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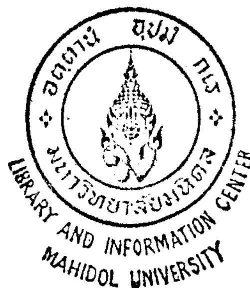

REGULATION OF A FLAGELLIN GENE IN *BURKHOLDERIA MALLEI* (A NON-MOTILE SPECIES)

ATCHARA PAEMANEE

อภินันทนาการ
จาก
บัณฑิตวิทยาลัย มหาวิทยาลัยมหิดล

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***BURKHOLDERIA MALLEI* (A NON-MOTILE SPECIES)**

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for the degree of Master of Science (Biochemistry)

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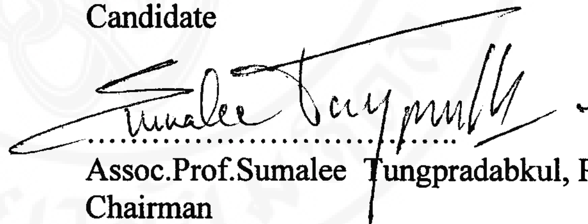
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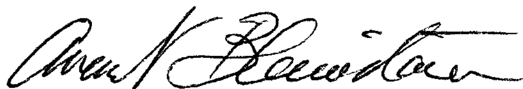
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MissAtchara Paemanee

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Burkholderia mallei is a gram negative rod-shape bacteria and it is a sole species in genus *Burkholderia* which is non-motile. *B. mallei* is considered to be the parasite on equines, in which it causes glanders and the infection can be transmissible to a large variety of animals. *B. mallei* is genetically related with *B. pseudomallei*, a causative agent of melioidosis, due to 16S rRNA sequence showing 100% homology. Although the motility property can be used to distinguish the two species, the genetic markers might be a rapid and simple method for accurate identification of the species. We therefore attempted to search for a genetic difference between the two species. Based on motility property, specific primers for amplification of flagellin gene from *B. pseudomallei* were used. A 1.1 kb of PCR product was obtained. Surprisingly, sequencing analysis of the 1.1 kb product showed a 99% nucleotide sequence homology or 100% amino acid sequence identity to *B. pseudomallei*. Thus, we tried to investigate the regulation of the gene expression in transcription and translation level in *B. mallei* compared with *B. pseudomallei*. The results suggested that mRNA was not transcribed and we were not able to detect flagellin protein from *B. mallei* by Western blot analysis. The 5′upstream sequence of *B. mallei* flagellin gene, which is nearly the same as the regulatory region of *B. pseudomallei* strains reported by Neubauer et al. Southern blot hybridization was performed to determine GATC methylation of 5′ untranslated sequence. The methylated upstream region of flagellin gene in both *B. mallei* and *B. pseudomallei* sequence show the same pattern. In addition, chemotaxis protein CheW gene and Chemotaxis response regulator CheY gene were able to be isolated from chromosomal DNA of *B. mallei* by PCR. The sequence analysis of both genes is also identical to CheW and CheY of *B. pseudomallei* respectively. Existence of the same methylation pattern on upstream region of flagellin gene and the appearance of both chemotaxis involving genes lead one to believe that expression of *B. mallei* flagellin gene is under control of upper operon in flagellar hierarchy. In addition, *Sau*3AI and *Mbo*I restriction patterns of *B. mallei* and *B. pseudomallei* are different, especially DNA fragment of above 1.2 kb. As no sequence data have been available, these patterns are worthy to elucidate the genetic dissimilarities among this two *Burkholderia* species.

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Burkholderia mallei เป็นสปีชีส์เดียวในจีนัส *Burkholderia* ที่เคลื่อนที่ไม่ได้และเป็นสาเหตุของโรคมองคล่อพิษในสัตว์หลายชนิดโดยเฉพาะสัตว์จำพวกม้า ลำดับเบสของ 16S rRNA ที่เหมือนกันร้อยเปอร์เซ็นต์แสดงความใกล้ชิดทางพันธุกรรมกับ *B. pseudomallei* ที่ก่อโรคเมลิออยโดสิสในคน ความแตกต่างทางความสามารถในการเคลื่อนที่ถูกนำมาใช้เป็นแนวทางการแยกเชื้อทั้งสองชนิดโดยคุณสมบัติทางพันธุกรรมบนสมมุติฐานที่ว่า *B. mallei* ซึ่งไม่เคลื่อนที่ควรจะขาดยีนแฟลกจิลินที่เกี่ยวข้องกับการเคลื่อนที่ แต่จากการศึกษาโดยวิธีพีซีอาร์พบว่า *B. mallei* มียีนที่เป็นรหัสของโปรตีนแฟลกจิลินที่มีขนาดและลำดับกรดอะมิโนเท่ากับที่พบใน *B. pseudomallei* งานวิจัยนี้จึงทำการสำรวจการควบคุมการแสดงออกของยีนแฟลกจิลินของ *B. mallei* โดยวิธี Westhern blot immunoassay ศึกษาการแปลรหัสของยีนเป็นผลผลิตโปรตีนและตรวจหา mRNA ในระดับการถอดรหัสโดยวิธี RNA dot blot hybridization แต่ไม่ให้ผลบวกในการศึกษาดังกล่าว จากผลที่ได้แสดงว่าแฟลกจิลินใน *B. mallei* ถูกควบคุมในระดับการถอดรหัส จึงพิจารณาจากการรายงานลำดับเบสด้านปลาย 5′ของยีนแฟลกจิลิน *B. mallei* ของ Neubauer และคณะ ที่ตรงกับบริเวณควบคุมของยีนแฟลกจิลินใน *B. pseudomallei* เป็นแนวทางนำไปสู่การศึกษา GATC methylation ในบริเวณดังกล่าวโดยเทคนิค Southern hybridization พบว่าให้แบบแผนที่เหมือนกันทั้ง *B. mallei* และ *B. pseudomallei* แสดงว่า methylation ไม่มีส่วนในการควบคุมการแสดงออกของยีนแฟลกจิลินดังกล่าว เพื่อทดสอบว่ายังมียีนที่เกี่ยวข้องในขบวนการเคลื่อนที่เพื่อตอบสนองต่อภาวะแวดล้อมใน *B. mallei* เทียบกับ *B. pseudomallei* งานวิจัยนี้จึงทดลองแยกยีน chemotaxis response regulator CheY และ chemotaxis protein CheW โดยวิธีพีซีอาร์ และพบว่า *B. mallei* มียีนทั้งสองเช่นเดียวกับที่พบใน *B. pseudomallei* แสดงว่าการแสดงออกของยีนแฟลกจิลินของเชื้อนี้อยู่ภายใต้การควบคุมของยีนที่เกี่ยวข้องกับการเคลื่อนที่ในโอเปอรอนที่สูงขึ้นไป แม้ว่าการตัดดีเอ็นเอจากโครโมโซมด้วย *Mbo*I และ *Sau*3AI ของ *B. mallei* ให้ชิ้นดีเอ็นเอย่อยๆไม่ต่างกัน แต่รูปแบบของดีเอ็นเอดังกล่าวแตกต่างกับชิ้นดีเอ็นเอที่ได้จาก *B. pseudomallei* อย่างชัดเจน โดยเฉพาะชิ้นดีเอ็นเอที่มีขนาดมากกว่า 1.2 กิโลเบส ผลดังกล่าวแสดงให้เห็นถึงความแตกต่างในเชิงพันธุกรรมระหว่างเชื้อทั้งสองได้เป็นอย่างดี

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LIST OF ABBREVIATIONS

A	absorbance
ATCC	American Type Culture Collection, Rockville, Maryland 20852 USA
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
BSU	BioService Unit
cm	centimeter
^{o}C	degree Celsius
dATP	deoxyadenosine 5′- triphosphate
dCTP	deoxycytosine 5′- triphosphate
dGTP	deoxyguanosine 5′- triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
DMS	Department of Medical Sciences, Ministry of Public Health, Thailand
dTTP	deoxythimidine 5′- triphosphate
dUTP	deoxyuridine 5′- triphosphate
EDTA	ethylene diamine tetraacetic acid
GTE	glucose-tris-ethylene diamine tetraacetic acid
hr	hour
IPTG	isopropylthio-β-D-galactoside
kb	kilobases

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LIST OF ABBREVIATIONS (CONTINUED)

LB	Luria-Bertani
l	liter
LPS	lipopolysaccharide
M	molar
min	minute
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
N	normal
NBT	nitroblue tetrazolium
NCBI	National Center for Biotechnology Information
ng	nanogram
nm	nanometer
NSTDA	National Science and Technology Development Agency
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
rpm	revolution per minute
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid

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LIST OF ABBREVIATIONS (CONTINUED)

SDS	sodium dodecyl sulfate
sec	second
SSC	standard saline citrate
TAE	tris-acetate-ethylene diamine tetraacetic acid
TBE	tris-borate-ethylene diamine tetraacetic acid
T_a	annealing temperature
T_m	melting temperature
U	unit
μg	microgram
μl	microliter
uv	ultraviolet
v/v	volume by volume
w/v	weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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CHAPTER I

INTRODUCTION

B. mallei and *B. pseudomallei* are the causative agents of infectious diseases, namely, glanders and melioidosis respectively. *B. pseudomallei* can be found in soil and surface water in rice paddies. Humans and a large variety of animals can be infected by soil contamination through skin abrasion, ingestion and inhalation. Melioidosis or glanderslike disease is endemic in northern Australia and Southeast Asia including the Northeast Region of Thailand (1). Sporadic cases can be found throughout the world between 20 degrees north and south latitude (2). Septicemic melioidosis is a major cause of morbidity and mortality in northeastern Thailand (3). In the other hand, glanders is a serious infectious disease in equine (4). Human glanders is rare but can be found primarily in veterinarians, horse and donkey caretakers, abattoir workers (5) and laboratory workers (6). The spectrum of both diseases ranges from a symptomatic infection to fulminate septicemia (7, 8), which need rapid detection to decrease the mortality rate. *B. mallei* and *B. pseudomallei* are closely related. They are very similar with respect to their nutritional and biochemical properties (9). Even through *B. mallei* is a permanently non-motile bacterium. DNA-DNA hybridization presents DNA similarity of more than 80% in this two species (10). 16S rRNA sequences also show 100% homology, and confirm a relationship between them (11). As describe above, melioidosis bears a striking resemblance to glanders both clinically and pathologically but they are

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epidemiologically dissimilar. However, genetic identification of these two species still to be investigated.

Bacteriological aspects

1. History and Nomenclature

The etiological agent of glanders was discovered in 1882 by Loeffler and Schutz and subsequently named *Bacillus mallei* by Zopf. It has been placed at various times in *Pfeifferella*, *Mallesmyces*, *Actinobacillus*, *Loefflerella*, *Acinetobactor*, and *Pseudomonas* (9,12). Recently, on the basis of DNA-rRNA hybridization, DNA-DNA hybridization and 16S rRNA sequence *Pseudomonas mallei* has been classified as member of *Burkholderia* genus, namely *Burkholderia mallei* (11). Since aerosol spread is efficient, *B. mallei* has been viewed as a potential biological weapon agent. During World War I glanders was believed to be spreaded deliberately by agents of the Central Powers to infect large members of Russian horses and mules. This had an effect on troop and supply convoy as well as on artillery movement, which were depending on horses and mules. The Japanese deliberately infected horses, civilians, and prisoners of war with *B. mallei* at the Pinfang (China) Institute during World WarII. The low transmission rates of *B. mallei* to man from infected horses is examplified by the fact that in China, during World war II, thirty percent of tested horses were positive for glanders, but human cases were rare (5).

2. Characterization

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2.1 Physiology and morphology

B. mallei is a non-motile, gram-negative rod shape bacteria that stain irregularly with methylene blue (4,5). In contrast to *B. pseudomallei*, *B. mallei* grows more slower on nutrient agar. Normally, this organism grows on most common meat infusion bacteriological media but requires glycerol for optimum growth at 37 ^{O}C for 48 hours (5, 7, 9). Like other *Burkholderia* genus, *B. mallei* is a obligate aerobe (9). The form and colour of growth of *B. mallei* on solid media do not show the variations found with *B. pseudomallei*. Colonies ranged from smooth cream to smooth white. An occasional rough white is rare (9,10). In addition its distinctive colonial morphology *B. mallei* is generally very scanty (9,10). The colonial appearance of *B. mallei* is overlap in appearance with 24 hours *B. pseudomallei* colonies and it is difficult to distinguish them.

2.2 Biochemistry

There is usually no growth under strictly anaerobic condition in a complex medium containing glucose, but *B. mallei* can grow under strictly anaerobic condition in a complex medium containing nitrate by reduction nitrate to nitrite (13). Growth occurred at 42 ^{O}C but not at 5 ^{O}C (9). Most of *B. mallei* strains produce a arginine dihydrolase activity. However, it fails to oxidize sucrose or maltose. Although spectrum of organic compounds utilizable by *B. mallei* as sole sources of carbon and energy resemble that of *B. pseudomallei*, it is less extensive (9). This may cause it grows more slowly than *B. pseudomallei* and it clearly lacks many enzymic potentialities possessed by *B. pseudomallei*.

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Table 1 Physical and biochemical characteristic of *B. mallei* and *B. pseudomallei*.(13)

Test	% Positive strains	
	B. pseudomallei (n = 70)	B. mallei (n = 8)
Oxidase	100	25
Growth		
MacConkey	100	88
Cetrimide	0	0
42°C	100	0
Nitrate reduction	100	100
Gas from nitrate	100	0
Arginine dihydrolase	100	100
Lysine decarboxylase	0	0
Ornithine decarboxylase	0	0
Hemolysis	0	0
Hydrolysis		
Urea	13	12
Citrate	77	0
Gelatin	79	0
Esculin	59	0
Acid from:		
Glucose	100	100
Xylose	86	12
Lactose	99	12
Sucrose	66	0
Maltose	99	0
Mannitol	94	62
Motile	100	0
No. of flagella	> 2	0

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Table 2 Difference in nutritional spectrum between *B. mallei* and *B. pseudomallei* strains (9)

Compound	Fraction of position strains	
	B. pseudomallei	B. mallei
D-Ribose	26/26	0/15
D-Xylose	0/26	14/15
L-Arabinose	0/26	8/15
Isobutyrate	26/26	0/15
Valerate	26/26	0/15
Isovalerate	26/26	0/15
Caproate	26/26	0/15
Heptanoate	26/26	0/15
Pelargonate	26/26	0/15
Caprate	26/26	0/15
Levulinate	24/26	0/15
Erythritol	26/26	0/15
Glycine	0/26	14/15
L-Isoleucine	25/26	0/15
L-Lysine	25/26	0/15
DL-α-Aminobutyrate	0/26	13/15
L-Kynurenine	26/26	0/15
Kynurenate	26/26	0/15
Ethanolamine	26/26	0/15
Butylamine	24/26	0/15
α-Amylamine	24/26	0/15

3. Mode of transmission

Glanders was at one time widespread throughout Europe and still occurs in Southeast Asia, the Middle East, Africa and South America (14,15). It is a serious infection disease of livestock, particularly horses, mules and donkeys. Other animals become infected through inoculation of broken skin of the nasal mucosa with contaminated discharges. The most susceptible of these occasionally infected species

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are ferrets, moles, field mice, cat and dogs. Sheep, goats, hogs, rabbits, white mice and house mice are reported to be less susceptible, and cattle are immune (16). A number of instances of airborne infection have been reported in laboratory workers (5,14).

4. Clinical Manifestation

The route of infection, the dose and virulence of inoculated organisms are the determining factors in the severity of the disease, which consists of two basic manifestations (1) the nasal-pulmonary form (glanders) and (2) the cutaneous form (farcy) which may be present simultaneously and are usually accompanied by systemic disease. Clinical disease may be acute or chronic, or the disease may be subclinical and even latent (14,16).

5. Treatment

In experimental infections, 3 weeks of therapy gave better results than 1 week did. Sulfadiazine 100 mg/kg per day in divided doses for 3 weeks has been found to be effective in experimental animals and in human. Doxycycline, rifampin, trimethoprim-salfamethoxazole, and ciprofloxacin have effective in experimental infection in hamsters (6,16,17).

6. Virulence determinants

The bacteria that cause the infection possess the degree of their ability to induce diseases. These pathogenic bacteria have special properties, referred to as virulence factors, which enhance the ability to cause disease. These factors may be common to all bacteria of a genus or species, or they may be unique to some strains (18). However, the virulence factors have their roles in many steps for infection as colonization, invasion, survival and growth with in host. In addition, pathogenic

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bacteria produce various toxins and enzymes to disrupt host cell functions and generally destroy host cells.

Virulence factors that determine the ability of many pathogens to attach to surface of host cells are termed adhesins or colonization factors. Adhesin are protein or polysaccharides on the surface of bacterial cells that bind bacteria to epithelial cells within the host. One of cell-surface structures responsible for adherence is fimbriae (pili) which bind tightly to receptors on the surface of host cell. Capsules and slime layers also contribute to the bacteria to attach to particular host cells. Gram-positive bacteria do not posses fimbriae but they have other surface adhesins that bind extracellular protein on host cells (19). Unlike many adhesins, filamentous hemeagglutinin is a free molecule that makes two different attachments, one to a molecule on the bacterial cell surface and the other to a carbohydrate receptor on a host cilium (20). Some pathogenic bacteria produce different adhesin at different times. Having a repertoire of adhesins helps a pathogen evade the body 's immune defenses and allows the pathogen to attach more than one cell type.

Flagellum seems to be a potential virulence factor in several steps for bacterial infection in host. Motility or adhesin-associated with flagellin is needed for the colonization of *Vibrio cholerae* (21,22), *Compylobacter jejuni* and *Helicobacter pylori* (23). The studies in some bacteria, as *P. aeruginosa*, indicate that motility contributes to the invasive capacities. One of four flagellin subunits, like flagellin A of *V. anguillarum*, that constitute the flagellar filament is needed for virulence after invasion of the host (21,22,24).

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Many pathogenic bacteria are called intracellular pathogens due to their ability to penetrate and grow inside the host cells (19). Some pathogens use enzyme-containing pilli to penetrate host tissue (18). Some other bacteria, *N. gomorrhes*, have a surface protein called Protein I to trigger its uptake into host cell (20). Before multiplication of these bacteria, they must evade from host 's immune system by using several antiphagocytic mechanisms. Some cases, bacteria such as *Shigella*, *Salmonella*, *Listeria* and *Yersinia* are able to invade nonprofessional phagocytic cells (i.e. epithelial cells), where they can escape from the endocytic vacuole and grow in the cytoplasm of host cell. Some cases in phagocytic monocytes or neutrophils, bacteria, as tubercle bacillus and *Brucella abortus*, can inhibit the phagosome-lysosome fusion and grow within the phagosome (19).

In addition, secretion of a long polysaccharide polymer surround the bacteria, called capsule, prevent a leukocyte from making direct contact with the pathogen (18,19,25). The capsule may prevent the interaction between antibody and C3 bound to the outer membrane and their receptors on phagocytic cells (19). Alternatively, the capsule may prevent C3b, Bb formation (18). Like capsules, M proteins, which appear as hairlike projection on surface of *Streptococcus pyogenes*, prevent phagocytosis by blocking the alternate pathway of complement activation (19,20). In the same way, fimbriae also impede phagocytosis through their ability to bind to a surface component phagocyte. However, toxic substances called leukocidins, produced by some bacteria, can destroy neutrophils that are very active in phagocytosis by degradation lysosomes and cause the death of white blood cell (19,26).

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Environmental conditions at most body sites limit the growth of the pathogens. Pathogens often have virulence factors that enable them to overcome. The ability of bacterial to grow within blood is limited by the lack of available iron. Binding iron to high affinity glycoprotein, transferrin and lactoferrin, are host defense mechanisms that prevent establishing an infection in the blood. Some pathogens produce low-molecular-weight compound with a higher affinity for iron, called siderophores. Siderophores are able to capture iron and bind to specific membrane receptors on bacteria, releasing the iron for microbial growth (19). There are 2 types of siderophores, which are catechols and hydroxymates (18). In both types, the ferric ion is chelated between two hydroxyl groups or between hydroxyl group and an amino group. *Neisseria meningitidis* and *Mycobacterium tuberculosis*, instead, synthesize outer membrane protein to remove iron directly from transferrin (25). Bacterial endotoxins are heat-stable lipopolysaccharide derived from gram-negative bacterial cell walls. Endotoxins of different bacteria vary in their potency and ability to cause characteristic symptoms. Toxicity of the molecule resides in its lipid A. This toxin is mot secreted by pathogen, but is released when bacterial cell dies. Endotoxins are responsible for the development of sepsis and septic shock (20).

Exotoxins are highly destructive proteins produced by both gram-positive and gram-negative pathogens. Most exotoxins are composed of two subunits a B, or binding, component and an A, or active, component. The B component attaches the exotoxin to molecular receptors on curtain type of host cells. It is the B component that accounts for the specificity of exotoxins. After binding, the A component enters the cell and disrupts cell function usually by inhibiting one

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specific metabolic reaction (20). These can be divided into 3 types, based on their modes of action (1) cytotixins, which kill host cells by enzymatic attack or by blocking essential cellular metabolism. (2) neurotoxins, which interfere with normal nerve impulse transmission; and (3) enterotoxins which effect cells lining the gastrointestinal tract (25,26).

Many bacteria produce and secrete enzymes, called exoenzymes or extracellular enzymes (20) which may play an important pathogenic role by a variety of mechanisms. These enzymes can be divided into 3 groups. First, there are extracellular enzymes that lyse cells. The cytolysins attack cell menbranes, causing host cells to lyse. Hemolysins lyse red blood cells. Leukocidins lyse leukocytes, white blood cells that are among the body's most powerful defenders against microbial infection. The second type of extracellular enzyme breaks down the materials that hold cells together to form tissues. Hyaluronidase degrades the polysaccharide hyaluronic acid that cements cells together in many different tissues. Collagenase degrades the protein collagen, a major structural component of connective tissue. The third type of extracellular enzymes affect the delicate balance between formation and destruction of blood clots. Coagulases split the serum protein fibrinogen to form fibrin, thus creating blood clots. In contrast, kinases split fibrin, dissolving clots. (One of these bacterial kinases, streptokinase, is used clinically to dissolve the blood clots that block coronary arteries during a heart attack.)

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Extracellular enzymes and other toxic proteins are capable of harming cells, but they do not necessarily mean they play roles in causing the signs and symptoms that are recognized as disease. That is, although they are toxins, they

are not known for certain that all of them are virulence factors, substances that help cause disease (20).

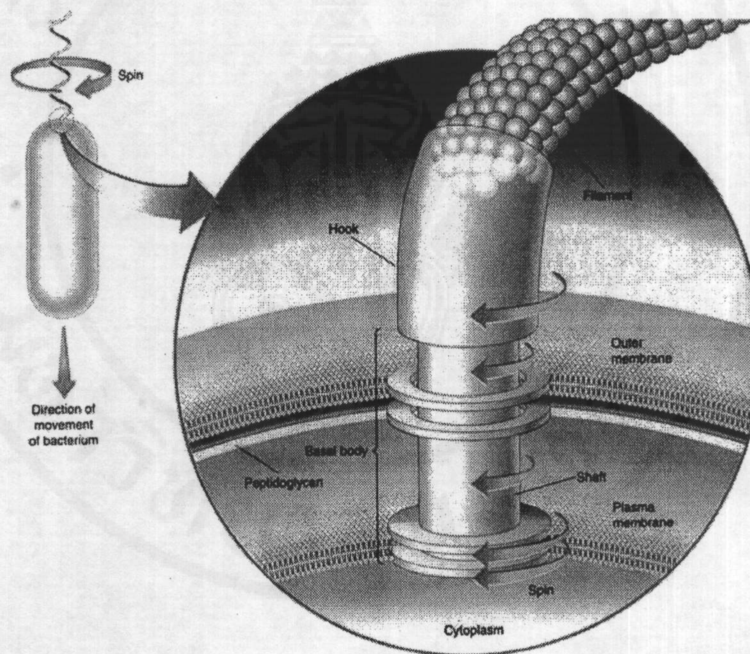

Figure 1 Drawing of gram-negative bacterial flagellar component. Ultrastructure of flagella compose of basal bodies, hooks and filaments which consist of flagellin proteins (27).

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Figure 2 EM of flagellar basal bodies and hooks in gram-negative bacteria. All four rings (L, P, S, and M) can be clearly seen. The uppermost arrow is at the junction of the hook and filament. Bar = 30 nm. (28).

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7. The flagellar gene system

Based on genetic analysis of *Salmonella typhimurium* and *Escherichia coli*, there are nearly 50 genes involved in flagellar function and formation. Most of the flagellar genes are clustered in three regions of the bacterial chromosome termed region I, II and III. Genes, which are responsible for flagellar formation are called flg flh fli or flj. The base symbol starts with fl. And the flagellar region within the chromosome determines its third letter. Thus, genes in flagellar region I are assigned the symbol flg (for flagellum). Those in region II are assigned the symbol flh, those in region III are assigned the symbol fli and those in the phase 2 flagellin region of *S. typhimurium* are assigned the symbol flj. Genes are given alphabetical extensions (flgA, flgB, etc.) on the basis of genome order (29). These clustered genes constitute 14 and 13 different operon in *E. coli* (30) and *S. typhimurium* (31). There are three kinds of genes, which are responsible for flagellar function, including flagellar rotation (mot), chemotaxis (che), and transmembrane signal transduction of chemotaxtic stimuli (tar, trg, tsr, etc).

Flagellar operons form a coordinated system of expression, or regulon and are arranged in a hierarchy in which expression of operons in a given class is necessary for expression of operons in lower classes (32). Four classes can be defined: class1, 2, 3a and 3b. Class 1 comprises only the flhD operon. This operon contains two genes, flhD and flhC. It is not known whether the master operon itself is transcribed using the primary cellular initiation factor (σ^{70}) or whether it employs a special one. Control of transcription is provided by the c-AMP/CAP system. Class 1

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contains only the master operon, whose expression is necessary for the expression of all other operons.

Class 2 operons include seven operons, flgA, flgB, flhB, fliA, fliE, fliF and fliL. These operons involve in hook-basal body complexes in the morphogenic pathway of flagellar structure. The class 1 protein, FlhC and FlhD are positive regulators of the class II operons (33). The mechanism is not clear, FlhD may combine with FlhC act as σ^{C2} factor The expression of class 2 genes are required for expression of the class 3 operons.

Class 3 operons comprise seven operons, flgM, flgK, fliD, fljB, fliC, motA and tar. Class 3 can be divided into 3a and 3b. Class 3a contains flgM, flgK, fliD and class 3b contains fliC, fljB, motA and tar operon. These operons are responsible of the filament, the final stop of flagellar assembly and function of the complete flagellum. The consensus promote sequences for class 3a operons and class 3b operons appear indistinguishable, and both classes expression can occur using FliA as σ factor (σ^{C3} or σ^{F}). Only class 3a operons have appreciable levels of expression, even in the absence of FliA. This expression is dependent on expression of the flhCD operon, like class 2 operon. The identical –10 region produce binding of σ^{C2} to both class 2 and class 3a. The absence of the –35 consensus in class 2 operon presumably provides the specificity that prevents recognition of σ^{C3}. In addition, although there are the identical promoter, inability of recognition by of σ^{C2} to 3b. This leads to the discrimination of class 3 into 2 classes above. Their expression needed functions of

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class I and class II operon, and their function were not responsible for expression of the other operons (34).

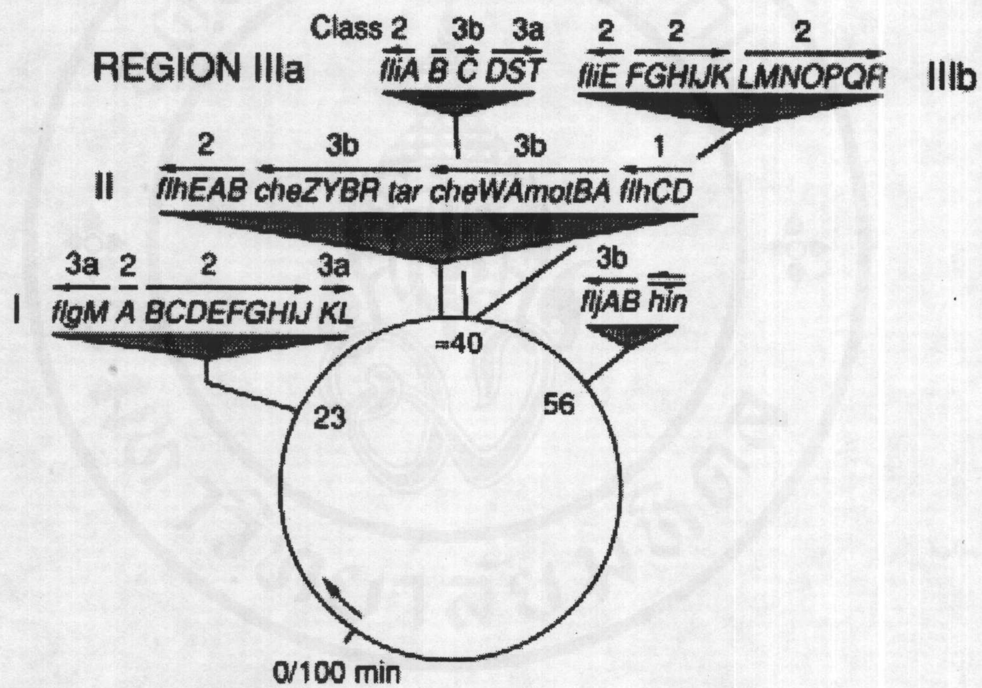

Figure 3 Chromosomal organization of flagellar and related genes in *S. typimurium*. (The organization for *E. coli* is essentially the same except for the presence of the tap receptor gene adjacent to tar, and the lack of flj region). (34)

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8. The process of flagellar assembly

Nearly 50 gene products are expressed in three stages. The membrane embedded rotary device (basal body) is constructed first and followed by the construction of structure external to the cells, such as the hook and the helical filament. Flagellar assembly is tightly coupled with gene expression. In the beginning, the products of flhD and flhC, which positively control the transcription of a second class of flagellar genes, are needed in synthesizing the early stages proteins that act antagonistically. FliA is held in an inactive state by the anti-σ factor FlgM until the stage in flagellar assembly which FliA activity is needed (35).

By isolation of ring structure from strains overproducing FliF and by electron microscopy. FliF is the first structure, which is inserted at the inner membrane by the polymerization of a single polypeptide. It self-assembles into the MS ring complex, which is a core structure of the basal body (36). When the MS ring complex has been built, a bell-shaped structure will start accumulating at the rim of the M ring extending towards the cytoplasm and thus is called the C-ring. The C-ring contains all three components of switch complex (FliG, FliM, and FliN) (37,38). Flagellar proteins seem to go through specialized flagellum-specific pathway, the channel in the filament and hook, for assembly outside the cell membrane. There is also the apparatus that actively and selectively transfers flagellar protein to the channel. Since FliI amimo acid sequence is homologous to that of proteins of the antigen-export system, it could be a component of this apparatus, which have an affinity for flagellar proteins and probably consume energy for function (39).

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Once the export apparatus is installed at the cytoplasmic side of M ring, the protein export can begin. FlgB, FlgC and FlgF are formed the proximal rod. FlgG, which is the major component in the rod, form, the distal part of the rod extends to the outer membrane. To overcome this physical barrier, two more rings, P (peptidoglycan) and L (lipopolysaccharide), encircle the rod in the plane of the peptidoglycan layer and the outer membrane, respectively. The former protein encoded by *flgI* and the latter one is encoded by *flgH*. These two proteins are believed to be secreted in a conventional signal peptide-dependent manner.

Outside the cell, the hook protein subunits (FlgE) will polymerize into hooks. This polymerization requires FlgD scaffolding protein. During polymerization, FlgD subunits stay at the tip of growing hook and nascent hook proteins seem to be inserted beneath a FlgD cap. When the hook reaches its mature length. FlgR replaces FlgD. Not any FliK but also FlhB is involved in hook-length control but not the molecular ruler. Completion of hook assembly signals the release of a scaffolding protein and the beginning of export the negative regulator, FlgM. Removal of FlgM relieves the inhibition of FliA, the σ factor that turns on class 3 genes such as the flagellin, filament cap, junction protein gene, etc (37, 40).

The flagellar filament does not grow from the hook directly, instead, FlgK and flgL are inserted between the two structure. These minor proteins are also called HAP1 and HAP3 (Hook-associated protein). The filament consists of single protein, flagellin. Filament formation requires HAP2 or FliD, which adds and polymerizes new flagellin subunits beneath a HAP2 cap. Then, HAP3 plays a role in stabilizing the filament structure (41). It seems that regulation is governed at the

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export level. Two factors may prevent the filament from getting excessively long: the rate of elongation decreases with length due to increasing of the frictional coefficient for passage of a column of monomer along length and long flagellar filament are relatively fragile and so breakage resets the length.

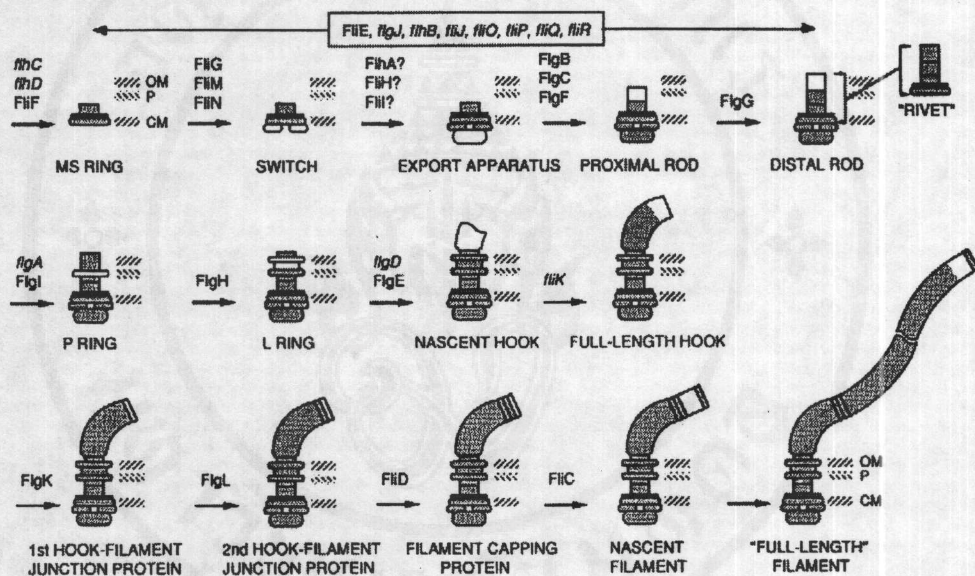

Figure 4 The steps in flagellar assembly (34).

9. The chemosensory pathway

Cells of bacteria swim using flagella which comprise thin helical filament, hooks and basal bodies, each driven at the base by a rotary motor. This motor extends through the cytoplasmic membrane and the energy for rotation comes from the transmembrane proton gradient (Some bacteria use sodium ion instead). Bacterial motor can switch direction and produce a random pattern of motility. When the motors

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turn counterclockwise (CCW), the filaments join into a bundle that propels the cells in smooth trajectory called "run". Whereas if motors reverse to clockwise direction, the bundle disperses and causes rapid somersaulting called "tumble". Unstimulated cells alternate 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 in a new direction that almost uncorrelated with the original trajectory. Bacterial cells that can migrate in moist surfaces "swarming" are also likely to have same random character (42, 43).

The random walk would be a poor strategy for migration because the net distance is proportional (on average) to only the square root of the time spent. Cells adhere in a chosen direction by controlling the frequency of motor reversals in response to sensory cues. An attractant stimulus causes momentary suppression of CW rotation and prolongs the run that carries the cell up the gradient. Conversely, a repellent stimulus increases CW rotation, shortening runs in unfavorable direction. The cell measures the concentration encountered during the past second and compares it with that encountered during the previous three or four seconds. This short-term memory allows the cell to make the temporal comparisons that guide its choices to run or tumble called "chemotaxis". In addition to time-limited action employed, two features of the bacterial chemotactic response are important. First, the sensing apparatus is highly adaptable to different effector concentrations. Second, the response is very sensitive. The binding of a few attractant molecule(s) to a receptor on the surface of the cell can change the reversal probability of the flagellar motor (44).

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The bacterial response begins after a chemoeffector (a small molecule, metal ion, or periplasmic receptor occupied by its ligand) binds to either a receptor protein within the cytoplasmic membrane or a periplasmic protein, then binds to a transmembrane receptor or transducers which each mediate responses to a specific set of chemoeffectors. (The receptors are clustered near the poles of the cell, forming a chemical-sensing “nose”.) When a chemoeffector binds to the transducer, it undergoes a conformation change that signal is transmitted across the membrane to components in the cytoplasm. Four cytoplasmic proteins then relay the signal to the flagellar motors and two proteins, which determine the extent of covalent modification of the membrane receptors at specific amino acid residue (42).

The state of the receptors is responded by a sensor kinase, CheA, which can phosphorylate itself slowly in the absence of other proteins but the reaction is accelerated several hundred fold in the presence of membrane receptors, CheW and ATP. The stimulatory effect of the receptors is inhibited by binding of an attractant. CheA, CheW and receptors together form a stable ternary complex with a stoichiometry of 2:2:2. The receptor-CheW-CheA complex also can bind to CheY. When stimulated by the receptors, Che A can rapidly phosphorylate many molecules of CheY and that phosphorylated CheY induced CW rotation of the flagellar motor by binding to a motor protein called FliM which involve in controlling the direction of motor rotation. CheZ antagonizes CW signaling because it bind to phospho-CheY and accelerates it dephosphorylation.

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Bacteria must adapt then migrate up and down chemical gradients and remain responsive to local gradients. CheR is a methyltransferase that catalyzes

the transfer of methyl groups from S-adenosyl methionine to specific amino acid residues in the cytoplasmic domains of the receptors, forming methyl esters. CheB is a methylesterase that catalyzes the removal of methyl groups and deamidation amino acid residue, producing methanol and restoring the amino acid side chain. Methylation of the receptors enhances their stimulatory effect on CheA and increases CW signals, whereas demethylation has the opposite effect. These two activities of both CheR and CheB can maintain the number of methyl groups on receptors and gives an intermediate CW-CCW motor bias. So, the bacteria can respond to changes in attractant or repellent concentration even in the presence of high static concentration of chemoeffectors.

The level of receptor methylation is controlled globally and locally. Global control involves the modulation of CheB activity by CheA, CheW and receptors. In the unphosphorylated CheB molecule, N-terminal domain acts as an inhibitor of C-terminal domain, which contain the esterase active site. Whereas an increase in the autokinase activity of CheA causes increased CW rotation of motor, it also decreases methylation of receptors. The later reduces its stimulatory effect on CheA and counteract initial stimulus. This global mechanism is controlling which methylation level of all the other receptors can be produced from stimulation through any of the receptors, by affecting CheB activity.

In the other hand, the local mechanism is the sense which the binding of attractant to a receptor increases the level of methylation that receptor more than others. The binding induce a conformation change that alters the exposure of methylation sites to CheR and CheB. This adaptation mechanism enables a cell

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swimming through multiple chemoeffector gradients to adapt to each gradient independently (cause CCW signaling) and also generates negative feedback which then lead to methylation of receptor, increasing CheA activity.

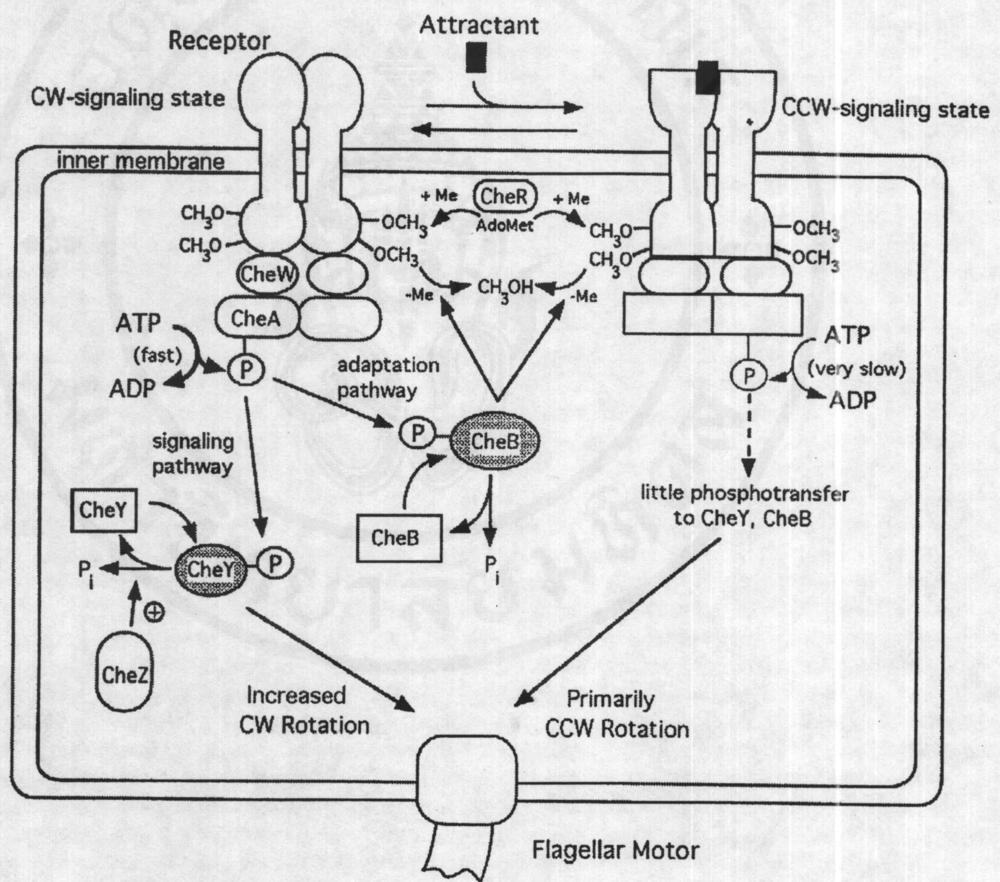

Figure 5 The chemotactic signaling and adaptation pathways of *E. coli* (44).

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10. Bacterial DNA Methylation

Methylation at adenine residues by DNA adenine methylase (Dam) controls biological process such as replication (45, 46), methyl direct mismatch repair (MDMR) (47), and transposition (48). Moreover, Dam controls the expression of operons, which involve virulence determinants, since the defection of Dam reduces the ability in colonization deeper tissue (49). Several experiments with *Salmonella* showed that Dam is essential for bacterial virulence. Both *dam* insertion and deletion have the same reduced virulence (49). Although Dam plays role of methyl directed mismatch repair, pathogenesis is not affected through an increased mutation rate. DNA adenine methylation directly controls virulence gene expression, not via any methylated gene mediated pathway. However, the study in *Salmonella* indicates that Dam and the other regulatory pathway constitute an overlapping global regulatory network controlling pathogenesis. Binding of some regulatory proteins to Dam target sites blocks Dam methylation at specific GATC-containing sites. Therefore, Dam methylation patterns are formed in 5′ upstream noncoding DNA regions of the genes (50,51,52). Methylation pattern in the regulatory regions of virulence genes modulates the binding of regulatory proteins to DNA. The alteration of Protein-DNA interactions can affect virulence gene expression (53,54,55,56).

Preliminary experiments indicate that attenuated Dam$^-$ *S. typhimurium* may express multiple immunogens and could elicit a cross-protective immune response between related bacterial strains. Consequently, DNA adenine methylases are potentially excellent targets for vaccines and antimicrobial drugs development.

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CHAPTER II

OBJECTIVES

Among the several similar characters between *B. mallei* and *B. pseudomallei*, the motility property is obvious to distinguish the two species, and this genetic differentiation was studied to understand the pathogenesis of these bacteria. Therefore, the aims of the thesis were

1. To investigate the mechanism that involves in the regulation of the flagellin gene expression of *B. mallei* by using molecular procedures.

2. To search for the genetic difference between *B. mallei* and *B. pseudomallei* that might be used for rapid identification of these species.

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CHAPTER III

MATERIALS AND METHODS

Materials

1. Bacteria strains and plasmids

Table 3. Bacteria species, strains and plasmid used in this experiment

Species, strains and plasmid	Properties	References and sources
Burkholderia mallei		
ATCC10399	Arabinose nonassimilators (Ara-)	Infected horse
Burkholderia pseudomallei		
NF47/38	Arabinose nonassimilators (Ara-)	Human blood
E271	Arabinose nonassimilators (Ara-)	Environment
Pseudomonas putida		
DMS2704	Non-identified	National Institutes of Health, Nonthaburi, Thailand
Escherichia coli		
JM109	(F' traD36 lac1^{q}Δ(lacz)M15 proABrecA1 endA1 gyrA96(Nalr) thi hsdR17(r_k-m_k) supE44 relA1Δ (lac-proAB))	New England Biolabs
Plasmid pUC19	Plasmid carrying a 54 basepairs multiple cloning site polylinker. The physical map was shown in figure 6.	New England Biolabs

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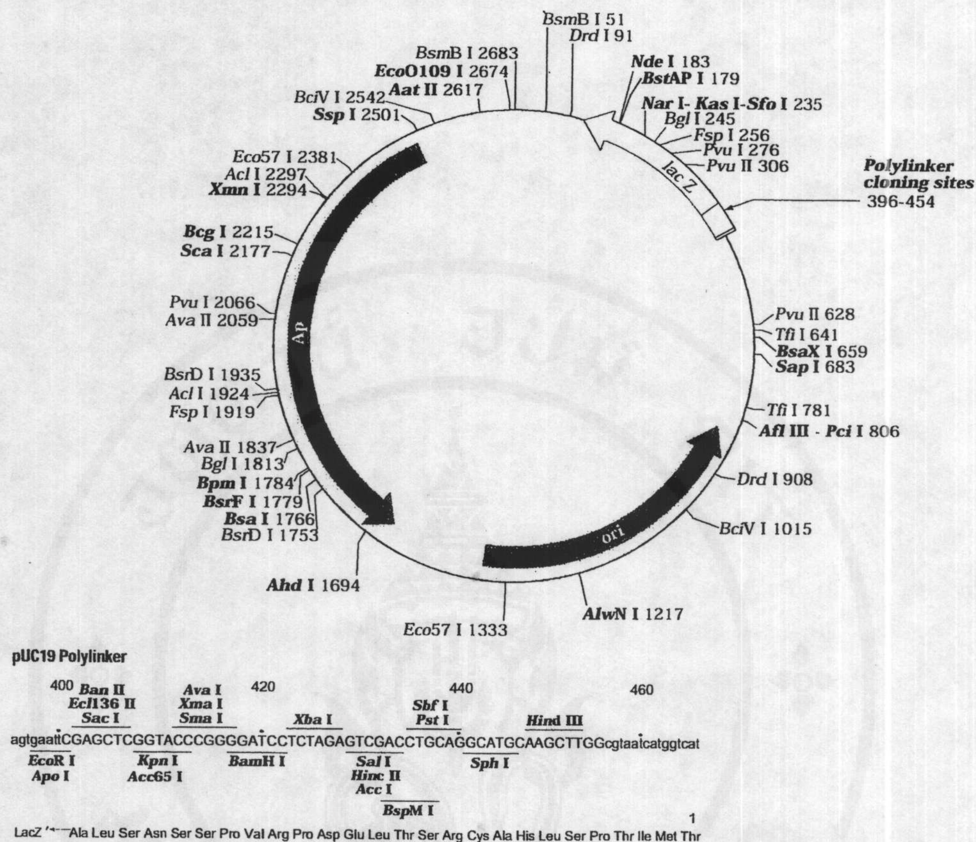

Figure 6 The physical map of pUC19 (57)

2. Antiflagellin antibody raised against *Pseudomonas putida* DMS 3052 (58)

The blended flagella of *Pseudomonas putida* DMS 3052 were used to immunized the rabbits. Rabbit polyclonal antiserum raised against *P. putida* flagellin protein was obtained. Due to the capable of recognizing with *B. pseudomallei* flagellin protein, rabbit antiserum raised against the *P. putida* flagellin protein was used for detection the flagellin protein from *Burkholderia* species in Western blot immunoassay.

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3. Oligonucleotide primers for gene amplification by PCR

The oligonucleotide primers for flagellin gene, chemotaxis protein CheW gene and chemotaxis response regulator CheY gene were designed from DNA sequence at the 5′ and 3′ terminal of genes from *B. pseudomallei* strain 1026b in GeneBank Database accession number U73848, U92493 and U78087, respectively. The primers were supplied from the Bioservice Unit (BSU), National Science and Technology Development Agency (NSTDA), Thailand.

Table 4 The oligonucleotide primers and their melting temperature used in this study.

Gene	Primer name	Primer sequence	T_m (°C)
fliC	PPM1	5' ATGCTCGGAATCAACAGCAACAT 3'	66
	PPM2	5' CAGGAGCTTCAGCACTTGCTG 3'	66
cheW	CHEWFO	5' GTGTCCGAAGTCCAAACG 3'	56
	CHEWRE	5' TTATGCGCCAAGTGTCTCG 3'	54
cheY	CHEYFO	5' ACAGACGATGGACAAGAGC 3'	58
	CHEYRE	5' TCAACGTCAGCTCCCCGC 3'	60

4. Chemicals

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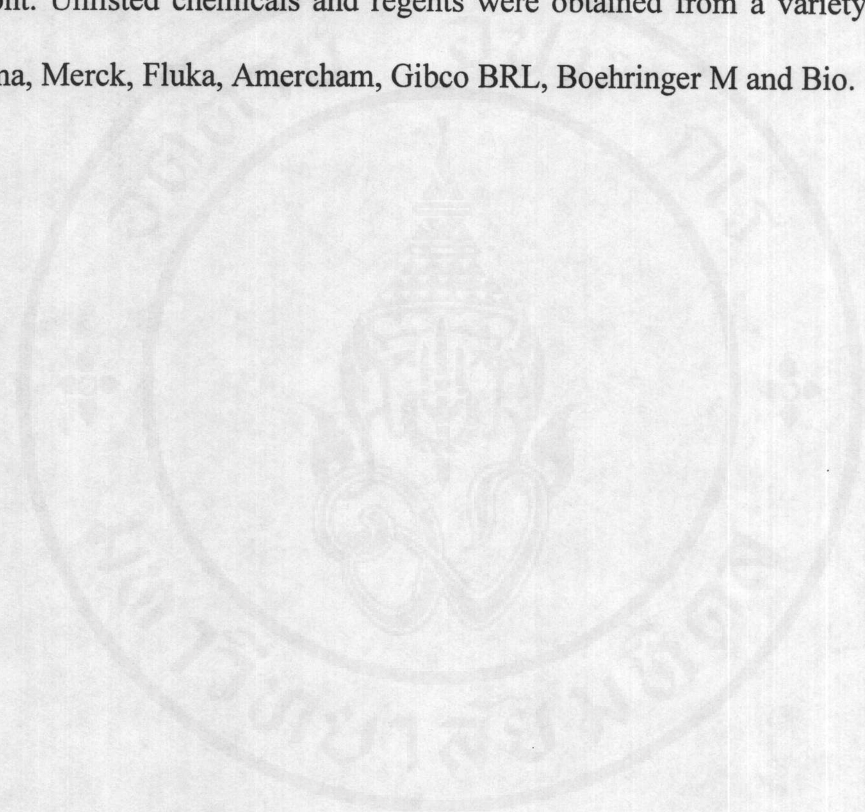
Bacterial culture media were purchased from Difco. Ampicilin, dNTP, IPTG, X-gal agarose and mineral oil were obtained from Sigma. Developer, Fixer and Tri-X 100 were purchased from Kodak. Restriction endonucleases and other enzymes used

in this experiment were supplied from New England Biolabs, Boehringer M. and Gibco BRL. Cellulosenitrate transfermedium was supplied by Schleicher & Schuell, Germany. Positively charge nylon membrane was obtained from GeneScreenPlus™, Dupont. Unlisted chemicals and regents were obtained from a variety of companies (Sigma, Merck, Fluka, Amercham, Gibco BRL, Boehringer M and Bio. 101).



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Methods

1. Bacterial cultures

1.1 *B. pseudomallei* was grown on LB agar plate overnight at 37 °C. A single colony was picked up and then grown in LB broth medium at 37 °C for 16-18 hours with shaking.

1.2 *B. mallei* was grown on LB agar plate 30-36 hours at 37 °C. A single colony was picked up and then grown in LB broth medium at 37 °C for 24-30 hours with shaking.

1.3 *P. putida* was grown on LB agar plate overnight at 37 °C. A single colony was picked up and then grown in LB broth medium at 37 °C for 16-18 hours with shaking.

1.4 *E. coli* JM 109 for competent preparation was grown on MM agar plate at 37 °C for 30-36 hours.

1.5 *E. coli* JM 109 with flagellin clone was grown in LB agar containing 100 μl/ml of ampicillin. A single colony was picked up and then grown in LB broth medium at 37 °C for 16-18 hours with shaking.

2. Preparation of Chromosomal DNA from *B. mallei* and *B. pseudomallei*

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2.1 High Pure PCR Template Preparation Kit

Bacterial cells were collected by low speed centrifugation (3,000xg) for 5 min. The cell pellet was resuspended in 200 μl PBS. 15 μl lysozyme (10 μg/ml in Tris-HCl pH 8.0) was added. After incubation at 37 °C 15 minute, 200 μl of Proteinase K (20 mg/ml) were added, mixed immediately and incubated for 10 min at 72 °C. Subsequently, samples were mixed with 100 μl isopropanol. Sample was taken in high pure filter tube combined with collection tube, then centrifuged at 8,000 rpm 1 min. Discarded the flow through and washed fiber tube with 500 μl of wash buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5, 80% ethanol) and centrifuged for 1 min at 8,000 rpm. Discarded the flowthrough, and fiber tube was washed with washing buffer again, then centrifuged for 1 min at 8,000 rpm. Finally centrifuged for 10 sec at 13,000 rpm to remove residual wash buffer. New 1.5 ml reaction tube replace. The chromosomal DNA was eluted with 200 μl of 70 °C prewarmed elution buffer (10 mM Tris, pH 8.5) and centrifuged at 8,000 rpm for 1 min. The nucleic acids ware stored at 4 °C.

2.2 Phenol-Chloroform Extraction (59)

2.2.1 Small scale preparation

Burkhoderia culture was grown in LB medium to an OD_{600} value of 0.5 on spectrophotometer (Spectronic 21). Bacterial 1.5 ml of culture was collected by centrifugation at 3,000-5,000 xg for 5-10 min. The bacteria pellet was resuspended in 100 μl of Glucose-lysozyme solution (50% Glucose, 25 mM Tris, 10 mM EDTA pH 8.0, 2 mg/ml lysozyme), gentle mixed and incubated for 1 hour at

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37 °C. 100 μl lysis solution (2% SDS, 200 μg/ml Proteinase K, 0.01 M NaCl) was added, gentle mixed and incubated for 1 hour at 37 °C. Subsequently, 10 mg/ml RNaseA was added, and incubated for 1 hour at 37 °C. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed until an emulsion formed, then a mixture was centrifuged at 10,000 rpm 10 min. Aqueous phase formed upper phase was kept and equal volume of phenol: chloroform (1:1) was added. After centrifugation at 10,000 rpm 10 min, then upper phase was kept. Extraction with phenol chloroform was performed two or more times. 0.1 volume of 3 M Sodium acetate pH 5.5 was added and mixed gently. Chromosomal DNA was precipitated by adding 2 volume of cold absolute ethanol and mixed gently. Mixture was left for 1 hour at –20 °C. The precipitated DNA was recovered by centrifugation 10,000 rpm for 10 min. The supernatant was removed carefully. DNA pellet was washed with 70% ethanol and dissolved in 100 μl of TE Buffer (10 mM Tris, 1 mM EDTA; pH 7.4).

2.2.2 Large scale preparation

The same method as the small-scale extraction was used but reagents were proportional to bacteria culture. After adding 2 volume of cold absolute ethanol, the polypropylene tube with mixture was inverted several times and precipitated DNA was spooled by using a glass rod. The spooled DNA was washed in 70% ethanol briefly and transferred to tube containing 0.1-2 ml of TE pH 7.4. The dissolved DNA was left to volatile the trace of ethanol.

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3. Quantitation of DNA and RNA (60)

The two methods which are widely used for measurement amount of nucleic acid are spectrophotometric measurement of ultraviolet irradiation absorbed by bases and estimation from the intensity of fluorescence emitted by ethidium bromide. The latter methods comprise Saran Wrap method, Agarose Plate method, and Mini Gel method. The ultraviolet spectrophotometric determination and Mini Gel method were only choose for these experiments

3.1 Spectrophotometric determination

Nucleic acid was diluted 1:200 and 1:400 in sterile distilled water and quantitated at wavelengths of 260 nm and 280 nm by using UV-160; Shimadzu. OD_{260} of 1.0 normally corresponds to approximately 50 ng/ml for double-stranded, 40 µg/ml for single-stranded DNA and RNA. The ratio between the reading at 260 nm and 280 nm (OD_{260}/OD_{280}) provides estimate of the purity of nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 and 2.0, respectively.

3.2 Mini Gel method

Electrophoresis through minigels provides a rapid and convenient way to measure the quantity of DNA and to analyze it physical state at the same time. Electrophoresis carries out as description in 6. The gel was photographed under short-wavelength ultraviolet irradiation. Compare the intensity of fluorescence of the

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unknown DNA with that of the DNA standards and estimated the quantity of DNA in the sample.

4. Designation of oligonucleotide primers

The oligonucleotide primers were designed and modified from both ends of *B. pseudomallei* strain1026b flagellin gene, chemotaxis protein CheW gene and chemotaxis response regulator CheY gene which reported in GeneBank database accession number U73848, U92493 and U78087, respectively. The estimated T_m (melting temperature) can be calculated as $T_m = 4(G+C)+2(A+T)$ (61).

5. Isolation of genes from *B. mallei* chromosomal DNA by PCR

Polymerase Chain Reactions were performed in 50 μl reaction mixture contained 100-300 ng of chromosomal DNA template, *Vent* DNA Polymerase buffer, 200 μM of each dNTP, 0.4 μM of each primer, 1 unit of *Vent* DNA Polymerase which posses 3′→5′proofreading exonuclease activity and sterile distilled water. Each reaction was overlaid with 50 μl of light mineral oil and heat 100 °C 10 minute before adding *Vent* DNA Polymerase. The amplification was performed for 30 cycles in an automated DNA Thermal Cycler. One cycle composes of denaturation, annealing and extension stop as below;

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94 °C 5 min T_a °C 1 min 72 °C 1 min 1 cycle

94 °C 5 min T_a °C 1 min 72 °C 1 min 28 cycles

94 °C 5 min T_a °C 1 min 72 °C 1 min 1 cycle

T_a of each genes as shown in table 5

Table 5 PCR condition for gene amplifications and the size of PCR products

Gene	Primers	T_a (°C)	Size of PCR product
fliC	PPM1, PPM2	65	1,161
cheW	CHEWFO, CHEWRE	55	528
cheY	CHEYFO, CHEYRE	59	395

PCR products were analyzed by 0.8-2.0% agarose gel electrophoresis and DNA bands were revealed by ethidium bromide staining.

6. Agarose Gel Electrophoresis

This method is a standard technique used to separate and identify DNA fraction. Agarose gel can be prepared in various concentration (0.3-3.0%) depend on the moleclar size of DNA. Agarose Gel can be dissolved either in TBE (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA) or TAE (40mM Tris base, 20 mM Glacial acetic acid, 200 mM EDTA, pH 8) and heated to melt. The 60 °C warmed solution was poured into the chamber set with a comb of a desired thickness and number of wells, then leave to harden. After equilibration of gel into the same buffer as dissolve buffer, DNA sample and DNA size standards (*λHin*dIII or 100 bps ladder marker) were mixed with volume of loading dye (0.1% bromophenol blue, 40% ficoll and 5 mM

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EDTA) to sink the DNA to the bottom of the wells. Electrophoresis was carried out at constant voltage 80-120 volts at room temperature until the tracking dye had moved to 2 cm far from the edge of the gel. Then, the gel was stained with ethidium bromide solution and destained in distilled water. DNA patterns were visualized under a short wavelength UV transilluminator and photographed through a red filter.

7. Recovery of DNA fragment from agarose gel by GENECLEAN II kit (BIO 101 Inc.)

Pieces of the excised DNA gel were dissolved in 3 volumes of 6 M NaI solution or kept the final concentration of NaI above 4 molar and placed in a 45 °C to 55 °C water bath incubator. For agarose with TBE, 0.5 volume of TBE modifier and 4.5 volumes of NaI were added. The mixture was left to room temperature and 5 μl of GLASSMILK® suspension containing very small silica particle was added then mixed every1-2 min through 5 min on ice. The suspension was spinned for approximately five seconds. The supernatant was removed and the white pellet was rinsed with 10-50 volumes of ice cold NEW WASH (NaCl/ethanol/water WASH) 3 times. The pellet was resuspended by pipetting and spinned for 5 seconds. DNA was eluted from GLASSMILK® by resuspension pellet with TE buffer or water or low-salt buffer. Suspension was incubated at 45 °C to 55 °C for 2-3 min and then centrifuged about 30 seconds. The supernatant containing the eluted DNA was removed into a new tube.

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8. Strategies for cloning and transformation (62)

8.1 Digestion of plasmid pUC19 with *Sma*I

Plasmid pUC19 vector contains ampicillin resistant gene and *lac*z with multiple cloning sites. These vectors were digested with *Sma*I to generate the blunt end linear plasmid. The reaction consists of 1 μg of pUC19. 1x NEBuffer, steriled distilled water and 20 units of *Sma*I in total volume 50 μl. Mixed well and was incubated at 25 °C 2 hours. When digestion was complete, the sample mixture was purified by GENECLEAN II kit and redissolved in 10-20 μl of TE buffer.

8.2 DNA ligation

The reaction which produce phosphodiester bond between 5′-phosphate and 3′ hydroxyl group of inserted DNA and pUC19 plasmid vector consists of digested plasmid to inserted DNA equal 1:3 or 2:3 molar ratio, 1x T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM ATP, 50 μg/ml BSA; pH 7.8) and 400 units of T4 DNA ligase and sterile distilled water to 10 μl final volume. Mix gently and incubated at 16 °C 14-16 hours.

8.3 Preparation of competent *E. coli* using calcium chloride

A single colony of *E. coli* JM109 from a MM agar plate was grown in 3 ml LB broth for 14-16 hours at 37 °C with vigorous shaking. 1% inoculum in SOB (2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$,

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10 mM $MgSO_4$) was incubate for 2-3 hours at 37 °C with shaking 300 cycles /min. Culture was grown to an OD_{600} value of 0.3-0.4 (It is essential that the number of viable cells should not exceed 10^8 cell/ml). The cells was transferred aseptically to sterile, disposable, ice-cold 15 ml polypropylene tube (Falcon 2001). The cultures were cool by storing the tubes on ice for 10 min. The cells were recovered by centrifugation at 3,000 rpm for 10 min at 4 °C. The cell pellets were resuspended in 0.5 volume of ice-cold $CaCl_2$ solution (50 mM $CaCl_2$, 10 mM Tris-HCl pH 8.0) and stored on ice 10 min. The cells were recovered by centrifugation at 3,000 rpm for 10 min at 4 °C. Subsequently, the cell pellets were resuspended in 1/15 volume of ice-cold $CaCl_2$ solution containing 10% glycerol. The suspension was stored at 4 °C for 12-14 hours and the competent cells were obtained. The competent cells were aliquoted and stored at –80 °C. This method is frequently used to prepare the competent between that yield $5x10^6$ to $2x10^7$ transformed colonies per μg of supercoiled plasmid DNA

8.4 Bacterial transformation

The ligated DNA (no more than 50 ng in a volume of 10 μl or less) was added into 1.5 ml chill sterile microfuge tube containing 50 μl of competent cells. The content of the tubes were mixed by swirling gently and stored on ice for 30 min. The tubes was heat shocked for exactly 90 seconds in a circulating water bath that has been prewarmed to 42 °C. The cells were chilled on ice for 1-2 min. 150 μl of SOC medium (SOB containing 0.02 M glucose) was added to each tube.

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9. Identification of bacterial colonies that contain recombinant plasmids

The pUC series, carry a short segment of *E. coli* DNA that contains the regulatory sequences and the coding in formation for first 146 amino acid of β-galactosidase gene (*lac*Z). Vectors of this type are used in host cells that code for the carboxy terminal portion of β-galactosidase. Host-encoded and plasmid-encoded fragments can associate to form enzymatically active protein. Deletion mutants of the operator-proximal segment of the *lacZ* gene are complemented by β-galactosidase-negative mutant that have the operator-proximal region intact is called ∝-complementation. The Lac$^-$ bacteria that result from ∝-complementation are easily recognized because they form blue colonies in the presence of the chromogenic substrate 5-bromo-4chloro-3-indolyl-β-D-galactoside (X-gal) and inducer isopropylthio-β-D-galactoside (IPTG). However, insertion of a fragment of foreign DNA into the polycloning site of the plasmid almost invariably results in production of an amino-terminal fragment that is not capable of ∝-complementation. Bacteria carrying recombinant plasmids therefore form white colonies. The development of this simple color test has greatly simplified the identification of recombinants constructed in plasmid vectors of this type. It is easily possible to screen many thousands of colonies visually and to recognize colonies that carry putative recombinant plasmids by following procedure. The transformed cells were spreaded over the surface of the LB- agar plate containing 100 mg/ml ampicillin which was spread with 50 μl of

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20 g/ml X-gal in dimethylformamide and 50 μl of 100 mM IPTG. The plates were incubated at 37°C for 12-16 hours. The structure of recombinant plasmids were then verified by restriction analysis of minipreparations of plasmid DNA.

10. Extraction of plasmid DNA

10.1 Phenol-Chloroform extraction

This method was used for rapid screening of a great number of recombinant but plasmid DNA from this step can not be used for further analysis. This method is performed in the following procedure. One ml of bacteria culture was collected at 10,000 rpm for 20 sec. Cell pellets were loosen by vortexing 30 sec, then 50 μl of phenol-chloroform (1:1v/v) was added and mix by vortexing 30 sec until an emulsion formed. The mixture was centrifuged at 10,000 rpm for 10 min. The upper phase containing genomic and also recombinant plasmid were analyzed by 8% agarose gel electrophoresis.

10.2 Alkaline lysis method

The culture cells were collected by centrifugation at 10,000 rpm for 20 sec. The supernatant was removed and left the pellet as dry as possible. The cell pellets were resuspended in 100 μl of GTE solution (50 mM glucose, 25 mM This pH 8.0, 10 mM EDTA) and incubate on ice for 10 min. This suspension was added 200 μl of freshly prepared lysis solution (0.2 N NaOH, 1% SDS) ,then mixed by inverting the tube. The mixture was stored on ice 10 min. The viscous bacterial lysate was

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neutralized with 150 μl of 3 M NaOAc pH 4.8. This reaction mixture was incubated on ice 10 min and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred into a new microfuge tube and added 2 volumes of cold absolute ethanol to precipitate double-stranded DNA. This mixture was mixed and stored at –20 °C for 1-2 hours. The plasmid DNA was collected by centrifugation at 10,000 rpm 10 min. Subsequently, the DNA pellets were washed with 500 μl of 70% ethanol and dried in the air for 15-30 min. Finally, the plasmid DNA was redissolved in 20-50 μl of TE.

10.3 QIAGEN Plasmid midi Kit

QIAGEN Plasmid purification protocols are based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to QIAGEN anion-exchange resin. The culture cells were grown to a cell density of approximately 1×10^9 cell/ml (OD_{600} = 1.0-1.5) and harvested by centrifugation at 6,000 rpm for 15 min at 4 °C. The bacterial pellet was resuspended completely in 4 ml of Buffer P1. The bacterial cell are lysed in 4 ml of Buffer P2 (200 mM NaOH, 1% SDS) with incubating at room temperature for 5 min. The lysate was neutralized by adding 4 ml of chilled Buffer P3 (3 M potassium acetate, pH 5.5), mixed immediately but gently, and incubated on ice for 15 min. The precipitated debris was removed by centrifugation twice at 12,000 rpm for 30 min and 15 min at 4 °C. After equilibration, a QIAGEN-tip 100 was washed with 4 ml of Buffer QBT. The clear lysate was applied to the QIAGEN-tip and allowed to flow by gravity. The QIAGEN-tip was washed twice with 10 ml of Buffer QC to remove all contaminants. 5 ml of Buffer QF was applied to QIAGEN-tip to elute plasmid DNA. This plasmid DNA was precipitated

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with 0.7 volume of room-temperature isopropanol. The precipitated DNA was collected by centrifugation immediately at 9,500 rpm for 30 min at 4 ^{o}C. Plasmid DNA was washed with 2 ml of 70% ethanol, then air-dry for 5 min, and redissolved in suitable volume of TE buffer.

11. Restriction Endonuclease digestion (63)

Genomic DNA or plasmid DNA digestion with restriction enzyme was carried out according to the manufacturer's recommendation. Restriction endonuclease was the enzyme that recognizes specific base sequence of 4-8 bases of double stranded DNA and cleaves both of the duplex. The efficiency of enzymatic activity depends on digestion condition, which is controlled by the buffer. Buffers for the different restriction enzymes differ in the concentration of NaCl that they contain. Therefore, restriction endonucleases can be divided into 3 groups followed amount of NaCl requirement at suitable digestion condition (low, medium and high). One another group is the number of restriction enzymes that require specific salt as KCl. If the enzymes have different requirements, two alternatives are possible. (1) The DNA should be digested first with the enzyme that work best in the buffer of low ionic strength. The appropriate amount of NaCl and the second enzyme can be added and incubation continued. (2) A single buffer, potassium glutamate buffer (KGB), can be used.

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The digestion of genomic DNA should increase the time of digestion or amount of restriction enzyme to get rid residual material. Moreover, the restriction buffer and enzyme should distribute homogeneity.

Table 6 Recognition sequence of restriction enzymes and their optimum temperature used in this study.

Restriction enzyme	Recognition sequence	Optimum temperature (°C)
AvaII*	G▼GWCC	37
BstEII	G▼GTNACC	60
ClaI**	AT▼CGAT	37+BSA
EcoRI	G▼AATTC	37
HindIII	A▼AGCTT	37
MboI**	▼GATC	37
MboII**	GAAGA$(N)_8$▼	37
Sau3AI	▼GATC	37+BSA
Sau96I*	G▼GNCC	37
SmaI	CCC▼GGG	25
XhoI	C▼TCGAG	37+BSA

*blocked by *dcm* methylation **blocked by *dam* methylation

12. DNA sequencing

The DNA sequencing was analyzed by automated sequencing analyzer (Perkin Dlmer ABI No. 377). The sequencing method was followed PRISM™ Dye Terminator Cycle Sequencing Ready kit (FS) protocol.

12.1 Template preparation

The recombinant plasmids in *E. coli* JM109 were extracted and purified by alkaline lysis method and by QIAGEN kit.

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12.2 Sequencing reactions

The sequencing reaction consisted of 8 μl of terminator Ready Reaction Mix (A-Dye Terminator, C-Dye terminator, G-Dye Terminator, T-Dye Terminator, dITP, dATP, dCTP, dTTP, Tris-HCl pH 9.0), $MgCl_2$ thermal stable pyrophosphate and Ampli Taq DNA polymerase, FS), 200 ng DNA template, 3.2 pmol primer and adjusted final volume with distilled water to 20 μl. The reaction mixture was placed in the thermal cycler, begin the thermal cycling as follow;

-rapid thermal ramp to 96 °C

-96 °C for 10 sec

-rapid thermal ramp to 50 °C

-rapid thermal ramp to 60 °C

-60 °C for 4 min

This step was repeated for 1-25 cycles and hold at 4 °C for further preparing and loading process.

12.3 Preparing, loading and analysis

Twenty microlitres of PCR product was added into 80 μl of H_2O. Then 100 μl of phenol : H_2O : chloroform (68:18:14) was added to eliminate the excess dye terminator dNTP. The supernatant was collected, followed by adding 10 μl of 3 M sodium acetate pH 5.5 and 2.5 volume of ethanol. The DNA was centrifuged down at room temperature at 10,000 rpm for 15 min, washed twice with 70% ethanol and dried. Four microlitre deionized formamide : 50 M EDTA; pH 8.0 (5: 1) (50 mM EDTA containing 50 mg/ml blue dextran) was added to sequencing reaction tube,

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agitated vigorously to dissolve the dried residue. The sample was load on 5% denaturing gel and for 10 hrs and analyzed by ABI 377 sequencing analysis software.

13. Computer sequencing analysis

The nucleotide sequences were compared to search for homology using BLAST searching (64) and translated from nucleic acid to amino acid sequences using ORF Finder by the NCBI e-mail server. Amino acid sequences were aligned among different isolates using CLUSTAL W Multiple Sequence Alignment Program version 1.7 (65). The restriction maps were determined using Webcutter by Carolina Biological Supply Co. server.

14. Western blot immunoassay with anti-flagellin antibody

14.1 Preparation of first antibody for detection (66)

100 ml of culture of *B. pseudomallei* NF47/38 was collected by centrifugation at 7,000 g 20 min. The cell pellets were washed with 100 ml of 0.9% sodium chloride solution. The pellets were resuspended in 100 ml of 50 μM sodiumphosphate buffer solution. The suspension was blended 3 min to release tail of *B. pseudomallei* and then centrifuged at 12,000 g for 20 min. The cell pellets containing non-tail *B. pseudomallei* were collected and weigh to get the same amount of *E. coli* JM 109, *P. putida* DMS 0638, DMS 3056 grown in 3 ml of LB. 200 μl of sterile distilled water were added in to the pellet mixture. This mixture was boiled

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10 min and allowed to cool down to room temperature. Subsequently, 500 μl of flagellin-immunized antibody Rab1 was added, then mixed by flip-flop at 4°C overnight. The suspension was centrifuged 10,000 rpm for 10 min. The supernatant has been the absorbed antibody for Western immunodetection.

14.2 Preparation of whole cell extract

A single colony of *B. mallei* ATCC10399, *B. pseudomallei* NF47/38 and *P. putida* DMS 2704 were grown in LB medium and 1% inoculum in LB medium were incubated to an OD_{600} values of 0.5. 1.5 ml of cell cultures were collected and resuspended in 50 μl of sterile distilled water. Subsequently, 50 μl of SDS gel loading buffer (50 mM Tris-HCl pH 6.8, 150 mM dithiothretol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) was mixed, boiled in water bath 10 min and centrifuged briefly. 20 μl of these sample proteins were run into Polyacrylamide Gel Electrophoresis.

14.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The denaturing protein gel electrophoresis was performed according to Laemmi et at. The resolving or separating gel was prepared from 10% acrylamide which consists of 3 ml of 30% T, 2.7 % C acrylamide mix (29% acrylamide, 1% N,N′ methylene-bis-acrylamide), 3 ml of lower buffer(1.5 M Tris-HCl pH 8.8, 0.4% SDS) 3 ml of distilled water, 100 μl of 10% ammonium persulfate and 4 μl of TEMED (N, N, N′, N′-tetramethylethylenediamine). This lower part of resolving gel was overlaid with a small volume of distilled water and allowed to polymerized for

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30 min. After polymerization, water was removed and the 5% stacking gel was set. This upper gel contained 3 ml of 30% T, 2.7% C acrylamide mix, 2.5 ml of upper buffer (0.5 M Tris-HCl pH 6.8, 0.4% SDS, 6 ml of distilled water, 100 μl of ammonium persulfate, 6 μl of TEMED. The comb was plugged into the surface of stacking gel and left to polymerize. Around 20 μl of each sample was applied to the gel. Electrophoresis was carried out in Tris-glycine electrophoresis buffer (25 mM Tris, 250 ml glycine pH 8.3, 0.1%SDS) at constant 100 volts until the tracking dye approached to the end of the gel. The protein patterns were visualized by staining with coomassie brilliant blue solution (0.1% Coomassie Brilliant Blue R250, 40% methanol, 10% glacial acetic acid)

14.4 Coomassie brilliant blue staining

The electrophoretic gel was stained with coomassie brilliant blue solution for 1 hour. The excess stained color was removed out by soaking the gel in destaining solution (25% ethanol, 25% glacial acetic acid). The treated gel was placed between 2 cellophane and kept at room temperature for drying.

14.5 Western blotting

Proteins in SDS polyacrylamide gel were transferred to a nitrocellulose filter membrane (Cellulosenitrate transfer media, Schleicher and Schuell) by an electrophoresis apparatus for electrode blotting (Hoeffer Transphor Power model TE 50). The electrophoretic gel and attached filter membrane were sandwiched between Whatman 3 MM paper, and two porous pads as submerging in

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the transfer buffer or Towbins buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% (v/v) methanol). This sandwich was squeezed out any air bubbles by using a glass pipette as a roller, and placed between two plastic supports. The entire construction is then immersed in an electrophoresis tank containing cold Towbin's buffer. Electrophoresis was carried out at constant voltage 100 volts and 198 mA for 1 hour, during this time, the proteins migrate from the gel toward the anode and became attached to the nitrocellulose filter.

14.6 Detection by immunoassay

The sensitivity of Western blotting depends on reducing the background of nonspecific binding by blocking potential binding sites with irrelevant proteins. The blotted membrane was therefore placed in blocking solution (5% skim milk in phosphate buffer saline) and incubated for 1.5-2 hours at room temperature with gentle agitation on a platform shaker. Thereafter, the blocking solution was discarded. The membrane was immediately incubated in blocking solution containing first antibody from method 14.1 (1:560) for 2 hours with gentle agitation. The membrane was then washed three times with PBS-0.1% tween 20 for 7 min/time. The washed membrane was then incubated with blocking solution containing the alkaline phosphatase conjugated anti-rabbit IgG (1:10,000) for 1.5 hour with shaking. The membrane was washed once with PBS-0.1% tween 20 and then wash three times with PBS for 7 min/time. Subsequently, the washed membrane was developed color by 9.8 ml of carbonate buffer (10 mM $NaHCO_3$, 1 mM $MgCl_2 \cdot 6 H_2O$) containing 100 µl of each chromatic substrate NBT/BCIP (5-bromo-4-chloro-3-indolyl phosphate/nitro

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blue tetrazolium). When the bands were achieved to the desired intensity, rinsed the membrane with distilled water.

15. RNA dot blot hybridization

15.1 RNA extraction by using RNeasy Mini kit

B. mallei ATCC 10399 and *B. pseudomallei* NF47/38 were grown to OD_{600} value of 0.3. The appropriate numbers of cells (max. $1x10^9$) were harvested by centrifugation at 5,000 xg for 3-5 min, and supernatant was completely removed. Cell pellets were loosen by flicking the bottom of the tube. The cells were resuspended in 100 µl of lysozyme-containing TE buffer (400 µg/ml lysozyme) and incubated at room-temperature for 3-5 min. 350 µl of RLT (guanidinium isothiocyanate-containing lysis buffer) buffer was then added and cleared lysate could be obtained. For suitable binding to column, 250 µl of absolute ethanol was added and mix well by pipetting. The samples were applied to each RNeasy mini spin column sitting in a 2-ml collection tube and centrifuged at 10,000 rpm for 15 sec. The RNeasy column was washed with 700 µl of RW_1 and centrifuged at 10,000 rpm. RNeasy column was transferred into a new 2-ml collection tube then washed twice with 500 µl of RPE buffer and centrifuged at 10,000 rpm for 15 sec and 2 min respectively. RNA was eluted into 1.5-ml collection tube by 30-50 µl of RNase-free water and centrifuged at 10,000 rpm for 1 min.

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15.2 Preparation of nonradiolabeled DNA probe by DIG Labeling and Detection Kit

DIG-labeled probe was prepared according to DIG DNA Labeling and Detection kit, but PPM replace hexanucleotide primer mix. 1.2 μl of flagellin gene using as template DNA was denatured by heating for 10 min in a boiling water bath, quickly chilled on ice and briefly centrifuged. 3 μg of PPM2, 2 μl of dNTP labeling mixture (1 mM dNTP, 1 mM dCTP, 1mM dGTP, 0.65 mM dTTP, 0.35 mM alkali-labile DIG-dUTP; pH 6.5) were added on ice. Digoxiginin-11-dUTP was incorporated by 2 units of Klenow enzyme. The sterile distilled water was added to adjust volume to 20 μl reaction. The reaction mixture was briefly centrifuged and incubated overnight at room temperature. 2 μl of 0.2 M EDTA, pH 8.0 was added to stop the reaction. The labeled DNA was precipitated by adding 2.5 μl of 4 M LiCl, 75 μl of prechilled absolute ethanol and then mixed well. The mixture was stored at –20 °C for 2 hours. The precipitate DNA was collected by centrifugation 10000 rpm for 15 min and rinsed with 70 % ethanol. Subsequently, pellet DNA was dried and dissolved in 20 μl of TE buffer. Before adding probe in hybridization buffer, the probe was heat-denatured by boiling for 5 min and chilled on ice water.

15.3 RNA Dot Blotting (67)

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RNA dot blot is a rapid method for the qualitative screening of RNA. RNA samples were diluted in RNA dilution buffer (DEPC-treated H_2O : 20X SSC : formaldehyde (5 : 3: 2)). One μl of each RNA concentration was spotted onto a dry

Nylon membrane (Genescreen Plus) by using a micropipettor. Then RNA was fixed to the membrane by baking in an oven at + 120 °C for 30 min.

15.4 Prehybridization and Hybridization

The samples on the dot blot were hybridized according to the recommendations described in "The DIG system User's Guide for Filter Hybridization of Boehringer Mannheim GmbH Biochemica." The blot was placed in a hybridization bag containing 20 ml High SDS buffer (7% SDS, 50% formamide; deionize, 5XSSC, 2% blocking reagent, 50mM sodiumphosphate; pH 7.0, 0.1% N-lauroylsarcosine) per 100 cm^2 of membrane surface area and flip-flop at 42 °C for 2 hours. The prehybridization buffer was discarded from the bag. High SDS buffer containing 30 ng/ml of the DIG-labeled probe was added and allowed to hybridize for 18 hours. Unbound probe was removed from membrane by washing twice with 2X wash solution (2X SSC, 0.1% SDS) for 15 min at room temperature. After that the membrane was washed twice in 0.1X wash solution (0.1X SSC, 0.1% SDS) for 15 min at +68 °C under constant agitation.

15.5 Immunological detection (Colorimetric Detection with NBT and BCIP)

After hybridization and stringency washes, the membrane was rinsed in washing buffer (100 mM maleic acid, 150 mM NaCl pH 7.2, 0.3% Tween20) for 1 min. The membrane was then blocked in blocking solution (1% blocking reagent in maleic acid buffer) by gently agitating for 1 hour. The membrane was incubated for

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30 min in antibody solution (anti-Dig-Alkaline phosphatase conjugate antibody (1:10,000) in blocking solution) with gently agitation. Unbound antibody was removed by twice washing with washing buffer for 15 min. Subsequently, the washed membrane was equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$ pH 9.5) for 2-5 min. The freshly prepared color substrate solution (45 μl NBT and 35 μl BCIP solution in 10 ml of detection buffer) was added and color was developed in the dark without shaking. When desired spots were achieved, the reaction was stopped by washing in the distilled water.

16. Southern blot hybridization

16.1 Southern blotting

The digested genomic DNA on agarose gel was transferred to the nylon membrane (GeneScreenPlus™ nylon membrane) by following procedures. The gel was submerged in denaturation solution (0.5N NaOH, 1.5M NaCl) for 2X 15 min at room temperature with gently agitate. The single-stranded on the gel was rinsed with distilled water and submerged twice in the neutralization solution (0.5 M Tris-HCl, pH 7.5, 3 M NaCl) for 15 min at room temperature. A piece of membrane was placed on the neutralized gel without air pubbles, then covered with dry paper towel and light weight at the top. The digested DNA fragment was blot to nylon membrane by capillary transfer using 20X SSC buffer (3 M NaCl, 300 mM Sodium citrate pH 7.0) overnight. The membrane was removed to dry at room temperature, then store at 4 °C before hybridization.

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16.2 Preparation of nonradiolabeled DNA probe by DIG DNA labeling and Detection Kit

The flagellin gene PCR product that was template DNA was labeled with Digoxigenin-11-dUTP using the random primed method. The 20 µl reaction yielded a digoxigenin-labeled DNA probe from 1.2 µg of denatured flagellin gene. 2 µl of Hexanucleotide mixture (1.56 mg/ml random hexanucleotides, 500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mM Dithioerythritol, 2 mg/ml BSA; pH 7.2) and 2 µl of dNTP labeling mixture (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM alkali-labile DIG-dUTP; pH 6.5) was added on ice. 2 units of Klenow enzyme were added for dNTP incorporation. The total volume was adjusted by adding sterile distilled water. The reaction mixture was briefly centrifuged and incubated overnight at room temperature. 2 µl of 0.2 M EDTA, pH 8.0 was added to stop the reaction. The labeled DNA was precipitated by adding 2.5 µl of 4 M LiCl, 75 µl of prechilled absolute ethanol and then mixed well. The mixture was stored at –20 °C for 2 hours. The precipitate DNA was collected by centrifugation 10,000 rpm for 15 min and rinsed with 70% ethanol. Subsequently, pellet DNA was dry and dissolved in 20 µl of TE buffer. Before adding probe in hybridization buffer, the probe was heat-denatured by boiling for 5 min and rapidly cooling on ice water.

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16.3 Prehybridization and Hybridization

This step followed the procedure in 15.4. Except for probe concentration which equal to 20 ng/ml. After hybridization, membrane was washed non-stringency three times, then once washed stringency.

16.4 Immunological detection (Colorimetric Detection with NBT and BCIP)

This step followed the procedure in 15.5.

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CHAPTER IV

RESULTS

1. Isolation of gene for flagellin filament protein by PCR

Burkholderia flagellin genes were isolated from chromosomal DNA by amplification with PPM1 and PPM2 flagellin-specific primers (68). Under 65 °C of annealing temperature, the single band of approximately 1.1 kb PCR product was obtained from chromosomal DNA of *B. mallei* ATCC10399 as well as positive control.

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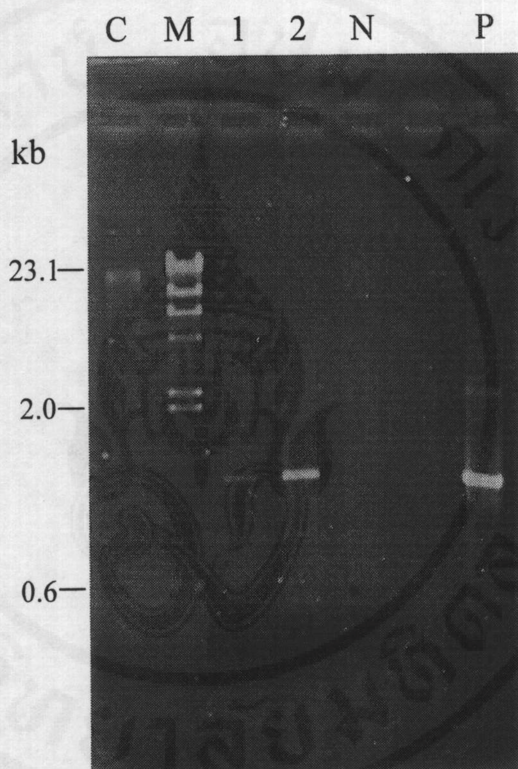

Figure 7 Agarose Gel Electrophoresis analysis of flagellin gene from *B. mallei* ATCC10399 and *B. cepacia*. Lane C, chromosomal DNA of *B. mallei* ATCC10399; land 1 to 2, PCR product with 100 and 200 ng of *B. mallei* template DNA; lane N, negative control without template DNA; and lane P, positive control with *B. cepacia* compare to λDNA digested with *Hin*dIII in lane M.

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2. Restriction enzyme analysis of the PCR product

PCR product from amplification using flagellin-specific primers was digested with restriction endonuclease to roughly define DNA fragment comparing with *B. pseudomallei* flagellin gene. The *Sau*3AI and *Mbo*II restriction enzymes, which digest a 5′ end and middle part of flagellin sequence were chosen. The restriction patterns presented the digested fragments corresponding to the restriction map of *B. pseudomallei* flagellin gene.

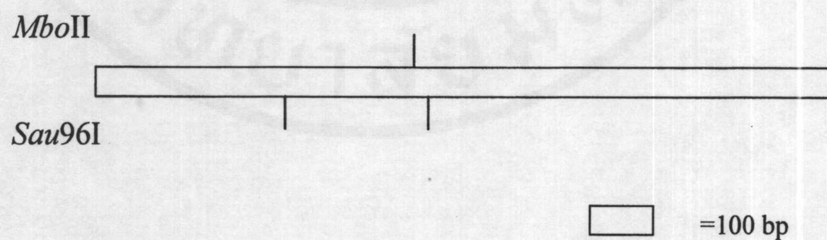

Figure 8 *Sau*96I and *Mbo*II restriction map of *B. pseudomallei* 1026b flagellin nucleotide sequence.

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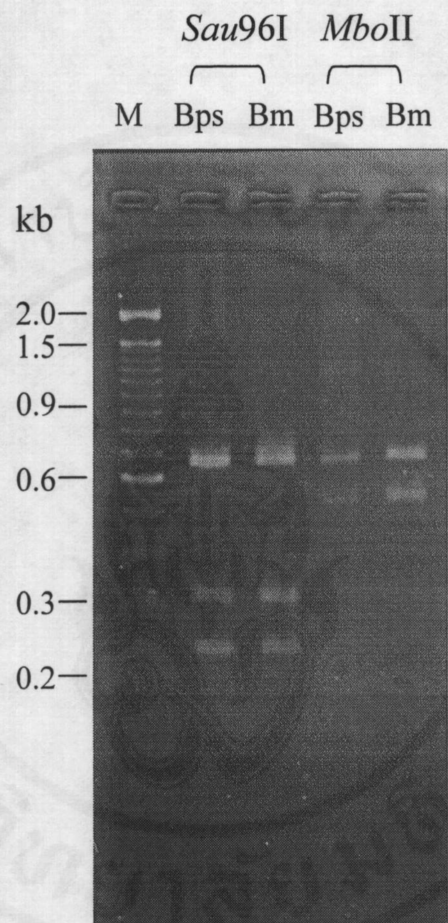

Figure 9 Restriction patterns of flagellin PCR product from *B. pseudomallei* NF47/38 and *B. mallei* ATCC10399. Flagellin PCR from *B. pseudomallei* (Bps) and *B. mallei* (Bm) digested with *Sau*96I and *Mbo*II compare to 100 bp ladder marker in lane M.

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3. Cloning and Identification for flagellin gene

The amplified PCR product was purified by using GENECLEAN II kit. Then, about 1.1 kb of purified fragment was ligated to 2,686 bp of pUC19 digested with *Sma*I. Ligated products were transformed into *E. coli* JM109 and the transformants containing recombinant plasmids were identified using α–complement property. White colonies were screened for recombinant plasmids by Phenol-Chloroform extraction. Recombinant plasmid from the corresponding colony was extracted by alkaline lysis method. After digestion recombinant plasmid with *Eco*RI, about 4 kb digested fragment, which correspond to the combination of insert fragment and plasmid was obtained. Subsequently, the fragment whose size is nearly inserted fragment size was separated from plasmid pUC19 by digestion of *Hind*III and *Eco*RI as shown in Figure 10.

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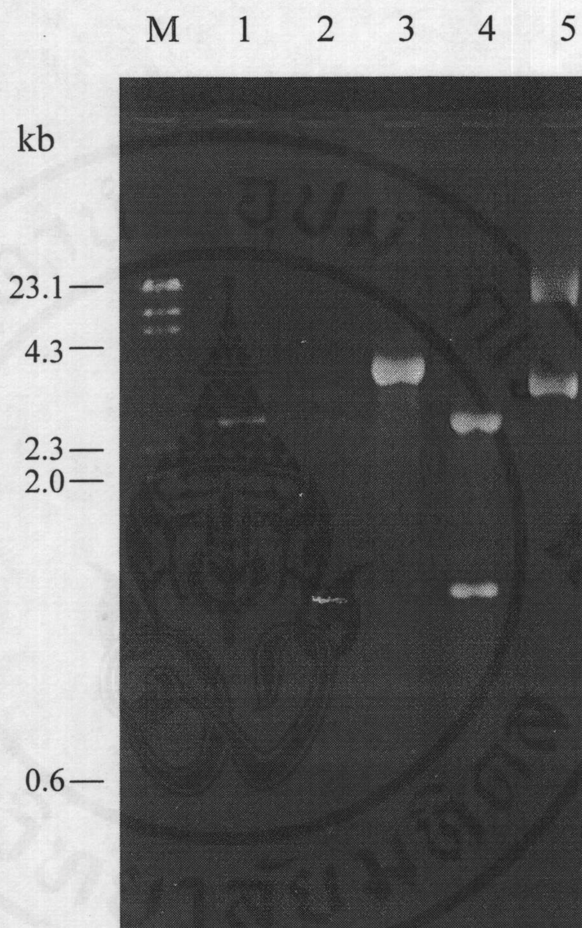

Figure 10 Restriction analysis of putative recombinant plasmid containing flagellin gene from *B. mallei* ATCC10399. Lane 1, pUC19 vector digested with *Sma*I; lane 2 flagellin PCR product; lane 3 putative recombinant DNA digested with *Eco*RI; lane 4, pututive recombinant DNA double digested with *Eco*RI and *Hin*dIII; lane 5, putative recombinant extracted using alkaline lysis method.

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4. DNA sequencing of putative *B. mallei* flagellin gene

The recombinant plasmid was automatically sequenced both directions by the sequencing analyzer. The 1,161 bp of putative flagellin sequence contains 65.72% GC content. The nucleotide sequence and translated amino acid sequence of *B. mallei* flagellin gene was showed in Fig 11.

```
  1 atgctcggaatcaacagcaacattaactcgttggtcgctcaacagaacctcaacggc
    M  L  G  I  N  S  N  I  N  S  L  V  A  Q  Q  N  L  N  G

 58 tcgcaaggcgccctgtcccaagcgatcacccgcctgtcgtcgggcaagcgcatcaac
    S  Q  G  A  L  S  Q  A  I  T  R  L  S  S  G  K  R  I  N

115 agcgcggcggacgatgcggccggcctcgcgatcgccacccggatgcaaacgcagatc
    S  A  A  D  D  A  A  G  L  A  I  A  T  R  M  Q  T  Q  I

172 aacggcctgaaccagggcgtgtcgaacgcgaacgacggcgtgtcgatcctgcaaacg
    N  G  L  N  Q  G  V  S  N  A  N  D  G  V  S  I  L  Q  T

229 gcatcgagcggcctgacctcgctcaccaacagcctgcagcgtatccgccagctcgcc
    A  S  S  G  L  T  S  L  T  N  S  L  Q  R  I  R  Q  L  A
                Sau96I
286 gtgcaggcctcgaacggcccgctgagcgcgagcgacgcgtcggcgctgcaacaggaa
    V  Q  A  S  N  G  P  L  S  A  S  D  A  S  A  L  Q  Q  E

343 gtcgcgcagcagatctcggaagtgaaccgtatcgcttcgcagacgaactacaacggc
    V  A  Q  Q  I  S  E  V  N  R  I  A  S  Q  T  N  Y  N  G

400 aagaacatcctcgacggctcggcaggcacgctgagcttccaggtcggcgcgaacgtc
    K  N  I  L  D  G  S  A  G  T  L  S  F  Q  V  G  A  N  V
                                               MboII
457 ggccagacggtctccgtcgacctcacgcaaagcatgtcggcggcgaagatcggcggc
    G  Q  T  V  S  V  D  L  T  Q  S  M  S  A  A  K  I  G  G
                Sau96I                                  XhoI
514 ggcatggttcagacgggccagacgctcggcacgatcaaggtggcgatcgactcgagc
    G  M  V  Q  T  G  Q  T  L  G  T  I  K  V  A  I  D  S  S

571 ggcgcggcctggtcgtcgggcagcaccggccaggagacgacgcagatcaacgtcgtg
    G  A  A  W  S  S  G  S  T  G  Q  E  T  T  Q  I  N  V  V

628 tcggacggcaagggcggcttcacgttcaccgatcagaacaaccaggcgctgtcgtcg
    S  D  G  K  G  G  F  T  F  T  D  Q  N  N  Q  A  L  S  S

685 acggccgtgaccgccgtgttcggctcgtcgaccgccggcacgggcacggcggcctcg
    T  A  V  T  A  V  F  G  S  S  T  A  G  T  G  T  A  A  S

742 ccgtcgttccagacgctggcgctgtcgacttcggcaaccagcgcgctgtccgcgacc
    P  S  F  Q  T  L  A  L  S  T  S  A  T  S  A  L  S  A  T

799 gaccaggcgaacgccacggcgatggttgcgcagatcaacgcggtcaacaagccgcaa
    D  Q  A  N  A  T  A  M  V  A  Q  I  N  A  V  N  K  P  Q
```

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Figure 11 For legend see page 61

```
 856 acggtctcgaacctcgacatcagcacgcagacgggcgcgtaccaggcgatggtatcg
     T  V  S  N  L  D  I  S  T  Q  T  G  A  Y  Q  A  M  V  S

 913 atcgacaacgcactcgcgacggtcaacaatctgcaggcaacgctcggcgcggcgcaa
     I  D  N  A  L  A  T  V  N  N  L  Q  A  T  L  G  A  A  Q

 970 aaccgcttcaccgcgatcgcgacgacgcagcaagccggctcgaacaacctcgcgcag
     N  R  F  T  A  I  A  T  T  Q  Q  A  G  S  N  N  L  A  Q

1027 gcgcaatcgcaaatccagagcgcggactttgctcaggaaaccgcgaacctgtcgcgc
     A  Q  S  Q  I  Q  S  A  D  F  A  Q  E  T  A  N  L  S  R

1084 gcgcaagtgctccagcaagccggcatctcggtgctcgcgcaagcgaactcgctgccg
     A  Q  V  L  Q  Q  A  G  I  S  V  L  A  Q  A  N  S  L  P

1141 cagcaagtgctgaagctcctg
     Q  Q  V  L  K  L  L
```

Figure 11. The nucleotide and putative amino acid sequence of flagellin gene from *B. mallei* ATCC10399

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5. Computer analysis

The nucleotide sequence of putative flagellin gene was aligned with *B. pseudomallei* flagellin gene. The alignment has shown 100% homology between *B. mallei* ATCC10399 and *B. pseudomallei* strains like NF105/37 and ATCC15682 as shown in Figure 12. In addition, the translated amino acid sequence was aligned with amino acid sequences of flagellin protein from the relative species in Genebank using CLUSTALW (Japan) program version 1.7. These alignments showed this amino acid sequence has the characteristic of flagellin protein, which is conserved in 5′ terminal end and highly conserved in 3′ terminal end as show in Figure 13.

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```
 Bm: atgctcggaatcaacagcaacattaactcgttggtcgctcaacagaacctcaacggctcg  60
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: atgctcggaatcaacagcaacattaactcgttggtcgctcaacagaacctcaacggctcg  60

 Bm: caaggcgccctgtcccaagcgatcacccgcctgtcgtcgggcaagcgcatcaacagcgcg 120
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: caaggcgccctgtcccaagcgatcacccgcctgtcgtcgggcaagcgcatcaacagcgcg 120

 Bm: gcggacgatgcggccggcctcgcgatcgccacccggatgcaaacgcagatcaacggcctg 180
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: gcggacgatgcggccggcctcgcgatcgccacccggatgcaaacgcagatcaacggcctg 180

 Bm: aaccagggcgtgtcgaacgcgaacgacggcgtgtcgatcctgcaaacggcatcgagcggc 240
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: aaccagggcgtgtcgaacgcgaacgacggcgtgtcgatcctgcaaacggcatcgagcggc 240

 Bm: ctgacctcgctcaccaacagcctgcagcgtatccgccagctcgccgtgcaggcctcgaac 300
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: ctgacctcgctcaccaacagcctgcagcgtatccgccagctcgccgtgcaggcctcgaac 300

 Bm: ggcccgctgagcgcgagcgacgcgtcggcgctgcaacaggaagtcgcgcagcagatctcg 360
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: ggcccgctgagcgcgagcgacgcgtcggcgctgcaacaggaagtcgcgcagcagatctcg 360

Bm: gaagtgaaccgtatcgcttcgcagacgaactacaacggcaagaacatcctcgacggctcg 420
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: gaagtgaaccgtatcgcttcgcagacgaactacaacggcaagaacatcctcgacggctcg 420

Bm: gcaggcacgctgagcttccaggtcggcgcgaacgtcggccagacggtctccgtcgacctc 480
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: gcaggcacgctgagcttccaggtcggcgcgaacgtcggccagacggtctccgtcgacctc 480

 Bm: acgcaaagcatgtcggcggcgaagatcggcggcggcatggttcagacgggccagacgctc 540
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: acgcaaagcatgtcggcggcgaagatcggcggcggcatggttcagacgggccagacgctc 540

 Bm: ggcacgatcaaggtggcgatcgactcgagcggcgcggcctggtcgtcgggcagcaccggc 600
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: ggcacgatcaaggtggcgatcgactcgagcggcgcggcctggtcgtcgggcagcaccggc 600
```

Figure 12 For legend see page 64

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```
 Bm: caggagacgacgcagatcaacgtcgtgtcggacggcaagggcggcttcacgttcaccgat 660
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: caggagacgacgcagatcaacgtcgtgtcggacggcaagggcggcttcacgttcaccgat 660

 Bm: cagaacaaccaggcgctgtcgtcgacggccgtgaccgccgtgttcggctcgtcgaccgcc 720
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: cagaacaaccaggcgctgtcgtcgacggccgtgaccgccgtgttcggctcgtcgaccgcc 720

 Bm: ggcacgggcacggcggcctcgccgtcgttccagacgctggcgctgtcgacttcggcaacc 780
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: ggcacgggcacggcggcctcgccgtcgttccagacgctggcgctgtcgacttcggcaacc 780

 Bm: agcgcgctgtccgcgaccgaccaggcgaacgccacggcgatggttgcgcagatcaacgcg 840
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: agcgcgctgtccgcgaccgaccaggcgaacgccacggcgatggttgcgcagatcaacgcg 840

 Bm: gtcaacaagccgcaaacggtctcgaacctcgacatcagcacgcagacgggcgcgtaccag 900
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: gtcaacaagccgcaaacggtctcgaacctcgacatcagcacgcagacgggcgcgtaccag 900

 Bm: gcgatggtatcgatcgacaacgcactcgcgacggtcaacaatctgcaggcaacgctcggc 960
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: gcgatggtatcgatcgacaacgcactcgcgacggtcaacaatctgcaggcaacgctcggc 960

 Bm: gcggcgcaaaaccgcttcaccgcgatcgcgacgacgcagcaagccggctcgaacaacctc1020
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: gcggcgcaaaaccgcttcaccgcgatcgcgacgacgcagcaagccggctcgaacaacctc1020

 Bm: gcgcaggcgcaatcgcaaatccagagcgcggactttgctcaggaaaccgcgaacctgtcg1080
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: gcgcaggcgcaatcgcaaatccagagcgcggactttgctcaggaaaccgcgaacctgtcg1080

 Bm: cgcgcgcaagtgctccagcaagccggcatctcggtgctcgcgcaagcgaactcgctgccg1140
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: cgcgcgcaagtgctccagcaagccggcatctcggtgctcgcgcaagcgaactcgctgccg1140

 Bm: cagcaagtgctgaagctcctg1161
     |||||||||||||||||||||
Bps: cagcaagtgctgaagctcctg1161
```

Figure 12 Alignment of flagellin nucleotide sequence of *B. mallei* (Bm) and *B. pseudomallei* strain NF47/38 and ATCC15682 (Bps).

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```
Bm10399 -MLGINSNINSLVAQQNLNGSQGALSQAITRLSSGKRINSAADDAAGLAIATRMQTQING
Bp23343 -MLGINSNINSLVAQQNLNGSQGALSQAITRLSSGKRINSAADDAAGLAIATRMQTQING
Bp1026b -MLGINSNINSLVAQQNLNGSQGALSQAITRLSSGKRINSAADDAAGLAIATRMQTQING
Bp15682 -MLGINSNINSLVAQQNLNGSQGALSQAITRLSSGKRINSAADDAAGLAIATRMQTQING
BthE264 -MLGINSNLNSLVAQQNLNGSQSALSQAITRLSSGKRINSAADDAAGLAIATRMQTQING
BcpE243 -MLGINSNINSLVAQQNLNGSQNALSQAITRLSSGKRTNSAADDAAGLAISTRMQTQING
BcpE242 -MLGINSNINSMVAQQNLNGSQNVLSQAITRLSSGKRINSAADDAAGLAIATRMQTQING
Pa15691 MALTVNTNIASLNTQRNLNNSSASLNTSLQRLSTGSRINSAKDDAAGLQIANRLTSQVNG
Pa1128  MALTVNTNIASLNTQRNLNNSSASLNTSLQRLSTGSRINSAKDDAAGLQIANRLTSQVNG
RsolK60 MSLSLNTNISSLQTQQALSTSQSALQKSLQRLSTGMRVNSAQDDAAAYASASSLTTTLNA
          * :*:*: *: :*: *. *.  *. :: ***:* * *** ****.    :. : : :*.

Bm10399 LNQGVSNANDGVSILQTASSGLTSLTNSLQRIRQLAVQASNGPLSASDASALQQEVAQQI
Bp23343 LNQGVSNANDGVSILQTASSGLTSLTNSLQRIRQLAVQASNGPLSASDASALQQEVAQQI
Bp1026b LNQGVSNANDGVSILQTASSGLTSLTNSLQRIRQLAVQASNGPLSASDASALQQEVAQQI
Bp5682  LNQGVSNANDGVSILQTASSGLTSLTNSLQRIRQLAVQASNGPLSASDASALQQEVAQQI
BthE264 LNQGVSNANDGVSILQTASSGLTSLTNSLQRIRQLAVQASNGPLSASDASALQQEVAQQI
BcpE243 LNQGVSNANDGVSMIQTASSALSSLTNSLQRIRQLAVQASTGTMSSTDQAALQQEVAQQI
BcpE242 LNQGVSNSNDGVSMIQTASSGLSQITSSLQRIRQLAVQASSGSLSPTDQQALQQEVSQQI
Pa15691 LNVATKNANDGISLAQTAEGALQQSTNILQRMRDLSLQSANGSNSDSERTALNGEVKQLQ
Pa1128  LNVATKNANDGISLAQTAEGALQQSTNILQRMRDLSLQSANGSNSDSERTALNGEVKQLQ
RsolK60 QTQGIQNANGANSYLQTADSYLGQVENNLQRMRQLAVEANNGGLSAADQTNLDKEYQQLA
         . . .*:*.. *  ***.. * .  . ***:*:*:::: .*  * ::   *: *  *

Bm10399 SEVNRIASQTNYNGKNILDGSAGTLSFQVGANVGQTVSVDLTQSMSAAKIGGGMVQTGQT
Bp23343 SEVNRIASQTNYNGKNILDGSAGTLSFQVGANVGQTVSVDLTQSMSAAKIGGGMVQTGQT
Bp1026b SEVNRIASQTNYNGKNILDGSAGTLSFQVGANVGQTVSVDLTQSMSAAKIGGGMVQTGQT
Bp15682 SEVNRIASQTNYNGKNILDGSAGTLSFQVGANVGQTVSVDLTQSMSAAKIGGGMVQTGQT
BthE264 SEVNRIASQTNYNGKNILDGSAGTLSFQVGANVGQTVTVDLSQSMSAAKIGGGLVQTGQT
BcpE243 QEVNRIASQTTYNGTNILDGSAGIVSFQVGANVGQTMSLDLSQSMSAAKIGGGLVQKGQT
BcpE242 SEVNRIASQTNYNGKNLLDGSAGNVSFQVGANVGQTISLNLSQSVSAASLGTGLPTNGAT
Pa15691 KELDRISNTTTFGGRKLLDGSFGVASFQVGSAANEIISVGIDEMSAESLNGTYFKADGGG
Pa1128  KELDRISNTTTFGGRKLLDGSFGVASFQVGSAANEIISVGIDEMSAESLNGTYFKADGGG
RsolK60 TANKNIETNANYNGNKLFDGSVASTTFQYG------------------------------
           ..* . :.:.* :::*** .  :** *

Bm10399 LGTIK-VAIDSSGAAWSSGSTGQETTQINVVSDGKGGFTFTDQNNQALSSTAVTAVFGS-
Bp23343 LGTIK-VAIDSSGAAWSSGSTGQETTQINVVSDGKGGFTFTDQNNQALSSTAVTAVFGS-
Bp1026b LGTIK-VAIDSSGAAWSSGSTGQETTQINVVSDGKGGFTFTDQNNQALSSTAVTAVFGS-
Bp15682 LGTIK-VAIDSSGAAWSSGSTGQETTQINVVSDGKGGFTFTDQNNQALSSTAVTAVFGS-
BthE264 LGTFK-VAVDSSGAAWTASSTGQETTQINVLSDGKGGFTFTDQNNQTLSSTAVTALFGA-
BcpE243 VGTVTGLSLDNAGAYVSSGAT---ITAINVISDGQGGYTFTDQNGQSISSGAATAVFGSN
BcpE242 LGQLTGLSLTSAGAATTGTQTP-AITTINILSDGQGGFKFTDQNNQALASGAVTNLFGA-
Pa15691 AVTAA---TASGTVDIAIGITG--GSAVNVKVDMKG--NETAEQAAAKIAAAVNDANVGI
Pa1128  AVTAA---TASGTVDIALGITG--GSTVNVKVDMKG--NETAEQAAAKIAAAVNDANVGI
RsolK60 ------------------------------------------QNAATDVAT-VT--NVDM
                                                  :: : :  ..
```

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Figure 13 For legend see page 67

```
Bm10399 STAGTGTAASPS------------------------------------------------
Bp23343 STAGTGTAASPS------------------------------------------------
Bp1026b STAGTGTAASPS------------------------------------------------
Bp15682 STAGTGTAASPS------------------------------------------------
BthE264 STAGSGTAL---------------------------------------------------
BcpE243 ATTGSGTAVG--------------------------------------------------
BcpE242 NTAGSGTALSLTPVATGALGSITSVPAAPSAATTSSVTAINATNAGNGSTVVGRAASGTA
Pa15691 GAFTDGAQISYV------------------------------------------------
Pa1128  GAFSDGDTISYV------------------------------------------------
RsolK60 SAY---------------------------------------------------------
                :

Bm10399 ---------------------------------------FQ-------------------
Bp23343 ---------------------------------------FQ-------------------
Bp1026b ---------------------------------------FQ-------------------
Bp15682 ---------------------------------------FQ-------------------
BthE264 ------------------------------------------------------------
BcpE243 ------------------------------------------------------------
BcpE242 LGTITGLSLDSNGGFIAPNESGATITSISVLSDGAGGFTFQDQNGNALTAGVTSKVFSIT
Pa15691 ----------------S-------------------------------------------
Pa1128  ----------------S-------------------------------------------
RsolK60 ------------------------------------------------------------

Bm10399 --------TLALSTSATSALS-ATDQANAT-AMVAQINAVNKPQTVSNLDISTQTGAYQA
Bp23343 --------TLALSTSATSALS-ATDQANAT-AMVAQINAVNKPQTVSNLDISTQTGAYQA
Bp1026b --------TLALSTSATSALS-ATDQANAT-AMVAQINAVNKPQTVSNLDISTQTGAYQA
Bp15682 --------TLALSTSATSALS-ATDQANAT-AMVAQINAVNKPQTVSNLDISTQTGAYQA
BthE264 --------TVTLNSAATSSLS-AADQAAAA-AMQTQVNAVNQPQTVSNLNISTQTGAYQA
BcpE243 --------ALSLQPSATGANT-TAAQLTAINNAIAQINAVNKPVTVSGLDISTVSGANVA
BcpE242 AATTTAGASLTLNATIGSATTNATTQGLASQSAISSANLANVPPRVADINISTTAGANQA
Pa15691 ----KASADGTTSAVSGVAITDTGSTGAGTAAGTTTFTEAND--TVAKIDISTAKGAQSA
Pa1128  ----KASADGTTSAVSGVVITDTGSTGAGAAASTSTFTEAND--TVAKIDISTAKGAQSA
RsolK60 ----------------------------GTLAGTSVTSVAN----------ATAAQAA--
                                    .      : . .*          :*   *

Bm10399 MVSIDNALATVNNLQATLGAAQNRFTAIATTQQAGSNNLAQAQSQIQSADFAQETANLSR
Bp23343 MVSIDNALATVNNLQATLGAAQNRFTAIATTQQAGSNNLAQAQSQIQSADFAQETANLSR
Bp1026b MVSIDNALATVNNLQATLGAAQNRFTAIATTQQAGSNNLAQAQSQIQSADFAQETANLSR
Bp15682 MVSIDNALATVNNLQATLGAAQNRFTAIATTQQAGSNNLAQAQSQIQSADFAQETANLSR
BthE264 MVSIDNALATVNNLQATLGAAQNRFTAIATTQQAGSNNLAQAQSQIQSADFAQETANLSR
BcpE243 MVSIDNALQTVNNLQAALGAAQNRFTAIATAQQAESTDLSSAQSQITDANFAQETANMSK
BcpE242 MESIDNALATVNNIQATFGAAQNRFTAISTTQQAQATNLSQAQSQIQDANFAQETANLSK
Pa15691 VLVIDEAIKQIDAQRADLGAVQNRFDNTINNLKNIGENVSAARGRIEDTDFAAETANLTK
Pa1128  VLVIDEAIKQIDAQRADLGAVQNRFDNTINNLKNIGENVSAARGRIEDTDFAAETANLTK
RsolK60 ---IDTDLTSLKAARASLGAQQSGLASTINTLTSNNTALSAAKSTLVDTDYASETSNMTR
           **  :  :.  :* :** *. :    .        :: *:. : .:::* **:*:::
```

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Figure 13 For legend see page 67

```
Bm10399  AQVLQQAGISVLAQANSLPQQVLKLL--
Bp23343  AQVLQQAGISVLAQANSLPQQVLKLLQ-
Bp1026b  AQVLQQAGISVLAQANSLPQQVLKLLQ-
Bp15682  AQVLQQAGISVLAQANSLPQQVLKLLQ-
BthE264  AQVLQQAGISVLAQANSLPQQVLKLLQ-
BcpE243  NQVLQQAGISVLAQANSLPQQVLKLLQ-
BcpE242  AQVLQQAGISVLAQANSLPQQVLKLLQ-
Pa15691  NQVLQQAGTAILAQANQLPQSVLSLLR-
Pa1128   NQVLQQAGTAILAQANQLPQSVLSLLR-
RsolK60  QNILQQAGTAMLAQANSAPNNILNLLKG
          ::***** ::*****. *:.:*.**
```

Figure 13 Amino acid sequence alignment of flagellin proteins between *B. mallei* (Bm) ATCC10399 and the related bacteria genus *B. pseudomallei* (Bp) ATCC23343, ATCC1026b, ATCC15682, *B. cepacia* (Bcp) E243, E242, *P. putida* (Ppd) *B.thailandensis* (Bth) E264, *P. aeruginosa* (Pa) ATCC15691, DSM1128 and *Ralstonia solanacearum* (Rsolan). Dark boxes represent the conserved regions on 5′ and 3′ terminal end.

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6. Western Immunoblotting for flagellin protein expression

Whole cell protein from *Burkholderia* species and *P. putida* DMS2704 containing loading dye were applied in 10% resolving gel. The gel was stained with coomassie blue and the other one was simultaneously blotted on nitrocellulose membrane. Because the flagellin gene of *P. putida* is equal to 834 bp corresponding to the 30.58 kDa of flagellin protein and the flagellin gene of *B. pseudomallei* NF47/38 contains more than 1,161 bp corresponding to more than 39.13 kDa but not excess 42 kDa (68), *P. putida* and *B. pseudomallei* flagellin protein can be detected at about 30 and 40 kDa respectively. While *B. mallei* loaded lane displayed a characteristic smear-like pattern as shown in Figure 14. Whether or not the smear-like pattern is a time-dependent degraded product of flagellin protein. We had followed proteins expression pattern at various time intervals as shown in Figure 15. The result suggests that the smear-like pattern may not be occurred by the degradation of flagellin protein.

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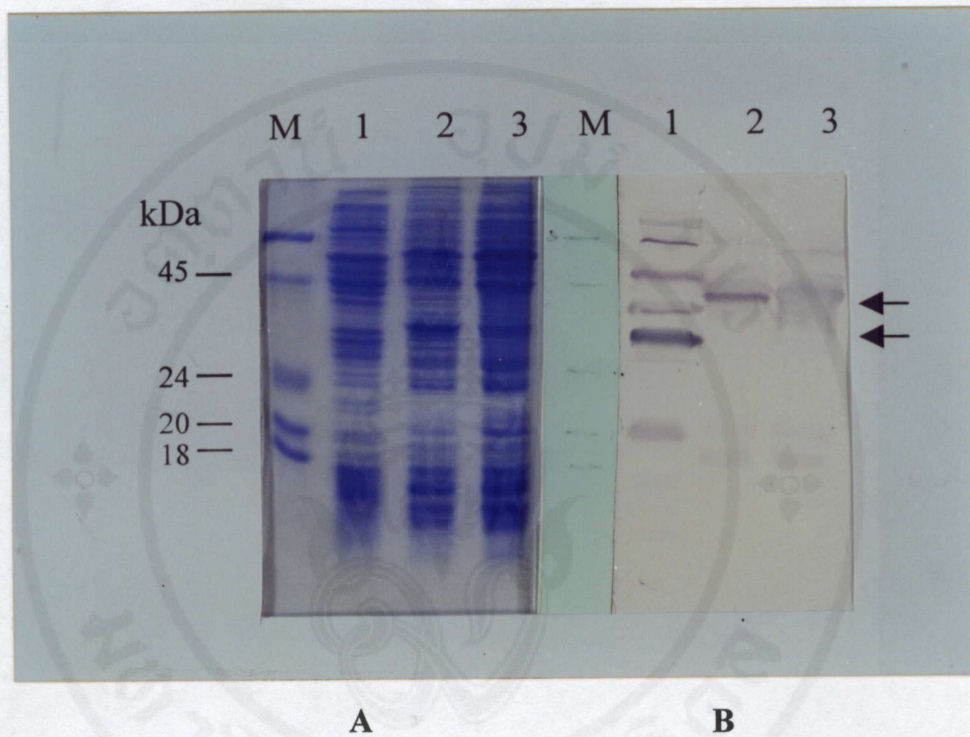

Figure 14 Western blot immunoassay for flagellin protein. (A) Coomassie blue-stained SDS-PAGE and (B) Colorimetric detection of Western blot of SDS-PAGE of *Burkholderia* flagellin from whole cell extract protein of *P. putida* DMS3052 flagellin clone (lane 1), *B. pseudomallei* NF47/38 (lane 2), *B. mallei* ATCC10399 (lane 3) against anti-flagellin antibody diluted 1: 345. Lane M indicated Protein Broad Range Marker stained with fast green solution. (The upper arrow denotes the position of flagellin from *B. pseudomallei* and the expected position of flagellin from *B. mallei* and the lower arrow denotes the position of flagellin from *P. putida*.)

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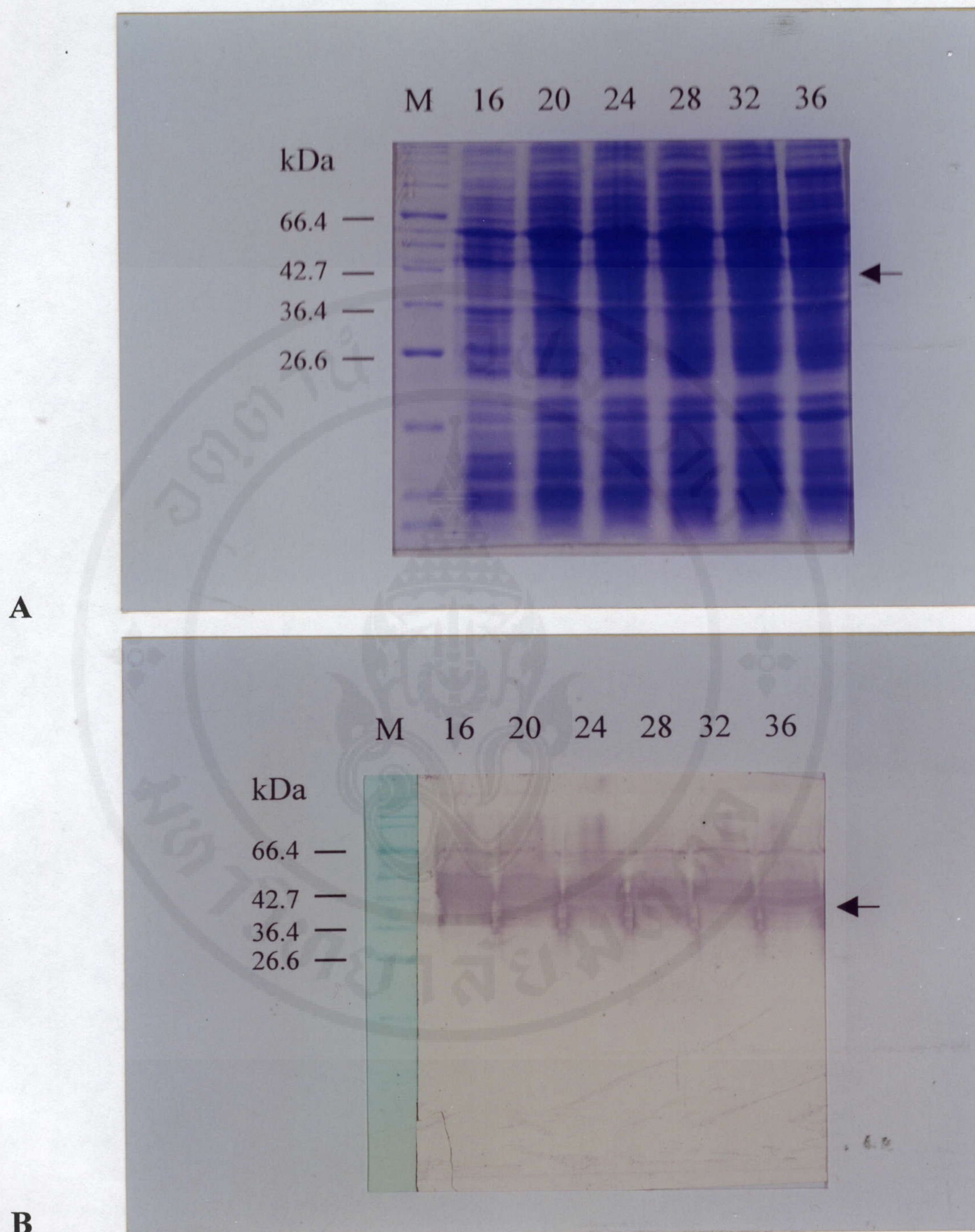

Figure 15 Western blot immunoassay of *B. mallei* ATCC10399 whole cell extracts varied the collection time from 16 hours to 36 hours. (A) Coomassie blue stained SDS-PAGE. (B) Colorimetric detection of Western blot. Arrows denote the expected signals from flagellin protein.

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7. RNA dot blot analysis for transcription of the flagellin gene

Total RNA from *Burkholderia* species was extracted by using RNeasy Mini kit. Total RNA was spotted on the positively charge nylon membrane and hybridized with DIG labeled flagellin gene. In several times, the detection presents signals from total RNA of *B. pseudomallei* only.

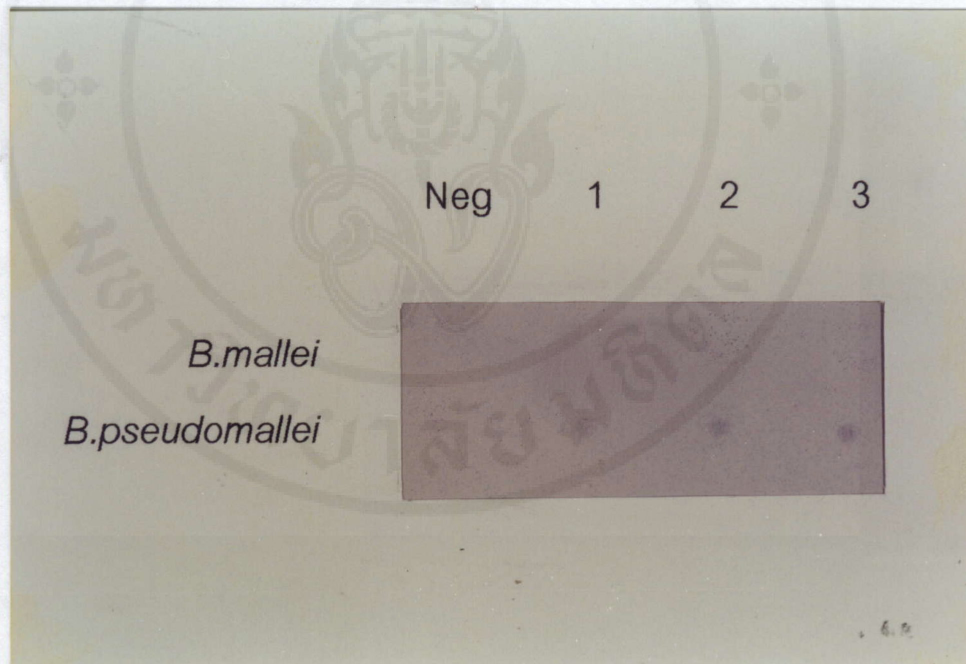

Figure 16 Dot blot hybridiztion of *B. mallei* ATCC10399 mRNA compared to *B. pseudomallei* NF47/38 mRNA. The amount of total RNA range from 200 ng to 800 ng (from left to right). Upper panel showed no signals of *B. mallei* total RNA. Lower panel showed signals *B. pseudomallei* total RNA.

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8. Isolation of genes for chemotaxis proteins by PCR

The presence of flagellin gene in *B. mallei* genomic DNA but not be transcribed to be mRNA might be occurred because the lack of genes on upstearm region of flagellin gene such as *cheW* and *cheY* which are in the same class of flagellar operon. This suspect brought about the study in existing of the genes coded for chemotaxis proteins, which are useful only after the complete flagellar assembly. Chemotaxis protein CheW gene and Chemotaxis response regulator CheY gene, which normally embedded in chromosomal DNA of motile *B. pseudomallei* were isolated from chromosomal DNA of *Burkholderia mallei* by PCR using *cheW* and *cheY*-specific primers, respectively. Amplification with CHEWFO and CHEWRE primers gave approximately 500 bp of PCR products as shown in Figure 17. Purified DNA fragments were cloned using pUC19 vector by method described in strategies for cloning and transformation. Recombinant plasmids were obtained as shown in Figure 19. After automated sequencing, 528 bp nucleotide sequence was translated to be amino acids sequence as shown in Figure 20. This amino acids sequence show 100% homology to chemotaxis protein CheW from *B. pseudomallei* 1026b as reported by DeShazer et al. (see appendix). On the other hand, amplification with CHEYFO and CHEYRE primers gave approximately 400 bp of PCR product as shown in Figure 18. Purified DNA fragments were also cloned and recombinant plasmids were obtained as shown in Figure 19. After cloning and sequencing, 396 bp nucleotide sequence was obtained. In addition, nucleotide sequence was translated to be amino acids sequence (Figure 21). This nucleotide sequence show 100% homology to chemotaxis response

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regulator CheY gene from *B. pseudomallei* 1026b as reported by DeShazer et al (see Appendix). Such results showed that the non- expression of flagellin gene does not occurred by the lack of chemotaxis gene.

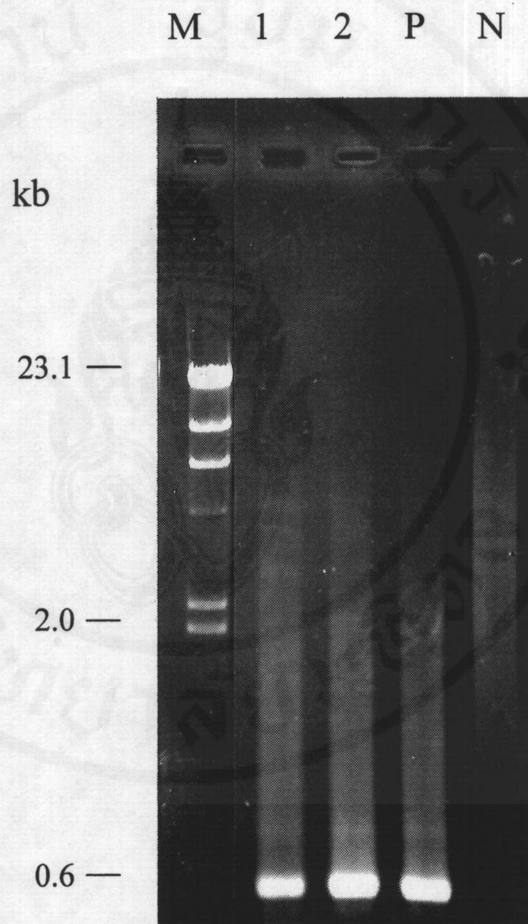

Figure 17 Agarose Gel Electrophoresis analysis of chemotaxis protein CheW gene amplification from *B. mallei* ATCC10399 and *B. pseudomallei* E271. PCR product with 100 ng and 200 ng of *B. mallei* DNA template (lane 1, 2), positive control with 250 ng of *B. pseudomallei* DNA template (lane P), negative control without template DNA (lane N) compare to λDNA digested with *Hin*dIII in lane M.

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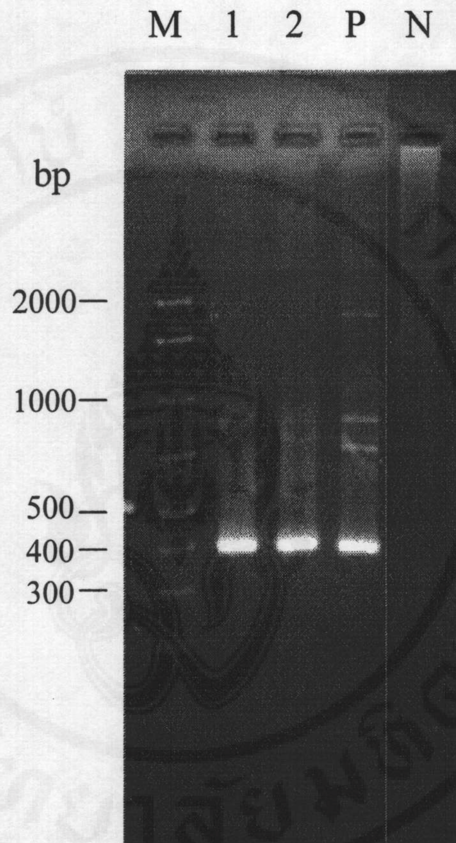

Figure 18 Agarose Gel Electrophoresis analysis of chemotaxis response regulator CheY gene amplification from *B. mallei* ATCC10399 and *B. pseudomallei* E271. PCR product with 100 ng and 200 ng of *B. mallei* DNA template (lane 1 to 2), positive control with 250 ng of *B. pseudomallei* DNA template (lane P), negative control without template DNA (lane N) compare to PCR molecular weight marker in lane M.

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9. Cloning and identification for chemotaxis genes

The amplified PCR products were purified by using GENECLEAN II kit. Then, about 500 bp and 400 bp of purified fragment was ligated to 2,686 bp of pUC19 digested with *Sma*I. Ligated products were transformed into *E. coli* JM109 and the transformants containing recombinant plasmids were identified using α–complement property. White colonies were screened for recombinant plasmids by Phenol-Chloroform extraction. Recombinant plasmid from the corresponding colony was extracted by alkaline lysis method and the extracted plasmids were analyzed by gel electrophoresis as shown in Figure 19. Subsequently, plasmids whose sizes were larger than the purified plasmid from blue colony were sequenced by automated sequencer.

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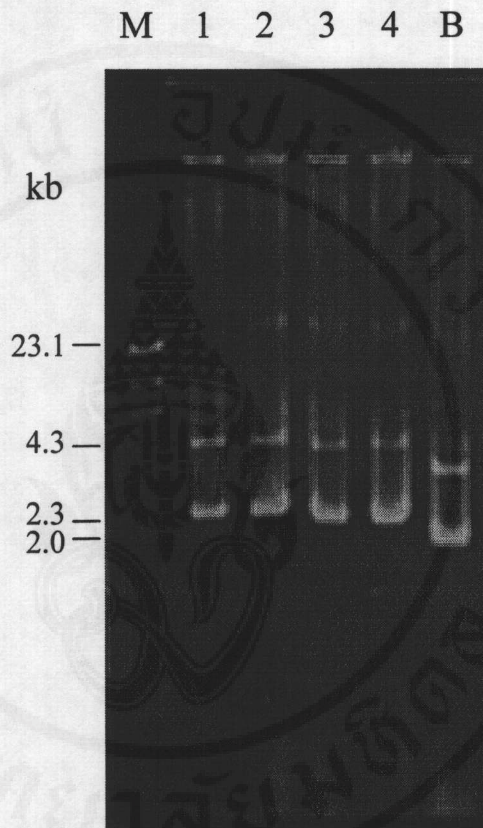

Figure 19 Extracted recombinant plasmids carrying the possible chemotaxis protein CheW gene and chemotaxis protein regulatory response CheY gene of *B. mallei* ATCC10399. The size of recombinant plasmids containing the possible CheW gene (lane 1 to 2) and the possible CheY gene (lane 3 to 4) compare to blue colony plasmid (lane B) and λ*Hin*dIII marker (lane M).

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After automated sequencing, 528 bp nucleotide sequence was translated to be amino acids sequence as shown in Figure 20. This amino acids sequence show 100% homology to chemotaxis protein CheW from *B. pseudomallei* 1026b as reported by DeShazer et al. (see Appendix).

```
  1 gtgtccgaagtccaaacgaatcatccggccgcgccgaacgcggccagccgccgcgac
    V  S  E  V  Q  T  N  H  P  A  A  P  N  A  A  S  R  R  D
 52 gccgaacagggcgacgccgcgggccaggagtttctcgtcttcacgctcggcgacgag
    A  E  Q  G  D  A  A  G  Q  E  F  L  V  F  T  L  G  D  E
115 gaatacggcatcgacattctgaaagtacaggaaatccgcggctacgacagcgtcacg
    E  Y  G  I  D  I  L  K  V  Q  E  I  R  G  Y  D  S  V  T
172 cggatcgcgaacgcgcccgatttcatcaagggcgtgatcaacctgcgcggcatcatc
    R  I  A  N  A  P  D  F  I  K  G  V  I  N  L  R  G  I  I
229 gtgccgatcgtcgacatgcggatcaagttccacctcggccgcgtcgagtacgaccat
    V  P  I  V  D  M  R  I  K  F  H  L  G  R  V  E  Y  D  H
286 cagacggtcgtgatcatcctgaacgtcgcgcatcgcgtcgtcgggatggtcgtcgac
    Q  T  V  V  I  I  L  N  V  A  H  R  V  V  G  M  V  V  D
343 ggcgtgtccgacgtgctcacgctgtcgaccgagcagatcatgcccgcgccggaattc
    G  V  S  D  V  L  T  L  S  T  E  Q  I  M  P  A  P  E  F
400 ggcggcgtgctgacgaccgagtacctgacgggcctcggcacggtcgacggccggatg
    G  G  V  L  T  T  E  Y  L  T  G  L  G  T  V  D  G  R  M
457 ctgatcctgatggacatcgagaagctgatgacgagcaaggagatggcgctgatcgag
    L  I  L  M  D  I  E  K  L  M  T  S  K  E  M  A  L  I  E
514 acacttggcgcataa 528
    T  L  G  A  *
```

Figure 20 The nucleotide and deduced amino acid sequence of chemotaxis protein CheW gene from *B. mallei* ATCC10399.

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After cloning and sequencing, 396 bp nucleotide sequence was obtained. In addition, nucleotide sequence was translated to be amino acids sequence as shown in Figure 21. This nucleotide sequence shows 100% homology to chemotaxis response regulator CheY gene from *B. pseudomallei* 1026b as reported by DeShazer et al. (see Appendix).

```
  1 atggacaagagcatgaagattctggtggtggacgattttccgacgatgcgtcggatc
    M  D  K  S  M  K  I  L  V  V  D  D  F  P  T  M  R  R  I
 58 gtccgcaacttgctgaaagagttgggctattcgaacgtcgacgaggcggaggacggc
    V  R  N  L  L  K  E  L  G  Y  S  N  V  D  E  A  E  D  G
115 ctggccggcctcgcgcggctgcgcggcggcggctacgacttcgtgatctccgactgg
    L  A  G  L  A  R  L  R  G  G  G  Y  D  F  V  I  S  D  W
172 aacatgccgaacctcgacggtctcgcgatgttgaaggagatccgcgcggacgcgtcg
    N  M  P  N  L  D  G  L  A  M  L  K  E  I  R  A  D  A  S
229 ctcacgcacctgccggtgctgatggtgacggccgagtcgaagaaggagaacatcatc
    L  T  H  L  P  V  L  M  V  T  A  E  S  K  K  E  N  I  I
286 gcggctgcgcaggcgggcgcgagcggctacgtcgtgaaaccgtttacggcggcgacg
    A  A  A  Q  A  G  A  S  G  Y  V  V  K  P  F  T  A  A  T
343 ctcgacgagaagctcaacaagattctggaaaagatggcgaaagcggggagctga 396
    L  D  E  K  L  N  K  I  L  E  K  M  A  K  A  G  S  *
```

Figure 21 The nucleotide and deduced amino acid sequence of chemotaxis response regulator CheY gene from *B. mallei* ATCC10399.

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10. Methylation analysis for flagellin regulatory region of *B. mallei*

5′ upstream region of flagellin genes were aligned to compare the sequence among *B. mallei* and *B. pseudomallei* strain's as shown in Figure 22. Promoters and Shine-Dalgarno sequence of these sequences are nearly identical. So, effect of methylation on the control region were expected because adenine methylation on control region was reported in regulation of gene expression in *E. coli*. GATC recognition sites were considered for adenine methylation. Two positions of GATC were expected in the upstream region of flagellin gene. After digestion with *Sau*3AI and *Mbo*I, restriction DNA fragment were separated on agarose gel electrophoresis. (Figure 23(A)) Separated fragments were transferred into nylon membrane and hybridized with DIG-labeled flagellin gene. The flagellin probe can hybridized with expected fragment V, Z, W of *Sau*3AI digested and expected fragment V, Y, W genomic DNA from *B. mallei* as well as genomic DNA from *B. pseudomalle*i as shown in Figure 23(B).

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```
Bm23344   CATGTCGTTCCTAATTTGCTTGATACGGTCAAAAACGTAGCGGCCAAGCGCCACGCACCG
Bps1026b  CATGTCGTTCCTAATTTGCTTGATACGGTCAAAAACGTAGCGGCCAAGCGCCACGCACCG
Bps15682  CATGTCGTTCCTAATTTGCTTGATACGGTCAAAAACGTAGCGGCCAAGCGCCACGCACCG
Bps23343  CATGTCGTTCCTAATTTGCTTGATACGGTCAAAAACGTAGCGGCCAAGCGCCACGCACCG
Bm15310   CATGTCGTTCCTAATTTGCTTGATACGGTCAAAAACGTAGCGGCCAAGCGCCACGCACCG
          ************************************************************

Bm23344   GCCTTCCGGGCGTTTCCACGAACAGGCGAAACGAGCGGGTAGCGGCCTCGGAGGCCGGAA
Bps1026b  GCCTTCCGGGCGTTTCCACGAACAGGCGAAACGAGCGGGTAGCGGCCTCGGAGGCCGGAA
Bps15682  GCCTTCCGGGCGTTTCCACGAACAGGCGAAACGAGCGGGTAGCGGCCTCGGAGGCCGGAA
Bps23343  GCCTTCCGGGCGTTTCCACGAACAGGCGAAACGAGCGGGTAGCGGCCTCGGAGGCCGGAA
Bm15310   GCCTTCCGGGCGTTTCCACGAACAGGCGAAACGAGCGGGTAGCGGCCTCGGAGGCCGGAA
          ************************************************************

Bm23344   AAGAGAGGCGGAATGCACCGCGAAACGCTCGGCAACCAACACCAGGCGCCGCGTTTGACT
Bps1026b  AAGAGAGGCGGAATGCACCGCGAAACGCTCGGCAACCAACACCAGGCGCCGCGTTTGACT
Bps15682  AAGAGAGGCGGAATGCACCGCGAAACGCTCGGCAACCAACACCAGGCGCCGCGTTTGACT
Bps23343  AAGAGAGGCGGAATGCACCGCGAAACGCTCGGCAACCAACACCAGGCGCCGCGTTTGACT
Bm15310   AAGAGAGGCGGAATGCACCGCGAAACGCTCGGCAACCAACACCAGGCGCCGCGTTTGACT
          ************************************************************

Bm23344   GTCAGCAGACATGCCCTACGGGTAGCCGTAAACGGCAAAGGCGTGACAGAAATCTCAATT
Bps1026b  GTCAGCAGACATGCCCTACGGGTAGCCGTAAACGGCAAAGGCGTGACAGAAATCTCAATT
Bps15682  GTCAGCAGACATGCCCTACGGGTAGCCGTAAACGGCAAAGGCGTGACAGAAATCTCAATT
Bps23343  GTCAGCAGACATGCCCTACGGGTAGCCGTAAACGGCAAAGGCGTGACAGAAATCTCAATT
Bm15310   GTCAGCAGACATGCCCTACGGGTAGCCGTAAACGGCAAAGGCGTGACAGAAATCTCAATT
          ************************************************************

Bm23344   CAGCCCGCCATCATAACAGGTAAACGTCGGCTCGACCAGTGGTTTACCGCTTTTCCGCGC
Bps1026b  CAGCCCGCCATCATAACAGGTAAACGTCGGCTCGACCAGTGGTTTACCGCTTTTCCGCGC
Bps15682  CAGCCCGCCATCATAACAGGTAAACGTCGGCTCGACCAGTGGTTTACCGCTTTTCCGCGC
Bps23343  CAGCCCGCCATCATAACAGGTAAACGTCGGCTCGACCAGTGGTTTACCGCTTTTCCGCGC
Bm15310   CAGCCCGCCATCATAACAGGTAAACGTCGGCTCGACCAGTGGTTTACCGCTTTTCCGCGC
          ************************************************************
              Sau3AI
Bm23344   GGGCGATCCGTGTCCACGCCCGTTCGCGCCGCCTTGCGGGCCGCCG-ACGGCGCGCCGTT
Bps1026b  GGGCGATCCGTGTCCACGCCCGTTCGCGCCGCCTTGCGGGCCGCCGGACGGCGCGCCGTT
Bps15682  GGGCGATCCGTGTCCACGCCCGTTCGCGCCGCCTTGCGGGCCGCCGGACGGCGCGCCGTT
Bps23343  GGGCGATCCGTGTCCACGCCCGTTCGCGCCGCCTTGCGGGCCGCCGGACGGCGCGCCGTT
Bm15310   GGGCGATCCGTGTCCACGCCCGTTCGCGCCGCCTTGCGGGCCGCCGGACGGCGCGCCGTT
          ********************************************** *************
```

Figure 22 For legand see page 81

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```
                                                          Sau3AI
Bm23344   CGCATGCGCGATTCGTGCGCATCCAACGTTGGCTCACCGAACGATCGACACAAAAAATTC
Bps1026b  CGCATGCGCGATTCGTGCGCATCCAACGTTGGCTCACCGAACGATCGACACAAAAAATTC
Bps15682  CGCATGCGCGATTCGTGCGCATCCAACGTTGGCTCACCGAACGATCGACACAAAAAATTC
Bps23343  CGCATGCGCGATTCGTGCGCATCCAACGTTGGCTCACCGAACGATCGACACAAAAAATTC
Bm15310   CGCATGCGCGATTCGTGCGCATCCAACGTTGGCTCACCGAACGATCGACACAAAAAATTC
          ************************************************************
                                 -35                -10         +1
Bm23344   GGGCCGAATCTCGCCCTGCCCCCTCAAGTTTTTTTGGCGTACGCCGAAATCCTGCACAAG
Bps1026b  GGGCCGAATCTCGCCCTGCCCCCTCAAGTTTTTTTGGCGTACGCCGAAATCCTGCACAAG
Bps15682  GGGCCGAATCTCGCCCTGCCCCCTCAAGTTTTTTTGGCGTACGCCGAAATCCTGCACAAG
Bps23343  GGGCCGAATCTCGCCCTGCCCCCTCAAGTTTTTTTGGCGTACGCCGAAATCCTGCACAAG
BmA15310  GGGCCGAATCTCGCCCTGCCCC-TCAAGTTTTTTTGGCGTACGCCGAAATCCTGCACAAG
          ********************** *************************************

Bm23344   GCGGCCGGCAAAGAACGAGTGCTTCCGCCCGCGCCAATAGTGGCATTGATGGGCTTATAC
Bps1026b  GCGGCCGGCAAAGAACGAGTGCTTCCGCCCGCGCCAATAGTGGCATTGATGGGCTTATAC
Bps15682  GCGGCCGGCAAAGAACGAGTGCTTCCGCCCGCGCCAATAGTGGCATTGATGGGCTTATAC
Bps23343  GCGGCCGGCAAAGAACGAGTGCTTCCGCCCGCGCCAATAGTGGCATTGATGGGCTTATAC
Bm15310   GCGGCCGGCAAAGAACGAGTGCTTCCGCCCGCGCCAATAGTGGCATTGATGGGCTTATAC
          ************************************************************

Bm23344   AGCTCAATTTGGAGATTTTCATG
Bps1026b  AGCTCAATTTGGAGATTTTCATG
Bps15682  AGCTCAATTTGGAGATTTTCATG
Bps23343  AGCTCAATTTGGAGATTTTCATG
Bm15310   AGCTCAATTTGGAGATTTTCATG
          ***********************
```

Figure 22 Sequence alignment of 5′ upstream region of flagellin gene from 2 strains (ATCC) of *B. mallei* (Bm) and 3 strains (ATCC) of *B. pseudomallei* (Bps). Gray boxes denote *Sau*3AI recognition sites. Whereas dark boxes denote promoter sites and light boxes denote GGAG corresponding to ribosome binding sites.

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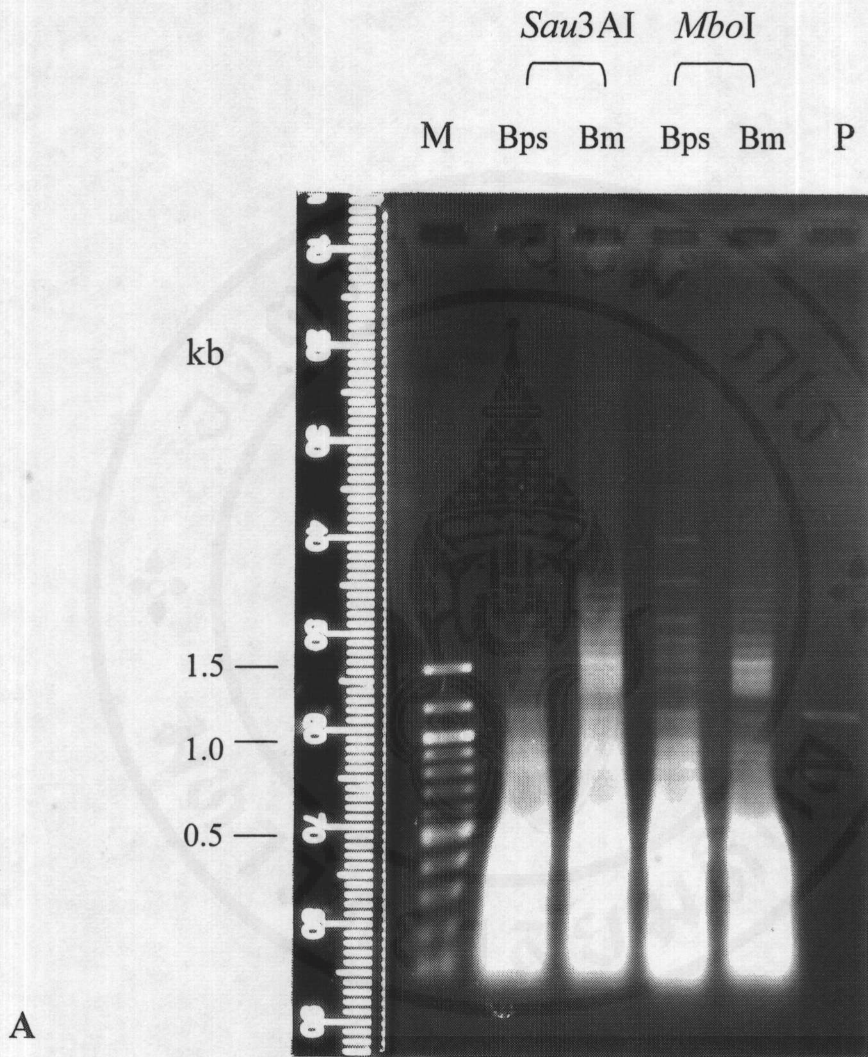

Figure 23 For legend see page 84

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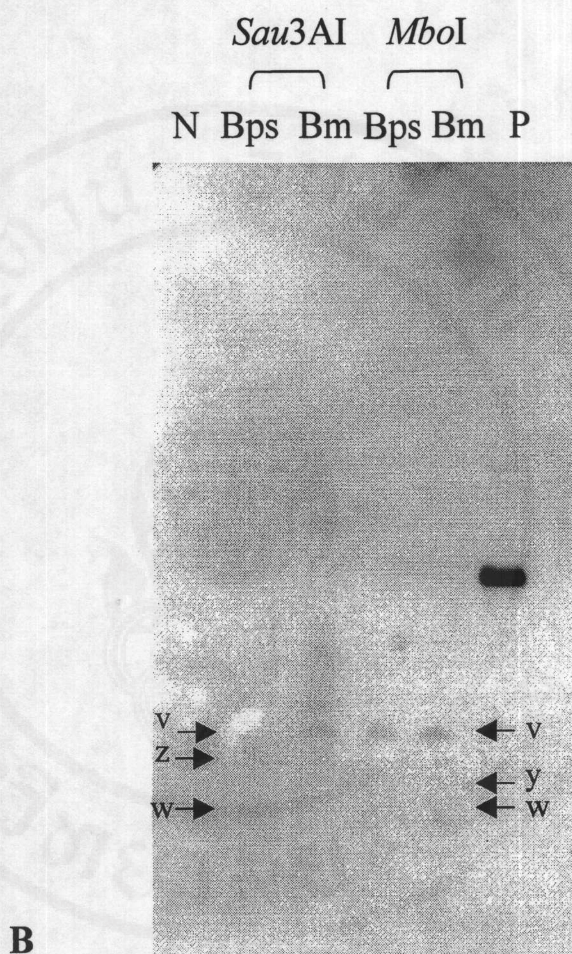

Figure 23 For legend see page 84

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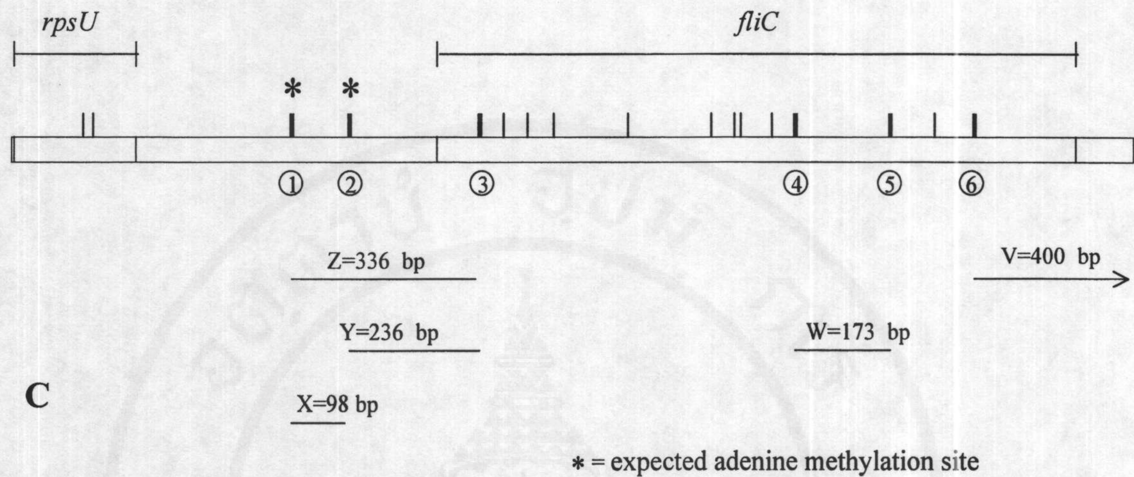

Figure 23 Analysis of the GATC Methylation of 5′ untranslated sequence of flagellin gene isolated from *B. mallei* ATCC10399 and *B. pseudomallei* NF47/38. Chromosomal DNAs were isolated from *B. mallei* (Bm) and *B. pseudomallei* (Bps) and digested with *Sau*3AI and *Mbo*I. DNA fragment were separated by electrophoresis on 0.8% agarose gel (A), transferred to nylon membrane, and probed using flagellin gene as described in experimental procedure. Each band in Southern blot showed in (B) has been given a letter designation that corresponds to the DNA fragment showed in (C). (In Figure 23 (C), thick lines with numbers denote the *Sau*3AI digested positions that give the DNA fragments corresponding to the hybridized bands in Figure (B) and thin lines donate the *Sau*3AI digested positions that give the smaller DNA fragments that the hybridized bands can not appear obviously)

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In addition, cytosine methylation sites and the other adenine methylation site were surveyed by *Ava*II, *Xho*I and *Cla*I restriction. Sizes of DNA fragments were expected by restriction map of *B. pseudomallei* 1026b as shown in Figure 24. *Ava*II digested genomic DNA gave about 3,000 bp of hybridized band. *Xho*I digestion gave the two hybridized bands at about 8,400 and 4,300 respectively. Finally, the *Cla*I digestion gave the signals of hybridized bands at 4,300 and 900 respectively. There was no the difference of hybridization pattern between *B. mallei* and *B. pseudomallei* as shown in Figure 25.

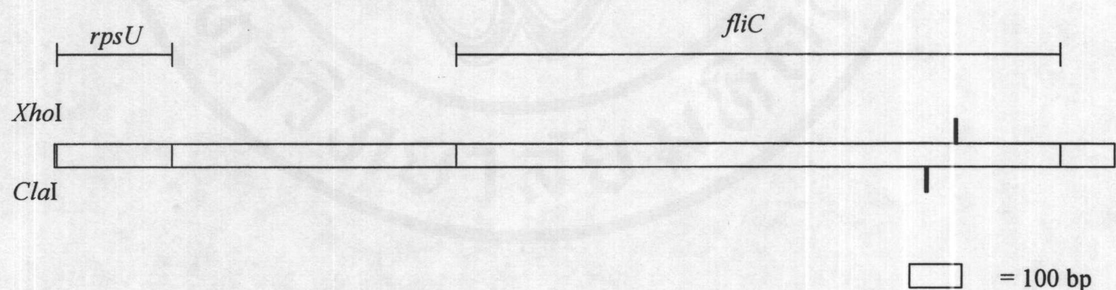

Figure 24 *Ava*II, *Xho*I and *Cla*I restriction map of *rpsU-fliC* nucleotide sequence in *B. pseudomallei* 1026b reported by DeShazer et al.

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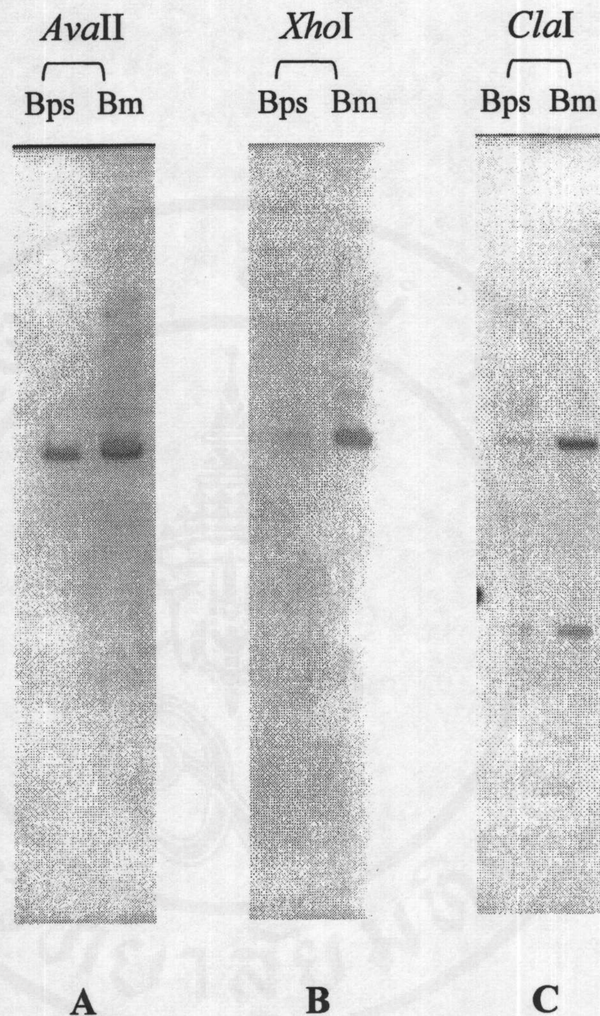

Figure 25. DNA methylation pattern of chromosomal DNA sequence isolated from *B. mallei* ATCC10399 and *B. pseudomallei* NF47/38. Chromosomal DNAs were isolated from *B. mallei* (Bm) and *B. pseudomallei* (Bps) and digested with *Ava*II (A), *Xho*I (B) and *Cla*I (C). DNA fragments were separated by electrophoresis on 0.8% agarose gel, transferred to nylon membrane, and probed using flagellin gene as described in experimental procedure.

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CHAPTER V

DISCUSSION

1. Isolation of genes for flagellin filament and chemotaxis proteins by PCR

Polymerase Chain Reaction is the repetitive bi-directional DNA synthesis via primer extension of a region of nucleotide sequence which requires two oligonucleotide primers, deoxynucleotide triphosphates (dNTP), magnesium ions in reaction buffer and a thermophilic DNA polymerase. The chromosomal DNA, extracted using High Pure PCR Template preparation kit and phenol/chloroform extraction, from *Burkholderia* species were used as template for amplification of flagellin gene and chemotaxis protein genes respectively. The denaturation of DNA template is needed to separate the two strand of DNA, especially GC rich DNA template. The predenaturation at 100 °C or hot start increases the efficiency of PCR, primers and DNA polymerase access the DNA strand more easily. Afterthat proceding enter the PCR cycles which composes of denaturation, annealing and extension step in each cycles. Denaturation and extension temperature are determined by type of DNA polymerase. The optimal annealing temperature for the primer should be examined from melting temperature in decreasing of 5 °C. The numbers of PCR cycles are normally determined from size of amplified product and copy number of the gene. There are several types of the thermostable DNA polymerase such as *Taq*, *Pfu* and

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Vent which have distinct characteristics suited for each research. Since cloning and sequencing are the application of PCR which requires the high accuracy. *Vent*$_R$ DNA Polymerase is needed because it contains an integral 3′→5′ proofreading exonuclease activity (69). In addition, proofreading are useful for PCR with the modified primers which are not completely identical to the desired terminal ends, like chemotaxis genes amplification in this study. *Vent*$_R$ DNA Polymerase also generates more than 95% blunt end fragments which can be directly cloned in *Sma*I digested vectors. In this study, flagellin gene was amplified by using primer pair designed from 5′ and nearly 3′ terminal of *B. pseudomallei* strain 1026b flagellin sequence. Because of base variation at the 3′ terminal, 6 bases of 3′ terminal end were not used for revert primer designation. For amplification of chemotaxis protein CheW gene and chemotaxis response regulater CheY gene, primers were also designed from *B. pseudomallei* strain 1026b at both terminal ends. Primer sequences, which are identical to sequence of gene tend to produce dimer and hair pin structure of primer pairs that lead to depletion in efficiency of PCR amplification. So, some bases were replace to get rid of these problems. Several amplification reactions here yield product with a smear, which involves many factors such as Mg^{2+} level, amount of enzyme, amount of DNA template, extension time and annealing temperatures. Especially, the DNA that was purified by Phenol-Chloroform extraction and alcohol precipitation, high salt in reaction can also a smear. A negative control with no template also presents a smear migrating out of the gel well or originating at the gel well. This occurs because DNA polymerases with low Km values for DNA, especially enzymes with proofreading exonuclease functions can cause primer artifacts to form because the DNA polymerase

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can not bind to its preferred substrate (a 3′ end of an annealed primer). In this study, flagellin, chemotaxis protein CheW gene and chemotaxis response regulator CheY gene from *B. mallei* ATCC10399 could be amplified at approximately 1.1, 0.5 and 0.4 kb of expected size, respectively. The results indicate that the chromosomal DNA of *B. mallei* ATCC10399 contains flagellin protein and chemotaxis protein genes.

2. Cloning and Identification for flagellin gene and chemotaxis gene

The construction of a recombinant plasmid, like the flagellin and chemotaxis genes in this research, involves a biomolecular reaction in which one end of a linearized plasmid vector is ligated to a target DNA fragment, after which the linear chimera is circularized by the ligation of the two remaining ends. This circularization was produced prior to the transformation of the bacteria by DNA ligase. Because of the availability of T4 DNA ligase and its blunt-end ligation capacity, this enzyme was chosen for these ligation reactions. T4 DNA ligase catalyses the formation of a phosphodiester bond between the 3′-hydroxyl and 5′-phosphate of two adjacent nucleotides and requires ATP as an energy source. The ligation reaction is a process that is dependent on several parameters including temperature, ionic concentration, the nature of the DNA ends, the relative concentration of the DNA ends, and the concentration and molecular weight of the DNA fragments. In contrast with cohesive-end ligations, the blunt-end ligations are more complex and significantly slower. One reason is blunt-end fragment can not anneal and so the time interval for the juxtaposition of 5′-phosphate to the 3′-hydroxyl is exceedingly small. This supports

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the requirement of T4 DNA ligase which increase 10 to 30 times more than cohesive-end ligation. So, 400 units of T4 DNA ligase were used for all ligation reaction here. The blunt-end ligation is less influenced by temperature than the cohesive-end ligation. However, the optimal temperature for this ligation should not exceed 37 °C, which is the temperature optimum for DNA ligase. Although ATP is needed for the blunt-end ligation, the higher ATP concentration than 25 mM can inhibit the ligation capacity of T4 DNA ligase. The standard buffer that obtained from supplier normally maintains the optimal condition for T4 DNA ligase. When a mixture of DNA fragment is ligated, it is also possible to affect the ligation products by manipulating the ratio of the fragment in ligation reaction. In addition, the optimal ratio can produce the hybrid molecules for transformation. Not only recombinant plasmids but also rejoined plasmids could be obtained and transformed into *E. coli*. Depend on the capable of α–complementation, white colonies which lack active β-galactosidase were selected and extracted by using Phenol/chloroform to screen for *E. coli* containing recombinant plasmid. Since the plasmids from this step have high impurities, they can not be used for further analysis. Thus the alkaline lysis method was required to extract plasmids for restriction analysis and sequencing.

3. Analysis sequences data

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Complete sequence of flagellin gene, chemotaxis protein CheW gene and chemotaxis response regulator CheY gene from *B. mallei* ATCC10399, which was isolated from lung and nose of horse, were obtained. All nucleotide sequences were

translated to amino acid sequences and compared with protein from many sources by using BLAST search program (64). For the putative flagellin gene, although nucleotide sequence displays a few nucleotides differences, amino acid sequence is identical to several *Burkholderia pseudomallei* strains such as strain 1026b, ATCC 23343 and ATCC15682. In contrast with flagellin protein, CheW and CheY were compared with less protein database. However, amino acid sequences alignment showed the CheW amino acid sequence from *B. mallei* ATCC10399 was identical to CheW protein from *B. pseudomallei* 1026b (see appendix). Whereas *cheY* nucleotide sequence from *B. mallei* was identical to *cheY* from *B. pseudomallei*. These sequences analysis showed the existence of flagellin gene, chemotaxis response regulator CheY gene and chemotaxis protein CheW gene in *B. mallei* ATCC10399 like the motile bacteria, especially *B. pseudomallei*.

4. Western Immunoblotting for flagellin protein expression

Rabbit antiserum raised against the flagella filament of *P. putida* DMS 3052 was showed to react strongly with flagellin protein from both *P. putida* DMS 2704 (30.58 kDa) and reveal the cross reaction with *B. pseudomallei* NF47/38 flagellin protein (<42 kDa) as demonstrated by Western blot (immunoblot) analysis. The antibody in rabbit antiserum that react with another protein were screened out by the homogenate from several gram-negative bacteria. It gave only a weak signal to another protein. Figure 14, lane 1 in panels B showed band at 30 kDa approximately. Lane 2 showed a distinctive band for flagellin protein of *B. pseudomallei* NF 47/38.

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Whereas the sodium dodecyl sulfate (SDS)-polyacrylamide gel with coomassie blue staining (Figure 14, lane 2 in panel A) can not distinguish the protein pattern between *B. mallei* and *B. pseudomallei*. The immunostaining for whole cell extract of *B. mallei* displayed a smear-like pattern from 55 to 36 kDa in range. However, intense signal was observed in the center of pattern. Whether or not the smear-like pattern is a time-dependent degraded product of flagellin protein. We had followed proteins expression pattern at various time intervals as shown in Figure 15. All of whole cell extracts gave the same ladder profile (Figure 15, panel B). The result suggests that the smear-like pattern may not be occurred by the degradation of flagellin protein. Although silver staining normally used for lipopolysaccharide (LPS) detection (70), these immunoblotting character are similar to the LPS immuno profile were reported in *B. pseudomallei* and gram-negative bacteria. Especially, *B. pseudomallei* was demonstrated two different types of LPS detected with antibody (71). The latter also revealed the pattern from 20 to 55 kDa in range, which near the observed pattern here. Reactions of whole cell extract with rabbit antiserum might react with both flagellin and a type of LPS pattern from *B. mallei* ATCC10399. Unfortunately, LPS appeared in range, which covered the expected position of flagellin protein of *B. mallei*. Such result can not explain the producing of flagellin protein. However, LPS which is the macromolecular composition of cell wall may associate with the flagellar, like *Vibrio cholera* which the sheath of flagellum coated with LPS (72). The question of whether LPS pattern presents only the existing of LPS antigen on cell wall or flagellar of *B. mallei* has been resolved by next step of this study.

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5. RNA dot blot analysis for transcription process of the flagellin gene

When expression of a new gene is initiated, its mRNA typically appears in the cell within few minutes and the corresponding protein appears. In bacteria, the transcription and translation processes are closely liked and they occur simultaneously. Then degradation of mRNA closely follows its translation. Consequently, bacterial mRNA is unstable and its half-life is less than 5 minutes. Thus, mRNA should be isolated from bacterial cell rapidly. It seems to be difficult to detect mRNA using hybidization technique due to instability of bacterial mRNA. However, the endonuclease degradation of mRNA prevents its use as a template, but not produce the alteration that impedes its hybridization. For example, a single cleavage in the mRNA would not affect its ability to hybridize with DNA, but could prevent its translation. So the detection of mRNA by using hybridization could be occurred (73). In this study, amounts of tatal RNA were spotted for Dot blot, which is the rapid method for the qualitative screening of RNA. The mRNA codes for flagellin polypeptide were hybridized with DIG-labeled flagellin DNA. Normally, only 1% mRNA exists in total RNA. However, the RNeasy Mini procedure isolates all RNA molecules longer than 200 nucleotides. Since low-molecular-weight RNA species make up 15-20% of total RNA, the extracted RNA consist of larger RNA molecules. The results showed that the detection did not present any mRNA from *B. mallei* ATCC10399, whereas total RNA extraction from *B. pseudomallei* was given a positive signal.

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6. Methylation analysis for flagellin regulatory region of *B. mallei*

There are several documents, which support the role of DNA methylation in the regulation of Pyelonephritis-associated pilus (Pap) expression in *E. coli*. The methylation of GATC in regulatory region can inhibit the formation of an active transcription complex (50,51). This events lead to the formation of two different pap methylation state. Thus, *E. coli* can attach to a variety of host tissues. For *B. mallei*, the transcription of the *fliC* operon may be blocked by adenine residue methylation, which is similar to the mechanism that control phase variation of the *Pap* operon. Southern blot hybridization was performed to determine the methylation of GATC site which are at 140 and 41 bp upstream of –35 sequence (denoted by asterisks 1, 2 in Figure 23(C) respectively) promoter of flagellin gene from *B. mallei* compared to *B. pseudomallei*. Control digestion of both *Burkholderia* species were cut with *Sau*3AI, which cut GATC sites regardless of methylation. Analysis of methylation was carried out using restriction enzyme *Mbo*I that cut only non-methylated GATC sites. The blots which hybridized with DIG-labeled flagellin probe showed significant signals in the *Sau*3AI-digested chromosomal DNA of both *B. mallei* and *B. pseudomallei* at 400 (V), 336 (Z) and 173 (W) bp fragments. As shown in Figure.23 (C), the blots hybridized with the same probe appear the signal of the *Mbo*I-digests at 400 (V), 238 (Y), 173 (W) bp fragments. The 98 (X) bp fragment was not seen in the Southern blot here. Since *Sau*3AI cut recognition sites regardless of methylation, large amount of enzyme must be used. The enzyme molecule cut only the sites that were easy for accessibility of enzyme. Consequently, the 336 bp DNA fragment revealed,

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instead the appearance of 98 bp and 238 bp fragments simultaneously. *Sau*3AI and *Mbo*I can cut and give the other fragments but signals can not see obviously because such fragments are too small. Southern blot with DIG-labeled flagellin probe can not display the difference of methylation pattern on regulatory region between *B. mallei* and *B. pseudomallei*. Existing of same-methylated upstream region of flagellin gene and the appearance of both chemotaxis involved genes tend to believe that expression of *B. mallei* flagellin gene is under control of the upper operon in hierarchy. As shown in Figure.25, *Xho*I, *Ava*II, *Cla*I digestion of both *Burkholderia* species also did not give the results that can distinguish *B. mallei* from *B. pseudomallei*. Interestingly, restriction endonuclease digests that were fractionated on 0.8%agarose gel electrophoresis in Figure 23 (A) present pattern difference. Although *Sau*3AI and *Mbo*I restriction patterns in each *Burkholderia* species were the same, the difference of digested pattern between *B. mallei* and *B. pseudomallei* was presented. Gel electrophoresis displays continuous bands from 0.7 to 1.5 kb approximately and discrete bands of above 1.5 kb fragments for *Sau*3AI and *Mbo*I digests of *B. pseudomallei*. Whereas *Sau*3AI and *Mbo*I digests of *B. mallei* display continuous bands in the same range. The distribution of *Sau*3AI and *Mbo*I restriction fragments from genomic DNA of *B. mallei* and *B. pseudomallei* are not adequate to explain the methylation difference between these two species, in particular the fragments below 1.0 kb in size. Southern blots with appropriate labeled probe should be used for detection. However, the *Mbo*I digested fragments of above 1.5 kb can distinguish *B. mallei* from *B. pseudomallei*. In addition to their biochemical activities (13), colony morphologies and epidemiologies of the diseases, these evidences provide the

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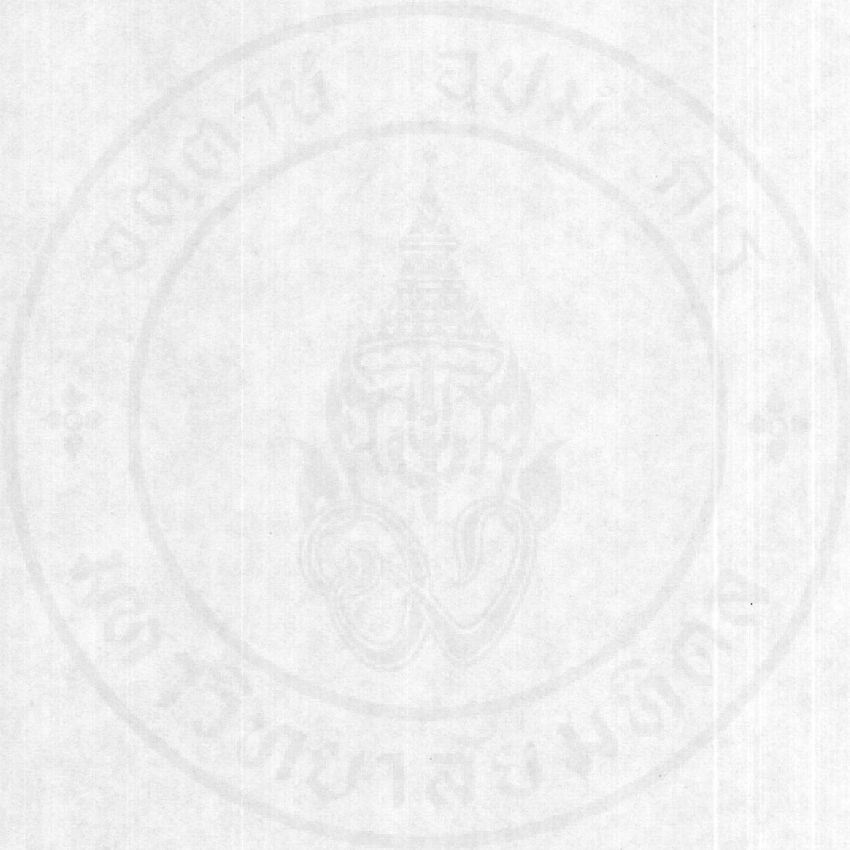
identification of *B. mallei* and *B. pseudomallei* as two distinct species. As no sequence data have been available, these patterns are worthy to elucidate the genetic dissimilarities among *B. mallei* and *B. pseudomallei*.



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CHAPTER VI

CONCLUSION

1. Approximately. 1.1 kb of PCR product from *B. mallei* ATCC10399 can be amplified as well as flagellin gene of *B. pseudomallei* using specific PCR primers. Thus, this amplified DNA fragment was cloned. Nucleotide sequence comprises 1,161 bp and contains 65.72% of GC content. DNA and translated amino acid sequence were aligned with flagellin genes and proteins from the several of relative species in NCBI database. The DNA sequence from *B. mallei* is homology to *Burkholderia* species 's and amino acid sequence is identical to the *B. pseudomallei* 's flagellin protein.
2. The translation of putative flagellin gene from *B. mallei* ATCC10399 was determined by using Western blot immunoassay. After detection with rabbit antiserum against flagellin protein of *P.putida* DMS3052, the distinct band from whole cell extract of *B. mallei* can not be obtained. The expected band at about 39 kDa was covered with smear-like pattern, which was believed to be lipopolysaccharide antigen.
3. The transcription of *B. mallei* flagellin gene was investigated by rapidly screening method, RNA Dot blotting. Detection with DIG-labeled flagellin DNA probe did not present any signal from total RNA of *B. mallei* ATCC10399, as compared with *B. pseudomallei* NF47/38.

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4. Upstream sequences of *B. mallei* flagellin genes were reported by Neubauer, H. et al. The sequence analysis shows that these 5′ upstream regions are rarely different from sequence of *B. pseudomallei* strain. Therefore, the GATC methylation of 5′ untranslated region of *B. mallei* flagellin gene was studied by using Southern blot hybridization. Difference of methylation pattern between *B. mallei* ATCC10399 and *B. pseudomallei* NF47/38 have not been detected by flagellin DNA probe.
5. Chemotaxis protein CheW gene and chemotaxis response regulator CheY gene from *B. mallei* ATCC10399 were also amplified as well as *B. pseudomallei* 's. After cloning and sequencing, these DNA fragments was translated to be amino acid sequence. The protein sequence alignment showed that both chemotaxis proteins compose of the amino acid sequences which are identical to chemotaxis protein and chemotaxis response regulator from *B. pseudomallei* 1026b, respectively.
6. Existing of same-methylated upstream region of flagellin gene and the appearance of both chemotaxis-involving genes lead to believe that expression of *B. mallei* flagellin gene is under control of the upper operon in hierarchy.

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APPENDIX

A. Composition of medium

MM medium

Na_2HPO_4	0.6 g
KH_2PO_4	0.3 g
NH_2Cl	0.1g
H_2O	50 ml

Sterize by autoclaving and leave to cool down for 55-60 ^{o}C, then add the following solution.

1.5% bacto agar	50 ml
1 M $MgCl_2z6H_2O$	200 µl
1 M $CaCl_2.2H_2O$	200 µl
1 M thiamine hydrochloride	200 µl
20% glucose	1000 µl

LB medium

Peptone	10 g
Yeast extract	5 g
NaCl	5 g
Distilled water	1 l
Bacto agar	15 g

Shake until the solutes have dissolved and sterilize by autoclaving

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SOB medium

Peptone	20 g
Yeast extract	5 g
NaCl	5 g
KCl	0.2 g
Distilled water	1 l

Sterilize by autoclaving. Just before use, add 5 ml of a sterile solution of 1 M $MgCl_2$ and 5 ml of a sterile solution of 1 M $MgSO_4$

SOC medium

SOB medium ad 20 mM glucose

B. Composition of solutions

Lysozyme solution in High Pure PCR Preparation kit : 10 μg/ml in Tris-HCl pH 8.0

wash buffer : 20 mM NaCl, 2 mM Tris-HCl, pH 7.5, 80% ethanol

elution buffer : 10 mM Tris, pH 8.5

Glucose-lysozyme solution : 50% Glucose, 25 mM Tris, 10 mM EDTA pH 8.0, 2 mg/ml lysozyme

lysis solution : 2% SDS, 200 μg/ml Proteinase K, 0.01 M NaCl

TE. Buffer : 0.01 M Tris, 0.001 M EDTA pH 7.4

Vent DNA Polymerase buffer

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TBE : 89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA

TAE : 40mM Tris base, 20 mM Glacial acetic acid, 200 mM EDTA, pH 8

Loading dye : 0.1% bromophenol blue, 40% ficoll and 5 mM EDTA

NEW WASH : NaCl/ethanol/water WASH

1X T4 DNA ligase buffer : 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM ATP, 50 μg/ml BSA; pH 7.8

$CaCl_2$ solution : 50 mM $CaCl_2$, 10 mM Tris-HCl pH 8.0

GTE solution : 50 mM glucose, 25 mM This pH 8.0, 10 mM EDTA

Lysis solution : 0.2 N NaOH, 1% SDS

Buffer P1 : 50 mM Tris-HCl, 10 mM EDTA, pH 8.0

Buffer P2 : 200 mM NaOH, 1% SDS

Buffer P3 : 3 M potassium acetate, pH 5.5

Buffer QBT : 750 mM NaCl, 50 mM MOPS, 15% ethanol, 0.15% Triton X-100, pH 7.0

Buffer QC : 100 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0

Buffer QF : 1.25 M NaCl, 50 mM Tris-HCl, 15% ethanol, pH 8.5

50 μM Sodiumphosphate buffer solution

SDS gel loading buffer : 50 mM Tris-HCl pH6.8, 150 mM dithiothretol, 2% SDS, 0.1% bromophenol blue, 10% glycerol

Tris-glycine electrophoresis buffer : 25 mM Tris, 250 ml glycine pH 8.3, 0.1%SDS

30% T, 2.7 % C acrylamide mix : 29% acrylamide, 1% N,N′ methylene-bis-acrylamide

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Lower buffer : 1.5 M Tris-HCl pH 8.8, 0.4% SDS

Upper buffer : 0.5 M Tris-HCl pH 6.8, 0.4% SDS

Tris-glycine electrophoresis buffer : 25 mM Tris, 250 ml glycine pH 8.3, 0.1%SDS

Coomassie brilliant blue solution : 0.1% Coomassie Brilliant Blue R250, 40% methanol, 10% glacial acetic acid

Destaining solution : 25% ethanol, 25% glacial acetic acid

Transfer buffer or Towbins buffer : 25 mM Tris-HCl pH 8.3, 192 mM glycine, 20%(v/v) methanol

blocking solution : 5% skim milk in phosphate buffer saline

Carbonate buffer : 10 mM $NaHCO_3$, 1 mM MgC_{l2}•6 H_2O

Lysozyme-containing TE buffer : 400 μg/ml lysozyme

RLT (guanidinium isothiocyanate-containing lysis buffer) buffer

RNA dilution buffer : DEPC-treated H_2O : 20X SSC : formaldehyde (5 : 3: 2)

High SDS buffer : 7% SDS, 50% formamide; deionize, 5XSSC, 2% blocking reagent, 50mM sodiumphosphate; pH 7.0, 0.1% N-lauroylsarcosine

2X wash solution : 2X SSC, 0.1% SDS

0.1X wash solution : 0.1X SSC, 0.1% SDS

Washing buffer : 100 mM maleic acid, 150 mM NaCl pH 7.2, 0.3% Tween20

Blocking solution : 1% blocking reagent in maleic acid buffer

Detection buffer : 0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$ pH 9.5

Color substrate solution : 45 μl NBT and 35 μl BCIP (in dimethylformamide) solution

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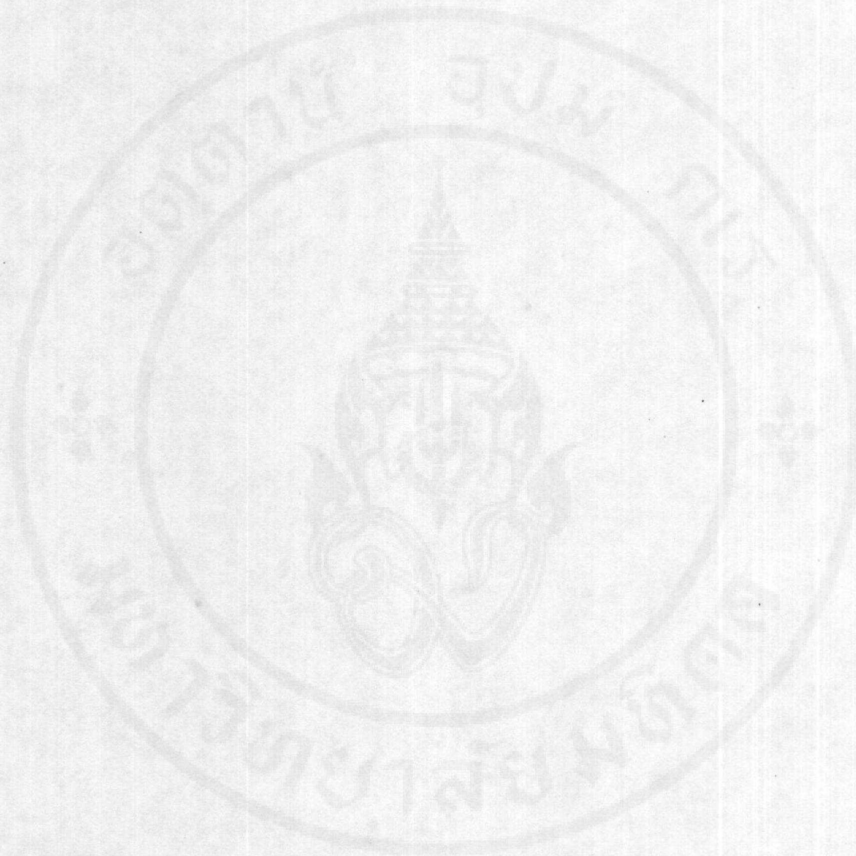
Denaturation solution : 0.5 N NaOH, 1.5M NaCl

Neutralization solution : 0.5 M Tris-HCl, pH 7.5, 3 M NaCl

<u>20X SSC buffer</u> : 3 M NaCl, 300 mM Sodium citrate pH 7.0



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C. Blast search results of amino acids sequences translated from *B. mallei* ATCC10399 genes

1. Blast search results of translated amino acids sequence of putative flagellin gene

```
                                                                 Score     E
Sequences producing significant alignments:                      (bits)  Value

gb|AAD24676.1|AF078151_1 (AF078151) flagellin [Burkholderia       725      0.0
gb|AAB40150.1| (U82286) flagellin [Burkholderia pseudomalle       725      0.0
gb|AAC71051.1| (AF098793) flagellin [Burkholderia mallei]         723      0.0
gb|AAD24677.1|AF078152_1 (AF078152) flagellin [Burkholderia       723      0.0
gb|AAD24679.1|AF078154_1 (AF078154) flagellin [Burkholderia       722      0.0
gb|AAD24678.1|AF078153_1 (AF078153) flagellin [Burkholderia       664      0.0
gb|AAC31966.1| (AF081500) flagellin [Burkholderia thailande       664      0.0
gb|AAD24680.1|AF078155_1 (AF078155) flagellin [Burkholderia       662      0.0
gb|AAC38199.1| (AF011370) type II flagellin [Burkholderia c       530    e-150
gb|AAD12053.1| (AF080259) flagellin [Burkholderia cepacia]        530    e-149
gb|AAC27442.1| (AF030240) flagellin [Burkholderia pseudomal       481    e-135
gb|AAC27443.1| (AF030241) flagellin [Burkholderia pseudomal       422    e-117
gb|AAC38200.1| (AF011371) type I flagellin [Burkholderia ce       366    e-100
gb|AAC38201.1| (AF011372) large flagellin [Burkholderia cep       331    6e-90
sp|P53606|FLA_LEGMI FLAGELLIN >gi|1073177|pir||S52444 flage       258    7e-68
gb|AAC28556.1| (L81146) flagellin [Pseudomonas aeruginosa]        257    9e-68
gb|AAC28557.1| (L81147) flagellin [Pseudomonas aeruginosa]        256    2e-67
gb|AAC63947.1| (AF034765) flagellin [Pseudomonas fluorescens]     251    7e-66
gb|AAC63946.1| (AF034764) flagellin [Pseudomonas aeruginosa]      250    2e-65
sp|Q06064|FLAA_BORBR FLAGELLIN >gi|538720|pir||A40594 major       248    6e-65
emb|CAA58234.1| (X83232) flagellin [Legionella pneumophila]       247    1e-64
gb|AAF32261.1|AF169322_1 (AF169322) flagellin [Escherichia        246    2e-64
gb|AAF32259.1|AF169320_1 (AF169320) flagellin [Escherichia        243    2e-63
dbj|BAA85082.1| (AB028473) flagellin [Escherichia coli]           243    3e-63
gb|AAB33952.1| flagellin {alternatively spliced} [Salmonell       240    2e-62
sp|P72151|FLIC_PSEAE FLAGELLIN >gi|1314747|gb|AAA99807.1| (       239    3e-62
gb|AAG04481.1|AE004540_1 (AE004540) flagellin type B [Pseud       239    4e-62
sp|P13713|FLIC_SERMA FLAGELLIN >gi|79151|pir||JU0056 flagel       239    4e-62
gb|AAB33953.1| flagellin {alternatively spliced} [Salmonell       238    7e-62
sp|P21184|FLAA_PSEAE FLAGELLIN >gi|94771|pir||A37853 flagel       233    2e-60
dbj|BAA06987.1| (D32256) flagellin [Serratia marcescens]          230    1e-59
dbj|BAA85081.1| (AB028472) flagellin [Escherichia coli]           228    6e-59
sp|P42272|FLC1_PROMI FLAGELLIN 1 >gi|1073658|pir||JC2559 fl       228    6e-59
pir||S78461 flagellin fleB, 37.4K - Yersinia enterocolitica       227    1e-58
pir||S69767 flagellin fleC, 39.6K - Yersinia enterocolitica       224    8e-58
gb|AAC27808.1| (AF069392) polar flagellin [Vibrio parahaemo       221    1e-56
pir||S78460 flagellin fleA, 36.7K - Yersinia enterocolitica       220    2e-56
sp|P42273|FLC2_PROMI FLAGELLIN 2 >gi|1073659|pir||JC2560 fl       219    3e-56
pir||D72335 flagellin - Thermotoga maritima (strain MSB8) >       218    8e-56
gb|AAF95289.1| (AE004287) flagellin FlaE [Vibrio cholerae]        217    1e-55
gb|AAB09433.1| (U52198) flagellin E [Vibrio anguillarum]          217    1e-55
gb|AAC01555.1| (AF007122) flagellin [Vibrio cholerae]             217    2e-55
gb|AAC01557.1| (AF007122) flagellin [Vibrio cholerae] >gi|9       217    2e-55
gb|AAB09435.1| (U52198) flagellin B [Vibrio anguillarum]          212    4e-54
gb|AAF19179.1|AF198617_3 (AF198617) FlaA [Aeromonas caviae]       210    2e-53
emb|CAA35488.1| (X17440) hsg48 [Escherichia coli]                 209    3e-53
gb|AAF95288.1| (AE004287) flagellin FlaD [Vibrio cholerae]        208    5e-53
gb|AAC27801.1| (AF069392) polar flagellin [Vibrio parahaemo       207    2e-52
gb|AAC01556.1| (AF007122) flagellin [Vibrio cholerae]             206    3e-52
```

2. Blast search results of translated amino acids sequence of chemotaxis protein CheW gene

Sequences producing significant alignments:	Score (bits)	E Value
gb|AAB53941.1| (U92493) chemotaxis protein [Burkholderia ps	343	6e-94
sp|P06110|CHEW_SALTY CHEMOTAXIS PROTEIN CHEW >gi|79056|pir|	219	2e-56
sp|P07365|CHEW_ECOLI CHEMOTAXIS PROTEIN CHEW >gi|72605|pir|	214	4e-55
sp|P21821|CHEW_ENTAE CHEMOTAXIS PROTEIN CHEW >gi|95554|pir|	208	4e-53
gb|AAG03567.1|AE004455_8 (AE004455) probable purine-binding	206	1e-52
gb|AAF96985.1| (AE004434) purine-binding chemotaxis protein	116	1e-25
sp|Q52881|CHEW_RHIME CHEMOTAXIS PROTEIN CHEW >gi|2120801|pi	114	5e-25
sp|Q60251|CHEW_RHOSH CHEMOTAXIS PROTEIN CHEW >gi|7442994|pi	108	5e-23
gb|AAC08070.1| (AF031898) CheW [Pseudomonas putida]	107	7e-23
sp|O87715|CHEW_CAUCR CHEMOTAXIS PROTEIN CHEW >gi|3387364|em	106	1e-22
pir||D69380 purine-binding chemotaxis protein (cheW) homolo	106	2e-22
gb|AAF95205.1| (AE004280) purine-binding chemotaxis protein	106	2e-22
gb|AAF96986.1| (AE004434) purine-binding chemotaxis protein	106	2e-22
gb|AAF32421.1| (AF069392) chemotaxis protein CheW [Vibrio p	106	2e-22
dbj|BAA33555.1| (AB012767) CheW [Pseudomonas aeruginosa] >g	105	3e-22
pir||S47260 cheW protein - Rhodobacter sphaeroides	100	2e-20
gb|AAD13199.1| (AF096317) putative chemotaxis protein CheW	97	1e-19
pir||D72341 purine-binding chemotaxis protein - Thermotoga	97	1e-19
pir||A75003 purine-binding chemotaxis protein (chew) PAB102	93	1e-18
pir||A71160 probable purine-binding chemotaxis protein (che	91	6e-18
sp|O83453|CHEW_TREPA CHEMOTAXIS PROTEIN CHEW >gi|7443002|pi	90	1e-17
gb|AAC33469.1| (AF074950) CheW [Treponema denticola]	90	2e-17
gb|AAF94559.1| (AE004218) purine-binding chemotaxis protein	89	3e-17
pir||B71335 probable purine-binding chemotaxis protein (che	87	1e-16
gb|AAD45221.1|AF105017_2 (AF105017) chemotaxis protein CheW	87	1e-16
pir||D70170 purine-binding chemotaxis protein (cheW-2) homo	84	1e-15
pir||A44130 chemotaxis protein CheW - Bacillus subtilis	82	3e-15
emb|CAB72750.1| (AL139074) chemotaxis protein [Campylobacte	81	6e-15
sp|P39802|CHEW_BACSU CHEMOTAXIS PROTEIN CHEW >gi|7442993|pi	80	1e-14
gb|AAD01689.1| (AF021091) chemotaxis protein CheW [Helicoba	79	3e-14
gb|AAB71330.1| (U64519) CheW [Rhodospirillum centenum (Rhod	78	6e-14
pir||G64568 purine-binding chemotaxis protein - Helicobacte	78	6e-14
pir||E70183 purine-binding chemotaxis protein (cheW-3) homo	78	7e-14
gb|AAB81649.1| (U61498) CheW [Borrelia burgdorferi]	78	7e-14
pir||G70138 purine-binding chemotaxis protein (cheW-1) homo	72	3e-12
pir||A71865 histidine kinase (mcp coupling protein) - Helic	72	3e-12
gb|AAB96836.1| (U34384) CheW [Borrelia burgdorferi]	71	7e-12
gb|AAG06737.1|AE004757_6 (AE004757) probable chemotaxis pro	68	8e-11
gb|AAF95347.1| (AE004291) chemotaxis protein CheV [Vibrio c	68	8e-11
gb|AAD42910.1|U12817_4 (U12817) chemotaxis CheV homolog [Vi	67	1e-10
emb|CAA13141.1| (AJ231099) z41f [Vibrio cholerae]	64	7e-10
emb|CAB72752.1| (AL139074) chemotaxis protein [Campylobacte	61	8e-09
sp|Q56311|CHEW_THEMA CHEMOTAXIS PROTEIN CHEW >gi|7443004|pi	59	4e-08
emb|CAA04431.1| (AJ000977) chemotaxis protein, CheWIII [Rho	59	4e-08
sp|P43498|FRZA_MYXXA FRZA PROTEIN (FRIZZY AGGREGATION PROTE	58	7e-08
gb|AAF96850.1| (AE004422) chemotaxis protein CheV, putative	56	3e-07
gb|AAF95154.1| (AE004275) chemotaxis protein CheV [Vibrio c	55	6e-07
sp|P37599|CHEV_BACSU CHEMOTAXIS CHEV PROTEIN >gi|1075832|pi	52	3e-06
gb|AAD01687.1| (AF021091) chemotaxis protein CheV [Helicoba	50	2e-05
emb|CAA04430.1| (AJ000977) chemotaxis protein, CheWII [Rhod	50	2e-05
pir||A64569 chemotaxis protein - Helicobacter pylori (strai	50	2e-05
pir||G71864 probable chemotaxis protein - Helicobacter pylo	48	6e-05
dbj|BAA83929.1| (AB024556) CHEV [Bacillus halodurans]	46	3e-04

3. Blast search results of translated amino acids sequence of chemotaxis response regulator CheY gene

```
                                                                 Score    E
Sequences producing significant alignments:                      (bits)  Value

gb|AAB36948.1| (U78087) CheY [Burkholderia pseudomallei]          258    2e-68
pdb|1CYE| Chey Mutant With Met 1 Deleted, Arg 1 Inserted          199    1e-50
pdb|1CEY| Chey Complexed With Magnesium (Nmr, 46 Structu          199    1e-50
pdb|3CHY| CheY >gi|5107492|pdb|1BDJ|A Chain A, Complex S          199    1e-50
sp|P06143|CHEY ECOLI CHEMOTAXIS PROTEIN CHEY >gi|72606|pir|       199    1e-50
sp|P06657|CHEY SALTY CHEMOTAXIS PROTEIN CHEY >gi|72607|pir|       197    3e-50
pdb|2CHE| Chey Complexed With Mg2+ >gi|515286|pdb|2CHF|           197    3e-50
pdb|5CHY| Structure Of Chemotaxis Protein Chey                    197    5e-50
gb|AAA23570.1| (M13463) cheY peptide [Escherichia coli]           197    5e-50
pdb|1VLZ|A Chain A, Chey Mutant With Thr 87 Replaced By Ile       196    6e-50
pdb|1D4Z|A Chain A, Crystal Structure Of Chey-95iv, A Hyper       196    6e-50
pdb|1EHC| Structure Of Signal Transduction Protein Chey           196    8e-50
pdb|1C4W|A Chain A, 1.9 A Structure Of A-Thiophosphonate Mo       196    8e-50
pdb|1YMU|A Chain A, Signal Transduction Protein Chey Mutant       196    1e-49
pdb|2CHY| CheY (Mutant With Ser 56 Replaced By Cys) (S56C)        195    1e-49
pdb|6CHY|B Chain B, Structure Of Chemotaxis Protein Chey >g       194    2e-49
pdb|1YMV| Signal Transduction Protein Chey Mutant With P          192    2e-48
pdb|1AB6|A Chain A, Structure Of Chey Mutant F14n, V86t >gi       189    8e-48
pdb|1AB5|A Chain A, Structure Of Chey Mutant F14n, V21t >gi       189    8e-48
pdb|1UDR|A Chain A, Chey Mutant With Lys 91 Replaced By Asp       189    8e-48
pdb|1HEY| Chey Mutant With Asp 12 Replaced By Gly, Asp 1          180    4e-45
gb|AAF32415.1| (AF069392) chemotaxis protein CheY [Vibrio p       178    2e-44
gb|AAF95211.1| (AE004280) chemotaxis protein CheY [Vibrio c       175    1e-43
sp|Q51455|CHEY PSEAE CHEMOTAXIS PROTEIN CHEY >gi|94801|pir|       159    8e-39
gb|AAC08062.1| (AF031898) CheY [Pseudomonas putida] >gi|767       156    7e-38
gb|AAB66375.1| (U97567) chemotaxis response regulator [Heli       113    6e-25
sp|P71403|CHEY HELPY CHEMOTAXIS PROTEIN CHEY HOMOLOG >gi|74       113    6e-25
pir||F71941 response regulator - Helicobacter pylori (strai       113    6e-25
emb|CAA57487.1| (X81897) cheY [Helicobacter pylori]               112    1e-24
sp|P71129|CHEY CAMJE CHEMOTAXIS PROTEIN CHEY HOMOLOG >gi|16       109    8e-24
emb|CAA07181.1| (AJ006687) CheYII [Caulobacter crescentus]         97    8e-20
gb|AAC44858.1| (U62038) chemotaxis protein [Campylobacter j        95    2e-19
gb|AAG03569.1|AE004455 10 (AE004455) probable two-component        90    6e-18
pir||A70171 chemotaxis response regulator (cheY-2) homolog         88    3e-17
gb|AAC25076.1| (AF044495) CheY homolog [Agrobacterium tumef        86    1e-16
pir||S61833 cheY1 protein - Rhizobium meliloti >gi|534836|g        83    1e-15
gb|AAD47845.1|AF171076 1 (AF171076) probable CheY1 protein         83    1e-15
gb|AAF96988.1| (AE004434) chemotaxis protein CheY [Vibrio c        83    1e-15
gb|AAG08749.1|AE004948 5 (AE004948) probable two-component         81    4e-15
emb|CAA04428.1| (AJ000977) chemotaxis response regulator [R        80    9e-15
pir||S49210 cheyY protein - Rhodobacter sphaeroides >gi|744        79    2e-14
gb|AAC25080.1| (AF044495) CheY homolog [Agrobacterium tumef        79    2e-14
emb|CAA07176.1| (AJ006687) CheYI [Caulobacter crescentus]          78    3e-14
emb|CAB76988.1| (AL159178) putative response regulator [Str        78    3e-14
pir||S61838 cheY2 protein - Rhizobium meliloti >gi|534841|g        78    3e-14
sp|P28835|YC27 PORAE PROBABLE TRANSCRIPTIONAL REGULATOR YCF        78    4e-14
sp|P24072|CHEY BACSU CHEMOTAXIS PROTEIN CHEY HOMOLOG >gi|98        77    6e-14
pir||F75002 chemotaxis response regulator (chey) PAB1330 -         76    1e-13
pir||S47262 cheY2 protein - Rhodobacter sphaeroides >gi|744        76    1e-13
sp|P35163|RESD BACSU TRANSCRIPTIONAL REGULATORY PROTEIN RES        76    1e-13
pir||E71160 probable chemotaxis protein (cheY) - Pyrococcus        76    1e-13
emb|CAB59609.1| (AL132662) putative response regulator [Str        75    4e-13
gb|AAG06197.1|AE004708 3 (AE004708) probable two-component         75    4e-13
```

D. Comparison of nucleotide sequences coded for chemotaxis proteins from *B. mallei* ATCC10399 and *B. pseudomallei* 1026b

>gb|U92493.1|BPU92493 Burkholderia pseudomallei 1026b chemotaxis protein CheW (cheW) gene,
Length = 528
Score = 1023 bits (516), Expect = 0.0
Identities = 525/528 (99%)
Strand = Plus / Plus

```
 Bm: gtgtccgaagtccaaacgaatcatccggccgcgccgaacgcggccagccgccgcgacgcc 60
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: gtgtccgaagtccaaacgaatcatccggccgcgccgaacgcggccagccgccgcgacgcc 60

 Bm: gaacagggcgacgccgcgggccaggagtttctcgtcttcacgctcggcgacgaggaatac 120
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: gaacagggcgacgccgcgggccaggagtttctcgtcttcacgctcggcgacgaggaatac 120

 Bm: ggcatcgacattctgaaagtacaggaaatccgcggctacgacagcgtcacgcggatcgcg 180
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: ggcatcgacattctgaaagtacaggaaatccgcggctacgacagcgtcacgcggatcgcg 180

 Bm: aacgcgcccgatttcatcaagggcgtgatcaacctgcgcggcatcatcgtgccgatcgtc 240
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: aacgcgcccgatttcatcaagggcgtgatcaacctgcgcggcatcatcgtgccgatcgtc 240

 Bm: gacatgcggatcaagttccacctcggccgcgtcgagtacgaccatcagacggtcgtgatc 300
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: gacatgcggatcaagttccacctcggccgcgtcgagtacgaccatcagacggtcgtgatc 300

 Bm: atcctgaacgtcgcgcatcgcgtcgtcgggatggtcgtcgacggcgtgtccgacgtgctc 360
     ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Bps: atcctgaacgtcgcgcaccgcgtcgtcgggatggtcgtcgacggcgtgtccgacgtgctc 360

 Bm: acgctgtcgaccgagcagatcatgcccgcgccggaattcggcggcgtgctgacgaccgag 420
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: acgctgtcgaccgagcagatcatgcccgcgccggaattcggcggcgtgctgacgaccgag 420

 Bm: tacctgacgggcctcggcacggtcgacggccggatgctgatcctgatggacatcgagaag 480
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: tacctgacgggcctcggcacggtcgacggccggatgctgatcctgatggacatcgagaag 480

 Bm: ctgatgacgagcaaggagatggcgctgatcgagacacttggcgcataa 528
     ||||||||||||||||||||||||||||||||||| || |||||||||
Bps: ctgatgacgagcaaggagatggcgctgatcgagacgctcggcgcataa 528
```

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>gb|U78087.1|BPU78087 Burkholderia pseudomallei chemotaxis response regulator CheY (cheY) gene,
gene, complete cds
Length = 509

Score = 785 bits (396), Expect = 0.0
Identities = 396/396 (100%)
Strand = Plus / Plus

```
 Bm: atggacaagagcatgaagattctggtggtggacgattttccgacgatgcgtcggatcgtc  60
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: atggacaagagcatgaagattctggtggtggacgattttccgacgatgcgtcggatcgtc 127

 Bm: cgcaacttgctgaaagagttgggctattcgaacgtcgacgaggcggaggacggcctggcc 120
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: cgcaacttgctgaaagagttgggctattcgaacgtcgacgaggcggaggacggcctggcc 187

 Bm: ggcctcgcgcggctgcgcggcggcggctacgacttcgtgatctccgactggaacatgccg 180
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: ggcctcgcgcggctgcgcggcggcggctacgacttcgtgatctccgactggaacatgccg 247

 Bm: aacctcgacggtctcgcgatgttgaaggagatccgcgcggacgcgtcgctcacgcacctg 240
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: aacctcgacggtctcgcgatgttgaaggagatccgcgcggacgcgtcgctcacgcacctg 307

 Bm: ccggtgctgatggtgacggccgagtcgaagaaggagaacatcatcgcggctgcgcaggcg 300
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: ccggtgctgatggtgacggccgagtcgaagaaggagaacatcatcgcggctgcgcaggcg 367

 Bm: ggcgcgagcggctacgtcgtgaaaccgtttacggcggcgacgctcgacgagaagctcaac 360
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bps: ggcgcgagcggctacgtcgtgaaaccgtttacggcggcgacgctcgacgagaagctcaac 427

 Bm: aagattctggaaaagatggcgaaagcggggagctga 396
     ||||||||||||||||||||||||||||||||||||
Bps: aagattctggaaaagatggcgaaagcggggagctga 463
```

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BIOGRAPHY

NAME	MissAtchara Paemanee
DATE OF BIRTH	20 October 1974
PLACE OF BIRTH	Songkhla, Thailand
INSTITUTIONS ATTENDED	Chulalongkorn University, 1992-1996: Bachelor of Science (Biochemistry) Mahidol University, 1996-2000: Master of Science (Biochemistry)
FELLOWSHIP/ RESEARCH GRANT	the Shell Grants Funds for Research Projects to Graduate Students, 1998-2000

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