CONSTRUCTION AND CHARACTERIZATION OF A *Burkholderia pseudomallei wzm* DELETION MUTANT

by

Yuen Chee Wah

Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

October 2010

ACKNOWLEDGEMENTS

I would like to thank Prof Dr. Nazalan Najimudin for giving me the opportunity to complete my masters degree in his laboratory and for serving as my supervisor. Prof. Nazalan has always been available for advice and support. I have greatly enjoyed working in his lab and I have received invaluable education in the field of molecular genetics.

I would like to thank the past and present members of the lab for their help and friendship during the whole duration of my masters degree. Many thanks go to Dr Suriani, Dr. Razip, Hok Chai, Ainihayati, Kamariah Hasan, Balqis, Yifen, Eng Keat, Tee and Afiqah for their invaluable advices in molecular biology and microbiology.

My acknowledgements would not be complete without expressing my endless gratitude to my family for their support they have offered me throughout the duration of my study. Special thanks to my Dad, Mom, grandfather, grandmother and my aunt. I would like to thank my girl friend, Mei Mei, for her patience and support. Thank you once again for their tremendous support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Symbols and Abbreviations

%	percentage
^{o}C	degree Celsius
λ	lambda clone
µg	micro gram
µl	micro litre
ABC	ATP Binding Cassette
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cat	chloramphenicol resistance gene
CFU	colony forming units
CPS	capsular polysaccharide
Da	Dalton
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonuclecoside triphosphate
EDTA	ethylenediaminetetraacetatic acid
EPS	exopolysaccharide
EtBr	ethidium bromide
et al.	*et alia* (and others)

g	gram
g	gravity
GDP	glucose diphosphate
GSP	general secretory pathway
HCl	hydrochloride acid
IPTG	isopropyl β-D-1- thiogalactopyranoside
IVET	*in vivo* gene expression technologies
kb	kilo base pair
kDa	kilo Dalton
KH_2PO_4	potassium dihydrophosphate
LB	Luria Bertani
L	litre
LD	lethal dose
LOS	lipooligosaccharide
LPS	lipopolysaccharide
M	molar
$MgSO_4$	magnesium sulphate
Min	minute
mL	mililitre
mM	milimolar
M	molar
Mϕs	macrophages
Mab	monoclonal antibody
Mb	mega base

N/A	not applicable
NaCl	sodium chloride
Na_2HPO_4	sodium hydrogen phosphate
NCBI	National Center for Biotechnology Information
NH_4Cl	ammonium chloride
nt	nucleotide
ntp	nucleotide position
OD	Optical Density
OPS	O-polysaccharide
ori	origin of replication
PC	phosphatidylcholine
PCR	polymerase chain reaction
PEG	polyethylene glycol
PLC	phospholipase C
PMNs	human polymorphonuclear cells
ppm	part per million
pmol	pico mole
PVC	polyvinyl chloride
rRNA	ribosomal ribonucleic acid
rpm	revolution per minute
*sac*B	levan sucrase gene
sec	second
SEM	standard error of mean
STM	signature-tagged mutagenesis

TAE	tris acetate acid EDTA
TTSS	type III secretion system
TSS	transformation and storage solution
UV	ultra violet
w/v	weight per volume
V	volt
VC	viable count
v/v	volume per volume
X-GAL	bromo-chloro-indolyl-galactopyranoside

PEMBINAAN DAN PENCIRIAN MUTAN DELESI *wzm*

Burkholderia pseudomallei

Abstrak

Dalam *Burkholderia pseudomallei*, patogen yang menyebabkan 'melioidosis', kumpulan kluster gen yang berfungsi mengekod polisakarida kapsul tersebut terletak pada kromosom pertama. Secara keseluruhannya, terdapat sejumlah 22 gen pada kluster ini dan di kalangan gen-gen kapsul dalam gen kluster ini, gen *wzm* masih tidak dikaji secara keseluruhan. Jujukan asid amino gen *wzm* menunjukkan similariti yang tinggi terhadap CtrC (*Neisseria Meningitidis*), BexB (*Haemophilus Influenzae*) dan KpsM (*Escherichia coli*). Untuk mengkaji fungsi gen *wzm*, mutan isogenik gen *wzm* dibentuk dan dianalisiskan secara *in vitro* serta dibandingkan dengan strain jenis liar. Daripada penemuan yang didapati, mutan menghasilkan kurang biofilm berbanding dengan strain jenis liar. Selain itu, mutan lebih sensitif terhadap pengeringan dan stres oksidatif daripada strain jenis liar. Namun, tiada perbezaan yang signifikan dilihat untuk kedua-dua mutan dan strain jenis liar dalam sensitivi terhadap osmolariti dan keadaan acid. Di samping itu, mikroskopi elektron penskanan menunjukkan permukaan sel-sel bakteria mutan didapati "kasar" berbanding dengan strain jenis liar. Akan tetapi, tiada perbezaan fenotip dapat diperhatikan dengan mikroskopi elektron transmisi apabila sel-sel bakteria untuk kedua-dua mutan dan strain jenis liar dibandingkan.

CONSTRUCTION AND CHARACTERIZATION OF A *Burkholderia pseudomallei wzm* DELETION MUTANT

Abstract

In *Burkholderia pseudomallei*, a pathogen that causes melioidosis, the gene cluster that encodes for capsular polysaccharide are located in chromosome one. There are 22 genes in this cluster and among the capsular genes in the cluster, the *wzm* gene has not been studied thoroughly. The amino acid sequence of *wzm* shows high similarity to CtrC (*Neisseria Meningitidis*), BexB (*Haemophilus Influenzae*) and KpsM (*Escherichia coli*). To study the function of *wzm* gene, an isogenic mutant of *wzm* gene was generated and analyzed in comparison with the wild type strain. From the findings, the mutant produced less biofilm compared to the wild type strain. Furthermore, the mutant was more sensitive towards desiccation and oxidative stress than the wild type strain. However, no significant difference was observed for both mutant and wild type strains in their sensitivity towards high osmolarity and acid conditions. Moreover, scanning electron microscopy showed that the bacterial cells of the mutant strain have "rough" surface compared to the wild type strain. However, no phenotypic difference could be observed using transmission electron microscopy when both bacterial cells of both mutant and wild type strains were compared.

Chapter 1

1.0 Introduction

Burkholderia pseudomallei is a gram negative rod-shaped bacterium that causes melioidosis to both human and animals. Among the signs and symptoms that are caused by this disease in human are septicaemia and pneumonia (Cheng and Currie, 2005). Study on the pathogenesis of this fastidious bacterium has attracted the attention of scientists as melioidosis has been reported to be endemic in various parts of the world and *B. pseudomallei* was reported to be resistant to many antibiotics (Cheng and Currie, 2005).

To understand the molecular and cellular basis of pathogenesis of *B. pseudomallei*, many researchers had started to identify virulence determinants at both proteomic and genetic levels. Among the virulence determinants that were identified were phopholipase C (Korbsrisate *et al.,* 1999), flagella (DeShazer, 1997) and lipopolysaccharide (DeShazer *et al.,* 1998)

Capsular polysaccharide which is one the virulence determinants in *B. pseudomallei* has the potential to be utitilized as a vaccine (Sarkar-Tyson *et al.,* 2007). The genetic organization of capsular polysaccharide in *B. pseudomallei* has been identified by Reckseidler-Zenteno *et al.* (2001) and Holden *et al.* (2004). The functions of the genes that are located in the cluster were annotated by the latter. Previous studies have shown that capsular polysaccharide is involved in the virulence of *B. pseudomallei*

in Syrian hamster and mice (Reckseidler *et al.,* 2001; Atkins *et al.,* 2002; Cuccui *et al.,* 2007). Apart from these *in vivo* animal studies, the capsular polysaccharide may protect the *B. pseudomallei* from phagocytosis by reducing levels of complement C3 deposition.

It has been shown in previous studies that inactivation using signature-tagged mutagenesis of *wzm* gene had led to the decrease in survival in mice (Cuccui *et al.,* 2007). The *wzm* gene was chosen for this study among the genes in the capsular polysaccharide cluster as this gene was not well studied compared to the other capsular genes in the cluster. Hence, in this study, *B. pseudomallei* UKMS-01, a local animal isolate from sheep is used to investigate the role of *wzm* gene in harsh conditions. The approach of this research is to construct a markerless and in-frame deletion of *wzm* mutant of *B. pseudomallei* and use a few *in vitro* studies which are osmotic stress assay, desiccation survival assay and biofilm formation assay to study the phenotypic characteristics of the Δ*wzm* mutant in comparison with the wild type strain. In addition, the sensitivity of Δ*wzm* mutant towards oxidative and acidic conditions is also investigated. Moreover, the surface and the internal structures of the bacterial Δ*wzm* mutant cells will be viewed using scanning and transmission electron microscopy to determine whether there are changes in cellular surface as well as the internal structures after the *wzm* gene is disrupted.

Chapter 2

2.0 Literature review

2.1 *Burkholderia pseudomallei*

Burkholderia pseudomallei is generally classified as a gram negative, facultative anaerobic, motile and rod shaped bacteria (Wiersinga *et al.*, 2006, Holden *et al.*, 2004, Dance, 2002). It is formerly known as *Bacillus pseudomallei* or *Pseudomonas pseudomallei*, and is later re-named as *Burkholderia pseudomallei* in 1992 based on 16S rDNA sequence (Currie *et al.,* 2004). *Burkholderia pseudomallei* has been documented by many researchers as a known causative agent of melioidosis, a disease that can cause acute illness in both humans and animals (Gilad *et al.*, 2007).

B. pseudomallei is motile, due to the existence of lophotrichous flagella (DeShazer *et al.*, 1997). This bacterium has the ability to grow on various types of organic matters such as in carbohydrates, amino acids and fatty acids. At the same time, the morphology the *B. pseudomallei* colony can be positively identified although it may vary from rough to smooth wrinkled and may either show white or brown pigmentation (Stone, 2007). It can grow in any growth medium with temperatures ranging from 18^{o}C to 42^{o}C, with the optimum temperature being 37^{o}C (Tong *et al.*, 1996). Nevertheless, the *B. pseudomallei* has been shown to have a rapid growth curve at 42^{0}C in the any growth medium and this in turn can cause a depletion or shortage of nutrients within 48 hours and will eventually form a layer of sediment on the plate.

The optimal pH for the growth of bacteria was reported to be in the range of pH 5 to 8. It was also reported that rapid bacterial inactivation occurs when the pH is below 4.5 (Tong *et al.*, 1996). In addition, studies have shown that *B. pseudomallei* is able to survive when exposed to solutions with less than 2.5% of sodium chloride. However, the bacteria tend to be inactive when exposed to solutions that are more than 2.5% sodium chloride (Inglis and Sagripanti, 2006). This is opposed to the study done by Pumirat *et al.* (2009), which mentioned that *B. pseudomallei* can survive in a salty concentration of more than 2.5%. So far, no other studies were conducted on the survival rate of the particular bacteria in seawater but there were reports of melioidosis in survivors of the tsunami disaster that occurred on the 26th December 2004. Those infected were believed to have directly contacted *B. pseudomallei* through their lungs by ingesting contaminated flood water supply (Chierakul *et al.*, 2005). There were reports regarding the use of chlorine to treat drinking water to eliminate the *B. pseudomallei*. Studies conducted by Howard and Inglis (2003) showed that a concentration of 1000 ppm of chlorine can eradicate *B. pseudomallei* in drinking water. However, this may not be commercially viable as 1000 ppm of chlorine may cause more harm to human health instead. Furthermore, this bacterium can only survive for 7.75 minutes after exposure to UV light (Tong *et al.*, 1996).

The genome of *B. pseudomallei* strain K96243, a clinical isolate from Thailand had been sequenced and analysed by Wellcome Trust Sanger Institute, United Kingdom. *B. pseudomallei* has two main chromosomes, namely, chromosome 1 and chromosome 2 which are 4.1Mb and 3.2Mb in size respectively (Holden *et al.*, 2004). A large percentage of coding sequence in chromosome 1 has been associated with core functions

like cell growth and metabolism while chromosome 2 entails more extensive coding sequences that are involved in accessory functions such as survival and adaptation to the environment (Holden *et al.*, 2004). In addition, the National Center for Biotechnology Information currently has listed another 20 genome sequences and among the 20, three were fully annotated. The three strains are strains 1106a, 1710a (Thai clinical isolates) and 668 (an Australian clinical isolate) (Adler *et al.*, 2009).

The genomic DNA of *B. pseudomallei* strain K96243 has 16 genomic islands that take up to 6% of the whole genome (Holden *et al.*, 2004). Tumapa *et al.* (2008) demonstrated that there is variation in the presence of genomic islands among different *B. pseudomallei* isolates. However, the presence of a specific island does not correlate with the virulence of the isolate. The paper by Tumapa *et al.* (2008) presented only a few representative islands and strains. There is a possibility that other studies may identify striking differences in terms of genomic characteristics between clinical and environmental isolates. By performing phylogenetic studies, it was demonstrated that *B. pseudomallei* is not so closely related to *B. thailandensis* than to *B. mallei.* This suggested that the evolution of *B. pseudomallei* is more recent compared to other members of the *Burkholderia* genus (Ou *et al.*, 2005).

By comparing the genomic, transcriptional and proteomic levels of strain *B. pseudomallei* K96243 and *B. pseudomallei* 15682, there were significant intrinsic differences between both strains (Ou *et al.*, 2005). From the data obtained, it was suggested that horizontal gene transfer or gene loss events had occurred as about 43% of the gene expression differences were associated with genes that are not present in one or

the other strain. Furthermore, about 38 % of the global proteomic differences were attributed to strain-specific isoforms of proteins expressed in these two strains (Ou *et al.*, 2005). These findings correlate with the results obtained in another two studies that observed the genome-wide variability between *B. pseudomallei* K96243 and 1026b strains (DeShazer, 2004) and between *B. pseudomallei* K96243 and *B. mallei* (Fushan *et al.*, 2005). From all these findings gathered, it can be deduced that variation in phenotypes that are related to growth rate, environmental resistance and virulence may be due to the molecular variation in different *B. pseudomallei* strains (Ou *et al.*, 2005).

2.2 Habitat

B. pseudomallei is reported to thrive through saprophytic means. It inhibits stagnant waters or soils, for example, in paddy fields or in flooded areas (Chaowagul *et al.*, 1989). This explains why it was prevalent among rice farmers and helicopter pilots during the Vietnam War, as most of them were exposed to contaminated soil through ingestion and open wounds (Sanford, 1995). The bacterium was also found to be endemic in Thailand (Leelarasamee 2000), Malaysia (Puthucheary *et al.*, 1992), Singapore (Chan *et. al.*, 1985; Lim *et al.*, 1997) and northern Australia (Cheng *et al.*, 2000; Currie *et al.,* 2000). Besides that, sporadic cases were also reported in Southern Taiwan (Shih *et al.*, 2009), North, Central and South America (Inglis *et al.*, 2006), India (Saravu *et al.*, 2008), Africa, the Caribbean, and the Middle East (Dance, 2000).

Incidence of melioidosis that occurred in certain parts of the world, however, remains woefully unknown. This is especially in third world countries. This could be

due to the fact that some reported cases were poorly documented, under reported or even unreported due to limited resources and unsuitable facilities in some laboratories (Dance, 2002).

2.3 Melioidosis

One of the reasons why *B. pseudomallei* generated a world wide interest is due to the fact that it is a potential agent of melioidosis, which can indirectly cause a broader spectrum of chronic illnesses in both humans and animals alike (Gilad *et al.*, 2007). A person could be infected easily by *B. pseudomallei* through simple means of ingestion of contaminated materials, open cuts or wounds, abrasions or even through inoculation of infected needles during laboratory experiments by researcher themselves (White, 2003). Once infected with *B. pseudomallei*, it may stay dormant in the host body for years without showing any obvious physical or bodily symptoms (Cheng and Currie, 2005). Examples of reported cases include cases of American soldiers who returned from the Vietnam War, only to start showing symptoms of melioidosis after 30 years. The mortality rate of melioidosis is high and relapses are not uncommon as the disease can affect almost every organ in the host body (Sanford, 1995). Up till now, there is still no vaccine for the prevention of *B. pseudomallei* infection and an infected person is likely to suffer from either acute septicaemia (blood poisoning) or pneumonia (lung infections) (Deshazer *et al.*, 1998, Reckseidler *et al.*, 2001). Wiersinga *et al.* (2006) also reported that this septicaemic form of melioidosis involves a rapid onset and death is inevitable usually during the first 12 to 24 hours.

Therefore, the incidence of melioidosis should not be taken lightly as it could spiral out of control as it can affect anyone. Furthermore *B. pseudomallei* has been classified as being a potential bio-agent to be used in future bioterrorism attacks.

2.4 Melioidosis in animals

Melioidosis affects animals as much as human. So far, there have been reports showing that animals such as birds, dogs, goats, kangaroos, pigs, camels, horses, cats, rats and dolphins are being infected by melioidosis caused by *B. pseudomallei* (Cheng and Currie, 2005). Although there is still no incidence of melioidosis being transmitted from animals to humans, precautions have to be observed at all times. For example, face masks and gloves should be worn during handling of infected animals. Meanwhile, farmers should also be prudent and not hesitant to cull infected live stock though loss is expected as it may cause serious loss of human lives later on.

2.5 Treatment of melioidosis

Treatment of melioidosis is challenging due to the fact that *B. pseudomallei* itself is resistant to certain antibiotics such as gentamicin and streptomycin (Chaowagul *et al.*, 1989). On top of that, it has also been reported that *B. pseudomallei* could survive in soil and water for many years, and therefore, must have a certain adaptive mechanism which allows them to continue to survive in harsh and stressful environments (Pumirat *et al.*, 2009). Proteomics studies conducted by Pumirat *et al.* (2009) showed the changes in protein secretions in *B. pseudomallei* under high salt (sodium chloride) concentration

stress. It was reported that bacteria induced with a high salt environment had a 19-fold increase in a beta-lactamase-like protein which render it resistant to beta-lactam antibiotics, and hence, increases its survival rate.

Despite this, treatment of melioidosis is not impossible and the percentage of recovery increases with early detection and proper diagnosis. There were many cases of patients being successfully treated with various types of antibiotics and these include penicillins and cephalosporins (Cheng and Currie, 2005). Generally, the treatment would be conducted in two stages; the antibiotic being administered intravenously in multiple doses until the steady state of drugs in blood level is achieved. This would then be followed by giving the patients drugs orally to prevent further recurrence or relapse (Cheng and Currie, 2005).

Current studies supporting successful treatments of melioidosis include a study conducted by Simpson *et al*. (1999), whereby ceftazidime or imipenem were administered intravenously, after the onset of the disease. Nevertheless, the easiest route of administration, the oral route, is still lacking data to support its effectiveness. Despite this, there are evidence in mice studies showing that animals administered with co-trimoxazole as pre-exposure and post-exposure prophylaxis had longer survival rate, compared to the control group (without administration of antibiotics) and those administered with amoxicillin or clavulanic acid (Cheng and Currie, 2005).

2.6 Diagnosis of melioidosis

To diagnose the incidence of melioidosis, an effective method is highly sought after for the fast delivery of correct dosage/ medicament to the infected patients. One of the most popular means to diagnose melioidosis is by utilizing the Ashdown medium (Ashdown, 1979). Ashdown medium is a highly selective medium that selects for *B. pseudomallei.* To carry this out, selective specimens were normally taken from patients' blood, urine, sputum, throat or pus afflicted areas and were then cultured on 5% blood agar (usually horse blood agar) to isolate the bacteria. The medium is then observed through phenotypic means for the appearance of *B. pseudomallei* colonies. The method is simple but the drawback is that the medium is not commercially available and not rapidly obtainable for patients from non-endemic countries like the United States or in western countries (Inglis *et al.*, 2006). Besides that, the procedure is also time consuming and may not be viable for clinically chronic patients (Kaestli *et al.*, 2007; Chaowagul *et al.*, 1989). Nevertheless, this method is still being used, and remains the 'gold standard' in diagnosing melioidosis due to its relative cheap cost and high efficacy.

A unique monoclonal antibody (Mab) which is specific to the 30-kDa protein of *B. pseudomallei* has been developed by Pongsunk *et al.* (1999). The specific monoclonal antibody has been proven in both *in vitro* and *in vivo* (mice) studies to directly agglutinate with 243 clinical isolates of the *B. pseudomallei* but not with other Gram negative bacteria except *B. mallei.* A closely related method involves a study in developing a rapid multiplex immunofluorescent assay method to detect antibodies

against *B. pseudomallei*. The method will detect antibodies against the lipopolysaccharide (LPS) layer of *B. pseudomallei* should they be present in the obtained clinical samples (Iihara *et al*., 2007).

There are various publications on molecular methods for the detection of *B. pseudomallei*. These methods involved PCR analysis of primers targeting regions in 16S and 23S rRNA genes, 16S-23S intergenic region, flagellin gene *fliC* and Type III secretion gene cluster (Lew and Desmarchelier, 1994; Dharakul *et al.*, 1996; Bauernfeind *et al.*, 1998; Gal *et al.*, 2005; Merritt *et. al.*, 2006). PCR-based method is reported to be accurate and produces fewer discrepancies in diagnosing melioidosis (Merritt *et al.*, 2006). Apart from the normal PCR methods, some scientists has evaluated real-time PCR to obtain more reliable and rapid results (Meumann *et al.,* 2006; Novak *et al.,* 2006; Supaprom *et al.,* 2007).

2.7 Virulence determinants of *Burkholderia pseudomallei*

The huge magnitude of the disease caused by this pathogen may imply that this bacterium utilizes a broad range of virulence factors as means of survival in the infection of animals. It is therefore essential to investigate the roles of virulence factors to understand the pathogenesis of *B. pseudomallei.* To date, the virulence factors in this fastidious bacterium are currently understudied compared to other pathogenic bacteria. Elucidation of these virulence determinants in *B. pseudomallei* is currently the main focus of study in the scientific community. A better understanding of these virulence factors can form a rational basis in producing novel vaccines and therapies in curing

melioidosis. The completion of *B. pseudomallei* K96243 genome sequencing by Sanger Institute, genomic sequencing analysis using bioinformatics and the development of genetic tools had facilitated identification and isolation of putative virulence factors (Holden *et al.*, 2004; Reckseidler-Zenteno *et al.*, 2003). Genetic techniques that were used in identifying genes associated with virulence factors include Tn*5* transposon mutagenesis (DeShazer *et al.*, 1997; Burtnick, *et al.*, 2001), counter-selection markers that facilitate allelic exchange (Moore *et al.*, 1999; Brown *et al.*, 2004; Burtnick and Woods, 1999, Lopez *et al.*, 2009) and signature-tagged mutagenesis using animal infection models (Atkins *et al.*, 2002, Moore *et al.*, 2004). Apart from the reported genetic manipulation methods, subtractive hybridization was also introduced in search for novel virulence factors (Brown and Beacham, 2000; Reckseidler *et al.*, 2001).

In order to study the virulence factors in *B. pseudomallei*, animal models had been developed. These animal models include BALB/c mice (Leakey *et al.*, 1998; Hoppe *et al.*, 1999; Liu *et al.*, 2002; Jeddeloh *et al.*, 2003;), Syrian hamster (Brett *et al.*, 1997), diabetic rats (Woods *et al.*, 1993), guinea pigs (DeShazer *et al.*, 1998), pigs (Najdenski *et al.*, 2004) and *Caenorhabditis elegans* (O' Quinn *et al.*, 2001; Gan *et al.*, 2002).

The diabetic rat model using intraperitonal route of infection was devised following the correlation of diabetes mellitus and melioidosis (Woods *et al.*, 1993). This model had been used by many researchers to study a number of putative *B. pseudomallei* virulence factors with some success (Sexton *et al.*, 1994; DeShazer *et al.*, 1997; Jones *et al.*, 1997; DeShazer *et al.*, 1998). DeShazer *et al.* (1998) also utilized guinea pig as an

animal model by using intraperitonal route of infection to compare the lipopolysaccharide mutants with the wild type *B. pseudomallei* 1026b strain. However, there is no subsequent use of guinea pigs in any literature reported for the study of virulence determinants. Brett *et al*. (1997) had reported that Syrian hamster were highly susceptible to *B. pseudomallei* when the animal was infected via intraperitonal route with an LD_{50} <10 CFU. Subsequent studies had reported the use of Syrian hamster to study the putative virulence factors (DeShazer *et al.*, 1997; Jones *et al.*, 1997; DeShazer *et al.*, 1998; DeShazer *et al.*, 1999; Moore *et al.*, 1999; Reckseidler *et al.*, 2001; Ulrich *et al.*, 2004, Warawa and Woods, 2005).

With the employment of genetic tools and animal studies, a number of virulence determinants were identified. The identified virulence factors that are involved in the pathogenesis of *B. pseudomallei* are as listed in Table 2.1.

Table 2.1 : List of virulence factors that are identified in *B. pseudomallei* (Adapted from Adler *et al.* (2009)

Virulence factors	References
Capsule	Reckseidler-Zenteno *et al.* (2005)
Lipopolysaccharide (LPS)	DeShazer *et al.* (1998); Burtnick and Woods (1999)
Flagella	DeShazer *et al.* (1997); Chua *et al.* (2003); Chuaygud *et al.* (2008)
Type III secretion systems (Bsa)	Stevens *et al.* (2002); Boddey *et al.* (2006)
Quorum Sensing	Valade *et al.* (2004); Song *et al.* (2005); Lumjiaktase *et al.* (2006)
Pili	Essex-Lopresti *et al.* (2005); Brown *et al.* (2002); Boddey *et al.* (2006)
Efflux pumps	Chan and Chua (2005)
Siderophore	Loprasert *et al.* (2000)
Exoproducts*	DeShazer *et al.*(1999); Gauthier *et al.* (2000)
Morphotype switching	Chantratita *et al.* (2007)

*Exoproducts are secreted factors which are proteases, lipases, lecithinases and haemolysins (Ashdown and Koehler, 1990).

2.7.1 Secreted Antigens

The identified extracellular virulence factors are thermolabile toxin, protease, antigens with lipase and lecithinase activities, haemolysin and water soluble siderophore. Although these putative virulence factors were identified, they are not well characterized.

Several studies that were published between the years 1955 and 1964 indicated that intraperitoneal (i.p.) injections of filter sterilized *B. pseudomallei* supernatants were lethal to both mice and hamsters. This experiment had suggested that *B. pseudomallei* produce a lethal toxin (Nigg *et al.,* 1955). Ismail *et al.* (1987) later had partially purified a 31 kDa thermolabile toxin. This protein was shown to be able to inhibit DNA and protein synthesis, and to be lethal in mice. However, subsequent researchers were unable to reproduce this finding using filter sterilized *B. pseudomallei* culture to test on animal models (Brett and Woods, 2000).

Haase *et al.* (1997) had identified another cytolethal toxin (CLT) in *B. pseudomallei* and the isolates from goat and patients with melioidosis encephalitis produced higher toxin as compared to the isolates from soil. Furthermore, Haubler *et al.* (1998) demonstrated that a 762 Da heat-stable toxin produced cytotoxic effects on phagocytic (HL60) and non-phagocytic (HeLa) cell lines. This toxin showed haemolytic activity when incubated with erythrocytes (Haubler *et al.*, 1998). O'Quinn *et al.* (2001) and Gan *et al.* (2002) had conducted studies using *C. elegans* which suggested that toxin produced by *B. pseudomallei* failed to have deleterious effect on this model organism. These findings were supported by Balaji *et al.* (2004) whereby filter sterilized *B.*

pseudomallei supernatants did not kill the worms but live bacteria were lethal to *C. elegans*. Even though *B. pseudomallei* had been shown to produce exotoxins, there were no reports on characterization of these toxins at the molecular level.

Apart from toxins, *B. pseudomallei* was shown to secrete biologically active proteins such as hemolysin, protease, lipase and lecithinase (Ashdown and Koehler, 1990) and iron-acquiring siderophores (Yang *et al.*, 1991). Protease and lipase as secreted antigens were further demonstrated by Sexton *et al.* (1994) and Korbsrisate *et al.* (1999) respectively by purifying and characterizing these proteins. DeShazer *et al.* (1999) generated mutants with complete loss of protease, lipase and lecithinase activities using transposon mutagenesis system. From the results obtained, the type II general secretory pathway (GSP) gene cluster was identified but no structural genes of protease, lipase or lecithinase were identified (DeShazer *et al.*, 1999). There was no significant difference between the generated mutants and wild type in terms of virulence when the *B. pseudomallei* GSP mutant was tested on Syrian hamsters (DeShazer *et al.*, 1999).

There are many reports in identifying proteases in *B. pseudomallei*. Among them are the studies conducted by Sexton *et al.* (1994). The findings revealed a purified 36 kDa metalloprotease showing optimal activity at 60^{o}C and pH 8 (Sexton *et al.*, 1994). A few years later, a 47 kDa *B. pseudomallei* serine metalloprotease designated as MprA was characterized by Lee and Liu (2000). N-terminal sequence of the protease that was purified by Sexton *et al.* (1994) existed in the predicted amino acid sequence of MprA, thus suggesting that these two proteins were the same proteases (Sexton *et al.*, 1994; Lee and Liu, 2000). Valade *et al.* (2004) demonstrated that Δ*mprA* mutant did not show any reduction in survival in mice via intraperitoneal and intranasal routes of entry and this

indicated that MprA was not a virulence determinant in mice (Valade *et al.*, 2004). In 2001, a serine metalloprotease with 52 kDa in size that was characterized by Ling *et al.* (2001) was highly dependent on calcium ions and showed optimal activity at 38^{o}C and pH 9. This 52 kDa metalloprotease was able to digest immunoglobulins, transferring, albumin, myosin and actin (Ling *et al.*, 2001). Gautheir *et al.* (2000) reported that there was no correlation between protease production in *B. pseudomallei* and virulence in BALB/c mice. Based on the findings by Gautheir *et al.* (2000) and DeShazer *et al.* (1999), it is suggested that exoproducts that are protease, lipase and lecithinase play a minor role in *B. pseudomallei* pathogenesis.

Based on the bioinformatic analysis of the *B. pseudomallei* K96243 genomic sequence, three phospholipase C (PLC) enzymes were identified and designated as PLC-1, PLC-2 and PLC-3 (Holden *et al.*, 2004). The enzymes PLC-1 and PLC-2 were acidic in nature and were able to hydrolyse lipids, phosphatidylcholine (PC) and sphingomyelin (Korbsrisate *et al.*, 2007). It had been demonstrated that Δ*plc-1*, Δ*plc-2* and a double knockout Δ*plc-1 plc-2* mutants showed decrease in PC-PLC activity (Korbsrisate *et al.*, 2007). Thus, this indicated that these genes encoded functional enzymes. However, there was no complete loss of PC-PLC activity, suggesting that PLC-3 was also likely to be functional. In addition, Tuanyok *et al.* (2006) demonstrated that the virulence of Δ*plc-3* mutant had decrease in hamsters. The Δ*plc-3* gene was shown to be highly upregulated in comparison to its expression in *in vitro* grown bacteria when DNA microarray analysis was used to analyse *B. pseudomallei* isolated from hamsters (Tuanyok *et al.*, 2006). Moreover, PC-PLC which was *in vivo* expressed in *E. coli* can induce IgM antibody production in patients with melioidosis using

Western blotting (Korbsrisate *et al.*, 1999). Hence, this indicates that this lipase might be involved in intracellular survival and cell-to-cell spread of *B. pseudomallei* in the host (Korbsrisate *et al.*, 1999).

Ashdown and Koehler (1990) managed to identify two hemolysins after assessing hemolytic activities of 100 *B. pseudomallei* strains. Among the 100 strains, 4% of the strains had high hemolytic activity on sheep blood agar plates while the rest demonstrated low hemolytic activity (Ashdown and Koehler, 1990). In 2007, three ATP-binding cassette transport systems that were involved in exporting hemolysins were predicted from *B. pseudomallei* K96243 genome (Harland *et al.,* 2007). To date, the roles of hemolysin are still not clear. Hence, characterization in the molecular level is important to understand the virulence of *B. pseudomallei*.

2.7.2 Cell-walled associated antigens

Besides secreting antigens as part of its strategy to impose virulence on other organisms, the *B. pseudomallei* is also known to produce cell-walled associated antigens such as flagella, pili, LPS and EPS. The flagella has long been hypothesized by many to be important to *B. pseudomallei* as it renders the cell motile and therefore, enabling it to move around freely in search of a conducive environment to thrive and spread virulence. Thus, Vorachit *et al.* (1995) has proven the existence of flagella and shown the variable expression of pili on *B. pseudomallei* by using electron microscopy. Furthermore, DeShazer *et al.* (1997) had identified 19 unique genetic loci that aid motility by performing mutagenesis. Bioinformatic analysis of *B. pseudomallei* K96342 genome further the identification of 13 gene clusters that are responsible for the synthesis of type

I fimbriae, type IV pili and Tad-like pili (Adler *et al.*, 2009). The synthesis of a 39.1 kDa flagellum protein is controlled by *fliC* gene (DeShazer *et al.*, 1997). There are conflicting evidences on the involvement of flagella towards virulence.

Studies conducted by DeShazer *et al.* (1997) and Wikraiphat *et.al* (2009) showed that Δ*fliC B. pseudomallei* 1026b transposon mutant failed to reduce virulence in BALB/c mice as compared to the wild type when the diabetic rat or Syrian hamster was infected through the intraperitoneal route. However, Chua *et al.* (2003) showed that *B. pseudomallei* KHW Δ*fliC* mutant reduce in survival through intranasal or intraperitoneal routes in BALB/c mice but not in the *C. elegans* model of infection. The factors that contribute to the conflicting data are the usage of different animal models, different modes of infection and different types of mutants generated.

As mentioned earlier, *B. pseudomallei* has also been reported to have structure on its surface known as pili. There are eight type IV pili-associated loci in the *B. pseudomallei* K96243 genome but only one type IV A pilin gene, *pilA*, exists on the genome (Essex-Lopresti *et al.,* 2005). *B. pseudomallei* strain K96243 *pilA* mutant demonstrated reduced adhesion to epithelial cell lines and reduced virulence in *C. elegans* animal model and in BALB/c mice that were infected through intranasal route but not intraperitoneal route (Essex-Lopresti *et al.*, 2005). Studies that were conducted by Boddey *et al.* (2006) showed that two *B. pseudomallei* Δ*pilA* mutants generated in strains 08 and K96243 had variation in in *pilA* expression, microcolony formation and cell adhesion. Nevertheless, *pilA* expression in strain 08 mutant was temperature regulated and is important for microcolony formation expression of pilA in strain 08 mutant was also not essential for adhesion to cultured human cells (Boddey *et al.*, 2006).

Meanwhile, the LPS of the *B. pseudomallei* consists of 2 types of *O*-polysaccharides, which is aptly named as type I-OPS and type II O-PS (Perry *et.al*, 1995). *B. pseudomallei* mutants without the type II O-PS was shown to be susceptible to 30% human serum killing through the bactericidal activities (DeShazer *et al.,* 1998). In 2006, a further identification of type III O-PS and type IV O-PS has been reported (Sarkar-Tyson *et.al*, 2007). Mice which were challenged with type III O-PS and type IV O-PS mutants showed increased mean times to death (approximately 11.6 days) as compared to those challenged with the wild type (3 days). Moreover, immunization with all polysaccharide mutant strains also resulted in delayed time to death, compared to the control group (Sarkar-Tyson *et al.*, 2007).

Another type of cell-walled associated antigen of *B. pseudomallei* is the exopolysaccharide (EPS). Up to date, there are a number of studies which identified the presence of these structures. One of the more well known EPS, a linear tetrasaccharide repeating galactose units, was shown to exist only in *B. pseudomallei* and not in *B. thailandensis* (Kawahara *et al.*, 1998; Masoud *et al.*, 1997; Steinmetz *et al.*, 1995). Since *B. thailandensis* does not cause virulence, it is then suggested that the EPS plays a role in causing virulence.

2.8 *Burkholderia pseudomallei* capsular polysaccharide

The polysaccharide capsule is the structure that encapsulates and 'surrounds' the outer-most layer of the bacterium. It interacts with the existing environment and therefore, plays an important role in protecting the bacterium from harsh conditions as

well as to give rise to causing virulence in other living beings.

B. pseudomallei extracellular polysaccharide has been classified into 2 major categories, (a) capsular polysaccharides (CPS), the polysaccharide closely in contact with the bacteria cell and (b) slime polysaccharides which is loosely associated with the cell respectively (Whitfield, 1995). It is difficult to differentiate between both types since CPS may sometimes resemble the slime polysaccharides when they are being 'released' from the particular cell (Whitfield, 1995). Moreover, CPS also contains lipopolysaccharides (LPS) which are similar to other types of polysaccharides found on the surface of the cell (Whitfield, 1995).

2.8.1 Roles of bacterial capsule in *Burkholderia pseudomallei*

Bacterial capsule in each pathogenic bacterium has its own function(s). Although the role of capsular polysaccharide in *B. pseudomallei* is still not satisfyingly clear, the capsule basically helps the bacteria in terms of survival as well as to cause virulence.

Previous studies have shown that capsular polysaccharide plays a vital role in virulence by using *in vivo* animal models such as hamsters and mice. Virulence studies conducted by Reckseidler *et al.* (2001) and Atkins *et al.* (2002) demonstrated that Δ*wcbB* and Δ*wcbE* mutants showed reduction in survival with more than 10^5 fold when the mutants were exposed to hamsters and mouse models intraperitoneally and intraveneously. In addition to that, there were also studies that utilize signature tagged mutagenesis to identify genes from the capsule operon that were critical in causing diseases and virulence in mice (Cuccui *et al.*, 2007). Inactivation of the genes *wcbB*,

wcbC and *wcbN* resulted in reduced survival of these mutants in mice (Cuccui *et al.*, 2007). More recently, Wikraiphat *et al*. (2009) did a comparative *in vivo* and *in vitro* analysis to determine the effects of 3 different types of *B. pseudomallei* mutants (without lipopolysacccharide, without capsule and without flagelin) in causing virulence in BALB/c mice and in both human polymorphonuclear cells (PMNs) and macrophages (Mϕs). The lipopolysaccharide and capsule mutants, which were generated through molecular means, were reported to demonstrate a significant decrease in virulence in both mice models as well as in the presence of human PMNs and Mϕs, suggesting that both lipopolysaccharide and capsule are highly associated with the virulence of the bacterium (Wikraiphat *et al*., 2009).

On the other hand, there were also studies which indicated the importance of capsular polysaccharide in protecting the *B. pseudomallei* from host serum cidal activity and opsonophagocytosis by reducing levels of complement C3 deposition (Reckseidler-Zenteno *et al.*, 2005). Another similar study was conducted by Wikraiphat *et al.* (2009) in which the acapsular *B. pseudomallei* mutant was exposed to human PMNs and Mϕs. The experiment showed a decreased level in bacteria residual numbers as compared to the wild type (with capsule), suggesting that without the presence of capsule, the bacteria is more susceptible to intracellular killing by the host's defence systems. Besides that, Wikraiphat *et al*. (2009) also reported that the presence of the *B. pseudomallei* capsule provided a mediated resistance towards histatin and lactoferrin, suggesting that the capsule itself is important for the resistance towards certain antimicrobial peptides.

On top of that, *B. pseudomallei* capsular polysaccharide mutant has also been identified as a possible candidate for vaccine therapy (Sarkar-Tyson *et al.*, 2007). Mice that were vaccinated with Δ*wcbH* mutants had higher levels of survival with 70% of mice surviving at day 35 as compared to mice that were vaccinated with wild type strain which showed 40% survivors (Sarkar-Tyson *et al.*, 2007). Thus, this suggested that immunization with killed capsular polysaccharide mutant strains may confer immunity against *B. pseudomallei*.

Other suggested functions of the bacterial capsule which have yet to be determined in *B. pseudomallei* include its involvement in the prevention of desiccation, adherence for colonization and resistance to specific host immunity (Roberts, 1996).

2.8.2 Genetic organization of capsular polysaccharide gene cluster of *B. pseudomallei*

According to the *B. pseudomallei* genomic sequence, the genes that are responsible for the synthesis and export of capsular polysaccharide in *B. pseudomallei* are located between nucleotides number 3327179 and 3359841 of the Chromosome 1 of *B. pseudomallei* K96243 (Holden *et al.*, 2004). There are 22 genes altogether in this cluster (Figure 2.1). The functions of these genes were annotated by Sanger Institute as shown in table 2.2 (Holden *et al.*, 2004). The capsular polysaccharide gene cluster was shown to have high percentage of similar in identity to the capsular clusters of other bacteria like *Escherichia coli, Neisseria meningitidis* and *Haemophilus influenzae* (Reckseidler-Zenteno *et al.*, 2009) This gene cluster, however, does not have genes

B. pseudomallei K96243 Chromosome 1

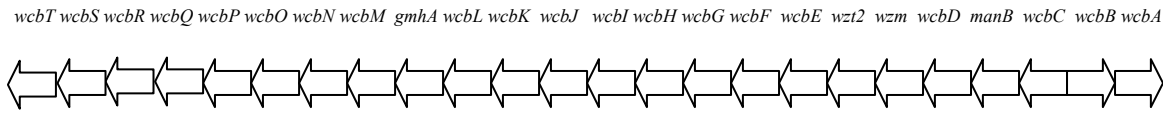

Figure 2.1: Genetic organization of *B. pseudomallei* K96243 capsular polysaccharide cluster. located at the sites 3327179 bp and 3359841 bp of the Chromosome 1 of *B. pseudomallei* K96243. This capsule cluster is approximately 30kb in size. Genes are not drawn to scale. (Adapted from Reckseidler-Zenteno *et al.*, 2001; Cuccui *et al.*, 2007)