Burkholderia pseudomallei SECRETORY VIRULENCE FACTORS: IDENTIFICATION AND ROLE IN HOST-PATHOGEN INTERACTIONS

KUMUTHA MALAR VELLASAMY

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
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KUMUTHA MALAR VELLASAMY

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DEDICATION

To “God”, the almighty
Mom and Dad
Grandparents
Husband and son
Siblings
Uncles, aunts, cousins, nephews and nieces
All my friends and every other person that is a part of my life
From the depths of my heart

Tvam-Eva Maataa Ca Pitaa Tvam-Eva
Tvam-Eva Bandhush-Ca Sakhaa Tvam-Eva
Tvam-Eva Viidyaa Dravinnam Tvam-Eva
Tvam-Eva Sarvam Mama Deva Deva

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Field of Study: **MEDICAL MICROBIOLOGY**

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ABSTRACT

Burkholderia pseudomallei, is the causative agent of melioidosis with features including latency and recrudescence. It poses a worldwide emerging infectious disease problem and a bioterrorism threat. Secreted products of *B. pseudomallei* have been identified as virulence factors. However, the pathogenesis of the disease due to these virulence factors still remains unclear. Therefore, the ability to characterize the virulence factors and understand host-pathogen interaction using proteomic, genomic and bioinformatic tools will facilitate a better understanding of the pathogenesis of *B. pseudomallei*.

In this study, the activities of selected extracellular enzymes of *B. pseudomallei* isolate CMS, were determined at different phases of growth and experimental conditions were optimized for efficient invasion and intracellular survival in human lung epithelial cell, A549. Proteomic approaches and MALDI-TOF analysis were used to map and identify stationary phase secretome proteins in order to identify proteins that may play a role in the pathogenesis of disease. *In silico* analysis were used to identify the localisation and function of the identified proteins and, western blot analysis were performed to identify the immunogenic proteins to ascertain potential diagnostic markers or putative candidate vaccines. In addition the gene regulation towards the exposure of *B. pseudomallei* and its secreted proteins on A549 cell were determined using microarray analysis.

The ability of *B. pseudomallei* to invade and survive intracellularly in A549 cells was found to correlate with the increase in the MOI and time of contact. Optimal activity of the six selected extracellular enzymes, i.e. phospholipase C, peroxidase, acid phosphatase, alkaline phosphatase, superoxide dismutase and catalase were found at varying time-points indicating expression and secretion at different phases of growth.

Secretome mapping demonstrated 113 protein spots of which 54 of the proteins including metabolic enzymes, transcription/translation regulators, potential virulence factors, chaperones, transport regulators, and hypothetical proteins were identified. In addition, 12 of the proteins were found to be immunogenic using hyperimmune mice sera raised against the *B. pseudomallei* secreted proteins.

Microarray studies revealed significant regulation of various pathways involved in metabolism, immune response and defense, cell communication and signaling and also proliferation and survival. The extracellular enzymes including phospholipase C, acid and alkaline phosphatase, catalase, peroxidase, superoxide dismutase, GAPDH and SCOT, monooxygenase and pyruvate dehydrogenase and other proteins identified in the secretome including FliC, GroEL and the type three secretion proteins BipC and BopA, may have contributed to the regulation of these pathways. Nevertheless, pathogenesis of *B. pseudomallei* infection is multifactorial and as such, whether these proteins and other factors act singly or in cascades remains to be elucidated.

In conclusion the *B. pseudomallei* (CMS) used in this study was found to secrete several virulence factors that may contribute to the ability to invade and survive intracellularly in the A549 cells. These identified proteins especially the immunogenic proteins may be used as potential diagnostic markers or putative candidate vaccines. Differential host gene expression upon exposure to *B. pseudomallei* live bacteria and secreted proteins provided preliminary insights into the pathogenesis mechanisms of *B. pseudomallei.*

ABSTRAK

Burkholderia pseudomallei, adalah agen penyebab melioidosis dengan ciri-ciri termasuk kependaman dan luapan. Ia menimbulkan masalah penyakit berjangkit di sedunia dan ancaman bioterorisme. Produk rembesan bakteria ini telah dikenalpasti sebagai faktor virulens. Namun, patogenesis penyakit disebabkan oleh faktor-faktor virulens ini masih tidak jelas. Oleh itu, keupayaan untuk mencirikan faktor virulens dan memahami interaksi hos-patogen menggunakan pendekatan proteomik, genomik dan bioinformatik dapat meningkatkan pemahaman terhadap patogenesis *B. pseudomallei*.

Dalam kajian ini, aktiviti enzim ekstraselular terpilih yang dirembeskan oleh *B. pseudomallei* isolate CMS, telah ditentukan pada fasa pertumbuhan yang berbeza dan kondisi eksperimental untuk kecekapan invasi dan keupayaan isolat ini untuk hidup intraselular di dalam sel epitelium paru-paru manusia, A549, juga telah dioptimasikan. Pendekatan proteomik dan analisis MALDI-TOF telah digunakan untuk memetakan dan mengenalpasti protein secretome fasa pegun untuk mengenalpasti protein yang mungkin terlibat dalam pathogenesis penyakit. Analisis in silico telah digunakan untuk mengenal pasti lokalisasi dan fungsi protein yang dikenalpasti dan analisis western blot telah dilakukan untuk mengenal pasti protein imunogenik dan menentukan potensinya sebagai penanda diagnostik atau calon putatif vaksin. Tambahan pula, regulasi gen sel A549, terhadap pendedahan kepada *B. pseudomallei* hidup dan protein yang dirembeskan olehnya telah ditentukan menggunakan analisis aturan mikro.

Keupayaan invasi dan hidup intraselular *B. pseudomallei* di dalam sel A549 didapati menunjukkan korelasi dengan peningkatan MOI dan masa pendedahan. Aktiviti optimum enam enzim ekstraselular terpilih, i.e. phospholipase C, peroxidase,

phosphatase asid, phosphatase alkali, superoxide dismutase dan catalase didapati pada masa berlainan menunjukkan ekspresi dan rembesan pada fasa pertumbuhan yang berbeza. Peta secretome menunjukkan 113 tompok protein di mana 54 protein termasuk enzim metabolik, pengawal selia transkripsi/terjemahan, faktor dengan potensi virulens, chaperones, pengawal selia pengangkutan, dan protein hipotetikal, telah dikenal pasti. Tambahan pula, 12 daripada protein tersebut didapati immunogenic menggunakan sera imun tikus yang dibangkitkan terhadap protein yang dirembeskan oleh *B. pseudomallei*.

Kajian mikro atur mendedahkan regulasi signifikan pelbagai laluan yang terlibat dalam metabolisme, tindak balas imun dan pertahanan, komunikasi sel dan isyarat dan juga pertumbuhan dan survival. Enzim ekstraselular termasuk phospholipase C, phosphatase asid and alkali, catalase, peroxidase, superoxide dismutase, GAPDH and SCOT, monooxygenase and pyruvate dehydrogenase dan protein lain yang dikenalpasti di dalam secretome termasuk FliC, GroEL dan protein rembesan jenis ketiga BipC and BopA, mungkin menyumbang kepada regulasi laluan-laluan tersebut. Namun demikian, pathogenesis infeksi *B. pseudomallei* adalah multifactorial, oleh itu, samada protein-protein ini dan faktor lain mungkin bertindak secara sendirian atau di dalam rangkaian masih tidak jelas.

Sebagai kesimpulan, *B. pseudomallei* (CMS) telah didapati merembeskan beberapa faktor virulens yang mungkin menyumbang kepada keupayaan untuk menyerang dan hidup intraselular di dalam sel A549. Protein-protein yang dikenalpasti terutamanya protein imunogenik mungkin berpotensi untuk digunakan sebagai penanda diagnostik atau calon vaksin putatif. Ekspresi gen berbeza oleh hos apabila didedahkan kepada *B. pseudomallei* hidup dan protein yang dirembeskannya memberikan pemahaman awal mengenai mekanisme patogenesis *B. pseudomallei*.

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2D-GE	two-dimensional gel electrophoresis
ºC	degree Celcius
g	gravity
h	hour
mins	minute
ml	milliliter
M	molar
mM	milimolar
MWr	relative molecular weight
nm	nanometer
rpm	rotation per minute
µg	microgram
µl	microliter
µM	micromolar
U	unit
V	volt
vs.	versus
BCMS	exposure to live *B. pseudomallei*
BLAST	basis local alignment search tool
BSA	bovine serum albumin
CCMS	Exposure to *B. pseudomallei* secreted proteins
cDNA	complementary deoxyribonucleic acid
cfu	colony forming units
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
FCS	fetal calf serum
ICD	isocitrite dehydrogenase
IEF	isoelectric focusing
IL	interleukin
IPG	immobiline pH gradient
LB	Luria-Bertani

LPS	lipopolysaccharide
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight
MIC	minimal inhibition concentration
MOI	multiplicity of infection
NA	nutrient agar
NCBI	National Centre for Biotechnology Information
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
SDS	sodium-dodecyl sulphate
TCA	trichloroacetic acid
TTSS	type III secretion system
T6SS	type VI secretion system
UMMC	University Malaya Medical Centre

CHAPTER 1

INTRODUCTION

1.1 The genus *Burkholderia*

Members of the genus *Burkholderia* was originally placed under the genus *Pseudomonas*. Based on the DNA-DNA and rRNA-DNA hybridization, the genus *Pseudomonas* was later split into five groups (I to V) (Palleroni *et al*., 1973; Palleroni *et al*., 1972). The genus *Burkholderia* was first defined by Yabuuchi *et al.*, (1992) to accommodate most of the former rRNA group II pseudomonads. Initially, it contained seven species including *B. caryophylli*, *B. cepacia*, *B. gladioli*, *B. mallei*, *B. pseudomallei*, *B. solanacearum* and *B. pickettii*. Both *B. solanacearum* and *B. pickettii* where subsequently transferred to the genus *Ralstonia* (Yabuuchi *et al*., 1995).

To date, there are 60 validly named *Burkholderia* spp. (http://www.bacterio.cict.fr/) and also several candidate species (LiPuma *et al*., 2011). Members of the genus range from zoonotic and plant pathogens as well as symbionts of fungi, insects and plants that inhabit remarkably diverse ecological niches including soil, water, plants, insects, industrial settings, hospital environments and humans (Coenye and Vandamme, 2003). Most bacteria of the genus *Burkholderia* are also noted for their metabolic plasticity in the ability to utilise a wide range of organic compounds as carbon sources. They also seem to be among the most versatile bacteria that occupy a surprisingly wide array of ecological niches (Coenye and Vandamme, 2003). Since the genus was first defined, the attention given to *Burkholderia* spp. has increased tremendously, leading to the description of many novel species. Properties of the members of *Burkholderia* spp. have been widely exploited in terms of biocontrol, bioremediation and plant growth promotion purposes. However, their role and mechanism used in human infections is still not fully understood.

According to Coenye and Vandamme (2003), two groups, in terms of pathology, taxonomy, and global distribution are the cause of most of the *Burkholderia* infections in humans. The first group consist of *B. pseudomallei and B. mallei*, the causative agents of melioidosis and glanders, respectively (Wiersinga *et al*., 2006) and the second group include the nine species originally defined as the *B. cepacia* complex (Bcc) (Mahenthiralingam *et al*., 2005).

1.2 *Burkholderia pseudomallei*

Burkholderia pseudomallei has been known by many different names over the past 100 years, and was generally well known as *Pseudomonas pseudomallei* until Yabuuchi and colleagues incorporated it into the new genus, *Burkholderia* in 1992 (Yabuuchi *et al.*, 1992). It was first described by Whitmore and Krishnaswami in the year 1912, in Rangoon, Burma (Whitmore and Krishnaswami, 1912).

The bacterium is a small (0.5 – 2µm), motile, non-spore forming, aerobic Gram-negative bacillus that exhibits bipolar staining that gives it the safety pin appearance (Figure 1.1) (Pandey *et al*., 2010; Howe *et al*., 1971). It is also oxidase positive and does not have the ability to assimilate arabinose (ara^-) as compared to its non-virulent counterpart, *B. thailandensis*, which has the ability to assimilate arabinose (ara^+) (Smith *et al*., 1997). It can be cultured on many common laboratory media with colony morphologies ranging from smooth in appearance to dry and wrinkled (Aldhous, 2005) and cream to orange in colour (Chantratita *et al.*, 2007). On the selective agar containing crystal violet, glycerol, and gentamycin, the Ashdown's agar, *B. pseudomallei* forms a characteristic wrinkled morphology (Ashdown, 1979).

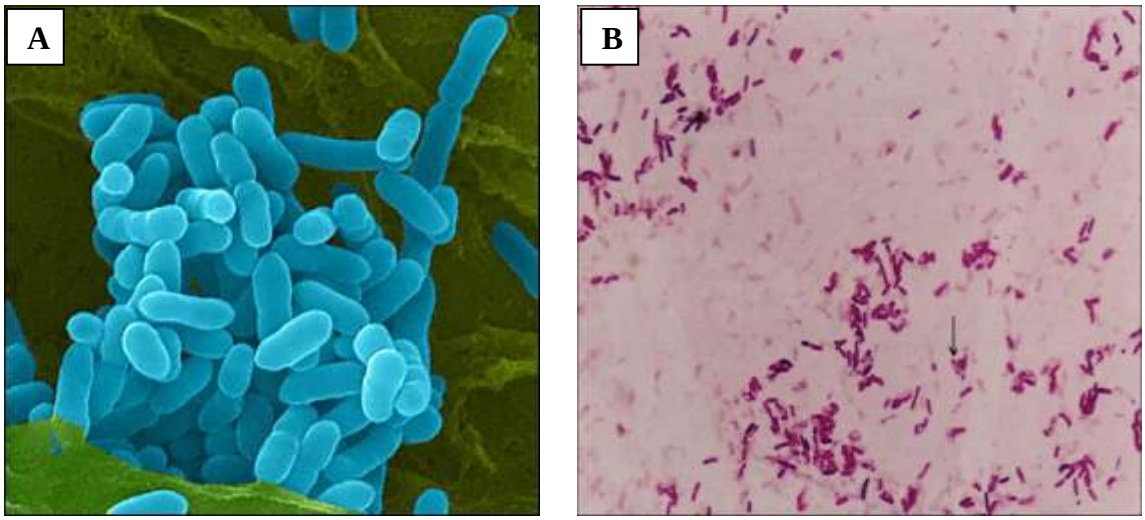


Figure 1.1: *B. pseudomallei*. A) Image from Wellcome Trust Sanger Institute press release (Dennis Kunkel Microscopy, Inc.); B) Gram stain showing safety pin appearance of bipolar stained *B. pseudomallei* (Pandey *et al*., 2010).

B. pseudomallei is a soil saprophyte and can be readily recovered from water and wet soils in endemic areas. Epidemiological surveys have confirmed that *B. pseudomallei* is mostly endemic to regions which usually border 20° north and south of the equator. However, the incidences of disease have been reported to be particularly high in South-East Asian and northern Australian regions (Dance, 1991; Leelarasamee and Bovornkitti, 1989; Chaowagul *et al*., 1989).

The genome of *B. pseudomallei* is relatively large with a size of 7.24 Mb and it consist of two chromosomes with sizes of 4.07 Mb and 3.17 Mb, respectively (Figure 1.2). Functional separation based on gene annotation indicates that the smaller chromosome carries genes associated with virulence and survival while the larger chromosome carries genes necessary for cell metabolism and growth (Holden *et al*., 2004). The G+C content of the genome is also high at approximately 68% (White, 2003).

B. pseudomallei is known to be a facultative bacterial intracellular pathogen. It can survive and multiply in human phagocytic and non-phagocytic cells *in vitro* and also persist in a dormant stage in macrophages for months or years (Jones *et al*., 1996; Pruksachartvuthi *et al*., 1990). It is among the most nutritionally versatile members of the genus *Burkholderia.* It has the ability to grow on a wide range of organic compounds including, carbohydrates, amino acids and fatty acids (Nigg *et al.*, 1955) and also survive in hostile conditions such as low pH, temperature extremes, osmotic stress and UV light (Inglis and Sagripanti, 2006; Inglis *et al*., 2000). *B. pseudomallei* has been listed as a category B critical agent by the US Centre for Disease Control and Prevention because it was considered to have potential in germ warfare and regarded as a potential bioterrorist weapon (White, 2003; Rotz *et al*., 2002).

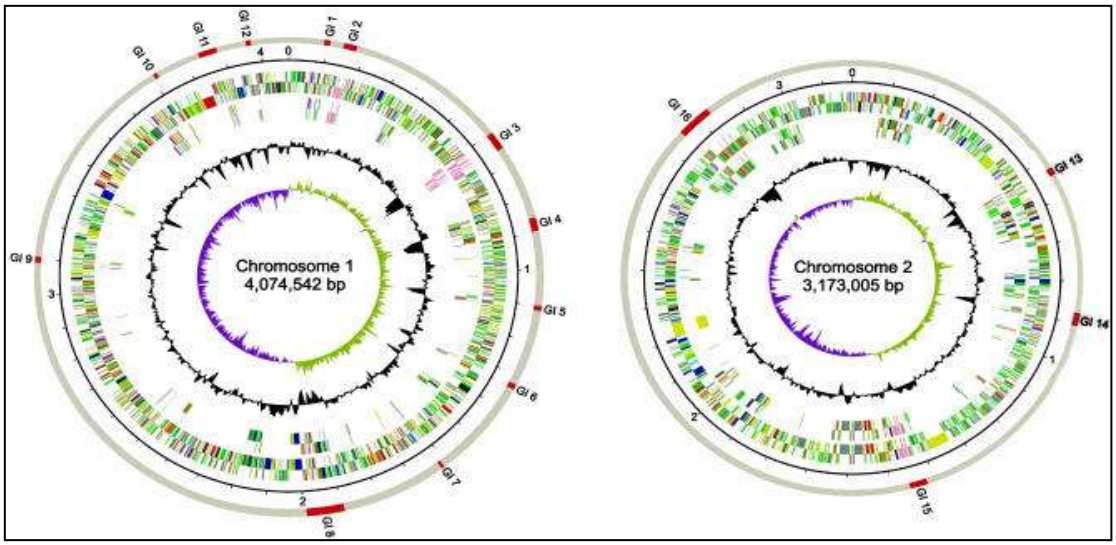


Figure 1.2: Schematic diagrams of *B. pseudomallei* genome. The genome consists of one large (4.07 Mb) and one small (3.17 Mb) chromosome (Holden *et al*., 2004).

1.3 Melioidosis

Melioidosis is caused by *B. pseudomallei*. The disease was originally called the Whitmore disease because it was first described by Whitmore and Krishnaswami in 1912 in Rangoon, Burma from fatal cases of pneumonia amongst the destitute and morphine addicts. In the year 1921, Stanton and Fletcher coined the term melioidosis from the Greek words "*melis*" meaning "a distemper of asses" and "*eidos*", resemblance. This is because melioidosis is a disease known to be glanders-like whereby it clinically and pathophysiologically resembles glanders, a chronic and debilitating disease of equines caused by *B. pseudomallei* sister strain, *B. mallei* (Stanton and Fletcher, 1932).

Melioidosis is more frequently observed in the tropical regions especially in South East Asia and Northern Australia (Figure 1.3). However, endemic distribution of this disease is expanding well beyond this known traditional endemic regions whereby cases of melioidosis is being reported from many other countries including, Madagascar, America, India, Mauritius, south Asia, China and Taiwan (Currie *et al*., 2008; Dance, 2000). Sporadic cases of melioidosis have also been reported from Philippines, Mexico, Papua New Guinea, Africa and South America. In general, melioidosis is rare in the western hemisphere, however, a limited number of cases have also been reported in North America. There is a high risk of the disease developing into an epidemic with high-rates of fatality in the non-endemic areas (Cheng and Currie, 2005). Melioidosis is also considered as an infection that can be acquired occupationally and recreationally (Currie *et al*., 2010). Most of the people affected with melioidosis are adults with one or more underlying predisposing conditions. Diabetes mellitus and renal disease are among the major risk factors of melioidosis (Galyov *et al*., 2010; Chaowagul *et al*., 1989).

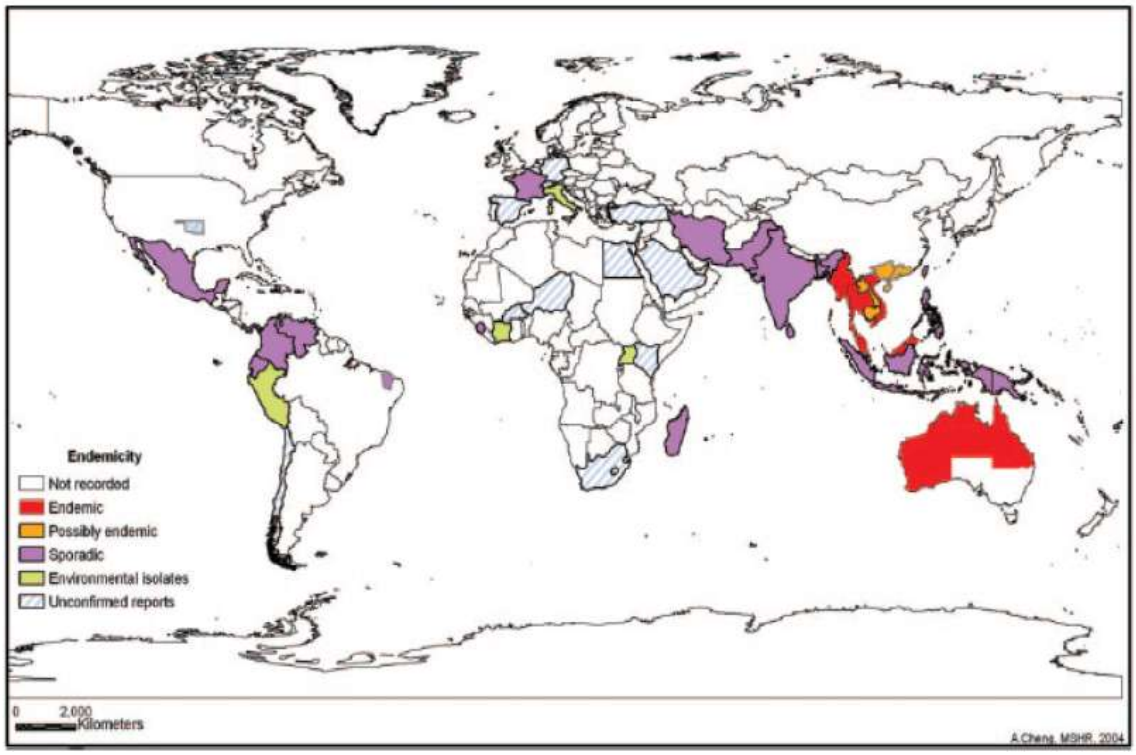


Figure 1.3: Melioidosis endemicity. World map showing the endemicity of melioidosis infection (Cheng and Currie, 2005).

1.3.1 Modes of acquisition

B. pseudomallei infection can occur through three different modes of acquisition, including, inhalation of contaminated soils, ingestion of the organisms or inoculation through damaged surface tissues. Zoonotic and person-to-person transmission or nosocomial and laboratory-acquired infection are rare (Currie *et al*., 2010). Initially, inhalation was considered as the primary route for acquiring *B. pseudomallei* due to the finding of increased melioidosis incidence in the helicopter crews within endemic regions, possibly attributed to aerosolisation of the bacteria from the soil (Howe *et al*., 1971). Inhalation of aerosolised bacteria is also possible during severe weather such as tropical storms (Cheng and Currie, 2005). However, inoculation via skin abrasions is regarded as the major mode of acquisition in endemic area mainly due to the high incidence of melioidosis among the paddy field workers and also reports on injury prior

to symptom onset in patients (Cheng and Currie, 2005; Currie *et al*., 2000). Aspiration, in near drowning, as well as ingestion especially in grazing animals and mastitis-associated infected breast milk have also been documented (Cheng and Currie, 2005; Ralph *et al*., 2004).

1.3.2 Clinical manifestation

Melioidosis can present itself with a wide variations of clinical manifestation ranging from chronic, sub-acute, or acute form of the disease, including acute septicaemia and acute pulmonary infection (Cheng and Currie, 2005; Currie, 2003; White, 2003). Chronic melioidosis is among the most common presentation and it is basically a carrier state and generally lacks clear symptoms. This form of melioidosis may also mimic tuberculosis, and as such, it is clinically challenging to differentiate between these two diseases (Puthucheary *et al*., 1992). Acute forms of melioidosis, if left untreated, can lead to death. This acute form of melioidosis is normally characterized by symptoms that occur quite rapidly including, high fever and pulmonary distress followed by visceral abscesses or septicemia. Sub-acute melioidosis are characterised by continuous febrile illness. The possibility of abscesses occurring on various organs and misdiagnosis is quite high. Melioidosis can also present as pneumonia with septicemia or without septicemia or a localized infection involving the skin and soft tissue organs (Figure 1.4) (Wiersinga *et al*., 2006). Latent infections are also established in some cases, whereby it may later reactivate to cause disease, usually in association with an intercurrent illness, typically pulmonary disease, surgery or trauma. Late-onset diabetes, renal failure and immunosuppressant drugs may also be among the contributing factor to reactivation of the infection (Short, 2002). The factors that influence disease manifestation and presentation are still unknown. However,

differences in the virulence of infecting strains coupled with the immunocompetence of the host might be a contributing factor to the outcome of infection (Sarkar-Tyson *et al*., 2007).

Additionally, 85% of individuals with melioidosis present with acute symptoms and have been symptomatic for less than two months when they enter care. Meanwhile, 11% of clinical cases are accounted by chronic melioidosis infections and the remaining 4% are caused by reactivation of the disease (Currie *et al*., 2010). In the tropical region where melioidosis is endemic, it is reported to cause up to 20% of all community-acquired sepsis and approximately 40% of the sepsis-related mortality in northern Thailand (White, 2003). The overall mortality for primary disease can be very high *i.e.*, up to 50% and ~20% in northeast Thailand and Northern Australia, respectively (Wiersinga *et al*., 2006; Cheng and Currie, 2005; White, 2003).

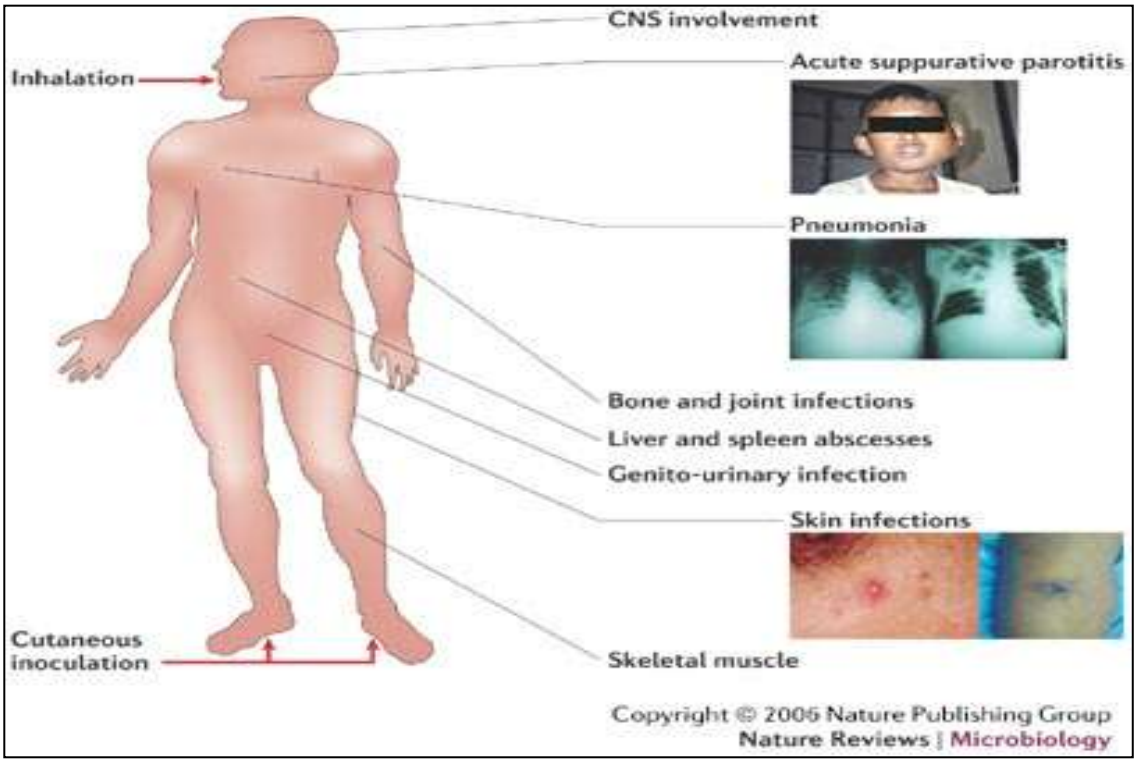


Figure 1.4: Clinical manifestation of melioidosis (Wiersinga *et al*., 2006).

1.3.3 Treatment of melioidosis

Treatment of melioidosis is very difficult due to the intrinsic resistance of *B. pseudomallei* to many different antimicrobial agents including first and second generations of cephalosporins, penicillins, colistin, macrolides, rifamycins, and aminoglycosides (Jenny *et al*., 2001; Dance *et al*., 1989). Current treatment for melioidosis includes ceftazidime, as well as the carbapenems such as imipenem and meropenem, and to a lesser degree amoxicillin-clavulanate. At present, standard treatment requires 2-4 weeks of parenteral therapy *e.g*., with ceftazidime. This is followed by 3-6 months of oral eradication therapy with other antibiotics including trimethoprim/sulfamethoxazole, doxycycline, chloramphenicol or a combination therapy. Despite being the antibiotic that is the most effective for treatment, mortality rate in ceftazidime treated patients is reported to be more than 40% (White, 2003).

1.3.4 Melioidosis in Malaysia

In Malaysia, melioidosis was first recognised in laboratory animals at the Institute for Medical Research, Kuala Lumpur in the year 1913 by Fletcher followed by the first description of infection in a human patient by Stanton in 1917 (Stanton and Fletcher, 1932). During the Second World War, melioidosis occurred in the Allied and Japanese soldiers in Burma, Malaysia and in Thailand. Following the war, sporadic cases of the disease were reported including 10 cases from Malaysia (Thin *et al*., 1970). Since then, meliodosis has been recorded from different states in Malaysia including, Johor Bahru (Pagalavan, 2005), Pahang (How *et al*., 2005) and Kuala Lumpur (Puthucheary *et al*., 1992). Subsequently, many other cases of melioidosis have been reported in humans

and animals including, buffalo, orang utan, sheep, deer, monkey, parrot, zebra, gibbon, hamster, and crocodile (Puthucheary, 2009).

More recently, in 2010, an outbreak of melioidosis and leptospirosis co-infection was reported, following a search and rescue operation of a drowned victim in Lubuk Yu, a natural recreational forest with waterfall and stream in Pahang, Malaysia. A total of 153 people, including 85 professional rescuers and 68 villagers, were exposed to this outbreak with ten cases of confirmed melioidosis and eight deaths were reported (Sapian *et al.*, 2012).

1.4 Pathogenesis and virulence factors of *B. pseudomallei*

The ability of *B. pseudomallei* to enter, survive, and replicate within mammalian host cells is among the key factor in its pathogenesis (Allwood *et al*., 2011). Many pathogens employ common virulence strategies for survival and infectivity in the host. Discovery and identification of these bacterial virulence factors are vital in understanding the pathogenesis of disease. However, to date, the pathogenesis of disease due to *B. pseudomallei* has not been well defined. Over the years, several virulence factors have been identified, however, they are poorly characterised. Additionally, *in vivo* and *in vitro* studies using melioidosis animal and cell culture models have aided in revealing a variety of bacterial factors that may contribute to survival, pathogenicity, and long-term persistence of *B. pseudomallei* within the host. Among the virulence factors that have been identified includes, the extracellular virulence factors such as endotoxin, exotoxin, protease, lipase, and lecithinase, peroxidase, superoxide dismutase, cytotoxic exolipid, lipase, hemolysin (Ashdown and Koehler, 1990; Sexton *et al*., 1994) as well as the cell-associated virulence

determinants such as lipopolysaccharide (LPS), pili, extracellular polysaccharide and flagella (Smith *et al*., 1987). The organism is also known to produce water-soluble siderophore for iron acquisition from the host, which contributes to its survival and maintenance (Yang *et al*., 1991).

Types II, III and VI protein secretion systems are also known to be virulence determinants (Johnson *et al*., 2007; Holden *et al*., 2004, Chua *et al*., 2003). Apart from that, *B. pseudomallei* is also known to be a facultative bacterial intracellular pathogen. It can invade both phagocytic and nonphagocytic cells followed by intracellular multiplication and intercellular spread (Pruksachartvuthi *et al*., 1990; Razak and Ismail, 1982). Additionally, *B. pseudomallei* are known to be resistant to the bactericidal action of normal human serum, which is a property that provides the bacterium with significant survival advantage since it may be directly introduced into the blood via cuts or skin abrasions (Ismail *et al*., 1988).

1.4.1 Membrane and surface proteins

It has been indicated in the recent studies that bacterial attachment to mucosal surfaces forms the primary event in the pathogenesis of most infectious diseases caused by bacteria (Beachey, 1981). In *B. pseudomallei*, adhesion to the host tissue may be mediated by individual proteins and carbohydrate molecules or sophisticated organelles such as pilus and non-pilus adhesins (Vellasamy *et al*., 2012). The presence of flagella and the variable expression of pili that aid in the attachment to host cells have been demonstrated (Vorachit *et al*., 1995).

Type IV pilus are reported to be important in the pathogenesis of *B. pseudomallei* (Essex-Lopresti *et al.*, 2005). Thirteen gene clusters involved in the synthesis of type I

fimbriae, type IV pili and Tad-like pili, have been predicted to be in the genome of *B. pseudomallei* K96243 (Holden *et al*., 2004). A *B. pseudomallei* strain K96243 pilA mutant displayed reduced adhesion to epithelial cell lines. However, Boddey *et al*. (2006) reported that the expression of pilA was temperature regulated and essential for microcolony formation, and not required for adhesion to cultured human cells. Thus, the type IV pilus may be involved in the natural competency of *B. pseudomallei* (Thongdee *et al*., 2008), but no direct role has been demonstrated.

Flagella proteins are also recognized to play a role in virulence as they allow motility of the bacterium to the cells at the target site of infection (Chua *et al*., 2003, Milton *et al*., 1996). In *B. pseudomallei*, a polar tuft of two to four flagella confers temperature independent motility. Synthesis of the flagella requires *fliC* gene, which encodes a 39.1-kDa flagellum protein (DeShazer *et al*., 1997). There have been conflicting reports on the involvement of flagella in the virulence of *B. pseudomallei*. DeShazer *et al.,* (1997) did not observe any attenuation of the *B. pseudomallei fliC* mutant in the diabetic rat or Syrian hamster melioidosis models. In contrast, Chua *et al.* (2003) reported the attenuation of a *fliC* mutant in BALB/c mice infected by either the intranasal or intraperitoneal routes. Similarly, a *fliC* mutant was also found to be unable to adhere to the cells of *A. astronyxis*, compared to the wild type Inglis *et al.* (2003).

1.4.2 Extracellular proteins

Bacterial secreted proteins are vital in the pathogenesis of diseases. These secreted proteins are known to be involved in a range of biological functions, from host cell toxicity to alterations of the host cell. Bacteria free culture supernatant of *B.*

pseudomallei was found to have cytotoxic effects on a range of eukaryotic cell (Balaji *et al*., 2004; Haase *et al*., 1997; Ismail *et al*., 1987). Some of the biologically active molecules that are secreted into the culture supernatant include proteases, lipases, lecithinases, haemolysins and siderophores (Ashdown and Koehler, 1990).

Bacterial proteases have the ability to catalyse the cleavage of host peptide bonds and as such, they normally act as a toxic factor to their host. Additionally, the pathogens also use proteases for growth and infection of the host cells (Miyoshi and Shinoda, 2000). Based on their catalytic mechanism, bacterial proteases can be categorized into five major classes *i.e.*, serine, threonine, cysteine, aspartate and metallo proteases (Lucy and Gros, 2002). Sexton *et al*. (1994) purified a 36-kDa metalloenzyme, protease from the culture supernatants of *B. pseudomallei* and reported that it was essential for the full virulence of the pathogen in a rat model of lung infection. However, when the mice were injected with *B. pseudomallei via* the intraperitoneal route, no correlation was observed between the virulence and the level of protease activity (Gauthier *et al.*, 2000) suggesting the role of protease in pathogenesis could be dependent on the route of infection (Valade *et al*., 2004). Serine MprA protease, initially produced in the *B. pseudomallei* cytosol and subsequently processed to produce the secreted mature active protease, has been associated with virulence and is reported to cause extensive damage to the mammalian physiological proteins that circumvent the damaging effects of bacterial secreted proteases (Chin *et al*., 2007; Lee and Liu, 2000). The pathogenic role of *B. pseudomallei* serine proteases in melioidosis has also been further confirmed using the *Caenorhabditis elegans* worm model (Lee *et al*., 2011). In a more recent study, MprA protease has been indicated as a promising prophylactic candidate against *B. pseudomallei* although the protection provided by this single protein was incomplete (Chin *et al*., 2012).

Phospholipase C is among the other important extracellular protein implicated in the virulence of *B. pseudomallei* with roles in nutrient acquisition and macrophage infection. *B. pseudomallei* is known to produce three different phospholipase C (PLC) enzymes (Plc-1, Plc-2 and Plc-3), whereby Plc-3 is required for full virulence of *B. pseudomallei* in a hamster melioidosis model (Tuanyok *et al.,* 2006). Plc-1 and Plc-2 are predicted to be acidic with the ability to hydrolyse lipids, phosphatidylcholine and sphingomyelin. A *plc-1 plc-2* double mutant was found to demonstrate reduced plaque formation in HeLa cells and decreased cytotoxicity in RAW 264.7 macrophage cells. Additionally, following starvation, the replication of the mutant was also reduced, suggesting a role for PLC in nutrient acquisition (Korbsrisate *et al.*, 2007).

Hemolysins may be involved in the intracellular survival and cell-to-cell spread of *B. pseudomallei* within the host. *B. pseudomallei* are known to produce two hemolysins *i.e.*, i) a heat-stable, weakly cytolytic hemolysin commonly found in majority of the strains, and ii) a heat-labile hemolysin which occurs infrequently and shows cytolytic activity that produces clear zones of a-hemolysis on sheep blood agar (Ashdown & Koehler, 1990). The heat-labile hemolysin was putatively identified as a 762-Da rhamnolipid, which demonstrated cytotoxic and haemolytic activity (Haussler *et al.*, 1998). More recently, Harland *et al.* (2007) have also demonstrated that the genome of *B. pseudomallei* K96243 encodes three ATP-binding cassette transport systems, which are predicted to be involved in the export of haemolysins. Nevertheless, the specific roles of hemolysins in the pathogenesis of *B. pseudomallei* infection have not been clearly established.

1.4.3 Cell wall and outer membrane components

Generally, in Gram-negative bacteria, LPS, an amphiphilic molecule, is believed to provide protection against complement-mediated lysis (Raetz and Whitfield, 2002). LPS also play two different roles in the pathogenesis *i.e.*, contribute to the antimicrobial resistance of pathogen and promote strong pro-inflammatory response. Bosshart and Heinzelmann (2007) have also reported that the LPS are among the most common bacterial components associated with sepsis initiation. Although the role of LPS in sepsis have been implicated, due to a delay in nitric oxide and tumor necrosis factor alpha (TNF-α) production, the LPS of *B. pseudomallei* have low-level macrophage-activating activity *in vitro* (Utaisincharoen *et al*., 2001; Utaisincharoen *et al*., 2000; Matsuura *et al*., 1996). This provides an advantage to the pathogen to evade macrophage killing. According to DeShazer *et al*., (1998), a lipopolysaccharide-deficient mutant was found to be attenuated in *in vivo* models of hamsters, guinea pigs and diabetic rats. This mutant was susceptible to complement-mediated killing by the alternative pathway. Additionally, mice immunized with *B. pseudomallei* LPS or capsular polysaccharide was found to provide protection against a subsequent challenge indicating their potential as vaccine candidates (Nelson *et al.*, 2004).

LPS is composed of an outer O-antigen-specific polysaccharide and an inner core oligosaccharide that is covalently linked to a lipophilic moiety termed lipid A, which is responsible for the endotoxic activity associated with LPS (Miller *et al*., 2005; Rietschel *et al*., 1994). In several aspects, the LPS of *B. pseudomallei* differ from other Gram-negative bacteria (Matsuura *et al*., 1996). The Lipid A of *B. pseudomallei* LPS is reported to consist of a biphosphorylated disaccharide backbone, which is modified with 4-amino-4-deoxy-arabinose at both phosphates and penta-acylated with fatty acids (FA) $C_{14:0}$(3-OH), $C_{16:0}$(3-OH), and either $C_{14:0}$ or $C_{14:0}$(2-

OH). This structural difference of lipid A, unique to *B. pseudomallei*, may contribute to the induction of weak immunological activities and thus evade early host defenses (Novem *et al*., 2009).

1.4.4 Secretion systems

In general, pathogens use macromolecular protein complexes known as secretion systems to export toxins and proteins across the inner and outer membranes. There are six different secretion systems described in the Gram-negative bacteria including, *B. pseudomallei*, known as the Type I - Type VI secretion systems (Figure 1.5). The type III secretion system (TTSS), which inject effectors into target host cells, are known to be major virulence determinants that play essential roles in their intracellular lifestyle of *B. pseudomallei*. Similarly, the Type VI secretion system (T6SS) is a newly-described secretion system. Despite implications that this secretion system plays key roles in pathogenesis, it is still poorly understood. In both the TTSS and T6SS, the proteins are not synthesised with a signal peptide but carry a secretion signal that varies from one system to the other (Bleves *et al*., 2010).

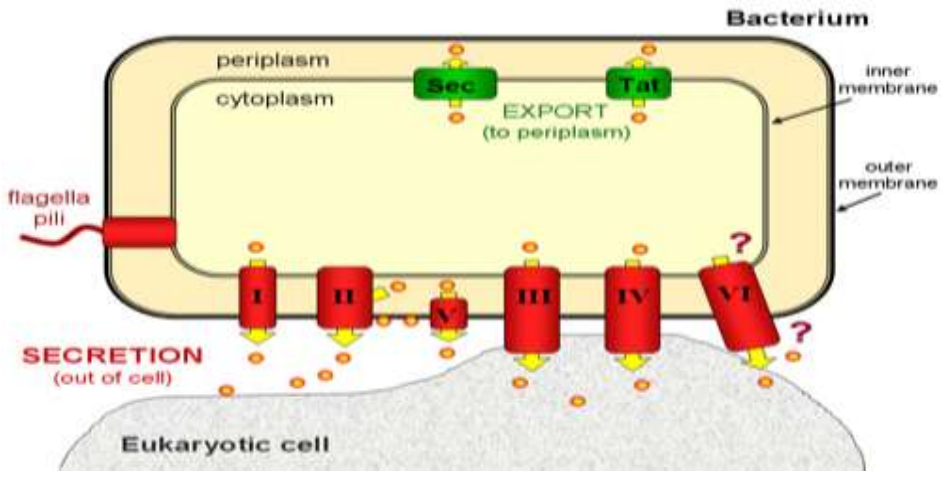


Figure 1.5: Schematic representation of protein secretion system in Gram-negative bacteria (http://www.lifesci.dundee.ac.uk/groups/sarah_coulthurst/research-areas).

The TTSS, when triggered by a close contact with host cells, mediate the transport and secretion of bacterial effectors directly into host cells using a molecular syringe, and thus, alter the physiological functions of the infected host cells (Finlay and Cossart, 1997). In the genome of *B. pseudomallei* K96243, presence of three TTSS (TTSS1, TTSS2 and TTSS3) operons have been reported (Sun and Gan, 2010). The role of TTSS1 and TTSS2 are not clear, however, they demonstrate similarity with the TTSS from the plant pathogen, *R. solanacearum* (Rainbow *et al*., 2002; Winstanley *et al.* 1999). On the other hand, the TTSS3 (also known as the *Burkholderia* secretion apparatus, Bsa) shows homology to the TTSS of human pathogens *Salmonella enterica* serovar Typhimurium and *Shigella flexneri* (Sun *et al*., 2005; Rainbow *et al*., 2002; Attree and Attree, 2001).

The translocator proteins of the TTSS interact with the eukaryotic cell membrane and mediate the delivery of the effectors into the cytosol of the target cell (Hueck, 1998). In the recent years, many studies have been performed in order to determine the role of these translocator and effector proteins of the TTSS3 in the virulence of *B. pseudomallei*. Stevens *et al.* (2002) demonstrated contribution of both the Bsa secretion apparatus and translocated effector proteins, to early vacuolar escape and replication of *B. pseudomallei*, within the murine macrophage-like cells. *B. pseudomallei* are known to induce formation of multinucleated giant cell (MNGC) in infection of cells lines (Kespichayawattana *et al.*, 2000). However, Suparak *et al.* (2005) have demonstrated that genetic inactivation of *bipB*, a type III translocator protein, reduced MNGC formation as well as cell-to-cell spreading of bacteria via actin-based motility, and induction of apoptosis of J774A.1 macrophages. Additionally, the *bipB* mutant was also significantly attenuated in BALB/c mice following intranasal challenge. Complementation with a functional *bipB* gene was found to fully restore virulence.

Similarly, Stevens *et al*., 2002 have reported that a bipD mutant was unable to escape from endocytic vacuoles, replicate or form actin tails in J774.2 cells. In addition, the bipD mutant was also found to be attenuated in BALB/c mice, with reduced bacterial loads in the spleens and livers of infected mice (Stevens *et al*., 2004).

Over the years, many other mutational studies on the effector and translocator proteins of *B. pseudomallei* TTSS have implicated the importance of this secretion system in the virulence of the pathogen. Reduced invasion efficiency of bsaQ mutant into the human lung epithelial cell (A549) (Muangsombut *et al*., 2008) and loss of cytotoxic activity against macrophage-like cell lines (Sun *et al*., 2005) was demonstrated, however, the bsa Q mutant in strain KHW did not demonstrated attenuation in invasion into the human embryonic kidney cells (HEK293T) (Hii *et al*., 2008). Stevens *et al*. (2002) found that a bsaZ mutant demonstrate reduced ability in escape from the endocytic vacuoles, replication or formation of actin tails following infection of J774.2 macrophage cells. Similarly, in an *in vivo* model of syrian hamsters, a bsaZ mutant was also attenuated for virulence (Burtnick *et al*., 2008; Warawa and Woods, 2005). Reduced LD_{50} and decreased bacterial load in the spleen, liver and lungs was also demonstrated by a bsaU mutant in the BALB/c mouse model (Pilatz *et al*., 2006). Additionally, other *in vitro* and *in vivo* studies using the TTSS3 effector proteins (bopA, bopB, bopE and bapC) also further strengthen the important role of these proteins in the pathogenesis of *B. pseudomallei* (Cullinane *et al*., 2008; Warawa and Woods, 2005; Stevens *et al*., 2004; Stevens *et al*., 2003).

Although there have been numerous studies on the TTSS3 of *B. pseudomallei*, its precise role in pathogenesis remains unclear. Whether the reduction in virulence observed in the above mentioned studies are specifically due to the defect observed in

vacuolar escape and its downstream effects or due to other undefined role of this TTSS3 in pathogenesis, remains to be answered.

Another important secretion system which has been newly identified in the Gram-negative bacteria is the T6SS (Bönemann *et al*., 2010). It is structurally similar to an inverted bacteriophage tail, and injects effectors directly into the cytosol of cells (Hood *et al.,* 2010). There are six T6SS gene clusters in *B. pseudomallei* (T6SS1-T6SS6) (Schell *et al*., 2007; Shalom *et al*., 2007). Studies on the Hcp proteins, which are integral surface-associated components of the T6SS apparatus, have suggested that the T6SS-1 is a critical *B. pseudomallei* virulence determinant, with important role in the intracellular lifestyle of *B. pseudomallei* (Burtnick *et al,* 2011). Using *in vivo* expression technology (IVET), T6SS-1 was also found to be induced inside murine macrophages (Shalom *et al*., 2007). Similarly, a mutant of the T6SS-1 component was also found to be highly attenuated in mice and demonstrated defect in cell to cell spread on epithelial cells (Pilatz *et al*., 2006). Further investigations are necessary in order to determine the role of the different *B. pseudomallei* T6SS in pathogenesis.

Additionally, many bacterial pathogens have also been reported to utilise outer membrane vesicles (OMVs) for delivery of virulence factors into host cells (Elmi *et al*., 2012). These OMVs are produced spontaneously during the normal growth of the bacteria and have been detected in infected human tissues (Mashburn-Warren and Whiteley, 2006; Kuehn and Kesty, 2005; Heczko *et al*., 2000; Keenan *et al*., 2000). Initially, the OMVs originate as a bulge in the bacterial outer membrane, and then separate from the cell as vesicles that are able to deliver hydrophobic proteins and also carry soluble proteins in the lumen of the vesicle (Kesty and Kuehn, 2004). The OMVs are involved in several biological functions including toxins delivery, immune system modulation, trafficking of signaling molecules between bacterial cells, and biofilm

formation (McBroom and Kuehn, 2007; Mashburn-Warren and Whiteley, 2006; Klieve *et al*., 2005). In B. *pseudomallei,* immunisation with OMVs *via.* sub-cutaneous route was found to produce protective immunity against pulmonary challenge with virulent *B. pseudomallei*. Interestingly, the OMVs failed to provide protection when the intranasal route of immunization was used and also did not protect against chronic infection (Nieves et al., 2011).

1.4.5 Other virulence factors

B. pseudomallei are known to form biofilms, an important aspect in bacterial pathogenesis due to its ability to promote bacterial survival or spreading within the host (Mohammed and Huenf, 2007; Dunne, 2002). It is also able to act as a matrix shield to protect the bacteria from the host defence factors and antimicrobial agents (Harrison *et al*., 2002). In a study by Taweechaisupapong *et al*. (2005), they did not find any correlation between the biofilm formation by *B. pseudomallei* and virulence in the BALB/c mouse. Additionally, attenuation of virulence was also not observed in *B. pseudomallei* mutants deficient for biofilm production. *B. pseudomallei* growing in biofilm was found to be significantly more resistant to antimicrobial agents compared to the planktonic cells (Olson *et al*., 2002). As such, biofilm formation by *B. pseudomallei* was considered not important as a virulence factor. However, it plays an important role in the persistence of the organism in harsh environments and also causing resistance to antibiotics.

However, regulation of biofilm formation has been associated with quorum sensing, a cell-density-dependent communication network (O'Toole *et al*., 2000). The coordination of gene expression in quorum sensing is dependent on N-acyl-homoserine

lactone (AHLs) (Fuqua and Greenberg, 2002). High cell densities in the biofilms provide an optimum site for quorum sensing activation. According to Ulrich *et al*. (2004), quorum sensing is among the putative virulence factors of *B. pseudomallei*. Nevertheless, further elucidations are necessary in order to elucidate the direct relationship between quorum sensing and biofilm formation in B. *pseudomallei*.

Additionally, iron is essential for bacterial growth and survival, as a cofactor for many metabolic enzymes (Peek *et al*., 2012). However, free iron is available in very low concentrations during infection. It is mainly found in iron-binding proteins such as transferrin, lactoferrin, and ferritin (Smith, 2007). Thus, bacteria, including *Burkholderia spp.* has evolved high-affinity iron uptake systems, involving the low-molecular-weight iron chelators, siderophores (Lamont *et al*., 2009; Visca *et al*., 2007). *B. pseudomallei* are known to produce malleobactin, a hydroxamate-type siderophore, which has the ability to remove iron from lactoferrin and transferrin. This provides advantage to the bacteria to grow under iron-limiting conditions (Alice *et al*., 2006; Yang *et al*., 1993; Yang *et al*., 1991). Several genes encoding a number of other iron acquisition systems including pyochelin (*pch*) gene cluster, a heme uptake locus (*hmu*) and plasma membrane iron transporters have been identified in its genome (Harland *et al*., 2007; Tuanyok *et al*., 2005; Holden *et al*., 2004; Ong *et al*., 2004).

1.5 Proteomic analysis

Proteomics is an emerging area of research of the post-genomic era, which is based on three technological platforms, high-resolution two-dimensional gel electrophoresis (2D-GE), highly sensitive biological mass spectrometry (MS), and the rapidly growing protein and DNA databases (Pandey and Mann, 2000). Proteome is a new word that

was proposed by Wilkins *et al*. (1996) to define all the different proteins occurring in an organism in space and time. During the past few years, proteomics has been extensively applied to several fields of medicine to better understand normal physiology, to define the pathophysiology of diseases, and to identify novel protein biomarkers and new therapeutic targets.

2D-GE which separates proteins on the basis of their isoelctric points (pI) and molecular weight may aid in the examination of the complete pattern of the gene expression of a cell as revealed by the levels of individual proteins. Proteomics have been used widely to compare the proteome pattern between microorganisms and also to identify proteins that are over-expressed or suppressed by different conditions of growth. Some of the studies carried out are comparative proteome analysis of different strains of *Mycobacterium* (Jungblut *et al*., 1999) and *Helicobacter pylori* (Jungblut *et al*., 2000). In a study to investigate the pathological effect of *Helicobacter pylori* on human hepatic cells, Zhang *et al*. (2005) used proteomic methods to find and identify proteins that were over expressed in HepG2 cells treated by *H pylori*. To date, knowledge of virulence genes in *B. pseudomallei* and their corresponding proteins is still fragmentary. The ability of *B. pseudomallei* to survive intracellularly is known but the genes or proteins involved in their survival strategies have not been defined. Therefore, precise analysis of the *B. pseudomallei* secretome is thought to facilitate identification of the secreted proteins with emphasis on their role in virulence and intracellular survival. The availability of *B. pseudomallei* K96243 genome sequence has facilitated the identification of many of these secretome proteins. It is hoped that the availability of *B. pseudomallei* proteome and ability to characterise the virulence factor using proteomic, genomic and bioinformatic tools will facilitate in the design of novel measures for prevention and identification of drug target and design of drugs and effecting molecules against disease caused by *B. pseudomallei*.

1.6 Host response to *B. pseudomallei*

Infection of a host by pathogenic bacteria is a complex interplay that iniatiates a cascade of events that determine the outcome of the interaction. During an infection, bacterial pathogens adhere, invade and survive in normally privileged sites within a host. They also produce factors that result in host damage, aid in avoiding the host's immune system, and acquiring necessary nutrients. The host, on the other hand, recognises the presence of the bacteria and mobilise specific immune defence mechanisms. The complementary technologies of both DNA microarrays and proteomics allow the response of bacterial pathogens to different environments and the response of the host towards the infection to be probed at the whole genome level.

High density DNA microarray analysis of host gene expression in response to pathogen infection which provides a powerful approach to examine microbial pathogens from the host perspective was used in this study. In recent years, many studies have been performed using DNA microarray analysis in order to determine the host response to bacterial infection (Koo *et al*., 2012; Blanco *et al*., 2012, Yang *et al*., 2002; Utaisincharoen *et al*., 2004; Moreilhon *et al*., 2005). The ability to survey the responses of a large subset of the host genome, and to find patterns among the profiles from many different microorganisms and hosts, allows fundamental questions to be addressed concerning the basis of pathogen recognition, the features of the interaction between host and pathogen, and the mechanisms of host defense and microbial virulence (Manger and Relman, 2000).

Generally, in response to bacterial infection, mammalian cells launch a transcriptional programme that generates an intracellular anti-bacterial state involving cytokines such as inteferons. This may result in rendering neighbouring cells resistant to bacterial infection. A genome-wide expression analyses will be useful to understand the

pathology induced by *B. pseudomallei* at the molecular level. Monitoring of gene expression profiling using a cDNA microarray is a powerful approach to characterising and understanding host-pathogen interaction. Gene expression profiling analysis of cells infected with *B. pseudomallei* can help to reveal the mechanisms by which the host responds to *B. pseudomallei* and some of the mechanisms by which the pathogens avoid host defense thereby surviving and growing in host cells. This information may help to identify new therapeutic targets.

1.6 Hypothesis and objectives

In this study, it is hypothesised that the secretory proteins of *B. pseudomallei* play essential roles in the virulence and host-pathogen interactions. Thus, the specific objectives of this study were:

a) To determine the virulence factors of *B. pseudomallei via* exoenzyme, invasion and intracellular survival assays

b) To perform proteome profiling and identify the putative virulence factors of *B. pseudomallei* secretory proteins using 2D-GE and bioinformatic analysis.

c) To identify immunodominant epitopes of secreted virulence factors of *B. pseudomallei*.

d) To study the host gene regulation in response to the virulence factors of *B. pseudomallei* using microarray analysis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains

2.1.1 Source of bacterial strains

The bacterial strain used in this study includes the clinical isolate of *Burkholderia pseudomallei* (CMS) and *Escherichia coli* ATCC 25922. The *B. pseudomallei* isolate was obtained from blood culture of a patient admitted at the University Malaya Medical Center (UMMC), with a history of many incidence of relapse of melioidosis. The *E. coli* is a non-invasive strain that was used as the negative control.

2.1.2 Identification of bacterial strains

The clinical isolate used was identified as *B. pseudomallei* based on morphological, biochemical and molecular methods:

1) colony morphology on Ashdown agar (Appendix 1). *B. pseudomallei* colonies appeared as purple coloured, wrinkled with rough edges on the Ashdown agar.
2) Gram-stain (Appendix 2). *B. pseudomallei* appeared as Gram-negative rods.
3) routine biochemical confirmatory tests. Using API20NE (Biomerieux, France), the isolate generated a profile of 1156577 which is typical for *B. pseudomallei*. The isolate was also characterised as oxidase positive and arabinose-negative (ara^-) biotypes.
4) PCR based methods using specific primers for detection of *Burkholderia* genus (*gro1*& *gro2*) and detection of *B. pseudomallei* (*mpr1* & *mpr2*) (Suppiah *et al*., 2010). The PCR amplification demonstrated the presence of 162 bp amplicon using the *gro* primers and 139bp amplicon for the *mpr p*rimers which confirms the isolate as *B. pseudomallei*.

Table 2.1: Primer sequences used for the confirmation of the isolates

Primer	Sequence (5' – 3')	Amplicon size (bp)
***gro1* (F)**	CTG GAA GAC ATC GCG ATC	**162**
***gro2* (R)**	CGT ATC ACA TCG CAT CGC	
***mpr1* (F)**	**TCT CCG ATA GCC GCC TTG**	**139**
***mpr2* (R)**	**CGT CGA TGA TCG TCG TGT T**	

F – forward primer; R - reserve primer

2.1.3 Bacterial maintenance and stock preparation

All bacterial cultures were grown overnight on nutrient agar (NA) (Appendix 1) (Stock culture from the glycerol stock was used each time in order to avoid continuous culturing and lab adaptation). A single colony was inoculated into 10 ml of Luria Bertani (LB) broth (Appendix 1) and grown aerobically at 37ºC overnight with an agitation of 150 rpm until OD_{600nm} of 0.8-1.0 is reached. Following incubation, 0.2 ml of the bacterial suspension was mixed with 0.3 ml of 50% sterile glycerol (Appendix 1) to prepare a glycerol stock with final concentration of 30% glycerol. The suspension was prepared in cryovials and stored at -80ºC for long term storage. A loopful of the stock suspension was streaked onto NA plates and incubated overnight at 37˚C. The purity of the isolate was checked. A single colony was streaked onto the NA slant and incubated overnight at 37˚C. The slants were used for short term storage up to 3 months.

2.2 *Burkholderia pseudomallei* growth curve and viable count

A single colony of *B. pseudomallei* CMS, from an overnight culture at 37ºC, was inoculated into 10 ml LB broth. The culture was grown aerobically overnight with an agitation of 150 rpm at 37ºC until OD_{600nm} of 0.8-1.0 was reached. The bacterial pellet was then recovered from the culture by centrifugation at 4,000*g* for 5 mins. The bacterial pellet was resuspended in fresh LB and used to inoculate a fresh LB broth medium to obtain an OD_{600nm} of 0.1. Subsequently, 10 µl of the culture was inoculated into 50 ml LB broth contained in 21 subsequent flasks and grown aerobically at 37ºC for a further 0-24 h with agitation at 150 rpm. Three of the flasks were taken out at 4 hour intervals (0, 4, 8, 12, 16, 20 and 24 h) and 1 ml of the culture was used to determine the optical density of the culture samples at OD_{600nm}. In order to determine the viable counts of the bacteria, serial dilutions (10-fold) of the culture were plated onto NA (Miles and Misra, 1938). Determination of the *B. pseudomallei* growth curve was carried out as triplicate in three independent experiments (Figure 2.1).

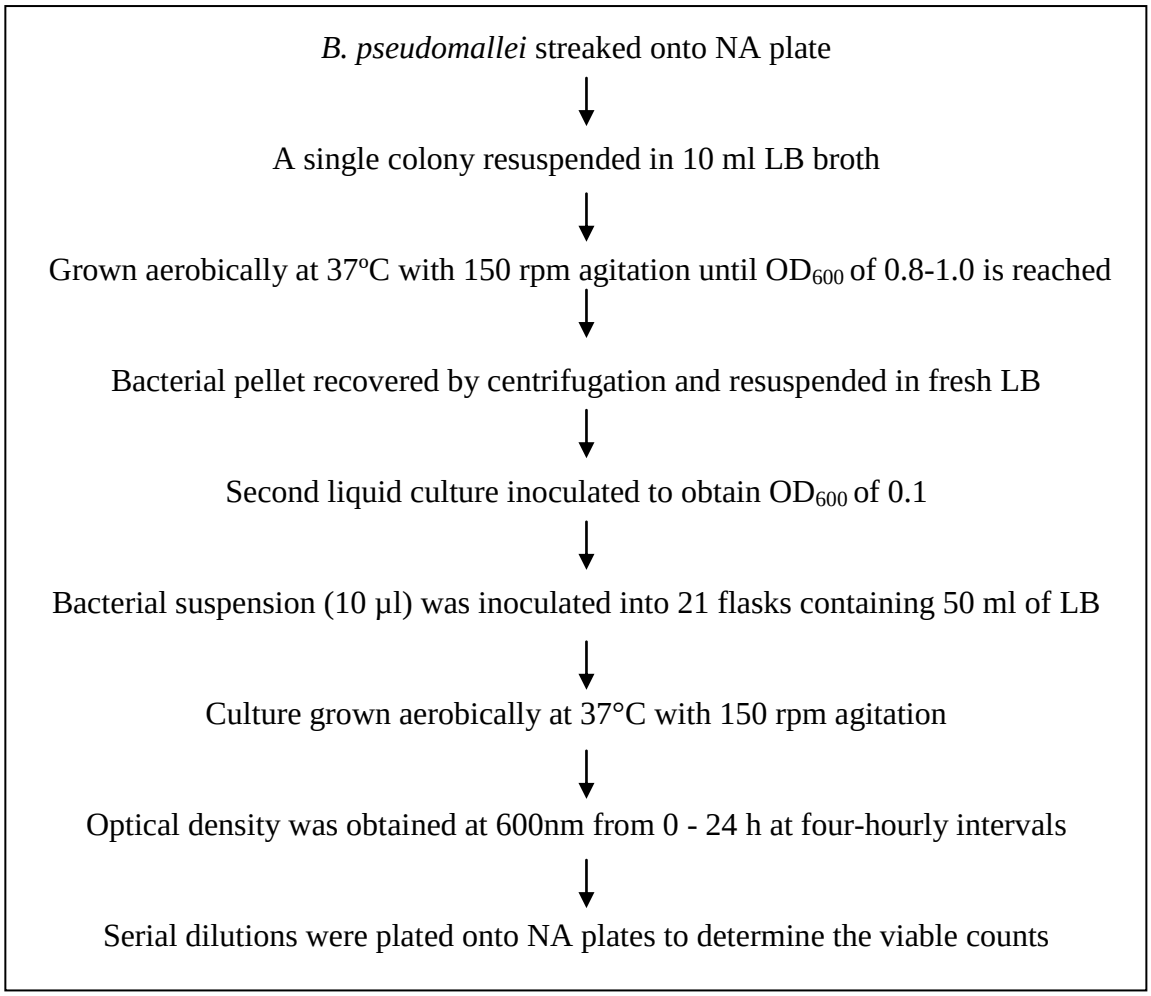


Figure 2.1: Flowchart of experimental procedures to generate optimal bacterial growth curve and determination of viable count.

2.3 Preparation of bacterial secreted protein for analysis

Bacterial secreted protein was prepared by concentration of the bacterial-free culture supernatant using two different methods *i.e.* i) ultrafiltration, and ii) chemical precipitation.

i) Concentration of the bacterial-free culture supernatant using ultrafiltration method was performed as previously described by Kumar *et al.* (1994) with minor modification. Briefly, bacterial inoculum was prepared as previously described (Section 2.2**)** and one liter of the stationary phase (20 h) culture in LB was centrifuged at 20,000*g* for 40 mins at 4ºC. The supernatant was collected and

filtered through a 0.22 mm filter (Millipore, USA) to obtain a bacterial-free culture supernatant. The bacterial-free culture supernatant was concentrated 20-fold using the Quixstand bench top system (GE Healthcare, Darmstadt, Germany). The supernatant obtained was further concentrated 50-fold by ultrafiltration employing 10 kDa centricon ultra-free centrifugal filter units (Millipore, Massachusetts, USA). The samples were then dialysed overnight using 0.1 M phosphate buffered saline and the protein concentration was determined using Bradford (1976) method (Appendix 3). The concentrated bacterial secreted protein was stored at -80°C in aliquots until further use. The secreted protein prepared was used to assay the extracellular enzyme activity (Section 2.5.1), enzyme-linked immunosorbent assay (ELISA) (Section 2.5.2), protein analysis (SDS-PAGE) (Section 2.7.1), antibody production (Section 2.4) and gene expression studies (Section 2.8) (Figure 2.2).

ii) Concentration of the bacterial-free culture supernatant using chemical precipitation method was also performed. Briefly, bacterial inoculum was prepared as previously described (Section 2.2**)** and one liter of the stationary phase (20 h) culture in LB was centrifuged at 20,000*g* for 40 mins at 4ºC. The supernatant was collected and filtered through a 0.22 mm filter (Millipore, USA) to obtain a bacterial-free culture supernatant. The bacterial-free culture supernatant was concentrated using two different chemicals i.e. ammonium sulphate (AS) and trichloroacetic acid (TCA).

AS precipitation method - AS was added to 15% saturation until fully dissolved in the bacterial-free culture supernatant, after which the homogenous solution was centrifuged at 18,000*g* for 20 mins at 4°C to collect the precipitated proteins. The

protocol was repeated in a step-wise manner to the remaining supernatant, with further addition of 15% AS followed by collection of the protein pellets through centrifugation. The addition of 15% AS was repeated until a saturation level of 75% was achieved. All protein pellets obtained from the 15% to 75% saturation were pooled and washed 3× with cold acetone and dried at room temperature for 10 mins. The dried protein pellets were solubilized in 100 µl of lysis buffer (Appendix 5) and stored in aliquots at -80°C until further use. The secretory protein prepared was used in the protein analysis (SDS-PAGE) (Section 2.7.1).

TCA precipitation method - prechilled 25% (w/v) TCA (Sigma, USA) was added to the bacterial-free culture supernatant at a ratio of 1:3. The proteins in the culture supernatant were left to precipitate on ice for 2 h, following which the precipitated proteins were collected by centrifugation at 10,000*g* for 20 mins at 4ºC. The resulting pellet was washed 3× with acetone and let to dry at room temperature to allow evaporation of residual acetone. The dried protein pellet was then resuspended in 100 µl of lysis buffer and stored in aliquots at -80°C until further use. The secretory protein prepared was used in the protein analysis (SDS-PAGE and 2D-GE) (Section 2.7.1 and 2.7.2).

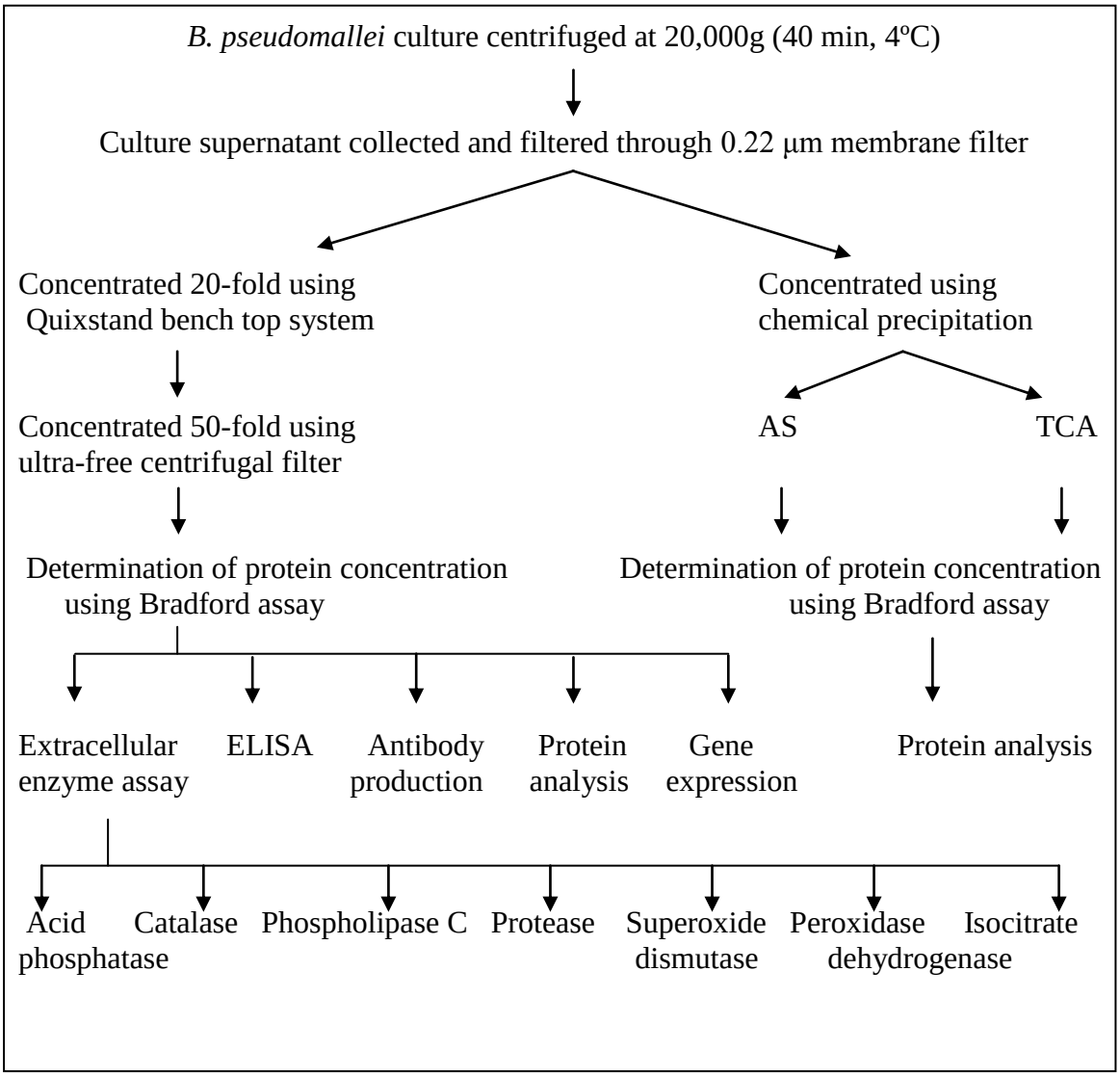


Figure 2.2: Flow chart of experimental procedures for bacterial culture supernatant collection, protein determination and virulence assay.

2.4 Production of mice anti-*B. pseudomallei* secreted proteins antibody

A secreted protein sample for immunisation was prepared for the production of polyclonal antibodies using the ultrafiltration method as previously described (Section 2.3). The secreted protein concentration was determined using the Bradford method prior to use in immunization of the Balb/C mice. The protocol for antibody production was performed according to Mariappan *et al.* (2009) with slight modifications. Briefly, six male Balb/C mice (6–8 weeks old) were divided into 2 groups of three mice each, Group A representing immunised mice and group B representing the control group. Pre-immunisation tail bleeds (100 – 250 µl) were performed from each mouse in each group and pooled prior to injection. Mice in the immunized group were immunised with 50 µg CFA in Freund's complete adjuvant via subcutaneous injection. Booster injections at every two-week interval for two months were given using 100 µg of antigen in Freund's incomplete adjuvant. Prior to booster immunisation, blood was collected using tail bleed from the mice in both groups (immunised and control). The blood from the three mice in each group were pooled and left at room temperature for 30 mins before centrifugation at 3,000*g* for 5 mins to collect the sera, after which detection of antibodies to the secreted proteins was performed using ELISA (Chenthamarakshan *et al.* 2001). Sera that elicited high antibody titer were collected and stored at -20ºC for further use. Sera from the control group were used as the negative control. Mice from both the groups were sacrificed on week 8 following booster (Figure 2.3).

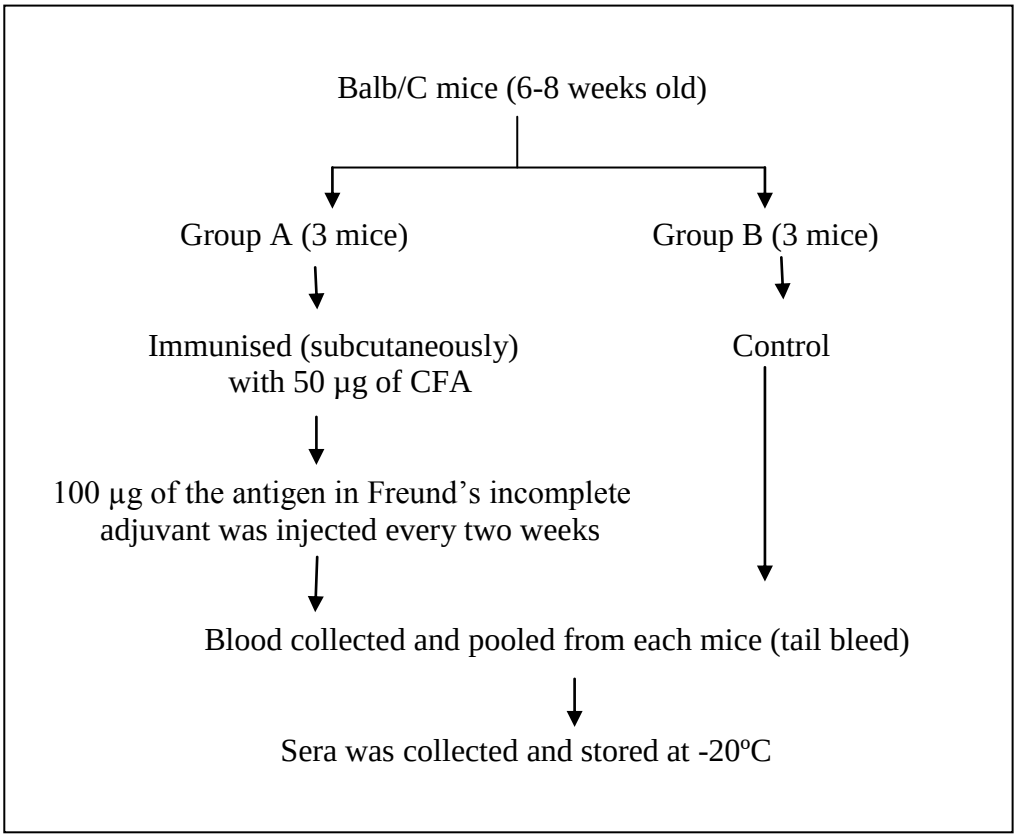


Figure 2.3: Flowchart of experimental procedures for mice antibody production.

2.5 Virulence factors

2.5.1 Extracellular enzyme assays

Extracellular enzyme activity in the secreted proteins produced over time was investigated. The activity of acid phosphatase, catalase, phospholipase C (PLC), protease, peroxidase, superoxide dismutase (SOD) were assayed to determine the presence of these virulence factors in the *B. pseudomallei* secreted proteins. The activity of isocitrate dehydrogenase (ICD) was also assayed in order to determine the degree of bacterial lysis at each time point sampled. All enzymatic activities were expressed as unit activity per ml of secreted proteins. Concentrated LB broth was used

as negative control in all the extracellular enzyme assays. Each enzymatic assay was carried out in triplicate. Enzyme activities (Unit/min) for acid phosphatase, peroxidase, phospholipase C and isocitrate dehydrogenase were determined using the following formula:

$$\frac{(\text{Test} - \text{Enzyme blank}) - \text{Substrate blank}}{\text{Assay time}} = \text{Unit / min}$$

2.5.1.1 Protease assay

The protease assay in the secreted proteins was performed as described previously by Chavira *et al.* (1984) with minor modifications. Briefly, varying concentrations of extracellular secreted products were incubated with 0.5 ml of 0.05 M phosphate buffer (pH 7.5) containing 5 mg/ml azocoll (Sigma, USA), overnight at 37ºC. The mixture was then centrifuged at 200*g* for 5 min, and the absorbance of the supernatant was measured at A_{540nm}. One unit of activity was calculated as the amount of protease needed to increase the absorbance per hour from 0.05 to 0.1.

2.5.1.2 Acid phosphatase assay

The acid phosphatase activity was assayed by measuring the release of p-nitrophenol (p-NP) from p-nitrophenyl phosphate (p-NPP) (Sigma, USA) at OD_{405nm} (Domenech *et al*., 1992). One ml of 100 mM sodium acetate buffer (pH 5.0) and 0.1 ml of 250 mM p-NPP were added to 0.1 ml of the secreted proteins to initiate the reaction. The reaction was terminated by the addition of 2 ml of 0.4 N NaOH. One unit of phosphatase activity was defined as the amount needed to release 1 mmol of p-nitrophenol per min.

2.5.1.3 Catalase assay

The catalase activity in the secreted proteins was determined by the decrease in the A_{240} of H_20_2 as described previously by Pine *et al.* (1984). The secreted proteins were added with 0.05–0.70 ml of freshly prepared 13.2 mM H_20_2 in 0.05 M potassium phosphate buffer (pH 7.0). The solution was mixed thoroughly, and a loss of absorbance was determined at A_{240nm} for 1–3 min. One unit of catalase activity was defined as 1 mmol of H_20_2 decomposed per min.

2.5.1.4 Peroxidase assay

Peroxidase activity in the secreted proteins was determined using o-dianisidine (Sigma, USA) (Abrams and Webster, 1990). Briefly, 0.05 ml of the secreted proteins was added to 0.75 ml of 0.01 M phosphate buffer (pH 6.0) containing o-dianisidine (10 mg/ml). The reaction mixture was then added with 0.05 ml of 0.3% freshly prepared H_20_2 in distilled water and the change of absorbance recorded at OD_{460nm} for 3-5 mins. One unit of activity was defined as 1 mmol of H_20_2 decomposed per min.

2.5.1.5 Superoxide dismutase assay

The SOD activity of the secreted proteins was assayed by monitoring the inhibition of pyrogallol (Sigma, USA) auto-oxidation at pH 8.0 as described previously by Steinman (1985). The change in optical density during the SOD mediated inhibition of oxygen free radicals was measured at OD_{420nm}. One unit of activity was defined as the amount of SOD needed to reduce the absorbance per minute from 0.02 to 0.01.

2.5.1.6 Phospholipase C assay

The phospholipase C activity of the secreted proteins was assayed using p-nitrophenyl phosphorylcholine (p-NPPC) (Sigma, USA) as the substrate (Geoffroy *et al*., 1991). Briefly, a 20 mM solution of p-NPPC was prepared in 0.25 M Tris–HCl buffer pH 7.0 containing 60% glycerol (v/v) and 1 mM $ZnCl_2$. The reaction was started by the addition of 50 ml of the secreted proteins into a total of 1 ml reaction mixture and incubated at 37ºC after which the absorbance was read periodically at OD_{405nm}. One unit of enzyme activity was calculated as the amount required for the release of 1 mmol p-nitrophenol per min.

2.5.1.7 Isocitrate dehydrogenase assay

ICD activity in the secreted proteins was determined by measuring the reduction of $NADP^+$ at room temperature as described previously by Vives-Rego *et al.* (1981). Three ml of reaction mixture consisting of 100 mM Tris-HCL (pH 8.0) buffer, 1.3 mM MnCl solution, 3.3 mM isocitrate solution, 0.33 mM $NADP^+$ solution and 0.1 ml of the secreted proteins was prepared. The mixture was added with either cell-free extract or $NADP^+$. One unit (U) of enzyme is the amount that converts one μmol of $NADP^+$ to NADPH per min at 37°C.

2.5.2 Enzyme-linked immunosorbent assay for IgG

Imuunogenic properties of the secreted proteins produced over time were also determined using the Enzyme-linked immunosorbent assay (ELISA) (Chenthamarakshan *et al.,* 2001). Briefly, secreted proteins (100 ng/well) in 100 µl of

Tris buffered saline (TBS) was applied into Maxisorp polysterene flat bottom microtitration plates (Nunc, Denmark) and incubated overnight at 4ºC. The wells were blocked with 1% bovine serum albumin (BSA) (Sigma) in phosphate buffered saline (PBS) at 37ºC for one hour. The wells were then washed with PBS supplemented with 0.05% Tween-20 (PBST). Following blocking, 100 µl of anti-*B. pseudomallei* secreted proteins sera (Section 2.4) diluted 1:1000 with PBST was added. After 1 hour incubation at room temperature, horseradish peroxidise (HRPO)-labelled goat anti-mouse IgG conjugate (Sigma, USA) in PBST (1:5000) was added and incubated at room temperature for 30 mins, after which the wells were washed 3× with PBST. Fifty microlitre of the substrate 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Sigma, USA) was added into each well and incubated for 15-20 minutes at room temperature. The colour development was read spectrophotometrically at 450 nm (BioRad, USA) (Figure 2.4). Unimmunised mice sera (1:1000 in PBST) were used as the negative control.

2.5.3 *In vitro* studies

Human alveolar epithelial carcinoma cells (A549) (ATCC, USA) was used for the *in vitro* studies. The cell line was routinely grown and maintained using the RPMI growth or maintenance medium, respectively, at 37ºC with 5% CO_2 atmosphere (Appendix 4). The cells were stored in RPMI medium containing the inhibiting agent, dimethyl sulfoxide (DMSO) in liquid nitrogen. All the solutions used were pre-warmed at 37ºC prior to use on the cells.

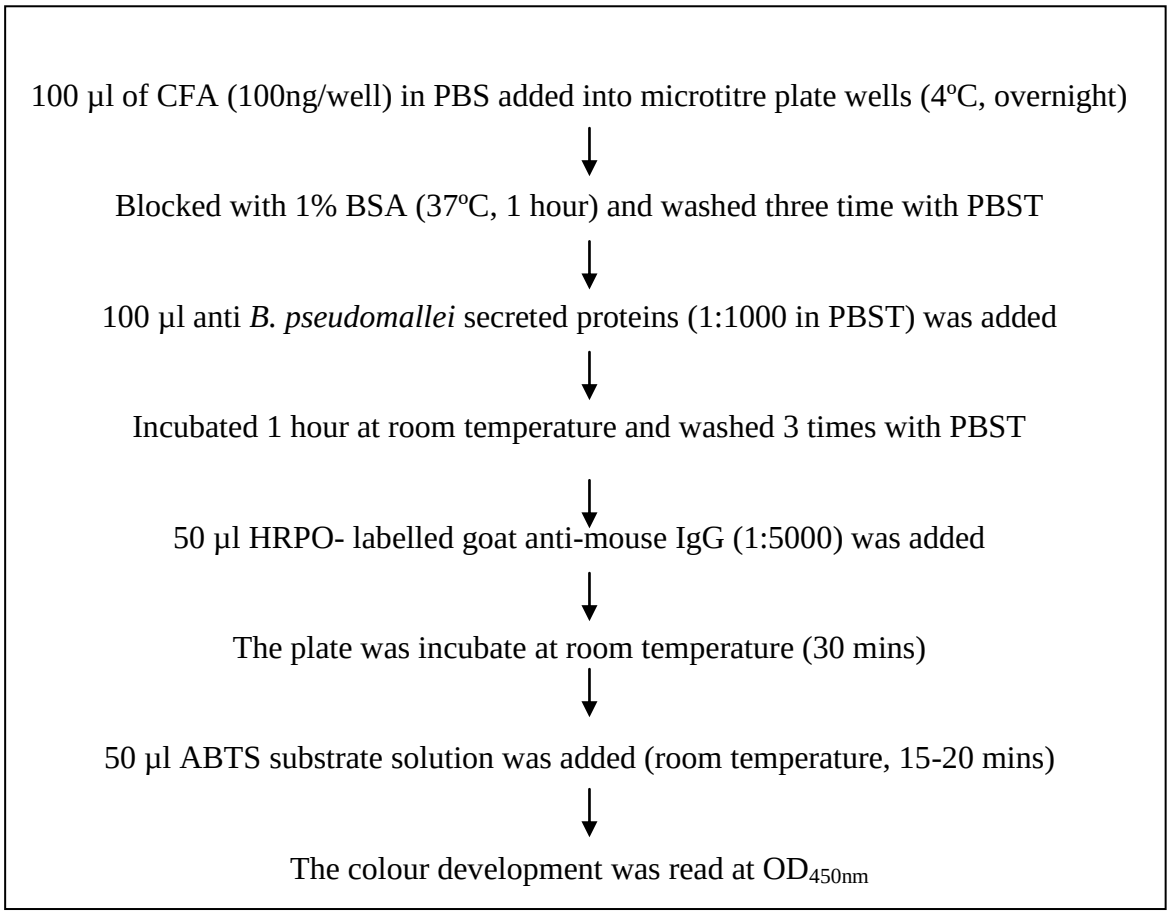


Figure 2.4: Flowchart of experimental procedures for ELISA.

2.5.3.1 Cell propagation and maintenance

A549 cells stored in liquid nitrogen were thawed at room temperature and mixed with fresh RPMI growth medium. The cells were centrifuged at 1,500 rpm at 4ºC for 5 mins and the resulting pellet was resuspended in fresh RPMI growth medium, transferred into the 75 cm^2 tissue culture flask and incubated at 37ºC with 5% CO_2 atmosphere. The cells were routinely subcultured when confluency was reached. To harvest the cells, the growth medium was removed and the cells washed with sterile PBS prior to the addition of trypsin-EDTA (1 ml) and incubation at 37ºC for 2 minutes. Gentle tapping was performed to aid the cell monolayer to detach from the surface of the flask. The resulting cell suspension was aspirated and pelleted by centrifugation at 1,500 rpm for 5

mins and resuspended in 1 ml of RPMI medium, after which the number of viable cells was enumerated using trypan blue and the haemocytometer.

2.5.3.2 Preparation of bacterial inoculum

B. pseudomallei bacterial inoculum was prepared as previously described (Section 2.2), and 1 ml of the culture at OD_{600nm} = 0.1 was transferred into 1.5 ml centrifuge tubes and centrifuged at 300*g* for 5 mins to pellet the bacteria. The resulting pellet was resuspended in 1 ml of RPMI maintenance medium and incubated at 37°C for 30 mins, after which the bacterial numbers in the suspension was adjusted to 1 x 10^8 cfu/ml using RPMI.

2.5.3.3 Infection of A549 cells

The infection of A549 cells was performed as described by Martin and Mohr (2000) with minor modifications. Briefly, A549 cells were seeded (5 X 10^5 cells per well) into a 24-wells tissue culture plate. The cells were incubated overnight at 37ºC with 5% CO_2 after which the confluent monolayers of the A549 cells obtained were washed 3× with PBS to remove the dead cells and fresh RPMI medium was added. The adjusted bacterial inoculum (Section 2.5.3.2**)** was added into the wells at multiplicity of infections (MOI) of 1:10, 1:100 and 1:200. The non-invasive *E. coli* was used as the negative control.

2.5.3.4 Invasion assay

The invasion assays were performed as previously described by Kespichayawattana *et al*. (2004) with slight modifications. Briefly, the infected cells (Section 2.5.3.3) were incubated for 1, 2, 3, 6, 12, 18 or 24 h respectively, at 37°C in 5% CO_2 to allow bacterial invasion. Following invasion at the respective time points, the monolayers were washed 3× using PBS and 1 ml of RMPI medium containing a combination of ceftazidime (1 mg/ml) and imipenem (1 mg/ml) was added into each well for 2 h at 37°C in order to completely eliminate the residual extracellular bacteria. After 2 h, the cell monolayers were washed three times with PBS. PBS used for the final wash was collected and plated onto a NA and no bacterial colony was observed indicating that all the residual extracellular bacteria have been eliminated at this stage. Three wells from each of the different MOI and incubation period were used to count the number of viable cells using trypan blue exclusion method. The A549 cells were then lysed using 0.5% tergitol, 1% BSA prepared in PBS and serial dilutions of the lysate were plated onto NA to determine the number of the intracellular bacteria (Miles and Misra, 1938). This experiment was performed in triplicates and the results were averaged (Figure 2.5).

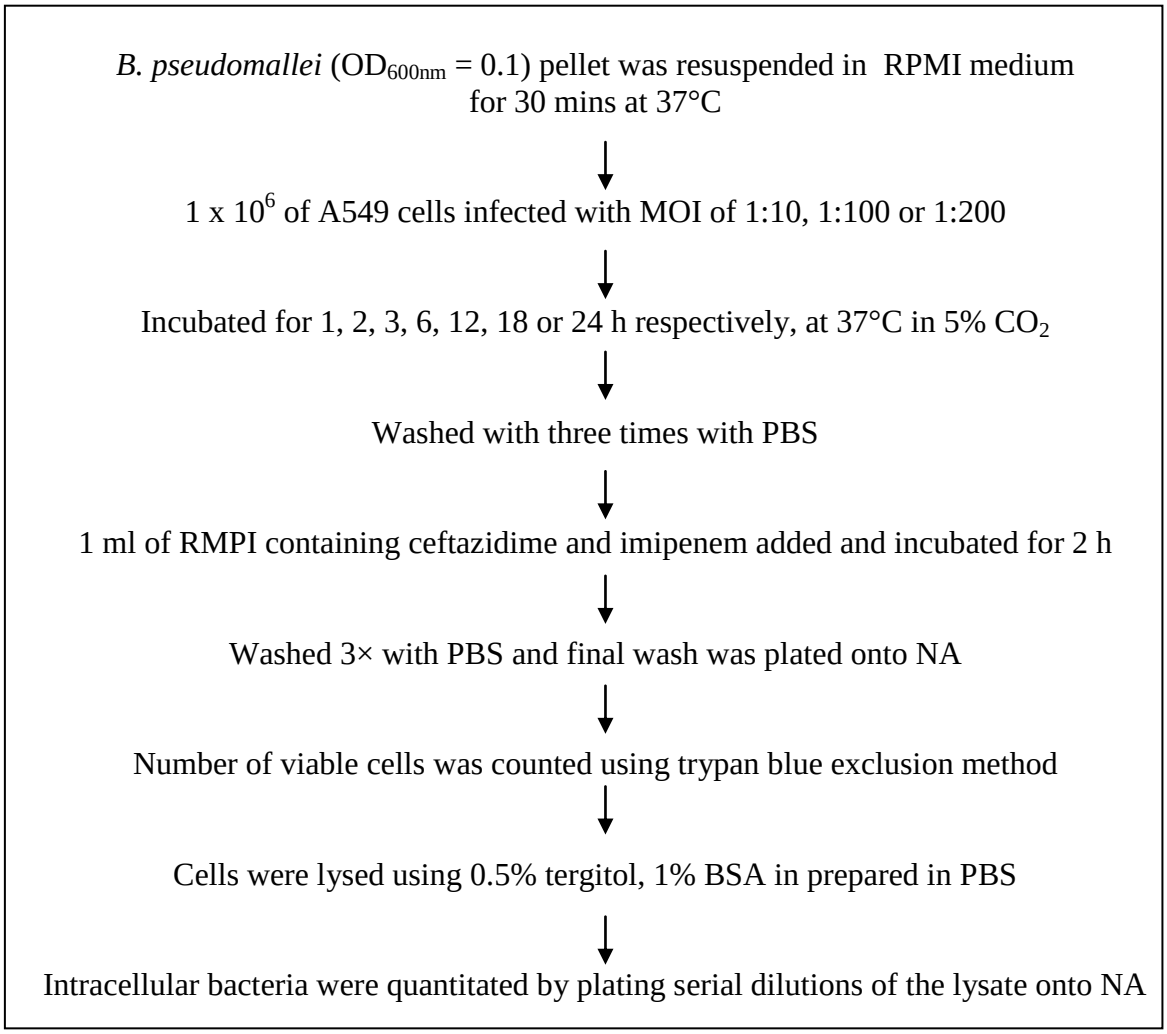


Figure 2.5: Flowchart of experimental procedures for invasion assay.

2.5.3.5 Intracellular survival assay

Intracellular survival assay was performed similarly as the invasion assay (Section 2.5.3.4). Following 2 h of incubation with RPMI containing antibiotic to kill the residual extracellular bacteria, the monolayers were washed 3× with PBS and the final wash was collected and plated onto a NA to determine the number of live extracellular bacteria. The monolayers were then further incubated for 1, 2, 3, 6, 12, 18 and 24 h in RPMI medium containing ceftazidime (10 µg/ml) and imipenem (10 µg/ml). At each time point of incubation, three wells from each of the different MOI and incubation

period were used to count the number of viable cells using trypan blue exclusion method. The A549 cells were then lysed using 0.5% tergitol, 1% BSA in prepared in PBS and serial dilutions of the lysate were plated onto nutrient agar to determine the number of the intracellular bacteria (Miles and Misra, 1938). This experiment was performed in triplicates and the results were averaged (Figure 2.6).

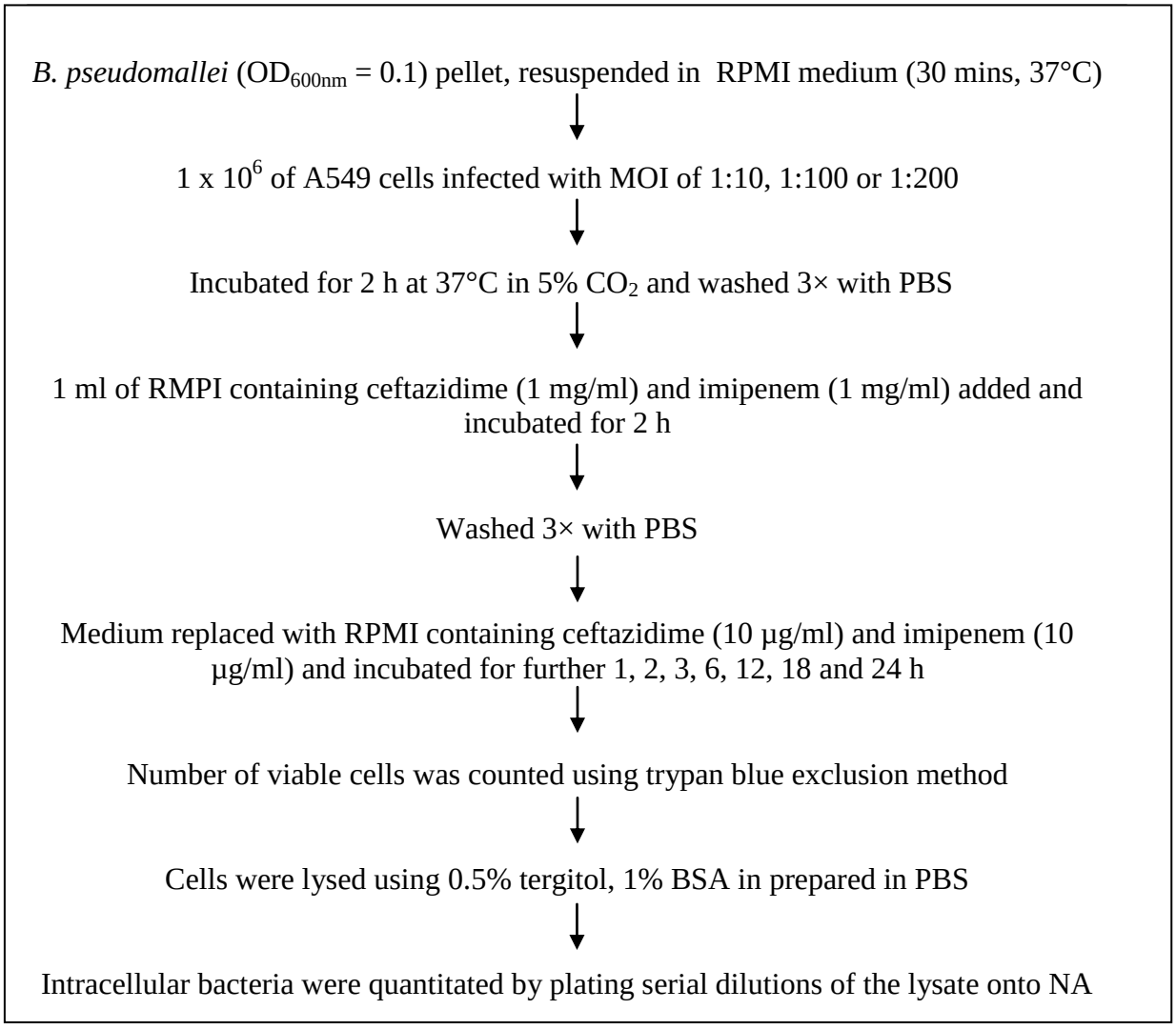


Figure 2.6: Flowchart of experimental procedures for intracellular survival assay.

2.6 Transmission Electron Microscopy (TEM)

A549 cells were infected with *B. pseudomallei* and allowed to invade as previously described (Section 2.5.3.3 and 2.5.3.4) with the MOI 1:10 for two hours. The infected cells were trypsinised using trypsin-EDTA (Flow labs, USA). Trypsinised cells were collected and pelleted by centrifugation at 1500 rpm for 5 mins. Resulting pellet was fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer solution for 30 mins followed by post fixation with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for an hour. The pellet was washed twice in 0.1 M sodium cacodylate buffer for five mins followed by three times washing of two mins each with phosphate buffer. The cells were then suspended in 2% molten agar and allowed to solidify. This is followed by the dehydration step using graded solutions of alcohol consisting of 50% ethanol (5-10 mins), 70% ethanol (5-10 mins), 95% ethanol (5-10 mins) and 100% ethanol (3 X 15 mins). The dehydrated pellet was then embedded in pure resin and allowed to polymerise at 60°C for 18 h. The polymerised blocks were trimmed and ultrathin sections (75-85nm) was made and mounted onto copper grids, stained in 2% uranyl acetate and viewed using a CM12 TEM at an accelerating voltage of 80 kW.

2.7 Proteomic analysis

Protein analysis consisted of three major steps *i.e.* i) the initial separation of the secreted proteins using sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), ii) two dimensional - gel electrophoresis (2D-GE) and iii) the Matrix-Assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOF) mass spectrophotometry and bioinformatics analysis.

2.7.1 SDS-PAGE

The SDS-PAGE was performed according to Laemmli (1970) using a 12% polyacrylamide gel. Two different concentrations of proteins (15 and 30 μg) obtained from the three different sample preparation methods (Section 2.3) were individually mixed with sample buffer at a ratio of 1:4. The mixture was then boiled for 10 mins, loaded into the 12% SDS-PAGE gel and electrophoresed using a constant current of 10 mA per gel until the dye front migrated into the running gel (~15 mins). The current was then increased to 25 mA per gel until the dye front reached the bottom of the gel (~45 mins). The resulting gels were stained using Coomassie Brilliant Blue (CBB) staining (Section 2.7.3). The gels were then scanned with an Image Scanner using the Image Master ^{TM} 2D Platinum version 5.0 (GE Healthcare, Uppsala, Sweden).

2.7.2 Two-dimensional gel electrophoresis (2D-GE)

2D-GE involves two major steps *i.e. the* first-dimension isoelectric focusing (IEF) followed by the second dimension gel electrophoresis.

First dimension isoelectric focusing

First dimension IEF was performed using the Ettan IPGphor II system (GE Healthcare, Darmstadt, Germany). Optimisation for 2D-GE was performed using the precast 13 cm linear IPG strip (GE Healthcare, Uppsala, Sweden) with a pH range of 3-10. The strips were first removed from storage at -20ºC and allowed to thaw at room temperature (~10 mins) prior to addition of the protein samples for rehydration. The protein samples were then mixed with the rehydration buffer (Appendix 5) and dithiothreitol **(**DTT) to a final concentration of 20 mM. (The maximum volume that can be used during

rehydration of the 13cm IPG strip is 250µl including the sample (Table 2.2). Excess rehydration solution can prevent complete sample uptake.

The rehydration solution was applied evenly into the groove in the reswelling tray and IPG strip was positioned into the rehydration solution gel side down ensuring that no air bubbles were formed underneath the strip. The strip was then overlaid with 3 ml of immobiline drystrip cover fluid in order to minimise evaporation and urea crytallisation. The cover fluid was then pipetted dropwise from one end of the groove in the reswelling tray until half of the IPG strip was covered. The fluid was then pipetted dropwise from the other end until the entire IPG strip was covered, the reswelling tray was then covered and the strip allowed to rehydrate passively overnight (minimum of 18 h) at room temperature.

Table 2.2: Preparation of in-gel rehydration solution volume per IPG strip.

IPG strip (13 cm)	**Protein amount (µg)**	**Volume per strip (µl)**			
		Sample	**RB**	**DTT**	**Total**
Silver staining:					
pH 3–10	150	20	227.5	2.5	250
Coomassie blue staining:					
pH 3–10	250	33	214.5	2.5	250
	350	47	200.5	2.5	250
	450	60	187.5	2.5	250

The first-dimension IEF was performed using the Ettan IPGphor II system (GE Healthcare, Darmstadt, Germany. The Ettan IPGphor II Manifold tray was positioned on the Ettan IPGphor II system. The IPG strip was removed from the reswelling tray using a forcep and immediately transferred into the grove of the manifold tray with the

gel side up. The strips were positioned with the acidic end at the anode and the basic end at the cathode. Electrode pads were soaked in deionised water and blotted on the tissue paper to ensure that the pads are damp and not wet because excess water may cause protein streaking. The electrode pads were then placed across the cathodic and anodic end of the IPG strips with partial contact with the gel. The electrode assemblies were then aligned onto the electrode strips. The strips were covered with approximately 105 ml of Immobiline DryStrip cover fluid by pouring it evenly into all the 12 grooves on the Manifold tray. The Ettan IPGphor II system safety lid was covered and the IEF started with the running parameter of at 500 V for 500 Vh, 1000 V for 1000 Vh, and 8000 V for 12 500 Vh (Görg *et al.*, 2000; Figure 2.7). After completion of the IEF, the strips were either subjected to the second-dimension separation immediately or stored at -80ºC in a screw cap culture tube.

Rehydration of IPG strips
IPG strips were rehydrated with the gel side down in the Immobiline DryStrip Reswelling tray using appropriate volume of sample in rehydration buffer. The IPG strips were overlayed with DryStrip Cover Fluid and rehydrated for 18 h.

Positioning of Manifold Tray onto IPGPhor III
Manifold Tray was placed on IPGPhor using the T-shaped protrusion as a guide

Transfer of IPG strips to Manifold Tray
IPG strips were transferred to the individual channels in the Manifold tray with the gel side up. The anodic (+) and cathodic (+) ends of the strips were positioned appropriately based on the mark etched on the IPGPhor III platform.

Moistening and placement of electrode pads
Wet electrode pads was moistened with deionised water and blotted until almost dry and placed on the ends of the IPG strips.

Positioning of electrode assembly
Electrode assembly was aligned and fixed on the electrode pads.

Setting of parameters and run
The Ethan IPGphor III lid cover was closed. Parameters were set and run.

Figure 2.7: Flowchart of experimental procedure summary for first dimension IEF. (Adapted and modified from 2D-Electrophoresis Principles and Methods, GE Healthcare)

Second-dimension gel electrophoresis

The second-dimension SDS-PAGE was performed in a vertical 2D-GE system employing a 1.5 mm thick homogenous, 12.5% SDS-PAGE gel (Appendix 5) using the method described by Laemmli (1970). Prior to the second dimension electrophoresis, the IPG strip was subjected to two equilibration steps. The first equilibration was performed for 15 mins in the equilibration buffer (Appendix 5) containing 100 mg of DTT. This was followed by the second equilibration for 15 mins in the equilibration buffer containing 250 mg of iodoacetamide (IAA). Both the equilibration steps were performed by placing the tubes on its side on a rocker.

After equilibration, the strip was washed with the cathode buffer (Appendix 5) prior to placing it on the surface of the SDS-PAGE gel. The empty space on the surface of the gel was also filled with the cathode buffer. The strip was placed with the plastic backing against the longer glass plate and gentling letting it slide into the cathode buffer until the entire lower edge of the strip is in contact with the top surface of the gel. Any air bubbles trapped in between the strip and the gel was removed by gently pushing the strip down using a flat forcep. The excess cathode buffer was then drained by tilting the gel cassette to one side and the empty space on top of the strip was sealed using the agarose sealing solution (Appendix 5). The agarose solution helped to prevent the IPG strip from moving or floating in the electrophoresis buffer.

The electrophoresis was carried out in a SE 600 Ruby system (GE Healthcare, Darmstadt, Germany) that was connected to an external power supply (EPS 601; GE Healthcare, Darmstadt, Germany) and thermostatic circulator (MultiTemp III Thermostatic Circulator; GE Healthcare, Darmstadt, Germany) to control the buffer temperature. The upper buffer chamber was filled with the cathode buffer and the lower buffer chamber was filled with anode buffer (Appendix 5). The running

conditions used for the electrophoresis consist of two steps. In step 1, a constant current of 15 mA/gel (50V, 25W/gel, for 30 mins) was applied and in step 2, the current was increased to 30 mA/gel (600V, 25W/gel, for 4 h). The run was eventually terminated when the dye front reaches approximately 1-5 mm from the bottom edge of the gel. The gels were then removed and subjected to visualisation using silver and/or CBB stain or western blotting. Three biological replicates were used and reproducibility of the results was determined.

2.7.3 Analysis of the protein spots

Analysis of the separated protein spots involved using two different staining methods i.e. Silver staining and CBB staining for visualisation of the protein spots on the gel, prior to selecting and picking the spots for MALDI-TOF analysis followed by bioinformatics analysis.

Silver Staining

Briefly, the gels were immersed in a fixing solution for 2 h with a change after the first hour, followed by incubation in the sensitising solution for a further one hour (Table 2.3). The gels were washed 5X with distilled water, the silver reaction added and incubated for one hour followed by four washes in distilled water. After the washing step, the developing solution was added to the gels and the staining reaction was visually monitored to ensure that the stain had reached the desired intensity and before the background became too dark. The gels were then transferred into fresh stop solution for 45 mins after which, the gels were washed 2X with distilled water and stored in the preservation solution for further image analysis.

Table 2.3: Silver staining method

Step	Solutions	Amount	Time
Fixing	Ethanol	200 ml	2 × 60[*] mins
	Acetic acid, glacial	50 ml	
	Made up to 500 ml with distilled water		
Sensitising	Ethanol	150 ml	60 mins
	Sodium acetate	34 g	
	Sodium thiosulphate, 5% (w/v)	20 ml	
	Glutaraldehyde[†], 25% (w/v)	2.5 ml	
	Made up to 500 ml with distilled water		
Washing	Distilled water		5 X 8 mins
Silver reaction	Silver nitrate solution, 2.5% (w/v)	50 ml	60 mins
	Formaldehyde[†], 37% (w/v)	0.2 ml	
	Made up to 500 ml with distilled water		
Washing	Distilled water		4 X 1 mins
Developing	Sodium carbonate	12.5 g	5 mins[¶]
	Formaldehyde, 37% (w/v)	0.2 ml	
	Made up to 500 ml with distilled water		
	Vigorously stirred to dissolve sodium carbonate		
Stop	EDTA-$Na_2.2H_20$	7.3 g	45 mins
Washing	Distilled water		2 X 30 mins
Preservation	Ethanol, 10% (v/v)	50 ml	20 mins
	Made up to 500 ml with distilled water		

[*]Fixation maybe prolonged to 3 days if desired
[†]Glutaraldehyde and Formaldehyde were added just before staining
[¶]Approximate time; gels can be visually monitored and transferred to stop solution when spots have reached desired intensity and before the background becomes too dark.

Coomassie Brilliant Blue (CBB) staining

Coomassie Brilliant Blue R-250 (0.1% (v/v)) (Biorad, USA) was used to stain the gels overnight (Sambrook *et al*., 1989). The following day, the gels were de-stained in 15% methanol (v/v) and 15% acetic acid (v/v) until a clear background was obtained. The gels were finally stored in a storage solution for image analysis (Table 2.4).

Table 2.4: Coomassie Brilliant Blue (CBB) staining method.

Step	Solutions	Amount	Time
Staining	CBB	0.1 mg	30 mins
	Destaining solution	1000 ml	
Destaining	Methanol	500 ml	variable
	Acetic acid	50 ml	
Storage	Acetic acid	50 ml	variable

2.7.4 MALDI-TOF mass spectrophotometry and bioinformatic analysis

The stained gels were scanned using the LabScan version 5.0 (GE Healthcare, Darmstadt, Germany). Images of the gels were captured and analysed using ImageMaster™ 2D Platinum Software version 5.0 (GE Healthcare, Darmstadt, Germany). Protein spots were picked for further analysis and identification using Ettan Spot Picker (GE Healthcare, Darmstadt, Germany). Each of the excised gel plugs were transferred into a microcentrifuge tube containing 200 µl deionised distilled water in order to keep them hydrated prior to analysis at the Biomolecular Research Facility, University of Newcastle, Australia for MALDI-TOF MS analysis and mass spectrometry analysis using Ettan MALDI-TOF Pro (GE Healthcare, Darmstadt, Germany).

Protein identification was based on peptide mass fingerprints obtained from MALDI-TOF mass spectrometer. The mass lists obtained were submitted to database search using MASCOT (http://www.matrixscience.com/) (Matrix Science, London, UK) as the search engine. The searching criteria exploited carboxymidomethylation of cysteine as fixed modification and oxidation of methionine as variable modification. In all searches, one missed cleavage per peptide was allowed and an initial peptide tolerance

of 50 ppm was used. All the searches were performed using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/) and a non-redundant NCBI library database comprising annotated proteins of *B. pseudomallei* K96243.

Functional class assignment of the identified proteins was based on cluster of orthologous groups of proteins (COGs) functional categories (http://www.ncbi.nlm.nig.gov/COG/old/palox.cgi?fun=all). In silico analysis was performed by utilising PSORTb v.2.0 (http://www.psort.org/psortb2/index.html), to predict the cellular location of the identified proteins, SignalP v.3.0 (http://www.cbs.dtu.dk/services/SignalP/) to infer the presence of signal peptides in the proteins and TMHMM v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) to predict the transmembrane proteins topology with a hidden Markov model. Protein similarities with other closely related bacteria including *B. thailandensis* and *B. mallei* were also performed using BLAST analysis.

2.7.5 Identification of immunogenic proteins using Western blot of 2D gels

The separated proteins on replicate 2D gels were transferred onto nitrocellulose membranes in a semi-dry transfer apparatus (GE Healthcare, Darmstadt, Germany) using 0.9 mA/cm^2 for two hours with at room temperature. Following transfer, the membranes were blocked with 3% gelatine in PBS for one hour and washed 2X (15 mins each) in PBST. The membranes were then incubated with 1:1000 dilutions of mice anti-*B. pseudomallei* secreted proteins sera for two hours, followed by incubation with 1:5000 dilution of alkaline phosphatase conjugated rabbit anti-mouse IgG secondary antibody (CalBiochem) in PBST for two hours. The membranes were

developed using Western Blue Stabilising Substrate (Promega). Unimmunised mice serum was used as the negative control.

The protein spots detected on the Western blot membrane were scanned and matched with the corresponding 2D gel of *B. pseudomallei* secreted proteins. Correlating spots were collected and analysed at Biomolecular Research Facility, University of Newcastle, Australia and bioinformatics analysis was performed as previously described in Section 2.7.3.

2.8 Gene expression studies

The HumanHT-12 v4 Expression BeadChip (llumina, USA), which contains more than 47,000 probes targeting >22,000 genes derived from the National Center for Biotechnology Information Reference Sequence (NCBI) RefSeq Release 38 (November 7, 2009) and other sources, was used as the platform of choice to investigate gene expression of the host (human lung epithelial cells, A549) in response to *B. pseudomallei* live bacteria or its secreted proteins (Figure 2.8). The protocol to investigate the differentially regulated host genes was performed using:

Step I: Exposure of *B. pseudomallei* live bacteria or secreted proteins to A549 cells

Stpe II: RNA extraction from the A549 cells,

Step III: Preparation of cRNA for hybridization on the microarray chip,

Step IV: Microarray analysis

Step V: Validation of the microarray results using the quantitative real time PCR (qRT-PCR) analysis.

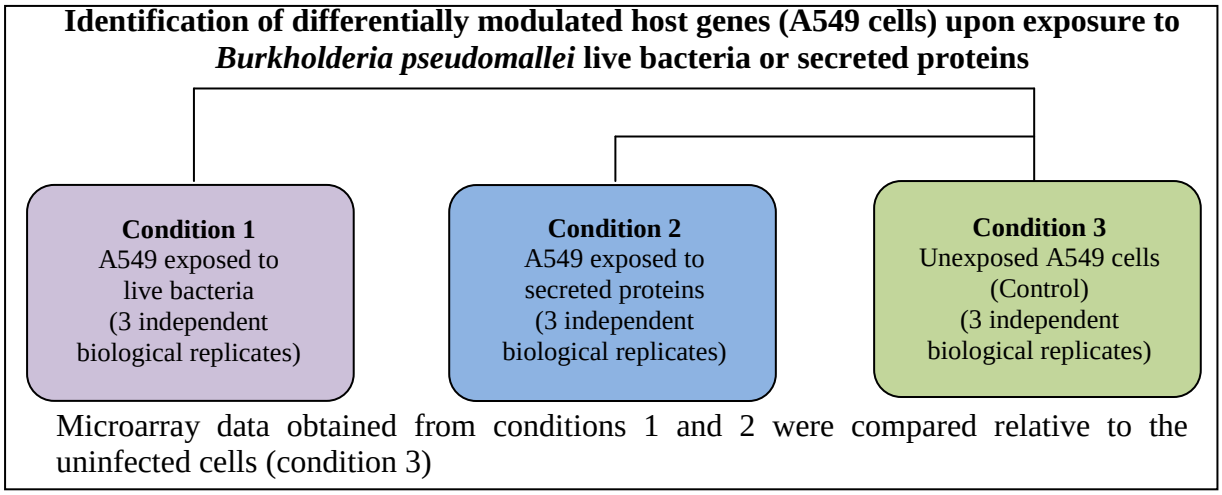


Figure 2.8: Schematic representation of the experimental design for the gene expression studies.

Exposure of A549 cells to live *B. pseudomallei* or secreted proteins was performed using A549 cells (1 X 10^6 cells/ml) seeded into T25 tissue culture flasks. The cells were grown to confluency (1 X 10^7 cells/ml) at 37ºC with 5% CO_2 after which, the monolayers were washed three times with PBS and exposed to *B. pseudomallei* live bacteria at MOIs of 1:10, 1:100 and 1:200, or filter sterilized secreted proteins at concentrations of 0.5, 1, 2, 5, 10, 25, 50 and 100 µg/ml. Following three hours of exposure at 37ºC with 5% CO_2, the cells were viewed using an inverted microscope at 40X, to examine any cytopathic effect produced. The cells in each of the flasks were then washed three times with PBS, trypsinised using 0.1% trypsin, collected into RNase-free microcentrifuge tubes and centrifuged at 300*g* for 5 mins. The resulting pellets were washed 3× with PBS through centrifugation at 300*g* for 5 mins and subjected to cell viability assay using trypan blue exclusion method. Briefly, the cells were resuspended in 1 ml of PBS and 20 µl of suspension was mixed with 80 µl of 0.4%

(w/v) Trypan blue solution. Ten microlitre of the Trypan blue-cell suspension mixture was transferred to the hemocytometer and the numbers of viable cells were counted.

$$\text{Cell viability (\%)} = \frac{\text{number of viable cells (unstained)}}{\text{total number of cells (stained and unstained)}} \times 100$$

Three replicate flasks containing the confluent monolayer were used as biological control for each of the different MOIs of live bacteria or secreted proteins used. Negative control (unexposed A549 cells) included six replicate flasks. The MOI (1:10) and secreted proteins concentration (5 µg/ml) that gave 95-100% cell viability was selected for the microarray experiment.

RNA extraction was performed using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instruction. All plasticwares and glasswares were treated overnight with 0.01% (v/v) DEPC (Sigma-Aldrich, USA) followed by autoclaving or surface decontamination, prior to RNA extraction. Briefly, A549 cells exposed *B. pseudomallei* live bacteria or the secreted proteins and control were trypsinised individually and pelleted by centrifugation at 300*g* for 5 mins, following which 600 µl of RTL buffer was added into each tube to lyse the cells. The lysate was then homogenized by passing it five times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. One volume (600 µl) of 70% ethanol was added into the lysate, transferred to an RNeasy mini spin column and centrifuged at ≥8000*g* for 15 sec. The flow-through was discarded and any DNA contamination was removed using 80 µl DNase I incubation mix followed by incubation at room temperature for 30 mins. Buffer RW1 was then added and the columns were centrifuged at ≥8000*g* for 15 sec and the flow-through was discarded. The column was washed with 500 µl of RPE buffer through centrifugation at ≥8000 *g* for 15 sec and the RNA was eluted using 50 µl of RNase-free water through centrifugation at ≥8000*g* for 1 min.

Quality control of the RNA was performed using two quality control measures: 1) a spectrophotometric analysis using the NanoPhotospectrometer (Implen, Germany) to confirm the concentration and assess the purity, and 2) a size fractionation procedure using capillary electrophoresis, *i.e*., the Bioanalyser (RNA 6000 Nano, Agilent, USA). Briefly, 3 µl of RNase-free water was pipetted onto the LabelGuard Microliter Cell of the NanoPhotospectrometer as blank, followed by 3 µl each of the sample to obtain the concentration of total RNA. The ratio of $A_{260\ nm}/A_{280\ nm}$ was also obtained as an indication of the purity. Good quality RNA will have $A_{260/280}$ ratio of 1.8-2.0 and $A_{260/230}$ ratio of ≥1.8, which indicate that the RNA sample was devoid of any protein, salt and solvent contaminations. Generally for microarray experiments, $A_{260/280}$ ratio of ≥1.8 and $A_{260/230}$ ratio of ≥2.0 generated better results compared to samples with lower ratios. Further confirmation of the concentration and purity was carried out using the Bioanalyser. A RIN of 7 or greater is necessary for a sample to be used for microarray. Thus, samples with RNA integrity number (RIN) ≥8 were selected. The RNA sample was aliquoted into 10 µl and stored in -80ºC until further use.

Preparation of cRNA for hybridization on the microarray chip was performed using the Illumina TotalPrep RNA Amplification Kit (Ambion, USA), according to the manufacturer's instructions (Figure 2.9). Briefly, the reverse transcription master mix was prepared in a nuclease-free tube at room temperature (Table 2.5) and nine µl of the master mix was added into 500 ng of total RNA. Following two hours incubation at 42°C, 80 µl of the second strand master mix (Table 2.6) was added and incubated for a further two hours at 16°C. Following incubation, the tubes were immediately placed on ice and 250 µl of cDNA Binding Buffer was added, pippeted into cDNA filter cartridge, centrifuged at 10,000 *g* for 1 min and the flow-through was discarded. The filter cartridge was washed using 500 µl of wash buffer through centrifugation at 10,000g for

~1 min and 20 µl of nuclease-free water preheated to 55°C was added. The double-stranded cDNA was eluted by centrifugation at 10,000*g* for 1 min.

cRNA was synthesised by adding 7.5 µl of the *In vitro* Transcription Master Mix (Table 2.7) and incubated at 37°C for 14 h. Following incubation, the reaction was stopped by adding 75 µl nuclease-free water and 350 µl of cRNA binding buffer was added followed by 250 µl of 100% ethanol. The sample was transferred into the cRNA Filter Cartridge and centrifuged at 10,000g for 1 min. The filter cartridge was washed with 650 µl of wash buffer through centrifugation at 10,000g for ~1 min and 200 µl of nuclease-free water preheated to 55°C was added. The purified cRNA was eluted by centrifugation at 10,000*g* for 1.5 mins.

Quality control of the cRNA was performed using two quality control measures: 1) a spectrophotometric analysis using the NanoPhotospectrometer (Implen, Germany) to confirm the concentration and assess the purity, and 2) a size fractionation procedure using a capillary electrophoresis, i.e. the Bioanalyser (RNA 6000 Nano, Agilent, USA) as described in the quality control of RNA**.**

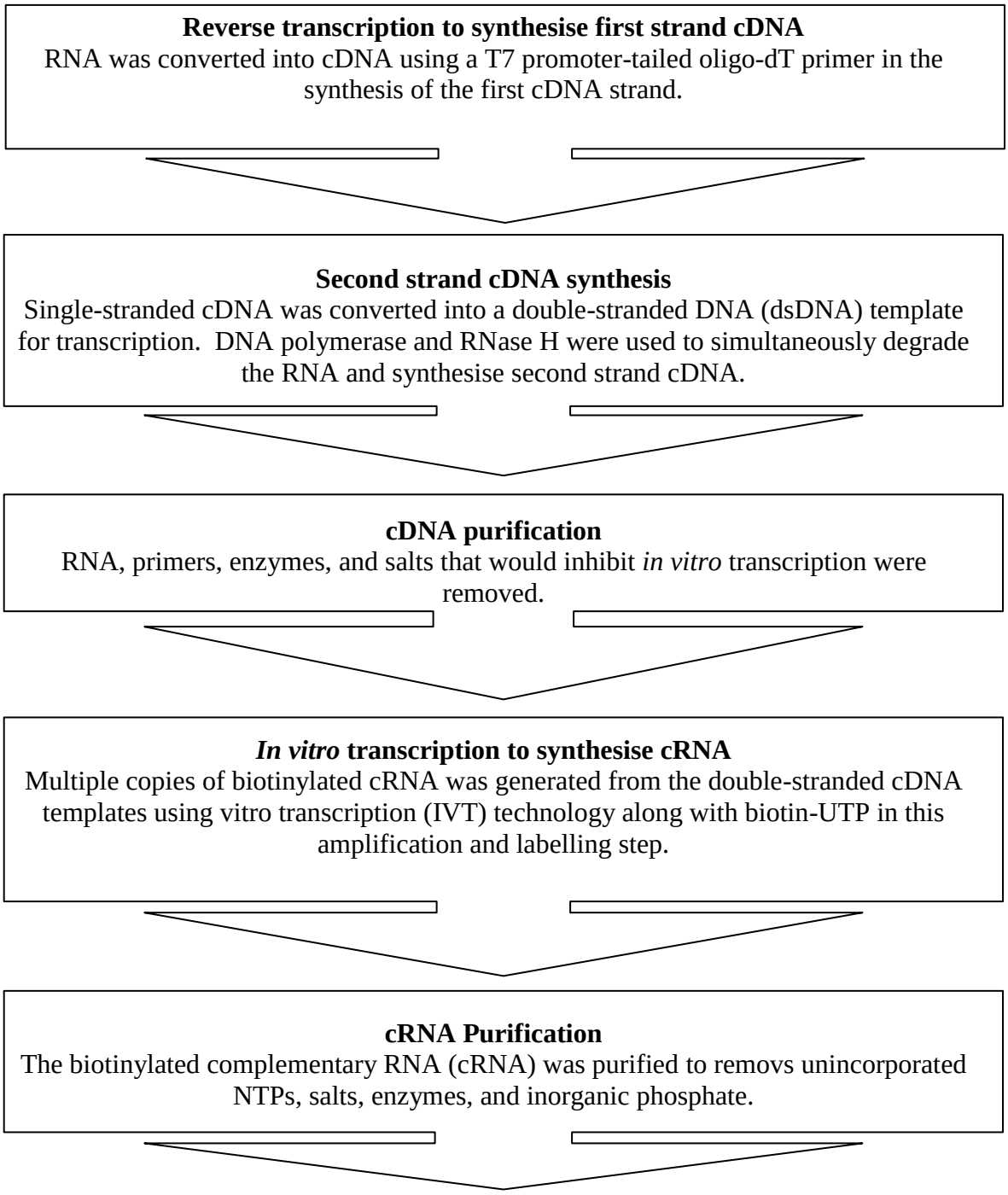


Figure 2.9: Illumina TotalPrep RNA amplification procedure.

Table 2.5: Reverse transcription master mix (for 9 samples) with 5% overage.

	Reagent volume/ reaction (µl)	**Reagent volume/ 9 reaction (with necessary overage) (µl)**
T7 Oligo(dT) Primer	1	9.45
10X First Strand Buffer	2	18.9
dNTP Mix	4	37.8
RNase Inhibitor	1	9.45
ArrayScript	1	9.45
Total	**9**	**85.05**

Table 2.6: Second strand master mix (for 9 samples) with 5% overage.

Component	**Reagent volume/ reaction (µl)**	**Reagent volume/ 9 reaction (with necessary overage; µl)**
Nuclease-free water	63	595.35
10X Second Strand buffer	10	94.5
dNTP Mix	4	37.8
DNA Polymerase	2	18.9
RNase H	1	9.45
Total (µl)	**80**	**756**

Table 2.7: *In vitro* transcription master mix (for 9 samples) with 5% overage.

Component	**Reagent volume/ reaction (µl)**	**Reagent volume/ 9 reaction (with necessary overage; µl)**
T7 10X Reaction buffer	2.5	23.63
T7 Enzyme Mix	2.5	23.63
Biotin-16-UTP	2.5	23.63
Total	**7.5**	**70.89**

Microarray analysis was performed using the Whole-Genome Gene Expression Direct Hybridization Assay. In general, the cRNA samples, as purified above, were applied to the arrays on the BeadChip and the BeadChip was hybridised at 58°C overnight. Signal was developed with streptavidin-Cy3 and the BeadChip was scanned with the Illumina BeadArray Reader (Illumina, USA) (Figure 2.10).

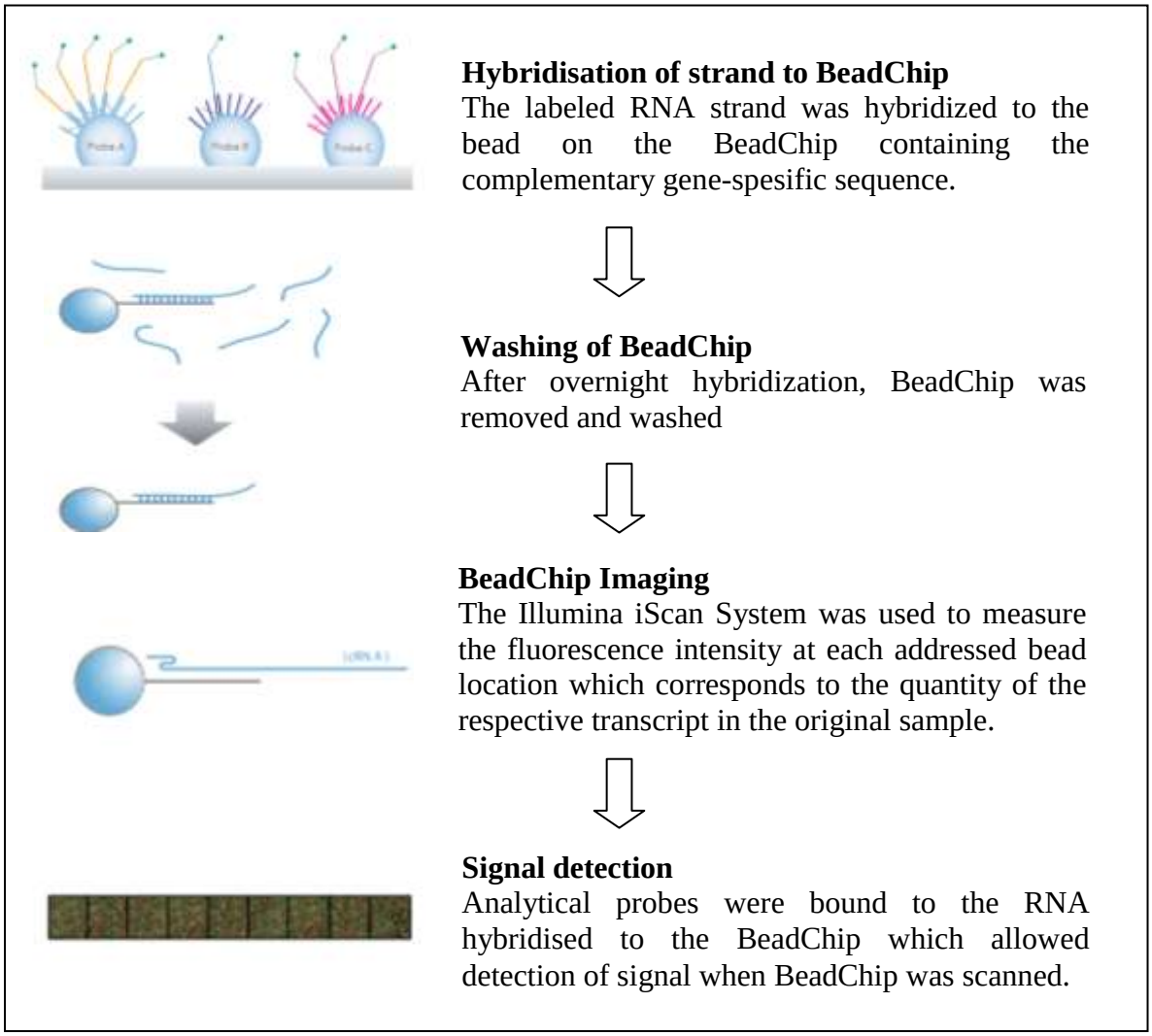


Figure 2.10: Flowchart of overview of the workflow for the microarray analysis using Direct Hybridization Assay (Adapted and modified from Whole-Genome Gene Expression Direct Hybridization Assay Guide, Illumina)

Briefly, the Illumina Hyb chamber gaskets were placed into the BeadChip Hyb chamber and 200 µl of HCB was dispensed into each of the two humidifying buffer reservoirs in the Hyb chamber. The cRNA sample tubes were preheated at 65°C for 5 mins and allowed to cool to room temperature. cRNA (750 ng) was pipetted into the hybridisation tube and up to 5 µl of RNase-free water was added. Ten microliter of the HYB mix was added to each of the cRNA sample. The BeadChips were placed in the Hyb chamber insert and 15 µl of the samples were pipetted to the center of each inlet port. The Hyb chamber inserts were loaded into the Hyb chamber and placed into the Illumina Hybridization Oven and incubated at 58°C for 18 h.

Following hybridisation, the BeadChip was submerged in one liter of Wash EIBC buffer and immediately transferred into 250 ml Wash E1BC solution. The BeadChip was then transferred into the High-Temp Wash buffer for 10 mins followed by the first room temperature wash in 250 ml fresh Wash E1BC and shaked at medium speed for 5 mins. Subsequently, an ethanol wash in 250 ml fresh 100% ethanol was performed for 10 mins at room temperature followed by the second room temperature wash in 250 ml fresh Wash E1BC buffer with shaking a for 2 mins.

The BeadChip was then transferred into 4 ml of Block E1 buffer and incubated with shaking at medium speed for 10 min. Following incubation, the BeadChip was transferred to a fresh wash tray containing Cy3-Streptavidin and shook at medium speed for 10 mins after which the third room temperature wash was performed in 250 ml fresh Wash E1BC buffer with medium shaking for 5 mins at room temperature. The BeadChip was then centrifuged at 1,400 rpm for 4 mins at room temperature and stored in a dark, ozone-free environment until ready to scan.

The BeadChip was scanned on the Illumina BeadArray Reader and the resulting image of decoded gene expression data was then subjected to further analysis using the GenomeStudio Gene Expression Module (Illumina, USA).

Analysis of microarray data was performed using Illumina's GenomeStudio Gene Expression Module. The initial step of the microarray data analysis was the quality control of the data in order to determine the quality of the hybridization. The Illumina GenomeStudio supports an easy and powerful data quality control using the internal controls present on the HumanHT-12 v4 Expression BeadChip. The quality control allows identification of outliers, the potential cause of outlier data and also aid to determine if a sample needs to be repeated or meets the expected quality standards.

Following quality control, raw microarray data were extracted whereby the signal intensity image decoded using BeadArray Reader was converted to numerical data. The raw microarray data was then recovered and subjected to standard normalization procedure for one-colour array data using GeneSpring GX version 11 (Agilent Technologies, USA). The data was normalised by dividing intensity of each probe by the median intensity for all samples. The Box Plot was then used to check for the presence of any outliers and sample hierarchical clustering was carried out. Statistical analysis (One-Way ANOVA) was used to obtain the number of significant differentially expressed genes ($p \leq 0.05$). The data were then filtered using the Volcano Plot to obtain the differentially expressed genes with an absolute change greater than 2-fold relative to the uninfected control.

A few of the free web-based software available was used for further analysis of the set of differentially expressed genes. The GeneSet Analysis (http://www.bioinfo.vanderbit.edu/) was used to identify Gene Ontology (biological process, molecular function and cellular component) of the differentially expressed

genes. The pathways that are significantly regulated by the genes were also identified using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) mapper database (http://www.genome.jp/kegg/). GOTerm Finder (http://go.princeton.edu/cgi-bin/GOTermFinder), GeneTrail (http://genetrail.bioinf.uni-sb.de/) and GATHER (http://gather.genome.duke.edu/) were also used to analyse significant pathways. The Cluster 3.0 and Java Treeview V1.1.3 softwares were used for hierarchical clustering and visualisation of the differentially expressed genes, respectively.

Validation of microarray data was performed using quantitative real-time PCR (qRT-PCR) analysis. Ten genes, including eight genes that were significantly regulated in the microarray analysis and two reference genes, were identified to be used for the validation. The genes identified were involved in functions, including chemokine receptors, protease inhibitors and complement activation. The genes *β-actin* and *GAPDH* were used as reference genes for normalisation. Primers for the ten genes identified were selected from a public resource for PCR primers, the PrimerBank (http://pga.mgh.harvard.edu/primerbank/) based on the amplicon size between 100bp - 200bp, which is the recommended fragment size for real-time PCR analysis (Table 2.8). All primers selected were synthesised at Nano Life Quest Laboratories (Malaysia).

Validation of the microarray data was performed using:

i) Gradient conventional PCR in order to determine the optimal temperature for the qRT-PCR
ii) Gradient qRT-PCR in order to identify the optimal temperature for the gene expression analysis.
iii) qRT-PCR to generate standard curve for each of the primers in order to determine primer efficiency
iv) qRT-PCR for gene expression analysis

Table 2.8: Primer sequences of the genes used for microarray validation

Primer	Sequence (5' – 3')	Amplicon size (bp)
B-actin (F) *β-actin* (R)	CAC CTT CAC CGT TCC AGT TT GAT GAG ATT GGC ATG GCT TT	102
GAPDH (F) *GAPDH* (R)	TGT TGC CAT CAA TGA CCC CTT CTC CAC GAC GTA CTC AGC G	102
G6PC2 (F) *G6PC2 (*R)	CAG AAG GAC TAC CGA GCT TAC T CCA ATC CCC AAT GAC TGC TAC	153
CES1 (F) *CES1* (R)	CAA GGC GGG GCA GTT ACT C TTT CTT GGT CAA GTC AGC AGG	118
CXCR7 (F) *CXCR7* (R)	TCT GCA TCT CTT CGA CTA CTC A GTA GAG CAG GAC GCT TTT GTT	130
LAYN (F) *LAYN* (R)	GCG TGG TCA TGT ACC ATC AG AGG TGT TGT CAG CTC TGT TTC	176
SERPINA3 (F) *SERPINA3* (R)	CCT GAA GGC CCC TGA TAA GAA GCT GGA CTG ATT GAG GGT GC	196
PYCARD (F) *PYCARD* (R)	TGG ATG CTC TGT ACG GGA AG CCA GGC TGG TGT GAA ACT GAA	110
FXYD2 (F) *FXYD2* (R)	ATC CTC CTC AGT AAG TGG GGT CTT GGC AAC TCC CGA AAG C	101
FST (F) *FST* (R)	ACG TGT GAG AAC GTG GAC TG CAC ATT CAT TGC GGT AGG TTT TC	151

F – forward primer; R - reserve primer

i) Prior to the qRT-PCR, all the primers selected for validation were optimized using the gradient conventional PCR. All PCR components were mixed accordingly (Table 2.9) and PCR was performed using a temperature gradient of 50-60ºC (Table 2.10). Agarose gel electrophoresis was performed on a 1.5% agarose gel at 90 V for 1 h. Five µl of each PCR product was mixed with one µl of 6X Loading Dye and the mixture was loaded into the wells in the agarose gel. A 100 bp ladder (iDNA, Singapore) was used as marker. Electrophoresis was performed at 90 V for 60 mins. The amplified DNA bands were visualized following staining with SybrSafe (Invitrogen) stain in the Gel Documentation System (Biorad, USA) under ultraviolet (UV) illumination.

ii) Gradient qRT-PCR was carried out using the primers with three different annealing temperatures (56ºC, 58ºC and 60ºC) in the ICycler IQ thermocycler (Biorad, USA). qRT-PCR master mix was prepared for each primer (Table 2.11) and 24 µl of the mixture was added with one µl of the respective templates. For the non-template control (NTC), template was substituted with one µl of RNase-free water as the negative control. qTR-PCR for each temperature was performed in triplicates (Table 2.12). The temperature that showed the optimal amplification was identified to be used in the following steps.

Table 2.9: Preparation of PCR reaction mixture.

Reaction component	**Volume (µl)**
5X Premix containing Taq DNA Polymerase, dNTP, buffer and $MgCl_2$	5
Reserve primer (10 µM)	0.5
Forward primer (10 µM)	0.5
Nuclease free water	17
DNA Template Premix reaction buffer (5X)	2
Total	**25**

Table 2.10: Cycling condition for the conventional PCR

Steps	**Running conditions**	**Time**
Initial denaturation	94ºC	2 minutes
Denaturation	94ºC	30 seconds
Annealing (Gradient)	50-60ºC	30 seconds
Elongation	72ºC	1 minute
Repeat steps denaturing, annealing and elongation	72ºC	10 minutes (30 cycles)
Extended elongation	72ºC	8 minutes

Table 2.11: Quantitative real-time PCR reaction master mix.

Reaction component	**Volume per reaction**
2X QuantiTech SYBR green	12.5 µl
RNase free water	10.5 µl
Forward primer (10 µM)	0.5 µl
Reverse primer (10 µM)	0.5 µl

iii) The primer efficiency was determined using 5-fold serially diluted cDNA of the A549 cells ranging from 30 ng/µl to 500 ng/µl. Twenty four µl of the master mix prepared was added with one µl of the template of each concentration (Table 2.11) and subjected to qRT-PCR amplification (Table 2.13). RNase-free water was used as the non-template control (NTC) (negative control). A standard curve with an acceptable slope equation (–3.3 to –3.1), correlation coefficient (0.9601-1) and percentage of efficiency (90-110%) for each primer was obtained.

iv) The qRT-PCR assay was performed on the RNA samples used in the microarray analysis. Twenty four µl of master mix containing each of the primer pairs (Table 2.11) was added with 1 µl of template and subjected to qRT-PCR amplification (Table 2.13). The qRT-PCR analysis was performed in triplicate using templates generated from RNAs extracted from independent experiments. An intra-plate control (IPC) using the housekeeping gene primers (*GAPDH)* with untreated template were also included in each of the qRT-PCR run for normalization purposes. NTC was also included for each set of primer tested.

The raw Ct values were obtained using the IQ5 real-time PCR software (Biorad, USA). Ct value of the housekeeping genes were analysed using different algorithms (Silver *et al*. 2006; Andersen *et al*. 2004; Brunner *et al*. 2004). The fold changes of the expression of the genes under both the BCMS and CCMS conditions were calculated using standard delta-delta-Ct method (Pfaffl, 2001).

Table 2.12: Gradient quantitative real-time PCR running parameter

Steps	Running conditions	Time	No. of cycle (s)
Initial denaturation	95ºC	3 minutes	1
Denaturation	95ºC	30 seconds	35
Annealing	56ºC, 58ºC and 60ºC	30 seconds	
Elongation	72ºC	50 seconds	
Extended elongation	72ºC	10 minutes	1
Determination of melting temperature for the respective amplicon	72ºC with increasing set-point temperature after cycle 2 by 0.5ºC	5 seconds	61

Table 2.13: Quantitative real-time PCR running condition

Steps	Running conditions	Time	No. of cycle(s)
Initial denaturation	95ºC	3 minutes	1
Denaturation	95ºC	30 seconds	35
Annealing	60ºC	30 seconds	
Elongation	72ºC	50 seconds	
Extended elongation	72ºC	10 minutes	1
Determination of melting temperature for the respective amplicon	72ºC with increasing set-point temperature after cycle 2 by 0.5ºC	5 seconds	61

CHAPTER 3

RESULTS

3.1 *Burkholderia pseudomallei* growth curve

Growth curve analysis at OD_{600nm} of *B. pseudomallei* CMS performed over 24 h in the LB medium revealed a typical growth. An initial lag phase of approximately four hours was observed. Following the lag phase, the optical density of the culture increased rapidly during the log phase and reached stationary phase at 16 h (Figure 3.1).

A good correlation (>95%) was also observed between the optical density value of the bacterial culture and the viable count of the bacteria over the 24 h (Figure 3.1). The initial inoculum used was 1.31±1.41 X 10^2 cfu/ml corresponding to OD_{600nm} of 0.01±0.002. The growth was slow during the first four hours, indicating a lag phase of growth. A rapid increase was observed from four up to 16 h, when the bacteria were at the logarithmic phase of growth with OD_{600nm} of 0.075±0.001 (2.67±0.06 X 10^3 cfu/ml) increasing to 1.531±0.009 (9.97±0.02 X 10^7 cfu/ml). After 16 h, the optical density of the culture started to decrease slightly when the bacteria entered the stationary phase of growth. At 20 h and 24 h, the bacterial growth was 1.565±0.006 (2.25±0.07 X 10^9 cfu/ml) and 1.653±0.006 (4.57±0.08 X 10^9 cfu/ml), respectively.

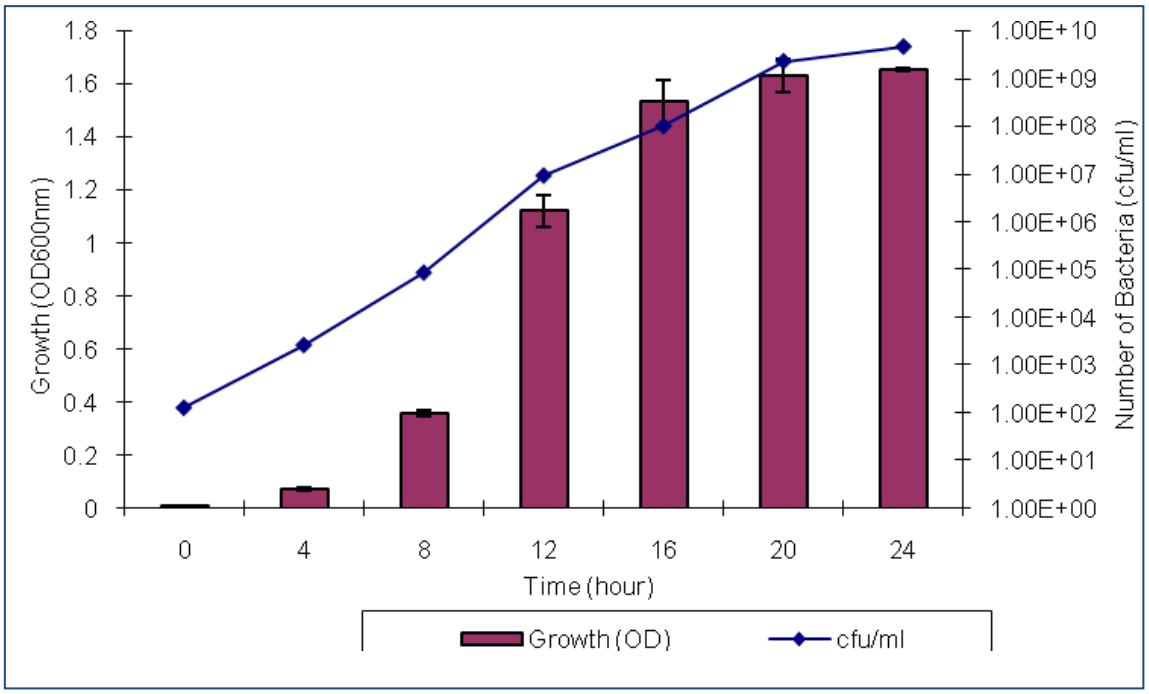


Figure 3.1: Growth curve of *B. pseudomallei* in LB broth. Data indicates OD_{600nm} and the corresponding number of bacteria (cfu/ml) measured over 24 h.

3.2 Concentration of protein in the culture supernatant

Protein concentration in the culture supernatant of *B. pseudomallei* CMS collected at different time-points of growth was determined using the Bradford method (1975). A standard curve was obtained using various bovine serum albumin (BSA) concentrations, ranging between 0 and 100 µg/ml. The protein concentration in the bacterial culture supernatant was found to increase in correlation with the duration of growth. The protein concentration increased from the initial concentration of 0.540±0.005 mg/ml to 0.248±0.006 mg/ml at four hours of growth. Subsequently, the protein concentration increased steadily during the logarithmic phase of growth to up to 0.556±0.012 mg/ml at 16 h of growth. As the culture entered the stationary phase of growth, the increase in the protein concentration was still observed, albeit at a lower level of 0.627±0.016 mg/ml at 20 h and 0.699±0.006 mg/ml at 24 h of growth (Figure 3.2).

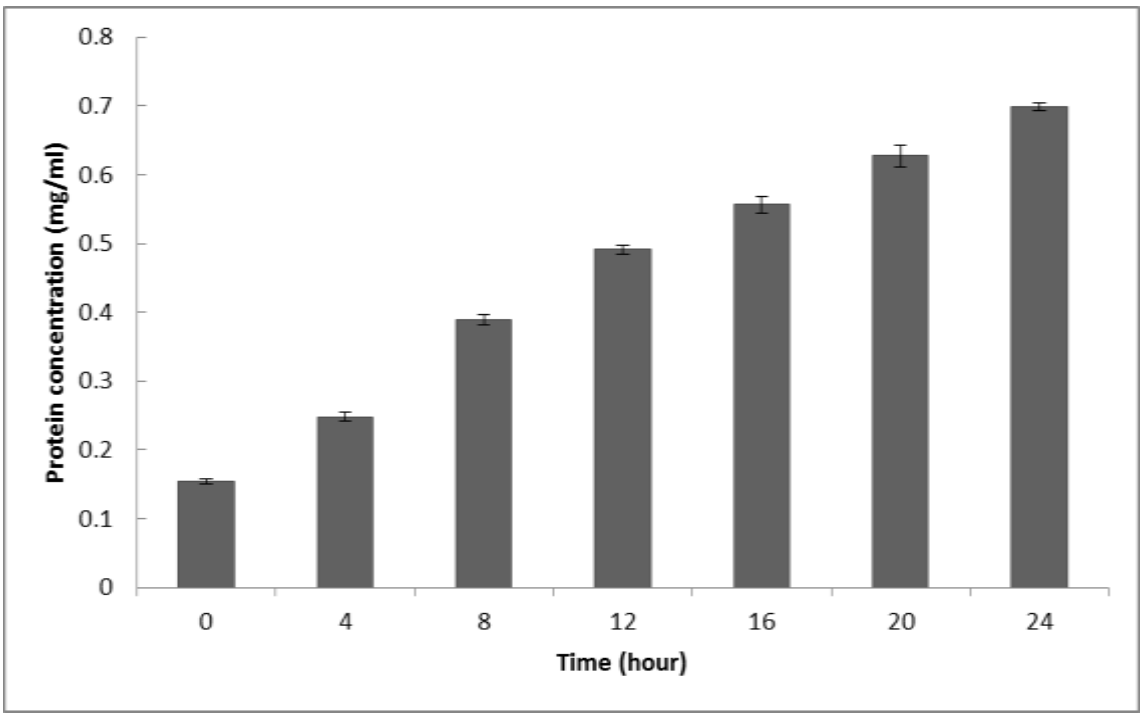


Figure 3.2: Protein concentrations of *B. pseudomallei* culture supernatant at various phases of growth. Protein concentration at each time-point of growth was determined in triplicate and the mean value was used. Error bars indicate standard deviation.

3.3 Virulence factors

3.3.1 Extracellular enzyme activity

Extracellular enzyme activities of proteases, phosphatases, phospholipase C, catalase, peroxidase and superoxide dismutase (SOD) were assayed every two hours. All six enzymatic activities were found in the culture supernatant of the *B. pseudomallei* isolate used in this study. It was also found that the first significant detection of any enzymatic activity was only detected in the culture medium at the fourth hour when the bacteria enter the logarithmic phase of growth. At this phase of growth, *B. pseudomallei* showed a similar profile of secretion of the protease, phosphatase and phospholipase C extracellular enzymes. The activities of these three enzymes were found to increase with the bacterial growth rate. After 16 h of growth, as the bacteria enters the stationary phase of growth, no further increase was observed in the activities of these enzymes.

Levels of the anti-oxidant enzymes, catalase, peroxidase and SOD were lower at the logarithmic phase of growth. However, the levels increased substantially following eight hours of growth. Overall, the activities of the anti-oxidant enzymes were significantly lower as compared to the activities of protease, phosphatase and phospholipase C. Activity of phospholipase C was found to be the highest of the enzymes assayed (0.2–27.00 Unit/ml) followed by protease (0.15–24.70 Unit/ml). The activity of phosphatase was found to be at the intermediate level (0.01–15.90 Unit/ml). The enzymes catalase (0–7.50 Unit/ml), SOD (0.01–4.05 Unit/ml) and peroxidase (0–3.00 Unit/ml) were found to be lowest in activity (Figure 3.3).

The levels of the intracellular enzyme isocitrate dehyrogenase (ICD) was also measured in order to ascertain that the extracellular enzymes activities measured above were genuinely secreted and not present in the culture supernatant due to cell lysis. The levels of ICD activity, despite increasing throughout the growth, was very low (0–0.152 Unit/ml) and insignificant (Figure 3.4).

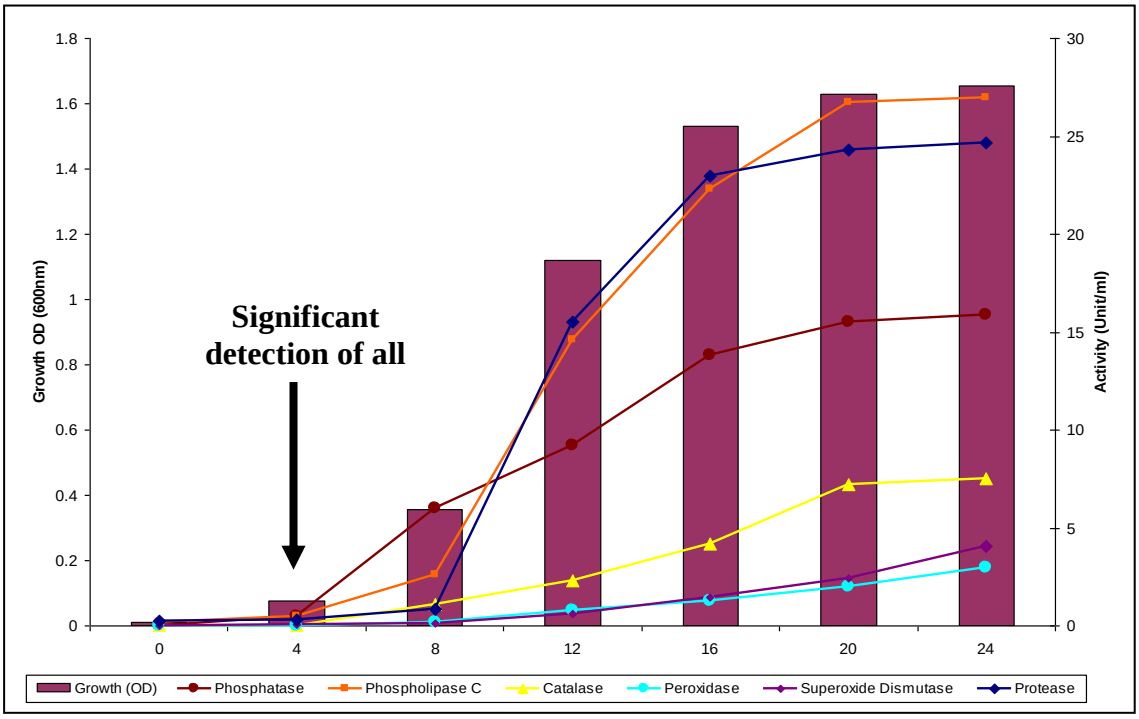


Figure 3.3: Extracellular enzyme activity of phospholipase C, protease, phosphatase, catalase, SOD and peroxidase in *B. pseudomallei* secretory proteins at different phases of growth.

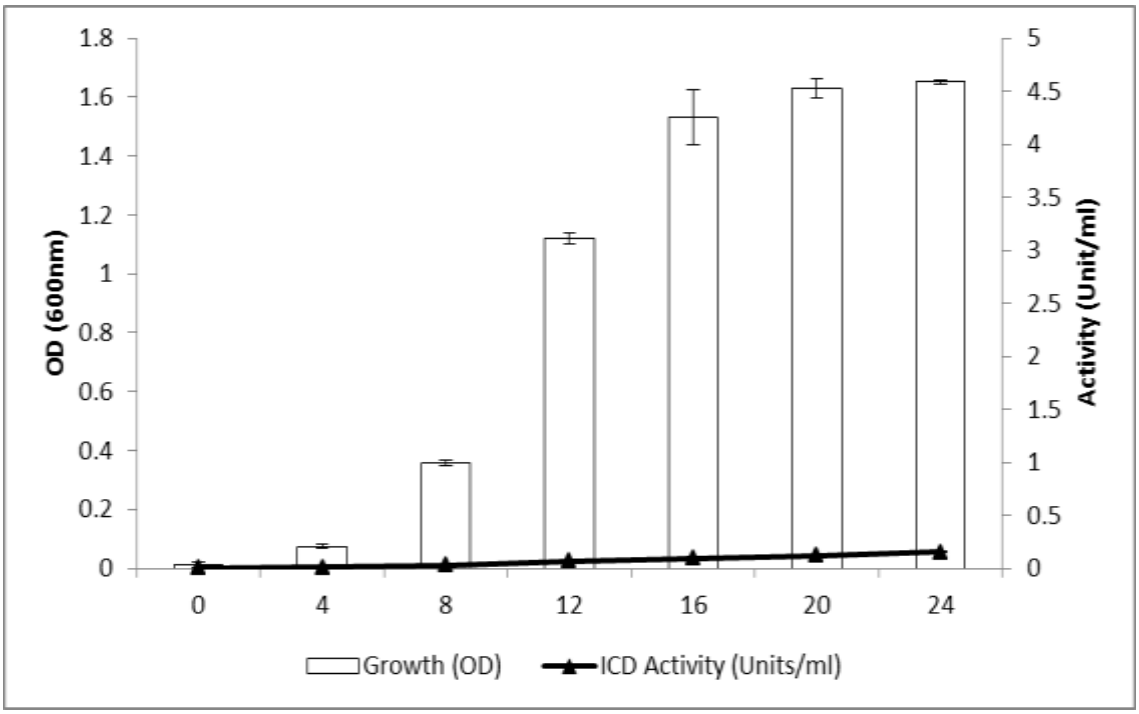


Figure 3.4: Extracellular enzyme activity of isocitrate dehydrogenase in *B. pseudomallei* secretory proteins at different phases of growth.

3.3.2 Invasion assay

The ability of *B. pseudomallei* to invade the human lung epithelial cells, A549, was determined using different multiplicity of infection (MOI) of 1:10, 1:100 and 1:200 (corresponding to 1 X 10^8 cfu/ml, 1 X 10^9 cfu/ml and 2 X 10^9 cfu/ml, respectively) over 1, 2, 3, 6, 12, 18 or 24 h post-infection to identify the invasion efficiency (in percentage) of *B. pseudomallei* (Table 3.1). The invasion efficiency of *B. pseudomallei* CMS was found to correlate with the inoculum size used, *i.e.*, the MOI. The invasion efficiency at one hour post-infection was found to be very low (0.000125± 0.00004% to 0.0002± 0.00011%) at all the MOIs used.

Generally, as the post-infection time increased from 1-12 h, a gradual increase in the invasion efficiency was observed at all the MOIs used. This was followed by a gradual decrease between 18 and 24 h post-infection. At MOI 1:10, invasion efficiency increased from 0.00232±0.00100% at three hours post-infection to 3.15±0.020000% at 12 h post-infection. However, the percentage of invasion decreased to 2.08± 0.070000% and 1.83±0.20000% at 18 and 24 h post-infection, respectively.

Invasion efficiency was also found to be increased from 0.00322±0.00085% at three hours post-infection to 3.28±0.09000% at 12 h post-infection, at MOI 1:100. However, the percentage of invasion decreased to 2.00± 0.05000% and 1.75± 0.15000% at 18 and 24 h post-infection, respectively.

Pattern of invasion similar to MOI 1:10 and 1:100 was also observed at MOI 1:200. Invasion efficiency was found to be increased from 0.0048±0.00093% at three hours post-infection to 3.65± 0.05200% at 12 h post-infection. However, the percentage of invasion decreased to 2.20±0.05000% and 1.82± 0.07500% at 18 and 24 h post-

infection, respectively. *E. coli* ATCC used as a negative control did not show any significant invasion into the epithelial cells.

Table 3.1: Invasion efficiency of *B. pseudomallei* at MOI 1:10, 1:100 and 1:200. Data presented as mean percentage of invasion (%) with standard deviation.

Hours	% Invasion (MOI 1:10)	% Invasion (MOI 1:100)	% Invasion (MOI 1:200)
1	0.000125±0.00004	0.000168±0.00002	0.0002±0.00011
2	0.00232±0.00100	0.00322±0.00085	0.0048±0.00093
3	0.385±0.01400	0.399±0.01400	0.535±0.01200
6	1.725±0.05500	1.888±0.02300	1.987±0.09900
12	3.15±0.020000	3.28±0.09000	3.65±0.05200
18	2.08±0.070000	2.00±0.05000	2.20±0.05000
24	1.83±0.20000	1.75±0.15000	1.82±0.07500

3.3.3 Intracellular survival assay

The ability of *B. pseudomallei* CMS to survive intracellularly in the human lung epithelial cells A549 at different MOIs and post-infection times are demonstrated in Figure 3.5. The intracellular survival at one hour post-infection was almost similar among the three different MOIs used i.e. $\log_{10}$ cfu values of 1.52, 1.66 and 1.80 for MOI 1:10, 1:100 and 1:200, respectively. Similarly, at two hours post-infection, no significant differences was observed among the three different MOI's used *i.e.*, $\log_{10}$ cfu values of 2.63, 2.90 and 2.98 for MOI 1:10, 1:100 and 1:200, respectively. However, at three and six hours post-infection, there was a marked difference between the MOI 1:10 ($\log_{10}$ cfu value of 3.29 at three hours and 4.42 at six hours post-infection) as compared to the MOI 1:100 ($\log_{10}$ cfu value of 3.91 at three hours and 5.39 at six hours post-infection) and 1:200 ($\log_{10}$ cfu value of 4.00 at three hours and 5.59 at six hours post-infection).

At 12 h post-infection, the number of intracellular bacteria observed at all three MOIs were almost the same with the $\log_{10}$ cfu values of 6.78, 6.95 and 6.99 at MOI 1:10, 1:100 and 1:200, respectively. However, all three MOIs showed decrease in the number of intracellular bacteria at 18 and 24 h post-infection. At MOI 1:10, there was a decrease of $\log_{10}$ cfu values of 0.41 and 1.03 at 18 and 24 h post-infection, as compared to 12 h post-infection. At MOI 1:100, a larger decrease of $\log_{10}$ cfu values of 0.89 and 1.59 was observed at 18 and 24 h post-infection, as compared to 12 h post-infection. MOI 1:200 demonstrated the largest decrease ($\log_{10}$ cfu values 1.08 and 2.00) at 18 and 24 h post infection, as compared to the 12 h post-infection.

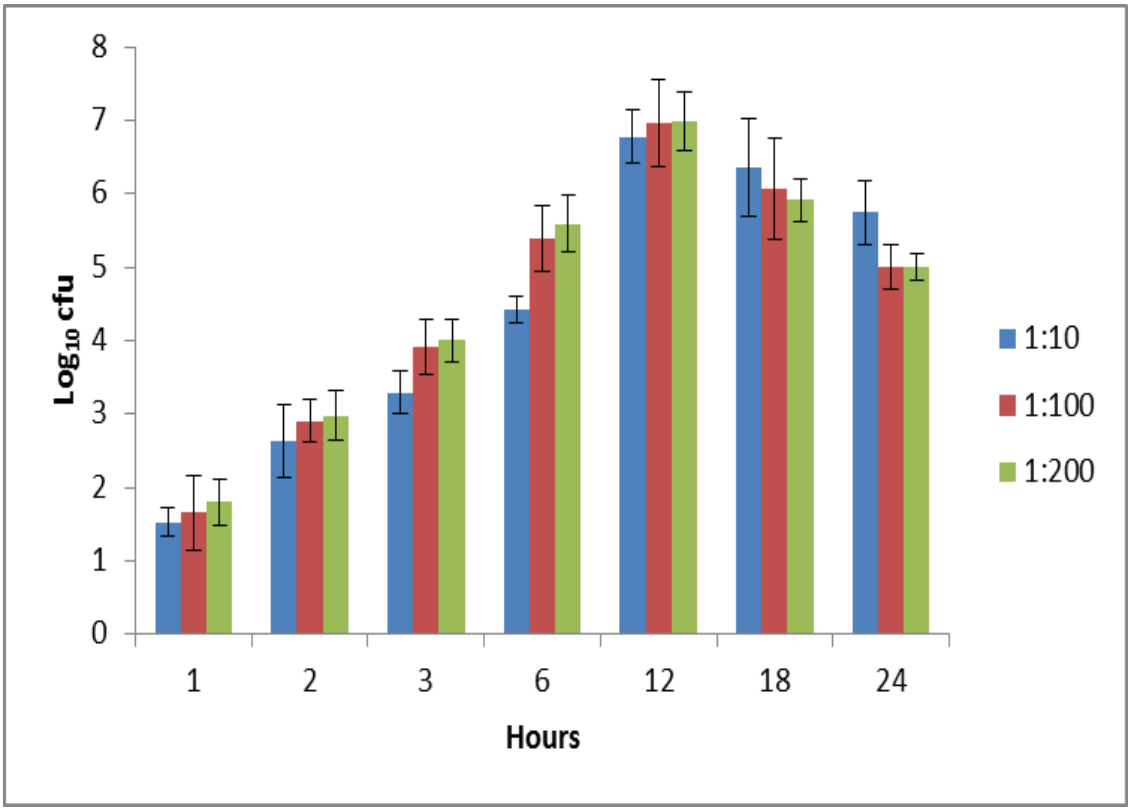
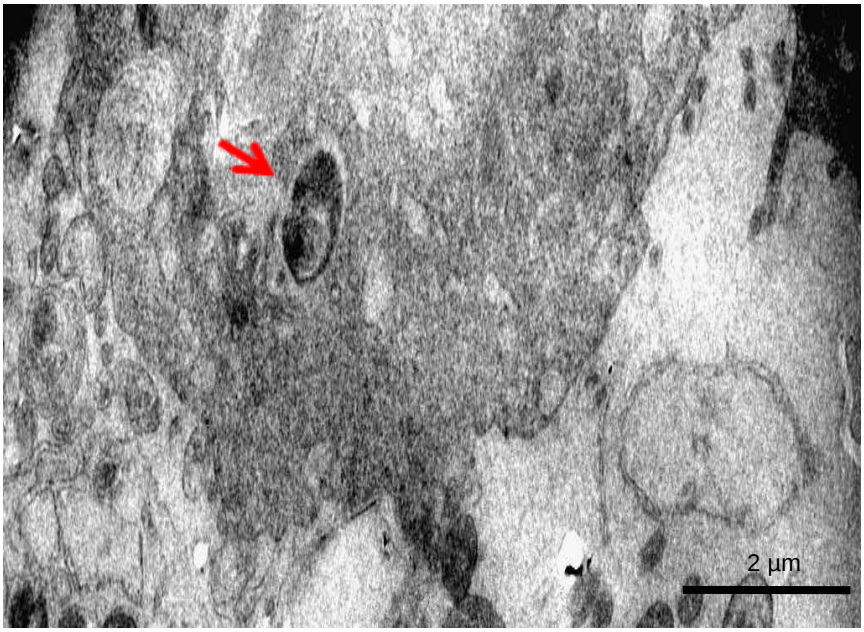


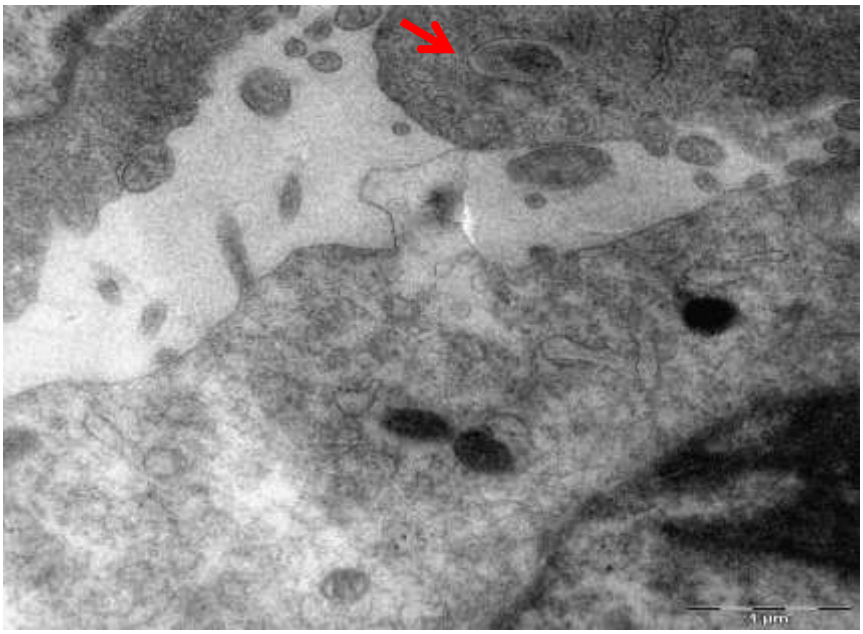
Figure 3.5: Intracellular survival of *B. pseudomallei* at different post-infection time points following two hours infection with the MOI of 1:10, 1:100 and 1:200. Error bars indicate standard deviation.

3.3.4 Transmission electron microscopy

Evidence for the presence of *B. pseudomallei* CMS in the cytoplasm of the A549 cells was observed using the TEM analysis. Panels A and B of Figure 3.6 show the transmission electron micrographs of *B. pseudomallei* at three hours infection and 24 h post-infection, respectively. Intracellular bacteria are clearly visible in cytoplasm of the cell. .



(A)



(B)

Figure 3.6: Transmission electron micrograph of *B. pseudomallei* CMS infecting the A549 human lung epithelial cell. A) three hours infection, and B) 24 h post-infection.

3.4 Protein analysis

3.4.1 Sample preparation

B. pseudomallei secreted protein samples were prepared using three different sample preparation methods, *i.e.*, the ultrafiltration method, the ammonium sulphate (AS) precipitation method and the trichloroacetic acid (TCA) precipitation method. Among these methods, TCA precipitation yielded the highest protein concentration compared to ultrafiltration and AS precipitation. The protein concentration of *B. pseudomallei* secreted proteins samples obtained were 0.18±0.02 mg/ml, 0.35±0.05 mg/ml and 0.38±0.10 mg/ml using ultrafiltration, AS precipitation and TCA precipitation, respectively (Table 3.2).

Table 3.2: Concentrations of *B. pseudomallei* secreted proteins

Protein extraction method	Mean protein concentration (mg/ml)
Ultrafiltration	0.18±0.02 (0.13 - 0.25)*
Ammonium sulphate precipitation	0.35±0.05 (0.27 - 0.43)*
Trichloroacetic acid precipitation	0.38±0.10 (0.33 – 0.45)*

* values in brackets are the range of the concentrations.

3.4.2 SDS-PAGE

SDS-PAGE analysis was carried out on the secreted protein samples prepared using the three different sample preparation methods. Two different concentrations, 15 µg and 30 µg of the protein from each of the different preparation were subjected to SDS-PAGE. The sample prepared using the ultrafiltration method did not yield any prominent bands on the 12% SDS PAGE gel at both the concentrations used. Similarly, AS precipitated proteins also did not yield very prominent bands at both the concentrations. TCA precipitation was found to be the most appropriate preparation method as protein separation was found to yield prominent, discrete and distinct bands (Figure 3.7).

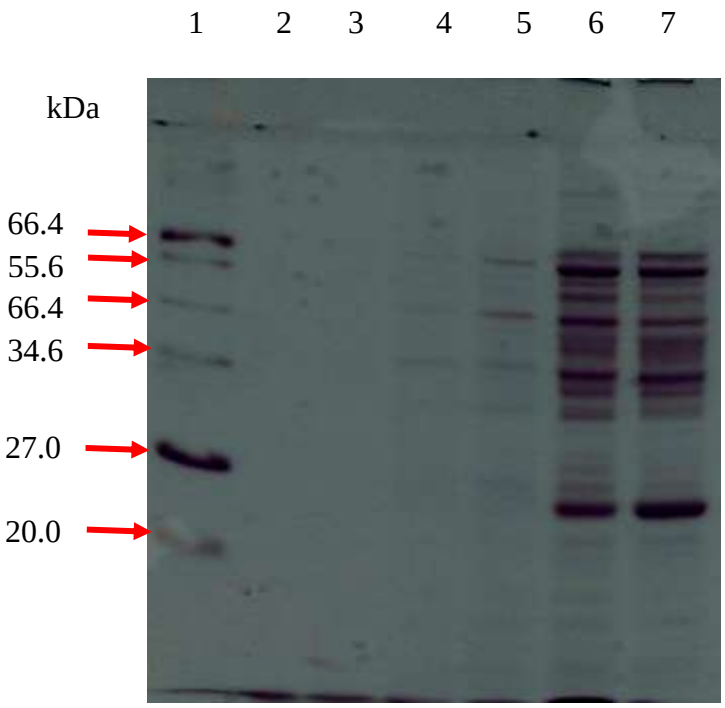


Figure 3.7: SDS-PAGE of *B. pseudomallei* stationary phase secreted proteins using 12% SDS-PAGE gel. L1: Protein marker, L2: *B. pseudomallei* secreted protein prepared by ultrafiltration (15 µg), L3: *B. pseudomallei* secreted protein prepared by ultrafiltration (30 µg), L4: *B. pseudomallei* secreted protein prepared by AS precipitation (15 µg), L5: *B. pseudomallei* secreted protein prepared by AS precipitation (30 µg), L6: *B. pseudomallei* secreted protein prepared by TCA precipitation (15 µg), L7: *B. pseudomallei* secreted protein prepared by TCA precipitation (30 µg).

3.4.3 Two dimensional gel electrophoresis

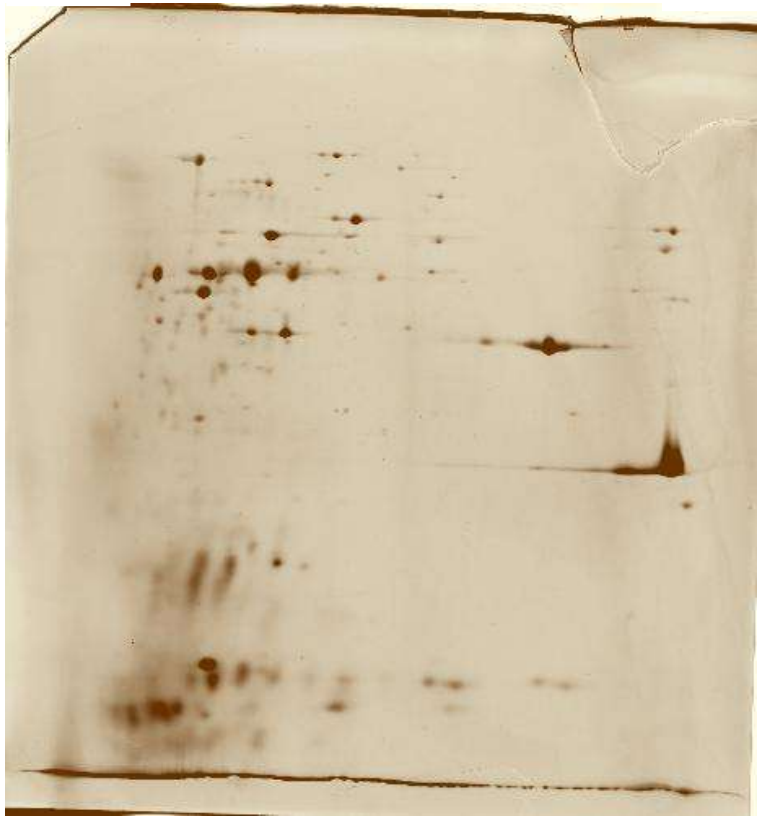
3.4.3.1 Optimisation of *B. pseudomallei* secreted protein concentration for proteomic analysis.

The *B. pseudomallei* secreted protein obtained through TCA precipitation was initially subjected to optimisation to determine the optimal concentration to be used for the proteomic analysis. Initial optimisation was carried out using different concentrations of the secreted proteins (150 μg, 250 μg, 350 μg and 450 μg) separated using IPG strip pH 3-10 and 12.5%SDS-PAGE gel. Both the silver staining and CBB staining methods methods were used for the initial visualization of the secreted proteins (Figure 3.8, panels A, B, C and D).

Approximately 125 protein spots were detected in the proteome profiles of *B. pseudomallei* mapped using 150 μg of stationary phase secreted proteins and visualized using silver staining (Figure 3.8, panel A). (It must be noted that at the point of time of this study, silver stain gels were not compatible with MALDI-TOF mass spectrometry analysis). Thus, the gels were stained with CBB using higher amount of the protein. However, when higher protein amounts were separated and visualized using CBB staining, 39, 52 and 113 spots were detected using 250 μg, 350 μg and 450 μg of proteins, respectively (Figure 3.8, panels B, C and D). Profiling of the *B. pseudomallei* stationary phase secreted proteins using 450 μg of proteins were selected for further analysis because the proteins visualized using CBB staining detected more prominent lower molecular weight proteins and increased number of protein spots as compared to the lower amounts of proteins used.

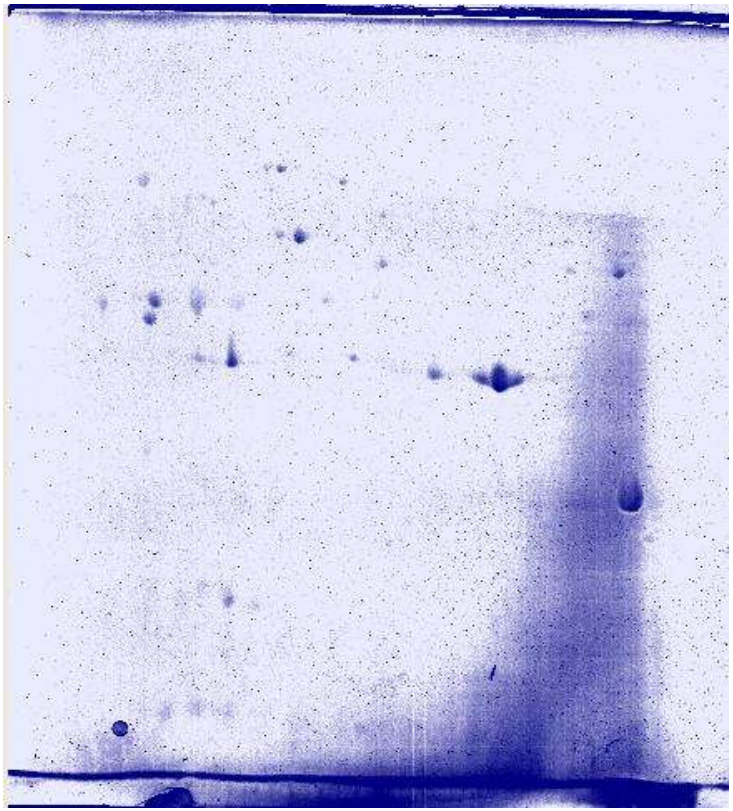
Subsequently, 450 µg of the *B. pseudomallei* secreted proteins were also subjected to the first dimension separation using the IPG strip pH 4-7 in order to ascertain that the protein spots detected on the separation using IPG strip pH 3-10 are well separated. However, the IPG strip pH 4-7 was found to be not suitable for separation of the *B. pseudomallei* secreted proteins because some of the prominent basic proteins were found to be missing (Figure 3.9).

pH 3 ⟶ pH 10



(A)

pH 3 ⟶ pH 10



(B)

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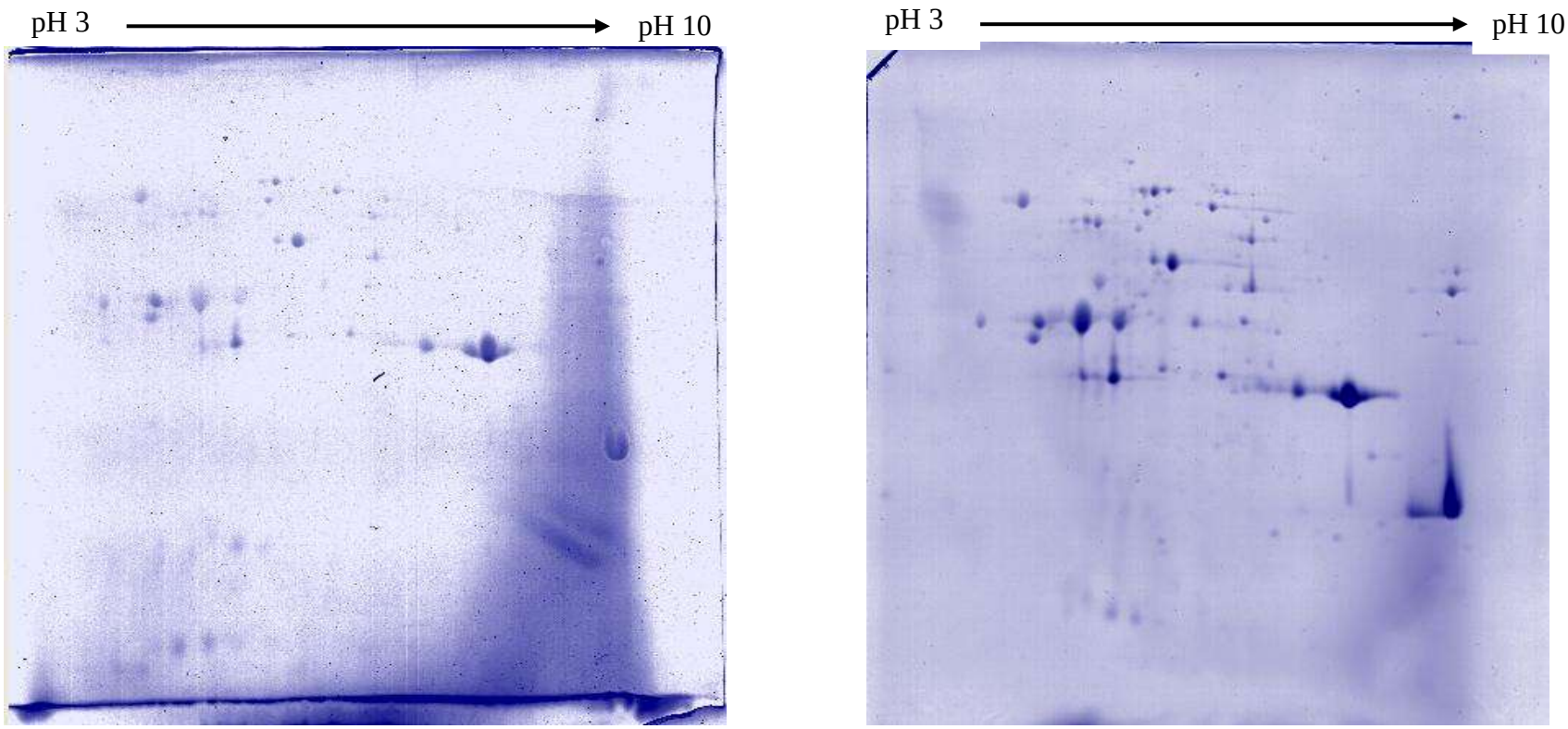


(C) (D)

Figure 3.8: Proteome profile of *B. pseudomallei* stationary phase secreted proteins. Different concentrations of the proteins were mapped on a 12.5% gel, pH 3 – 10 (A) 150 µg (silver staining), (B) 250 µg (CBB staining), (C) 350 µg (CBB staining) and (D) 450 µg (CBB staining).

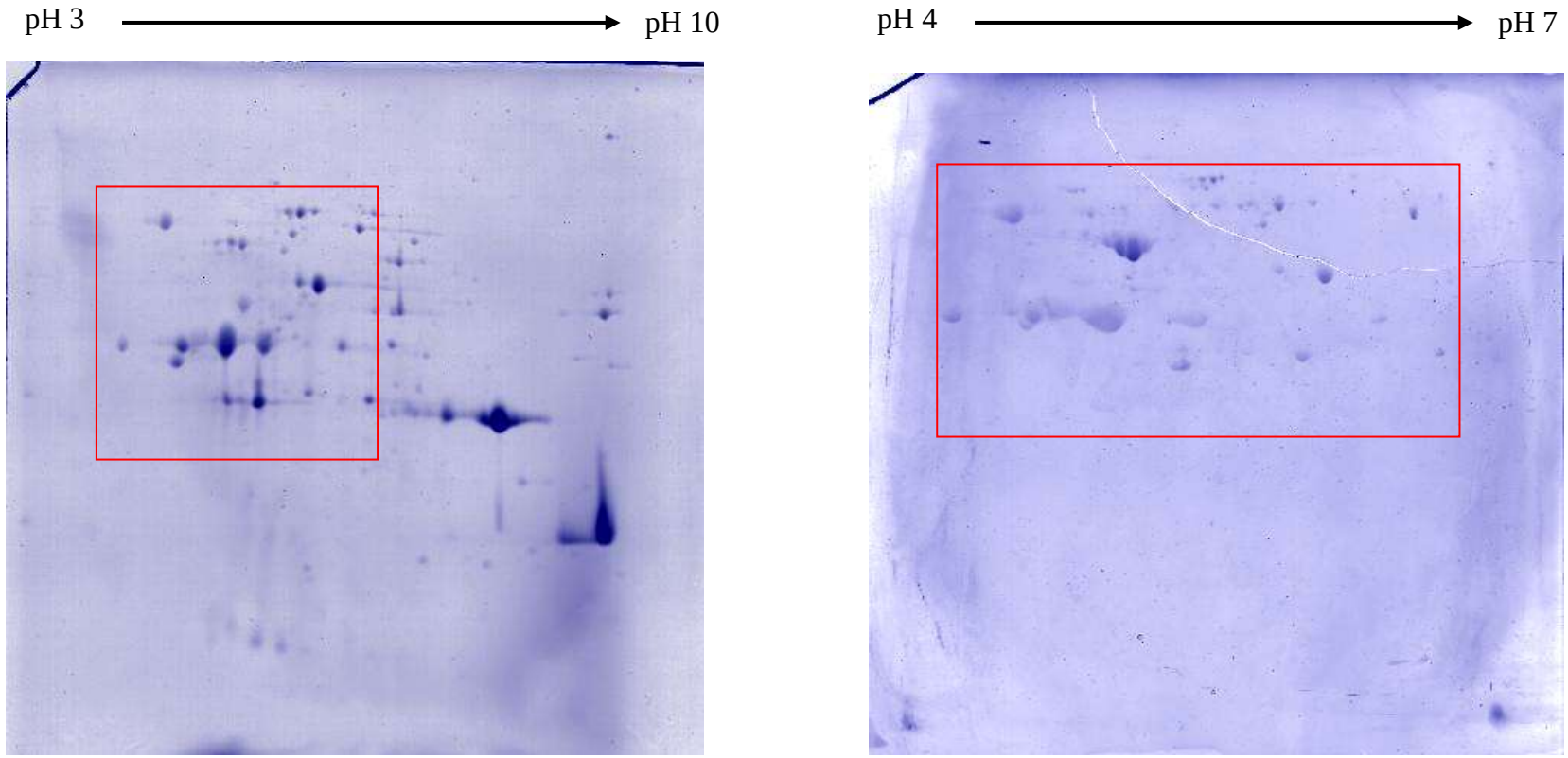
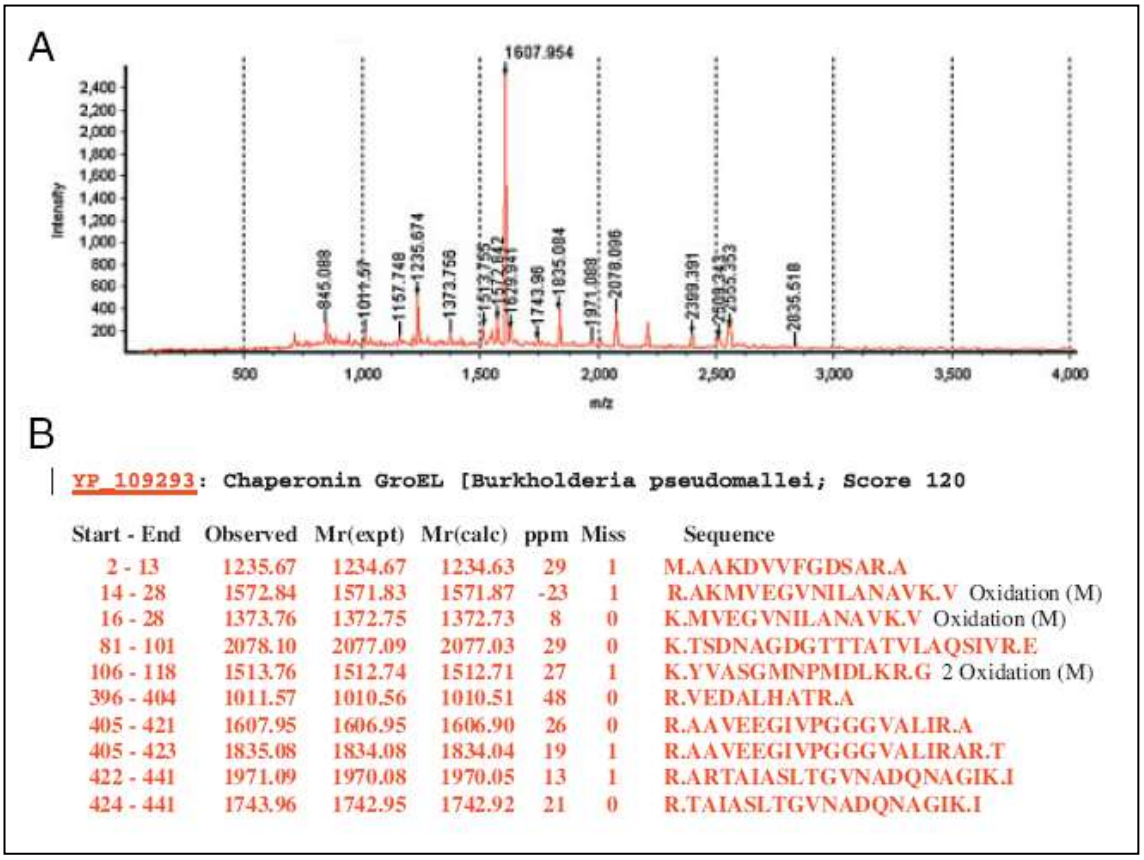


Figure 3.9: Proteome profile of *B. pseudomallei* stationary phase secreted proteins using different pH range. IPG strips with different pH was used for the first dimension separation of 450 µg of *B. pseudomallei* secreted proteins, (A) pH3-10 and (B) pH 4-7, followed by second dimension separation on 12.5% SDS-PAGE gel.

3.4.3.2 Identification of *Burkholderia pseudomallei* secreted proteins

More than one hundred spots were resolved when TCA precipitated proteins of *B. pseudomallei* culture supernatants were subjected to 2D-GE using IPG strip pH 3-10 and CBB staining. All the 113 spots were picked using a spot picker and sent for identification using MALDI-TOF analysis. Protein identification was based on the peptide fingerprint map obtained from MALDI-TOF mass spectrometer and the mass list generated. An example of a typical PMF result of spot number B10 (identified as chaperonin GroEL) and MASCOT search engine query result is shown in Figure 3.10. Among the 113 spots visualized, only 54 were able to be identified with confidence using the MALDI-TOF MS and database search (Figure 3.11, Table 3.3). The remaining 59 proteins could not be identified perhaps due to insufficient amounts of protein for MALDI-TOF analysis or the limitation of using MALDI-TOF instead of MALDI-TOF/TOF analysis for identification of the proteins. The MALDI-TOF/TOF analysis could have better facilitated identification of these remaining proteins due to its higher sensitivity in identifying and quantifying low-abundance proteins. Three proteins including putative hydrolase (C7 and B5), hyphothetical protein BPSL1622 (C1 and D1), and hyphothetical protein BPSL2466 (A6 and F5) were apparently resolved into more than one spots (Table 3.3).



Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
2 - 13	1235.67	1234.67	1234.63	29	1	M.AAKDVVFGDSAR.A
14 - 28	1572.84	1571.83	1571.87	-23	1	R.AKMVEGVNILANAVK.V Oxidation (M)
16 - 28	1373.76	1372.75	1372.73	8	0	K.MVEGVNILANAVK.V Oxidation (M)
81 - 101	2078.10	2077.09	2077.03	29	0	K.TSDNAGDGTTTATVLAQSIVR.E
106 - 118	1513.76	1512.74	1512.71	27	1	K.YVASGMNPMDLKR.G 2 Oxidation (M)
396 - 404	1011.57	1010.56	1010.51	48	0	R.VEDALHATR.A
405 - 421	1607.95	1606.95	1606.90	26	0	R.AAVEEGIVPGGGVALIR.A
405 - 423	1835.08	1834.08	1834.04	19	1	R.AAVEEGIVPGGGVALIRAR.T
422 - 441	1971.09	1970.08	1970.05	13	1	R.ARTAIASLTGVNADQNAGIK.I
424 - 441	1743.96	1742.95	1742.92	21	0	R.TAIASLTGVNADQNAGIK.I

Figure 3.10: MALDI-TOF MS analysis for spot number B10. (A) Illustration of representative PMF spectra typical for Chaperonin GroEL. (B) Mass list obtained from the PMF was subjected to the MASCOT search engine. Ten of the 17 queried masses were matched to the theoretical masses of with ±50 ppm tolerance and 0–1 missed cleavage. Peptide that caused oxidation at methionine residue is shown (Vellasamy *et al*., 2011).

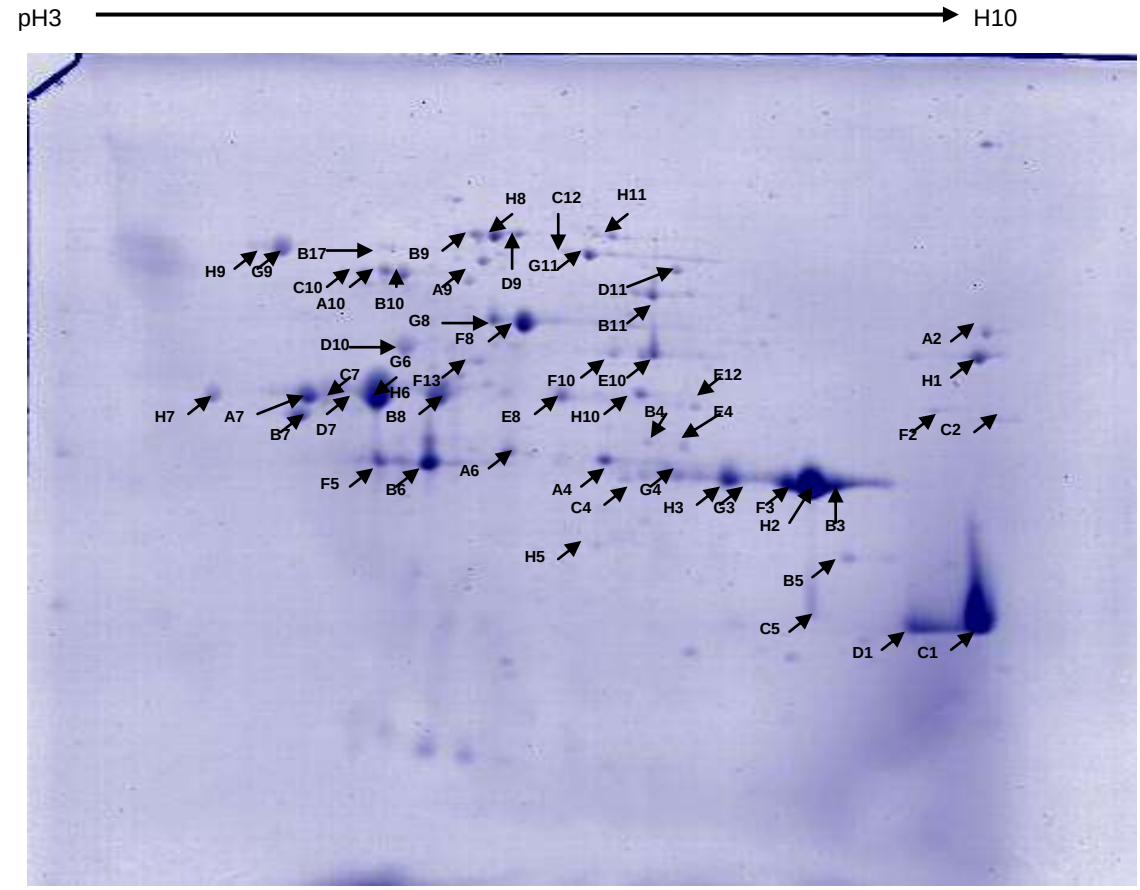


Figure 3.11: Secretome map of B. *pseudomallei* (CMS) proteins obtained using 2D-GE. Marked spots indicate 54 protein spots that were able to be identified with confidence using MALDI-TOF analysis (Vellasamy *et al*., 2011).

Table 3.3: Secreted proteins of *B. pseudomallei* identified using MALDI-TOF analysis (Vellasamy *et al*., 2011).

Spot No.[a]	Protein Name	Locus Tag	Sequence Coverage (%)	Peptides Matched	Exp/Theo MW	Exp/ Theo pI	SignalP[b]	TMHMM[c]	pSORT[d]
Information Storage and Processing									
- Transcription									
A2	Flagellar hook associated protein	YP_110870	23	7	52.1/52.0	8.78/8.79	+	-	Extracellular
H5	GntR family transcriptional regulator	YP_108300	11	3	25.5/24.8	5.29/6.71	-	-	Unknown
- Translation, ribosomal structure and biogenesis									
B7	Putative tRNA thiotransferase Protein MiaB	YP_107303	35	4	50.4/ 50.5	5.86/ 5.86	-	-	Cytoplasmic
D7	Hyphothetical protein BPSL1538	YP_108158	50	5	49.5/ 49.7	4.88/5.87	-	-	Cytoplasmic
- DNA replication, recombination and repair									
B11	Transposase	YP_110723	13	4	25.5/55.9	8.63/9.69	-	-	Cytoplasmic
C8	DNA gyrase subunit B	YP_106698	33	4	15.6/91.0	8.81/5.82	-	-	Cytoplasmic membrane
Metabolism									
- Energy production and conversion									
H3	Glycerophosphoryl diester phosphodiesterase family protein	YP_110428	63	4	32.4/33.4	5.91/6.44	+	1	Unknown
B17	Pyruvate dehydrogenase subunit E1	YP_108897	20	4	100.2/ 100.3	5.51/ 5.63	-	-	Unknown
H7	Succinate dehydrogenase flavoprotein subunit	YP_111724	57	1	42.7/64.4	4.21/6.55	-	-	Unknown
G4	NAD(P) transhydrogenase subunit alpha	YP_109481	52	4	21.6/39.1	6.02/6.33	-	-	Cytoplasmic membrane
- Carbohydrate transport and metabolism									
C4	ABC transporter, periplasmic binding protein	YP_110795	39	2	4.63/35.6	9.68/8.82	+	-	Periplasmic
A7	Glyceraldehyde 3-phosphate dehydrogenase	YP_109546	34	3	35.9/36.1	4.70/ 6.37	-	-	Cytoplasmic
B9	Phosphotransferase system, II BC component	YP_107124	10	2	61.1/61.2	8.09/8.70	-	10	Cytoplasmic membrane
C12	Putative transporter protein	YP_106875	17	2	30.1/45.5	10.0/9.65	-	12	Cytoplasmic membrane
- Lipid metabolism									
H9*	Monooxygenase	YP_110337	34	3	43.3/ 43.5	10.0/ 6.81	-	-	Unknown
G9*	Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A	YP_108553	32	5	25.1/ 25.2	5.85/ 5.56	-	-	Cytoplasmic
H10	Putative acyl-CoA dehydrogenase oxidoreductase protein	YP_107277	5	2	51.5/64.9	10.56/5.67	-	-	Unknown
B3	Phosphatidylserine decarboxylase	YP_107821	23	2	16.6/23.3	9.10/9.26	-	1	Unknown

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Spot No.[a]	Protein Name	Locus Tag	Sequence Coverage (%)	Peptides Matched	Exp/Theo MW	Exp/ Theo pI	SignalP[b]	TMHMM[c]	pSORT[d]
- Amino acid transport and metabolism									
C10	Peptidase	YP_109684	40	3	44.4/79.0	7.82/6.14	+	-	Unknown
C5	ABC transport system ATP- binding protein	YP_110365	19	2	10.7/38.8	8.52/6.90	-	-	Cytoplasmic
- Secondary metabolites biosynthesis, transport and metabolism									
F8	Hyphothetical protein BPSS1116	YP_111129	67	3	54.6/64.4	5.97/8.94	-	-	Cytoplasmic
G11	Multidrug efflux system exported	YP_110312	12	4	41.1/42.9	7.66/9.37	+	1	Cytoplasmic membrane
F13	Cytochrome monooxygenase related protein	YP_110047	6	2	25.4/51.5	6.21/8.97	-	-	Cytoplasmic
B8	Short chain dehydrogenase	YP_108558	45	5	27.6/ 27.7	6.60/ 6.97	-	-	Cytoplasmic
Cellular Processes									
- Cell envelope biogenesis, outer membrane									
C2	Putative ADP-heptose-LPS Heptosyltransferase II	YP_107416	14	4	39.1/37.8	9.07/9.08	-	-	Unknown
H6	Cell division protein FtsQ	YP_109618	58	4	27.9/ 28.1	4.85/ 6.34	+	1	Cytoplasmic membrane
H11	Peptidoglycan synthetase FtsI	YP_109627	14	1	54.6/66.2	5.04/ 9.46	+	1	Cytoplasmic membrane
E10	Transferase	YP_112247	6	2	38.7/50.0	9.71/ 9.50	-	-	Cytoplasmic
- Post translational modification, protein turnover and chaperones									
F2	Hyphothetical protein BPSL0264	YP_106891	21	3	21.7/23.8	5.20/6.31	-	-	Unknown
B10	Chaperonin GroEL	YP_109293	19	10	56.5/57.0	5.18/5.17	-	-	Cytoplasmic
F10	Putative heat shock protein	YP_109423	11	3	20.5/19.6	6.60/4.78	-	-	Unknown
- Cell motility and secretion									
G6	Flagellin	YP_109915	49		39.1/39.3	5.05/5.05	-	-	Extracellular
E8	Putative type II/IV secretory system ATP-binding protein	YP_108493	13	4	47.3/48.9	5.99/5.90	-	-	Cytoplasmic
D9	Chemotaxis related protein	YP_111880	30	4	42.6/60.3	4.22/5.27	-	2	Cytoplasmic membrane
D11	Chemotaxis-related methyltransferase protein	YP_111878	8	3	53.3/71.2	7.14/ 5.51	-	-	Cytoplasmic
- Cell division and chromosome partitioning									
H8	Hyphothetical protein BPSL0566	YP_107192	17	2	42.7/71.8	8.90/ 6.00	-	-	Cytoplasmic

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Spot No.[a]	Protein Name	Locus Tag	Sequence Coverage (%)	Peptides Matched	Exp/Theo MW	Exp/ Theo pI	SignalP[b]	TMHMM[c]	pSORT[d]	
Poorly Characterised										
C7	Putative hydrolase	YP_107999	8	2	17.3/16.2	5.88/ 8.00	-	-	Unknown	
B5	Putative hydrolase	YP_107999	8	2	17.3/16.2	5.88/ 8.00	-	-	Unknown	
D10	Hyphothetical protein BPSL2050	YP_108649	19	2	87.3/ 97.3	10.08/ 6.20	-	-	Unknown	
E4	Undecaprenyl pyrophosphate phosphatase	YP_109239	25	2	21.4/30.2	8.74/8.65	-	5	Cytoplasmic membrane	
A10	Putative lipoprotein	YP_108354	41	2	34.0/58.1	4.94/8.54	+	-	Unknown	
B4	Hyphothetical protein BPSL1094	YP_107715	14	4	16.2/16.0	5.16/5.55	-	-	Unknown	
A9	Hyphothetical protein BPSL2092	YP_108689	38	2	50.3/52.1	9.30/7.10	-	-	Unknown	
H2	Chitin binding protein	YP_110514	24	7	39.5/39.2	8.59/8.59	+	-	Unknown	
G3	Hyphothetical protein BPSS1981	YP_111987	26	3	10.4/10.1	6.96/6.50	-	-	Cytoplasmic	
C1	Hyphothetical protein BPSL1622	YP_108236	36	8	26.7/26.9	9.30/9.30	+	-	Unknown	
D1	Hyphothetical protein BPSL1622	YP_108236	47	10	26.7/26.9	9.30/9.30	+	-	Unknown	
H1	Cell invasion protein	YP_111537	34	10	44.2/44.2	8.67/8.01	-	-	Unknown	
F3	Hyphothetical protein BPSL0584	YP_107212	3	3	19.1/48.3	5.37/8.95	+	3	Cytoplasmic membrane	
A6	Hyphothetical protein BPSL2466	YP_109058	100	2	9.89/15.8	6.12/9.79	+	1	Unknown	
B6	Intracellular spread protein	YP_111530	19	3	13.4/56.7	9.80/ 9.85	-	-	Unknown	
F5	Hyphothetical protein BPSL2466	YP_109058	100	2	9.89/15.8	6.12/9.79	+	1	Unknown	
A4	Hyphothetical protein BPSL1182	YP_107804	12	3	23.1/22.7	6.75/6.13	-	-	Unknown	
D10	Hyphothetical protein BPSL0345	YP_106972	20	3	53.2/96.3	5.24/6.74	-	-	Unknown	

[a] Protein spot corresponding to position on gel (Fig.1)
[b] Output of computer algorithms that predict presence (+) or absence (-) of signal peptide
[c] Output of computer algorithms that predict transmembrane helices
[d] Output of computer algorithms that predict subcellular location of protein

3.4.3.3 *In silico* analysis of the identified proteins

In silico analysis was carried out on the 54 proteins identified in the secretome of *B. pseudomallei*. Initially, the proteins were assigned into functional classes based on "Clusters of Orthologous Groups" (COG). The proteins identified were found to be involved in three major functions including metabolism (18 proteins), cellular processes (13 proteins), and information storage and processing (6 proteins). However, the functions of 17 proteins were poorly characterised (Figure 3.12). Metabolic proteins were mainly found to be involved in energy production and conversion (22.2%), carbohydrate transport and metabolism, lipid metabolism (22.2%), secondary metabolites biosynthesis, transport and metabolism (22.2%), and amino acid transport and metabolism (11.1%) (Figure 3.13, Panel A). Among the proteins involved in cellular processes, 33.3% were found to play a role in cell envelop and outer membrane biogenesis. Similarly, 33.3% were also involved in cell motility and secretion and other functions including PTM and chaperones (25.0%) and cell division and chromosome partitioning (8.3%) (Figure 3.13, Panel B). Among the information storage and processes proteins, 33.3% of each were found to be involved in translation, ribosomal structure and biogenesis, transcription, and DNA replication, recombination and repair (Figure 3.13, Panel C).

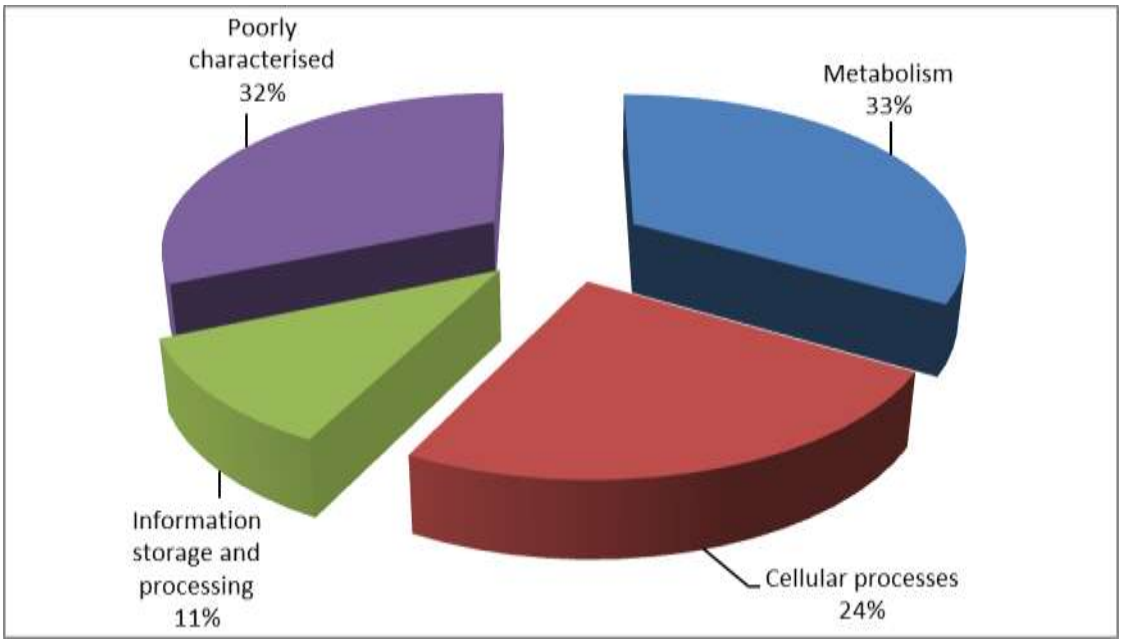
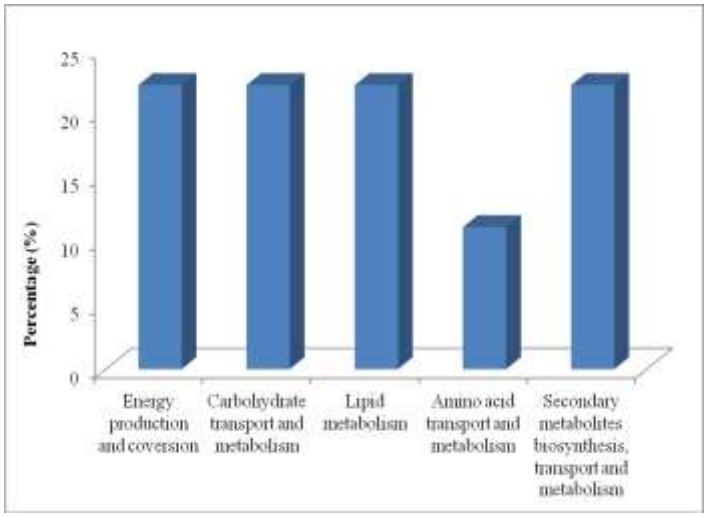
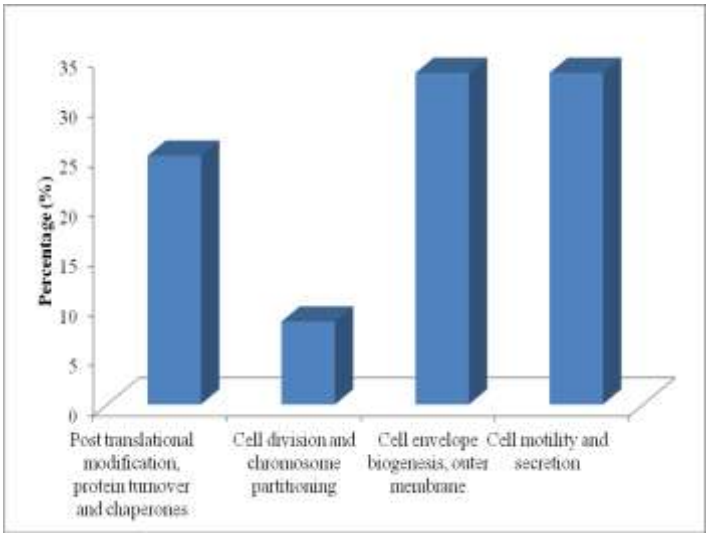


Figure 3.12: Functional classes of the stationary phase *B. pseudomallei* secreted proteins based on Clusters of Orthologous Groups.

(A)



(B)



(C)

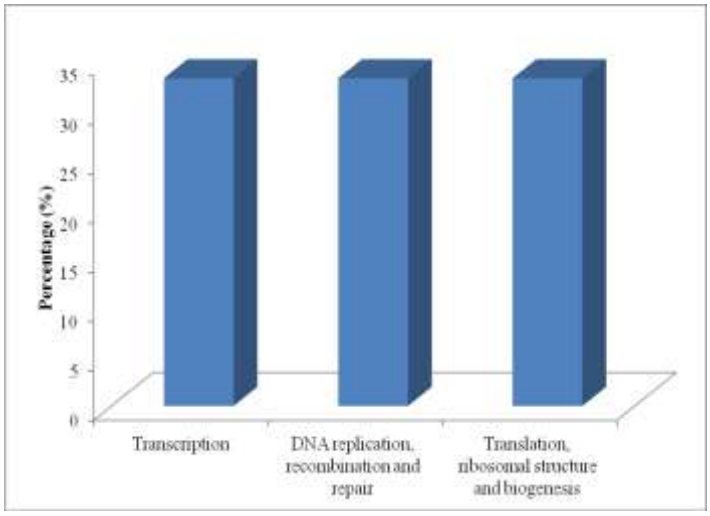


Figure 3.13: Major functional categories of proteins from the different functional classes. (A) metabolic proteins, (B) cellular processes proteins, and (C) information storage and processes proteins.

After assigning the proteins to their functional classes, PSORTb v.2.0 was used to predict the cellular locations of the identified proteins. Among the 54 proteins identified, only two, *i.e.*, flagellin and flagellar hook associated protein, were predicted as extracellular proteins. From the remaining 52 proteins, 15 were predicted to be cytoplasmic proteins, 10 cytoplasmic membrane-associated proteins and one periplasmic binding protein, identified as the ABC transporter. Twenty-six other proteins were from unknown location (Figure 3.14). Eleven proteins including eight predicted as cytoplasmic membrane protein by PSORT analysis were also predicted as cytoplasmic proteins by the TMHMM alogarithm (Table 3.3).

Further characterisation of the proteins was carried out using SignalP v3.0, whereby the presence of signal peptides was predicted in 25.9% of the proteins identified. The proteins predicted as secreted via the classical sec pathway includes ABC transport system ATP-binding protein, peptidase, peptidoglycan synthetase FtsI, putative lipoprotein, ABC transporter periplasmic-binding protein, glycerophosphoryl diester phosphodiesterase family protein, cell division protein FtsQ, chitin-binding protein, multidrug efflux system transported protein and hyphothetical proteins BPSS1116, BPSS1981, BPSL2466, BPSL1622, BPSL0584.

The presence of *B. pseudomallei* secretome proteins in other closely related *Burkholderia* species, *B. mallei* strain 23344 and *B. thailandensis* strain E264 were also determined using BLAST analysis. Forty-five of 54 proteins identified in the *B. pseudomallei* secretome showed high homology (≥80%) to the predicted proteins of *B. mallei* (23344). Similar homology was also demonstrated by 44 of the proteins with predicted proteins of *B. thailandensis* (E264) (Table 3.4). One of the proteins (hypothetical protein BPSS 1981) did not have any orthologue in *B. mallei* and one

protein (hypothetical protein BPSL 1622) did not have any orthologue in *B. thailandensis*.

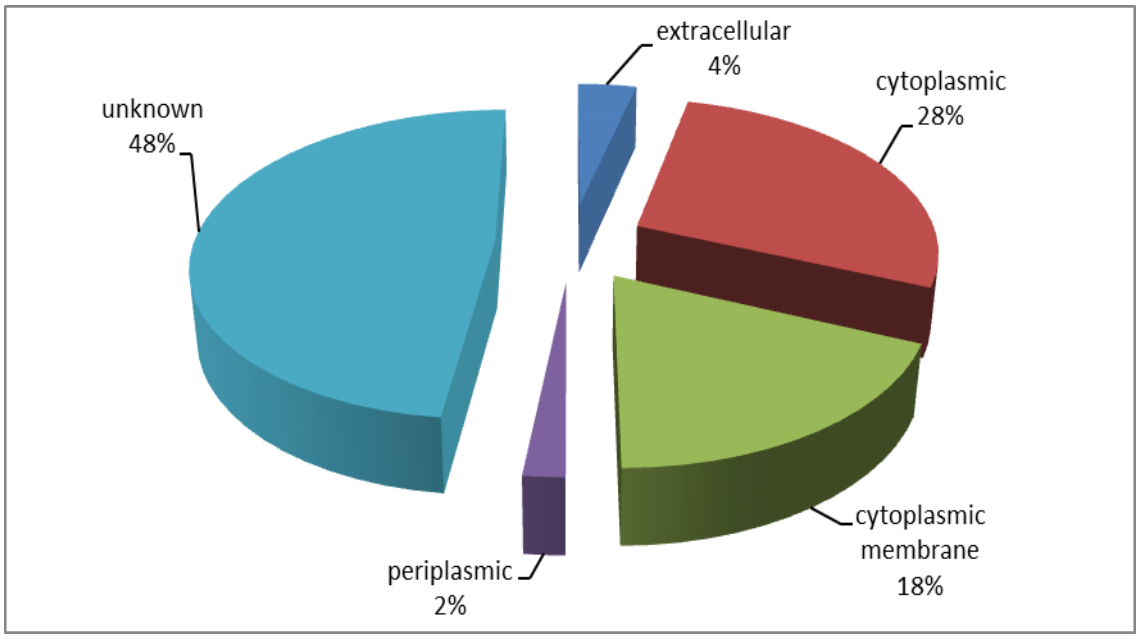


Figure 3.14: Cellular locations of the identified stationary phase *B. pseudomallei* secreted proteins using PSORTb v.2.0.

Table 3.4: Similarity of the *B. pseudomallei* proteins identified compared with *B. mallei* and *B. thailandensis*.

Spot Number [a]	Identity to *B. mallei* 23344 (%)	Identity to *B. thailandensis* E264 (%)
A2	99	93
H5	26	26
B7	100	96
D7	99	96
B11	99	35
C8	100	99
H3	42	33
B17	100	96
H7	99	99
G4	100	98
C4	99	95
A7	99	98
B9	98	96
C12	99	95
H9	97	94
G9	100	97
H10	99	99
B3	99	99
C10	100	96
C5	99	97
F8	99	96
G11	100	96
F13	100	83
B8	100	98
C2	99	98
H6	99	98
H11	99	96
E10	99	28
F2	100	95
B10	99	99
F10	100	93
G6	100	90
E8	29	98
D9	100	95
D11	99	82
H8	42	42
C7	99	94
B5	99	94
D10	99	93
E4	100	94
A10	99	87
B4	97	90
A9	99	91
H2	100	95
G3	No significant similarity	92
C1	97	No significant similarity
D1	97	No significant similarity
H1	100	86
F3	34	28
A6	29	85
B6	99	90
F5	29	85
A4	99	92
D10	26	27

[a] Identities of spots were as described in Table 1

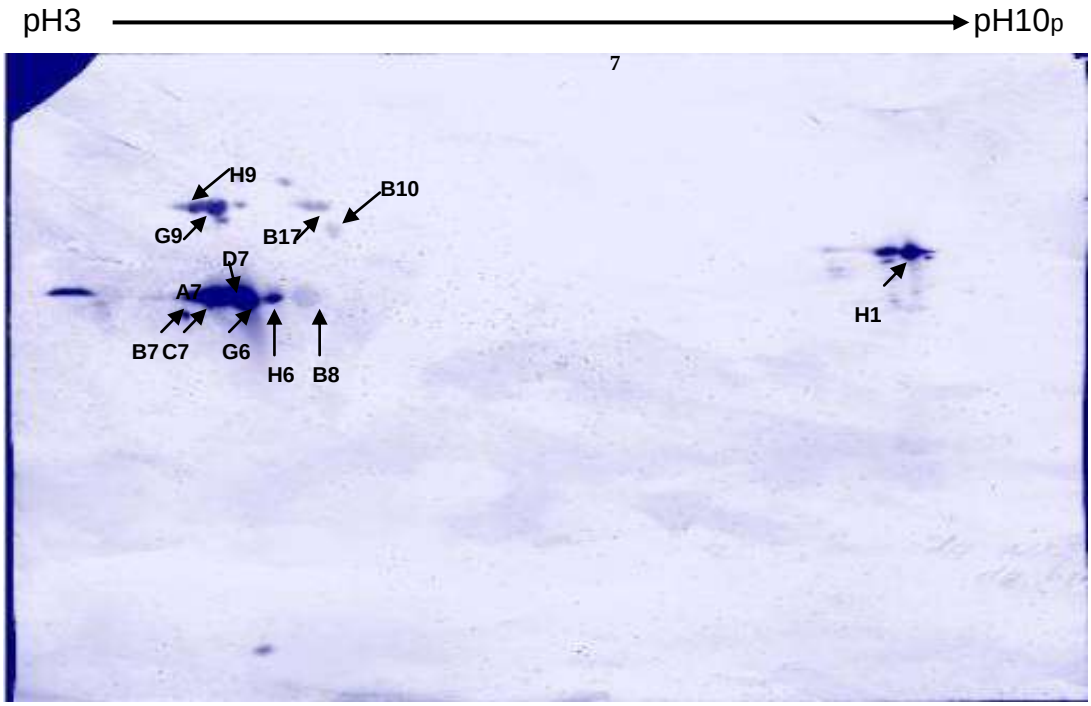
3.4.4 Immunogenic proteins

3.4.4.1 Immunised mice sera

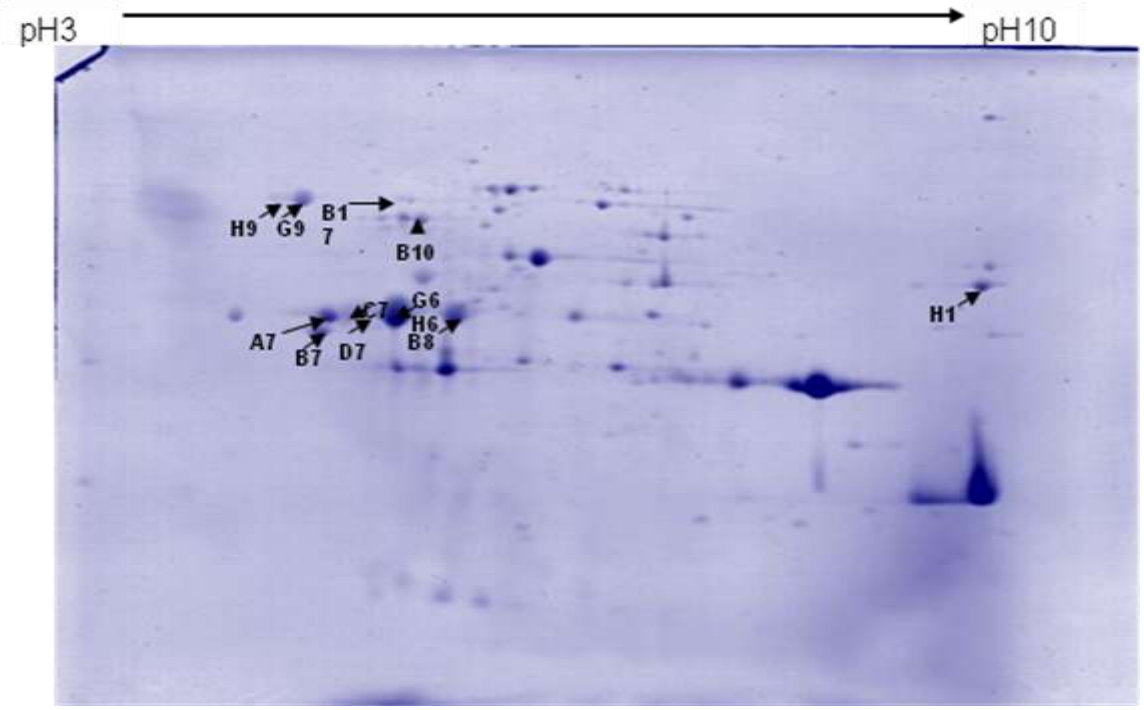
The immunogenic proteins present in the *B. pseudomallei* secretome was identified using western blot analysis. The secreted proteins separated using 2D-GE was detected using mice-anti *B. pseudomallei* sera raised against the secreted proteins. The concentration of the secreted proteins collected was 1.28±0.018 mg/ml. The mice-anti *B. pseudomallei* sera raised against the secreted proteins were tested for the presence of high antibody titers against *B. pseudomallei* secreted proteins using ELISA techniques prior to western blot. The antibody titres at a dilution of 1:1000 was high (OD_{410nm} = 1.735±0.074) compared to the unimmunised mice sera (OD_{410nm} = 0.112±0.035).

3.4.4.2 Detection of *B. pseudomallei* secreted proteins that are reactive to mice antisera

Two dimensional gel electrophoresis analyses of the *B. pseudomallei* secreted proteins detected approximately 113 protein spots using CBB staining (Figure 3.10). Twelve of these protein spots were found to be reactive to mice antisera raised against *B. pseudomallei* secreted proteins (Figure 3.15, panel A). The corresponding protein spots on the 2D-GE is shown in Figure 3.15, panel B). No protein spots were detected on the western blot membrane when probed with the control sera from unimmunized mice.



(A)



(B)

Figure 3.15: Western blot analysis of *B. pseudomallei* secretome using mice anti-*B. pseudomallei* secreted proteins sera. (A) Nitrocellulose membrane with marked spots indicating the proteins reactive to antisera of mice following immunisation with *B. pseudomallei* secreted proteins, (B) 2D gels showing spots corresponding to the proteins reactive to antisera of mice.

3.4.4.3 Identification of *B. pseudomallei* secreted proteins reactive to mice antisera

Twelve proteins reactive to specific mice antisera raised to *B. pseudomallei* secreted proteins were detected by Western blot analysis of the 2D-GE gel. All the 12 proteins were subjected to MALDI-TOF MS analysis and the MS data was matched against the non-redundant NCBI library database, comprising currently available annotated complete protein sequences of *B. pseudomallei*. The proteins reactive to mice antisera includes flagellin, cell invasion protein (BipC), putative hydrolase, chaperonin GroEL, pyruvate dehydrogenase, cell division protein (FtsQ), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), succinyl-CoA:3-ketoacid-coenzyme A transferase (SCOT), short-chain dehydrogenase, putative tRNA thiotransferase protein MiaB, hyphothetical protein BPSL1538, and monooxygenase. The proteins were found to be in the pH range of 4-10 and molecular weight ranging from 15 000 to 100 000 (Table 3.5).

Table 3.5: *B. pseudomallei* secreted proteins reactive to mice hyperimmune sera raised to *B. pseudomallei* secreted proteins using Mascot search and the non-redundant database from National Center for Biotechnology (NCBI).

Spot No.[a]	Protein Name	Locus Tag	Sequence Coverage (%)	Peptides Matched	Exp/Theo MW	Exp/ Theo pI	SignalP[b]	TMHMM[c]	pSORT[d]
Information Storage and Processing									
- Translation, ribosomal structure and biogenesis									
B7*	Putative tRNA thiotransferase Protein MiaB	YP_107303	35	4	50.4/ 50.5	5.86/ 5.86	-	-	Cytoplasmic
D7*	Hyphothetical protein BPSL1538	YP_108158	50	5	49.5/ 49.7	4.88/5.87	-	-	Cytoplasmic
Metabolism									
- Energy production and conversion									
B17*	Pyruvate dehydrogenase subunit E1	YP_108897	20	4	100.2/ 100.3	5.51/ 5.63	-	-	Unknown
- Carbohydrate transport and metabolism									
A7*	Glyceraldehyde 3-phosphate dehydrogenase	YP_109546	34	3	35.9/36.1	4.70/ 6.37	-	-	Cytoplasmic
- Lipid metabolism									
H9*	Monooxygenase	YP_110337	34	3	43.3/ 43.5	10.0/ 6.81	-	-	Unknown
G9*	Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A	YP_108553	32	5	25.1/ 25.2	5.85/ 5.56	-	-	Cytoplasmic
- Secondary metabolites biosynthesis, transport and metabolism									
B8*	Short chain dehydrogenase	YP_108558	45	5	27.6/ 27.7	6.60/ 6.97	-	-	Cytoplasmic

Continued on next page…

…continuation from previous page

Spot No.[a]	Protein Name	Locus Tag	Sequence Coverage (%)	Peptides Matched	Exp/Theo MW	Exp/ Theo pI	SignalP[b]	TMHMM[c]	pSORT[d]
Cellular Processes									
- Cell envelope biogenesis, outer membrane									
H6*	Cell division protein FtsQ	YP_109618	58	4	27.9/ 28.1	4.85/ 6.34	+	1	Cytoplasmic membrane
- Post translational modification, protein turnover and chaperones									
B10*	Chaperonin GroEL	YP_109293	19	10	56.5/57.0	5.18/5.17	-	-	Cytoplasmic
- Cell motility and secretion									
G6 *	Flagellin	YP_109915	49		39.1/39.3	5.05/5.05	-	-	Extracellular
Poorly Characterised									
C7*	Putative hydrolase	YP_107999	8	2	17.3/16.2	5.88/ 8.00	-	-	Unknown
H1*	Cell invasion protein	YP_111537	34	10	44.2/44.2	8.67/8.01	-	-	Unknown

[a] Protein spot corresponding to position on gel (Fig.1)
[b] Output of computer algorithms that predict presence (+) or absence (-) of signal peptide
[c] Output of computer algorithms that predict transmembrane helices
[d] Output of computer algorithms that predict subcellular location of protein

3.4.4.4 *In silico* analysis of proteins reactive to mice antisera

Five of the 12 proteins reactive to antisera of mice following immunisation with *B. pseudomallei* secreted proteins were found to be involved in metabolic functions which include specific functions of lipid metabolism (16.7%), carbohydrate transport and metabolism (8.3%), secondary metabolites biosynthesis, transport and metabolism (8.3%), and energy production and conversion (8.3%). Three proteins were involved in cellular processes with functions including cell envelope biogenesis and outer membrane (8.3%), PTM, protein turnover and chaperones (8.3%) and also cell motility and secretion (8.3%). Two of the reactive proteins were involved in information storage and processes with translation, ribosomal structure, and biogenesis functions (Figure 3.16).

However, functions of two other proteins were poorly characterised. *In silico* analysis of the proteins reactive to mice antisera using PSORT predicted 50.0% cytoplasmic proteins, 8.3% extracellular proteins, and another 8.3% cytoplasmic membrane protein. The remaining 33.3% of the reactive proteins were from unknown locations (Figure 3.17). Only one protein, cell division protein FtsQ, showed the presence of signal peptide indicating that it may be secreted via the classical Sec pathway. However, this protein was also predicted as cytoplasmic protein by the TMHMM algorithm with one transmembrane helix. BLAST analysis showed that all the 12 proteins reactive to antisera of mice following immunisation with *B. pseudomallei* secreted proteins had orthologues in *B. mallei* (23344) and *B. thailandensis* (E264) with more than 85% homology (Table 3.6).

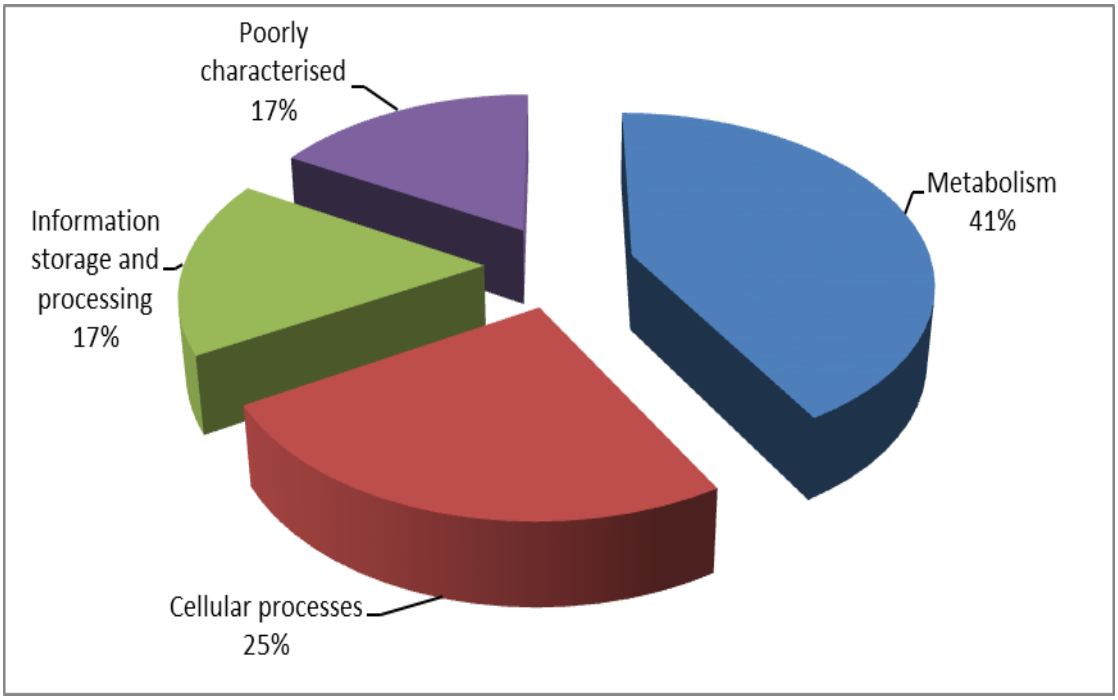


Figure 3.16: Functional classes (COGs) of the stationary phase *B. pseudomallei* secreted proteins reactive to antisera of mice following immunisation with *B. pseudomallei* secreted proteins.

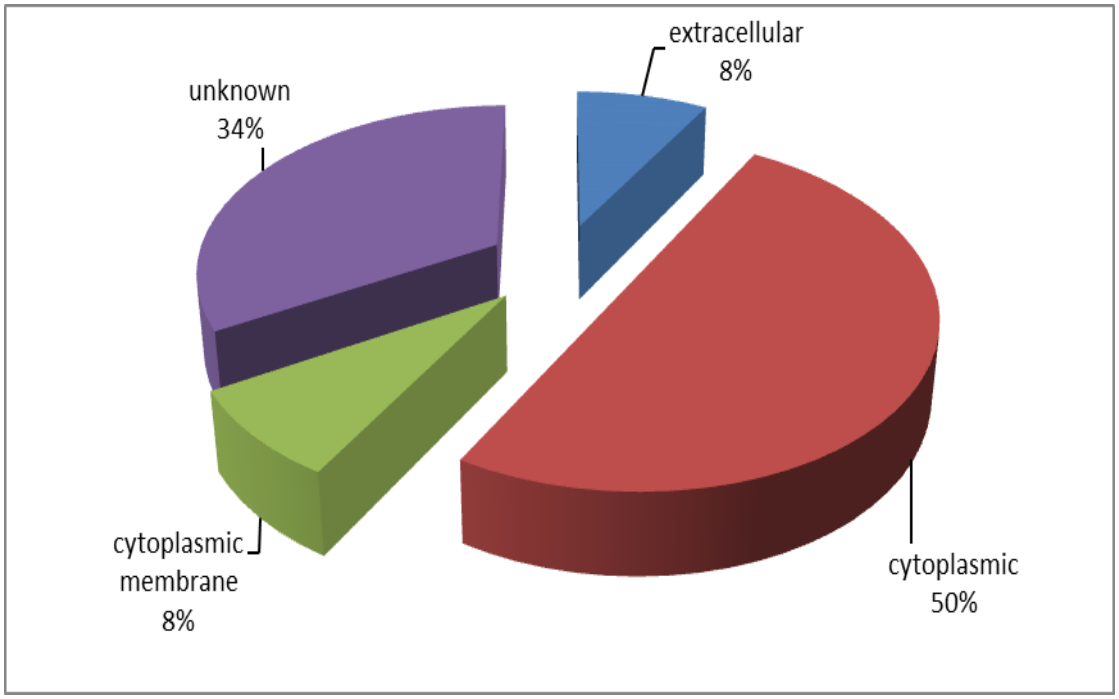


Figure 3.17: Cellular locations of the identified *B. pseudomallei* secreted proteins reactive to antisera of mice following immunisation with *B. pseudomallei* secreted proteins using PSORTb v.2.0.

Table 3.6: Similarity of the *B. pseudomallei* secreted proteins reactive to antisera of mice following immunisation with *B. pseudomallei* secreted proteins compared with *B. mallei* and *B. thailandensis*.

Spot Number [a]	Identity to *B. mallei* 23344 (%)	Identity to *B. thailandensis* E264 (%)
B7	100	96
D7	99	96
B17	100	96
A7	99	98
H9	97	94
G9	100	97
B8	100	98
H6	99	98
B10	99	99
G6	100	90
C7	99	94
H1	100	86

[a] Identities of spots are as described in Table 1

3.5 Gene expression analysis

3.5.1 Cell viability

Viability of A549 cells upon three hours exposure to (i) *B. pseudomallei* live bacteria (at MOIs of 1:10, 1:100 and 1:200) or (ii) secreted proteins of the bacteria (at concentrations of 0.5, 1, 2, 5, 10, 25, 50, and 100 µg/ml), was observed using Trypan blue exclusion assay. Infection of the A549 cells with *B. pseudomallei* at different MOIs demonstrated inverse correlation, *i.e*., as the MOI increased, decreasing number of cells were recovered (Table 3.7). At MOI 1:10, 2.7% of the cells were unable to be recovered. Higher number of cells, 7.3% and 14.7%, did not survive after three hours of exposure to *B. pseudomallei* at MOI of 1:100 and 1:200, respectively

Similarly, inverse correlation was also observed between the percentage of cell recovered after three hours exposure to *B. pseudomallei* secreted proteins and the concentration of the secreted proteins (Table 3.8). *B. pseudomallei* culture supernatant at a concentration of 0.5 µg/ml did not have any effect in terms of the A549 cell death after three hours of exposure. As the concentration of the culture supernatant increased from 1.0 to 5.0 µg/ml, the percentage of A549 cells recovered was slightly less (97.0±0.20 to 99.7±0.12 %) as compared to the concentration of 0.5 µg/ml. When the concentrations of *B. pseudomallei* secreted proteins were increased to 10 and 25 µg/ml, more cells were found to be unable to survive; with the percentage of cells recovered at 94.3±0.31 and 84.6±0.25%, respectively. Drastic reduction in the percentage of cell recovered was observed at secreted proteins concentrations of 50 and 100 µg/ml. At 50 µg/ml concentration, 48.7±0.15% cells were recovered. However, only 25.3% of the cells survived at 100 µg/ml secreted proteins concentration.

Table 3.7: Percentage of A549 lung epithelial cells recovered following three hours of exposure to live *B. pseudomallei at different MOIs.*

MOI	Percentage of cells recovered (%)
1:10	97.3±0.11
1:100	92.7±0.25
1:200	85.3±0.32

Table 3.8: Percentage of A549 lung epithelial cells recovered following three hours of exposure to *B. pseudomallei secreted proteins at different concentrations.*

Concentration (µg/ml)	Percentage of cells recovered (%)
0.5	100±0.09
1.0	99.7±0.12
2.0	99.0±0.15
5.0	97.0±0.20
10.0	94.3±0.31
25.0	84.6±0.25
50.0	48.7±0.15
100.0	25.3±0.23

Based on the cell viability assay, the MOI 1:10 and secreted protein concentration of 5 μg/ml was selected to be used in the microarray analysis to investigate the host response towards infection with *B. pseudomallei* live bacteria and its secretory proteins. These MOI and concentration of secreted proteins were selected due to the optimal (>95%) number of cells recovered. This helped to minimise the risk of DNA contamination due to cell lysis during RNA isolation.

3.5.2 Quality control and quantification of RNA

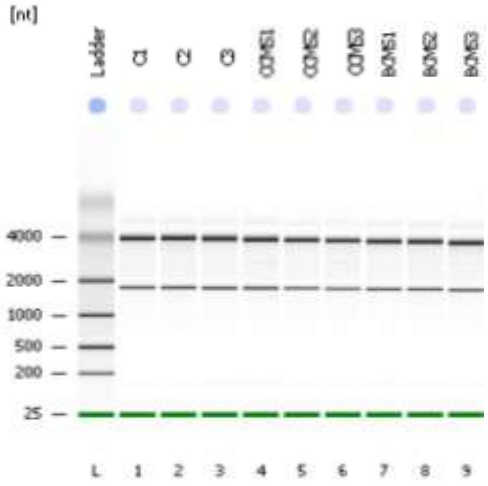
The sensitivity and accuracy of microarray data generated is dependent on the quality and composition of the input RNA. Two different measures were used for quality control and quantification of the extracted RNA. Initially the NanoPhotospectrometer (Implen, Germany) was used to determine the concentration and assess the purity. The RNAs from A549 control (C1-C3) were found to be higher (815 – 922 ng/ml) compared to the RNAs from the A549 cells exposed to 5 μg/ml of *B. pseudomallei* secreted proteins (CCMS1-CCMS3) and A549 cells exposed to *B. pseudomallei* live bacteria (BCMS1-BCMS3), which ranged between 147-197 ng/ml and 153-184 ng/ml, respectively. Purity of the RNA sample was determined using the $A_{260/280}$ and $A_{260/230}$ ratios. All samples were found to have $A_{260/280}$ ratio of $\geq$1.8 and $A_{260/230}$ ratio of ~2.0 (Table 3.9).

The use of the Bioanalyser (RNA 6000 Nano, Agilent, USA) confirmed that the purity of the RNA sample. Total RNA integrity was determined using RIN, which measures the degradation of the RNA. For microarray analysis, RNA samples with a RIN of $\geq$8 were preferred. All the RNA samples used in this study did not show extensive

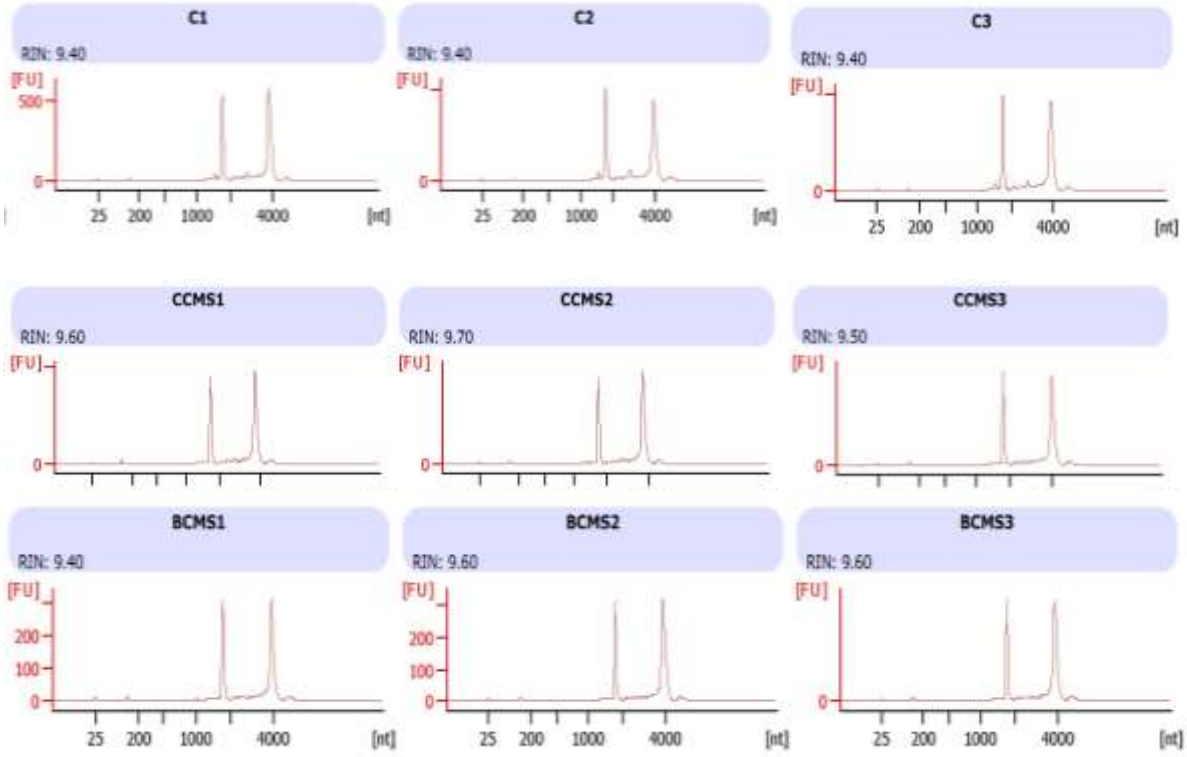
degradation, whereby the RIN of all samples were found to be ≥9 (Table 3.9, Figure 3.18).

Table 3.9: RNA concentration and purity measured using NanoPhotospectrometer and Bioanalyser.

Sample	NanoPhotospectrometer			Bioanalyser	
	RNA Concentration (ng/ml)	$A_{260/280}$	$A_{260/230}$	RNA Concentration (ng/ml)	RNA Integrity Number (RIN)
C1	815	1.825	1.921	1632	9.4
C2	922	1.835	1.917	2146	9.4
C3	834	1.843	1.993	1429	9.4
CCMS1	196	1.922	1.997	950	9.6
CCMS2	197	1.943	1.971	1187	9.7
CCMS3	147	1.999	1.915	763	9.5
BCMS1	180	1.968	1.982	1075	9.4
BCMS2	184	1.982	1.999	1085	9.6
BCMS3	153	2.000	2.107	760	9.6



(A)



(B)

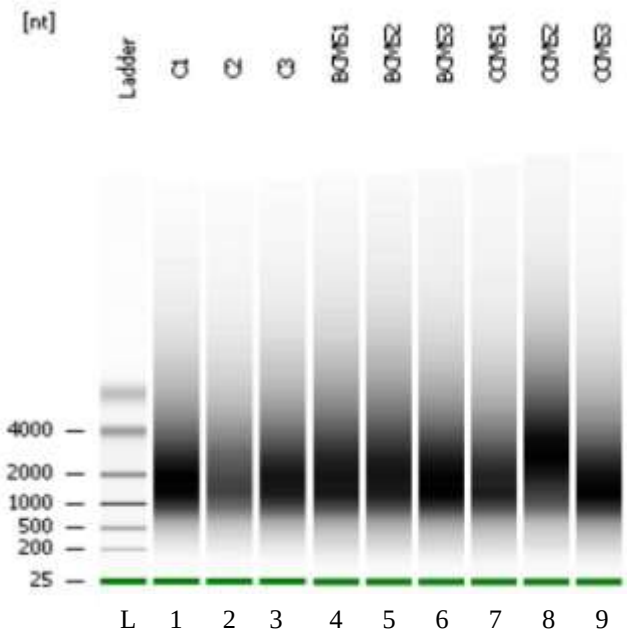
Figure 3.18: Analysis of the total RNA using Bioanalyser. (A) Densitometry plot, creating a gel-like image bands of high quality RNA, which appears as two distinct bands corresponding to the 18S and 28S ribosomal RNAs and (B) electropherograms, with peaks showing no contaminating genomic DNA peak between the 18S and 28S.

3.5.3 Quality control and quantification of cRNA

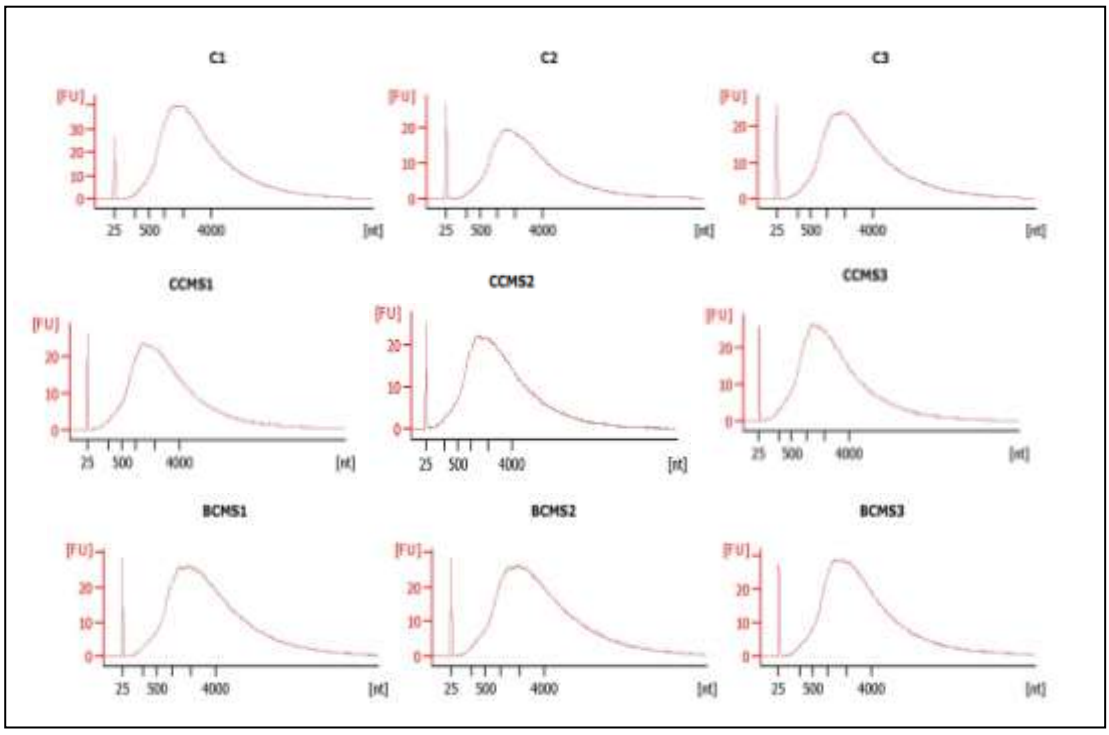
The RNA samples were then converted to cRNA using the Illumina TotalPrep RNA Amplification Kit (Ambion, USA). Concentrations of the cRNA samples measured using the Nanospectrophotometer ranged from 538-681 ng/ml. All the samples also gave $A_{260/280nm}$ ratios of ≥2.0 (Table 3.10). The cRNA samples were analysed using the Bioanalyser to confirm the purity. All samples were found to generate typical size distribution profiles indicating good quality of the cRNA samples (Figure 3.19).

Table 3.10: cRNA concentration and purity measured using NanoPhotospectrometer

Sample	NanoPhotospectrometer		
	cRNA Concentration (ng/ml)	$A_{260/280}$	$A_{260/230}$
C1	681	2.120	2.022
C2	606	2.138	1.967
C3	579	2.166	1.912
CCMS1	555	2.171	1.982
CCMS2	591	2.000	1.919
CCMS3	548	2.191	1.682
BCMS1	562	2.153	1.807
BCMS2	538	2.168	1.956
BCMS3	609	2.187	1.808



(A)



(B)

Figure 3.19: Analysis of the total cRNA using the Bioanalyser. (A) A densitometry plot, creating a gel-like image bands of high quality cRNA and (B) electropherograms showing broad peaks typical for cRNA, indicating no fragmentation of the cRNA.

3.5.4 Microarray analysis

Early transcriptional response of the A549 epithelial cells, were analysed using Illumina HumanHT-12 v4 microarray platform following three hours of exposure to *B. pseudomallei* live bacteria (BCMS) and its secreted proteins (CCMS). The HumanHT-12 v4 Expression BeadChip targets more than 31,000 annotated genes with more than 47,000 probes.

In order to determine the quality of the hybridization, the microarray data obtained was subjected to the quality control filters. The use of internal controls present on the HumanHT-12 v4 Expression BeadChip allowed identification of outliers, the potential cause of outlier data and also aided to determine if a sample needs to be repeated or meets the expected quality standards. While the poor performance of sample-independent controls indicates a general problem with hybridisation, washing and staining, the poor performance of sample-dependent controls indicates problems related to the sample or labelling. In this study, all the controls were found to be within the expected value of the control metrice as described in Table 2.10. The quality control data obtained confirmed that all the arrays on the BeadChip met the expected quality control whereby the hybridisation, washing and staining of the BeadChip had been carried out without any problems. There is also no problem detected in the sample and it's labelling (Figure 3.20).

Following quality control of the hybridisation and washing procedures, hierarchial clustering was performed and showed that the control and the treated group (BCMS and CCMS) were grouped into different clusters (Figure 3.21). The raw microarray data was then extracted from the Genome Studio software and subjected to further analysis using the Genespring GX 11 software. The latter software determines the quality of the samples using the Principal Component Analysis (PCA), which allows identification of

outlier samples that fall distal to cohort biological replicates or dataset at large. Using PCA, samples from the same experimental condition were found to group closer to each other than to samples from different conditions and without any outliers (Figure 3.22).

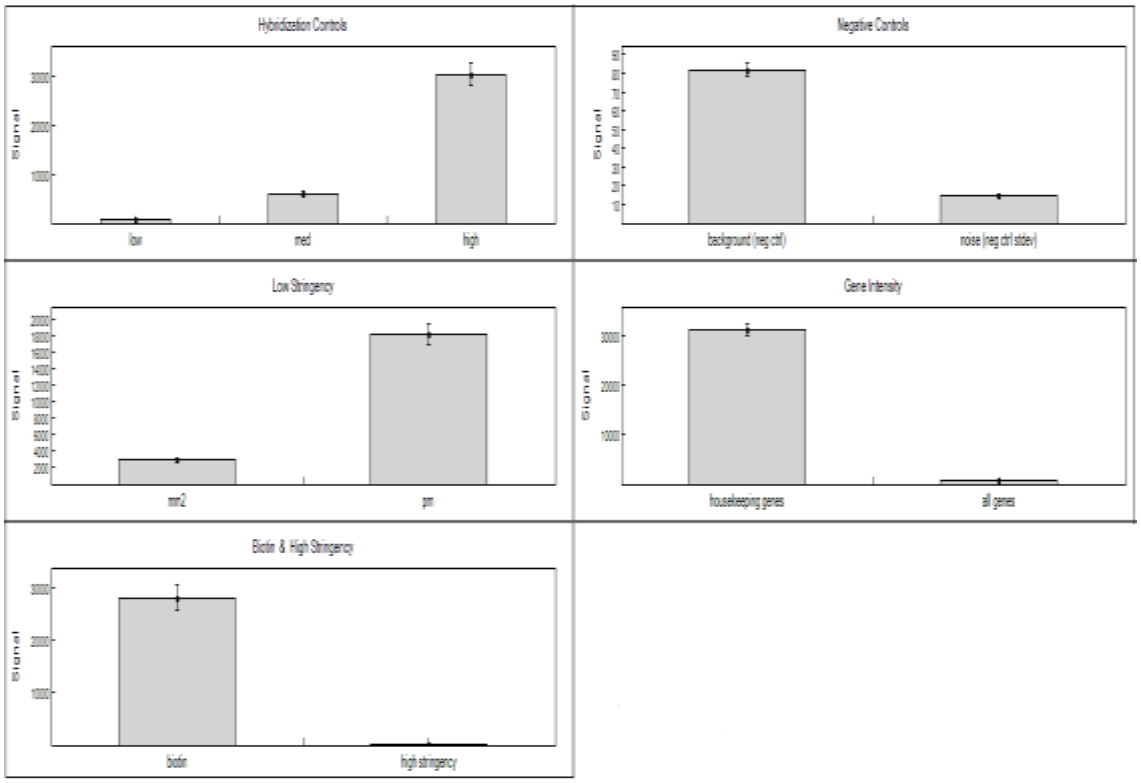


Figure 3.20: The hybridisation control plot generated using GenomeStudio GX software.

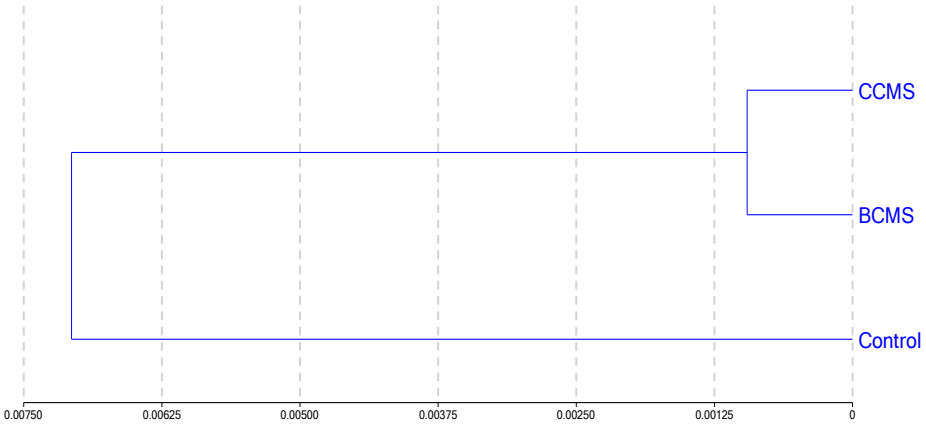


Figure 3.21: Hierarchical clustering of the control and treated (BCMS and CCMS) groups with different distance metrics using the Illumina GenomeStudio software.

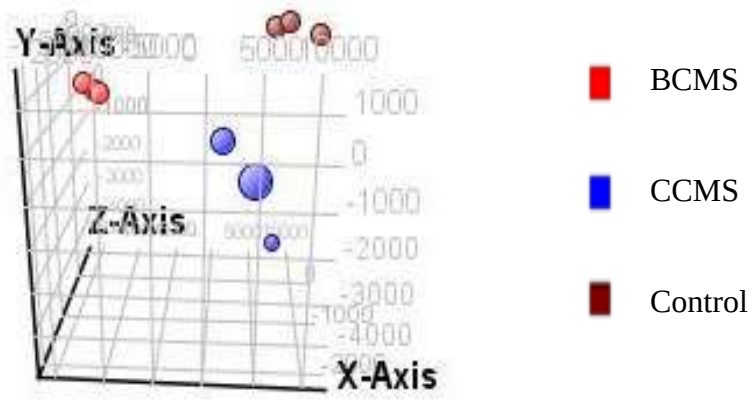


Figure 3.22: Quality control of samples using Principal Component Analysis (PCA).

3.5.4.1 Normalisation and filtration

The raw data was normalised in order to remove systematic effects, which arise from variation of non-biological origin rather than from biological differences between the RNA samples or between the printed probes. Normalisation is achieved by dividing intensities by the total intensity of the given array. Box Whisker plots were generated to evaluate the overall consistency and quality of the microarray data obtained. All the arrays showed the same scaling median and a uniform interquartile range (25% percentile and 75% percentile of a dataset as the respective lower and upper boundary of a box) generated between all the 9 arrays (Figure 3.23). These normalised data was then subjected to filtration in order to identify the entities that were present (> 0.08) or marginal (0.06 - 0.08) and eliminate the entities that were absent (< 0.06) (Figure 3.24). From a total of 47,323 genes, 32,339 genes (68.34%), having only the Present and Marginal cut-off in at least one sample were filtered and selected. Using the one-way ANOVA with Benjamini Hochberg (multiple testing corrections), 2,560 genes of the 32,339 genes with Present and Marginal cut-off were filtered and identified as significantly expressed ($p<0.05$).

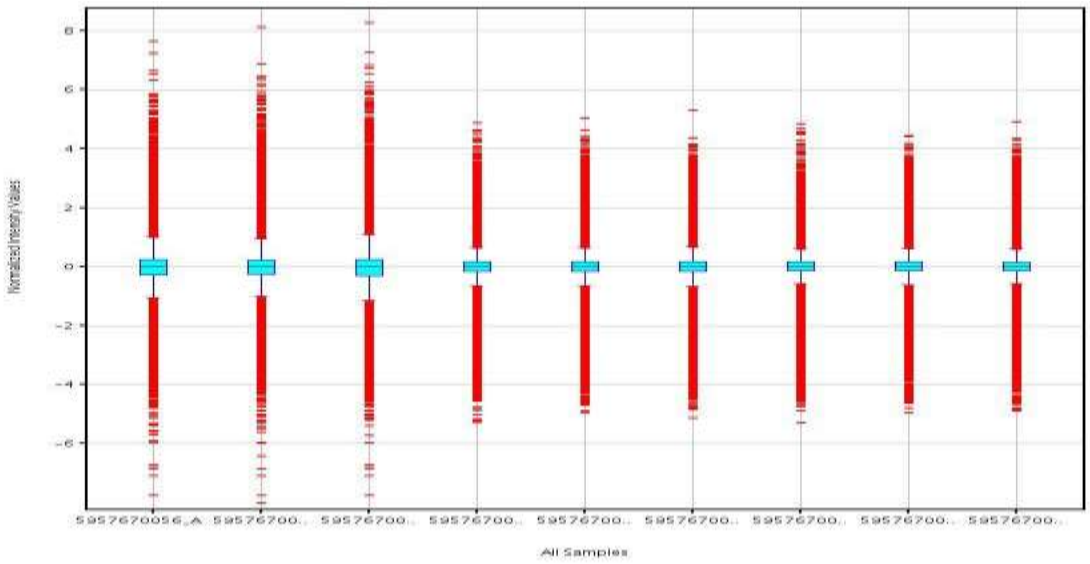


Figure 3.23: Box-Whisker plot of the data normalised using the GeneSpring software analysis.

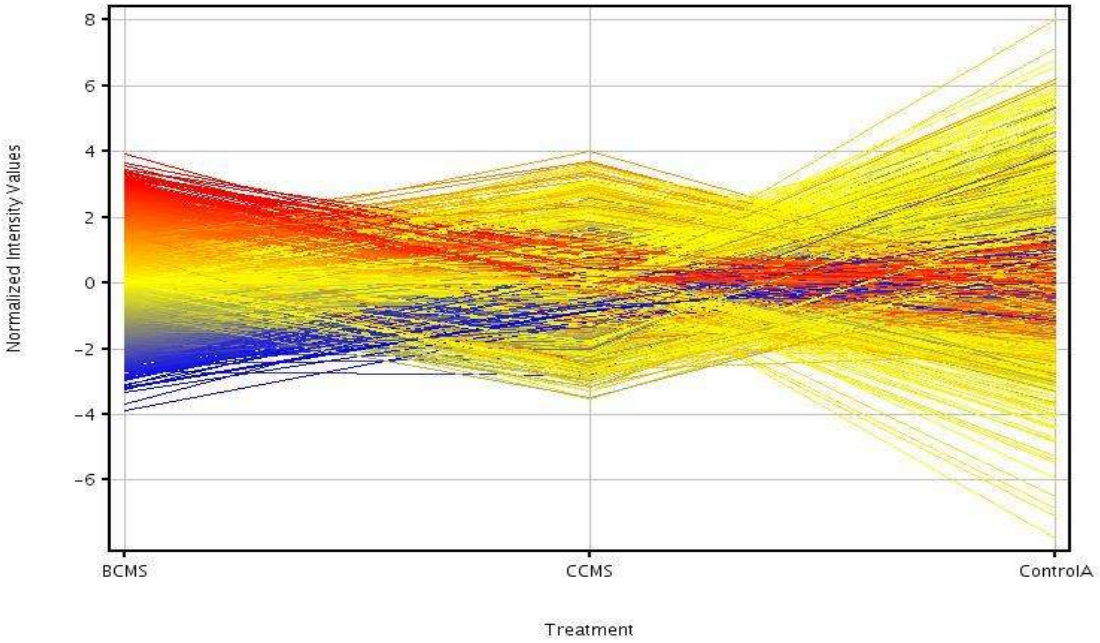


Figure 3.24: The profile plot generated from signal values that were normalised and filtered using the Present and Marginal flags.

3.5.4.2 Volcano plot for fold change analysis

The 9,115 significantly expressed ($p<0.05$) genes were further statistically analysed using the volcano plot which allows statistical and fold change analysis between two conditions. The live bacteria (BCMS) was found to differentially regulate 593 genes and the secreted proteins (CCMS) differentially regulated 624 genes as compared to the uninfected control cells, with the cut-off of two folds and p-value < 0.05. The genes were plotted on the volcano plot according to the fold change and p-value (Figure 3.25).

In general, under both the BCMS and CCMS conditions, the number of down-regulated genes outnumbered the number of up-regulated genes. The BCMS condition was found to down-regulate 418 genes and up-regulate 175 genes among the 593 genes differentially regulated genes. Of the 624 differentially regulated genes under the CCMS condition, 429 were down-regulated and 191 were up-regulated (Figure 3.26). Further analysis using the Venn diagram revealed the presence of 517 genes that were commonly regulated under both the BCMS and CCMS conditions, whereas, 76 and 107 genes were exclusively regulated by the BCMS and CCMS, respectively (Figure 3.27).

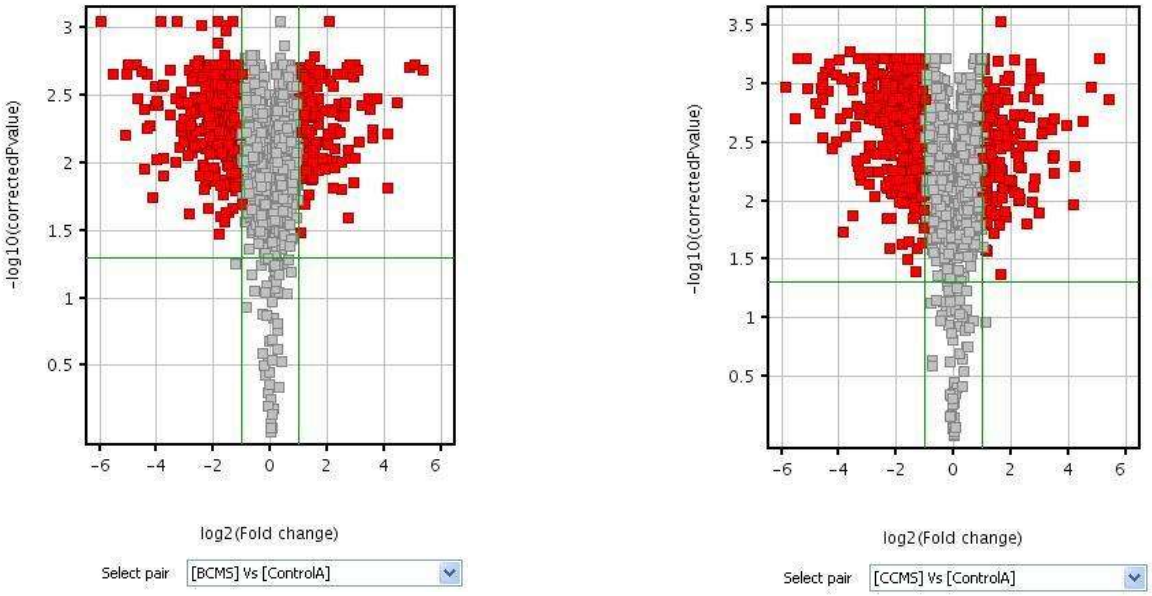


Figure 3.25: Volcano plot of the live bacteria (BCMS) and secreted proteins (CCMS) versus control. The red spots indicate differentially modulated genes that passed the filter with a fold change of two and p-value <0.05.

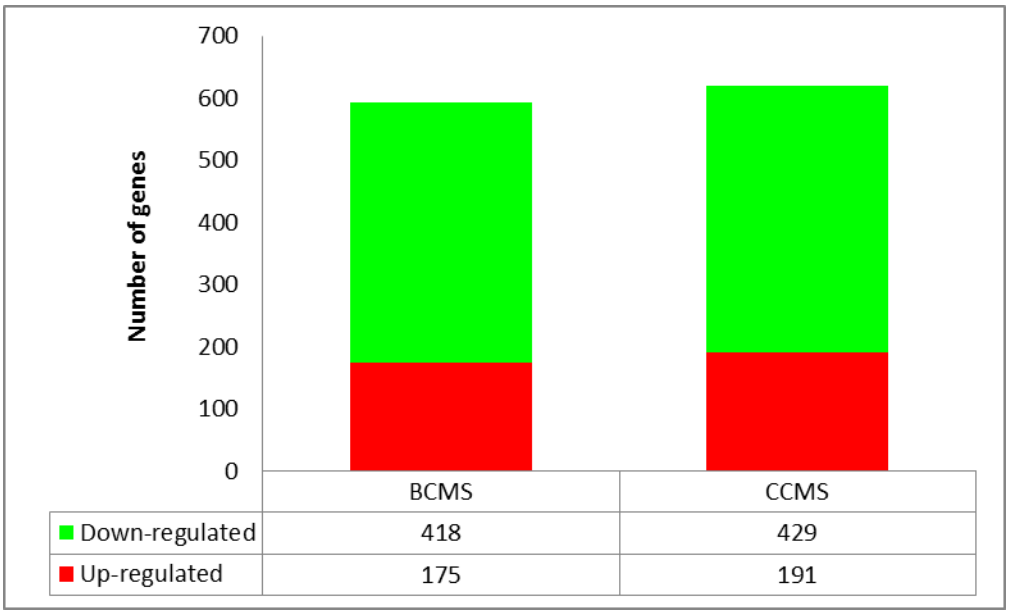


Figure 3.26: The number of genes up-regulated and down-regulated with fold change ≥ 2.0 under the BCMS and CCMS conditions.

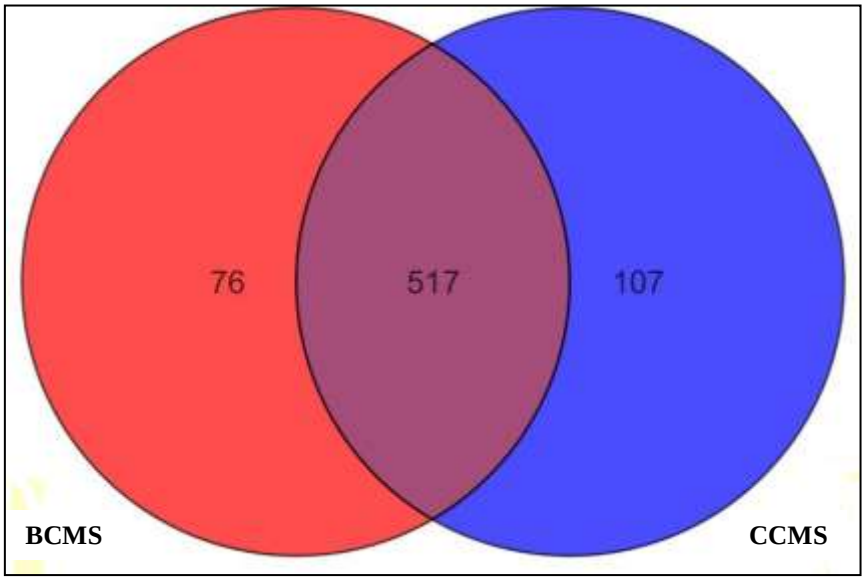


Figure 3.27: Venn diagram demonstrating the number of genes commonly regulated by both the BCMS and the CCMS conditions and the number of genes exclusively regulated by each of the conditions.

3.5.4.3 Gene Ontology analysis

In order to assess the gene products in terms of their associated biological processes, cellular components and molecular functions in a species-independent manner, the microarray data was further analysed using the Gene Ontology (GO) analysis. The 593 genes regulated (418 down-regulated and 175 up-regulated) by BCMS and 624 genes regulated (429 down-regulated and 191 up-regulated) by CCMS were subjected to GO analysis. This GO analysis describes the number of genes that are affected under each of the process.

Biological processes

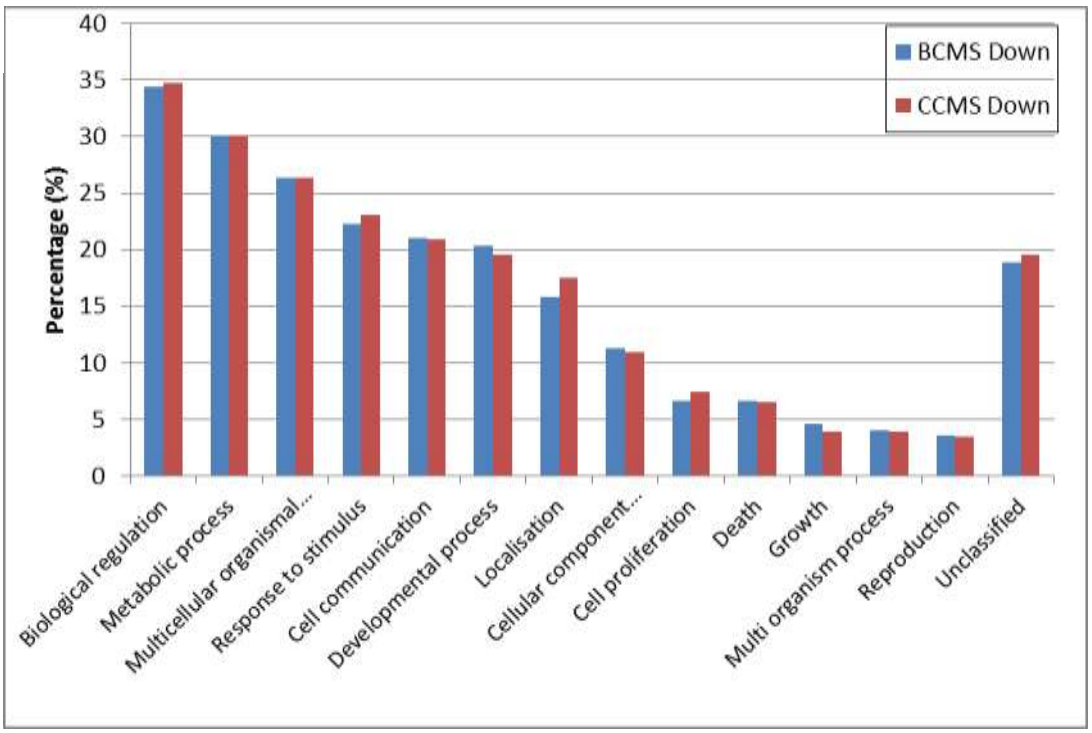
Among the down-regulated genes in the BCMS and CCMS conditions, similar percentage of genes were found involved in all the different biological processes (Figure 3.28). The highest percentages of down-regulated genes were involved in biological regulation (35%), metabolic process (30%) and multicellular organismal process (26%), response to stimulus (22-23%), cell communication (21%) and developmental process (20%). Less than 20% of the down-regulated genes under both the BCMS and CCMS conditions were involved in two other biological processes i.e. localisation (16-17%) and cellular component organisation (11%). However, only a small percentage (3.5-7%) of the down-regulated genes under both the BCMS and CCMS conditions were involved in rest of the biological processes, including cell proliferation, death, growth, multi organism process and reproduction.

A similar pattern of involvement in biological process was also observed among the up-regulated genes under both the BCMS and CCMS conditions. It was found that approximately 31% of the up-regulated genes under both conditions were found to be involved in biological regulation, 29-34% in metabolic process and 23% in multicellular

organismal process. Less than 20% of the up-regulated genes were also found to be involved in response to stimulus (15-16%), cell communication (16-18%), developmental process (18-19%) and localisation (13-16%) and cellular component organisation (10-11%). Similar to the down-regulated genes under both the BCMS and CCMS conditions, only a small percentage (2-9%) of the up-regulated genes under both conditions were involved in the rest of the biological process, including cell proliferation, death, growth, multi organism process and reproduction. In comparison between BCMS and CCMS conditions, significant difference was observed between the percentages of genes involved in death, whereby higher percentage of up-regulated genes under the CCMS conditions (4.2%) were found to be involved in death compared to the genes under the BCMS condition (1.7%).

There were approximately similar percentages of down-regulated and up-regulated genes under both conditions found to be involved in the different biological process. However, two fold higher percentages of up-regulated genes under both the conditions were found to be involved in growth compared to the down-regulated genes.

A)



B)

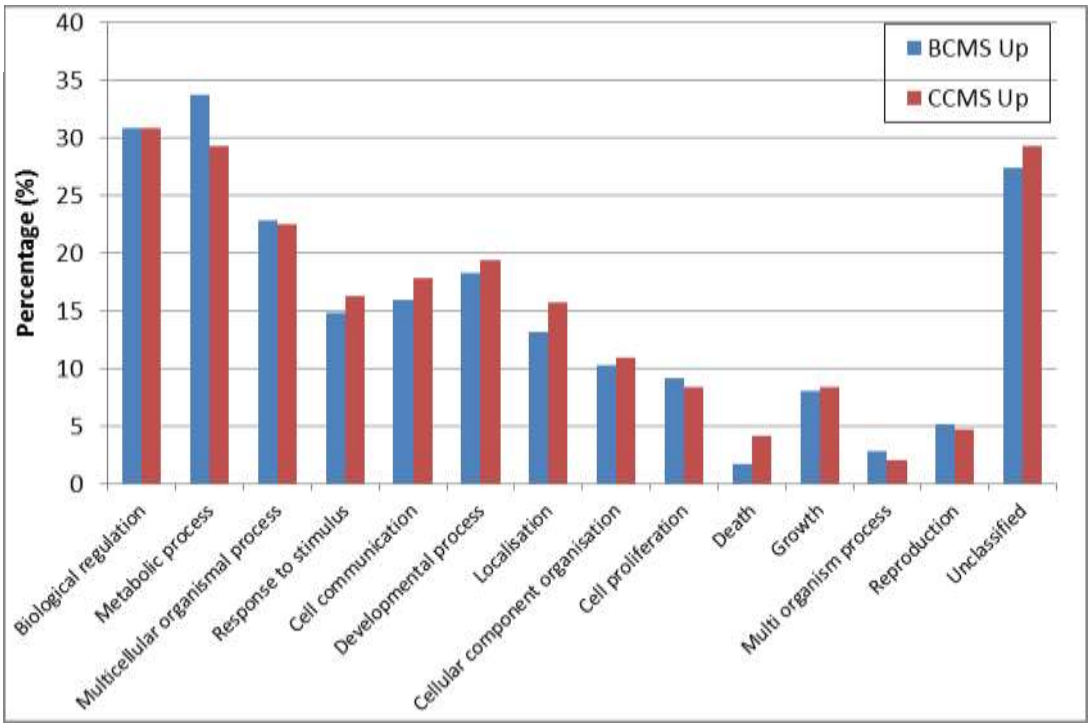


Figure 3.28: Significantly enriched biological processes GO categories under the BCMS and CCMS conditions. A) GO categories for BCMS and CCMS down-regulated genes and B) GO categories for BCMS and CCMS up-regulated genes. The total percentage of genes in the analysis do not sum to 100% (number of up or down-regulated genes) since some genes occur in multiple nodes.

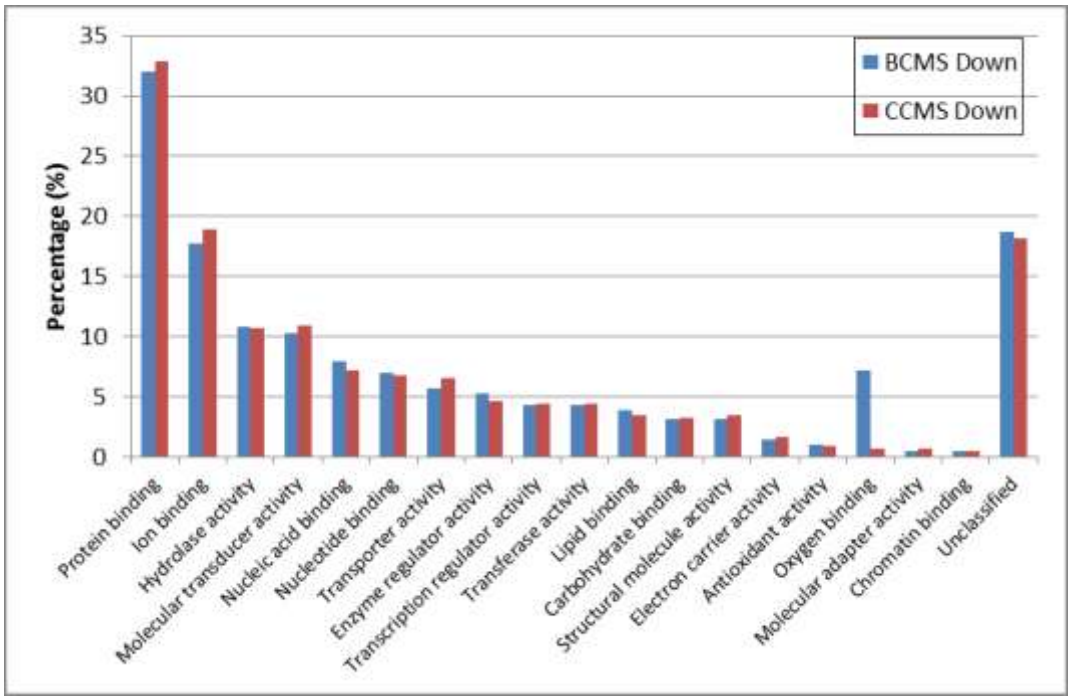
Molecular functions

Among the down-regulated genes under both the BCMS and CCMS conditions, similar percentage of genes were found to be involved in the various GO molecular function (Figure 3.29). However, oxygen binding function showed involvement of approximately 10-fold higher percentage of the BCMS down-regulated genes (7.2%) compared to the CCMS down regulated genes (0.7%). The highest percentage of genes was found to be involved in protein binding (32-32.8%). This is followed by ion binding (17.7-18.9%), hydrolase activity (10.7%) and molecular transducer activity (10.3-11%). Less than 10% (0.5 -7.9%) of the down-regulated genes under both the BCMS and CCMS conditions were found to be involved in the rest of the categories of molecular functions.

A similar percentage of up-regulated genes under both the BCMS and CCMS conditions were also found to be involved in the various GO molecular function categories (Figure 3.29). The highest percentage of the genes was found to be involved in protein binding (31.4 -34.5%), and this is followed by ion binding (12 -12.5%). Less than 10% genes were found to be involved in rest of the molecular function categories.

Comparison among the down-regulated and up-regulated genes showed that noticeably higher percentages of down-regulated genes were involved in almost all the molecular function categories. However, higher percentages of the up-regulated genes were found to be involved in the nucleic acid binding and transcription regulator activities as compared to the down-regulated genes.

A)



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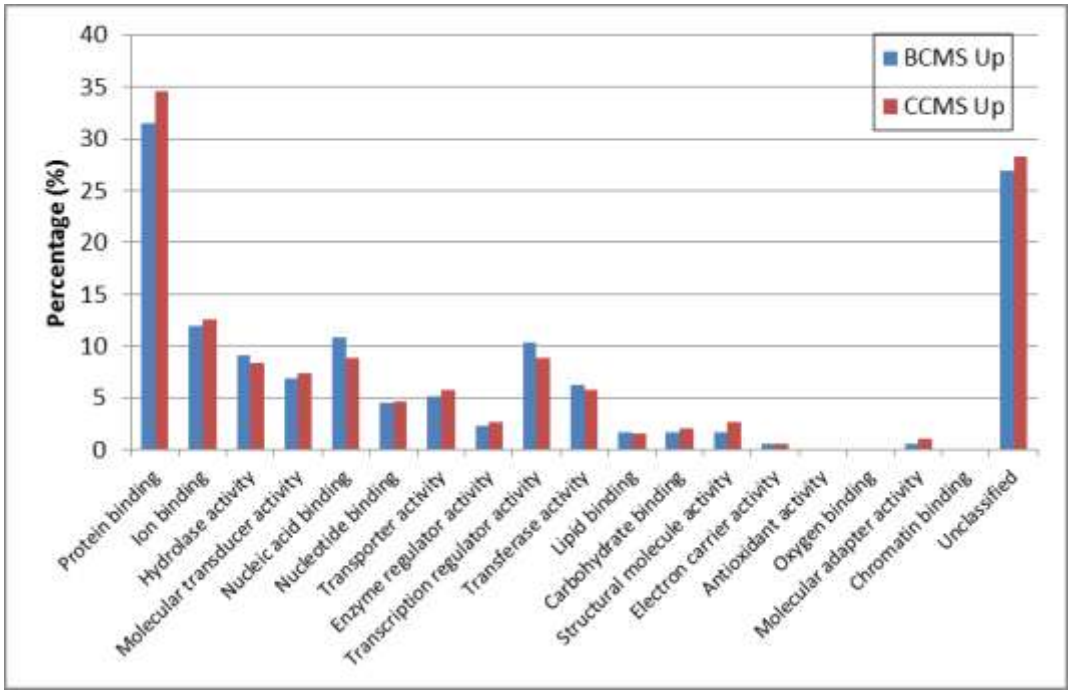
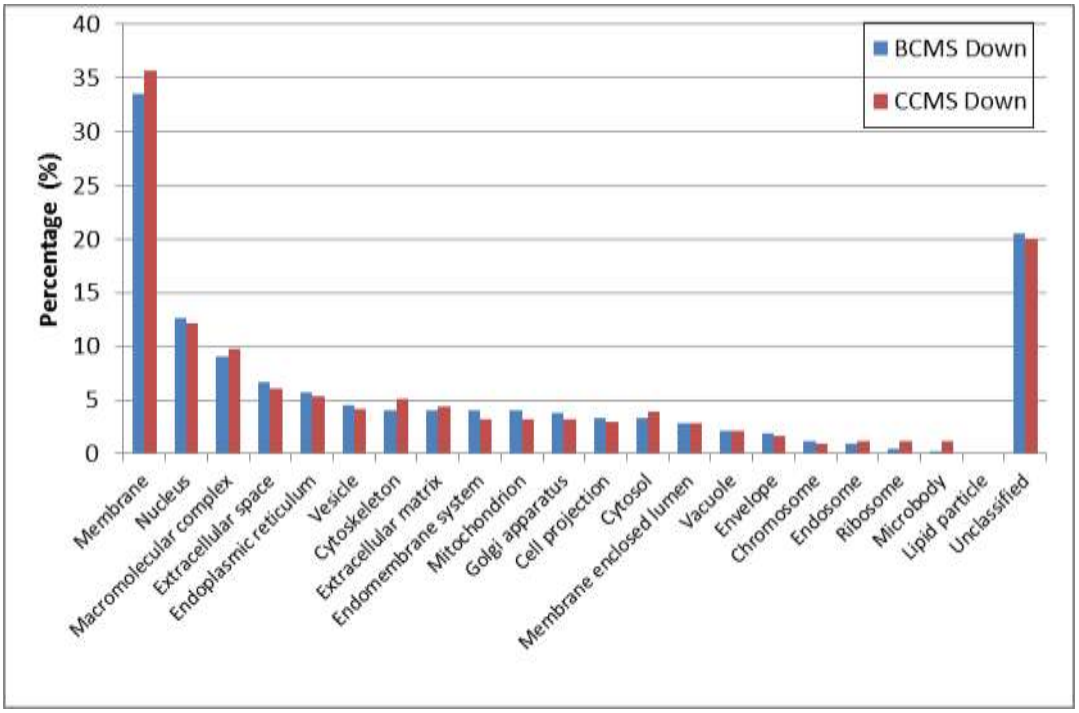


Figure 3.29: Significantly enriched molecular function GO categories under BCMS and CCMS conditions. (A) GO categories for BCMS and CCMS down-regulated genes and (B) GO categories for BCMS and CCMS up-regulated genes. As some genes occur in multiple nodes, the total percentage of genes in the analysis do not sum to 100% (number of up or down-regulated genes).

Cellular component

Comparison between the up-regulated and down-regulated genes revealed similar percentages of genes under both the BCMS and CCMS conditions were found to be associated with the cellular component GO categories (Figure 3.30). The highest percentage of genes was found to be associated with membrane (33.5-35.7% down-regulated genes; 26.9-30.9% up-regulated genes), and this is followed by nucleus (12.1-12.7% down-regulated genes; 15.1-17.1% up-regulated genes). Less than 10% of both the down-regulated and up-regulated genes were associated with the rest of the cellular components.



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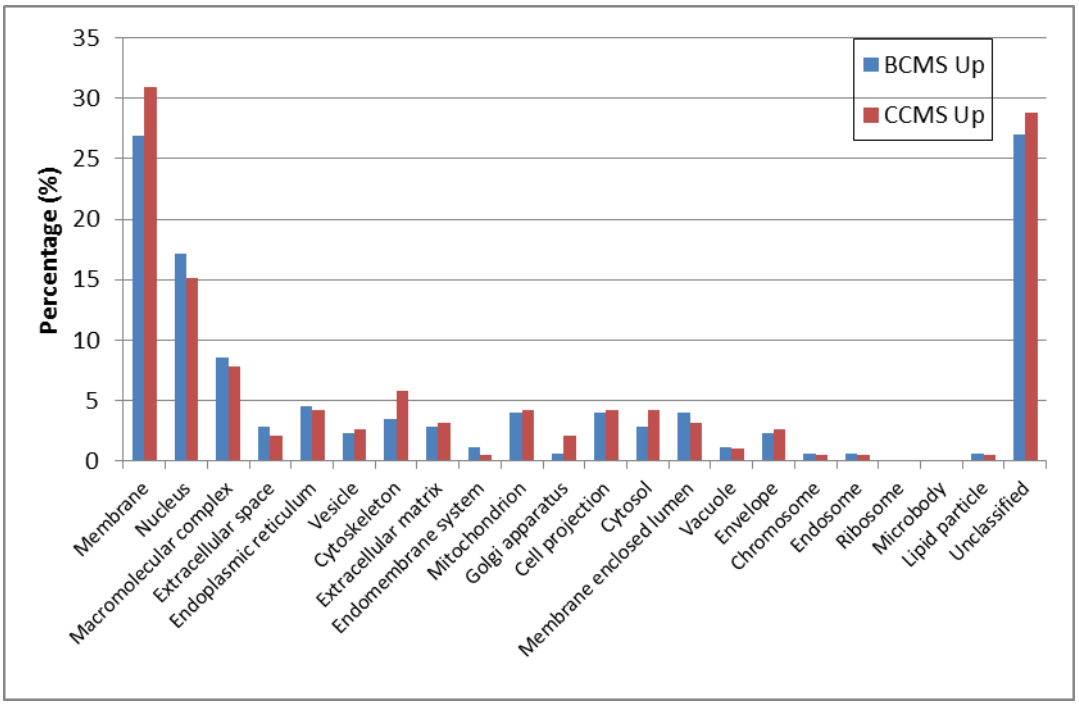

Figure 3.30: Significantly enriched cellular component GO categories under the BCMS and CCMS conditions. (A) GO categories for BCMS and CCMS down-regulated genes and (B) GO categories for BCMS and CCMS up-regulated genes. As some genes occur in multiple nodes, the total percentage of genes in the analysis do not sum to 100% (number of up or down-regulated genes).

3.5.4.4 Prediction of the A549 cells pathways effected by *Burkholderia pseudomallei* live bacteria and secreted proteins

Significant regulations of different pathways were analysed using the web-based Kyoto Encyclopaedia of Genes and Genomes (KEGG) databases. This pathway analysis allowed identification of the higher-level systemic functions of the genes that were significantly regulated ($p < 0.05$) under the BCMS and CCMS conditions. Both the conditions were found to differentially regulate various pathways.

Using GeneTrail, secreted proteins were found to regulate higher number of pathways (Table 3.11). However, both the live bacteria and secreted proteins significantly up-regulated ($p<0.05$) pathways associated with metabolism especially the starch and sucrose metabolism, ascorbate and aldarate metabolism and pentose and glucuronate interconversions. Similarly, pathways associated with cell signalling (neutrophin signalling, insulin signalling, TGF-β signalling and Hedgehog signalling) and cell adhesion (focal adhesion and cell adhesion molecules; CAMs) pathways were also up-regulated. Secretory proteins also exclusively regulated other signalling pathways (adipocytokine signalling, Fc epsilon RI signalling, Jak-STAT signalling, ErbB signalling, chemokine signalling and mTOR signalling), tight junction, Fc gamma R-mediated phagocytosis and apoptosis pathways.

Both the live bacteria and secreted proteins also showed similar significant down-regulation ($p<0.05$) of metabolic pathways (drug metabolism – cytochrome P450, arginine and proline metabolism, nicotinate and nicotinamide metabolism, glutathione metabolism) and pathways associated with complement and coagulation cascades, lysosome and phagosome. Secretory proteins also exclusively regulated pathways associated with antigen processing and presenting, ECM-receptor interaction and PPAR signalling.

In order to increase the extent of annotations based on analysis of a network of genes in the literature, GATHER was used with the activated functional network inference component to reveal a wider scope of functions not immediately evident in the signature and investigate further potential functional relationships of the pathways significantly regulated. This revealed significant associations with more pathways in KEGG, including up regulation of apoptosis and MAPK signaling pathways in BCMS and MAPK signaling pathway in CCMS conditions (Table 3.12). Similar down regulation of cytokine-cytokine receptor interaction, Jak-STAT signaling, oxidative phosphorylations and Toll-like receptor signaling pathways were identified in both the BCMS and CCMS conditions. Specific genes involved in the particular pathways are further reported in the heat map analysis section.

Table 3.11: KEGG pathways that were significantly regulated (p-value <0.05) by *B. pseudomallei* live bacteria (BCMS) and secreted protein (CCMS) conditions. The pathways were identified using GeneTrail software.

Condition	KEGG pathway	p-value
BCMS		
Up-regulated	Starch and sucrose metabolism	$1.87e^{-4}$
	Ascorbate and aldarate metabolism	$3.68e^{-4}$
	Pentose and glucuronate interconversions	$5.11e^{-4}$
	Neutrophin signalling pathway	$4.82e^{-3}$
	Insulin signalling pathway	$6.49e^{-3}$
	Metabolism of xenobiotics by cytochrome P450	$6.84e^{-3}$
	TGF-β signalling pathway	$1.12e^{-2}$
	Focal adhesion	$2.38e^{-2}$
	Cell adhesion molecules (CAMs)	$3.78e^{-2}$
	Hedgehog signalling pathway	$3.81e^{-2}$
Down-regulated	Complement and coagulation cascades	$2.87e^{-3}$
	Drug metabolism – cytochrome P450	$3.82e^{-3}$
	Arginine and proline metabolism	$4.92e^{-3}$
	Nitrogen metabolism	$1.13e^{-2}$
	Nicotinate and nicotinamide metabolism	$1.28e^{-2}$
	Glutathione metabolism	$1.94e^{-2}$
	Alanine, aspartate and glutamate metabolism	$2.78e^{-2}$
	Lysosome	$3.89e^{-2}$
	Phagosome	$4.49e^{-2}$

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CCMS

Up-regulated	Insulin signalling pathway	$2.89e^{-4}$
	Neurotrophin signalling pathway	$1.52e^{-3}$
	Tight junction	$2.00e^{-3}$
	Fc gamma R-mediated phagocytosis	$3.79e^{-3}$
	Starch and sucrose metabolism	$5.47e^{-3}$
	Hedgehog signalling pathway	$6.38e^{-3}$
	Adipocytokine signalling pathway	$1.05e^{-2}$
	Focal adhesion	$1.12e^{-2}$
	Cell adhesion molecules (CAMs)	$1.30e^{-2}$
	Ascorbate and aldarate metabolism	$1.34e^{-2}$
	Fc epsilon RI signalling pathway	$1.63e^{-2}$
	Pentose and glucuronate interconversions	$1.65e^{-2}$
	TGF-β signalling pathway	$1.99e^{-2}$
	Jak-STAT signalling pathway	$2.06e^{-2}$
	ErbB signalling pathway	$2.11e^{-2}$
	Apoptosis\	$2.17e^{-2}$
	Chemokine signalling pathway	$3.89e^{-2}$
	mTOR signalling pathway	$4.88e^{-2}$
Down-regulated	Complement and coagulation cascade	$7.70e^{-4}$
	Drug metabolism – cytochrome P450	$5.35e^{-3}$
	Antigen processing and presenting	$7.38e^{-3}$
	Phagosome	$8.14e^{-3}$
	ECM-receptor interaction	$1.05e^{-2}$
	Nicotinate and nicotinamide metabolism	$1.54e^{-2}$
	Lysosome	$1.75e^{-2}$
	PPAR signalling pathway	$1.80e^{-2}$
	Glutathione metabolism	$2.43e^{-2}$
	Histidine metabolism	$2.56e^{-2}$
	Arginine and proline metabolism	$2.78e^{-2}$

Table 3.12: KEGG pathways significantly regulated by *B. pseudomallei* live bacteria and secreted proteins using GATHER with the activated Infer from Network component.

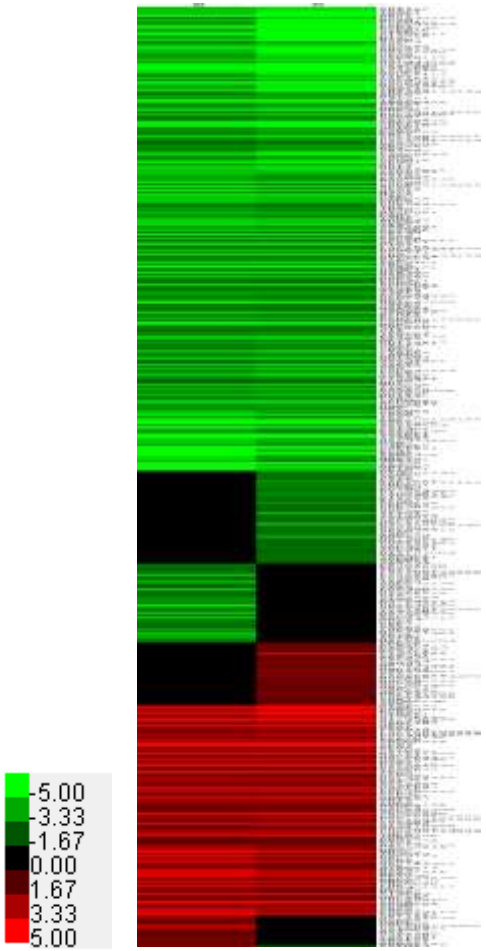
Condition	KEGG pathway	Number of genes	p-value	Bayes factor
BCMS				
Up-regulated	*Apotosis*	*38*	*0.003*	*23*
	MAPK signalling pathway	45	0.05	3
Down-regulated	Cytokine-cytokine receptor interactions	111	0.001	38
	Jak-STAT signalling pathway	59	0.01	11
	Toll-like receptor signalling pathway	40	0.02	8
CCMS				
Up-regulated	MAPK signalling pathway	46	0.05	3
Down-regulated	Cytokine-cytokine receptor interactions	110	0.001	35
	Jak-STAT signalling pathway	59	0.02	10
	Toll-like receptor signalling pathway	40	0.02	8

3.5.4.5 Heat map analysis

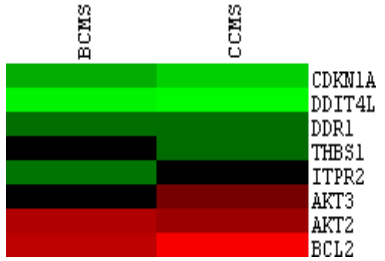
Heat map analysis was performed using the web-based software Cluster v3.0, and visualised using the Java Treeview v1.1.3. The heat map generated indicate the specific genes involved in each of the pathways regulated, whereby the up-regulation or down-regulation of the genes associated with the pathways and the fold-change of the regulation was used for clustering (Figure 3.31).

Four functional categories of regulation of A549 cell genes in response to *B. pseudomallei* live bacteria and secretory proteins upon three hours of exposure were identified:

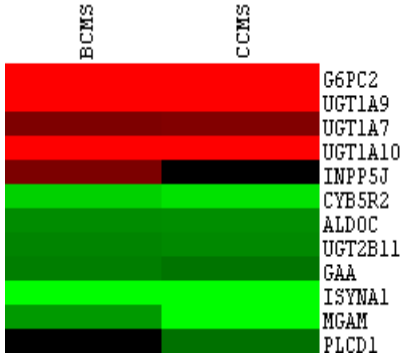
i) Host genes associated with carbohydrate metabolisms were up-regulated and amino-acid metabolisms were down-regulated in response to *B. pseudomallei* live bacteria and secreted proteins
ii) Host genes associated with immunity and defence were down-regulated in response to *B. pseudomallei* live bacteria and secreted proteins
iii) Host genes associated with cell communication and signalling molecules were altered in response to *B. pseudomallei* live bacteria and secreted proteins
iv) Host genes associated with proliferation and survival were altered in response to *B. pseudomallei* live bacteria and secreted proteins



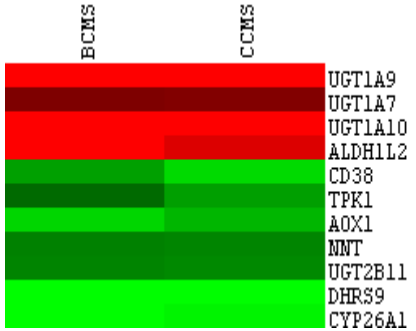
overall



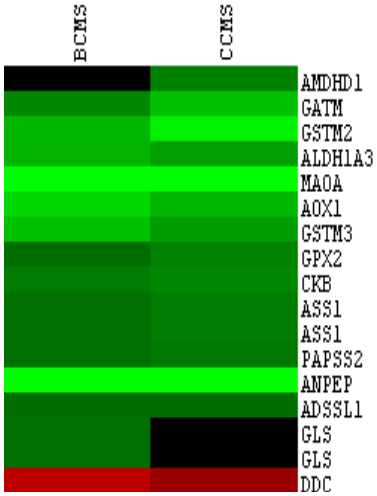
cell growth and death



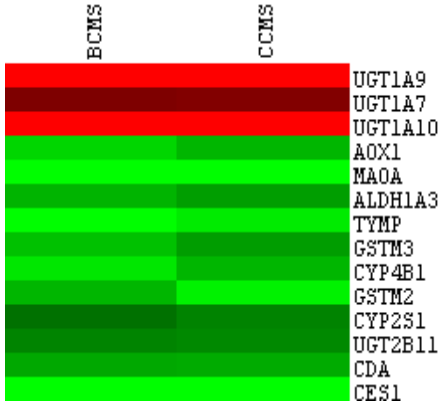
Carbohydrate metabolism



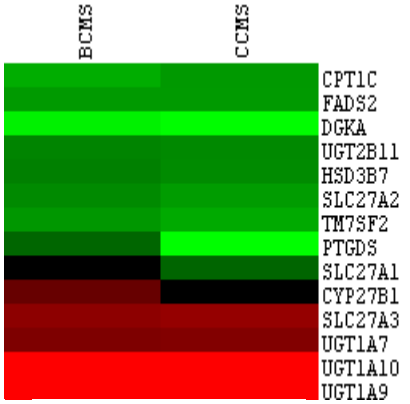
Metabolism of cofactors and vitamins



amino acid metabolism



Xenobiotic biodegradation and metabolism



Lipid metabolism

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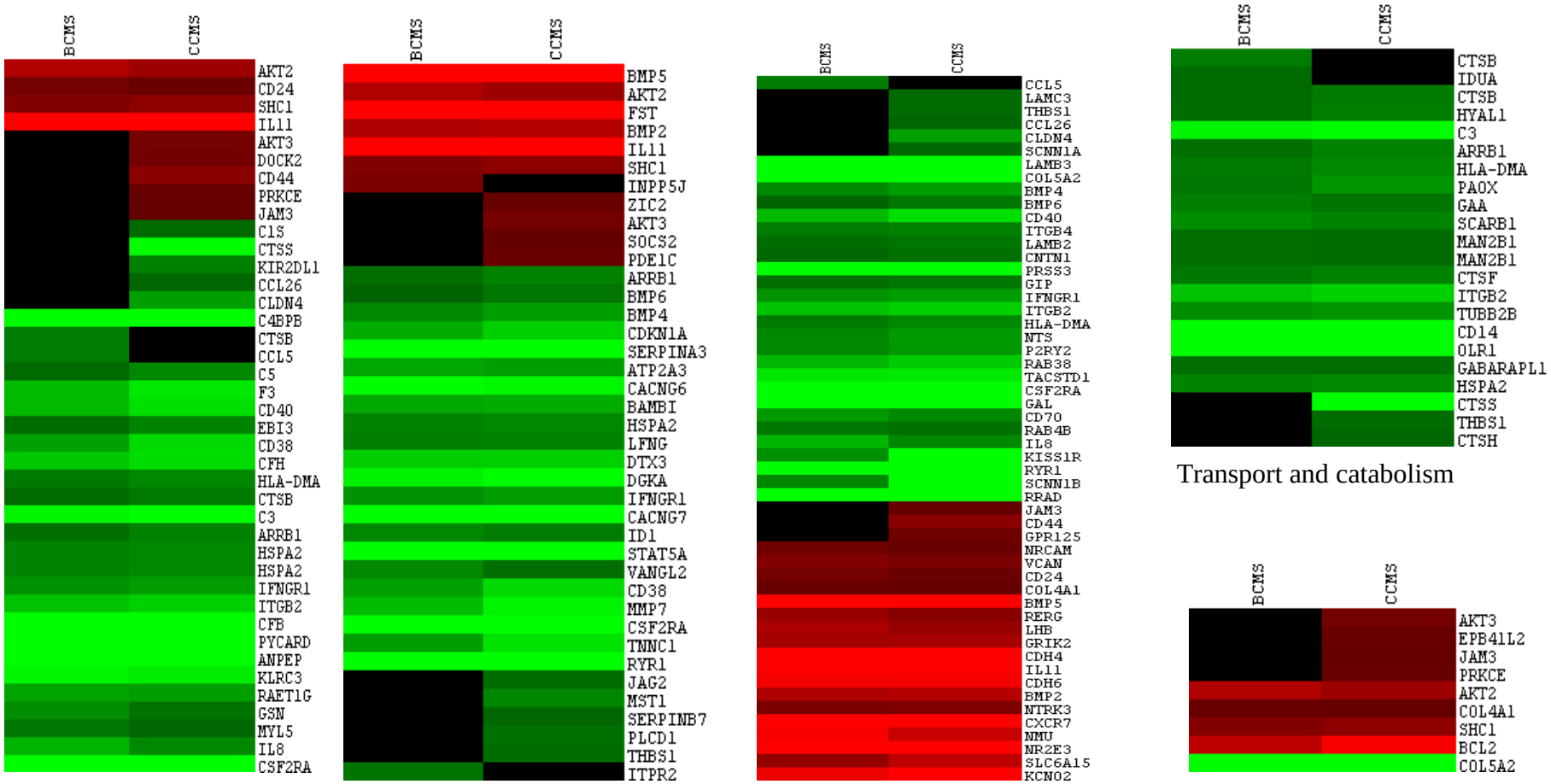


Figure 3.31: Heat map analysis

In general, both the BCMS and CCMS were found to up-regulate host genes involved in carbohydrate metabolism, a basic metabolic process that provides carbon and energy (Figure 3.31). Both conditions were found to highly up-regulate (13.2 and 6.6 fold up-regulation by BCMS and CCMS, respectively) glucose-6-phosphatase (G6PC2), the gene that encodes the enzyme that catalyses the hydrolysis of glucose-6-phosphate, which is the terminal step in gluconeogenic and glycogenolytic pathways. UDPGTs, which are important in the conjugation and subsequent elimination of potentially toxic xenobiotics and endogenous compounds including UDP glucuronosyltransferase 1 family, polypeptide A10, A7 and A9 (UGT1A10, UGT1A7, UGT1A9) were also up-regulated.

Down-regulation of the genes associated with amino acid metabolism, indicating amino acid starvation due to host membrane damage, were also observed under both the BCMS and CCMS conditions (Figure 3.31). Host genes, including argininosuccinate synthase 1 (ASS1), creatine kinase (CKB), glycine amidinotransferase (GATM), glutaminase (GLS), monoamine oxidase A (MAOA) and adenylosuccinate synthase like 1 (ADSSL1), were down-regulated under the BCMS condition. In addition to MAOA, ASS1, CKB, and GATM , CCMS condition was found to down-regulate other genes involved in the amino acid metabolism, including alanyl (membrane) aminopeptidase (ANPEP), glutathione peroxidase 2 (GPX2), glutathione S-transferase mu 2 and 3 (GSTM2, GSTM3), aldehyde dehydrogenase 1 family, member A3 (ALDH1A3) and amidohydrolase domain containing 1 (AMDHD1).

Generally, genes associated with the immune system are down-regulated under both the BCMS and CCMS conditions. Complement component 3 (C3), complement component 4 binding protein, beta (C4BPB), complement component 5 (C5), complement factor B (CFB), complement factor H (CFH) and coagulation factor III

(F3) were down regulated leading to the down-regulation of the complement and coagulation cascades pathways. Toll-like signalling pathway was also down-regulated with the down-regulation of chemokine (C-C motif) ligand 5 (CCL5), CD 14 molecule (CD14), CD40 molecule, TNF receptor superfamily member 5 (CD40) and interleukin 8 (IL8) genes. Down-regulation of antigen processing and presentation was also observed under the CCMS conditions, with the CTSB, CTSS, HLA-DMA HSPA2, KIR2DL1 and KLRC3 being down-regulated.

Additionally, genes involved in the lysosome pathway, including cathepsin B (CTSB), cathepsin F (CTSF), glucosidase alpha acid (GAA), hyaluronoglucosaminidase 1 (HYAL1), iduronidase, alpha-L- (IDUA) and mannosidase, alpha, class 2B, member 1 (MAN2B1) and genes associated with the phagosome pathway including C3, CD14 molecule (CD14), major histocompatibility complex, class II, DM alpha (HLA-DMA), integrin, beta 2 (ITGB2), oxidized low density lipoprotein (lectin-like) receptor 1 (OLR1), scavenger receptor class B, member 1 (SCARB1) and tubulin, beta 2B (TUBB2B) were also significantly down-regulated.

Genes associated with cell communication and signaling molecules were also generally up-regulated in response to *B. pseudomallei* live bacteria and secretory proteins. Both BCMS and CCMS conditions were found to up-regulate bone morphogenetic protein 2 (BMP2), bone morphogenetic protein 5 (BMP5) and follistatin (FST), leading to the up-regulation of TGF-β signaling pathway. Similarly, activation of the serine/threonine-protein kinases members v-akt murine thymoma viral oncogene homolog 2 and 3 (AKT2 and AKT3) in combination with B-cell CLL/lymphoma 2 (BCL2), collagen (COL4A1) and Src homology 2 domain containing transforming protein 1 (SHC1) led to the activation of focal adhesion pathway.

The cell adhesion molecule (CAMs) pathway was also activated under both BCMS and CCMS conditions, with the up-regulation of cadherin 4, type 1, R-cadherin (CDH4), neuronal cell adhesion molecule (NRCAM), which is a member of the immunoglobulin superfamily and versican (VCAN). Additionally, CCMS also up-regulated junctional adhesion molecule 3 (JAM3), which serves as a counter-receptor for integrin and alpha M (complement component 3 receptor 3 subunit) (ITGAM), that is involved in the regulation of transepithelial migration of polymorphonuclear neutrophils (PMN).

ErbB signaling pathway and mTOR signalling pathway were also up-regulated under the CCMS condition with the up-regulation of AKT2, AKT3 and SHC1. Coupling of binding of extracellular growth factor ligands to intracellular signaling pathways by the ErbB family of receptor tyrosine kinases (RTKs) regulated diverse biologic responses, including proliferation, differentiation, cell motility, and survival. At the same time, up-regulation of anti-apoptotic gene Bcl2 coupled with high down-regulation of the pro-apoptotic genes and regulators, including TP53I11, TP53INP1, TP63/TP73L and CDKN1A, indicate that early exposure of both *B. pseudomallei* and its secreted proteins led to the suppression of apoptosis of the A549 cells.

3.6.5 Validation of microarray results

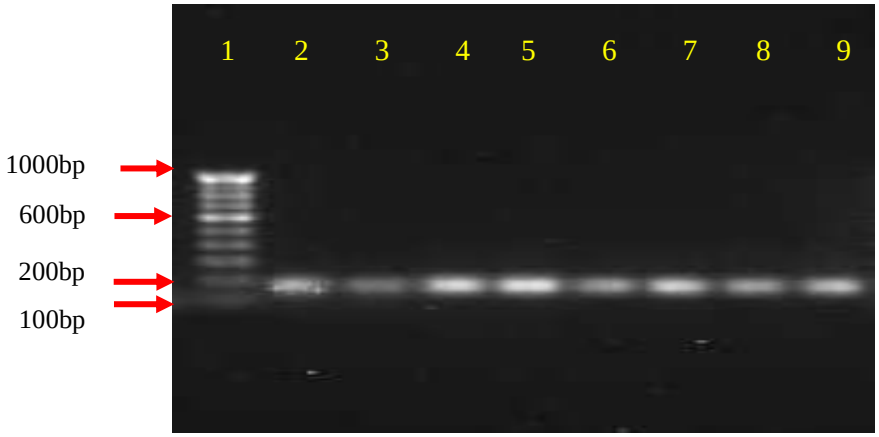
Validation of the microarray results were carried out using quantitative real-time polymerase chain reaction (qRT-PCR) assay. Eight target genes that were differentially expressed in the microarray analysis were selected and two housekeeping genes (*β-actin* and *GAPDH*) were used for normalization.

i) Gradient Conventional PCR

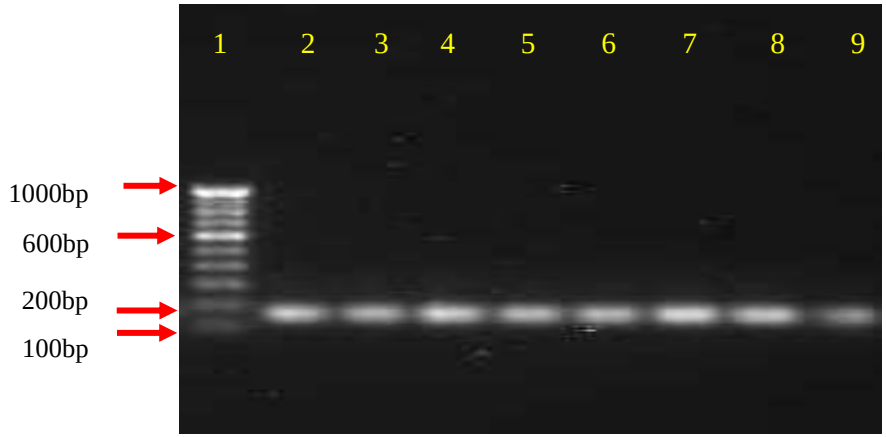
Annealing temperature gradient of 50-60ºC was used to perform gradient conventional PCR in order to identify the optimal annealing temperature for all the 10 identified primers. All primers showed amplification with the expected amplicon size of 162bp (ADAMST9), 153bp (G6PC2), 101bp (B-Actin), 130bp (CXCR7), 196bp (SERPINA3), 118bp (CES1), 151bp (FST), 101bp (GAPDH), 101bp (FXYD) and 110bp (PYCARD) (Figure 3.32). Multiple band amplification was observed for the primers SERPINA3 and FST at annealing temperature of 53.1-50ºC and 53.7-50ºC, respectively. However, annealing temperatures ranging from 56.4ºC to 60ºC showed the presence of single and clear band for all the genes used in this study. Consequently, the annealing temperatures of 56ºC, 58ºC and 60ºC were identified to perform the gradient qRT-PCR assay.

ii) Gradient qRT-PCR

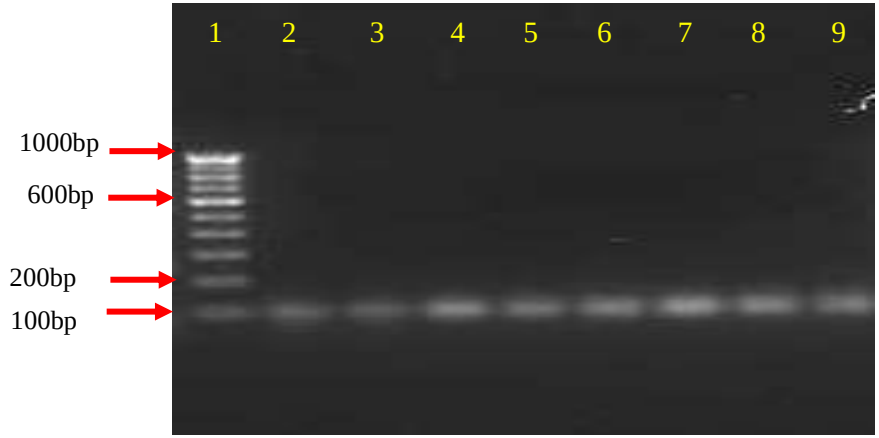
Similar single melting peaks were observed at the three different annealing temperatures of 56ºC, 58ºC and 60ºC used to perform the gradient qRT-PCR analysis using all the 10 selected primers (Figure 3.33). All the primer pairs used also showed the highest melting peak at the annealing temperature of 60ºC. The non-template control (NTC) did not show any amplification.



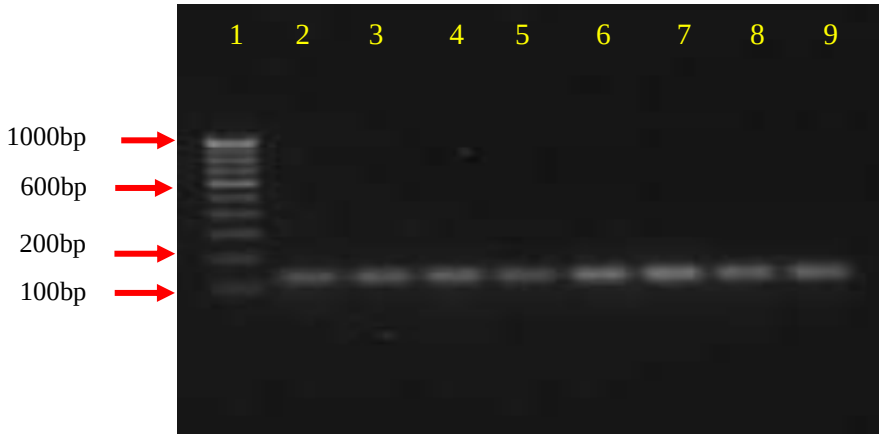
(A) ADAMST9 (164bp)



(B) G6PC2 (153bp)



(C) B-Actin (101bp)



(D) CXCR7 (130bp)

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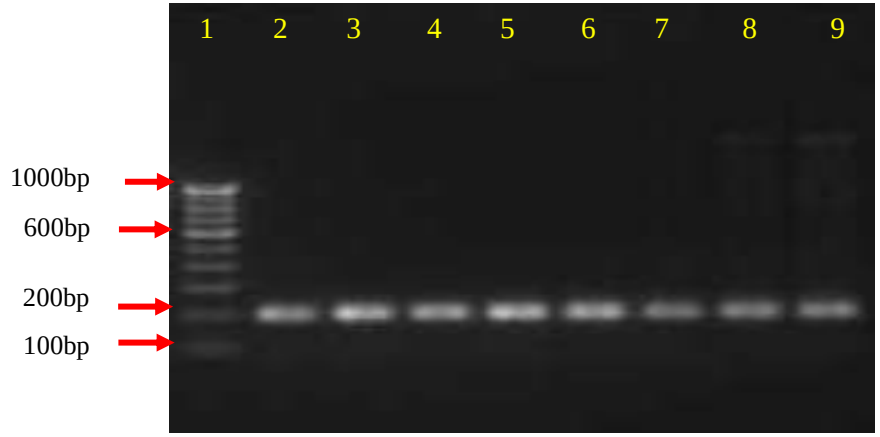
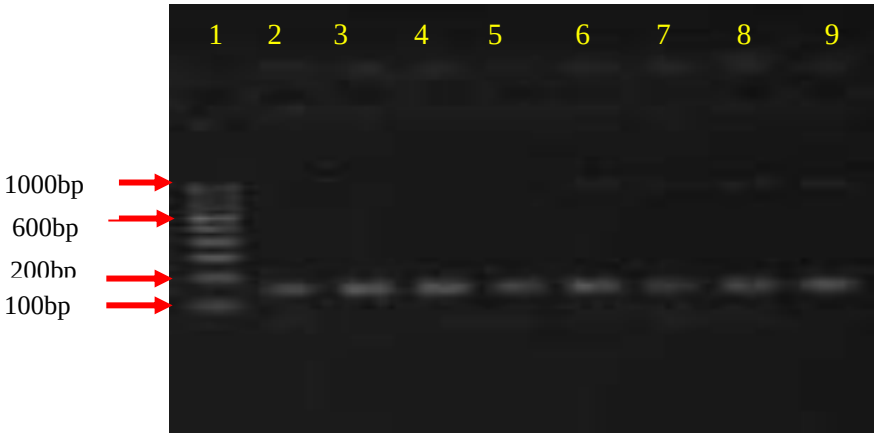
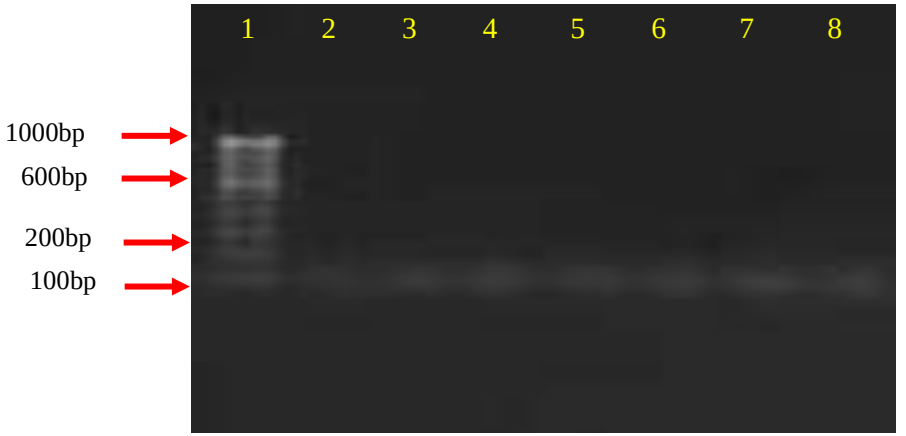


(E) SERPINA3 (196bp)

(F) CES1 (118bp)



(G) FST (151bp)



(H) GAPDH (101bp)

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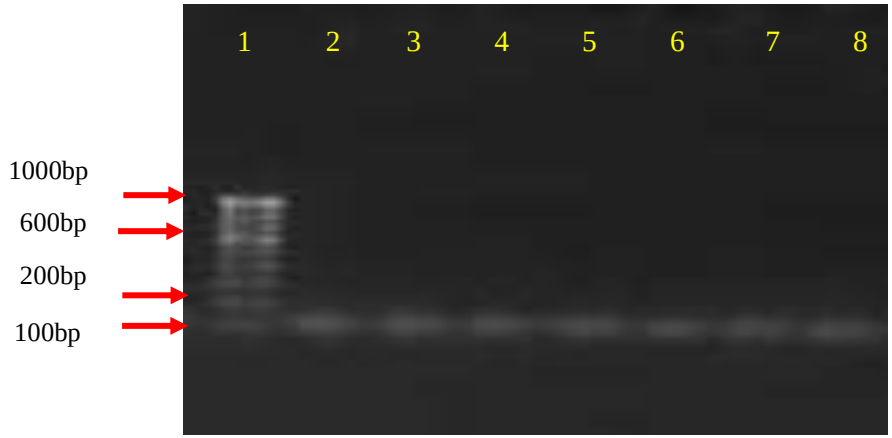
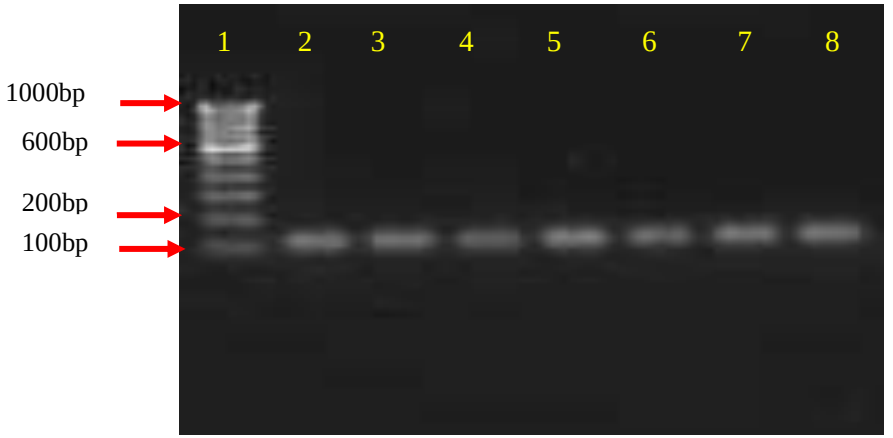


(I) FXYD (101bp)



(J) PYCARD (110bp)

Figure 3.32: Agarose gel showing gene fragment (100-200 bp) amplified from extracted genomic DNA of *B. pseudomallei*. Lane 1: DNA Ladder, Lane 2: 60ºC, Lane 3: 59.5ºC, Lane 4: 58.3ºC, Lane 5: 56.4ºC, Lane 6: 53.9ºC, Lane 7: 52.1ºC, Lane 8: 50.8ºC, Lane 9: 50ºC. All negative control did not show any amplification.

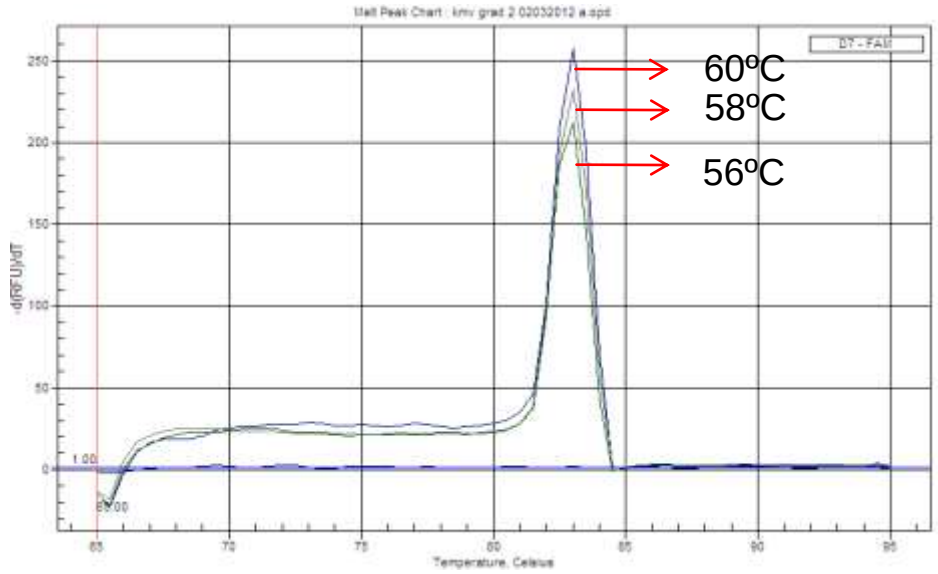


Figure 3.33: Melt peak chart showing gradient qRT-PCR at three different annealing temperatures of 56ºC, 58ºC and 60ºC.

iii) Standard curve for determination of primer efficiency

Standard curves were generated for each of the primer used in order to determine the efficiency of the primers before gene expression analysis was performed. The slope of the standard curve was used to estimate the PCR amplification efficiency of the qRT-PCR reaction. A real-time PCR standard curve is graphically represented as a semi-log regression line plot of CT value vs. log of input nucleic acid. A standard curve slope of –3.32 indicates a PCR reaction with 100% efficiency. Slopes which are more negative than –3.32 indicate reactions that are less than 100% efficient and slopes which are more positive than –3.32 may indicate problems associated with sample quality or pipetting. Generally, the standard curves of all primers used in this study were within the acceptable range, with slopes ranging between -3.347 to -3.157, correlation coefficient between 0.965-0.999 and efficiency percentage between 99.0%-107.4% (Table 3.13).

Table 3.13: Standard curve analysis showing the slope, correlation coefficient, efficiency percentage for all the primers used for validation of microarray results.

Gene	Slope	Correlation coefficient	Efficiency percentage
β-actin	-3.308	0.987	100.6%
GAPDH	-3.263	0.985	102.5%
G6PC2	-3.248	0.994	103.2%
CES1	-3.278	0.965	101.9%
CXCR7	-3.347	0.966	99.0%
LAYN	-3.299	0.985	101.0%
SERPINA3	-3.250	0.985	103.1%
PYCARD	-3.308	0.992	100.6%
FXYD2	-3.313	0.999	100.4%
FST	-3.157	0.998	107.4%

iv) Gene expression analysis

qRT-PCR performed on the same samples used for the microarray analysis verified the results obtained in the microarray analysis. The genes analysed was confirmed as up-regulated or down-regulated in correlation with the results obtained in the microarray analysis, indicating that the trend was comparable between both techniques used. However, the magnitude of the regulation obtained through qRT-PCR was different compared to the magnitude of regulation obtained using microarray analysis (Table 3.14). Using qRT-PCR, under the BCMS condition, four of the genes, namely G6PC2, CXCR7, FXYD2 and FST, were up-regulated with fold-changes of 16.33, 8.34, 65.13 and 35.11, respectively. Four other genes, namely CES1, LAYN, SERPINA3 and

PYCARD were down-regulated with fold-changes of -10.10, -15.2, -9.67 and -79.98, respectively. Similarly, G6PC2, CXCR7, FXYD2 and FST were also induced under the CCMS condition, with fold-changes of 11.26, 8.99, 68.52 and 35.00, respectively. CES1, LAYN, SERPINA3 and PYCARD were down-regulated, with fold-change values of -18.74, -12.59, -11.73 and -85.77, respectively.

Table 3.14: Validated genes regulated in response to live *B. pseudomallei* and its secreted proteins.

Symbol	**Description**	**BCMS**		**CCMS**	
		Array	**qRT-PCR**	**Array**	**qRT-PCR**
G6PC2	glucose-6-phosphatase, catalytic, 2	13.24	16.33	6.57	11.26
CES1	carboxylesterase 1	-14.14	-10.10	-24.44	-18.74
CXCR7	chemokine (C-X-C motif) receptor 7	5.78	8.34	6.23	8.99
LAYN	layilin	-13.80	-15.2	-10.65	-12.59
SERPINA3	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	-10.17	-9.67	-12.07	-11.73
PYCARD	PYD and CARD domain containing	-62.52	-79.98	-58.18	-85.77
FXYD2	FXYD domain containing ion transport regulator 2	41.36	65.13	41.63	68.52
FST	follistatin	34.02	35.11	33.22	35.00

CHAPTER 4

DISCUSSION

4.1 Invasion and intracellular survival of *Burkholderia pseudomallei*

Among the vital components of the pathogenesis of *B. pseudomallei* is the ability to enter, survive, and replicate within the host cells. Adherence of a pathogen to the host surface is an important and prerequisite step in the pathogenesis of diseases. This virulence mechanism is mediated by different bacterial factors, including carbohydrate molecules, pili, and nonpilus adhesins (Klemm and Schembri, 2000; Soto and Hultgren, 1999; Finlay and Falkov, 1997; St. Geme, 1997; Hultgren *et al*., 1993) that interact with host cell membrane molecules or extracellular matrix proteins (Hultgren *et al*., 1993). The initial adherence of the pathogen to the host cell surface is followed by intracellular invasion and survival. Many studies have already established that *B. pseudomallei* have the ability to invade the phagocytic cells, including polymorphonuclear leukocytes (Egan and Gordon, 1996; Jones *et al*., 1996; Pruksachartvuthi *et al*., 1990) and macrophages (Utaisincharoen *et al*., 2001; Kespichayawattana *et al*., 2000; Jones *et al*., 1996; Harley *et al*., 1994; Pruksachartvuthi *et al*., 1990). Similarly, the ability of *B. pseudomallei* to invade non-phagocytic cells, including HeLa, CHO, A549, and Vero cultured epithelial cell lines has also been documented (Jones *et al*., 1996; Harley *et al*., 1998; Kespichayawattana *et al*., 2000).

Over the last decade, many studies have been carried out to investigate the invasive and intracellular survival abilities of *B. pseudomallei* in cultured human epithelial cell, A549. Nevertheless, different *Burkholderia* strains have previously been shown to demonstrate a varied range of interactions with the human macrophages, from no effect, to host cell apoptosis and capase-1-dependent lysis (Adler *et al*., 2009; Sun *et al*., 2005). It has also been reported that the different strains of *B. pseudomallei* demonstrated marked variation in their virulence in murine models of the disease (Wand *et al*., 2010). Thus, in view of the strain dependent variation in the

invasive and intracellular survival abilities of the *B. pseudomallei* strains, the invasion and intracellular survival of the *B. pseudomallei* isolate CMS were investigated in this study.

In first part of this study, it was demonstrated that *B. pseudomallei* CMS was able to invade the A549 cells, and the percentage of invasion was found to directly correlate with the MOI up to 12 h as determined by viable counts (Miles and Misra, 1938). However, a decrease in this invasion efficiency was observed at all the MOI used post-12 h of contact with the A549 cells as indicated by the reduction in the numbers of culturable bacteria. This finding needs to be treated with caution as a reduction in numbers of bacterial counts may be due to the reduction in the cell viability of A549 cells post-12 h of infection as indicated by the Trypan blue assay. In addition, the presence of *B. pseudomallei* in the cytoplasm of the infected A549 cells observed in the TEM analysis further provided evidence that the invasion of *B. pseudomallei* was indeed a true event.

This invasive ability of *B. pseudomallei* could be partly facilitated by the flagellin protein (FliC), which is a pathogen-associated molecular pattern (PAMP) molecule (Chua *et al.*, 2003). In this study, presence of functional FliC protein was also demonstrated in the secretome of this strain (Results Section 3.4.3.2). This lends further evidence that *B. pseudomallei* FliC is indeed important in the pathogenesis of the disease, whereby the flagella are used as one of the factors that enable invasion of the host cells. In addition, flagellum motility is also known to facilitate penetration of the host epithelial cell barriers, thus enhancing the invasion of host cells (Chuaygud *et al.*, 2008; Tomich *et al.*, 2002; Ormonde *et al.*, 2000; Mobley *et al.*, 1996; Yao *et al.*, 1994). Following invasion, a crosstalk between the flagella and/or actin-mediated motility may then increase the frequency of contact between the pathogen and the host

cell internal membranes, leading to the intracellular spread of the organism (French *et al*., 2011).

In this study, presence of functional BipC protein, a TTSS3 component, was also detected in the secretome of *B. pseudomallei*. This protein may have partly contributed to the invasive ability of the isolate used. The TTSS3 have been reported to encode several groups of functional proteins, including effector proteins which are able to interfere with the host actin cytoskeleton and consequently facilitate invasion and intracellular survival (Galyov *et al.,* 2010; Steven *et al*., 2003). Earlier studies have also established that bacterial invasion is aided by the up-regulation of the MAPK pathway in the host (Kohler *et al.*, 2002). Bacterial components, such as the LPS or potentially the TTSS3 effector proteins, are known to activate the MAPK dependent invasive phenotype of *B. pseudomallei* (Utaisincharoen *et al.*, 2000). Thus, it is tempting to postulate that the functional BipC and/or BopA proteins observed in the secretome of *B. pseudomallei* in this study may have played a role in inducing the up-regulation of the host MAPK pathway (as observed in the microarray analysis, Section 3.5.4.4), and subsequently contributing to the invasion ability of the isolate used.

B. pseudomallei CMS was also found to have the ability to survive intracellularly and establish significant bacterial load. Similar to the invasive ability, intracellular survival ability of this isolate was found to increase with time and the MOI used. Following 12 h of intracellular survival, marked decrease in numbers of the intracellular bacteria was observed. This observation was parallel to the invasion, whereby the reduction in the number of intracellular bacteria may be attributed to the lack of host-cell viability due to invasion of the bacteria. Intracellular survival ability is of paramount importance to the bacterium as this feature will allow it to establish infection while avoiding the immune responses of the host. The ability of *B. pseudomallei* to survive intracellularly in both

phagocytic and non-phagocytic cells has been established and is considered as a significant component of its pathogenesis (Jones *et al*., 1996). Indeed, many features of melioidosis such as latency, recrudescing infection, and difficulty to treat the disease may be explained by the ability to survive intracellularly (Allwood *et al*., 2011). In the present study, another TTSS3 effector protein, BopA, was also identified in the secretome analysis of *B. pseudomallei* CMS. Cullinane *et al.* (2008) have reported that in *B. pseudomallei,* BopA plays a role to increase intracellular survival by mediating bacterial evasion of autophagy. In a study on the *B. pseudomallei* sister strain *i.e., B. mallei,* BopA has also been reported to contribute to its intracellular life cycle (Whitlock *et al*., 2009; Whitlock *et al*., 2008).

Despite accelerating research on factors that aid *B. pseudomallei* entry into the intracellular environment and its subsequent escape from endocytic vesicles into the cytoplasm, a comprehensive understanding of the precise mechanism of intracellular invasion, survival and other critical steps of pathogenesis is still lacking. The numerous strategies utilized by *B. pseudomallei* that enable it to survive in the intracellular niche remains to be elucidated. However, it is believed that this study has been able to further correlate and support the utilization of some of the virulence factors, especially the factors that are associated with the TTSS3, in aiding the invasion and intracellular survival of *B. pseudomallei* in the host cells.

Nevertheless, the data derived in this study have to be treated with caution due to the fact that the A549 cells used as the *in vitro* model are immortalized cell lines derived from tumors and are prone to genotypic and phenotypic drifting, causing them to lose tissue-specific functions and acquire different molecular phenotype compared to the cells *in vivo* (Pan *et al*., 2009; Masters *et al*., 2002). So, the question arises on why the A549 cells were chosen in this study compared to the primary cell lines or the animal

models? Primary cell lines have limited replicative potential and may become senescent in culture. Similarly, the cell culture models also hold an advantage over the animal models due to the ethical issues that are raised in the use of live animals. Cell culture models are easily manipulated and can support massive screening and cost effectiveness in contrast to the more expensive animal trials with limited screening capacity (Langerholc *et al*., 2011). With these factors in view, the A549 cell line was selected for this study as they are able to mimic the *in vivo* situation to some extent. A549 cells have also been widely used as the host cell model in intracellular bacterial infections studies (Wongprompitak *et al*., 2008) and host transcriptional studies of bacteria, including *Mycoplasma pneumonia* (Yang *et al*., 2002), *B. pseudomallei* (Utaisincharoen *et al*., 2004) and *Staphylococcus aureus* (Moreilhon *et al*., 2005).

In addition, *B. pseudomallei* infection can occur *via* multiple routes, which include ingestion, inhalation or inoculation *via* skin abrasions (White, 2003). However, infection through inhalation is often associated with a high rate of mortality (Currie and Jacups, 2003; Tan *et al*., 2008) and lung epithelial cells have been reported as important in the defense to infection as they are among the first cells that come into contact with the pathogen (Mendez-Samperio *et al*., 2008; Yang *et al*., 2002; O'Brien *et al*., 1999). As such, utilization of the human lung epithelial cells as an *in vitro* model has been recommended as it could provide clues to the pathogenesis events that can occur in the lung tissues (Smith *et al*., 1997).

4.2 *Burkholderia pseudomallei* secreted virulence factors

Various studies have demonstrated the presence of secreted proteins and their potential role in virulence of *B. pseudomallei* (Shang *et al*., 2001; Haubler *et al*., 1998; Haase *et al*., 1997). However, concentration and effect of the different secreted proteins of *B. pseudomallei* isolates have not been reported. To determine their patterns of secretion, and thus associate them with the virulence of *B. pseudomallei*, six housekeeping enzymes, which included phospholipase C, catalase, phosphatase, protease, superoxide dismutase and peroxidase, were selected for assays at different growth phases of the bacteria. These six enzymes were chosen due to their potential role in virulence of *B. pseudomallei* (Woods *et al*., 1999).

Proteases have been found to digest biologically important proteins involved in invasion, such as collagen and elastin (Rechnitzer *et al.*, 1992), and modulate immune response by digesting the cell surface markers, receptors, complements and immunoglobulins (Mintz *et al*., 1993). Acid phosphatases are a ubiquitous class of enzymes that catalyze the hydrolysis of phosphomonoesters at an acidic pH. In addition to mobilization of phosphate, some members of this class of enzymes perform many essential biological functions, including regulation of metabolism, energy conversion, and signal transduction (Rielly *et al*., 1996). Phospholipase C is known to cleave the phosphodiester bond of phospholipids to yield diacylglycerol and water soluble phosphate ester (Korbsrisate *et al*., 1999). This can facilitate host cell lysis because phospholipids, such as phosphatidylcholine, are primarily found in eukaryotic cell membranes and lung surfactans (Songer, 1997). This enzyme was also found to play a role in the escape of the pathogen from the phagosome membrane and invasion of adjacent cells (Smith *et al*., 1995).

Catalase, peroxidase and superoxide dismutase are known to be putative candidates that resist toxic oxygen intermediates such as superoxide anions (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) produced by host phagocytes (Gort *et al*., 1998). Catalase negative mutants of *Mycobacterium tuberculosis* have also been found to exhibit lower virulence in guinea pigs (Wilson *et al*., 1995).

In this study, all the six enzymes assayed *in vitro* were found to be present in the culture supernatant of the isolate tested. Phospholipase C, catalase, and phosphatase activities contributed to the major enzyme activity in the culture supernatant of the isolate, whereas protease, superoxide dismutase and peroxidase activities were detected at lower concentrations. All enzymatic activities were detected in the culture supernatant of the isolate after the first four hours of growth, indicating that the enzymes were secreted while the cells are actively growing in the exponential phase. Therefore, the enzymes detected in the culture supernatant were those that were released from intact viable cells and not as a result of release due to cell death and lysis.

Catalase and superoxide dismutase were found to be released at lower levels during the log phase but increased during the early stationary phase. This might be important *in vivo* as these enzymes have been reported to provide a higher protection to *B. cepacia* to establish a chronic infection, where the bacteria will be facing a physiological situation similar to the stationary phase of growth. Other conditions of stress, such as tissue inflammation, may also stimulate a bacterial response similar to that of the stationary phase and induce activity of these enzymes (Lefebre and Valvano, 2001).

In *in vivo* conditions in the mice, *B. pseudomallei* may be exposed to a variety of host killing mechanisms, including O^{2-}, H_2O_2 and OH^- generated by the respiratory burst as the bacterium remains bound within an endosome (De Melo *et al*., 1989). H_2O_2 has

bacteriocidal activity and additionally, interaction of H_2O_2 with myeloperoxidase, reduced iron, or products of nitric oxide synthase may lead to formation of more toxic intermediates (Fang, 1997). Thus, bacterial factors that inactivate H_2O_2, such as catalase, may interrupt production of these toxic species and aid persistence and survival within host cells and tissues (DeGroote *et al*., 1997). Acid phosphatases have also been predicted to play a role in virulence, most often in intracellular pathogens, by the inhibition of respiratory burst (Mohapatra *et al*., 2007; Rielly *et al*., 1996). Therefore, these enzymes could be important in persistence of a pathogen. This could also be one of the mechanisms used by *B. pseudomallei* to interrupt or modify pathways involved in the disease process.

Phospholipase C activity was found to be among the highest produced by the *B. pseudomallei* isolate. Phospholipase C is known to contribute to cytotoxicity (Korbsrisate *et al*., 2005). In an *in vivo* condition, phospholipase C might aid in the disruption of the host cell membrane and facilitate entry of the bacteria into the bloodstream resulting in systemic spread. Previous studies have also implicated phospholipase C as a virulence factor involved in infection of pathogenic bacteria including *B. pseudomallei* (Tuanyok *et al*., 2006), *Listeria monocytogenes* (Smith *et al*., 1995), *M. tuberculosis* (Johansen *et al*., 1996) and *P. aeruginosa* (Plotkowski *et al*., 1997).

The six enzymes assayed may not necessarily be the only enzymes responsible for bacterial virulence. Presence or absence of other extracellular enzymes such as alanine dehydrogenase, glutamine synthetase, nicotinamidase and alcohol dehydrogenase have also been reported to be correlated with the virulence (Raynaud *et al*., 1998). Further studies using proteomics approach to map the extracellular proteome of *B. pseudomallei* in order to understand other extracellular proteins that might be involved in

pathogenesis and virulence of *B. pseudomallei* was performed to complement the present study.

4.3 *Burkholderia pseudomallei* secretome mapping

Bacterial secretome, including those of *B. cepacia* (Mariappan *et al*., 2009), Bacillus anthracis (Chitlaru *et al*., 2007; Walz *et al*., 2007), *Staphylococcus aureus* (Sibbald *et al*., 2006), *P. aeruginosa* (Wehmhöner *et al*., 2003), and *H. pylori* (Bumann *et al*., 2002), has been the subject of recent proteomics and immunoproteomics studies. Interest in the secretome comes from the fact that some of these secreted proteins mediate important host–pathogen interactions when they come into direct contact with the host compartments during the course of infection.

In this study, the 2-DE separation and identification of proteins that are actively secreted by *B. pseudomallei* at the stationary phase of growth are reported. Many reports have been published on the 2-DE analysis of intracellular and surface proteins of *B. pseudomallei* (Wongtrakoongate *et al*., 2007; Thongboonkerd *et al*., 2007; Harding *et al*., 2007), and altered secretome of *B. pseudomallei* due to salt stress (Pumirat *et al*., 2009). However, to our knowledge, this is the first report on the proteomic mapping and identification of the whole secretome captured through TCA precipitation and also identification of the secretome proteins that are reactive to mice hyperimmune sera raised to *B. pseudomallei* secreted proteins.

Stationary phase culture was used to harvest the majority of the bacterial secreted proteins based on an earlier report by Wehmhöner *et al*. (2003). In addition, Lefebre and Valvano (2001) also reported that in an *in vivo* condition, bacterial cells that are

able to establish chronic infection might face a host physiological environment similar to the *in vitro* stationary phase of growth. Therefore, proteins secreted during the stationary phase were considered most suitable to obtain a complete secretome map.

Several measures were taken to ensure that the proteins detected in the *B. pseudomallei* secretome were purely secreted proteins and not proteins that were medium derived or released due to bacterial cell lysis. LB broth, containing minimal protein, was used for culture in order to limit medium derived contamination. TCA precipitation of the LB broth also confirmed the presence of insignificant amount of protein as compared to the amount of protein in the culture supernatant of *B. pseudomallei* cultured in LB broth. Furthermore, the ICD activity in the secretome was also monitored at different growth phases. ICD serves as an indicator of autolysis as it is an intracellular enzyme which is not secreted by the actively dividing cells (Andersen *et al*., 1991). Low level of ICD activity was detected at the stationary phase of growth indicating minimal contamination due to autolysis.

The secretome map of *B. pseudomallei* yielded 113 spots using linear IPG strip pH 3–10. Andersen and colleagues (1991) reported that the numbers and types of proteins released to the culture supernatant is highly dependent on the cultivation, growth time of the bacterial culture, the medium used and environmental factors such as temperature and aeration during culture. Among the 113 spots detected, only 54 were able to be identified using MALDI-TOF analysis. The low number of proteins identified using MALDI-TOF may be attributed to the problems faced with the identification of low molecular mass proteins or low abundance of certain proteins under the growth conditions used (Chen *et al*., 2007).

The majority of proteins identified in the *B. pseudomallei* secretome were predicted to be associated with the cell wall or the cytosol despite the low ICD activity, which suggests minimal cell lysis. Antelmann *et al*. (2005) and Riedel *et al*. (2006) had similarly reported that half of the identified secretome proteins were associated with intracellular or surface-related proteins. Abundant cytoplasmic proteins have also been reported in the secretome of other pathogens such as *B. cepacia* (Mariappan *et al*., 2009), *M. tuberculosis* (Malen *et al*., 2007), *L. monocytogenes* (Trost *et al*., 2005) and *S. aureus* (Sibbald *et al*., 2006). Among the detected proteins, GroEL, GAPDH, and flagellin have also been identified as natural components of the secretome in other studies (Holland *et al*., 2010; Sanchez *et al*., 2009; Debroy *et al*., 2006). However, Cole and colleagues (2005) reported GroEL and GAPDH, as cellular, cell wall associated and also secreted. In case of the *H. pylori* ribosomal protein L11, its presence in the culture media was demonstrated to occur by active secretion and not due to non-specific cell lysis (Kim *et al*., 2002). According to Bendtsen and collegues (2005), the presence of proteins without signal seqeuence in the secretome preparations may be due to secretion through non-classical secretion pathways, *e.g.* translocation via yet uncharacterized routes of protein transport. It has also been widely reported that cytoplasmic proteins of different bacterial species have been secreted thorugh incorporation into OMVs (Ferrari et al., 2006; Galka et al., 2008). Additionally, cytoplasmic-associated proteins may also have dual functions that can be targeted by the cell to different subcellular sites or secreted during certain stages of the cell growth (Song *et al*., 2009).

Based on the genomic annotation and translation, some proteins, including those involved in secondary metabolism, drug resistance, intracellular stress, motility and chemotaxis, have been associated with the survival of *B. pseudomallei*. On the other hand, types I, II, III, and IV secretion system proteins, surface components, exoproteins, fimbriae/pili and adhesion proteins have been associated with the virulence of *B.*

pseudomallei (Holden *et al*., 2004). Likewise, we also identified proteins that may be associated with survival and virulence in the *B. pseudomallei* secretome, including flagellar hook associated protein, flagellin, multidrug efflux system exported proteins, chaperonin GroEL, putative heat shock protein, chemotaxis-related methyltransferase protein and chemotaxis-related protein, cell invasion protein, intracellular spread protein, chitin-binding protein and putative lipoprotein.

BLAST analysis demonstrated that the hyphothetical protein BPSL1622 (poorly characterised protein) did not have any orthologue in *B. thailandensis*, a non-virulent counterpart of *B. pseudomallei*. This suggests that the protein may be involved in the virulence of *B. pseudomallei*. Obviously, this protein should be investigated further in order to identify its specific functions. Other proteins including the hyphothetical protein BPSL0584, glycerophosphoryl diester phosphodiesterase family protein, hyphothetical protein BPSL0345, hyphothetical protein BPSL0566, and GntR family transcriptional regulator proteins showed less than 50% homology with *B. mallei* strain 23344 and *B. thailandensis* strain E264. Based on this low homology, we predict that these proteins would not cross-react with proteins of the two closely related *Burkholderia* spp. and therefore, these proteins or antibodies towards these proteins may have the potential to be developed as diagnostic markers. However, further evaluation and characterisation of these proteins have to be carried out to confirm their potentials.

4.4 *Burkholderia pseudomallei* secretome proteins reactive to mice hyperimmune antisera raised to *B. pseudomallei* secreted proteins.

Identifying the secreted immunogenic proteins will allow the discovery of novel antigens that may be important for the development of diagnostics, vaccines, and passive immunotherapies since these proteins are able to provoke the immune response of the host. Western blot analysis was carried out using mice hyperimmune antisera raised to *B. pseudomallei* secreted proteins, in order to identify the potential diagnostic markers and/or putative vaccine candidates. Stationary phase secretome was used for immunobloting since detection of more immunogenic proteins in the secretome of stationary-phase cells as compared with that of logarithmic-phase cells has also been reported in a similar study (Singh *et al*., 2000).

Among the secretome proteins, 12 were found to be reactive to hyperimmune mice sera raised against the *B. pseudomallei* secreted proteins. Three of these proteins have already been described as highly immunoreactive in the secretome of other organisms including GroEL in *B. anthracis* (Chitlaru *et al*., 2007), GAPDH in *Streptococcus suis* (Geng *et al*., 2000), and flagellin in *P. aeruginosa* (Wehmhöner *et al*., 2003). Several of the reactive proteins identified are of interest due to their possible potential to be developed as diagnostic markers or putative vaccine candidates.

Flagelin, a bacterial flagellar subunit protein coded by the gene fliC, is known as a factor involved in the pathogenesis of *B. pseudomallei*. Brett and co-workers have reported that anti-flagella (flagellin) antibody was able to reduce bacterial motility *in vitro* and provide passive protection for diabetic rats infected with *B. pseudomallei* (Brett *et al*., 1994). In addition, bacterial flagellin has also been recognised as a strong immunostimulator capable of activating NF-κβ signalling (Eaves-Pyles *et al*., 2001) and

Chen *et al*. (2006) have used plasmid DNA encoding flagellin as a vaccination candidate against infection of *B. pseudomallei* in Balb/c mice.

It is surprising that cell invasion protein, BipC, a type III secretion protein that is common among bacterial pathogens and symbionts for delivery of effector proteins into eukaryotic host cells (Hueck, 1998; Lee, 1997) was present in the secretome albeit the absence of the host. However, Uchiya *et al.* (1999) reported that these proteins are commonly found in the culture supernatants of bacteria grown under laboratory conditions. One of the *B. pseudomallei* Type III secretion systems, *Burkholderia* secretion apparatus, shares high homology with type III secretion system of *Salmonella typhimurium* and *Shigella flexneri* (Stevens *et al*., 2002). Stevens *et al*. (2003) reported that the inactivation of *Burkholderia* secretion apparatus components resulted in impaired invasion and survival within eukaryotic cells, inability to escape from endocytic vacuoles, and failure to produce membrane protrusions and actin tails. Another potential vaccine candidate, chaperonin GroEL, is known to produce strong antigen–antibody response with melioidosis patient's sera (Woo *et al*., 2001). The role of GroEL in vaccination against tuberculosis (Tascon *et al*., 1999), brucellosis (Bae and Toth, 2000) and yersiniosis (Noll and Autenrieth, 1996) has been studied. In addition, a virulence property of GroEL has also been suggested (Woo *et al.,* 2001).

Four of the reactive proteins, pyruvate dehydrogenase, GAPDH, SCOT, and monooxygenase, were identified to have metabolic functions. Although metabolic proteins are known to play a major role in energy production for survival, their role in virulence has also been suggested (Bumann *et al*., 2002). In *P. aeruginosa* E1 and E2, components of pyruvate dehydrogenase were found to be involved in Type III secretion system-dependent cytotoxicity (Dacheux *et al*., 2002) but in *B. subtilis* they were

involved in regulation of sporulation (Gao *et al*., 2002) and transcriptional activation of protoxin genes (Walter and Aronson, 1999).

Meanwhile, GAPDH, a typical enzyme of the glycolysis pathway, might also play an important role in bacterial pathogenesis. In several fungi and Gram-negative bacteria, GAPDH is known to be a multifunctional protein displayed on the surface and contribute to their adhesion and virulence. It is associated with physiologic functions such as ADP-ribosylation (Pancholi and Fischetti, 1993), adhesion to fibronectin, myosin, and actin (Pancholi and Fischetti, 1992) as well as the ability to serve as a receptor for plasmin on the surface of *Streptococcus* (Lottenberg *et al.*, 1994). However, the role for extracellular localisation of GAPDH in the pathogenesis of Gram-negative bacteria has not been described (Egea *et al*., 2007). In addition, in a study on the intracellular proteins expression at stationary phase, Wongtrakoongate and colleagues (2007) reported that the metabolic enzyme SCOT is highly expressed in *B. pseudomallei* but not in the nonvirulent *B. thailandensis*. This suggests that SCOT might also be a potential protein marker although BLAST analysis shows a 97% homology with predicted proteins of *B. thailandensis*.

Six of the proteins identified in this study (SCOT, chaperonin GroEL, chitin-binding protein, putative heat shock protein, Cell division protein FtsQ, and NAD(P) transhydrogenase subunit α) have been previously identified in other proteome studies *of B. pseudomallei*. These include the proteome analysis of total cellular protein extracted from *B. pseudomallei* (Wongtrakoongate *et al*., 2007; Thongboonkerd *et al*., 2007; Ou *et al*., 2005), surface proteins of *B. pseudomallei* (Harding *et al*., 2007) and also secretome (Pumirat *et al*., 2009). Harding *et al*. (2007) identified the surface located proteins of *B. pseudomallei* using biotin labelling and also found GroEL to be immunogenic when probed with convalescent human sera. Protein microarray to

identify serodiagnostic and crossreactive antigens using a large number of melioidosis and other bacterial infection patient sera also identified GroEL to be seroreactive and giving the best single antigen discrimination to accurately distinguish melioidosis cases from control (Felgner *et al*., 2009). This suggests the suitability of GroEL to be used in serodiagnosis. Felgner *et al*. (2009) also identified several components of TTSS3 including BPSS1532 (BipB), BPSS1525 (BopE) to be potential for serodiagnostic. On the other hand, in this study, two TTSS3 components including BPSS1531 (BipC) and BPSS1524 (BopA) were identified in the secretome of *B. pseudomallei* and BipC was found to be reactive to hyperimmune mice sera raised to *B. pseudomallei* secreted proteins.

The BipC protein encoded by *bipC* gene has high homology with the *sipC* gene in the *S. typhimurium*, which encodes for a translocator protein involved in the TTSS. Research has shown that the mutation in the two proteins from the Bip family, BipB and BipD, has shown attenuation of *B. pseudomallei* virulence (Suparak *et al*., 2005; Stevens *et al*., 2004; Stevens *et al*., 2002). BipD mutants were found to exhibit impaired invasion of HeLa cells, reduced intracellular survival in murine macrophage-like cells and a marked reduction in actin-tail formation. Similarly, BipB was found to be important in the induction of MNGC, plaque formation, bacterial invasion, and killing of phagocytic cells in vitro. However, to date there are no reports regarding the role of the BipC protein on the *B. pseudomallei* virulence. Therefore, this warrants the investigation of the role of BipC in pathogenicity and virulence of *B. pseudomallei.*

4.5 Early responses of human lung epithelial cells, A549, on exposure to *B. pseudomallei* live bacteria and its secretory proteins.

In order to further understand the role of the secreted proteins in virulence of *B. pseudomallei* and elucidate the pathogenesis mechanism involved, early transcriptional responses of the A549 epithelial cells, were investigated using Illumina HumanHT-12 v4 microarray platform following three hours of exposure to *B. pseudomallei* live bacteria (BCMS) and its secreted proteins (CCMS). During an infection, the infected host recognises the presence of the pathogen and mobilises specific immune defense mechanisms. At the same time the pathogen will actively modulate host signaling pathways and enhance their persistence and survival. The use of microarray analysis will aid in better understanding of this host pathogen interactions.

In this study, the response of A549 cells to both *B. pseudomallei* live bacteria and its secreted proteins were elucidated. This comparison allows the identification of the dynamic of the actual bacterial infection as opposed to the exposure with the bacteria free secreted virulence factors. Generally, at three hours of exposure with the actively dividing bacteria growing at the logarithmic phase, some of the secreted virulence factors are secreted at lower levels as demonstrated earlier in the extracellular enzyme assay in this study. During this phase, the bacteria also synthesize mainly cell surface virulence factors as demonstrated by Moreilhon *et al*. (2005). During the logarithmic phase of growth, genes encoding secreted virulence factors are also reported to be transcribed at lower levels compared to the stationary phase of growth (Lowy, 1998; Novick and Muir, 1999). Thus, the secreted proteins collected at the stationary of growth are largely enriched in the soluble virulence factors. As such, it is believed that the use of both the actively dividing live bacteria and secreted proteins of *B. pseudomallei* collected at the stationary phase of growth will provide an advantage and

a more complete picture of the host response to the different virulence factors of the bacteria whereby the host cells are exposed to the surface and the secreted virulence factors of the bacteria. Some studies utilized the live and heat killed bacteria in order to identify the host response to static pathogen-associated molecular patterns and active virulence-associated processes (Boldrick *et al*., 2002). However, in this study, the use of heat-killed bacteria was not considered as it does not permit the study of host-pathogen interactions influenced by secreted bacterial molecules and the heat-sensitive cell wall components (Menzies and Kenoyer, 2005).

Host genes associated with carbohydrate and amino acid metabolism

In this study, the host carbohydrate metabolic pathways were up-regulated in response to both the live bacteria and the secreted proteins. Both the conditions were found to up-regulate starch and sucrose metabolism, ascorbate and aldarate metabolism and pentose and glucuronate interconversions. Similar to our findings, the up-regulation of metabolic pathways have also been reported in the human airway cells upon exposure to live *Staphylococcus aureus* and its bacterial soluble factors (Moreilhon *et al.*, 2005) and silkworm, *Bombyx mori*, infected with *Bacillus bombyseptieus* (Huang *et al.*, 2009). In general, the up-regulation of host metabolic pathways during early infection may suggest the active utilisation of host resources by the bacteria as means of adaptation and survival in the host. At the same time, up-regulation of host metabolic pathways also contributes to the maintenance of host homeostasis, where higher metabolic energy is required by the host. Therefore, these metabolic alterations observed in this study may be presumed to be of survival benefit to both the pathogen and the host.

On the other hand, this study also found that the host amino acid metabolism was down-regulated under both the conditions. We believe that the host membrane damage

caused by the infection with live bacteria and also exposure to the secreted proteins could be responsible for the down-regulation of amino acid metabolism. In support of this, Tattoli *et al*. (2012) have also observed using liquid chromatography/mass spectrometry (LC/MS) to analyze the cytosolic concentration of free L-leucine and L-isoleucine, the intracellular amino acid starvation during *Salmonella* and *Shigella* infections. The intracellular levels of L-leucine and L-isoleucine are known to play key roles in controlling mTOR. This amino acid starvation has been attributed to the host membrane damage caused by these bacteria. They have also indicated that pathogen-induced amino acid starvation can lead to the down-regulation of mTOR activity, and subsequently result in the induction of anti-bacterial autophagy. However, the mTOR pathway is also known to be activated in phagocytes in response to bacterial infection or the LPS of the bacteria (Allan, 2008). In this study, the up-regulation of the mTOR activity was observed only in the CCMS condition and not in the BCMS. It is believed that this may be attributed to the short exposure time of three hours contact with the live bacteria. However, the secreted proteins may be able to activate the mTOR pathway during the same duration of exposure due to the presence of concentrated secreted bacterial factors in contact with the A549 cells.

Interestingly, in this study, the host glutathione metabolism pathway was also significantly down-regulated under both the BCMS and CCMS conditions. In diabetic condition, which is among the major risk factors of melioidosis, deficiency in intracellular reduced glutathione concentrations is constantly observed, thus establishing a link between glutathione deficiency in diabetes and increased susceptibility to melioidosis (Tan *et al*., 2012). Low intracellular glutathione levels in diabetics have been reported to be critical in allowing better initiation of the intracellular infection and also affect disease progression and outcome. This provides an explanation for the increased susceptibility of diabetic individuals to melioidosis. A

similar observation has also been reported with the *Mycobacterium tuberculosis* infection, whereby the glutathione imbalance was reported to affect IL-12 production and subsequent bacterial control (Tan *et al*., 2012). Thus, it is believed that the down-regulation of glutathione metabolism of the A549 cells, as observed in this study, could provide a major advantage for the *B. pseudomallei* isolate to initiate intracellular infection.

Host genes associated with immunity and defence

In this study, the complement and coagulation cascades were found to be down-regulated upon three hours of exposure to both *B. pseudomallei* live bacteria and secreted proteins. The extracellular polysaccharide capsule of *B. pseudomallei* have been reported to cause the down-regulation of the complement factor C3b and the consequently interfere with the activation of the complement cascade (Reckseidler-Zenteno *et al.*, 2005). As such it is also tempting to postulate that the extracellular polysaccharide capsule of *B. pseudomallei* used in this study may have caused the down-regulation of complement factor C3 and C5 leading to the down-regulation of the complement and coagulation cascades observed in this study. This suppression of the host complement and coagulation pathways may increase the chances of the bacteria to evade the innate immune responses of the host.

In addition, *B. pseudomallei* and its secreted proteins were also found to suppress the lysosome and phagosome pathways. It is postulated that this will prevent the bacteria from being recognised by these pathways and thus escaping from engulfment in the phagolysosome, resulting in early escape of the bacteria from elimination by the host cells. Early escape of the bacteria may also provide protection from being killed by lysosomal constituents such as lysosomal defensins and free radicals (Galyov *et al*.,

2010). It has been reported in earlier studies that the early escape from phagocytic vacuoles and intracellular replication of *B. pseudomallei* is partly mediated by the *Burkholderia* Secretion Apparatus (Bsa), TTSS3 (Muangsombut *et al*., 2008; Ribot and Ulrich, 2006; Stevens *et al.*, 2002).

The anti-inflammatory cytokine, IL-11, was found to be highly up-regulated (approximately 6.5 folds) while the pro-inflammatory cytokine, IL-8, was found to be significantly down-regulated under both the BCMS and CCMS conditions. In a previous study, Dessus-Babus *et al.* (2000) also detected significantly higher concentrations of IL-11 in *C. trachomatis*-infected cultures of polarized HeLa cells. Interestingly, they also found that the level of IL-11 was significantly higher in infection with a disseminating serovar compared to that of the non-disseminating serovar. The immunosuppressive effect of IL-11 was suggested to allow the bacteria to escape from the host innate defenses for better dissemination. As such, it may be possible that the high concentration of IL-11 upon exposure to *B. pseudomallei* live bacteria and the secreted proteins observed in this study may also similarly aid escaping elimination by the host innate immune response and thus providing means for the pathogen to disseminate in the host.

In this study, the significant down-regulation of IL-8 was observed. However, detection of low levels of IL-8 production in the epithelial cell lines infected with *B. pseudomallei* compared to the cells infected with *S. Typhi* has also been previously reported (Utaisincharoen *et al*., 2004). Yet, in another study on A549 cells infected with *Chlamydia pneumonia*, IL-8 mRNA was found to remain at constitutive levels, and significant up-regulations were only observed at 7, 10, 16, and 24 h after infection (Yang *et al*., 2003). Thus, the down-regulation of IL-8 observed in this study should be treated with caution as this may be due to the short exposure time of three hours. This

is further supported by Sim *et al*. (2009), with the report that substantial increase in cytokine production was only observed after 24 h of infection with *B. pseudomallei* in the murine lung epithelial cell line, LA-4.

However, if down-regulation of IL-8, is among the strategies employed by *B. pseudomallei*, remains to be elucidated. Additionally, Peacock (2006) reported that BALB/c mice infected with *B. pseudomallei* died of septicemic disease, with the organs and blood demonstrating high bacterial loads as well as organ inflammation and necrosis a few days after infection. This observation was suggestive of the failure of the host innate immune response. Thus, based on the findings of this study it is questioned if:

i) B. pseudomallei is able to silence the host innate immune system?

Several lines of evidence indicate that bacterial pathogens employ mechanisms to downplay host immune system to facilitate entry. In this study, MX1, an important intrinsic host restriction factor, also known as myxovirus (influenza virus) resistance 1, was found to be down-regulated under both under the BCMS and CCMS conditions. MX1 is an intracytoplasmic innate immune protein produced in response to viral infections that responds to type I and III interferons, and negatively regulates viral replication (Verhelst *et al*., 2012). In agreement with this study, findings by others also are suggestive of potential interference of *B. pseudomallei* with innate immunity. Kobayashi et al., (2003) have also demonstrated that MX1 is down-regulated in neutrophils exposed to *B. cepacia*. More findings have established that neutrophils stimulated with bacterial LPS induced the expression of MX1 gene (Malcolm and Worthen (2003). In this study, these findings are further supported by the down-

regulation of IL-8 (also known as neutrophil chemotactic factor), a potent proinflammatory cytokine that plays an important role in the recruitment and activation of neutrophils during inflammation (Hammond *et al*., 1995). By down-regulating IL-8, is *B. pseudomallei* programming an anti-inflammatory cascade in the host immediately following entry? It is known that inflammation leads to the recruitment of cells and factors that could potentially arrest the establishment of infectious agents. Published findings show that IL-8 receptor, IL-8R1, is an appropriate target for therapeutic strategies to limit neutrophil influx in diseases where neutrophils contribute to inflammatory pathophysiology (Hammond *et al*., 1995). Blockade of IL-8 binding to IL-8R1 curtails neutrophil infiltration at the site of pathogen entry leading to marked alleviation of inflammation. Hence, it is reasonable to assume that *B. pseudomallei* possibly evade the innate immune responses to favour its intracellular establishment in the host, partly by silencing the recruitment of inflammatory mediators at the site of bacterial entry. It is also reasonable to hypothesize that following entry, by promptly silencing the mediators of acute inflammation viz. IL-8 and neutrophils, *B. pseudomallei* ensure to efficiently establish itself within the host system.

The up-regulation of IL-11 observed is seemingly supportive to this hypothesis as IL-11 is also generally known to be an immunoregulatory cytokine. However, this depends on to the STAT that IL-11 binds as exposure of oligodendrocytes to IL-11 enhances activity of STAT3 promoting survival and maturation (Zhang *et al*., 2011). On the contrary, activity of STAT1 predominates in dendritic cells exposed to IL-11, resulting in apoptosis and damping of the inflammatory response (Zhang *et al*., 2011), and therefore, the up-regulation of IL-11 in our experiments remain elusive although it should be assumed as supporting the anti-inflammatory saga initiated by *B. pseudomallei* given many other innate players described above are down-regulated, and that IL-11 could again be a pleiotropic cytokine with anti-inflammatory and

inflammatory roles. Furthermore, in this study, hypothesis suggestive of the role of *B. pseudomallei* in downplaying the innate immune system is further strengthened by the down-regulation of CD14, the surrogate marker of the monocyte/macrophage lineage. CD14 cooperates with other proteins to mediate the innate immune signalling to bacterial LPS and positively regulates cytokine secretion by innate immune cells. Contemporaneously, down-regulation of complement segment C3, a key innate immune glycoprotein factor synthesized by the liver was also observed in this study. C3 deficiency is known to be cause vulnerability to certain invasive bacterial infections. In agreement with this study, another intracellular pathogen, *Yersinia enterocolitica* was also found to mediate complement evasion by inactivating C3 factor following entry into the host. The most interesting finding is the up-regulation of suppressor of cytokine signaling 2 (SOCS2) following exposure of the cells to supernatants (possibly owing to the presence of some non-contact-dependent secreted factor inducing the expression of SOCS2), because SOCS2 is a well-known immunosuppressor (Lee et al., 2010). However, the STAT signaling pathway whereby SOCS2 operate still remains a grey area of investigation.

ii) B. pseudomallei could suppress the adaptive immune armory?

The down regulation of CD40 points to the notion of potential suppression of adaptive immune responses following bacterial entry into the cytosol. CD40 is a costimulatory molecule belonging to the TNF-receptor superfamily, conventionally found on DCs and macrophages, the innate immune cells are responsible for initiating adaptive immune responses, especially against intracellular pathogens. CD40 is constitutively expressed on B cells, endothelial cells, smooth muscle cells, fibroblasts and epithelial cells. Engagement of CD40 with CD40L leads to T-cell activation and eventual secretion of Th1 (IL-12), Th2 (IL-4) and Th17 (IL-6/TGF-β) polarizing cytokines (Mach *et al.*,

1997). CD40 is essential for T cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation (Kawabe *et al*., 1994). The down-regulation of CD40 signature following exposure to *B. pseudomallei* is important as it is a likely prelude to compromising the initiation of adaptive immune responses against the bacteria. Hence, it is likely that *B. pseudomallei* could harness the onset of negatively modulating the immune system to establish itself within the host. As expected, the CD40-CD70 axis has worked in concert as we also documented a corresponding down-regulation of CD70. The CD70 protein is a cytokine binding to TNFRSF27/CD27. It is a surface molecule expressed on activated T and B lymphocytes. It induces proliferation of costimulated T cells, enhances the generation of cytolytic T cells, and contributes to T-cell activation. Plasmacytoid DCs (pDCs) exhibit strong and stable expression of CD70, and promotes plasma cell differentiation and Ig secretion. Blockade of the CD70-CD27 interaction leads to reduced induction of B-cell proliferation and IgG secretion (Shaw *et al*., 2010). Hence, the likely role of *B. pseudomallei* in compromising the adaptive immune system is evident from this study.

Down-regulation of IFNGR1 is again suggestive of possible role of *B. pseudomallei* downplaying the innate and the adaptive immune systems. Rayamahji *et al.* (2010) have shown that *L. monocytogenes* down-regulates IFN-γ receptor (IFNGR) in line with our findings. It was shown that macrophages infected with *L. monocytogenes* were refractory to IFN-γ treatment as a result of down-regulation of the IFN-γ receptor (IFNGR), which potentially could suppress the adaptive immune system (Rayamahji *et al*., 2010). Down-regulation of CD38 in both the BCMS and CCMS conditions is another candidate notion supporting our viewpoint that *B. pseudomallei* could suppress the activation of the adaptive immune system, especially the memory T cells. CD38 is a novel multifunctional ectoenzyme widely expressed in

cells and tissues especially in leukocytes and is a marker of immune activation. It also functions in cell adhesion, signal transduction and calcium signaling.

Host genes associated with proliferation, survival and apoptosis

During infection, the ultimate goal of pathogens is to establish a replicative niche, in the host where the pathogen can multiply. In line with this, several studies have demonstrated using both the *in vitro* and *in vivo* models, that during infection, the pathogen prevents host apoptosis as a mechanism of immune evasion (Zou *et al.,* 2011; Wongprompitak *et al.,* 2008; Morales *et al.,* 2006). Induction of cell cycle was reported in another study using secreted proteins from *N. meningitidis* (Wells *et al.,* 2001). Other studies on *Chlamydia* and *Neisseria* have also reported to inhibit apoptosis by using their secreted factors to prevent the release of cytochrome C (Zhong *et al*., 2006; Massari *et al*., 2003). This inhibition of apoptosis was thought to provide a survival advantage by allowing the bacteria to replicate inside the host.

Conversely, in this study, it was found that the early exposure of *B. pseudomallei* and its secretory proteins to the A549 cells significantly up-regulated the host apoptosis pathway. Many microbial virulence factors have been shown to promote host apoptosis (DeLeo, 2004). Additionally, in this study, ERCC1, which is among the key genes involved in DNA replication and repair, was also down-regulated leading to damage in the DNA. DNA damage may act as a signal for apoptosis (Roos and Kaina, 2006). Alterations were also observed in the expression of the gene involved in the cell cycle progression or arrest, SPATA18, which was significantly down-regulated.

However, Bcl2 which is important for preventing permiabilisation of the mitochondrial membrane was found to be significantly up-regulated. Faherty and Maurelli (2008) have reported that the up-regulation of Bcl2 can enhance the pro-survival state of the

cell. Other regulators of cell survival, including AKT2 and AKT3 (Yang *et al.,* 2004), were also found to be significantly up-regulated, thus supporting that cell survival was being promoted.

The changes observed both in the expression of genes that promote apoptosis and also cell cycle progression may reflect the complex interplay between the bacteria and the host. It is postulated that intracellular pathogens like *B. pseudomallei* rely on the survival of the host cell. As such, continuous division of the eukaryotic cells are important to allow the bacteria to survive longer in the host (Faherty and Maurelli, 2008). Ultimately, the balance between these processes may determine whether the cell survives or undergoes apoptosis.

The gene expression responses of human A549 cells to live *B. pseudomallei* and its secreted proteins *in vitro* revealed vital features of responses that may be relevant to the initial stages of contact between the pathogen and the host. These responses suggest the efforts of host cells to recruit and activate various arms of the immune system. At the same time, the pathogen also works hard to suppress the host immune system. In this scenario, the host attempts to contain local infection while the pathogens pursue niches in which they can disseminate and continue to multiply or persist. Further analyses of the molecular interaction between the bacteria and the host will help illuminate the complex interplay between the host and the pathogen and provide a basis for the development of new strategies for detecting and preventing invading pathogens.

CHAPTER 5

CONCLUSION

The results of the present study demonstrated that the *B. pseudomallei* CMS was able to invade the A549 cells, survive intracellularly and establish significant bacterial load. It also showed different extracellular enzyme profiles at different phases of the growth cycle, which may partly contribute to the pathogenic potentials of the bacteria. These differences may influence the capacity of the strain to cause the disease in the host. However, further studies are necessary to increase our understanding of the exact roles of the enzymes in the pathogenesis of *B. pseudomallei* infections.

This study also mapped the whole secretome of *B. pseudomallei* and identified stationary phase secretome proteins that were reactive to mice hyperimmune sera raised to *B. pseudomallei* secreted proteins. Other proteome studies carried out on *B. pseudomallei* have emphasised on the cellular and surface proteins of the organism (Wongtrakoongate et al., 2007; Thongboonkerd et al., 2007; Ou et al., 2005). Although Pumirat and coworkers (2009) mapped the secretome of *B. pseudomallei*, they only identified the proteins that were altered under exposure to high salt-environment. Some of the identified proteins in the present study, especially proteins that were reactive to mice hyperimmune sera raised to *B. pseudomallei* secreted proteins, are potential to be used in serodiagnosis, as protein markers or developed as vaccine candidates. However, further *in vivo* experiments are needed to evaluate the potentials of these proteins since this conclusion is only based on comparison with other studies.

Microbes alone cannot be accused for pathogenicity since both microbes and host play important roles during infection or disease. Understanding the progression of the disease from the host's point of view helps to further elucidate the mechanism of infection utilized by the pathogen. More insight into the interrelation of *B. pseudomallei* and the A549 cells may lead to the development of new strategies to combat *B. pseudomallei* infection. In this study, the activation of host carbohydrate

metabolism and apoptosis, as well as suppression of amino acid metabolism and immune responses were identified using the microarray analysis. The understanding of the early response of the A549 cells towards *B. pseudomallei* infection generally allows a rational design for future functional experiments to further elucidate *B. pseudomallei* infection. Nevertheless, the data derived in this study have to be treated with caution since it only utilised an *in vitro* model. This warrants further functional studies using *in vivo* models which may better represent the actual scenario of *B. pseduomallei* infection.

CHAPTER 6

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APPENDIX

APPENDIX 1

Preparation of media

(a) Ashdown agar

Bacteriological agar - 7.5g

Tryptone Soy Broth - 5g glycerol

Glycerol - 20ml

Crystal violet - 2.5ml of 0.1%

Neutral red - 2.5ml of 1.0%

Distilled water - 475ml

Mixed, dissolved, 19 ml dispensed into glass universal containers and autoclaved. Cooled to 56°C, 1ml 100ug/ml gentamicin (freshly prepared) added, poured into petridish and stored at +4°C for no more than one week

(b) Nutrient agar (NA)

Nutrient agar (Oxoid, UK) - 28g

Suspend in 1000 ml double-distilled water, dissolved and autoclaved at 121ºC for 15 mins. Cooled to 50ºC and poured into steril petri dish.

(c) Luria-Bertani (LB) broth (1% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, pH 7.0)

Luria-Bertani (Difco, France) - 25g

Suspend in 1000 ml of double-distilled water, autoclaved at 121ºC for 15 mins.

(d) 30% glycerol stock

50% sterile glycerol (Sigma, USA) - 60 ml

LB broth - 40 ml

APPENDIX 2

Gram-stain

Gram-stain Solutions:

(a) Preparation of crystal violet solution

Crystal violet - 2% (w/v)

Ethanol (95%) - 20% (v/v)

Ammonium oxalate - 0.8%

The solution mix was left for 24 hrs, filtered and stored in a dark bottle.

(b) Preparation of Lugol's iodine solution

Potassium iodine - 0.7%

Iodine - 0.3%

(c) Preparation of Safranine solution

Safranine - 0.25% (w/v)

Ethanol (95%) - 10% (v/v)

(d) Preparation of acetone-alcohol

Acetone - 50% (v /v)

Ethanol - 50% (v/v)

Gram-stain Methods:

Bacterial colony was suspended in a drop of saline and smeared with a loop onto a glass slide surface

↓

The bacteria were fixed over a temperate flame.

↓

Crystal violet solution was added and left for 60s

↓

The slide was rinsed with tap water

↓

Lugol's iodine solution was added for 60s

↓

The slide was rinsed with tap water

↓

Aacetone-alcohol was added for 5s

↓

Safranine solution was added to the slide

↓

The slide was washed with tap water

↓

The slide was left to air-dry

APPENDIX 3

Bradford method

(a) Preparation of Bradford Reagent

Coomassie Blue G250 - 50mg

Methanol - 50ml

85% (w/v) H_3PO_4 - 100ml

The solution was made up to 1 L with double-distilled water, filtered and stored at 4^{o}C.

(b) Preparation of standard curve using bovine serum albumin (BSA)

Table Appendix 3: Preparation of standard curve using BSA solution (0 – 100 µg/ml)

Tubes	**1**	**2**	**3**	**4**	**5**	**6**
BSA (µl)	0	20	40	60	80	100
H_2O (µl)	100	80	60	40	20	0
Bradford reagent	1 ml added and incubated 10 mins Absorbance read at 595 nm					

APPENDIX 4

Preparation of tissue culture media

(a) 10X Roswell Park Memorial Institute (RPMI) stock solution (Sigma, USA)

RMPI - 3 bottles

$NaHCO_3$ - 6.0 g

Distilled water - 300 ml

The mixture was stirred on a magnetic stirrer until dissolved, filter sterilised and stored at 4˚C.

(b) RPMI growth medium

10X RPMI stock solution - 100 ml

Fetal calf serum (FCS) - 100 ml

2 mM L-Glutamine - 10 ml

Penicillin/Streptomycin - 2.0 ml

The solution was made up to 1000 ml using sterile double-distilled water

(c) RPMI maintenance medium

10X RPMI stock solution - 100ml

Fetal calf serum (FCS) (Sigma) - 50 ml

2 mM L-Glutamine - 10 ml

Penicillin/ Streptomycin - 2.0 ml

The solution was made up to 1000 ml using steril double-distilled water

APPENDIX 5

Two-dimensional gel electrophoresis

(a) Lysis buffer

Urea - 9 M

Dithiothreitol (DTT) - 10 mM

Pharmalyte pH 3–10 or pH 4–7 - 2% (v/v)

(b) Rehydration buffer (RB)

Urea - 8 M

Bromophenol blue - 0.002% (w/v)

CHAPS - 2% (w/v)

DTT (prior to use) - 12 mM

(c) Solution A - Monomer stock solution

Acrylamide - 30% (w/v)

N, N'-methylenebisacrylamide - 0.8% (w/v)

The solution was deinoised using amberlite MB-1, stirred until dissolved using a magnetic stirrer, filtered through a 0.45 µm filter and stored in a dark bottle at 4ºC.

(d) Solution B - 4X resolving buffer

Tris base - 1.5 M

pH was adjusted to 8.8 using HCl, filtered through a 0.45 µm filter and stored at 4ºC.

(e) Solution C - 10% SDS

10% (w/v) SDS

The solution was filtered through a 0.45 µm filter and stored at room temperature.

(f) Solution D - 10% ammonium persulfate (APS)

APS10% - (w/v)

The solution was prepared fresh prior to use.

(h) 30% Butanol

Butanol - 30% (v/v)

Table Appendix 5: Preparation of 12.5% SDS-PAGE solution

Solution	**Volume (ml)**
Solution A	375.0
Solution B	225.0
Solution C	9.0
Double-distilled water	285.7
Solution D	5.0
TEMED	0.25
Total	**900**

Note: TEMED and APS was added prior to gel casting

(i) SDS equilibration buffer

Urea - 6 M

Tris-HCl - 50 mM, pH 8.8

Glycerol - 30 % (v/v) of 87% (v/v)

SDS - 1% (w/v)

Bromophenol blue - 0.002% (w/v)

Stored in 1ml aliquots at -20˚C.

(j) Agarose sealing solution

Agarose - 0.5% (w/v) in SDS electrophoresis buffer

Bromophenol blue - 0.002% (w/v)

(k) SDS electrophoresis buffer (Cathode buffer, pH 6.7)

Tris - 25 mM

Glycine - 198 mM

SDS - 0.1% (w/v)

(l) Anode buffer

Tris - 0.375 M

pH adjusted to pH 8.8 with HCl

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AWARDS AND RECOGNITIONS

Welcome Trust Travel Award 2012, 15th ICID, Bangkok Thailand

Merck Young Scientist Award (MYSA), Bioscience Category (2010),
Top five finalist and consolation prize winner

Travel Award, Chulalongkorn University, Bangkok, Thailand. (2009)

Travel Award, National University of Singapore, Singapore (2008)