The identification and characterisation of PPIases from *Burkholderia pseudomallei* and *Burkholderia thailandensis*

Submitted by Isobel Harriet Norville, to the University of Exeter as a thesis for the degree of Doctor of Philosophy

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I certify that all material in this thesis which is not my own work has been identified and that no material has been previously submitted and approved for the award of a degree by this or any other University.

……..

Abstract

The aim of this study was to identify and characterise peptidyl-prolyl *cis-trans* isomerases (PPIases) from the bacterium *Burkholderia pseudomallei,* the causative agent of the disease melioidosis. The longer term goal was to assess their potential as vaccine candidates or antimicrobial targets.

Using bioinformatic approaches, six putative FK506-binding proteins (FKBPs) proteins and three putative parvulin proteins were identified in *B. pseudomallei*. Of these, six were expressed and purified as recombinant proteins. The purified proteins were used to immunise BALB/c mice, with some providing protection against a subsequent *B. pseudomallei* infection. These proteins could therefore be proposed as potential vaccine candidates.

Homologues of Mip or SurA, which are associated with virulence in other bacterial species, were identified in *B. pseudomallei* and closely related *B. thailandensis.* Recombinant Mip or SurA homologues from *B. pseudomallei* were shown to have characteristic PPIase enzyme activity. To evaluate the role of the Mip homologue from *B. pseudomallei* in virulence, an unmarked deletion mutant was constructed. The mutant had reduced intracellular survival; defects in putative virulence mechanisms and attenuated virulence in mice. To assess the role of a SurA homologue, closely related *B. thailandensis* was used as a model organism, with deletion of the gene resulting in defects in intracellular infection, outer membrane integrity and virulence. This indicates that PPIases from *B. pseudomallei* and *B. thailandensis* represent novel virulence determinants and potential antimicrobial targets for therapeutics against melioidosis.

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Publications

Papers

A novel FK-506 binding like-protein that lacks peptidyl-prolyl isomerase activity is involved in intracellular infection and *in vivo* virulence of *Burkholderia pseudomallei* - Isobel H. Norville, Katrin Breitbach, Kristin Eske-Pogodda, Nicholas J. Harmer, Mitali Sarkar-Tyson, Richard W. Titball, and Ivo Steinmetz. Submitted to Microbiology, Feb, 2011.

A *Burkholderia pseudomallei* Mip-like Protein has Rapamycin Inhibitable Peptidyl-Prolyl Isomerase Activity and has Pleiotrophic Effects on Virulence – Isobel H. Norville, Nicholas J. Harmer, Sarah V. Harding; Gunter Fischer, Karen E. Keith, Katherine A. Brown, Mitali Sarkar-Tyson, Richard W. Titball. Submitted to Infection and Immunity, Feb, 2011.

The structure of a *Burkholderia pseudomallei* immunophilin-inhibitor complex reveals new approaches to antimicrobial development - Isobel H. Norville, Katherine O'Shea, Mitali Sarkar-Tyson, Suxin Zheng, Richard W. Titball, Gabriele Varani and Nicholas J. Harmer. Submitted to Biochemical Journal, Feb, 2011.

Posters

Identification and characterisation of FK506-binding proteins in *Burkholderia pseudomallei* - I.H. Norville, T. P. Atkins, M. Sarkar-Tyson and R. W. Titball. Presented at the Spring SGM, 2009.

***Burkholderia pseudomallei* encodes a Mip-like protein which is involved in virulence** - I.H. Norville, N.J. Harmer, K.A. Brown, T. P. Atkins, M. Sarkar-Tyson and R. W. Titball. Presented at the European Melioidosis meeting, 2009

***Burkholderia pseudomallei* encodes a Mip-like protein which is involved in virulence** - I.H. Norville, N.J. Harmer, K.A. Brown, T. P. Atkins, M. Sarkar-Tyson and R. W. Titball. Presented at the South West and Wales Microbiology Forum, 2009.

Identification and characterisation of a Mip-like protein in *B. pseudomallei* - I.H. Norville, N.J. Harmer, K.A. Brown, T. P. Atkins, M. Sarkar-Tyson and R. W. Titball. Presented at the Texas/UK symposium: Controlling Emerging Infectious Diseases in the 21st Century, 2010 – awarded 'Outstanding pre-doctoral poster'.

Declaration

Unless otherwise stated, the results and data presented in this thesis were solely the work of Isobel Norville.

Hester Nichols produced and characterised recombinant BPSL0659 protein and produced and carried out initial characterisation of a *BTH_I0576* mutant strain. All of these experiments were designed by and carried out under the direct supervision of Isobel Norville.

Dr Nic Harmer carried out enzyme analysis; X-ray crystallography of BPSS1823 and assisted with PPIase assay development. Mass spectrometry was carried out by Kerry Anderson and circular dichroism was performed by Dr Tam Bui.

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Abbreviations

%	Percent
∆	Delta
ºC	Degrees centigrade
α	Alpha
A	Amps
aa	Amino acid
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ANOVA	Analysis of variance
AP	Alkaline phosphatase
β	Beta
BCA	Bicinchoninic acid
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
Bsa	*Burkholderia* secretion apparatus
cfu	Colony forming unit
Da	Daltons
DAB	3, 3'-diaminobenzidine
DIG	digoxigenin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
Dstl	Defence Science and Technology Laboratory
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme linked immunosorbant assay
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FKBP	FK506-binding protein
FPLC	Fast protein liquid chromatography
g	Grams
γ	Gamma
GST	Glutathione S-transferase
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPACC	Health Protection Agency Culture Collections
HRP	Horseradish peroxidise
IFN	Interferon
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISCOM	Immune stimulating complex
IV	intravenous
L	Litred
LB	Luria broth
LC-MS	Liquid chromatography mass spectrometry
LD50	Lethal dose, 50%
LF	Left flank
LiCl	Lithium chloride

LPS	Lipopolysaccharide
µ	Micro
m	Milli or metres
M	Molar
mAbs	Milliabsorbance
MIC	Minimum inhibitory concentraion
min	minute(s)
MLD	Median lethal dose
MOI	Multiplicity of infection
MW	Molecular weight
MTTD	Median time to death
N	Nano
NaOH	Sodium hydroxide
NaCl	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
NOESY	Nuclear Overhauser effect spectroscopy
OD	Optical denstiy
OMPs	Outer membrane proteins
O-PS	O-polysaccharide
P	Pico or probability
PPIase	Peptidyl-prolyl *cis/trans* isomerase
PAGE	Polyacrylamide gel electrophoresis
PBS(T)	Phosphate buffered saline (+Tween)
PCR	Polymerase chain reaction
pH	Potential of hydrogen-measure of hydrogen ions associated with acidity
PHYRE	Protein homology/analogy recognition engine
^{R}	Resistant
RT	Reverse transcriptase
RF	Right flank
^{S}	Sensitive
$^{\sigma}$	Sigma
SAS	Sigma adjuvant system
sec	Second(s)
SOC	Super optimal catabolite
SDS	Sodium dodecyl sulphate
SSC	Saline-sodium citrate
STM	Signiture tagged mutagenesis
TAE	Tris-acetate-EDTA
TFA	Trifluoroacetic acid
TTSS	Type III secretion system
U	Unit
UV	Ultraviolet
V	Volts
v	Volume
VBNC	Viable but non-culturable
WT	Wildtype
WW domain	a protein domain with two highly conserved tryptophans
x g	Centrifugal force

Chapter 1 – Introduction

1.1 The *Burkholderia* genus

A member of the proteobacteria phylum, the *Burkholderia* genus comprises more than 40 species (http://www.bacterio.cict.fr/b/burkholderia). These species occupy a range of ecological niches, with the majority inhabiting soil and plants. In addition to interactions with plant hosts, several *Burkholderia* species are pathogenic in humans, including *B. pseudomallei,* the causative agent of melioidosis; *B. mallei*, the causative agent of glanders and *B. cepacia* complex (Bcc) bacteria, which causes 'cepacia syndrome'. The Bcc is composed of 10 *Burkholderia* species, with *B. cenocepacia* most prevalent in cystic fibrosis patients (Lipuma, 2001; Mahenthiralingam *et al*., 2008). *B. cenocepacia* is an opportunistic pathogen and can lead to pneumonic illness with high fever and respiratory failure (Isles *et al.*, 1984). *B. thailandensis,* is closely related to *B. pseudomallei*, but has reduced virulence (Brett *et al*., 1997).

1.1.1 *B. pseudomallei*

B. pseudomallei was first described by Alfred Whitmore in Rangoon, Burma in 1911 (Whitmore, 1913). It is an aerobic, Gram negative, motile bacillus that exhibits bipolar staining. When grown on solid media it has differing colony morphology, ranging from smooth to wrinkled in form, and cream to orange in colour (Chantratita *et al*., 2007). It is oxidase positive and can not assimilate arabinose (Smith *et al.,* 1997). The genome of strain K96243 comprises of two chromosomes of 4.07 Mb and 3.17 Mb. Gene annotation indicates a functional separation between the two chromosomes, with the smaller carrying virulence and survival genes and the larger carrying genes needed for cell metabolism

and growth (Holden *et al.,* 2004). *B. pseudomallei* can replicate within protozoa and human cells and survive hostile conditions such as low pH, temperature extremes, osmotic stress and UV light (Jones *et al.,* 1996; Inglis *et al*., 2000; Inglis and Sagripanti, 2006). It is a soil saprophyte that can be isolated from the environment across much of Southeast Asia and Northern Australia (Cheng and Currie, 2005). Along with *B. mallei*, *B. pseudomallei* is listed as a category B agent by the US Centre for Disease Control and Prevention (Rotz *et al.,* 2002). This has lead to an increase in research to devise medical counter measures and vaccines (Warawa and Woods, 2002).

1.1.2 *B. thailandensis*

B. pseudomallei-like organisms were first isolated from a rice paddy field in central Thailand and were subsequently classed as *B. thailandensis* by phylogenetic analysis (Smith *et al*., 1995; Brett *et al.,* 1998). On solid media, *B. thailandensis* typically forms smooth, glossy colonies with pink pigmentation (Brett *et al*., 1998). Its genome comprises of two chromosomes of 3.8 Mb and 2.9 Mb (Kim *et al*., 2005). *B. thailandensis* has been shown to be closely related to *B. pseudomallei* by genome and 16S rRNA analysis but differs in its ability to assimilate L-arabinose, lack of capsular polysaccharide and type three secretion system-3 (TTSS; Wuthiekanun *et al*., 1996; Reckseidler *et al.,* 2001; Rainbow *et al*., 2002; Yu *et al.,* 2006). *B. thailandensis* exhibits reduced replication in human macrophages and reduced invasion of epithelial cells, compared to *B. pseudomallei* (Kespichayawattana *et al*., 2004; Charoensap *et al.,* 2009). It also has low virulence, with an LD_{50} of 10^6 cfu in a hamster model and 10^9 cfu in a BALB/c mouse model, which is $>10^5$ fold higher than *B. pseudomallei* (Brett *et al.,* 1997; Smith *et al.,* 1997). However, human infections have been reported (Lertpatanasuwan *et al.,* 1999; Glass *et al*., 2006).

1.1.3 *B. mallei*

B. mallei is a Gram negative, oxidative positive, aerobic, non-spore forming bacillus. Its genome consists of two chromosomes of 3.4 Mb and 2.3 Mb (Nierman *et al.,* 2004). *B. mallei* is widely regarded as a clonal derivative of *B. pseudomallei*, adapted for host survival (Godoy *et al.,* 2003; Holden *et al.,* 2004). More than 1400 *B. mallei* genes are either lost or variant compared to *B. pseudomallei*, with deletion of clusters of genes associated with environmental survival and a mutation in the *fliP* gene, rendering it immotile (Nierman *et al.,* 2004; Losada *et al.,* 2010). However, genes common to both species are highly conserved and similarly organised along the genome.

Unlike most *Burkholderia* species, *B. mallei* is incapable of persistence in the environment. Instead, it is an obligate pathogen and causes disease mainly disease in solipeds, where it can present as a nasal-pulmonary (glanders) or cutaneous (farcy) infection (Al-Ani and Roberson, 2007; Whitlock *et al.,* 2007). Several animal models have been developed to study *B. mallei* infection, including guinea pig, Syrian hamster and BALB/c mice (Miller *et al.,* 1948; Fritz *et al.,* 1999; Fritz *et al.,* 2000). In humans, infection is associated with occupations involving close contact with infected animals, such as farmers and veterinarians. Infection occurs by contamination of wounds, mucous membranes or ingestion (Whitlock *et al.,* 2007). *B. mallei* infection in humans can present as a pulmonary infection, with dissemination resulting in abscess formation (Srinivasan *et al.,* 2001). Factors required for *B. mallei* virulence include the capsule, TTSS and type six secretion system (T6S; DeShazer *et al*., 2001; Ulrich and DeShazer, 2004; Schell *et al.,* 2007).

1.2 Melioidosis

B. pseudomallei is the causative agent of melioidosis and is endemic to some tropical regions between 20ºN and 20ºS (Dance, 2000). In Darwin, Australia, *B. pseudomallei* is reported as the most common cause of community acquired, bacteremic pneumonia (Douglas *et al.,* 2004). In northeast Thailand, melioidosis is the most common cause of community-acquired bacteraemia and the third most common cause of death from infectious disease, after HIV/AIDS and tuberculosis (Suputtamongkol *et al*., 1994a; Limmathurotsakul *et al.,* 2010). Sporadic melioidosis cases have been reported in India, America, China and Taiwan (Hsueh *et al.,* 2001; Inglis *et al*., 2006b; Vidyalakshmi *et al.,* 2007; Yang, 2000). However, cases are thought to be widely underreported due to lack of awareness and incidence rates may be affected by imported melioidosis from travellers returning from areas of endemicity (Currie, 2003).

1.2.1 Risk factors

B. pseudomallei can be isolated from soil and water, with infection occurring by inhalation, ingestion or inoculation of wounds (Choy *et al.,* 2000; Wiersinga *et al.,* 2006). Because of these factors, there is a strong seasonal and occupational risk associated with melioidosis. In northeast Thailand, where most of the population belong to rice-farming families, *B. pseudomallei* can be isolated from rice paddy fields (Smith *et al*., 1995). Consequently, rice farmers constitute 81% of melioidosis patients, following exposure to *B. pseudomallei* by inoculation of wounds on their hands and feet or inhalation (Suputtamongkol *et al.,* 1999). Melioidosis cases were also reported in Southern Thailand after the 2004 tsunami, following near-drowning or penetrating injuries (Chierakul *et al.,* 2005).

In Northern Australia, rice farming does not occur and cases of acute, pneumonic disease are linked with the onset of heavy monsoonal rainfall (Currie and Jacups, 2003). Aerosols are created during heavy rain and may result in inhalation of *B. pseudomallei*. Cases of infection or positive serology cluster around extreme weather events such as cyclones, wind speeds greater than 40 kph and high rainfall (Cheng *et al*., 2006; Inglis *et al.,* 2009).

The incidence of melioidosis peaks in people over 45 year old, is most prevalent in men and is associated with several clinical risk factors (Suputtamongkol *et al*., 1999). Up to 60% melioidosis patients have pre-existing or newly diagnosed type 2 diabetes (Suputtamongkol *et al*., 1994a; Currie *et al.,* 2004). Other conditions, such as chronic renal disease and chronic lung disease are also recognised risk factors in endemic regions (Suputtamongkol *et al.,* 1999; Currie *et al*., 2004). The consumption of excessive alcohol has been documented in > 37% of Australian and 12% of Thai melioidosis patients (Suputtamongkol *et al.,* 1999; Currie *et al*., 2004; Malczewski *et al*., 2005).

1.2.2 Clinical presentation

The incubation period of melioidosis has been shown to range from 1 day to 62 years (Currie *et al.,* 2000a; Ngauy *et al.,* 2005). Infection with a high innoculum by near-drowning can result in a very short incubation period (Chierakul *et al.,* 2005). The manifestation of disease is varied and melioidosis can mimic other infections, such as tuberculosis (Overtoom *et al.,* 2008).

Serology indicates that infection with *B. pseudomallei* can be asymptomatic, with seroconversion occurring between 6 months and 4 years of age (Kanaphun *et al*., 1993). More than 50% melioidosis patients will present with pneumonia (Cheng and Currie*,* 2005). Acute septicaemia is the most severe form of infection, often occurring after dissemination to distant tissues from a localised abscess (Inglis *et al.,* 2006a). Infection may occur in soft tissue, bone, joints and hepatic and splenic abscesses are common (Puthucheary *et al.,* 1992; Currie *et al.,* 2000a). Acute parotitis accounted for up to 40% of paediatric cases in Thailand while 18% of male patients in Australia presented with prostatic abscesses (Lumbiganon and Viegnondha, 1995; Currie *et al.,* 2000b). Mortality has been reported to be 50% in northeast Thailand and <19% in Australia (Currie *et al.,* 2000a, 2004; Limmathurotsakul *et al.,* 2010).

Recurrent disease is common, occurring in 13 - 20% of patients (Currie *et al.,* 2000b; Deris *et al.,* 2010). Within 12 months of the initial diagnosis, relapse can occur but after 2 years, recurrence was equally due to reinfection as to relapse (Maharjan *et al*., 2005). Reinfection was associated with periods of heavy rainfall whereas relapse commonly occurred after a short duration of antibiotic treatment (Limmathurotsakul *et al*., 2008).

1.2.3 Intracellular lifecycle

B. pseudomallei is capable of surviving and replicating within a range of eukaryotic cells, including neutrophils, macrophages, dendritic cells and epithelial cells (Jones *et al.,* 1996; Charoensap *et al.,* 2009). Following internalisation of bacteria into cells, *B. pseudomallei* have been shown to reside inside plasma-membrane derived phagosomes (Puthucheary and Nathan, 2006). *B. pseudomallei* can survive in phagolysosomes and evade

macrophage killing and host immunity through several mechanisms, including resistance to host defensins; inhibition of cell protein synthesis and interference with iNOS production (Mohamed *et al.,* 1989; Jones *et al.,* 1996; Utaisincharoen *et al.,* 2001; Puthucheary and Nathan, 2006). As early as 15 minutes after infection, *B. pseudomallei* can destroy the phagolysome membrane, resulting in release of bacteria into the cytoplasm (Harley *et al.,* 1998b). The TTSS has been shown to be required for escape from the phagolysosome, with *bsaZ* or *bipD* mutants showing significantly reduced growth in J774.2 macrophages and a high association with lysosomes 6 h after infection (Stevens *et al*., 2002). Once in the cytoplasm, *B. pseudomallei* is able to form actin-based membrane protusions which can lead to direct cell-to-cell bacterial spread (Kespichayawattana *et al*., 2000). The actin-based motility of *B. pseudomallei* was abolished following inactivation of *bimA*, a bacterial protein located at the site of actin nucleation (Stevens *et al.,* 2005). Fusion of *B. pseudomallei* infected cells has been shown to induce the formation of multinucleated giant cells (MNGCs; Kespichayawattana *et al*., 2000). Inactivation of *bipB*, a type III translocator protein, or *rpoS* can inhibit MNGC formation and cell apoptosis (Suparak *et al.,* 2005; Utaisincharoen *et al.,* 2006).

1.2.4 Host immune response

As with all bacterial infections, the innate and adaptive immune responses are important for determining the outcome of melioidosis. Toll-like receptors (TLRs) detect initial pathogen invasion by recognising conserved surface motifs called ‘pathogen-associated-molecular-patterns’ or PAMPs. TLRs mediate the innate immune response and form a key link between innate and adaptive immunity (Takeda *et al.,* 2003). Melioidosis patients with septic shock have increased expression of TLR1, TLR2 and TLR4 (Wiersinga *et al.,* 2007). TLR4 recognises

LPS from a range of Gram-negative bacteria and has been shown to induce expression of several cytokines (Medzhitov *et al*., 1997; Hoshino *et al*., 1999). However, infection of TLR4 deficient mice with *B. pseudomallei* resulted in wildtype mortality and TLR2 has been implicated in recognising LPS instead (Wiersinga *et al*., 2007).

The production of proinflammatory cytokines is important in early defence against *B. pseudomallei* infection. During acute infection of BALB/c mice, cytokine expression peaks between 24-48 h post infection and is correlated to higher bacterial loads within the host (Ulett *et al.,* 2000; Ulett *et al*., 2002). In addition, concentrations of IFN-γ, lL-12 and TNF-α are elevated in melioidosis patients (Lauw *et al*., 1999). Inhibition of IL-12 or TNF-α, the predominant inducers of IFN-γ, resulted in increased mortality following infection of mice with *B. pseudomallei* (Santanirand *et al.,* 1999). In the same model, direct inhibition of IFN-γ with a neutralizing monoclonal antibody lowered the LD_{50} from >5 x 10^5 to 2 cfu, with 8500 fold increase in the bacterial burden in the liver (Santanirand *et al.,* 1999).

During *B. pseudomallei* infection, there is an influx of phagocytes to the site of infection. Following aerosol challenge of BALB/c mice, an infiltration of neutrophils in the alveolar spaces and hepatic lesions was observed 24-48h post infection (Lever *et al.,* 2009). Depletion of neutrophils in C57BL/6 mice lead to increased susceptibility of infection, with a median survival time of 6.5 days, compared to 49.5 days in control mice (Easton *et al.,* 2007). Similarly, depletion of macrophages in BALB/c or C57BL/6 mice increases mortality (Breitbach *et al*., 2006; Barnes *et al.,* 2008).

B. pseudomallei rapidly activates complement, resulting in opsonisation and deposition of C3 onto the bacterial surface (Egan and Gordan, 1996). Opsonisation of bacteria resulted in significantly greater phagocytosis (Egan and Gordan, 1996). Furthermore, the capsule of *B. pseudomallei* has been shown to provide resistance to phagocytosis by reducing the deposition of C3b (Reckseidler-Zenteno *et al*., 2005).

The adaptive immune response also plays an important role in protection from melioidosis, with increasing anti-LPS antibody titres correlating with non-septicemic disease and survival (Charuchaimontri *et al*., 1999). There are several components of *B. pseudomallei* that are immunogenic and mice immunized with capsule or lipopolysaccharide had increased protection against challenge (Nelson *et al.,* 2004). Passive immunisation is also possible with monoclonal antibodies showing protection against 10^4 cfu of *B. pseudomallei* (Jones *et al.,* 2002).

1.2.5 Diagnosis

Isolation of *B. pseudomallei* from blood, sputum or other sterile fluid using culture methods is the 'gold standard' of detection. Ashdown's Selective agar utilises the gentamicin and colistin resistance of *B. pseudomallei* and neutral red allows it to be distinguished from other bacteria (Ashdown *et al.,* 1979). The number of bacteria has been correlated to disease outcome, with >100 cfu/ml in the blood or $>10^5$ in the urine associated with 96% or 71% mortality, respectively (Walsh *et al*., 1995; Limmathurotsakul *et al.,* 2005). Following identification of a Gram negative bacillus from bodily fluid, a definitive diagnosis is only achieved if the culture is oxidase positive, gentamicin resistant, colistin

resistant, plus a positive serological or molecular based assay (Inglis *et al.,* 2005).

Serological evidence of *B. pseudomallei* infection can be obtained by detecting antigens or antibodies raised against the organism in clinical samples. One antigen detection test uses latex beads coated with monoclonal antibodies which recognise a 200 kDa surface antigen on *B. pseudomallei*. Blood samples positive for *B. pseudomallei* will agglutinate the latex beads. This test has been widely used in Thailand and showed 95% sensitivity and 99.7% specificity (Anuntagool *et al.*, 2000). Immunofluorescence can be used to detect *B. pseudomallei* in sputum, urine and pus, using whole-cell specific antibodies conjugated to FITC (Wuthiekanun *et al*., 2005a).

ELISAs have been developed to detect antibodies raised against *B. pseudomallei,* using antigens such as LPS, OmpA,and BipB (Druar *et al.,* 2008; Allwood *et al.,* 2008; Andadan*, et al*., 2010). An immunofluorescence method has also been developed to detect the presence of anti-LPS antibodies (Iihara *et al*., 2007). The indirect haemagglutination (IHA) assay uses fixed antigens to detect anti-*B. pseudomallei* antibodies in convalescent sera (Alexander *et al.,* 1970). Despite being regularly used, the IHA assay has some limitations. The presence of antibodies in healthy individuals from endemic areas has been noted, with 30-47% background seropositivity (Khupulsup and Petchclai, 1986). Seropositivity may be due to exposure to subclinical levels of *B. pseudomallei* or antigenically similar *B. thailandensis* (Gilmore *et al.,* 2007). Therefore, an IHA titre of > 160 is used as supportive, but not definitive, evidence of melioidosis (Inglis *et al.,* 2006a).

Molecular methods, such as PCR-based diagnostic tests, overcome some of the limitations of serological tests. Primers targeting 16S rRNA demonstrated sensitivity of 100% on culture-confirmed cases but low specificity, with positive results in 33% of patients without clinical melioidosis (Haase *et al.,* 1998). The TTSS genes of *B. pseudomallei* have also been targeted using real-time PCR, providing 100% sensitivity in sputum, urine, pus, and wound swabs (Meumann *et al*., 2006). 37 candidate diagnostic biomarkers have been identified by analysis of blood transcriptional profiles of patients with septic melioidosis, with 100% accuracy (Pankla *et al.,* 2009).

1.3 *B. pseudomallei* virulence factors

Several putative virulence factors have been characterised in *B. pseudomallei.* Roles for capsule, type IV O-PS, quorum sensing, TTSS, flagella and pili in pathogenesis have been demonstrated. Other putative virulence factors have been characterised but with limited experimental evidence, such as LPS, T6SS and secreted enzymes (reviewed by Adler *et al.,* 2009).

1.3.1 Quorum sensing

Quorum sensing is a cell-density regulated communication system, using signalling molecules such as N-acyl-homoserine lactones (HSLs; Swift *et al.,* 1996). *B. pseudomallei* K96243 has 3 *luxI* homologues, which encode HSL synthase proteins, and 5 *luxR* homologues, which encode transcription regulators of quorum sensing mediated genes, activated upon binding to HSLs (Ulrich *et al.,* 2004). Mutation of any *luxI* or *luxR* genes in *B. pseudomallei* resulted in an increased time to death in BALB/c mice and Syrian hamster

models of infection. Furthermore, reduced colonisation of the lungs and spleen was observed following inhalational challenge of BALB/c mice with the mutant strains (Ulrich *et al.,* 2004; Valade *et al.,* 2004).

A *luxR* homologue, *BPSS0887,* has been shown to regulate expression of the oxidative stress protein, DpsA. Deletion of *BPSS0887* resulted in increased sensitivity to hydrogen peroxide, showing the importance of quorum sensing in regulating the response to oxidative stress (Lumjiaktase *et al.,* 2006). Extracellular secretion of HSLs is dependent on the BpeAB-OprB efflux system in *B. pseudomallei* strain KHW (Chan *et al.,* 2007). Mutation of *bpeAB* significantly reduced invasion of macrophages and epithelial cells, which was restored upon addition of HSL (Chan and Chua, 2005). Quorum sensing has also been shown to be important for biofilm formation and secretion of sideophores and phospholipase C (Song *et al.,* 2005). In support of these findings, the *bpeAB* mutant exhibited a 50% reduction on siderophore production and a 77% reduction in biofilm formation (Chan and Chua, 2005). However, differing results have been reported for BpeAB-OprB mutants made in *B. pseudomallei* strain 1026b, with no effect on HSL export observed (Mima and Schweizer, 2010).

1.3.2 Polysaccharides

1.3.2.1 Capsule

The *B. pseudomallei* genome contains four operons encoding proteins required for capsular polysaccharide biosynthesis and the best characterised encodes a polysaccharide with the structure 2-O-acetyl-6-deoxy-β-D-manno-

heptopyranose, referred to as type I O-PS (Perry *et al*., 1995). Studies using STM or subtractive hybridisation identified genes located within a capsule operon, disruption of which reduced *B. pseudomallei* virulence in mice (Reckseidler *et al.*, 2001; Atkins *et al.,* 2002; Cuccui *et al.,* 2007; Warawa *et al*., 2009). Inactivation of a mannosyltransferase, encoded by *wcbB*, significantly attenuated virulence in a BALB/c mice and Syrian hamster models (Atkins *et al.,* 2002; Reckseidler-Zentano *et al.,* 2005; Cuccui *et al*., 2007). The *wcbB* mutant exhibited reduced growth in the blood and was more sensitive to killing by human serum. The presence of purified capsule significantly increased virulence of the *wcbB* mutant strain (Reckseidler-Zentano *et al.,* 2005). WcbC, a putative capsular polysaccharide export protein, has also been shown to be important for full virulence in a BALB/c mouse, but not Syrian hamster, model of infection (Reckseidler *et al.,* 2001; Cuccui *et al.,* 2007).

The capsule represents an important virulence factor of *B. pseudomallei* and *B. thailandensis* is acapsular, indicating the lack of a capsule may contribute to reduced virulence (Reckseidler *et al*., 2001). However, a naturally occurring *B. thailandensis* isolate has recently been shown to have a *B.pseudomallei*-like capsular cluster (Sim *et al.,* 2010). This strain expressed capsule and exhibited several *B. pseudomallei*-like phenotypes including colony wrinkling and resistance to human complement. However, virulence was not increased when compared to acapsular *B. thailandensis* type strains (Sim *et al.,* 2010). These results indicate that although the capsule is an important virulence determinant in *B. pseudomallei*, the reduced virulence in *B. thailandendsis* cannot be attributed entirely to a lack of capsule.

1.3.2.2 Lipopolysaccharide

The *B. pseudomallei* O-antigen has the structure -3)-β-D-glucopyranose-(1,3)-6-deoxy-α-L-talopyranose-(1-, referred to as type II O-PS (Perry *et al.,* 1995). Three different LPS serotypes have been identified using SDS-PAGE: smooth type A, smooth type B and rough type (Anuntagool *et al.,* 2006). In a study of 1327 *B. pseudomallei* isolates, while smooth type A accounted for 97% of strains, less common serotypes have been associated with clinical relapse and the rough type exhibited highest biofilm formation (Anuntagool *et al.,* 2006). Purified *B. pseudomallei* LPS has been shown to be a poor activator of macrophages, with NO and TNF-α release taking 30 mins, compared to < 5 min by *E. coli* LPS (Utaisincharoen *et al.,* 2000). As *B. pseudomallei* is less capable of activating immune cells, it may explain why TLR4 does not play a role in experimental melioidosis in mice (Wiersinga *et al.,* 2007).

Mutation of *wbiI,* a dehydratase gene, lead to reduced virulence in hamsters, guinea pigs and infant diabetic rats (DeShazer *et al.,* 1998). The mutant strain exhibited increased internalisation by macrophages but reduced intracellular replication 2 - 6 h post infection (Arjcharoen *et al*., 2007). The absence of the O-antigen resulted in upregulated IFN-β production, which regulates transcription factors required for expression of iNOS and subsequent bactericidal activity (Arjcharoen *et al*., 2007). Unlike wildtype *B. pseudomallei*, O-antigen mutants were sensitive to the bactericidal activity of normal human serum (DeShazer *et al.,* 1998). The role of LPS in serum survival was also indicated by a transposon mutation in *waaF*, which encodes a protein involved in LPS core biosynthesis, leading to polymyxin-B sensitivity (Burtnick and Woods, 1999).

1.3.2.3 Other polysaccharides

In addition to the capsule and LPS O-antigen, two further putative polysaccharide clusters have been identified: genes *BPSS0417 - BPSS0429* (type III O-PS) and *BPSS1825 - BPSS1832* (type IV O-PS; Holden *et al.,* 2004). These clusters show homology to polysaccharide biosynthesis and transport genes. These clusters are conserved in *B. thailandensis* but absent from *B. mallei,* suggesting an environmental role. However, inactivation of type IV O-PS significantly attenuated *B. pseudomallei,* with an extended mean time to death of 11.6 days (Sarkar-Tyson *et al.,* 2007).

1.3.3 Proteins

1.3.3.1 Adhesins

The adherence of bacteria to cells is an important virulence mechanism and is mediated by both pilus and non-pilus adhesins. Analysis of the *B. pseudomallei* genome identified 13 gene clusters predicted to be involved with pili formation, one of which contained a type IV A pilin gene, *pilA* (Holden *et al.,* 2004). Deletion of *pilA* in *B. pseudomallei* K96243 displayed reduced adhesion to epithelial cells and attenuated virulence following challenge of BALB/c mice via the intranasal, but not intraperitoneal, route (Essex-Lopresti *et al.,* 2005). In contrast, a *pilA* mutant made in *B. pseudomallei* 08 was not defective in adhesion to cells, but expression of *pilA* was temperature controlled and required for microcolony formation. *pilA* is not required for biofilm formation in either *B. pseudomallei* strains, suggesting the involvement of an alternative pilus (Boddey *et al.,* 2006).

Two putative autotransporter adhesins have been identified in *B. pseudomallei,* encoded by *boaA* and *boaB* (Balder *et al*., 2010). Inactivation of either gene significantly reduced attachment to human epithelial cells, when compared to the parent strain. However, a defect in intracellular growth was only observed with a *boaA/boaB* double mutant within macrophages and the exact role of these adhesins in intracellular replication is yet to be elucidated (Balder *et al.,* 2010).

1.3.3.2 Flagella

B. pseudomallei is a motile bacterium and the *fliC* gene is required for the synthesis of a 39.1 kDa flagellin protein (DeShazer *et al.,* 1997). Polyclonal sera raised against *B. pseudomallei* flagellin was shown to inhibit motility of *B. pseudomallei* (Brett *et al.,* 1994). Inactivation of *fliC* in *B. pseudomallei* 1026b resulted in defective adhesion to *Acanthamoeba astronyxis* cells however the mutant strain was not attenuated in a Syrian hamster or diabetic rat model of melioidosis (DeShazer *et al.,* 1997; Inglis *et al.,* 2003). In contrast, when *fliC* was inactivated in *B. pseudomallei* KHW, the mutant strain was attenuated in a BALB/c mouse following infection by either the intranasal or intraperitoneal routes. Furthermore, markedly reduced bacterial numbers were isolated from the lungs and spleens following intranasal challenge in this model (Chua *et al,* 2003).

1.3.3.3 Secretion systems

Three TTSS operons are encoded by the *B. pseudomallei* K96243 genome (Holden *et al*., 2004). TTSS1 and TTSS2 are homologous to a secretion-associated system from the plant pathogen *Ralstonia solanacearum*

(Winstanley *et al.,* 1999). Inactivation of TTSS1 did not affect virulence in a hamster model of infection, suggesting that instead it may be involved in plant-pathogen interactions during growth in the soil (Attree and Attree, 2001). The *B. pseudomallei* TTSS3 is similar to the virulence-associated SPI-1 locus of *S. typhimurium,* which was designated Burkholderia secretion apparatus (Bsa; Attree and Attree, 2001; Stevens *et al.,* 2002). Convalescent sera from a meliodiosis patient reacts with purified TTSS3 proteins BipB, BipC and BipD, indicating a functional expression of the Bsa *in vivo* (Stevens *et al.,* 2002).

Three TTSS3 secretion apparatus genes have been inactivated in *B. pseudomallei, bsaQ* (Sun *et al.,* 2005); *bsaU* (Pilatz *et al*., 2006) and *bsaZ* (Stevens *et al.,* 2002). *bsaQ* is the largest gene in the *bsa* locus and a homologue of the *invA* gene from *Salmonella*. Insertional disruption of *bsaQ* showed loss of cytotoxic activity against macrophage-like cells and exhibited reduced invasion of A549 human epithelial cells (Sun *et al*, 2005; Muangsombut *et al*., 2008). A *bsaU* mutant was constructed during a screen for *B. pseudomallei* genes required for intracellular lifecycle and virulence (Pilatz *et al.,* 2006). While the mutant strain was unable to escape endocytic vesicles 6 h post infection, by 12 h the bacteria were released into the cytoplasm and exhibited wildtype levels of intracellular replication. The *bsaU* mutant was also significantly attenuated in a BALB/c model of infection, with reduced bacterial loads in the spleen, liver and lungs (Pilatz *et al.,* 2006). The last gene in the secretion apparatus cluster, *bsaZ,* is similar to *spaS* from *Salmonella.* A *B. pseudomallei bsaZ* mutant was unable to replicate within J774 macrophages, exhibited delayed escape from endocytic vacuoles and actin tail production

(Stevens *et al*., 2002). Another *bsaZ* mutant was attenuated in a Syrian hamster model of melioidosis (Warawa and Woods, 2005).

Inactivation of TTSS3 translocation genes *bipB* or *bipD* resulted in attenuated virulence in a BALB/c mouse model (Stevens *et al.,* 2002; Suparak *et al*., 2005). A *B. pseudomallei bipD* mutant was unable to escape from endocytic vesicles, produce actin tails or replicate within J774 macrophage cells (Stevens *et al.,* 2002). A *bipB* mutant exhibited defects in MNGC formation, cell-to-cell spread and induction of apoptosis (Suparak *et al*., 2005). Characterisation of TTSS3 effector mutants revealed that inactivation of *bopA, bopB, bapC* or *bopE* had no significant effect on *B. pseudomallei* virulence (Stevens *et al*., 2004; Warawa and Woods, 2005).

Inactivation of the T6SS in *B. mallei* resulted in strains that were avirulent in hamsters (Schell *et al.,* 2007). The *B. pseudomallei* K96243 genome encodes six T6SS clusters and 3 genes from one of the clusters were upregulated during macrophage infection (Holden *et al.,* 2004; Shalom *et al.,* 2007). However, inactivation of one of these genes, *tssH-5*, had no affect on intracellular invasion and replication in macrophages (Shalom *et al.,* 2007). The role of other T6SS clusters on *B. pseudomallei* virulence requires further study.

1.3.3.4 Isocitrate lyase

Isocitrate lyase (ICL) is an enzyme of the glyoxylate shunt pathway, which is involved in the metabolism of fatty acids (Cozzone, 1998). A *B. pseudomallei icl* mutant was unable to establish a chronic infection, indicating ICL is a persistence factor in pulmonary melioidosis. The strain was hypervirulent in an

acute model of infection, which was correlated to increased cytotoxicity against macrophage cells. In addition, inhibition of ICL enzyme activity during a chronic infection forced the infection into an acute state, which could then be treated with antibiotics (Schaik *et al*., 2009).

1.3.4 Secreted factors

1.3.4.1 Proteases

B. pseudomallei secretes a range of extracellular enzymes, including haemolysins, lipases, lecithinases, peroxidases, superoxide dismutases and proteases (Ashdown and Koehler*,* 1990; Vellasamy *et al.,* 2009). 94% of *B. pseudomallei* isolates were found to produce extracellular proteases (Ashdown and Koehloer, 1990). A small 36 kDa metalloprotease was identified in *B. pseudomallei* and was shown to be required for full virulence in a rat model of lung infection (Sexton *et al.,* 1994). MprA, a 47 kDa serine metalloprotease has also been identified and was expressed at higher levels upon entry into stationary phase (Lee and Liu, 2000; Valade *et al.,* 2004). However, a *mprA* mutant was not attenuated following infection of BALB/c mouse by intraperitoneal, subcutaneous or intranasal routes (Valade *et al.,* 2004). In addition, no correlation between protease production and the virulence of six *B. pseudomallei* strains was observed (Gauthier *et al.,* 2000). While the importance of proteases of *B. pseudomallei* virulence requires further investigation, neutralizing antibodies may have potential as therapeutics against melioidosis (Nathan *et al.,* 2005).

1.3.4.2 Phospholipase C

B. pseudomallei K96243 encodes three phospholipase C (Plc) enzymes: two on chromosome 1 (Plc-1 and Plc-2) and one on chromosome 2 (Plc-3; Holden *et al*., 2004). Using starvation followed by subculture in media containing egg yolk to monitor Plc activity, it was observed that single mutants of *plc-1* or *plc-2* still had >93% wildtype Plc activity. However, a *plc-1*/*plc-2* double mutant had significantly reduced growth, indicating that Plcs are important for acquiring nutrients following starvation. The *plc-2* and *plc-1/plc-2* double mutant exhibited reduced plaque formation in HeLa cells and reduced cytotoxicity towards RAW254.7 cells (Korbsrisate *et al*., 2007). Furthermore, expression of *plc-3* was upregulated in the liver of hamsters infected with *B. pseudomallei* and a *plc-3* mutant was attenuated in a hamster model of infection (Tuanyok *et al.,* 2006).

1.3.4.3 Haemolysin

Following the characterisation of 100 clinical isolates, the majority of *B. pseudomallei* strains exhibited weak haemolysin activity which was only observed around areas of confluent growth (Ashdown and Koehler, 1990). However, 4% of strains displayed strong haemolysin activity, with a clear zone of haemolysis around individual colonies. This haemolysin was further characterised and shown to be optiminal at pH 5.5 and was active against a range of mammalian erythrocytes (Ashdown and Koehler, 1990). The haemolysin was also purified and shown to have haemolytic and cytotoxic activity (Häussler *et al*., 1998).

1.3.4.4 Siderophores

B. pseudomallei has been shown to produce a siderophore under iron-limited conditions, which was designated malleobactin. Malleobactin was shown to

remove iron from transferrin, lactoferrin and EDTA (Yang *et al*., 1991; Yang *et al*., 1993). The purified siderophore has subsequently been analysed by mass spectrometry, revealing it is a mixture of at least three compounds (Alice *et al.,* 2006). Transcriptional analysis of genes up-regulated under iron-limiting conditions identified an operon which was predicted to be involved in malleobactin biosynthesis (Alice *et al.,* 2006; Tuanyok *et al*., 2006). This was confirmed when inactivation of one of these genes, *mbaA,* resulted in inability to grow under low iron conditions, which was complemented upon the addition of purified malleobactin (Alice *et al.,* 2006).

1.3.4.5 Toxins

A lethal and necrotizing toxin has been reported in crude filtrates *B. pseudomallei* (Heckly and Nigg, 1958). The toxin was also shown to inhibit protein and DNA synthesis in macrophages (Mohamed *et al.,* 1989). The cytolethal activity of culture filtrates was reported to correlate with the source of the strain. For example, soil isolates exhibited lowest activity and clinical isolates from fatal meliodiosis exhibited highest activity (Haase *et al.,* 1997). The production of a toxin by *B. pseudomallei* has also been reported to cause paralytic killing in a *C. elegans* model of infection (O'Quinn *et al.,* 2001).

1.4 Treatment of melioidosis

There is currently no licensed vaccine available for the prophylaxis of melioidosis. Protective immunity against *B. pseudomallei* has been induced by a range of immunogens, including live or heat-inactivated bacteria and sub-unit antigens (reviewed by Sarkar-Tyson and Titball, 2010). The development of an

effective vaccine against melioidosis would provide protection to individuals living in endemic areas or following the release of *B. pseudomallei* from a bioweapon.

Following diagnosis of melioidosis, appropriate antimicrobial treatment should be commenced. Resistance of *B. pseudomallei* to several antibiotics can make treatment problematic. Clinical trials have established that an initial parenteral IV treatment followed by an oral eradication treatment is the most efficacious regime (reviewed by Wuthiekanun and Peacock, 2006).

1.4.1 Vaccine development

1.4.1.1 Killed whole cell

Killed whole-cell vaccines have been successfully used to vaccinate against bacterial diseases such as cholera and whooping cough (Vickers *et al.,* 2006; Mahalanabis *et al.,* 2008). Bacterial killing can be achieved by heat, irradiation or formaldehyde treatment. Immunisation of BALB/c mice with heat-inactivated *B. pseudomallei* has been shown to provide protection against challenge with live *B. pseudomallei* (Sarkar-Tyson *et al.,* 2009). 60% of immunised mice survived 45 days after challenge by the intraperitoneal route with 40 x the MLD. In addition, immunisation with heat-inactivated *B. pseudomallei* K96243 delayed the time to death after aerosol challenge with either *B. pseudomallei* or *B. mallei* (Sarkar-Tyson *et al.,* 2009). Following immunisation with heat-inactivated LPS or capsule *B. pseudomallei* mutants, higher protection was afforded when compared to heat-inactivated wildtype bacteria (Sarkar-Tyson *et al*., 2007). Irradiation has been used as an alternative method of inactivating *B. mallei*

however no protection against *B. mallei* challenge was observed (Amemiya *et al.,* 2006).

1.4.1.2 Live attenuated whole cell

Live attenuated vaccines are currently used in humans to provide protective immunity against diseases such as typhoid fever and tuberculosis (Hohmann *et al.,* 1996; Doherty and Anderson, 2005). Several attenuated mutant strains have been shown to provide protection against challenge of mice with *B. pseudomallei*. Inactivation of the *ilvI* gene by transposon mutagenesis resulted in a *B. pseudomallei* mutant (2D2) which was auxotrophic for branched chain amino acids. Immunisation of BALB/c mice with 2D2 provided significant protection against challenge with 10^6 cfu of *B. pseudomallei* strain 576 or BRI (Atkins *et al.,* 2002; Haque *et al.,* 2006). In addition, the auxotrophic mutant could not be isolated from lung, liver, spleen or kidney 25 days post infection (Atkins *et al.,* 2002). Adoptive transfer of T-cells from immunised mice to immunodeficient mice increased survival, indicating that immunisation with 2D2 generated T-cell mediated protection. Specifically, $CD4^+$ T-cells were shown to mediate immunity, with no protection observed following immunisation of $CD4^+$ depleted mice. Furthermore, splenic T-cells from immunised mice were shown to proliferate and produce IFN-γ in the presence of killed *B. pseudomallei* (Haque *et al.,* 2006).

A *B. pseudomallei purN* mutant was identified following screening of mutants defective in intracellular growth. The mutant strain was shown to be a purine auxotroph and was significantly attenuated in a BALB/c mouse model (Pilatz *et al*., 2006). Immunisation with the *purN* mutant provided significant protection

against a subsequent acute intranasal and intraperitoneal challenge with wildtype *B. pseudomallei,* but not against chronic forms of melioidosis (Breitbach *et al.,* 2008). Deletion of *aroC,* an enzyme in the *aro* biosynthetic pathway, resulted in LD_{50} values $>10^6$ fold higher than that of wildtype *B. pseudomallei.* While immunisation of BALB/c mice with the mutant strain afforded no protection, immunised C57Bl/6 mice were significantly protected against a challenge of 20 x LD_{50} (Srilunchang *et al.,* 2009).

1.4.1.3 Polysaccharide subunits

A correlation between high titres of anti-LPS antibodies and melioidosis patients who have survived or have had non-septicemic infections has been reported (Charuchaimontri *et al.,* 1999). Similarly, immunisation of BALB/c mice with purified LPS has been shown to generate IgM and IgG_3 responses. Following challenge of immunised mice with 2 x 10^4 cfu *B. pseudomallei,* 50% of immunised mice survived until the end of the experiment. Using the same model, mice immunised with purified capsule exhibited an increased mean time to death, however 100% had succumbed to infection 28 days after challenge (Nelson *et al.,* 2004). Polysaccharide conjugated to flagellin protein has also been shown to provide protection against infection, with a significantly higher LD_{50} compared to control mice (Brett and Woods, 1996).

1.4.1.4 Protein subunits

Proteins located on the surface of bacteria are likely to be exposed to the immune response during infection. Therefore, outer membrane proteins (Omps) from *B. pseudomallei* have been identified and evaluated as protective antigens (Harding *et al.,* 2007; Hara *et al.,* 2009). Purified Omp3 and Omp7 were used to immunise mice and were shown to elicit a strong antibody response. Following

challenge with *B. pseudomallei,* 50% of the immunised mice survived the experiment compared to none of the control mice (Hara *et al.,* 2009). LolC was selected for testing as a vaccine candidate as it is a membrane located protein and part of the ABC transporter system. Immunisation of BALB/c mice with recombinant LolC resulted in 80% mice surviving the subsequent *B. pseudomallei* challenge. The level of protection was shown to be dependent on the adjuvant used, with highest levels seen in combination with ISCOMs and CpG ODN (Harland *et al.,* 2007). While TTSS translocation proteins from *Yersinia* and *Pseudomonas* have been shown to be effective protective antigens, BipB, BipC and BipD from *B. pseudomallei* afforded no protection against infection (Leary *et al.,* 1995; Sawa *et al.,* 1999; Stevens *et al.,* 2004; Druar *et al.,* 2008). Flagella protein has not evaluated as a vaccine candidate, however immunisation with a DNA vaccine encoding *fliC* resulted in 83% survival 7 days after challenge with *B. pseudomallei* (Chen *et al*., 2006).

1.4.2 Antibiotic resistance

B. pseudomallei is intrinsically resistant to many antibiotics, including third generation cephalosporins, aminoglycosides and penicillins (Cheng and Currie, 2005). Clinical cases of emerging resistance are rare, but isolated cases have been reported. Study of 170 isolates from Royal Darwin Hospital revealed one isolate with primary resistance to amoxicillin-clavulanate. Resistance to oral antibiotics is more common with three out of 170 isolates from Darwin showing resistance to doxycycline (Jenney *et al.,* 2001). However, resistance is geographically variable, with trimethoprim-sulphamethoxazole (TMP-SMX) resistance reported as 2.5% in Australia, but 13% in North-East Thailand (Wuthiekanun *et al.,* 2005b). The development of resistance may be associated

with clinical relapse, with almost 24% of isolates from relapsed patients exhibiting resistance (Jenney *et al.,* 2001).

The *B. pseudomallei* K96243 genome encodes several putative resistance mechanisms, including β-lactamases, multidrug efflux systems and an aminoglycoside acetyltransferase (Holden *et al.,* 2004). A membrane associated β-lactamase was shown to be a cephalosporinase which had activity against carbenicillin, cefotaxime and cefuroxime (Livermore *et al.,* 1987). Inducible expression of β-lactamases has been observed in resistant strains, in particular, a class D β-lactamase was significantly expressed in a ceftazidime-resistant *B. pseudomallei* mutant (Godfrey *et al.,* 1991; Niumsup and Wuthiekanun, 2002).

Transposon mutagenesis identified AmrAB-OprA, an efflux system required for aminoglycoside and macrolide resistance. Inactivation of *amrA* or *amrB* increased susceptibility to a range of aminoglycoside antibiotics, with a 64 fold reduction in the MIC of streptomycin and >128 fold reduction in the MIC of gentamicin (Moore *et al.,* 1999). A second efflux pump is encoded by the *bpeAB-oprB* operon and while inactivation of *bpeAB* in strain KHW increased aminoglycoside sensitivity, inactivation of *bpeAB* in strain 1026b had no effect on aminoglycoside efflux (Chan *et al*., 2005; Mima and Schweizer, 2010). Insertional inactivation of a *lytB* homologue, *waaF* or *udg* in *B. pseudomallei* has been associated with reduced resistance to polymyxin B. All mutants were shown to have altered OMP profiles and the *waaF* and *udg* mutants exhibited truncated O-antigen (Burtnick and Woods, 1999).

1.4.3 Antibiotic regime

Following culture confirmed *B. pseudomallei* infection, the recommended antibiotic treatment regime in summarised in Table 1.1.

Initial parenteral therapy	**Duration of therapy**
Ceftazidime 50 mg/kg every 6 - 8 h	
OR meropenem 25 mg/mg every 8 h	Minimum of 10 - 14 days and 4 - 8 weeks for deep-seated infection
Oral eradication therapy	
TMP-SMX 8/40 mg/kg orally every 12 h	At least 3 – 6 months

Table 1.1. Treatment of melioidosis, adapted from Peacock *et al.,* 2008.

If exposure to *B. pseudomallei* is known to have occurred, post-exposure prophylaxis is recommended (Peacock *et al.,* 2008). This has been evaluated in BALB/c mice infected with aerosolised *B. pseudomallei.* Treatment with TMP-SMX 0, 10 and 24 h post infection resulted in 100% survival rate (Sivalingam *et al.,* 2008). The introduction of ceftazidime for initial parenteral therapy was shown to reduce mortality by 50%, compared to the previous combination of chloramphenicol, doxycycline and TMP-SMX (White *et al.,* 1989). Amoxicillin-clavulanate is the treatment of choice for pregnant women and results in a similar mortality rate as treatment with ceftazidime. However, out of the surviving patients treated with amoxicillin-clavulanate, 23% had to be switched to an alternative regime following an unsatisfactory clinical response after >72 h treatment (Suputtamongkol *et al.,* 1994b). The addition of granulocyte-colony stimulating factor (G-CSF) to ceftazidime-treated patients is recommended if the

patient has septic shock (Stephens *et al.,* 2002). However, although G-CSF treatment has been shown to increase the duration of survival, no effect on mortality rates has been reported (Cheng *et al*., 2007).

Following initial treatment, melioidosis patients undergo 3 – 6 months oral eradication therapy. Four-drug regimes (TMP-SMX, doxycycline and chloramphenicol) have been shown to be successful, with relapse rates of <10% after 1 year (Rajchanuvong *et al*., 1995; Chaowagul *et al.,* 2005). The use of doxycycline alone resulted in 26% relapse rate and a higher treatment failure rate compared to the four-drug regime (Chaowagul *et al.,* 1999). Although efficacious, the four-drug regime is often poorly tolerated, with 36% patients requiring a switch in therapy due to side effects. In contrast, treatment with TMP-SMX and doxycycline has been shown to significantly reduce the number of patients experiencing side effects, whilst maintaining the same levels of efficacy as the four-drug regime (Chaowagul *et al.,* 2005). Optimal dosing regimes for use of TMP-SMX alone have been investigated using pharmokinetic models (Cheng *et al.,* 2009).

1.5 Peptidyl-prolyl *cis-trans* isomerases (PPIases)

PPIases are a highly conserved superfamily of proteins, found in bacteria, fungi, plants and vertebrates and are widely expressed in many tissues. Most PPIases exhibit enzyme activity, which catalyses the slow, rate-limiting *cis*→*trans* isomerisation of peptidyl-prolyl bonds (Figure 1.1; Kiefhaber *et al*., 1990). Many proteins require PPIases for efficient folding which indicates the critical role of PPIases in a range of physiological situations. PPIases are divided into three main families of unrelated amino acid sequence, on the basis of binding

partners. FK506-binding proteins (FKBPs) bind to FK506 and rapamycin; cyclophilins bind to cyclosporine A; and parvulins bind to juglone (Göthel and Marahiel, 1999).

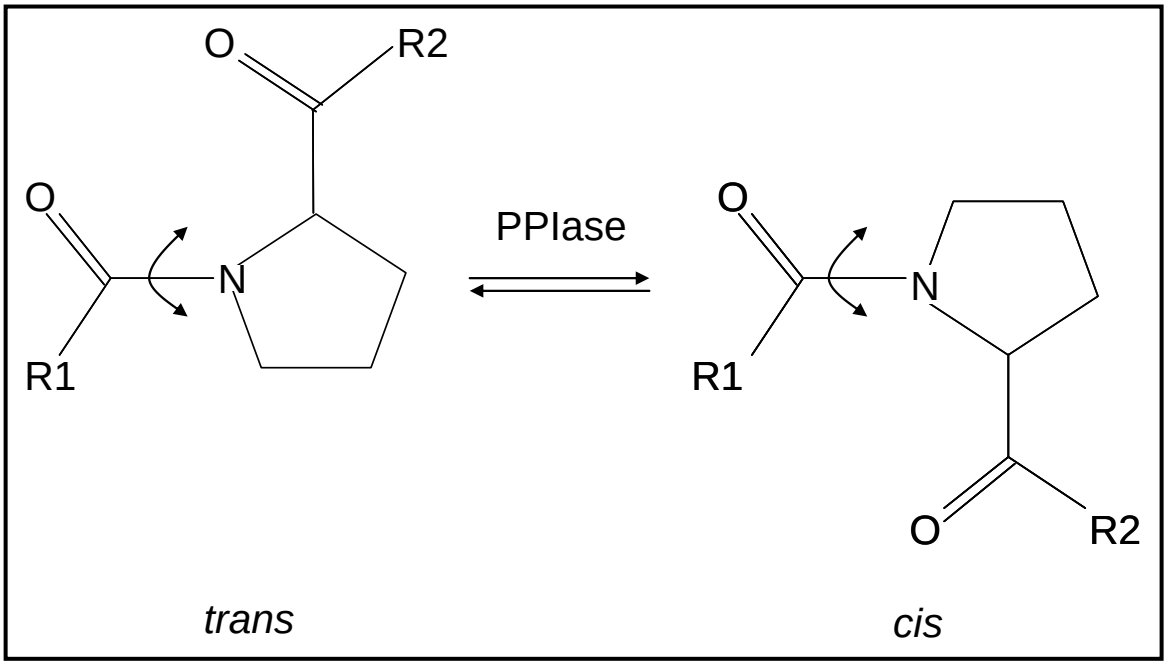


Figure 1.1 *Cis-trans* isomersation of a peptidyl-prolyl bond

1.5.1 FKBPs

FK506 is a fungal polyketide, produced by *Streptomyces tsukubasesis*, which was identified as a potent immunosuppressant (Kino *et al.,* 1987). To elucidate the mechanism of action of FK506, an affinity matrix was used to identify binding partners from bovine thymus and human spleen. A cytosolic 12 kDa FKBP was identified, purified from human T-cells and named FKBP12 (Harding *et al.,* 1989; Siekierka *et al.,* 1989). FKBP12 was shown to possess PPIase activity, inhibitable upon binding to FK506 and rapamycin (Harding *et al.,* 1989). FKBP12 was shown to consist of five β strands around a short α helix, with a

deep, hydrophobic pocket that included the active site and drug binding domains (Michnick *et al*., 1991).

FKBPs have subsequently been characterised in a range of organisms and although these proteins exhibit a diverse range of functions, the residues required for PPIase and drug binding are well conserved (Ikura & Ito, 2007; Ceymann *et al*., 2008; Löw *et al*., 2010). For example, a point mutation in Asp37 from human FKBP12, or the equivalent residue in a *L. pneumophilia* FKBP (Asp142), resulted in reduced PPIase activity in both cases (Wintermeyer *et al.,* 1995; Ikura and Ito, 2009). In addition to protein folding, the domain architecture of FKBPs enable these proteins perform a range of cellular functions. Structural analysis of 45 FKBPs suggested that FKBPs could be split into 6 groups based on their size and structural characteristics (Somarelli *et al*., 2008). The smallest FKBPs, such as human FKBP12, contain just one PPIase/drug binding domain (Michnick *et al.,* 1991). Additional accessory domains include a C-terminal domain in endoplasmic reticulum (ER) associated FKBPs; a central helix-loop-helix in nucleic acid binding FKBPs; tetratricopeptide repeats (TPR) in Hsp90 binding FKBPs; or additional drug binding domains (Nigam *et al.,* 1993; Rivière *et al*., 1993; Barent *et al*., 1998; Galat, 2003). Multiple domains have been shown to be functionally independent, with loss of TPR motifs having no effect on PPIase activity in FKBP35 from *Plasmodium falciparum* (Monaghan *et al*., 2005; Kumar *et al*., 2005). In addition, mutations in the PPIase domain of human FKBP52 did not affect TPR protein interactions (Barent *et al*., 1998). Small immunophilins, such as FkpA from *E. coli,* have been shown to exhibit multi-domain effects via dimerization. In a dimer, the PPIase and chaperone

active sites were shown to be simultaneously presented, which was required for full catalytic activity (Ramm and Pluckthun, 2001).

1.5.2 Cyclophilins

Cyclosporin A (CsA) is an undecapeptide produced by *Tolypocladium inflatum* and has been widely used as an immunosuppressant since the early 1980s (Borel and Gunn, 1986). In 1984, the first PPIase was isolated from porcine kidney (Fischer *et al.,* 1984). At the same time, in a bid to search for the intracellular receptor of CsA, an 18 kDa protein was purified from human spleen and was named cyclophilin (Harding *et al.,* 1986). Cyclophilin was subsequently shown to be identical to the previously identified PPIase, with CsA inhibitable enzyme activity (Fischer *et al.,* 1989). The structure of cyclophilin consists of 8 anti-parallel β strands that form a β barrel, with an α helix on either end (Kallen *et al*., 1991).

Although both FKBPs and cyclophilins possess PPIase activity and bind immunosuppressant drugs, the sequence and structure of the two families are dissimilar (reviewed by Barik, 2006). However, like FKBPs, the PPIase domain of cyclophilin is the same as the CsA binding site and the domain structure of cyclophilins is dependent on cellular location and function (Kallen *et al*., 1991). Human cyclophilin A represents a single domain cytosolic cyclophilin; cyclophilin B, C and D have an N-terminal signal sequence that targets them to the ER or mitochondria; and cyclophilin 19 has an RNA-binding domain and is located in the nucleus (Price *et al.,* 1991; Schneider *et al.,* 1994; Tanveer *et al.,* 1996; Teigelkamp *et al.,* 1998). In addition, some cyclophilins contain TPR domains or WD repeats which are associated with protein binding and chaperoning (Chen *et al.,* 1998).

1.5.3 Parvulins

A novel PPIase was identified in the periplasm of *E. coli* and shown to have enzyme activity that was not inhibitable by <5 µM CsA or FK506 (Rahfeld *et al*., 1994a). The recombinant protein was shown to be smaller than other PPIases, with a MW of 10.1 kDa and was named parvulin or Par10 (Rahfeld *et al*., 1994b). Although parvulins remain the smallest group of PPIases, several have been identified in mammals, plants, insects, yeast and bacteria (reviewed by Fischer and Aumüller, 2003). In addition, an inhibitor of parvulins was identified, named juglone (5-hydroxy-1,4-naphtoquinone; Hennig *et al.,* 1998). The structure of *E. coli* Par10 consists of four helical regions and a four-stranded anti-parallel β sheet, which closely resembles other human and plant parvulins (Kühlewein *et al.,* 2004).

Structural studies and site-directed mutagenesis experiments have identified several residues required for PPIase activity in parvulins (Mueller and Bayer, 2008). Cys113 from human Pin1 is in close proximity to the isomerisation site and substitution with an alanine residue reduced PPIase activity by 120 fold (Ranganathan *et al.,* 1997). Mutation of Asp15 in *B. subtilus* PrsA resulted in 50% wildtype PPIase activity *in vitro* (Tossavainen *et al*., 2006). In addition to PPIase domains, additional chaperoning domains or WW domains can mediate parvulin interactions within cells (Behrens *et al.,* 2001; Lu *et al.,* 2002)

1.6 PPIase activity

Following biosynthesis in the ribosome, most peptide bonds are connected in the *trans* conformation, resulting in folding events occurring in second or millisecond timescales (Brandts *et al.,* 1977). However, refolding experiments

highlighted that one conformational change can take significantly more time: the *cis/trans* isomerisation of a peptidyl-prolyl bond (Kiefhber *et al*., 1990). In this case, proline residues have a high intrinsic probability of existing in the *cis* conformation (approximately 10%), which can result in destabilisation of the native protein (Levitt, 1981). The spontaneous isomerisation from *cis*→*trans* is slow and rate limiting and therefore PPIases are thought to have evolved to speed up this process. The energy required for uncatalysed *cis*/*trans* isomerisation is high and PPIases reduce this requirement, resulting in efficient protein folding (Fischer, 1994).

1.6.1 Measuring PPIase activity

There have been several assays devised to measure PPIase activity, some of which have been adapted for specific proteins or applications (reviewed by Fischer and Aumüller, 2003). Most methods are based around polypeptides that contain proline residues or by studying refolding kinetics of denatured proteins such as ribonuclease T1 (RNase T1).

1.6.1.1 Protease coupled assay

The original assay developed to monitor PPIase activity used isomer-specific proteolysis using tetrapeptide derivatives based around the general structure Suc-Ala-Xaa-Pro-Phe-4-nitroanilide, where Xaa = any natural amino acid (Fischer *et al*., 1984). In this assay, α-chymotrypsin is used as a helper protease as it will specifically cleave the peptide in the *trans* conformation (Figure 1.2). This leads to a rapid release of a *p*NA chromophore from the 90% of the substrate in the *trans* conformation, followed by a slow kinetic phase of isomerisation from *cis*→*trans.* In the presence of a PPIase, the isomerisation is

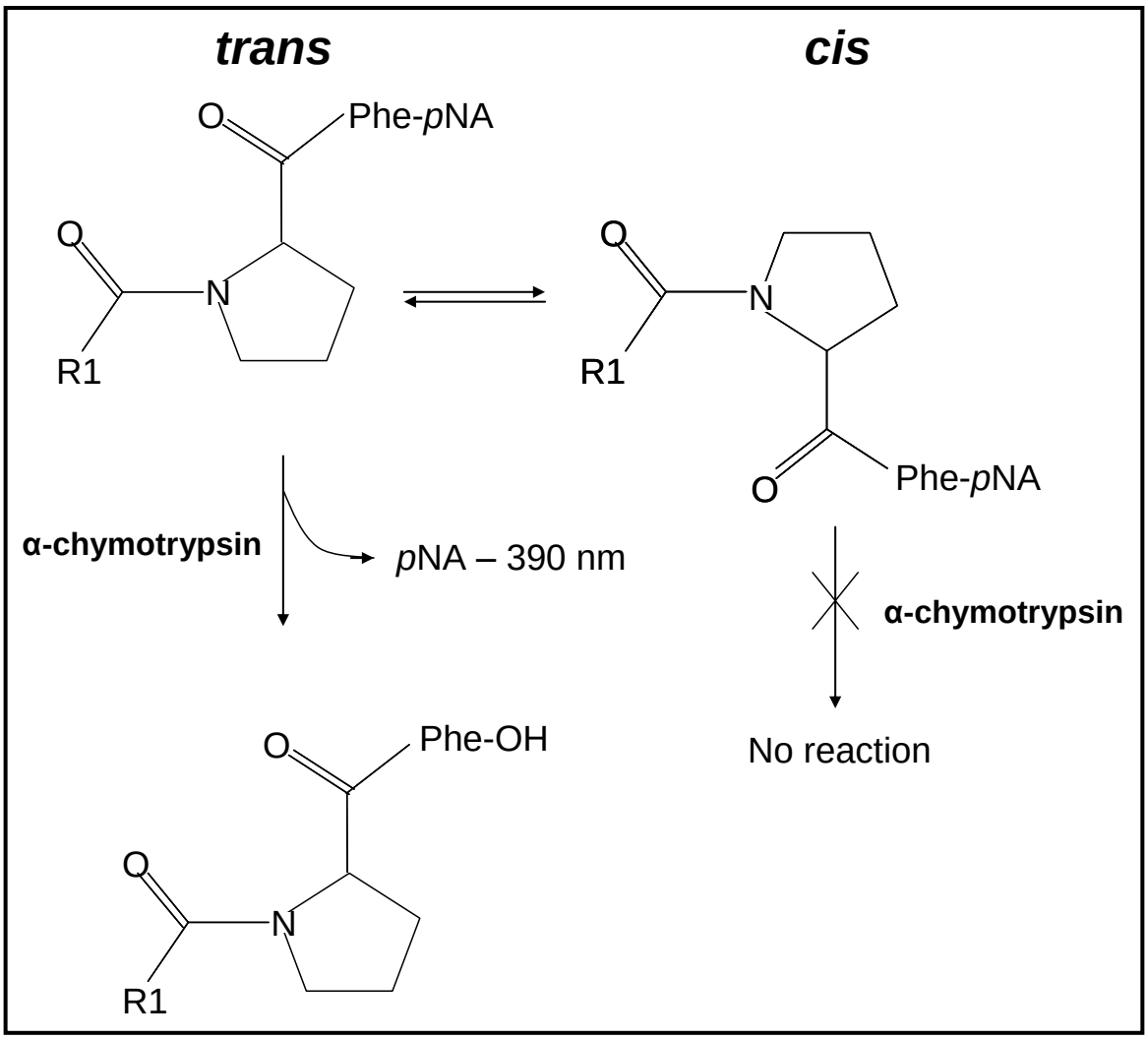


Figure 1.2. Isomer specific cleavage of *trans*-Phe-pNA by chymotrypsin. PPIases catalyse the *cis/trans* isomerisation of proline bonds, with the *trans* isomer being a substrate for chymotrypsin. Chymotrypsin cleaves the peptide, releasing a *p*NA chromophore which can be measured at 390 nm. Phe = phenylalanine; *p*NA = *p*-nitroanilide; R1 = side chain

accelerated and *p*NA production can be measured at 390 nm by an increase in absorbance (Fischer *et al*., 1984). The specificity constant (k_{cat}/K_m) of some well characterised PPIases are shown in Table 1.2.

Although the protease coupled assay is the most simple to perform, there are several limitations with its use. Only one direction of the reversible isomerisation is measured (*cis*→*trans*) and as only 10% of the substrate (*cis* content) can be

monitored, the signal-to-noise ratio can cause problems. This background can be minimised by dissolving the substrates in 0.48 M LiCl/trifluoroethanol (TFE), which shifts the *cis* content up to 70% (Kofron *et al*., 1991). However, although improved, the use of chymotrypsin in this assay can influence the PPIase activity of proteins that are highly susceptible to proteolytic degradation. SlyD, a metal ion regulated PPIase from *E. coli,* is highly sensitive to α-chymotrypsin digestion. Initially, when using the protease coupled assay, SlyD was not thought to have PPIase activity (Wülfing *et al*., 1994). To overcome protein degradation, trypsin was used as an alternative to chymotrypsin, and resulted in the successful measurement of PPIase activity (Hottenrott *et al.,* 1997).

Protein	k_{cat}/K_m **($M^{-1}s^{-1}$)**	**Substrate**	**Reference**
Human FKBP12	3.6×10^6	Suc-Ala-Leu-Pro-Phe-4-nitroanilide	Bossard *et al.,* 1994
Human cyclophilin A	3.2×10^7	Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide	Harrison and Stein, 1990
Human Pin1	1.9×10^7	Ala-Ala-pSer-Pro-Arg- *p*-nitroanilide	Yaffe *et al.,* 1997
***L. pneumophila* Mip**	1.01×10^6	Suc-Ala-Phe-Pro-Phe-4-nitroanilide	Wintermeyer *et al.*, 1995
***E. coli* SurA**	3.4×10^4	Suc-Ala-Leu-Pro-Phe-4-nitroanilide	Behrens *et al.,* 2001

Table 1.2. The enzyme activity of PPIases, determined using the protease coupled assay.

For the large scale screening of compounds, a 96-well plate assay has been developed (Kullertz *et al*., 1998). This allows for reliable determination of PPIase activity of up to 96 samples in less than 30 minutes and therefore can be useful for high through-put screening of potential inhibitors.

1.6.1.2 Protease free assay

A protease free assay was developed to overcome the problem of PPIase degradation (Janowski *et al.,* 1997). The assay is based around the small difference in absorbance determined for the *cis* (13100 $M^{-1}cm^{-1}$) and *trans* (12500 $M^{-1}cm^{-1}$) isomers of Suc-Ala-Xaa-Pro-Phe-4-nitroanilide. To increase the amplitude, the substrate was dissolved in 0.48M LiCl/TFE. On the addition of the substrate to an aqueous buffer, the *cis:trans* equilibrium will shift from 70:30 back to 10:90 and the change in absorbance measured at 330 nm. In the presence of a PPIase, the speed at which the shift occurs will be accelerated. This allowed the measurement of *cis/trans* isomerisation in both directions of human cyclophilin A and SlyD (Janowski *et al.,* 1997).

1.6.1.3 Nuclear magnetic resonance (NMR)

One-dimensional ^{1}H NMR experiments have been used as a direct measurement of the *cis/trans* isomerisation of tetrapeptide substrates. In an uncatalysed reaction, distinct signals occur for the *cis* and *trans* isomers in a spectrum. This method was used to monitor the PPIase activity of human cyclophilin A, where addition of the recombinant protein resulted in broadening of the NMR line, which was inhibitable upon addition of CsA (Hsu *et al.,* 1990). Two-dimensional NOESY NMR has also been used to more accurately monitor isomerisation in medium sized proteins (Mallis *et al*., 2002). Despite the successful application of NMR as a means for determining PPIase activity, it

requires large amounts of data analysis and technical equipment (Gothel and Marahiel, 1999).

1.6.1.4 Refolding experiments

Native RNase T1 contains four proline residues, two in the *cis* conformation (*cis*-Pro39 and *cis*-Pro55) and two in the *trans* conformation (*trans*-Pro60 and *trans*-Pro73). Therefore, the refolding of a reduced and carboxymethylated form of RNase T1 can be used to monitor the slow *trans*→*cis* isomerisation around the Tyr38-Pro39 bond (Mücke and Schmid, 1994). The refolding kinetics of RNase T1 and other proline containing proteins can be monitored by fluorescence, protolysis or electron microscopy (Davis *et al.,* 1989; Schiene-Fischer *et al.,* 2002).

1.6.2 Inhibition of PPIase activity

The diverse function and biological importance of PPIases (see section 1.7) has lead to the development of several PPIase inhibitors. The approaches used to identify novel inhibitors include: screening homologues of natural inhibitors; rational design based on scrutiny of crystal structure; or screening small molecule/chemical libraries (reviewed by Wang and Etzkorn, 2006).

1.6.2.1 FKBP inhibitors

The immunosuppressant drug FK506 binds strongly but reversibly to human FKBP12, with a Ki of 1.7 nM (Harrison and Stein, 1990). Beside FK506, ascomycin and rapamycin are natural inhibitors of FKBPs (Bierer *et al.,* 1990a; Kawai *et al.,* 1992). While several novel FKBP inhibitors have been tested, only V-10,267 can inhibit FKBP12 at nanomolar concentrations, with a Ki of 0.5 nM (Armistead *et al*., 1995). In addition, V-10,267 was shown to not have immunosuppressive properties. Similarly, an inhibitor based on common

regions of FK506 and rapamycin, named 506BD, was shown to inhibit PPIase activity and did not interfere with T cell activation (Bierer *et al.,* 1990b).

1.6.2.2 Cyclophilin inhibitors

CsA is a potent inhibitor of human cyclophilin A, with a Ki of 17 nM (Zydowsky *et al.,* 1992). Other natural cyclophilin inhibitors include cyclolinopeptide and sanglifehrin A, which both exhibit immunosuppressive activity (Wieczorek *et al.,* 1991; Fehr *et al.,* 1999). Virtual screening has been used to identify novel non-immunosuppressive inhibitors, with the most potent having an IC_{50} of 15 nM against cyclophilin A PPIase activity (Guichou *et al.,* 2006).

1.6.2.3 Parvulin inhibitors

Unlike FKBPs or cyclophilins, parvulins do not bind immunosuppressant drugs and some specifically interact with phosphorylated Ser-Pro or Thr-Pro motifs (Yaffe *et al.,* 1997). Therefore, inhibitor design has exploited this specificity and modified phopho-Ser-Pro residues to competitively inhibit PPIase activity (Zhang *et al*., 2002; Wang *et al.,* 2004). The best characterised parvulin inhibitor is called juglone, which irreversibly inhibits the PPIase activity of several parvulins (Hennig *et al*., 1998). Juglone target parvulins by covalent modification of two cysteine residues, one of which is required for PPIase activity (Hennig *et al.,* 1998).

1.7 Physiological role of PPIases

1.7.1 Human PPIases

Humans encode at least 16 cyclophilins, 15 FKBPs and 2 parvulins located in range of subcellular locations and vary in molecular weight from 12-52 kDa (Göthel and Marahiel, 1999). Identified as the original receptors of FK506 or CsA, FKBP12 and cyclophilin A have been extensively characterised to elucidate the mechanism of immunosuppression. The formation of FKBP12/FK506 or cyclophilin/CsA complexes inhibits the phosphatase activity of calcineurin (Figure 1.3 A, B). In turn, this prevents dephosphorylation of nuclear factor of activated T-cells (NF-AT) which fails to enter the nucleus, preventing T cell activation and IL-2 expression (Liu *et al.,* 1991; McCaffrey *et al*., 1993). In contrast, FKBP12/rapamycin complexes interact with mammalian target of rapamycin (mTOR) which inhibits cell cycle progression and protein synthesis (Figure 1.3 C; Heitman *et al.,* 1991; Dumont and Su, 1995).

In the absence of FK506, FKBP12 has other cellular targets such as ryanodine receptors (RyRs) and transforming growth factor-β (TGF- β; Brillantes *et al.,* 1994; Wang *et al.,* 1994). FKBP12 has been shown to bind to and stabilise RyR, a major $Ca2^{+}$-release channel in the sarcoplasmic reticulum (Brilliantes *et al.,* 1994; Mayrleitner *et al.,* 1994). Disruption of FKBP12 binding results in $Ca2^{+}$ leakage which has been associated with endothelial dysfunction and heart failure (Ono *et al.,* 2000; Marx *et al.,* 2000). FKBP12 also complexes with TGF-β and inhibits signalling function (Wang *et al.,* 1994).

Expression of human cyclophilin A has been shown to be induced by oxidative-stress or vascular injury and inhibits vascular smooth muscle cell apoptosis (Jin

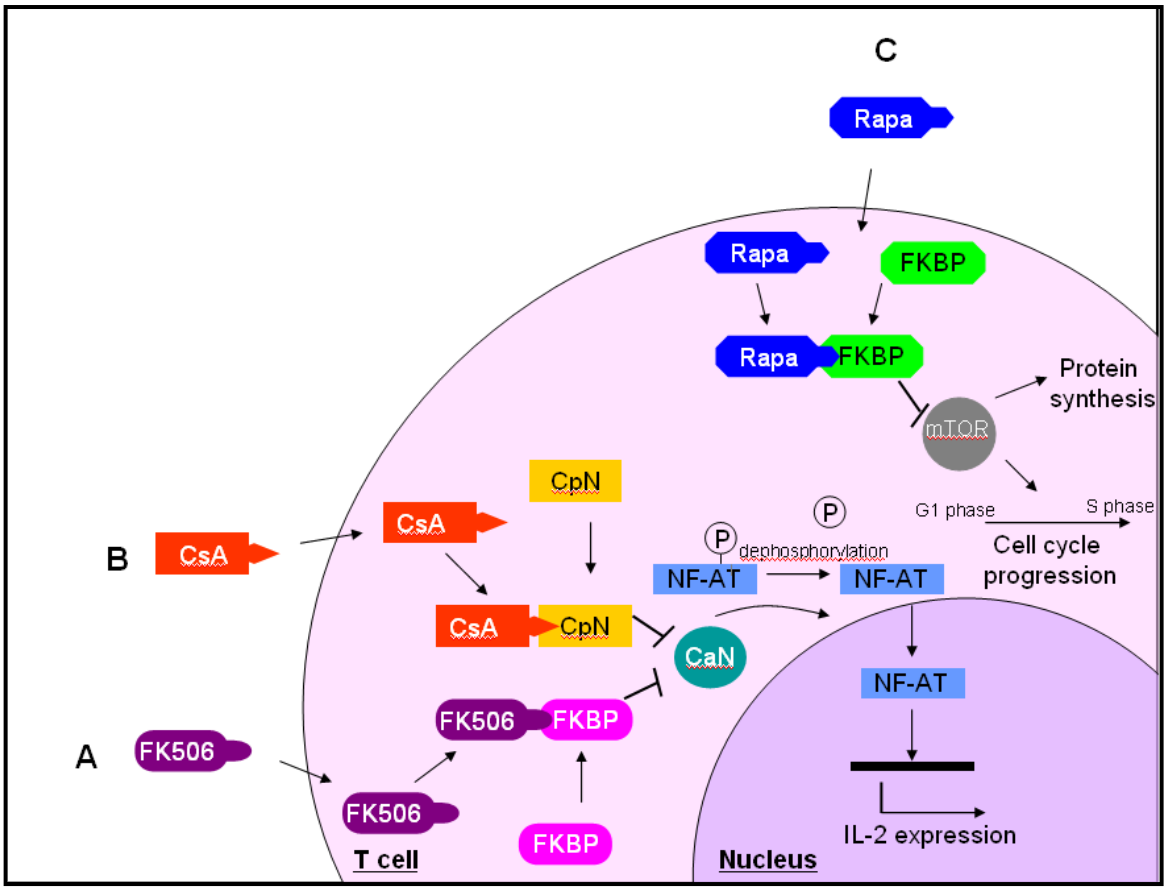


Figure 1.3. Mechanism of action of immunosuppressants FK506, CsA or rapamycin. Adapted from Stepkowski, 2000.

A – FK506 binds to human FKBP12 forming an FK506/FKBP complex which inhibits the phosphotase calcineurin (CaN). CaN fails to dephosphorylate nuclear factor of activated T cells (NF-AT) which fails to enter the nucleus. On entry into the nucleus, NF-AT usually binds to the promoter of IL-2 and initiates IL-2 production. Therefore, T-cells do not produce IL-2 and are not activated.

B – Cyclosporin A (CsA) binds to human cyclophilin A (CpN) forming a CsA/CpN complex which inhibits CaN activity. Phopho-NF-AT fails to enter the nucleus, resulting in inhibition of T-cell activation.

C – Rapamycin (Rapa) binds to human FKBP12, forming a Rapa/FKBP complex which binds to the mammalian target of rapamycin (mTOR). This inhibits biochemical pathways required for cell cycle progression and protein synthesis in T-cells.

et al., 2000). Cyclophilin A has also been implicated in the development of lung, endometrial and pancreatic cancers, with suppression of cyclophilin A reducing cell proliferation and inducing apoptosis (Campa *et al*., 2003; Shen e*t al.,* 2004; Zhao *et al.,* 2008). Cyclophilin A has been shown to be important for efficient replication of several pathogenic viruses, including HIV, HBC and HCV (Braaten and Luban*,* 2001; Nakagawa *et al.,* 2004; Tian *et al.,* 2010).

Pin1 is a well characterised human parvulin that plays a critical role in cell cycle progression, with deletion of Pin1 in HeLa cells inducing mitotic arrest (Lu *et al.,* 1996). Subsequently, Pin1 has been shown to be involved in cancer development and Alzheimer's disease (Ryo *et al.,* 2003; Bao *et al*., 2004; Butterfield *et al.,* 2006).

1.7.2 Plant PPIases

In *Arabidopsis thaliana,* 23 putative FKBPs, 29 putative cyclophilins and 3 putative parvulins have been identified*,* the largest number of PPIases encoded by organisms with sequenced genomes (He *et al*., 2004). 50% of FKBPs were predicted to be located in the chloroplast and AtFKBP13 was shown to regulate proteins required for photosynthetic electron transport (Gupta *et al.,* 2002). A homologue of human FKBP12, AtFKBP12, binds AtFIP37, which can be disrupted by the presence of FK506 (Faure *et al.,* 1998). AtFIP37 is a homologue of mammalian FAP48 and therefore indicates that FKBP12 interactions are conserved between plants and animals. In addition, high molecular weight *A. thaliana* FKBPs have been shown to bind Hsp90 via TPR domains, in a similar way to human FKBP52 (Kamphausen *et al.,* 2002; Aviezer-Hagai *et al*., 2007). FKBP42 has been shown to be involved in

Arabidopsis development, with a mutant strain exhibiting dwarfism and helical rotation of roots (Geisler *et al.,* 2003).

1.7.3 Fungal PPIases

S. cerevisiae encodes 4 FKBPs and 8 cyclophilins which have been shown to be individually and collectively dispensable for viability under laboratory conditions. The mutants exhibited a wildtype phenotype when grown at various temperatures, under stress conditions, on different carbon sources and had normal mating and sporulation properties (Dolinski *et al.,* 1997). However, the only yeast parvulin, Ess1, is essential for viability (Hanes *et al*., 1989; Hani *et al*., 1995). A double mutation in two cyclophilins from *C. neoformans* resulted in reduced growth and virulence (Wang *et al.,* 2001). Furthermore, CsA has been shown to enhance the antifungal activity of compounds against several medically important fungi *in vitro*, including *Aspergillus* species and *Candidia* species (reviewed by Blankenship *et al.,* 2003).

1.7.4 Microbial FKBPs

Three FKBPs have been well characterised in *E. coli* (Callebaut and Mornon, 1995; Horne *et al*., 1995; Hottenrott, *et al.,* 1997). Trigger factor acts as a ribosome-associated chaperone which binds to newly synthesised proteins (Hesterkamp *et al.,* 1996). SlyD is a metal-ion regulated PPIase, which is reversibly inhibitable by Ni^{2+} ions (Hottenrott *et al.,* 1997). FkpA is a periplasmic protein that has been shown to decrease protein misfolding and is required for the import of the toxic protein colicin M (Missiakas *et al*., 1996; Hullmann *et al.,* 2008). In addition, virulence-associated FKBPs have been indentified in several intracellular pathogens, named macrophage infectivity potentiators (Mips; Cianciotto *et al.,* 1989).

1.7.4.1 *Legionella pneumophila* Mip

L. pneumophila (Lp) is a bacterium which can multiply intracellularly and disrupt normal phagocytic activities (Horwitz, 1983). Inactivation of a gene encoding a 24 kDa surface-located protein resulted in reduced initiation of infection and intracellular replication in human alveolar macrophages. This gene was therefore named *mip*, a 'macrophage infectivity potentiator' (Cianciotto *et al*., 1989). The *mip* mutant was subsequently shown to exhibit reduced replication in protozoa and was significantly attenuated in a guinea pig model of infection (Cianciotto *et al*., 1990; Cianciotto and Fields, 1992).

Lp-Mip was shown to be an FKBP homologue, which exhibited characteristic PPIase activity and was inhibitable by FK506 (Fischer *et al*., 1992). The crystal structure of Lp-Mip was solved and revealed that the protein forms dimers, each consisting of an N-terminal domain which controls dimerisation and a C-terminal PPIase domain, connected by a long α-helix (Riboldi-Tunnicliffe *et al*., 2001). In addition, the structure of Lp-Mip bound to rapamycin showed that the hydrophobic cavity contained essential amino acids for PPIase activity and drug binding (Ceymann *et al*., 2008). Deletion of the N-terminal domain reduced refolding efficiency due to the loss of dimeric state, however PPIase activity was maintained, suggesting independent modular action of the two domains (Kohler *et al.,* 2003).

Several approaches have been used to analyse the contribution of the PPIase domain for function. Mutation of Asp-142 or Tyr-185 in recombinant Mip was shown to reduce PPIase activity to 5.3% or 0.6% of wildtype Mip, respectively. Complementation of the *mip* mutant with the wildtype gene restored intracellular

replication and introduction of the Asp-142 or Tyr-185 mutant gene also partially complemented growth (Wintermeyer *et al.,* 1995). This study concluded that the PPIase activity of Mip was not required for intracellular replication, however low levels of activity were still detected and complementation was incomplete. Some years later, a *mip* mutant was complemented with either a low PPIase Mip variant or an N-terminally truncated Mip variant and both were significantly attenuated in a guinea pig model, indicating both PPIase activity and the dimeric state are required for virulence (Kohler *et al.,* 2003). The importance of PPIase activity for function of Mip was also shown by the use of monoclonal antibodies to specifically target the active site, which lead to inhibition of infection in protozoa and human macrophages (Helbig *et al*., 2003).

Although the putative function has been well studied, the exact target(s) of Mip are yet to be elucidated. Inactivation of *mip* was shown to reduce the amount of type II secreted hydrolases in *L. pneumophila* culture supernatants by 40-70%. This was the first indication that Mip was involved in secretion of proteins beyond the outer membrane (DebRoy *et al.,* 2006). Lp-Mip has also been shown to interact with collagen from the extracellular cell matrix of the lung. Furthermore, Mip was shown to be necessary for bacterial transmigration of NCl-H292 lung epithelial cells and inhibition of migration was possible with anti-Mip antibodies, FK506 or rapamycin (Wagner *et al*., 2007). Mip has been shown to be expressed 24 h after infection of cells and reactive antibodies are present in convalescent sera from *Legionella* patients (Bangsborg *et al*., 1991; Wieland *et al.,* 2002).

1.7.4.2 *Chlamydia trachomatis* Mip

A 27 kDa membrane-located protein was identified in *C. trachomatis* (Ct), which showed 37% sequence identity to Lp-Mip (Lundemose *et al.,* 1991; Lundemose *et al.,* 1992). Immunofluoresence analysis of infected McCoys cells revealed synthesis of Ct-Mip was detected at 14 hours post-infection (Lundemose *et al.,* 1991). Ct-Mip was shown to have PPIase activity which is inhibitable by FK506 or rapamycin. In addition, pre-treatment of *C. trachomatis* or the presence of the drugs during the early stages of infection reduced bacterial infectivity of cells (Lundemose *et al.,* 1993). Characterisation of Ct-Mip revealed it is a lipoprotein exposed on the surface of elementary bodies (EB; Neff *et al*., 2007). Inactivated EBs or recombinant Ct-Mip was shown to induce release of IL-1β, TNF-α, IL-6 and IL-8 in human macrophages. TLR2/TLR1 and TLR6 were shown to be involved in Ct-Mip mediated activation which was attenuated in the presence of anti-Mip antibodies (Bas *et al*., 2008).

1.7.4.3 *Neisseria gonorrhoeae* and *Neisseria meningitidis* Mips

A surface-exposed lipoprotein from *N. gonorrhoeae* (Ng) was shown to have 43.8% sequence identity to Lp-Mip. In particular, the N-terminal domain was similar to the Lp-Mip dimerisation domain and HPLC analysis revealed Ng-Mip forms homodimers in solution. The recombinant protein exhibited PPIase activity, which was inhibitable by rapamycin. Inactivation of *mip* in *N. gonorrhoeae* did not affect binding to or internalisation by macrophages, however intracellular survival was significantly reduced (Leuzzi *et al*., 2005). A Mip homologue from *N.* **meningitidis** has also been implicated in intracellular infection, as it is upregulated following bacterial adhesion to epithelial cells (Grifantini *et al.,* 2002).

1.7.4.4 *Trypanosoma cruzi* Mip

Unlike other membrane associated Mips, a secreted Mip-like protein was identified in the intracellular protozoan parasite *T. cruzi* (Tc). With 29.6% sequence identity to Lp-Mp, Tc-Mip also exhibited PPIase activity which was inhibited by an anti-Mip antibody, FK506 or rapamycin. Addition of exogenous recombinant Tc-Mip significantly enhanced invasion of epithelial cells where-as anti-Mip antibodies or FK506 reduced infectivity (Moro *et al*., 1995). Furthermore, recombinant Lp-Mip could substitute for Tc-Mip in enhancing infection of cells, indicating functional similarities (Pereira *et al*., 2002).

1.7.4.5 Other bacterial Mips

Mips appear to be well conserved in a range of pathogenic bacteria and several have been at least partially characterised. *Coxiella burnetii* has been shown to produce a 23.5 kDa protein which has PPIase activity. Although this protein has been named Mip, a role in virulence has not yet been confirmed (Mo *et al*., 1995). A Mip-like protein was identified in *Salmonella typhimurium* and inactivation of the gene reduced intracellular survival in macrophages and epithelial cells (Horne *et al.,* 1997). Similarly, deletion of a Mip homologue from *Aggregatibacter actinomycetemcomitans* resulted in reduced invasion of epithelial cells and the Mip-like protein was shown to be expressed in sera from periodontal patients (Maeda *et al.,* 2010). The PPIase activity of the S. *typhimurium* or *A. actinomycetemcomitans* Mip-like proteins was not confirmed. The requirement of a Mip-like protein for virulence in a plant pathogen has also been reported. A Mip homologue from *Xanthomonas campestris* was shown to have FK506-inhibitable PPIase activity and was required for virulence and replication in Chinese radish (Zang *et al*., 2007).

1.7.5 Microbial cyclophilins

There has been limited functional characterisation of prokaryote cyclophilins, however enzymatically active cyclophilins have been identified in bacteria such as *E. coli* and *L. pneumophila* (Liu *et al,* 1990; Schmidt *et al.,* 1996). While the PPIase activity of bacterial cyclophilins is as high as eukaryotic cyclophilins, the affinity to CsA is weaker compared to human cyclophilin A (Schönbrunner *et al.,* 1991). CsA has been shown to attenuate pathogenesis of the intracellular parasite *Leishmania major* in BALB/c mice and reduce intracellular replication within murine macrophages (Behforouz *et al.,* 1986; Hoerauf *et al.,* 1997).

1.7.6 Microbial parvulins

E. coli encodes at least three parvulins: Par10, PpiD and SurA. Par10 is the smallest known parvulin and exhibits PPIase activity (Rahfeld *et al.,* 1994b). PpiD was shown to be required for the folding and production of OMPs in *E. coli* outer membrane. In addition, a double mutation in *ppiD* and *surA* was lethal (Dartigalongue and Raina, 1998). SurA has been shown to be required for β-barrel OMP synthesis, assembly and transport (Lazar and Kolter, 1996; Vertommen *et al.,* 2009). In addition, SurA is associated with virulence in uropathogenic *E. coli* (UPEC) and other pathogenic bacteria (Behrens-Kneip, 2010).

1.7.6.1 *E. coli* SurA

Survival protein A (SurA) was identified when it was shown to be required for stationary phase survival in the presence of a mutation of stationary phase sigma factor σ^S (Tormo *et al*., 1990; Lazar *et al.,* 1998). Recombinant SurA exhibited PPIase activity and facilitated the folding of OMPs, such as OmpA, OmpF and LamB (Lazar and Kolter, 1996; Rouvière and Gross, 1996; Vertommen *et al.,* 2009). In addition, in the *surA* mutant the σ^E stress response was induced, which can down-regulate *omp* transcript levels (Rouvière and Gross, 1996; Missiakas *et al*., 1996; Johansen *et al*, 2006). SurA is also required for biogenesis of, LptD, a β-barrel OMP which mediates insertion of LPS into the outer membrane (Wu *et al.,* 2006; Vertommen *et al.,* 2009; Denoncin *et al*., 2010). The *surA* mutant strain exhibited a phenotype associated with defects in the outer membrane, such as hypersensitivity to detergents and hydrophobic antibiotics (Rouvière and Gross, 1996).

SurA consists of an N-terminal region, two parvulin-like domains and a short C-terminal tail (Rouvière and Gross, 1996). The PPIase activity of SurA resides in only one parvulin-like domain and the chaperone activity resides in the N-terminal domain (Rouvière and Gross, 1996; Behrens *et al.,* 2001). However, proline containing peptides have been shown to be bind to the inactive parvulin-like domain (Xu *et al.,* 2007). Complementation of a *surA* mutant with the N-terminal chaperone domain was shown to restore wildtype OMP formation and resistance to detergents (Behrens *et al.,* 2001). This indicates that chaperone function is more important than PPIase function in OMP synthesis.

Inactivation of *surA* in UPEC was shown to reduce production of FimD, a type 1 pilus usher, which resulted in defective piliation in the mutant strain (Justice *et al.,* 2005). As *E. coli* uses type 1 pili to bind to and invade cells, the ability of the *surA* mutant to infect bladder epithelial cells was evaluated (Schaeffer *et al.,* 1987; Justice *et al.,* 2006). The *surA* mutant was shown to be defective in binding to and invasion of cells and was unable to persist in a murine cystitis model (Justice *et al.,* 2006). In addition, the *surA* mutant was found to abolish wildtype suppression of LPS-induced cytokine production in bladder epithelial cells (Hunstad *et al.,* 2005). Complementation of the *surA* mutant with the N-terminal chaperone domain partially restored *fimD* production, cell invasion and novobiocin resistance (Watts and Hunstad, 2008).

1.7.6.2 Other bacterial SurAs

The *surA* gene was identified as the insertion site of a transposon in an attenuated *Salmonella typhimurium* mutant (Miller *et al.,* 1989; Sydenham *et al*., 2000). A deletion mutant was further characterised and shown to exhibit defective adhesion to and invasion of epithelial cells. The *surA* mutant also showed reduced persistence in BALB/c mice and provided some protection against subsequent challenge with wildtype *S. typhimurium* (Sydenham *et al*., 2000). *S. typhimurium* SurA has been shown to be regulated by the σ^E regulon and is expressed in carbon-starved cell, under a range of environmental stresses (Dartigalongue *et al.,* 2001).

SurA from *Shigella flexneri* has been shown to be required for outer membrane localisation of IcsA, an autotransporter required for intracellular spread (Goldberg *et al.,* 1993). In addition, inactivation of *surA* resulted in reduced

plaque formation on cell monolayers, but no defect in cell invasion was observed (Purdy *et al*, 2007).

1.8 Aim of this project

The prevention and treatment of melioidosis can be problematic, with up to 40% mortality even with prolonged antibiotic treatment (White, 2003). Therefore, novel vaccine candidates and antimicrobial targets are required. Antigens that are likely to be 'seen' by the host immune response include surface proteins and virulence factors which are expressed *in vivo.* While traditional approaches to combat bacterial infections target processes required for bacterial growth *in vitro,* this may result in rapid selection of resistant sub-populations. Therefore, the identification of anti-virulence targets has several advantages, including reduction in development of resistance and increased specificity against pathogenic, rather than commensal, bacteria (Escaich, 2008).

Enzymes, such as PPIases, may represent novel vaccine candidates or anti-virulence targets in bacteria. Therefore, the aim of this project is to identify and characterise PPIases from *B. pseudomallei* and *B. thailandensis.* Specifically, FKBPs and parvulins will be evaluated as they have been shown to be virulence associated in other bacteria. Bioinformatics approaches will be used to identify and characterise FKBPs and parvulins in *Burkholderia* species. *B. pseudomallei* proteins will be recombinantly produced in *E. coli* and the PPIase activity will be analysed. The recombinant proteins will be used to immunise BALB/c mice and the protective efficacy will be determined following challenge with *B. pseudomallei.* The role of Mip and SurA homologues in *B. pseudomallei* and *B.*

thailandensis will be evaluated using deletion mutagenesis. The mutant phenotype will be characterised using *in vitro* and *in vivo* assays.

Chapter 2 – Materials and Methods

1 2.1 Bacterial strains and cell lines

2.1.1 *E. coli* strains

E. coli strains used in this study are listed in Table 2.1. Bacteria were typically grown in Luria Bertani (LB) broth (Table 2.3) at 37ºC overnight with agitation, unless otherwise stated.

2.1.2 *Burkholderia* strains

B. pseudomallei and *B. thailandensis* strains used in this study are listed in Table 2.2. Bacteria were grown in 50 ml LB broth at 37ºC overnight with agitation, unless otherwise stated.

2.1.3 Mammalian cell lines

Cell lines used in this study were J774A.1 mouse BALB/c macrophages and A549 human lung epithelial cells (HPACC). Cells were typically grown in 25 ml DMEM (Table 2.3) at 37ºC and 5 % CO_2 overnight, unless otherwise stated.

2.1.4 Growth media

Media used to grow bacteria and cell lines in this study are listed in Table 2.3.

***E.coli* strain**	**Use**	**Genotype**
Top 10 (Invitrogen)	Transformation	F⁻ **mcr**A 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 (StrR) **end**A1 **nup**G λ-
JM109 (Promega)	Transformation and glycerol stocks	*end*A1 *rec*A1 *gyr*A96 *thi hsd*R17 (r_k- m_k+) *rel*A1 *sup*E44 Δ(*lac-pro*AB) [F' *tra*D36 *pro*AB$^+$ *lac*I^{q}ZΔM15]
BL21 (DE3) (Invitrogen)	Expression	F$^-$ *omp*T *hsd*S_B (r_B^- m_B^-) *gal dcm* (DE3)
BL21 * pLysS (DE3) (Invitrogen)	Expression	F$^-$ *omp*T *hsd*S_B (r_B^- m_B^-) *gal dcm rne*131(DE3) pLysS (Cam^R)
DH5α λ*pir*	Transformation	F$^-$ endA1 *thi-1 rec*A1 *rel*A1 *gyr*A96 Φ80**lac**ZΔM15 Δ(**lac**ZYA-*arg*F) U169 *hsd*R17 (r_k- m_k+) λ *pir*
S17-1 λ*pir*	Conjugation	TpR SmR *rec*A *thi pro hsd*R$^-$ M$^+$RP4: 2-Tn7 λ *pir*
HB101 (pRK2013)	Conjugation	*sup*E44 Δ*mcrC-mrr) rec*A13 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20 *xyl*-5 *mtl*-1 *leu*B6 *thi*-1 pRK2013 (KmR oriColE1 RK2-Mob$^+$ RK2-Tra$^+$

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

***Burkholderia* strain**	**Comments**	**Source**
B. pseudomallei K96243	Clinical isolate; Gen^R	Prof S Songsivilai, Mahidol University
B. pseudomallei Δ*amrA*	K96243 derivative; unmarked deletion Δ*amrA*; Gen^S	Dr S Harding, Dstl
B. pseudomallei Δ*BPSS1823*	K96243 derivative; unmarked deletion *ΔBPSS1823*; Gen^R	This study
B. pseudomallei Δ*amrA* Δ*BPSS1823*	K96243 derivative; unmarked deletion Δ*amrA* *ΔBPSS1823*; Gen^S	This study
B. pseudomallei Δ*amrA* Δ*BPSS1823 pBR1823*	K96243 derivative; unmarked deletion Δ*amrA* *ΔBPSS1823*::pBBR1MCS2 Gen^S	This study
B. thailandensis E264	Environmental isolate; Gen^R	ATCC number 700388
B. thailandensis Δ*BTH_I0576*	Environmental isolate; unmarked deletion *ΔBTH_I0576*; Gen^R	This study

Table 2.2: *Burkholderia* species used in this study

Media	Composition	Treatment
Luria Bertiani (LB) broth	10 g Difco tryptone peptone, 5 g Difco Bacto yeast extract, 5 g sodium chloride, 1 L Milliq water	Autoclave at 121ºC for 15 min
LB agar	10 g Difco tryptone peptone, 5 g Difco Bacto yeast extract, 5 g sodium chloride, 20 g Difco Bacto agar, 1 L Milliq water	Autoclave at 121ºC for 15 min
0.3 % motility agar	10 g Difco tryptone peptone, 5 g Difco Bacto yeast extract, 5 g sodium chloride, 3 g Difco Bacto agar, 1 L Milliq water	Autoclave at 121ºC for 15 min
Skimmed milk agar	10 g Difco tryptone peptone, 5 g Difco Bacto yeast extract, 5 g sodium chloride, 5 g skimmed milk powder, 20 g Difco Bacto agar, 1 L Milliq water	Autoclave at 121ºC for 15 min
Sucrose agar	10 g Difco tryptone peptone, 5 g Difco Bacto yeast extract, 100 g sucrose, 1 L Milliq water	Autoclave at 121ºC for 15 min
Super Optimal broth with Catabolite repression (SOC)	2 % tryptone, 0.5 % yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate, 20 mM glucose	Filter sterilise using a 0.2 µm filter
Dulbecco's Modified Eagle Medium (DMEM)	4.5 g/l glucose, 10 % fetal calf serum, 1 % L-glutamine	n/a
Leibovitz's L-15 Medium (L15)	GlutaMAX™, 10 % fetal calf serum	n/a

Table 2.3: Growth media used in this study

2.1.5 Antibiotics

Final concentrations of antibiotics used in this study are listed in Table 2.4.

2.1.6 Preparation of reagents and buffers

Reagents and buffers used in this study are listed in Table 2.5-2.6. All chemicals were purchased from Sigma-Aldrich and Roche unless otherwise stated.

Antibiotic	Final concentration (μg/ml)	Solvent
Kanamycin	50	Water
Gentamicin	30	Water
Chloramphenicol	30	Ethanol
Ampicillin	50	Water

Table 2.4: Final concentrations of antibiotics used in this study

Reagent/buffer	Components	Use
Equilibration buffer	40 mM Tris, 750 mM NaCl, pH 7.5	Protein purification of His tagged proteins
Elution buffer	40 mM Tris, 750 mM sodium chloride, 500 mM of imidazole, pH 7.5	Protein purification of His tagged proteins
Equilibration buffer	PBS	Protein purification of GST tagged proteins
Elution buffer	50 mM Tris, 10 mM reduced glutathione, pH8	Protein purification of GST tagged proteins
Laemmli sample buffer (Sigma)	12.2 mM Tris-HCl (pH 6.8), 20 % glycerol, 4 % SDS, 10 % 2-mercaptoenthanol, 0.004 % w/v bromophenol blue	SDS-PAGE
Coomassie brilliant blue R250 stain (Pierce)		SDS-PAGE
Destain solution	30 % methanol, 10 % acetic acid	SDS-PAGE
Preservation solution	5 % acetic acid, 5 % glycerol	SDS-PAGE
Transfer buffer	37 mM glycine, 48 mM Tris, 0.037% SDS, 20 % methanol, pH 8.3	Western blot
PBST	PBS, 0.01 % v/v Tween 20	Western blot, ELISA
Hepes Buffer (Sigma)	35 mM Hepes in 250 ml deionised water, pH 7.8	PPIase assay
ABTS-citrate buffer	30 mg 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, 11.76 g/L citric acid, 12.49 g/L sodium hydroxide phosphate, pH 4.4	ELISA
Solution A	0.05% (v/v) trifluoroacetic acid (TFA) in water	Mass spectrometry
Solution B	0.05% TFA in water: acetonitrile 10:90 (v/v)	Mass spectrometry

Table 2.5: Buffers and reagents used for protein analysis

Reagent/buffer	Components	Use
TAE buffer	40 mM Tris-acetate, I mM EDTA	Gel electrophoresis
Denaturation solution (Roche)	0.5 M NaOH and 1.5 M NaCl	Southern hybridization
Neutralization solution (Roche)	0.5 M Tris-HCl and 1.5 M NaCl, pH 7.5	Southern hybridization
20× SSC (Sigma)	3 M sodium chloride and 0.3 M sodium citrate, pH 7. With/without 0.1 % SDS	Southern hybridization
Washing buffer (Roche)	10 x maleic acid buffer with 3-5 % Tween20.	Southern hybridization
Blocking solution (Roche)	10 ml liquid block to 90 ml 1 x maleic acid	Southern hybridization
Detection buffer (Roche)	1 M Tris-HCl, 1 M NaCl , pH 9.5,	Southern hybridization
Fixer solution		Southern hybridization
Developer solution		Southern hybridization

Table 2.6: Buffers and regents used for DNA analysis

2

3 2.2 Bioinformatic tools

The websites used to identify PPIases were:

1. http://www.sanger.ac.uk/Projects/B_pseudomallei/

2. http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/

3. http://blast.ncbi.nlm.nih.gov/. For all BLAST searches, only proteins with an e-value below 10^{-4} and sequence identity of more than 20% were retained.

The websites and programmes used to characterise PPIases were:

1. MegAlign (DNAstar Lasergene)

2. http://www.sbg.bio.ic.ac.uk/~phyre/

3. http://expasy.org/tools/pi_tool.html

4. http://www.psort.org/psorb/ (Yu *et al*., 2010)

5. http://www.cbs.dtu.dk/services/SignalP/ (Bendtsen *et al*., 2004)

7. http://www.cbs.dtu.dk/services/TMHMM/ (Sonnhammer *et al.,* 1998).

8. http://www.microbesonline.org (Price *et al.,* 2005).

4 2.3 Molecular biology

2.3.1 Plasmids

The plasmids used in this study are listed in Table 2.7.

2.3.2 Extraction of *B. pseudomallei* and *B. thailandensis* genomic DNA

A single colony was used to inoculate 5 ml LB broth and incubated at 37ºC overnight with agitation. 2 ml of overnight culture was centrifuged at 16 000 *x g* for 1 min and genomic DNA extracted using the Puregene DNA purification kit (Gentra), as per manufacturer's instructions.

2.3.3 Oligonucleotide primers

Oligonucleotide primers were synthesised by MWG and used to amplify DNA by PCR, sequence inserts or screen for plasmids after conjugation. Primers used in this study are listed in Tables 2.8 – 2.10.

Plasmid	Vector type	Antibiotic resistance	Source
pCR Blunt II-TOPO	Cloning	Kanamycin	Invitrogen
pET-15b	Expression	Ampicillin	Novagen
pGEX-4T-1	Expression	Ampicillin	GEhealthcare
pBBR1-MCS2	Complementation	Kanamycin	Kovach *et al*., 1995
pDM4	Suicide vector	Chloramphenicol	Milton *et al.,* 1996

Table 2.7: The plasmids used in this study

Oligonucleotide name	Use	Sequence 5' → 3'	Restriction site	Annealing temperature
BPSL0918.PET.F	PCR	CATATGAGCCTCATCGACCTT	*Nde*I	50ºC
BPSL0918.PET.R	PCR	GGATCCTTACAGAATCCCGATGAT	*Bam*HI	50ºC
BPSL2254.PET.F	PCR	CATATGAAAATTGCAAAAAAC	*Nde*I	50ºC
BPSL2254.PET.R	PCR	GGATCCTCAATGCAGGGTACGCGC	*Bam*HI	50ºC
BPSS1823.PET.F	PCR	CATATGACAGTCGTCACCACC	*Nde*I	50ºC
BPSS1823.PET.R	PCR	GGATCCTCAGACGTCGAGCAGTTC	*Bam*HI	50ºC
BPSL1402.PET.F	PCR	CATATGGCTAACGTTGTTGAA	*Nde*I	50ºC
BPSL1402.PET.R	PCR	GGATCCTTATGCTTGCGCCGTCGC	*Bam*HI	50ºC
BPSL0659.PET.F	PCR	CATATGGTGGCAATGAAGAAAATC	*Nde*I	45ºC
BPSL0659.PET.R	PCR	GGATCCTTACTGGGCGACGGGCAG	*Bam*HI	45ºC
BPSL0659.TRUNC.PET.F	PCR	CATATGCAGGCGTTGCGCGCGCAG	*Nde*I	45ºC
BPSL0659.TRUNC.PET.R	PCR	GGATCCTTACTGGGCGACGGGCAG	*Bam*HI	45ºC
BPSL1418.PGEX.F	PCR	GGATCCATGATCCTGAAATCTCCC	*Bam*HI	50ºC
BPSL1418.PGEX.R	PCR	GAATTCTTACTGAATCTTCGCCTG	*Eco*RI	50ºC

Table 2.8: Oligonucleotide primers used for cloning of genes for protein expression

Oligonucleotide name	Use	Sequence 5' → 3'
M13 forward	Sequencing of inserts in pCR- Blunt II-TOPO	GTAAAACGACGGCCAGTG
M13 reverse	Sequencing of inserts in pCR- Blunt II-TOPO	CAGGAAACAGCTATGAC
T7 forward	Sequencing of inserts in pET15b	GGGCTGGCAAGCCACGTTTGGTG
T7 reverse	Sequencing of inserts in pET15b	CCGGGAGCTGCATGTGTCAGAGG
pDM4.1823.F	Sequencing of inserts in pDM4	GCCGCCGACCTTTACATT
pDM4.1823.R	Sequencing of inserts in pDM4	CCAGTTGGCTGTTGTCGG
pDM4.0576.F	Sequencing of inserts in pDM4	GGTCTGACGAGCATCGAC
pDM4.0576.R	Sequencing of inserts in pDM4	GACATGCGCGTCGGCAAC
pBBR1.F	Sequencing of inserts in pBBR1-MCS2	TGTAGTCGACGCAACGCATAATTGTTGTCG
pBBR1.R	Sequencing of inserts in pBBR1-MCS2	TAGCGTCGACCTCGCCATCGTCCAGAAAAC

Table 2.9: Oligonucleotide primers used for nucleotide sequencing

Oligonucleotide name	Use	Sequence 5' → 3'	Restriction site	Annealing temperature
BPSS1823.PDM4.LFF	PCR	TCTAGAGC CGCCGACC TTTACATT	*Xba*I	50ºC
BPSS1823.PDM4.LFR	PCR	AGATCTGC TCGAATCG AACTTCTG	*Bgl*II	50ºC
BPSS1823.PDM4.RFF	PCR	AGATCTCT CGTGTTCG AAGTCGAA	*Xba*I	55ºC
BPSS1823.PDM4.RFR	PCR	TCTAGACC AGTTGGCT GTTGTCGG	*Bgl*II	55ºC
BTHI0576.PDM4.LFF	PCR	TCTAGAGG TCTGACGA GCATCGAC	*Xba*I	40ºC
BTHI0576.PDM4.LFR	PCR	AGATCTCG TCGCCTGC ACGGTTGC	*Bgl*II	40ºC
BTHI0576.PDM4.RFF	PCR	AGATCTCG CACCTACT CGCAGGAC	*Xba*I	60ºC
BTHI0576.PDM4.RFR	PCR	AGATCTCG CACCTACT CGCAGGAC	*Bgl*II	60ºC
BPSS1823.PBBR1.F	PCR	GAATTCAT GACAGTCG TCACCACC	*Eco*RI	50ºC
BPSS1823.PBBR1.R	PCR	TCTAGATCA GACGTCGA GCAGTTC	*Xba*I	50ºC
KAN.F	Screening complemented mutant	ATGATTGAA CAAGATGG ATTGC		55ºC
KAN.R	Screening complemented mutant	TCAGAAGA ACTCGTCA AGAAGGCG		55ºC

Table 2.10: Oligonucleotide primers for construction of mutant and complementation

2.3.4 Polymerase Chain Reaction (PCR)

A typical PCR using *Pyrococcus furiosus* (Pfu) polymerase (Promega) contained 1 x Pfu polymerase buffer, forward and reverse oligonucleotides (25 pmol/µl), dNTPs (2.5 mM), 0.1 µg genomic DNA, Pfu polymerase (2-3 units/µl), 55 µg dimethyl sulphoxide (DMSO) and distilled water to a final volume of 50µl. The PCR protocol is shown below. Gene specific optimisations were required for some reactions, stated in Table 3.4.

Typical reaction:

95ºC (denaturing) – 10 min	—	1 cycle
95ºC (denaturing) – 1 min	⎫	
50ºC (annealing) – 1 min	⎬	30 cycles
72ºC (extension) – 2 min per kb	⎭	
72ºC (extension) – 10 min	—	1 cycle
4ºC (hold)		

For colony PCR, a single bacterial colony was added to 100 µl dH_2O and boiled for 10 min. 2 µl boilate was used as the template in a standard PCR. To produce a DIG-labelled probe, 2.5 mM DIG NTPs were used instead of dNTPs.

2.3.5 Agarose gel electrophoresis

For a 1% w/v agarose gel, 1 g of agarose was added to 100 ml 1 x TAE. This was heated until the agarose was dissolved and allowed to cool to approximately 50ºC. Ethidium bromide was added to a final concentration of 1 µg/ml before the solution was poured into a gel tray and allowed to set. 1 x loading buffer was added to DNA samples prior to loading and the gel was then

run at 60 - 100 V for 45 min. The gel was visualised using a Syngene UV transilluminator.

2.3.6 DNA gel purification

DNA bands of interest were excised from a low melting point agarose gel and purified using the QIAquick gel extraction kit (Qiagen), as per manufacturer's instruction.

2.3.7 Restriction enzyme digest

A typical digest reaction consisted of 500 ng of prepared DNA, 10 x restriction enzyme digestion buffer, 10 U restriction enzymes and distilled water. The reaction was mixed and incubated at 37ºC (unless stated otherwise) for 2-16 hours. The digest products were subjected to agarose gel electrophoresis to visualise and purify fragments.

2.3.8 Ligation

A typical ligation reaction consisted of 20 ng digested plasmid, 5 x DNA ligase buffer and 5 U T4 DNA ligase (Invitrogen). The reaction was incubated on ice or at 15ºC overnight and then used in transformation reactions.

2.3.9 Heat shock transformation

2 µl ligation reaction was added to 50 µl chemically competent *E.coli* cells (Invitrogen) and incubated on ice for 30 min, 30-45 sec at 42ºC and returned to ice for 1 min. 250-900 µl SOC medium (Invitrogen) was added to the ligation

mix and incubated at 37ºC for 1 h with agitation. The mixture was plated onto LB agar plates containing appropriate antibiotics and incubated at 37ºC overnight.

2.3.10 Production of electrocompetent *E. coli*

A single bacterial colony was used to inoculate 10 ml of LB broth and incubated at 37ºC overnight with agitation. The next day, 2 ml overnight culture was added to 100 ml prewarmed LB broth. This was incubated at 37ºC with agitation until an absorbance reading of 0.5 was reached, using a WPA Colourwave colourimeter (model C07500) at 590 nm. Bacteria were chilled on ice, centrifuged at 10 000 *xg* for 10 min at 4ºC and pellets resuspended in 50 ml ice-cold H_2O. Centrifugation was repeated as before and pellets resuspended in 50 ml ice-cold H_2O. Centrifugation was repeated as before and pellets resuspended in 50 ml ice-cold 10 % glycerol. Centrifugation was repeated as before, pellets resuspended in 600 µl ice-cold 10 % glycerol and 50 µl aliquots stored at – 80ºC.

2.3.11 Electroporation transformation

1-2 µl ligation reaction was added to 50 µl electrocompetent *E.coli* cells and was transformed by electroporation using Gene pulser II electroporator (Bio-Rad) at 2.5 kV and a capacitance of 25 µF. 250 µl-1 ml SOC medium was added to the ligation mixutre and incubated at 37ºC for 1 h with agitation. The mixture was plated onto LB agar plates containing appropriate antibiotics and incubated at 37ºC overnight.

2.3.12 Extraction of plasmid DNA

A single bacterial colony was used to inoculate 5 or 100 ml LB broth with appropriate antibiotic and grown overnight at 37ºC with agitation. The bacteria were centrifuged at 10 000 *x g* for 10 min at 4ºC. The plasmid was purified using the QIAprep Spin Miniprep kit (<20 µg DNA) or HiSpeed Plasmid Midi kit (>20 µg DNA; Qiagen) as per manufacturer's instructions.

2.3.13 Nucleotide sequencing

Sequencing reactions were performed by Lark Technologies Inc., Essex or Geneservice, Cambridge. The reactions were performed using DNA obtained from using either the QIAprep Spin Miniprep kit or HiSpeed Plasmid Midi kit (Qiagen) as per manufacturer's instructions.

2.3.14 Southern hybridization

10 µg digested genomic DNA was separated by gel electrophoresis on a 0.8% agarose gel at 60 V for 4 hours. The gel was incubated for 20 min in 20 ml depurination solution, 20 min in 20 ml denaturation solution and 30 min in 20 ml neutralizing solution (see Table 2.5), all with gentle agitation. DNA was then transferred to a positively charged nylon membrane (Hybond-N+; Amersham Biosciences) by capillary transfer and exposed to UV in the cross linker (Stratalinker). To prepare for hybridization, 20 ml warmed DIG easy Hyb granules (Roche) was added to a hybridisation tube containing the membrane and incubated at 42ºC in a hybridisation oven for 1 h. 5 µl concentrated DIG-labelled probe was added to 50 µl of pre-warmed DIG easy Hyb granules and incubated at 100ºC for 10 min. The probe mixture was added to 20 ml DIG easy

Hyb granules and added to the hybridisation tube, then left on a hybridisation oven at 42ºC overnight. The hybridisation solution was removed, then the membrane was washed twice with 50 ml pre-warmed 2 x SSC, 0.1% SDS on a hybridisation oven at 42ºC for 30 min, then washed twice with 50 ml pre-warmed 0.1 x SSC, 0.1% SDS.

To process the blot, the DIG wash and block buffer set (Roche) was used. The membrane was washed with 1 x washing buffer at room temperature for 3 min with agitation. Blocking solution was added for 1 hour, then replaced with blocking solution containing 5 µl of anti-digoxigenin-AP antibody and incubated for 45 min. The membrane was washed 3 x for 10 min in washing buffer. To equilibrate the membrane, 50 ml 1 x detection buffer was added and incubated for 2 min. The membrane was placed on acetate and sprinkled with CDP-Star (Roche). This was exposed to film (GE healthcare) for 10-30min and the film washed with developer solution (Xograph) for 30 sec and fixer solution (Xograph) for 1 min.

5 2.4 Recombinant protein production

2.4.1 Protein expression

To determine the levels of protein expression over time, a single bacterial colony was used to inoculate 10 ml of LB broth containing appropriate antibiotics and incubated at 37ºC overnight with agitation. The next day, the overnight culture was diluted 1:10 with prewarmed LB broth containing appropriate antibiotics. This was incubated at 37ºC with agitation until an absorbance reading of 0.4-0.6 was reached, using a WPA Colourwave

colourimeter (model C07500) at 600 nm. IPTG was then added to a final concentration of 1 mM and the cultures incubated further at 37ºC with agitation. At 0, 2, 3 or 2 4h post induction, 1ml bacteria was harvested by centrifugation at 10 000 *x g* for 5 min and pellets resuspended in 100 µl PBS.

To determine solubility of the protein, cultures were induced and incubated at 37ºC with agitation for a period of time optimised for expression of the protein. 5 ml bacterial culture was harvested by centrifugation at 8 000 *x g* for 10 min and the pellet resuspended in 5 ml PBS. Cells were disrupted by a Soniprep 150 sonicator (MSE) 4 times for 15 sec and cell debris pelleted at 8000 *x g* for 30 min at 4ºC. Supernatants were retained and pellets resuspended in 5 ml PBS.

2.4.2 Large scale protein expression

A single bacterial colony was used to inoculate 50 ml of LB broth containing appropriate antibiotics and incubated at 37ºC overnight with agitation. The next day, 10 ml overnight culture was added to 400 ml prewarmed LB broth containing appropriate antibiotics. This was incubated at 37ºC with agitation until an absorbance reading of 0.4-0.6 was reached, using a WPA Colourwave colourimeter (model C07500) at 600 nm. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM and the cultures incubated further at 37ºC with agitation for a period of time optimised for expression of the protein. Bacteria were harvested by centrifugation at 8000 *x g* for 15 min at 4ºC and the supernatant discarded. Pellets were combined and stored at -20ºC until use. To extract the protein, the pellet was resuspended in 20 ml PBS, 100 mg/ml DNase I and an EDTA-free protease inhibitor tablet (Roche). Cells were disrupted using a Soniprep 150 sonicator (MSE) 4 times for

30 sec and cell debris pelleted at 8000 *x g* for 30 min at 4ºC. The supernatant was filtered through a 0.2 µm filter and loaded onto the purification column.

2.4.3 His-tagged protein purification

Proteins with a His_6 tag were purified using a 1 ml Histrap FF column (GE Healthcare) and a Fast Protein Liquid Chromatography system (GE Healthcare). The column was equilibrated with 40 mM Tris, 750 mM NaCl, pH 7.5; the sample loaded onto the column and washed using equilibration buffer. Fractions were eluted off the column with steps of 50 mM, 100 mM, 250 mM and 500 mM imidazole. Proteins were separated by SDS-PAGE and visualised by staining with Coomassie brilliant blue R250.

2.4.4 GST-tagged protein purification

Proteins with a GST-tag were purified using a 1 ml GSTrap FF column (GE Healthcare) and thrombin cleavage of the GST tag carried out on the column. The column was equilibrated with PBS, the sample loaded onto the column and washed using equilibration buffer. 80 U thrombin mixed with 920 µl PBS was loaded onto the column and incubated at room temperature overnight. The column was washed manually using a syringe with 1 ml equilibration buffer then the fractions eluted off the column. Proteins were separated by SDS-PAGE and visualised by staining with Coomassie brilliant blue R250.

2.4.5 Dialysis

Protein fractions were dialysed against PBS in a 10 000 MW Slide-A-Lyzer (Pierce) overnight at 4ºC. Glycerol was then added at a final concentration of 5% to the protein solution and stored at -80ºC.

2.4.6 Bicinchoninic acid assay

Protein concentrations were determined using a biochoninic acid (BCA) assay (Pierce). BCA solution was prepared by mixing 50 parts reagent A with 1 part reagent B. 50 µl either protein or BSA standard was added to 1 ml BCA solution and incubated at 37ºC for 30 min. The absorbance was read at 562 nm using a Ultraspec 4000 spectrophotometer (GE Healthcare) and protein concentration determined against a BSA standard curve.

2.4.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

10 µl bacteria or protein sample was mixed with 15 µl Laemmli sample buffer (Sigma) and boiled at 100ºC for 10 min. Samples were applied to 8-25% PhastGels (GE Healthcare) and run on a PhastSystem (GE Healthcare) at 250 V for 20 min.

2.4.8 Gel staining or transfer to nitrocellulose membrane

Phastgels were stained with 10 ml Coomassie brilliant blue R250 stain (Pierce) for 1 hour with agitation, followed by destaining solution for 1 h with agitation. Gels were preserved using preservation solution overnight with agitation. Replicate Phastgels were transferred to nitrocellulose membrane using the

PhastTransfer system (GE Healthcare) at 25 mA for 30 min, ready for Western blotting.

2.4.9 Western blot

The nitrocellulose membrane was blocked with 5% skimmed milk in PBS overnight with agitation. The membrane was incubated with 1:1000 dilution mouse anti-His antibody (GE Healthcare) or 1:1000 dilution mouse sera raised against recombinant protein for 1 h with agitation, then washed with 5% skimmed milk solution 3 x for 10 min each. The membrane was incubated with 1:1000 HRP-conjugated goat anti-mouse secondary antibody (GE Healthcare) for 1 h with agitation, then washed with 5% skimmed milk solution 3 x for 10 min each. The membrane was washed twice with PBS + 0.1% Tween 20 (PBST). The blot was developed using DAB (Sigma) following manufacturer's instructions.

2.4.10 Mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS) analyses were carried out using a Quattro II™ tandem quadruple mass spectrometer equipped with a Z-spray™ electrospray ionisation source (Micromass UK Ltd., Manchester, UK) together with an HP1100 Series LC system (Agilent Technologies UK Ltd., Stockport, Cheshire, UK). The HP1100 system was equipped with a 150 x 1 mm Jupiter C4, 300 Å, 5 μm column (Phenomenex, Macclesfield, UK). All data acquisition, mass spectrometer control and post run processing were carried out using MassLynx and MaxEnt software (Micromass).

Samples were diluted with Solution A to give a loading of approximately 2 μg. The elution gradient used was 25% Solution B for 1 min followed by a linear increase to 100% Solution B over 44 min. A 3 min wash at 100% Solution B was then followed by a return to initial conditions over 2 min. A 50 μl injection loop was used. Acquisition was commenced upon injection and was performed in continuum mode. Capillary voltage was 3.3 kV, desolvation temperature 250°C and source block temperature 80°C. Each sample was typically analysed over two different scan ranges, one broad (*m/z* 700 – 2000) and one narrow (dependant on the observed charge state envelope). All analyses were performed at 5 sec per scan. For BPSS1823 cone voltage was ramped from 48 V at *m/z* 700 to 87 V at *m/z* 2000. For BPSL0918 cone voltage was fixed at 45 V.

2.4.11 Circular dichroism

UV and CD spectra were determined at room temperature using a Chirascan spectrophotometer (Applied Photophysics Ltd.) in the ranges of 400-230 nm and 260-180 nm. Buffer baseline correction was applied and light scattering correction was performed using an in-house programme implemented in the GRAM/31AI software. Protein secondary structure contents were assessed using the Principle Component Regression method (M.K. Malik, PhD Thesis, University of London, 1997).

2.4.12 Crystallisation and structure solution

All crystals were grown using the microbatch method, prepared using an Oryx6 crystallization robot (Douglas Instruments). Recombinant BPSS1823 at 14 mg/ml was mixed with an equal volume of 2.2 M $(NH_4)_2SO_4$, 0.1 M Bis-Tris pH 5.5, and grown at 20°C. Prior to flash-freezing in liquid nitrogen, crystals were soaked for 30-60 seconds in a cryoprotectant solution of 1.1 M $(NH_4)_2SO_4$, 0.1 M Bis-Tris pH 5.5, 30 % (v/v) glycerol. Single wavelength X-ray diffraction data were collected at a wavelength of 0.861 Å. Data was processed using iMOSFLM version 1.0.3 (Leslie, 1992) and SCALA (Evans, 2005). Model building and refinement of the structures was performed using Coot version 0.6.1 (Emsley, 2010) and PHENIX version 1.6.1 (Adams, 2002). Structures were validated using PHENIX, Coot and MOLPROBITY (Davis, 2007). The Ramachandran plot for the final model showed 100% of residues in the favoured region. Structural images were prepared using the PyMOL molecular graphics system (Schrödinger).

6 2.5 Enzymology

2.5.1 Protease coupled PPIase assay

10 - 100 nM recombinant protein was incubated for 6 min at 6ºC in 1.2 ml 35 mM HEPES buffer, pH 7.8 with 10 µl succinyl-ala-xaa-pro-phe-*p*-nitroanilide (xaa=pro, leu or ala) (10 mg/ml; Bachem). 250 µl α-chymotrypsin (5 mg/ml) was added to the cuvette and mixed. Hydrolysis of the substrate was measured at 390 nm in a Shimadzu 1800 UV/Vis spectrophotometer at 1 sec intervals until there was no further change in absorbance.

2.5.2 Protease free PPIase assay

400 nM recombinant protein was incubated for 6 min at 6ºC in 1.5 ml 35 mM HEPES buffer, pH 7.8. The synthetic substrate succinyl-ala-phe-pro-phe-*p*-nitroanilide was dissolved in 0.48 M LiCl/anhydrous trifluroethanol (25 mg/ml). This substrate was added to the cuvette and mixed. The solvent jump was measured at 330 nm in a Shimadzu 1800 UV/Vis spectrophotometer for 300 s at 5 s intervals.

2.5.3 Inhibition by rapamycin or cycloheximide-N-ethylethanoate

Recombinant BPSS1823 was incubated with 100 pM – 1 pM rapamycin or 5 mM – 0.1 mM cycloheximide-N-ethylethanoate for 6 min at 6ºC in 1.2 ml 35 mM HEPES buffer, pH 7.8 with 10 µl succinyl-ala-phe-pro-phe-*p*-nitroanilide (10 mg/ml). 250 µl α-chymotrypsin (5 mg/ml) was added to the cuvette and mixed. Hydrolysis of the substrate was measured at 390 nm in a Shimadzu 1800 UV/Vis spectrophotometer for 100 sec at 5 sec intervals.

2.5.4 Kinetic analysis

All data fitting and statistical analyses were performed using SPSS v16.0 (IBM)

The pseudo first order rate constant was calculated using equation 1; data from 10-50 sec (which were always after the lag phase, and before substrate became limiting) were taken, and k_{obs} calculated by linear regression.

(1) $$\ln[A_\infty - A_t] = -k_{obs}t + \ln[A_\infty - A_0]$$

The enzymatic rate was determined by comparing the observed rate to the uncatalysed rate (equation 2).

(2) $$k_{enz} = k_{obs} - k_{uncat}$$

The specificity constant k_{cat}/K_M for the enzyme was calculated using equation 3 (Harrison and Stein, 1990): data were taken using 1 nM, 5 nM or 10 nM BPSS1823, and were fit using linear regression.

(3) $$\frac{k_{cat}}{K_M} = \frac{k_{enz}}{[PPIase]}$$

Data for inhibitor assays were fit to equation 4 (Williams and Morrison, 1979) using least squares non-linear fitting. [E] was treated as a constant (10 nM); v_0 and K_I^{app} were fit, using initial estimates based on the raw data.

(4) $$v = v_0 \frac{[E]-[I]-K+\sqrt{([E]-[I]-K)^2+4[E][K]}}{2[E]}$$

7 2.6 Mutant construction and complementation

2.6.1 Conjugation

Single colonies of *E.coli* S17 λpir containing pDM4 construct and *B. pseudomallei* K96243 or *B. thailandensis* E264 were used to inoculate 20 ml LB broth with appropriate antibiotics and grown at 37ºC overnight with agitation. 1 ml of each bacterial strain were centrifuged at 3000 *x g* for 2 min at RT and pellets resuspended in 500 µl PBS. 10 µl of each bacterial strain were spotted onto a sterile nitrocellulose membrane on an LB agar plate and incubated at 37ºC overnight. Filters were vortexed in 1 ml PBS, 100 µl conjugation mixture was plated onto LB agar plates containing appropriate antibiotics and incubated at 37ºC for 24 – 72 h. Integrants were confirmed by colony PCR.

2.6.2 Sucrose selection

A single integrant colony was used to inoculate 5 ml LB broth and incubated at 37ºC overnight with agitation. The overnight culture was diluted until an absorbance reading of 0.4-0.6 was reached, using a WPA Colourwave colourimeter (model C07500) at 590 nm then serially diluted in PBS. Appropriate dilutions were plated onto agar containing 10% sucrose and no salt and incubated at 24ºC for up to a week. Colonies were tested for chloramphenicol sensitivity and mutants confirmed by colony PCR and southern hybridization.

2.6.3 Complementation

The PBBR1-MCS construct was conjugated into *B. pseudomallei* K96243 Δ*amrA* Δ*BPSS1823* as described in section 2.6.1, alongside the helper strain *E. coli* HB101 (pRK2013). Conjugates were screened by colony PCR. The complemented mutant strain was grown in LB broth containing appropriate antibiotics and 1 mM IPTG.

8 2.7 *In vitro* assays

2.7.1 Growth curves

A single bacterial colony was used to inoculate 10 ml LB broth and incubated at 37ºC overnight with agitation. 2 ml overnight culture was added to 100 ml prewarmed LB broth and incubated at 37ºC for up to 216 h. At timepoints, 1 ml bacterial culture was removed and absorbance read using a WPA Colourwave

colourimeter (model C07500) at 590 nm. Alternatively, the bacterial culture was serially diluted, plated onto LB agar and incubated at 37ºC overnight.

2.7.2 Antimicrobial sensitivity assay

A single bacterial colony was used to inoculate 10 ml LB broth and incubated at 37ºC overnight with agitation. The overnight culture was adjusted to an absorbance of 0.01 using a WPA Colourwave colourimeter (model C07500) at 590 nm and grown for 2 h at 37ºC with agitation. 256 µg/ml HCl, NaCl, H_2O_2 or gentamicin were added to a 96-well plate and serially diluted to give final concentrations of 64 - 0.06 µg/ml. 100 µl adjusted bacterial culture (approximately 2 x 10^5 cfu/ml) was added and the plate was incubated at 37ºC overnight. Absorbance was read using a Thermo Multiskan EX reader at 620 nm.

2.7.3 Time kill assay

A single bacterial colony was used to inoculate 10 ml LB broth and incubated at 37ºC overnight with agitation. The overnight culture was adjusted to an absorbance of 0.01 using a WPA Colourwave colourimeter (model C07500) at 590 nm and grown for 2 h at 37ºC with agitation. 100 µl adjusted bacterial culture was added to 10 ml LB broth adjusted to pH 1 – pH 7 and incubated at 37ºC overnight with agitation. At timepoints, 100 µl bacterial culture was removed and serially diluted, plated onto LB agar and incubated at 37ºC overnight.

Alternatively, 100 µl adjusted bacterial culture was added to 10 ml LB broth with 256 µg/ml polymyxin B and incubated at 37ºC overnight with agitation. At 24 h post inoculation, 1 ml bacterial culture was removed and absorbance read using a WPA Colourwave colourimeter (model C07500) at 590 nm.

2.7.4 Motility assay

A single bacterial colony was used to inoculate 10 ml LB broth and incubated at 37ºC overnight with agitation. 1 µl overnight culture was stabbed into 0.3% motility agar using a sterile inoculating loop and the plates incubated at 37ºC overnight. Bacterial spread was measured using a Scienceware® vernier calliper (Sigma).

2.7.5 Protease assay

A single bacterial colony was used to inoculate 10 ml LB broth and incubated at 37ºC overnight with agitation. 2 ml overnight culture was added to 100 ml prewarmed LB broth and incubated at 37ºC for 24 h. At timepoints, 100 µl bacterial culture was removed and serially diluted, plated onto LB agar and incubated at 37ºC overnight. Alternatively, 1 ml bacterial culture was removed and pelleted at 15 000 *x g* for 5 min. 100 µl supernatant was added to 100 µl azocasein (5 mg/ml; Sigma) and incubated at 37ºC for 1h. The reaction was stopped with 10% trichloroacetic acid (Sigma) and non-hydrolysed azocasesin pelleted at 10 000 *x g* for 15 min. The supernatant was added to 500 mM NaOH and read using a WPA Colourwave colourimeter (model C07500) at 440 nm.

9 2.8 Tissue culture

2.8.1 Invasion assay

J774A.1 or A549 cells at a concentration of 4 x 10^5 cells/ml were seeded in DMEM onto a 24-well plate and incubated at 37ºC with 5% CO_2 for approximately 16 h. A single bacterial colony was used to inoculate 10 ml LB broth and incubated at 37ºC overnight with agitation. The overnight culture was diluted in L15 medium until an absorbance reading of 0.4-0.6 was reached, using a WPA Colourwave colourimeter (model C07500) at 590 nm then serially diluted in L15 medium. 1 ml bacterial culture was added to the cells at an MOI of 1:1 or 1:10 - 100 and incubated at 37ºC for 30 min or 1 h for J774s and A549s, respectively. Infected cells were incubated with L15 medium containing 30 µg/ml gentamicin or 1 mg/ml kanamycin for 30 min at 37ºC. Cells were then incubated with 10 µg/ml gentamicin or 250 µg/ml kanamycin at 37ºC for 24 h. At various timepoints, the cells were lysed with 1 ml dH_2O, serially diluted in PBS and plated onto LB agar and incubated at 37ºC overnight.

2.8.2 Adhesion assay

A549 cells and bacteria were prepared as per section 2.8.1. Cytochalasin-D (Sigma) was added to approximately 1 x 10^6 cells at a final concentration of 1 µg/ml and incubated at 37ºC with 5% CO_2 for 30 min. Cytochalasin-D was also added to approximately 1 x 10^7 cfu/ml bacteria at a final concentration of 1 µg/ml. 1 ml treated bacteria was added to the pretreated cells at an MOI of 1:10 and incubated at 37ºC for 1h. Cells were then washed 3 x times with warm PBS to remove non-adhered bacteria or incubated with L15 medium containing

30 µg/ml gentamicin. Cells were lysed with 1 ml dH_2O, serially diluted in PBS and plated onto LB agar and incubated at 37ºC overnight.

10 2.9 *In vivo* studies

2.9.1 *Galleria mellonella* challenge

Groups of 10 *G. mellonella* larvae (Livefood UK Ltd.) weighing 0.2 - 0.3 grams were injected into the right foremost proleg with 10 µl bacteria. Control groups were either injected with 10 µl PBS or nothing. Larvae were incubated at 37ºC, monitored over 4 days and scored as dead when they changed in colour from cream to black; ceased to move and failed to respond to touch.

2.9.2 Vaccine study

Groups of 6 female BALB/c age-matched mice (Charles River) were housed together with free access to food and water and subjected to a 12 h light/dark cycle. Mice were immunized intraperitoneally with 10 µg recombinant protein mixed 1:1 with SAS adjuvant (Sigma) on days 0, 14 and 28. One group was immunized with adjuvant only. All studies involving animals were carried out according to the requirements of the Animal (Scientific Procedures) Act 1986. On day 56, animals were challenged with approximately 1 x 10^4 cfu/ml *B. pseudomallei* K96243 under biosafety level III conditions within an isolator compliant with British Standard BS5726. The animals were monitored for signs of disease for 5 weeks and culled at predetermined humane end points.

2.9.3 Production of polyclonal antibodies

BALB/c mice were immunized intraperitoneally with 10 µg recombinant protein mixed 1:1 with SAS adjuvant (Sigma) on days 0, 14 and 28. On day 56, the mice were culled and blood collected by cardiac puncture. Following overnight incubation at 4ºC, the blood was centrifuged at 10 000 *x g,* the sera removed and stored at -20ºC.

2.9.4 Enzyme linked immunosorbant assay (ELISA)

96-well plates were coated with either 10 µg purified protein, heatkilled *B. pseudomallei* or SAS adjuvant (Sigma) and incubated at 4ºC overnight. Plates were blocked with 2% skimmed milk in PBS at 37ºC for 1 h then washed 5 x with PBST using an ELx405 auto plate washer. Plates were then incubated with sera (1:100 dilution, unless otherwise stated) in 2 % skimmed milk at 37ºC for 1.5 h. The plate was washed 5 x with PBST and incubated with HRP-conjugated anti-mouse or anti-rabbit IgG antibody (1:1000 dilution; GE Healthcare) in 2% skimmed milk at 37ºC for 45 min. The plate was washed 5 x with PBST and incubated with ABTS-citrate buffer plus 0.1 µl/ml hydrogen peroxide at 37ºC for 45 min. The reaction was then quantified using a Thermo Multiskan EX reader at an absorbance of 414 nm.

2.9.5 Determination of the Median Lethal Dose (MLD)

Groups of 6 female BALB/c age-matched mice (Charles River) were challenged intraperitoneally with approximately 1 x 10^1 – 1 x 10^6 cfu *B. pseudomallei*. The animals were monitored for signs of disease for 5 weeks and culled at predetermined humane end points. At the end of the experiment, all survivors

were culled and spleens aseptically removed. Spleens were homogenized in 1 ml PBS, serially diluted in PBS, plated on LB agar and incubated at 37ºC overnight. The MLD was determined by the method of Reed and Muench (Reed and Muench, 1938).

11 2.10 Statistical analysis

For PPIase activity characterisation, a paired student's t-test was used to determine confidence intervals (CI) for each group. For cell invasion and pH assays, a 2way ANOVA and Bonferroni's post test were used to compare bacterial numbers. For *B. pseudomallei* motility assays, a 1way ANOVA and Bonferroni's Multiple Comparison Test were used to compare bacterial spread. For *in vivo* mutant characterisation, a Log-rank (Mantel-Cox) Test was used for comparison of survival curves. For *B. thailandensis* motility and polymyxin B growth assays, a Mann-Whitney or unpaired t-test was used to compare bacterial growth.

Significances were referred as follows: * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$. Statistical analyses were performed using either GraphPad Prism version 4.0 or Microsoft Office Excel 2003.

Chapter 3 – Identification and characterisation of FK506-binding proteins and parvulins in *B. pseudomallei*

12 Introduction

Peptidyl-prolyl *cis-trans* isomerases (PPIases) are a highly conserved group of proteins that have a range of functions in eukaryotes and prokaryotes (reviewed by Fischer and Aumuller, 2003). The superfamily can be categorised into three groups: cyclophilins, FK506-binding proteins (FKBPs) and parvulins. There is no significant sequence homology between the three groups of PPIases and they differ in both active site architecture and structure of corresponding ligands. However, all PPIases exhibit substrate specificity for proline residues and can catalyse the *cis-trans* conformational change around prolyl bonds (Galat *et al.,* 2003).

In some pathogens, members of the FKBP and parvulin groups have been shown to be important for full virulence (Cianciotto *et al.,* 1989; Lundemose *et al*., 1993; Moro *et al.,* 1995; Horne *et al.,* 1997; Syndenham *et al.,* 2000; Justice *et al*., 2005; Leuzzi *et al.,* 2005). Macrophage infectivity potentiator (Mip) is a bacterial FKBP which possesses PPIase activity and is inhibitable by the immunosuppressant drugs FK506 or rapamycin (Fischer *et al*., 1992). Survival protein A (SurA) is a bacterial parvulin which possesses PPIase and chaperone activity (Rouvière and Gross*,* 1996; Behrens *et al.,* 2001). In addition, immunisation of mice with recombinant SurA affords protection against *Brucella abortus* challenge (Delpino *et al.,* 2007).

The aim of this chapter is to identify and characterise putative FKBPs and parvulins encoded by *B. pseudomallei*. Bioinformatic tools will be used for the identification of homologous genes and for characterisation of the protein sequences. The encoding genes will be cloned into an expression vector to allow purification of recombinant fusion proteins. These proteins will be tested for PPIase activity and their protective efficacy determined in a BALB/c mouse model of *B. pseudomallei* infection.

3.2 Results

3.2.1 Identification and characterisation of FKBPs and parvulins in *Burkholderia pseudomallei* using bioinformatic tools

3.2.1.1. Identification of FKBPs and parvulins in *B. pseudomallei*

Putative FKBPs and parvulins encoded by *B. pseudomallei* K96243 were identified using three bioinformatic approaches. Initially the genome of *B. pseudomallei* K96243 was scrutinised for genes annotated as 'FKBP', 'parvulin' or 'PPIase' (http://www.sanger.ac.uk). Six genes were identified with these annotations. Next, proteins containing FKBP-like domains were identified by scanning *B. pseudomallei* K96243 protein sequences against a collection of hidden Markov models (http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/). Nine PPIases from *B. pseudomallei* were predicted to contain an FKBP-like domain. In addition, four proteins were predicted as having multiple domains, including a 'Trigger factor/SurA peptide binding domain-like'. Finally, the amino acid sequence of a well characterised FKBP (FKBP12 from *H. sapiens*) and parvulin (Par 10 from *E. coli*) were used to search against a non-redundant NCBI database (http://blast.ncbi.nlm.nih.gov/), leading to the identification of eight proteins from *B. pseudomallei.* For each query protein, only the subject proteins with an e-value below 10^{-4} and sequence identity of more than 20% were retained. The genes identified using all bioinformatic approaches were amalgamated resulting in a list of 9 putative FKBPs and parvulins in *B. pseudomallei* (Table 3.1). Of these, six encode FKBP-like proteins and three encode parvulin-like proteins.

Gene name	Genome annotation	Predicted domains	Sequence identity to FKBP12	Sequence identity to Par10
BPSL0918	Putative FkpB-type peptidyl-prolyl *cis-trans* isomerase	8 - 150 aa - FKBP-like domain	25.4%	X
BPSL2254	Putative FkbP-type peptidyl-prolyl *cis-trans* isomerase	2 - 143 aa - FKBP-like domain	X	X
BPSS1823	Peptidyl-prolyl *cis-trans* isomerase	3 – 133 aa - FKBP-like domain	56.7%	X
BPSL1402	Trigger factor	1 – 128 aa - Trigger factor ribosome-binding domain 104 – 253 - FKBP-like domain 260 – 442 aa - Trigger factor/SurA peptide binding domain-like	22.7%	X
BPSL0659	Peptidyl-prolyl *cis-trans* isomerase SurA	41 – 182 aa - Trigger factor/SurA peptide binding domain-like 190 – 290 aa FKBP-like - domain 305 – 405 aa FKBP-like domain	X	37.8%
BPSL1418	Putative exported isomerase	25 – 80 aa - Trigger factor/SurA peptide binding domain-like 128 – 224 aa - FKBP-like domain	X	44.6%
BPSL1410	Putative peptidyl-prolyl *cis-trans* isomerase	42 – 173 and 207 – 239 aa - Trigger factor/SurA peptide binding domain-like 262 – 374 aa - FKBP-like domain	X	36.3%
BPSS1383	Rotamase	112 – 208 aa - FKBP-like domain	X	28.1%
BPSS1155	Rotamase/ peptidyl-prolyl *cis-trans* isomerase family protein	106 – 213 aa - FKBP-like domain	X	38.1%

Table 3.1. Putative FKBPs and parvulins encoded by *B. pseudomallei* K96243. Identified by annotation, conserved domains and amino acid identity to FKBP12 from *H. sapiens* and Par 10 from *E. coli.* X = < 20% identity and e-value < 10^{-4}

3.2.1.2 Conservation of *B. pseudomallei* FKBPs and parvulins in other *Burkholderia* species

Following identification of putative FKBPs and parvulins encoded by *B. pseudomallei,* their amino acid sequences were used to search for homologues in other *Burkholderia* species (http://blast.ncbi.nlm.nih.gov/). Homologues of some of these proteins were identified in *B. mallei* ATCC 23344 and *B. thailandensis* E264 (Table 3.2). Of these, eight putative proteins were highly conserved in *B. thailandensis* (identity of >80%) and five putative proteins were highly conserved in *B. mallei* (identity of >90%). *B. mallei* proteins with 29% and 30% identity have conserved FKBP-like domains. *B. mallei* does not encode a BPSS1823 homologue and neither *B. mallei* nor *B. thailandensis* encodes a BPSS1383 homologue.

3.2.1.3 Bioinformatic characterisation of PPIases encoded by *B. pseudomallei*

The theoretical molecular weight (MW); subcellular location; signal peptides and transmembrane helices of PPIases from *B. pseudomallei* was predicted using the ExPASY proteomics server (http://expasy.org/tools/pi_tool.html; http://www.psort.org/psorb/; http://www.cbs.dtu.dk/services/SignalP/; http://www.cbs.dtu.dk/services/TMHMM/; summarised in Table 3.3). The MWs of the mature proteins ranged in size from approximately 12 kDa to 67 kDa. PPIases were predicted to be located in most subcellular regions of the cell and proteins predicted to be located outside of the cytoplasm have a signal peptide (BPSL0659, BPSL1418 and BPSL1410). In addition, BPSL0659 and BPSL1410 were also predicted to have a transmembrane helix.

***B. pseudomallei* K96243 gene name**	**Putative homologue in *B. thailandensis* E264 (% identity)**	**Putative homologue in *B. mallei* ATCC 23344 (% identity)**
BPSL0918	BTHI0782 (97%)	BMA2229 (100%)
BPSL2254	BTHI1911 (98%)	BMA2229 (29%)
BPSS1823	BTHII0554 (98%)	N.I.
BPSL1402	BTHI2117 (96%)	BMA1466 (99%)
BPSL0659	BTHI0576 (97%)	BMA0209 (99%)
BPSL1418	BTHI2136 (98%)	BMA1444 (99%)
BPSL1410	BTHI2129 (95%)	BMA1453 (99%)
BPSS1383	N.I.	N.I.
BPSS1155	BTHI1253 (87%)	BMA0209 (30%)

Table 3.2. Putative homologues of *B. pseudomallei* FKBPs and parvulins encoded by *B. mallei* ATCC23344 and *B. thailandensis* E264. Identified using NCBI BLAST P. N.I. = not identified.

Protein name	Theoretical MW (Da)	Predicted subcellular location	Signal peptide?	Transmembrane domain?
BPSL0918	16076	Cytoplasm	N.I.	N.I.
BPSL2254	20086	Cytoplasm	N.I.	N.I.
BPSS1823	11931	Periplasm	N.I.	N.I.
BPSL1402	49768	Cytoplasm	N.I.	N.I.
BPSL0659	49088	Periplasm	Yes – cleavage at 29-30 aa	Yes – 7-29 aa
BPSL1418	26162	Periplasm	Yes – cleavage at 22-23 aa	N.I.
BPSL1410	66062	Cytoplasmic membrane/extracellular	Yes – cleavage at 25-26 aa	Yes – 13-32 aa
BPSS1383	17186	Unknown	N.I.	N.I.
BPSS1155	28655	Cytoplasm	N.I.	N.I.

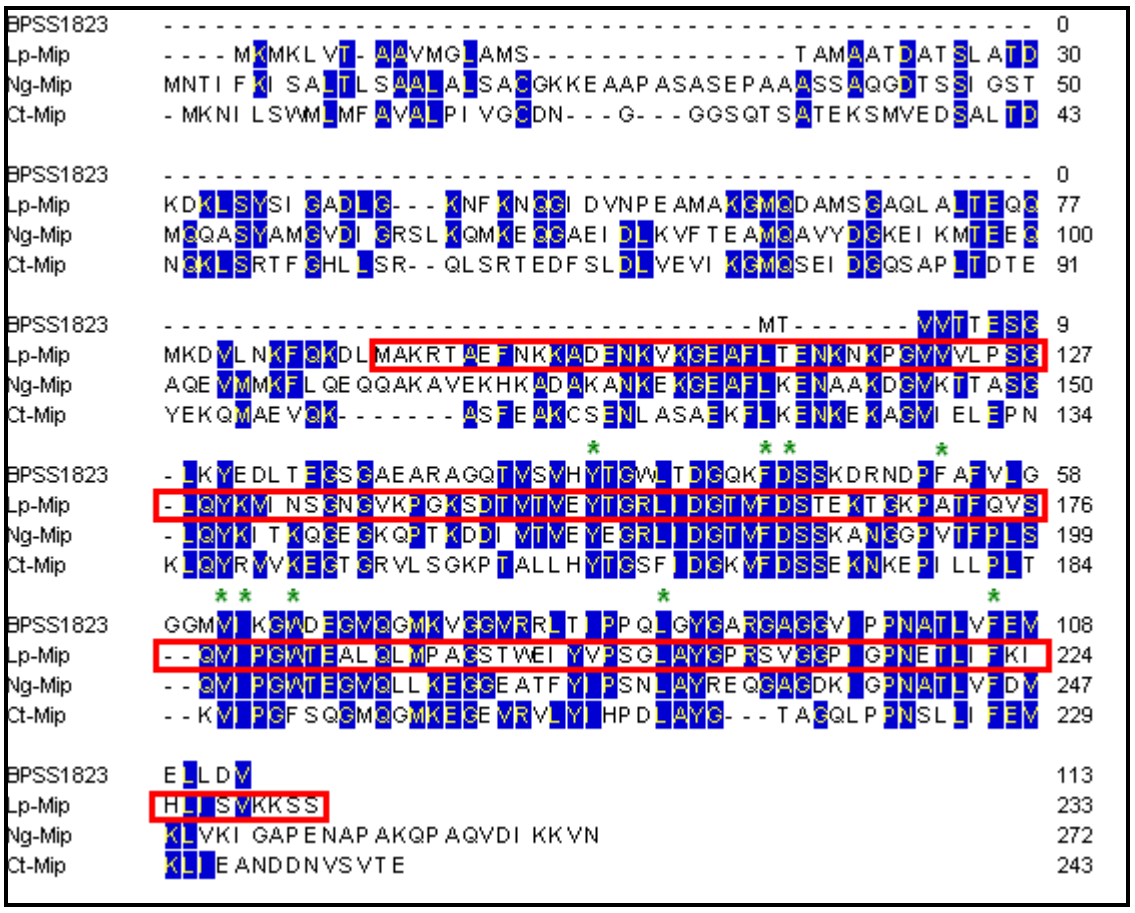
Table 3.3. Predicted mature molecular weights (MW); subcellular locations; signal peptides and transmembrane domains of putative PPIases from *B. pseudomallei.* Identified using the compute pI/MW tool; PSORTb tool; Signal P tool or TMHMM server from the ExPASY proteomics server. N.I. = not identified

3.2.1.4 Identification of a Mip homologue in *B. pseudomallei*

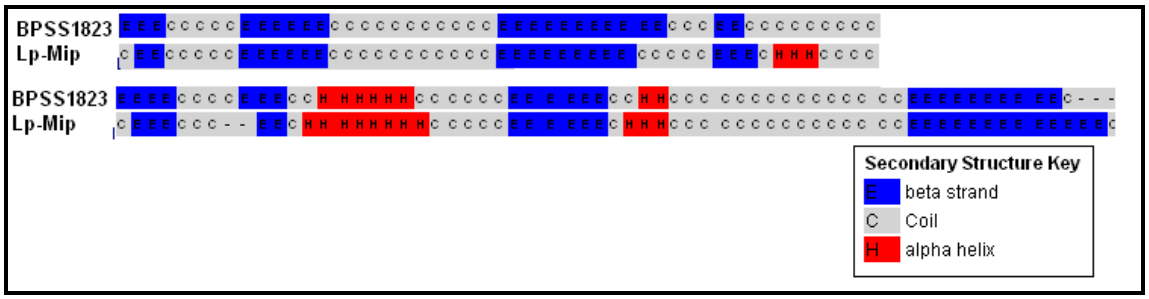
BPSS1823 from *B. pseudomallei* has 40%, 45% and 42% amino acid sequence identity to *L. pneumophila* Mip (Lp-Mip; YP_094827.1), *Neisseria gonorrhoeae* Mip (Ng-Mip; YP_208296.1) and *Chlamydia trachomatis* Mip (Ct-Mip; YP_001653886.1) respectively.

A multiple sequence alignment by Clustal W method of BPSS1823 against Lp-Mip, Ng-Mip and Ct-Mip is shown in Figure 3.1, A. BPSS1823 does not contain a putative N-terminal dimerization domain but has high homology to the C-terminal PPIase domain in other Mips, boxed in red in Figure 3.1, A. In addition, BPSS1823 contains residues required for PPIase activity in Lp-Mip and human FKBP12, highlighted by asterisks in Figure 3.1, A (Helbig *et al.,* 2003; Ikura and Ito, 2007; Ceymann *et al.,* 2008; Löw *et al*., 2010).

Further evidence that BPSS1823 encodes a putative Mip homologue was obtained by investigating the secondary structure using the Protein Homology/analogY Recognition Engine (PHYRE; http://www.sbg.bio.ic.ac.uk/phyre/; Kelley and Sternberg *et al.,* 2009). The predicted secondary structure of BPSS1823 was shown to have strong homology to the structure of the C-terminal domain of Lp-Mip, with a similar arrangement of β strands, coils and α- helices (Figure 3.1, B).



A



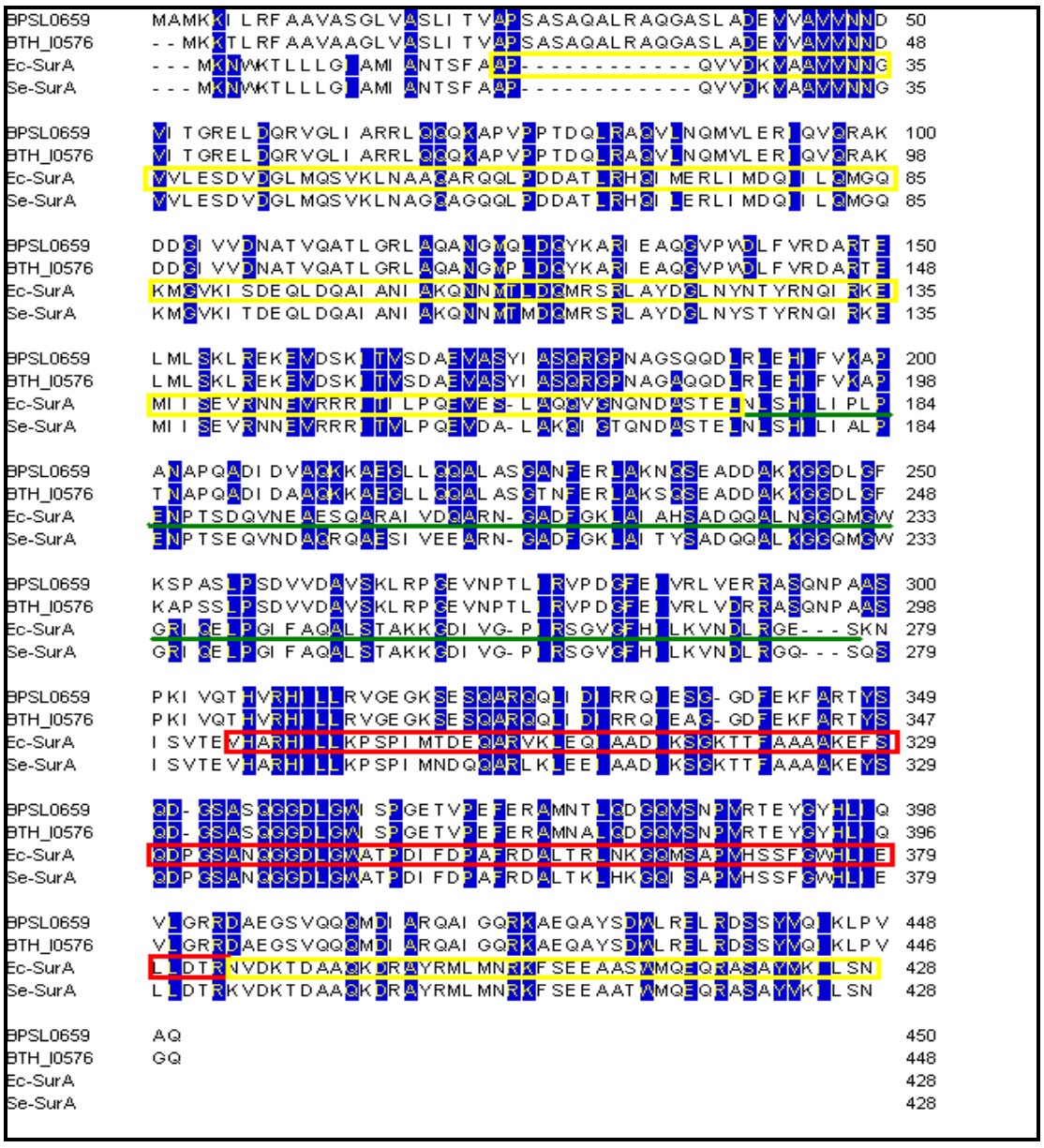
B

Figure 3.1. A) Multiple sequence alignment of *B. pseudomallei* BPSS1823, *L. pneumophila* (Lp), *N. gonorrhoeae* (Ng) and *C. trachomatis* (Ct) Mips. Identical amino acids are shaded in blue and the PPIase domain of Lp-Mip is boxed in red. Residues shown to be required for PPIase activity in hFKBP12 and Lp-Mip are highlighted by asterisks. B) Alignment of the predicted secondary structure of BPSS1823 against the predicted secondary structure of *L. pneumophilia* Mip (Lp-Mip).

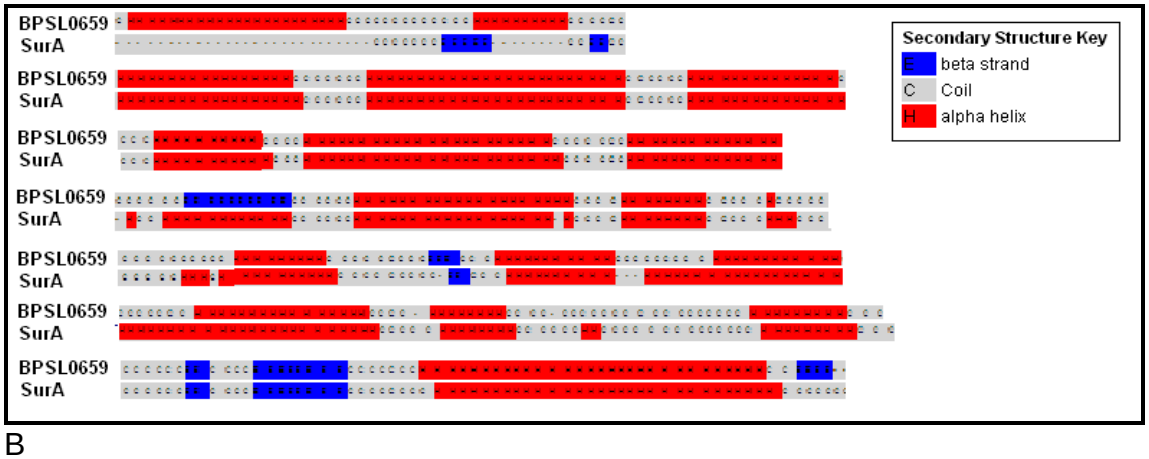
3.2.1.5 Identification of SurA homologues in *B. pseudomallei* and *B. thailandensis*

SurA homologues were identified in *B. pseudomallei* and *B. thailandensis* using the protein sequence of *E. coli* and *S. enterica* SurAs to search against the translated genomes using NCBI BLAST P. This revealed a homologue in *B. pseudomallei* (BPSL0659) and a homologue in *B. thailandensis* (BTH_I0576). BPSL0659 and BTH_I0576 have 32% amino acid sequence identity to *E. coli* SurA (Ec-SurA; NP414595.1) and 33% amino acid sequence identity to *S. enterica* SurA (Se-SurA; NP459097.1). In addition, BPSL0659 and BTH_I0576 are 97% identical to each other, with highly conserved PPIase and chaperone domains. BMA0209 from *B. mallei* has 33% sequence identity to Ec-SurA.

A multiple sequence alignment by Clustal W method of BPSL0659 and BTH_I0576 against Ec-SurA and Se-SurA is shown in Figure 3.2, A. BPSL0659 and BTH_I0576 possess N-terminal domains associated with chaperone function in SurA and highly conserved PPIase domains (Behrens *et al.,* 2001). Further confirmation that BPSL0659 and BTH_I0576 encode putative SurA homologues was obtained by investigating the secondary structure of BPSL0659 using PHYRE. The N-terminal domain of the predicted secondary structure of BPSL0659 had more α-helices than SurA, but the rest of the protein had a comparable predicted structure (Figure 3.2, B).



A



B

Figure 3.2. A) Sequence alignment of *B. pseudomallei* BPSL0659, *B. thailandensis* BTH_I06576, *E. coli* (Ec) and *S. enterica* (Se) SurAs. Identical amino acids are shaded in blue. The inactive PPIase domain of Ec-SurA is underlined in green and the active PPIase domain is boxed in red. The domains involved with chaperone activity are boxed in yellow. B) Alignment of the predicted secondary structure of BPSL0659 against the predicted secondary structure of *E. coli* SurA

3.2.2 Characterisation of recombinant FKBPs and parvulins in *B. pseudomallei*

3.2.2.1 Construction of expression plasmids

The cloning strategy used to construct expression plasmids is outlined in Figure 3.3. The genes encoding BPSL0918, BPSL2254, BPSS1823, BPSL1402, BPSL0659, BPSL1418 were PCR amplified from *B. pseudomallei* K96243 genomic DNA and cloned into pCR Blunt II-TOPO. To allow successful expression of BPSL0659, the first 29 amino acids encoding a transmembrane domain were removed. The sequence of the amplified genes was confirmed by nucleotide sequencing. The genes were digested out of pCR Blunt II-TOPO using restriction endonucleases and ligated into the *Bam*HI/*Nde*I or *BamHI/EcoRI* sites in pET-15b (BPSL0918, BPSL2254, BPSS1823, BPSL1402 and BPSL0659) or pGEX-4T-1 (BPSL1418) respectively. Ligations were transformed into *E. coli* JM109 cells and plated onto selective LB agar containing 50 µg/ml ampicillin. Colonies containing the correct construct were confirmed restriction digest and nucleotide sequencing.

3.2.2.2 Expression of recombinant proteins

The expression plasmids containing putative PPIase genes were transformed into either *E. coli* BL21 (DE3) or *E. coli* BL21 * PlysS (DE3). The bacteria were grown until an absorbance of 0.4 – 0.6 at 600 nm was reached, then expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM.

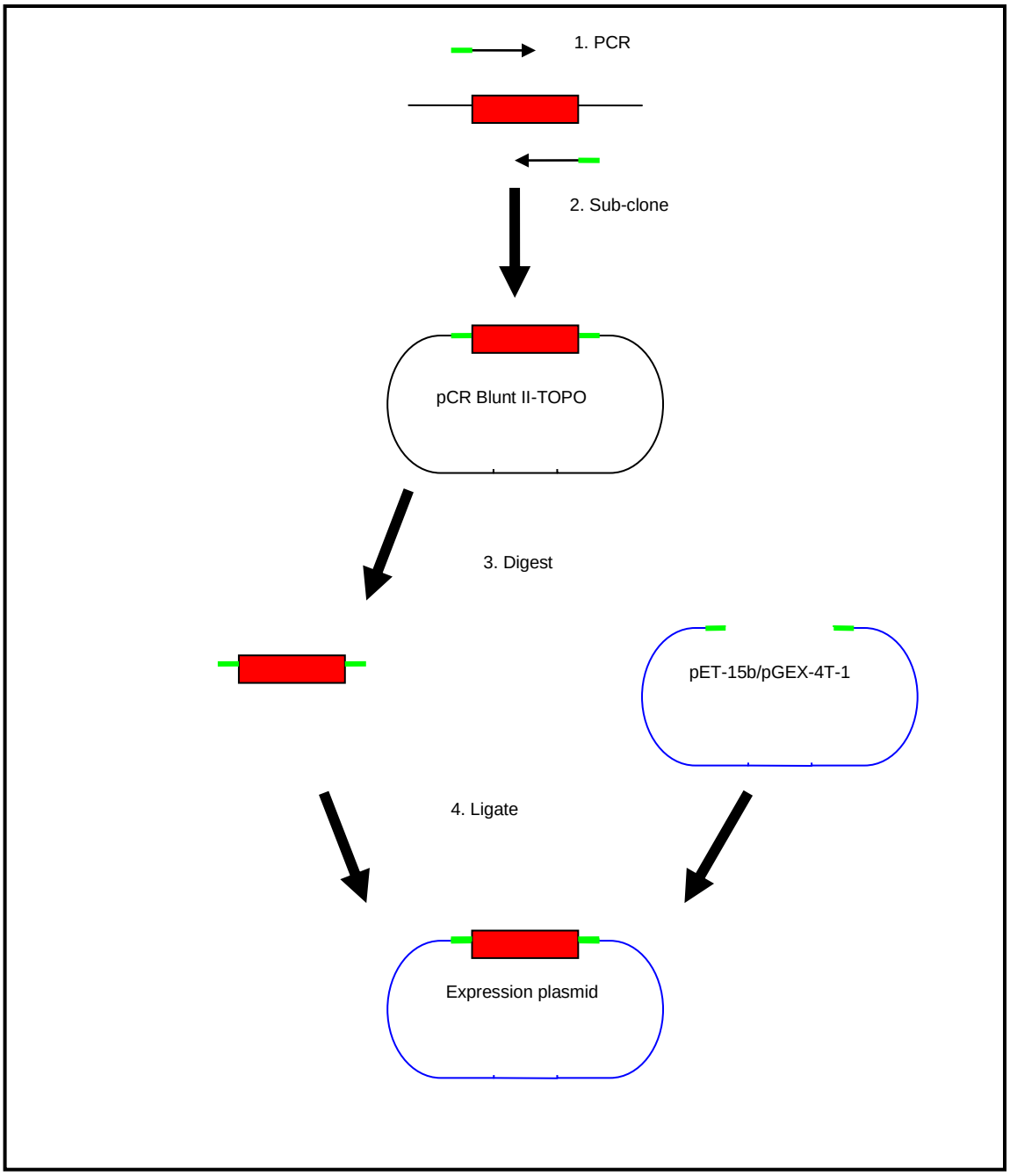


Figure 3.3. Cloning strategy used to construct expression plasmid. 1. Gene (shown in red) was PCR amplified from genomic DNA using primers containing specific restriction sites (shown in green) 2. PCR product was cloned into pCR Blunt II – TOPO 3. pET-15b/pGEX-4T-1 and insert were digested with specific restriction endonucleases 4. Insert was ligated into restriction sites in pET15-b/pGEX-4T-1.

Expression levels over time were determined by growing the bacterial cultures for 24 h, with samples taken at 0, 2, 4 and 24 h post induction (Figure 3.4). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane. The proteins were detected by western blot, using an anti-His or anti-GST antibody. Of the nine PPIases identified in *B. pseudomallei*, six were successfully expressed as recombinant, fusion tagged proteins: BPSL0918, BPSL2254, BPSS1823, BPSL1402, BPSL0659 and BPSL1418 (Figure 3.4). A band at the expected size of 18.5 kDa (BPSL0918); 22.5 kDa (BPSL2254); 14.5 kDa (BPSS1823); 52 kDa (BPSL1402); 51.5 kDa (BPSL0659) and 50 kDa (BPSL1418) was detected. The band for BPSL1418 was less distinct, indicating the protein may have degraded. Suitable expression levels were achieved between 4 – 24 h post-induction for all proteins. Although under the control of an inducible promoter, expression of BPSS1823, BPSL0659 and BPSL1418 was detected pre-induction with IPTG (Figure 3.4 C, E and F; Lane 1).

To determine the solubility of the recombinant proteins, the bacterial cultures were induced with IPTG, grown for the period of time optimised for expression, disrupted by sonication and separated into pellet and supernatant fractions. The proteins were detected by western blotting, using an anti-His or anti-GST antibody. All of the proteins were detected in the supernatant and pellet fractions (Figure 3.5). To increase solubility, the growth temperature post-induction was lowered to 20ºC for all proteins except BPSL0659. Proteins of multiple sizes were detected in fractions of BPSL2254, BPSL1402, BPSL0659 and BPSL1418, suggesting that the protein may have aggregated or degraded. The optimal growth conditions used for each protein in expression studies are summarised in Table 3.

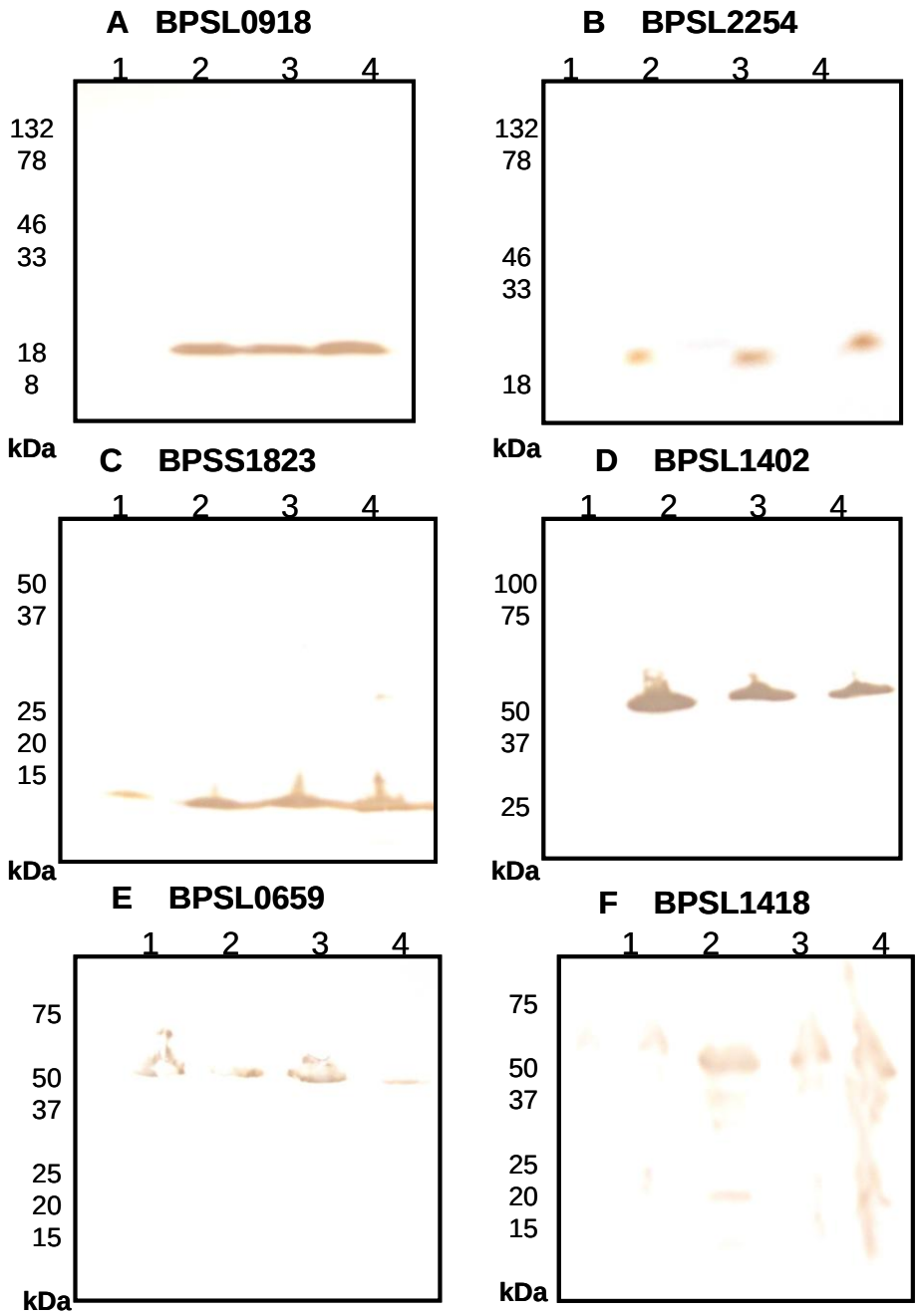


Figure 3.4. Western blots showing expression of His- tagged and GST-tagged proteins pre- and post-induction with IPTG, grown at 37ºC. Cell lysates were separated on a 8-25% gel using SDS-PAGE and transferred to nitrocellulose membrane. The proteins were detected using an anti-His (A-E) or anti-GST antibody (F). kDa ladder shows the size of marker proteins.

A – expression of BPSL0918; B – expression of BPSL2254; C – expression of BPSS1823; D – expression of BPSL1402; E – expression of BPSL0659; F – expression of BPSL1418.

Lane 1 – pre-induction; 2 – 2 h post-induction; 3 – 4 h post-induction; 4- 24 h post-induction

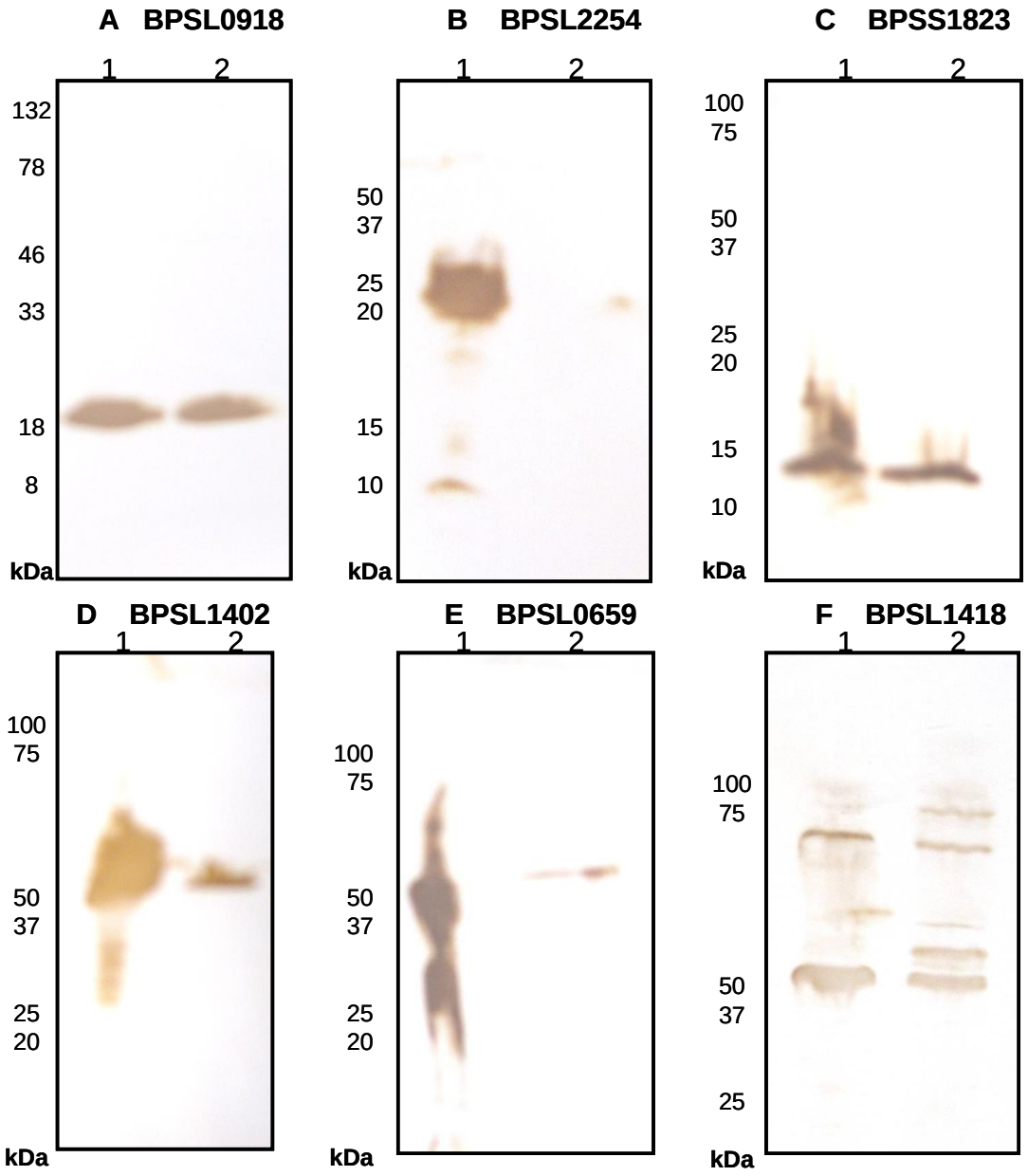


Figure 3.5. Western blots showing the cellular location of His- tagged and GST-tagged recombinant proteins. Cultures were induced and grown under conditions optimised for expression. Cells were disrupted by sonication and the supernatant and pellet separated on 8-25% gel using SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and detected using an anti-His (A-E) or anti-GST antibody (F). Proteins expressed in the supernatant were likely to be soluble. kDa ladder shows the size of marker proteins.

A – expression of BPSL0918; B – expression of BPSL2254;
C – expression of BPSS1823; D – expression of BPSL1402;
E – expression of BPSL0659; F – expression of BPSL1418.

Lane 1 – supernatant; 2 – pellet

Protein	***E. coli* strain**	**Concentration IPTG**	**Temperature post induction**	**Time post induction**
BPSL0918	BL21 (DE3)	1 mM	20ºC	24 h
BPSL2254	BL21*PlysS (DE3)	1 mM	20ºC	24 h
BPSS1823	BL21 (DE3)	1 mM	20ºC	4 h
BPSL1402	BL21 (DE3)	1 mM	20ºC	24 h
BPSL0659 (minus 1 – 29 aa)	BL21 (DE3)	1 mM	37ºC	4 h
BPSL1418	BL21 (DE3)	1 mM	20ºC	24 h

Table 3.4. Conditions used for the expression of recombinant proteins

3.2.2.3 Purification of recombinant proteins

Large scale protein purification was performed from 2 L of culture using the conditions shown in Table 3.4. The supernatant was applied to a Histrap or GSTrap FF column and washed with equilibration buffer. Samples of the proteins applied to the column (supernatant) and the unbound proteins washed off the column (flowthrough) were separated by SDS-PAGE and staining using Coomassie. In all cases, a protein of the expected size was present in the supernatant but reduced in the flowthrough (Figures 3.6-3.10, B; Lanes 1 and 2). For His-tagged proteins, fractions were eluted off the column using an imidazole gradient (50 – 500 mM) generated using an FPLC system. For GST-tagged protein, thrombin was used to cleave the GST tag on the column and the protein was eluted in PBS manually. All 6 proteins were purified (Figures 3.6-3.10). For each His-tagged protein, the elution profile was analysed to determine fractions containing proteins. Fractions were separated by SDS-PAGE and stained with Coomassie blue to identify proteins of the expected size. A single band was detected for all proteins, except BPSL1402 (Figure 3.9). For BPSL1402, two distinct bands were detected at the expected size of 50 kDa and at ~20 kDa (Figure 3.9), indicating that the protein had degraded. Fractions containing a purified protein of the correct size were then dialysed against PBS and the quantity of protein determined by a biochoninic acid (BCA) assay. The amount of each protein purified from 2 L LB broth is shown in Table 3.5.

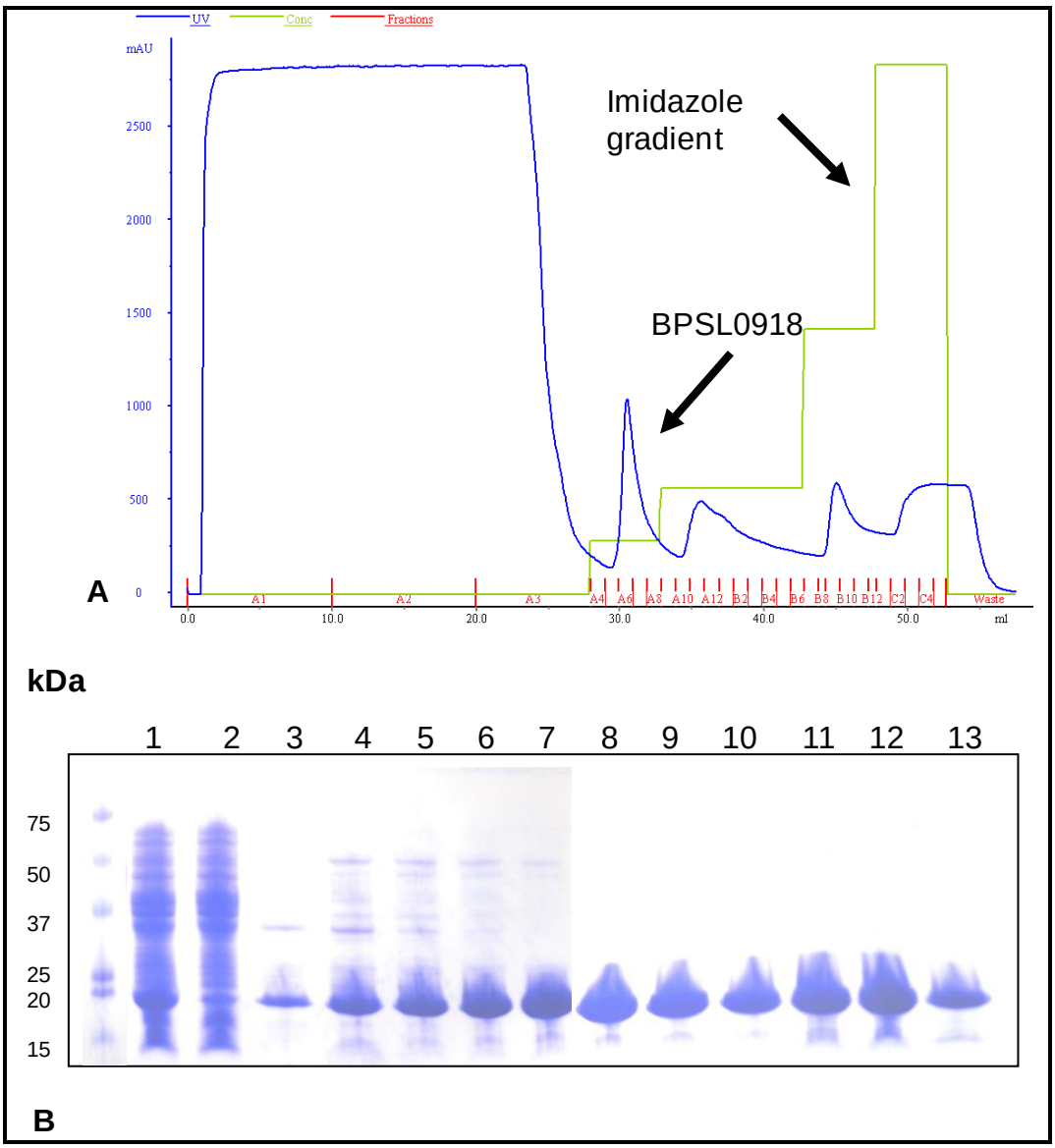


Figure 3.6. Production of recombinant BPSL0918 (16 kDa + His tag) from *B. pseudomallei* K96243, purified using affinity chromatography.

A – FPLC trace showing elution of purified BPSL0918. Supernatants containing soluble protein were applied to a HisTrap column and 1 ml fractions (shown in red) eluted using an imidazole gradient of increasing concentrations (shown in green). The absorbance of the fractions was measured using UV light (shown in blue).

B – Coomassie stained 8-25 % SDS-PAGE gel of purified BPSL0918. Selected protein fraction (shown by an arrow) were separated by SDS-PAGE and visualised by staining with Coomassie brilliant blue R250. KDa ladder shows size of marker proteins.

Lane 1 – supernatant loaded onto column;
2 – flow through;
3 to 13 – purified BPSL0918

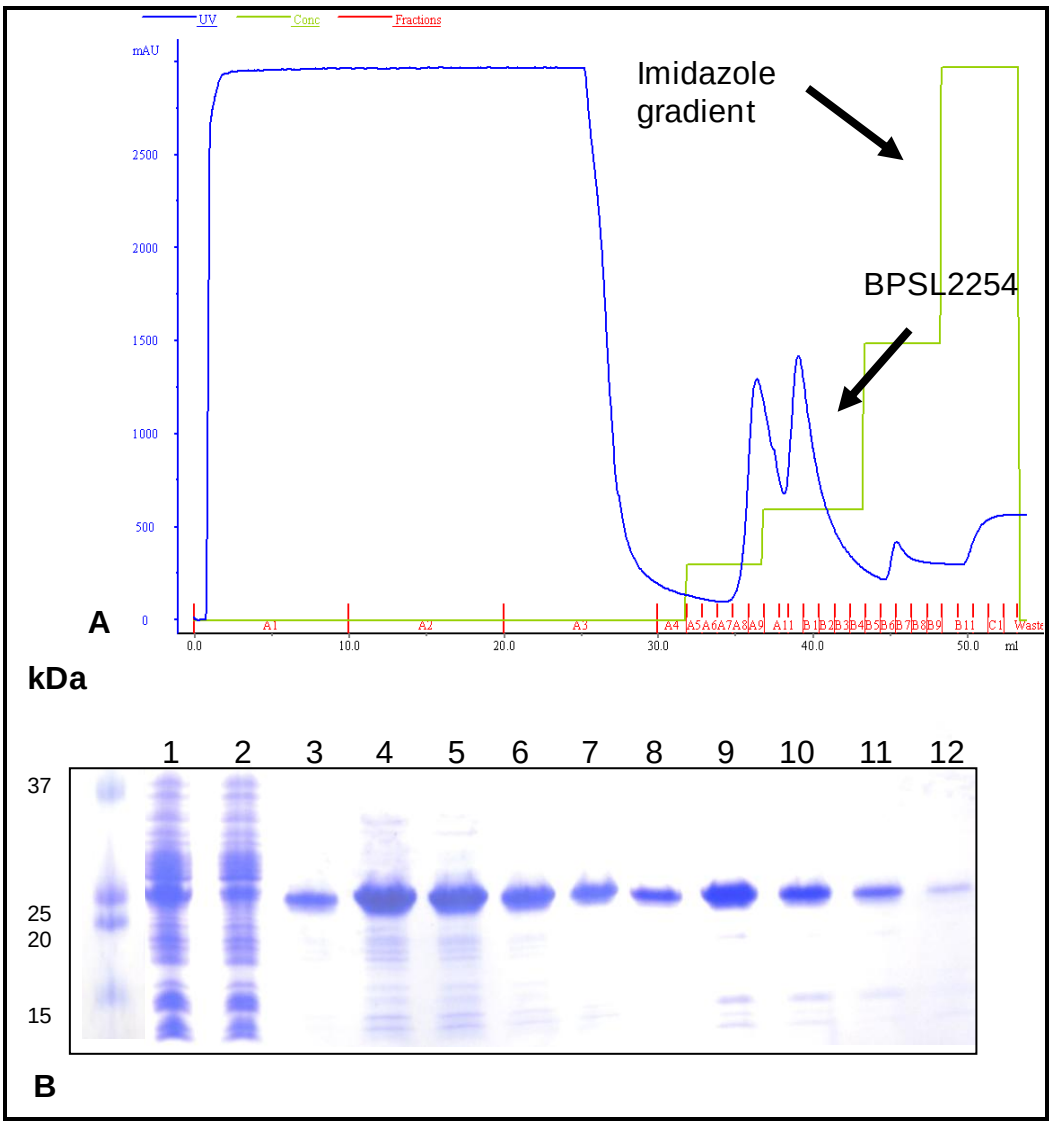


Figure 3.7. Recombinant BPSL2254 (20 kDa + His tag) from *B. pseudomallei* K96243, purified using affinity chromatography.

A – FPLC trace showing elution of purified BPSL2254. Supernatants containing soluble protein were applied to a HisTrap column and 1 ml fractions (shown in red) eluted using an imidazole gradient of increasing concentrations (shown in green). The absorbance of the fractions was measured using UV light (shown in blue).

B – Coomassie stained 8-25% SDS-PAGE gel of purified BPSL2254. Selected protein fractions (shown by an arrow) were separated by SDS-PAGE and visualised by staining with Coomassie brilliant blue R250. KDa ladder shows size of marker proteins.

Lane 1 – supernatant loaded onto column;
2 – flow through;
3 to 12 – purified BPSL2254

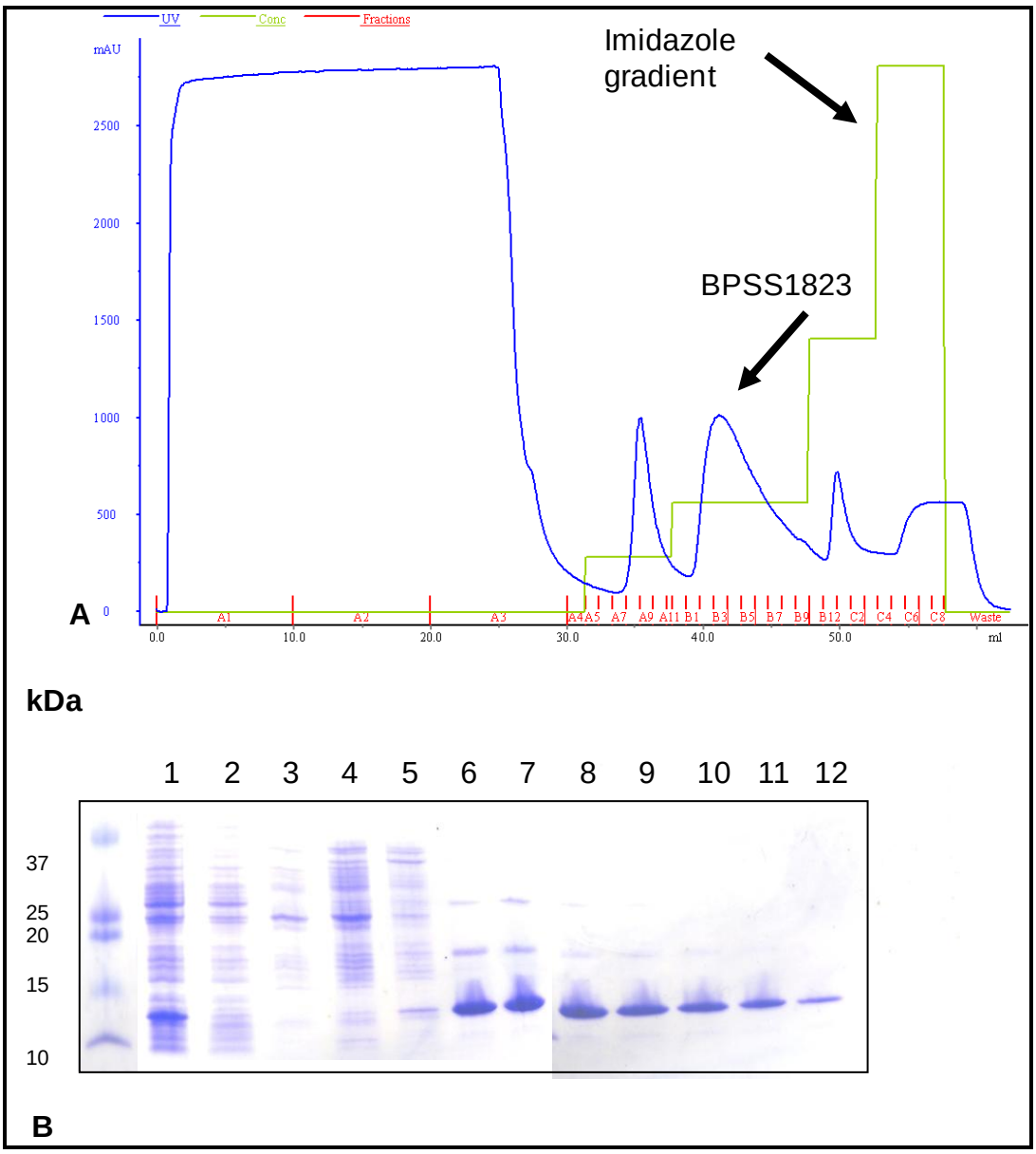


Figure 3.8. Recombinant BPSS1823 (12 kDa + His tag) from *B. pseudomallei* K96243, purified using affinity chromatography.

A – FPLC trace showing elution of purified BPSS1823. Supernatants containing soluble protein were applied to a HisTrap column and 1 ml fractions (shown in red) eluted using an imidazole gradient of increasing concentrations (shown in green). The absorbance of the fractions was measured using UV light (shown in blue).

B – Coomassie stained 8-25% SDS-PAGE gel of purified BPSS1823. Selected protein fractions (shown by an arrow) were separated by SDS-PAGE and visualised by staining with Coomassie brilliant blue R250. KDa ladder shows size of marker proteins.

Lane 1 – supernatant loaded onto column;
2 – flow through;
6 to 12 – purified BPSS1823

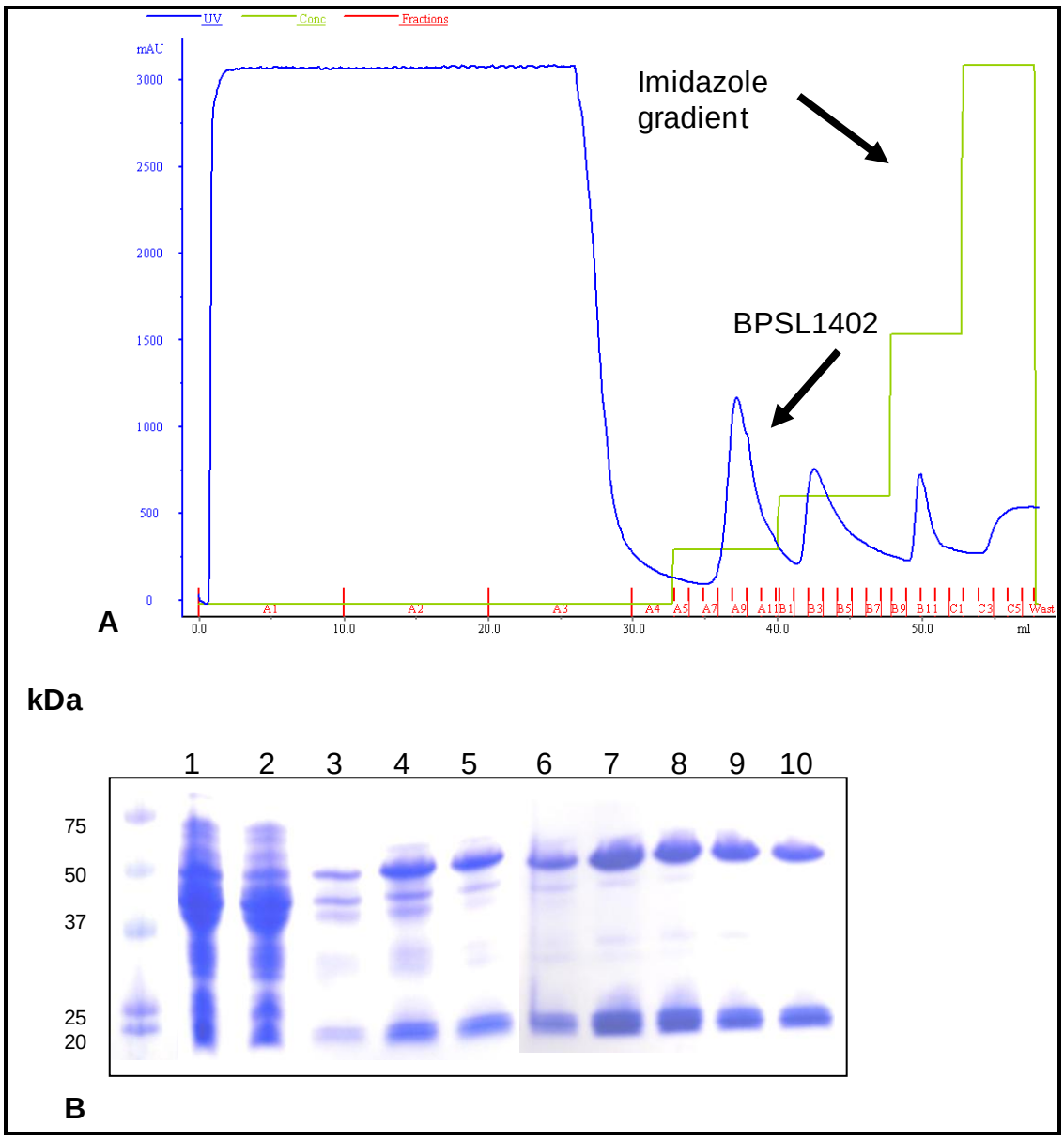


Figure 3.9. Recombinant BPSL1402 (50 kDa + His tag) from *B. pseudomallei* K96243, purified using affinity chromatography.

A – FPLC trace showing elution of purified BPSL1402. Supernatants containing soluble protein were applied to a HisTrap column and 1 ml fractions (shown in red) eluted using an imidazole gradient of increasing concentrations (shown in green). The absorbance of the fractions was measured using UV light (shown in blue).

B – Coomassie stained 8-25% SDS-PAGE gel of purified BPSL1402. Selected protein fractions (shown by an arrow) were separated by SDS-PAGE and visualised by staining with Coomassie brilliant blue R250. KDa ladder shows size of marker proteins.

Lane 1 – supernatant loaded onto column;
2 – flow through;
3 to 10 – purified BPSL1402

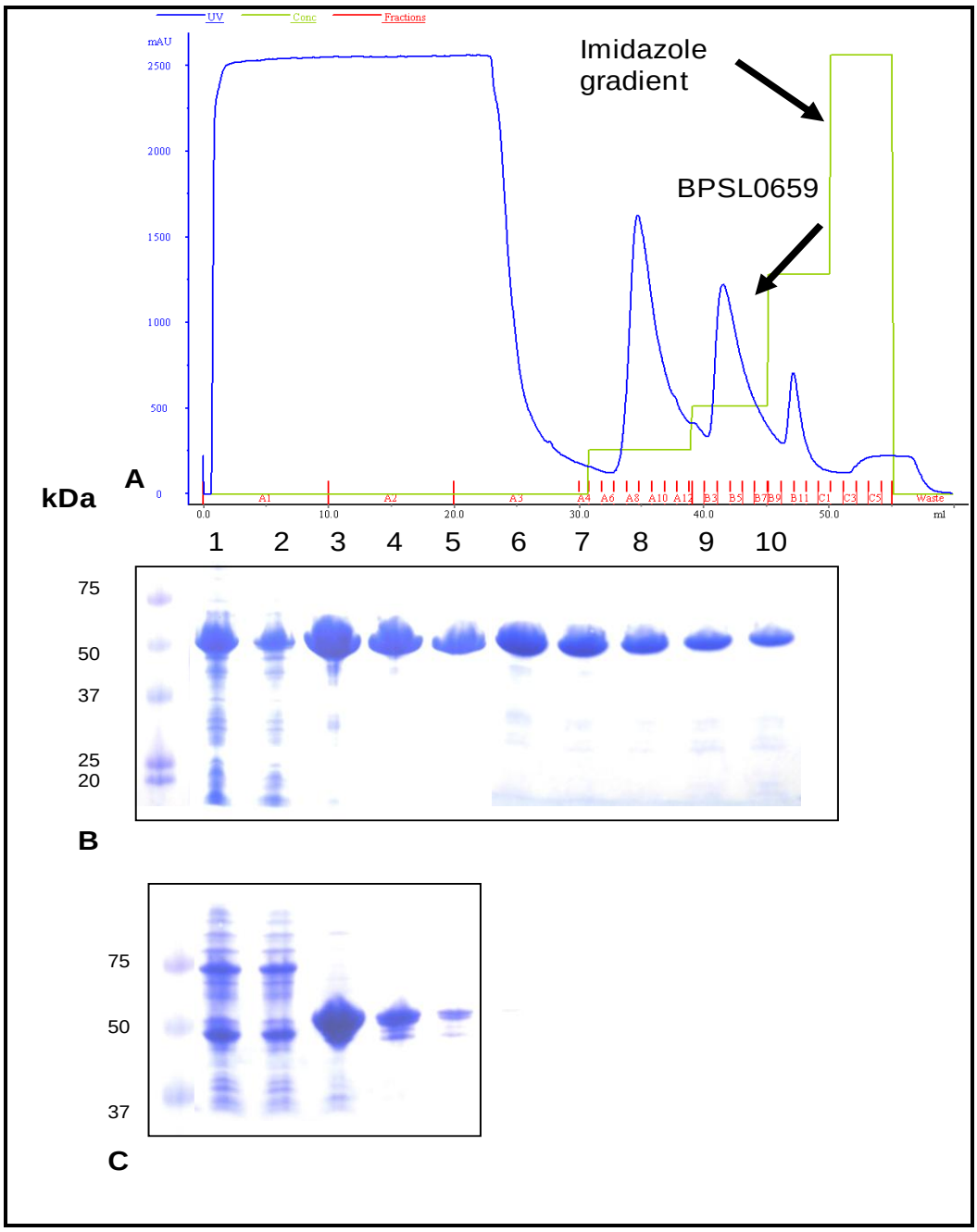


Figure 3.10. Recombinant BPSL0659 (49 kDa + His tag) and BPSL1418 (26 kDa + GST tag) from *B. pseudomallei* K96243, purified using affinity chromatography.

A – FPLC trace showing elution of purified BPSL0659. Supernatants containing soluble protein were applied to a HisTrap column and 1 ml fractions (shown in red) eluted using an imidazole gradient of increasing concentrations (shown in green). The absorbance of the fractions was measured using UV light (shown in blue).

B – Coomassie stained 8-25% SDS-PAGE gel of purified BPSL0659. Selected protein fractions (shown by an arrow) were separated by SDS-PAGE and visualised by staining with Coomassie brilliant blue R250. KDa ladder shows size of marker proteins.

Lane 1 – supernatant loaded onto column;
2 – flow through;
3 to 10 – purified BPSL0659

C – Coomassie stained 8-25% SDS-PAGE gel of purified BPSL1418
Lane 1 – supernatant loaded onto column;
2 – flowthrough
3-5 purified BPSL1418

Protein	Type of fusion tag	Quantity purified (mg)
BPSL0918	His	43.9
BPSL2254	His	29
BPSS1823	His	15.4
BPSL1402	His	6.3
BPSL0659	His	25
BPSL1418	GST (cleaved)	4

Table 3.5. Quantity and type of recombinant protein purified using 2 L *E. coli*

Protein	MW (Da, including His-Tag)	α-helix (%)	B-sheet (%)	Other (%)
BPSL0918	18582.1	11.7	32.5	55.8
BPSS1823	14436.3	31.2	23.9	44.9

Table 3.6. Molecular weight (MW) and secondary structure estimation of BPSL0918 and BPSS1823 recombinant proteins, determined using mass spectrometry and circular dichroism.

The molecular weight and secondary structure of two representative PPIases, BPSL0918 and BPSS1823, were analysed by liquid chromatography-mass spectrophotometry (MS) and circular dichroism spectroscopy (CD) (Table 3.6). Both samples contained a protein of the expected size with a secondary structure of both α-helices and β-sheets.

3.2.2.4 Determination of PPIase activity of recombinant FKBPs and parvulins from *B. pseudomallei*

To determine whether recombinant putative PPIases from *B. pseudomallei* have characteristic enzyme activity, a protease coupled or protease free assay was used (Fischer *et al.,* 1984; Kofron *et al.,* 1991). Both assays use synthetic tetrapeptides of the general structure Suc-Ala-Xaa-Pro-Phe-*p*-nitroanilide, where Xaa = any natural amino acid. In the protease coupled assay, the peptides are dissolved in DMSO, resulting in 10% in the *cis* conformation. Chymotrypsin is used as a helper protease to cleave the *p*-nitroanilide from *trans* isomers, resulting in initial rapid release from 90% in *trans*, whilst the remaining 10% undergoes slow *cis-trans* isomerisation. In the presence of a PPIase, this slow phase of isomerisation is accelerated (Figure 1.1). In the protease free assay, the peptides are dissolved in 0.48M LiCl/anhydrous trifluroethanol. This changes the ratio of *cis*:*trans* isomers from 10:90 to 40:60. Upon the addition of the substrate to an aqueous buffer, the equilibrium will shift back to 10:90 and in the presence of a PPIase, the speed at which the shift occurs will be accelerated.

BPSL0918, BPSS1823 and BPSL0659 were down selected for testing in this assay, as they were predicted to represent a single domain FKBP, a Mip

homologue and a SurA homologue, respectively. No enzyme was present in the negative control and 10 nM active FKBP12 from *H. sapiens* was used as a positive control. Using both the protease coupled or protease free assays, recombinant BPSL0918 had no measurable effect on a range of tetrapeptide substrates (Table 3.7). In the protease coupled assay, while addition of hFKBP12 had a significant effect on the rate of reaction (a mean change of 9.85 mAbs; lower limit of 7.67, upper limit of 12.04, 95 % CI; $P<0.05$), addition of 400 nM BPSL0918 had no significant effect (a mean change of -0.25 mAbs; lower limit of -2.54, upper limit of 2.03, 95 % CI; no significant differences). In the protease free assay, addition of hFKBP12 had a significant effect on the rate of reaction (a mean change of 13.28 mAbs; lower limit of 8.38, upper limit of 18.18, 95 % CI; $P<0.05$), addition of BPSL0918 had no significant affect (a mean change of -1.25 mAbs; lower limit of -3.54, upper limit of 1.04, 95 % CI; no significant difference).

Recombinant BPSL0659 was shown to possess PPIase activity using the protease coupled assay. Figure 3.11, A, shows a time course graph of *p*-nitroanilide release with no PPIase (yellow) or following addition of BPSL0659 (green), measured by absorbance at 390 nm. The positive control, hFKBP12, had a significant effect on the rate of reaction (a mean change of 16.2 mAbs; lower limit of 8.1; upper limit of 24.2, 95% CI; $P<0.05$) and addition of 10 nM BPSL0659 also had a significant effect (a mean change of 12.3 mAbs; lower limit of 3.9; upper limit of 20.7, 95% CI; $P<0.05$; Figure 3.11, B)

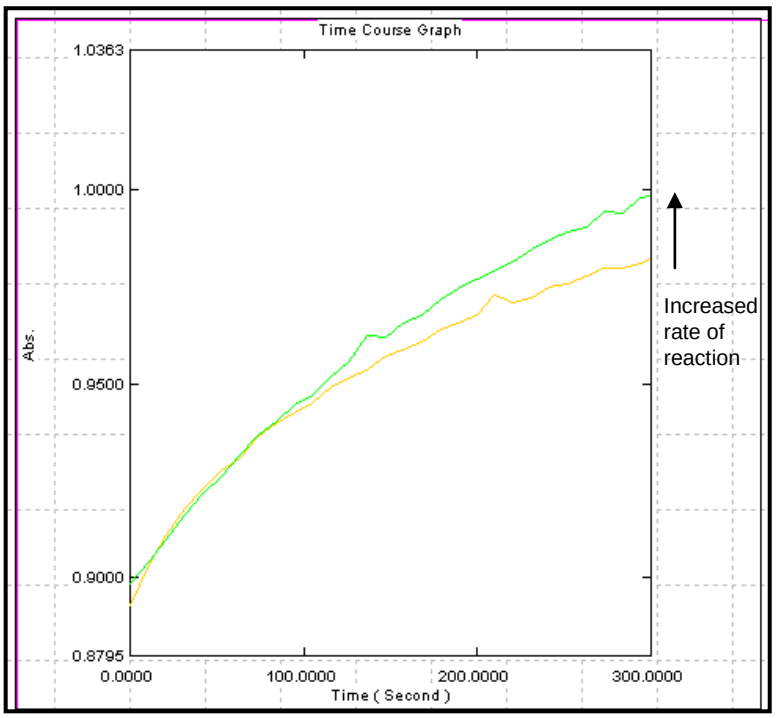
Test group	Protease coupled assay (mAbs/min)	Protease free assay (mAbs/min)
Control (w/o PPIase)	14.9 (± 0.2)	2.6 (± 0.9)
Human FKBP12 (Suc-Ala-Phe-Pro-Phe-*p*-nitroanilide)	24.7 (± 2.1)[a]	15.9 (± 4.4)[a]
BPSL0918 (Suc-Ala-Phe-Pro-Phe-*p*-nitroanilide)	14.6 (± 1.7)[b]	1.4 (± 1.2) [b]
BPSL0918 (Suc-Ala-Ala-Pro-Phe-*p* nitroanilide)	13.1 (± 2.1) [b]	n.d.[c]
BPSL0918 (Suc-Ala-Leu-Pro-Phe-*p* nitroanilide)	13.2 (± 1.3) [b]	n.d.

Table 3.7. The effect of hFKBP12 and BPSL0918 on isomerisation of Suc-Ala-Xaa-Pro-Phe-*p*-nitroanilide.

Rate of *cis-trans* isomerisation of substrates with the structure Suc-Ala-Xaa-Pro-Phe-4-nitroanilide using a protease coupled assay to monitor the rate of realise of *p*-nitroanilide upon addition of chymotrypsin or a protease free assay to monitor a solvent jump of the substrate. The values are means (± standard deviation) from three independent experiments.

[a] statistically significant compared to the control ($p<0.05$)
[b] not statistically significant compared to the control
[c] n.d. = not done

A



B

Test group	Protease coupled assay (mAbs/min)
Control (w/o PPIase)	6 (± 3.1)
Human FKBP12 (Suc-Ala-Phe-Pro-Phe-*p*-nitroanilide)	22.1 (± 2.5) [a]
BPSL0659 (Suc-Ala-Phe-Pro-Phe-*p*-nitroanilide)	18.3 (± 0.4) [a]

Figure 3.11. The PPIase activity of BPSL0659.

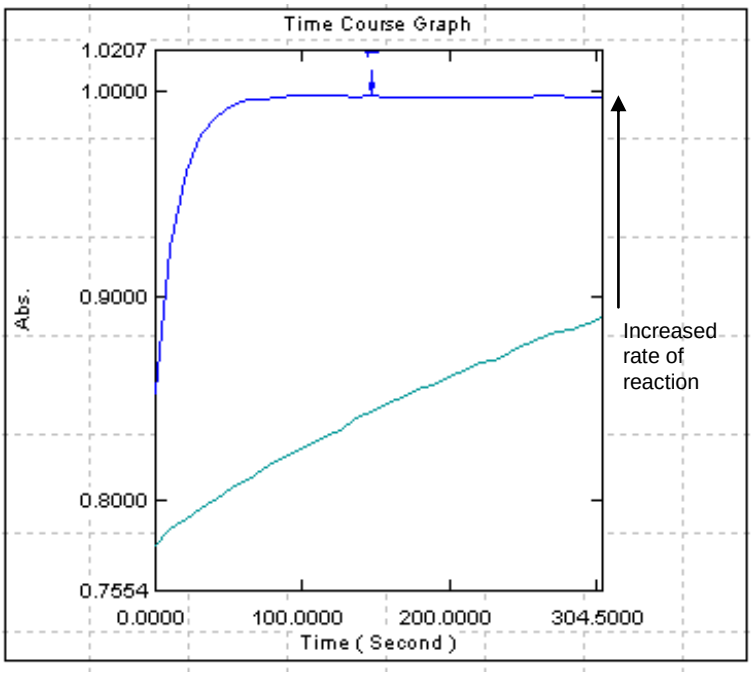
Rate of *cis-trans* isomerisation of Suc-Ala-Pro-Pro-Phe-4-nitroanilide using a protease coupled assay to monitor the rate of realise of *p*-nitroanilide upon addition of chymotrypsin. The values are means (± standard deviation) from three independent experiments.

A – a typical time course graph showing the release of *p*-nitroanilide from Suc-Ala-Pro-Phe-Pro-*p*-nitroanilide in the presence of no PPIase (yellow) or BPSL0659 (green)
B – the effect of hFKBP12 and BPSL0659 on isomerisation of Suc-Ala-Phe-Pro-Phe-*p*-nitroanilide.
[a] statistically significant compared to the control ($p<0.05$)

Recombinant BPSS1823 was also shown to possess PPIase activity using the protease coupled assay. Figure 3.12, A, shows a time course graphs of *p*-nitroanilide release with no PPIase (pale blue) or following addition of BPSS1823 (dark blue). The positive control, hFKBP12, had a significant effect on the rate of reaction (a mean change of 9.9 mAbs; lower limit of 5.1; upper limit of 14.7, 95% CI; $P<0.05$) and addition of 10 nM BPSS1823 also had a significant effect (a mean change of 7.5 mAbs; lower limit of 5.6; upper limit of 9.4, 95% CI; $P<0.01$; Figure 3.12, B). Furthermore, the calculated specificity constant k_{cat}/K_m was $6.7 \pm 0.4 \times 10^6\ M^{-1}\ s^{-1}$ (Figure 3.13).

The PPIase activity of FKBPs, but not parvulins, can be inhibited by the immunosuppressant drug rapamycin or cycloheximide-N-ethylethanoate (Schreiber, 1991; Christner *et al*., 1999). Therefore, recombinant BPSS1823 was incubated with increasing concentrations of rapamycin or cycloheximide-N-ethylethanoate. The PPIase activity of BPSS1823 was inhibited by nanomolar concentrations of rapamycin with a Ki of 5 ± 2 pM (Figure 3.14, A). The PPIase activity of BPSS1823 was inhibited by micromolar concentrations of cycloheximide-N-ethylethanoate, with a Ki of 6.5 ± 1.0 µM (Figure 3.14, B).

A



B

Test group	Protease coupled assay (mAbs/min)
Control (w/o PPIase)	14.9 (± 0.2)
Human FKBP12 (Suc-Ala-Phe-Pro-Phe-*p*-nitroanilide)	24.7 (± 2.1) [a]
BPSS1823 (Suc-Ala-Phe-Pro-Phe-*p*-nitroanilide)	22.4 (± 0.6) [b]

Figure 3.12. PPIase activity of BPSS1823

Rate of *cis-trans* isomerisation of Suc-Ala-Pro-Pro-Phe-4-nitroanilide using a protease coupled assay to monitor the rate of realise of *p*-nitroanilide upon addition of chymotrypsin. The values are means (± standard deviation) from three independent experiments.

A – a typical time course graph showing the release of *p*-nitroanilide from Suc-Ala-Pro-Phe-Pro-*p*-nitroanilide in the presence of no PPIase (pale blue) or BPSS1823 (dark blue)
B – a table summarising the effect of hFKBP12 and BPSS1823 on isomerisation of Suc-Ala-Phe-Pro-Phe-*p*-nitroanilide.
[a] statistically significant compared to the control ($p<0.05$)
[b] statistically significant compared to the control ($p<0.01$)

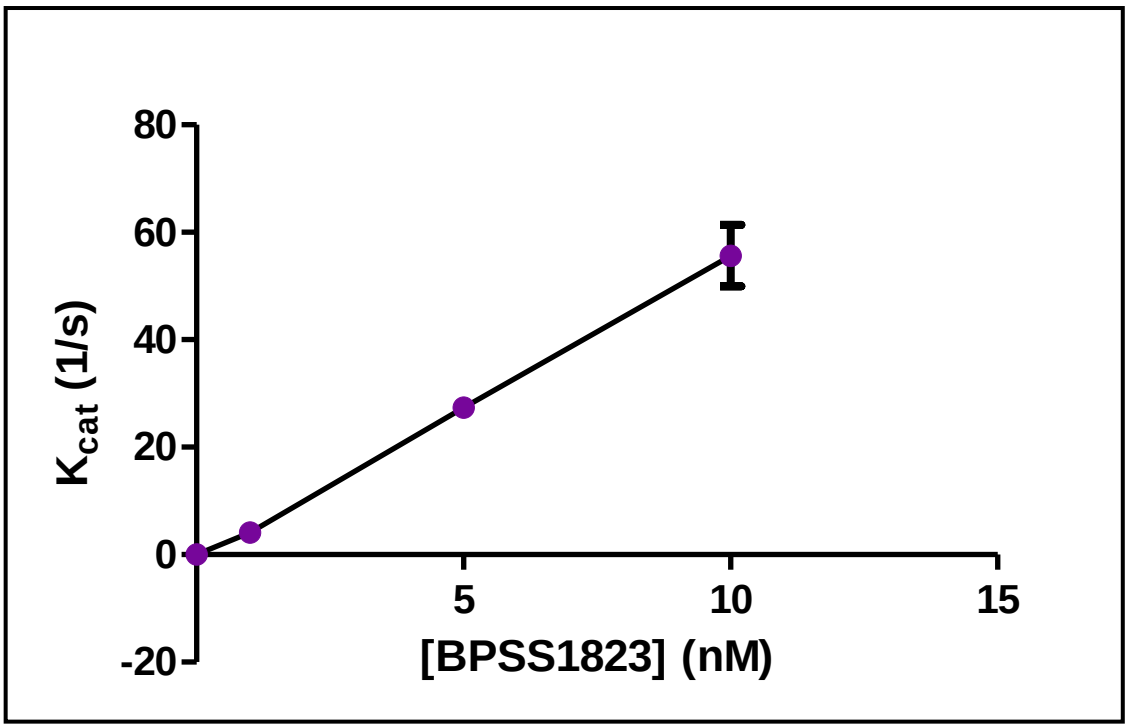


Figure 3.13. The first order rate constant of BPSS1823.

The rate constant (k) of the catalysed first-order *cis-trans* conversion upon addition of 0-15 nM of recombinant BPSS1823. The calculated specificity constant k_{cat}/K_m was 6.7± 0.4 x 10^6 M^{-1} s^{-1}. The values are means (± standard deviation) from at least three independent experiments.

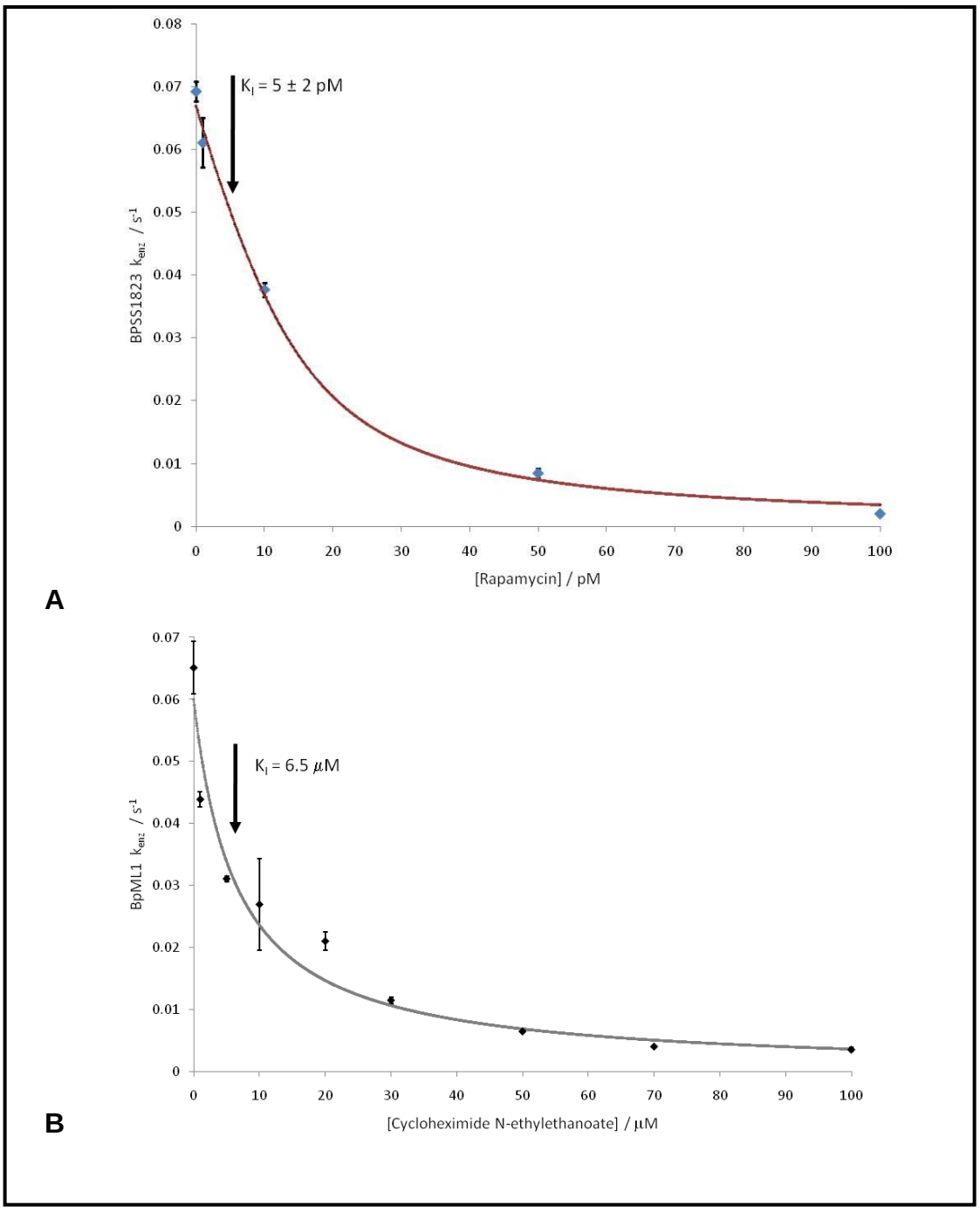


Figure 3.14. Inhibition of BPSS1823 PPIase activity by:
A – Rapamycin
B – Cycloheximide-N-ethylethanoate

Values are the means of at least three independent experiments ± standard error. The predicted inhibition curve for the fit data is shown.

3.2.2.5 Determination of the X-ray structure of BPSS1823

To provide further information on the putative function of BPSS1823, the X-ray structure of the recombinant protein was solved to 0.91 Å (Figure 3.15). The pET15b expression vector containing *BPSS1823* was used by Dr Nic Harmer to produce recombinant protein and determine the crystal structure. Analysis of the structure confirmed that BPSS1823 adopts a classical FKBP-like fold and has an active site configuration similar to Lp-Mip (PDB ID: 2DG3).

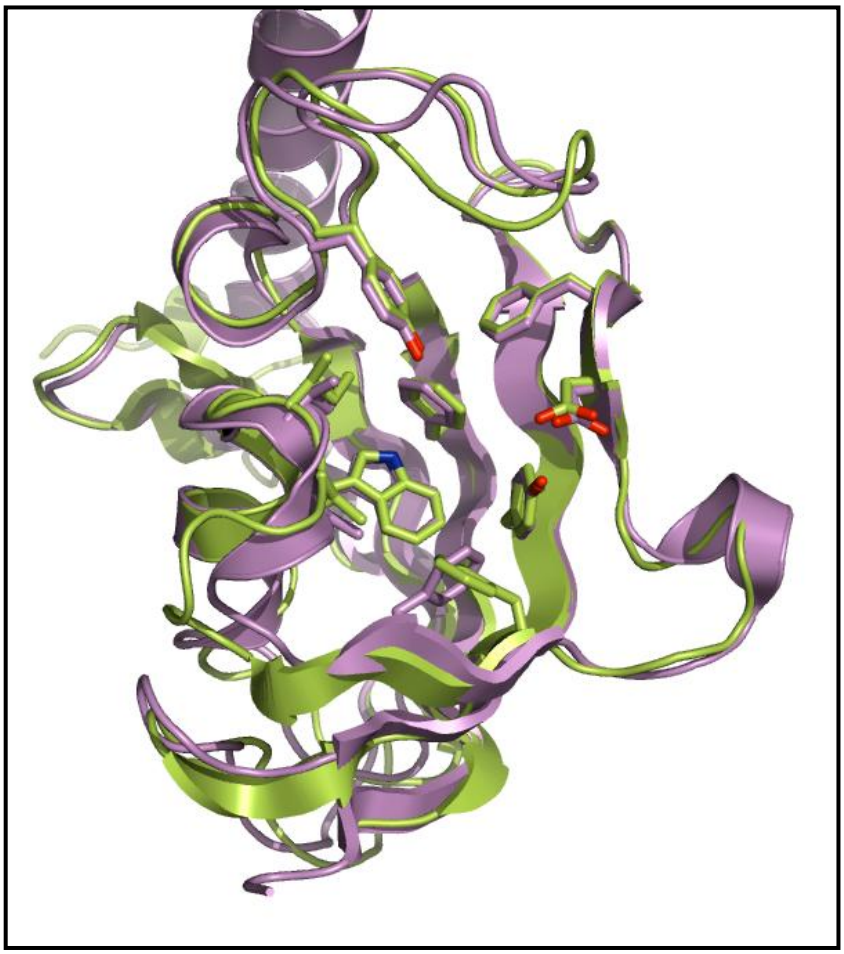


Figure 3.15. The X-ray structure of BPSS1823 (green) compared to the structure of Lp-Mip (Purple; PDB ID:2DG3).

3.2.3 Evaluation of FKBPs and parvulins from *B. pseudomallei* as protective antigens

3.2.3.1 The immune response and protection provided by recombinant proteins

Recombinant SurA has shown potential as a protective antigen against *B. abortus* challenge (Delpino *et al*., 2007). Therefore, the protective efficacy of recombinant PPIases was determined using a BALB/c mouse model of *B. pseudomallei* infection. Six mice per group were immunised with 10 µg recombinant protein adjuvanted with Sigma Adjuvant System (SAS), three times at two weekly intervals. The antibody response to proteins was determined in polyclonal sera collected from immunised BALB/c mice. ELISA plates were coated with 10 µg purified protein or SAS and probed with polyclonal sera to determine an antibody response. The approximate endpoint antibody titre is shown in Table 6.8.

The mice were challenged by the intraperitoneal route with 3.6×10^4 cfu (36 x MLD) or 3.4×10^4 (34 x MLD) of *B. pseudomallei* K96243, five weeks after the last immunisation. Between 66 – 80% of mice dosed with SAS only had died by day 30 and no significant immune response to SAS was detected by ELISA (Figure 3.16). Although immunisation of mice with BPSL0918 elicited an antibody response (Table 3.8), mice died faster than those immunised with SAS only (Figure 3.16, A). Mice immunised with BPSL2254 or BPSL1418 had high antibody titres but no protection against challenge was afforded (Figures 3.16, B, F). In contrast, mice immunised with recombinant BPSS1823, BPSL1402 or

BPSL0659 had an increased median time to death compared to the controls (Figures 3.15, C, D, E; Table 3.8). While immunisation with recombinant BPSL1402 or BPSL0659 elicited the highest antibody response (1:819200), mice immunised with BPSS1823 had the lowest antibody response (1:12800; Table 3.8). Despite differing antibody responses, there were four mice immunised with BPSS1823 or BPSL0659 still alive 35 days post challenge (Figure 3.16).

The mice were monitored for signs of disease for 5 weeks after which time survivors were culled, the spleens aseptically removed and plated onto L-agar. Colonies showing typical morphology to *B. pseudomallei* were recovered from all spleens except those immunised with BPSL2254, BPSL1418 and SAS only control (Table 3.8).

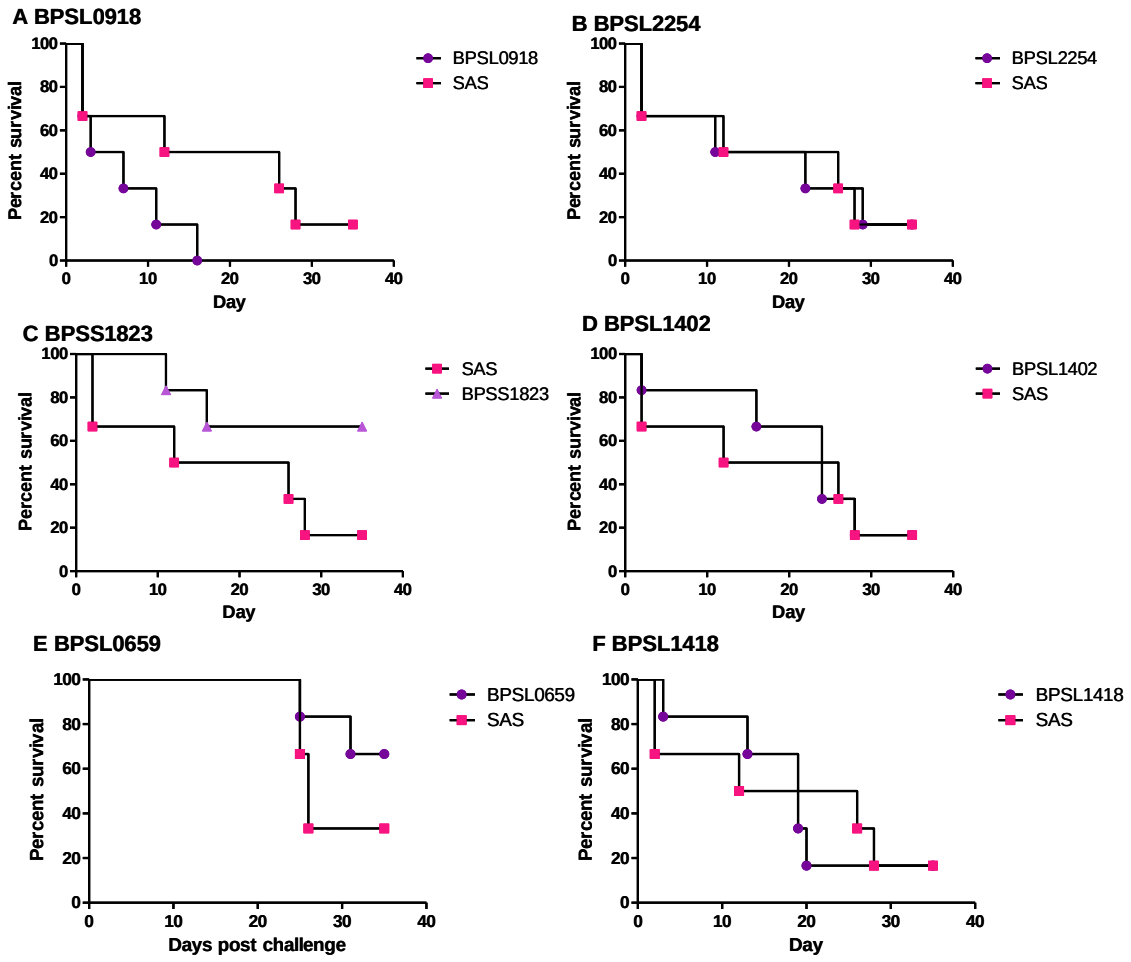


Figure 3.16. The protective efficacy following immunisation of BALB/c mice with recombinant PPIases.

Six mice per group were immunised three times with recombinant PPIases and SAS or SAS only, at two weekly intervals. Five weeks after the last immunisation the mice were challenged with 3.6 x 10^4 cfu/ml (36 x MLD; A, B, C, D and F) or 3.4 x 10^4 cfu/ml (34 x MLD; E) *B. pseudomallei* K96243 by the intraperitoneal route. The mice were monitored for signs of disease for five weeks after which the surviving animals were culled.

A – BPSL0918; B – BPSL2254;
C – BPSS1823; D – BPSL1402;
E – BPSL0659; F – BPSL1418.

Protein	Endpoint antibody titre	Median time to death (days)	Mean bacterial burden in the spleen (cfu/ml)
BPSL0918 *	1:102400	5	n/a
BPSL2254 *	1:102400	16.5	0
BPSS1823 *	1:12800	>35	3.8×10^6
BPSL1402 *	1: 819200	24	1×10^6
BPSL0659 **	1:819200	>35	2.5×10^7
BPSL1418 *	1:409600	19	0
SAS *	n.d.	19	0
SAS **	n.d.	26	5.4×10^7

Table 3.8. Immune responses and protective efficacy following immunisation of BALB/c mice with recombinant PPIases and challenge with *B. pseudomallei.*

The approximate endpoint antibody titres from mice immunised with recombinant PPIases. The endpoint was determined as the last diluted sample that gives positive results (>0.1 above background) by ELISA. The median time to death for mice immunised with recombinant PPIases. The mean bacterial burden in the spleens of the survivors.

***** - Mice challenged with 3.6×10^4 cfu/ml
** - Mice challenged with 3.4×10^4 cfu/ml

3.3 Discussion

The sequencing of *B. pseudomallei* K96243 genome has provided insights into pathogenesis mechanisms and putative virulence factors (Holden *et al.,* 2004; Chong *et al.,* 2006; Tumapa *et al.,* 2008; Sim *et al*., 2008). Bioinformatic tools have enhanced the comparative analysis of genomes, both between species and within isolates. A range of bioinformatics approaches were used to identify FKBPs and parvulins from *B. pseudomallei,* resulting in some overlap and any duplications were amalgamated. However, the possibility exists that some were not detected using these techniques. Higher eukaryotes such as *H. sapiens* encode at least 15 FKBPs and 2 parvulins and *A. thaliana* encodes 23 FKBPs and 3 parvulins (Galat, 2003; He *et al.,* 2004). Despite the small size of their genomes, prokaryotes encode a functionally diverse range of FKBPs and parvulins (Fischer and Aumuller, 2003). In *B. pseudomallei,* six genes encode FKBP-like proteins and three genes encode parvulin-like proteins. This is comparable to other prokaryotes, with *E. coli* K-12 encoding four FKBPs and one parvulin (Riley, 1998).

The conservation of *B. pseudomallei* PPIases in *B. mallei* or *B. thailandensis* was determined by searching their respective genomes. While most PPIases were well conserved, BPSS1823, a Mip homologue, was not identified in *B. mallei*. The absence of a Mip-like protein in *B. mallei,* but presence in *B. thailandensis* suggests that it is not required for the obligate pathogenic lifecycle of *B. mallei*. Sequence analysis of BPSS1823 revealed that it lacks the N-terminal domain, which is required for dimerization of Lp-Mip (Riboldi-Tunnicliffe *et al.,* 2001). However, BPSS1823 has high homology to the C-terminal PPIase domain of Lp-Mip and amino acids required for enzyme activity in FKBPs are

well conserved in BPSS1823 (Cianciotto *et al.,* 1989; Ikura and Ito, 2007; Ceymann *et al.,* 2008; Löw *et al*., 2010). Comparison of the crystal structure of BPSS1823 to the structure of Lp-Mip revealed a similar active site configuration. This sequence and structural homology indicates that BPSS1823 probably does not form dimers but is likely to be enzymatically active and may have a similar function as other bacterial Mips.

In addition to a Mip homologue, several proteins with putative SurA domains were identified in *B. pseudomallei.* BPSL0659 showed the highest sequence homology to SurAs from other bacteria and sequence analysis revealed an N-terminal chaperone domain and conserved PPIase domains (Behrens *et al.,* 2001), suggesting that BPSL0659 may possess both chaperone and enzyme activity. SurA homologues were also encoded by *B. mallei* and *B. thailandensis*, indicating that SurA may be required for both survival in the environment and as a pathogen.

B. pseudomallei PPIases were predicted to vary in size from single-domain PPIases to multi-domain PPIases. This size variation may correlate to the different targets of PPIases in *B. pseudomallei.* For example, BPSS1823 is a small protein, containing a single PPIase domain, indicating that enzyme activity alone may be crucial for function. PPIases from *B. pseudomallei* were also predicted to be located in most subcellular compartments, demonstrating the potential wide ranging function of these proteins. This is consistent with PPIases from other prokaryotes, which can vary in size, domain arrangement and cellular distribution (reviewed by Barik, 2006). Mip proteins are usually membrane located or secreted, while *E. coli* SurA is periplasmic (Cianciotto *et al.,* 1989; Moro *et al.,* 1995; Rouvière and Gross, 1996; Leuzzi *et al.,* 2005; Neff

et al., 2007). However, both BPSS1823 and BPSL0659 were predicted to be located in the periplasm of *B. pseudomallei.* BPSL0659 was also predicted to have an N-terminal signal peptide of 29 amino acids, further supporting that it is periplasmic or secreted. Future work to determine the cellular location could be done by detection in membrane, periplasmic or cytosolic preparations using an anti-BPSS1823 or anti-BPSL0659 antibody.

Using a protease coupled assay, the PPIase activity of BPSS1823 and BPSL0659 was successfully determined, indicating they represent a functional FKBP and parvulin from *B. pseudomallei*. Furthermore, the specificity constant k_{cat}/K_m of BPSS1823 is > 5 times higher than that of Lp-Mip under the same conditions (Wintermeyer *et al.,* 1995). This may indicate the importance of the PPIase activity in the function of BPSS1823.

In contrast, no activity was detected with recombinant BPSL0918 under these conditions. As some PPIases show varying substrate specificity, Suc-Ala-Ala-Pro-Phe-4-nitroanilide or Suc-Ala-Leu-Pro-Phe-4-nitroanilide were also tested (Fischer *et al.,* 1992). However, no activity was detected in BPSL0918 using these peptides. Although this assay is the simplest to perform, there are limitations to its use. Only the *cis* → *trans* isomerisation is measured and some PPIases are highly susceptible to proteolytic degradation. Therefore, a protease-free assay was developed to measure the PPIase activity of protease sensitive proteins (Kofron *et al*., 1991). To eliminate the possibility that BPSL0918 was digested by chymotrypsin, the recombinant protein was tested in this alternative assay. However, no PPIase activity was detected using protease free assay either. Scrutiny of the amino acid sequence of BPSL0918

indicates that several residues required for PPIase activity in other FKBPs are not conserved (Ikura & Ito, 2007; Ceymann *et al*., 2008) which may explain the lack of activity.

The PPIase activity of Lp-Mip has been shown to be directly related to its role in virulence (Lundemose *et al.,* 1993; Helbig *et al*., 2003; Wagner *et al*., 2007), therefore BPSS1823 may have a similar role in *B. pseudomallei* pathogenesis. However, the contribution of PPIase activity for physiological function of some proteins has been debated (Wintermeyer *et al.,* 1995; Behrens *et a*l., 2001; Zhang *et al.,* 2007; Weininger *et al.,* 2009). Studies suggest that that the chaperone activity of SurA is independent of its PPIase activity (Behrens *et al.,* 2001; Watts and Hunstad, 2008). Therefore, the PPIase activity exhibited by BPSL0659 may not be required for full function and its primary role is as a chaperone instead. Similarly, although BPSL0918 is annotated as a 'Putative FkpB-type peptidyl-prolyl *cis-trans* isomerase', has 25% amino acid sequence identity to hFKBP12 and contains an 'FKBP-like' domain, it perhaps acts as a chaperone and has lost PPIase activity due to selective pressure. Future work could involve verification of chaperone activity of both BPSL0659 and BPSL0918 using a citrate synthase aggregation assay (Buchner *et al*., 1998).

Rapamycin or cycloheximide-N-ethylethanoate were shown to inhibit the PPIase activity of BPSS1823 at low concentrations, with an observed Ki of 5 pM and 6.5 ± 1.0 µM, respectively. This is comparable to inhibition of hFKBP12 and Lp-Mip with rapamycin (0.2 nM; 15 nM) and hFKBP12 with cycloheximide-N-ethylethanoate (4.1 µM; Bierer *et al.,* 1990a; Christener *et al*., 1999; Kőhler *et al.,* 2003). FK506 and rapamycin do not bind to parvulins and there are no

inhibitors that bind to all members of the parvulin family (Rahfeld *et al*., 1994). However, a human Pin1 inhibitor, such as Juglone (Hennig *et al.,* 1998) or chaperone inhibitors could be used to target the PPIase activity of BPSL0659.

The protective efficacy of recombinant *B. pseudomallei* PPIases was determined using a BALB/c mouse model. Although mice were challenged with more than 30 x MLD, this was not sufficient to kill all control mice. This may indicate that the adjuvant alone affords protection. While ELISAs revealed that IgG antibody responses were specific to the recombinant protein and not SAS, a cellular immune response may have been elicited. Alternatively, this could be a feature of the model used. BALB/c mice are usually highly susceptible to *B. pseudomallei* infection, resulting in an acute infection (Hoppe *et al.,* 1999). However, individual variations in susceptibility and a lower challenge dose may have resulted in a chronic infection which could be overcome by repeating with a higher challenge dose.

Mice immunised with recombinant BPSL0918 had a reduced time to death when compared to the SAS only control group. It is possible that exposure to BPSL0918 protein increased sensitivity to infection by reducing the effectiveness of macrophages to recognise bacteria. Alternatively, anti-BPSL0918 antibodies could be used for passive immunisation to test whether their presence predisposes mice to infection. In contrast, mice immunised with recombinant BPSS1823, BPSL1402 or BPSL0659 had in increased median time to death compared to the control group, although bacteria were recovered from the spleens of all surviving mice. These proteins therefore represent potential novel vaccine candidates against melioidosis. To improve protection, the protein could be conjugated to an immunogenic polysaccharide, such as

LPS, or an alternative adjuvant could be used. In addition, this data is from a single experiment (n=6) so additional repeats are required.

Previous studies using other recombinant proteins as protective antigens have shown varying levels of protection. Mice immunised with OMP3 or OMP7 then challenged with 1 x 10^6 cfu/ml *B. pseudomallei* lead to 50% survival (Hara *et al.,* 2009). Protection was also observed following immunisation of mice with recombinant LolC protein, with 80% survival following challenge with 4 x 10^4 cfu/ml *B. pseudomallei* (Harland *et al.,* 2007). In contrast, mice immunised with components of TTSS, BipB, BipC and BipD, showed no protection against *B. pseudomallei* challenge (Druar *et al*., 2007). Some recombinant PPIases have been shown to provide protection against other bacterial infections, with immunisation with a SurA homologue resulting in 50% survival following challenge with *B. abortus* (Delpino *et al*., 2007).

In conclusion, nine putative FKBPs and parvulins have been identified in *B. pseudomallei*. Bioinformatic characterisation of the amino acid sequences indicate the proteins vary in size, cellular location and function. A Mip and SurA homologue were also identified using sequence and predicted secondary structure analysis. Six FKBPs and parvulins were successfully expressed and purified as recombinant proteins. The Mip and SurA homologue were shown to have characteristic PPIase activity. In addition, the enzyme activity of the Mip homologue was inhibitable with rapamycin and cycloheximide-N-ethylethanoate. The protective efficacy of six recombinant FKBPs and parvulins was determined using BALB/c mouse model of *B. pseudomallei* infection, with three increasing the median time to death post-challenge.

Chapter 4 – Evaluation of a Mip homologue in *B. pseudomallei*

4.1 Introduction

Macrophage infectivity potentiators (Mips) are virulence-associated FKBPs encoded by several human and plant pathogens (Cianciotto *et al.,* 1989; Lundemose *et al*., 1993; Moro *et al.,* 1995; Horne *et al.,* 1997; Leuzzi *et al.,* 2005; Zang *et al.,* 2007). The prototypic Mip was first identified in 1989 as a 24 kDa surface-located protein in *L. pneumophila*. Inactivation of the *mip* gene resulted in reduced initiation of infection in macrophages, epithelial cells and protozoa (Cianciotto *et al.,* 1989; Cianciotto and Fields*.,* 1992; Cianciotto *et al.,* 1995) and attenuated virulence in a guinea pig model of Legionellosis (Cianciotto *et al.,* 1990).

Mips have subsequently been characterised in other medically important pathogens with similar phenotypes observed. Deletion of *mip* from *N. gonorrhoeae* or *S. enterica* resulted in reduced intracellular survival in macrophages and epithelial cells (Horne *et al.,* 1997; Leuzzi *et al*., 2005). Addition of anti-Mip monoclonal antibodies or the inhibitors FK506 or rapamycin caused defects in invasion of cells by *T. cruzi* or *C. trachomatis* (Lundemose *et al.,* 1993; Moro *et al*., 1993).

BPSS1823 was identified in Chapter 3 as encoding a Mip homologue in *B. pseudomallei*. Furthermore, it has been shown to have characteristic PPIase activity, inhibitable by rapamycin or cycloheximide-N-ethylethanoate (Figures 3.12 -3.14). The aim of this chapter is to evaluate the role of BPSS1823 in

virulence of *B. pseudomallei*. The approach is to make unmarked deletion mutants of *BPSS1823* in *B. pseudomallei* K96243 and in an efflux pump mutant, *B. pseudomallei* Δ*amrA*. Efflux pump mutants are hypersusceptible to aminoglycosides and macrolides and an advantage to using these strains in cell infection assays is that a lower antibiotic concentration is required (Moore *et al*., 1999). Both mutant strains will be characterised for defects in intracellular infection of cell lines and virulence in a BALB/c model of *B. pseudomallei* infection.

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4.2 Results

4.2.1 Deletion of *BPSS1823* in *B. pseudomallei*

4.2.1.1 Production of a deletion construct

The cloning strategy used to produce the construct for deletion of *BPSS1823* is outlined in Figure 4.1. Upstream and downstream flanking regions of *BPSS1823* were PCR amplified from *B. pseudomallei* K96243 genomic DNA using BPSS1823.PDM4.LFF, BPSS1823.PDM4.LFR, BPSS1823.PDM4.RFF and BPSS1823.PDM4.RFR (Table 2.9). PCR products were cloned into pCR Blunt II-TOPO and the inserts confirmed by nucleotide sequencing. The flanking regions were digested out of pCR Blunt II-TOPO using *Bgl*II and *Xba*I and ligated into the *Xba*I site of pDM4. Ligations were transformed into *E. coli* DH5α λpir and plated onto selective LB agar containing 30 µg/ml chloramphenicol. Colonies containing the correct construct were confirmed after agarose gel electrophoresis of restriction digests and by nucleotide sequencing of the insert DNA.

4.2.1.2 Mutant production

The mutant making strategy adopted is outlined in Figure 4.2 (Logue *et al.,* 2009). First, the pDM4 deletion construct containing the *BPSS1823* flanking regions was transformed into *E. coli* S17 λpir. The plasmid was transferred by conjugation into wildtype *B. pseudomallei* K96243 or *B. pseudomallei* K96243 Δ*amrA*. The first cross-over event resulted integration of the deleted allele into the host chromosome by homologous recombination. Merodiploid integrants were identified on selective media containing 30 µg/ml chloramphenicol and confirmed by colony PCR. The *sacB* gene encoded by pDM4 rendered the integrants sensitive to sucrose, allowing counter-selection

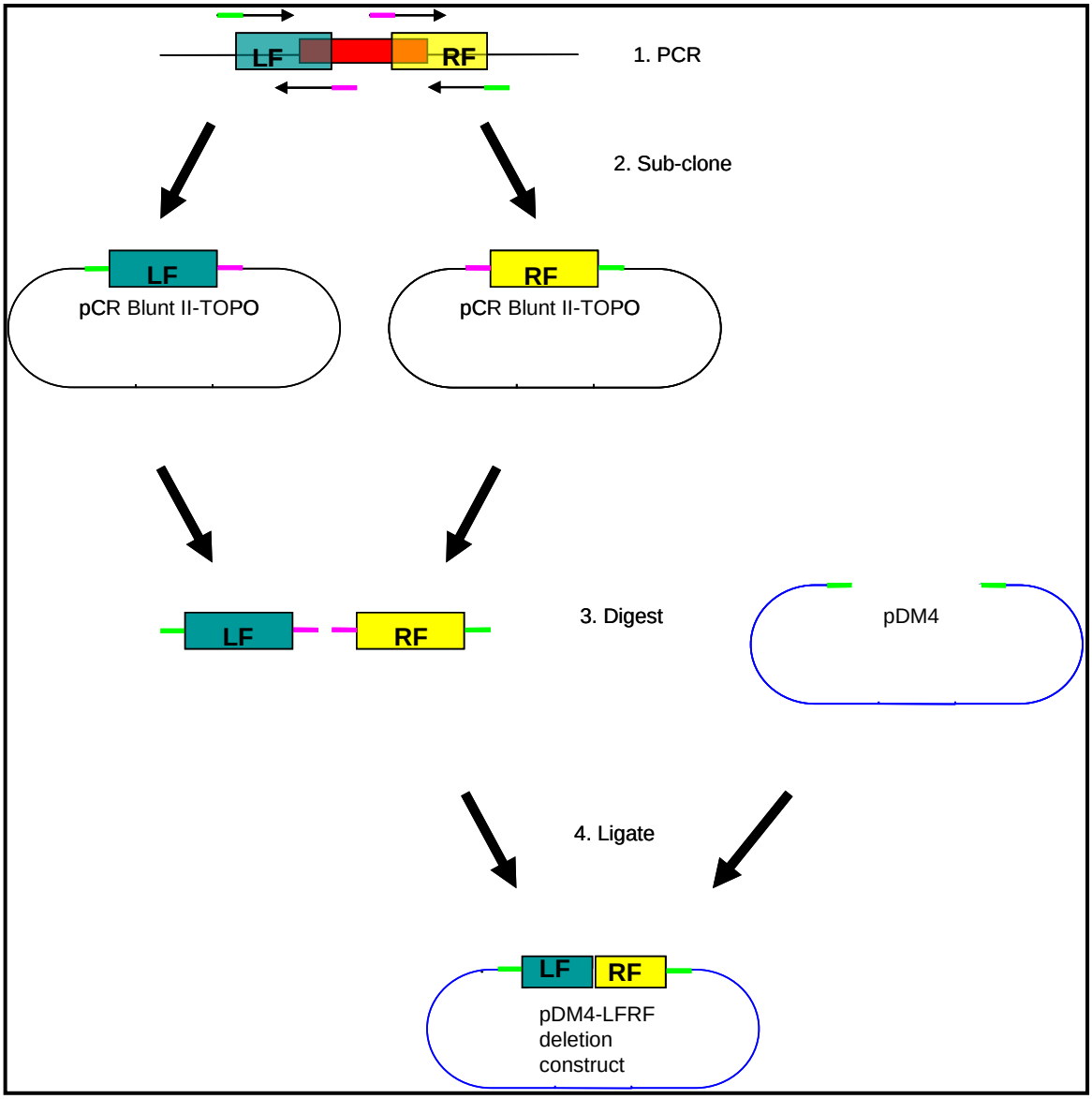


Figure 4.1. Cloning strategy used to produce deletion construct.

1. Flanking regions upstream (shown in yellow) and downstream (shown in turquoise) of the target gene (shown in red) were PCR amplified from genomic DNA using primers containing specific restriction sites (pink - *BlgII* and green - *XbaI*)
2. PCR product was cloned into pCR Blunt II – TOPO
3. pDM4 and inserts were digested with specific restriction endonucleases
4. Inserts were ligated into restriction sites in pDM4.

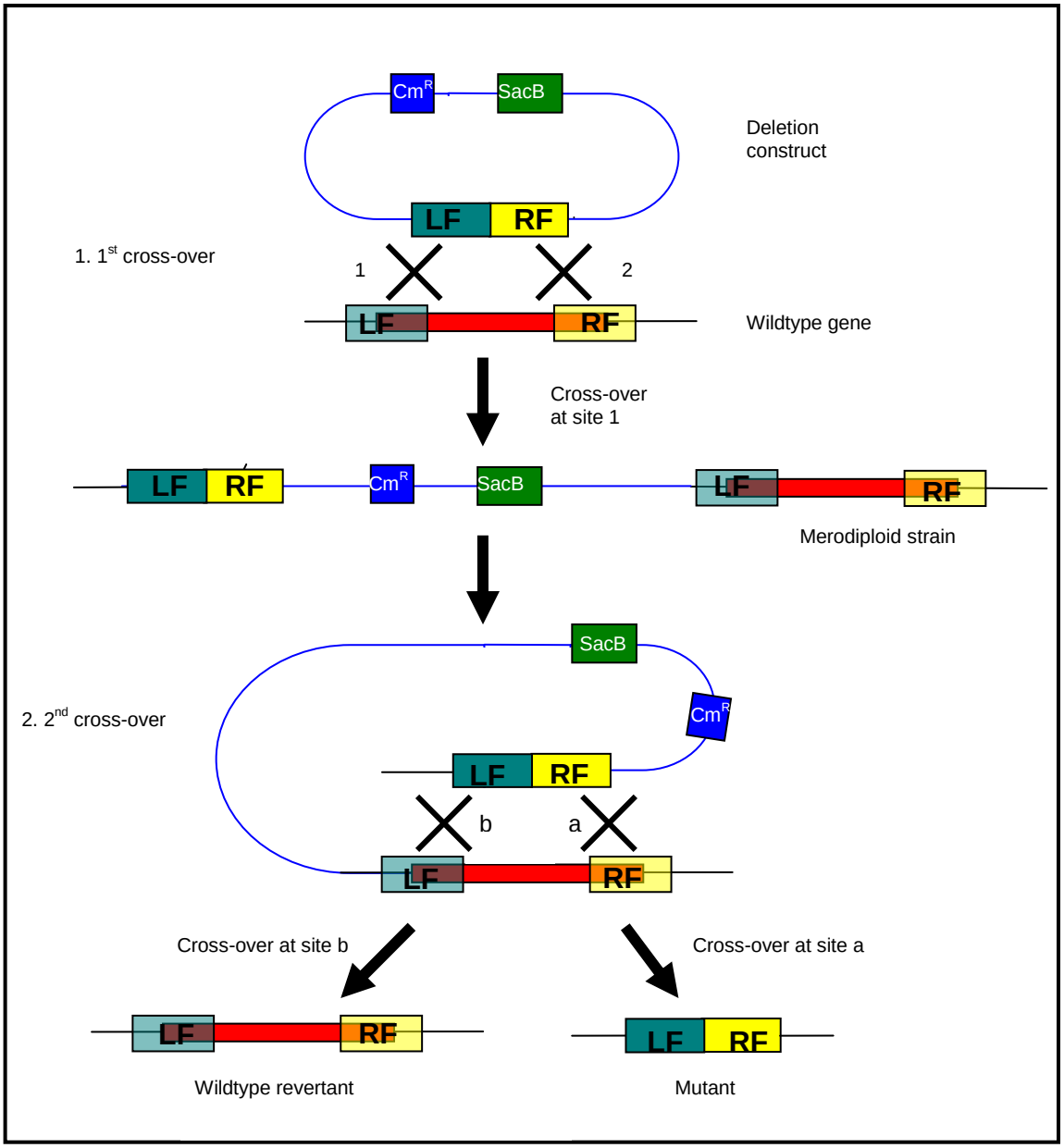


Figure 4.2. Production of a deletion mutant using pDM4, adapted from Logue *et al*., 2009.

1. Deletion construct was conjugated into wildtype *B. pseudomallei.* The deletion construct integrated into the genome by homologous recombination (shown for site 1, but can occur at site 2). The merodiploid integrant was selected for by resistance to chloramphenicol.
2. The pDM4 backbone was excised from the genome, resulting in allelic exchange (site a) and generation of a deletion mutant or a wildtype revertant (site b). Strains are selected for by resistance to sucrose and sensitivity to chloramphenicol.

Cm^R – chloramphenicol resistance cassette
SacB – SacB gene
___ - *B. pseudomallei* genome
___ - pDM4 vector

for excision of the vector by a second cross-over event. Colonies were screened for sensitivity to chloramphenicol and screened by PCR. From 18 chloramphenicol sensitive *B. pseudomallei* K96243 colonies screened, 4 mutants were identified. From 16 chloramphenicol sensitive *B. pseudomallei* Δ*amrA* colonies screened, 1 was confirmed as a mutant (data not shown). Southern hybridization was used to confirm a 171 bp deletion in the *BPSS1823* allele (Figure 4.3, A, B) and the mutant strains named *B. pseudomallei*Δ*BPSS1823* or *B. pseudomallei*Δ*amrA* Δ*BPSS1823.*

4.2.1.3 Complementation of mutant strain

The gene encoding *BPSS1823* was PCR amplified from *B. pseudomallei* K96243 genomic DNA and cloned into pCR Blunt II-TOPO. The sequence of the amplified gene was confirmed by nucleotide sequencing. The gene was excised from pCR Blunt II-TOPO using restriction endonucleases and ligated into the *Eco*RI/*Xba*I sites of pBBRI-MCS2. Ligations were transformed into *E. coli* JM109 and plated onto selective LB agar containing 50 µg/ml kanamycin. Colonies containing the correct construct were confirmed by restriction digest and nucleotide sequencing.

The complementation construct was transferred to *B. pseudomallei* Δ*amrA* Δ*BPSS1823* by three-way conjugation using *E. coli* HB101 (pRK2013) as a helper strain. Mutant bacteria containing the complementation plasmid were plated onto selective LB agar containing 50 µg/ml kanamycin and confirmed by colony PCR, using BPSS1823.PBBR1F; BPSS1823.PBBR1R; KAN.F and KAN.R (Table 2.10; Figure 4.3, C). Expression of BPSS1823 was induced by addition of 1 mM IPTG to the growth media. The complemented mutant was named *B. pseudomallei* Δ*amrA* Δ*BPSS1823 pBR1823.*

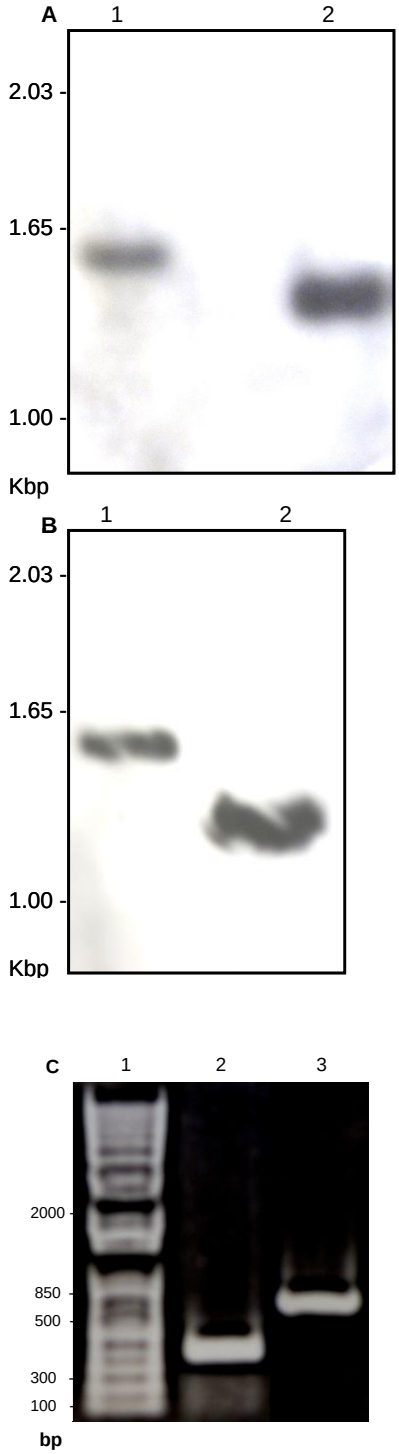


Figure 4.3. Confirmation of *BPSS1823* mutant and complemented mutant strains.

A – Southern hybridization showing deletion of *BPSS1823* in *B. pseudomallei* K96243
B – Southern hybridization showing deletion of *BPSS1823* in *B. pseudomallei* K96243 Δ*amrA*

Lane 1 – wildtype genomic DNA digested with *Bam*HI and *Cla*I (1.55 kbp); 2 – mutant genomic DNA digested with *Bam*HI and *Cla*I (1.38 kbp). Probed with Dig-labelled left-flank. Kbp ladder shows the sizes of marker DNA.

C – Colony PCR showing presence of *BPSS1823* and kanamycin cassette in *B. pseudomallei* Δ*amrA* Δ*BPSS1823*. Bp ladder shows the sizes of marker DNA.

Lane 1 – 1 Kb+ marker; 2 - BPSS1823 gene (342 bp) 3 – kanamycin resistance gene (750 bp)

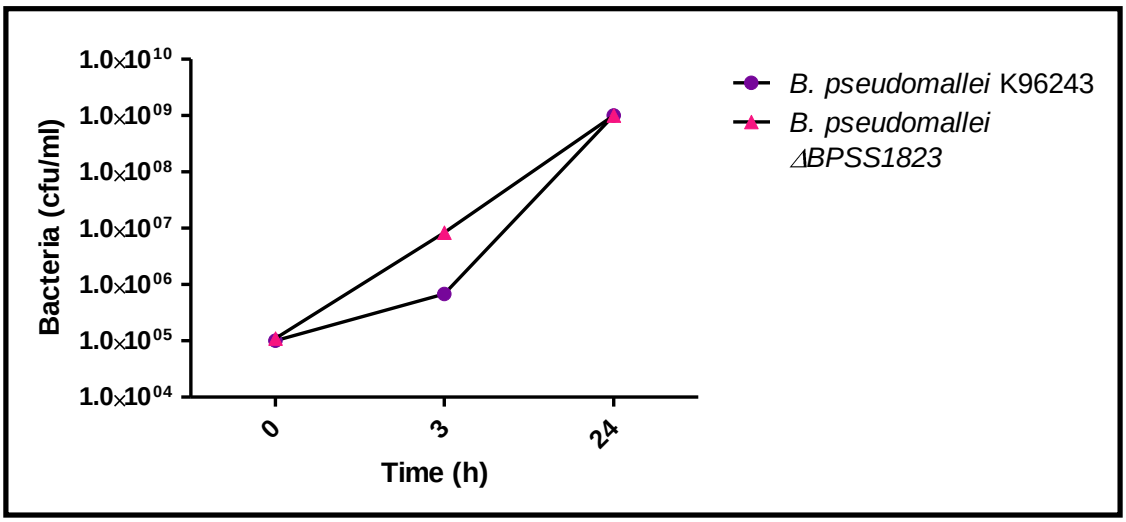
4.2.2 *In vitro* characterisation of *BPSS1823* mutant strains

4.2.2.1 Growth in media

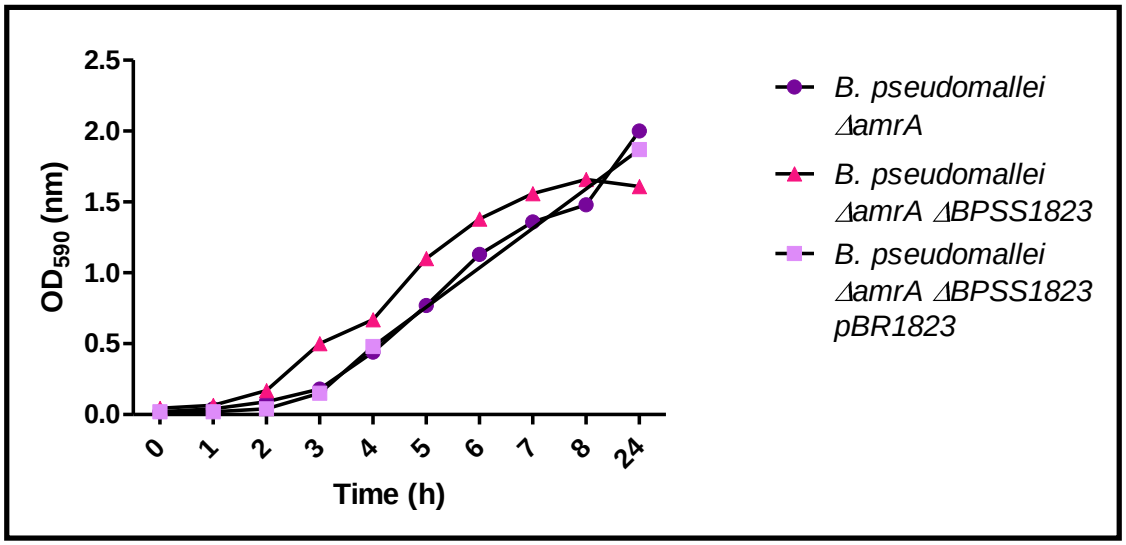
The growth rate of *B. pseudomallei*∆*BPSS1823*, *B. pseudomallei*Δ*amrA* ∆*BPSS1823* or *B. pseudomallei*Δ*amrA* ∆*BPSS1823 pBR1823* was determined in liquid media. A 2 ml overnight culture was used to inoculate 100 ml LB media and growth was monitored over 24 h. Deletion of *BPSS1823* or complementation of the mutant strain did not result in restricted growth (Figure 4.4) and both parent strains grew at the same rate (data not shown).

4.2.2.2 Growth in J774 macrophages

B. pseudomallei can survive and replicate in a range of cell lines (Jones *et al*., 1996). The effect of *BPSS1823* deletion on *B. pseudomallei* uptake, survival and growth in a phagocytic cell line was determined by infection of J774 macrophages with either *B. pseudomallei* K96243; *B. pseudomallei*∆*BPSS1823*; *B. pseudomallei*Δ*amrA*; *B. pseudomallei*Δ*amrA* ∆*BPSS1823* or *B. pseudomallei*Δ*amrA* ∆*BPSS1823 pBR1823,* at an MOI of 1. After incubation for 30 min to allow uptake of bacteria, the extracellular bacteria were killed with 1 mg/ml kanamycin (wildtype), for 1 h, or 30 µg/ml gentamicin (efflux pump mutant), for 30 min. At 0, 2, 4 and 24 h after extracellular killing, infected cells were lysed and the number of viable bacteria within the cells was determined. Following infection with *B. pseudomallei*∆*BPSS1823,* there was an average of 32% fewer bacteria present at each timepoint, when compared to *B. pseudomallei* K96243 (Figure 4.5, A). However this defect in intracellular growth was not significantly different. In contrast, there was significantly fewer *B. pseudomallei* Δ*amrA* ∆*BPSS1823* present following 24 h of growth, when compared to *B. pseudomallei* Δ*amrA* ($P<0.001$; Figure 4.5, B).



A

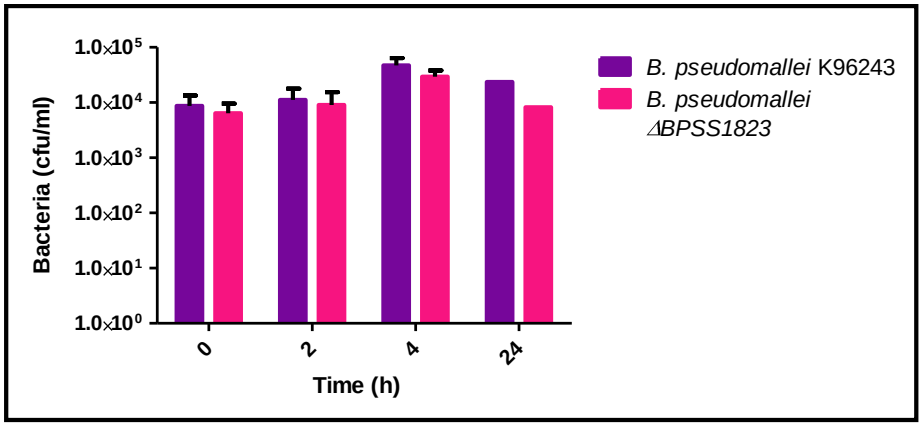


B

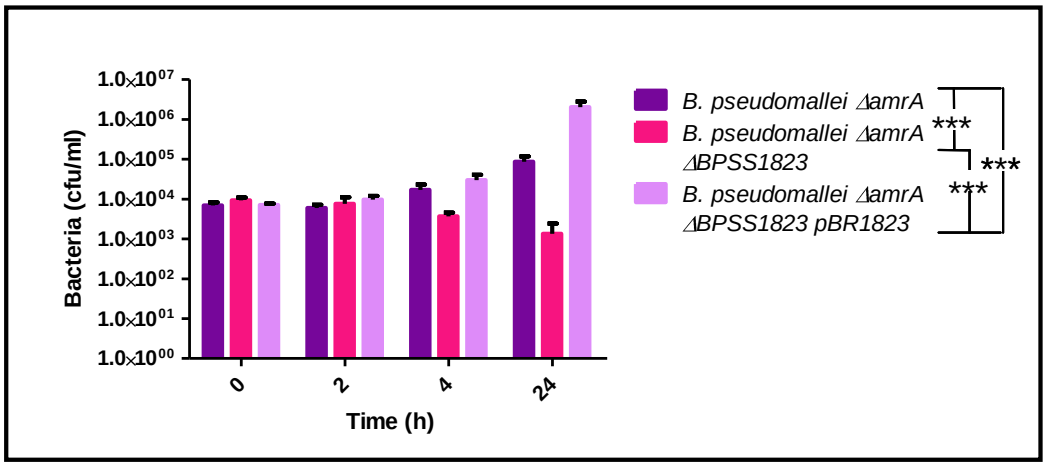
Figure 4.4. The growth of *BPSS1823* mutants in LB broth at 37ºC. Growth was monitored over 24 h by viable counts or absorbance at 590 nm.

A – The growth of *B. pseudomallei* Δ*BPSS1823* compared to *B. pseudomallei* K96243
B – The growth *B. pseudomallei* Δ*amrA* Δ*BPSS1823* compared to *B. pseudomallei* Δ*amrA*

Values are from a single experiment.



A



B

Figure 4.5. Survival of *BPSS1823* mutant strains in J774 macrophages over 24 h. Cells incubated with bacteria for 30 min at an MOI of 1. Extracellular bacteria were killed with 1 mg/ml kanamycin (A) or 30 µg/ml gentamicin (B). Intracellular bacterial numbers determined at 0, 2, 4 and 24 h after extracellular killing.

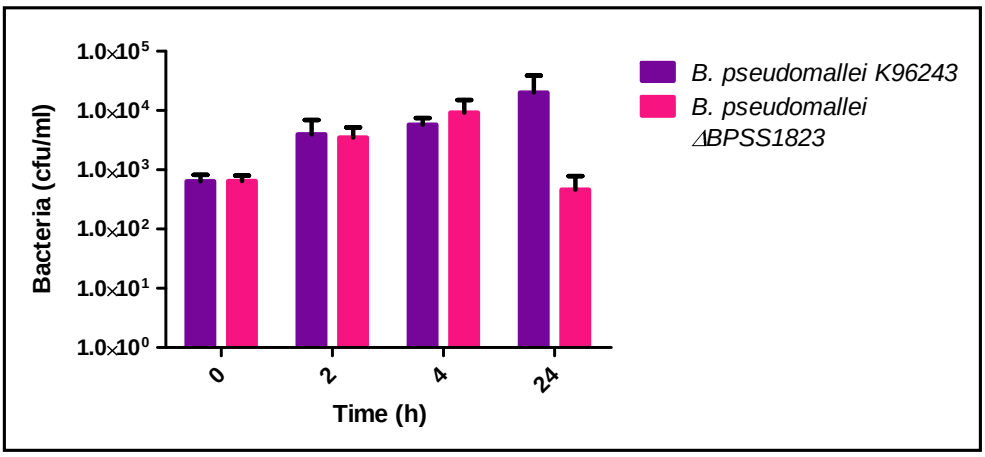
A – Cells were infected with *B. pseudomallei* K96243 or *B. pseudomallei* Δ*BPSS1823*
B – Cells were infected with *B. pseudomallei ΔamrA*; *B. pseudomallei ΔamrA* Δ*BPSS1823* or *B. pseudomallei ΔamrA* Δ*BPSS1823 pBR1823*

Values are the means from triplicate experiments, ± standard errors. P values are shown for the comparison of intracellular bacteria 24 h post infection.

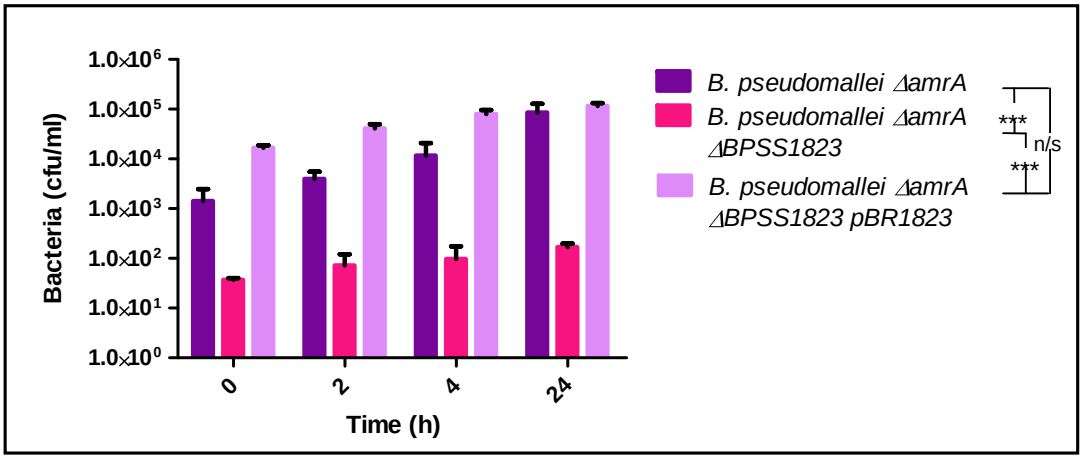
Complementation of *B. pseudomallei* Δ*amrA* ∆*BPSS1823* fully restored intracellular growth to wildtype levels (P<0.001). Deletion of *BPSS1823* had no effect on sensitivity to gentamicin (Figure 4.8, E).

4.2.2.3 Growth in A549 epithelial cells

To determine whether deletion of *BPSS1823* also affects *B. pseudomallei* invasion, survival and growth in a non-phagocytic cell line, A549 epithelial cells were infected with either *B. pseudomallei* K96243; *B. pseudomallei*∆*BPSS1823*; *B. pseudomallei*Δ*amrA*; *B. pseudomallei* Δ*amrA* ∆*BPSS1823* or *B. pseudomallei*Δ*amrA* ∆*BPSS1823 pBR1823,* at an MOI of 10. After incubation for 1 h to allow uptake of bacteria, the number of viable bacteria within the cells was determined as described in section 4.2.2.2. There was no defect in the ability of *B. pseudomallei*∆*BPSS1823* to infect cells and initial intracellular growth was comparable with wildtype. However, 24 h post infection, there were 10-fold fewer mutant bacteria present when compared to *B. pseudomallei* K96243 (Figure 4.6, A). Following infection with *B. pseudomallei* Δ*amrA* ∆*BPSS1823*, there was significant fewer bacteria within the cells 0 h after extracellular killing, compared to *B. pseudomallei* Δ*amrA* (P<0.01; Figure 4.6, B). Furthermore, while the number of intracellular *B. pseudomallei*Δ*amrA* increased 60-fold over 24 h, the *BPSS1823* mutant strain exhibited almost no replication (P<0.001; 24 h). Complementation of *B. pseudomallei* Δ*amrA* ∆*BPSS1823* fully restored both the ability to invade epithelial cells and intracellular replication to parent levels (P<0.001).



A



B

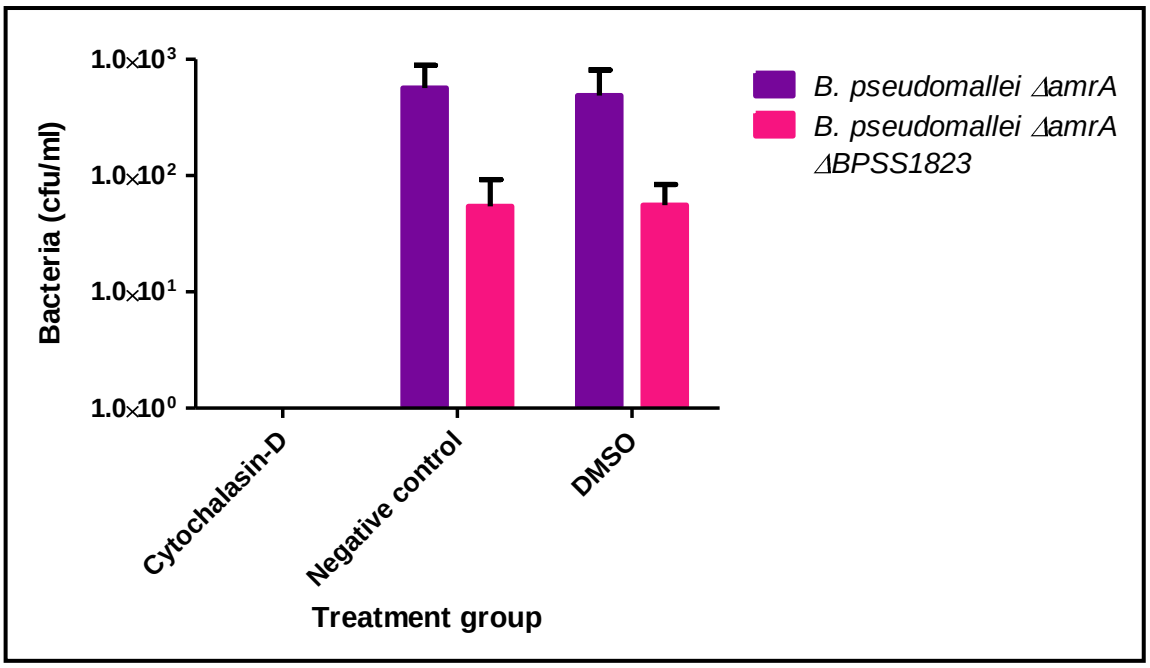
Figure 4.6. Survival of *BPSS1823* mutant strains in A549 epithelial cells over 24h. Cells incubated with bacteria for 30 min at an MOI of 10. Extracellular bacteria were killed with 1 mg/ml kanamycin (A) or 30 µg/ml gentamicin (B) for 1h. Intracellular bacterial numbers determined at 0, 2, 4 and 24 h after extracellular killing.

A – Cells were infected with *B. pseudomallei* K96243 or *B. pseudomallei* Δ*BPSS1823*
B – Cells were infected with *B. pseudomallei ΔamrA*; *B. pseudomallei ΔamrA* Δ*BPSS1823* or *B. pseudomallei ΔamrA* Δ*BPSS1823 pBR1823*

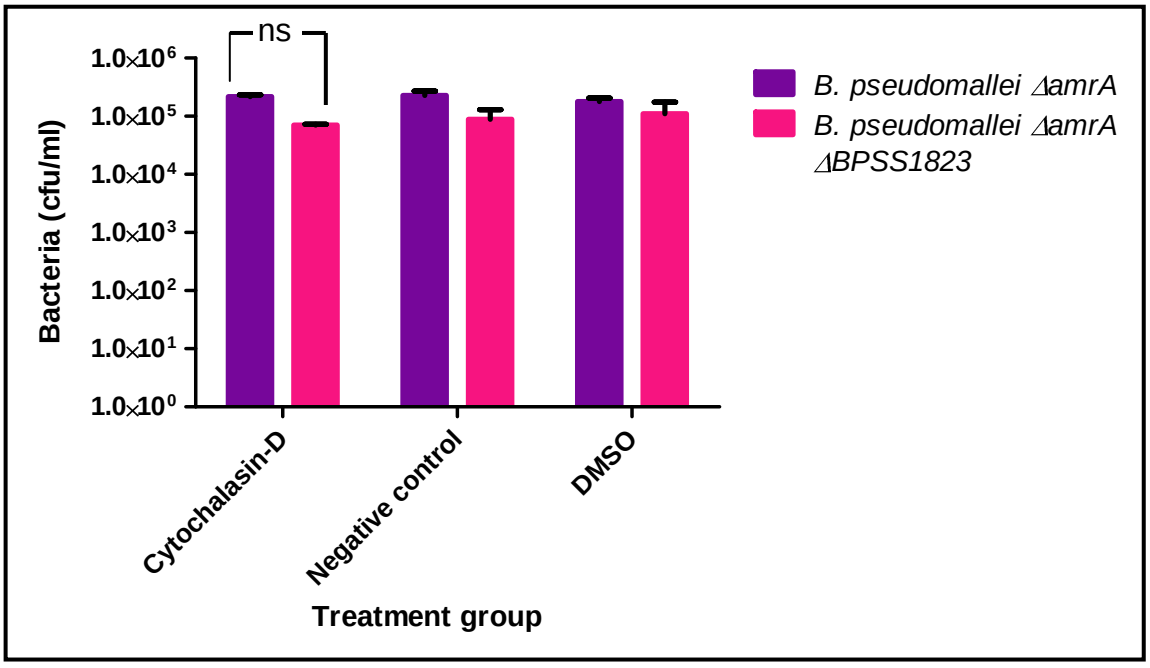
Values are the means from triplicate experiment, ± standard errors. P values are shown for the comparison of intracellular bacteria 24 h post infection.

4.2.2.4 Adherence to A549 epithelial cells

Following infection of A549 cells with *B. pseudomallei* Δ*amrA* ∆*BPSS1823,* significantly fewer intracellular bacteria were isolated 0 h after extracellular killing**,** compared to *B. pseudomallei* Δ*amrA* ($P<0.01$; Figure 4.6, B). To determine whether deletion of *BPSS1823* affects adherence of bacteria to the cell surface, cells were infected with either *B. pseudomallei* Δ*amrA* ∆*BPSS1823* or *B. pseudomallei* Δ*amrA*, at an MOI of 10. 30 min before infection, 1 µg/ml cytochalasin-D was used to inhibit phagocytosis so adherent bacterial numbers could be monitored. After incubation for 1 h to allow adherence of bacteria, non-adherent bacteria were removed by washing with PBS or, in parallel samples, extracellular bacteria were killed with 30 µg/ml gentamicin. Cells were lysed and the number of viable bacteria adhered to or inside of the cells was determined. Cytochalasin-D or DMSO was present throughout the assay. Cells treated with cytochalasin-D, infected with *B. pseudomallei* and extracellular bacteria killed had no intracellular bacteria, confirming that phagocytosis was fully inhibited (Figure 4.7, A). To monitor adherent bacteria, phagocytosis was inhibited with cytochalasin-D and non-adherent bacteria removed by washing. There was no significant difference between the amount of *BPSS1823* mutant bacteria adhered to the cells, compared to *B. pseudomallei*Δ*amrA* (Figure 4.7, B), indicating that *BPSS1823* is not involved in initial adherence to A549 cells.



A



B

Figure 4.7. Adherence of *B. pseudomallei ΔamrA* or *B. pseudomallei ΔamrA ΔBPSS1823* to A549 epithelial cells. Cells and bacteria were treated with cytochalasin-D or DMSO or untreated (negative control).

A – Cells were infected with *B. pseudomallei ΔamrA* or *B. pseudomallei ΔamrA ΔBPSS1823* for 1 h at an MOI of 10. Extracellular bacteria killed with 30 µg/ml gentamicin and intracellular bacterial numbers were determined 0 h after extracellular killing.
B – Cells were infected with *B. pseudomallei ΔamrA* or *B. pseudomallei ΔamrA ΔBPSS1823* at an MOI of 10. Non-adhered bacteria were removed by washing with PBS and bacterial numbers were determined 0 h after washing.

Values are the means from triplicate experiments, ± standard errors.

4.2.2.5 Exposure to environmental stresses

Deletion of *BPSS1823* in the efflux pump mutant of *B. pseudomallei* significantly reduced intracellular survival within cell lines compared with the parent strain (Figures 4.5 and 4.6). Therefore, the phenotype of *B. pseudomallei*Δ*amrA* Δ*BPSS1823* was characterised in more detail.

Host cells produce a range of antimicrobial compounds in response to infection and intracellular pathogens, such as *B. pseudomallei,* have adopted strategies to overcome host defence mechanisms. As BPSS1823 is important for intracellular survival of *B. pseudomallei*, the concentrations of different antimicrobial conditions required to inhibit growth of the mutant or parent strain were determined. For oxidative stress, bacteria were grown with H_2O_2. For high-osmolarity stress, bacteria were grown with NaCl. For low-pH stress, bacteria were grown with HCl. Both *B. pseudomallei*Δ*amrA* and *B. pseudomallei*Δ*amrA* Δ*BPSS1823* exhibited similar levels of growth in the presence of the diluent, H_2O (Figure 4.8, A). No sensitivity to high-osmolarity stress was observed with either strain (Figure 4.8, B). In contrast, neither strain grew under high oxidative stress, with a minimum inhibitory concentration of 1 – 0.5 µg/ml H_2O_2 (Figure 4.8, C). Interestingly, while *B. pseudomallei*Δ*amrA* grew well under low-pH stress, there was almost complete inhibition of growth of *B. pseudomallei* Δ*amrA* Δ*BPSS1823* at 64 µg/ml HCl (Figure 4.8, D).

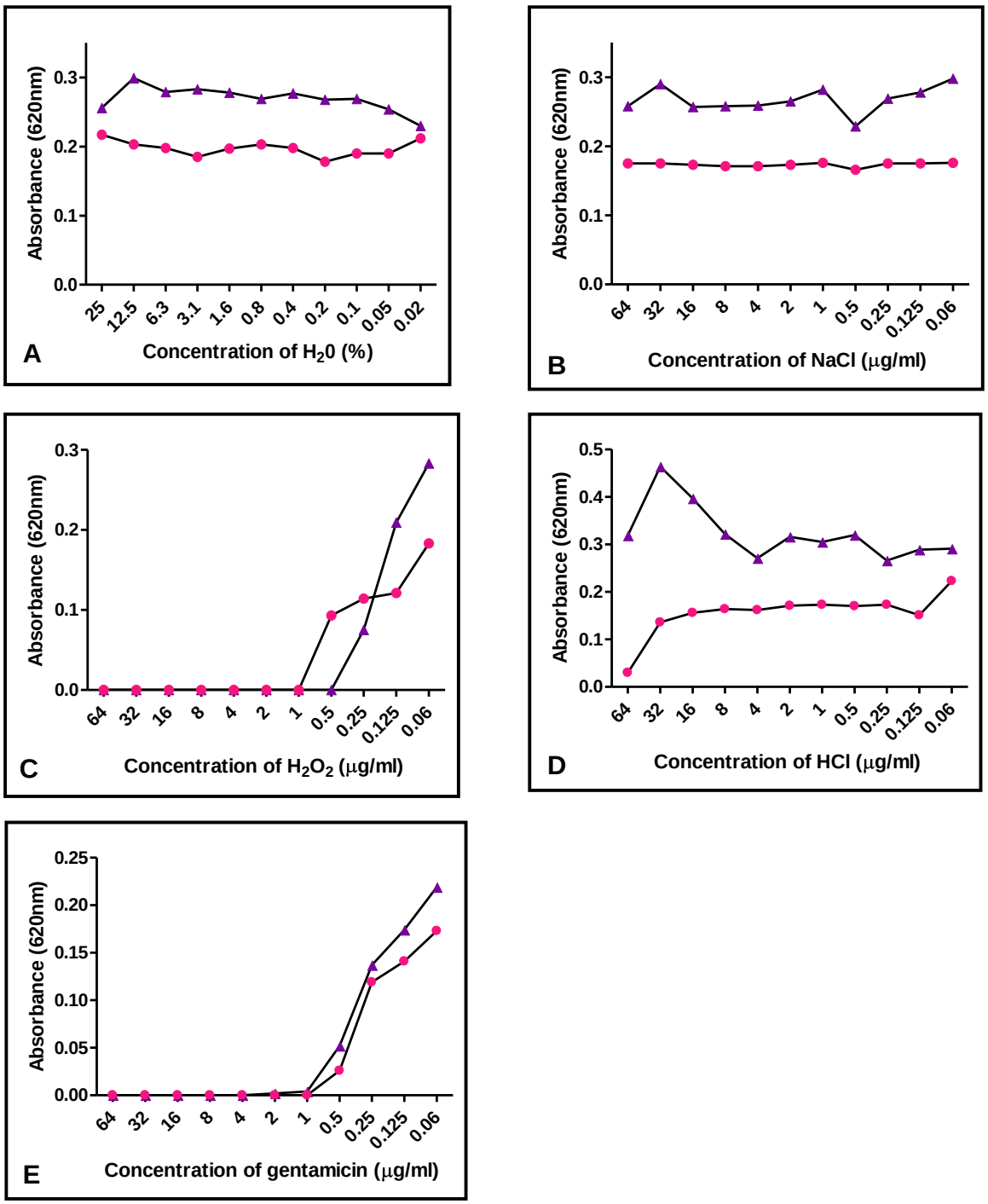


Figure 4.8. Growth of *B. pseudomallei* Δ*amrA* (purple triangles) or *B. pseudomallei* Δ*amrA* Δ*BPSS1823* (pink circles) in different environmental stresses. Compounds were diluted in LB broth to final concentrations of 64 – 0.06 µg/ml and 100 µl ~2 x 10^5 cfu/ml bacteria added. Plates were incubated at 37ºC for 24 h and absorbance read at 620 nm. A negative control with no bacteria and a positive control with no antimicrobials were included. Background values were subtracted. Experiments were carried out in triplicate and values from a representative experiment are shown.

A – water
B – sodium chloride
C – hydrogen peroxide
D – hydrochloric acid
E - gentamicin

4.2.2.6 Exposure to low pH

B. pseudomallei Δ*amrA* Δ*BPSS1823* had restricted growth in 64 µg/ml HCl (Figure 4.8) so this was investigated further by monitoring growth in pH 1 – 7 and viable counts taken at 0, 3 and 24 h post inoculation. Following 24 h growth, both *B. pseudomallei*Δ*amrA* and *B. pseudomallei*Δ*amrA* Δ*BPSS1823* had not grown at pH 1 -3 but had grown to $>10^9$ cfu/ml at pH 5, 6 and 7. At pH 4 however, there was significantly less growth of *BPSS1823* mutant strain (Figure 4.9, A; P<0.001). This difference was only observed 24 h post inoculation with similar levels of both strains present at 0 and 3 h (Figure 4.9, B). This suggests that *BPSS1823* may provide resistance against acidification as a host killing mechanism within cells.

4.2.2.7 Measurement of swarming motility

As *BPSS1823* is required for intracellular survival, virulence mechanisms such as swarming motility and protease secretion were investigated. To determine whether deletion of *BPSS1823* affects motility in *B. pseudomallei,* the swarming motility of *B. pseudomallei*Δ*amrA* Δ*BPSS1823* was analysed. 1 µl overnight culture was inoculated into the centre of a 0.3% agar plate, the plate was incubated overnight to allow bacterial swarming and diameter of growth was measured. While inoculation with *B. pseudomallei*Δ*amrA* resulted a mean bacterial spread of 21.4 mm, *B. pseudomallei*Δ*amrA* Δ*BPSS1823* resulted in localised growth of 5.4 mm at the site of inoculation and significantly less bacterial spread (Figure 4.10; P<0.0001). Complementation of the mutant strain fully restored bacterial motility, resulting in significantly increased bacterial spread compared to both *B. pseudomallei*Δ*amrA* and *B. pseudomallei*Δ*amrA* Δ*BPSS1823* (Figure 4.10; P<0.0001).

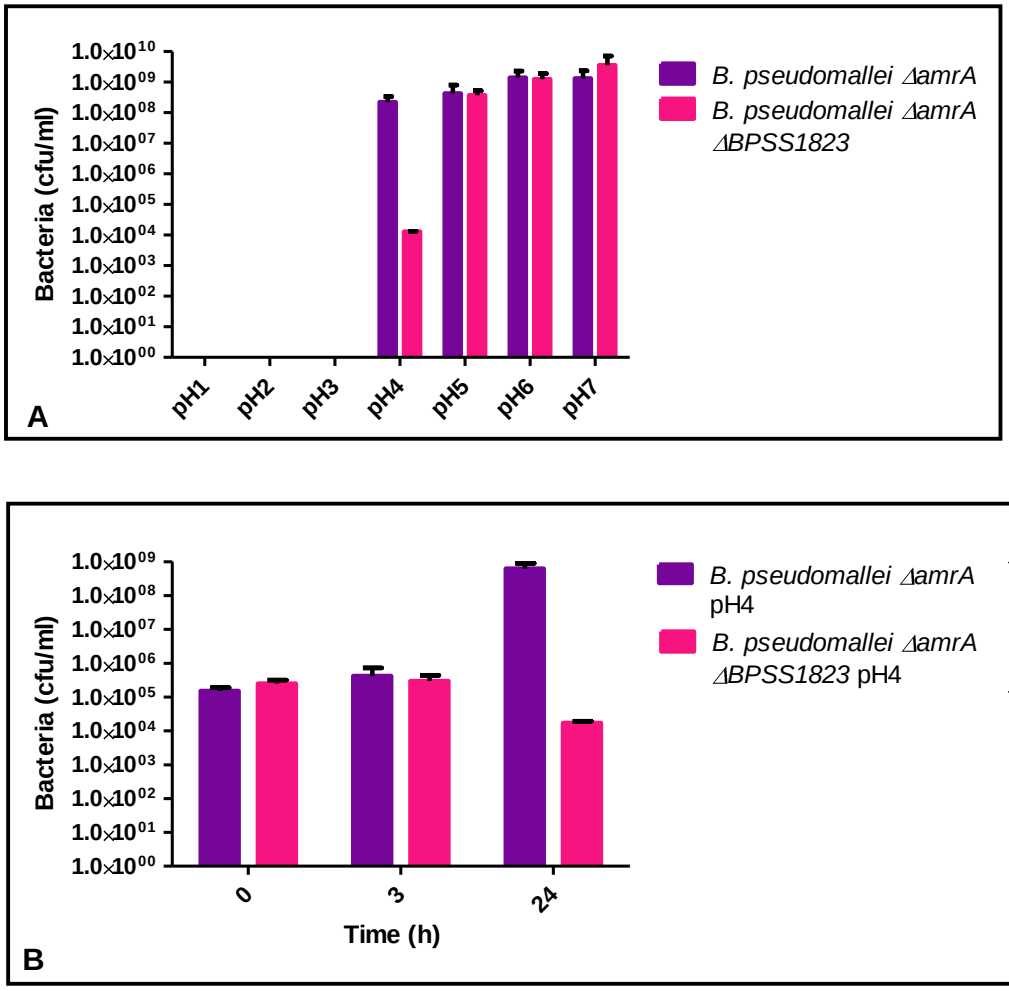


Figure 4.9. Growth of *B. pseudomallei ΔamrA* or *B. pseudomallei ΔamrA ΔBPSS1823* at different pHs. 100 µl ~2 x 10^5 cfu/ml bacteria was added to 10 ml LB broth adjusted to different pHs and incubated at 37ºC with agitation for 24 h. At timepoints post inoculation samples were removed to determine numbers of viable bacteria.

A – Bacteria grown in LB broth adjusted to pH 1 – 7 for 24 h. Values are the means from duplicate experiments, ± standard errors.
B – Bacteria grown in LB broth adjusted to pH 4 or pH7 at 0, 3 and 24 h post inoculation. Values are the means from triplicate experiments, ± standard errors. P values are shown for the comparison of strains 24 h post inoculation.

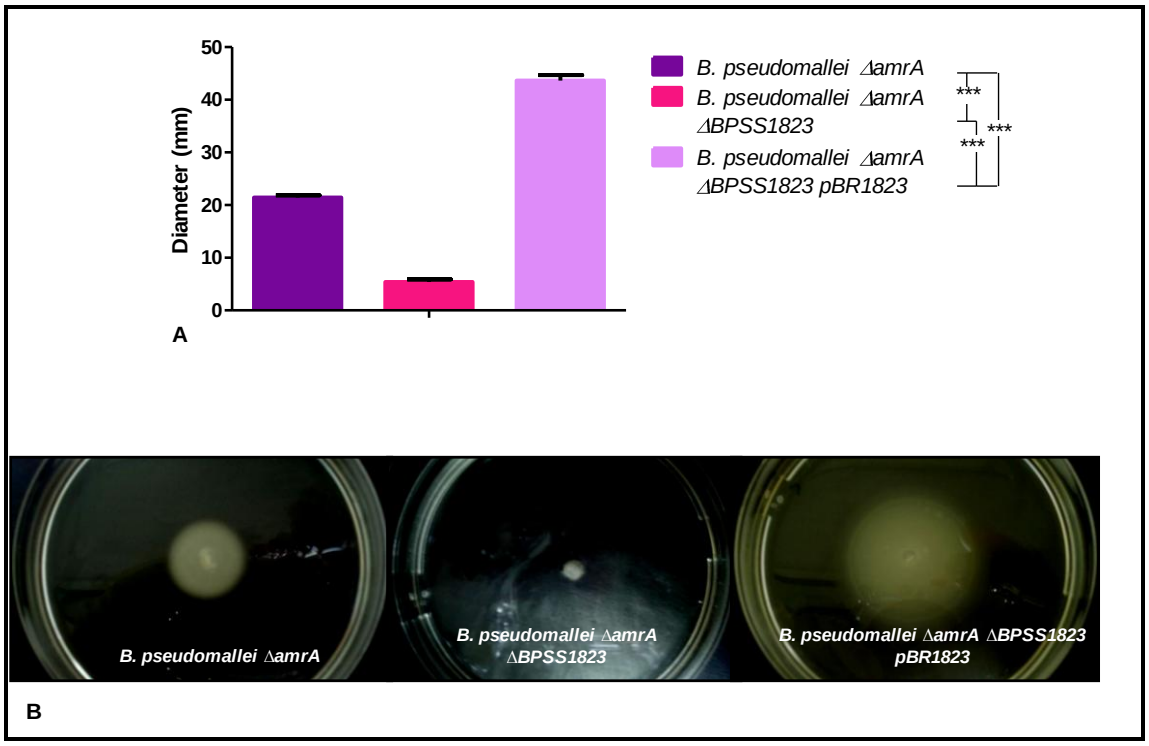


Figure 4.10. Determination of swarming motility of *B. pseudomallei ΔamrA ΔBPSS1823*. Bacteria were spotted onto 0.3% agar plates and incubated at 37ºC for 24 h. Values are the means from triplicate experiments, ± standard errors. P values are shown for the comparison of strains.

A – Zones of growth of *B. pseudomallei ΔamrA*; *B. pseudomallei ΔamrA ΔBPSS1823* or *B. pseudomallei ΔamrA ΔBPSS1823 PBR1823* spotted onto a 0.3% agar plate
B – Photographs of *B. pseudomallei ΔamrA*; *B. pseudomallei ΔamrA ΔBPSS1823* or *B. pseudomallei ΔamrA ΔBPSS1823 PBR1823* spotted onto a 0.3% agar plate

4.2.2.8 Measurement of secreted protease activity

To determine whether deletion of *BPSS1823* affects the secretion of proteases from *B. pseudomallei*, azocasein was used as a substrate (Brock *et al*., 1982). Bacterial supernatants were incubated with azocasein and the reaction stopped with trichloroacetic acid. Non-hydrolysed azocasein was pelleted and the azo dye in the supernatant was detected by absorbance at 440 nm. While both strains exhibited protease activity, hydrolysis of azocasein was almost 10-fold lower in the presence of *B. pseudomallei*Δ*amrA* Δ*BPSS1823* than in the presence of *B. pseudomallei*Δ*amrA* (Figure 4.11, B). Viable counts confirmed similar numbers of bacteria (data not shown).

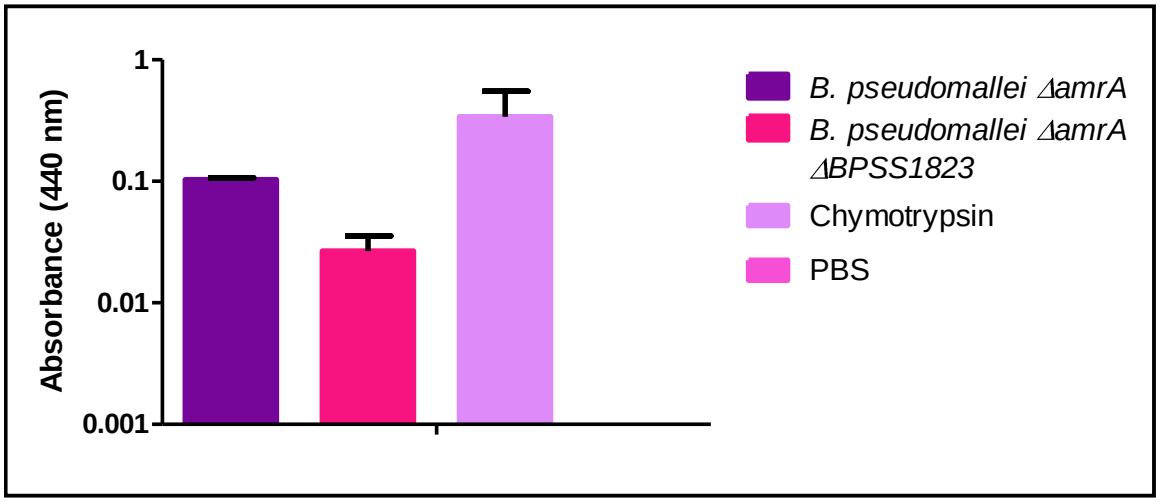


Figure 4.11. Determination of secreted protease activity of *B. pseudomallei* Δ*amrA* Δ*BPSS1823*.

100 µl *B. pseudomallei* Δ*amrA* or *B. pseudomallei* Δ*amrA* Δ*BPSS1823* supernatant was added to 100 µl azocasein (5 mg/ml) at incubated at 37ºC for 1h. The reaction was stopped with 10% TCA and absorbance of hydrolysed azocasesin read at 440 nm. Chymotrypsin (10 mg/ml) was used as a positive control and PBS as a negative control. Values are the means from triplicate experiments, ± standard errors.

4.2.3 Virulence of *BPSS1823* mutant strains *in vivo*

A BALB/c mouse model of *B. pseudomallei* infection was used to determine virulence of *BPSS1823* mutant strains. 6 mice per group were challenged by the intraperitoneal route with 4.8 x 10^4 cfu *B. pseudomallei* K96243; 2.9 x 10^4 cfu *B. pseudomallei*∆*BPSS1823*; 8.6 x 10^4 cfu *B. pseudomallei*Δ*amrA* or 2.5 x 10^4 cfu *B. pseudomallei*Δ*amrA* ∆*BPSS1823.* All mice challenged with *B. pseudomallei* K96243 succumbed to infection by day 15, with a median time to death (MTTD) of 2 days. In contrast, mice challenged with *B. pseudomallei*∆*BPSS1823* had a significantly increased MTTD of 29.5 days (Figure 4.12, A; P<0.01). Only 50% of mice challenged with *B. pseudomallei*Δ*amrA* had succumbed to infection by day 30, where as all mice challenged with *B. pseudomallei*Δ*amrA* ∆*BPSS1823* survived over the observed period (Figure 4.12, B).

To determine the median lethal dose (MLD) for *B. pseudomallei*∆*BPSS1823*, 6 mice per group were challenged with 4.8 x 10^1 – 10^6 cfu *B. pseudomallei* K96243 or 2.9 x 10^1 – 10^6 cfu *B. pseudomallei*∆*BPSS1823*. Using the method of method of Reed and Muench (1938), the MLD for *B. pseudomallei*∆*BPSS1823* was determined as 9.3 x 10^3 cfu/ml. Previous studies with *B. pseudomallei* K96243 determined the wildtype MLD to be 1 x10^3 cfu/ml. The mice were monitored for signs of disease for 5 weeks after which time survivors were culled and the spleens aseptically removed. Colonies showing typical morphology to *B. pseudomallei* were recovered from some spleens (Table 4.1).

B. pseudomallei*∆*BPSS1823 **challenge dose (cfu)**	**Mean bacterial burden in spleen (cfu/ml)**
2.9×10^1	1.7×10^3
2.9×10^2	0
2.9×10^3	0
2.9×10^4	$>3 \times 10^6$
2.9×10^5	n/a
2.9×10^6	n/a

A

B. pseudomallei*∆*amrA* ∆*BPSS1823 **challenge dose (cfu)**	**Mean bacterial burden in spleen (cfu/ml)**
2.5×10^1	0
2.5×10^2	0
2.5×10^3	$<3 \times 10^2$
2.5×10^4	4.2×10^2
2.5×10^5	0
2.5×10^6	$<3 \times 10^2$

B

Table 4.1. The mean bacterial burden in spleens of surviving mice, 5 weeks post challenge with *BPSS1823* mutant strains. n/a = not applicable; all mice succumbed to infection

A – Mice challenged with *B. pseudomallei*∆*BPSS1823*
B – Mice challenged with *B. pseudomallei*∆*amrA* ∆*BPSS1823*

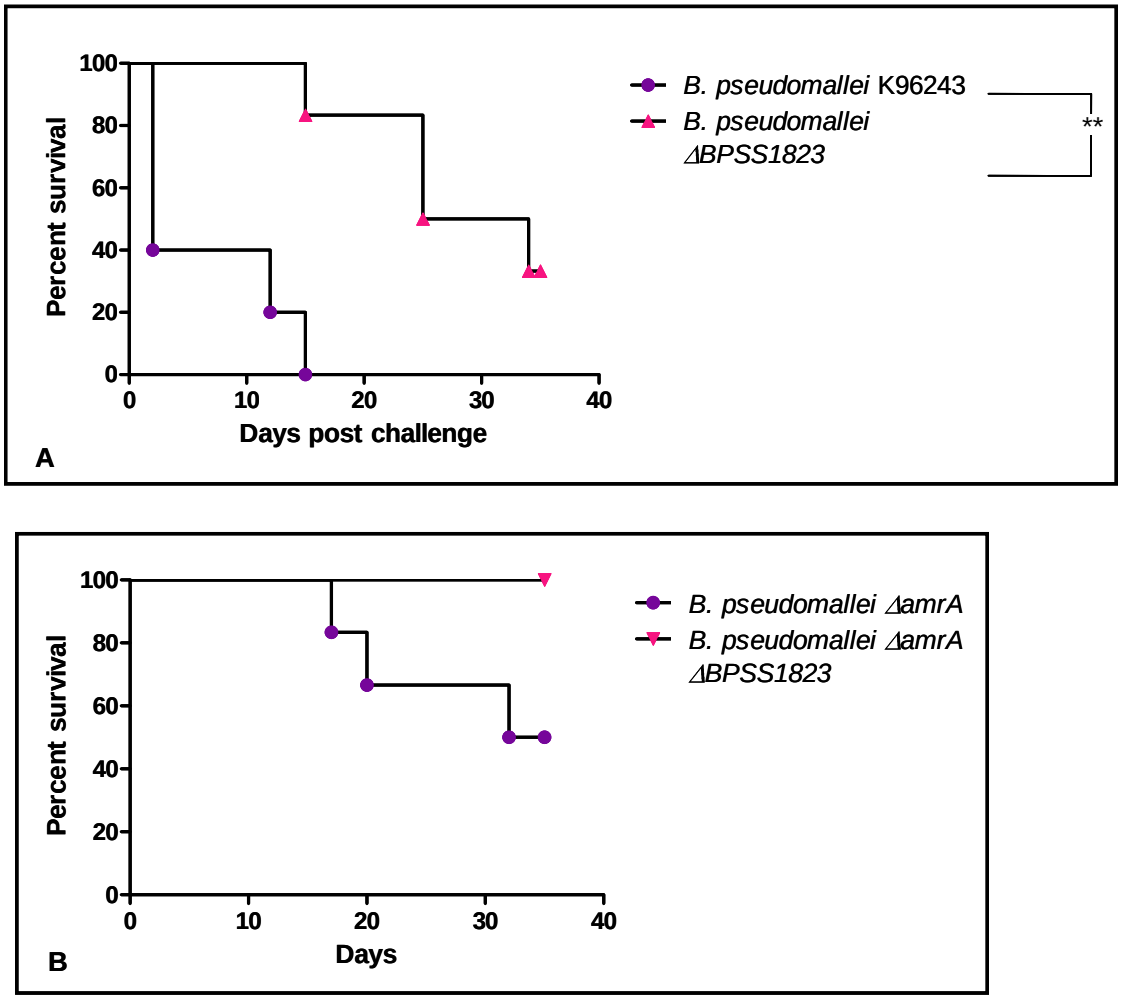


Figure 4.12. Virulence of *BPSS1823* mutants in BALB/c mice. Six mice were challenged with strains of *B. pseudomallei* by the intraperitoneal route. The mice were monitored for signs of disease for 5 weeks after which the surviving animals were culled.

A – Survival of mice challenged with 4.8 x 10^4 cfu/ml *B. pseudomallei* K96243 or 2.9 x 10^4 cfu/ml *B. pseudomallei* Δ*BPSS1823*

B – Survival of mice challenged with 8.6 x 10^4 cfu/ml *B. pseudomallei* Δ*amrA* or 2.5 x 10^4 cfu/ml *B. pseudomallei* Δ*amrA* Δ*BPSS1823*

P values are shown for comparison of survival curves over time.

4.3 Discussion

Most *B. pseudomallei* strains, including K96243, are intrinsically resistant to aminoglycosides, with an MIC of 16 µg/ml and 256 µg/ml for kanamycin and gentamicin, respectively (Moore *et al.,* 1999; Cheng and Currie, 2005). This intrinsic resistance is mediated in part by the AmrAB-OprA efflux pump (Moore *et al.,* 1999). *B. pseudomallei* 708a is a naturally occurring aminoglycoside susceptible strain, attributed to a 141 kb deletion on chromosome 1, which included deletion of the AmrAB-OprA efflux pump. Despite a large chromosomal deletion, this strain was fully virulent in an acute mouse model of infection (Trunck *et al.,* 2009).

Previously constructed *amrAB-oprA* mutants exhibit an MIC of < 1 µg/ml for kanamycin and 0.75 µg/ml for gentamicin (Moore *et al.,* 1999). This efflux pump mutant strain, named DD503, was shown to be as virulent as the parent strain 1026b in hamsters, but has recently been shown to be significantly attenuated in mice (Moore *et al*., 1999; Trunck *et al.,* 2009). DD503 has been used as a tool to characterise novel virulence determinants in *B. pseudomallei,* allowing the use of new selective markers or to reduce the amount of antibiotic required in infection assays (Moore *et al.,* 1999). DD503 has been used as the parent strain to characterise mutants in the TTSS (*bsaZ*); isocitrate lyase (*icl*); capsule (*wcbT/wcbA*); adhesion (*boaA*) and quorum sensing (*luxR/luxI*; Ulrich *et al.,* 2004; Burtnick *et al*., 2008; Schaik *et al.,* 2009; Warawa *et al.,* 2009). Therefore, a derivative of *B. pseudomallei* K96243, with an unmarked deletion in *amrA,* was used as an additional parent strain in this study.

Deletion of *BPSS1823* in wildtype *B. pseudomallei* K96243 revealed that BPSS1823 alone is important for intracellular survival and virulence in a mouse model of infection. *B. pseudomallei* ∆*BPSS1823* exhibited reduced intracellular survival over 24 h, indicating that *BPSS1823* may be involved in protecting the bacteria against intracellular killing. This is consistent with the phenotype reported for other *mip* mutants, which are also defective in intracellular growth and exhibit attenuated virulence (Cianciotto *et al.,* 1989; Horne *et al.,* 1997; Leuzzi *et al*., 2005).

The phenotype observed following deletion of *BPSS1823* in *B. pseudomallei* K96243 was more dramatically manifested following deletion of *BPSS1823* in *B. pseudomallei*Δ*amrA*. One reason for the greater reduction in the number of intracellular *B. pseudomallei*Δ*amrA*∆*BPSS1823* isolated may be a feature of the infection assay. Although high concentrations of kanamycin were required to kill extracellular *B. pseudomallei* K96243 ∆*BPSS1823*, some bacteria were still recovered from the supernatants, possibly increasing the numbers of 'intracellular' bacteria detected. In contrast, treatment of extracellular *B. pseudomallei* Δ*amrA* ∆*BPSS1823* with low concentrations of gentamicin resulted in no bacteria detected in the supernatant, resulting in a more accurate representation of intracellular numbers.

An alternative hypothesis is that there is a functional link between *BPSS1823* and *amrA* (Figure 4.13). BPSS1823 could be required for the correct folding or chaperoning of proteins with a direct role in virulence, which might explain why virulence is attenuated following deletion of *BPSS1823* alone. In addition, BPSS1823 may also be required for the correct folding and maturation of an

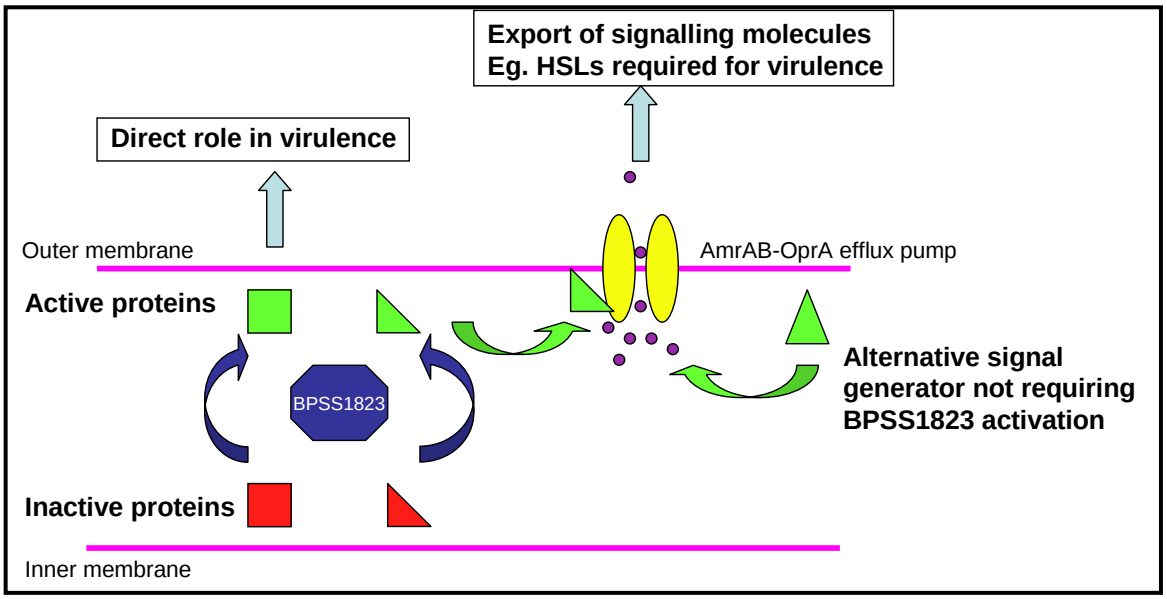


Figure 4.13. A putative mechanism of BPSS1823 and AmrAB-OprA interactions.

BPSS1823 'activates' proteins with a direct role in virulence, by mediating correct folding or chaperoning. BPSS1823 also 'activates' proteins that generate or are involved in the export of signalling molecules via efflux pumps, such as AmrAB-OprA.

enzyme(s) that are involved in the biosynthesis of small molecules, such as HSLs, that are then exported via the efflux pump or for the folding of a protein that forms part of the AmrAB-OprA efflux pump. This would explain the more marked manifestation of the *BPSS1823* mutant phenotype in the presence of an *amrA* deletion. Consistent with recent reports that inactivation of AmrAB-OprA reduced virulence in mice (Trunck *et al*., 2009), *B. pseudomallei* Δ*amrA* was attenuated compared to *B. pseudomallei* K96243. This provides further support that this efflux pump is not just involved in antibiotic resistance, but has a broader physiological role in *B. pseudomallei.* Several Gram-negative bacteria have been shown to require efflux pump expression for full virulence (reviewed by Piddock, 2006). For example, disruption of the AcrAB-TolC efflux pump in *Salmonella enterica* down-regulated expression of multiple virulence genes, including genes required for motility, TTSS and adhesion (Webber *et al.,* 2009). This study indicates that efflux pumps can affect a wide range of virulence associated mechanisms within bacteria. RT-PCR and microarray analysis could be used to identify differential transcription of genes from *B. pseudomallei*Δ*amrA* and *B. pseudomallei*Δ*amrA* Δ*BPSS1823.*

Another multidrug efflux pump from *B. pseudomallei*, BpeAB-OprB, has been shown to be important for secretion of HSLs and quorum sensing associated virulence in *B. pseudomallei* KHW (Chan *et al*., 2005; Chan *et al.,* 2007). However, the function of BpeAB-OprB has been shown to be strain dependent (Mima and Schweizer, 2010). While *B. pseudomallei* strain PP844 has been reported to produce up to six different types of HSLs, strain DD503 has been reported to only secrete three HSLs (Ulrich *et al.,* 2004; Lumjiaktase *et al.,* 2006; Chan *et al*., 2007). This indicates that the AmrAB-OprA efflux pump may

also be involved in HSL export and that a putative function for BPSS1823 is to fold proteins required for the biosynthesis or export of HSLs. This could be investigated further by detecting HSL production in *B. pseudomallei* ∆*amrA* or *B. pseudomallei* Δ*amrA* ∆*BPSS1823*.

If the *B. pseudomallei*Δ*amrA* ∆*BPSS1823* strain is defective in HSL export, this might explain why pleiotrophic effects on virulence mechanisms were observed. Quorum sensing has been shown to regulate the production of bacterial virulence determinants and *B. pseudomallei* Δ*amrA* ∆*BPSS1823* was hypersensitive to low pH, exhibited reduced motility and reduced protease production. The reduced growth of the mutant strain in low pH may explain why fewer numbers of bacteria were isolated from within cells, where acidification of the phagolysosome occurs as a host defence mechanism. Furthermore, environmental stimuli, such as low pH, have been shown to increase production of HSLs in some bacteria (Surette and Bassler, 1999). Inactivation of *luxS* in *Streptococcus mutans* reduced the tolerance of the mutant strain to acid stress compared to the wildtype strain (Wen and Burne, 2004). HSL production has also been shown to mediate swarming motility in other bacteria and inactivation of a *luxI* homologue in *Yersinia enterocolitica* lead to down-regulation of a flagellin structural gene (Atkinson *et al.,* 2006; Hussain *et al.,* 2008). Expression of a quorum sensing system has previously been shown to negatively regulate the production of a metalloprotease in *B. pseudomallei* (Valade *et al.,* 2004). However, the production of HSLs has been shown to correlate with protease production in *P. aeruginosa* and *Porphyromonas gingivalis* (Zhu *et al.,* 2002; Burgess *et al.,* 2002). Overall, many of the phenotypes exhibited by *B. pseudomallei* Δ*amrA* ∆*BPSS1823* are consistent with observations from other

bacterial species defective in quorum sensing, providing further support that BPSS1823 and the AmrAB-OprA efflux pump are involved in the synthesis or export of HSLs. To determine the effect of *BPSS1823* and *amrA* inactivation on protease production, 2D-PAGE could be used to identify secreted proteins or RT-PCR used to examine *mprA* expression. The flagella of the mutant strain could also be analysed by EM or expression of flagellum genes detected using RT-PCR.

In conclusion, a Mip homologue, encoded by *BPSS1823*, is required for full virulence in *B. pseudomallei.* Like Mips from other intracellular pathogens, a null mutation resulted in reduced survival within phagocytic and non-phagocytic cells and attenuated virulence in a BALB/c model of infection. Deletion of *BPSS1823* in an efflux pump mutant resulted in defective invasion of epithelial cells and further attenuation of intracellular survival and virulence. This strain was characterised in more detail and exhibited sensitivity to low pH and reduced virulence mechanisms, such as swarming motility and protease section. These results lead to the hypothesis that BPSS1823 and the AmrAB-OprA efflux pump are functionally linked, with both acting to fold or export of HSLs in quorum sensing. In addition, although natural and laboratory-constructed efflux pump mutants have been used previously to characterise virulence determinants in *B. pseudomallei*, the results presented in this study suggest that caution should be exercised when using these strains.

Chapter 5 – Evaluation of a SurA homologue in *B. thailandensis*

5.1 Introduction

Survival protein A (SurA) is a periplasmic parvulin which is involved in OMP folding and is required for virulence in some Gram-negative bacteria (reviewed by Behrens-Kneip, 2010). SurA was first identified in *E. coli* as a periplasmic chaperone and inactivation of the *surA* gene lead to a reduction in β-barrel OMPs in the cell membrane (Lazar and Kolter, 1996; Rouvière & Gross, 1996).

SurA was characterised as a virulence determinant in uropathogenic *E. coli*, where a *surA* mutant was shown to be defective in adherence to and invasion of cells and unable to persist in a murine cystitis model (Justice *et al*., 2006). SurA has also been shown to be important for virulence of the enteric pathogens *S. enterica* and *S. flexneri* (Sydenham *et al*., 2000; Purdy *et al.,* 2007).

BPSL0659 and BTH_I0576 were identified in Chapter 3 as encoding SurA homologues in *B. pseudomallei* and *B. thailandensis,* respectively. Amino acid and structural analysis revealed conserved putative chaperone and PPIase domains, with homology to other bacterial SurAs (Figure 3.2). In addition, BPSL0659 and BTH_I0576 are 97% identical to each other. BPSL0659 was purified as a recombinant protein and shown to have characteristic PPIase activity (Figure 3.11). The aim of the work described in this chapter was to evaluate the role of SurA in *B. thailandensis*. The approach used was construction of an unmarked deletion mutant of *BTH_I0576* in *B. thailandensis.*

B. thailandensis has been used as a model system to study *B. pseudomallei* virulence factors, such as TTSS (Haraga *et al.,* 2008). *B. thailandensis* ∆*BTH_I0576* was characterised for defects in infection of cell lines and virulence using the wax moth larvae, *G. mellonella* as a model of *B. thailandensis* infection.

5.2 Results

5.2.1 Comparison of *BPSL0659* and *BTH_I0576* genomic regions

To determine whether deletion of a *surA* homologue from *B. thailandensis* (*BTH_I0576*) would be comparable to deletion of a *surA* homologue from *B. pseudomallei* (*BPSL0659*), the up-steam and down-steam genes were analysed (Figure 5.1). The genomic organisation around both genes was identical, with the adjacent up-stream gene annotated as a 'hypothetical protein', a homologue of *lptD,* an outer membrane protein involved in LPS assembly. The adjacent down-stream gene was annotated as '*pdxA* (4-hydroxythreonine-4-phosphate dehydrogenase)', a homologue of a protein involved in vitamin B6 biosynthesis. In addition, both *surA* genes were predicted to be located in an operon, shown in blue in Figure 5.1 (http://www.microbesonline.org; Price *et al.,* 2005).

5.2.2 Production of a deletion construct

The cloning strategy used to produce the construct for deletion of *BTH_I0576* was outlined in Figure 4.1. Upstream and downstream flanking regions of *BTH_I0576* were PCR amplified from *B. thailandensis* E264 genomic DNA using BTHI0576.PDM4.LFF; BTHI0576.PDM4.LFR; BTHI0576.PDM4.RFF and BTHI0576.PDM4.RFR (Table 2.9). The PCR products were sub-cloned into pCR Blunt II-TOPO and sequence fidelity of inserts confirmed by nucleotide

sequencing. The flanks were digested out of pCR Blunt II-TOPO using *Bgl*II and *Xba*I and ligated into the *Xba*I site of pDM4. Ligations were transformed into *E. coli* DH5α λpir and plated onto selective LB agar containing 30 µg/ml chloramphenicol. Colonies containing the correct construct were confirmed after agarose gel electrophoresis of restriction digests and by nucleotide sequencing of the insert DNA.

5.2.3 Mutant production

The mutant making strategy adopted is outlined in Figure 4.2 (Logue *et al.,* 2009). First, the pDM4 deletion construct containing the *BTH_I0576* flanking regions was transformed into *E. coli* S17 λpir. The plasmid was then transferred by conjugation into *B. thailandensis* E264*.* The first cross-over event resulted in integration of the pDM4 construct into the host chromosome by homologous recombination. Merodiploid integrants were identified on selective LB agar containing 30 µg/ml chloramphenicol and confirmed by colony PCR. The *sacB* gene encoded by pDM4 allowed counter-selection for excision of the vector when grown on sucrose, via a second cross-over event. Colonies were screened for sensitivity to chloramphenicol and southern hybridization was used to confirm a 692 bp deletion in the *BTH_I0576* allele (Figure 5.2). From 4 chloramphenicol sensitive colonies analysed by southern hybridization, 1 strain was confirmed as a mutant (data not shown). This mutant strain was named *B. thailandensis* Δ*BTH_I0576.*

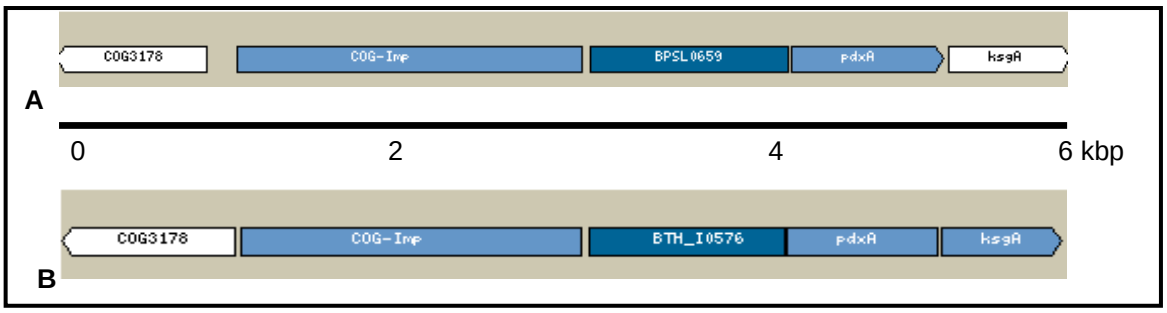


Figure 5.1. The genomic regions up-steam and down-stream of SurA homologues from A - *B. pseudomallei* and B - *B. thailandensis*.

BPSL0659 or *BTH_I0576* are shown in dark blue. Genes predicted to be in the same operon are shown in light blue. The arrowhead indicates transcription direction. Scale bar shows kilobases.

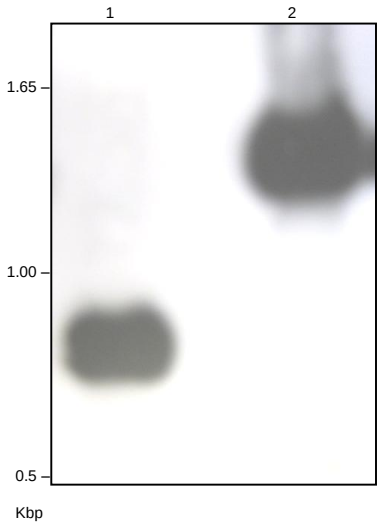


Figure 5.2. Confirmation of *BTH_I0576* mutant in *B. thailandensis.*

Southern hybridization showing deletion of *BTH_I0576* in *B. thailandensis*. Digested genomic DNA was probed with Dig-labelled left-flank. Kbp ladder shows the sizes of marker DNA.

Lane 1– mutant genomic DNA digested with *SmaI* (0.87 kbp); 2 – wildtype genomic DNA digested with *SmaI* (1.57 kbp)

5.2.4 *In vitro* characterisation of *BTH_I0576* mutant strain

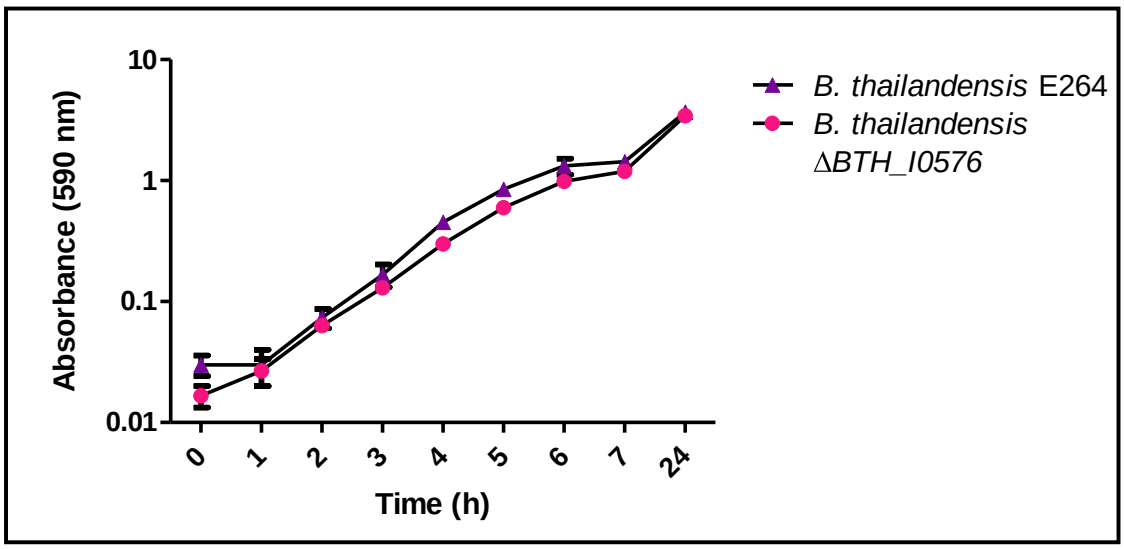
5.2.4.1 Growth studies

The growth rate of B. thailandensis ΔBTH_I0576 *was determined in liquid media. 2 ml overnight culture was used to inoculate 100 ml LB broth and growth was monitored at 37ºC for up to 216 h by viable counts or absorbance at 590 nm. Deletion of* BTHI_0576 *did not effect growth in LB broth, compared to the wildtype strain (Figure 5.3). However, the mutant bacteria exhibited reduced growth on L-agar, as shown by smaller colony size (data not shown).*

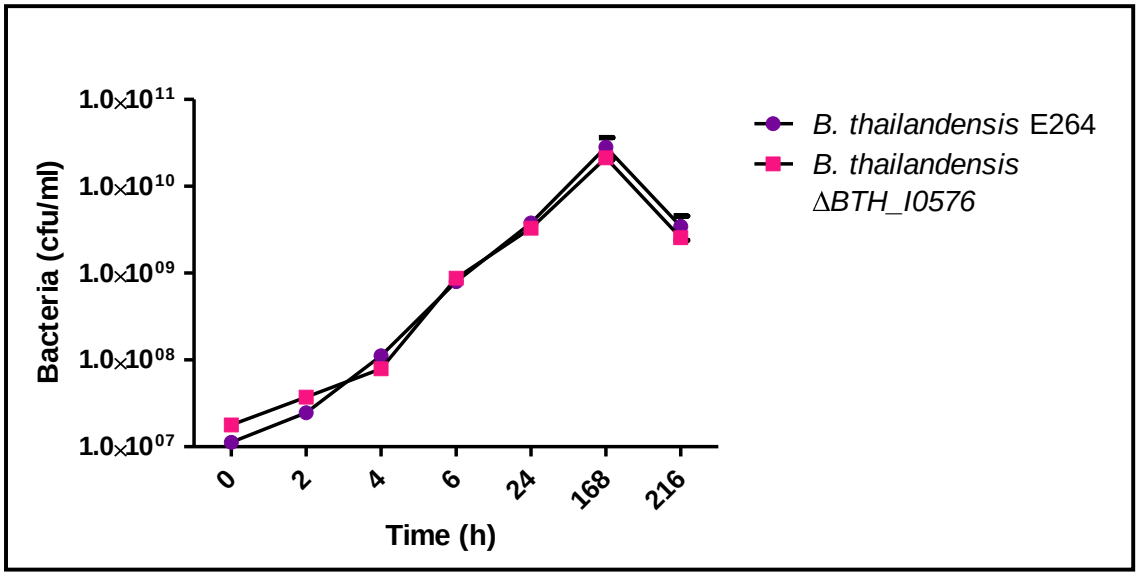
SurA mutants have been shown to have defective cell membranes and show increased sensitivity to compounds normally limited by the outer membrane (Rouvière and Gross, 1996). Therefore, B. thailandensis ΔBTH_I0576 *was grown in LB broth containing 256 µg/ml polymyxin B. Following 24 h growth, the OD as detected by absorbance at 590 nm was lower for the mutant strain, compared to the wildtype strain ($P<0.05$; Figure 5.4).*

5.2.4.2 Growth in cell lines

B. thailandensis has been shown to invade and replicate within eukaryotic cells (Harley *et al.,* 1998a). To determine whether deletion of *BTH_I0576* affected invasion, survival and growth in eukaryotic cells, J774 macrophages or A549 epithelial cells were infected with *B. thailandensis* Δ*BTH_I0576* or *B. thailandensis* E264*,* at an MOI of 1 or 100, respectively. After incubation for 30 min (J774) or 1h (A549) to allow uptake of bacteria, extracellular bacteria were killed with 1 mg/ml kanamycin for 1 h. At 0, 2, 4 and 24 h after extracellular killing, infected cells were lysed and the number of viable bacteria within the cells was determined.



A



B

Figure 5.3. The growth of *B. thailandensis* Δ*BTH_I0576* in LB broth at 37ºC. Growth was monitored over 24 h by viable counts or absorbance at 590 nm.

A – The growth of *B. thailandensis* Δ*BTH_I0576* compared to *B. thailandensis* E264, measured by absorbance at 590 nm. Values are the means from triplicate experiments, ± standard errors.
B – The growth *B. thailandensis* Δ*BTH_I0576* compared to *B. thailandensis* E264, measured by viable counts. Values at 0-24 h are from a single experiment; values at 168 - 216 h are from triplicate experiments.

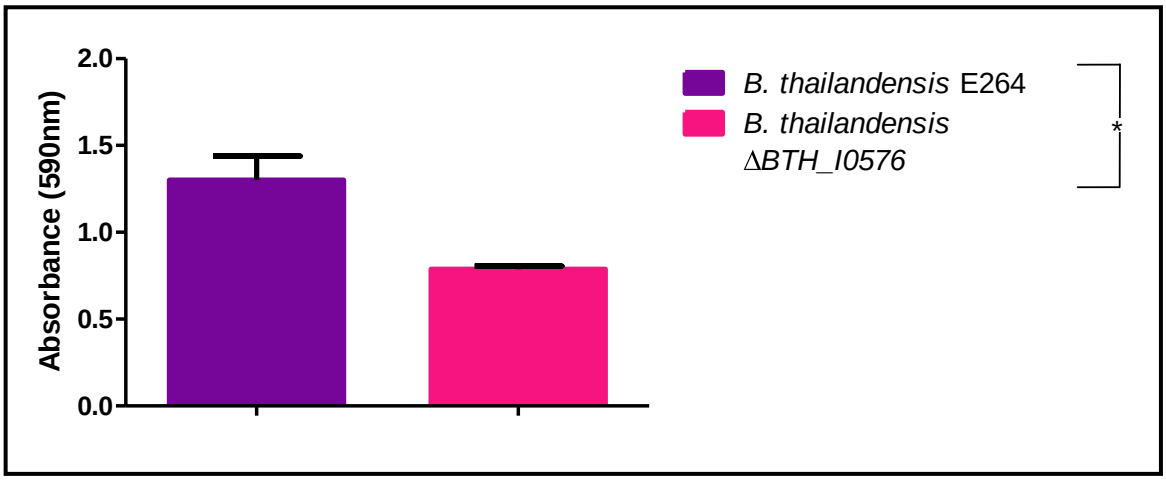
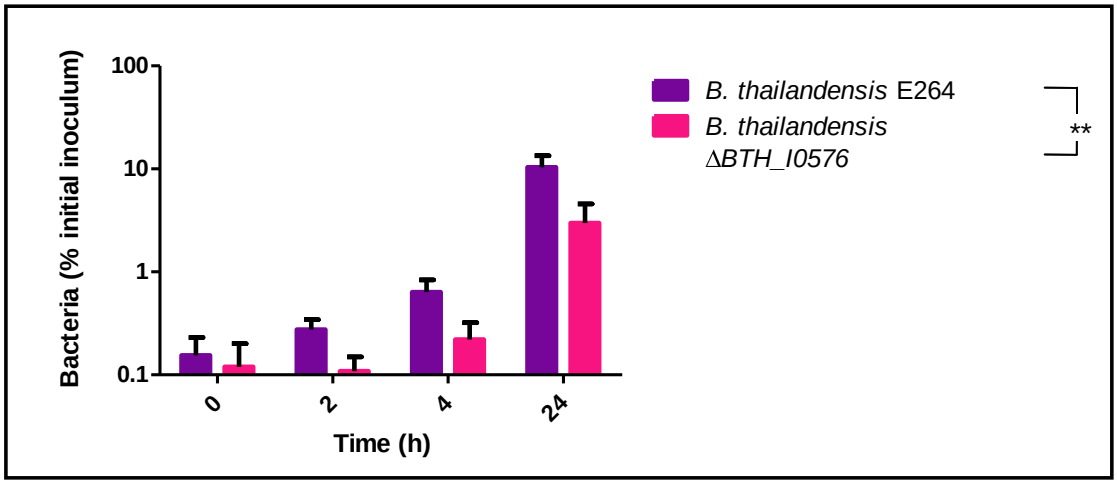


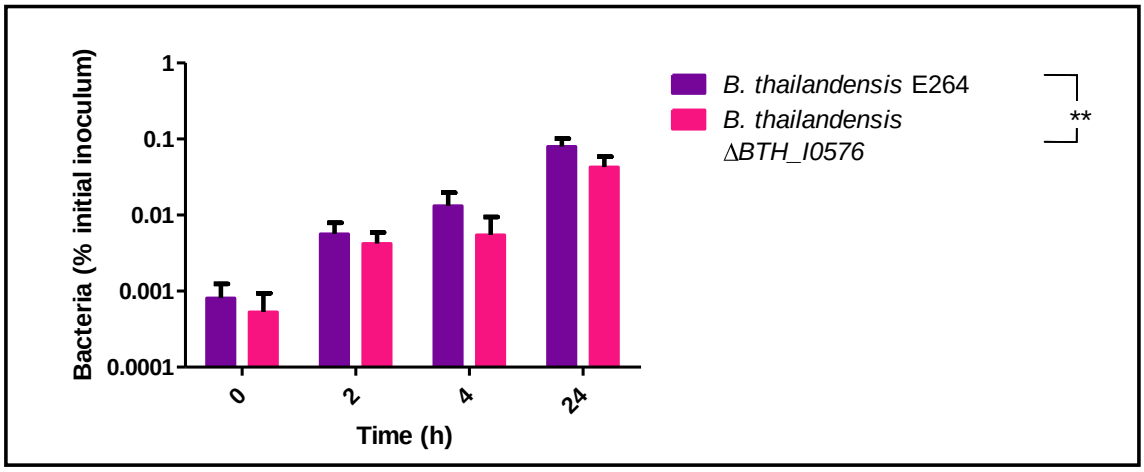
Figure 5.4. The growth of *B. thailandensis* Δ*BTH_I0576* in LB broth containing 256 µg/ml polymyxin B at 37ºC.

The growth of *B. thailandensis* Δ*BTH_I0576* compared to *B. thailandensis* E264, measured at 24 h by absorbance at 590 nm. Values are the means from triplicate experiments, ± standard errors. P values are shown for the comparison of absorbance 24 h post inoculation

Following infection of J774 macrophages with *B. thailandensis* Δ*BTH_I0576* or *B. thailandensis* E264, both strains were taken up into the cell. However, the numbers of mutant strain 0 h after extracellular killing was reduced compared to the wildtype (Figure 5.5, A). Furthermore, there was significantly fewer *B. thailandensis* Δ*BTH_I0576* detected at 2, 4 ($P<0.05$) and 24 h ($P<0.01$) post infection when compared to *B. thailandensis* E264 (Figure 5.5, A). Similarly, following infection of A549 epithelial cells with *B. thailandensis* Δ*BTH_I0576*, the mutant strain exhibited a defect in initial invasion and there were significantly fewer intracellular bacteria 24 h post infection when compared to *B. thailandensis* E264 (Figure 5.5, B; $P<0.01$).



A



B

Figure 5.5. Survival of *B. thailandensis* Δ*BTH_I0576* in A: J774 macrophages or B: A549 epithelial cells over 24 h. Cells incubated with bacteria for 30 min at an MOI of 1 (J774) or 1 h at an MOI of 100 (A549). Extracellular bacteria were killed with 1 mg/ml kanamycin for 1 h. Intracellular bacterial numbers were determined at 0, 2, 4 and 24 h after extracellular killing.

Data is presented as a percentage of the initial starting innoculum. Values are the means from triplicate experiments, ± standard errors. P values are shown for the comparison of intracellular bacteria 24 h post infection.

5.2.4.3 Measurement of swarming motility

Inactivation of *surA* in *E. coli* has been shown to reduce OMP production (Rouvière and Gross, 1996). To determine whether deletion of *BTH_I0576* affects motility in *B. thailandensis,* the swarming motility of *B. thailandensis*Δ*BTH_I0576* was analysed as previously described (Section 4.2.2.7). While inoculation of 0.3% agar with *B. thailandensis* E264 resulted in a mean bacterial spread of 45 mm, the spread was significantly reduced to 22 mm with the *BTH_I0576* mutant (Figure 5.6; P<0.0001).

5.2.5 Virulence of *B. thailandensis* Δ*BTH_I0576 in vivo*

As *B. thailandensis* shows low virulence in mammalian models of infection, a *G. mellonella* infection model was used to determine the virulence of the *BTH_I0576* mutant strain. Groups of 10 larvae were challenged with approximately 100 or 1000 cfu of *B. thailandensis* E264 or *B. thailandensis* Δ*BTH_I0576*. Control group larvae injected with PBS survived the course of the experiment, whilst all larvae challenged with *B. thailandensis* E264 succumbed to infection by 45 h post infection. Larvae challenged with either 100 or 1000 cfu of the *BTH_I0576* mutant strain had a significantly increased time to death when compared to larvae challenged with a similar number of *B. thailandensis* E264 (Figure 5.7 P<0.0001).

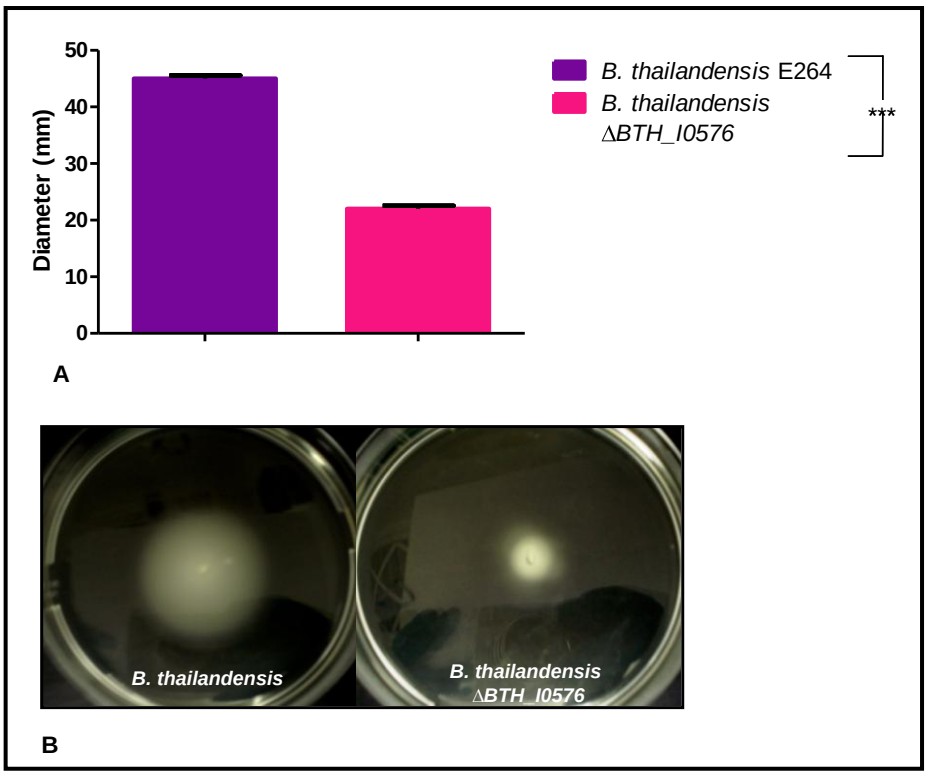


Figure 5.6 Determination of swarming motility of *B. thailandensis* Δ*BTH_I0576*. Bacteria were spotted onto 0.3% agar plates and incubated at 37ºC for 24 h. Values are the means from triplicate experiments, ± standard errors. P values are shown for the comparison of strains.

A – Zones of growth of *B. thailandensis* Δ*BTH_I0576* or *B. thailandensis* E264 spotted onto a 0.3% agar plate
B – Photographs of *B. thailandensis* Δ*BTH_I0576* or *B. thailandensis* E264spotted onto a 0.3% agar plate

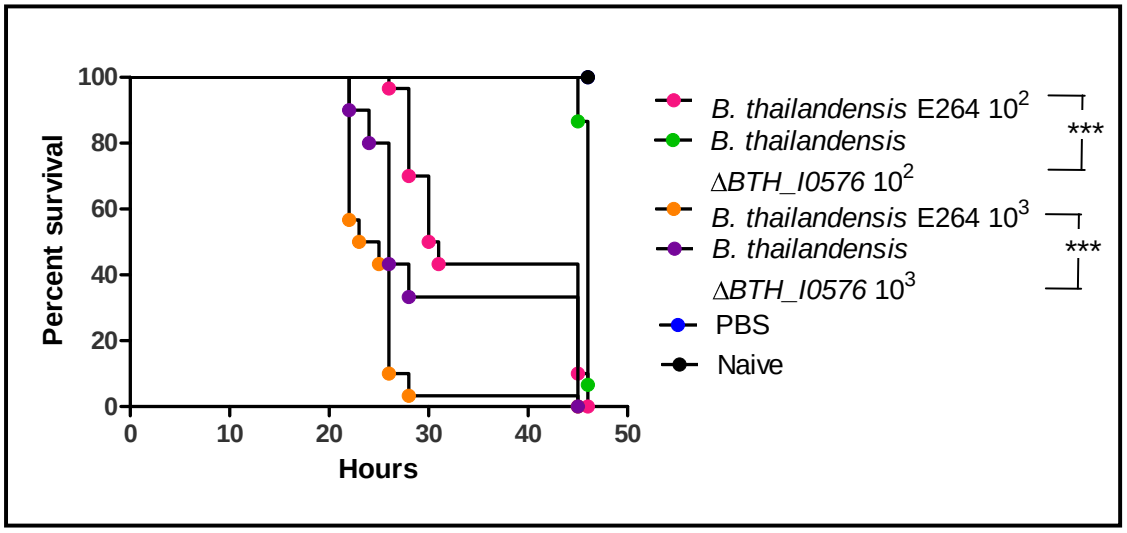


Figure 5.7. Virulence of *B. thailandensis* Δ*BTH_I0576* in a *G. mellonella* model. 10 larvae were challenged with approximately 100 or 1000 cfu of *B. thailandensis* E264 *or B. thailandensis* Δ*BTH_I0576* by injection into the uppermost right proleg. The larvae were monitored for signs of disease for 45 hours, after which time survivors were culled.

Values are the pooled data from triplicate experiments. P values are shown for comparison of survival curves over time.

5.3 Discussion

The *B. thailandensis* E264 genome is smaller than that of *B. pseudomallei* (6.7 compared to 7.2 Mbp) but encodes many homologues of known and putative *B. pseudomallei* virulence determinants (Kim *et al.,* 2005; Yu *et al.,* 2006). The high degree of similarly between the two species means that *B. thailandensis* has been used as a model system to study various aspects of *B. pseudomallei* biology. In particular, several studies have used *B. thailandensis* as a surrogate to characterise the role of *B. pseudomallei* virulence factors, including TTSS and quorum sensing (Haraga *et al*., 2008; Chandler *et al.,* 2009; Seyedsayamdost *et al*., 2010). An advantage of using *B. thailandensis* as a model system is that, in contrast to *B. pseudomallei*, manipulation does not require containment level 3 facilities. Although many virulence factors are well conserved between the species, *B. thailandensis* has low virulence in humans, which should be considered when extrapolating data to *B. pseudomallei* (Brett *et al.,* 1997; Smith *et al.,* 1997).

The gene encoding a SurA homologue in *B. pseudomallei* (*BPSL0659*), has 97% amino acid identity to *BTH_I0576* from *B. thailandensis* (Figure 3.2). The non-identical amino acids represent 14 residue substitutions, with two differing residues in the region homologous to the active PPIase domain from *E. coli* SurA (Behrens *et al.,* 2001). The genomic regions around both genes are also identical (Figure 5.1). To evaluate the role of SurA in *B.* thailandensis, and perhaps provide an indication of the role of SurA in *B. pseudomallei*, an unmarked deletion of *BTH_I0576* was made in *B. thailandensis.* Although this deletion was in-frame and unmarked, complementation of the mutant strain is required to confirm a *BTH_I0576*-specific phenotype. In addition, *BTH_I0576* is

predicted to be located in an operon, which may indicate that inactivation of one gene will affect the function of up-stream or down-stream genes. However, the specificity of the operon prediction method used was shown to be only 79.9% in *E. coli* (Price *et al*., 2005). Interestingly, directly up-stream of *BTH_I0576* is a homologue of *lptD,* which encodes an outer membrane protein which is dependent on SurA for assembly in *E. coli* (Vertommen *et al*., 2009; Denoncin *et al*., 2010).

E. coli SurA was initially identified when it was observed to be required for growth at stationary phase, in the presence of a mutation of σ^S, a stationary-phase-specific sigma factor. Inactivation of *surA* or *rpoS* alone in *E. coli* had no effect on stationary phase survival (Tormo *et al.,* 1990; Lazar *et al.,* 1998). Growth of *B. thailandensis* Δ*BTH_I0576* was monitored over 9 days in LB broth, with no defects observed when compared to wildtype (Figure 5.3). To determine whether there is a functional link between *B. thailandensis surA* and *rpoS*, a double mutant could be constructed and characterised.

Inactivation of *E. coli surA* results in a defective cell membrane, caused by a reduction in major β-barrel OMPs (Lazar and Kolter, 1996). This leads to pleiotrophic effects, such as hypersensitivity to hydrophobic antibiotics and loss of piliation (Rouvière and Gross, 1996; Justice *et al.,* 2005). *B. thailandensis* Δ*BTH_I0576* was shown to have increased sensitivity to polymyxin B, a cationic antimicrobial peptide (Figure 5.4). The polymyxin B MIC for *B. thailandensis* E264 has been shown to be >128 000 μg/ml, indicating it is unable to permeabilise the outer membrane (Burtnick and Woods, 1999). Polymyxin B binds to LPS prior to disruption of the outer membrane and inactivation of LPS

biosynthesis genes increased polymyxin B sensitivity in *B. pseudomallei* (Burtnick and Woods, 1999). The hypersensitivity of the *BTH_I0576* mutant suggests that it may have defect in outer membrane integrity or LPS production. In addition, *B. pseudomallei* polymyxin B sensitive mutants all showed altered outer membrane protein profiles (Burtnick and Woods, 1999). *B. thailandensis* Δ*BTH_*I0576 also exhibited reduced swarming motility, which may be due to a defect in the flagella (Figure 5.6). To determine whether SurA in *B. thailandensis* is required for OMP production, including flagella formation, the OMP profile of the mutant strain could be characterised using SDS-PAGE or immunoblots. The mutant LPS could also be purified and analysed and compared to wildtype LPS.

Following infection of eukaryotic cells with *B. thailandensis* Δ*BTH_I0576,* significantly fewer bacteria mutant bacteria were detected at 24 h post infection. This observation may be due to a defect in the initial amount of mutant bacteria entering the cells, rather than a defect in replication. These results are consistent to the phenotype reported for other *surA* mutants, which are defective in binding and invasion of epithelial cells (Sydenham *et al.,* 2000; Justice *et al.,* 2006). In *E. coli,* SurA has been shown to be required for production of type 1-fimbriae, an adhesin required for bacterial attachment and invasion (Justice *et al.,* 2005; Watts and Hunstad, 2008). To investigate why inactivation of *surA* in *B. thailandensis* affects infection of cells, pili formation could be characterised using microscopy or detected using antibodies to known adhesins such as PilA (Essex-Lopresti *et al.,* 2005). In addition, the ability of *B. thailandensis* Δ*BTH_I0576* to adhere to A549 cells could be studied using the assay described in 2.8.2.

A *G. mellonella* model was used to determine the role of *BTH_I0576* in *B. thailandensis* virulence. *B. thailandensis* has low virulence in mouse and hamster models. Therefore an alternative infection model was required to monitor the effect of *BTH_I0576* inactivation on pathogenesis (Brett *et al*., 1997; Smith *et al.,* 1997). Initial studies revealed that *G. mellonella* represents an acute model of *B. thailandensis* infection (data not shown). *G. mellonella* have been used to study virulence of several bacterial pathogens, including *F. tularensis*, *B. cepacia* and *B. mallei* (Aperis *et al*., 2007; Schell *et al.,* 2008; Seed and Dennis, 2008). The innate immune system of *G. mellonella* is structurally and functionally similar to that of mammalian innate immune systems, with responses such as clotting; antimicrobial peptide production and phagocytosis (Hoffmann, 1995). In addition, a correlation between virulence of pathogens in *G. mellonella* and mice has been established, indicating it represents a robust model of infection (Jander *et al*., 2000; Brennan *et al.,* 2002). *B. thailandensis* Δ*BTH_I0576* was significantly attenuated in this model, however all larvae had succumbed to infection by 46 h post infection (Figure 5.7). This indicates that although BTH_I0576 is important for full virulence *in vivo*, other factors are required for disease in the *G. mellonella* model of infection.

A link between SurA function and bacterial virulence *in vivo* has been established in *S. enterica* and UPEC (Sydenham *et al.,* 2000; Justice *et al.,* 2006). The UPEC *surA* mutant fails to suppress the epithelial cytokine response, resulting in a stronger inflammatory response, which may reduce its ability to establish infection (Hunstad *et al.* 2005). To determine whether BTH_I0576 is required for bacterial persistence within *G. mellonella,* the

haemolymph could be collected after infection and numbers of viable bacteria monitored over time. In addition, macrophages could be infected with wildtype or ∆*BTH_I0576* mutant strains *in vitro* and cytokines in the supernatant detected using antibodies.

In conclusion, a SurA homologue, encoded by *BTH_I0576,* represents a novel virulence determinant in *B. thailandensis*. Like SurA from other bacterial pathogens, inactivation of the gene increased sensitivity to compounds that are normally limited by the outer membrane, indicating a defect in OMP formation. In addition, the *BTH_I0576* mutant strain exhibited reduced intracellular invasion in eukaryotic cells and was attenuated in a *G. mellonella* model of infection. Due to the genetic similarity between *B. pseudomallei* and *B. thailandensis*, these results may be extrapolated to suggest that SurA may represent a putative virulence factor required for pathogenesis of melioidosis. However, further work is required to confirm this by construction of a *BPSL0659* mutant in *B. pseudomallei* and to determine the specific role of SurA in *Burkholderia* species.

Chapter 6 – General discussion

6.1 General discussion and future work

There is currently no vaccine available against melioidosis and intrinsic antibiotic resistance can make treatment complex. Even with prolonged therapy, up to 40% mortality has been reported (White, 2003). While *B. pseudomallei* is a major cause of severe bacteremic infections in endemic regions, it is also listed as a potential biowarfare agent by the US Centre for Disease Control and Prevention (Suputtamongkol *et al*., 1994a; Rotz *et al.,* 2002; Douglas *et al.,* 2004). Therefore, there is a requirement for the identification of novel vaccine candidates and antimicrobial targets to prevent and treat melioidosis.

Several approaches have been used to develop a melioidosis vaccine, including live attenuated vaccines, killed whole cell vaccines and subunit vaccines (reviewed by Sarkar-Tyson and Titball, 2010). Patients who have recovered from melioidosis have antibodies that react with *B. pseudomallei* proteins, with strong responses to virulence proteins or surface located proteins (Felgner *et al*., 2009; Suwannasaen *et al.,* 2011). Of the putative PPIases identified in *B. pseudomallei,* three were predicted to be located in the periplasm of the bacteria (BPSS1823, BPSL0659, BPSL1418) and two were predicted to be virulence associated (BPSS1823, BPSL0659; section 3.2.1). While BPSL1418 elicited high antibody responses after immunisation, no protection was afforded. In contrast, mice immunised with recombinant BPSS1823 or BPSL0659 showed an increased median time to death following challenge with *B. pseudomallei* (section 3.2.3.1). This supports previous evidence that the immune system is

more likely to recognise surface located or virulence associated antigens during infection. These proteins therefore have potential as novel vaccine candidates.

To improve the levels of protection, these proteins could be conjugated to an immunogenic *B. pseudomallei* polysaccharide such as the LPS, which could establish a synergistic immune response (Finn, 2004). An alternative adjuvant or animal model could be used, such as using C57BL/6 mice which are more resistant to *B. pseudomallei* infection than BALB/c mice (Leakey *et al.,* 1998). Furthermore, the immunogenicity of these proteins in humans could be assessed using convalescent sera from recovered melioidosis patients. Homologues of BPSL0659 and BPSL1402 are encoded by *B. mallei* so the level of cross protection could be determined, to evaluate the potential of a subunit vaccine against both melioidosis and glanders.

The importance of PPIases in a range of physiological processes and the ability to inhibit function with commercially available drugs has lead to extensive research evaluating PPIases as novel therapeutic targets. In particular, PPIase inhibitors have shown potential against many infectious diseases, including viral, parasitic, fungal and bacterial infections (Dugave, 2006). In this study, bioinformatic approaches were used to identify putative FKBPs and parvulins in *B. pseudomallei*, to evaluate their potential as antimicrobial targets. Furthermore, *B. pseudomallei* was shown to encode homologues of virulence associated PPIases, Mip and SurA.

The role of a Mip homologue in *B. pseudomallei* was evaluated in Chapter 4 by construction of a deletion mutation in *BPSS1823*. The mutant strain was shown

to have reduced intracellular survival and attenuated virulence in mice, a phenotype consistent with Mip mutants in other bacteria. Recombinant BPSS1823 protein was also shown to exhibit inhibitable PPIase activity, confirming it is a functional FKBP (section 3.2.2.4). While these results suggest that BPSS1823 is a novel target for antimicrobial development, the mutant phenotype was heightened upon deletion of *BPSS1823* in *B. pseudomallei* Δ*amrA.* While this was unanticipated, the pleiotrophic effects on virulence mechanisms in the double mutant provided interesting scope for hypothesis about the putative function of BPSS1823 and of the AmrAB-OprA efflux pump. *B. pseudomallei* Δ*amrA* Δ*BPSS1823* was shown to have defects in virulence mechanisms similar to phenotypes observed in quorum sensing mutants in other bacterial species. Combined with recent research implicating efflux pumps in the regulation of virulence factors and quorum sensing, it was hypothesised that both BPSS1823 and the AmrAB-OprA efflux pump are required for the formation or export of HSLs. Although efflux pump mutants have been used to characterise several virulence determinants in *B. pseudomallei,* caution should be exercised when using these strains until the role of the AmrAB-OprA pump has been fully characterised. Future work could involve measurement of HSLs produced in both *BPSS1823* mutant strains and characterisation of the proteome or secretome to identify potential targets of BPSS1823 and AmrAB-OprA.

The NMR structure of Lp-Mip has revealed information on the mode of binding to rapamycin and may provide a basis for structural design of Mip inhibitors to treat Legionnaires' disease (Ceymann *et al.,* 2008). Recently, novel small-molecule inhibitors of Lp-Mip have been identified using structural analysis (Juli

et al., 2011). The crystal structure of BPSS1823 and *in vitro* assay for PPIase activity could be used to aid inhibitor development. BPSS1823 therefore represents a novel target for the development of antimicrobials, which could be targeted in combination with antibiotics or efflux pump inhibitors to treat melioidosis.

The role of a SurA homologue was evaluated in Chapter 5 using closely related *B. thailandensis* as a model organism, with deletion of *BTH_I0576* resulting in defects in intracellular infection and virulence. Like SurA from *E. coli,* initial studies suggest that *B. thailandensis*Δ*BTH_I0576* may be defective in outer membrane integrity. To confirm the mutant strain has defects in OMP formation, the OMP profile could be visualised using SDS-PAGE and characterised using immunoblots. Furthermore, pilus formation could be investigated by monitoring adherence to cells or the bacterial cell surface visualised using electron microscopy. To confirm that the mutant phenotype is specifically due to deletion of *BTH_I0576,* the mutant strain could be complemented by introduction of a wildtype copy of the gene. Finally, a *BPSL0659* mutant could be constructed to confirm the role of a SurA homologue in *B. pseudomallei.*

Recombinant BPSL0659 was shown to exhibit PPIase activity (section 3.2.2.4), indicating that it is a functional PPIase. To determine whether the PPIase activity can be inhibited, parvulin inhibitors such as juglone could be tested. The function of BPSL0659 could be further characterised by determination of chaperone activity using a citrate synthase aggregation assay (Buchner *et al*., 1998). Furthermore, some studies have reported that the PPIase activity itself is not required for the physiological function of SurA (Behrens *et al*., 2001). An *E.*

coli surA mutant complemented with a SurA variant lacking PPIase domains revealed that features associated with chaperone mediated functions were restored, suggesting that the chaperone role of SurA is independent of its PPIase activity (Behrens *et al.,* 2001, Watts & Hunstad, 2008). Therefore, while BPSL0659 may represent a novel antimicrobial target in *B. pseudomallei,* inhibition of chaperone activity in addition to PPIase activity may be required.

In conclusion, the data presented in this thesis shows for the first time that PPIases from *B. pseudomallei* have potential as novel vaccine candidates and antimicrobial targets against melioidosis. Inhibition of virulence associated proteins, such as Mip and SurA, have been proposed as an alternative strategy to antibiotics (Escaich, 2008). As these targets are well conserved in several pathogenic bacteria, the characterisation of Mip and SurA in a range of bacteria may result in the development of novel broad spectrum therapeutics.

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