A STUDY OF *Burkholderia pseudomallei* K96243 HYPOTHETICAL BPSL3393 GENE PRODUCT AS A PUTATIVE CoA-BINDING PROTEIN

OOI GIM LUAN

UNIVERSITI SAINS MALAYSIA

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By

OOI GIM LUAN

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Celcius
%	percent
µg	microgram
µl	microlitre
µm	micrometer
Amp^r	ampicillin resistance
ATP	adenosine-5'-triphosphate
BLAST	Basic Local Alignment Search Tool
Blastp	protein BLAST
bp	base pair
Bsa	*Burkholderia* secretion apparatus
cat^R	chloramphenicol resistance gene
Cm^r	chloramphenicol resistance phenotype
CDP	critical point drying
CH_3CO_2K	potassium acetate
CoA	Coenzyme A
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DSF	downstream forward region
DSR	downstream reverse region
DSR-EXT	downstream reverse external region
EDTA	Ethylenediaminetetraacetic acid
EutC	ethanolamine ammonia lyase light chain
EutE	acetaldehyde dehydrogenase

eutK	putative gene of ethanolamine utilization carboxysome
eutL	putative gene of ethanolamine utilization carboxysome
eutM	putative gene of ethanolamine utilization carboxysome
eutN	putative gene of ethanolamine utilization carboxysome
eutS	putative gene of ethanolamine utilization carboxysome
FAD	flavin adenine dinucleotide
g	gram
HMDS	Hexamethyldisilazane
IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilo base pairs
kDa	kilodalton
LA	Luria Bertani agar
LB	Luria Bertani broth
M	molar
mg	milligram
$MgSO_4$	magnesium sulphate
min	minute
ml	millilitre
mM	millimolar
mob	mobilization gene
N	normality
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NaH_2PO_4	sodium dihydrogen phosphate
Na_2HPO_4	disodium hydrogen phosphate

NaOH	sodium hydroxide
ng	nanogram
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
PHB	polyhydroxybutyrate
pilA	type I pili gene
pmol	picomole
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
s	second
sacB	levansucrase gene
SDS	Sodium dodecyl sulfate
TCA	Tricarboxylic acid
TSS	transformation and storage solution
T3SSs	Type III secretion system
USF	upstream forward region
USF-EXT	upstream forward external region
USR	upstream reverse region
US-INT	upstream internal region
V	volt
w/v	weight per volume
xg	relative centrifugal force
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

KAJIAN MENGENAI PRODUK GEN HIPOTETIKAL BPSL3393 DARI *Burkholderia pseudomallei* K96243 SEBAGAI PROTEIN PENGIKAT CoA PUTATIF

ABSTRAK

Burkholderia pseudomallei merupakan agen penyebab penyakit melioidosis. Dengan menggunakan *Burkholderia pseudomallei* K96243 sebagai genom rujukan untuk perbandingan pelbagai genom, sebanyak 48 gen yang khusus dan umum telah dijumpai dalam *B. pseudomallei*. Antara gen ini, gen BPSL3393 yang dikenali sebagai gen hipotetikal telah disimpulkan untuk memiliki motif pengikat CoA berdasarkan analisis jujukannya. Walaubagaimanapun, gen ini masih tetap dianggap sebagai gen putatif kerana fungsinya belum diketahui lagi. Objektif kajian ini adalah untuk mengkaji fungsi biologi gen BPSL3393 dalam *B. pseudomallei* K96243. Satu mutan delesi tanpa penanda untuk gen BPSL3393 telah dijana menggunakan vektor swa-hapus pDM4. Vektor ini mengandungi gen kerintangan kloramfenikol (cat^R) yang dijadikan penanda pemilihan manakala gen levansukrase (*sacB*) telah digunakan sebagai penanda lawan-pemilihan. Sistem Biolog GN2 MicroPlate™ telah digunakan untuk mengkaji profil biokimia mutan ΔBPSL3393 dan jenis liar. Mutan ΔBPSL3393 menunjukkan pengurangan keupayaan dalam penggunaan 2-aminoethanol jika dibandingkan dengan jenis liar. Selain itu, mutan ΔBPSL3393 juga menunjukkan perbezaan fisiologi dalam corak pertumbuhannya. Mutan ΔBPSL3393 juga mempamerkan tanda-tanda pengagregatan ketika berada dalam media M9 pada fasa eksponen. Sel jenis liar tidak menunjukkan ciri tersebut. Kajian yang lebih lanjut adalah diperlukan untuk mengkaji fungsi gen BPSL3393.

A STUDY OF *Burkholderia pseudomallei* K96243 HYPOTHETICAL BPSL3393 GENE PRODUCT AS A PUTATIVE CoA-BINDING PROTEIN

ABSTRACT

Burkholderia pseudomallei is the causative agent of melioidosis. From the whole genomic comparison by using *B. pseudomallei* K96243 as reference genome, a total 48 genes were found specifically and common in *B. pseudomallei* strains. Amongst, the hypothetical gene, BPSL3393, was deduced to contain the CoA-binding motif based on its sequence analysis. However, this gene is still remaining as a putative gene where it is still functionally uncharacterized. The objective of this study is to elucidate the biological function of gene BPSL3393 in *B. pseudomallei* K96243. An unmarked deletion mutant of gene BPSL3393 was constructed by using pDM4 suicidal vector. This vector employed cat^R, chloramphenicol resistant gene as the selection marker and *sacB*, levansucrase gene as the counter-selection marker. The biochemical profiles of ΔBPSL3393 mutant and wild type strains were determined by using th Biolog GN2 MicroPlate™ system. The ΔBPSL3393 mutant showed significant reduction in 2-aminoethanol utilization as compared to wild type. Apart from this, the ΔBPSL3393 mutant also has shown some physiological difference as compared to wild type. The ΔBPSL3393 mutant aggregated at the exponential phase in M9 minimal media whereas the wild type did not. Thus, further study is needed to characterize this ΔBPSL3393 mutant and to uncover its biological role.

CHAPTER ONE

INTRODUCTION

Burkholderia pseudomallei is a potential biothreat agent which causes the fatal human disease, melioidosis. This disease was found to expand beyond its endemic area in Southeast Asia and Northern Australia to other continents such as India, Southern China, Hong Kong and Taiwan at past decade (Currie *et al*., 2008). Individuals with underlying immunocompromised disease such as diabetes, chronic renal failure, and alcohol addictions are more susceptible to melioidosis. Several modes of transmission such as subcutaneous inoculation, inhalation and ingestion have been suggested. Climatic changes are highly associated with the acquisition of melioidosis. During the monsoon season, high rainfall and strong wind cause the leaching of *B. pseudomallei* from soil. This will lead to an increase in aerosol infection. Rice farmers experience a higher exposure rate through their occupational routines (Currie and Jacups, 2003). There is no specific clinical manifestation of melioidosis. It varies from acute or subacute to local or systemic infection. The clinical features can be categorized into four groups: acute fulminant septicemia, subacute illness, chronic infection and subclinical disease. However, septicemia with or without pneumonia is the commonest clinical presentation (Cheng *et al*,. 2013). The complexity of melioidosis infection and resistance of *B. pseudomallei* toward many antibiotics makes the antimicrobial therapy problematic. Different combinations of drugs are needed depending on the severity and antimicrobial susceptibility of the infection (Inglis, 2010).

Burkholderia pseudomallei genome comprises 2 chromosomes namely, chromosome I and Chromosome II. Approximately 86% of the genes are common to

all strains and this is denoted as the “core genome”. The rest of it are present variably across the strains and is denoted as an “accessory genome”. This accessory genome is commonly localized in genomic islands where the gene clusters were imported from other bacteria through horizontal gene transfer (Holden *et al.*, 2004; Wiersinga *et al.*, 2006). These horizontal gene transfer events are believed to contribute to the presence of virulence-related genes in *Burkholderia pseudomallei* (Yu *et al.*, 2006). In recent study, approximately 34% of *Burkholderia pseudomallei* K96243 genes are categorized as hypothetical proteins (http://cmr.jcvi.org/cgi-bin/CMR/shared/GetNumAndPercentGenesInARole.cgi; last accessed February 2013) where these genes are computationally predicted and the gene functions are yet to be elucidated (Sivashankari and Shanmughavel, 2006). Gene BPSL3393 is one of these hypothetical genes which is specific and common in all *B. pseudomallei* strains. A conserved domain prediction showed that gene BPSL3393 contains the CoA-binding domain. However, it remains functionally uncharacterized.

B. pseudomallei employs several pathogenesis patterns to survive in a host. For example, *B. pseudomallei* forms a capsule to prevent the deposition of C3 complement factor (Reckseidler-Zenteno *et al.*, 2005). Besides, *B. pseudomallei* also forms Type-IV pili to enable better attachment to its host. The pili protein is encoded by the *pilA* gene and the *pilA* mutant shown reduced virulence towards nematode and mouse (Essex-Lopresti *et al.*, 2005). The *Bukholderia* secretion apparatus (Bsa), part of the Type III secretion system (T3SSs), is believed to play an important role in *B. pseudomallei* infection. It consists of translocator and effector proteins. The translocator proteins form a needle complex structure to deliver the effector protein into their host in order to subvert the host cell mechanism (Cornelis and Van Gijsegem, 2000). For cell proliferation, a *B. pseudomallei* cell which is engulfed in

phagosome is able to induce actin polymerization leading to cell membrane protrusion. This enables the pathogen to perform cell-to-cell spreading without triggering the host immune response (Stevens *et al.*, 2005). Apart from these, *B. pseudomallei* also secrete some other virulence molecules such as haemolysin, lecthinase, siderophores and proteases which lead to the complexity of melioidosis (Lazar Adler *et al.*, 2009).

Coenzyme A (CoA) is a vital cofactor in living organisms. It acts as a acyl group carrier and a carbonyl-activating group in cell metabolism. It consists of adenosine 3',5'-diphosphate, 4-phosphopantothenic acid (vitamin B5) and β-mercaptoethylamine (Baddiley *et al.*, 1953). The recognition site of CoA is located at the adenosine 3', 5'-diphosphate where it increases the binding affinity to target enzymes. The functional group of CoA is the thiol group which is located at the β-mercaptoethylamine moiety. Coenzyme A, especially in the form of acetyl-CoA, is closely associated with a relatively large number of cellular metabolisms such as the tricarboxylic acid (TCA) cycle, fatty acid metabolism and amino acid metabolism (Lipmann, 1953).

Various types of CoA-binding proteins exist in a living cell acting specifically on their substrates and involve in various kind of metabolic pathways. Different CoA-binding proteins exist in different patterns, either as a monomer or oligomer. One conserved feature that was observed in all CoA-binding proteins is that the adenine ring is pointed towards the protein whereas the 3'-phosphate is pointed towards the solvent. Some of binding modes might be non-conserved but what is frequently observed from most of CoA-binding proteins are the presence of a hydrogen bond between the amino group of adenine and the main chain protein and the presence of a salt bridge between lysine or arginine of the protein molecule to the

phosphate group of CoA (Engel and Wierenga, 1996). In *Burkholderia pseudomallei*, there are approximately 220 genes in the NCBI database (http://www.ncbi.nlm.nih.gov/gene; last accessed April 2013) that encoded for various kinds of CoA-binding proteins. Among these genes, some are computationally deduced to possess the CoA-binding capability. However, these are functionally uncharacterized. Gene BPSL3393 is one of the hypothetical proteins deduced with the CoA-binding capability. It is also one of the genes that are species specific and common in all *B. pseudomallei*. Since CoA is an important central intermediate of cellular metabolisms, a study of this CoA binding protein may uncover an important physiological pathway in this pathogen. Thus, the objectives of this study are:

1. To uncover species-specific genes in *Burkholderia pseudomallei*.
2. To investigate the role of BPSL3393 hypothetical protein in *B. pseudomallei*.
3. To observe the resulting effects of gene BPSL3393 deletion on growth pattern, cells surface and internal structures, biofilm formation and its physiology.

CHAPTER TWO

LITERATURE REVIEW

2.1 Genus *Burkholderia* and *Burkholderia pseudomallei*

The first discovered *Burkholderia* species was *Burkholderia cepacia* which reported by Walter H. Burkholder as the causing agent of the onion bulbs disease (Burkholder, 1942). Initially, this species was named as *Pseudomonas cepacia* (Burkholder, 1950). In 1992, seven species of the genus *Pseudomonas* were relocated to new genus *Burkholderia* based on their 16S rRNA, phenotypic characteristics, DNA-DNA homology values, cellular lipid and fatty acid compositions. The seven species were *Burkholderia cepacia, Burkholderia mallei*, *Burkholderia pseudomallei*, *Burkholderia caryophylli*, *Burkholderia gladioli*, *Burkholderia pickettii* and *Burkholderia solanacearum* (Yabuuchi *et al.*, 1992) . However, Burkholderia *pickettii* and *Burkholderia solanacearum* were later destined to the genus *Ralstonia* (Yabuuchi *et al.*, 1995).

The genus *Burkholderia* contains more than 30 species that occupy various ecological niches such as soil, water, rhizosphere, animals and human. Members of the genus *Burkholderia* are Gram-negative non-spore-forming bacilli with a diameter of 0.5 – 1.0 µm and length 1.0 – 5.0 µm. They are capable of utilizing nitrate as an electron acceptor under anaerobic conditions and metabolize glucose oxidatively (Garrity, 2005). This genus included species that are useful in bioremediation and promotion of plant growth, plant pathogens, zoonotic pathogen and opportunistic human pathogen (Payne *et al.*, 2005). Several *Burkholderia* species show significant commercial values and ecological importance. For example *B. xenovorans* strain LB400 was notoriously known as an effective polychlorinated biphenyl (PCB)

degrader due to its capability to degrade a wide range of PCBs aerobically (Vial *et al.*, 2007).

Plant pathogens within this genus include *B. caryophylli* which is a pathogen for carnations and also causes onion rot; *B. andropogonis* for stripe disease of sorghum and leaf spot of velvet bean; *B. plantarii* for seedling blight of rice, *B. glumae* for bacterial seedling and grain rot in rice and other grasses, and *B. gladioli* for lesion on *Gladiolus* spp., *Crocus* spp., *Freesia* spp., *Iris* spp., ferns and orchids (Gitaitis and Nischwitz, 2006). Some *Burkholderia* species develop symbiotic relationship with their plant hosts. *B. caribensis*, *B. tuberum* and *B. phymatum* provide aid in the nitrogen fixation process through the formation of tuber. *B.vietnamiensis*, *B. unamae*, *B. tropica* provide their plant host nitrogen source through the fixation of the atmospheric nitrogen (Stoyanova *et al.*, 2007). *B. vietnamiensis* was firstly isolated from rice plants rhizophere (Gillis *et al.*, 1995) and later gradually isolated from more and more plant hosts such as maize and coffee plants (Estrada-De Los Santos *et al.*, 2001). Other than the beneficiary characteristic towards environment, *B. vietnamiensis* is also an opportunistic pathogen as shown by the isolate obtained from cystic fibrosis patients. Due to its phenotypic similarity with *B. cepacia*, it was proposed as a member of genomovar II in *Burkolderia cepacia* complex (BCC) (Vandamme *et al.*, 1997). The term "genomovar" was introduced by Ursing *et al.*, (1995) which refers to strains that are genotypically distinct based on genomic data but shared similar phenotypic characteristics.

B. cepacia complex consist of nine genomovars: *B. cepacia* (genomovar I), *B. multivorans* (genomovar II), *B. cenocepacia* (genomovar III), *B. stabilis* (genomovar IV), *B. vietnamiensis* (genomovar V), *B. dolosa* (genomovar VI), *B. ambifaria* (genomovar VII), *B. anthina* (genomovar VIII) and *B. pyrrocinia* (genomovar IX). In

most of the time, BCC bacteria have been found to associate with immunocompromised patients, particularly patients with cystic fibrosis (CF) and chronic granulomatous disease. BCC bacteria are common contaminant of cosmetics, pharmaceutical solutions, water supplies and even sterile solutions (Coenye and Vandamme, 2003).

B. cepacia emerged in the last 20 years as an opportunistic pathogen to patients with underlying disease particularly in cystic fibrosis (CF) or chronic granulomatous disease patients. The clinical manifestation of the infection is inconsistent and varies from asymptomic to fatal pneumonia. The first clinical syndrome was described by Isles *et al.*, (1984) and given the name "cepacia syndrome". The multiresistancy of *B. cepacia* against antimicrobial agents makes the treatment more challenging.

B. mallei is the etiologic agent of glanders which occurs mainly in equines, but most of the mammals are also susceptible. Horse is the primary carrier of this disease, other mammals such as human, monkey, mice and guinea pig may acquired this disease via zoonatic transmission. Food sharing and crowding in farm may increase the transmission rate (Stoyanova *et al.*, 2007).

Burkholderia pseudomallei was first discovered by Alfred Whitmore which caused a 'glanders-like' disease on a mophine addict patient in Rangoon, Burma (Whitmore, 1913). The disease was later given the name melioidosis, a fatal disease in human. In the past century, *B. pseudomallei* caused major public health importance in Southeast Asia and Northern Australia. It is also classified as a category B agent by the US Centers for Disease Control and Prevention (CDC) (Rotz *et al.*, 2002). In genus *Burkholderia*, the utility of 16S rRNA in phylogenetic analysis is limited. Thus, *recA*-based phylogenetic analysis was used and it was found to

accurately distinguish the species in genus *Burkholderia*. From the phylogenetic analysis of the *recA* gene sequences, *Burkholderia pseudomallei* is closely related to *Burkholderia mallei* and *Burkholderia thailandensis* (Karlin *et al.*, 1995; Tom & Peter, 2003; Payne *et al.*, 2005). Unlike *B. pseudomallei* and *B. mallei*, *B. thailandensis* is an avirulent strain and does not correlate with the disease in the Syrian golden hamster model (Brett *et al.*, 1997). *B. thailandensis* display similar phenotypic characteristic with *B. pseudomallei* except for the capability to assimmilate L-arabinose. *B. thailandensis* can readily assimilate L-arabinose as the sole carbon source while *B. pseudomallei* cannot. The eight genes involved in arabinose assimilation operon in *B. thailandensis* are replaced by a two-protein cluster containing one hypothetical protein and one MarR family regulatory protein in the *B. pseudomallei* genome (Moore *et al.*, 2004; Yu *et al.*, 2006).

B. pseudomallei is an facultative anaerobic, motile soil dwelling saprophyte living in common niches such as moist soils, stagnant water, rice paddies and plant roots in the tropical and subtropical regions. The genomic plasticity of *B. pseudomallei* confers resilience with capability to adapt and overcome harsh environment. It is able to survive in nutrient deficiency condition (Wuthiekanun *et al.*, 1995), salty condition (Pumirat *et al.*, 2009), acidic condition, low water content environment except UV radiation (Tong *et al.*, 1996). The highest case occurrence of melioidosis is observed during monsoonal season. Higher rainfall may associate with melioidosis due to leaching of the saprophyte from the soils and causing occupational and recreational infection.

The recovery of *B. pseudomallei* from clinical specimens is tedious due to overgrowth and masking by other commensal flora. Thus, several selective media were design for the isolation of *B. pseudomallei*. Ashdown selective medium was

specifically designed for rapid screening of the *B. pseudomallei* from clinical specimens (Ashdown, 1979). Normally, it appears as a dry, wrinkled and violet-to-purple colony on the Ashdown selective medium after 48 hours incubation (Wuthiekanun *et al.*, 1990). However, the morphological switching from dry and rough to smooth colonies was observed in clinical specimens. The switching of phenotypic expression of *B. pseudomallei* is interrelated with its adaptation stratag in order to improve their survivability in various conditions (Chantratita *et al.*, 2007; Tandhavanant *et al*., 2010).

2.2 The *Burkholderia pseudomallei* K96243 genome

Burkholderia pseudomallei K96243 is referred to as the main reference genome of *B. pseudomallei* because it was the first to be sequenced (Holden *et al*., 2004). The complete *B. pseudomallei* K96243 genome sequence was compared with that of *B. mallei* and both were found to have features of genome plasticity. The genome comprises two circular replicons designated as chromosome I with the size of 4.07 Mb and chromosome II with the size of 3.17 Mb. Relatively large proportions of genes in chromosome I encoded for the essential housekeeping functions such as metabolism, nucleotides and proteins biosynthesis, cell wall synthesis and mobility. The genes in chromosome II mostly encoded for accessory functions involved in adaptation and survival enhancement. Chromosome II harbors greater portion of coding genes which either match to hypothetical genes or no databases at all. The G+C content of *B. pseudomallei* is relatively high making up 68% of the overall genome (Holden *et al,*. 2004). Through whole-genome *B. pseudomallei* microarrays analysis, 86% of the *B. pseudomallei* K96243 genes is common to all strains and this set of genes is denoted as the “core genome”. In contrast, the other 14% of the genes are present variably across the isolates and this is denoted as an “accessory genome”

(Wiersinga and van der Poll, 2009). The accessory genome is commonly localized in genomic islands. It is mainly augmented with paralogous genes and genes encoding for hypothetical proteins (Sim *et al.*, 2008). One remarkable feature that was observed in *B. pseudomallei* was, approximately 6% of the *B. pseudomallei* K96243 genome was contributed by the genomic islands (GIs). A genomic island is the region of genome that indicates horizontal gene transfer activity. Besides, this region of DNA also displayed anomalies in its G+C content and carried coding genes that are similar to those belonging to mobile genetic elements. The integration of GIs is a systemic process where the 3' end of tRNA genes of *B. pseudomallei* was predicted to mediate the integration. Genomic comparison revealed that the genomic islands vary greatly among the *B. pseudomallei* strains and this leads to the great diversity among *B. pseudomallei* strains (Tuanyok *et al.*, 2008). The great genomic diversity was believed to play a crucial role in shaping transcriptomic pattern and proteomic expression pattern in *B. pseudomallei*. This may in turn lead to variable in the phenotypic traits displayed across the strains (Ou *et al.*, 2005).

2.3 Melioidosis

Melioidosis, also known as Whitmore's disease, is a well recognized endemic disease in Southeast Asia and Northern Australia, which corresponded to approximately tropical latitudes 20°N and 20°S. Besides, sporadic cases were reported from South Africa, middle east, Caribbean, central and South America (Dance, 1991). However, severe global weather events and environmental disasters such as tsunami in recent years exposed several locations to sporadic cases. To date, the endemic regions include the major part of the Indian subcontinent, southern China, Hong Kong and Taiwan (Currie *et al,*. 2008). Melioidosis was found to be responsible for 20% of all community-acquired septicaemias and 40% of sepsis-

related mortality in northeast Thailand. Other than human, a wide variety of animals such as camels, horses, kangaroos, sheep and guinea pigs are also susceptible to melioidosis.

Melioidosis often infect immunocompromised individuals with pre-existing diseases such as diabetes, chronic kidney failure, thalassemia and alcoholic addiction (Wiersinga *et al.*, 2006). Melioidosis affected all range of ages but the highest incidence was found to fall between 40-60 years old (Raja *et al.*, 2005).

There are three acquisition modes, namely, inhalation, ingestion and inoculation. Initially, inhalation was suggested as the primary mode of acquisition due to a higher incidence of the disease occurring on the U.S. helicopter crews in the Vietnam War. The infection was also highly associated with weather events. In Northern Australia, the case of infection was relatively higher during the summer rainy season. During this period, a relatively higher case of pneumonic melioidosis was observed. The higher rainfall caused leaching of *B. pseudomallei* from soil which may lead to a higher aerosol infection rate (Currie and Jacups, 2003).

Rice farmers who work in flooded paddies have a relatively higher exposure rate to *B. pseudomallei*. In Thailand, during planting and harvesting season rice farmers are used to work with bare foot and hand. Thus, they may get injured or a wound cut during their work. Lesions, skin abrasions, ulcers or wounds may increase of the chance of infection through subcutaneous inoculation (Chaowagul *et al.*, 1989).

The ingestion mode is relatively less common in melioidosis. However, several case studies implicated contaminated water as one of the causes of outbreak (Currie *et al.*, 2001). Besides, this organism has been isolated from microabscesses

from stomach tissues surrounding the ulcer suggesting that ingestion can be one of the mode of transmission (Currie *et al.*, 2000).

The incubation period of *B. pseudomallei* varies from months to years without causing any clinical symptoms. *B. pseudomallei* is able to stay dormant within their host. The immunity status, environmental variables and stress may provoke the reactivation of the pathogens. When the surrounding favors or the host being immunocompromised, proliferation begins and they start causing disease on their host (Raja *et al.*, 2005). The longest period of incubation reported was 62 years in which a U.S soldier collapsed after 62 years from the Vietnam War. Just as other infectious disease, the infection of melioidosis is also highly depending on the size of inoculum. The size of inoculum determines the severity and patterns of disease. The size of inoculum is also associated with the organism's incubation period in which high inoculum leads to shorter incubation period (Cheng and Currie, 2005).

The clinical manifestation varies and ranges from acute to chronic and could be either a local or systemic infection. This disease can be divided into four catogeries: acute fulminant septicemia, subacute illness, chronic infection and subclinical disease (Renella *et al.*, 2006). Immunocompromised human with diabetes mellitus, chronic renal disease or thalassemia accounts higher risk for infection (Jain *et al.*, 2007). The most common clinical picture of melioidosis is septicemia with bacterial dissemination to distant organs such as spleen, liver and lung causing abscess (Holden *et al*., 2004).

Treatment of melioidosis is problematic due to the resistance of *Burkholderia pseudomallei* to a diverse group of antibiotics such as the third generation of cephalosporins, penicillin, macrolides, rifamycins and aminoglycosides. Several factors have to consider during drug prescription such as the illness severity, illness

symptoms and also the susceptibility of infection (Inglis, 2010). Besides, lengthy courses of treatment are needed in order to fully eradicate this pathogen. However, there was still approximately 2-9% of patients who experienced recurrent infection either relapsing with the same strain of *B. pseudomallei* or re-infection with a new strain (Jain *et al.*, 2007).

2.4 Putative pathogenesis pattern

Burkholderia pseudomallei employs a wide range of virulent factors as the means of survival in their host. Capsule formation is one of the common protections among bacteria. The production of capsule in *Burkholderia pseudomallei* is regulated by the presence of serum by using a *lux* reporter fusion to the *wcbB* capsule gene. *Burkholderia pseudomallei* produces -3)-2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose-(1- extracellular polysaccharide capsule that act as a protective barrier of the cells. The capsule mutant strain showed lower survivability in blood as compared to the wild type. This is because the capsular layer protects the pathogens from phagocytosis and deposition of complement C3 factor which in turn occlude the pathogens from the opsonization targeting (Reckseidler-Zenteno *et al.*, 2005). Besides, they also undergo several mechanisms to resist human defense mechanisms such as the invasion macrophages by inhibiting nitric-oxide synthase in these macrophages. The nitric-oxide synthase is responsible in producing reactive nitrogen intermediates for bacterial killing (Wiersinga *et al*., 2006).

Similar to other pathogens, the initial step and the most fundamental one for invasion is the adherence of bacteria to its host. Type IV pilin is a common virulence determinant in Gram-negative bacteria. *B. pseudomallei* genome contains several type-IV encoding loci including the *pilA* gene which encodes for the putative pili structural protein. The *pilA* mutant showed reduced adherence ability to human

epithelial cells, less virulence towards nematode model and the murine of the model of melioidosis (Essex-Lopresti *et al.*, 2005). Besides, the adherence of bacteria is believed to be regulated by temperature. At 30°C, the *B. pseudomallei* showed better adherence as compared to 37°C. However, the mechanism of temperature-regulation adhesion remains unknown (Brown *et al*., 2002).

Following adhesion, *Burkholderia pseudomallei* is internalized into the target cells with the coordination of type III secretion systems (T3SSs). *Burkholderia pseudomallei* is able to invade either phagocytic or non-phagocytic cells lines. They are able to survive intracellularly and proliferate within phagocytes without activating the host immune system of their host. The T3SS are common in pathogenic Gram-negative bacteria which helps bacteria to deliver the secreted effector proteins into their host cytosol in order to subvert the host cell mechanism (Cornelis and Van Gijsegem, 2000). One of the T3SSs in *B. pseudomallei* is known as *Burkholderia* secretion apparatus (Bsa) which is involved in cell virulence such as cellular invasion, phagosome lysis and intercellular spreading (Warawa and Woods, 2005). The BipB, BipC and BipD are three important translocator proteins of the Bsa T3SS. These translocator proteins form a needle complex protruding from bacterial membrane to interact with the host cell membrane and forming a path for the delivery of the effector proteins such as BopE and BopA (Mueller *et al*., 2008). The T3SS was also found to be involved in the formation of multinucleated giant cells (MNGCs). The BipB protein and RpoS protein was showed to induce the formation of multinucleated giant cells by cell fusion. The formations of MNGC have been observed from both phagocytic and no-phagocytic cell lines. The formation of MNGC was speculated as the strategy of cell-to-cell spreading. However, the actual mechanism still remains undefined (Suparak *et al.*, 2005; Utaisincharoen *et al.*,

2006). The *bipD* mutant showed absence of action formation and attenuated virulence following the intranasal and intraperitoneal challenge. It is also showed impaired bacterial proliferation in the liver and spleen of BALB/c mice (Stevens *et al.*, 2004; Pilatz *et al.*, 2006).

The BopE protein was found to be involved in the actin re-arrangement by acting as the guanine-nucleotide exchange factors. The *bopE* mutant showed less invasive to HeLa cells as compared to wild type (Stevens *et al.*, 2003). Meanwhile, the BopA protein was found to play a role in mediating the avoidance from its host mechanism. The *bopA* mutant showed impaired ability in intracellular survival and increased in colocalization with LC3, an autophagy marker protein (Cullinane *et al.*, 2008). However, both of the effector mutants showed only a slight attenuation in the BALB/c mice model suggesting that these two effectors might only play a minor role in the host virulence (Stevens *et al.*, 2004). Following internalization, *B. pseudomallei* proliferate inside phagosomes and escaped from the phagosome into the cytoplasm by lysing the phagosome membrane of the infected cells (Jones *et al.*, 1996; Wiersinga *et al.*, 2006). Inside the cytoplasm, *B. pseudomallei* induced actin polymerization at one pole leading to membrane protrusion. This enables the *B. pseudomallei* to perform cell-to-cell spreading without exposure to immunoactive molecules. The BimA protein was found to be located at the cellular poles and it is involved in actin formation where the mutants showed abolished actin-based motility (Stevens *et al.*, 2005).

Apart from this, *B. pseudomallei* also secrete several virulence molecules such as haemolysin, lipases, proteases, lecthinases and siderophores. However, the role of these secreted virulence molecules in the pathogenesis of human melioidosis still remains to be elucidated (Lazar Adler *et al.*, 2009).

Quorum sensing is a cell-density mediated communication system adopted by Gram-negative bacteria in which they produce, release and detect the autoinducers or signaling molecules, *N*-acyl-homoserine lactones (AHLs). The quorum sensing networks have been shown to be involved in the expression or repression of the virulence genes and secretion of exoproducts (Fuqua *et al.*, 1994). *Burkholderia pseudomallei* was reported to produce a wide range of signaling molecules including *N*-octanoyl-homoserine lactone (C_8HSL), *N*-(3-hydroxy)-octanoyl-homoserine lactone (3-hydroxy-C_8HSL), *N*-(3-oxo)-octanoyl-homoserine lactone (3-oxo-C_8HSL), *N*-decanoyl-homoserine lactone (C_{10}HSL), *N*-(3-hydroxy)-decanoyl-homoserine lactone (3-hydroxy-C_{10}HSL), and *N*-(3-hydroxy)-dodecanoyl-homoserine lactone (3-hydroxy-C_{12}HSL). However, the composition of the signaling molecules varied from strain to strain (Chan *et al.*, 2007). The *Burkholderia pseudomallei* QSS system is encoded by five *bpsR* genes and three *bpsI* genes and together they are termed as the BpsIR quorum sensing system. The BpsIR quorum sensing system serves as a LuxIR quorum sensing homolog (Song *et al.*, 2005; Kiratisin and Sanmee, 2008). However, the induction of quorum sensing system (QSS) itself is regulated by the stationary phase and stress response sigma factor, RpoS (Wongtrakoongate *et al.*, 2012).

2.5 Allelic exchange in *Burkholderia pseudomallei*

In this postgenomic era, the increased number of sequenced genomes has also led to an increasing number of uncharacterized ORFs. Characterization of gene functions is fundamental in understanding bacterial metabolism. Gene disruption is one of the most direct ways of elucidating gene function (Winzeler *et al.*, 1999; Alberts *et al.*, 2002). Generally, mutations can be categorized as either random or

site-specific (Schweizer, 2008). Random mutation can be achieved through chemical or transposon mutagenesis whereas site-specific mutation usually constructed through allelic exchange on the bacterial chromosome. The allelic exchange is achieved through homologous recombination whereby a segment of DNA flanking the altered targeted site is delivered into the recipient cells. The DNA fragment delivered into the recipient cells can either be in a linear form or carried by a suicide vector (Jasin and Schimmel, 1984; Liu *et al.*, 2007; Barrett *et al.*, 2008). A selection marker, usually an antibiotic resistance gene and a counter-selection marker, are introduced into the vector in order to, firstly, select for recipient cells which had received the plasmids via single site recombination and, subsequently, to remove the plasmid backbone after the mutagenizing replacement recombination event occured. Several types of counter-selection markers have been developed. For example, the fusaric acid-sensitivity system which contains the counter-selectable gene, *tetAR*, contributes to tetracycline resistance as well as hypersensitivity of the host towards fusaric acid. Loss of *tetAR* gene in turn causes increased resistance towards fusaric acid. Unfortunately, this system generates polar effects on the downstream genes (Maloy and Nunn, 1981). The streptomycin-sensitivity system employs the *rpsL* gene which encodes the ribosomal protein S12, the target of streptomycin. This system is applied on the streptomycin-resistant strain, where the streptomycin-resistant clones indicate the successful resolution of co-integrants (Dean, 1981). Another commonly used counter-selection method is the sucrose-sensitivity system. In this system, the *Bacillus subtilis sacB* gene which encodes the enzyme levansucrase causes lethality in most Gram-negative bacteria in sucrose-containing medium. The colonies that grew on the sucrose-containing medium indicate

successful allelic exchange and successful removal of the vector backbone from the merodiploid (Gay *et al*., 1983).

Allelic exchange in *Burkholderia pseudomallei* is relatively challenging due to its resistance towards a wide range of antibiotics and strict regulations on the choice of selection agents for genetic manipulations. Only a few antibiotics such as kanamycin, gentamicin and zeocin are approved as the genetic manipulation markers in this bacterium. However, the wild type *Burkholderia pseudomallei* is intrinsicly resistant towards these antibiotics (Cheng and Currie, 2005). Another obstacle in genetic manipulation of *B. pseudomallei* is the limited numbers of effective counter-selectable markers for the selection of plasmid-excised clones. Since most antibiotic markers are prohibited from being applied to *Burkholderia* strains, only non-antibiotic selectable markers such as *sacB* are employed in their genetic manipulations. However, *sacB* selection has very limited success due to the presence of the *sacB* operon in *B. pseudomallei* genomes. Consequently, several attempts were made to optimize the *sacB* counter-selectable system in genetic manipulation of *B. pseudomallei*. Suicide plasmid is one of the choices widely used and it has the advantage of producing a "scarless" chromosome mutation. It also enables the accumulation of several mutations in the same choromosome (Philippe *et al*., 2004). An example of a *sacB* counter-selection suicide plasmid is pDM4, a derivative of the pNQ705 suicide vector that contained a chloramphenicol resistance gene as a selective marker (Milton *et al*., 1996). Another example of a suicide plasmid is the vector pMo130. It was designed to carry the kanamycin selection gene (*aphA* gene), *xylE* reporter gene which allows the visualization of transformants and a modified *sacB* counter-selectable gene (Hamad *et al.*, 2009).

2.6 Coenzyme A

Coenzyme A is a thermal-stable essential cofactor which plays a crucial role as the acyl group carrier and carbonyl-activating group in cell metabolism. The CoA cofactor consists of adenosine 3',5'-diphosphate linked to 4-phosphopantothenic acid (vitamin B5) and β-mercaptoethylamine (Baddiley *et al.*, 1953). The biosynthesis of CoA comprises nine steps which involved various kinds of enzymes. A study regarding the phylogenetic profiling of the CoA biosynthesis genes by using *E. coli* as the reference strain revealed the orthologous relationship of these genes within the bacteria, archaea and eukaryotes. This suggest that the CoA synthesis pathway is an ancestral biosynthesis pathway where the genes are widely conserved across domains (Genschel, 2004). The adenosine 3',5'-diphosphate is the recognition site of CoA which in turn increases the CoA binding efficiency to the target enzymes. The functional group of CoA is the thiol group which is located at the β-mercaptoethylamine moiety. The thiol group connects to the acyl group via a high energy bond which is the thioester bond. The CoA is intimately associated in a number of intermediary metabolisms such as tricarboxylic acid metabolism, fatty acid metabolism and amino acids metabolism where the acetyl-CoA is the most common CoA intermediate (Lipmann, 1953).

The acetyl-CoA is involved in several pathways such as the oxidative decarboxylation of the pyruvate; the catabolism and synthesis of amino acids; β-oxidation and synthesis of fatty acids and the Tricarboxylic acid cycle (TCA) (Lipmann, 1953). Tricarboxylic acid cycle is an amphibolic central metabolic pathway by linking the anabolic and catabolic pathways. In prokaryote, the TCA cycle occurs in the cytosol while in the eukaryote, it is occurs in mitochondria. The first step of the TCA cycle is the condensation of acetyl-CoA with the oxaloacetate

to form citrate by the condensing enzyme citrate synthase. The whole cycle will release 2 molecules of carbon dioxide and electrons are transferred to the electron acceptor (commonly NAD^+ or FAD) which will pass through several stage of electron transport chain with different redox reaction to generate energy (Campbell and Farrell, 2006).

The end product of glycolysis is pyruvate and it is subjected to the oxidative decarboxylation under aerobic conditions. The pyruvate dehydrogenase complex is responsible to the oxidative decarboxylation of pyruvate. This reaction is exergonic where pyruvate is degraded into acetyl-CoA, carbon dioxide and NADH. The acetyl-CoA will subsequently be incorporated into the TCA cycle for energy generation. The carbon can also be used for amino acid synthesis or fatty acid synthesis. The NADH is subsequently subjected to electron transport chain for energy generation (Reed and DeBusk, 1953).

Some of the bacteria are able to perform nitrogen fixation by converting nitrogen (N_2) into ammonium ion (NH_4^+). Ammonium ion is an important precursor of many organic compounds. In the synthesis of amino acids, $NH4^+$ molecule is coupled with α-ketoglutarate, which is one of the components of TCA cycle to form glutamate (Berg *et al*., 2002b). The acetyl-CoA is firstly incorporated into the TCA cycle and subsequently into the synthesis pathway of amino acids. On the other hand, in the catabolism of amino acids, the amine group (NH_2) of the amino acid must firstly be transferred or deaminated from the carbon skeleton. The transamination is important to equilibrate the amino groups among available α-keto acids (Berg *et al*., 2002c). This permits the synthesis of non-essential amino acids and also ensures the balance of various amino acids within the cells. The deamination is important in net removal of nitrogen from cell where the carbon skeleton is hydrolyzed and the

ammonium is released (Krebs, 1935). Amino acids are classified into three categories based on the product of the carbon skeleton degradation: the glucogenic amino acids, the ketogenic amino acids and the glucogenic and ketogenic amino acids. The degradation of glucogenic amino acids will produce oxaloacetate or pyruvate whilst the degradation of ketogenic amino acids will produce acetyl-CoA or acetoacetyl-CoA. The catabolism of amino acids give rise to the metabolic intermediates that are intimately connected with the TCA cycle such as oxaloacetate, pyruvate and acetyl-CoA (Berg *et al.*, 2002; Campbell and Farrell, 2006).

Fatty acid consists of a hydrocarbon chain and a carboxylate group attached at the end. It provides the cells energy source, as the structural foundation of the cell membranes and others. Fatty acids are categorized according their number of carbon: short-chain fatty acids (SCFA) which have 2-4 carbons, medium-chain fatty acids (MCFA) which have 6-12 carbons, long-chain fatty acids (LCFA) which have 14-18 carbons and very-long chain fatty acids (VLCFA) which is the derivatives of 18-carbons molecules (Agostoni and Bruzzese, 1992). Among the fatty acid chains, the long-chain fatty acids gain more attention because they are believed to influence a myriad of cellular process such as intracellular signaling and gene expression (Graber *et al.*, 1994). In fatty acid synthesis, acetyl-CoA is highlighted as the 'primer' molecule where the carbon dioxide (CO_2) is incorporated acetyl-CoA into the molecule to form malonyl-CoA. The reaction is known as carboxylation and is catalyzed by acetyl-CoA carboxylase complex. The malonyl-CoA is a key intermediate in the elongation of the fatty acid chain. The elongation of fatty acids involved the successive addition of two-carbon molecule to the growing chain which is the two-carbon from malonyl-CoA. The β-oxidation of fatty acid must firstly couple with acyl-CoA by using enzyme acyl-CoA synthetase. The acyl-CoA then

enters the β-oxidation metabolism to break down into acetyl-CoA. The acetyl-CoA is subsequently incorporated into the TCA cycle for energy generation (Heath *et al.*, 2002; DiRusso and Black, 2004).

2.7 CoA-binding proteins

There are various types of CoA-binding proteins in cell which have their own specific role in metabolism. The CoA-binding proteins show high diversity in cell where they may be present as a monomer or an oligomer. The CoA-binding pocket is commonly present between the subunit to subunit interface except the monomeric acyl-CoA binding protein and the monomeric malonyl-CoA ACP transacylase. A conserved binding mode where the adenine ring is pointed towards the binding protein and the 3'-phosphate is facing towards the solvent is commonly observed in all CoA-binding species. There are some binding modes that are non-conserved but frequently observed in certain CoA-binding species, such as: hydrogen bond as the linkage between amino group of adenine base and the main-chain protein. Besides, the salt bridge between lysine or arginine of protein to the phosphate group of CoA is also commonly observed except the chloramphenicol acetyltransferase and acyl-CoA dehydrogenase. Different CoA-binding species perform different binding mode. Some binding modes are specifically observed in certain CoA-binding species, but some of the binding modes are commonly employed by certain CoA-binding species where functionally varies (Engel and Wierenga, 1996).

Acyl-CoA binding protein (ACBP) is a 10 kDa protein which is important in trafficking and utilization of long chain fatty acyl-CoA esters. It non-covalently binds the acyl-CoA ester with high specificity and affinity. The long chain acyl-CoA ester is not just involved in the lipid metabolism, but it also plays a pivotal role in modulating cellular function (Færgeman *et al.*, 2007). Apart from this, ACBP was

also reported to as the diazepam binding inhibitor to the γ-amino butyric acid ($GABA_A$) receptor (Guidotti *et al.*, 1983; Knudsen and Nielsen, 1990). The ACBP homologues have also been identified in eukaryotic kingdom and eubacteria. Thus, this highly conservative protein was believed to be a housekeeping gene in the organism. Generally, the ACBP consists of 4 α-helix organized into a bowl-like structure with a polar rim and the inside being a non-polar region (Andersen and Poulsen, 1993; Kragelund *et al.*, 1996).

Succinyl-CoA synthetase is the key enzyme in the TCA cycle. It is responsible for the hydrolysis of succinyl-CoA to succinate and CoA (Baccanari and Cha, 1973). It consists of two different subunits, namely, the α and β-subunit. The active enzyme is assembled in the form of $\alpha_2\beta_2$ heterotetramer, or more specifically, the dimer of αβ-dimer. The α-subunit interacts with the β-subunit forming the αβ-subunit which has the active pocket of CoA binding. The β-subunit is responsible for the formation of the dimerization of the αβ-dimers (Teherani and Nishimura, 1975; Nishimura, 2009). Succinyl-CoA synthetase binds CoA in a noncompact conformation in which the pantetheine arm of CoA is extended. The 3'-phosphate-ADP of CoA is bonded to the α-subunit of the enzyme in the classical Rossmann fold (Wolodko *et al.*, 1994).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Bacterial strains, vectors and culturing conditions

The bacterial strains and vectors used in this study are listed in **Table 3.1**. *Burkholderia pseudomallei* K96243 (Holden *et al*., 2004) and *E. coli* strains were cultured in Luria Bertani broth (LB) (Appendix A) or M9 minimal medium (Appendix A) (Sambrook & Russell, 2001) at 37°C with shaking at 180 rpm unless stated otherwise. For semi-solid media, agar powder was added to the respective media to a concentration of 1.5%. For mutant construction, bacterial strains harbouring pDM4 (Milton *et al*., 1996) or pUD3393 plasmids were cultured in Blomfield medium (Appendix A) (Blomfield *et al.*, 1991) at 37°C. Antibiotics were supplemented according to the following concentrations when applicable: ampicillin (75 µg/ml), chloramphenicol (20 µg/ml or 150 µg/ml) and gentamicin (25 µg/ml) (Appendix B). When needed, the 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) and isopropyl β-D-1-thiogalactopyranoside (IPTG) were added to a final concentration of 40 µg/ml and 0.1 mM respectively (Maas, 1999; Sambrook and Russell, 2001).

3.2 Multi-genomes homology comparison

A multi-genomes homology comparison was performed in order to obtain the specifically present yet conserved genes in *Burkholderia pseudomallei*. A multi-genome homology comparison tool from Comprehensive Microbial Resource (CMR) was employed (Peterson *et al.*, 2001) (http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi; last accessed February 2013). Firstly, comparison