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Evaluation of Two Novel Antimicrobial Targets in *Burkholderia pseudomallei*

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Abstract

Burkholderia pseudomallei is a Gram-negative bacterium and the causative agent of the disease melioidosis. Melioidosis is endemic in regions of Southeast Asia and northern Australia, with human infection associated with a high mortality rate. The disease can manifest in several forms, including pneumonia, septicaemia or a chronic infection which can affect multiple organs and persist for months or years. This makes treatment of *B. pseudomallei* infection extremely problematic, complicated further by its inherent antibiotic resistance. For this reason new antimicrobials are required that target novel pathways within the cell.

The aim of this study was to evaluate two proteins in *B. pseudomallei* as future targets for antimicrobial drugs. These proteins included an essential target, inhibition of which would result in cell death, and a second that was predicted to be crucial for virulence. The Min system is responsible for the correct placement of the cell division apparatus. It is made up of three proteins; MinC, MinD and MinE, where MinE is predicted to be essential in *B. pseudomallei*. The virulence target chosen was PspA, the main effector of the Phage-shock protein (Psp) response. The Psp response is an extracytoplasmic response system that is vital for maintenance of the inner membrane when the cell encounters stressful conditions.

In order to validate MinE as an essential target in *B. pseudomallei*, a number of conditional mutagenesis techniques were used to inactivate the gene. This study found that the *min* operon was not essential when all three genes were inactivated, but an imbalance any of the *min* genes did have a detrimental effect on the survival of the bacteria, indicating that this would provide an ideal target for inhibitors. The Psp response was fully characterised by creating a knockout mutant in the *pspA* gene. Deletion of *pspA* caused a growth defect during prolonged growth in a liquid culture, also displaying reduced survival in a macrophage infection during this stage of its lifecycle. However, the Δ*pspA* mutant did not show attenuation when tested in multiple infection models and so was not thought to play a major role in the virulence of *B. pseudomallei*. The results from this study indicate the PspA would not make an effective candidate for an antimicrobial target.

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Submitted to PLOS One, March 2014.

Posters

Role of Phage-shock protein A during stationary phase in *Burkholderia pseudomallei*.
S. J. Southern, T. S. Milne, M. Sarkar-Tyson, A. Tavassoli and P. C. F. Oyston.
Presented at the 7th World Melioidosis Congress, 2013.

Role of Phage-shock protein A during stationary phase in *Burkholderia pseudomallei*.
S. J. Southern, T. S. Milne, M. Sarkar-Tyson, A. Tavassoli and P. C. F. Oyston.
Presented at the SGM Spring Conference, 2013.

The Phage-shock protein response as a drug target in bacteria.
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Presented at the SET for BRITAIN exhibition in the Biological and Biomedical Sciences section, 2011.

The Phage-shock protein response as a therapeutic target.
S. J. Richards, T. S. Milne, M. Sarkar-Tyson, A. Tavassoli and P. C. F. Oyston.
Presented at the Gordon Research Seminar and Conference on Chemical and Biological Terrorism Defense, 2011.

Declaration of authorship

I, Stephanie Jayne Southern, declare that the thesis entitled "Evaluation of Two Novel Antimicrobial Targets in *Burkholderia pseudomallei*" and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research.

I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
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Animal work was carried out by Dstl members of staff under the Animals (Scientific Procedures) Act 1986, licence number 30/2623.

Signed: ……..

Date:…….

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Abbreviations

AAA	ATPases associated with diverse cellular activities
ABC	ATP-binding cassette
ACDP	Advisory committee on dangerous pathogens
ACGM	Advisory committee on genetic modification
AHL	*N*-acyl-homoserine lactone
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
Bsa	*Burkholderia* secretion apparatus
C-	Carboxy-terminal
cDNA	Complementary deoxyribonucleic acid
cfu	Colony forming units
Cm^R	Chloramphenicol resistance
Da	Dalton
dhfr	Dihydrofolate reductase (trimethoprim resistance cassette)
DIG	Digoxigenin
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides
EBP	Enhancer-binding protein
EDTA	Ethylenediaminetetra acetic acid
FCS	Foetal calf serum
GEF	Guanine nucleotide exchange factor
HD	Helical domain
Hsp	Heat shock protein
HTS	High-throughput screening
IFN-γ	Interferon-gamma
IL	Interleukin

i.p.	Intra-peritoneal
IVET	*In vivo* expression technology
Km^R	Kanamycin resistance
L-15	Leibovitz's L-15 medium
LB	Luria Bertani
LDH	Lactate dehydrogenase
LF	Left flank
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
Min	Minicell
Mip	Macrophage infectivity potentiator
MLD	Median lethal dose
MNGC	Multi-nucleated giant cell
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MTS	Membrane targeting sequence
MTTD	Mean time to death
N-	Amino-terminal
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMF	Proton motive force
Psp	Phage-shock protein
QS	Quorum sensing
RF	Right flank
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNAP	Ribonucleic acid polymerase
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase
RTHS	Reverse two-hybrid system
RT-PCR	Reverse transcription polymerase chain reaction

SDS	Sodium dodecyl sulphate
Sec	Secretion
SEM	Standard error of the mean
SICLOPPS	Split intein-mediated circular ligation of peptides and proteins
SOC	Super optimal catabolite
SSC	Saline-sodium citrate
STM	Signature-tagged mutagenesis
T3SS	Type 3 secretion system
T6SS	Type 6 secretion system
TAE	Tris-acetate-EDTA
Tat	Twin-arginine translocation
TLR	Toll-like receptor
TMP-SMX	Trimethoprim-sulfamethoxazole
Tm^R	Trimethoprim resistance
TraDIS	Transposon directed insertion-site sequencing
UV	Ultraviolet
WT	Wild-type

Chapter 1: **Introduction**

1.1 *Burkholderia pseudomallei*

1.1.1 The *Burkholderia* genus

Burkholderia belong to the subdivision beta-Proteobacteria, which contains a diverse range of Gram-negative species, including *Ralstonia, Pandoraea* and *Inquilinus sp.* (Coenye and Vandamme, 2003). The genus *Burkholderia* contains over 40 species including both pathogenic and non-pathogenic bacteria (Ussery *et al.*, 2009). Most species of *Burkholderia* are non-pathogenic soil bacteria, however, several species are known plant pathogens including *Burkholderia plantarii*, *Burkholderia caryophylli* and *Burkholderia andropogonis* (Coenye and Vandamme, 2003). The *Burkholderia* also contains several bacteria that have the ability to fix nitrogen, such as *Burkholderia vietnamiensis* (Gillis *et al.*, 1995), and includes endosymbionts which form beneficial interactions with plants. For example, *Burkholderia tuberum* can fix nitrogen and form nodules in tropical legume plants (Moulin *et al.*, 2001). Other *Burkholderia* are opportunistic pathogens in humans: *Burkholderia cepacia*, *Burkholderia gladioli* and *Burkholderia fungorum* are particular risks for certain groups such as cystic fibrosis patients (Coenye and Vandamme, 2003). The two primary mammalian pathogens belonging to the *Burkholderia* species are *Burkholderia mallei*, which causes glanders in solipeds, and *Burkholderia pseudomallei*, the causative agent of melioidosis in humans (Whitmore and Krishnaswami, 1912). *B. pseudomallei* is an opportunistic pathogen and usually exists as a saprophytic soil bacterium (Cheng and Currie, 2005).

Burkholderia genomes consist of two or three chromosomes and often contain plasmids. *B. pseudomallei* strain K96243 contains two chromosomes of 4.07 Mb and 3.17 Mb, and is one of the largest known prokaryotic genomes (Holden *et al.*, 2004). The larger chromosome contains mainly housekeeping genes, such as genes involved in cell growth and metabolism. The second chromosome is believed to encode genes required for survival in different environments and a number of virulence factors, including several secretion systems. The genome of *B. pseudomallei* also contains sixteen genomic islands, accounting for 6.1% of its content (Holden *et al.*, 2004). These sequences are absent in *B. mallei* which contains two smaller chromosomes of 3.51 Mb

and 2.32 Mb (Nierman *et al.*, 2004). *B. mallei* is thought to have evolved from *B. pseudomallei* as it contains extensive deletions and rearrangements in its genome relative to *B. pseudomallei* (Godoy *et al.*, 2003). *Burkholderia thailandensis* is also descended from *B. pseudomallei* but separately to *B. mallei* (Brett *et al.*, 1998). It coexists with *B. pseudomallei* in the soil in Thailand but only rarely causes disease (White, 2003). *B. thailandensis* contains an arabinose assimilation operon consisting of nine genes that is not present in *B. pseudomallei* (Moore *et al.*, 2004). This allows it to utilise L-arabinose as a carbon source. The operon is suggested to have a regulatory role as it down-regulates several genes in the type 3 secretion system (T3SS) when incorporated into the *B. pseudomallei* genome (Moore *et al.*, 2004).

1.1.2 Melioidosis

The name "melioidosis" comes from the Greek word "melis" meaning a "distemper of asses" and "eidos" meaning resemblance, as it causes a similar disease to glanders in horses (White, 2003). It was first isolated by the pathologist Alfred Whitmore at the Rangoon Central Hospital in Burma (Whitmore and Krishnaswami, 1912). He recorded a previously undescribed "glanders-like" disease in humans. The new bacterium differed from the organism that caused glanders as it was able to grow more rapidly, was motile, and did not induce the characteristic Strauss reaction.

1.1.2.1 Epidemiology

Melioidosis is endemic to regions of Southeast Asia and northern Australia with the main endemic areas considered to be between 20°N and 20°S (Figure 1.1; Dance, 1991). In northern Australia melioidosis is an important cause of sepsis in humans and animals and is the commonest cause of community-acquired bacteremic pneumonia (Currie *et al.*, 2000b). Melioidosis is endemic in Thailand where septicaemic melioidosis is a major cause of morbidity and mortality (Chaowagul *et al.*, 1989), although the incidence of the disease is unevenly distributed throughout the country: the majority of cases occur in the Ubon Ratchathani province in Northeast Thailand. Here the incidence of the disease is 4.4 cases per 100,000 people (Suputtamongkol *et al.*, 1994), although a more recent estimate has put the figure at 21.3 per 100,000 people in this province and 8.6 per 100,000 people in Northeast Thailand as a whole (Limmathurotsakul *et al.*, 2010a). The majority of cases occur in older members of the

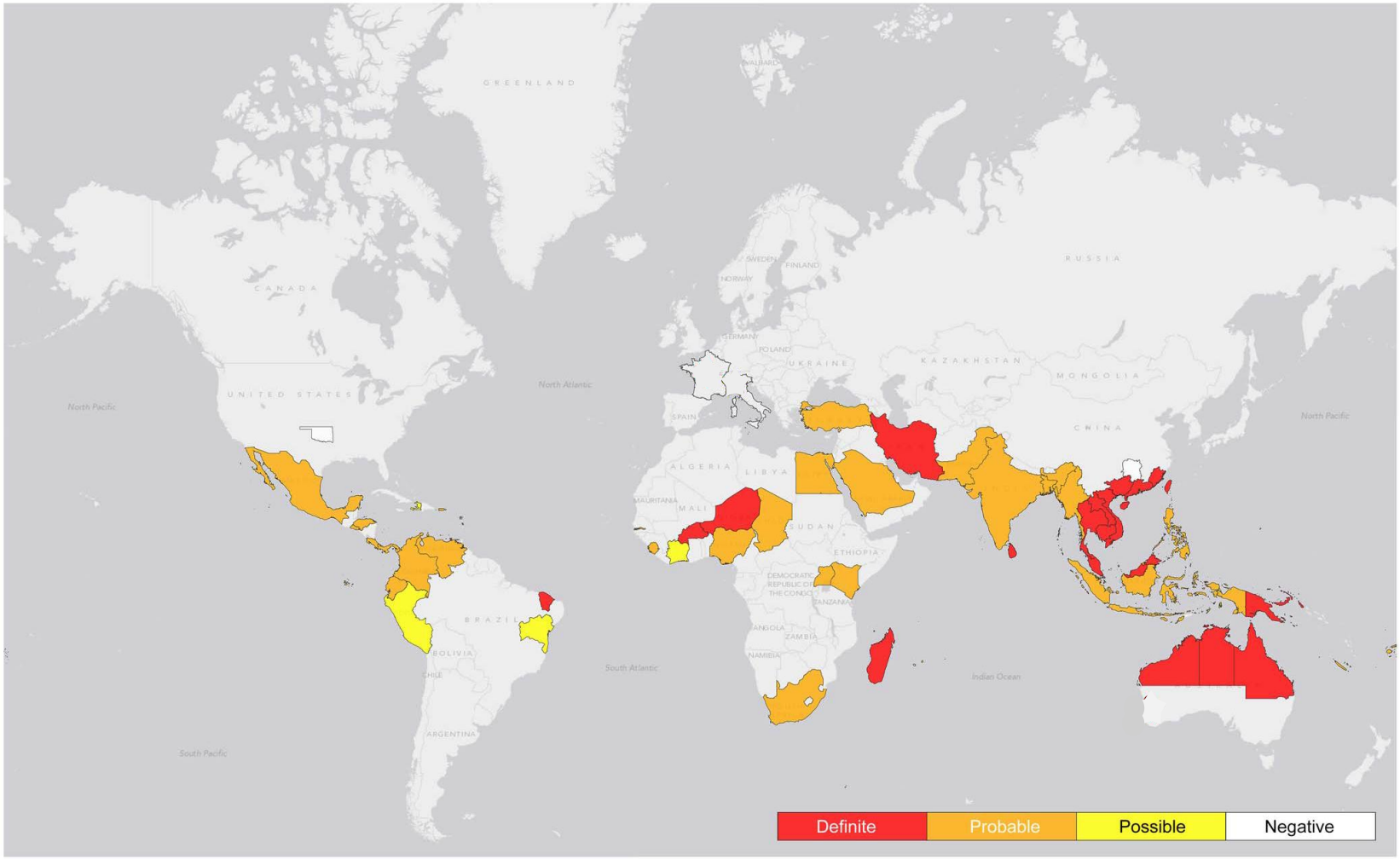


Figure 1.1 Worldwide distribution of environmental *B. pseudomallei* (Limmathurotsakul *et al.*, 2013).

population (Suputtamongkol *et al.*, 1994) and approximately 80% children in this region have antibodies against *B. pseudomallei* by the age of 4 years old (Kanaphun *et al.*, 1993). Melioidosis is also endemic to Burma, where it was first described, Malaysia, Singapore, Vietnam and southern China (Dance, 1991). There have been a number of isolated cases of melioidosis in India over the years, but a recent study has shown that it may be an emerging infectious disease in some regions of the country (Saravu *et al.*, 2010). Melioidosis also appears to be an emerging disease in Brazil (Rolim *et al.*, 2005;Rolim *et al.*, 2009). In other parts of the world most reports of melioidosis occur in travellers returning from endemic areas. Sporadic cases have occurred throughout the world in Africa, the Middle East, the Caribbean and Central and South America (Cheng and Currie, 2005).

1.1.2.2 **Risk factors**

B. pseudomallei is widely distributed in soil in Thailand and is mainly found in agricultural land which has been cleared and cultivated (Limmathurotsakul *et al.*, 2010b;Vuddhakul *et al.*, 1999;Dance, 2000). The major route of infection is thought to be percutaneous, through cuts and abrasions on the limbs of people working in endemic areas. Rice farmers are therefore at a high risk of contracting the disease, with one study recording 85% of melioidosis patients having this occupation (Suputtamongkol *et al.*, 1999). However, in the majority of cases the source of infection is unaccounted for with only 6% of infections involving a known penetrating injury (Suputtamongkol *et al.*, 1994).

The number of people contracting melioidosis shows a dramatic increase during the rainy season in Thailand and Australia (Suputtamongkol *et al.*, 1994). In fact, the majority of cases of melioidosis in Australia occur during the monsoon season (Currie *et al.*, 1993;Currie and Jacups, 2003). Transmission via the aerosol route is thought to be a significant hazard during this time due to the increase in pneumonia cases. In addition, patients that are diagnosed with melioidosis after heavy rainfall frequently develop a more severe disease (Currie and Jacups, 2003). These cases are often associated with exposure to surface water and mud in endemic areas where the bacteria may be aerosolised during intense rainfall and strong winds. In light of the transmissibility of *B. pseudomallei* via the aerosol route, this organism has been

classified as a Category B biological threat agent by the Centres for Disease Control and Prevention.

There are a number of other risk factors associated with *B. pseudomallei* infection. The disease is often contracted in patients with underlying conditions, the most common being diabetes mellitus (Suputtamongkol *et al.*, 1999). Other risk factors include alcoholism, chronic lung disease and chronic renal failure (Currie *et al.*, 2000b;Chou *et al.*, 2007;Saravu *et al.*, 2008;2010).

1.1.2.3 **Clinical presentation and diagnosis**

The disease melioidosis may result from primary exposure to the organism, re-infection or re-activation of a latent infection (Currie *et al.*, 2000a) The bacterium can be transmitted via inhalation, ingestion or inoculation, with melioidosis subsequently manifesting in different forms depending on the route of exposure (Cheng and Currie, 2005). A common route of infection is through percutaneous inoculation, with the average time between an inoculating event and symptomatic disease being 9 days (Currie *et al.*, 2000b). Alternatively, the infection can lie latent for several months or years. The longest recorded case occurred in a man who presented with symptoms of the disease 62 years after returning from an endemic country (Ngauy *et al.*, 2005). The disease can vary in severity and the symptoms of the disease are also very broad (Ip *et al.*, 1995).

A common form of the disease is cutaneous melioidosis (Gibney *et al.*, 2008). This can present with a range of symptoms from purulent ulcers to multiple lesions or subcutaneous abscesses (Gibney *et al.*, 2008;Wootton *et al.*, 2013). This type of melioidosis tends to cause chronic infection, but is also more localised and less severe than many other forms of the disease (Gibney *et al.*, 2008). However, there is evidence to suggest that skin inoculation can progress to pneumonia, sepsis and abscess formation through haematogenous spread (Currie *et al.*, 2000b). In severe cases of chronic melioidosis the disease often causes numerous suppurative abscesses which can form in many organs, most commonly the lungs, skin, liver, spleen, skeletal muscle and prostate (Ip *et al.*, 1995;White, 2003). In Thailand, melioidosis commonly presents as

parotitis in children, although this is rare in Australia (Dance *et al.*, 1989;Currie *et al.*, 2000c).

Acute melioidosis is frequently indistinguishable from other acute bacterial infections. It is often seen in endemic regions where melioidosis is a common cause of community-acquired bacteraemic pneumonia (Currie *et al.*, 2000b;Chaowagul *et al.*, 1989). In Australia, half of all cases present as pneumonia, which frequently leads to septicaemia (Currie *et al.*, 2000c). Septicaemic melioidosis is the most severe form of melioidosis. In one review, 86% of melioidosis patients with septic shock died, usually as a result of multi-organ failure (Currie *et al.*, 2000c). However, the mortality rates caused by melioidosis vary between countries. For example, in Thailand the mortality rate from *B. pseudomallei* infection is 40% (Limmathurotsakul *et al.*, 2010a), compared to Australia where it is reported to be 19% (Currie *et al.*, 2000c). This is probably due to the difference in availability of intensive-care facilities in the two countries.

Because of the range of symptoms that it causes, melioidosis is known as "the great mimicker" and is often mistaken for other diseases such as tuberculosis, typhoid, and other bacterial infections (Overtoom *et al.*, 2008;Valsalan *et al.*, 2008). This makes diagnosis difficult, a fact demonstrated by one case where an initial diagnosis of tuberculosis was given based on chest radiographs, before being diagnosed with melioidosis over 10 years later (Overtoom *et al.*, 2008). A single colony in a clinical sample can be conclusive for diagnosis of melioidosis when isolated from a patient presenting with a febrile illness, as *B. pseudomallei* is not part of the normal flora (Wuthiekanun and Peacock, 2006). When melioidosis is suspected, antibiotic therapy is started immediately due to the severity of some forms of the disease and the high mortality rate. Once melioidosis is confirmed, routine tests are carried out to detect the onset of renal failure, abnormal liver function and anaemia, which can occur during severe melioidosis infections (Wuthiekanun and Peacock, 2006).

1.1.3 Immune response to *B. pseudomallei* infection

B. pseudomallei expresses a number of surface-associated molecules, such as lipopolysaccharide (LPS), which are recognised by the Toll-like receptors (TLRs). This is a family of proteins that form a crucial part of the innate immune response as they are

responsible for detecting invading pathogens. They do this through recognition of conserved motifs expressed on a pathogen's cell surface. The TLRs then initiate the immune response via activation of a number of pathways. In melioidosis patients, expression of TLRs is up-regulated during sepsis, most notably TLR1, TLR2 and TLR4 (Wiersinga *et al.*, 2007). The LPS from *B. pseudomallei* signals through TLR2 rather than TLR4 (Wiersinga *et al.*, 2007), which is traditionally thought of as the Gram-negative receptor for recognition of LPS (Beutler and Rietschel, 2003). This receptor appears to be extremely important for host defence against melioidosis as TLR2 knock-out mice had reduced mortality and dissemination of the bacteria to the organs after an intranasal infection (Wiersinga *et al.*, 2007).

The involvement of neutrophils and macrophages is crucial for controlling the early stages of infection. When either of these cell types are depleted shortly before or after administration of *B. pseudomallei* it leads to exacerbation of disease and higher bacterial loads, resulting in a faster time to death (Breitbach *et al.*, 2006;Easton *et al.*, 2007). Activation of macrophages results in the up-regulation of pro-inflammatory cytokines. The presence of interleukin (IL)-12 and, to a lesser extent, IL-18 is essential for controlling the initial stages of infection (Haque *et al.*, 2006). These cytokines trigger the production of interferon (IFN)-γ from natural killer cells and T cells, among others. This is a key protective cytokine as depletion of IFN-γ results in mice that are highly susceptible to infection (Easton *et al.*, 2007;Haque *et al.*, 2006). Conversely, high levels of cytokine production triggered by the presence of LPS also contribute significantly to the pathogenesis of the disease (Wiersinga *et al.*, 2007). This is demonstrated to some extent in species of mice with different susceptibilities to *B. pseudomallei* (Ulett *et al.*, 2000). In acute melioidosis the increase in pro-inflammatory cytokines peaks at 24 to 48 hours after infection, which can cause excessive inflammation and tissue damage. However, in a chronic infection there is a less severe increase in the production of cytokines, allowing more time for the adaptive immune response to react (Ulett *et al.*, 2000).

The adaptive immune response to *B. pseudomallei* is less well understood but it is known that protection requires a strong humoral response as well as cellular immunity (Healey *et al.*, 2005). Individuals infected with *B. pseudomallei* produce antibodies to

the O-antigen of LPS (Ho *et al.*, 1997) and there is some evidence to suggest that higher levels of anti-LPS antibody correlate with survival in melioidosis patients (Charuchaimontri *et al.*, 1999). In addition, different subclasses of IgG antibodies are present in patients that have a different outcome of the disease. An example of this is the IgG3 response, which is only seen in patients that have survived septicaemic melioidosis (Ho *et al.*, 1997). It is thought that antibodies against LPS are protective by promoting phagocytic killing and that their action is significantly enhanced by complement (Ho *et al.*, 1997). However, despite this evidence supporting the protective role of antibodies, when passive immunisation was carried out against melioidosis in mice it resulted in only limited protection (Jones *et al.*, 2002). In this study a cocktail of monoclonal antibodies against capsular polysaccharide, LPS and protein antigens had some protective effect in animals infected with a dose of 1×10^4 cfu of *B. pseudomallei*. Many people living in endemic regions already possess antibodies towards *B. pseudomallei* either from environmental exposure or previous disease (Kanaphun *et al.*, 1993) but this is insufficient to protect from further episodes or re-infection, and recurrence of infection can occur even in the presence of high antibody levels (Vasu *et al.*, 2003).

1.1.4 Intracellular lifecycle

B. pseudomallei infection can occur through initial attachment on the epithelial cell layer as it is able to adhere to a number of cell types, such as lung epithelial cells (Kespichayawattana *et al.*, 2004). This is mediated through the presence of type IV pili on the surface of *B. pseudomallei*, although it is likely that other adhesins are also present (Essex-Lopresti *et al.*, 2005;Allwood *et al.*, 2011). Attachment to the epithelial surface is quickly followed by intracellular invasion, where the bacteria can survive and replicate inside both phagocytic and non-phagocytic cell lines (Jones *et al.*, 1996).
The genome of *B. pseudomallei* contains three T3SS gene clusters (Holden *et al.*, 2004), one of which is homologous to the Inv/Spa/Prg and Ipa/Mxi/Spa systems in *Shigella enterica* and *Salmonella flexneri* (Attree and Attree, 2001). This cluster, named the *Burkholderia* secretion apparatus (*Bsa*), plays a significant role in the intracellular lifestyle of *B. pseudomallei*. It secretes a number of factors that enable the bacteria to invade host cells, escape from endocytic vacuoles, survive within macrophages and spread to neighbouring cells (reviewed by Allwood *et al.*, 2011). A number of its

components and secreted factors have been investigated in order to determine the specific role of these proteins during infection.

Several components of the T3SS have a role in intracellular survival. The structural components of this system are clearly important, for example, inactivation of *bsaQ*, the largest gene in the *bsa* locus, reduces the ability of the bacterium to invade cells (Muangsombut *et al.*, 2008). This gene is homologous to the *invA* gene in *Salmonella*, which forms a structural component at the base of the needle (Sun *et al.*, 2005). Without this gene *B. pseudomallei* is prevented from causing cell death after internalisation, which requires a functional T3SS.

BopE is an effector secreted by the Bsa T3SS that plays a key role in invasion. A mutant is unable to invade epithelial cells as effectively as wild-type bacteria and is therefore important in mediating uptake of the bacteria into non-phagocytic cells (Stevens *et al.*, 2003). BopE is homologous to the *Salmonella* SopE effector proteins which act as guanine nucleotide exchange factors (GEFs). These proteins induce rearrangements in the actin cytoskeleton by exhibiting GEF activity which acts on the eurkaryotic Rho GTPases Cdc42 and Rac1 (Stevens *et al.*, 2003). Another effector secreted by the T3SS is BopC. A mutation in this gene results in reduced invasion of epithelial cells (Muangman *et al.*, 2011). Therefore, it is likely that effective invasion of epithelial cells relies on several secreted Bsa proteins that act in partnership.

Many bacteria are facultative intracellular bacteria that occupy different niches within the host cell. For example, *Salmonella* Typhimurium can invade host cells through the action of its T3SS (Galán *et al.*, 1992). Once inside, it resides in *Salmonella*-containing vacuoles where it can survive and replicate (reviewed by Bakowski *et al.*, 2008). Other bacteria, such as *Shigella* and *Listeria*, escape from the vacuole compartment to replicate in the cytoplasm (Ogawa and Sasakawa, 2006;de Chastellier and Berche, 1994). The intracellular lifecycle of *B. pseudomallei* follows the latter model by using its T3SS to secrete factors that facilitate rapid vacuolar escape, a process that can occur within 15 minutes after internalisation (Stevens *et al.*, 2002). BopA is an effector that is required for efficient escape from phagosomes. In a *bopA* mutant, although phagosomal

escape is delayed, once free it is can proliferate and spread between cells (Gong *et al.*, 2011).

B. pseudomallei that have entered the cytoplasm of a host cell can replicate rapidly and spread to other cells through actin-mediated motility. The manipulation of the host actin cytoskeleton is a well established model for dissemination of bacteria. Several bacterial species including *Shigella, Listeria* and *Rickettsia* express surface proteins that trigger the polymerisation of actin at one pole of the bacterial cell, enabling propulsion into neighbouring host cells (Gouin *et al.*, 1999). *B. pseudomallei* is also able to manipulate the host actin-associated proteins to produce membrane protrusions (Kespichayawattana *et al.*, 2000). This can lead to cell fusion which causes the formation of multi-nucleated giant cells (MNGCs). Formation of MNGCs is important for intracellular spread and for progression of infection as it allows *B. pseudomallei* to spread between cells while remaining hidden from the immune system. BimA is a putative Type V secreted protein that is essential for actin-based motility (Stevens *et al.*, 2005). A *bimA* mutant is able to escape from endocytic vacuoles but cannot form membrane protrusions, characteristic of actin polymerisation, and therefore is deficient in its ability to spread between cells (Stevens *et al.*, 2005).

An alternative secretion system, the type 6 secretion system (T6SS), also plays a role in the formation of MNGCs. There are six T6SS in *B. pseudomallei*, only one of which is apparently important for virulence (Shalom *et al.*, 2007). This T6SS has been shown to be up-regulated during macrophage infection, although it is not essential for intracellular survival (Shalom *et al.*, 2007). Instead, it is thought to play a role in the formation of MNGCs as a mutant deficient for an integral surface-associated component (Hcp) of the T6SS was able to form actin tails but not MNGCs (Burtnick *et al.*, 2011). The T6SS is up-regulated following internalisation but prior to vacuolar escape (Burtnick *et al.*, 2010) and it is thought that the low iron and zinc concentrations in this environment may be a trigger for its induction (Burtnick and Brett, 2013).

As well as the secretion systems, there are a number of other factors that contribute to the intracellular survival of *B. pseudomallei*. For example, several biosynthetic pathways are essential for intracellular growth *in vitro*. These include the purine, histidine and *para*-aminobenzoate biosynthetic pathways (Pilatz *et al.*, 2006). Mutants

in components of these pathways have either impaired or abolished growth in macrophages, resulting in varying degrees of attenuation in mice. The ability to invade cells and stimulate formation of actin tails is unaffected (Pilatz *et al.*, 2006).

1.1.5 Virulence factors

B. pseudomallei possesses a multitude of virulence factors which contribute to its pathogenesis. These include several components that are present on the cell surface, such as flagella, pili and LPS. In *B. pseudomallei* the *fliC* gene encodes the molecule flagellin, which forms the filament structure of the flagellum (DeShazer *et al.*, 1997). The flagella in *B. pseudomallei* are thought to aid in the dissemination of bacteria from the site of infection as mice infected intranasally with a *fliC* deletion mutant had fewer bacteria present in the lungs and spleen compared to infection with the wild-type strain (Chua *et al.*, 2003). However, this attenuation is not seen in a more acute infection model (DeShazer *et al.*, 1997). As well as flagella, there are a number of gene clusters on the genome of *B. pseudomallei* which are predicted to be involved in the synthesis of pili (Essex-Lopresti *et al.*, 2005). A mutation in one of these genes, *pilA*, results in reduced adherence to epithelial cells and the mutant is therefore attenuated in mice infected intranasally.

A number of surface polysaccharides are expressed by *B. pseudomallei*. The capsule polysaccharide produced by *B. pseudomallei* (Puthucheary *et al.*, 1996) is encoded by several biosynthesis loci on the genome (Holden *et al.*, 2004) and is essential for virulence in mice (Atkins *et al.*, 2002a;Sarkar-Tyson *et al.*, 2007). In addition, *B. pseudomallei* produces three antigenic types of LPS, two smooth serotypes, A and B, and a rare rough serotype, with serotype A accounting for 97% of strains (Anuntagool *et al.*, 2006). *B. pseudomallei* LPS is different to that of most other Gram-negative bacteria as it contains an unusual chemical structure in the inner core (Matsuura *et al.*, 1996). This is predicted to decrease the level of interaction with CD14 on the macrophage cell surface and therefore cause a reduced inflammatory response (Utaisincharoen *et al.*, 2000). Its role in virulence was demonstrated using an LPS-deficient mutant, which was found to be attenuated in several animal models and was more susceptible to complement-mediated killing (DeShazer *et al.*, 1998). This mutant also displayed

decreased intracellular survival and was more sensitive to polymixin-B, a host cationic peptide (Burtnick and Woods, 1999).

As described above, the Bsa T3SS is extremely important for intracellular survival in *B. pseudomallei*. In other bacteria, such as *Salmonella* Typhimurium, defects in the structural components of the T3SS resulted in attenuation due to a loss of ability to invade and survive inside host cells (reviewed by Wallis and Galyov, 2000). Similarly in *B. pseudomallei*, mutations in T3SS structural proteins led to secretion defects, which subsequently caused a reduction in virulence. For example, inactivation of *bsaU* resulted in partial attenuation in mice, correlating with a decreased bacterial burden in the spleen, liver and lungs (Pilatz *et al.*, 2006). Mice infected with *B. pseudomallei* with a mutation in the structural *bsaZ* gene, the last in the *Bsa* cluster, also showed a delayed mean time to death (MTTD) compared to mice infected with the wild-type (Stevens *et al.*, 2004).

In order to successfully deliver the effector proteins of the T3SS into the host cell, the T3SS requires a specific subset of proteins known as the translocators (Håkansson *et al.*, 1996). These proteins are thought to form a channel through the membrane and into the cytosol. In *Yersinia* species, disruption to the translocator proteins resulted in mutants that were unable to survive in a mouse infection (Mecsas *et al.*, 2001). In *B. pseudomallei*, the translocator proteins BipB and BipD are expressed at the tip of the T3SS apparatus and are thought to interact with the eukaryotic cell to aid delivery of T3SS effectors (Stevens *et al.*, 2002). Inactivation of *bipB* and *bipD* led to reduced intracellular invasion, replication and loss of the ability to escape from endocytic vesicles (Suparak *et al.*, 2005;Stevens *et al.*, 2002). Consequently, a mutant with a deletion in the *bipD* gene was found to be attenuated in mice, vaccination with which conferred partial protection against a subsequent challenge with wild-type bacteria, although the purified protein did not (Stevens *et al.*, 2004).

Quorum sensing (QS) is a cell density-dependent process whereby bacteria use *N*-acyl-homoserine lactones (AHLs) to co-ordinate gene expression within a community (Fuqua *et al.*, 1994). The *B. pseudomallei* genome contains a number of genes associated with QS, including three *luxI* homologues, encoding AHL synthase proteins

which generate the QS signalling molecules, and five *luxR* transcriptional regulator homologues (Ulrich *et al.*, 2004). The deletion of individual QS genes in *B. pseudomallei* caused an increase in the MTTD of mice challenged with the aerosolised mutants and reduced colonisation in the lungs. In addition, disruptions to genes within the QS network caused a significant increase in the MLD in a Syrian hamster model of infection for acute melioidosis, with a mutation in a AHL synthase, *bpmI3*, resulting in the highest attenuation (Ulrich *et al.*, 2004).

Although *B. pseudomallei* contains a wide variety of virulence factors, there are a number of proteins that, while not directly contributing to the pathogenesis of the disease, are important for virulence due to their role in the survival of the organism during infection. For example, peptidylprolyl *cis-trans* isomerases are enzymes that catalyse the folding of proteins containing proline residues. These enzymes have been implicated in virulence in *Legionella pneumophilia* and subsequently termed macrophage infectivity potentiator (Mip) proteins due to their importance in macrophage infection (Cianciotto *et al.*, 1989). A *B. pseudomallei* Mip protein is required for a number of virulence-associated functions. For example, deletion of a *B. pseudomallei mip* gene caused decreased resistance to low pH, reduced protease production, reduced motility and, consequently, led to reduced intracellular survival in macrophage cells and attenuation in a mouse model of infection (Norville *et al.*, 2011). Similarly, the disulfide oxidoreductase, DsbA, is vital for the proper folding of a number of secreted proteins through the oxidation of sulphur-containing residues to form disulfide bonds (reviewed by Heras *et al.*, 2009). Deletion of *dsbA* in *B. pseudomallei* resulted in defects in processing of a range of virulence factors, which resulted in motility defects, attenuation in macrophages and reduction in protease secretion, ultimately leading to a reduction in virulence in a mouse model of infection (Ireland *et al.*, 2013).

As described above, the large genome of *B. pseudomallei* encodes many genes associated with adaption to different environmental conditions (Holden *et al.*, 2004). Many of these are virulence-associated factors that have enabled *B. pseudomallei* to colonise mammalian cells. Consequently, this has allowed an environmental bacterium to become a significant mammalian pathogen.

1.1.6 Current therapeutic strategies for *B. pseudomallei* infection

1.1.6.1 Antibiotic treatment

Infection with *B. pseudomallei* is notoriously difficult to treat as the bacterium is inherently resistant to many classes of antibiotics (summarised in Cheng and Currie, 2005). In Thailand, where the disease is endemic, there is an extremely high rate of relapse (Maharjan *et al.*, 2005), therefore successful treatment requires a prolonged course of antibiotics. Because of this, melioidosis treatment is biphasic consisting of a short-term acute parenteral phase and a long-term oral eradication phase. The long-term treatment regimens vary between different parts of the world, but usually involve regular dosing with antibiotics for at least 3 months.

In the initial treatment phase bactericidal antibiotics are administered via the intravenous route, where a typical therapy consists of ceftazidime given in 8 hourly doses (Wuthiekanun and Peacock, 2006). This treatment continues for at least 10 to 14 days but is extended for more severe cases until a clinical improvement is seen in the patient. The use of carbapenems, such as meropenem or imipenem, can also be given at this stage but their use is generally reserved for when ceftazidime therapy is unsuccessful (Cheng *et al.*, 2004b). In Australia, therapy is sometimes complemented by a cytokine, granulocyte colony-stimulating factor (G-CSF), which has been shown to reduce mortality in cases of septic shock (Cheng *et al.*, 2004a).

During the oral eradication phase, trimethoprim-sulfamethoxazole (TMP-SMX) is taken twice daily for at least 3 to 6 months (Wuthiekanun and Peacock, 2006). The second-line choice of antibiotic is amoxicillin-clavulanic acid, which is given to patients who are either intolerant to TMP-SMX or where *B. pseudomallei* is resistant (Cheng *et al.*, 2008). Failure to complete at least twelve weeks of therapy means that relapse is likely (Estes *et al.*, 2010). However, even when therapy is completed, reoccurrence in survivors is common over the following 10 years (Currie *et al.*, 2000a). Some prophylaxis treatments have been tested in animals, with TMP-SMX being the most effective (Sivalingam *et al.*, 2008), suggesting that this treatment is an appropriate therapy for treatment and prevention of acute and chronic disease.

1.1.6.2 **Antibiotic resistance**

The prolonged treatment of melioidosis can lead to the development of resistance. This can occur by a number of mechanisms, including alteration of the drug target, modification of antibiotics, or through efflux of drugs out of the cell. *B. pseudomallei* has inherent resistance to several classes of antibiotic, conferred through the presence of resistance genes. The *B. pseudomallei* strain K96243 genome encodes seven β-lactamases, six multi-drug efflux systems and an aminoglycoside acetyltransferase (Holden *et al.*, 2004), and hence is resistant to a diverse range of antibiotics, including second generation cepholasporins, penicillins, rifamycins and aminoglycosides (Cheng and Currie, 2005).

The expression of efflux pumps makes a significant contribution to the resistance of *B. pseudomallei* to a broad range of antibiotics. The AmrAB-OprA efflux pump was the first to be characterised in *B. pseudomallei* and was identified by transposon mutatgenesis in a search for genes responsible for resistance to aminoglycosides (Moore *et al.*, 1999). Although a deletion in this system did not have an effect on virulence, the mutant was more sensitive to kanamycin and gentamycin. A deletion in the *amrA* and *amrB* genes also increased the susceptibility of these mutants to erythromycin and clarithromycin, which are both macrolide antibiotics (Moore *et al.*, 1999). A second efflux pump in *B. pseudomallei*, BpeAB-OprB, was also reported to be involved in the efflux of aminoglycosides and macrolides (Chan *et al.*, 2004b). This second multi-drug efflux pump was shown to be QS-regulated and to have an important role in the production of virulence factors such as siderophores and phospholipase C. This was supported by the observation that *bpeAB* mutants demonstrated reduced cell invasion and cytotoxicity in human cell lines (Chan and Chua, 2005). However, studies carried out since have shown that the BpeAB-OprB system is not required for export of AHLs or siderophore production (Mima and Schweizer, 2010). BpeAB-OprB is a broad-spectrum multidrug efflux pump that, along with AmrAB-OprA, contributes to resistance to fluoroquinolones, macrolides and tetracyclines but is not responsible for the efflux of aminoglycosides as previously thought. A third efflux pump, the BpeEF-OprC efflux pump, confers trimethoprim resistance to *B. pseudomallei*, but does not affect susceptibility to TMP-SMX (Podnecky *et al.*, 2013).

The initial stage of melioidosis treatment relies heavily on the drug ceftazidime, as it is used as the first line of defence against melioidosis in both Thailand and Australia (Wuthiekanun and Peacock, 2006). This drug may need to be used for prolonged periods as treatment is often extended until clinical symptoms begin to improve. Although primary resistance to ceftazidime is rare, secondary resistance has been observed in a number of case studies. For example, several cases have been documented where ceftazidime resistance has developed through mutations in the *penA* gene. This gene in *B. pseudomallei* encodes a membrane-associated β-lactamase, which is important for resistance to ceftazidime, but is susceptible to β-lactamase inhibitors such as clavulanic acid (Godfrey *et al.*, 1991). A single-nucleotide polymorphism in the promoter region causes an increased level of expression of *penA*, consequently increasing the minimum inhibitory concentration (MIC) of ceftazidime by 10-fold (Sarovich *et al.*, 2012). Amino acid substitutions in conserved motifs of PenA can cause increased affinity for the substrate or reduced susceptibility to clavulanic acid inhibition, leading to increased resistance (Tribuddharat *et al.*, 2003). One unusual mechanism of resistance to ceftazidime has been observed in *B. pseudomallei* through the deletion of a 150 kb region of DNA on chromosome 2 (Chantratita *et al.*, 2011). This mutation was first identified in a patient who was unresponsive to treatment after a prolonged course of ceftazidime. One of the genes encoded in this region is a penicillin binding protein, PBP3, which was found to confer ceftazidime resistance upon deletion. This has important implications for treatment and diagnosis of melioidosis as this mutation subsequently arose independently in a number of different clinical isolates and resulted in an almost undetectable variant of *B. pseudomallei* when analysed using the usual diagnostic tests (Chantratita *et al.*, 2011).

B. pseudomallei also has a number of other innate characteristics that affect its antimicrobial susceptibility. The LPS of *B. pseudomallei* acts as a protective barrier which allows the exclusion of certain antimicrobial peptides, such as polymixin B, from the cell (Burtnick and Woods, 1999). It is able to form biofilms that reduce the activity of antibiotics (Sawasdidoln *et al.*, 2010). It is also able to enter into a latent phase where it most likely resides intracellularly in a non-replicating form, making treatment more difficult (Gan, 2005).

1.1.7 Alternative treatment strategies for *B. pseudomallei* infection

Due to the natural resistance of *B. pseudomallei* to a number of antibiotic drugs, extensive work has been carried out to find alternative treatment methods. As discussed, the current therapy available is often ineffective and frequent cases of relapse occur. In these cases a prophylactic treatment, such as a vaccine, could provide better protection against melioidosis. The development and distribution of a vaccine to at risk populations could provide valuable protection against the disease. This could provide much needed protection to vulnerable groups living in endemic areas, such as Northeast Thailand and northern Australia, who come in to contact with the environmental bacteria. In addition, melioidosis is considered an emergent disease in several other countries such as India and Brazil (Saravu *et al.*, 2010;Rolim *et al.*, 2005) where a vaccine would also be beneficial to the population. A prophylactic treatment could also prove useful for people travelling to endemic countries, especially those at high risk, such as diabetics.

1.1.7.1 Inactivated whole cell vaccines

Killed whole cell vaccines have been used effectively to immunise populations against a number of bacterial diseases in the past. For example, successful killed whole cell vaccines include the cholera and whooping cough vaccines (Pace *et al.*, 1998). One problem with this type of vaccine is that multiple doses and frequent boosters are often required to promote long-lasting immune protection. In addition, this type of vaccine can be highly reactogenic and poorly protective, such as the early typhoid and cholera vaccines, meaning that new approaches tend to focus on the production of acellular vaccines (Pace *et al.*, 1998). However, killed whole cell vaccines are relatively simple to produce and so have been investigated as a method for protecting against melioidosis.

Several studies have been conducted in order to test the efficacy of both *B. pseudomallei* and closely related species as potential inactivated whole cell vaccine candidates for protection against melioidosis. In one study, heat-killed *B. pseudomallei* was found to induce protection in mice against an intra-peritoneal (i.p.) challenge after repeated immunisation over the course of 6 weeks (Sarkar-Tyson *et al.*, 2009). Immunisation with two strains (K96243 and 576) expressing serologically different forms of LPS was able to protect against a subsequent infection with either strain.

Vaccination with heat-killed cells also increased the MTTD after an aerosol challenge. In addition, mice immunised with heat-killed *B. thailandensis* and *B. mallei* showed a delayed time to death when challenged with *B. pseudomallei* (Sarkar-Tyson *et al.*, 2009). Immunisation with killed whole cells was more effective than immunisation with LPS alone (Nelson *et al.*, 2004) and it was hypothesised that this may be due to the presence of additional protective antigens on the surface of the bacteria (Sarkar-Tyson *et al.*, 2009). This is supported by a previous study which showed that the immunogenicity of heat-killed *B. pseudomallei* was affected by loss of the O-antigen of LPS or the capsular polysaccharide, indicating that other immunogens may be exposed on the surface when these molecules are missing (Sarkar-Tyson *et al.*, 2007).

1.1.7.2 **Live attenuated vaccines**

Live attenuated vaccines can be generated either by empirical methods or by the targeted mutation of specific genes, making it possible in some cases for the mode of attenuation to be defined. This type of vaccine can generate an appropriate immune response as the bacteria are able to infect and replicate in a relevant manner, unlike inactivated whole cell vaccines. For example, the BCG vaccine for immunisation against tuberculosis is an attenuated strain of a related mycobacterium strain (Doherty and Andersen, 2005). However, the risk associated with vaccinating immuno-compromised recipients with live vaccines such as this needs to be considered. In addition, there is a potential risk associated with these vaccines due to the possibility of the attenuated strain reverting back to full virulence, although this can be reduced by including multiple mutations. For example, a current vaccine for typhoid fever carries a mutation in the *galE* gene and Vi antigen, along with other unknown mutations, lowering the probability of it reverting to a virulent form (Germanier and Fürer, 1975).

A number of attenuated strains of *B. pseudomallei* have been identified using transposon mutagenesis. For example, an acapsular mutant of *B. pseudomallei*, strain 1E10, was created by transposon mutagenesis of a gene encoding a putative mannosyltransferase enzyme (Atkins *et al.*, 2002a). Although the mutant showed significant attenuation in the mouse compared to the wild-type when given i.p., it was not able to induce protection against a subsequent challenge. This lack of a protective immune response to 1E10 may be because the capsular polysaccharide is a key

protective antigen (Atkins *et al.*, 2002a). This was confirmed in a recent vaccine study testing a live strain of the less virulent *B. thailandensis* E555, which expresses a capsule antigenically similar to *B. pseudomallei* (Scott *et al.*, 2013). Immunisation with this strain was able to protect mice against a dose of 6000 median lethal doses (MLD) of *B. pseudomallei* given i.p., compared to immunisation with an acapsular strain of *B. thailandensis* which was only protective up to a dose of 600 MLD.

The *B. pseudomallei* strain 2D2 was an attenuated auxotrophic strain produced by transposon mutagenesis with an interruption in the *ilvI* gene (Atkins *et al.*, 2002b). This encodes the large subunit of the acetolactate synthase enzyme which is vital for the biosynthesis of the branched chain amino acids (Felice *et al.*, 1974). The 2D2 strain was able to colonise the spleen, liver, kidneys and lungs of vaccinated mice, but did not persist for more than 30 days. When mice were vaccinated with 2D2, most were able to survive challenge with fully virulent *B. pseudomallei*, but it did not fully protect against melioidosis (Atkins *et al.*, 2002b). Similarly, other auxotrophic transposon mutants with insertions in the genes *purM*, *hisF* and *pabB* were shown to have a limited protective effect against a challenge with wild-type *B. pseudomallei* in mice, indicating that these mutants did not induce an appropriate immune response (Breitbach *et al.*, 2008). However, a *purN* mutant was able to induce protection against acute melioidosis in mice when challenged either by the i.p. or intranasal route, although this was unable to protect against the onset of chronic melioidosis (Breitbach *et al.*, 2008).

1.1.7.3 **Subunit vaccines**

An alternative approach to developing a new vaccine is to immunise with a purified isolated antigen, combined with an adjuvant to increase the immunogenicity. This is beneficial as it reduces the likelihood of having an adverse reaction caused by the toxicity of a crude whole cell vaccine. It is also safer than a live attenuated vaccine as there is no chance of it reverting to a virulent form. However, the design of this type of vaccine does provide several challenges, such as selection of an appropriate antigen, delivery method and adjuvant type to induce the correct immune response for protection.

A second Typhi vaccine currently in use is a subunit vaccine, composed of the purified Vi capsule polysaccharide (Klugman *et al.*, 1987). In *B. pseudomallei*, several polysaccharides have also been tested as potential subunit vaccine candidates. These surface molecules, including the polysaccharide capsule and LPS, would be first to encounter the host immune system during an infection. In one study, a capsule polysaccharide from strain NCTC 4845^{T}, along with the O-polysaccharide of the LPS from strain K96243, was used to immunise mice challenged with *B. pseudomallei* NCTC 4845^{T} by either the i.p. or aerosol route (Nelson *et al.*, 2004). When challenged via the i.p. route, both candidates were able to increase the MTTD of the mice compared to the unvaccinated controls, although LPS provided a higher level of protection. However, challenge via the aerosol route is thought to result in a much more acute disease and, consequently, neither the capsule nor the LPS subunit vaccines were able to confer any protection against the aerosol challenge (Nelson *et al.*, 2004). It is thought that combining the polysaccharides with a protein subunit could improve the immune response and boost the protective efficacy of the vaccine. An example of this is the meningococcal vaccine consisting of capsular polysaccharide conjugated with the diptheria toxoid protein (Poland, 2010).

In *B. pseudomallei* a number of proteins associated with ATP-binding cassette (ABC) transporters have also been tested as vaccine candidates (Harland *et al.*, 2007). These were chosen based the known immunogenicity and virulence properties of the proteins in other pathogens. The LolC protein is an outer membrane protein which is associated with the transport of lipoproteins between the inner and outer membranes (Yakushi *et al.*, 2000). When LolC was administered to mice it was able to protect 5 out of 6 mice against an i.p. challenge of *B. pseudomallei* (Harland *et al.*, 2007). This protein was able to produce both humoral and cell-mediated immune responses, required for effective protection against *B. pseudomallei*. Two other ABC system proteins, PotF, a periplasmic binding protein, and OppA, an oligopeptide-binding protein, were also evaluated. PotF was able to protect 3 out of 6 mice against *B. pseudomallei* challenge, but OppA was unable to induce significant protection (Harland *et al.*, 2007).

As described above, the T6SS has been identified as a major virulence determinant in *B. pseudomallei* (Shalom *et al.*, 2007). The Hcp proteins are components of the T6SS that

are found on the cell surface, making them accessible to the host immune response. Six of the Hcp proteins were tested as vaccine candidates for protection against *B. pseudomallei* in a mouse model of infection (Burtnick *et al.*, 2011). When immunised with Hcp1-6, Hcp1, Hcp2, Hcp3 and Hcp6 improved survival of the mice, although most mice were still heavily colonised at the end of the experiment. The one exception was with Hcp6-vaccinated mice where no bacteria were present in the spleens of surviving animals. However, the protection afforded by the individual Hcp proteins was not high enough for them to be considered as suitable vaccine candidates. Only Hcp1 was found to be immunogenic as it reacted with sera from melioidosis patients, and therefore could potentially be used for the serological diagnosis of melioidosis (Burtnick *et al.*, 2011).

1.2 Selection of novel antimicrobial targets

Although there has been some progress towards developing a vaccine for melioidosis, there is still no candidate that induces sterilising immunity against *B. pseudomallei*. Consequently, antibiotics remain the only defence against infection with *B. pseudomallei*. The increasing incidence of resistance to drugs currently used in the clinic means that new strategies are required for combating infectious disease. The future of antimicrobial treatments may lie in the development of new drugs that are aimed at disrupting previously unexplored targets.

Traditional antibiotics have a very limited range of targets within the bacterial cell. The main classes of antibiotics fall into one of four categories: β-lactams, aminoglycosides, macrolides or fluoroquinolones, which target cell wall biosynthesis, protein synthesis or DNA replication (Kohanski *et al.*, 2010). All of these drugs are either bacteriocidal, causing cell death, or bacteriostatic, resulting in cell growth arrest. Therefore there is a high selective pressure for resistance. Despite the issues caused by antibiotic resistance in the clinic, there has been very little progress in recent years in the development of new antibiotic classes since the last new class brought to market in 1979, namely the oxazolidinones (Bozdogan and Appelbaum, 2004), and the introduction of linezolid in 1999 (Brickner *et al.*, 1996). Daptomycin is a recent addition that has a unique mechanism of action which targets the Gram-positive plasma membrane (Canepari *et*

al., 1990). However, other compounds in development mainly consist of second or third generation derivatives of the original classes with the same mode of action as antibiotics currently in use.

In recent years the publication of bacterial genome sequences has allowed the cataloguing of every gene possessed by an organism. This has provided a wealth of new information that can be used to identify new targets in bacteria. Bioinformatics can be used to select proteins that are well conserved and therefore have the potential to be targeted by broad-spectrum antimicrobials. This strategy can also be used to predict the essentiality of a particular target by comparing a common list of conserved genes between species. Proteins can be further down-selected by filtering out any candidates that have human homologues or proteins that have duplicates in the genome. Potential virulence targets can also be predicted by comparing the genomes of pathogenic and non-pathogenic bacteria and preferentially selecting for proteins that are only present in the pathogenic species. An example of this type of down-selection is the *B. pseudomallei* LolC protein, which was selected as a potential vaccine candidate using similar bioinformatic techniques (Harland *et al.*, 2007). The use of bioinformatics is supported by global screening technologies, such as signature-tagged mutagenesis (STM) and *in vivo* expression technology (IVET), and by the increasing number of data processing tools that can be used to identify promising antimicrobial targets in bacteria.

One system that was developed in order to identify suitable antimicrobial targets in bacteria is STM, which uses transposon technology to identify virulence targets (Hensel *et al.*, 1995). This technique can be used to identify virulence genes that cause attenuation when passed through an animal model. The mutants are produced using random transposon mutagenesis, resulting in a library with each member containing an insertion in an individual gene. The different mutants can be identified through unique tags incorporated into the transposon. When the libraries are passed through an infection model, any mutants that are not recovered from the output pool when compared with the input pool are known to have a fitness disadvantage. This allows screening for genes that are essential for virulence that would not otherwise be indentified under laboratory culture conditions. This method is useful for identifying attenuating targets for live attenuated vaccine candidates, such as those identified in *B. pseudomallei*, as described

above (Atkins *et al.*, 2002a;Cuccui *et al.*, 2007;Breitbach *et al.*, 2008). Transposon directed insertion-site sequencing (TraDIS) is a modification of STM that can be used to identify genes that are essential for growth due to its high transposon coverage of the genome (Langridge *et al.*, 2009). This technique can also be used to identify genes that are either essential for growth under *in vitro* conditions or those that are essential for virulence *in vivo* by using modern high-throughput sequencing to identify the site of the transposon insertion (Chaudhuri *et al.*, 2013).

Similarly, IVET is used to identify genes that are specifically expressed during infection (Mahan *et al.*, 1993). This technique uses the transcriptional fusion of random genomic sequences to a reporter gene to identify these genes. A library is created by cloning random genomic fragments upstream of the reporter, which usually consists of either a complementation for an essential auxotrophy mutant or an antibiotic resistance marker. The transcriptional fusion is incorporated onto a suicide plasmid that must recombine with the genome in order to be maintained. The library is then transformed into a bacterial strain and used to infect the selected animal model. The relevant strains are selected for by an insertion of the transcription fusion downstream of a promoter which is driving expression of an induced gene. The necessary complementation or resistance marker is then expressed also, allowing the strains expressing the *in vivo*-induced genes to survive. This technique was used to identify genes in *B. pseudomallei* that were required to survive in macrophages, resulting in the identification of a T6SS gene cluster essential for intracellular survival (Shalom *et al.*, 2007). This has subsequently been investigated as subunit vaccine candidate (Burtnick *et al.*, 2011).

Despite the common use of these technologies for the identification of interesting targets, all have their disadvantages. One disadvantage is the presence of bottlenecks in animal models of infection which result in the loss of mutant pool complexity before *in vivo* selection can take place (Mecsas, 2002). The route of infection may also have an impact on the virulence factors that are identified. For example, if the early infection stages are bypassed by using an i.p. route of infection then genes required for adherence will not be identified (Darwin and Miller, 1999). In addition, many genes will not be identified as virulence targets if their loss can be complemented by homologues present in the genome. Conversely, transposons located in operons are likely to have polar

effects on downstream genes, resulting in the misidentification of essential genes. Therefore, all potential targets identified by these methods need to be verified by experimental methods to ensure their authenticity.

Once a suitable target has been selected, it must then be fully characterised to determine its usefulness as an antimicrobial target. If the protein selected is a broad-spectrum target then it is important to characterise the protein in several species of bacteria to verify the impact of inactivating different homologues on growth or virulence. The validation of targets can be carried out by a number of methods. In order to confirm the essential nature of a gene, conditional mutagenesis can be carried out in order to inactivate the gene in question. This can be achieved either by complementation with an inducible copy of the gene before deletion of the chromosomal copy (Cardona and Valvano, 2005), or by using an inducible promoter to directly regulate the gene (Cardona *et al.*, 2006). Using this approach, the down-regulation of the inducible promoter causes a conditional lethal phenotype when the essential gene is inactivated. Virulence targets can be easier to study as they can be characterised by direct mutagenesis. This is because *in vitro* growth is still possible after the gene has been inactivated. The mutant can then be tested in an animal model to confirm attenuation.

The identification of a wealth of new targets in bacteria can be combined with high-throughput screening (HTS) in order to develop novel antimicrobial compounds. Once the target has been selected and validated by the methods described above an assay can then be developed to screen for inhibition of the target before HTS of compounds is carried out. Whole bacterial cell screening is also performed to identify compounds with the ability to inhibit growth, using genomics to help identify the target for these inhibitors. The advantage of this method is the ability to screen for inhibitors with antimicrobial characteristics, such as cell penetration, as a prerequisite. However, it is still extremely difficult to identify the specific target and elucidate a mechanism of action using a whole cell assay, making the targeted approach a more attractive method for identifying novel drugs.

Despite the advances in the technologies used to identify and screen novel targets, there has been little progress towards the discovery of new antimicrobial compounds since

the dawn of the post-genomic era. Several reasons have been proposed for this. One drawback to HTS is the identification of "false positives", compounds that are active against the membrane, such as detergents, or compounds that are extremely cytotoxic (Payne *et al.*, 2007). Additionally, once a hit has been identified, it is difficult to transform this into a successful lead possessing the necessary qualities for whole cell antibacterial activity. Often the compound is unable to bypass the membrane, with no reliable method for optimisation of this property. This chemical optimisation of new hits is far more difficult than creating new derivatives of old antibiotics. There has also been some discussion on the chemical diversity of compounds being screened. It has been suggested that the drug-like properties defined by Lipinski (Lipinski, 2000) differ for antimicrobial drugs compared to those in other therapeutic areas (Chan *et al.*, 2004a). These and other reasons have meant the progress made in this area has been slower than anticipated since HTS technologies became available.

A novel approach to developing antimicrobial drugs is the identification of compounds that are able to inhibit specific protein interactions, rather than inhibiting enzyme activity. There are thousands of protein networks within the cell that regulate a huge range of processes, vastly broadening the range of proteins that can be targeted. Potential inhibitors would be aimed at disrupting vital interactions required for viability or virulence. A novel technology, termed split intein-mediated circular ligation of peptides and proteins (SICLOPPS), enables the rapid generation of libraries of cyclic peptide compounds, which are then screened for their ability to inhibit a specific protein interaction (Scott *et al.*, 2001). The technique is linked to a reverse two-hybrid system (RTHS) which allows the use of a genetic selection methodology for the identification of inhibitors (Horswill *et al.*, 2004). This system is modified to link the disruption of specific protein interactions to the transcription of genes essential for survival of the bacteria (Figure 1.2). The library of cyclic peptide compounds can then be screened in the RTHS, where any positive hits allow transcription of essential genes and therefore bacterial growth to occur (Horswill *et al.*, 2004). The ability to screen for inhibitors to interactions between proteins opens up a new realm of potential networks that can be targeted in bacteria. This is because the site of action of the inhibitors is not restricted to the active site. Instead, the small molecule inhibitor can potentially block any part of the interacting face of a protein, preventing further interactions.

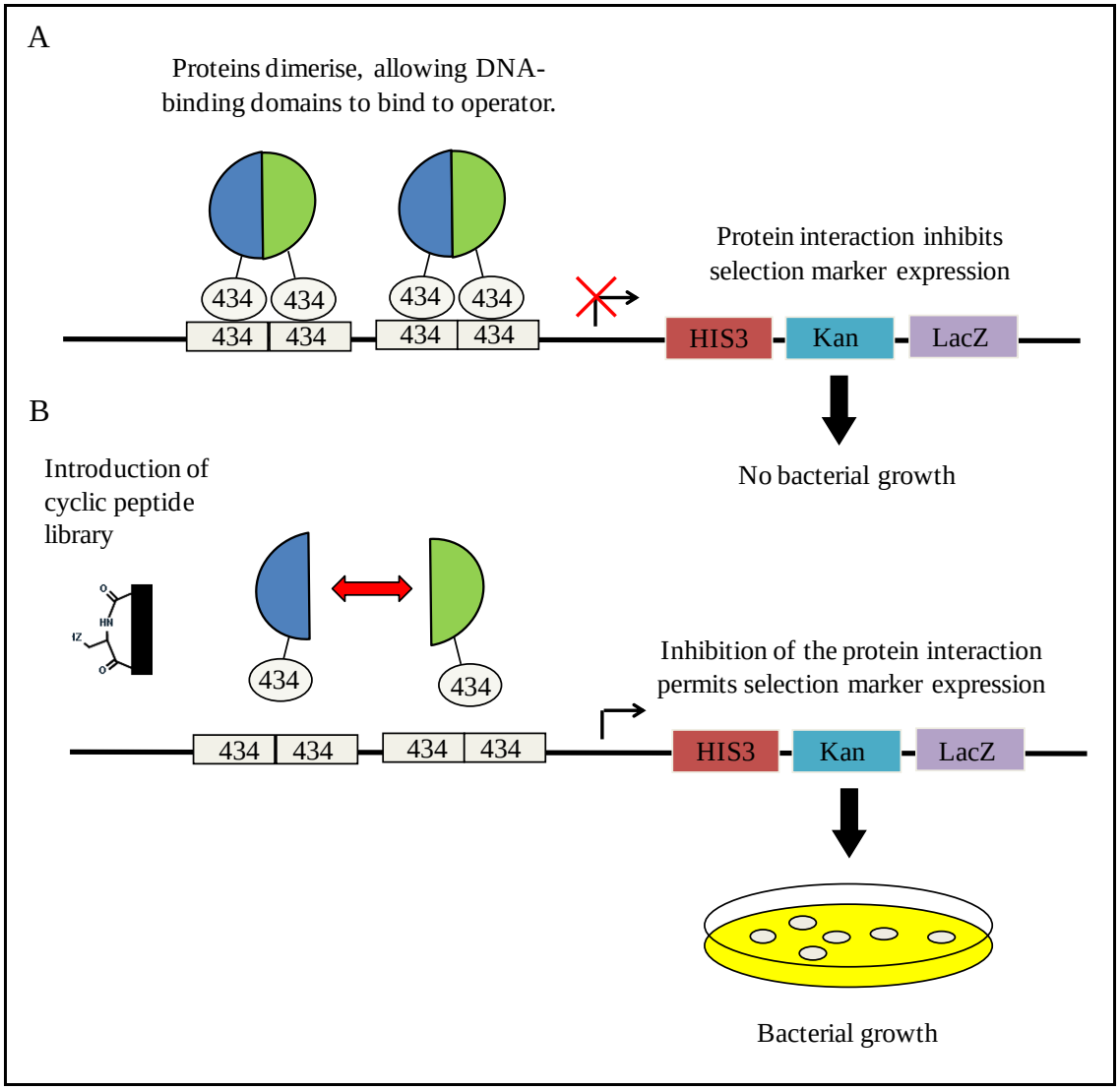


Figure 1.2 Representation of the RTHS in *Escherichia coli* used for the genetic selection of cyclic peptide inhibitors (adapted from Tavassoli *et al.*, 2008).

The protein of interest is cloned as a fusion with a DNA-binding domain (434) from a bacteriophage repressor (Hu *et al.*, 1990). Upon forming a complex, the DNA-binding domains associate with the 434 operator upstream of the reporter genes (A). This acts as a repressor and blocks the expression of the reporter genes, causing cell death. If the interaction between the two proteins is interrupted then the DNA-binding domains are unable to bind to the operators (B). This allows selection marker expression and therefore bacterial growth. Reporter genes: HIS3, imidazole glycerol phosphate dehydratase required for catalysis of the sixth step in the histidine biosynthesis pathway; Kan, kanamycin resistance cassette; LacZ, β-galactosidase, used to quantify the protein interaction.

1.2.1 Targeting virulence factors

A new strategy in the production of novel antimicrobial compounds is to target bacterial virulence factors rather than essential functions (Clatworthy *et al.*, 2007). Instead of preventing growth or causing death of the cell this would effectively disarm the bacteria from causing disease upon infection. In theory, this approach would slow the development of resistance as there would be no immediate effect on cell viability and therefore less selective pressure on the bacteria. This strategy would also prevent the disruption of the normal flora caused by traditional antibiotics which can result in undesirable side effects.

A number of virulence factors have been investigated as novel antimicrobial targets in several pathogens. Extracellular components are attractive targets for investigation as potential drugs against these would avoid the need to bypass the bacterial cell membrane. This also avoids the problem of efflux from the cell as the inhibitors do not act on cytoplasmic targets. For example, the T3SSs are important for virulence in many species of bacteria, including *B. pseudomallei*, as they allow the translocation of effectors into host cells (Hueck, 1998). Potential inhibitors of the T3SS would prevent the release of these virulence factors, necessary for the alteration of the host cellular functions, and therefore block virulence. The T3SSs are an attractive target for future anti-virulence drugs as the structure and mechanism of many systems are conserved among Gram-negative pathogens, although the secreted proteins themselves are highly diverse (Hueck, 1998). The salicylideneacylhydrazides are a new class of antimicrobial compounds that have specific activity against the T3SS of *Yersinia in vitro* (Kauppi *et al.*, 2003). The fact that T3SS systems are structurally highly conserved implies that these compounds could be used to target the T3SS in a range of Gram-negative pathogens. Accordingly, salicylidenehydrazide compounds have been tested *in vitro* against *Chlamydia* where they were shown to prevent intracellular growth (Muschiol *et al.*, 2006). Inhibitors to T3SSs have also been tested *in vivo* against another intracellular pathogen. Inhibitors were able to reduce enteric virulence in a bovine animal model for *Salmonella* infection (Hudson *et al.*, 2007). However, these compounds have yet to be tested in pseudomonads such as *B. pseudomallei*.

During infection, the expression of virulence factors in many pathogens is regulated by QS (Bassler, 1999). As such, there has been a significant interest in the development of inhibitors to target QS in bacteria as a novel antimicrobial approach. Recently, compounds were designed to target a QS system in *Burkholderia cenocepacia* (Riedel *et al.*, 2006). The *cep* system is important for regulation of virulence factors such as motility, protease production and biofilm formation (reviewed by Subramoni and Sokol, 2012). Compounds were produced that were able to inhibit certain QS-regulated functions in *B. cenocepacia* and therefore reduced virulence in a *Caenorhabditis elegans* pathogenesis model (Riedel *et al.*, 2006). This evidence demonstrates the importance of QS for full virulence and therefore antimicrobials targeting QS in *B. pseudomallei* could potentially form a beneficial therapy if combined with conventional antibiotics. Comparatively, disruption to QS in *Pseudomonas aeruginosa*, a member of the Proteobacteria, resulted in enhanced clearance of the bacteria in the lungs of infected mice due to inhibition of QS-controlled expression (Hentzer *et al.*, 2003). *P. aeruginosa* possesses two QS systems, both of which were inhibited by a furanone derivative, which resulted in reduction of virulence factor expression, demonstrating the potential to inactivate multiple QS receptors.

One disadvantage of developing anti-virulence compounds is the narrow-spectrum nature of the targets. Virulence factors are not as well conserved, nor as widely distributed as essential targets in bacteria and therefore individual therapies may need to be developed against specific diseases. Traditional methods, for example MICs, would be ineffective when determining the susceptibility of infectious agents to an anti-virulence drug as growth cannot be used as a measure of efficacy. Timing is also an important factor when considering an anti-virulence therapy, as different bacterial virulence factors come into play at different stages of infection. To increase their effectiveness, anti-virulence drugs could potentially be used as a combination therapy in conjunction with conventional antibiotics. Consequently, essential components in the cell still make attractive targets for antimicrobial compounds as disruption causes cell death or growth arrest. These proteins are more conserved across bacterial species and therefore drugs targeting these proteins tend to be broad-spectrum. However, this does mean that the target is also likely to be conserved in non-pathogenic species, resulting in unwanted side effects and more widespread resistance.

1.2.2 Selection of targets in *B. pseudomallei*

In this study, two targets were chosen for characterisation as potential antimicrobial targets. A transposon based technology, known as TraDIS (Langridge *et al.*, 2009), was used to identify a putative essential target in *B. pseudomallei*. This target, MinE, forms part of the Min system which is essential for proper placement of the cell division machinery. MinE is therefore important for the viability and fitness of the cell during normal growth. A second protein, PspA, was identified as a potential virulence target through literature searches and bioinformatics. PspA forms part of the Phage-shock protein (Psp) response, which is thought to play a role in the virulence of *B. pseudomallei* and adaption to stressful conditions, such as those encountered when inside the host. PspA is therefore is considered as a virulence target but is not essential for viability. The two main criteria for the selection of targets were therefore; 1) proteins that are essential for either growth or virulence and 2) proteins that from part of a known protein network, disruption of which would inhibit the function of the selected protein.

Chapter 2: **Materials and Methods**

2.1 Bacterial strains

2.1.1 *E. coli* strains

E. coli were grown in Luria Bertani (LB) broth at 37˚C overnight with agitation, unless otherwise stated. All manipulations with *E. coli* were carried out in ACDP/ACGM class II laboratories. The *E. coli* strains used in this study are listed in Table 2.1.

***E. coli* strain**	**Genotype**
E. coli DH5-λ *pir*	F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_κ^- m_κ^+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1 λ *pir*
E. coli HB101	F^-, *thi*-1, *hsd*S20 (r_B^-, m_B^-), *sup*E44, *rec*A13, *ara*-14, *leu*B6, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 (Str^R), *xyl*-5, *mtl*-1
E. coli JM109 (Promega)	*end*A1 *rec*A1 *gyr*A96 *thi hsd*R17 (r_κ^- m_κ^+) *rel*A1 *sup*E44 Δ(*lac-pro*AB) [F' *tra*D36, *pro*AB $lacI^qZ$ΔM15]
E. coli One Shot TOP10 (Invitrogen)	F^- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ^-
E. coli S17-λ *pir*	λ *pir rec*A *thi pro hsd*R^- RP4-2(Km::Tn7 Tc::Mu) Tp^R Sm^R

Table 2.1 *E. coli* strains used in this study.

2.1.2 *Burkholderia* strains

B. pseudomallei strains used in this study were all derived from *B. pseudomallei* K96243. All manipulations with *B. pseudomallei* were carried out in class III microbiological safety cabinets located in designated ACDP/ACGM class III laboratories. All work carried out using *B. thailandensis* E264 and its derivatives was performed in ACDP/ACGM class II laboratories. Bacteria were grown in LB broth at 37˚C with agitation, unless otherwise stated.

2.2 Growth media

The media used for growing bacteria and cell lines in this study are listed in Table 2.2.

Media	Components
LB broth	10 g Difco tryptone peptone, 5 g Difco Bacto yeast extract, 5 g NaCl, 1 L Milliq water.
LB agar	10 g Difco tryptone peptone, 5 g Difco Bacto yeast extract, 5 g NaCl, 20 g Difco Bacto agar, 1 L Milliq water.
M9 minimal media	11.28 g of 5 x M9 salts, 20 mM succinic acid, 2 mM $MgSO_4$, 1 μM $CaCl_2$, 1 L dH_2O.
Sucrose (10% w/v) agar	10 g Difco tryptone peptone, 5 g Difco Bacto yeast extract, 100 g sucrose, 20 g Difco Bacto agar, 1 L Milliq water.
Super Optimal Catabolite (SOC) Medium (Sigma)	2% tryptone (w/v), 0.5% yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, 20 mM glucose.
Dulbecco's modified eagle medium (DMEM; Fisher)	4.5 g/l glucose, 10 % foetal calf serum (FCS), 1% L-glutamine.
Leibovitz's L-15 Medium (L-15; Fisher)	GlutaMAX^{TM} (L-alanyl-L-glutamine dipeptide), 10% FCS.

Table 2.2 Growth media.

2.3 Antibiotics

The following antibiotics were used at a final concentration, unless otherwise stated, of 25 μg/ml kanamycin, 50 μg/ml chloramphenicol, 100 μg/ml ampicillin, 50 μg/ml trimethoprim, 50 μg/ml gentamycin.

2.4 Reagents and buffers

The reagents and buffers used in this study are listed in Table 2.3.

Reagents	Components	Use
Tris-Acetate-Ethyl (TAE)-EDTA buffer (Sigma)	40 mM Tris-acetate, 1 mM EDTA.	Gel electrophoresis
Denaturation buffer (Roche)	1.5 M NaCl, 0.5 M NaOH.	Southern hybridisation
Neutralisation solution (Roche)	1.5 M NaCl, 1 M TRIS, pH 7.4 with HCl.	Southern hybridisation
20 x Saline-sodium citrate (SSC) buffer (Sigma)	0.3 M sodium citrate, 3 M NaCl, pH 7.	Southern hybridisation
Maleic acid buffer (Digoxigenin (DIG) Wash and Block Buffer Set, Roche)	0.1 M maleic acid, 0.15 M NaCl, pH 7.5.	Southern hybridisation
Washing buffer (DIG Wash and Block Buffer Set, Roche)	Maleic acid buffer with 0.3-0.5% Tween20 (v/v).	Southern hybridisation
Blocking solution (DIG Wash and Block Buffer Set, Roche)	5 ml liquid block (containing milk powder), 45 ml of 10% maleic acid buffer.	Southern hybridisation
Detection buffer (DIG Wash and Block Buffer Set, Roche)	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5.	Southern hybridisation

Table 2.3 Buffers and solutions used for DNA analysis.

2.5 Molecular biology techniques

2.5.1 Extraction of genomic DNA

A single bacterial colony was used to inoculate 10 ml LB broth and grown overnight at 37˚C with agitation. A 500 µl aliquot of this was pelleted and genomic DNA purified using the Gentra Puregene Yeast/Bact. Kit (Qiagen) according to manufacturer's instructions.

2.5.2 Extraction of plasmid DNA

A single bacterial colony was used to inoculate 10 ml LB broth and incubated overnight at 37˚C with agitation. 1-10 ml of the overnight culture was pelleted at 13,000 x *g* for 5 minutes. The plasmid was purified using the Wizard Plus SV Minipreps DNA Purification System (Promega). Alternatively, 100 ml LB broth was inoculated with a single colony and incubated overnight at 37˚C with agitation. The cells were pelleted by centrifugation at 6,000 x *g* for 10 minutes and the plasmid purified using the HiSpeed Plasmid Midi Kit (Qiagen), according to manufacturer's instructions.

2.5.3 Agarose gel electrophoresis

DNA was separated by gel electrophoresis using 1% agarose gels. These were prepared by addition of 1 g agarose per 100 ml TAE buffer. For the production of Southern blots, a 0.8% agarose gel was used instead and prepared accordingly. The agarose and TAE were heated until the agarose had dissolved. Following this, 1 µg/ml ethidium bromide was added and the gel allowed to set. Loading buffer was added to the DNA samples before separation on the gel at 100 V for 30-45 minutes. The gel was visualised on a GelVue UV Transilluminator (Syngene).

2.5.4 Gel purification

DNA bands were excised from the agarose gel and purified using Wizard SV Gel and PCR Clean-up System (Promega) according to manufacturer's instructions.

2.5.5 Polymerase chain reaction

A typical polymerase chain reaction (PCR) using Herculase II fusion DNA polymerase (Agilent Technologies) contained 1 x Herculase buffer, dNTPs (200 μM), forward and reverse oligonucleotides (0.5 μM), DNA (0.1 μg), Herculase II, dimethyl sulphoxide (DMSO; 6%) and dH_2O to a final volume of 25 μl.

The PCR protocol was as follows (amplification conditions were optimised for each primer pair):

95˚C denaturing for 5 minutes
95˚C denaturing for 30 seconds
55-68˚C annealing for 30 seconds } x 30 cycles
72˚C extension for 1 minute
72˚C extension for 7 minutes

For colony PCR, individual colonies were suspended in 100 μl dH_2O and heated at 96˚C for 10 minutes. 2 μl of this was used as the template in a standard PCR:

96˚C denaturing for 10 minutes
95˚C denaturing for 1 minute
55˚C annealing for 1 minute } x 30 cycles
72˚C extension for 1 minute
72˚C extension for 10 minutes

DNA probes for Southern hybridisation were produced by using 200 μM DIG-labelled NTPs instead of dNTPs.

2.5.6 Restriction enzyme digestion

A typical restriction enzyme digest contained DNA (plasmid or genomic), 1 unit of each restriction enzyme and 10 x restriction enzyme digestion buffer diluted in dH_2O. The reactions were incubated at 37˚C for either 1-3 hours or overnight. The digest products were separated by gel electrophoresis followed by visualisation on a GelVue UV Transilluminator.

2.5.7 Plasmid dephosphorylation

Digested DNA was dephosphorylated as required by addition of 1 unit rAPid alkaline phosphatase (AP; Roche) with 2 x buffer, diluted to 1 x in a total volume of 20 µl and incubated at 37˚C for 30 minutes. This was followed by incubation at 75˚C for 2 minutes to inactivate the phosphatase.

2.5.8 Ligation

A typical ligation reaction consisted of DNA, DNA ligase buffer, 2 units T4 DNA ligase (Roche), made up to 10-20 µl with dH_2O. The reaction was incubated at 4˚C overnight followed by transformation into *E. coli* competent cells.

2.5.9 Heat shock transformation

A transformation reaction was carried out by adding 2 µl ligation product to 50 µl chemically competent *E. coli* JM109 cells. The cells were incubated on ice for 20 minutes, followed by 45 seconds at 42˚C and 2 minutes recovery on ice. 250-900 µl SOC Medium was added to the cells and incubated at 37˚C for 1 hour with agitation. The cells were then plated onto LB agar containing appropriate antibiotics and incubated overnight at 37˚C.

2.5.10 Production of electrocompetent *E. coli*

10 ml LB broth was inoculated with a bacterial colony and incubated overnight at 37˚C with agitation. The following day, 125 ml LB broth was inoculated with 1 ml overnight culture and incubated at 37˚C with agitation until an optical density at 600 nm of 0.4-0.6 had been reached, measured by a Cecil CE1021 spectrophotometer (Eco Scientific Ltd.). Bacteria were chilled on ice for 15 minutes, followed by centrifugation at 6,000 x *g* at 4˚C for 15 minutes. Pellets were resuspended in 200 ml ice cold 10% glycerol. The centrifugation step was repeated and the pellet was resuspended in 100 ml of 10% glycerol. The centrifugation was repeated as before and the pellet was resuspended in 5 ml of 10% glycerol. Centrifugation was repeated as before and the pellet was resuspended in 1 ml of 10% glycerol. The bacteria were divided into 50 µl aliquots and frozen at -80˚C.

2.5.11 Electroporation transformation

An aliquot of 2 µl ligation product was added to 50 µl electrocompetent cells. Cells were electroporated at 2.5 V (200 Ω). 250-400 µl SOC Medium was added and the cells were recovered by incubation at 37˚C with agitation for 1 hour. The cells were then plated onto LB agar containing appropriate antibiotics and incubated overnight at 37˚C.

2.5.12 Southern hybridisation

Genomic DNA was digested with selected restriction enzymes overnight and separated on a 0.8% agarose gel (w/v) at 80 V for 4 hours. The gel was processed by incubation with gentle agitation for 20 minutes in acid depurination solution, followed by denaturation solution for 30 minutes and neutralisation solution for 30 minutes. The DNA was transferred to a positively charged nylon membrane (Immobilon-P transfer membrane, Millipore) overnight with 20 x SSC buffer by capillary transfer. The DNA was immobilised by heating for 30 minutes at 120˚C.

Hybridisation was carried out by incubating the membrane at 42˚C with 40 ml pre-warmed DIG Easy Hyb Solution (Roche) for 1 hour. The DNA probe was prepared by PCR amplification of the gene with DIG-labelled NTPs, 10 µl of which was added to 50 µl DIG Easy Hyb solution. This was boiled for 10 minutes and added to 40 ml DIG Easy Hyb solution. The pre-hybridisation solution was replaced with hybridisation solution containing the DNA probe and incubated overnight at 42˚C with gentle agitation.

The membrane was washed twice in 50 ml of 2 x SSC, 0.1% SDS at 42˚C for 30 minutes, followed by washing twice in 50 ml of 0.1 x SSC, 0.1% SDS at 68˚C for 30 minutes, and once in 1 x washing buffer for 3 minutes, all with gentle agitation. 1 x blocking solution was added for 1 hour, which was replaced by blocking solution containing anti-DIG-AP antibody for 45 minutes. The membrane was washed three more times with 1 x washing solution for 10 minutes each and equilibrated for 2 minutes in 50 ml of 1 x detection buffer. The membrane was placed on acetate and sprinkled with 0.25 mM CDP-star® (Roche) and exposed to film (Amersham Hyperfilm ECL, GE Healthcare) for 10-60 minutes. The film was developed using RG Universal RTU X-Ray Developer followed by RG Universal RTU X-Ray Fixer (Champion).

2.5.13 Extraction of RNA

For RNA isolation, overnight cultures were diluted in 100 ml LB broth to an optical density at 590 nm of 0.01. The cultures were incubated at 37˚C with shaking until they had reached an optical density at 590 nm of 0.4-0.6. A 1 ml aliquot of each RNA sample was collected at selected time points by addition of 2 ml RNAprotect (Qiagen). RNA was recovered with the RNeasy Mini kit (Qiagen) as instructed by the manufacturer. This resulted in a final product of 100 µl RNA in nuclease-free H_2O at a concentration of 100-400 ng/µl, quantified using a NanoDrop 1000 Spectrophotometer.

2.5.14 Ethanol precipitation of RNA

RNA was concentrated by ethanol precipitation. 7 µl of 3 M sodium acetate was added to 70 µl RNA, followed by addition of 154 µl of 100% ethanol. The RNA was incubated at -20˚C for 30 minutes, followed by centrifugation at 13,000 x *g* for 30 minutes to pellet the RNA. The supernatant was discarded and the RNA pellet resuspended in 0.5 ml of 70% ethanol. This was centrifuged for 5 minutes. The supernatant was discarded and the pellet air dried. RNA was resuspended in the appropriate volume of RNase-free dH_2O (Sigma) to give a final concentration of 500 ng/µl.

2.5.15 Reverse transcription polymerase chain reaction

Residual DNA was removed by treating the RNA with TURBO DNA-free DNase (AmBion, Inc) and the RNA adjusted to a concentration of 125 ng/µl. The samples were then reverse transcribed using Enhanced Avian Reverse Transcriptase (RT; Sigma) according to manufacturer's instructions. The resulting cDNA was amplified by PCR using Herculase II fusion DNA polymerase in a standard reaction. For each PCR, the appropriate controls with water and RNA in the absence of RT were included to ensure that amplifications were from template cDNA and not contaminating genomic DNA. A further internal control, 16S rRNA, was also used to ensure the cDNA was at a uniform concentration across all samples. The results were visualised following gel electrophoresis.

2.6 Statistical analysis

All data are reported as mean ±SEM. Results were statistically analysed using a two-way ANOVA and Bonferonni post-tests. A p value of <0.05 was considered statistically significant.

Chapter 3: **Evaluation of a putative essential target in *B. pseudomallei*: The Min system**

3.1 Cell division

During the course of the cell cycle, rod-shaped bacteria such as *E. coli* grow and divide to form two new cells. These new daughter cells are mostly equal in size as cell division usually occurs at midcell (Nanninga and Woldringh, 1985). FtsZ is a key protein during division as it is crucial for the assembly of the cell division proteins (reviewed by Errington *et al.*, 2003). The Z ring is formed by polymerisation of FtsZ, brought about by its GTPase activity (Mukherjee and Lutkenhaus, 1994). This structure is thought to be fairly dynamic as it is made up of a meshwork of shorter FtsZ polymers which form bundles, subunits of which undergo rapid turnover with the FtsZ proteins in the surrounding cytoplasm (Stricker *et al.*, 2002). FtsA is another essential protein in cell division, which assembles early on in the process due to its direct interaction with FtsZ (Addinall and Lutkenhaus, 1996). This protein, along with ZipA, is thought to tether the Z ring to the membrane (Pichoff and Lutkenhaus, 2002). FtsA also plays a key role in assembly of the late cell division proteins to form the divisome (reviewed by Errington *et al.*, 2003). The late division proteins assemble in an organised manner in *E. coli*, with each protein being dependent on the one before to bind to the complex (Errington *et al.*, 2003). This divisome is the site where the new cell wall forms in the final stages of cell division. Lastly, the division septum closes and the two cells separate. In *E. coli* the site of Z ring formation is determined by the Min system (de Boer *et al.*, 1989).

3.2 The Min system

The Min system was first described in 1989 when a deletion in the *minB* locus in *E. coli* was found to cause the formation of spherical minicells and short filaments (de Boer *et al.*, 1989). Deletion of the *min* operon causes the minicell phenotype because the site of Z ring formation is not spatially restricted in the cell and therefore division can occur at the cell poles. The *min* operon contains three genes which encode for proteins MinC, MinD and MinE. These proteins work together to direct FtsZ to midcell during cell

division. This is achieved by inhibition of division at the poles, mediated by a MinCD bipartite inhibitor. This inhibitor is topologically regulated by MinE, allowing division to take place at midcell. In the absence of the Min system, the Z ring can form at the cell poles leading to the production of minicells which do not contain any genetic material (de Boer *et al.*, 1989).

A model for the MinCDE system is depicted in Figure 3.1. In this system the Min proteins undergo an oscillation between the poles of the cell. At the beginning of a cycle MinD assembles at the membrane, allowing the recruitment of MinC and the activation of the division inhibitor (de Boer *et al.*, 1991). MinD polymerises around the periphery of the cell towards midcell together with its partner MinC. As the MinCD complex approaches the polar zone, MinE binds to the ends of the MinD polymer forming a ring structure (Raskin and de Boer, 1997). This activates the ATPase activity of MinD, causing it to dissociate from the membrane and therefore also disassembling the MinCD complex (Hu and Lutkenhaus, 2001). The MinCD complex retreats back towards the pole, accompanied by the MinE ring. As the proteins dissociate from one pole they reassemble at the opposite pole and the process is repeated (Raskin and de Boer, 1999b). As a result, the lowest concentration of the division inhibitor is at midcell but is highest at the polar regions of the cell, preventing division at these sites.

The oscillation of the Min proteins between the poles was first described by Raskin and de Boer (Raskin and de Boer, 1999b). This remarkable characteristic of the Min system, essential for the system to function, is dependent on two proteins, MinD and MinE. During an oscillation cycle, MinD is present at the membrane of one pole, a segregation imposed by the topological regulator MinE (Hu and Lutkenhaus, 1999;Raskin and de Boer, 1999b). MinD is prevented from extending past midcell due to the presence of the MinE ring, which binds to the edge of the MinD polar zone (Raskin and de Boer, 1997). This results in the release of MinD from the membrane, which subsequently disperses into the cytoplasm and reassembles on the membrane at the opposite pole. The cycle repeats continuously with a complete oscillation interval of approximately 40 seconds (Raskin and de Boer, 1999b). MinC has no role in the oscillation but follows the same oscillatory pattern as MinD. Without MinD, MinC remains dispersed throughout the cytoplasm (Hu and Lutkenhaus, 1999;Raskin and de Boer, 1999a;1999b).

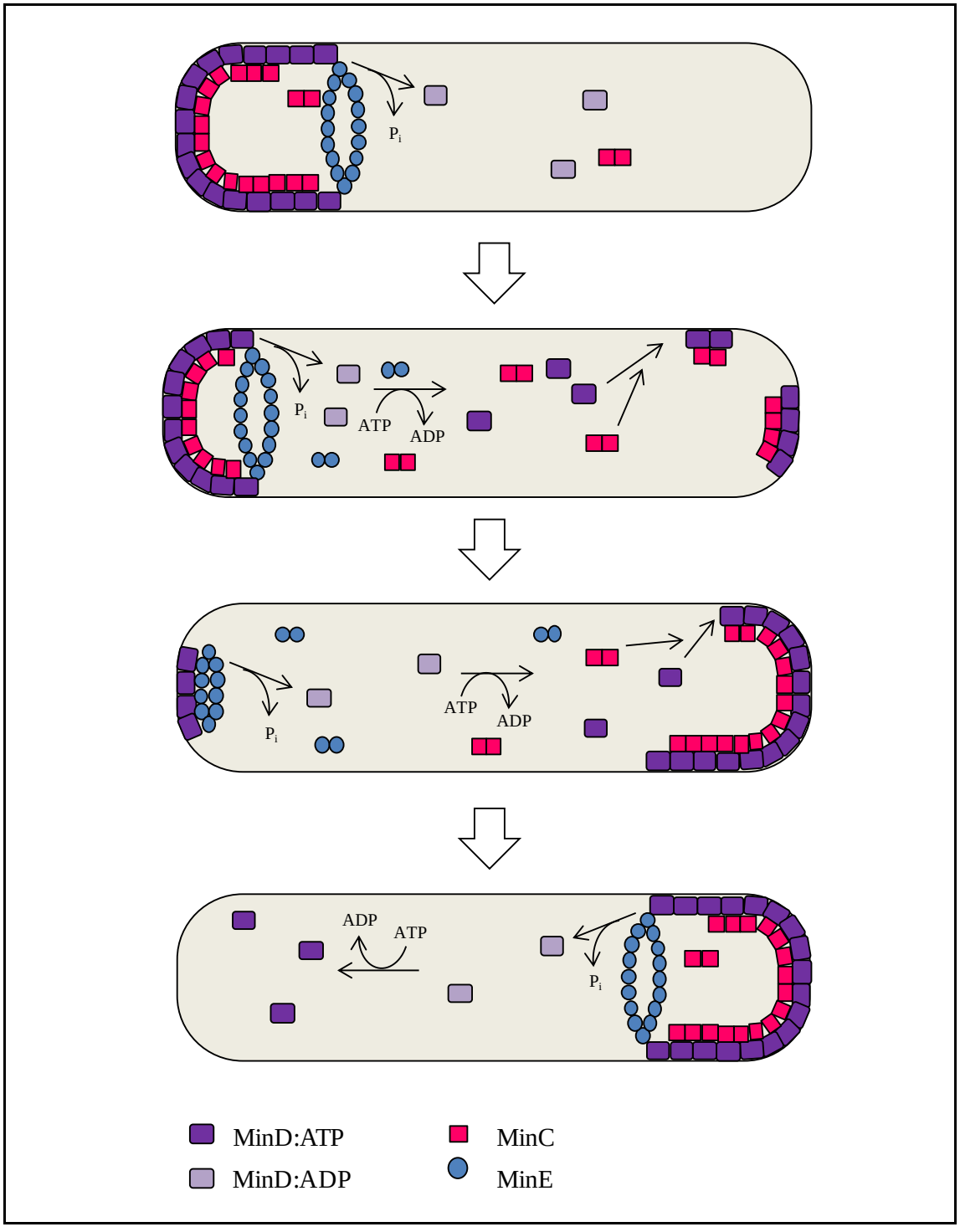


Figure 3.1 Oscillation of the Min proteins in *E. coli* (adapted from Lutkenhaus, 2007).

At the beginning of an oscillation cycle MinD:ATP binds to the membrane, which allows the recruitment of MinC. MinE binds to MinD at the edge of the inhibitor zone, displacing MinC. This activates the ATPase activity of MinD which results in its dissociation from the membrane along with MinC. As MinD:ADP undergoes nucleotide exchange and the concentration of MinD:ATP increases at the opposite pole, it binds to the membrane once again and repeats the cycle.

It has been hypothesised that oscillation of the MinD and MinE proteins is caused by the fact that MinD:ATP preferentially binds to the membrane when MinD:ATP is already present (Huang *et al.*, 2003). MinE then reacts with membrane-bound MinD:ATP, releasing MinD:ADP. The concentration of MinD:ATP in the cytoplasm is lower in the cytoplasm in this pole due its preferential membrane binding, whereas MinD:ATP at the opposite pole binds less efficiently due to the absence of already membrane-bound MinD:ATP. This leads to an increase in cytoplasmic MinD:ATP at the opposite pole which eventually binds to the membrane, resulting in the formation of a new polar zone of inhibition (Huang *et al.*, 2003). Oscillation also occurs in spherical cells where the rod shape of *E. coli* has been lost by deletion of the actin homologue MreB (Shih *et al.*, 2005). This is thought to be because there is still some level of asymmetry in these cells dictating the direction of the Min oscillation pattern.

3.2.1 MinC

The main division inhibitor activity of the Min system is supplied by MinC. It is able to block division in the absence of MinD if it is overexpressed by around 50 fold (de Boer *et al.*, 1992). However, its activity is enhanced by recruitment to the membrane by MinD, which increases the efficiency of the division inhibition by around 25 fold due to an increase in the local concentration of MinC (Hu and Lutkenhaus, 1999). MinC is able to directly inhibit FtsZ from forming polymers, observable in the production of bacterial filaments lacking Z rings during overexpression of MinCD (Bi and Lutkenhaus, 1993). This inhibition can be overcome by the simultaneous overproduction of FtsZ (de Boer *et al.*, 1992).

MinC contains two independent domains connected by a linker: one (the D domain) mediates the interaction with MinD and the other (the Z domain) contains the FtsZ inhibitor function. The Z domain towards the N-terminus of MinC is responsible for the interaction with FtsZ as a mutation in this region allows Z rings to form, whereas over-expression blocks division (Hu *et al.*, 1999;Hu and Lutkenhaus, 2000). Conversely, over-expression of the D domain at the C-terminus of the protein cannot inhibit FtsZ polymerisation (Hu and Lutkenhaus, 2000). The Z domain of MinC prevents Z rings from forming by a reduction in the number and length of FtsZ filaments by an unknown mechanism (Hu *et al.*, 1999). This is a separate mechanism to the main inhibitor of

FtsZ, SulA, which is a member of the SOS response (Huisman and D'Ari, 1981). The SOS response is induced by DNA damage and results in cell cycle arrest (Little and Mount, 1982). During induction of this response, SulA interacts with FtsZ directly to prevent formation of the FtsZ ring (Huang *et al.*, 1996). This protein acts by inhibiting the GTPase activity of FtsZ, preventing its assembly into polymers (Mukherjee *et al.*, 1998). Its action is independent of the Min system and non-specific in the cell as SulA is capable of blocking FtsZ in all locations (Bi and Lutkenhaus, 1993). It is thought that MinC acts at a later stage by destabilising FtsZ polymers after they have formed (Hu *et al.*, 1999;Pichoff and Lutkenhaus, 2001).

3.2.2 MinD

MinD is a peripheral membrane protein from the ATPase family of proteins (de Boer *et al.*, 1991). It contains a membrane targeting sequence (MTS), which associates with phospholipid bilayers through a C-terminal amphipathic helix of 10 residues (Hu and Lutkenhaus, 2003;Szeto *et al.*, 2002). This is inserted into the hydrophobic interior of the bilayer when MinD is bound to the membrane (Zhou and Lutkenhaus, 2003). The MTS is highly conserved between MinD proteins from different species but has been shown to have a higher affinity for its native membrane surface, the composition of which can vary between bacteria (Mileykovskaya *et al.*, 2003;Szeto *et al.*, 2003). When this sequence is deleted MinD can no longer bind and, consequently, is found distributed throughout the cytoplasm (Taghbalout *et al.*, 2006). This also prevents effective activation of MinC as it can no longer be recruited to the membrane (Hu and Lutkenhaus, 2003).

MinD binds to the other proteins in the Min system in an ATP-dependent manner. A mutation in the C-terminus Walker A motif, responsible for nucleotide binding, results in a protein that is deficient in binding to phospholipid vesicles, perhaps due to a hindered ATP-dependent conformational change (Hu *et al.*, 2002). This interaction with the membrane is required for MinD self-association and the interaction with MinC. Consequently, a deletion or mutation in this region also leads to the loss of ability to activate the MinC-dependent division inhibitor (de Boer *et al.*, 1991;Zhou and Lutkenhaus, 2004).

The ATPase activity of MinD is stimulated by approximately 10 fold by MinE in the presence of phospholipid vesicles (Hu and Lutkenhaus, 2001). Stimulation of the ATPase acitivity of MinD by MinE results in the disassembly of MinD polymers and the release of MinD from the membrane (Hu *et al.*, 2002). In the process of activating the ATPase activity of MinD, MinE is able to displace MinC from MinD and the membrane, resulting in the disruption of the MinCD bipartite inhibitor (Hu *et al.*, 2003). This is due to overlapping binding sites, meaning MinE must compete with MinC when binding to MinD. However, MinE has a higher affinity for MinD as MinC is unable to displace it once bound. As a result of the interaction with MinE, the position of a significant lysine in the P-loop of the ATPase region of MinD is altered (Ma *et al.*, 2004;Wu *et al.*, 2011;Hu and Lutkenhaus, 2003). This is thought to relay the ATPase activation signal of MinD, causing the release of MinC and MinD from the membrane. The two proteins that form the inhibitor can then proceed to the opposite end of the cell and repeat the process.

The interaction of MinD with MinC is mediated through the Switch I and II regions of MinD (Zhou and Lutkenhaus, 2004). These regions undergo nucleotide-dependent conformational changes which affect their binding partners. A mutation in the Switch I region, also known as the MinD box as it is highly conserved among MinD proteins, specifically affects the interaction with MinC and not MinE. MinE is still able to stimulate the ATPase activity in these mutants, indicating that the two proteins have a distinct effect on MinD despite their binding sites being in close proximity. The Switch II region also has a potential role in activating MinC (Zhou and Lutkenhaus, 2004).

3.2.3 MinE

The MinE protein is a small protein of 88 amino acids (de Boer *et al.*, 1989). It has two distinct activities; the N-terminus is responsible for its interaction with MinD and therefore its anti-MinCD activity, whereas the C-terminus is required for dimerisation and topological specificity (Pichoff *et al.*, 1995;Raskin and de Boer, 1997). At the N-terminus MinE contains an essential lysine required to activate the ATPase activity of MinD (Hu and Lutkenhaus, 2001). A mutation causes partial loss of interaction with MinD and therefore the rate of MinD disassembly from the membrane is much lower.

As a result, the rate of pole-to-pole oscillation decreases, leading to filamentation of the cell (Hu and Lutkenhaus, 2001;Ma *et al.*, 2003).

The model for the MinD and MinE interaction is based on the importance of dimer formation by both proteins (Pichoff *et al*., 1995; Hu and Lutkenhaus, 2003). MinE is thought to undergo a conformational change upon sensing a MinD dimer at the membrane (Park *et al*., 2011). This releases the N-terminal domain of MinE containing an α-helix, which is stabilised by the interaction with MinD. The equivalent domain in the other half of the dimer interacts with the membrane through an MTS. This therefore allows MinE to "swing" onto the next MinD dimer once the preceding MinD has been released following stimulation of its ATPase activity (Park *et al*., 2011).

The ratio between the concentrations of the Min proteins in this system is extremely important as any variation has an influence on the physiology of the daughter cells (de Boer *et al.*, 1989). If the entire Min system is deleted it results in viable cells with a frequent occurrence of the minicell phenotype. However, when individual proteins are either disrupted or produced in excess it results in a variety of different phenotypes. For example, over-expression of MinE causes a disruption to the MinCD division inhibitor throughout the cell, leading to the production of minicells (de Boer *et al.*, 1989). Conversely, in a *minB* deletion strain the cell cannot sustain a copy of *minCD* without a functional copy of *minE* also present (Pichoff *et al.*, 1995;Hu and Lutkenhaus, 2001). Clearly, this indicates the importance of the role MinE plays in the Min system and provides evidence that deletion of this gene has a profound effect on the viability of the cell.

3.2.4 Nucleoid occlusion

During the course of normal cell division the Min system directs FtsZ to polymerise at midcell (de Boer *et al.*, 1989). However, there is a second mechanism that is able to regulate Z ring formation in *E. coli*. In a *min* mutant the Z rings are still only able to form in specific locations, either at the poles or between nucleiods (Yu and Margolin, 1999). In cells where the replication or segregation of the nucleoid is prevented, the nucleoid remains at midcell. Nucleoid occlusion is the mechanism that prevents the Z ring from forming across the nucleoid (Woldringh *et al.*, 1991). It stops guillotining of

the chromosome and consequent uneven distribution of the genetic material in the daughter cells.

The first specific nucleoid exclusion factor, Noc (YyaA), was indentified in *Bacillus subtilis* (Wu and Errington, 2004). This is a non-specific DNA binding protein that binds to the nucleoid. In *E. coli* the SlmA protein was identified as a nucleoid exclusion factor shortly afterwards (Bernhardt and de Boer, 2005). It causes severe filamentation when it is absent in a *min* mutant, but this can be overcome by overexpression of FtsZ. Like Noc in *B. subtilis*, SlmA is a DNA binding protein that associates with the nucleoid. It also interacts directly with FtsZ (Bernhardt and de Boer, 2005).

The mechanism of nucleoid exclusion is unclear, but steric hindrance is unimportant as MinE rings are able to form across unsegregated nucleoids (Sun and Margolin, 2002). Instead, the concentration of proteins around the nucleoid is likely to be an important factor. Therefore supercoiling, condensation and proper nucleoid structure are important for nucleoid exclusion to occur (Sun and Margolin, 2004).

3.2.5 Selection of the Min system as an antimicrobial target

In order to design new antimicrobials, bacterial targets need to first be identified that are either essential for growth, pathogenesis or viability. STM is one technique that has been used to identify genes that are essential for virulence (Hensel *et al.*, 1995). This uses unique sequence tags to identify transposon mutants that are present in the inoculum but not in the output pool after mutants have been passed through an animal model. Modern high-throughput sequencing has allowed the development of more efficient techniques, such as TraDIS, that can be used to identify genes that are essential for growth. TraDIS is a modification of STM that combines the generation of transposon mutant libraries with high-throughput sequencing to identify the location of the transposon on the genome (Langridge *et al.*, 2009). Using this method thousands of transposon mutants can be generated, allowing coverage of the entire genome with multiple mutants per gene. This allows every gene to be simultaneously assessed for its essentiality. By screening the libraries under different negative selection criteria it is possible to identify genes required for specific conditions, for example, bile tolerance or intestinal colonisation and virulence (Langridge *et al.*, 2009;Chaudhuri *et al.*, 2013).

TraDIS has recently been applied to *B. pseudomallei* K96243 to identify essential genes under different assayable conditions (Moule *et al.*, 2014). A number of genes were found to have no transposon insertions, indicating their essentiality: one of these genes was *minE*.

Homologues of the three proteins that make up the Min system in *E. coli*, MinC, MinD and MinE, were identified in *B. pseudomallei*. Deletion of the Min system in other bacteria results in minicell formation due to incorrect positioning of the Z ring during cell division. Previous work attempted to delete the *min* genes in *B. pseudomallei* in order to evaluate their potential as antimicrobial targets. However, repeated attempts to produce individual deletion mutants in the three *min* genes were unsuccessful (data not shown), indicating that one or more of the *min* genes may be essential for growth. This and the data from the TraDIS analysis provided evidence that *minE* is essential in *B. pseudomallei*, making it an attractive target for novel antimicrobial therapies.

The aim of this study was to produce definitive proof that the Min system is essential for *B. pseudomallei* growth and would therefore provide a good target for novel antimicrobials. This was achieved by conditional mutagenesis of the *min* genes by a number of methods. The ability of these mutants to grow in permissive and non-permissive conditions was then assessed.

3.3 Materials and Methods

3.3.1 *Burkholderia* strains

The *Burkholderia* strains used in this study are listed in Table 3.1. *B. pseudomallei* strains used in this study were all derived from *B. pseudomallei* K96243. All manipulations with *B. pseudomallei* were carried out in a class III microbiological safety cabinet located in a designated ACDP/ACGM class III laboratory. The abbreviations are as follows: Cm, chloramphenicol; Km, kanamycin; Tm, trimethoprim; R, resistance.

Strain	**Characteristics**
B. thailandensis E264	Environmental isolate, ATCC number 700388.
B. thailandensis pSJS01.RFP	E264 derivative, complemented with pSJS01.RFP, Km^R.
B. pseudomallei::pDM4.Δ*minE*	K96243 derivative, insertion of pDM4.Δ*minE* in *BSPL2595*, Cm^R.
B. pseudomallei::pDM4.Δ*minE* pSJS01.*minE*	K96243 derivative, insertion of pDM4.Δ*minE* in *BSPL2595*, complemented with pSJS01.*minE*, Cm^R, Km^R.
B. pseudomallei::pSC807.$minC^{300}$	K96243 derivative, insertion of pSC807.$minC^{300}$ in *BPSL2597*, Km^R.
B. pseudomallei::pSC807.$pyrH^{250}$	K96243 derivative, insertion of pSC807.$pyrH^{250}$ in *BPSL2157*, Km^R.
B. pseudomallei Δ*minCDE*	K96243 derivative, unmarked deletion in *BPSL2595*, *BPSL2596* and *BPSL2597*.
B. pseudomallei pSJS01.*minCD*	K96243 derivative, complemented with pSJS01.*minCD*, Km^R.

Table 3.1 Bacterial strains used in this study.

3.3.2 Plasmids

The plasmids used in this study are listed in Table 3.2.

Plasmid	**Characteristics**	**Source**
pBBR1-MSC2	Cloning vector used for PCR amplification of Km cassette, Km^R.	Kovach *et al.*, 1995
pBHR4-groS-RFP	pBHR1 derivative, *turboFP635*, P_{groES}, *rrnB*, Cm^R. Cloning vector used for PCR amplification of RFP.	Wand *et al.*, 2011
pBHR5-GroS-RFP	pBHR1 derivative, *turboFP635*, P_{groES}, *rrnB*, Km^R. Cloning vector used for the generation of pSJS01.	University of Exeter
pCR Blunt II-TOPO	Cloning vector for blunt-end PCR products, Km^R.	Invitrogen
pDM4	pNQ705 derivative, *ori*R6K, *mob*RP4, *sacBR*, Cm^R. Suicide vector.	Milton *et al.*, 1996
pDM4.Δ*minE*	pDM4 derivative with *BPSL2595* flanking regions for mutagenesis, Cm^R.	This study
pDM4.Δ*minCDE*	pDM4 derivative with *BPSL2595* and *BPSL2597* flanking regions for mutagenesis, Cm^R.	This study
pRK2013	RK2 derivative, *ori*colE1, *mob tra*, Km^R. Helper plasmid.	Figurski and Helinski, 1979
pSC200	pGpΩTp derivative, *ori*R6K, *rhaR rhaS* P_{rhaB}, Tm^R. Integration vector used for the generation of pSC807 and pSJS01.	Ortega *et al.*, 2007
pSC807	pSC200 derivative, Km^R. Integration plasmid for conditional mutagenesis.	This study
pSC807.$minC^{300}$	pSC807 derivative, containing 300 bp of *BPSL2597*, Km^R.	This study
pSC807.$pyrH^{250}$	pSC807 derivative, containing 250 bp of *BPSL2157*, Km^R.	This study

pSC807.*minE*^{200}	pSC807 derivative, containing 200 bp of *BPSL2595*, Km^R.	This study
pSC807.*minDE*^{250}	pSC807 derivative, containing 250 bp of *BPSL2596* and *BPSL2595*, Km^R.	This study
pSJS01	pSC200 and pBHR5 derivative, *rhaR rhaS* P_{rhaB}, Km^R. Conditional complementation plasmid.	This study
pSJS01.RFP	pSJS01 derivative, expressing *turboFP635* under P_{rhaB} promoter control, Km^R.	This study
pSJS01.*minE*	pSJS01 derivative, expressing *BPSL2595* under P_{rhaB} promoter control, Km^R.	This study
pSJS01.*minCD*	pSJS01 derivative, expressing *BPSL2597* and *BPSL2596* under P_{rhaB} promoter control, Km^R.	This study

Table 3.2 Plasmids used in this study.

3.3.3 Oligonucleotide Primers

Primers were synthesised by Eurofins MWG. Primers used in this study are listed in Table 3.3.

Name	**Sequence**	**Restriction site**
***B. pseudomallei* Δ*minE* and Δ*minCDE* mutant construction**		
minE LF F	GCTAGCTCGCGATGGACTTCGAGTTC	*Nhe*I
minE LF R	CCATGGTTTCTCACCGAGCAGAAACG	*Nco*I
minE RF F	CCATGGTTGGAAGTGCTCGAAGTGAA	*Nco*I
minE RF R	GCTAGCGATGCGCTCGACTTCCCAAT	*Nhe*I
minE screen F	GGAGGCGTACAAGGACATCG	-
minE screen R	TTACCGGAGGCCGGAAAGTG	-

minB LF F	CATATGGCGTTCGTGAGCGAGCTGTG	*Nde*I
minB LF R	ACTAGTGCTGCGCAGTTCGAAGAATG	*Spe*I
minB RF F	ACTAGTCGCCAGGACGATTTGGAAGT	*Spe*I
minB RF R	CATATGTCGTGATCCCAGCGCCAGTG	*Nde*I
minB screen F	GTCAGGCTTGCGGAATCTCG	-
minB screen R	ATCGCCATTCTTCGAACTGC	-
Complementation with pSJS01		
RFP F	CATATGGTGGGCGAGGACTCGGTGCT	*Nde*I
RFP R	TCTAGATCAGCGCGAGCCCGTGCTGC	*Xba*I
minE ATG F	CATATGATGTCGATTCTGTCGTTTCT	*Nde*I
minE comp R	TCTAGATCAGGCTTGCGGAATCTCGA	*Xba*I
minC ATG F	CATATGATGTCGCTTAAAAAATCGCC	*Nde*I
minD comp R	GATATCTTACTTGGAGCCGAAGAGGC	*Eco*RV
Construction of pSC807 and derivatives		
Kan F	ATGCATTCAGCTACTGGGCTATCTGG	*Nsi*I
Kan R	CTCGAGGCTCAGAAGAACTCGTCAAG	*Xho*I
minC ATG F	CATATGATGTCGCTTAAAAAATCGCC	*Nde*I
minC 300 R	TCTAGACGCGCCTCGAGAAGCGGCAG	*Xba*I
minE ATG F	CATATGATGTCGATTCTGTCGTTTCT	*Nde*I
minE 200 R	TCTAGATCGAGGCTCACGCGGATGTC	*Xba*I
pyrH ATG F	GCCCATATGCCCAATGCCTATAAACG	*Nde*I
pyrH 250 R	TCTAGAAGCGCGAGCGCGTTCATCAT	*Xba*I
minDE pSC F	CATATGGACGTCGCGGAGGCGTACAA	*Nde*I

minDE pSC R	TCTAGAGCAACGCGGGCAGATAATCG	*Xba*I
minC screen	ATGAAGTCGCTCTTCGTCTG	-
pyrH screen	GATCGAGCAGATGCTCAAGG	-
1300	TAACGGTTGTGGACAACAAGCCAGGG	-
RT-PCR		
RT RFP F	TCCCGGATTTCTTCAAGCAG	-
RT RFP R	TTTCCTTGTCCGCTTCCTTG	-
RT minCD F	CGGAAGGCAACATCCACATC	-
RT minCD R	AGATCGAGATTGCGCAGACC	-
RT minDE F	ACCTGCTCATCACCCGCTAC	-
RT minE F	AAGAGCGCCTGCAGCTCATC	-
RT minE R	TCACGCGGATGTCATCGTTC	-
RT 16S rRNA F	GATGACGGTACCGGAAGAATAAGC	-
RT 16S rRNA R	CCATGTCAAGGGTAGGTAAGGTTT	-

Table 3.3 Primers used in this study.
Restriction sites in oligonucleotide primers are underlined.

3.3.4 Mutant construction

3.3.4.1 Conjugation with pSJS01

A single colony of either *B. thailandensis* E264 or *B. pseudomallei* K96243 was used to inoculate 10 ml LB broth and grown overnight at 37˚C. *E. coli* S17-λ *pir* containing the pSJS01 derivative was grown in LB broth containing 25 μg/ml kanamycin and 0.5% glucose, which was incubated overnight at 37˚C. A 1 ml aliquot of each culture was pelleted by centrifugation and reuspended in 500 μl LB broth. The conjugation was carried out on a nitrocellulose membrane placed on a LB agar plate containing 0.5% glucose, except in the case of pSJS01.*minE* where 0.5% rhamnose was used instead.

The glucose ensured reduced expression of the relevant gene under control of the rhamnose-inducible promoter, whereas rhamnose was added to the media where up-regulation of expression from the plasmid was required. A 10 µl aliquot of either *B. pseudomallei* or *B. thailandensis* and *E. coli* was added to the membrane and incubated overnight at 37˚C. Filters were vortexed in 1 ml PBS to remove the cells from the membrane. This was plated onto LB agar containing appropriate antibiotics to select for the presence of the plasmid in the relevant *Burkholderia* strain.

3.3.4.2 **Conjugation with pDM4**

Conjugation with pDM4 constructs was carried out by filter mating as described above with some modifications. *B. pseudomallei* K96243 was grown in LB broth overnight at 37˚C. The *E. coli* S17-λ *pir* donor containing the pDM4-based plasmid was grown in LB broth containing 50 µg/ml chloramphenicol. The conjugation was carried out on a nitrocellulose membrane placed on an LB agar plate. The cells recovered from the filters after overnight conjugation were plated onto LB agar containing 50 µg/ml chloramphenicol, to select for the presence of pDM4, and either 50 µg/ml gentamycin or 100 µg/ml ampicillin to kill the *E. coli* donor.

3.3.4.3 **Sucrose selection**

After conjugation with pDM4-based plasmids, sucrose selection was carried out in order to identify deletion mutants. An integrant colony of *B. pseudomallei* containing the pDM4 plasmid was used to inoculate 10 ml LB broth without antibiotics and incubated at 37˚C overnight. The cultures were diluted in LB broth to an optical density at 590 nm of between 0.35 and 0.4, followed by serial dilution to a concentration of approximately 1 x 10^4 cfu/ml. Appropriate dilutions were plated onto LB agar supplemented with 10% sucrose, no NaCl and incubated for up to a week at 24˚C. In order to select for *B. pseudomallei* deletion mutants also carrying the pSJS01 complementation plasmid, selection was carried out on LB agar containing 10% sucrose, no NaCl, 250 µg/ml kanamycin and 0.2% rhamnose. Resulting colonies were checked for chloramphenicol sensitivity and mutants identified by colony PCR and Southern hybridisation.

3.3.4.4 **Conjugation with pSC807**

A single colony of *B. pseudomallei* K96243 was used to inoculate 10 ml LB broth which was incubated overnight at 37˚C. In addition, the *E. coli* S17-λ *pir* strain containing the pSC807 construct was grown overnight in 10 ml LB broth supplemented with 25 μg/ml kanamycin and 0.5% glucose. The *E. coli* HB101 helper strain was grown overnight in 10 ml LB broth with 25 μg/ml kanamycin. Aliquots of 1 ml from each culture were centrifuged and the pellets re-suspended in 500 μl LB broth containing 0.5% rhamnose. A 10 μl aliquot of each was added to a single nitrocellulose membrane placed on a LB agar plate containing 0.5% rhamnose to allow tri-parental mating to take place. The following day, filters were removed from the plates, vortexed in 1 ml PBS and 100 μl plated onto LB agar containing appropriate antibiotics and 0.5% rhamnose.

3.3.5 Growth curves

3.3.5.1 **Induction of RFP in *B. thailandensis***

A colony of *B. thailandensis* E264 containing pSJS01.RFP was used to inoculate 10 ml LB broth containing 25 μg/ml kanamycin. This was incubated overnight at 37˚C with agitation. The overnight culture was used to inoculate flasks of 40 ml of the same media and incubated for 4 hours at 37˚C. In order to modify the expression of RFP from pSJS01, 1% rhamnose was added to one culture and 1% glucose added to the second. After a further 2 hours of growth the fluorescence of each culture was measured using a Glomax Multi JR Detection System (Promega).

3.3.5.2 **Growth of pSC807 conditional mutants**

Two methods were used to grow conditional mutants of *B. pseudomallei* with slight variations. *B. pseudomallei* was either grown in a 5 ml volume or, alternatively, growth was measured during incubation in a 96-well plate with shaking.

In the first method, a single colony of *B. pseudomallei*::pSC807.$minC^{300}$ was used to inoculate 5 ml LB broth containing either 0.5% rhamnose or 0.5% glucose. The cultures were incubated overnight at 37˚C and 10 μl of each was used to inoculate two further cultures: 5 ml LB broth containing 0.5% rhamnose and 5 ml LB broth containing 0.5%

glucose. These were incubated at 37˚C for a further 24 hours. The optical density at 590 nm was measured at each stage. A similar experiment was carried out with *B. pseudomallei*::pSC807.*pyrH*^{250} grown in M9 minimal media instead of LB broth.

In order to measure growth in a 96-well plate, a single colony of *B. pseudomallei*::pSC807.*minC*^{300} was used to inoculate 10 ml M9 minimal media containing either 0.5% rhamnose or 0.5% glucose and 25 μg/ml kanamycin and grown overnight at 37˚C. A wild-type control was also grown in M9 minimal media containing rhamnose or glucose without antibiotics. The cultures were pelleted, washed twice in PBS and resuspended in M9 minimal media with no additional sugars. These cultures were used to seed 5 ml of M9 minimal media containing 0.2% rhamnose or 0.2% glucose to an optical density at 590 nm of 0.03-0.04 (see Table 3.4).

Species	**Starting sugar (overnight)**	**Secondary sugar (continuous measurement)**
B. pseudomallei ::pSC807.*minC*^{300}	Rhamnose	Rhamnose
	Glucose	Glucose
B. pseudomallei	Rhamnose	Rhamnose
	Glucose	Glucose

Table 3.4 Sugars used to supplement M9 minimal media during growth.

Overnight cultures of either the mutant or wild-type were grown in M9 minimal media containing the starting sugar. These cultures were pelleted, washed and resuspended in M9 minimal media with no additional sugars. These suspensions were used to inoculate a culture containing the secondary sugar.

The cultures containing the secondary sugar were grown in a 96-well plate format in aliquots of 200 µl. The 96-well plate was set up with one culture condition per column, with each column containing six similar wells. 200 µl of M9 minimal media was added to 6 wells of a seventh column to provide a negative control. The surrounding wells were filled with water. The plate was incubated at 37˚C for 20 hours while shaking using a Multiskan® FC (Thermo Scientific). The optical density of each well was measured automatically at 15 minute intervals.

3.3.5.3 **Induction of *minCD* in *B. pseudomallei***

A single colony of *B. pseudomallei* pSJS01.*minCD* was used to inoculate 10 ml LB broth containing 25 µg/ml kanamycin and 0.2% glucose, grown overnight at 37˚C. A wild-type control was also grown in LB broth containing glucose without antibiotics. The *B. pseudomallei* pSJS01.*minCD* cultures were pelleted, washed twice in PBS and resuspended in LB broth. These cultures were used to seed 5 ml LB broth containing 25 µg/ml kanamycin and either 0.2% rhamnose or 0.2% glucose to an optical density at 590 nm of 0.03-0.04. The wild-type cultures were treated similarly but seeded in LB broth with either 0.2% rhamnose or 0.2% glucose without antibiotics. Each culture was divided into 200 µl aliquots in a 96-well plate, with 6 replicates per condition. A negative control was set up using LB broth alone. The surrounding wells were filled with water. The plate was incubated at 37˚C for 20 hours while shaking using a Multiskan® FC. The optical density of each well was measured automatically at 15 minute intervals.

3.4 Results

3.4.1 Conditional complementation of a *minE* deletion mutant

3.4.1.1 Construction of a conditional complementation vector

Conditional mutagenesis is a useful technique for the characterisation of essential genes as it allows the gene to be manipulated without adverse effect to the cell. One method for the manipulation of essential genes is by complementation with a plasmid expressing the active version of the gene (Bhavsar *et al.*, 2001). The chromosomal copy can then be deleted without detrimental effect to the cell. If the plasmid copy of the gene is under the control of an inducible or repressible promoter, this can then be manipulated in order to determine the effect of inactivating the gene completely. This method was adopted to evaluate whether *minE* is essential for growth in *B. pseudomallei*.

The construction of the plasmid used for conditional complementation of genes in *B. pseudomallei* was achieved by combining the backbones of two plasmids, pSC200 and pBHR5-groS-RFP (Ortega et al., 2007;Wand et al., 2011). The *rhaS* operon and rhamnose-inducible promoter, P_{rhaB}, from pSC200 were joined with the replication and mobilisation components and a kanamycin resistance cassette from pBHR5-groS-RFP. Both plasmids were cut with the restriction enzymes *Sac*I and *Kpn*I before a ligation was carried out to join the relevant fragments (Figure 3.2). The ligation product was transformed by heat shock into *E. coli* JM109, following which any colonies were checked for the presence of the plasmid by antibiotic selection and restriction digest. The resulting plasmid, pSJS01, was sequenced. On sequencing it was found that there was a base pair mutation in the *rhaS* gene, causing an amino acid change from asparagine to aspartic acid (Figure 3.3). This mutation was also present in the original pSC200 plasmid. The function of the *rhaS* operon and inducible promoter was therefore tested before further cloning was carried out.

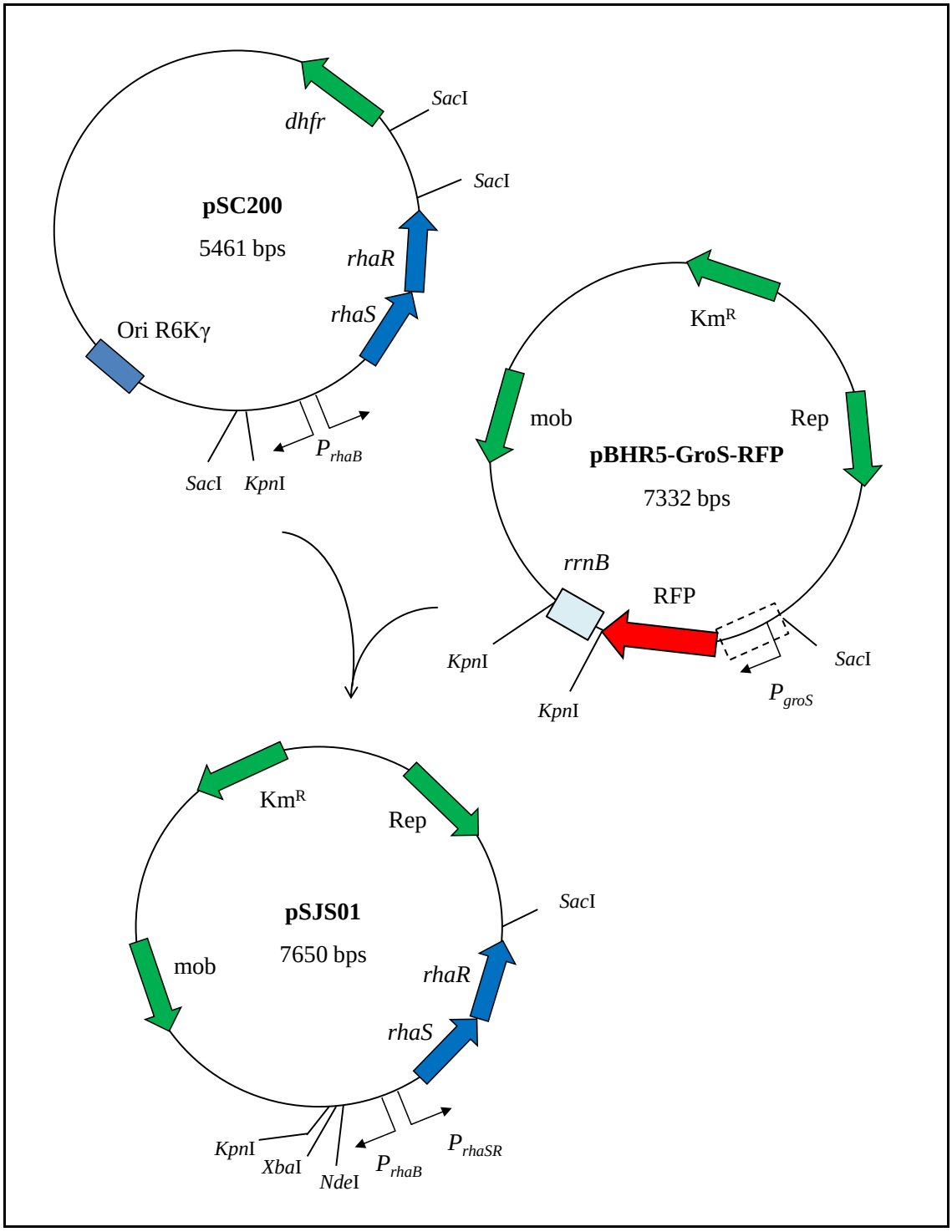


Figure 3.2 Cloning map of pSJS01.

Plasmids pSC200 and pBHR5-groS-RFP were digested with *Kpn*I and *Sac*I. The fragment containing P_{rhaB} and *rhaS* operon of pSC200 along with the replication, mobilisation and resistance components of pBHR5-groS-RFP were ligated to form pSJS01. The RFP gene was cloned from pBHR4-groS-RFP and ligated with pSJS01 using the restriction sites *Nde*I and *Xba*I.

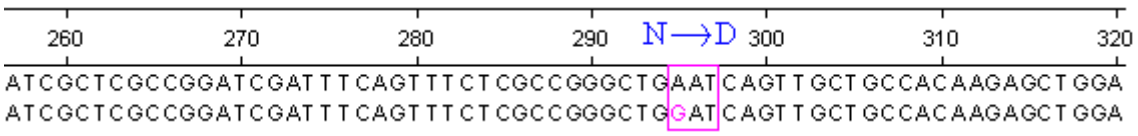


Figure 3.3 Sequence alignment of the *rhaS* gene from *E. coli* (top row) with *rhaS* from pSJS01 (bottom row).

The base pair change from A to G (highlighted in pink) resulted in an amino acid change from asparagine to aspartic acid.

In order to test if the promoter was inducible by addition of rhamnose, RFP was cloned downstream of P_{rhaB}. The codon optimised RFP gene was amplified from pBHR4-groS-RFP by PCR using primers RFP F and RFP R. This incorporated *Nde*I and *Xba*I restriction sites at the 5' and 3' end of the PCR product respectively. The PCR product was cloned into pCR-Blunt II-TOPO before digestion with *Nde*I and *Xba*I, followed by ligation in order to transfer the gene into pSJS01. The presence of RFP in the resulting plasmid, pSJS01.RFP, was confirmed by PCR and sequencing.

Plasmid pSJS01.RFP was transformed into *E. coli* S17-λ *pir*, followed by conjugation into *B. thailandensis* E264. Conjugates were selected for on LB agar containing 250 μg/ml kanamycin and 100 μg/ml ampicillin. The kanamycin selected for bacteria containing pSJS01.RFP, while ampicillin selected against the *E. coli* donor. Colonies were visible after incubation at 37˚C for 48 hours. *B. thailandensis* pSJS01.RFP colonies were confirmed by resistance to kanamycin and ampicillin, as well as by PCR amplification of both RFP and the kanamycin resistance cassette.

The presence of the *rhaS* operon and P_{rhaB} allow the expression of RFP to be up-regulated by addition of rhamnose or down-regulated by glucose. To test the regulatory function of the plasmid, *B. thailandensis* pSJS01.RFP was grown to exponential phase in LB broth before inducing expression of RFP from P_{rhaB} by addition of 1% rhamnose. The fluorescence produced by *B. thailandensis* pSJS01.RFP was measured after two hours using a fluorometer. This was compared to the fluorescence produced when cultures were grown in the presence of glucose instead. The result showed no difference between the culture supplemented with rhamnose compared to glucose or a wild-type

control, which did not contain the pSJS01.RFP plasmid (data not shown). This may be because RFP was not expressed sufficiently in 2 hours to be detected. A further experiment was carried out to measure the fluorescence of RFP in cultures that had been grown in either inducing or repressing conditions for up to 144 hours. However, there was no significant difference in fluorescence between the cultures after this time (data not shown).

A second induction experiment was carried out to measure expression of RFP from pSJS01 using RT-PCR. Overnight cultures of *B. thailandensis* E264 containing pSJS01.RFP were grown in 10 ml LB broth containing either 1% rhamnose or 1% glucose, alongside a control with neither sugar. After 24 hours RNA was collected from each culture. Residual DNA was removed before the RNA was reverse transcribed. The cDNA was amplified by PCR using primers RT RFP F and RT RFP R and the results visualised by gel electrophoresis (Figure 3.4). RFP was expressed at a higher level in the cultures supplemented with 1% rhamnose compared to cultures with added glucose or LB broth alone. This demonstrated that the point mutation in *rhaS* did not affect the function of the rhamnose-inducible promoter. There was also less transcript present in the culture grown with glucose compared to the no sugar control, indicating that expression of RFP was repressed but that the system was still leaky in the presence of glucose.

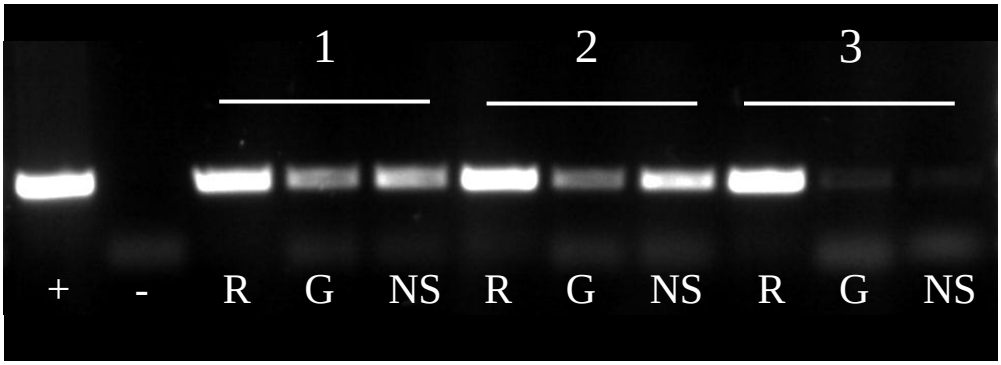


Figure 3.4 Amplification of RFP by RT-PCR from *B. thailandensis* pSJS01.RFP.

B. thailandensis cultures containing pSJS01.RFP were incubated for 24 hours in 10 ml LB broth containing additional sugars. Primers RT RFP F and RT RFP R were used to amplify RNA from cultures containing either 1% rhamnose (R), 1% glucose (G) or no additional sugars (NS). The figure represents three replicates for each condition. A plasmid DNA positive control (+) was included as well as a negative control (-) containing water. Product size is 399 base pairs.

3.4.1.2 **Creating a conditionally complemented *minE* deletion mutant**

In order to complement a *minE* deletion in *B. pseudomallei*, *minE* was cloned into the pSJS01 plasmid. The *minE* gene was amplified by PCR using primers minE ATG F and minE comp R. This produced a fragment of 267 base pairs flanked by *Nde*I and *Xba*I restriction sites. The PCR product was cloned into pCR-Blunt II-TOPO before digestion with *Nde*I and *Xba*I, followed by ligation with the similarly digested pSJS01 plasmid. This produced pSJS01.*minE* containing the full length *minE* gene under the control of P_{rhaB}, allowing *minE* to be conditionally expressed.

The *B. pseudomallei* Δ*minE* unmarked deletion mutant was created by homologous recombination using the suicide vector pDM4. Flanking regions of *minE* were amplified and cloned separately into a pCR-Blunt II-TOPO plasmid. The flanking regions were then joined to create an inactive version of *minE* in pDM4 with an in-frame deletion between 33 and 214 base pairs. The method is outlined in Figure 3.5.

The pDM4.Δ*minE* plasmid was constructed by ligation of *Nhe*I-linearised pDM4 with the left flank (LF) and right flank (RF) of the *minE* gene. The construct for the in-frame deletion of the gene was produced by amplifying upstream and downstream flanking regions of *BPSL2595* from *B. pseudomallei* K96243 genomic DNA using primers minE LF F and minE LF R (upstream), minE RF F and minE RF R (downstream). This produced two products, 539 and 514 base pairs in size respectively, flanked by *Nhe*I and *Nco*I restriction sites to aid manipulation. These flanks were ligated into pCR-Blunt II-TOPO followed by digestion with *Nhe*I and *Nco*I for subsequent ligation with the similarly digested pDM4 suicide vector.

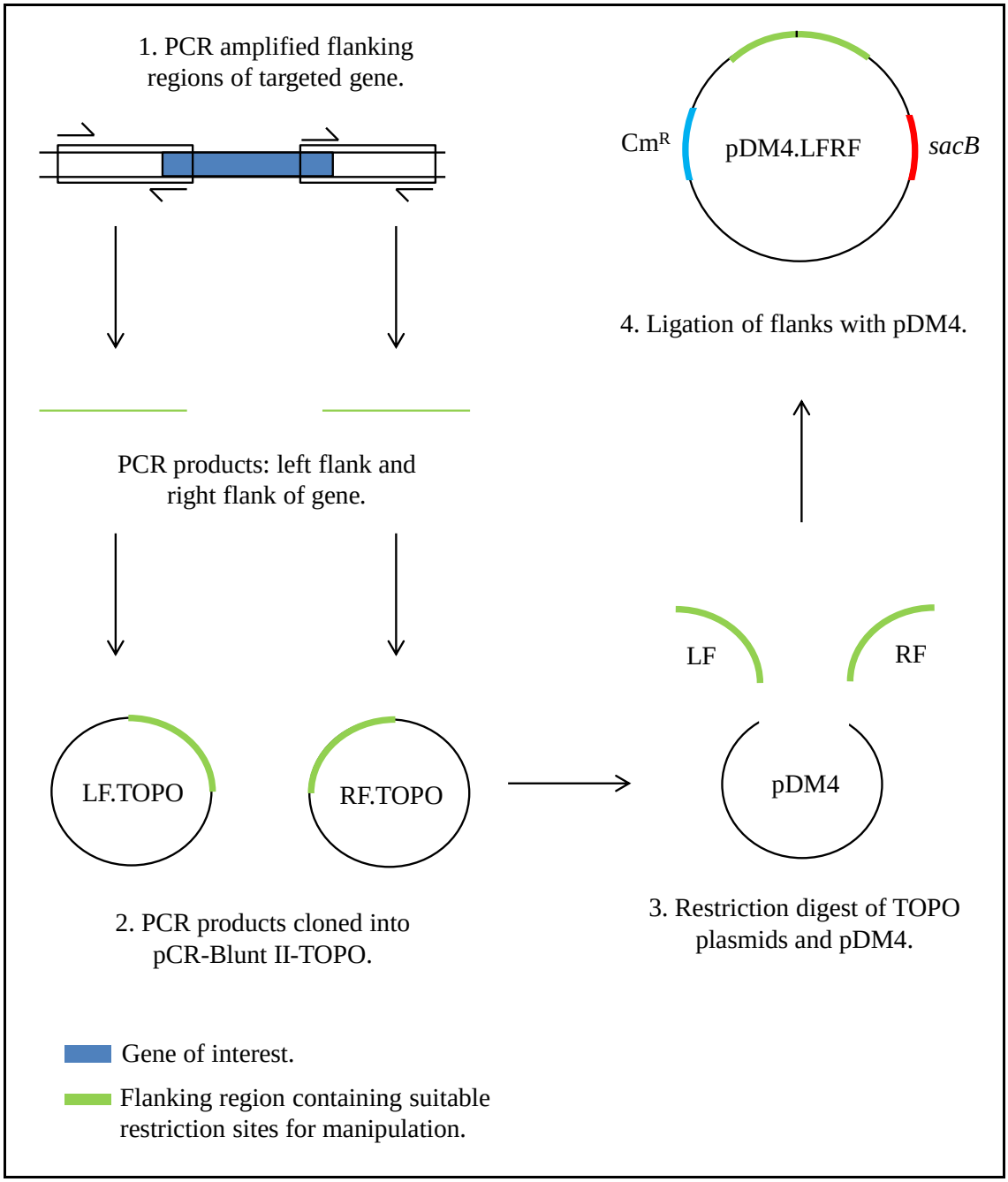


Figure 3.5 Cloning strategy used to create constructs for the targeted deletion of genes in *B. pseudomallei*.

Primers were used to amplify the LF and RF of the gene of interest, which were then sub-cloned into pCR-Blunt II-TOPO. The TOPO plasmids containing LF and RF were digested using the relevant restriction enzymes to isolate the inserts, which were then purified and cloned into pDM4. The pDM4 suicide vector was linearised by restriction enzyme digest to create compatible ends with the two flanks. Following this, the pDM4 construct was created by carrying out a three way ligation with the LF and RF of the gene and linearised pDM4.

For the construction of *B. pseudomallei* Δ*minE*, the pDM4.Δ*minE* plasmid was transformed into *E. coli* S17-λ *pir* cells before conjugation into *B. pseudomallei* K96243. This was carried out using a similar method to conjugation with *B. thailandensis* as described above. The two strains were filter mated on LB agar and the *B. pseudomallei*::pDM4.Δ*minE* integrants selected for on LB agar containing 50 μg/ml chloramphenicol and 100 μg/ml ampicillin. *B. pseudomallei* is naturally resistant to ampicillin, allowing selection over the *E. coli* donor strain. The merodiploid strain was selected by chloramphenicol resistance, present on the pDM4 backbone, and PCR amplification of the *minE* region using primers minE screen F and minE screen R (Figure 3.6).

The *B. pseudomallei*::pDM4.Δ*minE* integrant contained both an intact and inactivated copy of *minE*. This allowed normal growth of the integrant as the chromosome still contained one functional copy of the gene. The generation of *B. pseudomallei* Δ*minE* was attempted before complementation with pSJS01.*minE* to determine if mutagenesis of *minE* was possible and if the gene was therefore not essential. Sucrose selection was carried out to select for a second recombination event where the pDM4 backbone was lost from the chromosome. The second cross-over event can result in either creation of a deletion strain or reversion to wild-type. After six sucrose selections with *B. pseudomallei*::pDM4.Δ*minE*, 120 colonies were recovered that were sensitive to chloramphenicol but had a wild-type genotype when screened by PCR. Therefore the gene appeared to be essential.

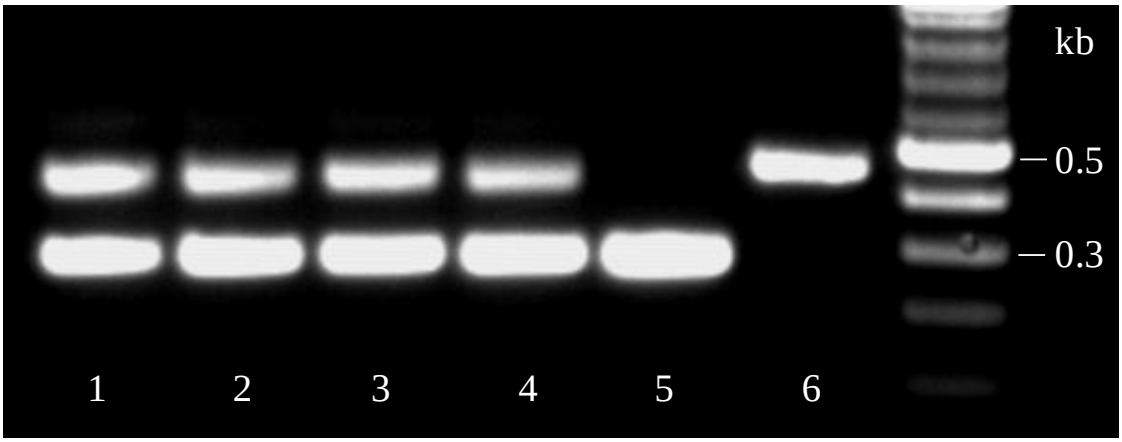


Figure 3.6 Screening PCR for *B. pseudomallei*::pDM4.Δ*minE*.

Primers minE screen F and minE screen R were used to amplify the *minE* gene from the integrant. The expected product sizes of the full length gene and in-frame deletion were 458 bp and 284 bp, respectively. Lanes 1-4, *B. pseudomallei*::pDM4.Δ*minE* integrant clones 1-4; lane 5, pDM4.Δ*minE* plasmid DNA; lane 6, *B. pseudomallei* genomic DNA; lane 7, 100 bp ladder.

The pSJS01.*minE* plasmid was conjugated into *B. pseudomallei*::pDM4.Δ*minE* by filter mating, with successful conjugations indicted by growth on 250 μg/ml kanamycin and 50 μg/ml chloramphenicol. The resulting colonies were screened for kanamycin resistance by plating on LB agar containing up to 1 mg/ml, indicating the presence of pSJS01.*minE*. Sucrose selection was then carried out as before. Briefly, an overnight culture was grown in 10 ml LB supplemented with 25 μg/ml kanamycin. This was diluted to approximately 1 x 10^8 cfu/ml, followed by serial dilution to 1 x 10^4 cfu/ml. Various dilutions were then plated onto selective LB agar containing 10% sucrose, no NaCl, 250 μg/ml kanamycin, and 0.2% rhamnose and incubated at 24˚C for several days. The presence of the kanamycin permitted selection for the pSJS01.*minE* complementation plasmid, while rhamnose ensured expression of *minE* was induced. The sucrose selected for successful second cross-over events where pDM4 had been excised from the chromosome. Nine sucrose selections were carried out and resulting colonies were screened for sensitivity to chloramphenicol and by PCR using primers minE screen F and minE screen R to determine the genotype of chromosomal *minE*. However, after screening 200 colonies only wild-type or integrant colonies were recovered.

3.4.2 Conditional mutagenesis of the Min system by promoter control

3.4.2.1 Construction of the pSC807 plasmid

The pSC200 plasmid was originally constructed for use in *B. cenocepacia* to confirm the essentiality of the Ara4N biosynthesis cluster (Ortega *et al.*, 2007). The plasmid allows the integration of the rhamnose-inducible promoter, P_{rhaB}, into the chromosome upstream of the gene of interest. A truncated version of the target gene is inserted into the plasmid, which integrates into the chromosome by homologous recombination. The plasmid therefore integrates downstream of the native promoter, allowing the expression of the active gene to be driven by P_{rhaB} (Figure 3.7). Expression can therefore be controlled by addition of rhamnose or glucose, and thus the outcome of increasing or reducing expression of the gene can be determined.

The pSC200 plasmid contains in addition to P_{rhaB}, the operon containing *rhaR* and *rhaS*, a multiple cloning site, an origin of replication and *mob* genes. It also contains the *dhfr*

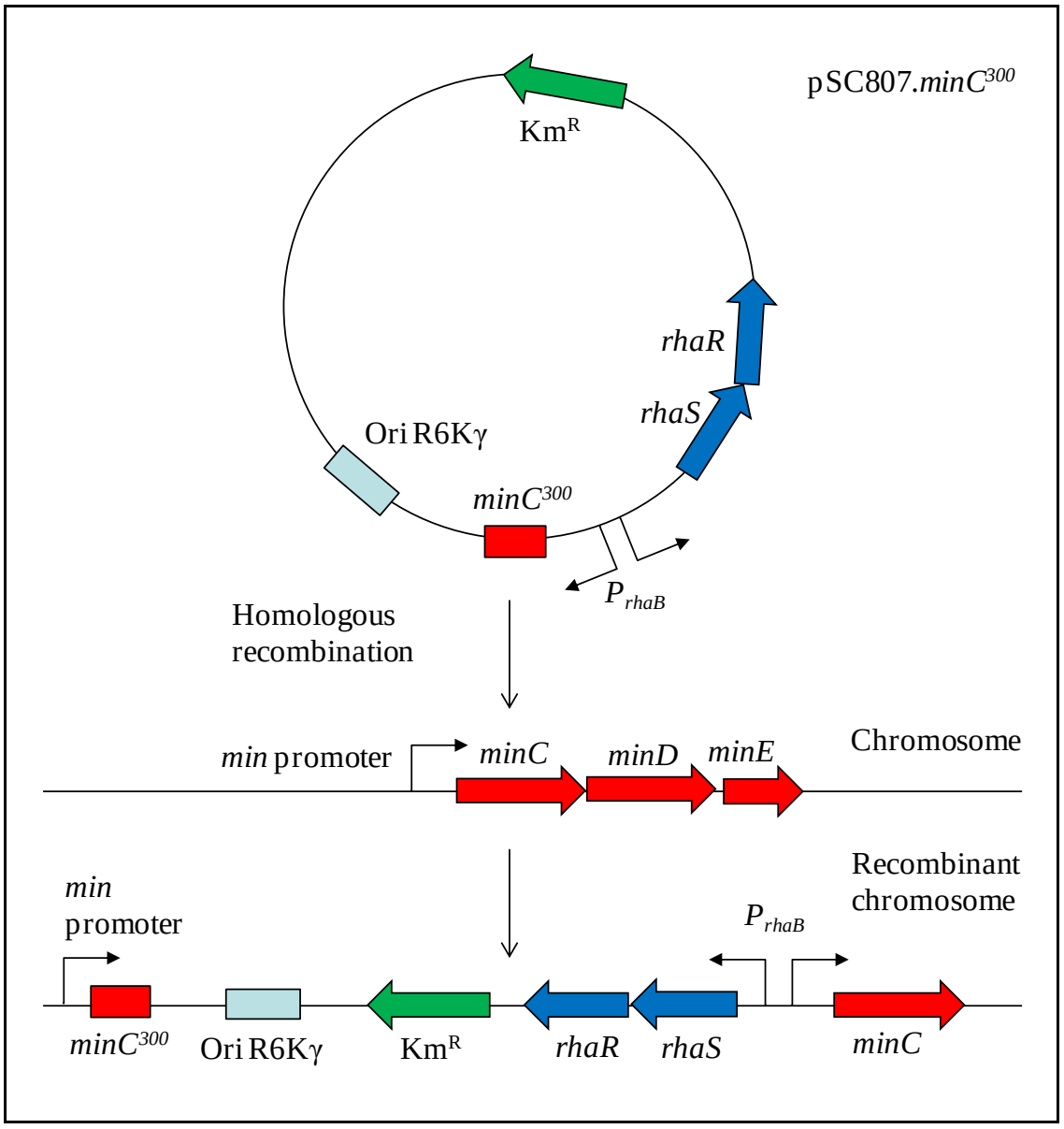


Figure 3.7 Integration of pSC807 into the chromosome by homologous recombination.

The truncated version of *minC* undergoes homologous recombination with the chromosomal copy of *minC*. The plasmid integrates into the chromosome alongside this, leaving $minC^{300}$ under control of the native promoter and the *minC* operon under control of the rhamnose-inducible promoter.

cassette for trimethoprim resistance from pSCrhaB2 (Cardona and Valvano, 2005). Trimethoprim is not permitted for use in *B. pseudomallei* as it is used to treat melioidosis and, consequently, the antibiotic cassette needed to be replaced before use. The *dhfr* cassette was replaced with a kanamycin cassette, amplified from pBBR1-MSC2 using primers Kan F and Kan R, which incorporated an *Nsi*I and *Xho*I restriction site at opposite ends of the region. The cassette was cloned into pCR-Blunt II-TOPO before restriction digests were carried out using *Nsi*I and *Xho*I to isolate the fragment containing the antibiotic cassette and linearise pSC200 by removing *dhfr*. The two fragments were joined by ligation, resulting in pSC807 (Km^R).

Following the replacement of the trimethoprim cassette with the kanamycin cassette, a *minC* fragment was cloned into pSC807. A 300 base pair fragment of *minC* beginning at the start codon was amplified by PCR using primers minC ATG F and minC 300 R, which incorporated an *Nde*I site at the 5' end and *Xba*I at the 3' end. This was cloned into pCR-Blunt II-TOPO before digestion with *Nde*I and *Xba*I. These fragments were ligated into similarly digested pSC807 to create pSC807.$minC^{300}$ (Figure 3.7).

The pSC807.$minC^{300}$ plasmid was transformed by electroporation into *E. coli* DH5α-λ *pir* and the bacteria recovered on LB agar containing 50 μg/ml kanamycin and 0.2% glucose. The glucose was required in order to prevent expression of the truncated *minC* under the control of P_{rhaB}. Transformants were confirmed by restriction digest and nucleotide sequencing.

A similar method was used for the construction of pSC807.$minE^{200}$ and pSC807.$pyrH^{250}$. The initial 200 base pairs of *minE* were amplified from genomic DNA by primers that incorporated the *Nde*I and *Xba*I restriction sites at the 5' and 3' end respectively. The *minE* gene is 255 base pairs in length and so in order to conserve enough of the gene to undergo homologous recombination only a fifth of the gene was removed. Because of the short length of the gene, it was thought that this was sufficient to impair its function within the cell. Similarly, the 250 base pair fragment of *pyrH* was amplified from *B. pseudomallei* genomic DNA by PCR using primers pyrH ATG F and pyrH 250 R. The fragments were cloned into pCR-Blunt II-TOPO before being transferred into pSC807 by digest and ligation.

3.4.2.2 Creating a conditional mutant by integration of a rhamnose-inducible promoter

The pSC807.*minC*^{300} plasmid was transformed into *E. coli* S17-λ *pir* by electroporation and conjugated with *B. pseudomallei* K96243 by tri-parental mating. The potential conjugates were plated onto LB agar containing 250 μg/ml kanamycin, 50 μg/ml gentamycin and 0.5% rhamnose to select for integrants. Successful integrants were confirmed by kanamycin and gentamycin resistance, indicating the presence of pSC807.*minC*^{300} in *B. pseudomallei*, which is naturally gentamycin resistant, unlike *E. coli*. Rhamnose was included in the media in order to allow expression of the *min* operon from P_{rhaB}, as disruption caused by the integration of the plasmid may inhibit growth. Integration into the chromosome was confirmed by PCR using primers minC screen and 1300 to amplify a region of 1063 base pairs, extending from upstream of the integration site into the plasmid. One conditional mutant, colony 13, was identified in this way, which contained an integrated copy of pSC807.*minC*^{300} downstream of the *min* promoter (Figure 3.8). A Southern blot was carried out to confirm the integration (Figure 3.9).

The pSC807.*minE*^{200} and pSC807.*pyrH*^{250} constructs were conjugated with *B. pseudomallei* as described above. The pSC807.*pyrH*^{250} integrant was confirmed by PCR using primers pyrH screen and 1300.

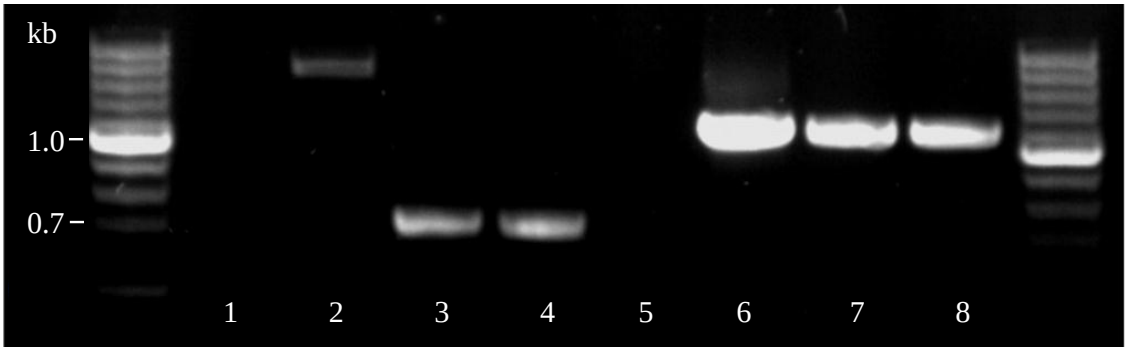


Figure 3.8 Screening PCR for pSC807.*minC*^{300} integrants of *B. pseudomallei*.

In lanes 1-4 primers minC screen and 1300 were used to amplify a region upstream of the *min* promoter extending into the plasmid. There was no expected product for the plasmid DNA control, and a band of 699 bp for the integrant. In lanes 5-8 a region of the kanamycin cassette was amplified with an expected product size of 1063 bp for the plasmid DNA control and integrant. A product was not expected for the wild-type in either PCR. Lanes 1 and 5, wild-type genomic DNA; lanes 2 and 6, pSC807.*minC*^{300} plasmid DNA; lanes 3, 4, 7 and 8, colony 13 genomic DNA.

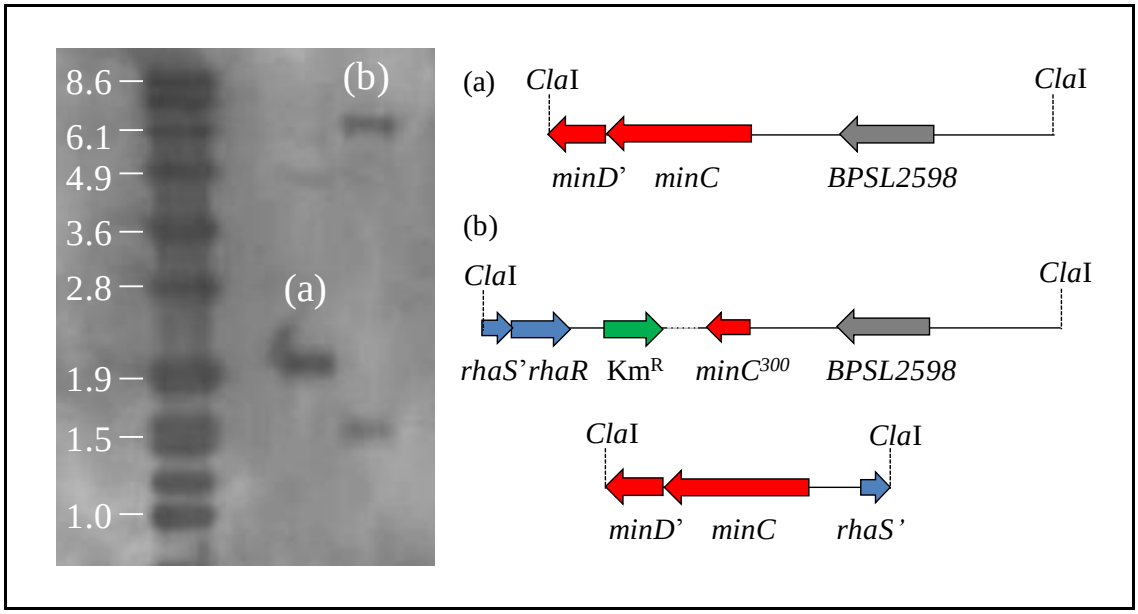


Figure 3.9 Southern blot confirming the integration of pSC807.*minC*^{300} in *B. pseudomallei*.

Wild-type and mutant genomic DNA was digested with the *Cla*I restriction enzyme, which cuts either side of *minC* and within the pSC807 plasmid backbone. Primers minC ATG F and minC 300 R were used to make the DIG-labelled probe. (a) Wild-type operon (2016 bp); (b) integrant operon from clone 13 (6507 and 1542 bp).

3.4.2.3 **Essentiality of the *min* operon in *B. pseudomallei***

The pSC807.*minC*^{300} plasmid was successfully integrated into the chromosome of *B. pseudomallei*, creating *B. pseudomallei*::pSC807.*minC*^{300}. In this strain an inactive *minC* is under the control of the native promoter and, therefore, in the absence of rhamnose the operon is non-functional. An experiment to determine the essentiality of the *min* operon was carried out by growing *B. pseudomallei*::pSC807.*minC*^{300} under either permissive or non-permissive conditions. An overnight culture of *B. pseudomallei*::pSC807.*minC*^{300} was grown in LB broth containing either 0.5% rhamnose or 0.5% glucose. The cultures were grown at 37˚C with agitation and passaged after 24 hours into either rhamnose or glucose, followed by a further 24 hours growth. The optical density at 590 nm was measured to compare growth (Table 3.5A). The results showed no significant difference between the optical density of cultures grown in rhamnose and those grown in glucose. This suggests that the *min* operon is not essential for growth in *B. pseudomallei*.

In order to confirm that the inducible promoter P_{rhaB} was functional, a second conditional mutant was constructed in the *pyrH* gene. This is known to be an essential gene in several species of bacteria (Akerley *et al.*, 2002;Thanassi *et al.*, 2002) and was also shown to be essential in *B. pseudomallei* using P_{rhaB} as a conditional mutagenesis tool (Moule *et al.*, 2014). The *B. pseudomallei*::pSC807.*pyrH*^{250} strain was created using the same method as described for the conditional *minC* mutant. A similar growth experiment was carried out to determine the essentiality of *pyrH* for growth in M9 minimal media. *B. pseudomallei*::pSC807.*pyrH*^{250} was grown with either rhamnose or glucose for 48 hours and the optical density of the cultures measured every 24 hours. The *B. pseudomallei*::pSC807.*pyrH*^{250} conditional mutant was unable to grow after a second passage in glucose (Table 3.5B), demonstrating the functionality of the pSC807 plasmid and the essentiality of *pyrH* in *B. pseudomallei*. Overall, the optical density of the *B. pseudomallei*::pSC807.*pyrH*^{250} cultures was lower compared to *B. pseudomallei*::pSC807.*minC*^{300} due to slower growth in the more defined media.

A

Primary culture		Secondary culture	
R	1.84	R	1.62
		G	1.75
G	1.90	R	1.68
		G	1.80

B

Primary culture		Secondary culture	
R	0.80	R	0.76
		G	0.28
G	1.69	R	0.50
		G	0.00

Table 3.5 Optical density at 590 nm of cultures grown with either rhamnose or glucose.

A *B. pseudomallei*::pSC807.*minC*^{300}

B *B. pseudomallei*::pSC807.*pyrH*^{250}

Each primary culture was used to inoculate two sub-cultures containing either rhamnose or glucose and grown for a further 24 hours. The optical density was measured after 24 hours growth in the primary culture and after 24 hours growth in the secondary cultures. Average of two experiments. R = rhamnose, G = glucose.

Because the growth of *B. pseudomallei*::pSC807.*pyrH*^{250} was measured during growth in M9 minimal media, a second growth experiment was carried out to investigate the growth of *B. pseudomallei*::pSC807.*minC*^{300} in the more defined growth medium. The mutant and wild-type were grown in M9 minimal media overnight, supplemented with either rhamnose or glucose. These cultures were pelleted, washed and resuspended in M9 minimal media with no additional sugars, followed by inoculation into M9 minimal media containing either 0.2% rhamnose or 0.2% glucose. The cultures were grown in a 96-well plate at 37˚C for 20 hours with the optical density measured at 15 minute intervals (Figure 3.10). Therefore, the growth of the mutant and wild-type was measured after a primary and secondary passage in permissive or non-permissive conditions. The growth of *B. pseudomallei*::pSC807.*minC*^{300} was similar to that of the wild-type under all conditions. The optical density of both cultures increased when grown in either rhamnose or glucose, indicating that *minC* is not essential for growth. Cultures containing glucose had a growth advantage over those containing rhamnose.

The *B. pseudomallei*::pSC807.*minC*^{300} integrant controls the expression of *minC* by replacing the native promoter at the beginning of the *min* operon. This also controls the expression of the other genes in the operon, *minD* and *minE*. However, there is also a second promoter present in the operon to give differential control of *minD* and *minE* alone (de Boer *et al.*, 1989). It was hypothesised that only one of the *min* genes was essential for growth, namely *minE*. The expression from an internal promoter would bypass the dependence on rhamnose for expression. Hence if *minE* was not under control of the rhamnose-inducible promoter in *B. pseudomallei*::pSC807.*minC*^{300} then it would still be expressed, allowing growth under repressing conditions. To address this, RT-PCR was used to determine the levels of mRNA transcribed from the *min* operon during expression and repression by the rhamnose-inducible promoter.

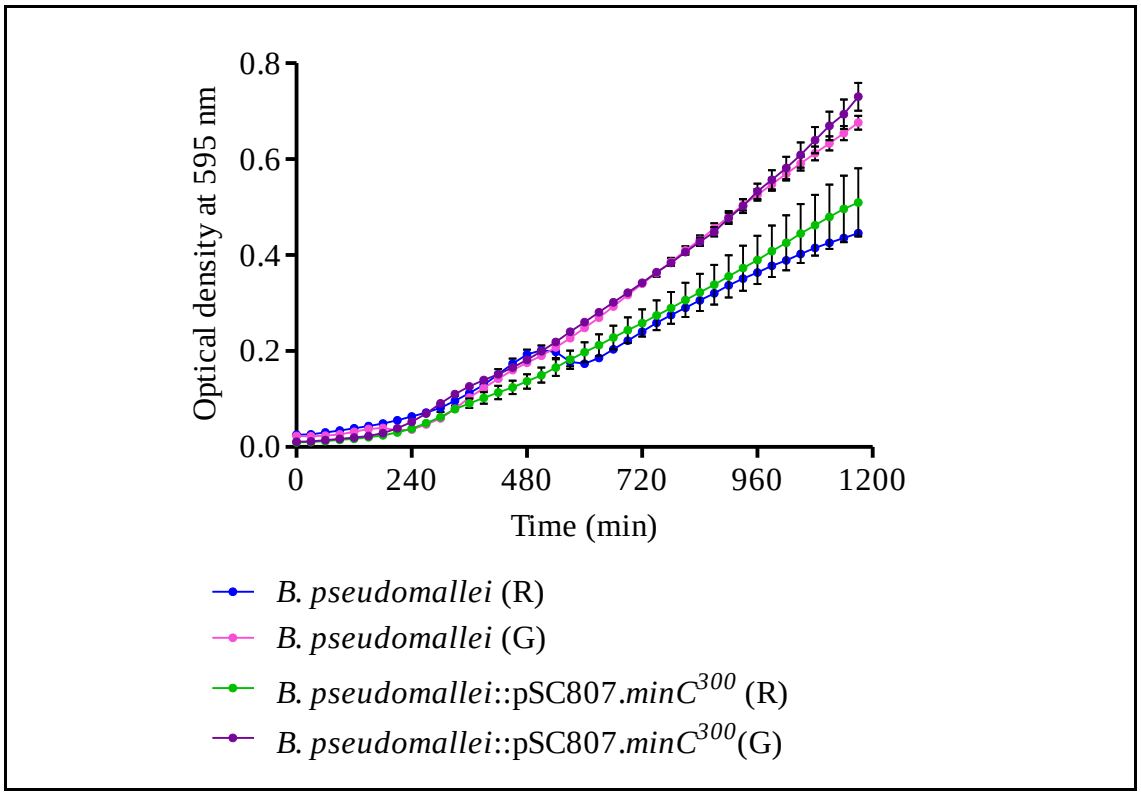


Figure 3.10 Growth of *B. pseudomallei* wild-type and *B. pseudomallei*::pSC807.*minC*^{300} in 0.2% rhamnose or 0.2% glucose.

Cultures were grown in a 96-well plate at 37˚C with continuous shaking for 20 hours. Optical density was measured at 590 nm at 15 minute intervals (30 minute intervals plotted); R = rhamnose, G = glucose. Error bars represent SEM from 6 wells of one experiment.

B. pseudomallei::pSC807.*minC*^{300} was grown overnight in either 0.5% rhamnose or 0.5% glucose. These cultures were passaged after 24 hours into fresh LB broth containing either 0.5% rhamnose or 0.5% glucose. RNA was collected from the cultures after a further 24 hours growth. Equivalent RNA samples were collected from wild-type cultures grown in either rhamnose or glucose as a control. The purified RNA was reverse transcribed and PCRs were carried out using primers that amplified regions from within the *min* operon. Primers were used to determine if the *min* genes were co-transcribed as polycistronic RNA or if *minE* was transcribed as a separate cistron Figure 3.11A). An internal control was carried out using primers RT 16S rRNA F and RT 16S rRNA R. The PCR product was visualised using gel electrophoresis, which provided a semi-quantitative result (Figure 3.11B).

The PCRs carried out with all primers demonstrated that addition of rhamnose was able to increase expression of the *min* genes when under the control of P_{rhaB}. However, the addition of glucose did not reduce expression to below the wild-type level in *B. pseudomallei*::pSC807.*minC*^{300}. This showed that *minC* and *minD* are co-transcribed, but their expression was not prevented by addition of glucose. Similarly, *minD* and *minE* are co-transcribed but expression was seen when grown in both rhamnose and glucose.

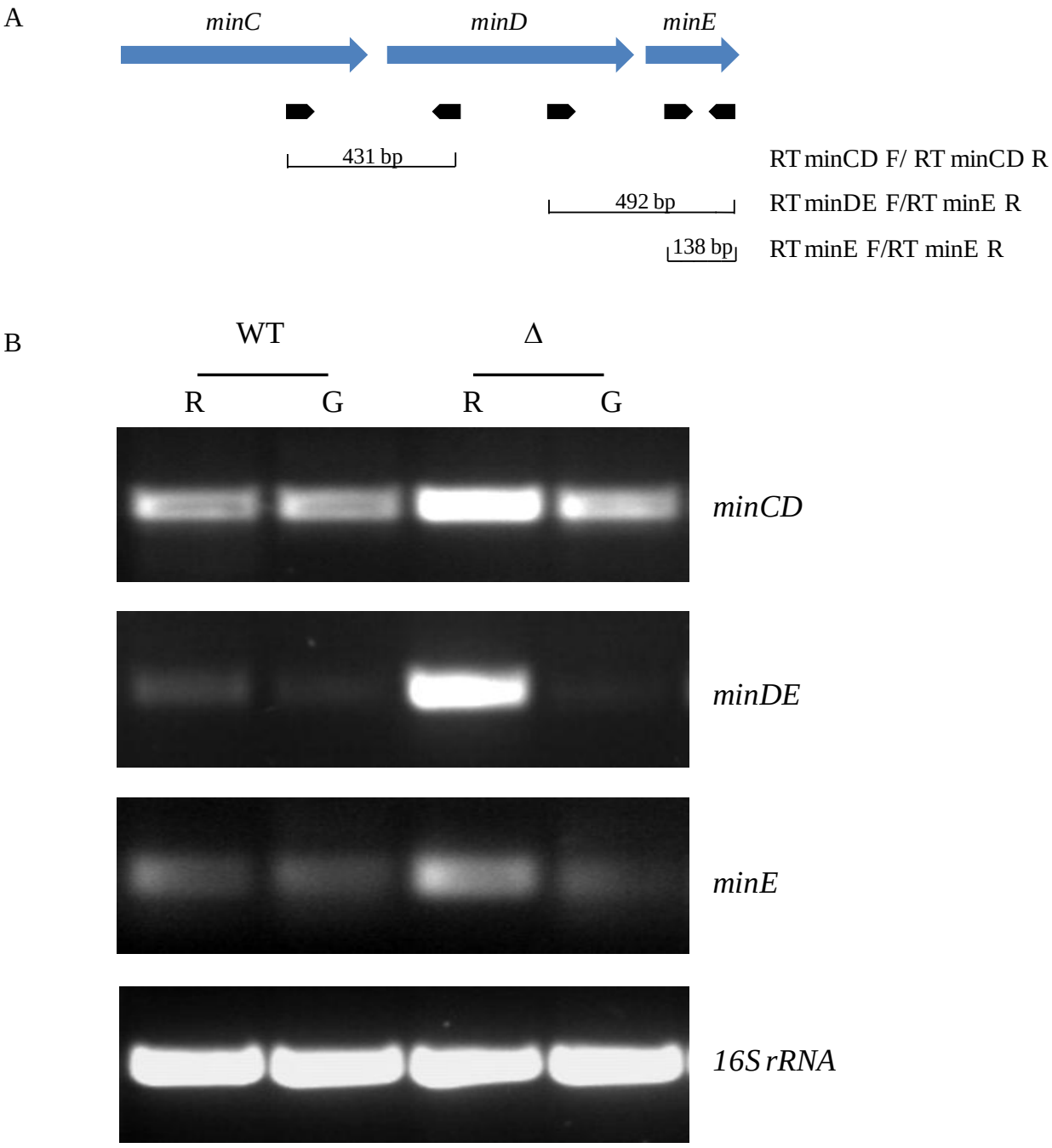


Figure 3.11 RT-PCR amplification of *min* genes under control of P_{rhaB}.

A Representation of the *min* operon. Arrows indicate the site of the primers used in RT-PCR.

B RT-PCR amplification of *min* genes under control of P_{rhaB}. RNA samples were collected from wild-type (WT) and *B. pseudomallei*::pSC807.*minC*^{300} (Δ) cultures after 48 hours growth in either rhamnose (R) or glucose (G). A combination of primers was used to amplify the *min* genes in order to show co-transcription. Control reactions were carried out using RNA in the absence of RT (not shown).

3.4.2.4 **Essentiality of *minE* in *B. pseudomallei***

In order to examine the essentiality of *minE* alone, a second construct was made using the pSC807 plasmid. The pSC807.$minE^{200}$ construct contained the initial 200 base pairs of the *minE* gene, which is a total of 255 base pairs in length. This was conjugated with *B. pseudomallei* as described previously. A total of 20 conjugations were carried out and over 200 colonies were screened for kanamycin resistance, which would have indicated that integration had occurred. However, there were no successful integration events detected, hypothesised to be due to the short region of homology. Therefore, a second construct was created to incorporate a larger region of homology within the *minD* and *minE* genes to encourage a recombination event with the chromosome.

A 250 base pair fragment of *minD* and *minE* was amplified by PCR using primers minDE pSC F and minDE pSC R. The primers incorporated an *Nde*I site at the 5' end and *Xba*I at the 3' end of the PCR fragment. This fragment contained an in-frame region of *minD* (117 bp) and the beginning of the *minE* gene (142 bp). This was cloned into pCR-Blunt II-TOPO before being transferred to pSC807. The plasmid pSC807.$minDE^{250}$ was transformed into *E. coli* S17-λ *pir*. This was conjugated with *B. pseudomallei* K96243 using the method described previously. Unfortunately, after several conjugation attempts, no colonies with the integrated plasmid were recovered.

3.4.3 Complementation of a *minCDE* deletion mutant

In *E. coli* the *min* operon is non-essential for growth. Cells with a deletion in the whole operon are viable but are unable to regulate the site of FtsZ ring formation (de Boer *et al.*, 1989). This leads to the production of minicells, which occur when the FtsZ ring forms at one pole of the cell producing uneven cell sizes when cell division takes place. However, expression of the MinCD division inhibitor in the absence of MinE leads to a cell division block (de Boer *et al.*, 1989). This is because the division inhibitor is no longer contained at the cell poles by the regulatory action of MinE. This gives the MinCD inhibitor free-reign in the cell and therefore prevents cell division along the entirety of the cell, resulting in filamentation. In a cell where the whole operon has been deleted, the loss of *minE* does not cause an adverse effect as the MinCD inhibitor is no longer present.

The deletion of *minE* by the methods discussed previously had proved unsuccessful. An alternative method of verifying the effect of MinE was therefore devised, whereby the entire *min* operon was deleted using the suicide vector pDM4. The *minC* and *minD* genes were then reintroduced under the control of the rhamnose-inducible promoter, P_{rhaB}. Upon induction it was hypothesised that the over-expression of MinC and MinD would cause a cell division block, leading to growth inhibition. The absence of MinE when the MinCD inhibitor is expressed would therefore produce a *minE* null phenotype. When these genes are down-regulated, normal growth should be possible.

3.4.3.1 **Creating the *minCDE* deletion mutant**

The Δ*minCDE* deletion mutant was produced by allelic replacement. The deletion construct was created in pDM4. Two flanking regions of the gene were amplified by PCR using primers minB LF F and minB LF R for the upstream flank and minB RF F and minB RF R for the downstream flank. The primers incorporated the restriction sites *Nde*I and *Spe*I at each end of the flanking regions. The resulting PCR products were each sub-cloned into pCR-Blunt II-TOPO before being transferred to pDM4. This was achieved by a three-way ligation after digestion of the two TOPO clones with *Nde*I and *Spe*I to isolate the inserts and linearization of pDM4 with *Nde*I, resulting in pDM4.Δ*minCDE*. The presence of the truncated operon was confirmed by restriction digest and sequencing.

The pDM4.Δ*minCDE* construct was transformed into *E. coli* S17-λ *pir* cells and then conjugated into *B. pseudomallei* K96243. Integrants were selected for on LB agar containing 50 μg/ml chloramphenicol and 50 μg/ml gentamycin. These colonies were screened by PCR using primers for the chloramphenicol resistance cassette and *sacB* gene. An integrant colony was used to inoculate 10 ml LB broth and grown overnight at 37˚C. This was diluted and sucrose selection carried out by plating onto LB agar containing 10% sucrose with no NaCl. This isolated colonies that had undergone a second cross-over event to remove the pDM4 vector from the chromosome. The colonies were screened for chloramphenicol sensitivity to verify the loss of pDM4 and by PCR to check for the deletion of the *min* genes. Two colonies, clones 32 and 34 were confirmed to have unmarked deletion mutants in the *min* operon. The *B. pseudomallei* Δ*minCDE* strain was confirmed by Southern blot (Figure 3.12).

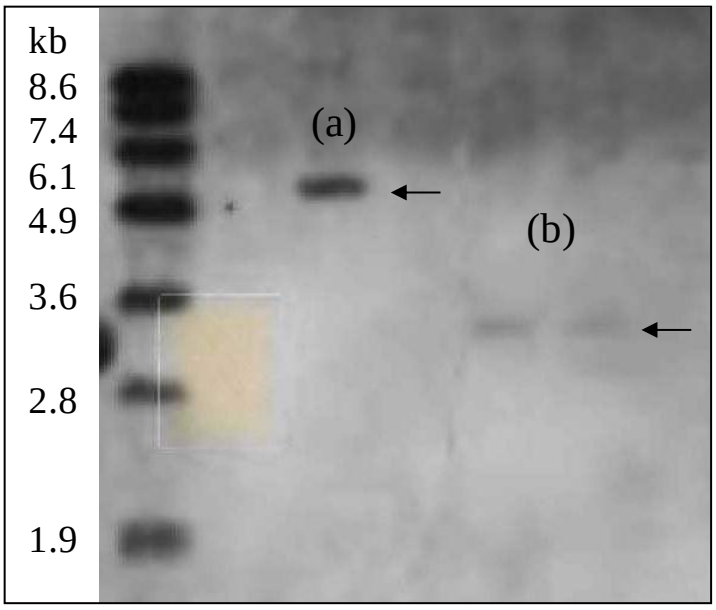


Figure 3.12 Southern blot confirming the deletion of the *min* operon in *B. pseudomallei*

Wild-type and mutant genomic DNA was digested with the *Eco*RV and *Nhe*I restriction enzymes, followed by separation on a 0.8% agarose gel. Primers minB LF F and minB LF R were used to make the DIG-labelled probe. (a) Wild-type operon (5097 bp); (b) Δ*minCDE*, clones 32 and 34 (3242 bp).

3.4.3.2 **Conditional complementation of *B. pseudomallei* Δ*minCDE***

The *minC* and *minD* genes were cloned into the pSJS01 plasmid to allow conditional expression of the division inhibitor under the control of the rhamnose-inducible promoter, P_{rhaB}. The primers minC ATG F and minD comp R were used to amplify the two genes by PCR using genomic DNA as a template. These primers incorporated an *Nde*I and *Eco*RV restriction site at the 3' and 5' ends of the genes, respectively. The PCR product was cloned into pCR-Blunt II-TOPO before digesting with *Nde*I and *Bam*HI. This produced a fragment of DNA encoding MinC and MinD with an extra 56 base pairs from the TOPO plasmid on the 5' end of the gene. This fragment was cloned into pSJS01 that had been linearised by restriction digest with *Nde*I and *Bam*HI to produce pSJS01.*minCD*. This plasmid encodes the *minC* and *minD* genes under the control of a rhamnose-inducible, glucose-repressible promoter (Figure 3.13).

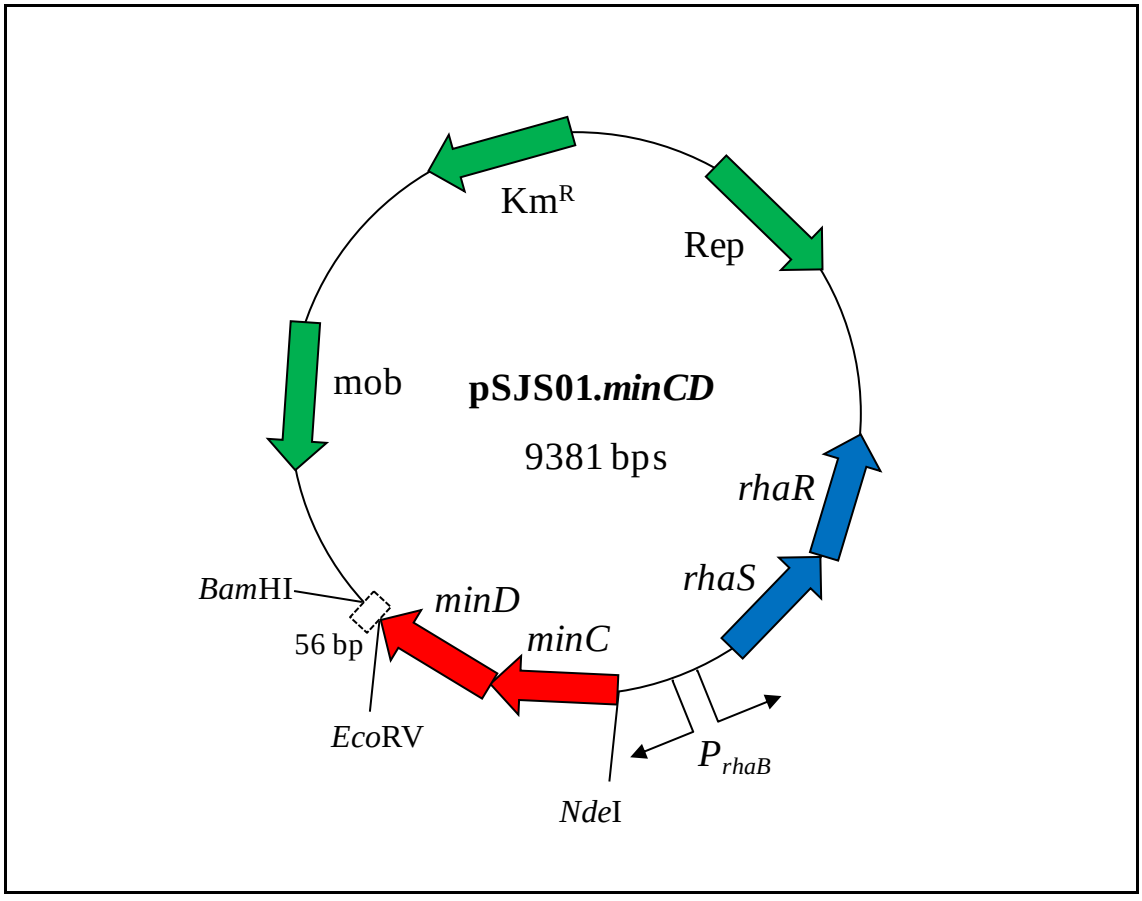


Figure 3.13 Plasmid map of pSJS01.*minCD*.

A conjugation was carried out in order to transform pSJS01.*minCD* into *B. pseudomallei* Δ*minCDE*. An overnight culture of *B. pseudomallei* Δ*minCDE* was grown in 10 ml LB broth along with an overnight culture of *E. coli* S17-λ *pir* containing the pSJS01.*minCD* plasmid in LB broth supplemented with 25 μg/ml kanamycin and 0.5% glucose. These were filter mated on LB agar containing 0.5% glucose. After 18 hours incubation at 37˚C the cells were resuspended in 1 ml PBS and the bacteria plated onto LB agar plates containing 250 μg/ml kanamycin, 50 μg/ml gentamycin and 0.5% glucose. The glucose was included to prevent expression of the *minCD* genes. However, no kanamycin resistant colonies were isolated.

In order to reduce the residual concentration of MinCD expressed from pSJS01.*minCD* during the conjugation, the *E. coli* strain containing the inducible plasmid was passaged twice in media containing 25 μg/ml kanamycin and 0.5% glucose before a second conjugation was performed with *B. pseudomallei* Δ*minCDE*. As a control, a conjugation was also carried out using wild-type *B. pseudomallei*. This expresses MinE and therefore would be able to regulate the activity of MinCD expressed from the inducible plasmid. Following conjugation and selection there were approximately ten fold more colonies on the wild-type plates. When these were gridded onto LB agar with 750 μg/ml kanamycin, all were found to be kanamycin resistant. However, unlike the wild-type, all the colonies produced from the conjugation with *B. pseudomallei* Δ*minCDE* were kanamycin sensitive, indicating that the conjugation was unsuccessful and these colonies were likely to be breakthrough.

As no *B. pseudomallei* Δ*minCDE* colonies were isolated that contained pSJS01.*minCD*, a growth experiment was carried out to investigate the growth of wild-type *B. pseudomallei* containing pSJS01.*minCD*. It was hypothesised that the up-regulation of *minCD* in from the rhamnose-inducible promoter would overcome the negative regulation imposed by MinE and would therefore result in a growth defect, demonstrating the essential role of MinE in the presence of an active MinC and MinD. *B. pseudomallei* pSJS01.*minCD* was grown overnight in LB broth supplemented with glucose, to prevent expression of *minC* and *minD*, and with kanamycin, to prevent loss of the plasmid. A wild-type control was grown in LB broth with glucose. These cultures were pelleted, washed and resuspended in LB broth, then used to inoculate LB broth

containing either 0.2% rhamnose or 0.2% glucose. The cultures were grown in a 96-well plate at 37˚C for 20 hours with the optical density measured at 15 minute intervals (Figure 3.14). *B. pseudomallei* pSJS01.*minCD* showed a growth defect when the *minC* and *minD* genes were induced by addition of rhamnose to the media compared to those grown in glucose. The results support the hypothesis that MinE is essential for growth when MinC and MinD are present.

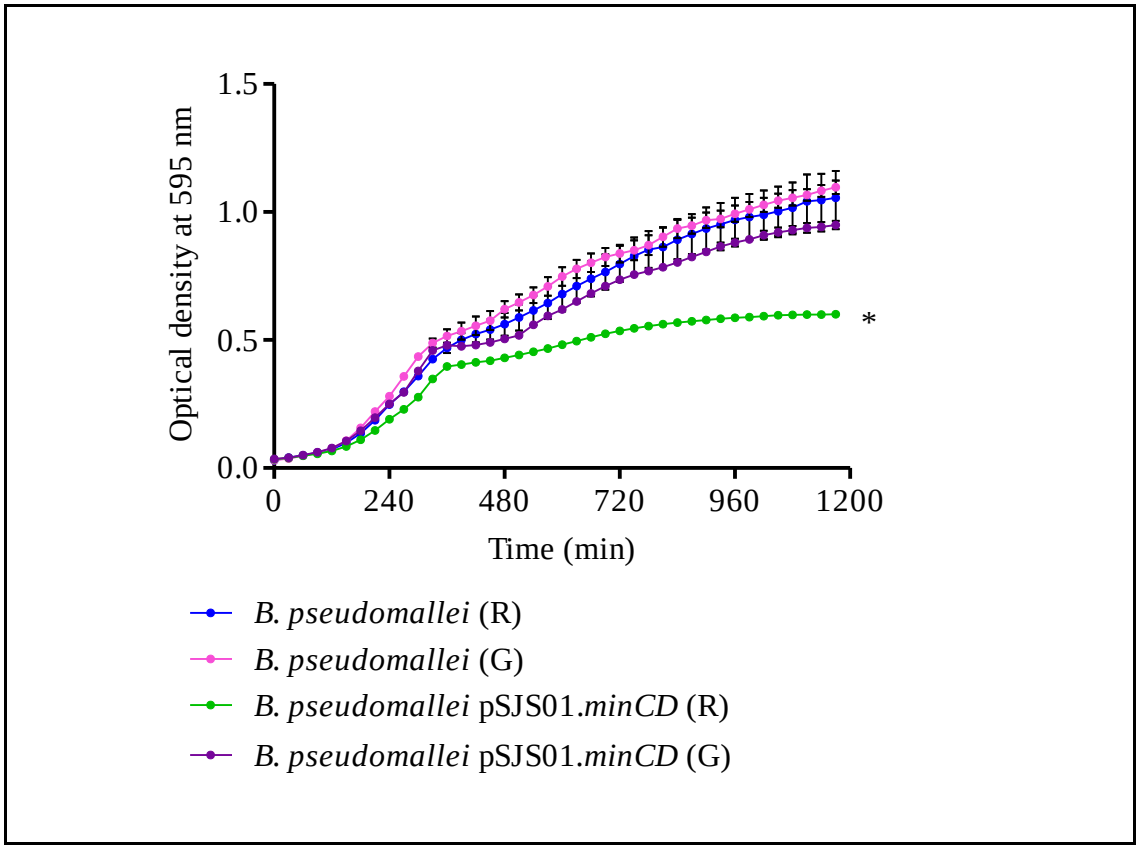


Figure 3.14 Growth of *B. pseudomallei* wild-type and *B. pseudomallei* pSJS01.*minCD* in 0.2% rhamnose or 0.2% glucose.

Cultures were grown in a 96-well plate at 37˚C with continuous shaking for 20 hours. Optical density was measured at 590 nm at 15 minute intervals (30 minute intervals plotted); R = rhamnose, G = glucose. Error bars represent SEM from 6 wells of one experiment. * indicates $P < 0.05$ for optical density values after 420 minutes compared to wild-type grown in rhamnose.

3.5 Discussion

In Gram-negative bacteria the Min system consists of three proteins, MinC, MinD and MinE. MinC and MinD form a dimer which inhibits the polymerisation of FtsZ (de Boer *et al.*, 1992;Bi and Lutkenhaus, 1993). MinE regulates the position of this inhibitor within the cell (Raskin and de Boer, 1997). Deletion of the Min system in bacteria, such as *E. coli*, results in minicell formation (de Boer *et al.*, 1988) due to incorrect positioning of the Z ring during cell division (Bi and Lutkenhaus, 1993). Therefore, the complete operon is not thought to be essential. However, in *B. pseudomallei* repeated attempts to mutagenise individual genes in the *min* operon failed, suggesting that one or more of the genes may be essential for growth in the presence of the remaining genes. This was supported by evidence using a high-throughput transposon based method (TraDIS) for identifying essential genes in *B. pseudomallei* (data not shown). This data supported the essential nature of the *minE* gene. For this reason several techniques were used in order to generate conditional *min* mutants in *B. pseudomallei*.

In order to create conditional mutants in bacteria, an inducible promoter system is required to maintain control over the expression of the gene of interest. There are several available promoter systems that have been used for this purpose. One particular system, the P_{BAD} promoter system, has been investigated for its potential use in validating essential genes in *B. cenocepacia* (Lefebre and Valvano, 2002). In this system the promoter is regulated by addition of arabinose to the growth media. When an essential gene is under the control of this promoter, it results in a conditional-lethal growth phenotype depending on the presence or absence of arabinose. However, there are several disadvantages to using this system. Experiments using this promoter in *B. cenocepacia* to control essential genes by insertion of the promoter into the chromosome were unable to reproduce wild-type expression levels when the promoter was induced (Cardona *et al.*, 2006). In addition, the concentration of arabinose required to induce adequate expression levels was relatively high due to the poor transport of the sugar into the cell (Lefebre and Valvano, 2002). The high concentration of arabinose caused damage to the cell, typical of that seen during osmotic stress. In contrast, the rhamnose-inducible system is much more sensitive (Cardona and Valvano, 2005). This system is regulated by the *rhaS* and *rhaR* genes, under the control of the P_{rhaB} promoter.

This requires concentrations of rhamnose of between 0.02% and 0.2% (w/v) to induce expression, compared to between 2% and 3% for the arabinose-inducible system (Cardona and Valvano, 2005). The rhamnose-inducible promoter therefore allows higher expression at lower concentrations and is more tightly regulated than the arabinose system. This was demonstrated when using the arabinose-dependent system to express essential genes in *B. cenocepacia*, as the absence of arabinose did not provide a conditional-lethal phenotype and was therefore shown to be leaky (Cardona *et al.*, 2006). For this reason, the rhamnose-inducible system was chosen for carrying out condition mutagenesis of the Min system in *B. pseudomallei*.

The pSJS01 plasmid was created by combining the rhamnose-inducible system from the pSC200 plasmid with the replication and mobilisation components of the pBHR5 plasmid. The pBHR5 plasmid was chosen as it is currently used for complementation of deletion mutants in *B. pseudomallei* and would therefore enable the new hybrid plasmid to be stable. This plasmid was designed for the purpose of in-trans expression of the gene of interest. Removal of rhamnose would then allow the essential nature of the chosen gene to be assessed by lack of expression from the plasmid-borne copy. The RFP gene was chosen to test the control of expression from P_{rhaB} by measuring the increase in fluorescence. However, the level of fluorescence was too weak to detect upon induction. The pSJS01 plasmid has a medium copy number of around 30 copies per cell and so the expression from the P_{rhaB} should be sufficient to express observable levels of fluorescence. Therefore an alternative method was used to measure the level of gene expression from P_{rhaB}.

RT-PCR was carried out on RNA samples taken from overnight cultures containing the pSJS01.RFP plasmid grown under either permissive or non-permissive conditions for the promoter. The results showed an increase in expression of RFP upon addition of rhamnose to the media, verifying that the rhamnose-inducible promoter was functional. This suggests that the RFP mRNA was being expressed but the translation into protein was less efficient, hence there was no detectable fluorescence despite the increased expression of RFP.

The results from the RT-PCR also revealed that there was residual expression of RFP in cultures grown without rhamnose or with glucose. This may be due to a low level of expression from the promoter even in non-inducing conditions. Varying levels of transcript indicated that the level of expression may depend on growth rate and sugar usage. In some repeats the glucose may have been metabolised, for example, due to higher growth, allowing breakthrough expression of RFP when grown in glucose-containing media.

The pDM4 suicide method has been used successfully for deletion of non-essential genes in *B. pseudomallei* according to the method of Logue et al (2009). This method was adopted in order to delete the chromosomal copy of *minE* after complementation with pSJS01.*minE*. The creation of the merodiploid strain, encoding an integrated pDM4 construct, was possible due to the presence of both a non-functional and functional copy of *minE* on the chromosome. However, after sucrose selection was carried out only wild-type or integrant colonies were recovered. The presence of the *sacB* on pDM4 makes the integrant strain sucrose sensitive, although wild-type *B. pseudomallei* K96243 also encodes a *sacB* gene. Therefore, the growth of the integrant colonies on the sucrose-containing media may have been due to a high density of cells, allowing breakthrough to occur.

The *minE* gene is hypothesised to be essential and hence if the second cross-over event in the *B. pseudomallei* pDM4 merodiploid strain resulted in a lethal mutation then the mutant would never be recovered. Despite complementation of *minE* from a plasmid, the cell tended to revert back to wild-type rather than delete the *minE* gene. It was thought that the loss of *minE* from the chromosome may select for the plasmid to be retained and therefore selection was carried out without kanamycin. This allowed more favourable conditions for growth in the absence of any antibiotics. Under these conditions the bacteria lost the pSJS01 plasmid and reverted to wild-type. Thus, any cells that had undergone the cross-over to delete *minE* did not survive and therefore no colonies were recovered with a mutant genotype. When kanamycin was included in the media, the cell was able to retain the plasmid but no mutants were isolated. The cost of maintaining the plasmid, along with the loss of an essential gene, may have reduced the fitness of a Δ*minE* mutant so that no colonies were isolated. This is a novel method of

conditional complementation for this species and needs further optimisation to allow successful selection of unmarked deletion mutants.

An alternative method for the validation of essential genes is by direct promoter control of the gene of interest. The disadvantage of this technique is the inability to control individual genes within an operon. Essential genes can often be misidentified when in operons using screening techniques such as random transposon mutagenesis. This is because the act of disrupting one gene may have downstream effects on other genes within the operon. If the operon happens to encode an essential gene then upstream genes may be misidentified as being essential. The *minE* gene is the third gene in the *minB* locus, following on from the *minC* and *minD* genes (de Boer *et al.*, 1989). In *B. pseudomallei* the three genes are transcribed from a promoter upstream of *minC*, although there is also a putative promoter present within the *minC* gene. Therefore, in order to control the expression of the *minE* gene, the rhamnose-inducible promoter was integrated upstream of the *min* operon. However, this does not allow more targeted validation of the individual genes within the operon.

When the pSC807 plasmid was integrated into the chromosome in place of the *min* promoter there was no effect on cell growth. If the promoter in this position was indeed able control expression of the entire *min* operon then growth would be unaffected as deletion of the entire *min* operon does not cause a growth defect in other species, such as *E. coli* (de Boer *et al.*, 1989). This is because in the absence of all three genes cell division can take place, although it is not spatially regulated when the Min system is absent. When *minE* alone is deleted, MinC and MinD form a bipartite inhibitor that prevents formation of the Z ring, required for cell division (Raskin and de Boer, 1997). Therefore, deletion of all three *min* genes does not affect cell growth, unlike deletion of *minE* alone (de Boer *et al.*, 1989). Because of this, ideally the inducible promoter from pSC807 would be integrated upstream of the *minE* gene, which is the gene of interest.

RT-PCR was carried out to determine the level of gene expression when the P_{rhaB} promoter was integrated into the chromosome upstream of *minC*. The results showed that *minCD* and *minDE* are co-transcribed, indicating a single polycistronic RNA. All three genes were up-regulated by addition of rhamnose, indicating that P_{rhaB} was able to

influence expression of the entire operon. The over-expression of *minC* alone would likely cause a cell division block as *minC* is active as a cell division inhibitor when over-expressed by around 50 fold (de Boer *et al.*, 1992). Similarly, the over-expression of *minCD* would cause a cell division block due to the action of the bipartite inhibitor. However, the conditional expression of all three genes simultaneously is unlikely to cause a growth defect, further indicating that these genes are expressed as a single RNA. Despite the presence of glucose, there was some expression of the downstream genes when under the control of the rhamnose-inducible promoter. If an essential gene is placed under the control of P_{rhaB}, the presence of glucose in the growth medium should sufficiently reduce expression to cause cell death or growth arrest. For this reason, the suitability of this system for validating essential genes was tested by integrating pSC807 upstream of a known essential gene.

PyrH is a kinase enzyme that catalyses an important step in the pyrimidine metabolic pathway. This gene has previously been shown to be essential in several species of bacteria such as *Haemophilus influenzae* and *Streptococcus pneumoniae* (Akerley *et al.*, 2002;Thanassi *et al.*, 2002). Consequently, the *pyrH* gene provided a useful control to test the function of the pSC807 plasmid and to check that the promoter was able to confer enough control to switch off the desired gene, thus preventing growth due to the loss of an essential component. When the pSC807 plasmid was integrated upstream of this gene, the inducible promoter was able to prevent growth when glucose was added to the media, although a second passage was needed in order to titrate out residual *pyrH* in the cell. After prolonged growth under non-permissive conditions the growth of the culture did recover, indicating that there was a low level of expression which allowed some cells to survive until the glucose had been completely metabolised. Nonetheless, the addition of glucose was able to prevent growth, indicating that this plasmid is a suitable tool for the validation of essential genes in *B. pseudomallei*.

A second pSC807 plasmid was constructed in order to determine the effect of disrupting expression of *minE* directly. The *B. pseudomallei minE* gene is 255 base pairs in length and so the plasmid only encoded a small region of homology of 200 base pairs. This encodes a large proportion of the gene and so it was possible that integration of this region under promoter control would not affect the function of the gene to the same

extent as a full deletion. After several conjugations, no successful integrants were isolated. This may have been because the homologous region was not large enough to undergo efficient recombination. A second construct was made that included a larger region of the *min* operon. This plasmid encoded an in-frame section of the *minD* gene directly in front of the *minE* start codon so as to facilitate integration into the chromosome upstream of *minE*. Despite increasing the region of homology, conjugations with this plasmid were still unsuccessful. This may be due to the deregulation of the *minD* and *minE* genes, which may have an adverse effect on growth following integration.

The deletion of the entire *min* operon is not lethal in species such as *E. coli*, therefore a back-to-front approach was used to prove the essential nature of the MinE protein. The MinC and MinD proteins form a bipartite cell division inhibitor, which, in the absence of MinE, causes a cell division block (de Boer *et al.*, 1989). A deletion of the *min* operon in *B. pseudomallei* does not kill the cell as MinE is only essential in the presence of MinCD. However, the over-expression of MinCD in a Δ*minCDE* mutant would demonstrate the essential nature of MinE. This is because the cell division inhibitor activity provided by MinCD would be unregulated by MinE and would therefore lead to a cessation of division throughout the cell. However, when an attempt was made to reintroduce the *minCD* genes on the pSJS01 plasmid in *B. pseudomallei* Δ*minCDE*, no recombinants were isolated. *B. pseudomallei* has an inherent level of resistance to kanamycin so a high concentration had to be used for selection, but it appeared that there was still a level of breakthrough. The lack of recombinants may have been due to a low level of *minCD* expression from the plasmid. Even after repeated passages in glucose to prevent expression from P_{rhaB} the plasmid was not retained in the Δ*minCDE* mutants. This suggests that there was not sufficient repression of the *minC* and *minD* genes to prevent expression of the cell division inhibitor. Hence the cells were unable to grow when *minE* was not provided in-trans with *minCD*.

The pSJS01.*minCD* plasmid was supported in a wild-type background. This is likely to be because the low level of the expression of *minCD* from P_{rhaB} was regulated by the wild-type *minE* expressed from the chromosome. When the expression from P_{rhaB} was up-regulated by addition of rhamnose, there was a significant decrease in the growth of

B. pseudomallei pSJS01.*minCD* compared to the wild-type, thus proving that MinE is essential for sustainable growth when MinC and MinD are active.

This study has attempted to demonstrate the essential nature of *minE* in *B. pseudomallei*. A multitude of techniques have been used to regulate expression of the individual *min* genes. Ultimately, these techniques have demonstrated the importance of maintaining the correct concentration of the Min proteins within the system as disruption or altered levels of expression of a single gene had a dramatic effect, often leading to inhibition of growth. Previous studies have shown that the *min* operon relies on the well-balanced expression of all three genes in order to fulfil its function correctly. When this ratio is altered it upsets the normal oscillation pattern of the cell and the Min system is therefore ineffective at regulating proper placement of the Z ring. For example, over-expression of MinD compared to MinE increases the length of the oscillation cycle and creates a MinC-dependent division block (Raskin and de Boer, 1999b). Conversely, over-expression of MinE prevents MinCD inhibitor activity and division can therefore occur at the poles (de Boer *et al.*, 1989;Pichoff *et al.*, 1995). Alternatively, when MinD is disrupted, the cell division inhibitor is no longer active unless MinC is over-expressed to compensate (de Boer *et al.*, 1992;Hu and Lutkenhaus, 2003). Mutations in MinE that prevent its interaction with MinD cause cell-wide division inhibitor activity, preventing further growth (Pichoff *et al.*, 1995). The interactions between all three Min proteins are therefore essential in maintaining their role within the cell and balancing the levels of expression of each component is key.

In conclusion, although the *minE* gene resisted mutation when it was specifically targeted for deletion, it was not found to be essential when disrupted along with the other two genes belonging to the *min* operon. Therefore, the *min* operon is not essential in *B. pseudomallei* as deletion of the entire operon had no effect on growth. It was hypothesied that MinE in *B. pseudomallei* was essential in the presence of a functional MinC and MinD and a number of methods were used in order to confirm this. However, the two methods used to delete *minE*; integration of an inducible-promoter and in-trans complementation of *minE*, were unsuccessful. The over-expression of MinC and MinD from an inducible-promoter in a Δ*minCDE* strain was also unsuccessful but did produce a growth defect when over-expressed in a wild-type background. In summary, the

accumulation of evidence does suggest that *minE* is essential for growth when the Min system is functional. The construction of a conditional lethal mutant would be definite proof of this, but gaining sufficient control of the relative levels of MinC, MinD and MinE in the cell, not achieved in this study, is key to achieving this outcome.

Chapter 4: **Evaluation of a putative virulence target in *B. pseudomallei*: The Phage-shock protein response**

4.1 The Phage-shock protein response

The Psp response is an extracytoplasmic stress response in bacteria that functions to maintain cell membrane integrity during stress. The Psp response was initially discovered in *E. coli*, with first reports describing a protein produced at a high concentration during filamentous phage infection, subsequently termed PspA (Brissette *et al.*, 1990). The Psp response is known to have a number of inducers, all of which are thought to have a detrimental effect on the proton motive force (PMF) and therefore the Psp response is thought to have an important physiological role in maintaining the PMF across the inner membrane (Kleerebezem *et al.*, 1996).

The Psp systems in *E. coli* and *Yersinia enterocolitica* are considered the paradigms for the Psp response. The *E. coli psp* operon (Figure 4.1) contains five open reading frames, which encode for proteins PspA, PspB, PspC, PspD and PspE, and is driven by a σ^{54} promoter. A second, divergently transcribed operon codes for PspF, the transcriptional activator (Model *et al.*, 1997). The *Y.enterocolitica* operon is smaller and contains only the *pspA*, *pspB* and *pspC* genes (Figure 4.1). A new member of the Psp response, PspG, was discovered in 2004 by transcriptional profiling of *E. coli* during expression of the

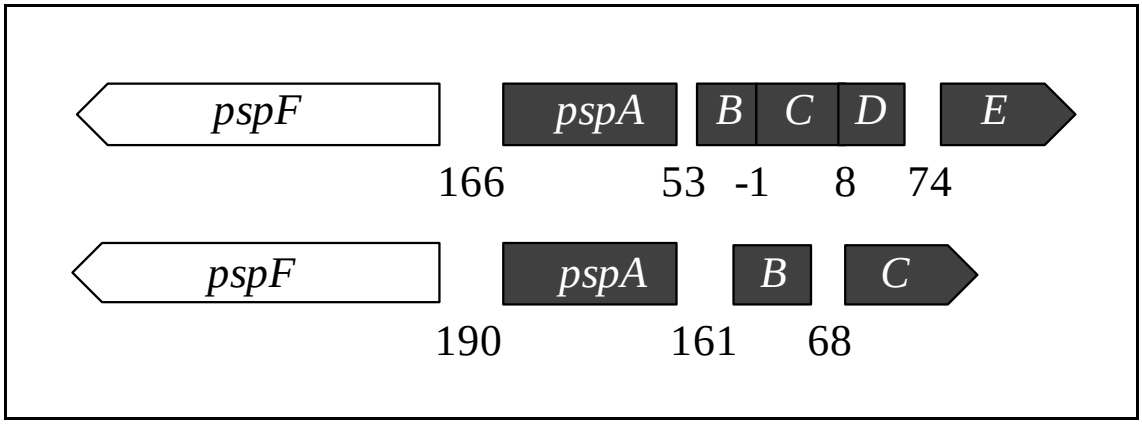


Figure 4.1 Representation of the *psp* operon in *E. coli* (top) and *Y. enterocolitica* (bottom).

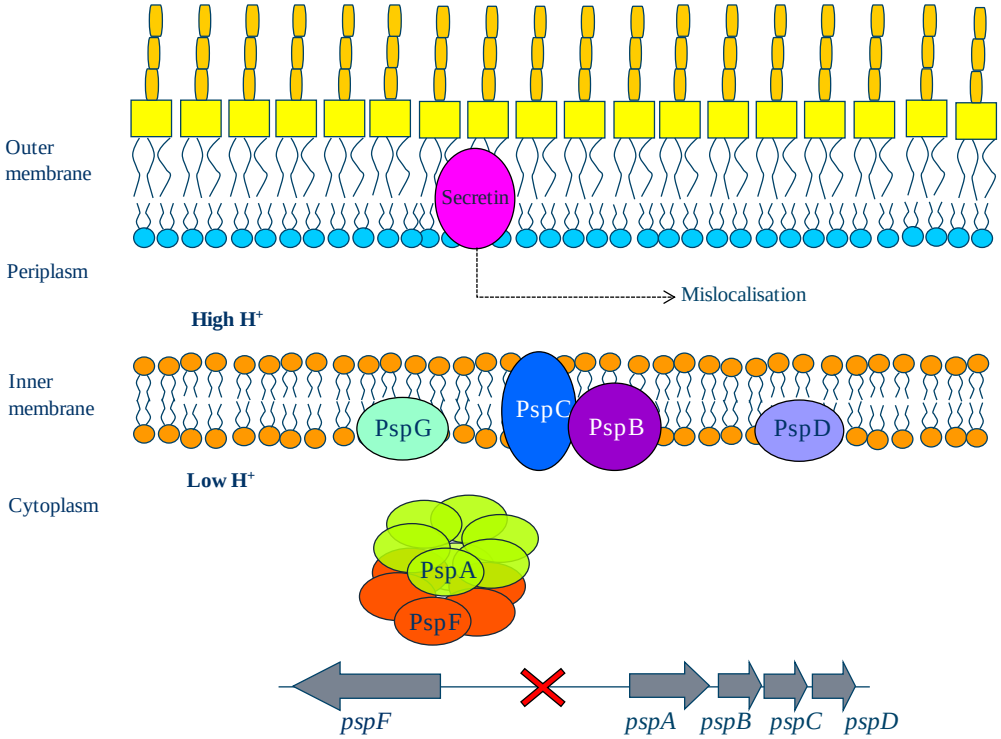
filamentous phage protein, pIV (Lloyd *et al.*, 2004). The only genes found to be significantly up-regulated under these conditions were the *psp* operon and an unlinked gene, *pspG*, both under control of a σ^{54} promoter. There is evidence to suggest that PspG may act as an effector of the Psp response in addition to PspA.

Figure 4.2 illustrates a working model of the Psp response as it is understood in *E. coli*. PspA negatively regulates transcription from the *psp* operon by binding to PspF, which is a transcriptional activator from the enhancer-binding protein (EBP) family (Dworkin *et al.*, 2000;Elderkin *et al.*, 2002). PspB and PspC are inner membrane proteins that form a transmembrane complex thought to detect and transduce signals to PspA, stimulating it to release control of PspF (Maxson and Darwin, 2006). Once an inducing signal is received, the newly freed EBP activates transcription from the σ^{54}-dependent *psp* promoter allowing up-regulation from the *pspA* operon (Jovanovic *et al.*, 1996;Jovanovic *et al.*, 1999). Consequently, PspA is expressed in higher concentrations in the cell and acts as effector by forming large oligomeric rings (Hankamer *et al.*, 2004). These structures are able to bind to membrane phospholipids and prevent further proton leakage through the damaged membrane (Kobayashi *et al.*, 2007).

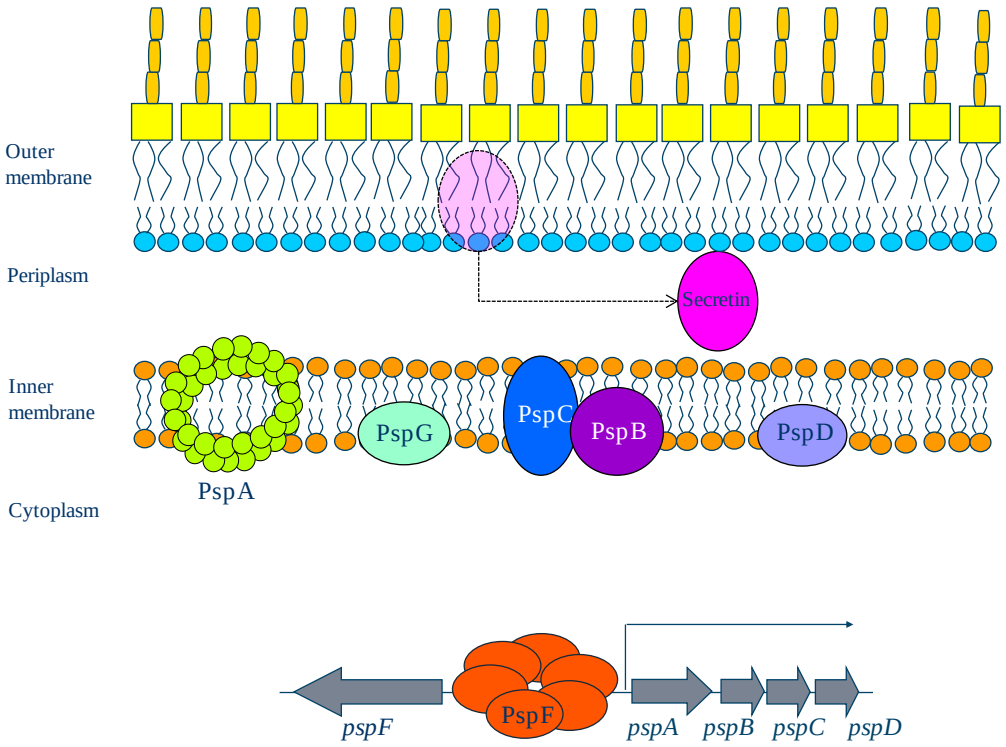
Figure 4.2 Model of the Psp response in *E. coli* (see next page; adapted from Darwin, 2005).

PspA acts as a negative regulator of the system by binding to the transcriptional regulator PspF. When the Psp response is induced, for example by mislocalisation of a secretin, PspA acts as an effector by associating with the cytoplasmic membrane, preventing further dispersion of the PMF. PspF activates transcription of the *psp* operon which further up-regulates the expression of *pspA*.

Uninduced conditions



Induced conditions



4.1.1 Regulation of the Psp response

4.1.1.1 Transcriptional activation by PspF

The transcription of the *psp* operon is controlled by a σ^{54}-dependent promoter. This type of promoter requires a bacterial EBP to activate transcription. PspF is a 37 kDa protein which acts as the EBP for the *psp* operon. It was initially identified by random transposon mutagenesis where it was found that an insertion in the *pspF* gene prevented induction of the *psp* operon (Jovanovic *et al.*, 1996). The σ^{54}-RNA polymerase (RNAP) requires the EBP to catalyse the formation of an open promoter complex for initiation of transcription. The EBP uses ATP hydrolysis to initiate the formation of the open promoter complex and is made up of two functional domains, an AAA^+ domain with ATPase activity at the N-terminal, and a C-terminal DNA-binding domain. PspF lacks a regulatory domain that also forms part of most EBPs and is instead regulated by PspA. PspA acts directly on the AAA^+ domain of PspF forming an inhibitory complex which represses the expression of *pspA* and the rest of the *psp* operon (Dworkin *et al.*, 2000;Elderkin *et al.*, 2002;2005). However, over-expression of PspF can overcome the negative regulation imposed by PspA and is therefore able to activate transcription of the *psp* operon without the need for any inducing conditions (Jovanovic *et al.*, 1996; 1999).

PspF transcription regulation is highly specific for the *psp* genes as it does not control any other known σ^{54}-dependent promoters. This is because the ATPase activity of PspF is dramatically increased by the presence of specific DNA. The C-terminal DNA-binding helix-turn-helix motif of PspF recognises two upstream activation sequences, which confer specificity on the initiation of transcription (Jovanovic *et al.*, 1996; 1999;Dworkin *et al.*, 1997;Lloyd *et al.*, 2004). This increases the local concentration of the activator upstream of the σ^{54} promoter to increase the efficiency of the interaction with σ^{54}-RNAP. Transcription is also partially dependent on the integration host factor, which enables looping of the DNA to bring the activator into the vicinity of RNAP, increasing the chances of a successful interaction (Weiner *et al.*, 1995;Jovanovic *et al.*, 1999;Dworkin *et al.*, 1997). An exception to this occurs during hyper-osmotic shock. In this case, transcription of the *psp* operon can be triggered in the absence of the upstream activation sequences and integration host factor. This is probably due to supercoiling of

the DNA caused when the cell suffers osmotic shock, which reduces the need for the precise alignment of σ^{54}-RNAP with the enhancer sequences in order for transcription to take place (Jovanovic *et al.*, 1999).

4.1.1.2 **Negative regulation by PspA**

PspA negatively regulates the Psp response by inhibiting the transcriptional regulator PspF (Dworkin *et al.*, 2000). It does this by forming an inhibitory complex with PspF in a dodecameric structure, where both proteins form hexameric rings. This allows quick release of PspA-mediated negative control as PspF is already in a hexameric form for σ^{54}-RNAP activation (Joly *et al.*, 2009). PspA inhibition functions by blocking the productive interaction of PspF with σ^{54}, preventing the formation of an open promoter complex (Elderkin *et al.*, 2002). It acts directly on PspF by inhibiting its ATPase activity, which is required for the activation of transcription (Jovanovic *et al.*, 1999;Elderkin *et al.*, 2002). PspA is able to bind to PspF before ATP has bound, blocking ATP from gaining access to the active site of PspF to undergo hydrolysis. It is also able inhibit an intermediate state of PspF bound to a transition state analogue of ATP. Therefore, PspA can act before or after ATP binding to cause inhibition of PspF activity (Elderkin *et al.*, 2002).

PspA is predicted to be a coiled-coil protein comprising of four helical domains (HD; Elderkin *et al.*, 2005;Dworkin *et al.*, 2000). However, not all HDs are required for interaction with PspF. The major PspF-binding determinants are found in HD-1-2-3, where HD-2-3 are responsible for hexamer formation (Elderkin *et al.*, 2005;Joly *et al.*, 2009). HD-1 modulates HD-2-3 interactions, leading to the production of dimers, but is vital for the formation of the PspA-PspF regulatory co-complex. Overall, no single HD is responsible for the inhibition of PspF as all the HDs function together to regulate the ATPase activity of PspF (Joly *et al.*, 2009). The PspA binding site on PspF is proposed to be close to the protomer-protomer interface and the ATPase active site. It was found that a single amino acid close to this site, W56, was required for PspA binding to PspF and that PspA was unable to negatively regulate PspF when the tryptophan was substituted for alanine (Elderkin *et al.*, 2005).

4.1.1.3 **The effector function of PspA**

PspA is the central component of the Psp response and acts as the main effector of the system in addition to its regulatory role (Kobayashi *et al.*, 2007). Once the Psp response has been induced, PspF activates transcription from the *psp* operon (Jovanovic *et al.*, 1996). Consequently, PspA is up-regulated and can become one of the most abundant proteins in the cell (Model *et al.*, 1997). PspA, the effector, functions by forming large oligomeric rings, which are thought to be involved in maintaining membrane integrity. It is predicted to form a 36-mer ring containing nine domains, each composed of four PspA subunits (Hankamer *et al.*, 2004). All four HDs of PspA are required for formation of the oligomer but HD-4 is specifically needed to overcome the destabilising effect caused by HD-1, which preferably forms dimers in its absence (Joly *et al.*, 2009). Therefore, as a result of the interaction between its helical domains, PspA can form at least two different oligomeric structures. This suggests that the regulation of PspA is likely to be dependent on these interactions, affecting the formation of PspA oligomers and allowing it to switch between its different roles in the cell.

Upon induction, PspA is recruited to the membrane where it assembles into an oligomeric form (Kobayashi *et al.*, 2007). In *Y. enterocolitica* this relocalisation to the membrane has been shown to be partially dependent on the integral membrane proteins PspB and PspC (Yamaguchi *et al.*, 2010). These proteins can form highly motile complexes with PspA, perhaps aiding in the detection and mitigation of membrane damage (Yamaguchi *et al.*, 2013). The proposed function for the 36-mer form of PspA is to suppress proton leakage by stabilisation of the inner membrane. The PspA oligomer can also bind directly to liposomes and reduce proton leakage in membrane vesicles damaged by ethanol (Kobayashi *et al.*, 2007). A further study demonstrated that *E. coli* PspA proteins form large scaffold-like structures that extend beyond the 36-mer ring (Standar *et al.*, 2008). This arrangement would be able to cover large regions of the inner membrane and stabilise any holes by preventing them from expanding.

4.1.1.4 **PspBC signal transduction**

The integral membrane proteins PspB and PspC play an important role in the positive regulation of the Psp response in *E. coli* and *Y. enterocolitica*. It has been proposed that the protein-protein interactions between PspB, PspC and PspA provide a method of

signal transduction from PspBC sensors to PspA (Adams *et al.*, 2003). The dependence of the Psp system on protein-protein interactions is supported by the presence of a leucine zipper-like amphipathic helix in PspC (Brissette *et al.*, 1991), a structure which is present in many eukaryotic transcriptional activators and is also involved in protein dimerisation. Similarly, the cytoplasmic region of PspB in *Y. enterocolitica* is predicted to contain an amphipathic helix, which is thought to be important for dimerisation with PspC (Gueguen *et al.*, 2011). Recent evidence has shown that PspB and PspC form both homodimers and heterodimers, which may play an important part in their role as regulators (Gueguen *et al.*, 2011). However, these PspBC complexes remain constant regardless of the state of induction and, therefore, dimerisation of PspB and PspC is unlikely to be a switching mechanism to trigger up-regulation of the Psp response. Gueguen *et al.* (2011) revealed that multiple domains are implicated in dimer formation and a change in induction state is therefore more likely to be caused by more subtle regulation than initially thought. For this reason, further work is needed to elucidate the mechanism by which the Psp system detects an inducing signal and how this is transduced.

There are many known inducers of the Psp response but its activation is not always dependent on the two membrane proteins. In *E. coli* the induction of the Psp response by heat shock is independent of PspC, whereas induction by ethanol and hyperosmotic shock is enhanced by, but not entirely dependent on, PspB and PspC (Brissette *et al.*, 1990;Weiner *et al.*, 1991). The over-expression of the outer membrane protein PhoE also partially induces the Psp response in a *pspC* null mutant (Kleerebezem *et al.*, 1996). The induction of the Psp response in the absence of the proposed signal transducers indicates that these proteins may be non-essential or have a partially redundant function with another protein (Maxson and Darwin, 2006;Gueguen *et al.*, 2009).

4.1.2 Induction of the Psp response

The organisation of the Psp response has been extensively characterised in *E. coli* and *Y. enterocolitica* and so is considered the paradigm for this system in other species. In these species the Psp response is induced by a number of conditions which compromise the integrity of the inner membrane. One example of this is heat shock, in fact, in *E. coli*

it was initially thought that PspA may form part of the heat shock protein (Hsp) regulon. The heat shock response is induced by an increase in temperature up to 42˚C in *E. coli* and can be up-regulated by infection with phage. However, it was found that PspA synthesis was independent of σ^{32}, needed for induction of Hsps, and was up-regulated by higher temperatures than the traditional heat shock response (Brissette *et al.*, 1990). Furthermore, PspA was found to be specifically induced by synthesis of the protein encoded by phage gene *IV*, pIV, alone whereas Hsps were only induced by the full phage infection.

There are many conditions that activate the Psp response. For example, the Psp response is induced by addition of ethanol (10% v/v), hyper-osmotic shock or treatment with a proton ionophore, carbonylcyanide *m*-chlorophenylhydrazone, which eliminates the transmembrane electrochemical proton gradient and uncouples oxidative phosphorylation (Brissette *et al.*, 1990;Weiner and Model, 1994). This induces synthesis of large amounts of PspA. Similarly, inhibition of lipid and fatty acid biosynthesis and addition of organic solvents, such as n-hexane and cyclooctane, induce synthesis of PspA (Bergler *et al.*, 1994;Kobayashi *et al.*, 1998). Organic solvents are able to intercalate into biological membranes which reduces the lipid interaction, disturbing the membrane structure. The common feature of these conditions is their potential to affect the PMF and, as such, the consensus is that this may be a direct or indirect inducer of the Psp response.

The Psp response is also induced during stationary phase in *E. coli* when the energy status of the cell is relatively low (Weiner and Model, 1994). This further supports the role of the Psp response in maintaining the PMF in response to stress. In late stationary phase at pH 9 PspA becomes one of the most abundant proteins in the cell. When the *psp* operon is deleted the mutant is unable to compete with wild type in a stationary phase culture, indicating a reduction in the relative fitness of the bacteria.

4.1.3 The role of the Psp response in virulence

Many of the conditions described above are encountered during bacterial interaction with a mammalian host, for example, a rise in temperature or change in osmolarity. These environmental conditions in the host can have a significant effect on the integrity

of the cell membrane. In addition to this, during the infection process bacteria secrete a number of proteins, such as lipases and toxins, and up-regulate other secretion systems such as the T3SS (Tuanyok *et al.*, 2006), resulting in changes to the cell envelope. The Psp response is an extracytoplasmic stress response that is required to respond to these changing conditions. The important role of the Psp response to countering membrane damage caused by extracellular conditions therefore also translates into a role during virulence. In support of this, the Psp response has been shown to be important in survival and virulence in several species of bacteria (Darwin, 2013).

An important process during infection is the secretion of virulence factors. The twin-arginine translocation (Tat) pathway transports secreted proteins across lipid bilayer membranes (Lee *et al.*, 2006). The translocation of proteins by this pathway is dependent on the PMF. When the expression of substrates is increased, the transport machinery can become saturated. Increasing the expression of *pspA* can relieve the block and increase the efficiency of protein export when this occurs (DeLisa *et al.*, 2004). Similarly, mutations in Tat export components that prevent protein export have been found to increase the expression of *pspA*. PspA is also required for efficient translocation of proteins via the general secretion (Sec) pathway, such as PhoE (Kleerebezem and Tommassen, 1993). It can relieve blockages of the export machinery caused by overproduction or mutant forms of these secreted proteins (Kleerebezem and Tommassen, 1993;Jones *et al.*, 2003). The expression of PspA is probably triggered in part by a reduction in the PMF due to the accumulation of precursor proteins in the export machinery, causing ion leakage through the open export channel (Kleerebezem *et al.*, 1996).

The T3SS in *Y. enterocolitica* plays a crucial role during the infection process (Cornelis and Wolf-Watz, 1997). In this species, a Δ*pspC* mutant has a severe growth defect when the T3SS is active. In *Yersinia*, the T3SS delivers effector proteins into the cytosol of a host cell where they can then subvert various aspects of the eukaryotic machinery, such as the cytoskeleton and signalling pathways (Trosky *et al.*, 2008). This system is essential for virulence and this most likely explains why the Δ*pspC* mutant is attenuated in a mouse model of infection (Darwin and Miller, 1999;2001). Specifically, it is the secretin YscC, a component of the outer membrane apparatus of the T3SS, which is

toxic in a Δ*pspC* mutant (Darwin and Miller, 2001). The toxicity is caused by the mislocalisation of this protein to the inner membrane as deletion of its pilot protein, YscW, exacerbates the situation. This mislocalisation of the secretin causes increased permeability of the inner membrane to larger molecules, caused by leakage through a central pore in the secretin multimer, which causes lethal cytoplasmic membrane permeabilisation (Horstman and Darwin, 2012). The requirement for PspC to maintain growth during secretin expression is unrelated to its regulatory role in the Psp response, demonstrating a dual role for this protein during extracytoplasmic stress (Maxson and Darwin, 2006;Gueguen *et al.*, 2009).

The Psp response is also important for biofilm formation in *E. coli*. The *psp* operon in *E. coli* is up-regulated during biofilm growth and proper formation of a biofilm is prevented by a deletion in the *pspF* gene (Beloin *et al.*, 2004). Further evidence supporting a role for the Psp response in virulence is demonstrated in *S. enterica* and *S. flexneri* where the *psp* genes are up-regulated during macrophage infection (Eriksson *et al.*, 2003;Lucchini *et al.*, 2005).

4.1.4 Selection of the Psp response as an anti-virulence target

The Psp response was chosen as a putative antimicrobial target due to its role in virulence in the cell. It was shown to be an attenuating target in *Y. enterocolitica* (Darwin and Miller, 1999;2001), indicating its potential for exploitation as an antimicrobial target. In addition, the extensive interactions of PspA with itself and other members of the Psp response provide a range of sites for inhibitors to act. The disruption to different aspects of the system has a broad range of effects on the physiology of the bacterial cell and therefore an inhibitor targeting this system would have pleiotropic effects on virulence. The Psp response was therefore chosen for characterisation in *B. pseudomallei* to evaluate its potential as an attenuating target that could be exploited by novel antimicrobial compounds.

As a drug target, the Psp response has the potential to be targeted by a broad-spectrum antimicrobial as experimental evidence indicates that it is highly conserved amongst bacteria. In a functional *psp* operon the genes *pspFABC* are always conserved, whilst the other genes appear to be dispensable (Darwin, 2005). It is possible that the *psp*

operon may have evolved from the two genes, *pspA* and *pspF*, as these are the most common pair of co-conserved genes (Huvet *et al.*, 2010). However, many bacteria contain more complex systems, in such groups as the Gammaproteobacteria, Deltaproteobacteria and Alphaproteobacteria, which contain a number of other members of the Psp system (Darwin, 2005). Equally, a large number of bacteria contain *psp* homologues not adjacent to any other *psp* genes, for example, many bacterial genomes contain only a *pspA* homologue but no other *psp* genes. The Gram-positive bacterium *Streptomyces lividans* contains a Psp system which consists solely of the *pspA* gene (Vrancken *et al.*, 2008). *B. subtilis* also contains a stress response system (Lia) comparable to the Psp response in other bacteria, also containing a PspA homologue (Wolf *et al.*, 2010). This conservation of PspA indicates that it may have an important function in these bacteria.

The Psp response has not previously been characterised in *B. pseudomallei* and therefore the aim of this chapter was to first identify Psp homologues in *B. pseudomallei* using bioinformatics, followed by characterisation of the response as a potential target for anti-virulence therapies. In order to verify the function of the Psp response in *B. pseudomallei*, a series of investigations were carried out to determine changes in expression of the putative *psp* genes under known Psp-inducing conditions. Mutagenesis of the *psp* genes was carried out to determine the effect on cell viability when grown under stressful conditions, as well as the effect on virulence and pathogenesis.

4.2 Materials and methods

4.2.1 Bioinformatics

The following websites were used to identify Psp homologues:

http://www.ncbi.nlm.nih.gov/guide/proteins/

http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome

The following website was used to identify conserved domains in the Psp homologues:

http://stash.mrc-lmb.cam.ac.uk/SUPERFAMILY

The following website was used to identify Psp homologues, identify domains and predict operons:

http://www.microbesonline.org

4.2.2 *Burkholderia* strains

The *B. pseudomallei strains* used in this study were all derived from *B. pseudomallei* K96243. The mutant strains constructed in this study are listed in Table 4.1. All manipulations with *B. pseudomallei* were carried out in class III microbiological safety cabinets located in designated ACDP/ACGM class III laboratories.

***B. pseudomallei* strain**	**Comments**
B. pseudomallei Δ*pspA*	K96243 derivative, unmarked deletion in *BPSL2105*.
B. pseudomallei Δ*BPSS2250*	K96243 derivative, unmarked deletion in *BPSS2250*.
B. pseudomallei Δ*pspA* pBHR4.*pspA*	K96243 derivative, unmarked deletion in *BPSL2105*, complemented with pBHR4.*pspA*, Cm^R.

Table 4.1 *B. pseudomallei* mutants.

4.2.3 Plasmids

The plasmids used in this study are listed in Table 4.2.

Plasmid	Characteristics	Source
pBHR4-groS-RFP	pBHR1 derivative, *turboFP635*, P_{groES}, *rrnB*, *cat*, Cm^R. Cloning vector used for PCR amplification of RFP.	Wand *et al.*, 2011
pBHR4.*pspA*	pBHR4-groS-RFP derivative, expressing *BPSL2105* under native promoter control, Cm^R.	This study
pCR Blunt II-TOPO	Cloning vector for blunt-end PCR products, Km^R.	Invitrogen
pDM4	pNQ705 derivative, *ori*R6K, *mob*RP4, *sacBR*, *cat*, Cm^R. Suicide vector.	Milton *et al.*, 1996
pDM4.Δ*pspA*	pDM4 derivative with *BPSL2105* flanking regions for mutagenesis, Cm^R.	This study
pDM4.Δ*BPSS2250*	pDM4 derivative with *BPSS2250* flanking regions for mutagenesis, Cm^R.	This study

Table 4.2 Plasmids used in this study.

4.2.4 Oligonucleotide Primers

Primers were synthesised by Eurofins MWG. Primers used in this study are listed in Table 4.3.

Primer name	Sequence	Restriction site
***B. pseudomallei* Δ*pspA* mutant construction**		
BPSL2105 LF F	AGATCTTGAACGCGTGCATGGAATCG	*Bgl*II
BPSL2105 LF R	CATATGTTTGATCGTGCGCGAAATAG	*Nde*I
BPSL2105 RF F	CATATGGACCGCCTCGAAGCGCTGAA	*Nde*I

BPSL2105 RF R	AGATCTCGAGCATGCCGCCCGAGGTC	*Bgl*II
BPSL2105 screen F	GTCGGTCCATACGGCTATCC	-
BPSL2105 screen R	GCGATCGATTGCTGGATTTG	-
***B. pseudomallei* Δ*BPSS2250* mutant construction**		
BPSS2250 LF F	AGATCTCAGGCGAGCTGCTGGGTATG	*Bgl*II
BPSS2250 LF R	CATATGTCTGCCGTCACTGCTGTGTT	*Nde*I
BPSS2250 RF F	CATATGATGCGCGAGCACGACGGGGA	*Nde*I
BPSS2250 RF R	AGATCTGTCGAGGCGATGACGATCGG	*Bgl*II
BPSS2250 screen F	CGTTCGTTCCGGTCAATTGC	-
BPSS2250 screen R	TCTGCTCGTCGGAGAGGATG	-
***B. pseudomallei* pDM4 integrant screening**		
CAM-screen F	ATCCCAATGGCATCGTAAAG	-
CAM-screen R	TAAGCATTCTGCCGACATGG	-
SacB F	CGGCTACCACATCGTCTTTG	-
SacB R	GCAATCAGCGGTTTCATCAC	-
***B. pseudomallei* species screening**		
SR1	ACCGCGTATGAAGGGATGTC	-
SR5	ACGCGCACGCACCTGCTGAAC	-
14F5	ACCTGCTGCCGGGCTACGACTTCA	-
14R5	CACCTTGCCGACCCACGAGATGC	-
SRT5	AAAGCTGCGCGCTCGGCATC	-
***B. pseudomallei* Δ*pspA* complementation**		
BPSL2105 prom F	CGATCGGCGCTGAACGCGTGCATGGA	*Pvu*I

BPSL2105 comp R	GGATCCTTACTGCGCCGGCGTGTTCA	*Bam*HI
pBBR1 F2	TGTAGTCGACGCAACGCATAATTGTTGTCG	-
pBBR1 R4	TAGCGTCGACCTCGCCATCGTCCAGAAAAC	-
RT-PCR		
RT BPSL2105 F	CGCGCACGATCAAAGGTCTG	-
RT BPSL2105 R	GCCGCTGTTCGTATCGGCTG	-
RT BPSL2106 F	CAGATCGAACGGGCGAAGTC	-
RT BPSL2106 R	TCGGCCTCCTGAGCAAATAC	-
RT BPSS2250 F	CGTTCGTTCCGGTCAATTGC	-
RT BPSS2250 R	TCTGCTCGTCGGAGAGGATG	-
RT 16S rRNA F	GATGACGGTACCGGAAGAATAAGC	-
RT 16S rRNA R	CCATGTCAAGGGTAGGTAAGGTTT	-

Table 4.3 Primers used in this study.
Restriction sites in primers are underlined.

4.2.5 Mutant construction and complementation

4.2.5.1 Conjugation

A single colony of *B. pseudomallei* K96243 was used to inoculate 10 ml LB broth which was incubated overnight at 37˚C. The *E. coli* S17-λ *pir* strain containing the relevant deletion construct was grown overnight in 10 ml LB broth supplemented with 50 μg/ml chloramphenicol. A 1 ml aliquot of each culture was centrifuged and the pellet resuspended in 500 μl LB broth with no antibiotics. Aliquots of 10 μl of both *B. pseudomallei* K96243 and *E. coli* S17-λ *pir* were added to a nitrocellulose membrane placed on a LB agar plate. This was incubated overnight at 37˚C to allow conjugation to take place. The following day filters were removed from the plates and vortexed with 1

ml PBS to remove the bacteria. This was then plated onto LB agar containing appropriate antibiotics and incubated at 37˚C for 48 hours to select for integrants. Selected integrant colonies were plated onto 250 μg/ml chloramphenicol to check for resistance. Colonies were also screened by PCR using speciation primers to ensure a *B. pseudomallei* integrant and not an *E. coli* colony had been selected.

4.2.5.2 **Sucrose selection**

The chosen pDM4 integrant was used to inoculate 10 ml LB broth and incubated at 37˚C overnight. The cultures were diluted to an optical density at 590 nm of between 0.38 and 0.4 in LB broth and serially diluted. Appropriate dilutions were plated onto LB agar supplemented with 10% sucrose, no NaCl and incubated for up to a week at 24˚C. Resulting colonies were checked for chloramphenicol sensitivity and mutants identified by colony PCR and Southern hybridisation.

4.2.6 *In vitro* assays

4.2.6.1 **Growth curves**

A single colony was used to inoculate 10 ml LB broth. This was incubated at 37˚C overnight with gentle agitation. In the morning, 1 ml of the overnight was added to 99 ml pre-warmed LB broth. A growth curve was performed by measuring optical density at 590 nm using a WPA Colourwave CO7500 colorimeter once an hour for 7 hours. Where viable cells were enumerated, 1 ml culture was removed and serially diluted in PBS. Appropriate dilutions were plated onto LB agar and incubated overnight at 37˚C.

The growth medium was modified when measuring growth under Psp-inducing conditions. Growth in ethanol was carried out in LB broth containing either 2% or 5% ethanol (v/v). The period of growth was extended to 72 hours, with viable cell counts carried out daily. Growth under conditions of hyper-osmotic shock was conducted by addition of 0.3 M or 0.75 M NaCl to LB broth. The number of viable cells was counted after 24 hours.

For stationary phase survival experiments, a single colony was used to inoculate 10 ml LB broth, which was incubated at 37˚C overnight with gentle agitation. In the morning

the culture was diluted in pre-warmed 100 ml LB broth to an optical density at 590 nm of 0.01. This was incubated at 37˚C with gentle agitation for up to 10 days. Viable cells were enumerated every 24 hours by removing 1 ml culture and serially diluting in PBS. Appropriate dilutions were plated onto LB agar and incubated overnight at 37˚C. The pH was measured daily using a Piccolo Plus pH meter (Hanna). Stationary phase survival experiments at alkaline pH were carried out as above but cultures were grown in media that had been adjusted to pH 9 by addition of NaOH.

4.2.6.2 **Heat shock survival assay**

Overnight cultures were diluted in 10 ml LB broth to an optical density at 590 nm of 0.01. Cultures were incubated at 37˚C for 4 hours and then divided into 1 ml aliquots. The 1 ml aliquots were incubated at 50˚C, 60˚C, 70˚C or 80˚C in a water bath for 10 minutes. Following this, the bacteria were serially diluted in PBS and plated onto LB agar in order to enumerate viable cells.

4.2.6.3 **pH survival assay**

Overnight cultures were prepared as previously described and diluted in 10 ml LB broth to an optical density at 590 nm of 0.01. After two hours growth at 37˚C, 100 µl of this culture was diluted in LB broth that had been adjusted to pH 3.5-9.5 by addition of HCl or NaOH. The cultures were incubated at 37˚C for 24 hours, after which viable cell counts were performed as described previously.

4.2.6.4 **Minimum inhibitory concentration determination**

Stock solutions of kanamycin, H_2O_2, HCl, NaCl, lysozyme and deferoxamine were prepared in water to give a final concentration of 10 mg/ml. These solutions were diluted in LB broth to a concentration of 256 µg/ml. A 100 µl aliquot of the above solutions was added to 100 µl LB broth in the first column of a 96-well plate and further diluted 1:2 into LB broth across each column (total 100 µl per well). The assays for testing the MIC of NaOH and NH_4OH were set up as described above but with some variations. A 1 M NaOH stock was prepared and diluted to a concentration of 4096 µg/ml (approximately 0.1 M) in LB broth. The NH_4OH assay was set up by preparing a 0.15 M stock solution, which is equivalent to approximately 5 mg/ml NH_4OH. The

above solutions were diluted in a 96-well plate by adding a 100 µl aliquot of the stock solution to 100 µl LB broth in the first column and serially diluting across the plate.

The bacterial inoculum was prepared by growing *B. pseudomallei* overnight at 37˚C in LB broth. This was used to inoculate a 100 ml LB broth and incubated at 37˚C for 6 days. The culture was adjusted to an optical density at 590 nm of 0.35-0.40 in LB broth, equivalent to approximately 1 x 10^8 cfu/ml, and then serially diluted to a concentration of 1 x 10^6 cfu/ml. A 100 µl aliquot of this culture was added to each compound dilution that had been dispensed into the test wells. This provided a final bacterial concentration of 5 x 10^4 cfu/ml and a compound dilution range of 64-0.03125 µg/ml for kanamycin, H_2O_2, HCl, NaCl, lysozyme and deferoxamine. The concentration of NaOH ranged from 1024 µg/ml to 0.5 µg/ml and approximately 1270-0.62 µg/ml for NH_4OH (equivalent to 18 mM-9 µM). A positive control was included where bacteria were grown in media without the test reagent, as well as a negative control of LB broth.

The inoculated dilution plates were incubated at 37˚C for 18 hours. The optical density was recorded for each well and the MIC was defined as the dilution of antimicrobial compound where the percentage growth was reduced to below 10% relative to growth of *B. pseudomallei* in media only (positive control).

4.2.7 Tissue culture

4.2.7.1 **Mammalian cell lines**

The cells used in this study were J774A.1 mouse BALB/c macrophages. J774A.1 cells were grown in 25 ml DMEM at 37˚C with 5% CO_2. Cells were passaged every 3-4 days by scraping and re-suspending in fresh media.

4.2.7.2 **Macrophage intracellular survival assay**

One colony of wild-type *B. pseudomallei* K96243, *B. pseudomallei* Δ*pspA* or *B. pseudomallei* Δ*pspA* pBHR4.*pspA* was used to inoculate 10 ml LB broth and incubated overnight with agitation. The complemented strain was grown overnight in 10 ml LB broth with 50 µg/ml chloramphenicol. The overnight cultures were diluted in L-15 to give an optical density at 590 nm of between 0.35-0.40. This was serially diluted to 1 x

10^6 cfu/ml. Where stationary phase cells were used to infect J774A.1 macrophages, an overnight culture was diluted in 100 ml LB broth to give an initial optical density at 590 nm of 0.01. The cultures were incubated with agitation at 37˚C for 6 days. On the sixth day the cultures were diluted in L-15 media to an optical density at 590 nm of between 0.35-0.4 and then serially diluted to 1 x 10^7 cfu/ml.

J774A.1 cells were seeded in a 24-well plate in DMEM at a concentration of 4 x 10^5 cells/ml. The cells were incubated at 37˚C with 5% CO_2 overnight until they had reached a density of approximately 1 x 10^6 cells/ml. The DMEM was removed from the J774A.1 cells and replaced with L-15 media containing either 1 x 10^6 cfu/ml (exponential phase) or 1 x 10^7 cfu/ml (stationary phase) bacteria. The cells were incubated at 37˚C for 30 minutes to allow infection to take place. The media was removed and the cells washed three times with PBS. Following this, 1 ml of 1 mg/ml kanamycin in L-15 was added to each well and incubated at 37˚C in order to kill any extracellular bacteria. After 1 hour the antibiotic media was removed and replaced with 1 ml of 250 μg/ml kanamycin in L-15 and incubated at 37˚C for 24 hours. At various time points the antibiotic media was removed and the cells lysed by addition of 1 ml water with pipetting. The lysate was serially diluted and plated onto LB agar in order to enumerate viable cells. Duplicate wells were lysed for each *B. pseudomallei* strain at each time point.

4.2.7.3 **LDH assay**

The CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) was used to quantitatively measure the amount of lactate dehydrogenase (LDH) released upon cell lysis. A J774A.1 intracellular survival assay was carried out as described above. After 24 hours, 50 μl supernatant was removed from the wells and added to 50 μl substrate mix in a 96-well plate. Duplicate samples were taken per well. The 96-well plate was incubated at room temperature for 30 minutes, protected from light. 50 μl stop solution was added to each well and the absorbance recorded at 490 nm. The following controls were carried out with the CytoTox 96® Assay: cell spontaneous LDH release (uninfected J774A.1 cells), maximum LDH release (cells lysed with 100 μl lysis solution 45 minutes before the 24 hour time point) and culture medium background.

4.2.8 *In vivo* studies

4.2.8.1 Determination of median lethal dose in mice

Groups of 6 BALB/c mice were challenged intraperitoneally with *B. pseudomallei* ranging from 1 x 10^1 to 1 x 10^6 cfu. The mice were monitored for signs of disease for 5 weeks and culled at predetermined humane end points. At the end of the experiment, surviving mice were culled and the spleens removed aseptically. Spleens were homogenised in 1 ml PBS and serial dilutions were plated onto LB agar to determine the bacterial burden. The MLD was determined by the Reed and Muench method (Reed and Muench, 1938).

4.2.8.2 Infection of *Galleria mellonella*

The challenge dose was prepared by inoculating 100 ml LB broth with 1 ml of an overnight culture and incubating for 6 days at 37˚C with agitation. The culture was adjusted to an optical density at 590 nm of 0.4 in PBS. This was serially diluted in 9 ml PBS and relevant dilutions plated out to enumerate the viable cells. Groups of ten *G. mellonella* weighing 0.2-0.3 grams were challenged with strains of *B. pseudomallei* by injecting 10 µl bacteria into the right foremost proleg. The *G. mellonella* were challenged with doses ranging between 1 x 10^2 cfu and 1 x 10^4 cfu of either wild-type *B. pseudomallei*, *B. pseudomallei* Δ*pspA* or *B. pseudomallei* Δ*pspA* pBHR4.*pspA*. A control group of PBS-injected larvae was also included. The larvae were incubated at 37˚C and monitored over 3 days. Larvae were scored as dead when they changed in colour from cream to black, ceased to move and failed to respond to touch.

The number of bacteria colonising larvae after 24 hours was determined by infecting a further group of five *G. mellonella* as described above. These were then culled after 24 hours. Before being culled, the larvae were placed on ice in order to sedate them. The last 2 mm of the tail was removed with scissors and the larva placed upright in a microcentrifuge tube for up to 10 minutes to allow the haemolymph to drain. The volume of haemolymph recovered was estimated by pipetting. 20 µl of this was serially diluted in 180 µl PBS, 100 µl of which was plated onto LB agar supplemented with 50 µg/ml gentamycin to select for *B. pseudomallei* colonies.

4.3 Results

4.3.1 Identification of putative Phage-shock proteins in *B. pseudomallei*

An initial aim for the project was to identify Psp homologues in *B. pseudomallei*, followed by more in-depth characterisation of the Psp response. Therefore, a bioinformatics study was performed using a range of tools to search for Psp homologues. Firstly, a search was carried out using an online database to identify previously annotated Psp proteins in *B. pseudomallei*. The search revealed a number of annotated proteins, but these were not present in all strains of *B. pseudomallei*. For example, *B. pseudomallei* strains NCTC 13177 and BCC215 contained an annotated PspA homologue, whereas *B. pseudomallei* K96243 contained no such annotations.

A further search was conducted using a Basic Local Alignment Search Tool (BLAST). This was used to compare the translated amino acid sequences from the *E. coli* and *Y. enterocolitica* Psp systems with the annotated Psp proteins identified in *B. pseudomallei*. The first protein to be investigated was PspA as it is the most highly conserved protein in the Psp system. The *E. coli* and *Y. enterocolitica* PspA amino acid sequences were 79% identical and so both were used for comparison in BLAST searches. The search criteria for positive hits required an E value below 10^{-4} with proteins with an excess of 20% identity.

The PspA homologue in *B. pseudomallei* K96243 was identified as BPSL2105, a conserved hypothetical protein. This protein was designated as PspA for *B. pseudomallei* K96243. *B. pseudomallei* PspA is 226 amino acids in length and showed 22% identity to PspA from *Y. enterocolitica*. There was no significant homology with the *E. coli* PspA amino acid sequence. The gene *BPSL2015* was predicted to form an operon with another gene, *BPSL2106*, which is a putative membrane protein (Figure 4.3). The upstream gene, *mrcA*, is divergently transcribed and the downstream gene, *mscL*, is not predicted to be part of the *pspA* operon (Price *et al.*, 2005).

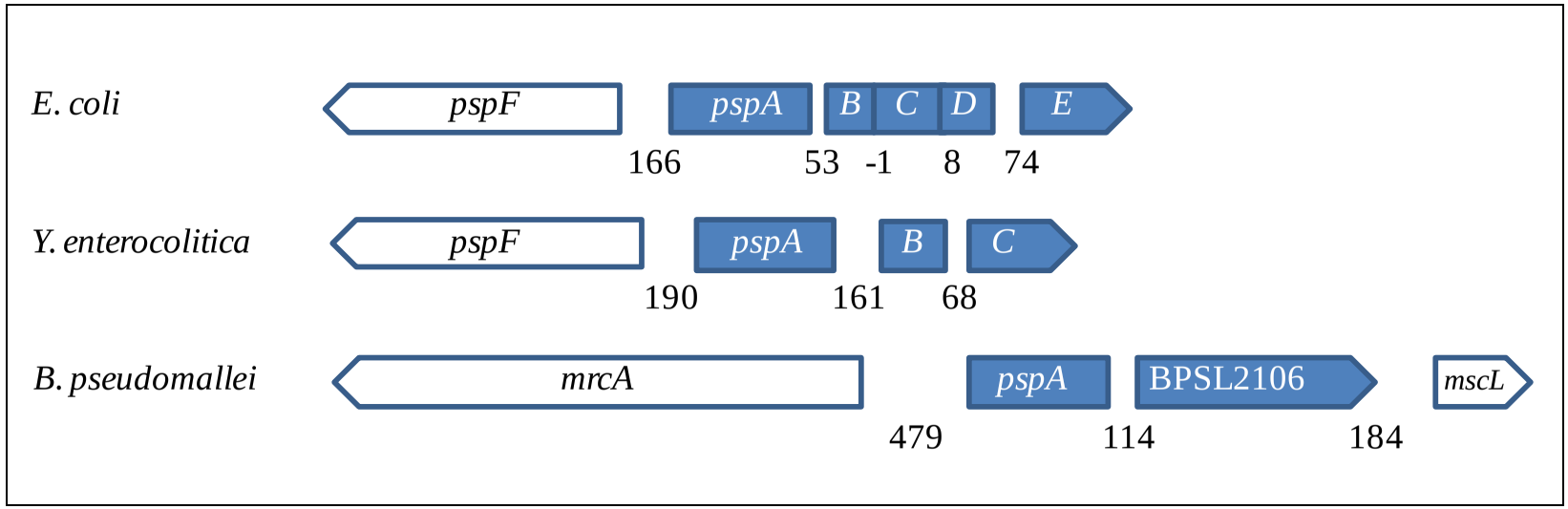


Figure 4.3 *B. pseudomallei pspA* operon.

The *B. pseudomallei psp* operon is found on chromosome 1 and contains two genes, *pspA* and *BPSL2106*. *mscL* is not predicted to be part of the *psp* operon. The gene upstream, *mrcA*, is divergently transcribed.

The PspA amino acid sequence from K96243 was compared to other strains of *B. pseudomallei*. The search found homologues in all strains with either 99% or 100% identity to BPSL2105. Homologues were also found in other *Burkholderia* species including the less pathogenic *B. thailandensis* species and close relative *B. mallei*, which both had 99-100% identity to BPSL2105. The search found PspA homologues throughout the *Burkholderia* genus, including some members of the *B. cepacia* complex with up to 93% identity to *B. pseudomallei* K96243, but many more distant relatives of *B. pseudomallei* did not contain any evident Psp systems.

A second BLAST search was carried out against *E. coli* and *Y. enterocolitica* PspF amino acid sequences, which are 80% identical. A number of possible PspF homologues were found in *B. pseudomallei* K96243. The putative PspF homologues had identities to *Y. enterocolitica* PspF ranging from 33% to 48% and most were annotated as transcriptional response regulators (Table 4.4). Searches were also performed in order to identify putative PspB and PspC proteins but no homologues were present in the *B. pseudomallei* genome within the set parameters.

PspF proteins contain several domains important for their function, including an AAA^+ domain at the N-terminal and a C-terminal DNA-binding domain. The putative PspF proteins located in *B. pseudomallei* K96243 all contained at least part of one or both domains. In order to refine the list a further search was carried out using an ExPASy domain search program (Gough *et al.*, 2001). When comparing the conserved domains in PspF, one protein, BPSS2250, was found to be most similar to the *Y. enterocolitica* PspF as it was of similar size and domain structure (Table 4.5). BPSS2250 was found to be 36% identical to *Y. enterocolitica* PspF and 38% identical to *E. coli*. The protein was 322 amino acids long, which is comparable to other known PspF proteins (Jovanovic *et al.*, 1996). BPSS2250 was annotated as a sigma-54 interacting transcriptional regulator and contained common domains found in all PspF proteins, such as AAA-type ATPase and DNA-binding domains. The ATPase domains and DNA-binding domain were located in the same region in all three proteins.

Protein	**Amino acids**	**Description**	**Identity to *E. coli***	**Identity to *Y. enterocolitica***
BPSS2249	456	Sigma-54 interacting response regulator	43%	43%
BPSL0427 (*dctD*)	451	C4-dicarboxylate transport transcriptional regulatory protein	47%	44%
BPSS0063 (*dctD*)	448	C4-dicarboxylate transport transcriptional response regulator	46%	39%
BPSL2475	486	Sigma-54 dependent regulatory protein	42%	41%
BPSL2316 (*glnG*)	511	Nitrogen regulation protein NR	46%	48%
BPSL0609	462	Fis family regulatory protein	37%	44%
BPSL2474	461	Sigma-54 interacting regulatory protein	44%	43%
BPSL1562	467	Putative transcriptional regulatory protein	44%	44%
BPSS2132	506	Sigma-54 dependent transcriptional regulator	44%	43%
BPSS2250	322	Sigma-54 interacting transcriptional regulator	38%	36%
BPSS0205 (*prpR*)	694	Sigma interaction transcriptional regulator	45%	40%

BPSS0596	646	Sigma-54 activated regulatory protein	45%	43%
BPSL1887	463	Putative sigma-54 related transcriptional regulatory protein	35%	41%
BPSL2423	465	Sigma-54 interacting response regulator protein	40%	33%

Table 4.4 Putative PspF homologues in *B. pseudomallei* K96243.

A BLAST search was carried out with PspA sequences from *E. coli* strain K-12 substrain MG1655 (GI: 90111246) and *Y. enterocolitica* subspecies enterocolitica 8081 (GI: 123442383).

	P-loop NTPase (N-terminal)		Homeodomain-like (C-terminal)	
	Region	E value	Region	E value
PspF (*Y. enterocolitica*)	8-248	1.2e-65	222-331	6.72e-10
PspF (*E. coli*)	9-220	1.27e-65	222-325	1.93e-6
BPSS2250 (*B. pseudomallei*)	12-224	1.23e-65	223-310	1.68e-9

Table 4.5 Similarities between conserved domains in PspF proteins.

An ExPASy domain search was carried out using *E. coli* and *Y. enterocolitica* PspF and *B. pseudomallei* BPSS2250 amino acid sequences.

4.3.2 Characterisation of the Psp response in *B. pseudomallei*

The Psp response reacts to changing conditions in the environment by up-regulating the *psp* operon. The increase in transcription results in a significant increase in the concentration of PspA in the cell (Brissette *et al.*, 1990). This is thought to counter the detrimental effect caused by unfavourable environmental conditions by forming large structures to stabilise the inner membrane (Hankamer *et al.*, 2004;Standar *et al.*, 2008). In order to characterise the Psp response in *B. pseudomallei*, RT-PCR was used to analyse the change in gene expression within the Psp system under stressful conditions. The conditions selected are known to cause up-regulation of PspA in *E. coli*.

4.3.2.1 Induction of *pspA* by heat shock

Heat shock at 50˚C causes a transient increase in the concentration of PspA in *E. coli* (Brissette *et al.*, 1990). To investigate the effect of temperature on PspA in *B. pseudomallei*, RNA was collected from cultures shocked at 50˚C. Equivalent samples were collected from cultures incubated at 37˚C as a control. The RNA was reverse transcribed and a PCR performed using primers RT BPSL2105 F and RT BPSL2105 R, which amplified a region within the *pspA* gene. An internal control was carried out using primers RT 16S rRNA F and RT 16S rRNA R. The PCR product was visualised using gel electrophoresis, which provided a semi-quantitative result. The results found that *pspA* expression increased at 50˚C during the 10 and 15 minute time points compared to the controls at 37˚C (Figure 4.4). RT-PCR was carried out on triplicate samples, with Figure 4.4 showing a representative gel.

4.3.2.2 Induction of *pspA* by ethanol and osmotic stress

In *E. coli* the Psp response is induced by many different conditions, including ethanol (10% v/v) and hyper-osmotic shock. An experiment was carried out using ethanol as an inducer of the Psp response. *B. pseudomallei* cultures were grown to exponential phase and shocked by addition of 10% ethanol. However, the RT-PCR results showed no increased expression of *pspA* relative to the controls without ethanol (data not shown).

A similar experiment was carried out using NaCl to cause PspA induction in response to hyper-osmotic shock. Previous work has shown that PspA is induced by concentrations

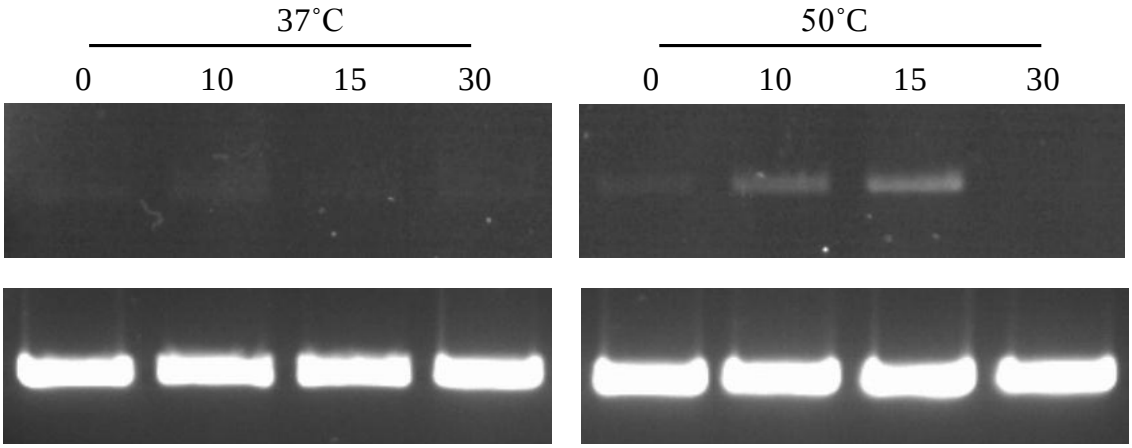


Figure 4.4 RT-PCR amplification of *pspA* in *B. pseudomallei* during induction by heat.

RNA samples were collected at 0, 10, 15 and 30 minute time points, purified and residual DNA removed. Following this, the samples were reverse transcribed and the resulting cDNA was amplified by PCR using primers amplifying a region either from the *pspA* gene (top row) or from 16S rRNA genes (bottom row). Controls were carried out using RNA in the absence of RT (not shown).

of 0.3 M and 0.75 M NaCl in *E. coli* (Brissette *et al.*, 1990), therefore, an experiment was carried out in order to study the effect of 0.3 M NaCl on *pspA* expression in *B. pseudomallei*. The cultures were grown to exponential phase as before and subjected to high salt conditions. RNA was extracted and RT-PCR was carried out to compare the concentration of *pspA* mRNA to un-shocked samples. The results showed no increased expression of *pspA* compared to controls (data not shown).

4.3.2.3 **Creation of *B. pseudomallei* Δ*BPSS2250***

In *E. coli* and *Y. enterocolitica* the *pspF* gene is found in close proximity to *pspA* as it is divergently transcribed from the *pspA* operon (Darwin, 2005). In *B. pseudomallei* a number of putative PspF homologues were identified, with BPSS2250 being the most similar in terms of amino acid sequence and conserved domains. PspF is the transcriptional regulator of the Psp response and is required for up-regulation of PspA in *E. coli* (Jovanovic *et al.*, 1996). Therefore the deletion of the putative PspF homologue in *B. pseudomallei* was predicited to prevent transcription from the *BPSL2105* operon when stimulated by Psp-inducing conditions. In order to investigate its role in the expression of *pspA* in *B. pseudomallei*, a deletion mutant was created in the *BPSS2250* gene.

A DNA construct for the deletion of *BPSS2250* in *B. pseudomallei* was made in *E. coli* by cloning a truncated version of the gene into the suicide plasmid pDM4. The truncated gene was produced by amplifying upstream and downstream flanking regions of *BPSS2250* by PCR using primers BPSS2250 LF F and BPSS2250 LF R (upstream), BPSS2250 RF F and BPSS2250 RF R (downstream). The *Nde*I and *Bgl*II restriction sites were incorporated into either end of the flanks to aid manipulation. The resulting flanks were cloned into pCR-Blunt II-TOPO followed by digestion with *Nde*I and *Bgl*II for ligation with the pDM4 suicide vector. Ligations were transformed into *E. coli* DH5-λ *pir* by electroporation and plated onto LB agar plates containing 50 μg/ml chloramphenicol. Transformants were confirmed by restriction digest and nucleotide sequencing. The pDM4.Δ*BPSS2250* construct (Figure 4.5) containing a truncated version of *BPSS2250* with a deletion from 29 to 933 base pairs was then transformed into *E. coli* S17-λ *pir* by electroporation.

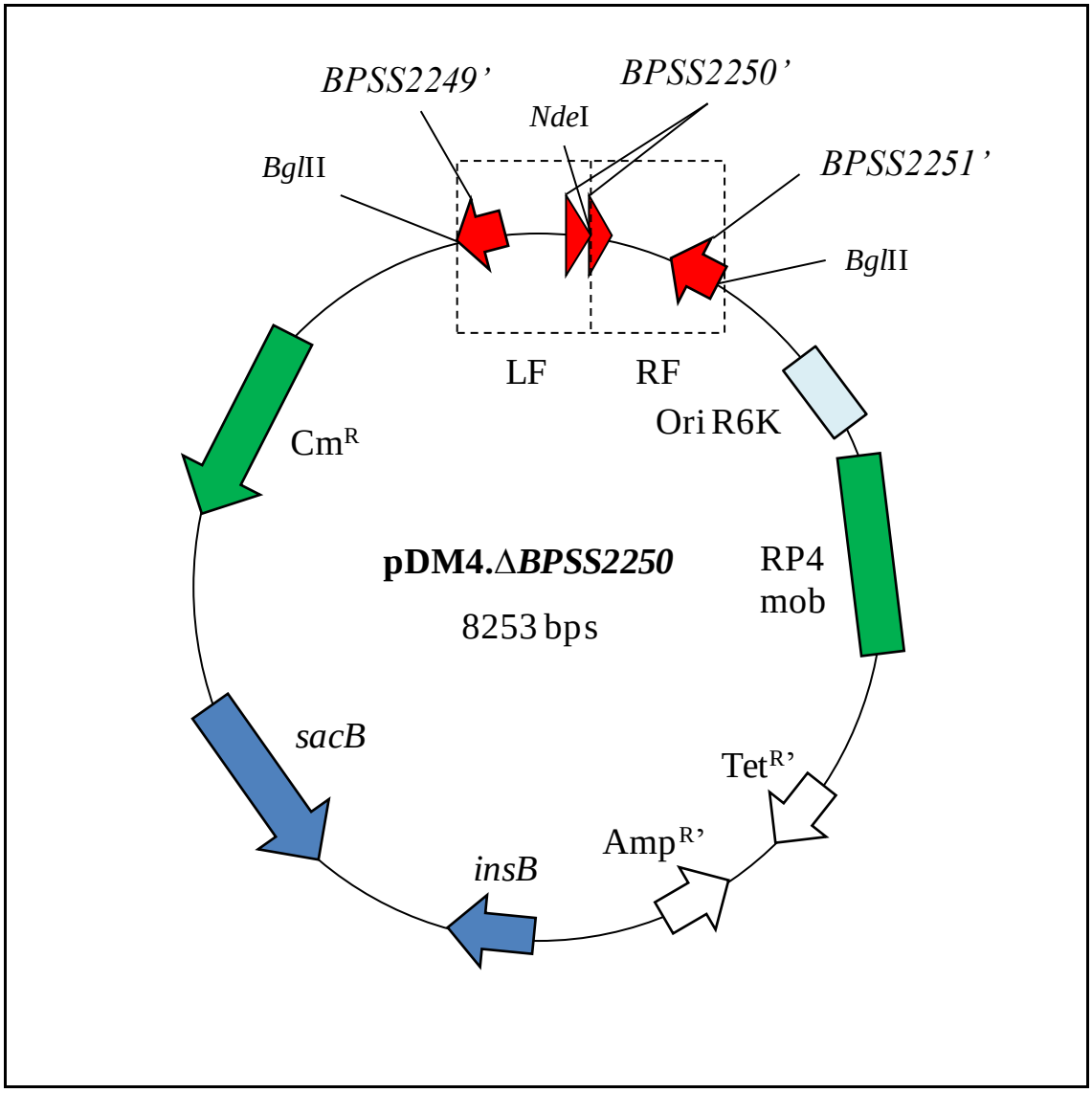


Figure 4.5 Plasmid map of pDM4.Δ*BPSS2250*.

Flanking regions of *BPSS2250* were amplified from genomic DNA. Restriction sites were introduced at either end of the PCR products by the primers. The resulting flanking regions were each cloned into pCR-Blunt II-TOPO. These plasmids containing left and right flanks were digested using *Bgl*II and *Nde*I restriction enzymes. The pDM4 suicide vector was linearised by digestion with *Bgl*II. Following this, the pDM4.Δ*BPSS2250* construct was created by carrying out a three way ligation with the left and right flanks of *BPSS2250* and linearised pDM4.

The pDM4.Δ*BPSS2250* construct was transferred by conjugation into *B. pseudomallei* K96243. *B. pseudomallei* integrants were selected for by plating onto LB agar containing chloramphenicol and ampicillin, where ampicillin was used to eliminate the *E. coli* donor. The first cross-over created a merodiploid strain, produced by the integration of the deletion construct into the genome by homologous recombination with the *BPSS2250* gene. Successful pDM4.Δ*BPSS2250* integration was confirmed by colony PCR using primers CAM-screen F, CAM-screen R, SacB F and SacB R to verify the presence of the *sacB* gene and chloramphenicol cassette, which are both present on the pDM4 vector backbone.

A sucrose selection was carried out with the *B. pseudomallei* pDM4.Δ*BPSS2250* integrants to isolate the deletion strain *B. pseudomallei* Δ*BPS2250*. The resulting colonies were screened for sensitivity to chloramphenicol by plating onto LB agar containing 50 μg/ml chloramphenicol. This method identified six potential *B. pseudomallei* Δ*BPSS2250* mutants.

The deletion of *BPSS2250* in *B. pseudomallei* Δ*BPSS2250* was confirmed by PCR using primers BPSS2250 screen F and BPSS2250 screen R. A Southern blot was also carried out to confirm the deletion of *BPSS2250* (Figure 4.6).

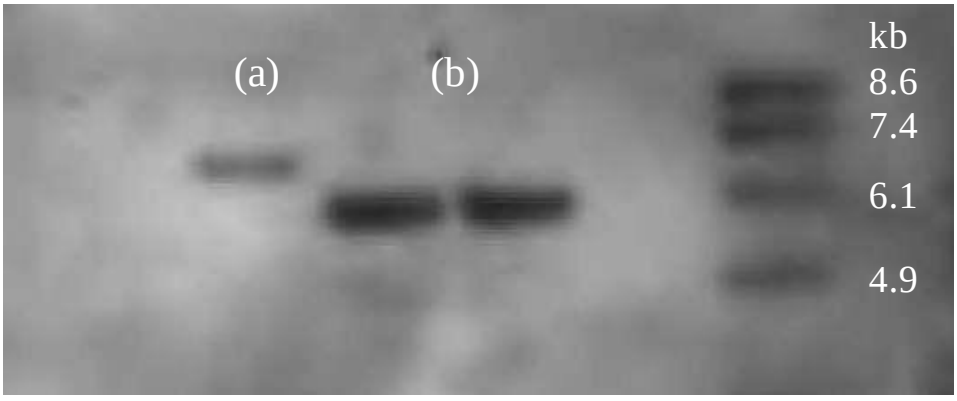


Figure 4.6 Southern blot confirming the deletion of *BPSS2250* in *B. pseudomallei*.

Wild-type and mutant genomic DNA was digested with *Eco*NI and *Cla*I restriction enzymes. Primers BPSS2250 LF F and BPSS2250 LF R were used to make the DIG-labelled probe. (a) wild-type *BPSS2250* (6923 bp); (b) truncated Δ*BPSS2250*, clones 5 and 24 (6023 bp).

4.3.2.4 **Induction of *pspA* in a Δ*BPSS2250* deletion mutant**

In *B. pseudomallei*, PspA is up-regulated by heat shock at 50˚C. In order to determine whether BPSS2250 is a transcriptional activator of PspA, RT-PCR was carried out to observe the expression of *pspA* under inducing conditions in a *B. pseudomallei* Δ*BPSS2250* mutant. It was predicted that the deletion of *BPSS2250* would result in no change in expression of *pspA* upon induction of the Psp response by heat shock.

Overnight cultures of *B. pseudomallei* and *B. pseudomallei* Δ*BPSS2250* were used to inoculate 100 ml LB broth. These cultures were grown at 37˚C with agitation for approximately 5 hours to allow the cells to reach exponential phase. The cultures were then shocked at 50˚C for up to 30 minutes and RNA collected at various time points. Equivalent samples were collected from cultures incubated at 37˚C. RT-PCR was performed using primers RT BPSL2105 F and RT BPSL2105 R to amplify a region within the *pspA* gene. The results showed that *pspA* was up-regulated at 50˚C regardless of whether *BPSS2250* was present or not (Figure 4.7), indicating that BPSS2250 is unlikely to be a PspF homologue.

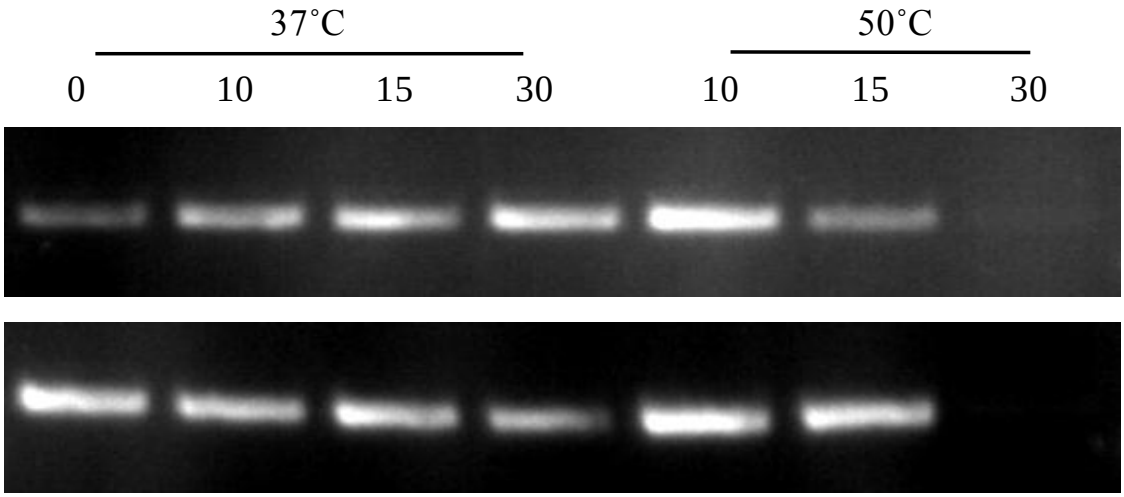


Figure 4.7 RT-PCR amplification of *pspA* in *B. pseudomallei* and *B. pseudomallei* Δ*BPSS2250* during induction by heat.

RNA samples were collected at 0, 10, 15 and 30 minute time points from *B. pseudomallei* wild-type (top) and *B. pseudomallei* Δ*BPSS2250* (bottom). The RNA was purified and residual DNA removed. Following this, the samples were reverse transcribed and the resulting cDNA was amplified by PCR using primers amplifying a region from the *pspA* gene. Controls were carried out using primers amplifying a region from 16S rRNA and RNA in the absence of RT (not shown).

4.3.3 Creation of *B. pseudomallei* Δ*pspA*

The results from the earlier induction experiments indicated that *pspA* expression is induced by heat shock in *B. pseudomallei*. This result is similar to the effect of heat shock on PspA in *E. coli* (Brissette *et al.*, 1990) and therefore it was decided to investigate the role of PspA in *B. pseudomallei* in more detail. In bacteria that possess several *psp* genes, such as *E. coli* and *Y. enterocolitica*, PspA acts as the main effector of the Psp response, as well as having an important role in the regulation of the system (Darwin, 2005). This makes it an ideal target as it is likely to have the greatest impact on the system when it is inactivated. In order to characterise the role of the Psp response in *B. pseudomallei*, an unmarked deletion mutation was generated in *BPSL2105*, encoding the PspA homologue identified by bioinformatics.

A DNA construct was created for deletion of *pspA* in *B. pseudomallei* using the same method as for *BPSS2250*. Primers BPSL2105 LF F, BPSL2105 LF R, BPSL2105 RF F and BPSL2105 RF R were used to produce the two flanking regions. These were sub-cloned into pCR-Blunt II-TOPO before being transferred into pDM4 by digestion and ligation. The final construct, pDM4.Δ*pspA* (Figure 4.8), was transformed into *E. coli* S17-λ *pir* by electroporation and selected for on LB agar containing 50 μg/ml chloramphenicol.

A conjugation was carried out with *B. pseudomallei* K96243. Integrants were selected for by plating onto 50 μg/ml chloramphenicol and 100 μg/ml ampicillin. Selected integrants were confirmed by colony PCR using primers CAM-screen F, CAM-screen R, SacB F and SacB R (Figure 4.9).

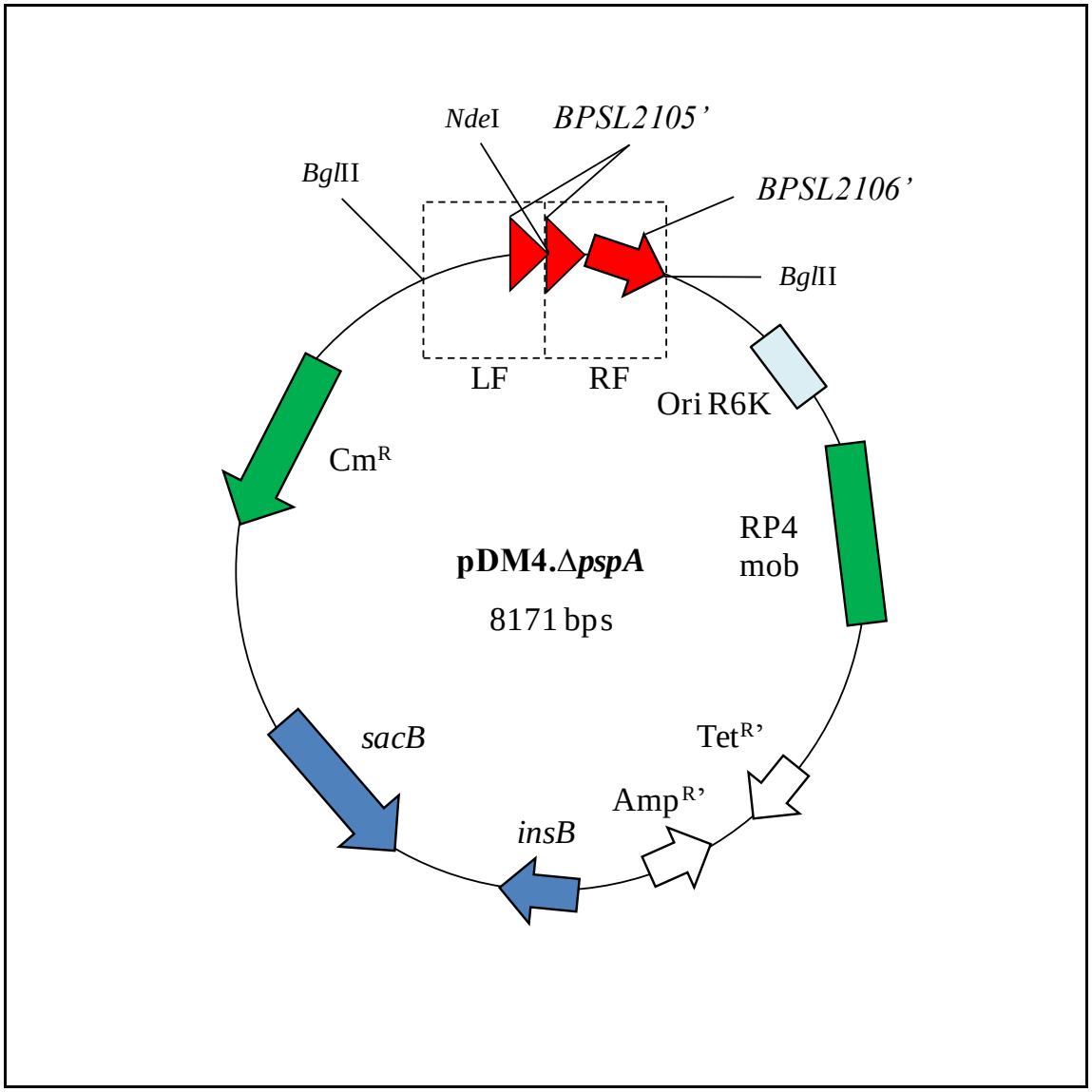


Figure 4.8 Plasmid map of pDM4.Δ*pspA*.

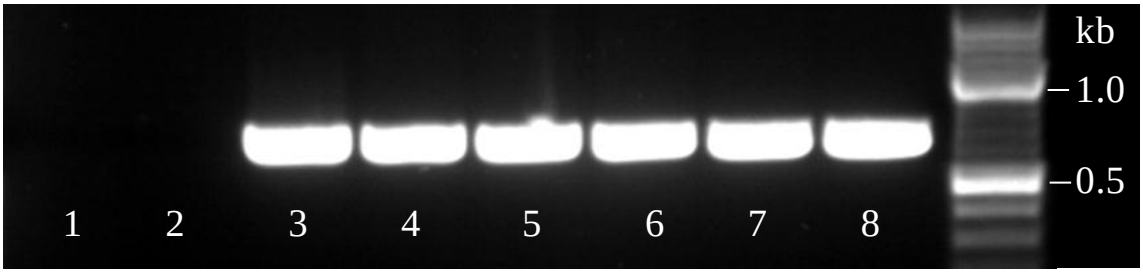


Figure 4.9 PCR confirmation of the *B. pseudomallei* pDM4.Δ*pspA* integrant.

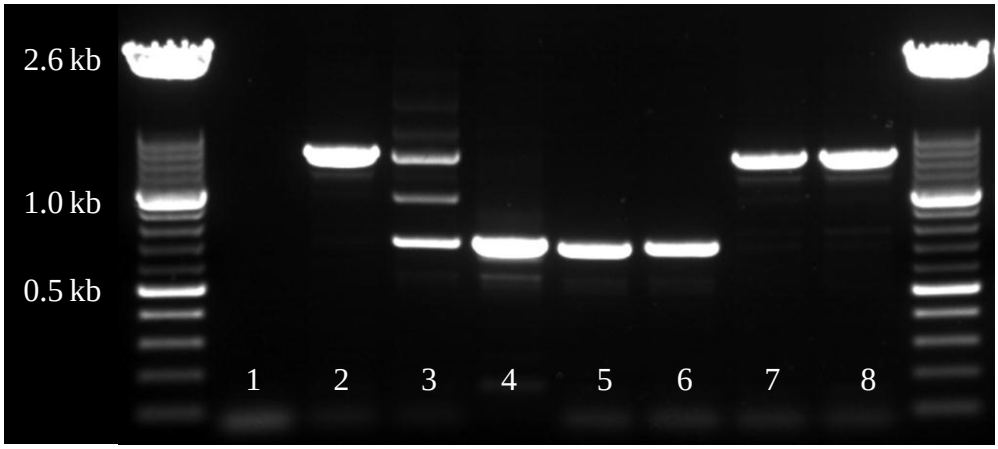
A PCR was carried out using primers SacB F and SacB R to confirm the presence of the *sacB* gene on pDM4. The template was produced by boiling a single colony in 100 µl H_2O, 2 µl of which was then used in the PCR. The predicted product size was 696 bp. Lane 1, *B. pseudomallei* K96243 genomic DNA; lane 2, water; lane 3, pDM4.Δ*pspA* plasmid DNA; lane 4-8, integrants 1-5.

A sucrose selection was carried out with a *B. pseudomallei* pDM4.Δ*pspA* integrant to isolate the deletion strain *B. pseudomallei* Δ*pspA*. Using this method of screening, four potential deletion mutants were produced that were sucrose-resistant but chloramphenicol sensitive.

B. pseudomallei Δ*pspA* has a deletion in *pspA* from bases 36 to 634. The deletion was confirmed by PCR using primers BPSL2105 screen F and BPSL2105 screen R (Figure 4.10). This generated a product 712 base pairs in size from *B. pseudomallei* Δ*pspA* (Figure 4.10A, lanes 5 and 6), or a product of 1303 base pairs from the full length *pspA*, (Figure 4.10A, lanes 7 and 8). Figure 4.10A confirms that clones 15 and 18 both had deletions in the *pspA* gene.

Southern hybridisation was also used to confirm the presence of the truncated *pspA* gene (Figure 4.10B). The *B. pseudomallei* Δ*pspA* clones 15 and 18 were sequenced to ensure there were no errors in either the upstream or downstream regions of *pspA*, which may cause polar effects on the downstream genes.

A



B

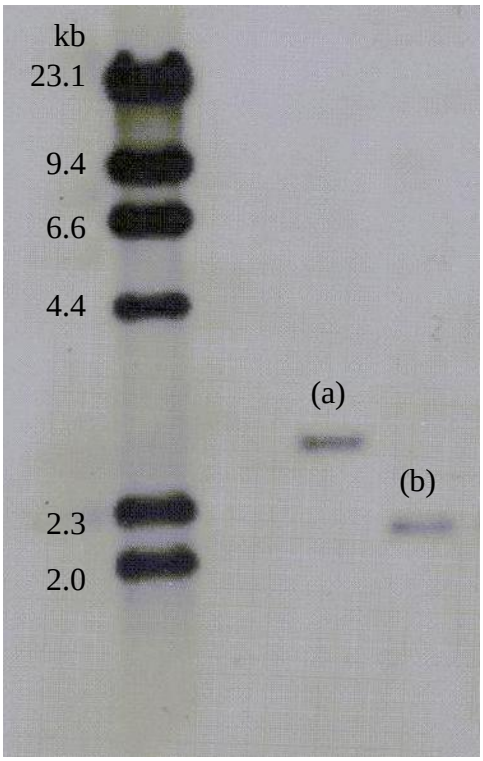


Figure 4.10 Confirmation of *B. pseudomallei* Δ*pspA* mutant strains.

A PCR screen for *pspA* in *B. pseudomallei*. Lane 1, water; lane 2, *B. pseudomallei* K96243 genomic DNA; lane 3, *B. pseudomallei*::pDM4.Δ*pspA* integrant genomic DNA; lane 4, pDM4.Δ*pspA* plasmid DNA; lanes 5-8, *B. pseudomallei* clones 15, 18, 19 and 21. Full length *pspA*, 1303 bp, truncated *pspA*, 712 bp.

B Southern blot confirming the deletion of *pspA* in *B. pseudomallei*. Wild-type and mutant genomic DNA was digested with *Bgl*II and *Bam*HI restriction enzymes. (a) wild-type *pspA* (2708 bp); (b) Δ*pspA*, clone 15 (2116 bp).

4.3.3.1 **Complementation of *B. pseudomallei* Δ*pspA***

In order to verify whether any phenotypes seen in *B. pseudomallei* Δ*pspA* were due to the in-frame deletion, a complemented strain of the mutant was created. *B. pseudomallei* Δ*pspA* was complemented by cloning full-length *pspA* into the plasmid pBHR4-groS-RFP (Wand *et al.*, 2011). The pBHR4-groS-RFP plasmid contains the RFP gene under the control of the constitutive promoter P_{groS} for the *groES* gene.

The *pspA* gene and promoter were amplified by PCR from *B. pseudomallei* genomic DNA using primers BPSL2105 prom F and BPSL2105 comp R. This introduced a *Pvu*I restriction site at the 5' end and a *Bam*HI site at the 3' end of the fragment. The PCR product was cloned into pCR-Blunt II-TOPO according to the manufacturer's instructions and transformed by heat shock into One Shot TOP10 cells. The transformed cells were plated onto LB agar containing 25 μg/ml kanamycin. A restriction digest using *Pvu*I and *Bam*HI was carried out with the TOPO product, followed by ligation with digested pBHR4-groS-RFP. The ligation product was transformed by heat shock into *E. coli* JM109 and plated onto LB agar containing 50 μg/ml chloramphenicol. This produced pBHR4.*pspA* containing the full length *pspA* gene preceded by the upstream intergenic region encoding the *pspA* promoter (Figure 4.11). At each step throughout the cloning procedure, accuracy was checked by restriction digest or sequencing.

The pBHR4.*pspA* plasmid was transferred by conjugation into *B. pseudomallei* Δ*pspA* and the complemented strain selected for by plating onto LB agar containing 50 μg/ml chloramphenicol. The presence of the plasmid was confirmed by colony PCR using primers pBBR1 F2, pBBR1 R4, BPSL2105 prom F, BPSL2105 comp R, CAM-screen F and CAM-screen R.

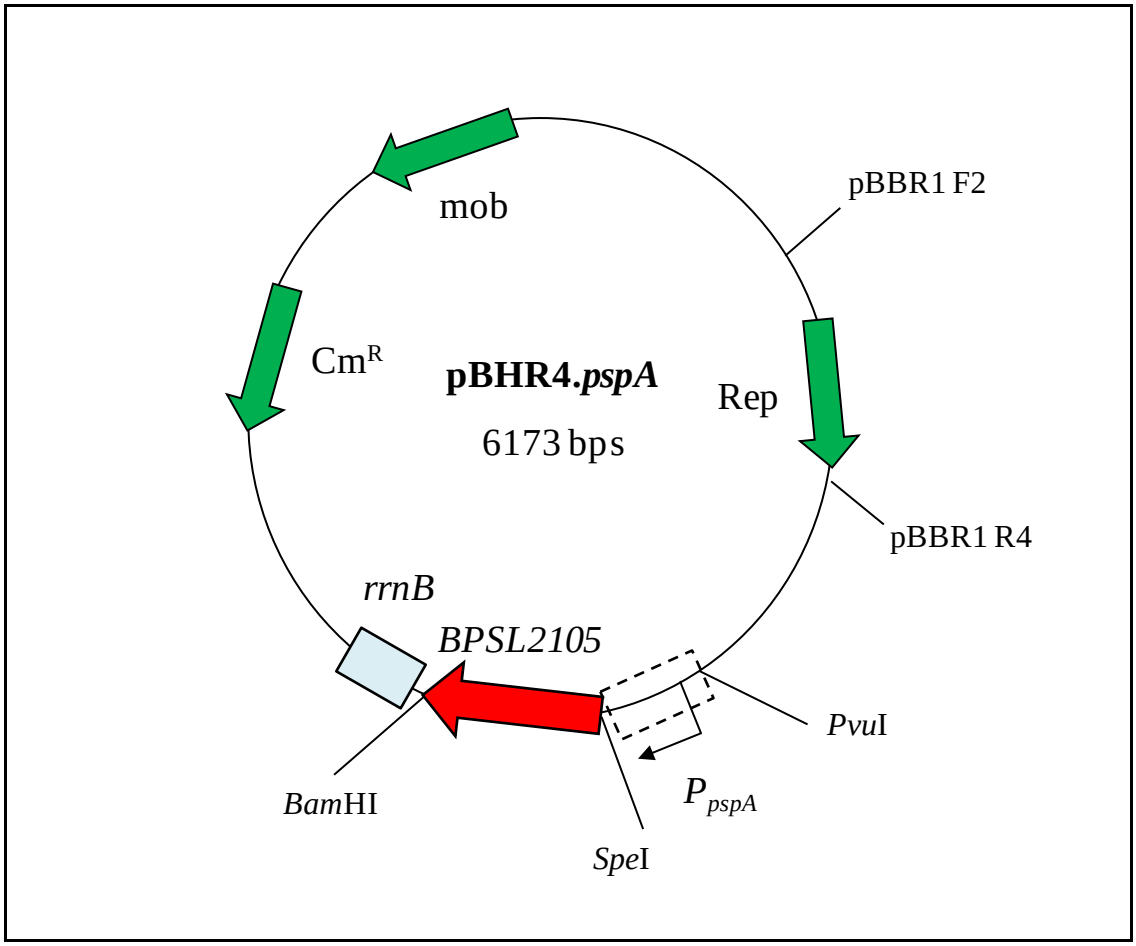


Figure 4.11 Plasmid map of pBHR4.*pspA*.

The construct was created by cloning *pspA* and its upstream region into the complementation plasmid, pBHR4-groS-RFP. The clone map shows the location of *pspA* and its promoter flanked by *Pvu*I and *Bam*HI restriction sites. A colony PCR using primers pBBR1 F2 and pBBR1 R4 was carried out to confirm the presence of the complementation plasmid in *B. pseudomallei* Δ*pspA*.

4.3.4 Characterisation of *B. pseudomallei* Δ*pspA*

Previously, induction experiments were carried out on *pspA* in order to determine if *B. pseudomallei* possessed a Psp response with a similar function to that of other bacteria. These studies found that *pspA* was up-regulated under certain conditions. A mutant was created with a deletion in the *pspA* gene using the pDM4 suicide vector and *sacB* counter-selection (Logue *et al.*, 2009) in order to determine if a deletion in *pspA* translated into a phenotype when grown under known PspA-inducing conditions. After the deletion mutant was constructed, the *B. pseudomallei* Δ*pspA* strain was subjected to a number of environmental stresses that are known to up-regulate PspA in other bacteria. Growth studies were carried out in order to determine whether disrupting *pspA* in *B. pseudomallei* had an effect on the phenotype.

4.3.4.1 **Growth at 37˚C**

The growth rate of *B. pseudomallei* and *B. pseudomallei* Δ*pspA* was compared when grown in LB broth at 37˚C. *B. pseudomallei* Δ*pspA* grew at the same rate as wild-type *B. pseudomallei* over 7 hours (Figure 4.12).

4.3.4.2 **Growth at 50˚C**

The RT-PCR results showed that *pspA* in *B. pseudomallei* was up-regulated at 50˚C compared to controls at 37˚C. This indicates PspA is important for surviving heat stress and therefore the mutant may demonstrate an altered growth phenotype under these conditions. *B. pseudomallei* and *B. pseudomallei* Δ*pspA* cultures were grown at 50˚C for 7 hours. However, there was no difference observed between the wild-type and the deletion strain when growth was measured by optical density (Figure 4.13A) or by number of viable bacteria (Figure 4.13B).

Growth of *B. pseudomallei* at temperatures higher than 50˚C was also examined. Cultures were grown to exponential phase at 37˚C and divided into aliquots. These were then transferred to water baths at temperatures ranging from 50˚C to 80˚C. However, no viable bacteria were recovered after 10 minutes incubation at 60˚C or above (data not shown).

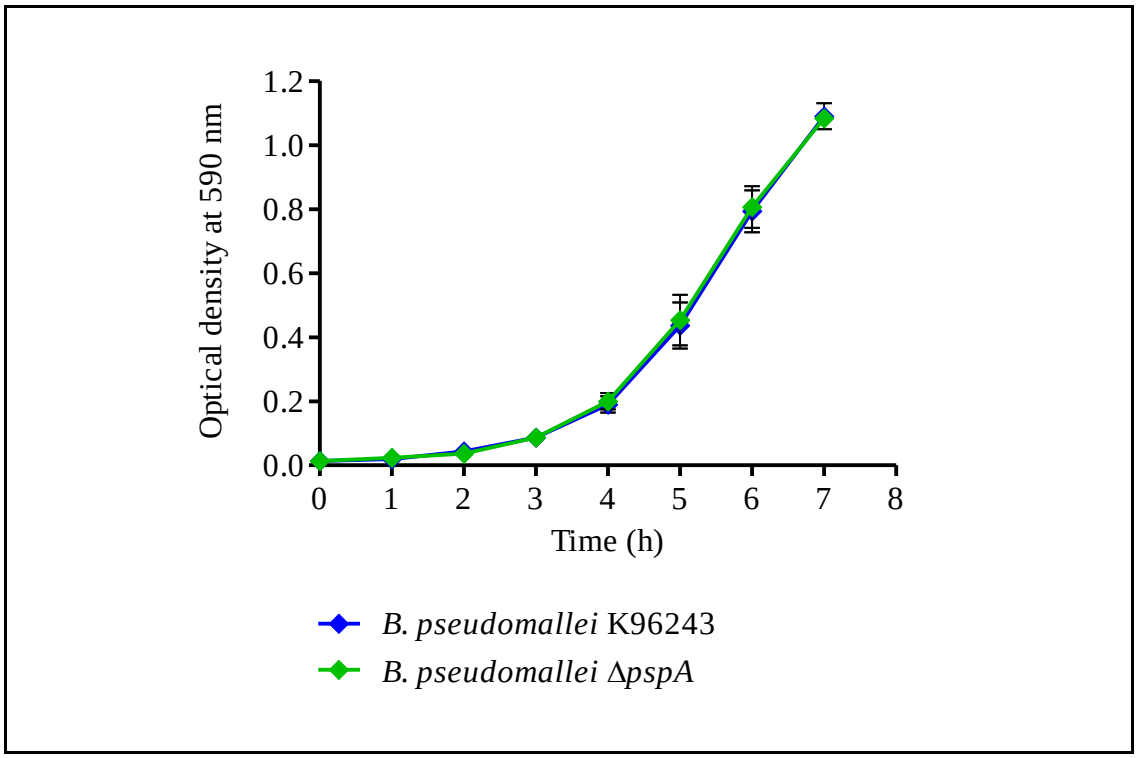


Figure 4.12 Growth of *B. pseudomallei* and *B. pseudomallei* Δ*pspA* at 37˚C.

Values represent the means from 3 experiments ± standard error.

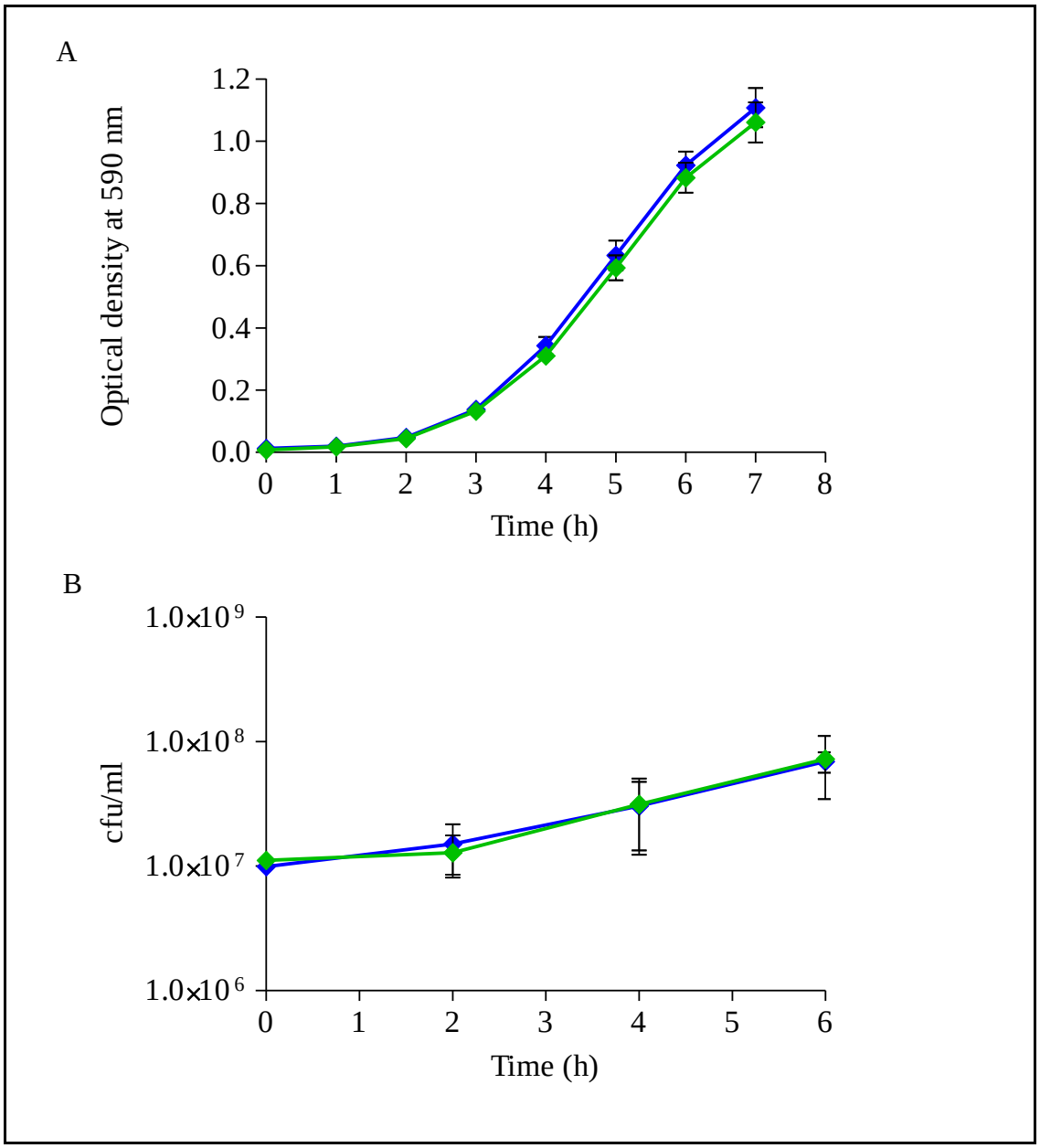


Figure 4.13 Growth of *B. pseudomallei* and *B. pseudomallei* Δ*pspA* at 50˚C.

A Growth was measured by optical density at 590 nm.

B Growth was measured by viable counts. 1 ml culture was removed and serially diluted in 9 ml PBS. Appropriate dilutions were plated onto LB agar and colonies counted.

Values represent the means from 3 experiments ± standard error.

4.3.4.3 **Growth in ethanol**

The addition of 10% ethanol to *E. coli* causes the concentration of PspA to increase within the cell (Brissette *et al.*, 1990), although the previous RT-PCR results indicate that this is not the case in *B. pseudomallei*. Nonetheless, the effect of ethanol on the growth of *B. pseudomallei* Δ*pspA* was measured. *B. pseudomallei* Δ*pspA* was grown in either 2% or 5% ethanol (v/v) for 48 hours. The results showed no difference between the wild-type or mutant strains when viable cells were counted (Figure 4.14).

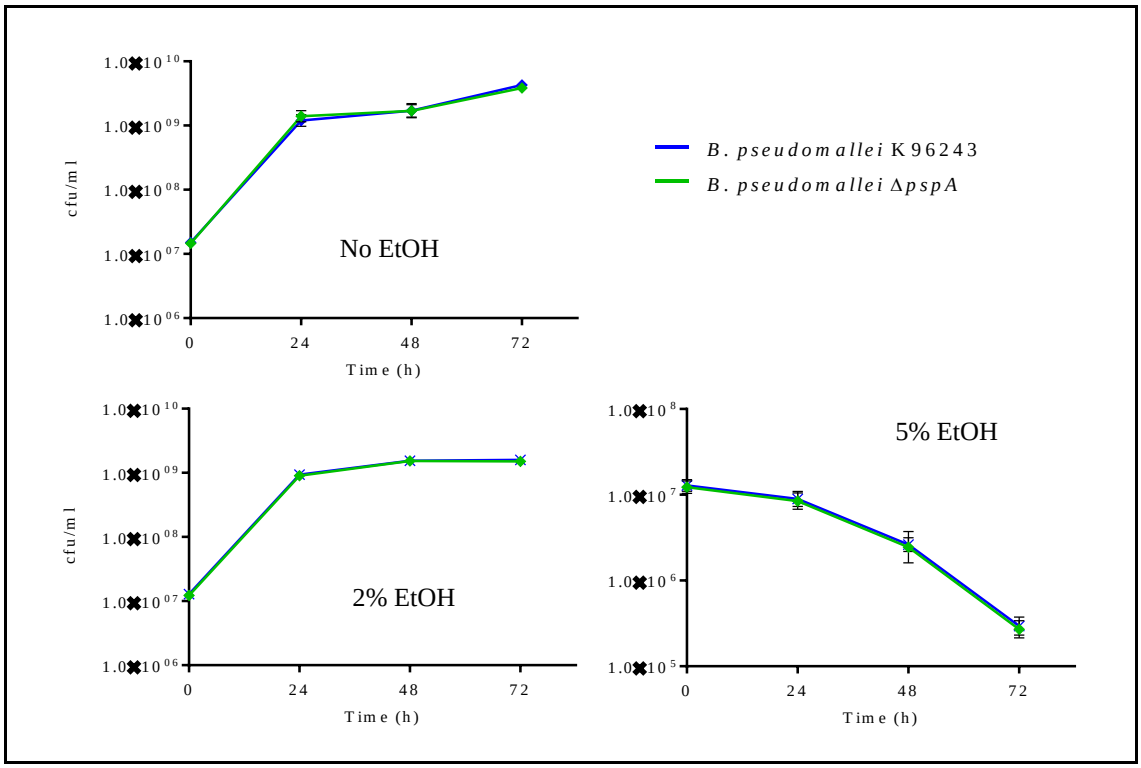


Figure 4.14 Growth of *B. pseudomallei* and *B. pseudomallei* Δ*pspA* in 2% and 5% ethanol.

Values represent the means from 3 experiments ± standard error.

4.3.4.4 **Growth under osmotic stress**

Hyperosmotic shock is a known inducer of the Psp response in *E. coli* (Brissette *et al.*, 1990). In these experiments osmotic shock was reproduced by addition of either 0.3 M or 0.75 M NaCl to a culture of *E. coli*. Therefore, a series of growth experiments was carried out to measure the effect of NaCl on the growth of *B. pseudomallei* Δ*pspA*. *B. pseudomallei* Δ*pspA* was grown in LB broth supplemented with NaCl at a concentration of either 0.3 M or 0.75 M NaCl. After 24 hours, samples were removed and the number of viable cells was measured by serial dilution and colony counting. No difference was observed in growth between the wild-type and the mutant under these conditions (Figure 4.15).

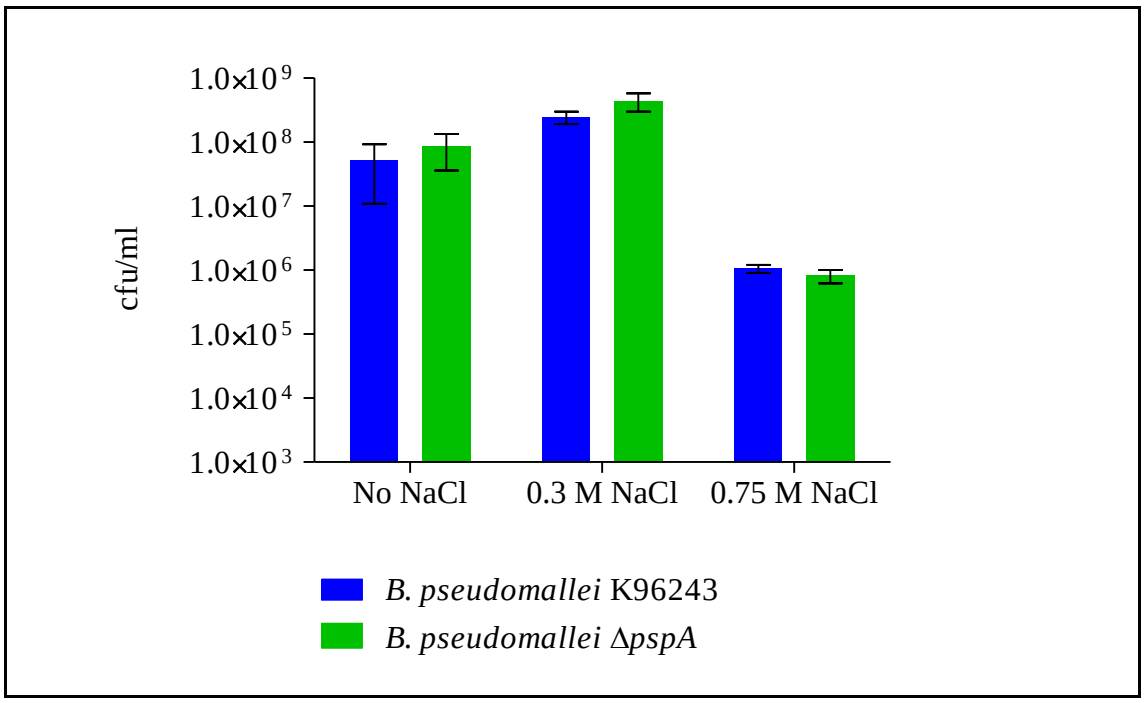


Figure 4.15 Survival of *B. pseudomallei* and *B. pseudomallei* Δ*pspA* in NaCl after 24 hours.

Values represent the means from 3 experiments ± standard error.

4.3.4.5 **Survival in stationary phase**

The changing conditions after a bacterial culture has entered the stationary phase of growth have been shown to be an important inducer of the Psp response. In *E. coli* PspA is rapidly accumulated in the cell after one day at stationary phase. Further to this, growth of an *E. coli* Δ*pspABC* strain shows a sharp decline in viability after the ninth day at stationary phase compared to the wild-type (Weiner and Model, 1994). For this reason a growth study was carried out in order to investigate the survival of *B. pseudomallei* Δ*pspA* at stationary phase. The cultures were grown to stationary phase by incubation at 37˚C in 100 ml LB broth for 9 days. The number of viable cells was enumerated every 24 hours. Both *B. pseudomallei* wild-type and mutant strains grew at the same rate and reached stationary phase after 48 hours. The cultures maintained a density of approximately 5 x 10^9 cfu/ml during stationary phase. On the eighth day (192 hours) *B. pseudomallei* Δ*pspA* began to decline in viability compared to the wild type (Figure 4.16). The wild-type growth phenotype was restored in the complemented strain. This trend continued until the experiment was terminated on the ninth day.

4.3.4.6 **Intracellular survival**

B. pseudomallei is an intracellular pathogen that is able to survive and replicate within phagocytic and non-phagocytic cells (Jones *et al.*, 1996). The Psp response also has a suggested role in virulence as *psp* genes are up-regulated during macrophage infection in bacteria such as *S. enterica* and *S. flexneri* (Eriksson *et al.*, 2003;Lucchini *et al.*, 2005). In order to investigate the intracellular survival ability *B. pseudomallei* Δ*pspA,* an infection assay was performed using J774A.1 murine macrophages. Cells were infected with a multiplicity of infection (MOI) of 1. At 0, 2, 4 and 23 hours the number of viable intracellular bacteria was measured by lysing the macrophages with water and plating on agar. Supernatants were also cultured to determine if there were any bacteria surviving extracellularly (data not shown). The results showed no significant difference in intracellular survival between the mutant and wild-type strains (Figure 4.17).

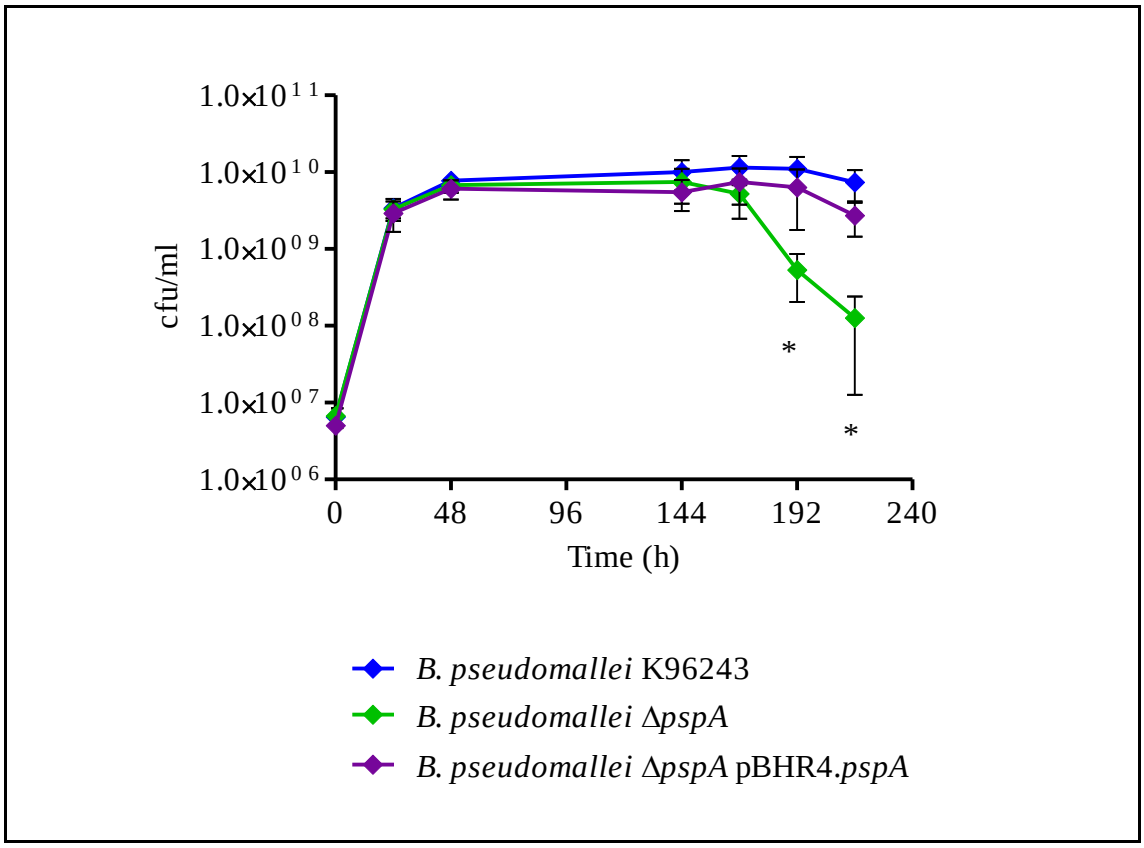


Figure 4.16 Growth of *B. pseudomallei* and *B. pseudomallei* Δ*pspA* over 9 days in 100 ml LB broth cultures.

Values are the means from 3 experiments, with error bars representing the standard errors; * indicates $P < 0.05$.

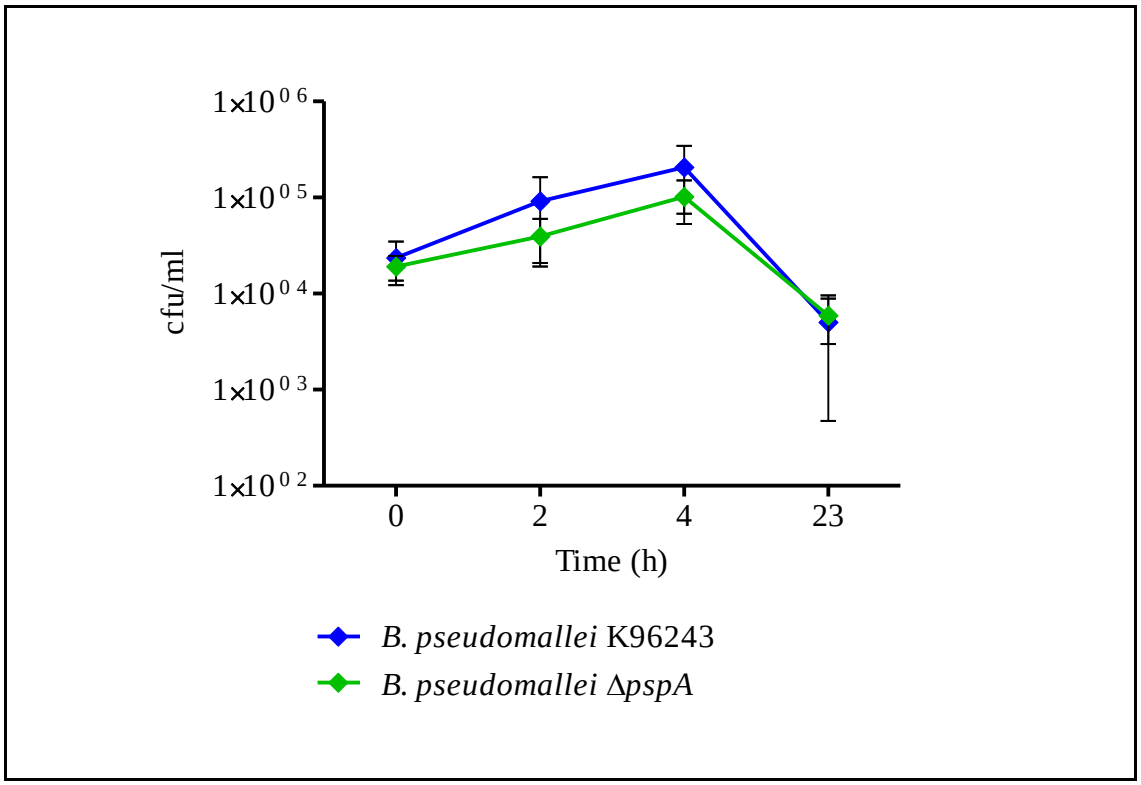


Figure 4.17 Intracellular survival of *B. pseudomallei* and *B. pseudomallei* Δ*pspA* in J774A.1 macrophages.

Cells were infected with *B. pseudomallei* wild-type or Δ*pspA* strains at an MOI of 1 and incubated at 37˚C for 30 minutes. Extracellular bacteria were killed by addition of 1 mg/ml kanamycin for 1 hour at 37˚C. Cells were lysed at 0, 2, 4 and 23 hours in order to enumerate the number of intracellular bacteria. Cells were lysed with 1 ml water and bacteria were serially diluted in 900 µl PBS. Appropriate dilutions were plated on LB agar and colonies counted. Values represent the means from 3 experiments ± standard error.

4.3.4.7 *In vivo* characterisation of *B. pseudomallei* Δ*pspA* in a murine infection model

An experiment was carried out to determine the effect of the deletion in the *pspA* gene on virulence in a BALB/c mouse model of infection. The challenge dose was prepared by inoculating 100 ml LB broth with a single bacterial colony and incubating overnight at 37˚C with agitation. The culture was adjusted to an optical density at 590 nm of 0.4 in LB broth. This was serially diluted in 9 ml LB broth and relevant dilutions plated onto LB agar to enumerate the viable cells. Groups of six mice were challenged with 4.7 x 10^4 cfu of *B. pseudomallei* Δ*pspA* and 4.2 x 10^4 cfu of *B. pseudomallei* by the i.p. route. The MLD of *B. pseudomallei* K96243 is 1 x 10^3 cfu via this route therefore mice were challenged with approximately 42 MLDs. The results showed no significant difference in virulence between the two strains (Figure 4.18).

The MLD of *B. pseudomallei* Δ*pspA* in a mouse model of infection was determined. Groups of six mice were challenged with 4.7 x 10^1-10^6 cfu of *B. pseudomallei* Δ*pspA* by the i.p. route. The MLD for *B. pseudomallei* Δ*pspA* was found to be 3 x 10^3 cfu as determined by the method of Reed and Muench (1938). The mice were monitored for 5 weeks after which the surviving animals were culled and spleens were removed for colonisation studies. The mice surviving for the duration of the experiment were found to have spleens colonised with *B. pseudomallei* Δ*pspA* (Table 4.6).

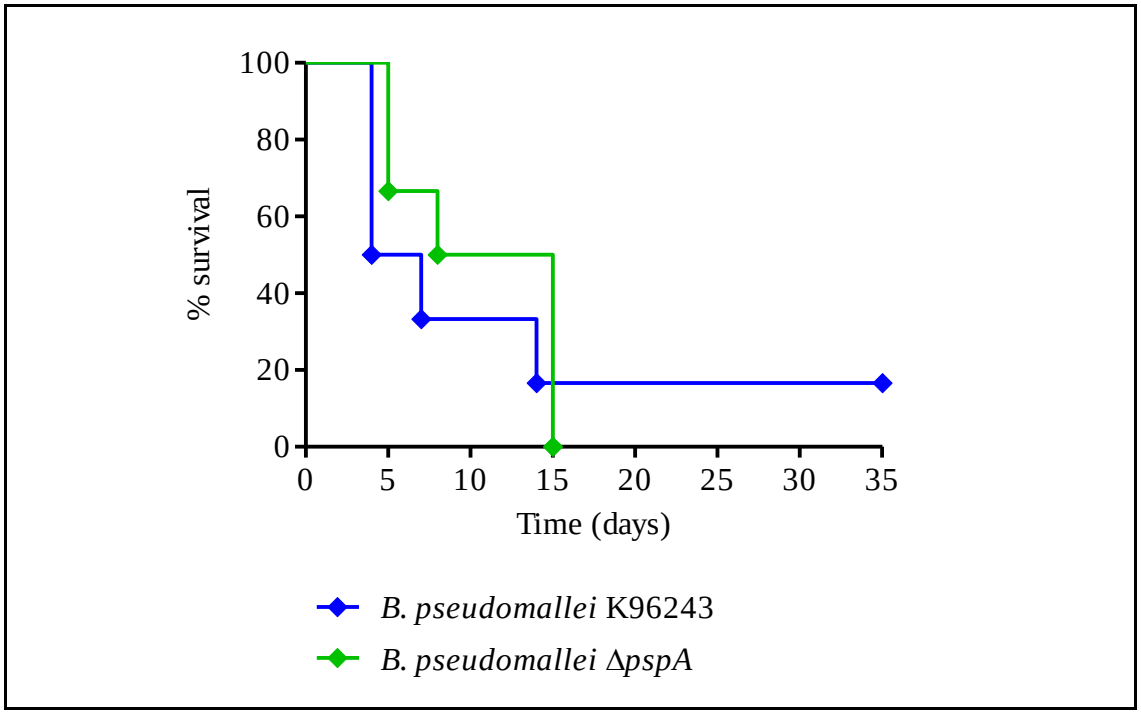


Figure 4.18 Virulence of *B. pseudomallei* Δ*pspA* in BALB/c mice.

Groups of 6 mice were infected by the i.p. route with 4.7 x 10^4 cfu of *B. pseudomallei* Δ*pspA* or 4.2 x 10^4 cfu of wild-type *B. pseudomallei*. Mice were monitored for signs of disease for 5 weeks.

***B. pseudomallei* Δ*pspA* challenge dose**	**Mean bacterial burden in spleen**
4.7 x 10^6 cfu	n/a
4.7 x 10^5 cfu	n/a
4.7 x 10^4 cfu	n/a
4.7 x 10^3 cfu	4.7 x 10^6 cfu/ml
4.7 x 10^2 cfu	2.9 x 10^6 cfu/ml
4.7 x 10^1 cfu	2.4 x 10^6 cfu/ml

Table 4.6 The mean bacterial burden in spleens of surviving mice 5 weeks post challenge with *B. pseudomallei* Δ*pspA*.

Spleens were removed aseptically and homogenised in 1 ml PBS. The homogenate was serially diluted and plated on LB agar in order to enumerate the number of viable cells. The mean bacterial burden was determined from four remaining mice infected with 4.7 x 10^3 cfu *B. pseudomallei* Δ*pspA*, four mice infected with 4.7 x 10^2 cfu and five remaining mice infected with 4.7 x 10^1 cfu. n/a – not applicable; all mice succumbed to infection.

4.3.5 Characterisation of a stationary phase phenotype

Stationary phase survival has been shown to be an important inducer of the Psp response in *E. coli*. In a *B. pseudomallei* Δ*pspA* culture there was a significant decrease in the number of viable cells compared to the wild-type after 8 days of continuous growth. An *E. coli* Δ*pspABC* strain shows a similar sharp decline in viability after the ninth day at stationary phase compared to the wild-type (Weiner and Model, 1994). A series of experiments was conducted to further investigate this phenotype observed in *B. pseudomallei*.

4.3.5.1 Growth at a range of pH

Weiner *et al* suggested that the decline in viability during *E. coli* growth at stationary phase was due to an increase in the pH of the culture over the course of the experiment (Weiner and Model, 1994). As this phenotype was reproduced in *B. pseudomallei* (Figure 4.16), an experiment was carried out in order to investigate whether pH had a significant effect on the survival of *B. pseudomallei* Δ*pspA*. The optimal growth conditions for *B. pseudomallei* are between pH 5 and pH 8 (Inglis and Sagripanti, 2006). Therefore, *B. pseudomallei* wild-type and mutant cultures were used to inoculate LB broth that had been adjusted to a pH of between 3.5 and 9.5 by addition of either HCl or NaCl. The cultures were incubated for 24 hours, after which the number of viable cells was enumerated. The results found there was no difference in survival between the two strains and that the permissive pH for *B. pseudomallei* Δ*pspA* growth is between pH 4 and pH 9 with optimal growth between pH 4 and pH 8 (Figure 4.19).

A second study was carried out to determine the inhibition of stationary phase bacteria at high pH. *B. pseudomallei* wild-type and mutant cultures were grown to stationary phase by continuous incubation at 37˚C for 6 days. The cultures were diluted to 1 x 10^5 cfu/ml and applied to a 96-well plate containing a range of concentrations of NaOH in LB broth. After 18 hours growth at 37˚C the MIC was calculated. The concentration of NaOH required to inhibit growth of both strains of *B. pseudomallei* was 512 μg/ml, which is equivalent to a pH of approximately 9.2 (Figure 4.20).

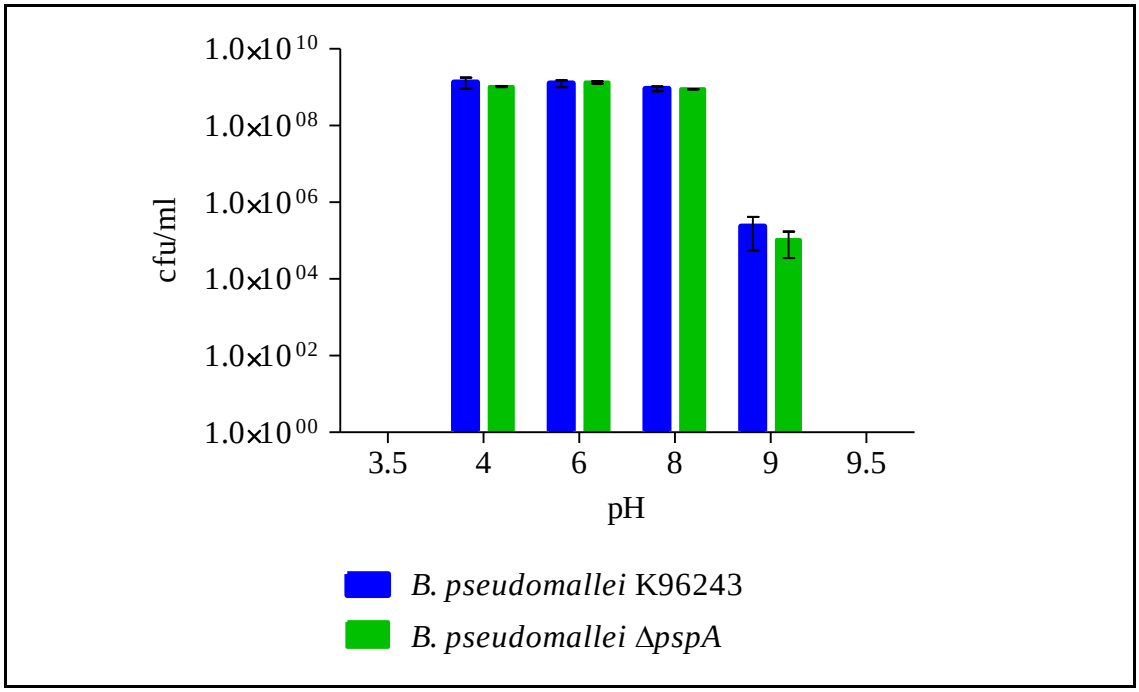


Figure 4.19 Survival of *B. pseudomallei* and *B. pseudomallei* Δ*pspA* at a range of pH after 24 hours.
Values represent the means from 2 experiments ± standard error.

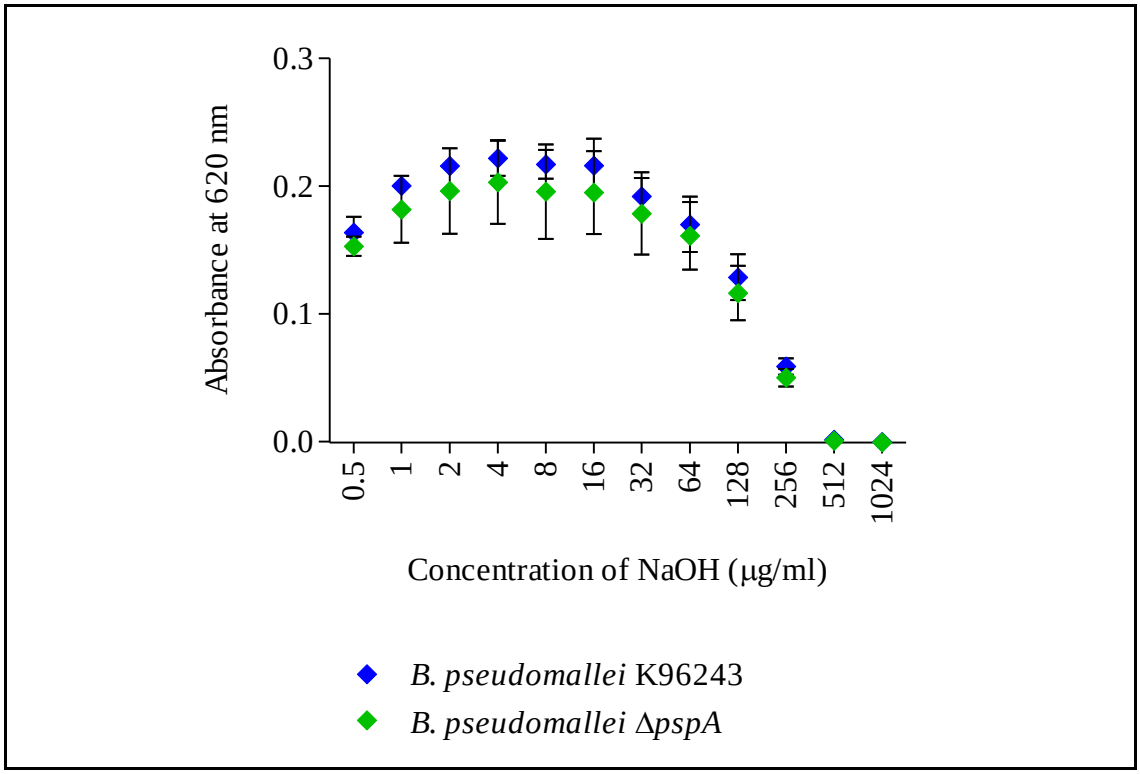


Figure 4.20 Growth of stationary phase *B. pseudomallei* and *B. pseudomallei* Δ*pspA* at range of concentrations of NaOH.
Values represent the means from 2 experiments ± standard error.

4.3.5.2 **Growth at stationary phase at pH 9**

Experiments carried out with an *E. coli psp* deletion mutant demonstrated a faster decline in viability when the bacteria were grown to stationary phase in media at pH 9 (Weiner and Model, 1994). The study carried out to investigate the ability of *B. pseudomallei* Δ*pspA* to survive at a range of pH showed no difference from the wild-type strain. However, this was carried out by measuring growth after 24 hours. *B. pseudomallei* Δ*pspA* does not show a decline in viability during stationary phase until the eighth day of continuous growth, thus the effect of pH on the growth of the mutant may not be apparent until after 24 hours. For this reason, the survival of *B. pseudomallei* Δ*pspA* at pH 9 was examined over an extended time into stationary phase.

B. pseudomallei Δ*pspA* was grown in 100 ml LB broth adjusted to pH 9 by addition of NaOH. This was incubated for 9 days at 37˚C with agitation and the number of viable cells monitored every 24 hours. *B. pseudomallei* Δ*pspA* showed a decline in viability on the eighth day (192 hours) compared to the wild-type strain (Figure 4.21). This was a sharper decline than when *B. pseudomallei* Δ*pspA* was grown in LB broth at neutral pH (Figure 4.16). The wild-type maintained a constant cell density at stationary phase of approximately 1 x 10^9 cfu/ml until the experiment was terminated after 9 days. This phenotype was restored in the complemented strain.

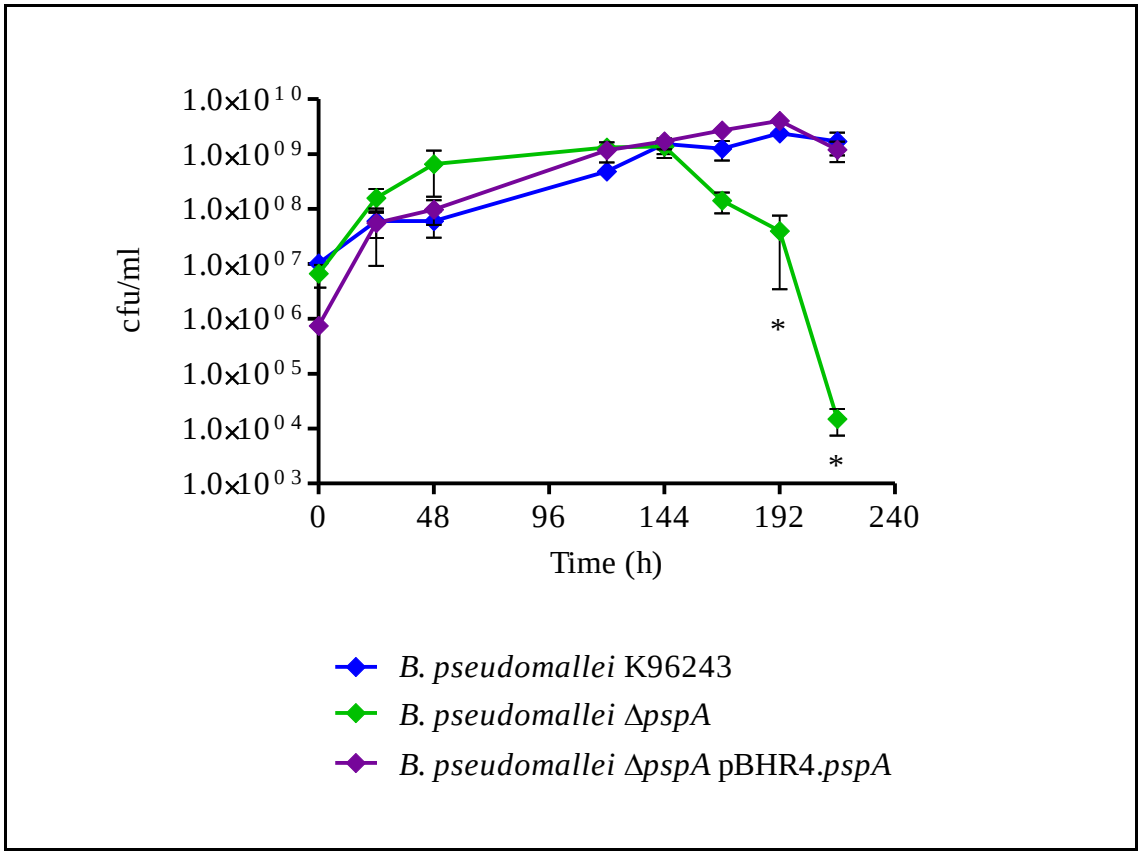


Figure 4.21 Growth of *B. pseudomallei* and *B. pseudomallei* Δ*pspA* over 9 days in 100 ml LB broth at pH 9.

Values are the means from 3 experiments, with error bars representing the standard errors; * indicates $P < 0.05$.

4.3.5.3 **Measurement of pH during stationary phase**

The pH of the media had a dramatic effect on the survival of an *E.coli psp* deletion mutant (Weiner and Model, 1994). Although, the growth phenotype in the *B. pseudomallei* Δ*pspA* mutant was more subtle, the pH of the media may have an important role in stationary phase survival. A stationary phase culture was set up by inoculating 100 ml LB broth at neutral pH with *B. pseudomallei* K96243 or *B. pseudomallei* Δ*pspA* alongside the complemented strain. This was incubated for several days at 37˚C with agitation. The pH of the media was measured every 24 hours using a Piccolo Plus pH meter. The results showed a gradual increase in pH of the media in the *B. pseudomallei* Δ*pspA* culture from pH 7.5 (pH of sterile LB broth) to approximately pH 8.5 over 8 days (Figure 4.22). Compared to this, the wild-type and complemented strains were able to maintain the pH between 6.5 and 7 over the course of the experiment.

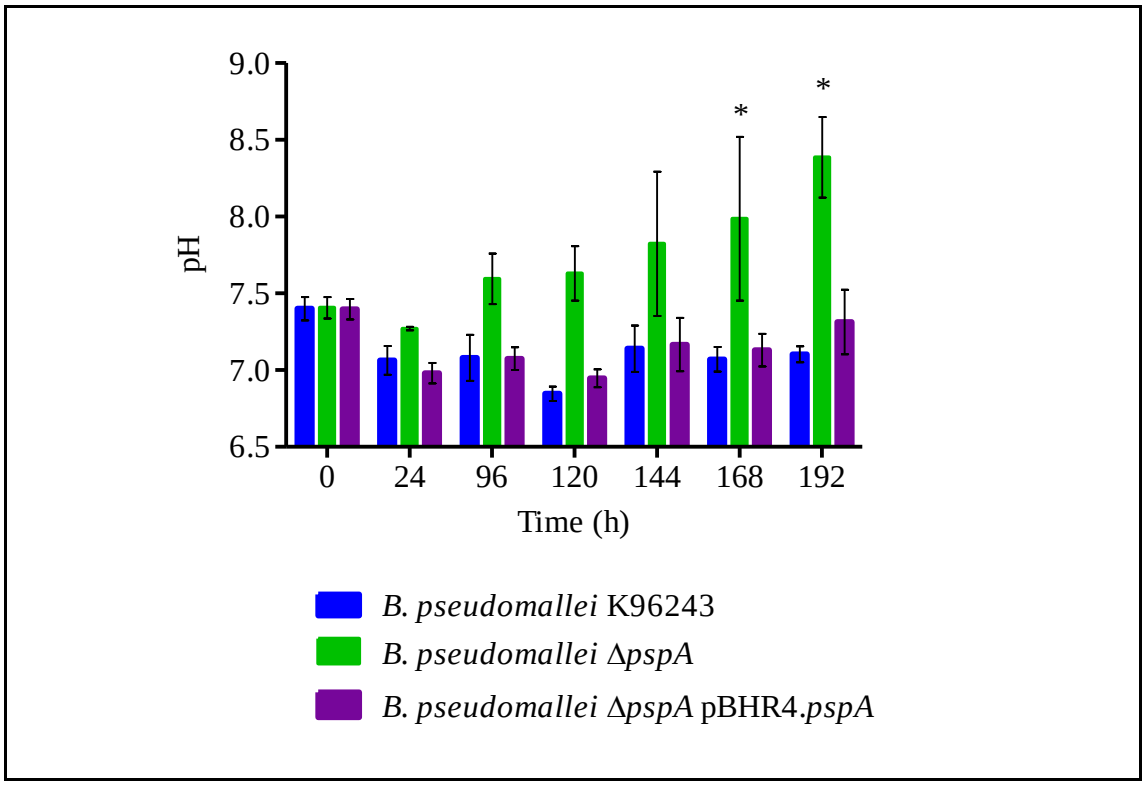


Figure 4.22 pH of *B. pseudomallei* cultures measured over 8 days.

Values are the means from 3 experiments, with error bars representing the standard errors; * indicates P < 0.05.

4.3.5.4 **Effect of ammonia on growth**

The pH of the media in a bacterial culture can be affected by the production of waste products, such as ammonia. The increase in the pH of the media during growth of the *B. pseudomallei* Δ*pspA* strain may be due to a reduced ability to regulate the production of these waste products or mitigate the effect they have on the culture once produced. Therefore an experiment was carried out to determine the ability of stationary phase *B. pseudomallei* Δ*pspA* to survive in a range of concentrations of NH_4OH compared to the wild-type strain.

Stationary phase cultures of the wild-type and mutant strains were grown by incubation at 37˚C in 100 ml LB broth for 6 days. The cultures were diluted to 1 x 10^5 cfu/ml and applied to a 96-well plate containing a range of concentrations of NH_4OH in LB broth. After 18 hours growth at 37˚C the MIC was calculated as 2.5 µg/ml for both strains of *B. pseudomallei* (Figure 4.23).

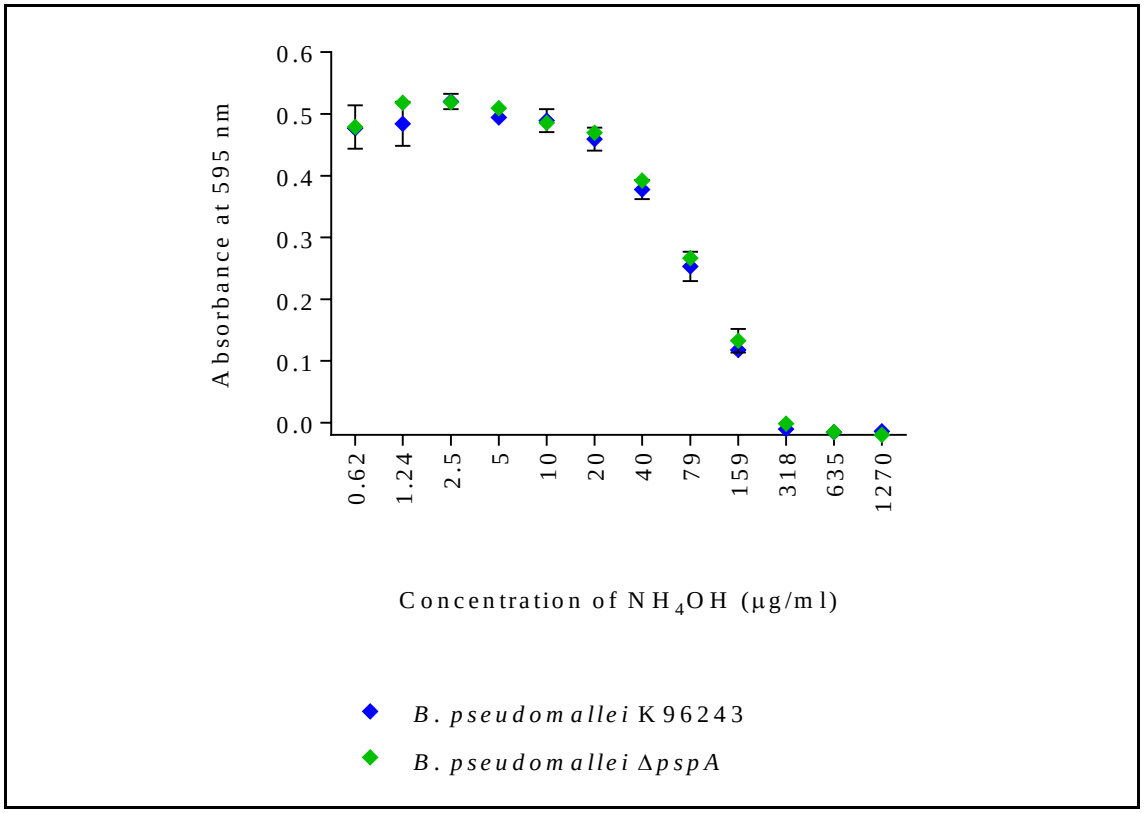


Figure 4.23 Growth of *B. pseudomallei* and *B. pseudomallei* Δ*pspA* at range of concentrations of NH_4OH.

Values represent the means from 2 experiments ± standard error.

4.3.5.5 **Intracellular survival of stationary phase cells**

In an initial intracellular survival assay with exponentially growing cells, there was no difference in viability between the wild-type and mutant strains. However, the *B. pseudomallei* Δ*pspA* strain showed a decrease in viability at stationary phase and, therefore, cells at this stage of growth may be less able to survive within a eukaryotic cell line. An intracellular survival assay was performed in order to investigate the ability of stationary phase *B. pseudomallei* Δ*pspA* to infect and survive within macrophages. *B. pseudomallei* strains were grown for 6 days in 100 ml LB broth. These cultures were used to infect a J774A.1 macrophage cell line at an MOI of 10. At 0, 2, 4 and 24 hours the number of viable intracellular bacteria was measured by lysing the macrophages with water. Bacteria in the supernatant were also counted to determine if there were any bacteria surviving outside the cells.

The results showed a 100 fold decrease in the number of intracellular *B. pseudomallei* Δ*pspA* compared to the wild-type at 24 hours (Figure 4.24A). The complemented strain was able to restore the wild-type phenotype. The reduction in viable cells may be due to lysis of the macrophage cells due to a higher bacterial burden, reducing the number of intact macrophages infected with *B. pseudomallei* Δ*pspA*. However, the number of bacteria surviving extracellularly was negligible at all timepoints (data not shown). To further investigate this, the survival assay was repeated by lysing macrophages at an earlier time point to determine if the intracellular cell counts matched or exceeded that of the wild-type before lysis may have occurred. The number of intracellular bacteria was therefore counted at 0 and 18 hours. The results still showed a significant reduction in the number of intracellular *B. pseudomallei* Δ*pspA* at 18 hours (Figure 4.24B).

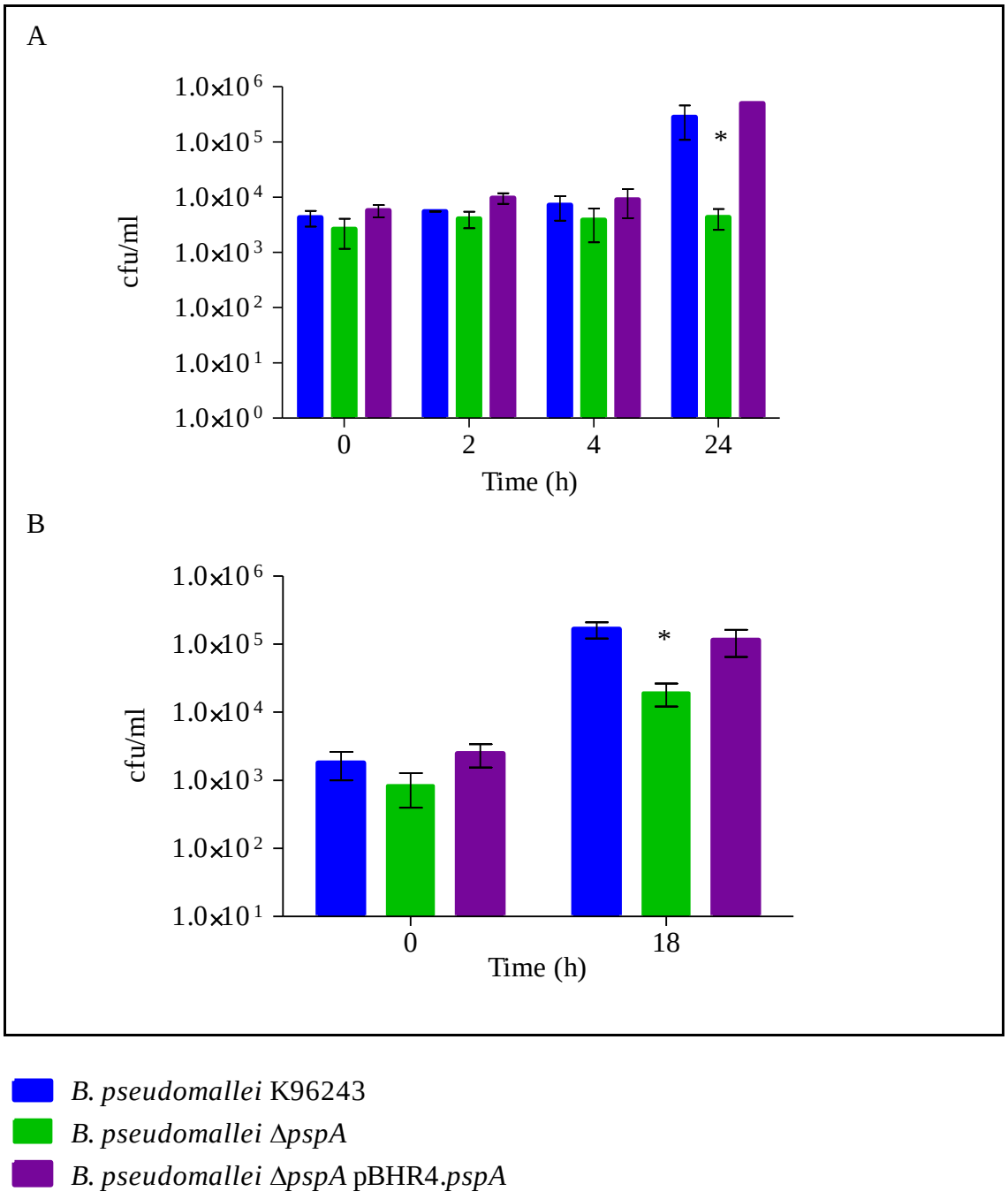


Figure 4.24 Intracellular survival of stationary phase *B. pseudomallei* in J774A.1 macrophages.

A Values are the means from 2 experiments, with error bars representing the standard errors; * indicates $P < 0.01$.

B Values are the means from 3 experiments, with error bars representing the standard errors. * indicates $P < 0.05$.

The LDH release from infected J774A.1 cells was measured using the CytoTox96 Non-Radioactive Cytotoxicity Assay to determine if the reduced bacterial burden seen in *B. pseudomallei* Δ*pspA* infected cells was caused by lysis, leading to the observed reduction in bacteria. The J774A.1 macrophage infection assay was carried out as described and a sample of the cell supernatant was removed at 24 hours, before lysing the macrophages for intracellular bacterial counts. The results showed that cells infected with *B. pseudomallei* Δ*pspA* released an equal concentration LDH compared to the wild-type at 24 hours (data not shown).

4.3.5.6 **Exposure to *in vitro* stress conditions**

Macrophages infected with *B. pseudomallei* Δ*pspA* had a lower bacterial burden compared to those infected with the wild-type after 18 hours. This suggests that the mutant is unable to grow intracellularly or is more susceptible to macrophage killing. Macrophages have a variety of mechanisms for killing phagocytosed bacteria, therefore, in order to determine the cause of the decreased intracellular survival, the resistance of *B. pseudomallei* Δ*pspA* to stress was characterised in more detail.

Bacteria were grown to stationary phase by incubation in 100 ml LB broth at 37˚C for 6 days. The concentration required to inhibit growth of stationary phase bacteria was determined for a range of antimicrobial compounds (Figure 4.25). H_2O_2 was used to represent the oxidative stress encountered inside a phagosome. High osmolarity stress was reproduced using NaCl, and the low pH inside macrophages was replicated by HCl. Other enzymes were tested, including lysozyme and an iron chelator, deferoxamine. Both strains of *B. pseudomallei* were able to grow well under conditions of osmotic stress, low pH and with lysozyme. Both strains were highly susceptible to oxidative stress, with an MIC of 0.125 – 0.25 μg/ml H_2O_2 (Figure 4.25F).

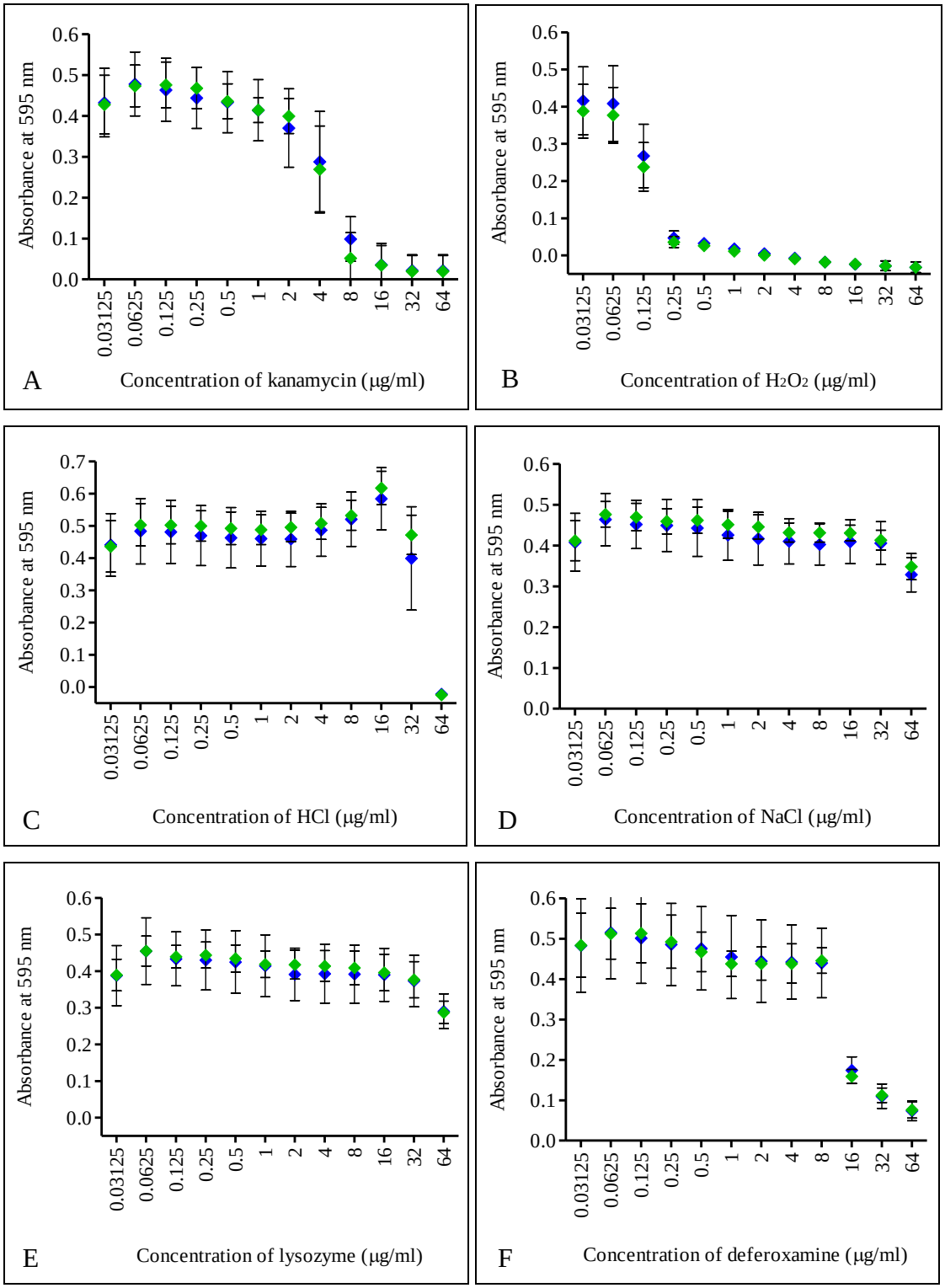


Figure 4.25 Growth of *B. pseudomallei* under different stress conditions.

Values represent the means from 3 experiments ± standard error.

4.3.5.7 *In vivo* characterisation of *B. pseudomallei* Δ*pspA* in an insect infection model

It was previously shown that *B. pseudomallei* Δ*pspA* is not attenuated in a mouse model of infection. However, the observed growth defect demonstrated in a macrophage infection model indicates that stationary phase *B. pseudomallei* Δ*pspA* may show attenuation in other infection models. An insect infection model using wax moth larvae (*G. mellonella*) has been shown to distinguish between different species of *Burkholderia*, which reflects the observed virulence in murine infection models (Wand *et al.*, 2011). Therefore, an experiment was carried out to determine the effect of a deletion in the *pspA* gene on pathogenesis in the *G. mellonella* model of infection.

The challenge dose was prepared by growing *B. pseudomallei*, *B. pseudomallei* Δ*pspA* and *B. pseudomallei* Δ*pspA* pBHR4.*pspA* strains for 6 days at 37˚C. On the sixth day the cultures were diluted and the *G. mellonella* challenged with doses ranging between 1.3×10^3 cfu and 1×10^4 cfu of the three *B. pseudomallei* strains (Figure 4.26A). The larvae were incubated at 37˚C and monitored over 3 days, after which any remaining insects were culled (Figure 4.26B). Over half of the *G. mellonella* infected with wild-type and mutant strains had succumbed to disease 30 hours post-infection. Most of the remaining insects had died by the end of the second day.

A

***B. pseudomallei* strain**	**Challenge dose (cfu)**
Wild-type	1.3×10^3 (1) 1.7×10^3 (2) 6.8×10^3 (3)
Δ*pspA*	1.4×10^3 (1) 1.6×10^3 (2) 7.7×10^3 (3)
Δ*pspA* pBHR4.*pspA*	8.2×10^2 (1) 3.1×10^3 (2) 1.0×10^4 (3)

B

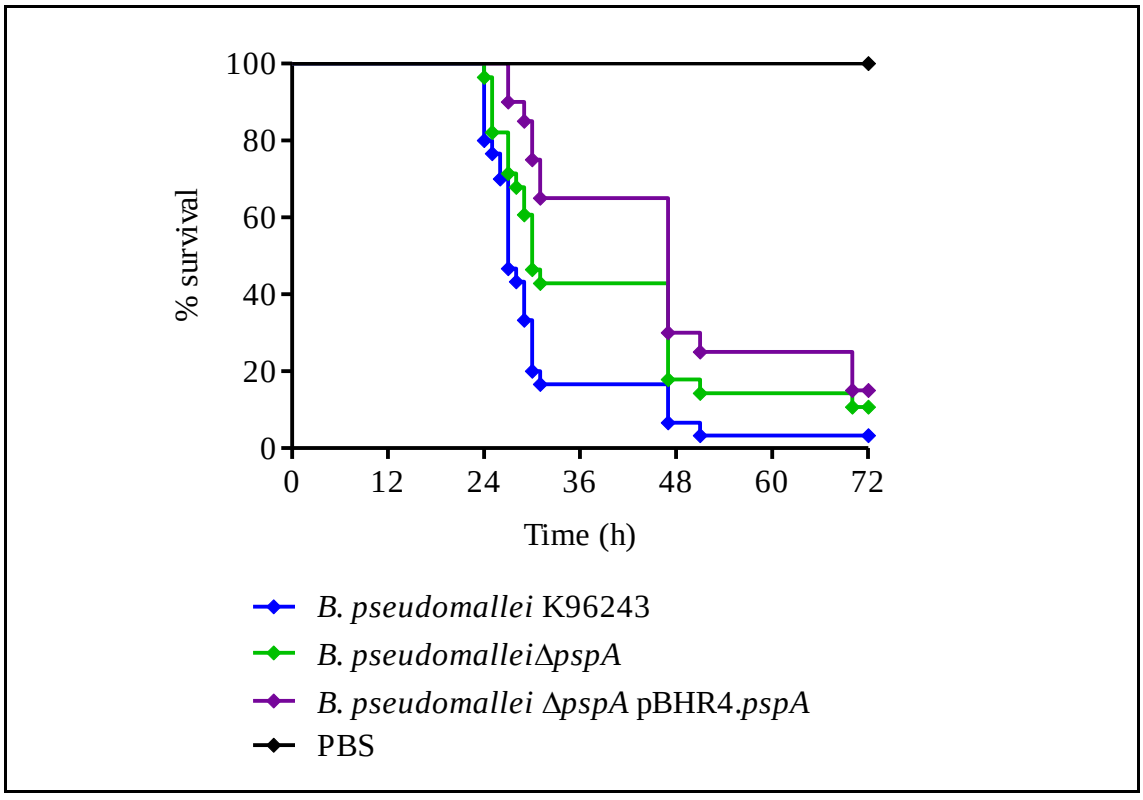


Figure 4.26 Virulence of *B. pseudomallei* Δ*pspA* in a *G. mellonella* model of infection.

Groups of 10 larvae were challenged with *B. pseudomallei* in the uppermost right proleg. Three challenges were carried out and the data pooled.

A Challenge doses given to larvae in individual experiments (replicate number in brackets).

B Survival of *B. pseudomallei*-infected *G. mellonella* monitored over 72 hours.

The number of bacteria colonising the larvae was enumerated in order to determine if there was a difference in bacterial burden between the wild-type and mutant strains. This was achieved by removing the haemolymph of infected *G. mellonella* after 24 hours. The material recovered from the larvae was serially diluted and plated onto LB agar supplemented with 50 μg/ml gentamycin in order to select for *B. pseudomallei* colonies. The results showed that the number of bacteria had increased up to 200-fold in the *B. pseudomallei* Δ*pspA*-infected larvae, with an average of a 95-fold increase (Figure 4.27). However, this was comparable to wild-type-infected larvae that showed an average of 64-fold increase in bacterial burden after 24 hours.

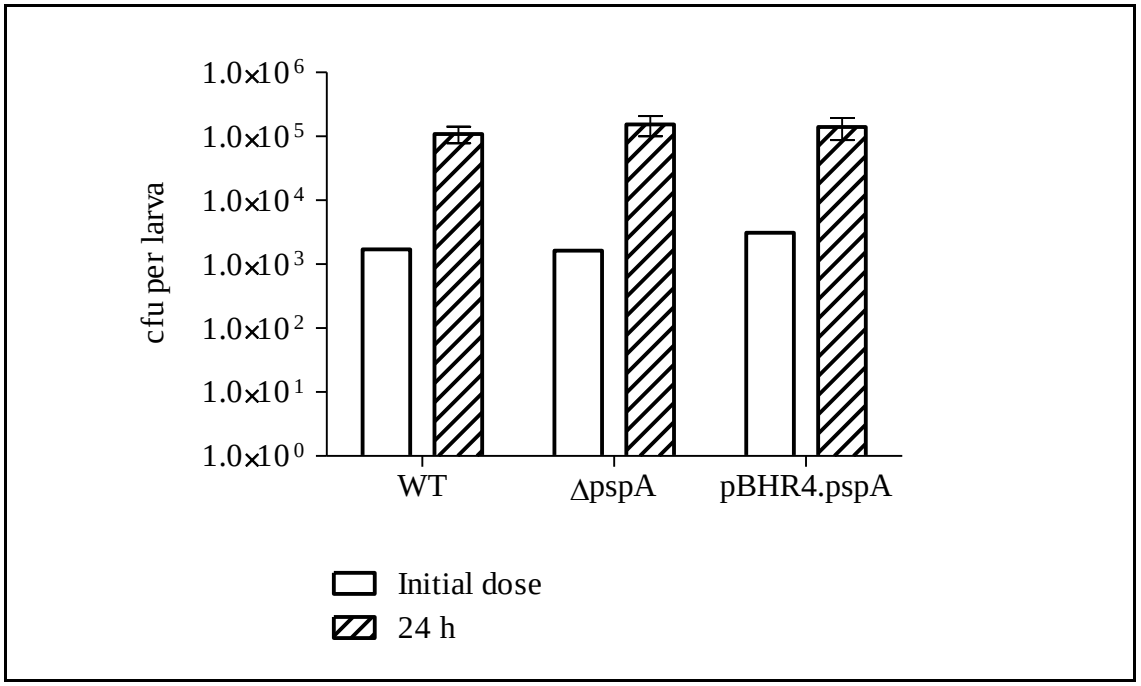


Figure 4.27 The bacterial burden in haemolymph of surviving *G. mellonella* 24 hours post challenge with *B. pseudomallei, B. pseudomallei* Δ*pspA* and *B. pseudomallei* Δ*pspA* pBHR4.*pspA*.

4.4 Discussion

A bioinformatics study was carried out initially to identify putative Psp homologues in *B. pseudomallei*. This identified two Psp homologues; a putative PspA homologue, BPSL2105, and a putative PspF homologue, BPSS2250. It was hypothesised that these proteins form a reduced Psp response in the bacteria alongside a further membrane protein, BPSL2106.

The PspA homologue in *B. pseudomallei* was identified as BPSL2105, a conserved hypothetical protein. This is predicted to form an operon with another gene, *BPSL2106*, encoding a putative membrane protein. This is very similar to another Psp system recently studied in the Gram-positive bacterium *S. lividans* (Vrancken *et al.*, 2008). The system in *S. lividans* is also made up of a putative PspA and a membrane protein. It has very similar characteristics to the classic Psp response as it is highly up-regulated under stress conditions such as ethanol treatment, hyperosmotic shock and treatment with SDS. In addition, a *S. lividans* Δ*pspA* mutant shows a severe decrease in viability compared to the wild-type strain in alkaline conditions (Vrancken *et al.*, 2008). Similarly, in *B. subtilis* the Lia system consists of the *liaIH* operon, which encodes a small membrane-bound protein, LiaI, and a PspA homologue, LiaH (Wolf *et al.*, 2010). Both these systems have similar characteristics to those of the *E. coli* Psp response. For example, LiaH can form large oligomeric rings (Hankamer *et al.*, 2004;Wolf *et al.*, 2010). This evidence supports the proposal of a reduced Psp system in *B. pseudomallei* that has a similar role to that of *E. coli* or *Y. enterocolitica*.

PspA expression is induced by a number of factors. In order to establish whether BPSL2105 in *B. pseudomallei* is a functional homologue of *E. coli* PspA, the level of its expression was measured under a series of stressful conditions. The first Psp-inducing condition investigated was heat shock at 50˚C. An early study of the *E. coli* Psp response found that PspA synthesis was strongly induced after a shift from 37˚C to 50˚C (Brissette *et al.*, 1990). This is higher than the traditional Hsps which are induced at 42˚C (Wada *et al.*, 1986), whereas at this temperature PspA is only moderately induced. The heat response in *E. coli* is also independent of the membrane proteins PspB and PspC, which are thought to be involved in signal transduction (Weiner *et al.*,

1991). During heat shock PspA must therefore receive the inducing signal either directly or indirectly through some other protein-protein interaction. Homologues of PspB and PspC were not identified in *B. pseudomallei* and so for this reason it was thought that there would be a similar Psp response under these conditions compared to *E. coli*.

In an initial investigation into the effect of high temperature on *B. pseudomallei*, it was found that *pspA* was up-regulated from 10 to 15 minutes during incubation at 50˚C. In previous experiments carried out in *E. coli*, the expression of *pspA* is a transient response that reaches a peak at 2 minutes after the temperature shift (Brissette *et al.*, 1990). Despite the slight time delay compared to *E. coli*, the transient increase in *pspA* expression observed in *B. pseudomallei* was comparable to that seen in *E. coli*. This evidence, taken together with the bioinformatics results, is indicative of a functional Psp response in *B. pseudomallei*, similar to that seen in *E. coli*. The slower increase in *pspA* expression was probably due to the fact that the samples were incubated in a heat block. In this situation the entire culture is not in contact with the heat and therefore convection of heat will be less efficient, resulting in a longer incubation time before the culture reaches 50˚C. The heat distribution is also impaired by the static nature and the uneven distribution of temperature in the instrument. This experimental design was chosen due to the constraints of working in a class III microbiological safety cabinet where it is impractical to work with water baths due to the large volume of waste produced.

A second potential Psp homologue was found in the *B. pseudomallei* genome. The protein BPSS2250 had 39% homology to *Y. enterocolitica* PspF and was therefore chosen for targeted deletion in *B. pseudomallei*. It was selected from a number of potential PspF homologues on the basis of its conserved domains, as these contained the most similarities to those of *E. coli* and *Y. enterocolitica*. The PspF protein plays a regulatory role in the Psp system in these species, where it acts as a transcriptional activator (Jovanovic *et al.*, 1996). It is expressed at a low level constitutively within the cell and is required for activating sigma factor RNA polymerase. It was thought that a deletion in *BPSS2250* in *B. pseudomallei* would prevent transcription of the *psp* operon as the RNA polymerase would be unable to initiate transcription and therefore would

show a similar phenotype to *B. pseudomallei* Δ*pspA*, as this is the main effector protein encoded by the operon. The *BPSS2250* gene in *B. pseudomallei* is not adjacent to the putative *pspA* in the chromosome and therefore its role as the putative PspF homologue was doubtful. When this gene was deleted it was found to have no effect on the expression of *pspA* under Psp-inducing conditions. This result indicates that BPSS2250 is not the transcriptional activator for the Psp response in *B. pseudomallei* but may activate alternative stress systems in the cell. No further investigations were carried out to characterise this protein. Although BPSS2250 did not have the highest homology of the putative PspF homologues identified, it was the only candidate to possess the relevant domains in a similar orientation to that of other known PspF proteins. In other bacteria the transcriptional regulator of the Psp response is found divergently transcribed from the *pspA* operon (Darwin, 2005). None of the putative PspF homologues were suitably positioned and therefore were thought unlikely to play a role in the Psp response in *B. pseudomallei*. For these reasons, no further putative PspF homologues were characterised.

The Psp response can be induced by a number of conditions, all of which are thought to have a detrimental effect on the integrity of the inner membrane and therefore also affect the PMF (Darwin, 2005). It was decided to investigate the effect that these conditions would have on the survival of a *B. pseudomallei* strain with a deletion in the *pspA* gene. An initial growth experiment was carried out in order to determine if the *B. pseudomallei* Δ*pspA* strain had any unusual growth phenotypes under normal growth conditions. When grown at 37˚C there was no change compared to the wild-type. This was an expected result as the Psp response is normally only expressed when the bacteria are stressed.

When the survival of the mutant strain at 50˚C was investigated it was found that there was no change in viability of *B. pseudomallei* Δ*pspA* compared to wild-type. This result demonstrates that although *pspA* is up-regualted at this temperature it does not show a phenotype when the gene is deleted from the bacterium. This observation has also been noted by Brissette *et al* during early characterisation of the *psp* operon in *E. coli* (Brissette *et al.*, 1991). This lack of a phenotype may be due to the presence of redundant systems that are able to compensate for the absence of *pspA*. Alternatively, it

could mean that the temperature challenge does not provide enough of a stress for *B. pseudomallei*. This organism is endemic to regions of Southeast Asia where it is mainly found in agricultural soil which has been cleared and cultivated (Cheng and Currie, 2005). It could easily be conceived that the temperature of the soil in a rice paddy field could climb above temperatures outside of the normal range of bacterial growth. To address this, a heat shock experiment was performed to investigate the limits of *B. pseudomallei* growth at temperatures above 50˚C, as it is still able to grow at this temperature. A temperature where the wild-type is at the limit of survival may show a growth defect in the *B. pseudomallei* Δ*pspA* strain at this extreme. However, when this was investigated further, no viable bacteria remained after 10 minutes at temperatures above 50˚C in either wild-type or mutant strains, indicating that *B. pseudomallei* was unable to survive at these higher temperatures. Therefore, the loss of *pspA* does not translate to an apparent growth phenotype, despite being up-regulated under these conditions.

A further possible reason for this lack of a phenotype is the transient nature of the Psp response when subjected to higher temperatures. It has been shown previously that the synthesis of PspA reaches a peak at 2 minutes after the temperature shift (Brissette *et al.*, 1990). This suggests that over a prolonged period the Psp response is ineffective and therefore there would be no obvious difference between the deletion mutant and wild-type. In *E. coli*, when grown at a 50˚C for a prolonged period the Δ*pspA* mutant dies as fast as the wild-type strain and shows no growth defect in comparison (Brissette *et al.*, 1991). As pointed out by the authors, this lack of an obvious phenotype does not mean that the operon is unimportant in response to stress, only that an appropriate test needs to be found to demonstrate the phenotype. Furthermore, *E. coli* has many redundant systems with which to compensate when coping with stress of this nature. The σ^{32}-dependent Hsps are induced by many similar stimuli to the Psps, although the Psp response is independent of this regulon. In a σ^{32} mutant, *pspA* transcription during heat shock is prolonged, suggesting that a product of the σ^{32} controlled heat shock system acts to suppress *psp* expression (Brissette *et al.*, 1990). This may account for the transient response in *E. coli* and implies that the heat shock response may act in parallel, reducing the effect of disrupting the Psp response on the bacteria.

An alternative inducing condition of the Psp response is treatment with 10% ethanol. Ethanol damages the cell by affecting the structure of proteins and lipids within the cytoplasmic membrane, which in turn affects the PMF. Psp-induction by treatment with ethanol has been studied previously in *E. coli* using a concentration of 10% ethanol (Brissette *et al.*, 1990) and in several other species of bacteria including *S. lividans* and *S.* Typhimurium (Vrancken *et al.*, 2008;Shoae Hassani *et al.*, 2009). These studies have shown that *pspA* and *pspG* are expressed during exposure to ethanol and that when *pspA* is inactivated it causes a growth defect compared to wild-type strains. However, in *B. pseudomallei* the *pspA* gene is not up-regulated by these conditions and there is no change in viability between the mutant and wild-type strains when grown in 2% or 5% ethanol. This indicates that *pspA* in *B. pseudomallei* does not play a role in protecting the cell from membrane stress caused by treatment with ethanol. This may be because *B. pseudomallei* has alternative strategies for protection against ethanol stress. For example, *B. pseudomallei* possesses a capsule, which has been implicated in protection against stressful conditions encountered both in the environment and during infection of a host (Puthucheary *et al.*, 1996).

Previous work has shown that PspA is induced by concentrations of either 0.3 M or 0.75 M NaCl in *E. coli* (Brissette *et al.*, 1990). When *B. pseudomallei* Δ*pspA* was grown in 0.3 M NaCl there was no change in viability compared to the wild-type. This indicates that PspA does not play a role in hyperosmotic shock in *B. pseudomallei*. Another possible explanation is that *B. pseudomallei* is able to tolerate higher salt concentrations than *E. coli*, the organism with which the original experiment was performed (Brissette *et al.*, 1990). *B. pseudomallei* also does not possess either PspB or PspC homologues, which are usually present in a functional *psp* operon (Darwin, 2005). In *E. coli* the induction of the Psp response by osmotic or ethanol stress is partially dependent on these proteins (Brissette *et al.*, 1990;Weiner *et al.*, 1991). The absence of PspB and PspC in *B. pseudomallei* may account for the lack of expression of *pspA* under similar stressful conditions.

B. pseudomallei is an intracellular pathogen that is able to survive and replicate within phagocytic and non-phagocytic cells (Jones *et al.*, 1996). The ability to survive within macrophages is an important part of the lifecycle of *B. pseudomallei*. A role for PspA in

macrophage infection has been implicated as it is up-regulated in *S. enterica* and *S. flexneri* (Eriksson *et al.*, 2003;Lucchini *et al.*, 2005). However, in a J774A.1 mouse macrophage cell line there was no significant difference between *B. pseudomallei* Δ*pspA* and wild-type survival. This indicates that PspA in *B. pseudomallei* is not essential for intracellular survival and thus is not important for virulence. This is supported by the characterisation of *B. pseudomallei* Δ*pspA* in a mouse model of infection. The MLD of the mutant was found to be 3×10^3 cfu, which is similar to the MLD of wild-type *B. pseudomallei* K96243. There was also no difference in the virulence of the mutant when analysed by MTTD or by bacterial burden in the spleens of surviving animals.

It has been noted that, although PspA is the most abundant protein of the Psp response, it does not have a role in virulence in *Y. enterocolitica* despite the fact that PspC is essential for virulence in a mouse model of infection (Darwin and Miller, 2001;Horstman and Darwin, 2012). However, in the intracellular bacterium *S.* Typhimurium, PspA is essential for virulence because it is required to maintain the PMF needed for metal ion import when inside the phagosome (Karlinsey *et al.*, 2010). This indicates that PspA has different roles in different species depending on its environmental niche. *B. pseudomallei* is an opportunistic intracellular pathogen and so its Psp response may have evolved to enable survival within different environmental niches not necessarily required for virulence.

It was decided to investigate growth of *B. pseudomallei* Δ*pspA* during stationary phase as this has been shown to be an important inducer of the Psp response (Weiner and Model, 1994). In *E. coli*, PspA is rapidly accumulated in the cell after one day at stationary phase in media at pH 7, and at pH 9 it shows constant expression during exponential and early stationary phase (Weiner and Model, 1994). Further to this, growth of an *E. coli* Δ*pspABC* strain shows a sharp decline in viability after the ninth day at stationary phase compared to the wild-type. In *B. pseudomallei* it was found that the wild-type maintained growth in stationary phase up to the ninth day, whereas the mutant strain showed a 100-fold drop in viability on the eighth day. This indicates that the deletion of *pspA* in *B. pseudomallei* has a similar effect to a Δ*pspABC* deletion mutant in *E. coli* due to the reduced system in *B. pseudomallei*.

The stationary phase survival defect seen in the *E. coli psp* mutant was linked to an increase in pH of the growth media (Weiner and Model, 1994). Therefore the survival defect also seen in *B. pseudomallei* Δ*pspA* indicated that PspA may also play a key role during survival at alkaline pH. A growth phenotype is also observed in a *S. lividans* Δ*pspA* strain. This mutant was less able to survive compared to wild-type when exposed to 30 mM NaOH, corresponding to a pH of 8.9 to 9.5 (Vrancken *et al.*, 2008). The pH also plays a significant role in the life of intracellular bacteria as the pH of phagocytosing cells decreases. *B. pseudomallei* is able to survive and proliferate inside macrophages where conditions are more acidic (Jones *et al.*, 1996). Therefore an experiment was carried out to determine the survival of *B. pseudomallei* wild-type and Δ*pspA* strains at a range of pH. The results found that there was no difference in survival between the two strains and that the permissive pH for *B. pseudomallei* was between pH 4 and pH 9 with optimal growth between pH 5 and 8, comparable to previous results (Inglis and Sagripanti, 2006). Similarly, *B. pseudomallei* Δ*pspA* at the stationary phase of growth was able to survive up to a pH of 9.2. This suggests that the decrease in survival during stationary phase may not be due directly to an increase in pH. This is supported by the fact that *B. pseudomallei* Δ*pspA* and wild-type strains were able to grow for a prolonged period of time in LB broth at pH 9. The mutant was able to survive for up to eight days before a rapid decline in viability was observed, a similar result to that seen when grown in media at pH 7.

Despite the similarity in survival rates of the two strains at high pH, the *B. pseudomallei* Δ*pspA* culture showed a marked rise in extracellular pH coinciding with a loss of viability during prolonged growth at 37˚C. This survival defect must therefore be caused by something more complex than just a change in pH. Stationary phase cultures tend to become more alkaline as they release amine-containing compounds due to amino acid metabolism. It was hypothesised that the loss of PspA in *B. pseudomallei* may result in a reduced ability to counter the effects of an increase in ammonia in the media. To test this, an MIC assay was carried out with stationary phase cultures applied to a range of NH_4OH concentrations. However, this resulted in an identical MIC to that of the wild-type. Despite the lack of a differential phenotype when *B. pseudomallei* Δ*pspA* was exposed to elevated concentrations of ammonia, the increase in pH may still have been caused by a by-product of amino acid metabolism. The loss of viability at

stationary phase in the *B. pseudomallei* Δ*pspA* culture indicates that there may be additional elements causing cell death, not necessarily linked to pH. This may be caused by the toxic build up of other waste products either in the extracellular milieu or inside the cell itself, however, the cause of this was not elucidated during this study.

Although *B. pseudomallei* Δ*pspA* was able to infect macrophages to the same extent as wild-type in an initial study, the mutant was less able to survive intracellularly when the inoculum was at stationary phase. Stationary phase wild-type bacteria were able to infect cells and replicate in the cell up to 24 hours post infection. The stationary phase *B. pseudomallei* Δ*pspA* was also able to infect and replicate at the same rate as wild-type in the initial 4 hours post infection, but at some point between 4 and 18 hours the number of intracellular bacteria began to decline. The reason for this was investigated by assessing growth in a range of *in vitro* stress conditions selected to mimic stresses experienced in macrophage cells. The conditions encountered within a macrophage include low pH, change in osmolarity and oxidising conditions. In addition, macrophages also produce enzymes that digest the cell wall or sequester iron from the bacteria to defend against infections (Forman and Thomas, 1986). The ability of *B. pseudomallei* Δ*pspA* to grow in these conditions was compared to the wild-type. However, none of the conditions tested showed any significant difference in survival between these two strains, indicating that either the relevant stress condition was not tested in this study or that it is not necessarily an individual stress causing cell death but may be an accumulation of the environmental conditions. Alternatively, it may be that the external conditions have a less significant impact on viability compared to the internal state of the bacterial cell.

G. mellonella was chosen as an infection model to further evaluate the virulence of the stationary phase mutant as it has been shown to differentiate between species and mutants of *Burkholderia* (Wand *et al.*, 2011). In addition, *G. mellonella* possesses an immune system similar to the innate immune system of mammals as it is able to produce antimicrobial peptides and can carry out phagocytosis (Hoffmann, 1995). However, the reduction in virulence of stationary phase *B. pseudomallei* Δ*pspA* seen in the macrophage infection model was not translated when tested in this insect model. There appeared to be a slight delay in the time to death of *G. mellonella* infected with *B.*

pseudomallei Δ*pspA* compared to the wild-type during the second day and for this reason the bacterial burden within the insect was measured 24 hours post-infection. However, this did not reveal any difference in the level of bacteria colonising the larvae. Therefore, the deletion of *pspA* does not affect the virulence of *B. pseudomallei* in a *G. mellonella* infection model.

In conclusion, this study has identified and characterised a reduced Psp-like system in *B. pseudomallei* consisting of PspA and potentially one other protein. This system has similar characteristics to that described in other bacteria, such as its up-regulation during heat shock and its importance for stationary phase survival. Although it does not appear to have an important role in virulence in the mouse or insect models used in this study, the Psp response in *B. pseudomallei* may play a more significant role in environmental survival, where this species is more commonly found.

Chapter 5: **General discussion**

B. pseudomallei is an emerging disease in many parts of the world and is a major cause of death in endemic regions (Cheng and Currie, 2005). In Thailand it is the third most common cause of death from an infectious agent after HIV and tuberculosis (Limmathurotsakul *et al.*, 2010a). Melioidosis can manifest in a number of forms and is treated with a prolonged course of antibiotics for up to six months (Wuthiekanun and Peacock, 2006). Despite this, relapse is a frequent problem when dealing with this disease (Maharjan *et al.*, 2005). The difficulty in treating melioidosis lies in its intrinsic antibiotic resistance mechanisms. These include the production of β-lactamses and multiple drug efflux systems, which confer resistance to a diverse range of antibiotics (Holden *et al.*, 2004;Moore *et al.*, 1999;Chan *et al.*, 2004b). For this reason, the purpose of this study was to evaluate two new proteins in *B. pseudomallei* for their use as therapeutic targets, inhibitors to which could potentially bypass the resistance mechanisms present in this bacterium.

Targeting virulence factors opens up a new realm of possibilities for the development of new antimicrobial drugs. The discovery and validation of these new virulence targets in bacteria has been aided greatly by the development of genomics. However, there is an ongoing debate about whether inhibitors targeting proteins required for virulence would be as effective as those targeting survival. An advantage of anti-virulence therapies is the potential for emergence of resistance to be much slower due to the reduction in selective pressure on the bacteria. This also reduces the likelihood of resistance developing in commensals, preventing them from passing on these traits to pathogenic bacteria, as well as reducing the side effects caused by destruction of the native gut flora. One drawback to this method of attack is the narrow spectrum of targets available across species. Virulence factors are much less well conserved in pathogenic bacteria compared to essential targets. This means that any therapies developed to combat disease would have to be tailored to infections with specific species. This complicates treatment strategies as it requires identification of the bacterium to confirm the species before the appropriate treatment can be prescribed. Essential proteins therefore still make desirable targets, although the range of pathways that are targeted in bacteria needs to be broadened to enable the production of new weapons against bacterial

infection. Two proteins were chosen from *B. pseudomallei* for evaluation as antimicrobial targets. One target was chosen as a predicted essential gene and the other was chosen due to its predicted role in virulence.

A number of techniques have been developed in recent years that have been designed to probe the role of individual genes on a large-scale platform. For example, STM and IVET are techniques that can be used to identify genes that are required for survival within a host (Hensel *et al.*, 1995;Mahan *et al.*, 1993). More recently, the development of high-throughput sequencing has lead to more advanced techniques, such as TraDIS (Langridge *et al.*, 2009). Due to the high coverage of the genome, TraDIS can be used to identify essential genes through the identification of those without transposon insertions. This method was used to identify *minE* as an essential gene in *B. pseudomallei*, which was then selected for further validation.

In this study a number of conditional complementation strategies were used to confirm data produced from TraDIS. A new plasmid was constructed in order to create a complementation plasmid for *B. pseudomallei* that could be controlled by the regulation of a rhamnose-inducible promoter. Complementation of *minE* was carried out in order to permit deletion of the chromosomal copy, leaving the functional copy under the control of the inducible promoter. Alongside this, a second method of complementation was carried out with a plasmid that allowed conditional control of an integrated promoter upstream of the gene of interest. This verified that the Min system was not essential when all three genes were down-regulated when compared to a conditional mutant of a known essential gene, *pyrH*. Lastly, the introduction of *minC* and *minD* under the control of an inducible-promoter into a Δ*min* strain was also attempted in order to demonstrate the filamentation phenotype in the absence of MinE. The methods used in this study were ultimately unsuccessful in the targeted deletion of *minE* alone, and so have not definitively proven its essentiality in *B. pseudomallei*. However, the evidence from previous studies of the *E. coli* Min system has indicated that the loss of this gene is detrimental to the cell (de Boer *et al.*, 1989;Pichoff *et al.*, 1995). Indeed, the inability to mutate this gene could be considered as evidence that it is vital to the normal growth of *B. pseudomallei*.

The future of antimicrobial drugs may lie in the ability to inhibit more than one target at once. This might be possible in the case of the Min system due to the fact that multiple interactions between the three proteins are required in order to maintain a fully functional system. In particular, the development of inhibitors that disrupt protein-protein interactions may be particularly relevant to the Min system. An example of this is the MinC and MinE interactions with MinD, which have overlapping binding sites (Hu *et al.*, 2003). Disruption of either one of these interactions leads to a change in cell morphology, either by the production of chromosomeless minicells or through filamentation (de Boer *et al.*, 1992;Hu and Lutkenhaus, 2003;Pichoff *et al.*, 1995). If an inhibitor were able to block this region of MinD then this multi-targeting antimicrobial would make the emergence of resistance less likely. Technologies, such as SICLOPPS (Scott *et al.*, 2001), could be applied to identifying molecules capable of inhibiting these physiologically relevant protein-protein interactions. However, the importance of fully understanding interactions between proteins is fundamental in informing inhibitor design to this type of target. For example, the MinE domain responsible for the interaction with MinD is sequestered within the MinE dimer interface when not involved in binding MinD (Park *et al*., 2011). Therefore, this interacting region would have a transient availability to potential inhibitors, making it less desirable as a target.

Due to its important role in cell division, the Min system is conserved among many Gram-negative species, making it a broad-spectrum target (Pinho *et al.*, 2013). Therefore, this study has produced data highly indicative of an essential role for MinE in *B. pseudomallei* and it may still prove a suitable target for development of future antimicrobial therapies. Targeting MinE alone could lead to the emergence of resistance through inactivation of the entire *min* operon, thus nullifying the loss of an essential function. However, the loss of the *min* operon and resulting minicell phenotype may have implications for virulence instead. Previous studies carried out with enterohemorrhagic *E. coli* have shown that strains containing a deletion in the *min* operon have reduced adherence to human epithelial cells (Parti *et al.*, 2011b). In *E. coli*, a deletion in *minD* had a dramatic effect on the cell morphology, causing a mixture of minicells and filaments. This was predicted to reduce the rate of microcolony formation and, consequently, reduce the adherence of the mutant, which is a vital step in intestinal colonisation (Parti *et al.*, 2011b). A similar phenomenon was observed in *min* mutants

of *Neisseria gonorrhoeae* (Parti *et al.*, 2011a). In this species deletion of either *minD* or *minC* resulted in reduced adherence and invasion of epithelial cells. These interactions would be essential for the bacteria to initiate an infection *in vivo*. Therefore, the inhibition of protein interactions within the system would potentially lead to attenuation, if not complete inhibition of growth.

As demonstrated, it is difficult to prove the essential nature of a gene due to the difficultly of inactivating it. This is especially difficult in bacteria where the tools for molecular characterisation are not as well established. The Min system requires a fine balance between the concentration of the three proteins in order to function properly (de Boer *et al.*, 1989), therefore a more effective strategy for carrying out conditional mutagenesis would perhaps require the integration of a single copy of *minE* elsewhere on the chromosome to maintain the homeostasis between gene products when expressed. Virulence targets can be more easily deleted and their effect on virulence determined by *in vivo* studies. The Min system could instead be characterised as a potential virulence target in *B. pseudomallei*. It would be interesting to determine if the minicell morphology has an impact on invasion of epithelial cells, necessary for *B. pseudomallei* to establish an infection *in vivo* (Kespichayawattana *et al.*, 2004). This would potentially result in attenuation in an animal model of infection. Therefore future work could focus on the *B. pseudomallei* Δ*min* strain and the importance of cell morphology in virulence.

The increasing interest in virulence factors has provided a new avenue for the development of antimicrobial drugs. The Psp response is one such factor that has been associated with a number of virulence roles within the cell. It is an extracytoplasmic stress response system that is required for maintaining the integrity of the inner membrane. Loss of this function results in reduced efficiency in functions such as protein secretion and biofilm formation (Kleerebezem and Tommassen, 1993;DeLisa *et al.*, 2004). Other extracytoplasmic stress responses are known to have an important role in pathogenesis (reviewed by Rowley *et al.*, 2006). This may be linked to the fact that the harsh conditions encountered when inside a host often have an effect on the integrity of the cell membrane, for example increased osmolarity, pH or temperature changes. The Psp response may therefore have an important role in this environment, supported

by previous work where it has been implicated in macrophage infection (Eriksson *et al.*, 2003).

The system identified as the Psp response in *B. pseudomallei* was much condensed compared to the systems seen in *E. coli* and *Y. enterocolitica* (Brissette *et al.*, 1990). Nonetheless, *B. pseudomallei pspA* was up-regulated during heat stress and stationary phase, similar to the response seen in *E. coli*. (Brissette *et al.*, 1990;Weiner and Model, 1994). However, this reduced system may have a diminished role within the cell compared to its *E. coli* counterpart as it was not induced during either osmotic or ethanol stress. *B. pseudomallei* is an environmental bacterium with a large genome which allows it to adapt to a wide variety of conditions. It is able to survive between a pH of 4 and 8, can withstand high salt concentrations, as well as being able to persist for long periods of time in water, soil and the human body (Inglis and Sagripanti, 2006). *B. pseudomallei* is therefore well equipped to deal with stressful conditions and is likely to have redundant stress response systems that compensate for the loss of *pspA*.

This study has shown that the Psp response in *B. pseudomallei* is not essential for the virulence of this bacterium. When tested in a mouse model of infection, *B. pseudomallei* Δ*pspA* was not attenuated compared to the wild-type. Although important for stationary phase survival, when an infection was carried out with bacteria in this stage of the lifecycle no attenuation was observed in a *G. mellonella* infection model, despite showing reduced intracellular survival in macrophages. This indicates that PspA is not an ideal candidate as an anti-virulence target and also demonstrates the difficulty in assessing virulence targets in multiple models of infection due to the range of host susceptibilty to infection. However, the importance of the Psp response during stationary phase survival suggests that loss of *pspA* may have a more significant impact during a chronic infection. The mouse and insect models tested here represent a more acute version of the disease, whereas C57BL/6 mice are less susceptible to *B. pseudomallei* but usually die from extensive bacterial growth in the organs (Leakey *et al.*, 1998). If *B. pseudomallei* Δ*pspA* were tested in C57BL/6 mice as a model of chronic melioidosis, there may be a reduction in bacterial burden after an extended period of infection.

Proteins that are important for stationary phase survival are gathering more interest as potential antibacterial targets. An example of this is a multistage vaccination strategy that has recently being investigated in *Mycobacterium tuberculosis*. This employs the use of multiple antigens that are expressed at different points during the infection process (Aagaard *et al.*, 2011). Vaccines that combined a latency-associated protein with early antigenic proteins promoted a stronger immune response and resulted in lower bacterial numbers in the lungs of mice compared to those vaccinated with BCG. Melioidosis is another disease that frequently causes a chronic infection, which is difficult to clear using the antibiotics currently prescribed. As discussed above, none of the novel treatment strategies being investigated to counter infection with *B. pseudomallei* have the ability to produce sterilising immunity. After immunisation with the vaccine candidates described, such as the live attenuated strain *purN* mutant, many bacteria were still found colonising the spleen, lungs and liver, indicating the onset of a chronic infection (Breitbach *et al.*, 2008). In order to create a more attenuated strain for vaccination against melioidosis, the current vaccine candidates could be further mutated in a gene important for stationary phase survival in order to further reduce the likelihood of bacteria remaining present in the body after immunisation. Additionally, if an anti-virulence target were identified, there may be some utility in combining a treatment against this protein with traditional antibiotics to produce a biphasic treatment strategy that targets both the exponential growth and chronic phases of the bacteria's lifecycle.

In conclusion, while anti-virulence treatments provide a promising new avenue for antibacterial treatment, new drugs targeting such proteins are yet to come to market. In this study, the essential target, MinE, has shown more promise as an antimicrobial target compared to the anti-virulence target, PspA. While the Psp response is present in *B. pseudomallei*, its importance for virulence is less well defined in this species. However, the interesting dynamics of the proteins within the Min system provide a second avenue of research as a potential anti-virulence target as well as a putative essential target. This system makes an exciting new prospect for inhibitors, although further work is required to validate it as an essential or anti-virulence target in *B. pseudomallei*. The overall importance of validating targets before development of inhibitors is clear.

Bibliography

Aagaard,C., Hoang,T., Dietrich,J., Cardona,P.J., Izzo,A., Dolganov,G. Schoolnik,G.K., Cassidy,J.P., Billeskov,R., Andersen,P. (2011) A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nature Medicine* 17: 189-194.

Adams,H., Teertstra,W., Demmers,J., Boesten,R., Tommassen,J. (2003) Interactions between Phage-shock proteins in *Escherichia coli*. *Journal of Bacteriology* 185: 1174-1180.

Addinall,S.G., and Lutkenhaus,J. (1996) FtsA is localized to the septum in an FtsZ-dependent manner. *Journal of Bacteriology* 178: 7167-7172.

Akerley,B.J., Rubin,E.J., Novick,V.L., Amaya,K., Judson,N., Mekalanos,J.J. (2002) A genome-scale analysis for identification of genes required for growth or survival of *Haemophilus influenzae*. *Proceedings of the National Academy of Sciences of the United States of America* 99: 966-971.

Allwood,E., Devenish,R., Prescott,M., Adler,B., Boyce,J. (2011) Strategies for intracellular survival of *Burkholderia pseudomallei*. *Frontiers in Microbiology* 2: Article 170.

Anuntagool,N., Wuthiekanun,V., White,N.J., Currie,B.J., Sermswan,R.W., Wongratanacheewin,S., Taweechaisupapong,S., Chaiyaroj,S.C., Sirsinha,S. (2006) Lipopolysaccharide heterogeneity among *Burkholderia pseudomallei* from different geographic and clinical origins. *The American Journal of Tropical Medicine and Hygiene* 74: 348-352.

Atkins,T., Prior,R., Mack,K., Russell,P., Nelson,M., Prior,J., Ellis,J., Oyston,P.C.F., Dougan,G., Titball,R.W. (2002a) Characterisation of an acapsular mutant of *Burkholderia pseudomallei* identified by signature tagged mutagenesis. *Journal of Medical Microbiology* 51: 539-547.

Atkins,T., Prior,R.G., Mack,K., Russell,P., Nelson,M., Oyston,P.C.F, Dougan,G., Titball,R.W. (2002b) A mutant of *Burkholderia pseudomallei*, auxotrophic in the branched chain amino acid biosynthetic pathway, is attenuated and protective in a murine model of melioidosis. *Infection and Immunity* 70: 5290-5294.

Attree,O., and Attree,I. (2001) A second type III secretion system in *Burkholderia pseudomallei*: who is the real culprit? *Microbiology SGM* 147: 3197-3199.

Bakowski,M.A., Braun,V., Brumell,J.H. (2008) *Salmonella*-containing vacuoles: directing traffic and nesting to grow. *Traffic* 9: 2022-2031.

Bassler,B.L. (1999) How bacteria talk to each other: regulation of gene expression by quorum sensing. *Current Opinion in Microbiology* 2: 582-587.

Beloin,C., Valle,J., Latour-Lambert,P., Faure,P., Kzreminski,M., Balestrino,D., Haagensen,J.A.J., Molin,S., Prensier,G., Arbeille,B., Ghigo,J.M. (2004) Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. *Molecular Microbiology* 51: 659-674.

Bergler,H., Abraham,D., Aschauer,H., Turnowsky,F. (1994) Inhibition of lipid biosynthesis induces the expression of the *pspA* gene. *Microbiology UK* 140: 1937-1944.

Bernhardt,T.G., and de Boer,P.A.J. (2005) SlmA, a nucleoid-associated, FtsZ binding protein required for blocking septal ring assembly over chromosomes in *E. coli*. *Molecular Cell* 18: 555-564.

Beutler,B., and Rietschel,E.T. (2003) Innate immune sensing and its roots: the story of endotoxin. *Nature Reviews Immunology* 3: 169-176.

Bhavsar,A.P., Zhao,X., Brown,E.D. (2001) Development and characterization of a xylose-dependent system for expression of cloned genes in *Bacillus subtilis*: conditional complementation of a teichoic acid mutant. *Applied and Environmental Microbiology* 67: 403-410.

Bi,E., and Lutkenhaus,J. (1993) Cell division inhibitors SulA and MinCD prevent formation of the FtsZ ring. *Journal of Bacteriology* 175: 1118-1125.

Bozdogan,B., and Appelbaum,P.C. (2004) Oxazolidinones: activity, mode of action, and mechanism of resistance. *International Journal of Antimicrobial Agents* 23: 113-119.

Breitbach,K., Klocke,S., Tschernig,T., van Rooijen,N., Baumann,U., Steinmetz,I. (2006) Role of inducible nitric oxide synthase and NADPH oxidase in early control of *Burkholderia pseudomallei* infection in mice. *Infection and Immunity* 74: 6300-6309.

Breitbach,K., Kohler,J., Steinmetz,I. (2008) Induction of protective immunity against *Burkholderia pseudomallei* using attenuated mutants with defects in the intracellular life cycle. *Transactions of the Royal Society of Tropical Medicine* 102: 89-94.

Brett,P.J., DeShazer,D., Woods,D.E. (1998) *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. *International Journal of Systematic Bacteriology* 48: 317-320.

Brickner,S.J., Hutchinson,D.K., Barbachyn,M.R., Manninen,P.R., Ulanowicz,D.A., Garmon,S.A. Grega,K.C., Hendges,S.K., Toops,D.S., Ford,C.W., Zurenko,G.E. (1996) Synthesis and antibacterial activity of U-100592 and U-100766, two oxazolidinone antibacterial agents for the potential treatment of multidrug-resistant Gram-positive bacterial infections. *Journal of Medicinal Chemistry* 39: 673-679.

Brissette,J.L., Russel,M., Weiner,L., Model,P. (1990) Phage-shock protein, a stress protein of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* 87: 862-866.

Brissette,J.L., Weiner,L., Ripmaster,T.L., Model,P. (1991) Characterization and sequence of the *Escherichia coli* stress-induced *psp* operon. *Journal of Molecular Biology* 220: 35-48.

Burtnick,M.N., and Woods,D.E. (1999) Isolation of polymyxin B-susceptible mutants of *Burkholderia pseudomallei* and molecular characterization of genetic loci involved in polymyxin B resistance. *Antimicrobial Agents and Chemotherapy* 43: 2648-2656.

Burtnick,M.N., and Brett,P.J. (2013) *Burkholderia mallei* and *Burkholderia pseudomallei* cluster 1 type VI secretion system gene expression is negatively regulated by iron and zinc. *PLoS One* 8: e76767.

Burtnick,M.N., Brett,P.J., Harding,S.V., Ngugi,S.A., Ribot,W.J., Chantratita,N. Scorpio,A., Milne,T.S., Dean,R.E., Fritz,D.L., Peacock,S.J., Prior,J.L., Atkins,T.P., DeShazer,D. (2011) The cluster 1 type VI secretion system is a major virulence determinant in *Burkholderia pseudomallei*. *Infection and Immunity* 79: 1512-1525.

Burtnick,M.N., DeShazer,D., Nair,V., Gherardini,F.C., Brett,P.J. (2010) *Burkholderia mallei* cluster 1 type VI secretion mutants exhibit growth and actin polymerization defects in RAW 264.7 murine macrophages. *Infection and Immunity* 78: 88-99.

Canepari,P., Boaretti,M., Lleo,M.D., Satta,G. (1990) Lipoteichoic acid as a new target for activity of antibiotics - mode of action of daptomycin (Ly146032). *Antimicrobial Agents and Chemotherapy* 34: 1220-1226.

Cardona,S.T., Mueller,C.L., Valvano,M.A. (2006) Identification of essential operons with a rhamnose-inducible promoter in *Burkholderia cenocepacia*. *Applied and Environmental Microbiology* 72: 2547-2555.

Cardona,S.T., and Valvano,M.A. (2005) An expression vector containing a rhamnose-inducible promoter provides tightly regulated gene expression in *Burkholderia cenocepacia*. *Plasmid* 54: 219-228.

Chan,P.F., Holmes,D.J., Payne,D.J. (2004a) Finding the gems using genomic discovery: antibacterial drug discovery strategies - the successes and the challenges. *Drug Discovery Today: Therapeutic Strategies* 1: 519-527.

Chan,Y.Y., and Chua,K.L. (2005) The *Burkholderia pseudomallei* BpeAB-OprB efflux pump: expression and impact on quorum sensing and virulence. *Journal of Bacteriology* 187: 4707-4719.

Chan,Y.Y., Tan,T.M.C., Ong,Y.A., Chua,K.L. (2004b) BpeAB-OpRB, a multidrug efflux pump in *Burkholderia pseudomallei*. *Antimicrobial Agents and Chemotherapy* 48: 1128-1135.

Chantratita,N., Rholl,D.A., Sim,B., Wuthiekanun,V., Limmathurotsakul,D., Amornchai,P. Thanwisai,A., Chua,H., Ooi,W., Holden,M.T., Day,N.P., Tan,P., Schweizer,H.P., Peacock,S.J. (2011) Antimicrobial resistance to ceftazidime involving loss of penicillin-binding protein 3 in *Burkholderia pseudomallei*. *Proceedings of the National Academy of Sciences of the United States of America* 108: 17165-17170.

Chaowagul,W., White,N.J., Dance,D.A.B., Wattanagoon,Y., Naigowit,P., Davis,T.M.E., Looareesuwan,S., Pitakwatchara,N. (1989) Melioidosis - a major cause of community-acquired septicemia in northeastern Thailand. *Journal of Infectious Diseases* 159: 890-899.

Charuchaimontri,C., Suputtamongkol,Y., Nilakul,C., Chaowagul,W., Chetchotisakd,P., Lertpatanasuwun,N., Intaranongpai,S., Brett,P.J., Woods,D.E. (1999) Anti-lipopolysaccharide II: an antibody protective against fatal melioidosis. *Clinical Infectious Diseases* 29: 813-818.

Chaudhuri,R.R., Morgan,E., Peters,S.E., Pleasance,S.J., Hudson,D.L., Davies,H.M. Wang,J., van Diemen,P.M., Buckley,A.M., Bowen,A.J., Pullinger,G.D., Turner,D.J., Langridge,G.C., Turner,A., Parkhill,J., Charles,I.G., Maskell,D.J., Stevens,M.P. (2013) Comprehensive assignment of roles for *Salmonella* Typhimurium genes in intestinal colonization of food-producing animals. *PLoS Genetics* 9: e1003456.

Cheng,A.C., Chierakul,W., Chaowagul,W., Chetchotisakd,P., Limmathurotsakul,D., Dance,D.A., Peacock,S.J., Currie,B.J. (2008) Consensus guidelines for dosing of amoxicillin-clavulanate in melioidosis. *The American Journal of Tropical Medicine and Hygiene* 78: 208-209.

Cheng,A.C., and Currie,B.J. (2005) Melioidosis: epidemiology, pathophysiology, and management. *Clinical Microbiology Reviews* 18: 383-416.

Cheng,A.C., Stephens,D.P., Anstey,N.M., Currie,B.J. (2004a) Adjunctive granulocyte colony-stimulating factor for treatment of septic shock due to melioidosis. *Clinical Infectious Diseases* 38: 32-37.

Cheng,A.C., Fisher,D.A., Anstey,N.M., Stephens,D.P., Jacups,S.P., Currie,B.J. (2004b) Outcomes of patients with melioidosis treated with meropenem. *Antimicrobial Agents and Chemotherapy* 48: 1763-1765.

Chou,D.W., Chung,K.M., Chen,C.H., Cheung,B.M.H. (2007) Bacteremic melioidosis in southern Taiwan: clinical characteristics and outcome. *Journal of the Formosan Medical Association* 106: 1013-1022.

Chua,K.L., Chan,Y.Y., Gan,Y.H. (2003) Flagella are virulence determinants of *Burkholderia pseudomallei*. *Infection and Immunity* 71: 1622-1629.

Cianciotto,N.P., Eisenstein,B.I., Mody,C.H., Toews,G.B., Engleberg,N.C. (1989) A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates initiation of intracellular infection. *Infection and Immunity* 57: 1255-1262.

Clatworthy,A.E., Pierson,E., Hung,D.T. (2007) Targeting virulence: a new paradigm for antimicrobial therapy. *Nature Chemical Biology* 3: 541-548.

Coenye,T., and Vandamme,P. (2003) Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environmental Microbiology* 5: 719-729.

Cornelis,G.R., and Wolf-Watz,H. (1997) The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells. *Molecular Microbiology* 23: 861-867.

Cuccui,J., Easton,A., Chu,K.K., Bancroft,G.J., Oyston,P.C.F., Titbal,R.W., Wren,B.W. (2007) Development of Signature-Tagged Mutagenesis in *Burkholderia pseudomallei* to identify genes important in survival and pathogenesis *Infection and Immunity* 75: 1186-1195.

Currie,B.J., Fisher,D.A., Anstey,N.M., Jacups,S.P. (2000a) Melioidosis: acute and chronic disease, relapse and re-activation. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 94: 301-304.

Currie,B.J., Fisher,D.A., Howard,D.M., Burrow,J.N.C., Selvanayagam,S., Snelling,P.L., Anstey,N.M., Mayo,M.J. (2000b) The epidemiology of melioidosis in Australia and Papua New Guinea. *Acta Tropica* 74: 121-127.

Currie,B.J., and Jacups,S.P. (2003) Intensity of rainfall and severity of melioidosis, Australia. *Emerging Infectious Diseases* 9: 1538-1542.

Currie,B., Howard,D., Nguyen,V.T., Withnall,K., Merianos,A. (1993) The 1990-1991 outbreak of melioidosis in the Northern Territory of Australia: clinical aspects. *Southeast Asian Journal of Tropical Medicine and Public Health* 24: 436-443.

Currie,B.J., Fisher,D.A., Howard,D.M., Burrow,J.N.C., Lo,D., Selva-Nayagam,S., Anstey,N.M., Huffam,S.E., Snelling,P.L., Marks,P.J., Stephens,D.P., Lum,G.D., Jacups,S.P., Krause,V.L. (2000c) Endemic melioidosis in tropical northern Australia: a 10-year prospective study and review of the literature. *Clinical Infectious Diseases* 31: 981-986.

Dance,D.A.B. (1991) Melioidosis - the tip of the iceberg. *Clinical Microbiology Reviews* 4: 52-60.

Dance,D.A.B. (2000) Ecology of *Burkholderia pseudomallei* and the interactions between environmental *Burkholderia* spp. and human-animal hosts. *Acta Tropica* 74: 159-168.

Dance,D.A.B., Davis,T.M.E., Wattanagoon,Y., Chaowagul,W., Saiphan,P., Looareesuwan,S., Wuthiekanun,V., White,N.J. (1989) Acute suppurative parotitis caused by *Pseudomonas pseudomallei* in children. *Journal of Infectious Diseases* 159: 654-660.

Darwin,A.J. (2005) The Phage-shock protein response. *Molecular Microbiology* 57: 621-628.

Darwin,A.J., and Miller,V.L. (1999) Identification of *Yersinia enterocolitica* genes affecting survival in an animal host using signature-tagged transposon mutagenesis. *Molecular Microbiology* 32: 51-62.

Darwin,A.J., and Miller,V.L. (2001) The *psp* locus of *Yersinia enterocolitica* is required for virulence and for growth in vitro when the Ysc type III secretion system is produced. *Molecular Microbiology* 39: 429-444.

Darwin,A.J. (2013) Stress relief during host infection: the Phage-shock protein response supports bacterial virulence in various ways. *PLoS Pathogens* 9: e1003388.

de Boer,P.A.J., Crossley,R.E., Hand,A.R., Rothfield,L.I. (1991) The MinD protein is a membrane ATPase required for the correct placement of the *Escherichia coli* division site. *The EMBO Journal* 10: 4371-4380.

de Boer,P.A.J., Crossley,R.E., Rothfield,L.I. (1988) Isolation and properties of *minB*, a complex genetic locus involved in correct placement of the division site in *Escherichia coli*. *Journal of Bacteriology* 170: 2106-2112.

de Boer,P.A.J., Crossley,R.E., Rothfield,L.I. (1989) A division inhibitor and a topological specificity factor coded for by the minicell locus determine proper placement of the division septum in *Escherichia coli*. *Cell* 56: 641-649.

de Boer,P.A.J., Crossley,R.E., Rothfield,L.I. (1992) Roles of MinC and MinD in the site-specific septation block mediated by the MinCDE system of *Escherichia coli*. *Journal of Bacteriology* 174: 63-70.

de Chastellier,C., and Berche,P. (1994) Fate of *Listeria monocytogenes* in murine macrophages: evidence for simultaneous killing and survival of intracellular bacteria. *Infection and Immunity* 62: 543-553.

DeLisa,M.P., Lee,P., Palmer,T., Georgiou,G. (2004) Phage-shock protein PspA of *Escherichia coli* relieves saturation of protein export via the Tat pathway. *Journal of Bacteriology* 186: 366-373.

DeShazer,D., Brett,P.J., Carlyon,R., Woods,D.E. (1997) Mutagenesis of *Burkholderia pseudomallei* with Tn5-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. *Journal of Bacteriology* 179: 2116-2125.

DeShazer,D., Brett,P.J., Woods,D.E. (1998) The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. *Molecular Microbiology* 30: 1081-1100.

Doherty,T.M., and Andersen,P. (2005) Vaccines for tuberculosis: novel concepts and recent progress. *Clinical Microbiology Reviews* 18: 687-702.

Dworkin,J., Jovanovic,G., Model,P. (1997) Role of upstream activation sequences and integration host factor in transcriptional activation by the constitutively active prokaryotic enhancer-binding protein PspF. *Journal of Molecular Biology* 273: 377-388.

Dworkin,J., Jovanovic,G., Model,P. (2000) The PspA protein of *Escherichia coli* is a negative regulator of σ^{54}-dependent transcription. *Journal of Bacteriology* 182: 311-319.

Easton,A., Haque,A., Chu,K., Lukaszewski,R., Bancroft,G.J. (2007) A critical role for neutrophils in resistance to experimental infection with *Burkholderia pseudomallei*. *Journal of Infectious Diseases* 195: 99-107.

Elderkin,S., Bordes,P., Jones,S., Rappas,M., Buck,M. (2005) Molecular determinants for PspA-mediated repression of the AAA^+ transcriptional activator PspF. *Journal of Bacteriology* 187: 3238-3248.

Elderkin,S., Jones,S., Schumacher,J., Studholme,D., Buck,M. (2002) Mechanism of action of the *Escherichia coli* Phage-shock protein PspA in repression of the AAA^+ family transcription factor PspF. *Journal of Molecular Biology* 320: 23-37.

Eriksson,S., Lucchini,S., Thompson,A., Rhen,M., Hinton,J.C.D. (2003) Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Molecular Microbiology* 47: 103-118.

Errington,J., Daniel,R.A., Scheffers,D.J. (2003) Cytokinesis in bacteria. *Microbiology and Molecular Biology Reviews* 67: 52-65.

Essex-Lopresti,A.E., Boddey,J.A., Thomas,R., Smith,M.P., Hartley,M.G., Atkins,T., Brown,N.F., Tsang,C.H., Peak,I.R.A., Hill,J., Beacham,I.R., Titball,R.W. (2005) A type IV pilin, PilA, contributes to adherence of *Burkholderia pseudomallei* and virulence *in vivo*. *Infection and Immunity* 73: 1260-1264.

Estes,D., Dow,S.W., Schweizer,H.P., Torres,A.G. (2010) Present and future therapeutic strategies for melioidosis and glanders. *Expert Review of Anti-Infective Therapy* 8: 325-338.

Felice,M.D., Guardiola,J., Esposito,B., Iaccarino,M. (1974) Structural genes for a newly recognized acetolactate synthase in *Escherichia coli* K-12. *Journal of Bacteriology* 120: 1068-1077.

Figurski,D.H., and Helinski,D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proceedings of the National Academy of Sciences* 76: 1648-1652.

Forman,H.J., and Thomas,M.J. (1986) Oxidant production and bactericidal activity of phagocytes. *Annual Review of Physiology* 48: 669-680.

Fuqua,W.C., Winans,S.C., Greenberg,E.P. (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology* 176: 269-275.

Galán,J.E., Ginocchio,C., Costeas,P. (1992) Molecular and functional characterization of the *Salmonella* invasion gene *invA*: homology of InvA to members of a new protein family. *Journal of Bacteriology* 174: 4338-4349.

Gan,Y.H. (2005) Interaction between *Burkholderia pseudomallei* and the host immune response: sleeping with the enemy? *Journal of Infectious Diseases* 192: 1845-1850.

Germanier,R., and Fürer,E. (1975) Isolation and characterization of GalE mutant Ty21a of *Salmonella typhi*: a candidate strain for a live, oral typhoid vaccine. *Journal of Infectious Diseases* 131: 553-558.

Gibney,K.B., Cheng,A.C., Currie,B.J. (2008) Cutaneous melioidosis in the tropical top end of Australia: a prospective study and review of the literature. *Clinical Infectious Diseases* 47: 603-609.

Gillis,M., Van Van,T., Bardin,R., Goor,M., Hebbar,P., Willems,A. Segers,P., Kersters,K., Heulin,T., Fernandez,M.P. (1995) Polyphasic taxonomy in the genus *Burkholderia* leading to an amended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N_2-fixing isolates from rice in Vietnam. *International Journal of Systematic Bacteriology* 45: 274-289.

Godfrey,A.J., Wong,S., Dance,D.A.B., Chaowagul,W., Bryan,L.E. (1991) *Pseudomonas pseudomallei* resistance to beta-lactam antibiotics due to alterations in the chromosomally encoded beta-lactamase. *Antimicrobial Agents and Chemotherapy* 35: 1635-1640.

Godoy,D., Randle,G., Simpson,A.J., Aanensen,D.M., Pitt,T.L., Kinoshita,R., Spratt,B.G. (2003) Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, *Burkholderia pseudomallei* and *Burkholderia mallei*. *Journal of Clinical Microbiology* 41: 2068-2079.

Gong,L., Cullinane,M., Treerat,P., Ramm,G., Prescott,M., Adler,B., Boyce,J.D., Devenish,R.J. (2011) The *Burkholderia pseudomallei* type III secretion system and BopA are required for evasion of LC3-associated phagocytosis. *PLoS One* 6: e17852.

Gough,J., Karplus,K., Hughey,R., Chothia,C. (2001) Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure. *Journal of Molecular Biology* 313: 903-919.

Gouin,E., Gantelet,H., Egile,C., Lasa,I., Ohayon,H., Villiers,V., Gounon,P., Sansonetti,P.J., Cossart,P. (1999) A comparative study of the actin-based motilities of the pathogenic bacteria *Listeria monocytogenes, Shigella flexneri* and *Rickettsia conorii*. *Journal of Cell Science* 112: 1697-1708.

Gueguen,E., Flores-Kim,J., Darwin,A.J. (2011) The *Yersinia enterocolitica* Phage-shock proteins B and C can form homodimers and heterodimers *in vivo* with the possibility of close association between multiple domains. *Journal of Bacteriology* 193: 5747-5758.

Gueguen,E., Savitzky,D.C., Darwin,A.J. (2009) Analysis of the *Yersinia enterocolitica* PspBC proteins defines functional domains, essential amino acids and new roles within the Phage-shock protein response. *Molecular Microbiology* 74: 619-633.

Håkansson,S., Schesser,K., Persson,C., Galyov,E.E., Rosqvist,R., Homblé,F., Wolf-Watz,H. (1996) The YopB protein of *Yersinia pseudotuberculosis* is essential for the translocation of Yop effector proteins across the target cell plasma membrane and displays a contact-dependent membrane disrupting activity. *The EMBO Journal* 15: 5812-5823.

Hankamer,B.D., Elderkin,S.L., Buck,M., Nield,J. (2004) Organization of the AAA^+ adaptor protein PspA is an oligomeric ring. *Journal of Biological Chemistry* 279: 8862-8866.

Haque,A., Easton,A., Smith,D., O'Garra,A., Van Rooijen,N., Lertmemongkolchai,G., Titball,R.W., Bancroft,G.J. (2006) Role of T cells in innate and adaptive immunity against murine *Burkholderia pseudomallei* infection. *Journal of Infectious Diseases* 193: 370-379.

Harland,D.N., Chu,K., Haque,A., Nelson,M., Walker,N.J., Sarkar-Tyson,M., Atkins,T.P., Moore,B., Brown,K.A., Bancroft,G., Titball,R.W., Atkins,H.S. (2007) Identification of a LolC homologue in *Burkholderia pseudomallei*, a novel protective antigen for melioidosis. *Infection and Immunity* 75: 4173-4180.

Healey,G.D., Elvin,S.J., Morton,M., Williamson,E.D. (2005) Humoral and cell-mediated adaptive immune responses are required for protection against *Burkholderia pseudomallei* challenge and bacterial clearance post-infection. *Infection and Immunity* 73: 5945-5951.

Hensel,M., Shea,J.E., Gleeson,C., Jones,M.D., Dalton,E., Holden,D.W. (1995) Simultaneous identification of bacterial virulence genes by negative selection. *Science* 269: 400-403.

Hentzer,M., Wu,H., Andersen,J.B., Riedel,K., Rasmussen,T.B., Bagge,N., Kumar,N., Schembri,M.A., Song,Z., Kristoffersen,P., Manefield,M., Costerton,J.W., Molin,S., Eberl,L., Steinberg,P., Kjelleberg,S., Høiby,N., Givskov,M. (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *The EMBO Journal* 22: 3803-3815.

Heras,B., Shouldice,S.R., Totsika,M., Scanlon,M.J., Schembri,M.A., Martin,J.L. (2009) DSB proteins and bacterial pathogenicity. *Nature Reviews Microbiology* 7: 215-225.

Ho,M., Schollaardt,T., Smith,M.D., Perry,M.B., Brett,P.J., Chaowagul,W., Bryan,L.E. (1997) Specificity and functional activity of anti-*Burkholderia pseudomallei* polysaccharide antibodies. *Infection and Immunity* 65: 3648-3653.

Hoffmann,J.A. (1995) Innate immunity of insects. *Current Opinion in Immunology* 7: 4-10.

Holden,M.T.G., Titball,R.W., Peacock,S.J., Cerdeno-Tarraga,A.M., Atkins,T., Crossman,L.C., Pitt,T., Churcher,C., Mungall,K., Bentley,S.D., Sebaihia,M., Thomson,N.R., Bason,N., Beacham,I.R., Brooks,K., Brown,K.A., Brown,N.F., Challis,G.L., Cherevach,I., Chillingworth,T., Cronin,A., Crossett,B., Davis,P., DeShazer,D., Feltwell,T., Fraser,A., Hance,Z., Hauser,H., Holroyd,S., Jagels,K., Keith,K.E., Maddison,M., Moule,S., Price,C., Quail,M.A., Rabbinowitsch,E., Rutherford,K., Sanders,M., Simmonds,M., Songsivilai,S., Stevens,K., Tumapa,S., Vesaratchavest,M., Whitehead,S., Yeats,C., Barrell,B.G., Oyston,P.C.F., Parkhill,J. (2004) Genornic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. *Proceedings of the National Academy of Sciences of the United States of America* 101: 14240-14245.

Horstman,N., and Darwin,A.J. (2012) Phage-shock proteins B and C prevent lethal cytoplasmic membrane permeability in *Yersinia enterocolitica*. *Molecular Microbiology* 85: 445-460.

Horswill,A.R., Savinov,S.N., Benkovic,S.J. (2004) A systematic method for identifying small-molecule modulators of protein-protein interactions. *Proceedings of the National Academy of Sciences of the United States of America* 101: 15591-15596.

Hu,J.C., O'Shea,E.K., Kim,P.S., Sauer,R.T. (1990) Sequence requirements for coiled-coils: analysis with lambda repressor-GCN4 leucine zipper fusions. *Science* 250: 1400-1403.

Hu,Z.L., Gogol,E.P., Lutkenhaus,J. (2002) Dynamic assembly of MinD on phospholipid vesicles regulated by ATP and MinE. *Proceedings of the National Academy of Sciences of the United States of America* 99: 6761-6766.

Hu,Z.L., and Lutkenhaus,J. (1999) Topological regulation of cell division in *Escherichia coli* involves rapid pole to pole oscillation of the division inhibitor MinC under the control of MinD and MinE. *Molecular Microbiology* 34: 82-90.

Hu,Z.L., and Lutkenhaus,J. (2000) Analysis of MinC reveals two independent domains involved in interaction with MinD and FtsZ. *Journal of Bacteriology* 182: 3965-3971.

Hu,Z.L., and Lutkenhaus,J. (2001) Topological regulation of cell division in *E. coli*: spatiotemporal oscillation of MinD requires stimulation of its ATPase by MinE and phospholipid. *Molecular Cell* 7: 1337-1343.

Hu,Z.L., and Lutkenhaus,J. (2003) A conserved sequence at the C-terminus of MinD is required for binding to the membrane and targeting MinC to the septum. *Molecular Microbiology* 47: 345-355.

Hu,Z.L., Mukherjee,A., Pichoff,S., Lutkenhaus,J. (1999) The MinC component of the division site selection system in *Escherichia coli* interacts with FtsZ to prevent polymerization. *Proceedings of the National Academy of Sciences of the United States of America* 96: 14819-14824.

Hu,Z.L., Saez,C., Lutkenhaus,J. (2003) Recruitment of MinC, an inhibitor of Z-ring formation, to the membrane in *Escherichia coli*: role of MinD and MinE. *Journal of Bacteriology* 185: 196-203.

Huang,J., Cao,C., Lutkenhaus,J. (1996) Interaction between FtsZ and inhibitors of cell division. *Journal of Bacteriology* 178: 5080-5085.

Huang,K.C., Meir,Y., Wingreen,N.S. (2003) Dynamic structures in *Escherichia coli*: spontaneous formation of MinE rings and MinD polar zones. *Proceedings of the National Academy of Sciences* 100: 12724-12728.

Hudson,D.L., Layton,A.N., Field,T.R., Bowen,A.J., Wolf-Watz,H., Elofsson,M., Stevens,M.P., Galyov,E.E. (2007) Inhibition of type III secretion in *Salmonella enterica* serovar Typhimurium by small-molecule inhibitors. *Antimicrobial Agents and Chemotherapy* 51: 2631-2635.

Hueck,C.J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiology and Molecular Biology Reviews* 62: 379-433.

Huisman,O., and D'Ari,R. (1981) An inducible DNA replication-cell division coupling mechanism in *E. coli*. *Nature* 290: 797-799.

Huvet,M., Toni,T., Sheng,X., Thorne,T., Jovanovic,G., Engl,C. Buck,M., Pinney,J.W., Stumpf,M.P.H. (2010) The evolution of the Phage-shock protein (Psp) response system: interplay between protein function, genomic organization and system function. *Molecular Biology and Evolution* 28: 1141-1155.

Inglis,T.J.J., and Sagripanti,J.L. (2006) Environmental factors that affect the survival and persistence of *Burkholderia pseudomallei*. *Applied and Environmental Microbiology* 72: 6865-6875.

Ip,M., Osterberg,L.G., Chau,P.Y., Raffin,T.A. (1995) Pulmonary melioidosis. *Chest* 108: 1420-1424.

Ireland,P., McMahon,R.M., Marshall,L.E., Halili,M., Furlong,E., Tay,S., Martin,J.L., Sarkar-Tyson,M. (2013) Disarming *Burkholderia pseudomallei*: structural and functional characterisation of a disulfide oxidoreductase (DsbA) required for virulence *in vivo*. *Antioxidants & Redox Signaling* 20: 606-617.

Joly,N., Burrows,P.C., Engl,C., Jovanovic,G., Buck,M. (2009) A lower-order oligomer form of Phage-shock protein A (PspA) stably associates with the hexameric AAA^{+} transcription activator protein PspF for negative regulation. *Journal of Molecular Biology* 394: 764-775.

Jones,A.L., Beveridge,T.J., Woods,D.E. (1996) Intracellular survival of *Burkholderia pseudomallei*. *Infection and Immunity* 64: 782-790.

Jones,S.E., Lloyd,L.J., Tan,K.K., Buck,M. (2003) Secretion defects that activate the Phage-shock response of *Escherichia coli*. *Journal of Bacteriology* 185: 6707-6711.

Jones,S.M., Ellis,J.F., Russell,P., Griffin,K.F., Oyston,P.C.F. (2002) Passive protection against *Burkholderia pseudomallei* infection in mice by monoclonal antibodies against capsular polysaccharide, lipopolysaccharide or proteins. *Journal of Medical Microbiology* 51: 1055-1062.

Jovanovic,G., Rakonjac,J., and Model,P. (1999) *In vivo* and *in vitro* activities of the *Escherichia coli* σ^{54} transcription activator, PspF, and its DNA-binding mutant, PspFΔHTH. *Journal of Molecular Biology* 285: 469-483.

Jovanovic,G., Weiner,L., Model,P. (1996) Identification, nucleotide sequence, and characterization of PspF, the transcriptional activator of the *Escherichia coli* stress-induced *psp* operon. *Journal of Bacteriology* 178: 1936-1945.

Kanaphun,P., Thirawattanasuk,N., Suputtamongkol,Y., Naigowit,P., Dance,D.A.B., Smith,M.D., White,N.J. (1993) Serology and carriage of *Pseudomaonas pseudomallei*: A prospective study in 1000 hospitalized children in Northeast Thailand. *Journal of Infectious Diseases* 167: 230-233.

Karlinsey,J.E., Maguire,M.E., Becker,L.A., Crouch,M.L., Fang,F.C. (2010) The Phage-shock protein PspA facilitates divalent metal transport and is required for virulence of *Salmonella enterica* sv. Typhimurium. *Molecular Microbiology* 78: 669-685.

Kauppi,A.M., Nordfelth,R., Uvell,H., Wolf-Watz,H., Elofsson,M. (2003) Targeting bacterial virulence: inhibitors of type III secretion in *Yersinia*. *Chemistry & Biology* 10: 241-249.

Kespichayawattana,W., Rattanachetkul,S., Wanun,T., Utaisincharoen,P., Sirisinha,S. (2000) *Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. *Infection and Immunity* 68: 5377-5384.

Kespichayawattana,W., Intachote,P., Utaisincharoen,P., Sirisinha,S. (2004) Virulent *Burkholderia pseudomallei* is more efficient than avirulent *Burkholderia thailandensis* in invasion of and adherence to cultured human epithelial cells. *Microbial Pathogenesis* 36: 287-292.

Kleerebezem,M., Crielaard,W., Tommassen,J. (1996) Involvement of stress protein PspA (Phage-shock protein A) of *Escherichia coli* in maintenance of the proton motive force under stress conditions. *The EMBO Journal* 15: 162-171.

Kleerebezem,M., and Tommassen,J. (1993) Expression of the PspA gene stimulates efficient protein export in *Escherichia coli*. *Molecular Microbiology* 7: 947-956.

Klugman,K.P., Koornhof,H.J., Schneerson,R., Cadoz,M., Gilbertson,I.T., Robbins,J.B., Schulz,D., Armand,J. (1987) Protective activity of Vi capsular polysaccharide vaccine against typhoid fever. *The Lancet* 330: 1165-1169.

Kobayashi,H., Yamamoto,M., Aono,R. (1998) Appearance of a stress-response protein, Phage-shock protein A, in *Escherichia coli* exposed to hydrophobic organic solvents. *Microbiology UK* 144: 353-359.

Kobayashi,R., Suzuki,T., Yoshida,M. (2007) *Escherichia coli* Phage-shock protein A (PspA) binds to membrane phospholipids and repairs proton leakage of the damaged membranes. *Molecular Microbiology* 66: 100-109.

Kohanski,M.A., Dwyer,D.J., Collins,J.J. (2010) How antibiotics kill bacteria: from targets to networks. *Nature Reviews Microbiology* 8: 423-435.

Kovach,M.E., Elzer,P.H., Hill,D.S., Robertson,G.T., Farris,M.A., Roop,R.M., Peterson,K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1-MCS, carrying different antibiotic-resistance cassettes. *Gene* 166: 175-176.

Langridge,G.C., Phan,M.D., Turner,D.J., Perkins,T.T., Parts,L., Haase,J., Charles,I., Maskell,D.J., Peters,S.E., Dougan,G., Wain,J., Parkhill,J., Turner,A.K. (2009) Simultaneous assay of every *Salmonella* Typhi gene using one million transposon mutants. *Genome Research* 19: 2308-2316.

Leakey,A.K., Ulett,G.C., Hirst,R.G. (1998) BALB/c and C57BL/6 mice infected with virulent *Burkholderia pseudomallei* provide contrasting animal models for the acute and chronic forms of human melioidosis. *Microbial Pathogenesis* 24: 269-275.

Lee,P.A., Tullman-Ercek,D., Georgiou,G. (2006) The bacterial twin-arginine translocation pathway. *Annual Review of Microbiology* 60: 373-395.

Lefebre,M.D., and Valvano,M.A. (2002) Construction and evaluation of plasmid vectors optimized for constitutive and regulated gene expression in *Burkholderia cepacia* complex isolates. *Applied and Environmental Microbiology* 68: 5956-5964.

Limmathurotsakul,D., Dance,D.A., Wuthiekanun,V., Kaestli,M., Mayo,M., Warner,J., Wagner,D.M., Tuanyok,A., Wertheim,H., Cheng,T.Y., Mukhopadhyay,C., Puthucheary,S., Day,N.P., Steinmetz,I., Currie,B.J., Peacock,S.J. (2013) Systematic review and consensus guidelines for environmental sampling of *Burkholderia pseudomallei*. *PLoS Neglected Tropical Diseases* 7: e2105.

Limmathurotsakul,D., Wongratanacheewin,S., Teerawattanasook,N., Wongsuvan,G., Chaisuksant,S., Chetchotisakd,P., Chaowagul,W., Day,N.P.J.; Peacock,S.J. (2010a) Increasing incidence of human melioidosis in Northeast Thailand. *The American Journal of Tropical Medicine and Hygiene* 82: 1113-1117.

Limmathurotsakul,D., Wuthiekanun,V., Chantratita,N., Wongsuvan,G., Amornchai,P., Day,N.P.J., Peacock,S.J. (2010b) *Burkholderia pseudomallei* is spatially distributed in soil in Northeast Thailand. *PLoS Neglected Tropical Diseases* 4: e694.

Lipinski,C.A. (2000) Drug-like properties and the causes of poor solubility and poor permeability. *Journal of Pharmacological Toxicological Methods* 44: 235-249.

Little,J.W., and Mount,D.W. (1982) The SOS regulatory system of *Escherichia coli*. *Cell* 29: 11-22.

Lloyd,L.J., Jones,S.E., Jovanovic,G., Gyaneshwar,P., Rolfe,M.D., Thompson,A., Hinton,J.C., Buck,M. (2004) Identification of a new member of the Phage-shock protein response in *Escherichia coli*, the Phage-shock protein G (PspG). *Journal of Biological Chemistry* 279: 55707-55714.

Logue,C.A., Peak,I.R.A., Beacham,I.R. (2009) Facile construction of unmarked deletion mutants in *Burkholderia pseudomallei* using *sacB* counter-selection in sucrose-resistant and sucrose-sensitive isolates. *Journal of Microbiological Methods* 76: 320-323.

Lucchini,S., Liu,H., Jin,Q., Hinton,J.C.D., Yu,J. (2005) Transcriptional adaptation of *Shigella flexneri* during infection of macrophages and epithelial cells: insights into the strategies of a cytosolic bacterial pathogen. *Infection and Immunity* 73: 88-102.

Lutkenhaus,J. (2007) Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z ring. *Annual Review of Biochemistry* 76: 539-562.

Ma,L.Y., King,G., Rothfield,L. (2003) Mapping the MinE site involved in interaction with the MinD division site selection protein of *Escherichia coli*. *Journal of Bacteriology* 185: 4948-4955.

Ma,L.Y., King,G.F., Rothfield,L. (2004) Positioning of the MinE binding site on the MinD surface suggests a plausible mechanism for activation of the *Escherichia coli* MinD ATPase during division site selection. *Molecular Microbiology* 54: 99-108.

Mahan,M.J., Slauch,J.M., Mekalanos,J.J. (1993) Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* 259: 686-688.

Maharjan,B., Chantratita,N., Vesaratchavest,M., Cheng,A., Wuthiekanun,V., Chierakul,W., Chaowagul,W., Day,N.P.J., Peacock,S.J. (2005) Recurrent melioidosis in patients in Northeast Thailand is frequently due to reinfection rather than relapse. *Journal of Clinical Microbiology* 43: 6032-6034.

Matsuura,M., Kawahara,K., Ezaki,T., Nakano,M. (1996) Biological activities of lipopolysaccharide of *Burkholderia* (*Pseudomonas*) *pseudomallei*. *FEMS Microbiology Letters* 137: 79-83.

Maxson,M.E., and Darwin,A.J. (2006) PspB and PspC of *Yersinia enterocolitica* are dual function proteins: regulators and effectors of the Phage-shock protein response. *Molecular Microbiology* 59: 1610-1623.

Mecsas,J. (2002) Use of signature-tagged mutagenesis in pathogenesis studies. *Current Opinion in Microbiology* 5: 33-37.

Mecsas,J., Bilis,I., Falkow,S. (2001) Identification of attenuated *Yersinia pseudotuberculosis* strains and characterization of an orogastric infection in BALB/c mice on day 5 post-infection by signature-tagged mutagenesis. *Infection and Immunity* 69: 2779-2787.

Mileykovskaya,E., Fishov,I., Fu,X.Y., Corbin,B.D., Margolin,W., Dowhan,W. (2003) Effects of phospholipid composition on MinD-membrane interactions *in vitro* and *in vivo*. *Journal of Biological Chemistry* 278: 22193-22198.

Milton,D.L., O'Toole,R., Horstedt,P., Wolf-Watz,H. (1996) Flagellin A is essential for the virulence of *Vibrio anguillarum*. *Journal of Bacteriology* 178: 1310-1319.

Mima,T., and Schweizer,H.P. (2010) The BpeAB-OprB efflux pump of *Burkholderia pseudomallei* 1026b does not play a role in quorum sensing, virulence factor production, or extrusion of aminoglycosides but is a broad spectrum drug efflux system. *Antimicrobial Agents and Chemotherapy* 54: 3113-3120.

Model,P., Jovanovic,G., Dworkin,J. (1997) The *Escherichia coli* Phage-shock protein (*psp*) operon. *Molecular Microbiology* 24: 255-261.

Moore,R.A., DeShazer,D., Reckseidler,S., Weissman,A., Woods,D.E. (1999) Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. *Antimicrobial Agents and Chemotherapy* 43: 465-470.

Moore,R.A., Reckseidler-Zenteno,S., Kim,H., Nierman,W., Yu,Y., Tuanyok,A., Warawa,J., DeShazer,D., Woods,D.E. (2004) Contribution of gene loss to the pathogenic evolution of *Burkholderia pseudomallei* and *Burkholderia mallei*. *Infection and Immunity* 72: 4172-4187.

Moule,M.G., Hemsley,C.M., Seet,Q., Guerra-Assunção,J.A., Lim,J., Sarkar-Tyson,M., Clark,T.G., Tan,P.B.O., Titball,R.W., Cuccui,J., Wren,B.W.. (2014) Genome-wide saturation mutagenesis of *Burkholderia pseudomallei* K96243 predicts essential genes and novel targets for antimicrobial development. *mBio* 5(1):e00926-13. doi:10.1128/mBio.00926-13.

Moulin,L., Munive,A., Dreyfus,B., Boivin-Masson,C. (2001) Nodulation of legumes by members of the β-subclass of Proteobacteria. *Nature* 411: 948-950.

Muangman,S., Korbsrisate,S., Muangsombut,V., Srinon,V., Adler,N.L., Schroeder,G.N., Frankel,G., Galyov,E.E. (2011) BopC is a type III secreted effector protein of *Burkholderia pseudomallei*. *FEMS Microbiology Letters* 323: 75-82.

Muangsombut,V., Suparak,S., Pumirat,P., Damnin,S., Vattanaviboon,P., Thongboonkerd,V., Korbsrisate,S. (2008) Inactivation of *Burkholderia pseudomallei bsaQ* results in decreased invasion efficiency and delayed escape of bacteria from endocytic vesicles. *Archives of Microbiology* 190: 623-631.

Mukherjee,A., and Lutkenhaus,J. (1994) Guanine nucleotide-dependent assembly of FtsZ into filaments. *Journal of Bacteriology* 176: 2754-2758.

Mukherjee,A., Cao,C., Lutkenhaus,J. (1998) Inhibition of FtsZ polymerization by SulA, an inhibitor of septation in *Escherichia coli*. *Proceedings of the National Academy of Sciences* 95: 2885-2890.

Muschiol,S., Bailey,L., Gylfe,A., Sundin,C., Hultenby,K., Bergstrom,S., Elofsson,M., Wolf-Watz,H., Normark,S., Henriques-Normark,B. (2006) A small-molecule inhibitor of type III secretion inhibits different stages of the infectious cycle of *Chlamydia trachomatis*. *Proceedings of the National Academy of Sciences of the United States of America* 103: 14566-14571.

Nanninga,N., and Woldringh,C.L. (1985) Cell growth, genome duplication, and cell division. In Molecular Cytology of *Escherichia coli*. Nanninga,N. (ed). Academic Press, London, pp. 259-318.

Nelson,M., Prior,J.L., Lever,M.S., Jones,H.E., Atkins,T.P., Titball,R.W. (2004) Evaluation of lipopolysaccharide and capsular polysaccharide as subunit vaccines against experimental melioidosis. *Journal of Medical Microbiology* 53: 1177-1182.

Ngauy,V., Lemeshev,Y., Sadkowski,L., Crawford,G. (2005) Cutaneous melioidosis in a man who was taken as a prisoner of war by the Japanese during World War II. *Journal of Clinical Microbiology* 43: 970-972.

Nierman,W.C., DeShazer,D., Kim,H.S., Tettelin,H., Nelson,K.E., Feldblyum,T., Ulrich,R.L., Ronning,C.M., Brinkac,L.M., Daugherty,S.C., Davidsen,T.D., Deboy,R.T., Dimitrov,G., Dodson,R.J., Durkin,A.S., Gwinn,M.L., Haft,D.H., Khouri,H., Kolonay,J.F., Madupu,R., Mohammoud,Y., Nelson,W.C., Radune,D., Romero,C.M., Sarria,S., Selengut,J., Shamblin,C., Sullivan,S.A., White,O., Yu,Y., Zafar,N., Zhou,L.W., Fraser,C.M. (2004) Structural flexibility in the *Burkholderia mallei* genome. *Proceedings of the National Academy of Sciences of the United States of America* 101: 14246-14251.

Norville,I.H., Harmer,N.J., Harding,S.V., Fischer,G., Keith,K.E., Brown,K.A., Sarkar-Tyson,M., Titball,R.W. (2011) A *Burkholderia pseudomallei* macrophage infectivity potentiator-like protein has rapamycin-inhibitable peptidylprolyl isomerase activity and pleiotropic effects on virulence. *Infection and Immunity* 79: 4299-4307.

Ogawa,M., and Sasakawa,C. (2006) Intracellular survival of *Shigella*. *Cellular Microbiology* 8: 177-184.

Ortega,X.P., Cardona,S.T., Brown,A.R., Loutet,S.A., Flannagan,R.S., Campopiano,D.J., Govan,J.R., Valvano,M.A. (2007) A putative gene cluster for aminoarabinose biosynthesis is essential for *Burkholderia cenocepacia* viability. *Journal of Bacteriology* 189: 3639-3644.

Overtoom,R., Khieu,V., Hem,S., Cavailler,P., Te,V., Chan,S., Lau,P., Guillard,B., Vong,S. (2008) A first report of pulmonary melioidosis in Cambodia. *Transactions of the Royal Society of Tropical Medicine* 102: 21-25.

Pace,J.L., Rossi,H.A., Esposito,V.M., Frey,S.M., Tucker,K.D., Walker,R.I. (1998) Inactivated whole-cell bacterial vaccines: current status and novel strategies. *Vaccine* 16: 1563-1574.

Park,K., Wu,W., Battaile,K.P., Lovell,S., Holyoak,T., Lutkenhaus,J. (2011) The Min oscillator uses MinD-dependent conformational changes in MinE to spatially regulate cytokinesis. *Cell* 146: 396-407.

Parti,R.P., Biswas,D., Helgeson,S., Michael,F.S., Cox,A., Dillon,J.A. (2011a) Attenuated virulence of *min* operon mutants of *Neisseria gonorrhoeae* and their interactions with human urethral epithelial cells. *Microbes and Infection* 13: 545-554.

Parti,R.P., Biswas,D., Wang,M., Liao,M., Dillon,J.A. (2011b) A MinD mutant of enterohemorrhagic *E. coli* O157:H7 has reduced adherence to human epithelial cells. *Microbial Pathogenesis* 51: 378-383.

Payne,D.J., Gwynn,M.N., Holmes,D.J., Pompliano,D.L. (2007) Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nature Reviews Drug Discovery* 6: 29-40.

Pichoff,S., and Lutkenhaus,J. (2001) *Escherichia coli* division inhibitor MinCD blocks septation by preventing Z-ring formation. *Journal of Bacteriology* 183: 6630-6635.

Pichoff,S., Vollrath,B., Touriol,C., Bouche,J.P. (1995) Deletion analysis of gene MinE which encodes the topological specificity factor of cell division in *Escherichia coli*. *Molecular Microbiology* 18: 321-329.

Pichoff,S., and Lutkenhaus,J. (2002) Unique and overlapping roles for ZipA and FtsA in septal ring assembly in *Escherichia coli*. *The EMBO Journal* 21: 685-693.

Pilatz,S., Breitbach,K., Hein,N., Fehlhaber,B., Schulze,J., Brenneke,B., Eberl,L., Steinmetz,I. (2006) Identification of *Burkholderia pseudomallei* genes required for the intracellular life cycle and *in vivo* virulence. *Infection and Immunity* 74: 3576-3586.

Pinho,M.G., Kjos,M., Veening,J.W. (2013) How to get (a)round: mechanisms controlling growth and division of coccoid bacteria. *Nature Reviews Microbiology* 11: 601-614.

Podnecky,N.L., Wuthiekanun,V., Peacock,S.J., Schweizer,H.P. (2013) The BpeEF-OprC efflux pump is responsible for widespread trimethoprim resistance in clinical and environmental *Burkholderia pseudomallei* isolates. *Antimicrobial Agents and Chemotherapy* 57: 4381-4386.

Poland,G.A. (2010) Prevention of meningococcal disease: current use of polysaccharide and conjugate vaccines. *Clinical Infectious Diseases* 50: S45-S53.

Price,M.N., Huang,K.H., Alm,E.J., Arkin,A.P. (2005) A novel method for accurate operon predictions in all sequenced prokaryotes. *Nucleic Acids Research* 33: 880-892.

Puthucheary,S.D., Vadivelu,J., Ce-Cile,C., KumThong,W., Ismail,G. (1996) Electron microscopic demonstration of extracellular structure of *Burkholderia pseudomallei*. *American Journal of Tropical Medicine and Hygiene* 54: 313-314.

Raskin,D.M., and de Boer,P.A.J. (1997) The MinE ring: an FtsZ-independent cell structure required for selection of the correct division site in *E. coli*. *Cell* 91: 685-694.

Raskin,D.M., and de Boer,P.A.J. (1999a) MinDE-dependent pole-to-pole oscillation of division inhibitor MinC in *Escherichia coli*. *Journal of Bacteriology* 181: 6419-6424.

Raskin,D.M., and de Boer,P.A.J. (1999b) Rapid pole-to-pole oscillation of a protein required for directing division to the middle of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* 96: 4971-4976.

Reed,L.J., and Muench,H. (1938) A simple method of estimating fifty per cent endpoints. *American Journal of Epidemiology* 27: 493-497.

Riedel,K., Köthe,M., Kramer,B., Saeb,W., Gotschlich,A., Ammendola,A., Eberl,L. (2006) Computer-aided design of agents that inhibit the *cep* quorum-sensing system of *Burkholderia cenocepacia*. *Antimicrobial Agents and Chemotherapy* 50: 318-323.

Rolim,D.B., Rocha,M.F.G., Brilhante,R.S.N., Cordeiro,R.A., Leitao-Junior,N.P., Inglis,T.J.J., Sidrim,J.J.C. (2009) Environmental isolates of *Burkholderia pseudomallei* in Ceara State, northeastern Brazil. *Applied and Environmental Microbiology* 75: 1215-1218.

Rolim,D.B., Vilar,D.C.F.L., Sousa,A.Q., Miralles,I.S., de Oliveira,D.C.A., Harnett,G., O'Reilly,L., Howard,K., Sampson,I., Inglis,T.J.J. (2005) Melioidosis, northeastern Brazil. *Emerging Infectious Diseases* 11: 1458-1460.

Rowley,G., Spector,M., Kormanec,J., Roberts,M. (2006) Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens. *Nature Reviews Microbiology* 4: 383-394.

Saravu,K., Mukhopadhyay,C., Vishwanath,S., Valsalan,R., Docherla,M., Vandana,K.E., Shastry,B.A., Bairy,I., Rao,S.P. (2010) Melioidosis in southern India: epidemiological and clinical profile. *Southeast Asian Journal of Tropical Medicine and Public Health* 41: 401-409.

Saravu,K., Vishwanath,S., Kumar,R.S., Barkur,A.S., Varghese,G.K., Mukhyopadhyay,C., Bairy,I. (2008) Melioidosis - a case series from South India. *Transactions of the Royal Society of Tropical Medicine* 102: S18-S20.

Sarkar-Tyson,M., Thwaite,J., Harding,S., V, Smither,S., Oyston,P., Atkins,T., Titball,R.W. (2007) Polysaccharides and virulence of *Burkholderia pseudomallei*. *Journal of Medical Microbiology* 56: 1005-1010.

Sarkar-Tyson,M., Smither,S.J., Harding,S., V, Atkins,T.P., Titball,R.W. (2009) Protective efficacy of heat-inactivated *B. thailandensis*, *B. mallei* or *B. pseudomallei* against experimental melioidosis and glanders. *Vaccine* 27: 4447-4451.

Sarovich,D.S., Price,E.P., Von Schulze,A.T., Cook,J.M., Mayo,M., Watson,L.M., Richardson,L., Seymour,M.L.; Tuanyok,A., Engelthaler,D.M., Pearson,T., Peacock,S.J., Currie,B.J., Keim,P., Wagner,D.M. (2012) Characterization of ceftazidime resistance mechanisms in clinical isolates of *Burkholderia pseudomallei* from Australia. *PLoS One* 7: e30789.

Sawasdidoln,C., Taweechaisupapong,S., Sermswan,R.W., Tattawasart,U., Tungpradabkul,S., Wongratanacheewin,S. (2010) Growing *Burkholderia pseudomallei* in biofilm stimulating conditions significantly induces antimicrobial resistance. *PLoS One* 5: e9196.

Scott,A.E., Laws,T.R., D'Elia,R.V., Stokes,M.G.M., Nandi,T., Williamson,E.D., Tan,P., Prior,J.L., Atkins,T.P. (2013) Protection against experimental melioidosis following immunisation with live *Burkholderia thailandensis* expressing manno-heptose capsule. *Clinical and Vaccine Immunology*: CVI.00113-13.

Scott,C.P., Abel-Santos,E., Jones,A.D., Benkovic,S.J. (2001) Structural requirements for the biosynthesis of backbone cyclic peptide libraries. *Chemistry & Biology* 8: 801-815.

Shalom,G., Shaw,J.G., Thomas,M.S. (2007) *In vivo* expression technology identifies a type VI secretion system locus in *Burkholderia pseudomallei* that is induced upon invasion of macrophages. *Microbiology SGM* 153: 2689-2699.

Shih,Y.L., Kawagishi,I., Rothfield,L. (2005) The MreB and Min cytoskeletal-like systems play independent roles in prokaryotic polar differentiation. *Molecular Microbiology* 58: 917-928.

Shoae Hassani,A., Malekzadeh,F., Amirmozafari,N., Hamdi,K., Ordouzadeh,N., Ghaemi,A. (2009) Phage-shock protein G, a novel ethanol-induced stress protein in *Salmonella* Typhimurium. *Current Microbiology* 58: 239-244.

Sivalingam,S.P., Sim,S.H., Jasper,L.C.W., Wang,D., Liu,Y., Ooi,E.E. (2008) Pre- and post-exposure prophylaxis of experimental *Burkholderia pseudomallei* infection with doxycycline, amoxicillin/clavulanic acid and co-trimoxazole. *Journal of Antimicrobial Chemotherapy* 61: 674-678.

Standar,K., Mehner,D., Osadnik,H., Berthelmann,F., Hause,G., Lunsdorf,H., Bruser,T. (2008) PspA can form large scaffolds in *Escherichia coli*. *FEBS Letters* 582: 3585-3589.

Stevens,M.P., Friebel,A., Taylor,L.A., Wood,M.W., Brown,P.J., Hardt,W.D., Galyov,E.E. (2003) A *Burkholderia pseudomallei* type III secreted protein, BopE, facilitates bacterial invasion of epithelial cells and exhibits guanine nucleotide exchange factor activity. *Journal of Bacteriology* 185: 4992-4996.

Stevens,M.P., Haque,A., Atkins,T., Hill,J., Wood,M.W., Easton,A., Nelson,M., Underwood-Fowler,C., Titball,R.W., Bancroft,G.J., Galyov,E.E. (2004) Attenuated virulence and protective efficacy of a *Burkholderia pseudomallei bsa* type III secretion mutant in murine models of melioidosis. *Microbiology SGM* 150: 2669-2676.

Stevens,M.P., Stevens,J.M., Jeng,R.L., Taylor,L.A., Wood,M.W., Hawes,P., Monaghan,P., Welch,M.D., Galyov,E.E. (2005) Identification of a bacterial factor required for actin-based motility of *Burkholderia pseudomallei*. *Molecular Microbiology* 56: 40-53.

Stevens,M.P., Wood,M.W., Taylor,L.A., Monaghan,P., Hawes,P., Jones,P.W., Wallis,T.S., Galyov,E.E. (2002) An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen. *Molecular Microbiology* 46: 649-659.

Stricker,J., Maddox,P., Salmon,E.D., Erickson,H.P. (2002) Rapid assembly dynamics of the *Escherichia coli* FtsZ-ring demonstrated by fluorescence recovery after photobleaching. *Proceedings of the National Academy of Sciences* 99: 3171-3175.

Subramoni,S., and Sokol,P.A. (2012) Quorum sensing systems influence *Burkholderia cenocepacia* virulence. *Future Microbiology* 7: 1373-1387.

Sun,G.W., Lu,J.H., Pervaiz,S., Cao,W.P., Gan,Y.H. (2005) Caspase-1 dependent macrophage death induced by *Burkholderia pseudomallei*. *Cell Microbiology* 7: 1447-1458.

Sun,Q., and Margolin,W. (2002) Roles of chromosome structure and associated activities on nucleoid-mediated inhibition of FtsZ ring assembly. *Abstracts of the General Meeting of the American Society for Microbiology* 102: 256.

Sun,Q., and Margolin,W. (2004) Effects of perturbing nucleoid structure on nucleoid occlusion-mediated toporegulation of FtsZ ring assembly. *Journal of Bacteriology* 186: 3951-3959.

Suparak,S., Kespichayawattana,W., Haque,A., Easton,A., Damnin,S., Lertmemongkolchai,G., Bancroft,G.J., Korbsrisate,S. (2005) Multinucleated giants cell formation and apoptosis in infected host cells is mediated by *Burkholderia pseudomallei* type III secretion protein BipB. *Journal of Bacteriology* 187: 6556-6560.

Suputtamongkol,Y., Hall,A.J., Dance,D.A.B., Chaowagul,W., Rajchanuvong,A., Smith,M.D., White,N.J. (1994) The epidemiology of melioidosis in Ubon Ratchatani, Northeast Thailand. *International Journal of Epidemiology* 23: 1082-1090.

Suputtamongkol,Y., Chaowagul,W., Chetchotisakd,P., Lertpatanasuwun,N., Intaranongpai,S., Ruchutrakool,T., Budhsarawong,D., Mootsikapun,P., Wuthiekanun,V., Teerawatasook,N., Lulitanond,A. (1999) Risk factors for melioidosis and bacteremic melioidosis. *Clinical Infectious Diseases* 29: 408-413.

Szeto,T.H., Rowland,S.L., Habrukowich,C.L., King,G.F. (2003) The MinD membrane targeting sequence is a transplantable lipid-binding helix. *Journal of Biological Chemistry* 278: 40050-40056.

Szeto,T.H., Rowland,S.L., Rothfield,L.I., King,G.F. (2002) Membrane localization of MinD is mediated by a C-terminal motif that is conserved across eubacteria, archaea, and chloroplasts. *Proceedings of the National Academy of Sciences of the United States of America* 99: 15693-15698.

Taghbalout,A., Ma,L.Y., Rothfield,L. (2006) Role of MinD-membrane association in Min protein interactions. *Journal of Bacteriology* 188: 2993-3001.

Tavassoli,A., Lu,Q., Gam,J., Pan,H., Benkovic,S.J., Cohen,S.N. (2008) Inhibition of HIV budding by a genetically selected cyclic peptide targeting the Gag-TSG101 interaction. *ACS Chemical Biology* 3: 757-764.

Thanassi,J.A., Hartman-Neumann,S.L., Dougherty,T.J., Dougherty,B.A., Pucci,M.J. (2002) Identification of 113 conserved essential genes using a high-throughput gene disruption system in *Streptococcus pneumoniae*. *Nucleic Acids Research* 30: 3152-3162.

Tribuddharat,C., Moore,R.A., Baker,P., Woods,D.E. (2003) *Burkholderia pseudomallei* class A beta-lactamase mutations that confer selective resistance against ceftazidime or clavulanic acid inhibition. *Antimicrobial Agents and Chemotherapy* 47: 2082-2087.

Trosky,J.E., Liverman,A.D.B., Orth,K. (2008) *Yersinia* outer proteins: Yops. *Cellular Microbiology* 10: 557-565.

Tuanyok,A., Tom,M., Dunbar,J., Woods,D.E. (2006) Genome-wide expression analysis of *Burkholderia pseudomallei* infection in a hamster model of acute melioidosis. *Infection and Immunity* 74: 5465-5476.

Ulett,G.C., Ketheesan,N., Hirst,R.G. (2000) Cytokine gene expression in innately susceptible BALB/c mice and relatively resistant C57BL/6 mice during infection with virulent *Burkholderia pseudomallei*. *Infection and Immunity* 68: 2034-2042.

Ulrich,R.L., DeShazer,D., Brueggemann,E.E., Hines,H.B., Oyston,P.C., Jeddeloh,J.A. (2004) Role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*. *Journal of Medical Microbiology* 53: 1053-1064.

Ussery,D., Kiil,K., Lagesen,K., Sicheritz-Ponten,T., Bohlin,J., Wassenaar,T. (2009) The genus *Burkholderia*: analysis of 56 genomic sequences. In Microbial Pathogenomics. de Reuse,H., and Bereswill,S. (eds). Basel, Karger, pp. 140-157.

Utaisincharoen,P., Tangthawornchaikul,N., Kespichayawattana,W., Anuntagool,N., Chaisuriya,P., Sirisinha,S. (2000) Kinetic studies of the production of nitric oxide (NO) and tumour necrosis factor-alpha (TNF-α) in macrophages stimulated with *Burkholderia pseudomallei* endotoxin. *Clinical and Experimental Immunology* 122: 324-329.

Valsalan,R., Seshadri,S., Pandit,V.R. (2008) Melioidosis masquerading as enteric fever. *Transactions of the Royal Society of Tropical Medicine* 102: 117-118.

Vasu,C., Vadivelu,J., Puthucheary,S.D. (2003) The humoral immune response in melioidosis patients during therapy. *Infection* 31: 24-30.

Vrancken,K., Van Mellaert,L., Anné,J. (2008) Characterization of the *Streptomyces lividans* PspA response. *Journal of Bacteriology* 190: 3475-3481.

Vuddhakul,V., Tharavichitkul,P., Na-Ngam,N., Jitsurong,S., Kunthawa,B., Noimay,P., Binla,A., Thamlikitkul,V. (1999) Epidemiology of *Burkholderia pseudomallei* in Thailand. *American Journal of Tropical Medicine and Hygiene* 60: 458-461.

Wada,M., Sekine,K., Itikawa,H. (1986) Participation of the *dnaK* and *dnaJ* gene products in phosphorylation of glutaminyl-tRNA synthetase and threonyl-tRNA synthetase of *Escherichia coli* K-12. *Journal of Bacteriology* 168: 213-220.

Wallis,T.S., and Galyov,E.E. (2000) Molecular basis of *Salmonella*-induced enteritis. *Molecular Microbiology* 36: 997-1005.

Wand,M.E., Muller,C.M., Titball,R.W., Michell,S.L. (2011) Macrophage and *Galleria mellonella* infection models reflect the virulence of naturally occurring isolates of *B. pseudomallei*, *B. thailandensis* and *B. oklahomensis*. *BMC Microbiology* 11: 11.

Weiner,L., Brissette,J.L., Model,P. (1991) Stress-induced expression of the *Escherichia coli* Phage-shock protein operon is dependent on σ-54 and modulated by positive and negative feedback mechanisms. *Genes & Development* 5: 1912-1923.

Weiner,L., Brissette,J.L., Ramani,N., Model,P. (1995) Analysis of the proteins and cis-acting elements regulating the stress-induced Phage-shock protein operon. *Nucleic Acids Research* 23: 2030-2036.

Weiner,L., and Model,P. (1994) Role of an *Escherichia coli* stress response operon in stationary phase survival. *Proceedings of the National Academy of Sciences of the United States of America* 91: 2191-2195.

White,N.J. (2003) Melioidosis. *The Lancet* 361: 1715-1722.

Whitmore,A., and Krishnaswami,C.S. (1912) An account of the discovery of a hitherto undescribed infective disease occuring among the population of Rangoon. *Indian Medical Gazette* 47: 262-267.

Wiersinga,W., Wieland,C.W., Dessing,M.C., Chantratita,N., Cheng,A.C., Limmathurotsakul,D., Chierakul,W., Leendertse,M., Florquin,S., de Vos,A.F., White,N., Dondorp,A.M., Day,N.P., Peacock,S.J., van der Poll,T. (2007) Toll-like receptor 2 impairs host defense in Gram-negative sepsis caused by *Burkholderia pseudomallei* (melioidosis). *PLoS Medicine* 4: 1268-1280.

Woldringh,C.L., Mulder,E., Huls,P.G., and Vischer,N. (1991) Toporegulation of bacterial division according to the nucleoid occlusion model. *Research in Microbiology* 142: 309-320.

Wolf,D., Kalamorz,F., Wecke,T., Juszczak,A., Mader,U., Homuth,G., Jordan,S., Kirstein,J., Hoppert,M., Voigt,B., Hecker,M., Mascher,T. (2010) In-depth profiling of the LiaR response of *Bacillus subtilis*. *Journal of Bacteriology* 192: 4680-4693.

Wootton,C.I., Elliott,I., Sengdetkha,D., Vongsouvath,M., Phongmany,S., Dance,D. (2013) Melioidosis: an unusual cause of recurrent buttock abscesses. *Clinical and Experimental Dermatology* 38: 427-428.

Wu,L.J., and Errington,J. (2004) Coordination of cell division and chromosome segregation by a nucleoid occlusion protein in *Bacillus subtillis*. *Cell* 117: 915-925.

Wu,W., Park,K.T., Holyoak,T., Lutkenhaus,J. (2011) Determination of the structure of the MinD-ATP complex reveals the orientation of MinD on the membrane and the relative location of the binding sites for MinE and MinC. *Molecular Microbiology* 79: 1515-1528.

Wuthiekanun,V., and Peacock,S.J. (2006) Management of melioidosis. *Expert Review of Anti-Infective Therapy* 4: 445-455.

Yakushi,T., Masuda,K., Narita,S.i., Matsuyama,S.i., Tokuda,H. (2000) A new ABC transporter mediating the detachment of lipid-modified proteins from membranes. *Nature Cell Biology* 2: 212-218.

Yamaguchi,S., Gueguen,E., Horstman,N., Darwin,A.J. (2010) Membrane association of PspA depends on activation of the Phage-shock protein response in *Yersinia enterocolitica*. *Molecular Microbiology* 78: 429-443.

Yamaguchi,S., Reid,D.A., Rothenberg,E., Darwin,A.J. (2013) Changes in Psp protein binding partners, localization and behaviour upon activation of the *Yersinia enterocolitica* Phage-shock protein response. *Molecular Microbiology* 87: 656-671.

Yu,X.C., and Margolin,W. (1999) FtsZ ring clusters in *min* and partition mutants: role of both the Min system and the nucleoid in regulating FtsZ ring localization. *Molecular Microbiology* 32: 315-326.

Zhou,H., and Lutkenhaus,J. (2003) Membrane binding by MinD involves insertion of hydrophobic residues within the C-terminal amphipathic helix into the bilayer. *Journal of Bacteriology* 185: 4326-4335.

Zhou,H., and Lutkenhaus,J. (2004) The switch I and II regions of MinD are required for binding and activating MinC. *Journal of Bacteriology* 186: 1546-1555.