Polar, Monotrichous (single flagellum at one end)

B. Polar, Amphitrichous (single flagellum at each end)

C. Lophotrichous (tuft of flagella at one or both ends)

D. Peritrichous (distributed all over)

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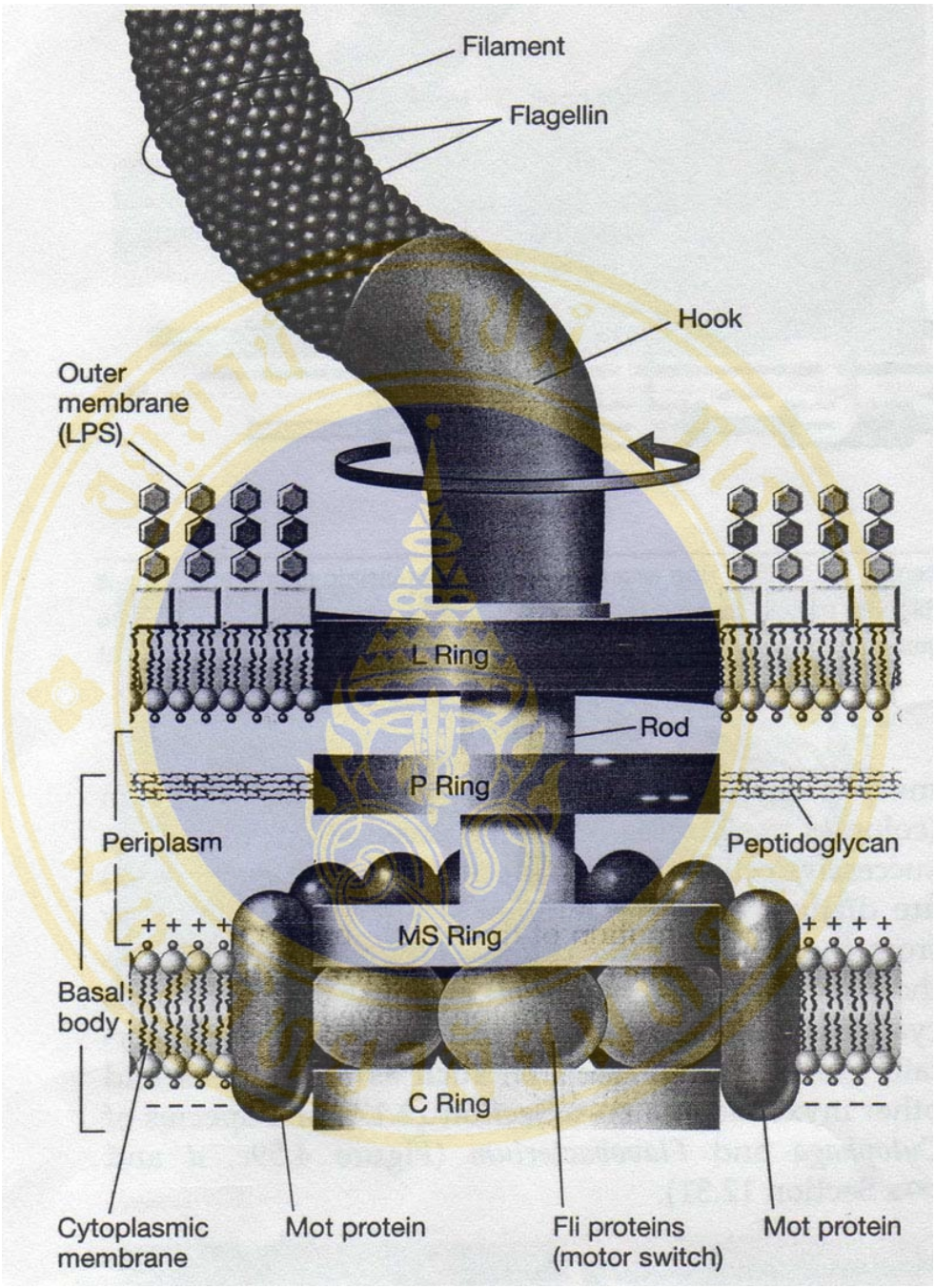

Figure 2. Structure of the prokaryote flagellum in gram-negative bacterium (76)

The flagellum is composed of three basic parts: a basal body, a hook, and a filament.

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into three domains as shown in Figure 3 (77). The innermost domain (D1) has the highest density and is responsible for most of the filament formation. The middle domain (D2) seems to have some interaction with the outermost domain (D3) of the neighbouring subunit in the clockwise direction, but this interaction is not as close as that between domain 1 of neighbouring subunits. Domain 3 is on the filament surface and is presumed to determine the antigenic properties (H antigen). Domain 1 was assigned to the amino (N)- and carboxyl (C)- terminal segments because a proteolytic fragment of flagellin missing portions of the terminal regions cannot polymerize into the filament (77). A part of Domain 2 could be contributing to the filament core because the amino acid replacements which affect filament shape are in domain 2 region and the interspecific sequence homology of this region is as high as that of domain 1 (78). The flagllin subunits form a chain that twists into a helical structure with a hollow core. A section of the filament contains 11 subunits in two helical turns is shown in Figure 3 (79). The overall structure of the filament is shown in Figure 4 (80).

3.2 Flagellar assembly

Flagellar assembly is initiated by the insertion of the MS ring into the cytoplasmic membrane. Then other anchoring proteins are synthesized along with the hook and the cap before filament formation occurs as shown in Figure 5 (76). Flagellin molecules synthesized in the cytoplasm pass up through a 3-nm channel inside the filament and add on at the terminus to form a mature flagellum. At the end of the growing flagellum, a protein called cap exist. Cap proteins assist flagellin molecules that have diffused through the channel to organize at the flagellum termini to form new filament. Growth of the flagellum occurs more-or-less continuously until the flagellum reaches its final length (approximately 20,000 copies are needed to make one filament). Broken flagella still rotate and can be repaired with new flagellin units passing through the filament channel to replace the lost ones.

3.3 Flagellar motility

A majority of motile prokaryotes move by means of rotating flagella. The energy required for rotation of the flagellum comes from the proton motive force. A

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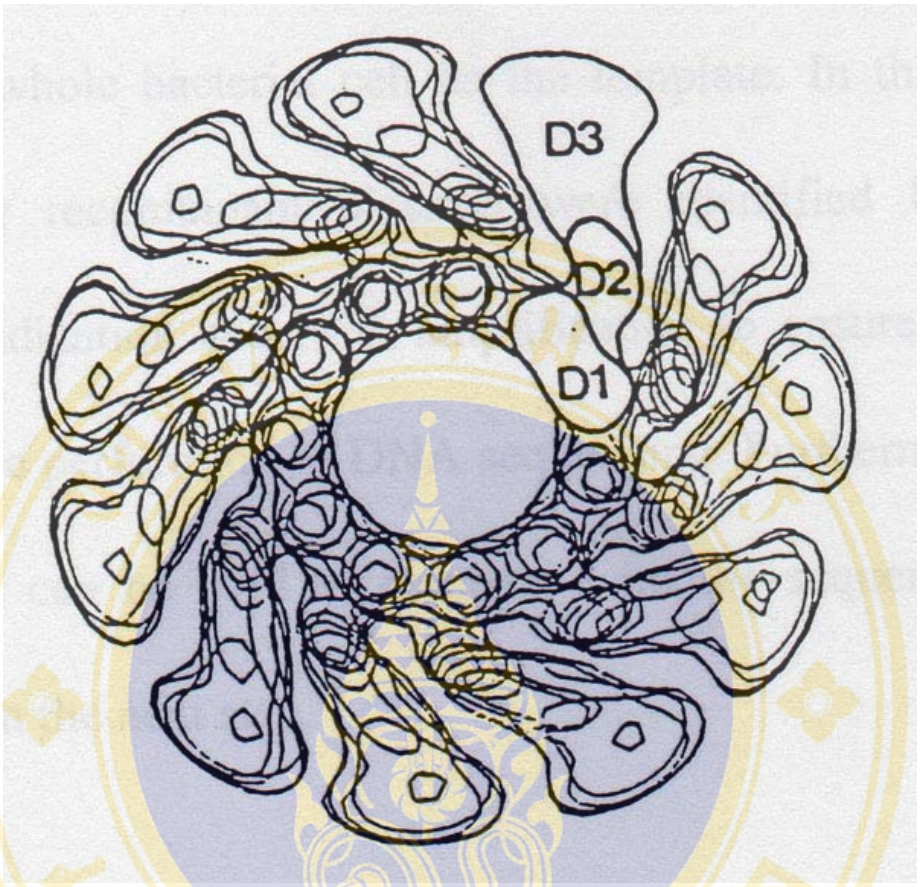

Figure 3. The assignment of a flagellin subunit (79)

A flagellin subunit can be divided into three domains; the innermost domain (D1), the middle domain (D2), and the outermost domain (D3).

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Figure 4. The overall surface structure of the flagellin (80)

The figure is shown as a shaded solid model.

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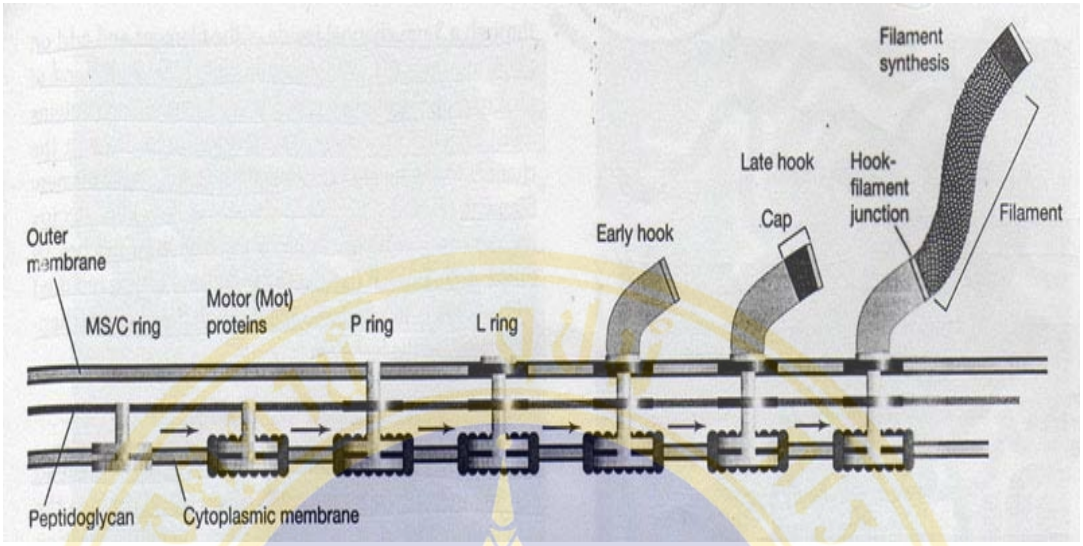

Figure 5. Summary of steps in flagella biosynthesis (76)

Synthesis begins with MS/C rings assembly in the cytoplasmic membrane. This is followed by formation of the other rings, the hook, and the cap. At this point, the flagellin protein flows through the hook to form the filament. Flagellin molecules are guided into position by cap protein to ensure that the growing filament develops evently.

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proton turbine model has been proposed to explain rotation of the flagellum. In this model, protons flowing through channel in mot protein exert electrostatic forces on helically arranged charges on the MS and C rings (76, Figure 6). Attractions between positive and negative charges would cause the basal body to rotate as proton flow. The speed of rotation can increase or decrease in relation to the strength of the proton motive force. Flagella rotation can move bacteria through liquid media at speeds of up to 60 cell lengths per second which is much faster than large organisms.

The motions of polarly flagellated organisms are different from those of peritrichously flagellated organisms (76, Figure 7). For, the peritrichously flagellated bacterium, in the absence of a gradient, the cell moves in a random fashion. When the flagella rotate counterclockwise (CCW), the filaments joint into a bundle that propels the cells in smooth trajectory called run. After a brief period, the direction of rotation of the flagella is reversed to clockwise (CW), the bundle disperses and causes the cell to stop and roll called tumble. Unstimulated cells alternated between two modes of swimming, running in more or less straight line for about a second, then tumbling for a fraction of a second and start in linear motion on a new direction that almost uncorrelated with the original trajectory (76, Figure 8). In the presence of stimulus, bacteria sense and respond by moving in a certain direction, a phenomenon called chemotaxis. Movement toward an attractant is due to runs of longer duration and tumble less frequently when bacteria sense they are moving closer to an attractant (by comparison the chemical or physical state of their environment with that sensed a few second before). In contrast, bacteria tumble more frequently when they sense they are moving closer to a repellent. The situation with polarly flagellated cells is somewhat different. Many polarly flagellated bacteria such as *Pseudomonas* species, CCW rotation of flagella cause cell to move forward, liked peritrichously flagellated bacteria. However, CW rotation**,** the bacterial cell is pulled instead of pushed, and causes cell changes the direction. Some polarly flagellated bacteria such as *Rhodobacter sphaeroides*, have unidirectional flagella that rotate only in a CW direction causing the cell to move forward. When rotation stops, the cell becomes randomly reoriented by Brownian motion. As the flagellum begins to rotate once again, the cell moves again but in a new direction (Figure 7).

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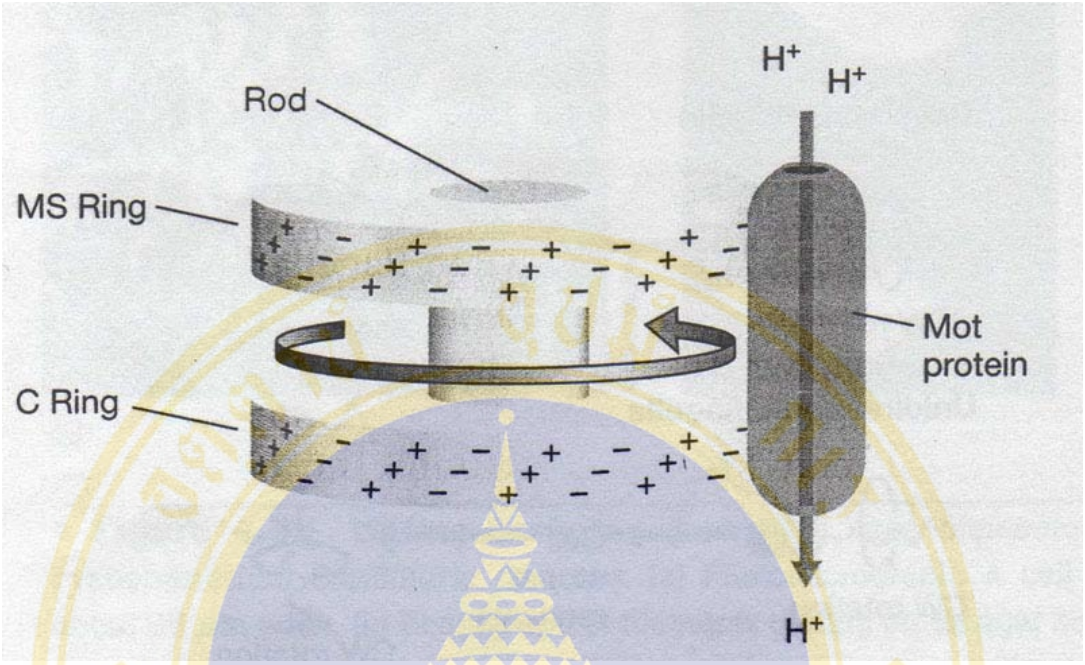

Figure 6. A proton turbine model (76)

Protons, flowing through the Mot proteins, may exert forces on charges present on the C and MS rings, thereby spinning the rotor.

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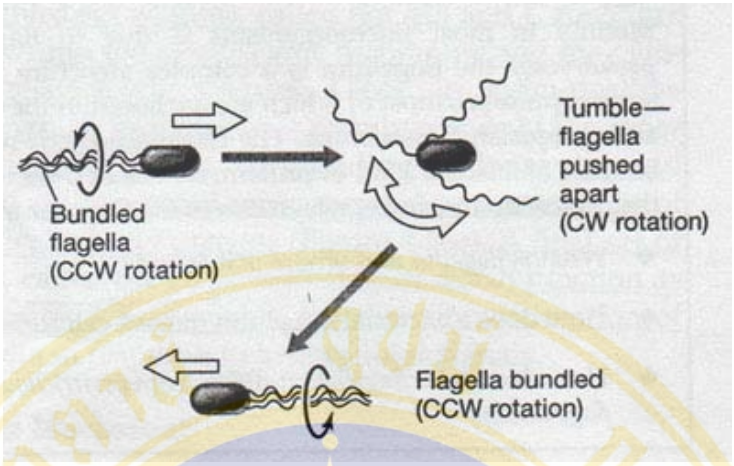
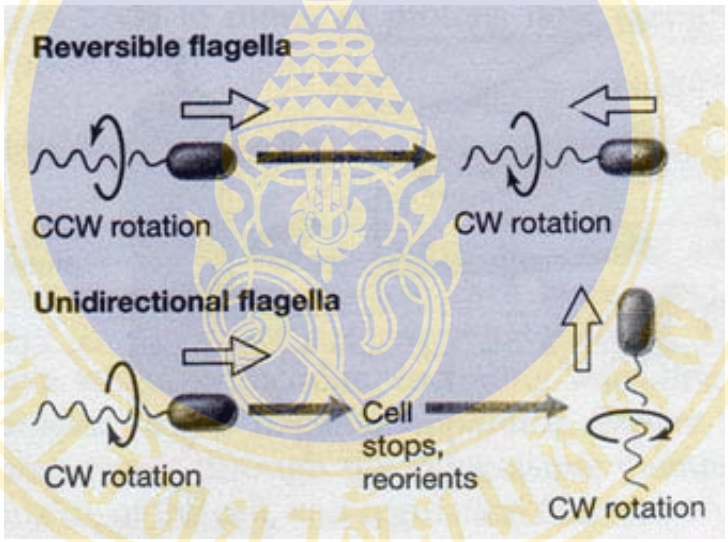


A. Peritrichous



B. Polar

Figure 7. Manner of movement in polarly and peritrichously flagellated bacteria (76)

A. Peritrichous: Forward motion is imparted ay all flagella rotating counterclockwise in a bundle. Clockwise rotation causes the cell to tumble, and then a return to counterclockwise rotation leads the cell off in a new direction.

B. Polar: Cells change direction by reversing flagellar rotation, or in unidirectional flagella, by stopping periodically to reorient, and then moving forward by clockwise rotation of its flagella. The arrows show the direction the cells traveling.

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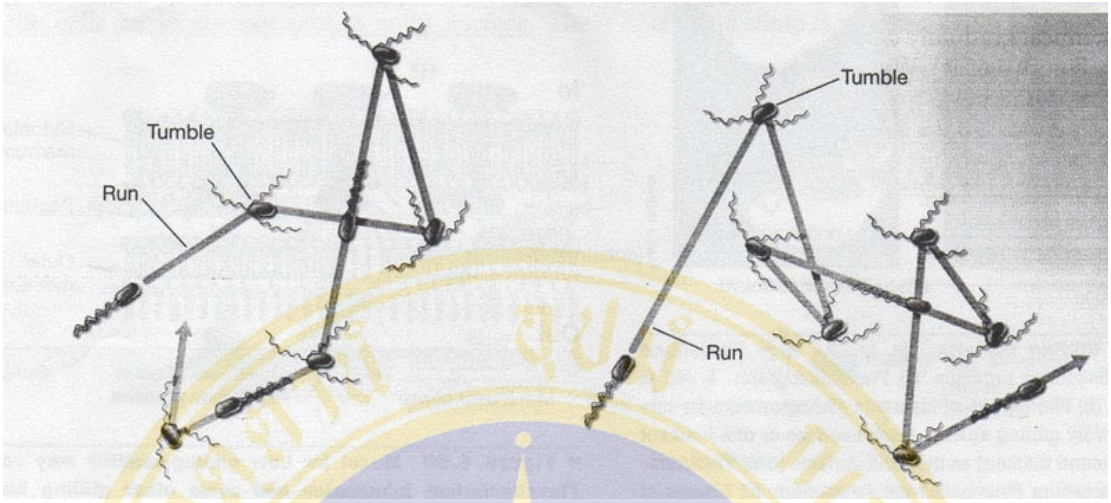

A. No attractant **B. Attractant present**

Figure 8. Chemotaxis in bacteria (76)

A. In the absence of a chemical attractant, the cell swims randomly in the runs, changing direction during tumbles.

B. In the presence of an attractant, the runs become biased, and the cell moves up the gradient of the attractant.

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The mechanism of chemotaxis involves sensory proteins in cytoplasmic membrane, called chemoreceptors. These chemoreceptors sense the chemical gradient over time and interact with cytoplasmic proteins to affect flagellar motor direction.

3.4 *B. pseudomallei* flagellin

B. pseudomallei flagellar filaments compose of a single subunit type of flagellins. SDS-PAGE analysis of the purified extracts yielded a flagellin monomer with an apparent M_r of 43,400 (42). Liked other bacteria, *Burkholderia* flagellins have a distinctive dominal structure comprising conserved N- and C- terminal regions and the central variable region that may vary considerably in both amino acid sequence and size. A flagellin protein composes of about 394 amino acid residues with 40% of the relative hydrophobicity (42). The structural flagellin gene is approximately 1.1 kb in length with about 65% of GC-content. Sequencing of the *B. pseudomallei* flagellin gene indicates that it is highly conserved among clinical isolates. *B. thailandensis* and *B. pseudomallei* exhibit different sequence within the variable domain of flagellin gene by a 15-bp deletion (4). A 15-bp deletion conserved in the flagellin gene of the *B. thailandensis* can be observed on 3.5% agarose gel electrophoresis (4). The deletion was not present in any of the *B. pseudomallei* isolates. This 15-bp sequence encodes for 5 amino acids (two of Serine, one of each Proline, Phenylalanine and Glutamine) at position 247-251 (from total 387 amino acids in length) of the flagellin polypeptide (Figure 9). This is indicative that the flagellin gene sequences of *B. pseudomallei* isolates are stable and related to arabinose assimilating property.

4. Virulence factors

Virulence factor denotes a bacterial product or strategy that contributes to virulence or pathogenicity. The virulence factors may be common to all bacteria of a genus or species, or may be unique to only certain strains. These factors have their roles in many steps for infection as adhesion, colonization, invasion, survival and growth in the host.

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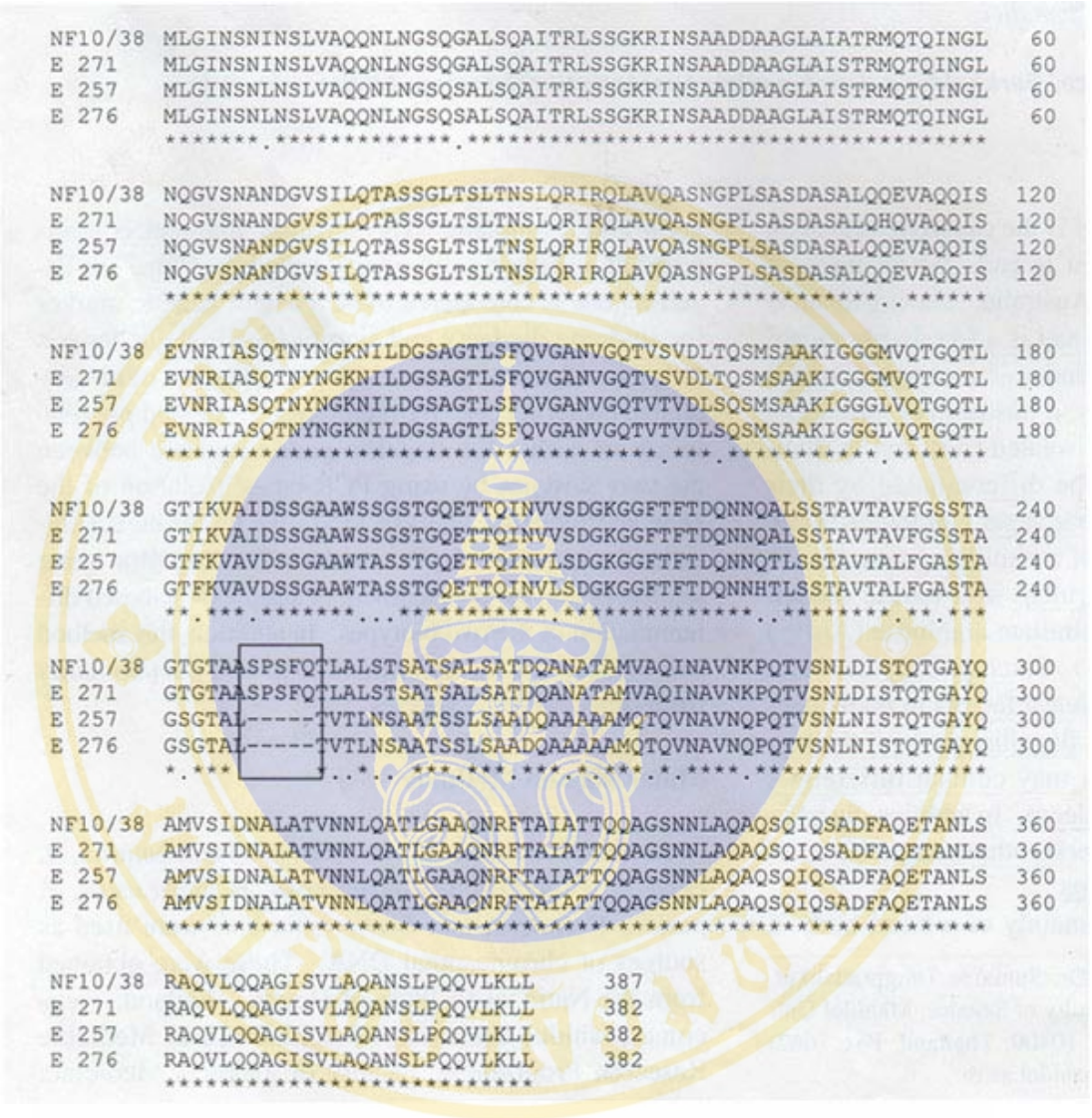

Figure 9. Alignment of the amino acid sequences of *Burkholderia* flagellin (4)

The figure shows the alignment of the amino acid sequences of *B. pseudomallei* flagellin from clinical isolate NF10/38 (Ara-), and environmental isolated E271 (Ara-), E257 (Ara+), and E276 (Ara+). Each of the identical amino acids is indicated by an asterisk, similar amino acids are marked by a dot and the distinctive sequence deletion is boxed.

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4.1 Flagella

Flagella are commonly recognized as an important virulence determinant expressed by bacterial pathogens since the motility phenotype with impaired flagella often correlated with the ability of an organism to cause disease. A nonmotile mutant of *Burkholderia cepacia* was found to be less invasive for lung epithelial cells (81). It has been suggested that *B. cepacia* might use flagellum-mediated motility to facilitate penetration of the viscous mucus layer in cystic fibrosis lung airways in order to establish contact with the underlying epithelial tissues. Another function of flagella that facilitates infection is their role as an adhesin. In *Pseudomonas aeruginosa*, flagellin protein was shown to act like an adhesin which is responsible for the binding of bacteria to Muc1, a mucin secreated by respiratory epithelial cells (82). For *Clostridium difficile*, there appear to be a receptor specific for flagella in the mucus of murine intestines that facilitates colonization and establishment of an infection in the gut (83). However, in *B. pseudomallei*, it has been reported that the wild type and nonmotile flagellin mutant of *B. pseudomallei* were equally invasion. Thus, flagella are not essential for entry of *B. pseudomallei* into the lung epithelial cells during in vitro assays. However, flagella are an important and necessary virulence determinant of *B. pseudomallei* during intranasal and intraperitoneal infection of mice by means of motility function or adhesion or both (8).

4.2 Adhesin

Adhesins are protein or polysaccharides on the surface of bacterial cells that bind bacteria to the host cells. One of the cell-surface structures responsible for adherence is pili (or fimbriae). Pili are rod-shaped protein structures that extend outward from the bacterial surface and act to establish contact with the surface of the host cell. Host cell receptors for pili are commonly carbohydrate residues of glycoproteins or glycolipids. Binding of a pilus to its host cell target is quite specific. Pili allow the bacteria to make an initial loose contact with a host cell surface, then followed by a tighter binding involving surface proteins such as afimbrial adhesins that bring the bacterial and host cell surfaces together (84). Afimbrail adhesins recognize protein on host cell surface rather than carbohydrate and allow the bacteria to attach to and invade host cell. Some pathogenic bacteria produce different adhesins

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at different times, having a repertoire of adhesins which allows the pathogen to attach to more than one cell type. In *B. pseudomallei*, a *pilA* (encoding a putative pilus structure protein) deletion mutant has been shown to have reduced adherence to human epithelial cells and it is also less virulent in the nematode model of virulence and the murine model of melioidosis (85).

4.3 Lipopolysaccharide (LPS)

LPS is the endotoxin from gram-negative bacterial outer membrane. It composes of lipid A, polysaccharide core and repeated polysaccharide side chains (O antigen). *B. pseudomallei* LPS has been reported to differ in several aspects from the LPS of other gram-negative bacteria, such as it exhibits weaker pyrogenicactivity in rodents than enterobacterial LPS but stronger mitogen activity in murine splenocytes (86). Moreover, LPS-mediated activation of a mouse-macrophage cell line is slower for *B. pseudomallei* LPS compared with *E. coli* LPS (87). It has been reported that the O-polysaccharide moiety of *B. pseudomallei* LPS interfered with the stimulation of macrophage bactericidal activities by decreasing the bacterial uptake (88).

4.4 Capsular polysaccharide

A capsule is a loose, unstructured network of polymers that covers the surface of a bacterium. Capsule is composed polysaccharide or protein or protein-carbohydrate mixture. Capsular polysaccharide appears to have a role in environmental protection, immune system evasion, and attachment to epithelial cells (89, 90). It is also found to be required for virulence of *B. pseudomallei* in the Syrian hamster model of acute melioidosis because the mutant strain lacking the capsule was 10,000-fold more attenuated for virulence than the wild-type strain (72). Capsule production by *B. pseudomallei* contributes to its persistence in the blood through the reduction of complement factor C3b deposition on the surface of the bacterium and phagocytosis (91). The presence of the capsule enables *B. pseudomallei* to survive in the blood and spread to other organs, which may result in the overwhelming septicemia in melioidosis patients.

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4.5 Type three secretion system (TTSS)

Type III secretion system is one of the mechanisms which bacteria use to export proteins from the cell. *B. pseudomallei* contains three TTSS gene clusters. The TTSS1 gene cluster is homologous to a TTSS found in the plant pathogen *Ralstonia solanacearum* but is absent from *B. thailandensis* (74). In *B. thailandensis*, there are six Bt-unique genes instead of the TTSS1 cluster. The GC content of the genes is not different from the neighboring genes, suggesting that these genes are indigenous. In contrast, the genes of TTSS1 in *B. pseudomallei* have a distinctly lower GC content indicating that they may have been recently acquired. TTSS2 and TTSS3 are present in both *B. pseudomallei* and *B. thailandensis*. The role of TTSS1 and TTSS2 in pathogenesis is not known. TTSS3 shares homology with TTSS of *Salmonella typhimurium* and *Shigella flexneri* (74, 93). The TTSS3 cluster encodes a secretion apparatus that functions like a molecular syringe. A subset of type III secreted proteins (translocators) interact with the eukaryotic cell membrane and inoculate type III secreted effecter proteins into the target-cell cytosol in order to facilitate invasion, survival in phagosomes, intercellular spread and pathogenesis (93). Bacterial products secreted by this TTSS (termed Bsa, *Burkholderia* secretion apparatus), including BopE protein (a TTSS protein that is encoded adjacent to the *B. pseudomallei bsa* locus and is homologous to *Salmonella typhimurium* SopE) induce cytoskeleton rearrangements to facilitate host cell invasion (94). *B. pseudomallei* mutants the Bsa and BopE system are confined to the endosome and are unable to gain access to cell actin, suggesting that this system is also important in mediating endosomal membrane lysis (94). Both *B. pseudomallei* and *B. thailandensis* share TTSS3, which is required for the full virulence of *B. pseudomallei* in a hamster model of infection (92). However, it has been reported that arabinose exposure may downregulate TTSS3 expression and activity (95). The absence of an arabinose assimilation operon in *B. pseudomallei* might thus have contributed to the increased virulence of this species.

5. Intracellular survival of *B. pseudomallei*

B. pseudomallei can survive and multiply inside both cultured phagocytic and nonphagocitic cells by using many mechanisms to escape macrophage killing and

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evade host immunity. These include resistance to human defensins and inhibition of DNA and protein synthesis within the host cell (96). Ultrastructural studies suggested that *B. pseudomallei* might reside within membrane-bound compartments, particularly phagolysosomes, making use of its ability to survive and grow in acidic environments (97, 98). The two major microbicidal mechanisms for phagocytic cells are reactive oxygen intermediates (ROI), consisting of superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen, and reactive nitrogen intermediates (RNI), such as nitric oxide (NO). The killing mechanisms of IFN-γ activated macrophages are mediated by one or more of these oxidants. The contributions of ROI and RNI may differ from one pathogen to another. In *B. pseudomallei*, the RNI pathway seemed to be more marked than that of ROI (99). It has been reported that *B. pseudomallei* is able to invade mouse macrophages without activating inducible nitric oxide synthase (iNOS) production (100). This enzyme is known to activate NO production which plays an essential role in controlling the intracellular survival and multiplication of *B. pseudomallei*. The expression of iNOS was markedly enhanced when macrophages were preactivated with IFN-γ or exposed to microbial products, e.g., LPS, leading to production of IFN-β (100, 101). IFN-β served in an autocrine fashion, mediating interferon regulatory factor 1(IRF-1) production (101). The expression of this transcriptional activator and the presence of a crucial IRF-1 binding site within the promoter of the iNOS gene are necessary for the induction of iNOS in murine macrophage (102, 103). *B. pseudomallei* minimally activated IFN-β production, which led to a low level of IRF-1 in the macrophage, in parallel with poor iNOS expression, thus allowing the bacteria to survive inside the cells (104). *B. pseudomallei* can escape from endocytic vesicles into the cytoplasm by lysing the endosome membranes as early as 15 minutes after internalization by phagocytic cells (93, 105, 106). Moreover, intracellular *B. pseudomallei* can induce polymerization of actin at one pole of the bacterial cell, which results in the formation of membrane protrusions in host cells with the bacteria at the tip (107, 108). These protrusions can project into adjacent cells, thus facilitating the spread *B. pseudomallei* from one eukaryotic cell to another (107). A *B. pseudomallei*-specific protein termed BimA is required for the formation of actin tails. BimA is located at the pole of the bacterial cell where actin polymerization occurs (109). Mutation of the *bimA* gene in *B. pseudomallei* abolished

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actin-based motility of intracellular bacteria in a macrophage-like cell line. Actin-tail formation could be restored by inducible expression of the *bimA* gene on a plasmid, indicating the essential role for this gene in actin-tail formation. Multinucleated giant cells are occasionally seen in human tissue infected with *B. pseudomallei* and might represent giant macrophages (110). It is possible that macrophages are the site of intracellular survival, given their relatively long half-life and reduced microbicidal capacity in comparison to neutrophils and monocytes (97). *B. pseudomallei* might evade killing by macrophages through the induction of caspase-1-dependent host-cell death; this process requires a functional *bsa* TTSS (111). Cell death was accompanied by the release of IL-1β and IL-18 (111). Survival within microcolonies encapsulated in a protective biofilm is an alternative explanation for prolonged quiescent survival within the host.

B. thailandensis can also survive and multiply inside both cultured phagocytic and nonphagocitic cells, and can induce cell fusion and MNGC formation. However, these phenomena were found at a lower rate and to a much lesser extent (107).

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CHAPTER III
MATERIALS AND METHODS

MATERIALS

1. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. *B. pseudomallei* 1026b, MM35, and *B. thailandensis* E257 were from Wellcome-Mahidol University-Oxford Tropical Medicine Research Program, Faculty of Tropical Medicine, Mahidol University, Thailand. *E. coli* DH5αλpir and pUCP28T plasmid were obtained from Department of Biochemistry, Faculty of Medicine, National University of Singapore, Singapore.

2. Cell culture

2.1 Mouse macrophage cell line (RAW 264.7) was obtained from American Type Culture Collection.

2.2 Human alveolar lung epithelial cell line (A549) was obtained from American Type Culture Collection.

3. Oligonucleotide primers

The oligonocleotide primers used in this study were designed for the full- length of flagellin gene and its promoter of *B. pseudomallei* 1026b and *B. thailandensis* E257. The primers were supplied from the BioService Unit (BSU), National Science and Technology Development Agency (NSTDA), Thailand.

Primer : Promoter1F 5′ AATTCAGCCCGCCATCATAA 3′

Primer : FliCSTSacI 5′ GGTGGAGCTCTTATTGCAGGAGCTTCAG 3′

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Table 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Reference
Bacterial strains		
B. pseudomallei		
1026b	wild type, clinical isolate, Sm^r , Tc^s	112
MM35	non-motile, derivative of 1026b with Tn5-OT182 mutagenesis of the *fliC* gene, Sm^r , Tc^r	112
Cp	complementation of MM35 with plasmid pUCP28TCp, Sm^r , Tc^r , Tp^r	this study
Ct	complementation of MM35 with plasmid pUCP28TCt, Sm^r , Tc^r , Tp^r	this study
B. thailandensis		
E257	wild type, environmental isolate	4
Escherichia coli		
DH5αλpir	λpir lysogen of DH5α for replication of *ori*R6K, *ori*T and *mob* region of RP4	N. Judson, GIBCO-BRL
Plasmids		
pUCP28T	Broad-host-range vector, *ori*T and *mob* region from RP4, Tp^r (Figure10)	113
pUCP28TCp	pUCP28T contains the full-length *fliC* gene and promoter of *B. pseudomallei* 1026b	this study
pUCP28TCt	pUCP28T contains the full-length *fliC* gene and promoter of *B. thailandensis* E257	this study

r = resistant, s = sensitive, Sm = streptomycin, Tc = Tetracyclin, Tp = Trimethoprim

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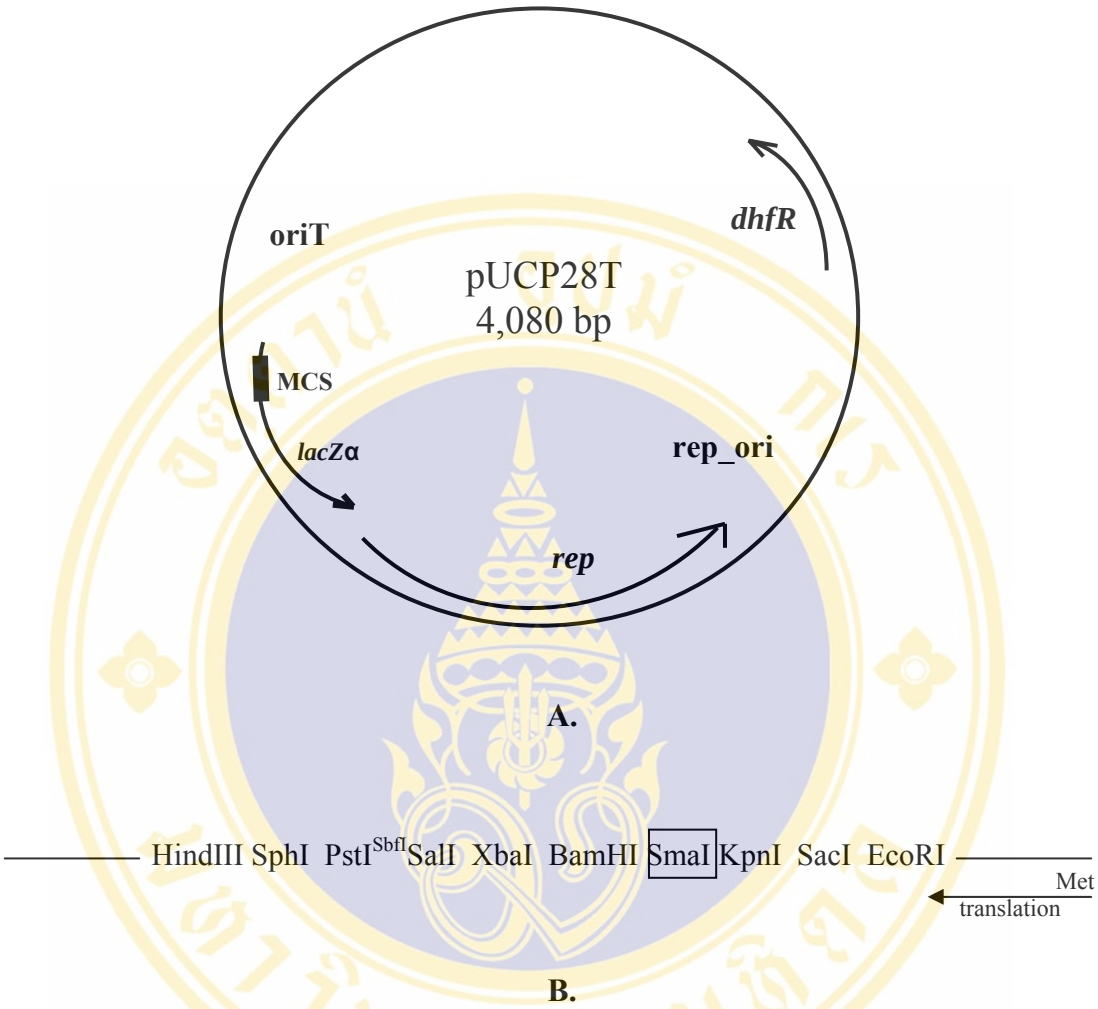

Figure 10. Map of the pUCP28T plasmid

A. The pUCP28T vector containing the *dhfR*II gene encoded for dihydrofolate reductase (trimethoprim resistant DHFR), rep_ori (the origin of replication), *rep* gene encoded for replication protein, *LacZα* gene containing multiple cloning site (MCS) for screening the insertion using α-complementation, and oriT (origin of transfer).

B. Restriction endonuclease sites in MCS of the *LacZα* gene, *Sma*I (in box) enzyme was used to digest the plasmid, generating the linearized plasmid. Arrow shows the translation direction.

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Promoter 1F

5′ aattcagccc gccatcataa caggtaaacg tcggctcgac cagtggttta 50
ccgcttttcc gcgcgggcga tccgtgtcca cgcccgttcg cgccgccttg 100
cgggccgccg gacggcgcgc cgtccgcatg cgcgattcgt gcgcatccaa 150
cgttggctca ccgaacgatc gacacaaaaa attcgggccg aatctcgccc 200
tgccccctca agttttttg gcgtacgccg aaatcctgca caaggcggcc 250
ggcaaagaac gagtgcttcc gcccgcgcca atagtggcat tgatgggctt 300
atacagctca atttggagat tttcATGctc ggaatcaaca gcaacattaa 350
ctcgttggtc gctcaacaga acctcaacgg ctcgcaaggc gccctgtccc 400
aagcgatcac ccgcctgtcg tcgggcaagc gcatcaacag cgcggcggac 450
gatgcggccg gcctcgcgat cgccacccgg atgcaaacgc agatcaacgg 500
cctgaaccag ggcgtgtcga acgcgaacga cggcgtgtcg atcctgcaaa 550
cggcatcgag cggcctgacc tcgctcacca acagcctgca gcgtatccgc 600
cagctcgccg tgcaggcctc gaacggcccg ctgagcgcga gcgacgcgtc 650
ggcgctgcaa caggaagtcg cgcagcagat ctcggaagtg aaccgtatcg 700
cttcgcagac gaactacaac ggcaagaaca tcctcgacgg ctcggcaggc 750
acgctgagct tccaggtcgg cgcgaacgtc ggccagacgg tctccgtcga 800
cctcacgcaa agcatgtcgg cggcgaagat cggcggcggc atggttcaga 850
cgggccagac gctcggcacg atcaaggtgg cgatcgactc gagcggcgcg 900
gcctggtcgt cgggcagcac cggccaggag acgacgcaga tcaacgtcgt 950
gtcggacggc aagggcggct tcacgttcac cgatcagaac aaccaggcgc 1000
tgtcgtcgac ggccgtgacc gccgtgttcg gctcgtcgac cgccggcacg 1050
ggcacggcgg cc*tcgccgtc gttccag*acg ctggcgctgt cgacttcggc 1100
aaccagcgcg ctgtccgcga ccgaccaggc gaacgccacg gcgatggttg 1150
cgcagatcaa cgcggtcaac aagccgcaaa cggtctcgaa cctcgacatc 1200
agcacgcaga cgggcgcgta ccaggcgatg gtatcgatcg acaacgcact 1250
cgcgacggtc aacaatctgc aggcaacgct cggcgcggcg caaaaccgct 1300
tcaccgcgat cgcgacgacg cagcaagccg gctcgaacaa cctcgcgcag 1350
gcgcaatcgc aaatccagag cgcggacttt gctcaggaaa ccgcgaacct 1400
gtcgcgcgcg caagtgctcc agcaagccgg catctcggtg ctcgcgcaag 1450
cgaactcgct gccgcagcaa gtgctgaagc tcctgcaaTA Acgtgtaccg 3′ 1500

FliCSTSacI

Figure 11. The full-length flagellin gene sequence of *B. pseudomallei*

The map of *B. pseudomallei fliC* gene composes of the binding sites for primer Promoter 1F and FliCSTSacI as underline, a 15-bp deletion site in *B. thailandensis* as indicated in the italic alphabets, ATG is the start codon, and TAA is the stop codon.

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The end of FliCSTSacI primer has *Sac*I enzyme cutting site.

4. Restriction Endonuclease

The plasmid DNA digestions with restriction enzymes were carried out according to the manufacturer's recommendation. The restriction enzymes used in this experiment including their sequences recognition and optimum temperature are shown in Table 2.

METHODS

1. Bacterial culture condition

B. pseudomallei, *B. thailandensis* and *E. coli* were grown at 37 °C in Luria-Bertani (LB) broth or on LB agar overnight. When appropriate, antimicrobial agents were added at the following concentrations: 25 µg of trimethoprim per ml for *E. coli*, and 50 µg of tetracycline, 100 µg of trimethoprim per ml for *B. pseudomallei*.

2. Preparation of genomic DNA (gDNA)

2.1 Genomic DNA extraction

The gDNA of *B. pseudomallei* 1026b and *B. thailandensis* E257 were isolated by phenol-chloroform extraction method. Single colony was picked up and cultured in LB broth overnight at 37 °C with shaking at 150 rpm. The cells were harvested in a microcentrifuge tube by centrifugation at 10,000 rpm for 2 min. The pellet was resuspened in 100 µl of lysozyme solution (appendix) and incubated at 37 °C for 1 h. The cells were lysed with 100 µl of lysis solution at 37 °C for 1 h. Before the lysate was added 2 µl of 10 mg/ml RNaseA and incubated at 37 °C for 1 h. The protein was removed from the gDNA by extraction with equal volume of phenol. After centrifugation at 10,000 rpm for 10 min, the upper phase was kept and extracted twice with equal volume of phenol:chloroform (1:1 v/v). The gDNA solution was precipitated with 1/10 volume of 3 M sodium acetate pH 5.5 and 2 volumes of cold

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Table 2. Restriction enzymes

Restriction enzyme	Recognition sequence	Incubation temperature (°C)	Company
*BamH*I	5′ G▼GATCC	37	Promega
*Hind*III	5′ A▼AGCTT	37	Promega
*Nar*I	5′ GG▼CGCC	37	Promega
*Pst*I	5′ CTGCA▼G	37	Promega
*Sac*I	5′ GAGCT▼C	37	Promega
*Sma*I	5′ CCC▼GGG	25	Promega
*Xho*I	5′ C▼TCGAG	37	Takara

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absolute ethanol at least 1 h at -20 °C. The pellet was collected by centrifugation at 10,000 rpm for 10 min, washed with cold 70% ethanol and again pelleted by centrifugation. The dried-gDNA pellet was dissolved in TE buffer pH 8.0 and stored at 4 °C. The gDNA was further analyzed by agarose gel electrophoresis and UV spectrophotometry.

2.2 Genomic DNA measurement

Two methods are widely used to measure the amount of nucleic acid in a preparation. If the sample is pure without significant amounts of contaminants such as protein and phenol, spectrophotometric measurement using UV irradiation is simple and accurate. If the quantity of DNA is very small or if the sample is impure, the amount of nucleic acid can be estimated from the intensity of fluorescence emitted by ethidium bromide.

2.2.1 Spectrophotometry

The concentration of gDNA was measured using UV spectrophotometer (UV-160; Shimadzu) with the dilution of 1:100 and measured absorbance at 260 (OD_{260}) nm and 280 (OD_{280}) nm. The value of OD_{260} was used for DNA quantification. An OD of 1 corresponds to ~50 µg/ml for double-stranded DNA or 40 µg/ml for RNA. The lower limit of detection by spectrophotometry is generally about 0.5 µg of nucleic acid. All bases that comprise nucleic acids absorb light in the UV range with purine bases generally absorbing more strongly than pyrimidines. Absorbance, as measured by spectrophotometry, is a function of the molar extinction coefficient of the base which defined by the Beer-Lambert Law. The OD_{280} was used for measurement of the protein contamination because aromatic amino acids absorb strongly at 280 nm. The OD_{260}/OD_{280} ratio was used as a measure of purity of isolated nucleic acids. Pure preparation of DNA has OD_{260}:OD_{280} value of 1.8. If there is significant contamination with protein or phenol, the OD_{260}:OD_{280} will be less than the value given above.

2.2.2 Agarose gel electrophoresis

This method is a standard technique used to separate, identify, and purify DNA fraction. Agarose gel can be prepared in various concentrations depended on the molecular size of DNA. The agarose was dissolved in TBE buffer and heated.

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The warm agarose solution was poured into the chamber set with a comb of a desired thickness and number of wells, then allowed to harden. The DNA sample and DNA marker (containing loading dye) were added to the wells. Electrophoresis was carried out at constant voltage of 80-120 volts at room temperature until the tracking dye moved to near the edge of the gel. After electrophoresis, the gel was stained with 0.5 µg/ml ethidium bromide solution and destained in distilled water. The DNA pattern was visualized under a long wavelength UV transilluminator.

3. Plasmid extraction by alkaline lysis method

The bacterial cells which carried the plasmid were cultured in LB broth supplemented with appropriate antibiotic overnight at 37 °C. The cells were harvested by centrifugation at 10,000 rpm for 2 min. The pellet was resuspened in 100 µl of GTE solution and 200 µl of lysis solution. Then 150 µl of neutralize solution was added into the mixture before centrifugation at 10,000 rpm for 10 min. The supernatant was mixed with 2 volumes of cold absolute ethanol, kept at -20 °C for 1-2 h or at -80 °C for 5 min. The plasmid pellet was collected by centrifugation at 10,000 rpm for 10 min, washed with cold 70% ethanol and pelleted by centrifugation. The dried-plasmid pellet was dissolved in TE buffer pH 8.0 and stored at 4 °C.

4. Purification of plasmid DNA by QIAGEN plasmid Midi Kit

The plasmid purification by QIAGEN plasmid Midi kit based on the binding of the plasmid to a QIAGEN anion exchange resin column. The culture was grown to a cell density of approximately 1×10^9 cells/ml (OD_{600} = 1.0) and harvested by centrifugation at 3,000x g for 15 min at 4 °C. The bacterial cells were resuspended in 4 ml of Buffer P1. To lyse the bacterial cells, 4 ml of Buffer P2 was added, mixed gently and incubated at room temperature for 5 min. The lysate was neutralized by adding 4 ml of chilled Buffer P3, mixed and incubated on ice for 15 min. The mixture was mixed again and centrifuged at 12,000 rpm for 30 min at 4 °C. The clear supernatant was separated and loaded onto a QIAGEN-tip. Before loading the supernatant, the QIAGEN-tip was equilibrated by adding 4 ml of Buffer QBT and allowed to empty by

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gravity flow. After the supernatant loading, the QIAGEN-tip was washed with 10 ml of Buffer QC to remove contamination. The plasmid was eluted by adding 5 ml of buffer QF onto the QIAGEN-tip. The eluted plasmid was precipitated by adding 0.7x volume of room temperature isopropanol and the mixture was centrifuged immediately at 12,000 rpm for 30 min at 4 °C. The plasmid was washed with 70% ethanol, air-dried and resuspended in TE buffer.

5. Preparation of full-length *fliC* and its promoter

5.1 Amplification of the *fliC* gene by polymerase chain reaction (PCR)

The PCR reaction was performed in 50 µl of the mixture containing 100-200 ng of DNA template, 0.6 µM of each primer, 200 µM of dNTPs, 1X thermopolymerase buffer with 1.5 mM $MgCl_2$, 1 unit of *Vent* DNA polymerase (New England Biolabs). The PCR conditions were 1 cycle of denaturation at 99°C for 5 min and 94 °C for 4 min, 30 cycles of 94 °C for 1 min, 56 °C for 30 s, and 72 °C for 2 min, followed by 72 °C for 15 min. The PCR products were analyzed by agarose gel electrophoresis and were purified by GENECLEAN II Kit.

5.2 Recovery of DNA fragment from agarose gel by GENECLEAN II

The desired DNA band was excised from ethidium bromide-stained TAE agarose gel while under long wavelength UV light illumination and transferred into a microcentrifuge tube. Three volumes of 6 M NaI were added to the tube, incubated at 50 °C for 5 min or until TAE agarose gel completely dissolved. The solution was mixed with 5 µl of glassmilk suspension (silica gel). The amount of glassmilk added depended on the DNA binding capacity of silica matrix (1-2 µg DNA per 1 µl of glassmilk suspension). The tube was incubated at room temperature for at least 5 min to allow binding of DNA to the silica matrix. To remove NaI, the silica matrix with the bound DNA was collected by centrifugation at 10,000 rpm for 5 sec before washing three times with new wash solution. The DNA was eluted from the matrix by adding TE buffer before centrifugation for 30 sec. The supernatant containing the DNA was transferred to a new tube and stored at 4 °C.

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6. Preparation of the competent cells for electroporation technique

E. coli DH5αλpir was cultured overnight in LB broth. The bacterial culture was subcultured by inoculating with 1% of overnight culture medium into new LB medium and cultured further until the OD_{600} reached 0.3-0.4. The cells were stored in microcentrifuge tube.

7. Construction of the *fliC* pUCP28TCp and pUCP28TCt

7.1 Preparation of *Sma*I pUCP28T

The pUCP28T plasmid was extracted by using plasmid extraction kit (QIAGEN), before digestion with *Sma*I enzyme to generate the blunt end linearized plasmid. The reaction consisted of 500 ng of plasmid DNA, 1X buffer J, 10 units of *Sma*I and sterile distilled water to a final volume of 10 µl. The enzyme reaction was performed at 25 °C for 2 h. The digested plasmid was checked size by agarose gel electrophoresis.

7.2 DNA ligation

*Sma*I pUCP28T and purified full-length *fliC* were ligated by using T4 DNA ligase (Promega). The 10-µl reaction consisted of plasmid and inserted gene at molar ratio of 1:3, T4 DNA ligase buffer and 1 µl of T4 DNA ligase. The reaction was performed at 16 °C for 16 h.

7.3 Transformation of the recombinant plasmids to *E. coli* DH5αλpir by electroporation technique

The 3 µl-recombinant plasmid (100ng) was mixed with the 100-µl of competent *E. coli* DH5αλpir cell before transferring to the cold cuvette. To allow the plasmid attach to the bacterial membrane, the mixture was chilled on ice for 20 minutes before electroporation with 1.8 kV, 3 mS by Bio-RAD MicroPulser machine. The 800-µl of the warm SOC medium was immediately added to the cuvette and mixed. The mixture was transferred into the culture tube and incubated at 37 °C for 1 h to allow the bacterial cells to recover and express the antibiotic resistance marker

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encoded by the plasmid. The culture medium was spread onto LB agar plates supplemented with 25 µg/ml of trimethoprim, 50µl of 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), and 50 µl of 100 mM isopropylthio-β-D-galactoside (IPTG). The plates were incubated at 37 °C overnight.

7.4 Identification of *E. coli* DH5αλpir containing the recombinant plasmid

The pUCP28T vector carries the gene that encodes the amino-terminal fragment of β-galactosidase (*lacZ*) which is able to α-complementation with a defective form of β-galactosidase encoded by the host. This association generates an enzymatically active protein. The Lac^+ bacteria obtained from α-complementation can be easily recognized as they form blue colonies in the presence of chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and inducer isopropylthio-β-D-galactoside (IPTG). However, insertion of a 1.5 kb of full-length *fliC* gene and its promoter into the multiple cloning sites (MCS) of the pUCP28T almost invariably results in production of the amino-terminal fragment that is not capable of α-complementation. The bacteria *E. coli* DH5αλpir carrying the recombinant plasmid pUCP28TCp or pUCP28TCt therefore form white colonies on the selective plate. The white colonies on the selective plates were selected for the plasmid extraction by rapid alkaline lysis extraction. The inserted DNA fragment in the recombinant plasmids were determined by digestion with the endonuclease enzymes. The number of cuts and the molecular sizes from endonuclease digestion are shown in Table 3 and Figure12 and 13.

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Table 3. The expected result of endonuclease restriction analysis

(1) The fragment sizes of cutting when the orientation of the *fliC* gene locates in the same direction with the *LacZα* gene.

(2) The fragment sizes of cutting when the orientation of the *fliC* gene locates in the opposite direction with the *LacZα* gene.

Plasmid	Endonuclease enzyme	Number of cut	Molecular size (bp)
pUCP28T (4,080 bp)	*Sma*I	1	4,080
	*Sac*I	1	4,080
pUCP28TCp (5,581 bp)	*HindIII*	1	5,581
	*Sac*I	2	(1) 1,511+4,070 (2) 10+5,571
	*Hind*III+*Xho*I	2	(1) 646+4,935 (2) 925+4,656
	*Pst*I	3	(1) 253+681+4,647 (2) 611+681+4,289
pUCP28TCt (5,552 bp)	*BamH*I+*Nar*I	2	(1) 291+5,261 (2) 1,191+4,361
	*Pst*I	3	(1) 255+670+4,627 (2) 593+670+4,289

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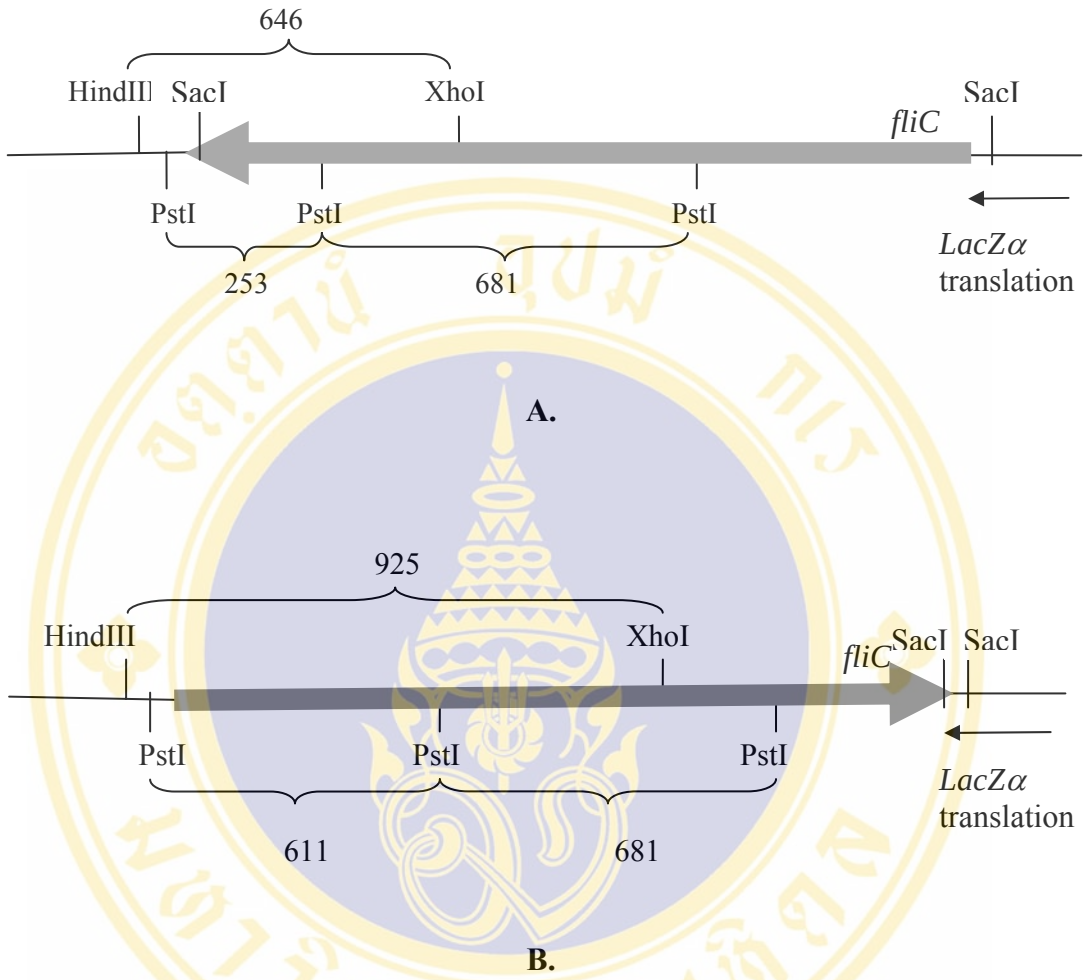

Figure 12. The *B. pseudomallei fliC* insertion in the multiple cloning site (MCS) of the *LacZα* gene pUCP28T

The orientation of the *fliC* gene may be the same direction with the *LacZα* gene (A) or the opposite direction (B). The line is the *LacZα* gene and the arrow indicates the translation direction. The big gray arrow is *fliC* fragment. The endonuclease restriction sites for checking the orientation are indicated.

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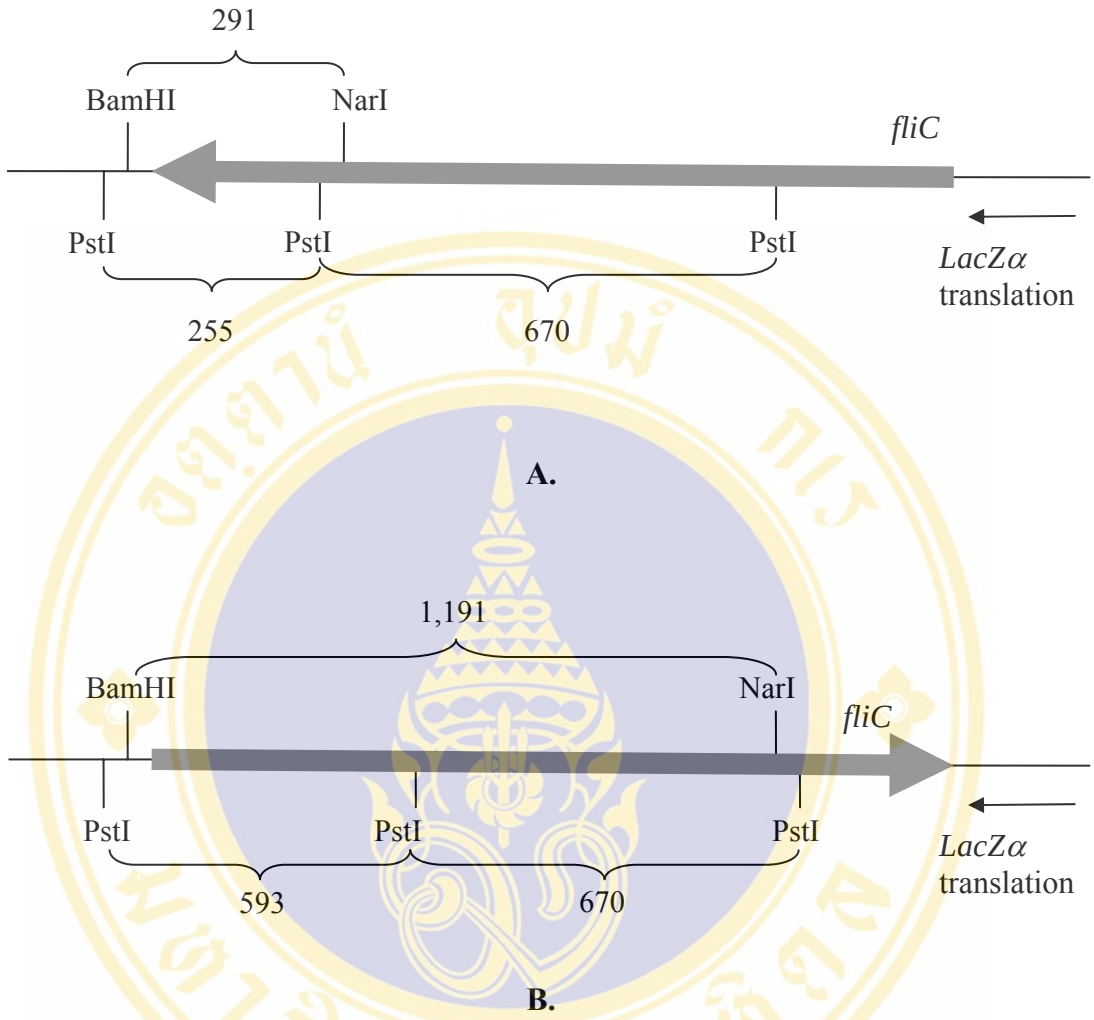

Figure 13. The *B. thailandensis fliC* insertion in the MCS of the *LacZα* gene pUCP28T

The orientation of the *fliC* gene may be the same direction with the *LacZα* gene (A) or the opposite direction (B). The line is the *LacZα* gene and the arrow indicates the translation direction. The big gray arrow is *fliC* fragment. The endonuclease restriction sites for checking the orientation are indicated.

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8. Transference of recombinant plasmid to *B. pesudomallei* MM35 by conjugation method

E. coli DH5αλpir harboring the *fliC* plasmid was used as the donor for conjugation with the recipient *B. pesudomallei* MM35. Single colony of MM35 and DH5αλpir were inoculated at 37 °C overnight in LB broth supplemented with 50 µg/ml of tetracycline for MM35 or supplemented with 25 µg/ml of trimethoprim for DH5αλpir. One percent of overnight cultures were grown in fresh LB medium until OD_{600} = 0.3-0.4. An equal amount of donor and recipient were mixed in the ratio of 1:1 and washed twice with PBS. The mixture was spotted onto a piece of membrane filter (pore size 0.2 µm) on LB agar plate. The plate was incubated at 37 °C overnight to allow the transference of the plasmid from *E. coli* DH5αλpir to *B. pesudomallei* MM35. After incubation, the bacteria were resuspended in 500 µl of PBS buffer before plating on LB agar supplemented with 50 µg/ml of tetracycline and 100 µg/ml of trimethoprim.

9. Motility assay

The colonies of *B. pesudomallei* Cp and Ct were selected to assess the motility phenotype by motility assay (112). The number of viable cell in overnight broth cultures were adjusted to 10,000 cells/ml. Then 4-µl of broth cultures were spotted and grown on 0.3% LB agar plates. Motility plates were incubated at 37 °C overnight before the motility phenotype was determined.

10. Cell culture condition

10.1 Mouse macrophage cell line (RAW 264.7)

The macrophages were cultured and maintained in 25 or 75-cm^2 flask (Costar, Cambridge, MA) with DMEM (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone) and 1 % L-glutamine (Biological Industries, Israel) at 37 °C in 5% CO_2 atmosphere and were subcultured at 80-90% confluency. The cells were subcultured by gently scraping the cells from plastic surface. The cell suspension

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was centrifuged at 4 °C with 1,000 rpm for 5 min. The pellet was resuspended in fresh medium and counted Trypan blue stained cells with heamacytometer.

10.2 Human alveolar lung epithelial cell line (A549)

Human alveolar lung epithelial cell line (A549) was culture in Ham F-12 culture medium (Hyclone) containing 2 mM L-glutamine in 1.5g/l sodium bicarbonate (Sigma) and 10% fetal bovine serum at 37 °C in 5% CO_2 atmosphere. The cultured medium was removed and cells washed with PBS. Trypsin-EDTA solution was added and allowed the solution to trypsinize for 5 min 37 °C in 5% CO_2 atmosphere. To stop the reaction, an equal volume of fresh medium was added and followed by PBS. The mixture was centrifuged at 4 °C with 1,000 rpm for 5 min. The cells were counted Trypan blue stained cells with heamacytometer.

11. Internalization, intracellular survival and replication of *Burkholderia* in phagocytic cells

The macrophages (1×10^6) were plated in 6-well plates and cultured overnight at 37 °C in 5% CO_2 atmosphere. The confluent macrophage monolayers were infected with bacteria at a multiplicity of infection (MOI) of 2:1 (when appropriate the coculturing was immediately centrifuged at 30 °C with 170 ×g for 5 min to enhance contact with the host cells). After 1 h, the bacteria were removed and the infected cells were washed twice with PBS, before fresh culture medium containing 250 µg/ml of kanamycin was added. The cells were incubated for one more hour to kill residual extracellular and attached bacteria. Thereafter, the infected cell monolayers were washed and lysed with 0.1% Triton X-100. The intracellular bacteria released were quantitated by plating serial dilutions of the lysate and the bacterial colonies were counted.

To determine the ability of *Burkholderia* to replicate intracellularly, a similar protocol was conducted but the infected macrophages were kept incubating in the culture medium containing 20 µg/ml of kanamycin for 4, 6 and 8 h before the number of intracellular bacteria was determined. The viability of the infected cells, judged by

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tryphan blue dye exclusion test, was more than 90%. The experiments were performed in three independent experiments, each performed in duplicate.

12. Invasion, intracellular survival and replication of *Burkholderia* in nonphagocytic cells

Human lung epithelial cells (4×10^5) were plated in 6-well plates and cultured overnight at 37 °C in 5% CO_2 atmosphere. The confluent human epithelial cell monolayers were infected with bacteria at MOI of 10:1 (when appropriate the coculture was immediately centrifuged at 30 °C with 170 ×g for 5 min to enhance contact with the host cells). After 2 h, the bacteria were removed and the infected cells were washed three times with PBS, before fresh culture medium containing 250 µg/ml of kanamycin was added. The cells were incubated for one more hour. Thereafter, the infected cell monolayers were washed and lysed with 0.1% Triton X-100. The intracellular bacteria released were quantitated by plating serial dilutions of the lysate and the bacterial colonies were counted.

To determine the ability of *Burkholderia* to replicate intracellularly, a similar protocol was conducted but the infected human lung epithelial cells were kept incubating in the culture medium containing 20 µg/ml of kanamycin for 5, 7 and 9 h before the number of intracellular bacteria was determined. The viability of the infected cells, judged by tryphan blue dye exclusion test, was more than 90%. The experiments were performed in three independent experiments, each performed in duplicate.

13. Immunoblotting

The cells were infected with bacteria for 15 min, 30 min, 1 h, and 2 h before lysing in lysis buffer (appendix). The lysates containing 30 µg of protein were electrophoresed using 10% polyacrylamide and were then electrotransferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was blocked with 10% blocking reagent (Roche, Mannheim, Germany), for 1 h before incubating overnight with polyclonal rabbit antibody to IkBα (Santa Cruz, USA).

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Blots were then reacted with horseradish peroxidase-conjugated goat antirabbit IgG (Pierce, USA). Protein bands were detected with an enhanced chemiluminescence kit (Roche).

14. Statistical analysis

Student's *t*-test or one-way ANOVA test by SPSS 11.5 was used to determine if there was a statistically significant difference ($P<0.05$) of the mean invasion frequencies and intracellular replication time.

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CHAPTER IV
RESULTS

1. Genomic DNA extraction

The gDNA of *B.pseudomallei* 1026b and *B. thailandensis* E257 were extracted by phenol-chloroform extraction method before determining the DNA content of samples by spectrophotometry and gel electrophoresis. Since the DNA solution was so viscous, therefore the sample had to be diluted with the dilution of 1:100. The absorbance at 260 nm allows calculation of the concentration of nucleic acid in the sample while the absorbance at 280 nm indicates the presence of protein. The ratio between the readings at 260 nm and 280 nm provides an estimate of the purity of the nucleic acid. The result of analysis are shown below;

Strains	OD_{260}	OD_{280}	OD_{260}:OD_{280}	DNA concentration $100X^{-1}$ (μg/ml)
1026b	0.108	0.057	1.89	5.4
E257	0.126	0.067	1.88	6.3

Although the UV light absorption at 260 nm is a simple method for determining the DNA content of sample, the sample may be contaminated from other compounds such as RNA, EDTA, and phenol that also absorb significantly at 260 nm. Gel electrophoresis was used to chech the purity of DNA by checking the DNA shearing, DNA concentration, and RNA contamination. The result of gel electrophoresis as shown in Figure 14 indicated that there was no DNA shearing or RNA contamination in the gDNA samples.

2. Full-length *fliC* gene and its promoter amplification

The PCR products of the full-length *fliC* gene are shown in Figure 15. The sizes of the structural *fliC* and its promoter were 1,501 bp for *B. pseudomallei*, and 1,472 bp for *B. thailandensis*.

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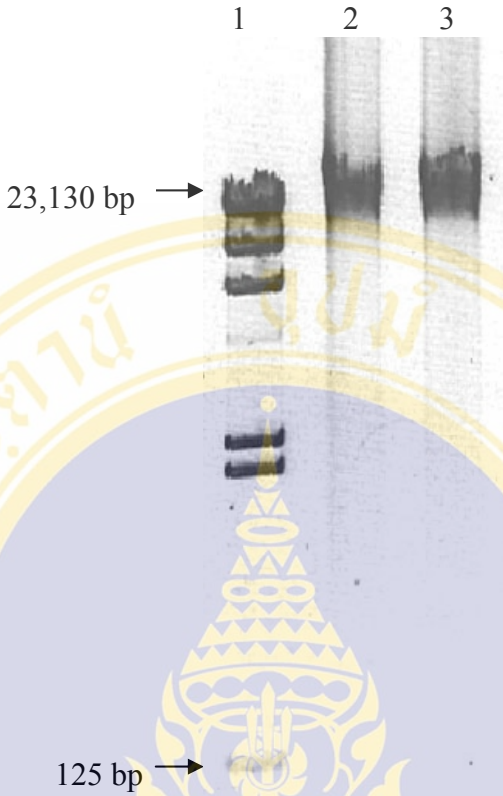

Figure 14. The gDNA of *B.pseudomallei* 1026b and *B. thailandensis* E257

The gDNA of the bacteria were extracted by phenol-chloroform extraction method.

Lane 1: DNA Marker λ/HindIII

Lane 2: The gDNA of *B. pseudomallei*

Lane 3: The gDNA of *B. thailandensis*

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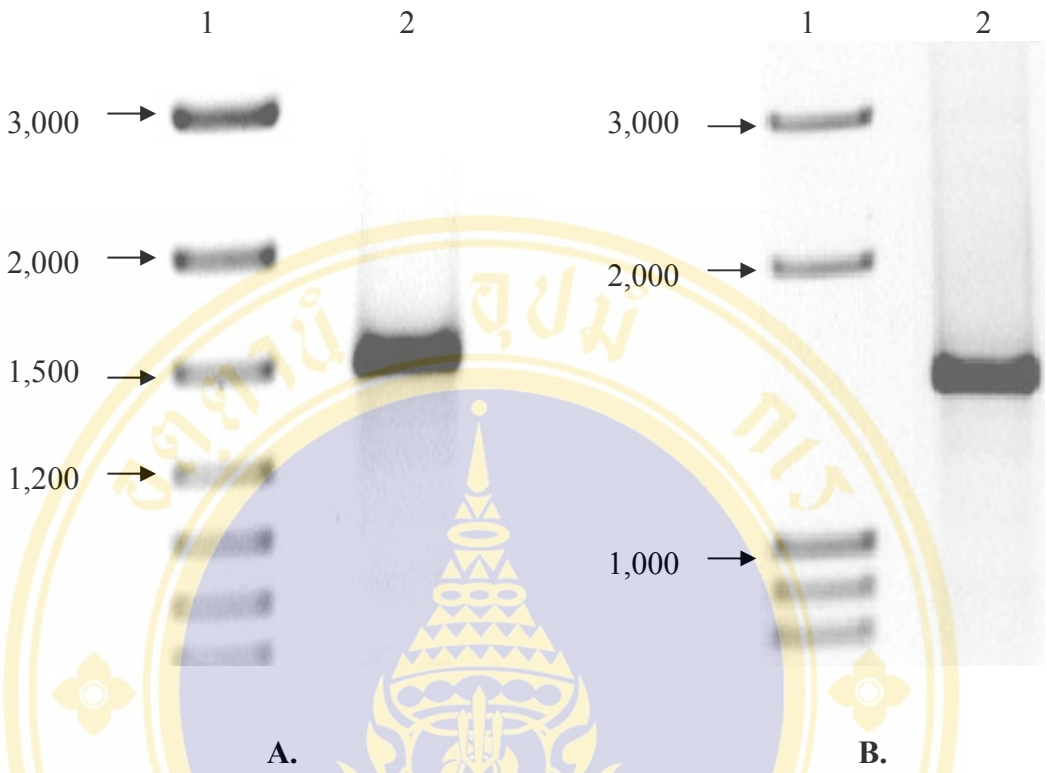

Figure 15. PCR products of the full length *fliC* gene and its promoter from the gDNA of the bacteria

A. PCR product of *B. pseudomallei* 1026b *fliC* gene and its promoter

Lane 1: DNA marker

Lane 2: The 1,501-bp *fliC* gene and promoter of *B. pseudomallei* 1026b

B. PCR product of *B. thailandensis* E257 *fliC* gene and its promoter

Lane 1: DNA marker

Lane 2: The 1,472-bp *fliC* gene and promoter of *B. thailandensis* E257

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3. *fliC* pUCP28TCp and pUCP28TCt construction

The pUCP28T plasmid was extracted by using plasmid extraction kit (QIAGEN). To obtain the blunt ends, the plasmid was digested with *Sma*I enzyme and the result of digestion is shown in Figure 16A. The *Sma*I pUCP28T and the *fliC* gene were ligated to construct the recombinant plasmid pUCP28TCp and pUCP28TCt. The ligation products were transferred into the host *E. coli* DH5αλpir. White colonies on the selective plates were selected for further analysis of the plasmids and used as a template for checking the insertion of *fliC* gene by PCR and endonuclease restriction enzyme analysis. The PCR results showed that the 1.5-kb *fliC* had been successfully inserted into the plasmid to generate a recombinant plasmid (Figure 17). Since PCR of the *fliC* gene generated the blunt end products, the orientation of the inserted fragments could be any of the two directions; the same direction as the *LacZα* gene or the opposite direction as previously mentioned in the method (Table 3 and Figures 12, 13). Approximately 10 clones of pUCP28TCp and 23 clones of pUCP28TCt were checked for the *fliC* gene insertion and the orientation by restriction enzyme analysis. The pUCP28TCp clones were classified into two groups; pUCP28TCp1 and pUCP28TCp2, according to the orientation of the *fliC* gene. Eight clones were grouped in pUCP28TCp1 whose *fliC* gene orientation was in an opposite direction to the *LacZα* gene. The two remainders were in pUCP28TCp2. It was surprising that *Pst*I and *Sac*I digestion for pUCP28TCp1 showed contradictious results (Figure 18A). The result of *Pst*I digestion indicated that the insertion of the *fliC* gene was in opposite direction to the *LacZα* gene but *Sac*I digestion showed the unexpected result. However, the digestions with *Hind*III and *Xho*I were consistent with that of *Pst*I (Figure 18B). Because of the plasmid pUCP28TCp1 digested with *Sac*I showed the fragment sizes which were not correlated with the map of enzyme cutting, the empty plasmid pUCP28T was checked with *Sac*I, *Aat*II and *Kpn*I digestion. The enzyme *Aat*II and *Kpn*I digestion showed result as expected. However, the *Sac*I showed unexpected result, that is there was more than 1 cutting of *Sac*I digestion (Figure 16B). For pUCP28TCt, all 23 clones showed the *fliC* gene orientation in an opposite direction to the *LacZα* gene (Figure 19).

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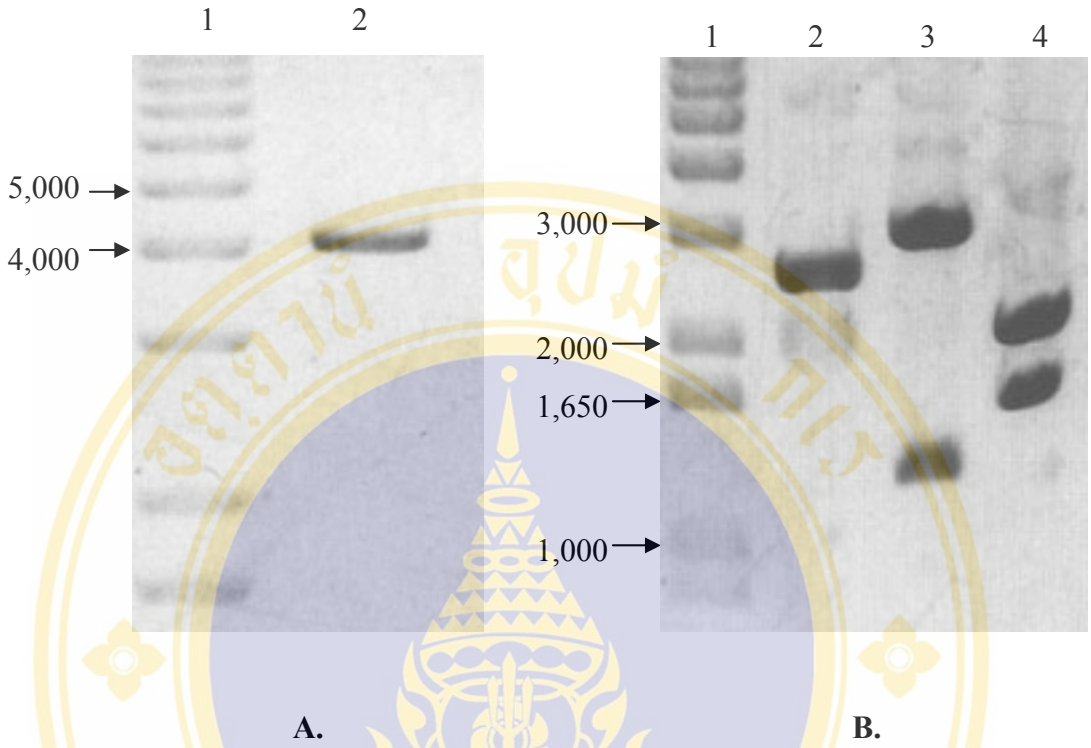

Figure 16. The pUCP28T plasmid with restriction enzyme digestion

A. The pUCP28T plasmid was digested with *Sma*I enzyme generated the blunt end pUCP28T (4,080 bp).

Lane 1: DNA marker

Lane 2: pUCP28T with *Sma*I enzyme digestion

B. The restriction enzyme digestion of pUCP28T with *Aat*II, *Kpn*I, and *Sac*I enzyme

Lane 1: DNA marker

Lane 2: pUCP28T uncut

Lane 3: pUCP28T with *Aat*II and *Kpn*I digestion generated a 2,852-bp and a 1,228-bp fragments.

Lane 4: pUCP28T *Sac*I digestion generated an approximately 2,500- bp and a 1,580-bp fragments.

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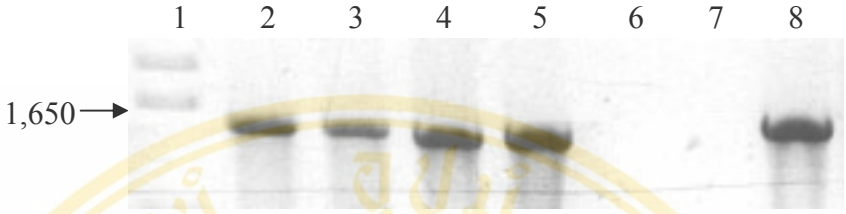

Figure 17. PCR products of the *fliC* gene from the recombinant plasmid pUCP28TCp and pUCP28TCt

Lane 1: DNA Marker

Lane 2: The 1,501-bp *fliC* gene from pUCP28TCp1

Lane 3: The 1,501-bp *fliC* gene from pUCP28TCp2

Lane 4: The 1,472-bp *fliC* gene from pUCP28TCt1

Lane 5: The 1,472-bp *fliC* gene from pUCP28TCt2

Lane 6: Negative control; no DNA template

Lane 7: Negative control; the empty pUCP28T template

Lane 8: Positive control; *B. thailandensis fliC* template

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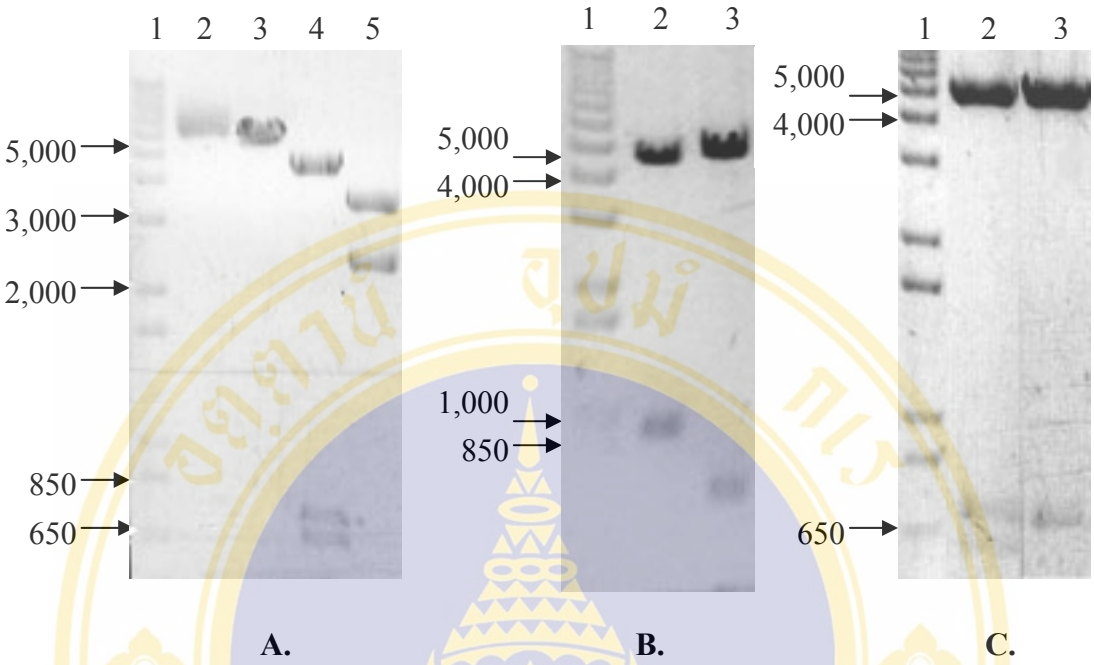

Figure 18. The plasmid pUCP28TCp with restriction enzyme analysis

A. pUCP28TCp1 with restriction enzyme analysis

Lane 1: DNA marker, Lane 2: pUCP28TCp uncut, Lane 3: *Hind*III digestion generated a 5,581-bp fragment, Lane 4: *Pst*I digestion generated a 611-bp, a 681-bp, and a 4,289-bp fragments, Lane 5: *Sac*I digestion generated an approximately 3,000- bp and a 2,500-bp fragments.

B. pUCP28TCp1 and Cp2 with *Hind*III and *Xho*I digestion

Lane 1: DNA marker, Lane 2: pUCP28TCp1 with *Hind*III and *Xho*I digestion generated a 925-bp, and a 4,656-bp fragments., Lane3: pUCP28TCp2 with *Hind*III and *Xho*I digestion generated a 646-bp, and a 4,935-bp fragments.

C. pUCP28TCp1 and Cp2 with *Pst*I digestion

Lane 1: DNA marker, Lane 2: pUCP28TCp1 with *Pst*I digestion generated a 611-bp, a 681-bp, and a 4,289-bp fragments., Lane 3: pUCP28TCp2 with *Pst*I digestion generated a 681-bp, a 253-bp, and a 4,647-bp fragments.

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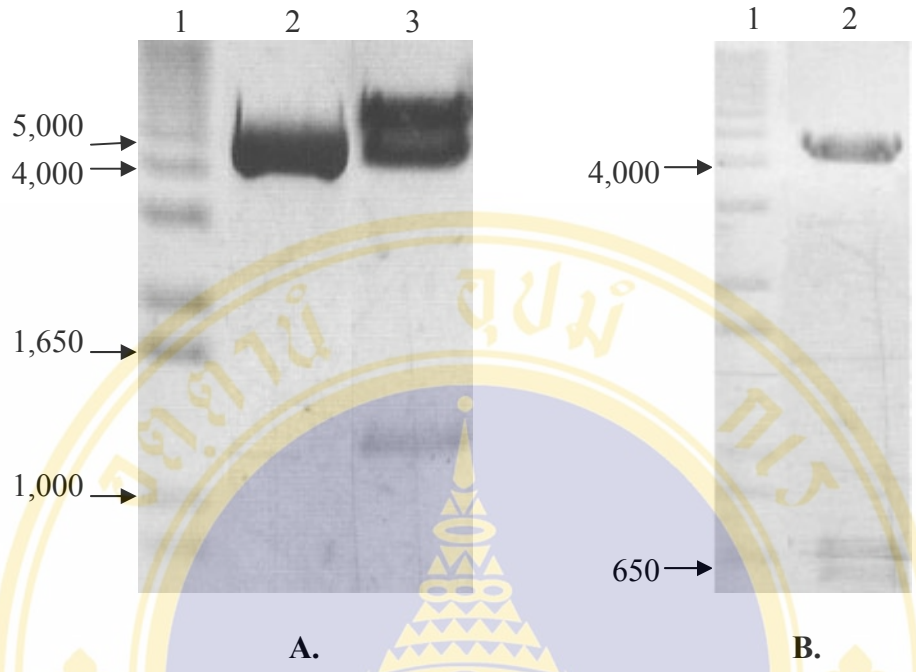

Figure 19. The plasmid pUCP28TCt with restriction enzyme analysis

A. pUCP28TCt with *BamH*I and *Nar*I digestion

Lane 1: DNA marker

Lane 2: pUCP28TCt uncut

Lane 3: pUCP28TCt with *BamH*I and *Nar*I digestion generated a 1,191-bp and a 4,361-bp fragments.

B. pUCP28TCt with *Pst*I digestion

Lane 1: DNA marker

Lane 2: pUCP28TCt with *Pst*I digestion generated a 593-bp, a 670-bp, and a 4,289-bp fragments.

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E. coli DH5αλpir which harbor the recombinant plasmid pUCP28TCp and pUCP28TCt were then conjugated to *B. pesudomallei* MM35 to generate the complemented mutants *B. pesudomallei* Cp and Ct.

4. Identification of complemented mutants *B. pesudomallei* Cp and Ct

B. pesudomallei Cp and Ct were assessed for their motility phenotypes on 0.3% LB agar plates. The results of motility assay showed that the pUCP28T derivatives, pUCP28TCp1, pUCP28TCp2 and pUCP28TCt were able to complement the mutation in MM35 and that there was no difference in motility phenotype between pUCP28TCp1, pUCP28TCp2, and pUCP28TCt (Figure 20A, B and C). The complemented mutants *B. pesudomallei* Cp and Ct restored the motility phenotype resulting in a larger area of growth than nonmotile MM35 (Figure 20D). However, the swarming area was smaller than that of the wild type. The empty pUCP28T plasmid (no inserted fragment) did not affect the motility (112). It should be noted that the swarming area of the wild type *B. thailandensis* was larger than wild type *B. pseudomallei* (Figure 20D and 21). The doubling times of growth of these bacteria were determined from the slopo of growth curve calculated during the log phase (Figure 22). *B. pseudomallei* 1026b had a doubling time of 42 min which was equaled to *B. thailandensis*, while that of *B. pseudomallei* Cp and Ct was slightly longer, i.e. 65 min. Although the data showed that the replication time between *B. pseudomallei* 1026b and *B. thailandensis* are not different but slightly shorter than that of *B. pseudomallei* Cp and Ct.

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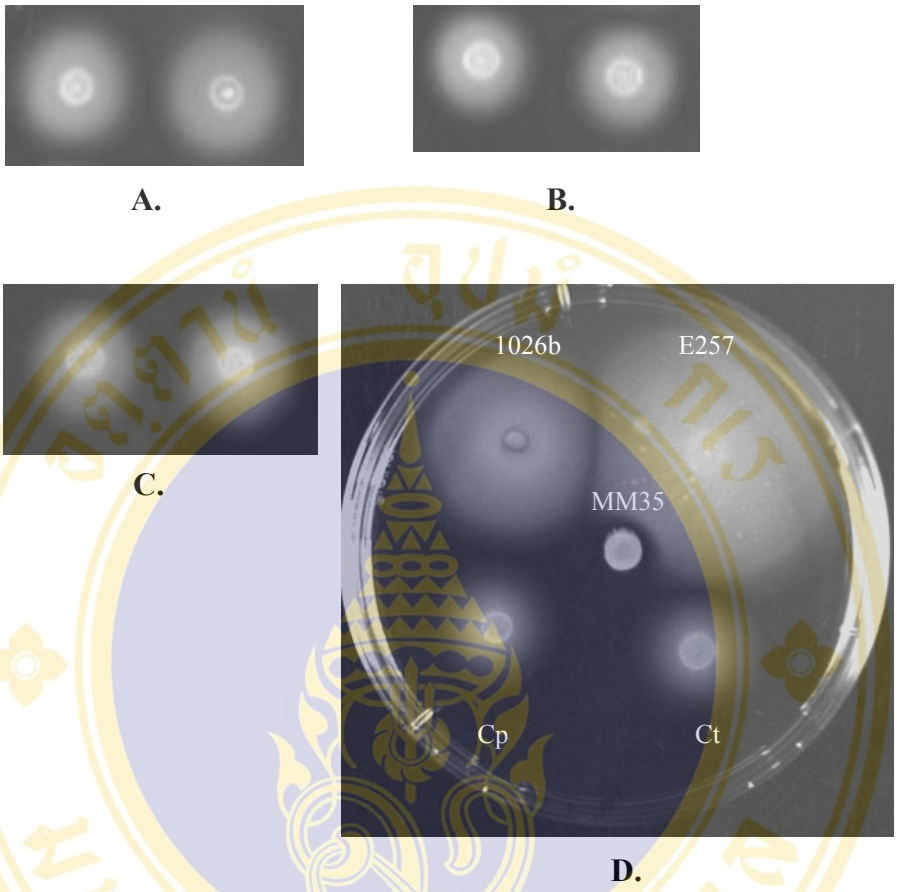

Figure 20. Motility assay of the complemented mutants Cp and Ct

The motility phenotype was assessed by the examining the circular swarming from the site of inoculation on 0.3% LB plates.

A. The motility phenotype of *B. pseudomallei* Cp1

B. The motility phenotype of *B. pseudomallei* Cp2

C. The motility phenotype of *B. pseudomallei* Ct

D. The motility phenotype of *B. pseudomallei* wild type1026b, *B. thailandensis* wild type E257, *B. pseudomallei* Δ*fliC* MM35, *B. pseudomallei* complemented mutant Cp, and *B. pseudomallei* complemented mutant Ct

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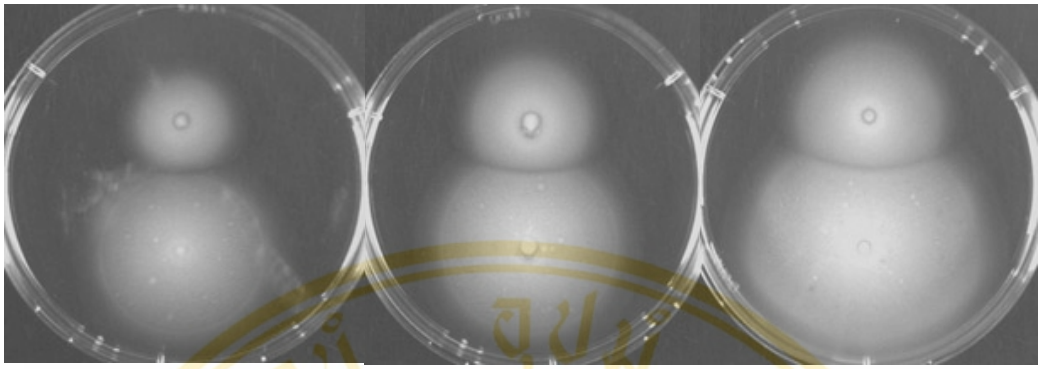

Figure 21. Motility assay of wild type *B. pseudomallei* wild type1026b and *B. thailandensis* E257

The upper swarming area is *B. pseudomallei* and the lower is *B. thailandensis*. The volumes and concentrations used were 2 µl of 10^4 cells/ml, 4 µl of 10^4 cells/ml, and 4 µl of 10^6 cells/ml, from left to right respectively.

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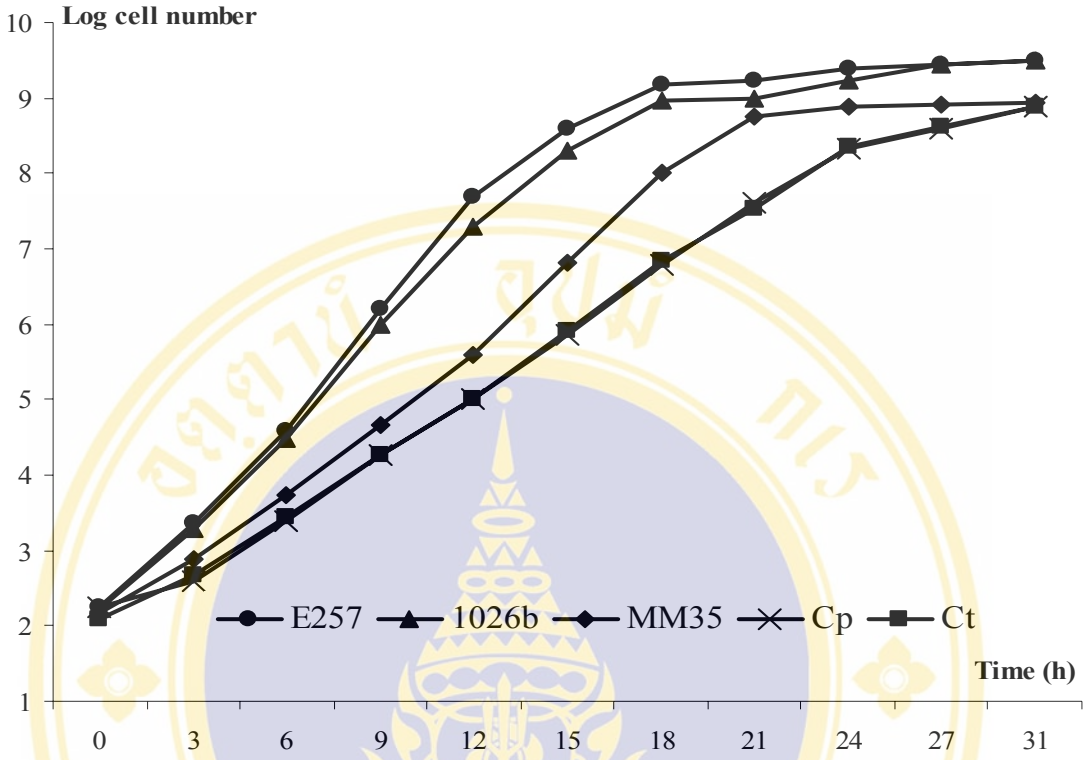

Figure 22. Growth Curve of *Burkholderia*

The bacteria were cultured in LB broth at 37 °C with 150 rpm shaking. The bacterial cells were collected every three hours. The doubling times of the bacteria were determined from the growth curve; 42 min for 1026b and E257, 51 min for MM35, 65 min for Cp and Ct.

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

Since the recombinant plasmid pUCP28TCps generated could be classified into two groups, pUCP28TCp1 and pUCP28TCp2, according to the orientation of the *fliC* gene, these two groups were investigated to determine whether they were different in susceptibility to macrophage uptake. The result in Figure 23 showed that there was no difference in bacterial uptake by the macrophages between *B. pseudomallei* Cp1 and Cp2 ($P>0.05$). Because the objective of this study is to compare the uptake of *B. pseudomallei* with different flagellin proteins by macrophage, therefore the complemented mutant *B. pseudomallei* with pUCP28TCp1 was selected for this purpose.

Both *B. pseudomallei* Cp and Ct could be readily internalized by the macrophages at nearly equal capacity (Figure 24). In contrast, the MM35 was internalized significantly less than 1026b, E257, Cp or Ct ($P<0.05$). However, with an additional step of centrifugation, the MM35 internalization was increased to the levels similar to those of other strains. The results suggested that flagella may facilitate the bacteria to contact the host cells. By comparing the wild type 1026b with E257, the results from Figure24 showed that pathogenic *B. pseudomallei* 1026b could be internalized significantly more than nonpathogenic *B. thailandensis* E257 ($P<0.05$).

The intracellular replication times of *Burkholderia* in mouse macrophages were calculated at 4, 6, and 8 h after infection and found to be not different from one another (60 min) (Figure 25).

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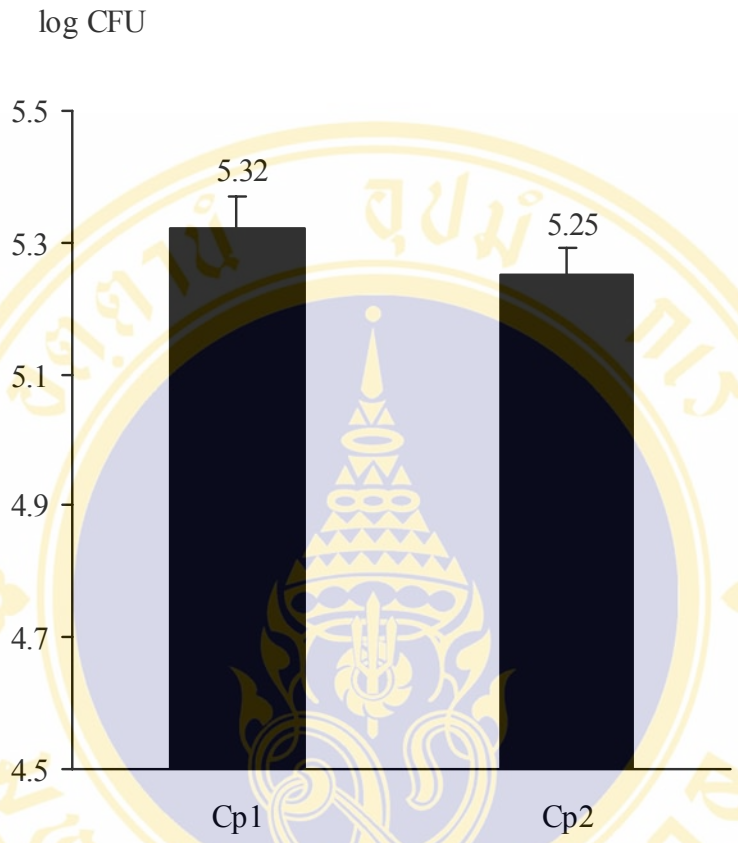

Figure 23. *B. pseudomallei* Cp1 and Cp2 uptake by macrophages

The cells (1× 10^6 cells/well) were infected with *Burkholderia* at MOI of 2:1. Internalization was determined at 2 h after infection by standard antibiotic protection assay. Data represent means and standard errors of three separate experiments, each experiment carried out in duplicate. $P<0.05$ by Student's *t*-test.

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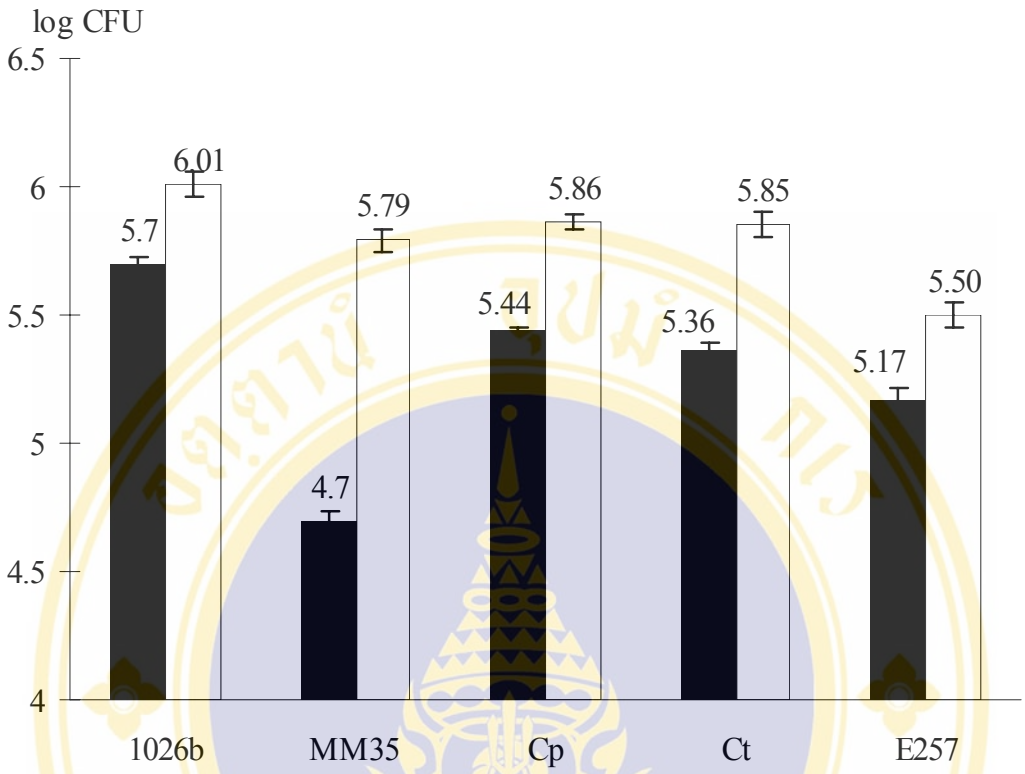

Figure 24. Bacterial uptake by macrophages

The cells (1× 10^6 cells/well) were infected with *Burkholderia* at MOI of 2:1. Internalization was determined at 2 h after infection by standard antibiotic protection assay. Black bars represented the invasive capacity without centrifugation during the infection. White bars represented the invasion with centrifugation. Data represent means and standard errors of three separate experiments, each experiment carried out in duplicate. $P<0.05$ by one-way ANOVA test.

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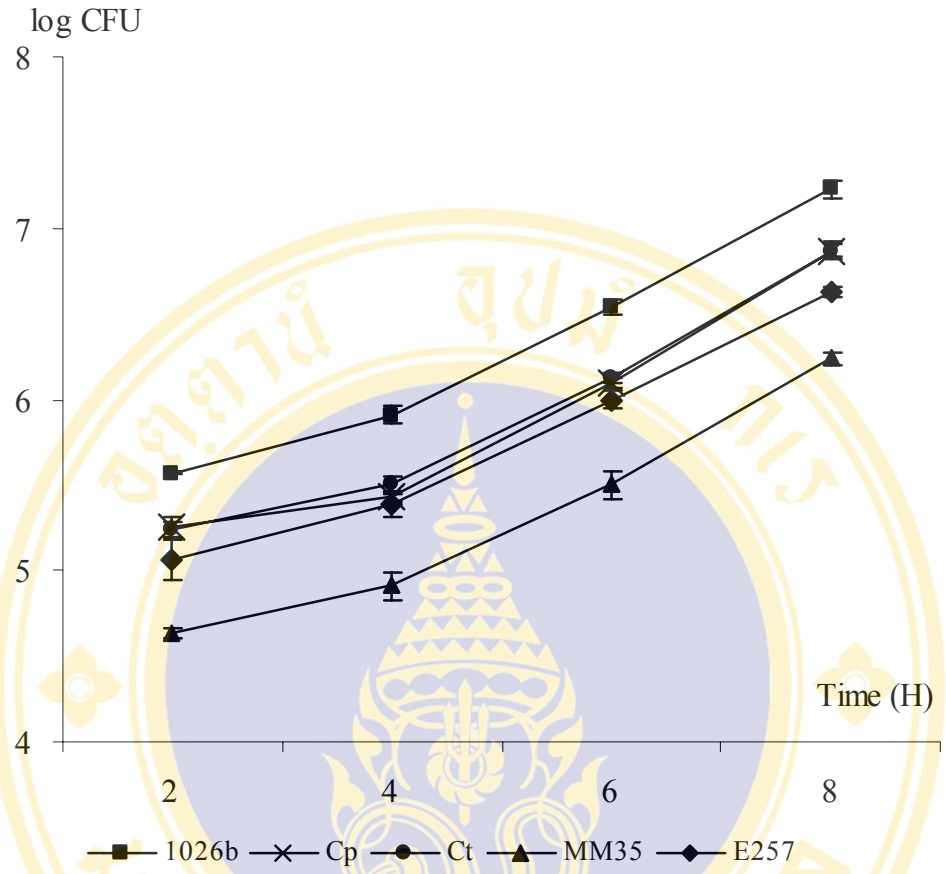

Figure 25. Intracellular survival and replication of the bacteria in mouse macrophages

The cells (1× 10^6 cells/well) were infected with *Burkholderia* at MOI of 2:1. Replication times of the bacteria were determined after a total of 4, 6, and 8 h of incubation. Data represent means and standard errors of three separate experiments, each experiment carried out in duplicate.

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

The complemented mutant *B. pseudomallei* carring the recombinant plasmid pUCP28TCp1 and pUCP28TCp2 were also investigated whether they were different in the invasive capacity by studying their interaction with human lung epithelial cells. The results in Figure 26 also showed that there was no difference in the invasive capacity between *B. pseudomallei* Cp1 and Cp2 ($P>0.05$). Therefore, *B. pseudomallei* Cp1 could be used to investigate the bacterial invasion.

As shown in Figure 27, there was no significant different in the invasive capacity between the complemented mutants *B. pseudomallei* Cp and Ct ($P>0.05$). Although *B. pseudomallei* 1026b, Cp, and Ct exhibited invasive capacity significantly higher than MM35 ($P<0.05$), the latter showed significantly higher invasive capacity than E257 ($P<0.05$). However, when bringing the bacteria into intact with the host cells by short centrifugation, there was no significant difference in the invasive capacity among all *B. pseudomallei* strain tested. It should be noted that the invasive capacity of *B. thailandensis* E257 was lower than all *B. pseudomallei* ($P<0.05$). These results confirmed previous data suggesting that pathogenic *B. pseudomallei* is more invasive than nonpathogenic *B. thailandensis*.

The intracellular replication times of *Burkholderia* in human lung epithelial cells were calculated at 5, 7, and 9 h after infection and found to be not different from one another (51 min) (Figure 28).

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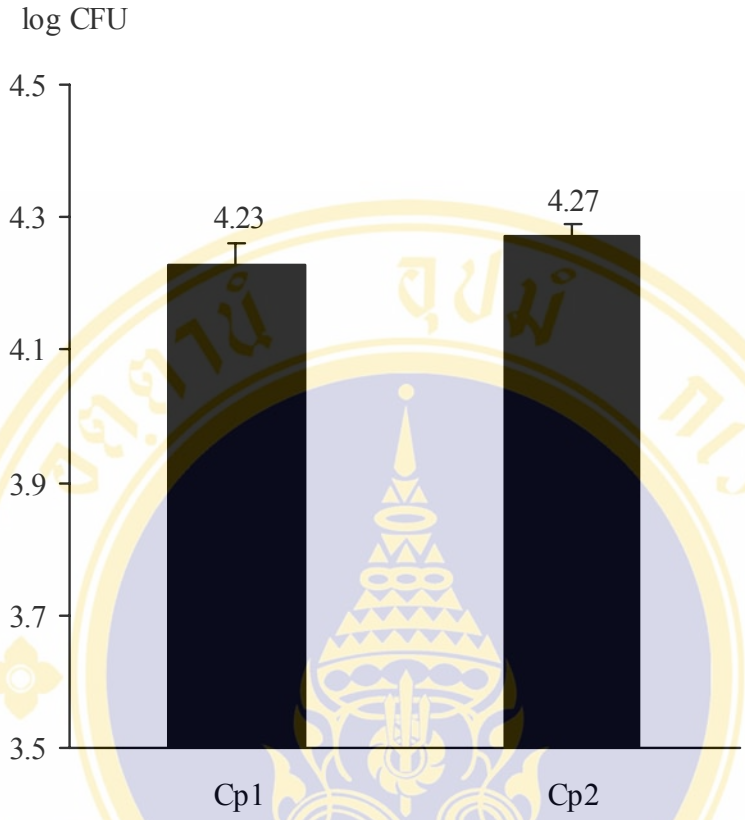

Figure 26. Invasion of human lung epithelial cells by *B. pseudomallei* Cp1 and Cp2

The cells (4×10^5 cells/well) were infected with *Burkholderia* at MOI of 10:1. Invasion was determined 3 h after infection by standard antibiotic protection assay. Data represent means and standard errors of three separate experiments, each experiment carried out in duplicate. $P<0.05$ by Student's *t*-test.

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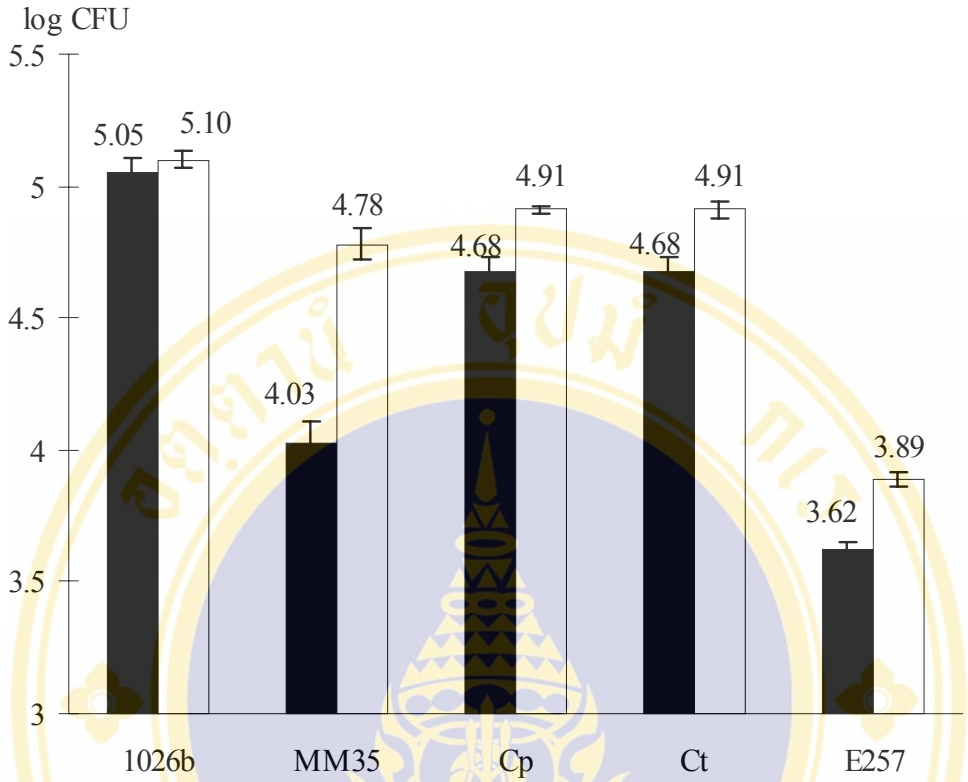

Figure 27. Invasion of human lung epithelial cells

The cells (4×10^5 cells/well) were infected with *Burkholderia* at MOI of 10:1. Invasion was determined 3 h after infection by standard antibiotic protection assay. Black bars represented the invasive capacity without centrifugation during the infection. White bars represented the invasion with centrifugation. Data represent means and standard errors of three separate experiments, each experiment carried out in duplicate. $P<0.05$ by one-way ANOVA test.

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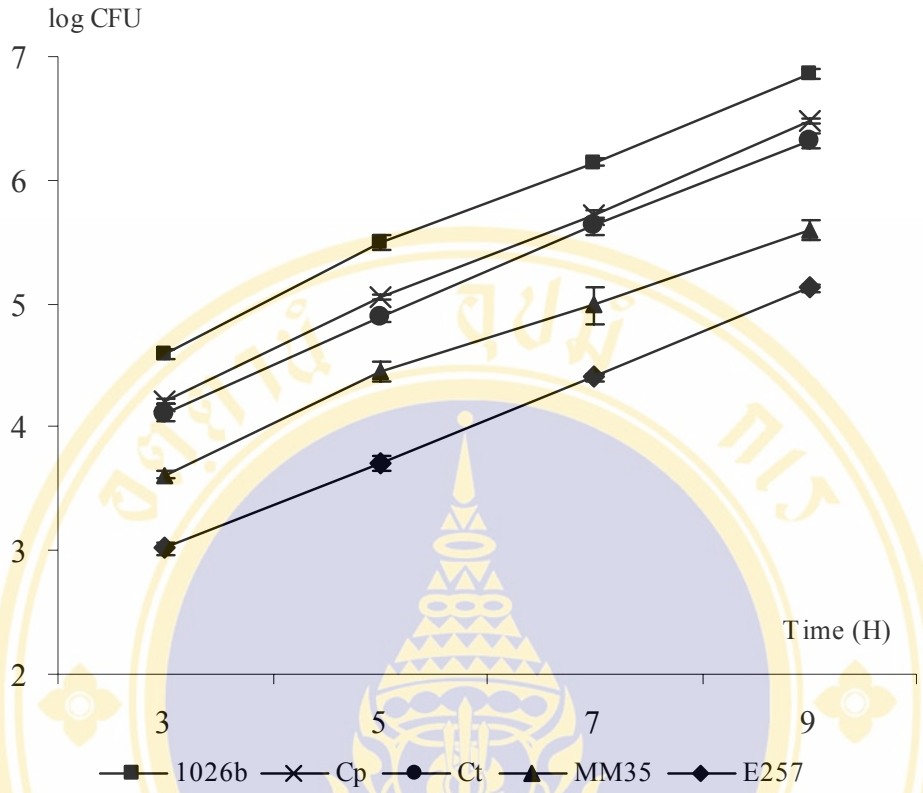

Figure 28. Intracellular survival and replication of the bacteria in human lung epithelial cell (A549)

The cells (4× 10^5 cells/well) were infected with *Burkholderia* at MOI of 10:1. Replication times of the bacteria were determined after a total of 5, 7 and 9 h of incubation. Data represent means and standard errors of three separate experiments, each experiment experiment carried out in duplicate.

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7. IκBα degradation in mouse macrophages

The macrophages (1×10^6 cells/well) were infected with *Burkholderia* at MOI of 2:1. At time intervals (15 min, 30 min, 1 h, 2 h), the cells were lysed and the supernatants were used for analysis of IκBα degradation by immunoblotting. The results presented in Figure 29 showed that the level of IκBα in all *Burkholderia*-infected cells was degraded after 15 min and reappeared within 1 h. The wild type *B. pseudomallei* 1026b and *B. thailandensis* E257 showed the similar pattern of IκBα degradation as in the complemented mutant Cp and Ct. However, in the *fliC* mutant MM35, the IκBα degradation in an early stage (30 min) was slightly less than the wild type. This phenotype was compensated in the complement mutant.

8. IκBα degradation in human lung epithelial cells

Human lung epithelial cells (4×10^5 cells/well) were infected with *Burkholderia* at MOI of 10:1. At time intervals (15 min, 30 min, 1 h, 2 h, or 4h), the cells were lysed and the supernatants were analysed for IκBα degradation by immunoblotting. IκBα in the *Burkholderia*-infected cells was gradually degraded after 30 min of infection before reappearing at 2 h, except in *B. thailandensis* whose IκBα reappearance was delayed to 4 h after infection (Figure 30). Similar to the phagocytic cells, IκBα degradation in an early stage (30 min) of the mutant MM35 was slightly less than that of the wild type but this phenotype could be compensated in the complemented mutant.

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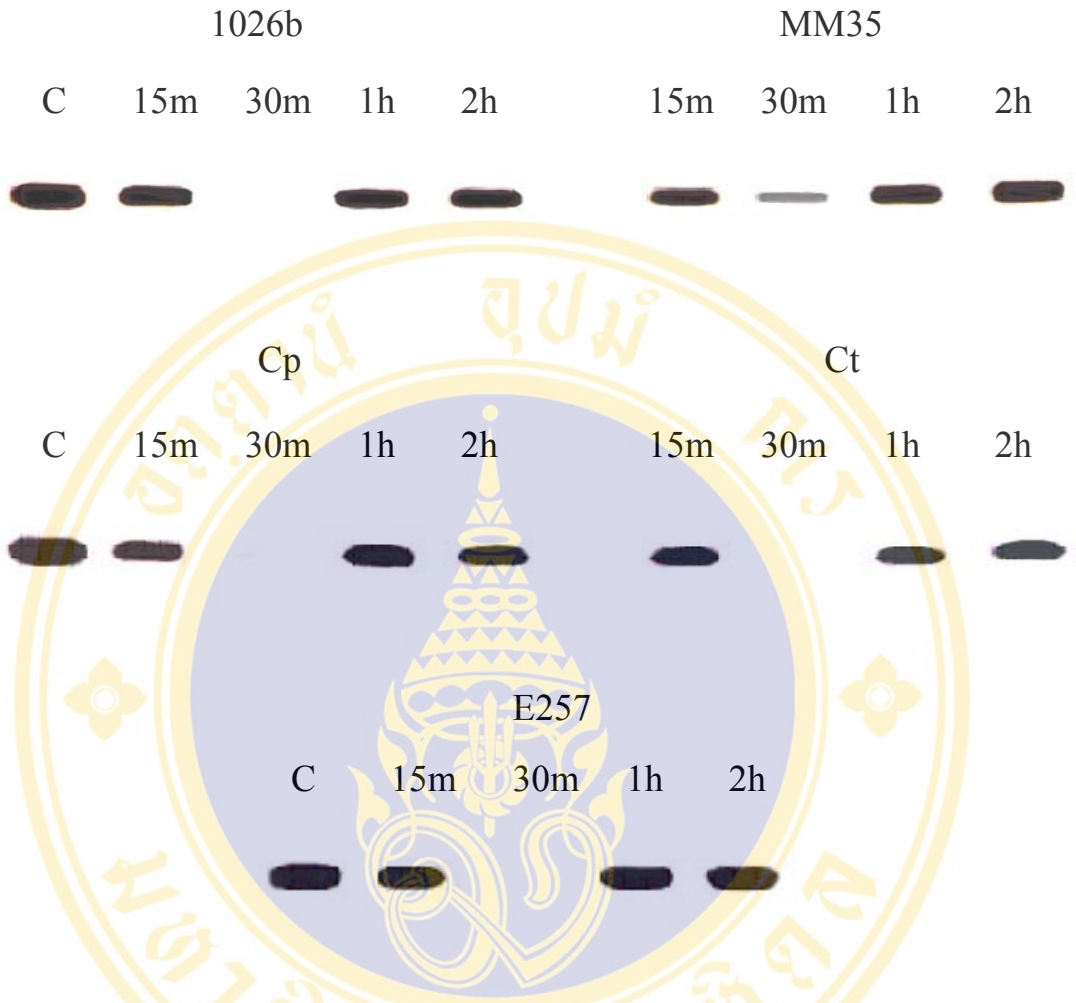

Figure 29. IκBα degradation in mouse macrophages

The cells (1× 10^6 cells/well) were infected with *Burkholderia* at MOI of 2:1. Uninfected cells were used as a control (C). At times indicated, the cells were lysed and used for analysis of IκBα degradation by immunoblotting.

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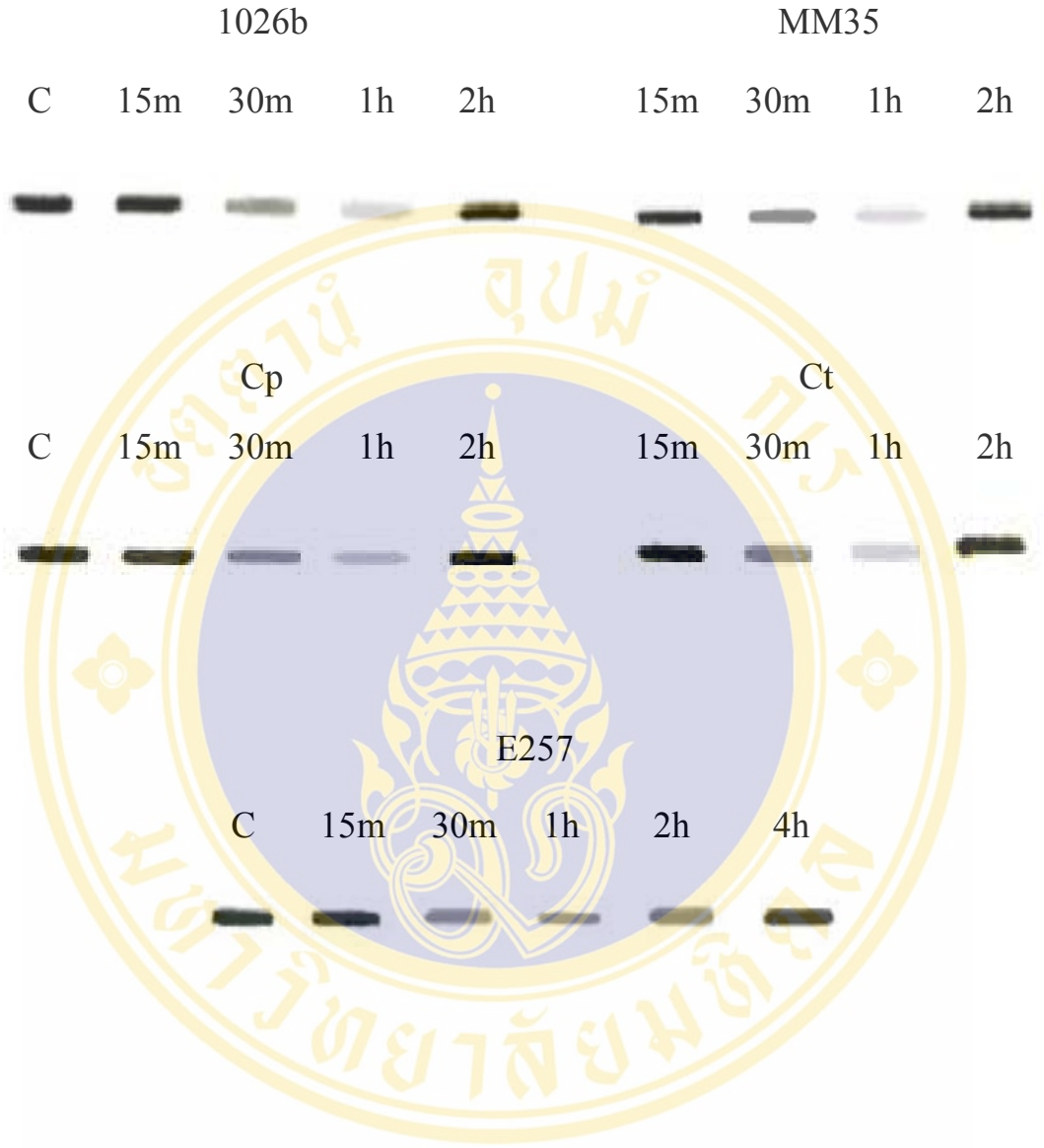

Figure 30. IκBα degradation in human epithelial cells

The cells (4× 10^5 cells/well) were infected with *Burkholderia* at MOI of 10:1. Uninfected cells were used as a control (C). At times indicated, the cells were lysed and used for analysis of IκBα degradation by immunoblotting.

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CHAPTER V
DISCUSSION

Flagella are considered to be a virulence factor in many bacteria since their presence correlates with the ability of an organism to cause disease (5-9). In *B. pseudomallei*, adherence by flagella was required to initiate bacterial entry into the amoebic trophozoite (9). The importance of flagella in the pathogenicity of *B. pseudomallei* in a mouse model was also evaluated and found that the flagellum is an important and necessary virulent determinant of *B. pseudomallei* during intranasal and intrapertoneal infection of mice (8). *B. pseudomallei* and *B. thailandensis* are very closely related to each other, except the capacity to cause disease. Although both bacteria contain flagella, the flagellin gene of *B. thailandensis* showed a 15-base pairs (encodes for five amino acids) missing in the variable domain (4). Therefore, the difference in the flagellin genes may be associated with difference in virulence in these two species.

The pUCP28T plasmid is a high copy number plasmid that has been used as a vector for construction of the complemented mutant flagellin gene. Generally, the recognition site of enzyme in the multiple cloning site (MCS) of the plasmid should has only 1 site to support the gene cloning. The finding of two cutting sites of *Sac*I digestion presented in Figure 16 suggested that some nucleotide sequences of the plasmid have been mutated. Although, the mutation may have occurred, the plasmid should restore the motility phenotype of the mutant as shown in Figure 20. This may be explain by the fact that mutation is not the important site for replication and gene expression. Both *B. pseudomallei* Cp1 and Cp2 were successful in motility complementation (Figure 20A and B) because the inserted fragment composed of its own promoter. Therefore, flagellin gene in the plasmid can be expressed under the regulation of its promoter. However, the result in Figure 20D showed that the complemented mutant Cp and Ct could not fully restore the motility function of the *fliC* mutant MM35 and this may be related to insufficiency of flagellin protein

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production. In general, the bacteria would need approximately 20,000 copies of the flagellin protein to make one filament (76). This is different from other bacterial proteins that only one protein unit can satisfactory 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 noted that the swarming growth area of wild type *B. thailandensis* was larger than wild type *B. pseudomallei* (Figures 20, 21). The fact that the bacterial growth rates in free-cell cultures between *B. thailandensis* and *B. pseudomallei* wild types were not different (Figure 22) indicates that the growth rate is not a factor responsible for a larger growth area of *B. thailandensis* in motility assay. The data from genomic analysis revealed that in addition to *fliC*, *B. thailandensis* (strain E264) possesses another flagellin gene called flagellin D (65). The flagellin D gene encodes 296 amino acids in length which is shorter than FliC, and is located in the flagellar system on chromosomeII which is not present in *B. pseudomallei* (strain K96243). Like FliC, the function of flagellin D protein is chemotaxis and motility (65) and its presence may result in its larger growth area.

The complemented mutant *B. pseudomallei* Cp were classified into two groups, Cp1 and Cp2, according to the orientation of the *fliC* gene. In spite of this, there was no different between Cp1 and Cp2 uptake by macrophages and human epithelial cells (Figures 23, 26), and both possessed similar motility phenotype on the motility assay (Figure 20). However, the objective of the study is to investigate the difference in the invasive capacity of *B. pseudomallei* which harbors different flagella (i.e. *B. pseudomallei* flagella or *B. thailandensis* flagella). Therefore, *B. pseudomallei* Cp1 whose *fliC* gene orientation was the same as in *B. pseudomallei* Ct should be selected for further analysis. The invasion assay of Cp and Ct in both cultured phagocytic and nonphagocytic cells indicated that the difference in the variable region of *B. pseudomallei* and *B. thailandensis fliC* is not associated in the invasive capacity (Figures 24,27). Therefore, the 15-base pair in the variable region of *B. pseudomallei fliC* is not essential for cell invasion. A brief centrifugation was performed to confirm that flagella facilitated the bacterial invasion and bacterial uptake which may enhance

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the adhesion of the bacteria to the cells. However, it was surprising that the invasive capacity of *B. pseudomallei fliC* mutant for human lung epithelial cells was significantly higher than *B. thailandensis*, thus suggesting that in addition to the flagella, *B. pseudomallei* may need some other factor(s) such as type three secretion system, which is presented only in *B. pseudomallei* (65), to facilitate invasion of nonphagocytic cells.

It has been documented that upon pathogen infection, NF-κB-dependent gene expression plays an important role in regulating apoptosis, inflammatory, and other immune related responses (114, 115). Nuclear factor (NF-κB) is a universal transcription factor that interacts with IκBα inhibitor in the cytosol and the later is the main cellular mechanism preventing NF-κB–dependent transcription under normal conditions. Degradation of IκBα protein in response to stimuli like cytokines, growth factors, and pathogen components is an obligatory step in NF-κB activation. This degradation is mediated by the Ub-proteasome pathway and requires that the IκB molecules are phosphorylated at specific serine residues (116). NF-κB then translocates to the nucleus to activate transcription of responsive genes. In normal circumstances, the IκBα is rapidly resynthesized. Therefore, the detection of IκBα degradation is an indirect method to determine NF-κB activation. The results presented in Figures 29 and 30 showed a similar pattern of IκBα degradation in both phagocytic and nonphagocytic cells infected with wild type *B. pseudomallei*, *B. thailandensis*, and the complemented mutant Cp and Ct. However in the flagellin mutant (MM35), the level of IκBα degradation was slightly less than that in the wild type (at 30 min after infection), suggesting that flagellin protein of *Burkholderia* may participate in NF-κB activation. In *Salmonella*, flagellin has been reported to be a potent mediator for inducing epithelial activation. It induces IκBα degradation, NF-κB translocation, and iNOS expression in culture intestinal epithelial cells (117). Similar results were found in *Pseudomonas aeruginosa*. Exposure of corneal epithelial cells to purified *P. aeruginosa* flagellin resulted in NF-κB activation by assessing IκBα degradation, transcriptional expression and subsequent secretion of IL6 and IL8 (118). It has been reported that the signaling response to bacterial flagellin is mediated by Toll-like receptor 5 (TLR5), resulting in the activation of NF-κB (118, 119). TLRs are an evolutionarily conserved family of receptors that function in innate immunity

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through recognition of pathogen-associated molecular patterns (PAMP). Pattern recognition by TLRs then leads to cytokine production and expression of costimulatory molecules, which can constitute protective innate and adaptive immunity. The fact that TLR5 recognized the conserved domain (D1 and D2 in Figure 3), but not the variable domain (D3) on the flagellin (120, 121), suggested a similar pattern of IκBα degradation may have occurred in *B. pseudomallei* Cp and Ct. In nonphagocytic cells, it seemed to be that NF-κB activation of all *B. pseudomallei* strains were more rapidly inhibited than *B. thailandensis* (Figure 30). This characteristic is unlikely to be associated with a difference in the flagella of *B. pseudomallei* and *B. thailandensis* because there was no difference in the pattern of IκBα degradation between *B. pseudomallei* Cp and Ct. Therefore, other factor likes the TTSS may be involved and this is consistent the well known fact that a virulence factor that is only presented in *B. pseudomallei* but not in *B. thailandensis*. In other organisms such as *Bordetella bronchiseptica* and *Yersinia pestis*, TTSS was found to respond to inhibition of NF-κB activation (122, 123). Infection of an epithelial cell line with wild type, but not TTSS deficient *B. bronchiseptica*, resulted in inhibition of NF-κB translocation to the nucleus by inducing the aggregation of NF-κB into large cytoplasmic complexes which were refractory to activation. This suggests that one or more type III-secreted effector molecules may be targeted into the host cell to specially inactivate this transcription factor (122). In *Yersinia*, it was found that YopJ, one virulence factor secreted via a TTSS, bound directly to the superfamily of MAP (mitogen-activated protein) kinases which inhibited c-Jun amino-terminal kinase, p38, and NF-κB signaling pathways (123). NF-kB plays a central role in the transcriptional regulation of pro-inflammatory cytokine genes, therefore, suppression of NF-kB activation could provide an effective mechanism to down regulate a broad range of immunostimulatory responses in the infected host.

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CHAPTER VI
CONCLUSION

Since flagella are considered to be one of virulence factors in many pathogens, the difference within the variable region of the flagellin gene between virulent *B. pseudomallei* and avirulent *B. thailandensis* were investigated. The motility function of *B. pseudomallei fliC* mutant was complemented with its own *fliC* or with *B. thailandensis fliC* and then comparing the invasiveness capacity, internalized ability, intrcellular replication, and NF-κB activation with the *fliC* mutant and wild type *B. pseudomallei*, and *B. thailandensis* in both phagocytic (mouse magcrophage cell line RAW264.7) and nonphagocytic (human lung epithelial cell line A549) cells. The results from this study showed that both complemented *fliC* mutants Cp and Ct were equally invasive in human lung epithelial cells, and were also not different in being internalized by mouse macrophage cells. Thus, the 15-base pair region which is only present in the *fliC* variable region of *B. pseudomallei* appears not to be essential for facilitating of *B. pseudomallei* into the phagocytic and nonphagocytic cells. However, the presence of flagella resulted in a higher invasive capacity of *B. pseudomallei* than the *fliC* mutant. This indicates the importance of flagella for both phagocytic and nonphagocytic cell invasion. The pathogenic *B. pseudomallei* 1026b showed higher invasive capacity than nonpathogenic *B. thailandensis* E257 in both phagocytic and nonphagocytic cells, suggesting that *B. pseudomallei* may need some other factor(s) including TTSS to facilitate host cell invasion. The observation of IκBα degradation is an indirect method to determine activation of the nuclear transcription factor NF-κB. The wild type of *B. pseudomallei* and *B. thailandensis*, and both complemented mutants showed similar pattern of IκBα degradation, which was different from that of in the flagellin mutant. The level of IκBα degradation in the mutant was slightly less than the wild type in both phagocytic and nonphagocytic cells, suggesting that involvement of flagellin in NF-κB activation. However, in nonphagocytic cells, NF-κB activation of all *B. pseudomallei* strains was more rapidly inhibited than *B.*

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thailandensis. This characteristic may be due to the presence of type III secretion system which only present in *B. pseudomallei* but not in *B. thailandensis*.

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APPENDIX

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APPENDIX

Reagents for Bacteria culture

LB broth	**1,000**	**ml**
Peptone	20	g
Yeast extract	5	g
NaCl	5	g
Distilled water to a final volume of	1,000	ml
SOC medium	**1,000**	**ml**
Peptone	20	g
Yeast extract	5	g
NaCl	5	g
$MgCl_2$	5	mM
$MgSO_4$	5	mM
Glucose	20	mM
Distilled water to a final volume of	1,000	ml

Reagents for cell culture

Supplemented DMEM medium	**100**	**ml**
Unsupplemented DMEM medium	89	ml
Heat-inactivated Fetal Bovine serum	10	ml
(FBS was heated at 56 °C for 30 min)		
L-glutamine	1	ml
Supplemented Ham F-12	**100**	**ml**
Unsupplemented Ham F-12 medium	89	ml
Heat-inactivated Fetal Bovine serum	10	ml
L-glutamine	1	ml

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Reagents for gDNA extraction

Lysozyme solution

Lysozyme	2	mg/ml
Glucose	50	mM
Tris-HCl pH 8.0	25	mM
EDTA pH 8.0	20	mM

Lysis solution

Sodium dodecyl sulphate-polyacrylamide (SDS)	2%	w/v
Proteinase K	200	µg/ml
NaCl	10	mM

TE

Tris-HCl pH 8.0	100	mM
EDTA pH 8.0	10	mM

Rnase A 10 mg/ml

Reagents for plasmid extraction

Rapid alkaline lysis method

Lysis solution

SDS	1%	w/v
NaOH	0.2	N

Neautralize solution

Sodium acetate (NaOac) pH 4.8	3	M

GTE solution

Glucose	50	mM
Tris-HCl pH 8.0	25	mM
EDTA pH 8.0	10	mM

QIAGEN Plasmid Midi Kit

Buffer P1

Tris-HCl pH 8.0	50	mM
EDTA	10	mM

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RNase A	0.1	mg/ml
Buffer P2		
NaOH	200	mM
SDS	1%	w/v
Buffer P3		
NaOAc pH 5.5	3	M
Buffer QBT		
NaCl	750	mM
MOPS pH 7.0	50	mM
Ethanol	15%	v/v
Triton X-100	0.15%	v/v
Buffer QC		
NaCl	1.0	mM
MOPS pH 7.0	50	mM
Ethanol	15%	v/v
Buffer QF		
NaCl	1.25	mM
Tris-HCl pH 8.5	50	mM
Ethanol	15%	v/v

Reagents for SDS-PAGE and Western blot

Acrylamide 30% w/v	**500**	**ml**
Acrylamide	150	g
N,N-bis-methylene acrylamide	4.5	g
Distilled water to a final volume of	500	ml
Tris-HCl pH 8.8	**1.875**	**M**
Tris-HCl pH 6.8	**1.875**	**M**
10X SDS-PAGE buffer	**1,000**	**ml**
Trizma base	3	g
Glycine	14.4	g
SDS	1	g

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Distilled water to a final volume of	1,000	ml
Towbin's buffer	**3,000**	**ml**
Trizma base	9.1	g
Glycine	43.2	g
Methanol	600	ml
Distilled water to a final volume of	3,000	ml
10X Phosphage buffer saline (PBS)	**1,000**	**ml**
NaCl	8.9	g
Na_2HPO_4	1.28	g
$Na_2HPO_42H_2O$	0.156	g
Distilled water to a final volume of	1,000	ml
5X Sample buffer pH 6.8	**10**	**ml**
Trizma base	0.3784	g
SDS	0.5	g
Glycerol	5	ml
2-mercaptoethanal (2-ME)	2.5	ml
1N HCl	1.5	ml
Bromophenol blue	0.005	g
Distilled water to a final volume of	10	ml

PBST

1% v/v of Tween 20 in 1X PBS

Reagents for agarose gel electrophoresis

10X TBE buffer pH 8.2-8.4	**1,000**	**ml**
Trizma base	108	g
Boric acid	55	g
EDTA	9.3	g
Distilled water to a final volume of	1,000	ml

Agarose gel

0.7-1.2 % of agarose powder dissolved in 1X TBE buffer

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