The identification and characterisation of novel antimicrobial targets in *Burkholderia pseudomallei*

Submitted by Laura Emma Marshall, to the University of Exeter as a thesis for the degree of
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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 previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

The bacterium *Burkholderia pseudomallei* causes the disease melioidosis, a significant public health threat in endemic regions and is a potential biowarfare agent. Treatment of melioidosis is intensive and prolonged and there is no licensed vaccine to protect against it. The aim of this study was to characterise novel targets for antimicrobials to improve treatment of melioidosis.

A holistic down selection process was undertaken in order to identify a range of possible novel and exploitable antimicrobial targets in *Burkholderia pseudomallei*. Four targets: FtsA, FtsZ, MraW and TonB were selected for characterisation by mutagenesis study.

FtsA and FtsZ are early effectors of cell division and are considered potential antimicrobial drug targets in other pathogenic bacteria. Genes for both were shown likely to be essential for viability in *Burkholderia pseudomallei,* following attempted deletion of the genes, thus confirming their potential for drug targeting for treatment of melioidosis.

MraW, a highly conserved methyltransferase, and TonB, the energiser for high affinity iron uptake in Gram negative bacteria, were also selected for characterisation as antimicrobial targets. In-frame deletions of the genes encoding these targets were constructed in *B. pseudomallei* K96243. In order to determine the roles played by MraW and TonB during infection, these mutants were characterised in several models of *Burkholderia pseudomallei* infection.

Deletion of *mraW* rendered the bacteria non-motile and led to attenuation during infection of Balb/C mice. A small growth defect was seen early during infection of macrophages by this mutant, whilst no attenuation was seen on deletion of *mraW* in *Galleria mellonella. Burkholderia pseudomallei ΔtonB* required free iron supplementation for growth. This mutant had an improved ability to invade murine macrophages, though the mutant was attenuated in both *Galleria mellonella* and Balb/C mice.

Attenuation of both mutants in a mammalian model of infection, support the strategy to target either of these proteins as novel targets for inhibition with small molecules during *Burkholderia pseudomallei* infection. However, an improved ability to infect macrophages by *Burkholderia pseudomallei ΔtonB* and non-complementation of this mutant by iron supplementation to *Galleria mellonella* suggests additional roles to iron uptake alone for TonB in *Burkholderia pseudomallei*, such as bacterial iron sensing and signalling.

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Declaration

Unless otherwise stated, the results and data presented in this thesis were solely the work of Laura Marshall

Dr Phillip Ireland conducted mutagenesis of *ftsZ* in *B. pseudomallei* K96243 and initial characterisation of the mutant strain. Stephanie Richards constructed *ΔtonB* pDM4 and *ΔmraW* pDM4 suicide plasmids.

Dr Simon Smith aided with electron microscopy.

Animal work was carried out by Dstl members of staff under the Animals (Scientific Procedures) Act 1986.

All of these experiments were designed and carried out under the direct supervision of Laura Marshall

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always knowing what to say and for believing in me from the beginning. This one’s for both of you.

Abbreviations

%	per cent
°C	degrees Celcius
µ	micro
AIDS	acquired immunodeficiency syndrome
ANOVA	analysis of variance
ATP	adenosine triphosphate
Bcc	*Burkholderia cepacia* complex
BLAST	basic local alignment search tool
bp	base pair
BPSL	*Burkholderia pseudomallei* large (chromosome)
BPSS	*Burkholderia pseudomallei* small (chromosome)
CcrM	cell cycle regulated methyltransferase
CDC	Centres for Disease Control
cDNA	complementary DNA
CF	cystic fibrosis
cfu	colony forming units
CHBP	cif homolog in *Burkholderia pseudomallei*
CM	cytoplasmic membrane
CPS	capsular polysaccharide
CTD	carboxy terminal domain
DAM	DNA adenine methyltransferase
dcw	division and cell wall
DIG	digoxigenin
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucletide triphosphates
EDTA	ethylenediaminetetracetic acid
EM	electron microscopy
erm-	erythromycin resistance methylases
F	Farad (capacitance)
FDA	Food and drug administration
FPN	ferroportin
fts-	filamentous thermo-sensitive
fur	ferric uptake regulation
g	gram
G-CSF	granulocyte colony-stimulating factor
gDNA	genomic DNA
GI	genetic island
GTP	guanosine triphosphate
h	hour
HIV	human immunodeficiency virus
i.g.	intragastric
i.m.	intramuscular

i.n.	intranasal
i.p.	intraperitoneal
IFN	interferon
IL	interleukin
irl	invasion related locus
IVET	in vitro expression technology
k	kilo
L	litre
LB	Luria Bertani
LFF	left flank forward
LFR	left flank reverse
LPS	lipopolysaccharide
M	molar
m	metres
m	milli
min	minute
mip	macrophage infectivity potentiator
ml	millilitre
MLS	macrolide, lincosamide, spectogramin
MNGC	multi-nucleated giant cells
MOI	multiplicity of infection
mra-	murein region A
mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation primary response gene 88
n	nano
NCBI	National Centre for Biotechnology Information
Nramp	natural resistance associated macrophage protein 1
NTD	amino terminal domain
OD	optical density
OM	outer membrane
ORF	open reading frame
PABA	paraminobenzoic acid
PAMP	pathogen associated molecular pattern
Pbp3	penicillin binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	potential of hydrogen
pmf	proton motive force
PPIase	peptidylprolyl cis/trans isomerase
RFF	right flank forward
RFR	right flank reverse
RNA	ribonucleic acid
RND	resistance, nodulation, division
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RT	room temperature

RT	reverse transcription
SAM	S-adenosine-L-methionine
SDS	sodium dodeco sulphate
Slc11a1	solute carrier 11a1
SOC (media)	super optimal catabolite media
SOD	super oxide dismutase
STM	signature-tagged mutagenesis
TAE	tris, acetic acid, EDTA
TBDT	tonB dependent transporter
TCA	trichloric acid
TFP	type IV pili
TLR	toll-like receptor
TMP-SMX	trimethoprim-sulphamethoxazole
TNF	tumour necrosis factor
TraDIS	transposon directed insertion site sequencing
TSSS	type VI secretion system
TTSS	type III secretion system
U	units
UV	ultraviolet
V	volts
WT	wildtype
x *g*	gravitational acceleration
Δ	delta

Chapter 1. Introduction

1.1 The history of antimicrobial discovery

1.1.1 Development of antimicrobials

In modern times, the idea of treating infectious disease with chemicals which would “be able to exert their full action exclusively on the parasite harboured within the organism’’, was postulated by Paul Ehrlich in the late 19th century (Foster and Raoult, 1974). Ehrlich, along with others, chemically synthesised panels of derivatives of the synthetic toxic dye atoxyl and tested them for treatment of syphilis in rabbits (Bosch and Rosich, 2008). Using this approach, the drug Salvarsan was discovered in 1909, and quickly became the standard treatment for syphilis (Bosch and Rosich, 2008). Screening panels of synthetic chemicals for structures which killed or inhibited infectious organisms whilst remaining non-toxic to the host became the strategy of choice for the pharmaceutical industry of the day. This approach also led to the discovery of Prontosil, a precursor of the antimicrobial sulfanilimide. Prontosil was shown to possess antimicrobial activity against a range of bacteria (Domagk, 1935). Administration of these synthetic molecules which have a systemic antimicrobial action has been referred to as chemotherapy (Greenwood, 2003).

The synthetic approach to anti-infective research was soon supplemented by a breakthrough by Florey and Chain in 1940 (Chain *et al*., 1940). The pair described the ability to purify enough of the antibiotic penicillin from the mould *Penicillium chrysogenum* for clinical testing. The antibiotic properties of mould had long been known, and had been demonstrated by Alexander Fleming in the late 1920s. Fleming understood the potential of the active substance

produced by *P. chrysogenum,* which curbed the growth of other organisms, as a therapeutic. Florey and Chain's purification method made it possible to examine toxicity, establish activity, study distribution, excretion and prove efficacy of the drug in animals (Chain *et al*., 1940). By 1945, penicillin was mass produced and distributed as an antimicrobial treatment. Subsequently, molecules from microorganisms which are antagonistic to the growth or life of others in high dilution have been termed 'antibiotics' (Greenwood, 2003).

Using these two screening approaches for discovery of antimicrobial molecules, the 1940s, 50s and 60s became a golden era for antibiotic discovery. Several new classes of antibiotic were either synthesized, or identified from nature during these years. Primarily, new compounds were identified by screening of compounds produced by actinomycetes species from soil samples (Aminov, 2010). Modes of action of these compounds fell into five main groups, targeting essential processes including: cell wall synthesis, DNA synthesis, protein synthesis (primarily targeting the ribosome), cell membrane stability and folate synthesis.

Antimicrobials are generally considered to fall into two main types: bactericidal and bacteriostatic. As the names suggest, bactericidal agents act to kill bacteria, whilst bacteriostatic agents halt growth of the bacteria. In clinical practice however, these two categories are much more fluid. A bactericidal agent is unlikely to kill every single bacterium within a host and a bacteriostatic agent may actually kill a high proportion but not all of the disease causing bacteria within the host. Traditionally, bactericidal agents

were considered to be of greater benefit during infection than bacteriostatic agents. However, the disadvantages of bactericidal agents, such as rapid lysis of bacteria leading to an endotoxin surge and resultant inappropriate inflammatory response has been recognised (Jackson and Kropp, 1992). The advantages of using a bacteriostatic agent to stem production of endotoxic shock causing toxins such as by staphylococci has also been investigated.

After the 1960s, few compounds were discovered which targeted alternative pathways and research became focussed on improving the properties of those chemical scaffolds which had already been discovered. Chemists altered structures to make compounds more stable and less toxic, change solubility and absorption rates and improve antimicrobial properties. Antibiotic structures which were discovered in nature and altered or added to, to improve their pharmacological properties, are known as semi-synthetics. A huge range of drug variants were produced which were minor improvements on previous structures (Greenwood, 2003).

1.1.2 Antimicrobial resistance

The short lifecycles of infectious bacteria means evolutionary change to circumvent the effect of a compound which kills or curbs growth is inevitable. Resultantly and as predicted by Fleming, resistance to all antimicrobial compounds introduced for therapy since the nineteenth century has emerged (Aminov, 2010; Hopwood *et al*., 2007). This effect is exacerbated when treatment with antimicrobials is not at a sufficient concentration or for a sufficient period of time, or when an inappropriate antibiotic is used (Tenover, 2006). Many genes conferring antimicrobial resistance are of ancient origin,

suggesting they emerged as a way of a bacterial species defending against antibiotic producing organisms living in the same ecosphere (Allen *et al*., 2010).

There are four main mechanisms bacteria have evolved to circumvent the effects of an antimicrobial drug. First, pathways to degrade or modify the anti-infective drug applied have emerged. For example, β-lactamase enzymes degrade β-lactam antibiotics and are a widespread cause of β-lactam resistance among human pathogens (Struelens, 2003).

Secondly, bacteria have been shown to alter the site which is targeted by the anti-infective of choice, to prevent its action (Tenover, 2006). An example of this type of resistance is of compounds targeting penicillin binding proteins (Pbps). These proteins are the major target for β-lactam antibiotics. Structural modification of Pbps to prevent β-lactam binding, is a major resistance mechanism to these drugs (Contreras-Martel *et al*., 2006). Another example of target modification by an enzymatic mechanism utilises erythromycin resistance methylases (Erm-) (Vester and Douthwaite, 2001). These enzymes modify 23S rRNA in the ribosome of Gram negative bacteria (Vester and Douthwaite, 2001). This modification confers resistance to a range of antimicrobials which target the ribosome as their mechanism of action.
The third main mechanism of antibiotic resistance is to modify the metabolic pathway which a drug targets. For example, *Bacillus anthracis* has been shown to encode a dihydrofolate reductase enzyme which has a low affinity

for trimethoprim, conferring resistance to this antibiotic (Barrow *et al*., 2004).

Finally, reducing accumulation of a drug within the bacteria has been demonstrated as a mechanism for resistance (Struelens, 2003). Many bacteria achieve this by efflux of antibiotic, through pumps specific to that class of antibiotic (Struelens, 2003). As previously described, efflux is major mechanism for *B. pseudomallei* antibiotic resistance.

1.1.2.1 Inhibitors of resistance mechanisms

To combat emergence of resistance among disease causing bacteria, anti-infective drugs have been developed to specifically combat resistance mechanisms. These compounds can then be co-administered with the drug which would otherwise be useless as a result of the resistance mechanism. For example, β-lactamase inhibitors such as clavulanic acid, prevent the action of bacterial β-lactamases to degrade β-lactam antibiotics, thus allowing the β-lactam drug to have its desired effect (Drawz and Bonomo, 2010). Clavulanic acid was identified from *Streptococcus clavuligerus* in 1972, and reached the UK clinic in combination with amoxicillin, a broad spectrum β-lactam antibiotic, in 1981 (Geddes *et al*., 2007).

In order to defeat efflux pump-mediated antibiotic resistance, efflux pump inhibitors have been developed, though remain unlicensed for use in humans (Lomovskaya and Hecker, 2010). Again, this type of drug would be administered in combination with an antibiotic which would otherwise be effluxed by the bacteria, thus allowing accumulation of the drug within the cell, enabling the intended action of the antimicrobial.

1.1.3 The latest antimicrobials

By the 1990s, the rate of discovery of new anti-infective agents had diminished greatly (Figure 1.1). Few new antibiotics have been licensed in the last ten years. Some of the most recent antibiotics brought to market include daptomycin, licensed in 2003, tigecycline, licensed for use by the FDA in 2005 and fidaxomicin, licensed for use to treat *Clostridium difficile* in 2011. Daptomycin is a cyclic lipopeptide produced by *Streptomyces roseosporus* which has a distinct anti-microbial mechanism of action to interfere with Gram positive bacterial cell membranes (Nguyen *et al*., 2006). This drug is primarily used to treat methicillin resistant *S. aureus* and is considered a 'last resort' drug. Evolution of resistance to daptomycin during treatment of *S. aureus* has been documented (Hayden *et al*., 2005). Tigecycline was modified from a tetracycline scaffold and acts on the 30S ribosomal subunit preventing protein synthesis. However, antibiotic resistance to tigecycline has also been documented in *Acinetobacter baumanni* (Peleg *et al*., 2007) and *Pseudomonas aeruginosa* (Dean *et al*., 2003). Fidaxomicin is a macrolide which inhibits RNA synthesis (Sergio *et al*., 1975). It is poorly absorbed (Swanson *et al*., 1991) and specifically acts on Gram positive bacteria, making it an ideal candidate for the treatment of *C. difficile*-associated diarrhoea (Ackermann *et al*., 2004). Since licensure in 2011, there have been no reported cases of resistance to this novel antimicrobial (Lancaster, 2012).

1.1.4 Post genomic antimicrobial discovery

With the decline in novel antimicrobial structures and emergence of resistance to all of the existing anti-infectives, the requirement for novel antimicrobial is increasingly pressing. In 1995, the first bacterial genome, of *Haemophilus*

influenza, was fully sequenced (Fleischmann *et al*., 1995). Subsequently, many bacterial genomes have been fully sequenced (www.sanger.ac.uk). The

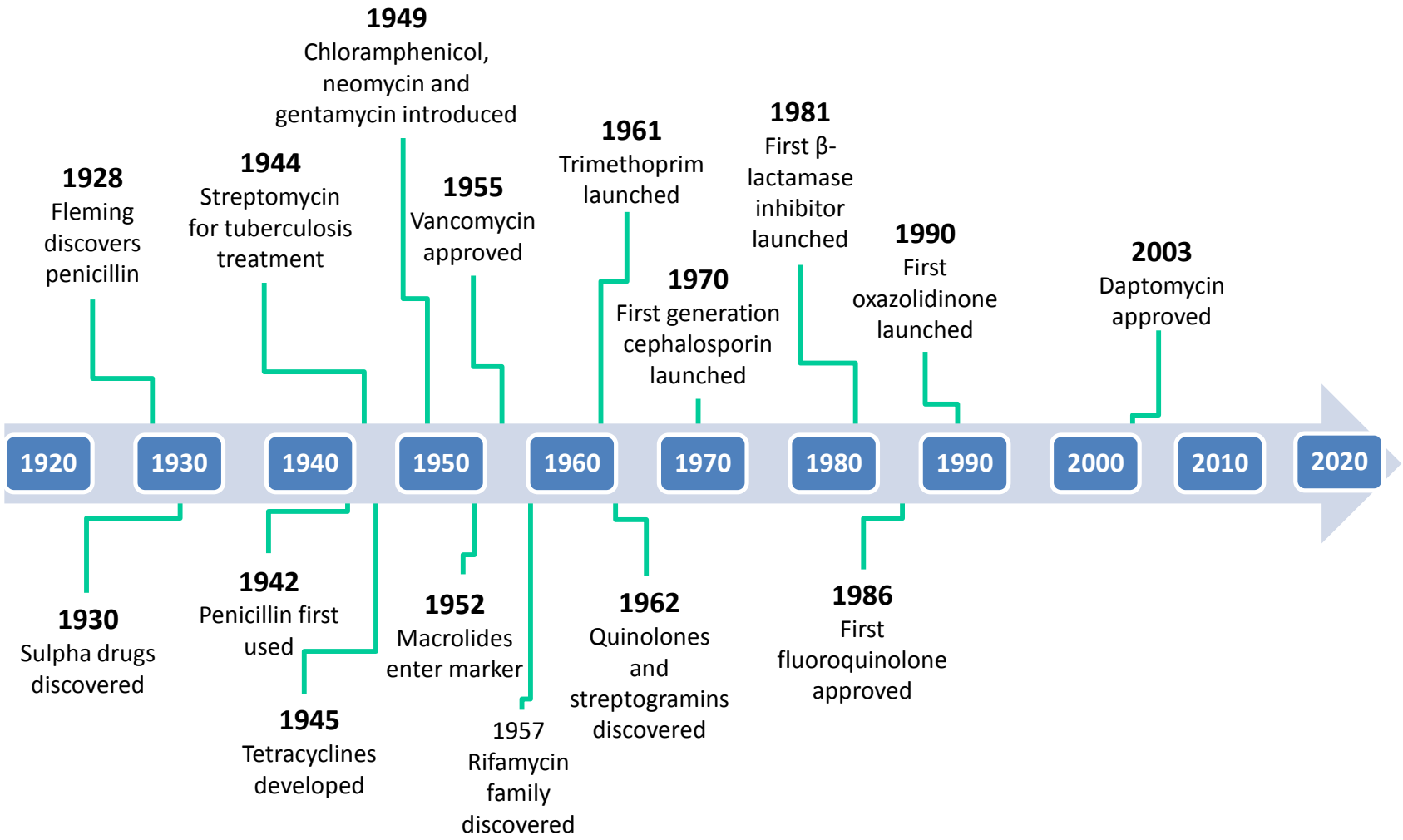

Figure 1.1 Milestones in antibiotic drug development. Adapted from Hopwood *et al.*, 2007.

arrival of the genomic era brought optimism to the field of antimicrobial discovery. It was considered that the vast arrays of pathogen genomic data, would reveal a mine of novel mechanisms and points which could be targeted with antimicrobials. Since the first sequenced bacterial genomes, the ability to manipulate both pathogen and host genomes *in silico*, *in vitro* and *in vivo* has expanded massively. The data from these studies adds to the amount of potential information which can be gathered concerning host pathogen interactions. In addition it was hoped that genomic data regarding the antibiotic producing actinomycete species would highlight novel chemistries for antimicrobial drug structures.

1.1.5 Experimental methods for identification of novel antimicrobial targets

Genomic data has been used to identify conserved novel antimicrobial targets in disease causing bacteria (Payne *et al*., 2008). A range of criteria can be applied to genomes *in silico* to identify new targets. For example, genes which are highly conserved among a wide range of pathogenic species, theoretically present targets which are important for basic bacterial cellular function. In addition, genes conserved among pathogens and absent in non-pathogens have been sought. These targets would elicit a selective drug affect against those pathogens which encode the target, whilst leaving commensal bacteria undisturbed.

Combining genomic data with *in vitro* screening can also yield new targets. An example of using molecular and genomic techniques, named signature-tagged mutagenesis (STM), was first described in 1995 for *Salmonella*

enterica serovar Typhimurium. STM uses negative selection to identify genes essential under a particular condition (Hensel *et al.,* 1995). Hensel *et al*., used a pool of transposon mutants, each containing a 'bar-coded' transposon insertion and inoculated the pool into a disease model of choice. Those mutants which were not recovered following infection were assumed to have been outcompeted and therefore the gene inactivated in those mutants was considered a virulence determinant. An updated STM technique known as transposon-directed insertion-site sequencing (TraDIS) uses the same negative selection principle as STM, but uses high-throughput sequencing techniques to screen much larger pools of mutants. The first study of this kind screened approximately 1.1 million *S. typhimurium* serovar Typhi mutants during both *in vitro* and *in vivo* growth to identify genes required for bile tolerance (Langridge *et al*. 2009). The ability to screen this many mutants ensures all (non-essential) genes can be screened through any possible bacterial niche of interest.

1.1.6 Anti-virulence targets

Whilst those factors considered indispensable for bacterial viability, known as essential gene products, are the traditional targets for novel antimicrobials, there is a modern paradigm which suggests factors key in virulence may present more attractive targets for novel antimicrobials (Rasko and Sperandio, 2010). Specifically inhibiting bacterial effectors of virulence creates a scenario in which the bacteria become disarmed and are unable to cause damage in the host. This in turn would allow the host immune system to clear the infection (Clatworthy *et al*., 2007). A potential advantage of this type of antimicrobial is the reduced selection pressure when compared to targeting of

essential processes, for emergence of resistance. Several features of this strategy support this hypothesis. First, the drug would not kill the bacteria on which it exerts it effect, only disarm it, within the host. Furthermore, these targets are generally only active during infection, reducing the opportunity for development of resistance at other times in the bacterial lifecycle. Until recently, this hypothesis was unproven. However, a recent study demonstrated the anti-virulence targeting of the quorum sensing response in *P. aeruginosa* leads to a reduced rate of emergence of resistance (Mellbye and Schuster, 2011). This phenomenon occurs as inhibitor sensitive bacteria are enriched over resistant bacteria because they are able to utilise QS regulated factors synthesized by the resistant clones, whilst not producing them themselves. This is the first report of a reduction in resistance emergence as a result of targeting virulence over essential factors in bacteria.

The ideal ‘anti-virulence’ drug would target common virulence associated mechanisms in pathogens, reducing the effect of traditional broad spectrum antibiotics on commensal bacteria in the host. However, in reality, individual pathogen species have distinct sets of virulence factors which are expressed following host specific cues and any potential anti-virulence drugs may be relatively narrow spectrum.

Targeting of effectors of virulence with new inhibitory compounds over essential processes also presents challenges for their development. For example, determination of a compounds minimal inhibitory concentration *in vitro* has been a traditional decision making criteria to further investigation of a

novel anti-infective compound (Brown and Wright, 2005). Universally recognised methods to measure effectiveness of an anti-virulence therapy *in vitro* are required to further this field of research.

Furthermore, the concept of use of hypothetical anti-virulence compounds should be defined. If growth of the pathogen is not inhibited in the host, maintenance of the therapy until a significant reduction of bacterial load in the host would be required to ensure effectiveness of the treatment. In reality, an anti-virulence drug would likely be administered in combination with traditional antimicrobials.

1.1.7 Anti-virulence target discovery

To capture those genes specifically regulated in the host, microarray had previously been used to compare the transcripts differentially regulated under one condition versus another. Many of the genes identified in these studies are hypotheticals, and the reason for their induction remains unknown. An alternative technique to investigate genes regulated by host cues is known as in vitro expression technology (IVET). IVET is a promoter trap technique which randomly fuses promoter regions to a reporter. Expression of the reporter thus identifies promoters active under the condition of interest, be it *in vivo* or *in vitro* infection (Angelichio and Camilli, 2002).

1.1.8 Target validation

Mutagenesis studies to inactivate the genes encoding the target of interest, whether essential or virulence-associated, can validate essentiality or the role of the gene product. Confirming essentiality ensures there is no hidden redundant mechanism for the gene which is being targeted (Payne *et al*.,

2008). Further, whilst up-regulation of virulence factors suggest they play an important role during infection, they may not in fact be essential during this process and therefore inhibiting that target as an anti-virulence strategy would be worthless. Mutagenesis of a virulence target and investigation of that gene product's role at various stages of infection can therefore elucidate the role played by that factor and the viability of an antimicrobial strategy to target it.

1.1.9 Target exploitation

Once a target has been characterised there are multiple strategies for discovery of target inhibiting molecules. A source of purified target is important for development of inhibitors following characterisation. Structural determination of a target by X-ray crystallography, nuclear magnetic resonance and homology modelling can be utilised for structure based drug design (Singh *et al*., 2006). Fragments and compounds from a database can then be scored for binding likelihood *in silico*. Alternatively, or in conjunction with *in silico* determination of target/compound interaction, *in vitro* biochemical studies can be undertaken. Targets with *in vitro* activity which can be measured, ideally in a high throughput format allow for screening of libraries of possible inhibitory molecules.

The source and quality of compound libraries will play a big role in future antimicrobial drug discovery (Payne *et al*., 2008). A return to screening compounds produced by actinomycetes species is becoming a more popular strategy for discovery of novel antimicrobial compounds (Baltz, 2008). The antimicrobial molecules produced by these species have evolved to penetrate other organisms and inhibit target molecules. Whilst screening of natural

products was abandoned by many drug discovery programmes, it has recently been acknowledged there is still untapped potential from natural sources. Advances in technology to miniaturise high throughput fermentation of actinomycetes (Baltz, 2007), isolation of new species from the marine environment (Bull and Stach 2007), discovery of cryptic biochemical pathways, and the possibility for combinatorial chemistries, together present new opportunities for antimicrobial drug discovery from natural sources (Baltz, 2008).

1.2 *Burkholderia pseudomallei*

Burkholderia pseudomallei is a Gram negative, proteobacterium which was originally identified by Alfred Whitmore in 1911 as the causative agent of a 'glanders-like' disease observed in heroin addicts in Yangon, the capital of Myanmar (Whitmore, 1913). The bacterium is a saprophyte which can be isolated from soil and water in endemic regions, as well as being the etiological agent of the human disease melioidosis (Cheng and Currie, 2005). *B. pseudomallei* is aerobic and non-spore forming. It grows well in either liquid broth or solid media. On Ashdown's media a range of colony morphologies are observed (Chantratita *et al.*, 2007). The bacterium is oxidase positive and is unable to assimilate the sugar arabinose (Smith *et al.,* 1997).

1.2.1 Taxonomy

B. pseudomallei is in the genus *Burkholderia,* which is comprised of greater than 40 species (http://www.bacterio.cict.fr/b/burkholderia.html). These bacteria inhabit a large range of biological niches including soil, water, plants and fungal mycelia*. Burkholderia* species are traditionally known as plant pathogens and soil inhabitants. However, the closely related *B. pseudomallei* and *Burkholderia mallei,* are both pathogens of humans and animals. *B. pseudomallei* and *B. mallei* are classified as level three pathogens by the Advisory Committee on Dangerous Pathogens. Another group of bacteria within this genus which are recognised for their ability to infect humans are the *Burkholderia cepacia* complex (Bcc) species (LiPuma, 2003). The Bcc comprises seventeen species (Vanlaere et al., 2008), which can cause 'cepacia syndrome', commonly observed in human cystic fibrosis (CF) patients. This disease includes fever, pneumonic illness and can lead to

respiratory failure (Isles et al*.,* 1984). Generally, these species are opportunistic pathogens of humans, and many of them usually occupy an alternative niche.

B. pseudomallei is closely related to *B. mallei*, the causative agent of glanders. This infection primarily causes disease of equines, though it has also been documented to infect humans (Godoy *et al*., 2003, Whitlock *et al*., 2007). Another closely related species, *Burkholderia thailandensis,* was previously considered to be a bio-type of *B. pseudomallei. B. thailandensis* was accepted as a separate species due to differences in the species 16S RNA sequences, biochemical profiles and general avirulence compared to *B. pseudomallei* (Brett *et al.* 1998). There are reports of *B. thailandensis* causing disease in humans, but this is considered extremely rare (Glass *et al*., 2006; Lertpatansuwan *et al*., 1999). *B. pseudomallei* is primarily distinguished from *B. thailandensis* by its inability to assimilate arabinose whilst *B. thailandensis* can (Smith *et al*., 1997).

1.2.2 Genomics

The genome of *B. pseudomallei* was first fully sequenced by the Sanger Centre (Holden *et al.*, 2004). The sequenced strain, K96243 was obtained from a Thai melioidosis patient and was shown to be composed of two circular chromosomes totalling 7.25 megabase-pairs (Holden *et al.*, 2004). Chromosome 1 contains a higher degree of coding sequences for core functions such as macromolecule biosynthesis and amino acid metabolism. Chromosome 2 contains a higher proportion of open reading frames (ORFs) associated with accessory functions, mainly adaptation to atypical conditions such as osmotic protection and iron acquisition. The *B. pseudomallei* genome

is larger and more diverse than that of its clonal derivative *B. mallei* which is on average is 5.7 megabase-pairs (Losada *et al*., 2010). *B. mallei* has lost sections of DNA and encodes a larger proportion of genes containing IS elements ranging from 166 to 218 in sequenced strains, compared to 42 in *B. pseudomallei* K96243 (Holden *et al*., 2004; Losada *et al*., 2010). *B. mallei* also contains more pseudogenes compared to *B. pseudomallei* (Losada *et al*., 2010). This extensive genome rearrangement and loss has curbed *B. mallei's* ability to survive outside of a mammalian host and is likely a result of an evolutionary bottleneck. Contrastingly *B. pseudomallei* contains a high number of genetic islands acquired from a variety of sources in its recent evolution. This reflects the flexibility of *B. pseudomallei* and its ability to survive and proliferate in both the environment and the human host (Holden *et al.*, 2004).

More recent sequencing efforts of a range of five *B. pseudomallei* strains from both Australia and South East Asia have revealed an enormous amount of diversity of origin and function in the recently acquired genomic islands among strains (Tuanyok *et al.*, 2008). Whilst all of the strains possessed a core set of genes, 71 different genomic islands (GIs) of between 3.91 Kb and 107.0 Kb were identified in these five strains alone. The GIs encoded by each strain varied greatly and GIs encoded at the same position often encoded different genes in different strains. The contents of the GIs included prophages, genes for metabolism, virulence associated genes and a high proportion of genes of unknown function. The huge genetic diversity provided by GIs may be associated with the host range and environmental niche of the

B. pseudomallei bacterium possessing them. Clustering isolates based on their accessory genome content was shown to group isolates into three main clades (SIm *et al*., 2008). Clinical strains were significantly over-represented in one of these clades, and animal isolates were over-represented in another of the clades. It is therefore suggested that strains associated with human melioidosis possess a distinct accessory genome compared to animal and environmental strains (SIm *et al*., 2008).

1.2.3 Melioidosis

Melioidosis is the name for the broad range of clinical presentations caused by infection with *B. pseudomallei*. The type of disease caused by this bacterium is dependent on the route of infection, health status of the host and the dose received (Barnes and Ketheesan, 2005; Srisurat *et al*., 2010; White, 2003). The most severe forms of the disease are acute septicaemia and acute pulmonary infection, both of which often progress rapidly to death (Currie *et al*., 2010). Sub-acute forms of melioidosis are characterised by febrile illness, and the formation of abscesses in the organs, including lungs, liver, spleen, bones and soft tissue (Currie *et al*., 2010). *B. pseudomallei* can also cause chronic localised infection in the host (Currie *et al*., 2010). Additionally, *B. pseudomallei* has been documented to re-activate in recently immuno-compromised patients many years after an initial exposure, in the most extreme documented case, 62 years following initial exposure in a World War II prisoner of war (Ngauy *et al*., 2005). The bacterium is therefore assumed to be able to lie dormant and undetectable as a latent infection for long periods of time.

Fifty percent of presentations of the disease have some lung involvement, either as a result of a primary lung abscess, or a secondary abscess due to septicaemic spread of the infection (Cheng and Currie, 2005). Other common documented presentations of the disease include acute parotitis in 38% of paediatric cases in Thailand (Dance *et al*., 1989). In Australia, presentation of prostatic abscess has been documented in 20% of male patients (Currie *et al*., 2010). In some cases melioidosis is only confirmed at post-mortem examination. Of note, in North East Thailand, seroconversion occurs in up to 75% of children under 15, with the proportion of children with detectable antibody titres peaking at age four. This indicates the majority of the population in this region are exposed to the bacterium at an early age and that asymptomatic infection is common (Wuthiekanun *et al.,* 2006). The range of manifestations of infection with *B. pseudomallei* makes the disease extremely difficult to diagnose and melioidosis is often mistaken for other diseases including tuberculosis (Vidyalakshmi *et al*., 2008).

1.2.4 Epidemiology

B. pseudomallei is traditionally regarded as endemic to South East Asia, mainly Thailand, and Northern Australia, between tropical latitudes 20°N and 20°S. Certainly the majority of cases are reported from these regions (Cheng and Currie, 2005). A study over 10 years to 2006 found melioidosis to be the third most common cause of death due to infectious disease after human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) and tuberculosis in a highly endemic region of northeastern Thailand (Limmathorotsakul *et al.,* 2010). Further, a recent study highlights a high incidence of the disease in other regions of Thailand, indicating that the

disease occurs throughout the country (Bhengsri *et al*., 2011). In tropical northern Australia, a prospective study over 20 years to 2010 revealed that melioidosis incidence ranged between 5.1/100,000 to 41.7/100,000 population, with a decreasing mortality rate over the progression of the study (Currie *et al.*, 2010). Following the Boxing Day tsunami in 2004, the number of reported cases of melioidosis increased from the entire tsunami affected region, indicating a wider endemnicity than traditionally accepted for this pathogen, including Aceh, Indonesia (Athan *et al.*, 2005).

It is becoming clear, as diagnostic facilities become more accurate and widespread, that lack of facilities with the ability to effectively identify *B. pseudomallei* is likely to have masked the real prevalence of this bacterium across the globe. Sporadic identification of *B. pseudomallei* infection indicates the bacterium is likely to be endemic to the Pacific Islands, the Indian subcontinent, Central and South America, and the Caribbean (Figure 1.2) (Dance, 1991; Currie *et al*., 2008; Currie, 2008). Further, cases of human and animal melioidosis have been reported in east and west Africa (Dance, 1991). Reported cases of melioidosis from all of these regions, as with many tsunami originating melioidosis reports, have often been identified in travellers exporting the disease to their home countries, where they can be accurately diagnosed (Currie *et al.,* 2008).

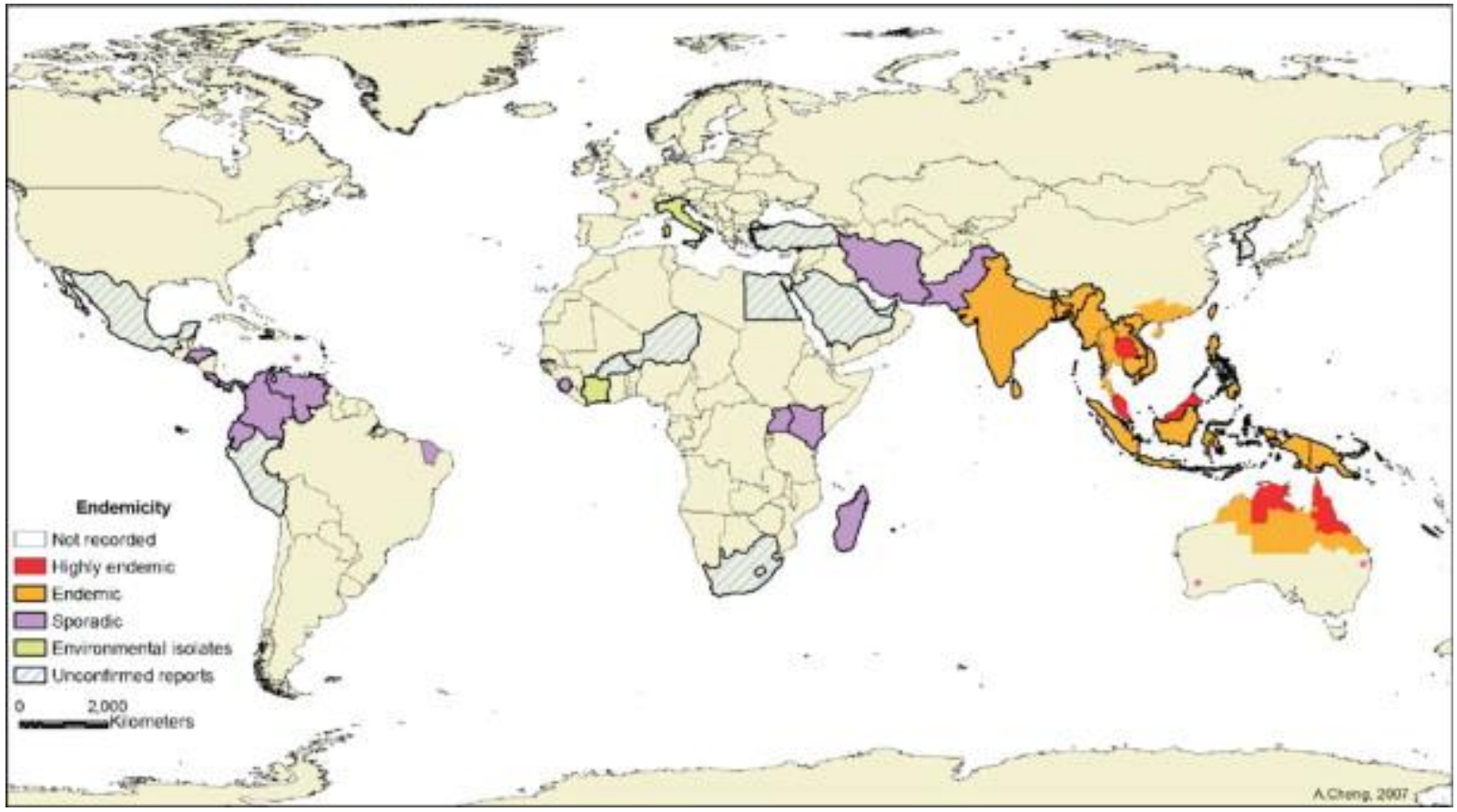

Figure 1.2 Worldwide distribution of melioidosis and *B. pseudomallei.* Pink asterisks indicate three documented temperate outbreaks of melioidosis (Currie *et al*., 2008)

1.2.5 Risk factors

There are several risk factors associated with melioidosis. Primarily, a high proportion of melioidosis patients are diabetic. In prospective studies, 39% in endemic North Eastern Thailand and 47% in Darwin, Australia co-presented with *B. pseudomallei* infection and diabetes (Currie *et al.* 2010; Limmathurotsakul *et al.,* 2010). Other risk factors for melioidosis include hazardous alcohol use, chronic lung disease and chronic renal disease (Currie *et al.,* 2010). Most recently, a case control study demonstrated association of certain host toll-like receptor (TLR) variants with development of melioidosis (West *et al.*, 2012).

Melioidosis is considered an occupational risk of rice paddy farmers in South East Asia. The environmental niche for *B. pseudomallei* is soil and water and the bacterium is readily cultured from these environments (Limmathurotsakul *et al.,* 2010). During a six month study in northeast Thailand during 1997, 84.9% of patients presenting with melioidosis were rice farm workers (Supputtamongkol *et al.,* 1999).

Interestingly, there is no association of melioidosis with HIV infection (Currie *et al.* 2010; Limmathurotsakul *et al.,* 2010). Additionally, a study of a small number of co-infected individuals from northeast Thailand demonstrated HIV infection does not affect presentation or outcome following infection with *B. pseudomallei* (Chierakul *et al.,* 2005).

1.2.6 Virulence mechanisms

B. pseudomallei is considered an intracellular pathogen and has many adaptations for adherence to, invasion of, survival within and escape from host cells (summarised Figure 1.3). Further, *B. pseudomallei* is well adapted for survival in host tissues, including survival in and resistance to serum. The mechanisms *B. pseudomallei* employs to infect and survive within the host are currently the subject of research and pathogenesis of this disease is becoming more fully understood.

1.2.6.1 Adherence to host cells

Type IV pili (TFP) have been shown to play a key role in the adherence of *B. pseudomallei* to epithelial cells. Multiple TFP associated loci have been identified in *B. pseudomallei*, one of which contains a full length *pilA* homolog. Deletion of this *pilA* from *B. pseudomallei* K96243 leads to reduced adhesion to epithelial cells as well as reduced virulence in *C. elegans* and a Balb/C mouse model of melioidosis at low doses (Essex-Lopresti *et al*., 2005).

More recently, the autotransporters BoaA and BoaB were shown to increase adherence of *E. coli* bacterium to three human epithelial cell lines (Balder *et al*., 2010). One of these culture models is a more representative culture of the lung known as normal human bronchial epithelium, which is grown in an air-liquid interface. Inactivation of *boaA* in *B. pseudomallei* strain DD503 decreased attachment to the same three types of epithelial cell. This indicates that BoaA plays a role in attachment to epithelial cells *(*Balder *et al.,* 2010*).*

1.2.6.2 Invasion of host cells

B. pseudomallei has been shown to invade the human respiratory epithelial cell line A549 more efficiently than *B. thailandensis,* suggesting that invasion of host cells is important during pathogenesis (Kespichayawattana *et al*., 2004). Several *B. pseudomallei* gene products have been shown to play a role in facilitating invasion of cultured cells. The two-component regulator and sensor system re-named invasion-related locus (*irlRS)* was identified by transposon mutagenesis of *B. pseudomallei* strain 1026b and the inactivation of this gene led to a 90% reduction in invasion levels of A549 cells (Jones *et al*., 1996). Although this system is key for invasion of cells, no loss of virulence was seen in diabetic rats, hamsters or C57BL/6 mice when this system was inactivated (Wiersinga *et al*., 2008).

The *B. pseudomallei* genome encodes three Type III Secretion Systems (TTSS), two of which are homologous to TTSSs in the plant pathogens *Ralstonia solanacearum* and *Xanthamonas* species (Winstanley *et al*., 1999; Rainbow *et al*., 2002). A third TTSS, shows greatest homology with the human pathogen *Salmonella enterica* serovar Typhimurium (Attree and Attree 2001). This third TTSS has been named *Burkholderia* secretion apparatus (*bsa*) in *B. pseudomallei* and plays an important role during mammalian infection (Stevens *et al*., 2003). Components of this third TTSS and the effector proteins it secretes into host cells, have been shown in several studies to play a role in a number of key host cell events during infection including invasion of cells. Inactivation of *bopE and bopC*, both type III secreted factors, led to a defect in invasion of epithelial cells (Stevens *et al*., 2003; Muangman *et al*., 2011).

1.2.6.3 Intracellular survival

B. pseudomallei has been shown to replicate within a range of cultured cells (Jones *et al*., 1996). A transposon mutagenesis study by Pilatz *et al*. (2006) identified genes which had no effect on invasion, but were required for replication in HeLa cells or J774A.1 macrophages and were required for virulence in Balb/C mice. First, BPSL1528, encodes a putative exported protein and its inactivation led to attenuation of virulence. The other four genes encoded proteins in the purine, histidine and para-aminobenzoate biosynthetic pathways. Products of these pathways appear to be limited in the intracellular environment, and therefore it is proposed these genes are essential for *B. pseudomallei* metabolism in the intracellular environment.

Escape from endocytic vesicles occurs early during infection of host cells and TTSS3 of *B. pseudomallei* has been shown to play a role in this process. The mutation of genes encoding structural and translocation components of the TTSS3, including BsaZ, BipD and BsaQ, have been shown to increase the association of these *B. pseudomallei* mutants with the phagosomal marker LAMP1 in macrophage cell lines. The association with marker suggests delayed or loss of the ability to escape from endocytic vesicles (Stevens *et al*., 2002; Burtnick *et al*., 2008; Muansombot *et al*., 2008).

Furthermore, disruption of *bipB*, also found in the *bsa* gene cluster led to a decrease in intracellular survival and a defect in induction of apoptosis in host cells (Suparak *et al*., 2005). Importantly, except for BopE, all of the *B. pseudomallei* TTSS3 proteins investigated to date have been shown to be

important for *in vivo* infection and the bacterium is attenuated in their absence (Stevens *et al*., 2004, Suparak *et al*., 2005;).

Another recently described intracellular effector is the macrophage infectivity potentiator (mip) like protein. This enzyme possesses peptidylprolyl cis/trans isomerase(PPIase) activity. Inactivation of this gene had a multitude of effects on cells including reduced acid tolerance and rendering the bacteria immotile. *B. pseudomallei* Mip mutants also demonstrated reduced replication within J774A.1 macrophages and A549 epithelial cells. This mutant was also attenuated in Balb/C mice (Norville *et al*., 2011).

The production of an unidentified toxin which inhibited protein and DNA synthesis in macrophages and causes paralytic killing of *C. elegans* by *B. pseudomallei* has previously been described (Mohamed *et al*., 1989; O'Quinn *et al*., 2001). Recently a potent toxin encoded by BPSL1549 was identified. This toxin was demonstrated to act intracellularly by promoting deamination of the human translation initiator eIF4A, abolishing its helicase activity and inhibiting translation. This protein is lethal to J774A.1 macrophages and to Balb/C mice via the intra-peritoneal route. Further, deletion of the gene from *B. pseudomallei* K96243 led to a 100 fold increase in the mean lethal dose of the bacteria (Cruz-Migoni *et al*., 2011).

B. pseudomallei encodes a second toxin, a type III secreted cycle inhibiting factor (cif), termed cif homolog in *B. pseudomallei* (CHBP). This toxin

functions to cause cell cycle arrest in HeLa cells (Yao *et al*., 2009), although a link to virulence for this factor is as yet unclear.

Moreover, whilst evidence for the role of intracellular reactive oxygen intermediates in resistance to *B. pseudomallei* remains conflicted, BPSL1001 has been shown to encode an active superoxide dismutase (SOD) known as SodC. This protein has been shown to play a role in protection against reactive intermediates (Vanaporn *et al*. 2011). On deletion of this gene, *B. pseudomallei* became more sensitive to extracellular, but not intracellular generated superoxide, suggesting it is used to protect against exogenous reactive oxygen species. This is further supported by the presence of a signal peptide and export of the protein across the cytoplasmic membrane. Deletion of *sodC* from *B. pseudomallei* K96243 leads to reduced survival in J774A.1 macrophages and attenuation of infection in Balb/C mice.

1.2.6.4 Cell to cell spread

A further feature of infection of host cells with *B. pseudomallei* is the bacteria's ability to spread intercellularly via actin based motility (Stevens *et al*., 2005). In this process bacteria hijack the host cell cytoskeleton, polymerising actin to propel themselves through the cytoplasm. These propelled bacteria form protrusions at the cell membrane, facilitating spread of bacteria between adjacent host cells (Gouin *et al*., 2005). BimA is a secreted autotransported factor which plays a key role in promoting actin polymerisation at one pole of *B. pseudomallei* bacteria in host cells (Stevens *et al.*, 2005). The inactivation of *bimA* completely abolishes actin based motility, formation of membrane protrusions in macrophages (Stevens *et al.*, 2005) and formation of plaques in

epithelial cell monolayers (Sitthidet *et al*., 2011). This suggests a role for BimA in cell to cell spread.

B. pseudomallei is also able to form multi-nucleated giant cells (MNGC) by inducing fusion of host cells in a poorly understood mechanism. MNGC have been observed both *in vitro* and during human infection (Wong *et al*., 1995; Harley *et al*., 1998). A Type VI secretion system (TSSS) has recently been shown to play a key role in formation of MNGCs. *B. pseudomallei* has been shown to encode six TSSSs, one of which is up-regulated during co-culture with macrophages (Shalom *et al*., 2007). Deletion of the gene encoding one of the components of this system, Hcp1, has been shown to lead to reduced intracellular growth in macrophages and a defect in induction of MNGCs in culture. Furthermore, this mutant was significantly attenuated in Syrian hamsters (Burtnick *et al*., 2011). It has been suggested that the formation of these MNGCs may have evolved to provide a niche for optimal replication and spread of the bacteria (Allwood *et al*., 2012).

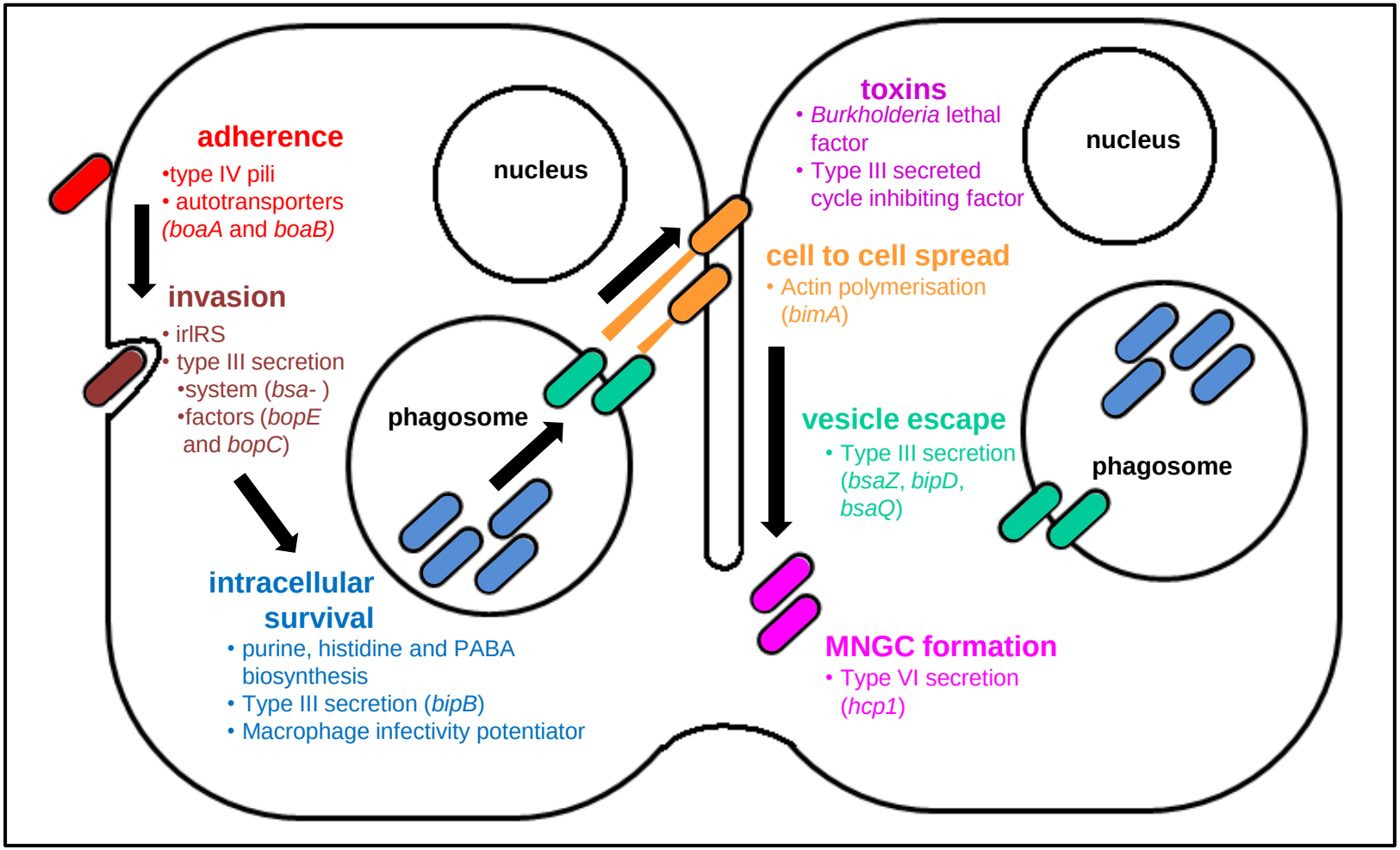

Figure 1.3 Effectors of the intracellular life cycle of *B. pseudomallei*

1.2.6.5 Secreted proteins

B. pseudomallei is also known to secrete exoproteins including proteases (Sexton *et al*., 1994), haemolysin, lecithinase, and lipases (Ashdown and Kohler, 1990) and phospholipase C (Korbsrisate *et al*., 1999). Transposon mutagenesis of ORFs with a high degree of homology to general secretory pathway encoding genes, led to abolition of secretion of protease, lipase and lecithinase. This inactivation had no effect on pathogenesis in an acute Syrian hamster model of disease (Ashdown and Kohler, 1990).

1.2.6.6 Quorum sensing

Quorum sensing is a cell density regulated system by which bacteria signal to each other and subsequently alter their gene expression by the production and detection of signalling molecules. Whilst Gram positive bacteria use a oligopeptide-two-component-type quorum sensing system, Gram negative bacteria signal with acyl-homoserine lactones (Li and Tian, 2012). Genes encoding a quorum sensing system have been identified in *B. pseudomallei* and production of homoserine lactones has also been demonstrated (Ulrich *et al*., 2004). Deletion of genes encoding the quorum sensing system has been shown to be attenuating in both Balb/C mice and hamsters (Ulrich *et al*., 2004). Whilst deletion of the system was shown not to affect secretion of lipase, protease or phospholipase C (Ulrich *et al*., 2004), *B. pseudomallei* mutants of quorum sensing show increased production of siderophore, an iron binding molecule (Song *et al*., 2005). To date the regulation of a single gene, *dpsA*, which plays a role in oxidative stress protection, has been shown to be regulated by this quorum sensing system (Lumjiaktase *et al*., 2006).

1.2.6.7 Biofilms

Biofilms are surface attached, multicellular communities surrounded by extracellular matrix formed by some species of Gram-negative bacteria. *B. pseudomallei* has been shown to form biofilms, though no association with virulence with biofilms has been reported to date (Taweechaisupapong *et al*., 2005).

1.2.6.8 Survival in serum

B. pseudomallei has been shown to resist the bactericidal activity of human serum and multiple factors have been identified in the bacterium which contribute to this resistance. First, surface polysaccharides expressed by *B. pseudomallei* have been shown to play roles in resistance to the host innate immune response. Four gene clusters encoding enzymes for synthesis of polysaccharides have been identified in *B. pseudomallei (*Holden *et al.,* 2004*).* Two of these clusters encode enzymes for synthesis of lipopolysaccharide (LPS) and capsular polysaccharide (CPS). LPS has been shown to provide resistance to serum killing (DeShazer et al., 1998). CPS has been shown to reduce deposition of complement factor C3b, therefore contributing to bacterial survival in serum (Reckseidler-Zenteno *et al*., 2005). Additionally, LPS O-antigen or CPS mutants of *B. pseudomallei* have been shown to be attenuated in animal models of melioidosis (Deshazer et al., 1998; Reckseidler *et al*., 2001; Atkins *et al.*, 2002). Further, immunisation with these cell surface located polysaccharides provided Balb/C mice with protection against lethal challenge with *B. pseudomallei* by the intra peritoneal (i.p.) route (Nelson *et al*., 2004). Passive immunisation with antibodies raised against LPS and CPS also reduced lethality of infection in animal models of disease (Nelson *et al*., 2004).

Additionally, the *B. pseudomallei* K96243 genome encodes an iron regulated cluster of genes responsible for the synthesis, secretion and uptake of a siderophore known as malleobactin (Alice *et al*., 2006). This hydroxamate type siderophore had been shown to be capable of removing iron from two iron binding glycoproteins found in human serum, transferrin and lactoferrin (Yang *et al*., 1993). A second gene cluster in *B. pseudomallei* K96243 shows similarity to that of a pyochelin synthesis and transport cluster in *P. aeruginosa* and *B. pseudomallei* was shown to be able to utilise pyochelin for growth (Alice *et al*., 2006). In a study of eighty four clinical isolates of *B. pseudomallei* in Thailand, all strains produced a siderophore under iron-limited culture conditions (Yang *et al*. 1991).

1.2.7 Host immune response

Both the innate and adaptive immune responses have been shown to play a role in control and outcome of melioidosis. *B. pseudomallei* expresses multiple potential 'pathogen associated molecular patterns' (PAMPS) including LPS, lipid-A, peptidoglycan, flagella, TTSS and DNA. These types of motifs are recognised by pattern recognition receptors including toll-like receptors (TLRs) on host cells (Kawai and Akira, 2006). The role of TLRs during infection with any pathogen is to detect the invasion and form a link between innate and adaptive responses. TLRs have been shown to be important during *B. pseudomallei* infection of mice. Mice lacking myeloid differentiation primary response gene 88 (MyD88), a key signalling adaptor for many TLRs undergo an accelerated time to death during experimental melioidosis (Wiersinga *et al*., 2008). Further, melioidosis patients have been shown to upregulate

expression of TLR1, TLR2, TLR3, TLR4, TLR5, TLR8 AND TLR10 as well as TLR4 co-receptors MD-2 and CD14 (Wiersinga *et al*., 2007).

Pro-inflammatory cytokines are also important for protection during early *B. pseudomallei* infection. Interferon (IFN)γ, interleukin (IL)12 and tumor necrosis factor (TNF)α have all been shown to be upregulated early during acute melioidosis infection in mice (Ulett *et al*., 2000). Neutralization of these cytokines also increased susceptibility to experimental melioidosis in an outbred strain of mice, demonstrating their importance *in vivo* (Santanirand *et al.,* 1999). In addition, treatment of mice with IFNγ in combination with antibiotic reduced bacterial burden and dissemination and significantly improved survival of the mice (Propst *et al*., 2010).

Early neutrophil recruitment is also key in protection against melioidosis. Infiltration of neutrophils to alveolar spaces and hepatic lesions was observed in Balb/C mice 24-48 h post inhalational challenge (Lever *et al*., 2009). Further, neutrophils have been shown to be the predominant cell type associated with *B. pseudomallei* in the lung following aerosol challenge with this bacterium (Laws *et al*., 2011). Knock-down of neutrophil recruitment to the lung during pulmonary melioidosis decreases production of inflammatory cytokines in the lung and accelerates the progression of infection (Easton *et al*., 2007).

The T cell response to primary *B. pseudomallei* infection is described to be biphasic (Haque *et al*., 2006). IFN-γ production to activate T cells was shown

to be cytokine rather than antigen dependent early during infection, though the activated T cells were functionally redundant for early bacterial clearance. Antigen-induced T cell activation, particularly CD4+ T cells, were shown to be important for host resistance later during *B. pseudomallei* infection (Haque *et al*., 2006).

1.2.8 Treatment

There is currently no licensed vaccine to protect against *B. pseudomallei* infection and treatment of melioidosis is complicated by the intrinsic and acquired antibiotic resistance of the bacterium. Ongoing research aims to produce a protective vaccine against *B. pseudomallei.* In addition, work to improve the antibiotic regimen as well as discovery of novel antibiotics in order to combat the disease more efficiently is ongoing.

1.2.8.1 Vaccines

All three of the main types of vaccine: live attenuated mutants, inactivated whole cells, and subunits have been explored as potential vaccine candidates to protect against infection with *B. pseudomallei.* Much of the vaccine research has been carried out by the bio-defence research community and a recent study has demonstrated the potential utilisation of a melioidosis vaccine in endemic regions (Peacock *et al*., 2012). To date, sterilising immunity by any of the vaccine candidates has not been achieved and optimisation of vaccine regimens and candidates remains a focus in melioidosis research.

First, a range of attenuated *B. pseudomallei* strains have been shown to be protective against subsequent lethal challenge with a fully virulent *B.*

pseudomallei strain in mice (Sarkar-Tyson and Titball, 2010). However, the development potential of this type of vaccine to protect against melioidosis is limited, since latency and persistence of this bacterium in the host is well established and is undesirable in a potential vaccine.

Additionally, Balb/C mice immunised with heat-killed *B. pseudomallei, B. thailandensis and B. mallei* cultures were partially protective against a lethal i.p. challenge with *B. pseudomallei* K96243 (Sarkar-Tyson *et al*., 2009).

Both LPS and capsular polysaccharide have been tested as possible sub-unit vaccine candidates to protect against *B. pseudomallei* (Nelson *et al*., 2004). Vaccination with either polysaccharide provided Balb/C mice with incomplete protection against a high i.p. challenge of approximately 250 mean lethal doses of *B. pseudomallei* K96243, though the degree of protection was reduced using lower challenge of approximately 2.5 mean lethal doses via the aerosol route (Nelson *et al*., 2004).

Finally, a number of *B. pseudomallei* protein subunits have been tested as potential vaccine candidates and shown to be protective. The most protective of these to date was LolC, a putative ATP binding cassette (ABC) system protein. LolC is proposed to be involved in lipoprotein sorting across the periplasm. When administered in combination with an adjuvant via the i.p route, this protein was partially protective to mice subsequently challenged with *B. pseudomallei* K96243 or *B. pseudomallei* 576 (Harland *et al*., 2007). Other protein subunits delivered as recombinant proteins or DNA vaccines

have also been tested as vaccine candidates with varying degrees of success (Chen *et al*., 2006; Hara *et al*., 2009).

1.2.8.2 Antibiotic regimen

Immediate post-exposure prophylaxis is recommended for those who suspect they have been exposed to *B. pseudomallei.* The current primary antibiotic regimen of choice following suspected laboratory exposure to *B. pseudomallei* is oral 12 hour dosing of trimethoprim-sulphamethoxazole (TMP-SMX). This treatment has been shown to protect 100% of Balb/C mice when initiated up to 24 hours post exposure to an aerosol challenge (Peacock *et al*.; 2008). Following culture confirmed melioidosis, initial intensive parenteral treatment with ceftazidime or meropenem for 10-14 days, followed by an eradication phase of oral TMP-SMX with or without doxycycline for up to six months is recommended (White, 2003). For pregnant women and those patients intolerant to β-lactam antibiotics, amoxicillin-clavulanate is the treatment of choice during initial and eradication antibiotic therapy (Suputtamongkol *et al*.; 1994).

Several clinical studies have compared antibiotic regimen options in the various phases of melioidosis treatment. Most significant of these was by White *et al*. in 1989, comparing initial parenteral ceftazidime treatment of melioidosis with the three drug regimen, chloramphenicol, doxycycline and TMP-SMX. A 50% reduction in mortality was demonstrated by ceftazidime compared to the triple drug treatment (White *et al*., 1989). Comparison of amoxicillin-clavulanate with ceftazidime treatment of acute disease demonstrated a similar mortality rate, but amoxicillin-clavulanate is associated

with a higher rate of treatment failure (Supputtamongkol *et al*., 1994). The carbapenems are also used in initial treatment of melioidosis. Treatment with the carbapenem, imipenem, has been shown to give an equivalent outcome to ceftazidime (Simpson *et al*., 1999). Meropenem has also been observed to provide a similar level of protection as ceftazidime (Cheng *et al*., 2004). The carbapenems have been suggested to be a preferable option to ceftazidime for melioidosis treatment for several other reasons, including higher activity *in vitro* (Jenney *et al*., 2001) and an association with reduced endotoxin release (Simpson *et al*., 2000).

1.2.8.3 Antibiotic resistance

B. pseudomallei is intrinsically resistant to many antibiotics including fluoroquinolones and aminoglycosides (Dance *et al.*, 1989; Thibault *et al.* 2004). In order to identify genes which play a role in antibiotic resistance, Moore *et al*. carried out a transposon mutagenesis study. Two transposon mutants which were more susceptible to aminoglycoside and macrolide antibiotics were identified. This susceptibility was shown to be due to inactivation of a multidrug efflux system known as AmrAB-OprA (Moore *et al*., 1999). The proteins which make up this efflux system were homologous to resistance, nodulation, division-type (RND type) multidrug resistance proteins which function to pump out potentially damaging antibiotics which gain access to the cell (Moore *et al.*, 1999). A clinically isolated strain of *B. pseudomallei* was described to have similar pattern of antibiotic susceptibility to the *amr* transposon mutants isolated by Moore *et al*.. More recently, this *B. pseudomallei* strain 708a was shown to contain a 131 kb deletion of greater than 90 genes compared to *B. pseudomallei* K96234, which included the

AmrAB-OprA operon, explaining the basis for this pattern of susceptibility (Trunck *et al*., 2009).

A second efflux system in the *B. pseudomallei* genome termed BpeAB-OprB also encodes proteins which have homology to RND type proteins. A mutant in this system in *B. pseudomallei* strain KHW was more susceptible to the aminoglycosides gentamicin and streptomycin and the macrolide erythromycin (Chan *et al.*, 2004). This mutant was also used to demonstrate a role for BpeAB-OprB in quorum sensing and virulence regulation. However, more recently a mutant in this system in *B. pseudomallei* strain 1026b was used to demonstrated that this efflux system is not responsible for efflux of aminoglycosides or involved in quorum sensing or virulence regulation. However, it was shown to mediate efflux of a diverse range of antibiotics including acraflavine, fluoroquinolones, macrolides and tetracyclines (Mima and Schweizer; 2010). The conflicting roles for this efflux system in different *B. pseudomallei* strains is yet to be explained. A third pump identified in *B. pseudomallei* 1026b was shown to efflux trimethoprim and chloramphenicol in a surrogate *P. aeruginosa* strain (Kumar *et al*., 2006).

The sporadic emergence of antibiotic resistant variants in the clinical setting has also been observed. Godfrey *et al*. investigated β-lactam resistance in three *B. pseudomallei* isolates in comparison to non resistant isolates from the same patient. Tribuddharat *et al*. went on to show that in two of these isolates the resistant phenotype was due to base pair changes in the β -lactamase gene *penA,* leading to changes in functionally important amino acids. This

resistance is therefore attributed to decreased susceptibility of PenA to the co-administered β -lactamase inhibitor, rather than resistance to the β -lactam itself (Tribuddharat *et al.*, 2003). More recently, clinically isolated ceftazidime resistant variants from Australian patients contained mutations in the *penA* gene or in the *penA* promoter (Sarovich *et al*., 2012). Additionally, a large scale genomic deletion containing the gene encoding penicillin-binding protein 3 (Pbp3) was shown to be responsible for ceftazidime resistance of strains isolated from six Thai patients who had undergone prolonged treatment with ceftazidime (Chantratita *et al*., 2011). Sporadically acquired resistance as in these cases is however, considered rare (White, 2003).

1.2.9 Targets of current *B. pseudomallei* antibiotic therapies

Current antimicrobial agents target well established essential processes within bacterial cells. These pathways include cell wall synthesis, DNA replication, RNA transcription and protein synthesis (Walsh, 2000). The current treatment for melioidosis involves initial intensive, intra-venous administration of ceftazidime. Ceftazidime is a third generation cephalosporin, and a type of β-lactam. β-lactam drugs target cell wall synthesis and can be inactivated by bacterial β-lactamases. Ceftazidime treatment is followed by long term oral administration of trimethoprim in combination with the sulphonamide, sulphamethoxazole or alternatively, one of the carbapenems imipenem or meripenem (White 2003). Carbapenems are also β-lactam antibiotics. Trimethoprim and sulphamethoxazole both function to inhibit the bacterial tetrahydrofolic acid synthesis pathway. Firstly, trimethoprim is a dihydrofolate reductase inhibitor, an enzyme which functions to convert dihydrofolic acid to tetrahydrofolic acid. Secondly, sulphonamides such a sulphamethoxazole are

structural analogs and competitive antagonists of para-aminobenzoic acid (PABA) which is an early substrate in the tetrahydrofolic acid synthesis pathway (Greenwood *et al*., 2003). Tetrahydrofolic acid is in turn required for DNA synthesis by bacteria.

It can therefore be seen that the primary treatment options for melioidosis target two pathways in the bacteria. Whilst targeting these pathways has clearly been effective in many patients to date, it exerts a selection pressure on the bacterium and can lead to the emergence of resistant strains *in vivo* (Godfrey *et al*., 1991; Wuthiekanun *et al*., 2005). Antimicrobials which target alternative pathways in *B. pseudomallei*, whether essential or virulence associated, may allow for improvement of current regimens and the option of alternative regimens if required.

1.3 Summary and project aims

This study aims to identify and characterise novel targets for antimicrobials in *B. pseudomallei*. Characterisation of the targets aims to determine their viability for inhibitory intervention during treatment of melioidosis.

First, genes were selected for investigation from *B. pseudomallei* targets identified in the published literature as essential or virulence associated. The criteria for selection of targets, considered the aim to investigate novel targets especially for improvement of treatment of *B. pseudomallei* infections.

Bioinformatic characterisation of the selected targets to compare them to other bacteria was undertaken. Directed mutagenesis of the targets was attempted to determine their essential nature. Lastly, any constructed mutants were characterised to determine the role of the target *in vitro* and *in vivo*.

Chapter 2. Materials and Methods

2.1 Bioinformatics

Websites and programmes used to identify and characterise genes and proteins throughout the study are listed below:

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

   For *B. pseudomallei* genome information

2. http://iioab-dgd.webs.com/

   Genomic target database, for identification of antimicrobial targets in *B. pseudomallei* (Barh *et al*., 2009)

3. http://blast.ncbi.nlm.nih.gov/. (Altschul *et al*., 1997)

   For Basic Local Alignment Search TOOL (BLAST) search of antibiotic targets of interest.

4. http://pfam.sanger.ac.uk/ (Punta *et al*., 2012)

   For searching for TBDT domains in the *B. pseudomallei* genome

5. MegAlign (DNAstar Lasergene)

   For aligment of protein amino acid sequences

6. Helical wheel construction

   A helical wheel diagram of amino acid sequences was constructed as described by Schiffer and Edmundson, 1967.

2.2 Strains, plasmids and primers

2.2.1 Bacterial strains

2.2.1.1 *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 shaking at 200rpm, unless otherwise stated.

2.2.1.2 *Burkholderia* strains

B. pseudomallei strains used in this study are listed in Table 2.2. All manipulations with *B. pseudomallei* were carried out at containment level 3 due to its classification by the Advisory Committee on Dangerous Pathogens as a level 3 agent. Bacteria were grown in 50 ml LB broth at 37ºC overnight with agitation, unless otherwise stated.

2.2.1.3 Mammalian cell lines

The cell line used in this study was J774A.1 mouse BALB/c macrophages (European Collection of Cell Cultures). Cells were typically grown in 25 ml DMEM (Table 2.3) at 37ºC and 5 % CO_2 overnight, unless otherwise stated.

***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]
DH5α λpir	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 λpir	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; GenR	Prof S Songsivilai, Mahidol University
B. pseudomallei ΔmraW	K96243 derivative; unmarked deletion ΔBPSL3033	This study
B. pseudomallei ΔtonB	K96243 derivative; unmarked deletion	This study

	ΔBPSS0368	
B. pseudomallei ftsZ integrant	K96243 derivative; Containing marked pDM4 suicide plasmid at BPSL3020	This study
B. pseudomallei ftsA integrant	K96243 derivative; Containing marked pDM4 suicide plasmid at BPSL3021	This study

Table 2.2 *B. pseudomallei* strains used in this study

2.2.2 Growth media

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

Media	Composition	Sterilisation
Luria Bertani (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
SOC medium	2% tryptone, 0.5% yeast	n/a

	extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate, 20 mM glucose	
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
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
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^{TM}, 10 % fetal calf serum	n/a

Table 2.3 Growth media used in this study

2.2.3 Antibiotics

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

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

Table 2.4 Antibiotics used in this study

2.2.4 Plasmids

Plasmids used in this study are listed in Table 2.5.

Plasmid	Vector type	Antibiotic resistance	Source
pCR Blunt II-TOPO	Cloning	Kanamycin	Invitrogen
pBHR4 groES	Complementation	Chloramphenicol	University of Exeter
pBBR1-MCS2	Complementation	Kanamycin	Kovach *et al*., 1995
pDM4	Suicide vector	Chloramphenicol	Milton *et al.,* 1996

Table 2.5 Plasmids used in this study

2.2.5 Primers

Primers used in this study are listed in Table 2.6.

Oligonucleotide name	Use	Sequence 5' → 3'	Restriction site	Annealing temperature
ftsZ LLF	PCR	TCTAGAGCGCG TCGAGGAGCTG TTCTCG	*Xba*I	68ºC
ftsZLFR	PCR	CATATGACCTG CACCGCCAACG CCGATCAC	*Nde*I	68ºC
ftsZ RFF	PCR	CATATGGACAC GTACGACATTC CGGCATTCC	*Nde*I	68ºC
ftsZ RFR	PCR	TCTAGACCCAA GCGCCCATCAC GAATGC	*Xba*I	68ºC
ftsA LFF	PCR	TCTAGAAACCA GGGCGAGCTC GATGC	*Xba*I	68ºC
ftsA LFR	PCR	CATATGGATGT CGAGGGCAAC CAGC	*Nde*I	68ºC
ftsA RFF	PCR	CATATGGGCCG CAAGGTCGCCG TGC	*Nde*I	68ºC
ftsA RFR	PCR	TCTAGAGGCGA TCTGCGCGACG ACAGG	*Xba*I	68ºC
camR F	PCR	ATCCCAATGGC ATCGTAAAG		55ºC
camR R	PCR	TAAGCATTCTG CCGACATGG		55ºC
sacB F	PCR	CGGCTACCACA TCGTCTTTG		55ºC
sacB R	PCR	GCAATCAGCGG TTTCATCAC		55ºC
tonB int screen F	Mutant screening	ATCGGTCAGAG TGAAGTCAT		65ºC

tonB int screen R	Mutant screening	GAATCCCCGCATCGTCACCG		65ºC
tonB F	Mutant screening	ATGAATCCCCGCATCGTCACC		66ºC
tonB R	Mutant screening	TAATCGGTCAGAGTGAAGTC		66ºC
tonB screen F	Mutant screening	CCGTCACGTTCTACGAACGC		65ºC
tonB screen R	Mutant screening	TCATGATCAGCGATTCGCCG		65ºC
mraW screen F	Mutant screening	ACCCGGACGGCTGCCTGTTGC		60ºC
mraW screen R	Mutant screening	GGCTCGATGCGAACTTCACG		60ºC
tonB F	PCR	ACTAGTATGAATCCCCGCATCGTCAC	*Spe*I	55ºC
tonB R	PCR	GGATCCTAATCGGTCAGAGTGAAGTC	*Bam*HI	55ºC
mraW F	PCR	ACTAGTATGGGAAACGAATTCCAGCATCG	*Spe*I	55ºC
mraW R	PCR	ATCGATCATGGCGCGACGCGCTCCGCGATGC	*Cla*I	55ºC
Kan F	Complementation screening	ATGATTGAACAAGATGGATTGC		55ºC
Kan R	Complementation screening	TCAGAAGAACTCGTCAAGAAGGCG		55ºC
pBBR1 F	Complementation screening	TGTAGTCGACGCAACGCATAATTGTTGTCG		55ºC
pBBR1 R	Complementation screening	TAGCGTCGACCTCGCCATCGTCCAGAAAAC		55ºC
mraW RT F	RT PCR	GATCGTCCATGACAGTTTCG		62ºC

mraW RT R	RT PCR	GCGAATGAAAG CTGATGACC		62ºC
16S RNA	RT PCR	GATGACGGTAC CGGAAGAATAA GC		55ºC
16S RNA	RT PCR	CCATGTCAAGG GTAGGTAAGGT TT		55 ºC

Table 2.6 Oligonucleotide primers used in this study

2.2.6 Reagents and buffers

Buffers and reagents used in this study are listed in Table 2.7. All chemicals were purchased from Sigma-Aldrich and Roche unless otherwise stated.

Reagent/buffer	Components	Use
TAE buffer	40 mM Tris-acetate, 1 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
RNA protect (Qiagen)		RNA extraction

Table 2.7 Buffers and reagents used in this study

2.3 Molecular Biology

2.3.1 Polymerase Chain Reaction (PCR)

A typical polymerase chain reaction was carried out using Herculase II Fusion DNA polymerase (STRATAGENE) and contained 5 µl 5x Herculase II reaction buffer, 10 pmol each forward and reverse oligonucleotides, 200µM dATPs, 200µM dCTPs, 200µM dGTPs 200µM dTTPs, 2 µl dimethyl sulphoxide, 0.1 µg genomic DNA, 0.5 µl Herculase II Fusion DNA polymerase and distilled water to a final volume of 25 µl using the PCR program below:

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)	

Gene specific optimisations were required for some reactions and are listed in tables 2.6.

For colony PCR a single bacterial colony was resuspended in 100 µl distilled water, and incubated at 96°C for 10 min. 2 µl of the resulting lysate was used as DNA template in the PCR reaction described above.

To produce a DIG-labelled probe for Southern blot 2.5 mM DIG NTPs were used instead of dNTPs.

2.3.2 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 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 at 312nm.

2.3.3 DNA gel purification

DNA bands of interest were excised from a 1% agarose gel and purified using the QIAquick gel extraction kit (Qiagen), as per manufacturer's instruction.

2.3.4 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 at 4°C overnight and then used in transformation reactions.

2.3.5 Transformation

2.3.5.1 Heat shock

Two µl of 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 2 min. Two hundred and fifty 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.

2.3.5.2 Electroporation

The ligation reaction was desalted by placing 10 µl reaction onto filter discs floated on distilled water for 30 min. One to two µl desalted ligation reaction was added to 50 µl electro-competent cells in electroporation cuvettes on ice and transformed by electroporation using Gene pulser II electroporator (Bio-Rad) at 2.5 kV and a capacitance of 25 µF. Two hundred and fifty µl SOC medium (Invitrogen) was added to the transformation mixture 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.

2.3.6 Plasmid DNA extraction

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.

2.3.7 Restriction digest

A typical digest reaction consisted of 500 ng of prepared DNA, 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 Nucleotide sequencing

Sequencing reactions were performed by Beckman Coulter Genomics, Essex. 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.

2.3.9 Genomic DNA extraction

A single bacterial colony was used to inoculate 10 ml LB broth and incubated overnight at 37°C 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.

2.3.10 Southern Hybridisation

Ten µg digested genomic DNA was separated by gel electrophoresis on a 0.8% agarose gel at 60 V for 4 h. 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 placed in a hybridisation tube containing the membrane and incubated at 42ºC in a hybridisation oven for 1 h. Five 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 placed in the hybridisation tube and incubated in a hybridisation oven at 42ºC overnight. The hybridisation solution was removed and the membrane washed twice with 50 ml pre-warmed 2 x SSC, 0.1% SDS in 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 of 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.

2.3.11 RNA extraction

A single bacterial colony was used to inoculate 10 ml LB broth and incubated at 37°C overnight with agitation. One ml of overnight culture was inoculated into fresh LB broth and grown at 37ºC with agitation until the absorbance at 590 nm reached 0.6-0.8. One ml of culture was then removed and 2 ml of RNAprotect (Qiagen) was added to 1 ml culture and vortexed briefly. The mixture was then incubated at room temperature for 5 min, followed by centrifugation for 10 minutes at 5000 x *g*. RNA was purified using the RNeasy Mini Kit (Qiagen) as per manufacturer's instructions. RNA was quantified using a NanoDrop (ThermoScientific).

2.3.12 Reverse transcription polymerase chain reaction

DNA contamination was removed from purified RNA using the TURBO DNA-free™ Kit as per manufacturer's instructions. DNase treated RNA was reverse transcribed into cDNA using Enhanced Avian First Strand Synthesis Kit

(Sigma Aldrich) as per manufacturer's instructions. cDNA was used in a typical PCR described above.

2.4 Mutant construction

2.4.1 Conjugation

Single colonies of *E. coli* S17 λpir containing the suicide vector pDM4 and *B. pseudomallei* K96243 were used to inoculate 10 ml of LB broth with appropriate antibiotics and grown at 37ºC overnight with agitation. One mililitre of each bacterial strain culture were centrifuged at 6000 *x g* for 2 min at RT and pellets resuspended in 500 µl of LB broth. Ten 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 of PBS, 100 µl of 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.

2.4.2 Sucrose selection

A single integrant colony was used to inoculate 10 ml of LB broth and incubated at 37ºC overnight with agitation. The overnight culture was diluted until an absorbance reading of 0.35 to 0.40 (~ 1 x 10^8 cfu/ml bacteria) 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.

2.5 Motility assay

A single bacterial colony was used to inoculate 10 ml of LB broth and incubated at 37ºC overnight with agitation. Five µl of overnight culture was stab inoculated into 0.3% motility agar using a sterile pipette tip and the plates

incubated at 37ºC overnight. Bacterial spread was measured using a Scienceware® vernier calliper (Sigma).

2.6 Electron microscopy

A single bacterial colony was used to inoculate 10 ml of LB broth and incubated at 37ºC overnight with agitation. Half a ml of overnight culture was added to 10 ml of prewarmed LB broth and incubated at 37ºC until an optical density reading of 0.4 was reached. One ml of culture was pelleted at 5000 *g* for 5 min. Pellets were fixed in 1 ml 4% formalin for 24 h. Samples were stained with 2% (wt/vol) uranyl acetate and examined in an FEI CM12 transmission electron microscope operating at 80 kV. Images were captured using a 1MP Keenview digital camera.

2.7 Growth studies

A single bacterial colony was used to inoculate 10 ml LB broth and incubated at 37ºC overnight with agitation. Two ml of overnight culture was added to 100 ml of prewarmed LB broth and incubated at 37ºC for up to 24 h. At time points, 1 ml of aliquots were removed and their optical density read using a WPA Colourwave colourimeter (model C07500) at 590 nm. Alternatively, the bacterial culture was assayed by plating samples onto LB agar, plus 40 mM $FeSO_4$ where appropriate and incubated at 37ºC overnight.

2.8 Tissue culture

2.8.1 Macrophage invasion and replication assay

J774A.1 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 or 100 ml of LB broth and incubated at 37ºC overnight with agitation. The overnight cultures were washed as described above in PBS, for cultures grown with additional $FeSO_4$, then diluted in L15 medium until an absorbance reading of 0.35-0.4 (1 x 10^8 cfu/ml) was reached, using a WPA Colourwave colourimeter (model C07500) at 590 nm then serially diluted in L15 medium. One ml of bacterial culture was added to the cells at an MOI of 1:1 and incubated at 37ºC for 30 min. Infected cells were then incubated with L15 medium containing 1 mg/ml of kanamycin for 1 h at 37ºC. Cells were then incubated with 250 µg/ml of kanamycin at 37ºC for 24 h. At various time points, 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 Macrophage adhesion assay

J774A.1 cells and bacteria were prepared as per section 2.8.1. In order to abrogate actin mediated phagocytosis of bacteria, 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 10^6 cfu/ml of bacteria at a final concentration of 1 µg/ml. One ml treated bacteria was added to the pre-treated cells at an MOI of 1:1 and incubated at 37ºC for 1 h. Cells were then washed 3 x times with PBS incubated at 37°C, to remove non-adhered bacteria or incubated with L15 medium containing 1000 µg/ml of kanamycin. Cells were lysed with 1 ml

of dH_2O, serially diluted in PBS and plated onto LB agar and incubated at 37ºC overnight.

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 1 x 10^2 bacteria in 10 µl of PBS. Control groups were either injected with 10 µl of PBS or nothing. Some groups were injected with 10 µl of 5mM $FeCl_3$ 30 min prior to bacterial challenge. 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 Mouse challenge

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. Animals were challenged with approximately 1 x 10^4 cfu/ml of *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 humanely at predetermined humane end points. At the end of the experiment, all survivors were culled and lungs, livers and spleens aseptically removed. Spleens were homogenized in 1 ml of PBS, serially diluted in PBS, plated on LB agar and incubated at 37ºC overnight.

2.10 Statistical analysis

For growth curves and macrophage assays, 2way ANOVA and Bonferroni's post tests were used to compare bacterial numbers. For motility assays, a 1way ANOVA was used to compare bacterial spread. For *in vivo* mutant characterisation, a log-rank (Mantel-Cox) test was used to compare survival curves.

Statistical analyses were performed using GraphPad Prism version 4.0.

Chapter 3. Target selection

3.1 Introduction

Many studies have identified essential and virulence targets in *B. pseudomallei* by manipulating genomic data *in silico, in vitro* and *in vivo*. In this study putative antimicrobial targets and their attributes will be considered for characterisation as novel targets for antimicrobial inhibition in *B. pseudomallei.* A selection of genes will be characterised for their potential as antimicrobial targets in *B. pseudomallei.* Therefore a down selection process was undertaken to obtain a shortlist of possible targets for characterisation. Antimicrobial research in recent years has tended to focus on Gram positive therapy (Schmid, 2006). Therefore in this study criteria were used which focused on improving *B. pseudomallei* therapy specifically. In addition, novel targets in *B. pseudomallei,* which are different to those already targeted by current antibiotics in the melioidosis treatment regimen such as the tetrahydrofolic acid pathway and cell wall synthesis, were selected. In addition, targets were selected which are not already targeted by current antibiotic therapies in general, such as DNA synthesis, ribosomal function and cell membrane stability. Furthermore, to inform characterisation of selected targets in *B. pseudomallei*, genes which have been characterised to some degree, especially as potential antimicrobial targets in other Gram negative bacteria will be prioritised.

To select final panel of targets for investigation from the short list, the potential for further characterisation and exploitation as drug targets was considered. Some general factors such as inhibitory potential and host toxicity information

will be briefly investigated. Some of this information will be taken from published data regarding these targets in other organisms, most importantly in other bacterial species.

3.2 Antimicrobial targets in *B. pseudomallei*

Several studies have described essential and virulence associated genes in *B. pseudomallei.* These targets represent possible points of intervention for novel inhibitory compounds. Various *in silico* analyses have been carried out on the fully sequenced *B. pseudomallei* K96243 genome. Comparison of the *B. pseudomallei* genome to that of *B. thailandensis* revealed broadly similar genomes (Yu *et al*., 2006). Many of the genes shown to be key for *B. pseudomallei* virulence, such as TTSS and LPS are also encoded by *B. thailandensis*. It is suggested a small number of horizontal gene transfer events into the *B. pseudomallei* and small-scale functional modification of proteins contribute to *B. pseudomallei* virulence when compared to the limited virulence *B. thailandensis* (Yu et al., 2006). In addition, Chong *et al* carried out an *in silico* analysis to identify potential drug targets. In this study, the 5855 protein coding genes present within the *B. pseudomallei* genome were screened by removal of genes within genomic islands, identified pseudogenes, duplicated genes, paralogs and genes with homology to human genes. The final set of genes was screened for matches in the database of essential genes (DEG) and a list of 312 genes considered to be potential drug targets were selected (Table 3.1) (Chong *et al*., 2006). All of these genes were considered for further investigation in this study.

Screen	Number of genes
Total number of protein coding sequences	5855
Non-genomic islands and non-pseudogenes	5331
Duplicates (>60% identical)	148
Non-paralogs	5181
Number of proteins without hits in *H. sapiens*	3723
Number of proteins with matches in DEG	312

Table 3.1 Summary of sequence analysis of *B. pseudomallei* genes by Chong *et al*., 2006

Additionally, Barh *et al* identified and listed twenty seven *B. pseudomallei* genes in the Genomic Target Database (www.iioab.webs.com/GTD.htm) as targets in pathogen specific unique metabolic pathways (Barh *et al*., 2009).

Several studies have identified conserved virulence or essential targets among a range of Gram negative bacteria (Stubben *et al*., 2009; Duffield *et al*., 2010). Stubben *et al.* identified one thousand and twenty four genes as broadly conserved among fourteen human pathogens, including *B. pseudomallei,* on the Centres for Disease Control (CDC) category A and B lists, whilst being absent from non-pathogens were identified (Stubben *et al*., 2009). This group selected targets for further characterisation by discarding genes with ≥ 50% identity to eukaryotic genes and/or ≥ 50% identity to 3 or more non-pathogens. A final seventeen genes were then tested for virulence association by mutagenesis using the model organism *Yersinia pseudotuberculosis* (Stubben *et al*., 2009). Putative targets from this study that are present in the *B. pseudomallei* genome were considered for investigation as antimicrobial targets.

Several negative selection transposon studies have been carried out to identify genes essential under host related conditions in *B. pseudomallei.* For example Cuccui *et al.* carried out STM in Balb/C mice in order to identify genes (Cuccui *et al*., 2007). Mutants of capsular polysaccharide genes were identified to be attenuated in this model. Further, auxotrophs of leucine, threonine, *p*-amino benzoic acid and aromatic compounds were also identified as attenuated indicating they are potential virulence targets. A transposon mutagenesis study to identify genes essential for intracellular survival of *B. pseudomallei* has also been undertaken (Pilatz *et al*., 2006). Mutants of 9 genes from a pool of 6000 mutants were shown to lead to defects in intercellular spreading by Pilatz *et al*.. Several of these genes were part of purine, histidine or *p*-amino benzoic acid biosynthetic pathways. These negatively selected targets which play a role in virulence were considered for further characterisation.

Studies to identify *B. pseudomallei* genes specifically regulated in the host have also been undertaken. Microarray analysis of the transcriptome of *B. pseudomallei* grown under salt stress was shown to up-regulate the Bsa locus for TTSS translation (Pumirat *et al*., 2010). Tuanyok *et al*., found that genes encoding siderophore-mediated iron uptake, heme transport and alternative metabolic pathways were up-regulated under iron restricted conditions (Tuanyok *et al*., 2005). IVET has also been applied to *B. pseudomallei* during infection of macrophages, demonstrating up-regulation of TSSS genes, as well as a gene for heme uptake and a gene for manganese uptake (Shalom *et al*., 2007). These virulence associated genes were also considered for characterisation.

These 375 genes previously identified in the literature as putative targets for novel antimicrobials in *B. pseudomallei* were considered for investigation in this study. General pathways containing many of these targets are summarised in Table 3.2.

Pathway	**Components**	**Essential/ Virulence**	**Target identified in *B. pseudomallei***	**Currently targeted by antibiotics**
Protein synthesis	Ribosome	E	Predicted essential *in silico* (Chong *et al*., 2006)	Tetracyclines, chloramphenicol Macrolides and aminoglycosides
	Amino acid synthesis	E	Essential intracellular by STM (Pilatz *et al*. 2006)	
	Transcriptional regulators	E/V	Predicted essential *in silico* (Chong *et al*., 2006)	
Dihydrofolate pathway		E	*pabB* - Essential intracellular by STM (Pilatz *et al*. 2006)	Sulphonamides
DNA synthesis	mRNA synthesis	E	Predicted essential *in silico* (Chong *et al*., 2006)	Rifampicin
	DNA modifying enzymes	E	Predicted essential *in silico* (Chong *et al*., 2006)	Quinolones
Cell division		E	Predicted essential *in silico* (Chong *et al*., 2006)	
Cell wall synthesis		E	Essential intracellular by STM (Pilatz *et al*. 2006)	β lactams, vancomycin and glycopeptides
Secretion pathways	TTSS	V	Up-regulated under salt stress (Pumirat *et al*., 2010)	
	TSSS	V	Up-regulated in macrophages (Shalom *et al*., 2007)	
	General secretory pathway	V	Predicted essential *in silico* (Chong *et al*., 2006)	
	Tat secretion	V	Predicted essential *in silico* (Chong *et al*., 2006)	
Capsule synthesis		V	Essential in mice by STM (Cuccui *et al*., 2007)	
Active uptake of	Iron uptake	V	Heme and siderophore uptake	

nutrients			upregulated on iron restriction (Tuanyok *et al*., 2005)	
	Manganese uptake	V	Up-regulated in macrophages (Shalom *et al*., 2007)	
Stress response	Stringent response	E	Predicted essential *in silico* (Chong *et al*., 2006)	
	Superoxide dismutases	V	Conserved in pathogens (Stubben *et al*., 2009)	
Hypothetical genes		E	Predicted essential *in silico* (Chong *et al*., 2006)	

Table 3.2 Long list of pathways with identified potential antimicrobial targets in *B. pseudomallei*

3.3 Down-selecting antimicrobial targets

Selection from the large number of potential targets was undertaken based on their predicted function, using the general criteria, as detailed in Figure 3.1. A holistic approach to bacterial pathways was taken in order to aid selection of targets, taking into account several factors. In order to characterise truly novel targets, the four main pathways targeted by current licensed antimicrobials to date: cell wall synthesis, protein synthesis, DNA synthesis and the dihydrofolate pathway were disregarded. In addition, targets which had already been investigated and shown to play a role in virulence by mutation study in *B. pseudomallei*, such as quorum sensing, capsule biosynthesis, TTSSs and many others were also disregarded. Further, many of the studies to discover potential antimicrobial targets in *B. pseudomallei* identified a large proportion of hypothetical genes. Developing biochemical assays to measure activity of a gene product with an unknown function is difficult. Therefore hypothetical genes were disregarded in order to increase the likelihood of the ability to characterise any mutated target. In their *in silico* study, Chong *et al*. grouped their potential targets by function. Groups of targets from this *in silico* study, which were disregarded by selection are indicated in Figure 3.1. A list of prioritised targets, containing eight genes broadly falling into cell division or metal uptake mechanisms, plus a secretion system was composed, following down-selection from the original target lists (Table 3.3).

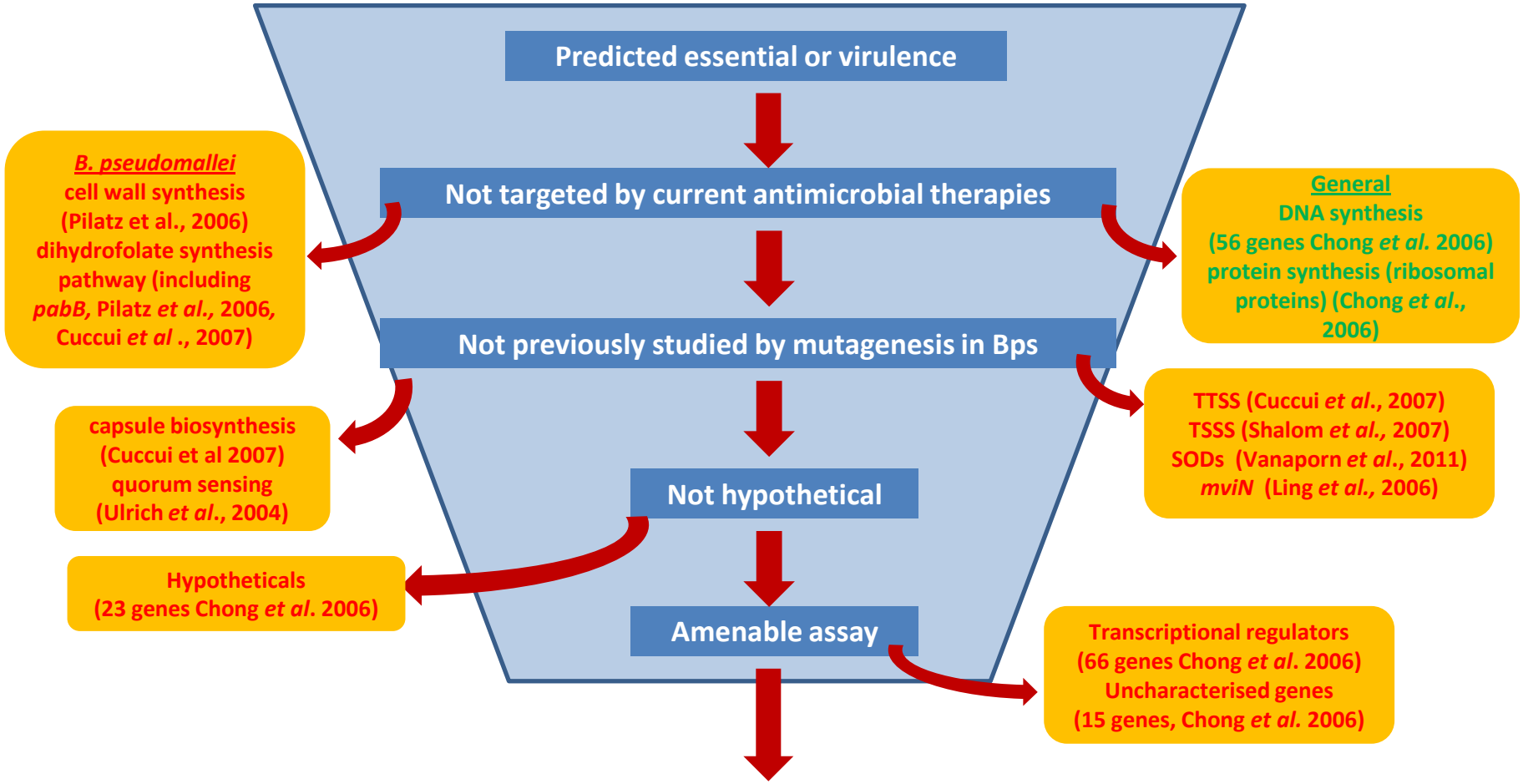

Figure 3.1 Down selection strategy for short-listing novel *B. pseudomallei* antimicrobial targets

Process		Target	Locus	Identified	Essential/ virulence	Purification	Assay	Inhibitors	Character-ised in bacteria
Cell division		FtsA	BPSL3021	Duffield *et al*., 2010, Chong *et al*., 2006	E	from *E. coli* and *B. subtilis*	ATPase	Yes	Yes
		FtsZ	BPSL3020	Chong *et al*., 2006	E	from *E. coli* and *B. subtilis*	GTPase	Yes	Yes
		MraW	BPSL3033		E	from *E. coli*	Methyltrans-ferase assay	Methyltrans-ferase inhibitors	Partially
Metal transport	Magnesium	MgtB	BPSL0973	Stubden *et al*., 2009	V	membrane protein	Magnesium utilisation	None	No
	Manganese	MntH	BPSL1554	Stubden *et al*., 2009	V	membrane protein	Manganese utilisation	None	Yes
	Iron	TonB	BPSS0368	Powers iron uptake Heme – Shalom *et al*., 2007 siderophore – Tuanyok *et al*., 2005	V	membrane protein – but domains expressed	Iron complex utilisation	Ton box peptide	Yes
	Heme	BhuRS TUV	BPSS0244-BPSS0240	Shalom *et al*., 2007	V	Membrane protein	Heme utilisation	None	Other TonB dependent transporters
Effector transport	Twin arginine transporter	TatC	BPSL3126	Chong *et al*., 2006	E	membrane protein – but circular dichroism undertaken	Effector secretion (complex)	None	Yes

Table 3.3 Properties of short listed antimicrobial targets. Highly desirable traits and informative data are highlighted in green. Incomplete information or data which could be applied from similar genes are highlighted in yellow. Undesirable traits or no information are highlighted red.

3.4 Short list

Further criteria, related to the ability to exploit targets following characterisation was also applied to down-select from the eight short-listed targets. First, the ability to express and purify the targets, as either domains or complete proteins was desirable for drug development. Therefore targets which have been expressed, purified and have had the structure of a bacterial species homologue determined, were considered. Structural information can allow intelligent design of novel inhibitors to the selected targets in the future. Comparison of structures to host proteins can also inform whether a novel inhibitory compound may have undesirable off target activity against host proteins. Gene products with assayable enzymatic activity i*n vitro*, or measurable activity *in vivo* were prioritised. Activities which are amenable to high-throughput screening with novel inhibitors are most desirable. Inhibitory potential demonstrated by existing small molecules toward the target is also advantageous for a novel antimicrobial target. Information regarding the ability of inhibitors to distinguish between host and pathogen targets is also useful.

Each of the factors considered, for each short-listed target, are described briefly in Table 3.3. A green, yellow or red ranking was given to each factor considered. Green factors represent highly desirable traits with significant supporting data. Incomplete information, or information applied to a target from similar targets is ranked yellow. Undesirable traits or a lack of data are ranked as red.

3.5 Target selection

A range of four targets with the greatest number of green and yellow ranked traits were selected for further characterisation by mutagenesis study. FtsA, FtsZ, TonB and MraW were chosen for further characterisation as antimicrobial targets in this study. The targets selected were either essential or virulence gene products.

Three of the genes for these targets, *ftsA*, *ftsZ* and *mraW,* are encoded by BPSL3021, BPSL3020 and BPSL3033 respectively are located within the division and cell wall (dcw) cluster of genes on chromosome one of *B. pseudomallei* K96243 (Figure 3.2). The fourth, *tonB,* encoded by BPSS0368 is found on chromosome two and its homologs in Gram negative bacteria play a key role in high affinity ferric iron uptake (Andrews *et al*., 2003).

3.6 Discussion

Formulaic approaches to target selection have previously been taken by high-throughput screening studies aiming to discover a novel broad spectrum antimicrobial inhibitor, with little success to date. This study aims to characterise targets which can be exploited to improve treatment of melioidosis. Therefore a holistic approach was taken to selection of novel targets from *B. pseudomallei* for investigation in this study. Characterisation of gene products will be informed by data about these targets in other bacterial species.

First, *ftsA* and *ftsZ* encode proteins which play key roles early on during the process of bacterial cell division (Errington *et al*., 2003). Both genes have been shown to be essential for viability in several bacterial species (Dai and Lutkenhaus, 1991; Sanchez, 1994) and are emerging as attractive therapeutic targets for antimicrobial inhibition.

FtsZ has been well characterised in *E. coli* and *B. subtilis* and shown to polymerise with associated GTPase activity into a ring at the bacterial mid-cell in the crucial first step of cell division (Lu *et al.,* 1998). FtsZ molecules interact directly with FtsA and the ‘Z’ ring acts as the scaffold onto which the cell division apparatus, formed by approximately twenty proteins, assembles (Errington, 2003). Determination of the structure of several bacterial FtsZ proteins has revealed it is a structural homolog of the mammalian cytoskeletal

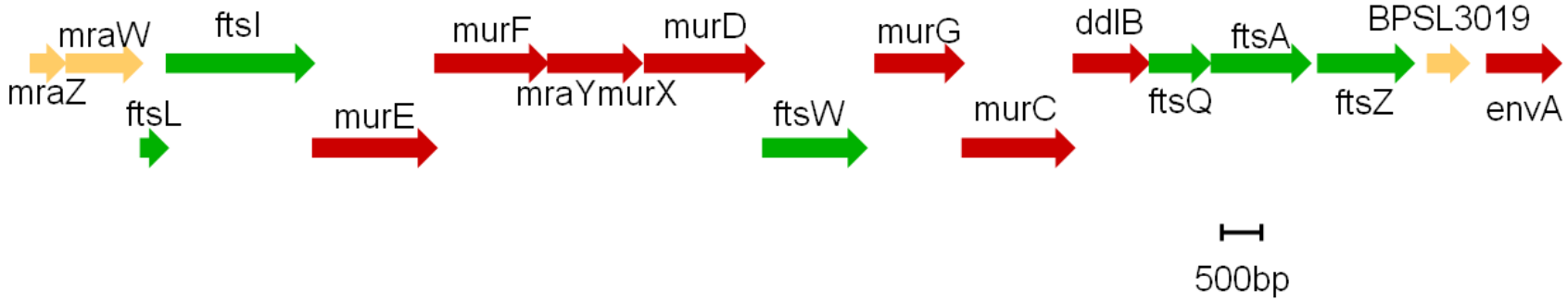

Figure 3.2 The annotated *B. pseudomallei* dcw cluster.

Genes involved in peptidoglycan synthesis are highlighted in red. Genes involved in cell division are highlighted in green. Genes involved in neither process or of unknown function are highlighted in orange.

protein tubulin (Lowe and Amos, 1998). With the aid of this structural information, many studies have identified small molecules which inhibit FtsZ GTPase enzyme activity and which also have antimicrobial activity (reviewed Vollmer, 2006; Lock *et al*., 2007, Kapoor *et al*., 2009).

FtsA has been shown to act as the anchor to the cell membrane of the FtsZ polymer 'Z ring' (Pichoff and Lutkenhaus, 2005). FtsA structural determination revealed it too is a homolog of a mammalian cytoskeletal element, in this case, actin (van den Ent and Lowe, 2000) and *E. coli* FtsA, like actin, has ATPase activity (Paradis-Bleau *et al.,* 2005). A single study has identified small peptide inhibitors of *P. aeruginosa* FtsA ATPase activity (Paradis-Bleu *et al*., 2005).

FtsA and FtsZ have been shown to be viable antimicrobial targets for mainly Gram positive bacteria. Their essential nature in *B. pseudomallei* will be assessed here.

The third target, MraW has been described as essential in *E. coli* due to an inability to mutate its encoding gene unless a second copy of the gene was present *in trans* in this bacteria (Carrion *et al*., 1999). Further, recombinant *E. coli* MraW has been shown to have methyltransferase activity (Carrion *et al*., 1999) and more recently, to methylate a specific ribosomal RNA (rRNA) base, and resultantly ensure fidelity of translation of mRNA transcripts (Kimura *et al*., 2010). This gene is well conserved among bacterial species, indicating the role it plays in rRNA methylation, and possibly in other functions are important

in many bacterial species. No studies have previously been conducted to determine if MraW is essential in *B. pseudomallei or* the role it plays during infection and therefore if it is a viable antimicrobial target.

Lastly, high affinity iron uptake is known to be a key virulence determinant in many bacterial pathogens. Ablation of high affinity TonB dependent ferric iron uptake by inactivation of *tonB* is attenuating in a range of pathogenic Gram negative bacteria (Gorbacheva *et al*., 2001; Reeves *et al*., 2000; Torres *et al*., 2001; Tsolis *et al*., 1996). TonB functions to harness cytoplasmic membrane proton motive force (pmf) and transduce it across the periplasm to power energy dependent uptake of substrates, mainly ferric iron, across the periplasmic membrane via TonB dependent transporters (TBDTs) (Postle and Kadner, 2003). Additionally, TonB has been shown to play a role in iron sensing and signalling (Koebnik, 2005) which is important for proper virulence factor expression during infection. Whilst the siderophore synthesis and uptake system has been investigated in *B. pseudomallei* (Alice *et al.,* 2006), the role of this system, TonB, or iron uptake in pathogenesis in general has not previously been investigated in *B. pseudomallei.* Iron uptake may present an attractive target for novel antimicrobial inhibitors for treatment of *B. pseudomallei* and will be investigated here.

Chapter 4. Mutagenesis of putative antimicrobial targets

4.1 Introduction

The deletion of a gene from an organism is one way to investigate the function of that individual gene. Deletion mutants can be tested in different models of infection in order to determine the role played by that gene product at specific stages of an infection. However essential genes pose their own conundrum as due to their nature, their deletion ablates viability of the cell therefore making determination of their role during infection or otherwise difficult. Pathogen specific essential gene products, have long been a holy grail for targeting of novel antimicrobials. However, whilst some genes are essential in one species of bacteria, this may not be the case in a different species, reducing the spectrum of activity of the hypothetical drug which targets that essential gene product, although single pathogen targeting may be an advantageous antibiotic strategy. Understanding the roles played by gene products during infection, as well as if a gene is essential, is extremely useful when considering novel antimicrobial targets.

In this study, four *B. pseudomallei* genes have been selected for mutagenesis and subsequent characterisation due to their potential as novel antimicrobial targets. The conservation of these four gene products amongst a range of pathogenic and non-pathogenic bacteria was investigated using bioinformatic methods. This analysis contributes to determining the possible spectrum of activity of an antimicrobial targeting them. In order to investigate the potential for inhibition by targeting these gene products in *B. pseudomallei,* attempts to

construct in-frame deletion mutants in all four genes in *B. pseudomallei* K96243 using the pDM4 suicide vector were undertaken, with a secondary aim to confirm and characterise any successfully mutated strains both *in vitro* and *in vivo*.

4.2 Results

4.2.1 Conservation of antimicrobial targets amongst bacterial species

In order to determine the possible spectrum of activity of a novel antimicrobial which targets one of the four gene products investigated here, multiple sequence alignments of the *B. pseudomallei* amino acid sequence of FtsA, FtsZ, MraW and TonB with closely and distantly related pathogens and non-pathogens were carried out using DNAStar Lasergene 8.0 SeqMan software. Sequence identity was calculated as a percentage, by identification of identical amino acids in the chosen species sequence, using the *B. pseudomallei* K96243 amino acid sequence as a reference.

4.2.1.1 Conservation of *B. pseudomallei* FtsA and FtsZ among bacterial species

A multiple sequence alignment of the primary amino acid sequences of FtsA and FtsZ from *B. pseudomallei* K96243, *B. thailandensis* E264, *B. mallei*, *E. coli* K-12, *B. subtilis* 168, *Y. pestis* CO92, *F. tularensis* subspecies *tularensis* Schu S4 and the species from which the first crystal structure of each protein was completed, was carried out by the Clustal W method (Figures 4.1 and 4.2). For both FtsA and FtsZ, key residues identified by mutational studies to play a role in interaction with other cell division proteins, or identified as conserved for enzyme activity by structural studies were annotated. Further, the degree of identity of *B. pseudomallei* FtsA and FtsZ was calculated using NCBI BLAST (Table 4.1 and Table 4.2).

Identity of *B. pseudomallei* FtsZ compared with the two closely related species *B. thailandensis* and *B. mallei* was high at 85% for both. Further, the primary protein sequence of *B. pseudomallei* FtsZ is well conserved when compared to all of the aligned sequences with identity values of around 40% for all of the non-*Burkholderia* species (Table 4.1). The FtsZ carboxy terminal domain region has been shown in *E. coli* to be important for interaction with FtsA and another important cell division molecule known as ZipA, and is therefore key to the progression of cell division following initiation of the process by formation of the Z ring (Ma and Margolin, 1999). A relatively high degree of conservation of this region was seen between the three *Burkholderia* species analysed and *E. coli* (Figure 4.1, green bar). The highly conserved P375 residue found in this region of *E. coli* FtsZ, shown to be key for FtsA recruitment (Ma and Margolin, 1999), is conserved in all aligned sequences.

Further, the majority of the residues identified as interacting with GDP in *M. jannaschii* FtsZ (Lowe and Amos, 1998) are conserved in all of the aligned *Burkholderia* FtsZ sequences (Figure 4.1, yellow boxes). Residues shown to be conserved in the T7 loop of the FtsZ mammalian homolog, tubulin and *E. coli* FtsZ, are also identical in the three *Burkholderia* species (Figure 4.1, green boxes). This loop has been shown to interact with adjacent monomers in the tubulin protofilament and is implicated in polymerisation-dependent GTPase activity of tubulin monomers (Lowe *and* Amos, 1999).

The primary sequences of FtsA are identical in *B. pseudomallei, B. thailandensis and B. mallei*, A high degree of identity, again of around 40% is seen between *B. pseudomallei* FtsA and the aligned non-*Burkholderia* species except for *Thermotoga maritima* (Table 4.2). Four out of seven residues described to play a role in interacting with FtsZ in *E. coli* (Pichoff and Lutkenhaus, 2007) are identical in *B. pseudomallei, B. thailandensis* and *B. mallei* FtsA sequences (Figure 4.2, red boxes). Overall, 18% identity of residues between *B. pseudomallei* FtsA and *T. maritima* FtsA exists. When looking specifically at *T. maritima* active site residues mapped from the mammalian homolog protein actin (van den Ent and Lowe, 2000), 25% are identical in the *Burkholderia* species sequences (Figure 4.2, yellow boxes). The carboxy terminal domain of *E. coli* and *B. subtilis* is loosely conserved and forms an amphipathic helix which is involved in membrane targeting of FtsA (Pichoff and Lutkenhaus, 2005). Whilst there is a low degree of identity of residues at this region between *Burkholderia* species, *E. coli* and *B. subtilis,*

the pattern of hydrophobic and hydrophilic residues which have the capacity to form an amphipathic helix is present in *B. pseudomallei* (Figure 4.3).

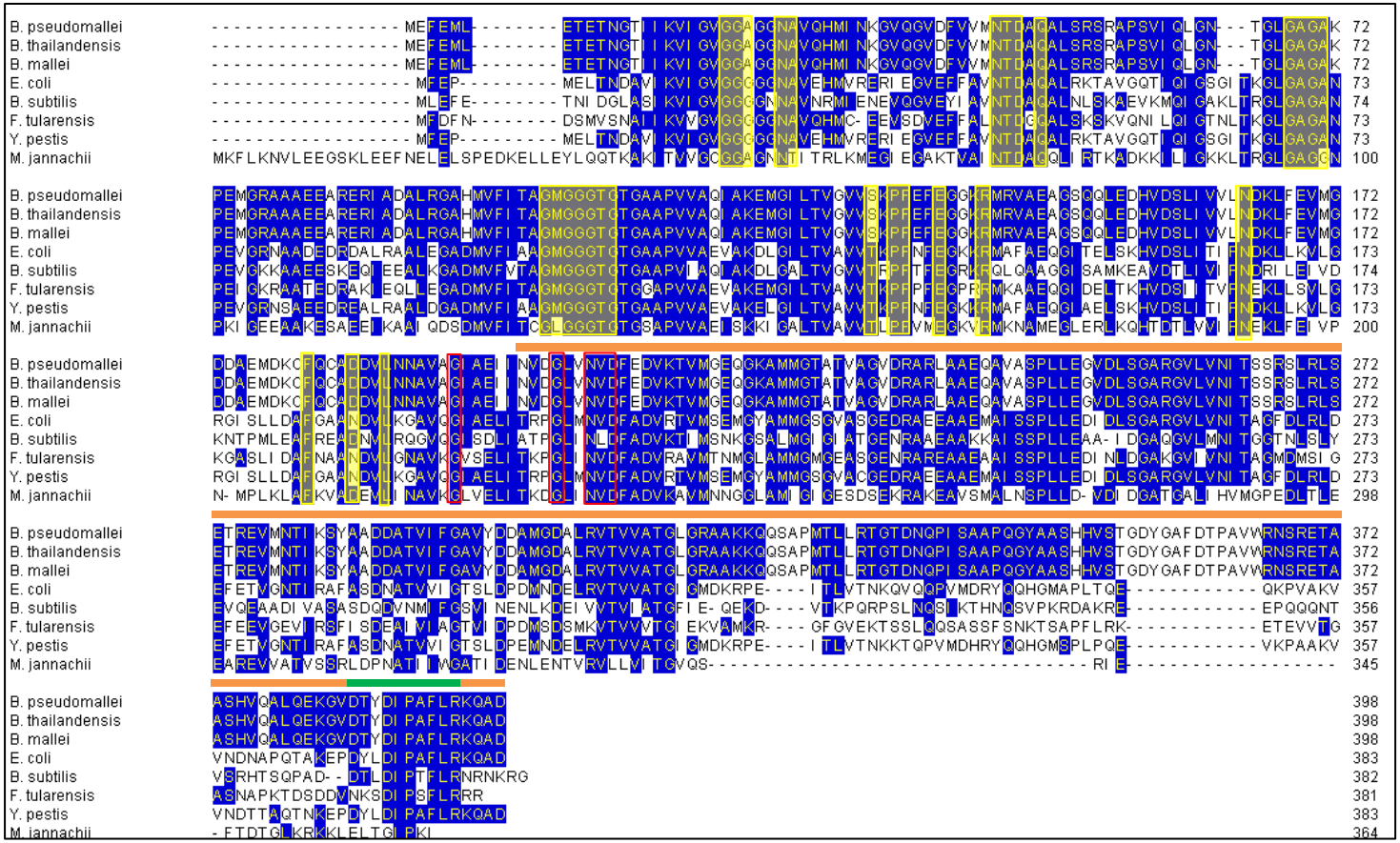

Figure 4.1 Sequence alignment of FtsZ from *B. pseudomallei*, *B thailandensis*, *B. mallei*, *E. coli* K-12, *B. subtilis* 168, *F. tularensis* Schu S4, *Y. pestis* CO92 and *M. jannaschii*. Identical amino acids are highlighted in blue. Residues boxed in red have been shown to be conserved in the T7 loop of M. *jannaschii* FtsZ and tubulin (Lowe and Amos,1999). Yellow boxed residues were identified in the *M. jannaschii* crystal structure to interact with GDP. The green bar denotes the extreme carboxy terminal motif which mediates interaction of FtsZ with FtsA and ZipA in *E. coli* (Ma and Margolin, 1999). The carboxy terminal domain of the protein is denoted by an orange bar (Lowe and Amos, 1998).

Species	Identity (%)
B. thailandensis	85
B. mallei	85
E. coli	44
B. subtilis	38
F. tularensis	39
Y. pestis	44
M. jannaschii	40

Table 4.1 *B. pseudomallei* FtsA homologues in *B thailandensis* and *B. mallei*, *E. coli* K-12, *B. subtilis* 168, *F. tularensis* Schu S4, *Y. pestis* CO92 and *M. jannaschii*. Calculated by NCBI BLAST.

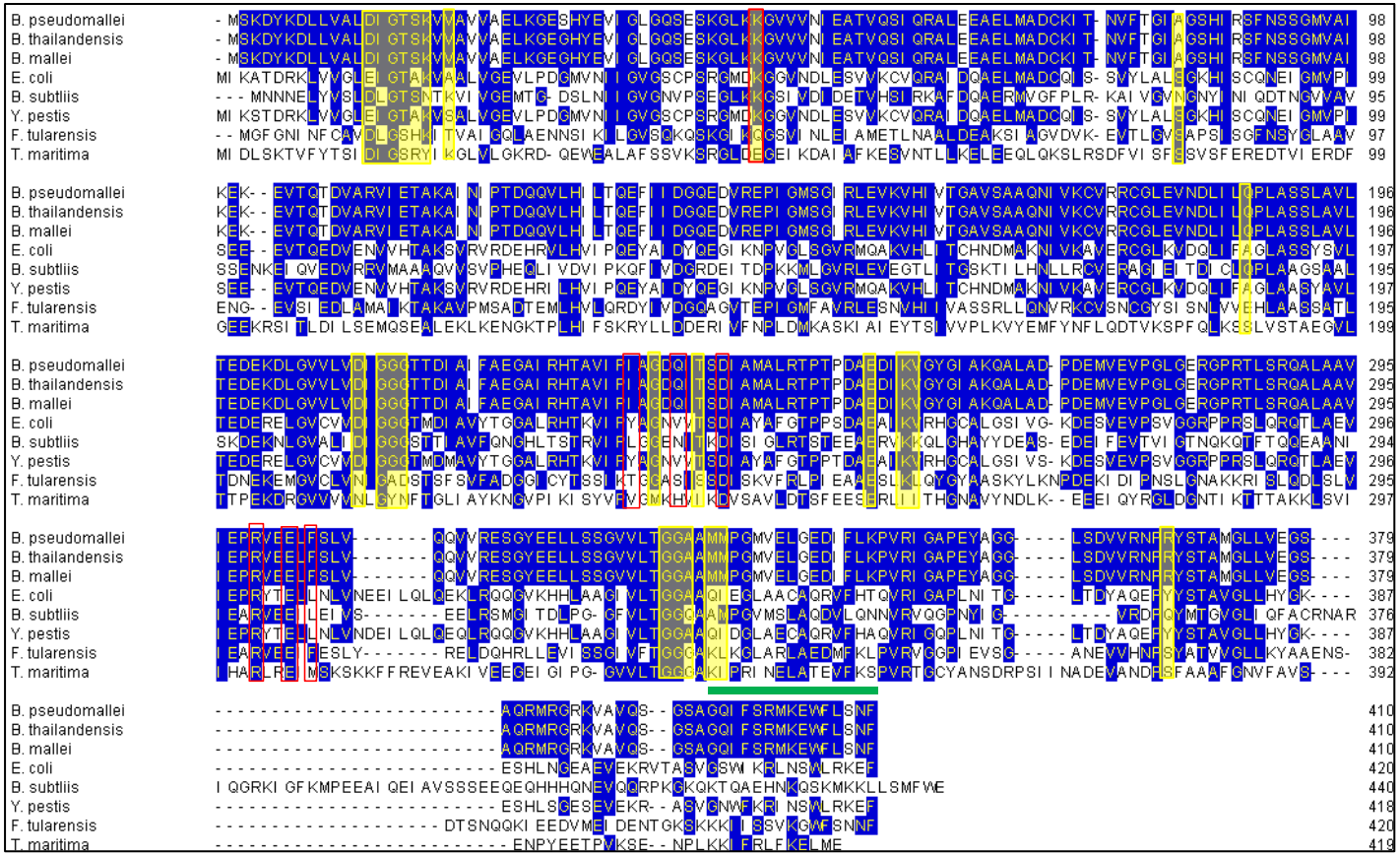

Figure 4.2 Sequence alignment of FtsA from *B. pseudomallei, B thailandensis*, *B. mallei*, *E. coli* K-12, *B. subtilis* 168, *F. tularensis* Schu S4, *Y. pestis* CO92 and *T. maritima*. Identical amino acids are highlighted in blue. Residues boxed in red were identified as required for interaction with FtsZ by Pichoff and Lutkenhaus (2007). Yellow shaded residues are active site residues identified in the crystal structure of *T. maritima* when comparing to the structure of mammalian actin (van den Ent, 2000). The green bar denotes the membrane targeting sequence which forms an amphipathic helix in *E. coli* (Pichoff and Lutkenhaus, 2005).

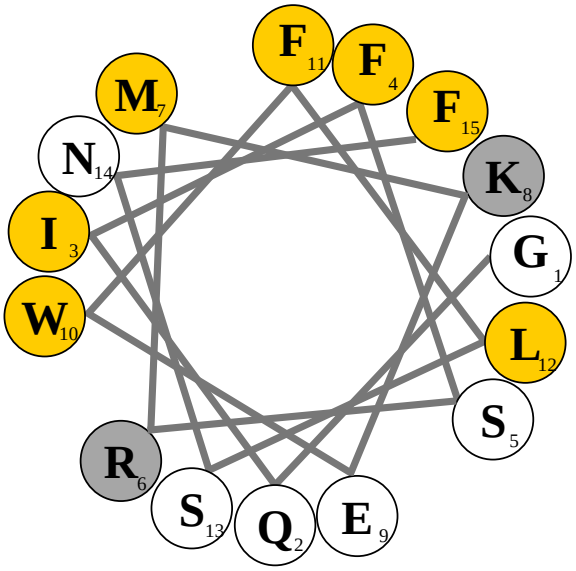

Figure 4.3 Helical wheel diagram of the conserved carboxy terminal amphipathic helix of *B. pseudomallei* FtsA. The wheel is a projection of the amino acid side-chains on a plane perpendicular to the axis of the helix (Schiffer and Edmundson, 1967). Large hydrophobic residues are highlighted in yellow, basic residues are highlighted in grey.

Species	Identity (%)
B. thailandensis	96
B. mallei	96
E. coli	46
B. subtilis	38
F. tularensis	38
Y. pestis	46
T. maritima	18

Table 4.2 *B. pseudomallei* FtsA homologues in *B thailandensis* and *B. mallei*, *E. coli* K-12, *B. subtilis* 168, *F. tularensis* Schu S4, *Y. pestis* CO92 and *T. maritima*. Calculated by NCBI BLAST.

4.2.1.2 Conservation of *mraW* among bacterial species

The sequences of MraW from different bacterial species were aligned using the Clustal W method to determine sequence similarity (Figure 4.4). Domains identified in *E. coli* MraW (Carrion *et al*. 1999) have a high proportion of identity of 84% in *B. pseudomallei* (Figure 4.4, green bars). Furthermore, all four residues required for methyltransferase activity in *E. coli* are conserved in *B. pseudomallei* (Figure 4.4, red boxes). Additionally, sequence identity between aligned species sequences and *B. pseudomallei* K96243 MraW was calculated using NCBI BLAST. Whilst both *Burkholderia* species tested are highly identical to the *B. pseudomallei* amino acid sequence, a high degree of identity of upwards of 40% is seen in the more distantly related species (Table 4.3).

Species	**Identity (%)**
B. thailandensis	97
B. mallei	100
E. coli	51
B. subtilis	43
F. tularensis	45
Y. pestis	54

Table 4.3 MraW homologue identity of *B. pseudomallei* with *B thailandensis*, *B. mallei*, *E. coli* K-12, *B. subtilis* 168, *F. tularensis* Schu S4 and *Y. pestis* CO92. Calculated by NCBI BLAST.

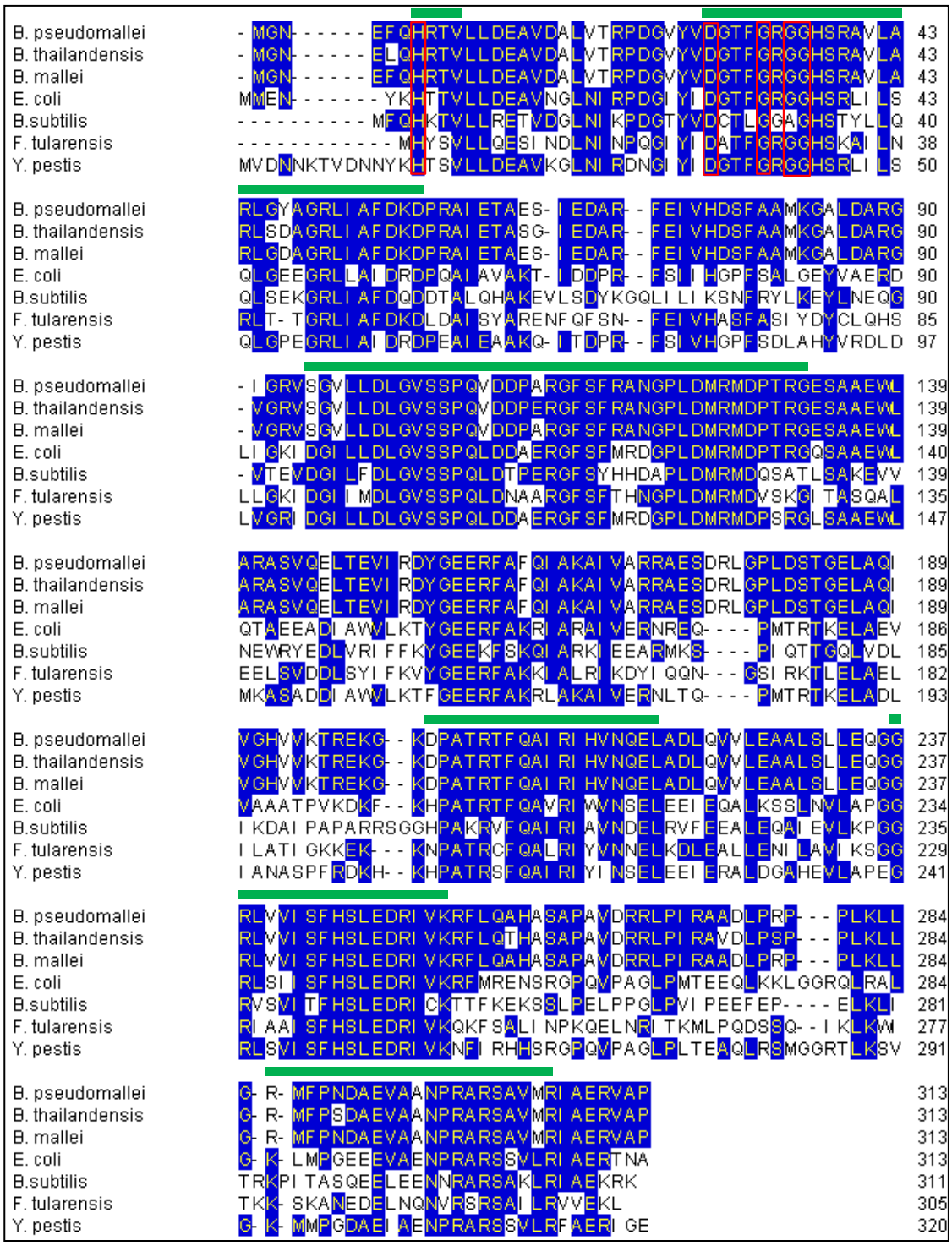

Figure 4.4 Sequence alignment of MraW from *B. pseudomallei, B thailandensis, B. mallei*, as well as more distantly related bacteria *E. coli* K-12, *B. subtilis* 168, *F. tularensis* Schu S4 and *Y. pestis* CO92. Identical amino acids are highlighted in blue. Residues boxed in red are conserved in methyltransferases as identified by Carrion *et al*. 1999. The six conserved MraW domains as identified by Carrion *et al.* 1999 are indicated by green bars.

4.2.1.3 Conservation of TonB among bacterial species

A multiple sequence alignment by the Clustal W method of the primary amino acid sequence of *B. pseudomallei* K96243, *B. thailandensis* E264, *B. mallei*, *E. coli* K-12, *Y. pestis* CO92, and *R. solanacearum* TonB was carried out (Figure 4.5) and identity between homologs calculated using NCBI BLAST or alignment by Clustal W (Figure 4.5). A high degree of identity was calculated between aligned *Burkholderia* species and the closely related *R. solanacearum*. Although the more distantly related *E. coli* and *Y. pestis* had a low degree of identity to *B. pseudomallei* TonB, the proline-rich region, as identified in *E. coli* and demonstrated to form a polyproline II helix in order to span the periplasm to energize TBDTs (Kohler *et al*., 2010) is clearly present in all aligned sequences (Figure 4.5, red bar).

Species	**Identity (%)**
B. thailandensis	65
B. mallei	57
E. coli	20.7
Y. pestis	19.1
R. solanacearum	35.4

Table 4.4 Homologue identity of *B. pseudomallei* TonB with *B thailandensis* and *B. mallei*, *E. coli* K-12, *Y. pestis* CO92 and *R. solanacearum.* Calculated using NCBI BLAST.

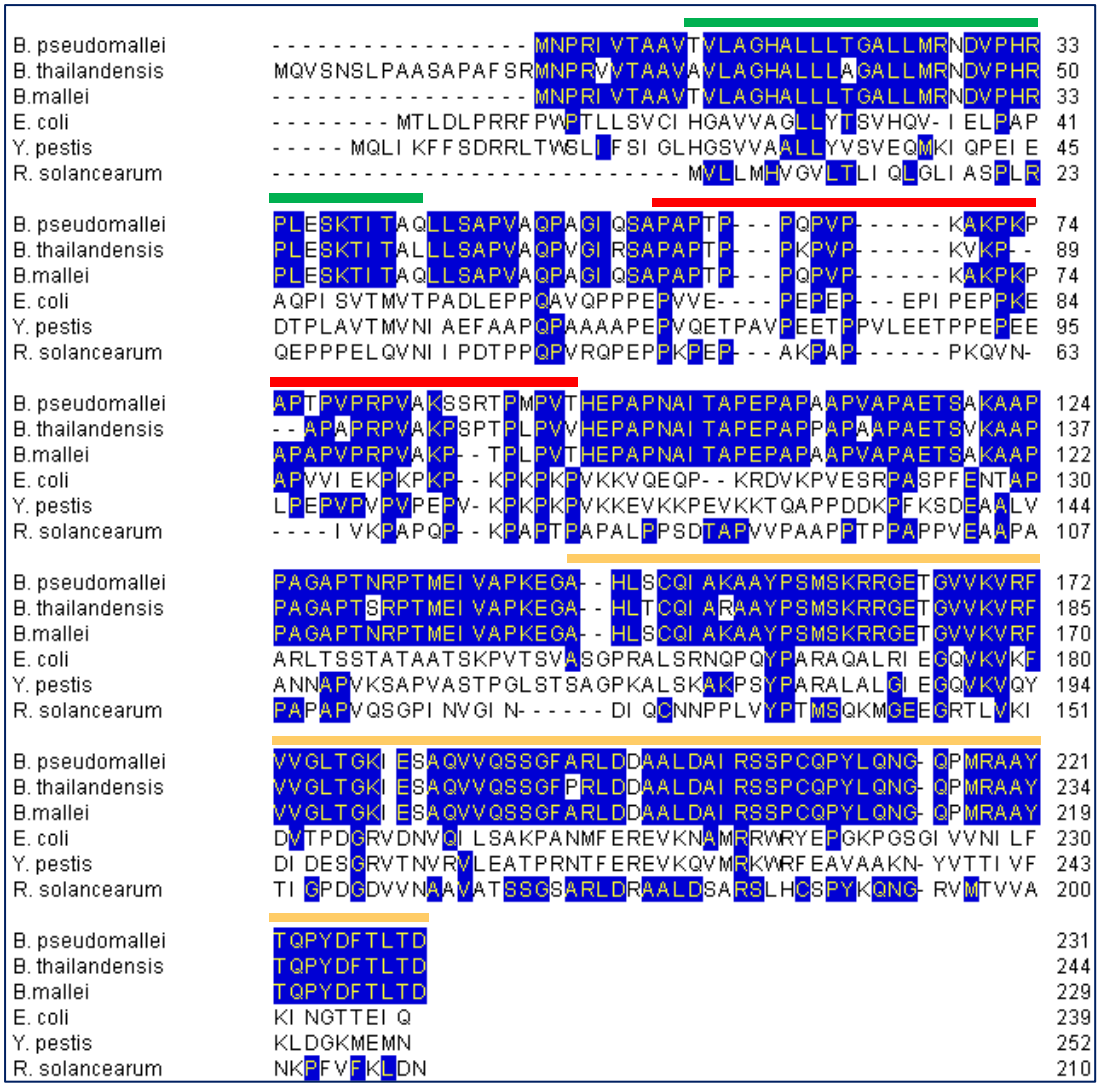

Figure 4.5 Sequence alignment of TonB from *B. pseudomallei, B thailandensis*, *B. mallei*, *E. coli* K-12, *Y. pestis* CO92 and *R. solanacearum*. Identical amino acids are highlighted in blue. Green bar indicates the *E. coli* hydrophobic signal sequence for targeting of TonB to the cytoplasmic membrane. The proline-rich spacer sequence is denoted by the red bar. Orange bar indicates the C-terminal domain essential for interacting with outer membrane transporters. All regions described in *E. coli* by Krewelak and Vogel, 2011.

4.3 Construction of deletion mutants

4.3.1 Mutant making strategy

The pDM4 suicide vector was used to attempt to delete the four genes of interest in this study. The strategy is outlined in Figure 4.6 . Briefly, suicide vectors were constructed containing a truncated version of each of the genes to be mutated, with additional flanking regions either side. The suicide vector integrated into the *B. pseudomallei* genome by homologous recombination at either of the flanking regions engineered into the vector. Integration of the vector afforded the recipient bacterium chloramphenicol resistance and sucrose sensitivity due to the *cam*^{r} and *sacB* genes encoded by the vector. Integration into the genome, and therefore creation of a merodiploid strain was screened for by PCR to amplify these two genes using primers camr F and camr R and sacB F and sacB R. Subsequently, merodiploid strains were selected for a second recombination event, which led either to reversion back to the parental strain genotype, or to deletion of the gene into which the vector had integrated. In both cases those clones which had undergone a second crossover event to excise the vector reverted to chloramphenicol sensitive phenotype. The genotype, either parental strain revertant, or deletion mutant was differentiated by the size of PCR product for the region in which the vector was integrated.

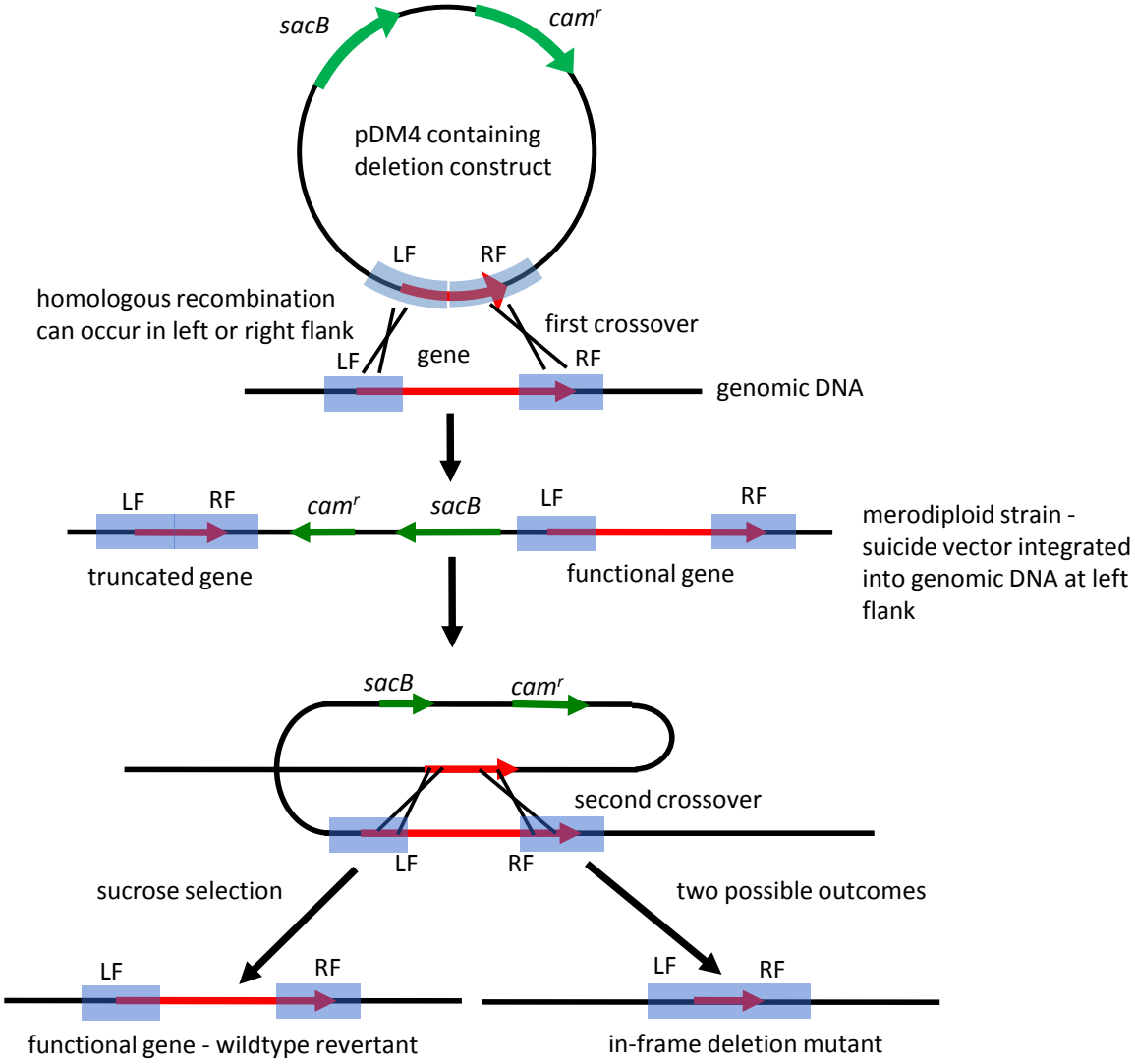

Figure 4.6 Mutant making strategy using pDM4 to construct in-frame un-marked deletion mutants in *B. pseudomallei.* Adapted from Logue *et al.*, 2009.

4.3.2 Construction of antimicrobial target merodiploid strains

Constructs for deletion of *ftsA* and *ftsZ* were produced by amplification of in-frame flanking regions from *B. pseudomallei* K96243 genomic DNA using primers ftsA LFF, ftsA LFR, ftsA RFF and ftsA RFR for *ftsA*, and ftsZ LFF, ftsZ LFR, ftsZ RFF and ftsZ RFR for *ftsZ* by PCR as described in section 2.3.1. Each flank was sub-cloned into the pCR Blunt II TOPO plasmid and transformed by heat shock into TOP10 *E. coli.* Sequences were verified by restriction digest and nucleotide sequencing. Flanking regions were subsequently excised from the pCR Blunt II TOPO plasmid using restriction enzymes *Xba*I and *Nde*I and ligated together with a *Xba*I linearised pDM4 plasmid. Ligated flank regions of *mraW* and *tonB* were synthesized by GeneArt, excised from the plasmid they were provided in using *Xba*I and ligated into *Xba*I linearised pDM4 plasmid.

The confirmed gene deletion pDM4 suicide vectors were introduced into *B. pseudomallei* K96243 by conjugative transfer as in section 2.4.1 and resulting merodiploid *B. pseudomallei* strains of *ftsA, ftsZ*, *mraW* and *tonB* were verified by amplification by PCR of *cam*^{r} or *sacB* genes (Figure 4.7).

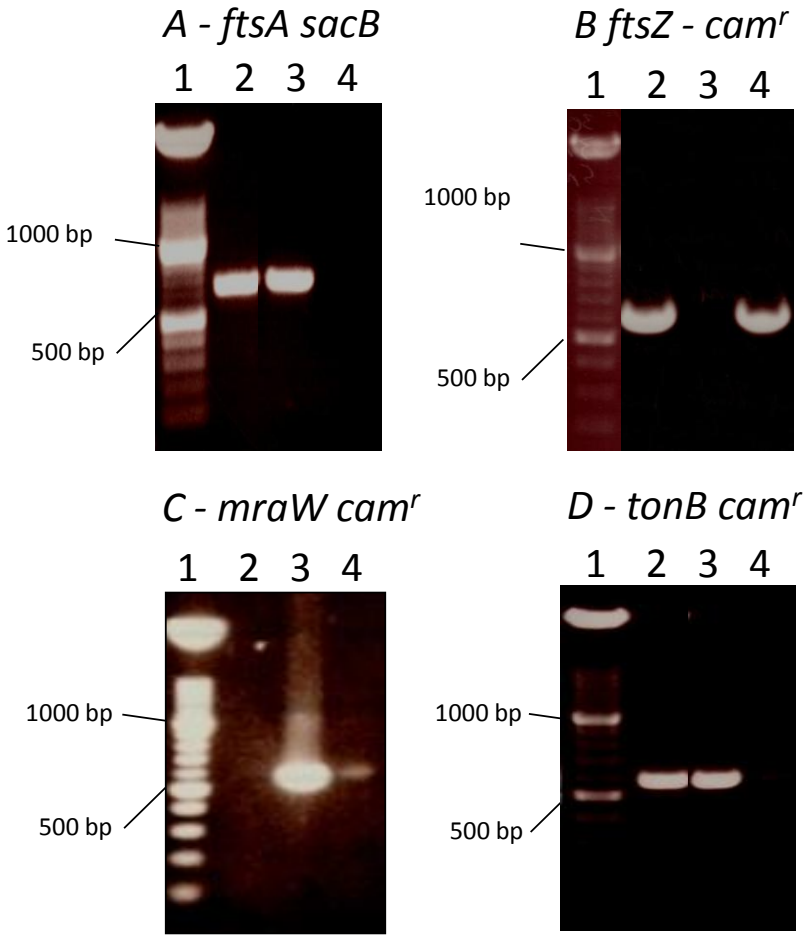

Figure 4.7 *B. pseudomallei* merodiploid *ftsA*, *ftsZ*, *mraW* or *tonB* strain confirmation by PCR of either *cam*^{r} or *sacB* genes.

A -
Lane 1 - 100 bp DNA marker;
lane 2 - *B. pseudomallei* K96243 merodiploid ftsA gDNA;
lane 3 - purified pDM4 plasmid DNA;
lane 4 - *B. pseudomallei* K96243 gDNA.

B -
Lane 1 - representative 100 bp DNA marker;
lane 2 - *B. pseudomallei* K96243 merodiploid ftsZ gDNA;
lane 3 - *B. pseudomallei* K96243 DNA;
lane 4 - purified pDM4 plasmid DNA.

C -
Lane 1 - 100 bp DNA marker;
lane 2 - *B. pseudomallei* K96243 gDNA;
lane 3 - purified pDM4 plasmid DNA;
lane 4 - *B. pseudomallei* K96243 merodiploid mraW gDNA.

D -
Lane 1 - 100 bp DNA marker;
lane 2 - *B. pseudomallei* K96243 merodiploid *tonB* gDNA;
lane 3 - purified pDM4 plasmid DNA;
lane 4 - *B. pseudomallei* K96243 gDNA.

4.3.2.1 Growth of merodiploid strains *ftsA* and *ftsZ*

In order to determine whether integration of the suicide vector into *ftsA* and *ftsZ* genes in *B. pseudomallei* K96243 affected viability of the bacteria, growth studies of the merodiploid strains of both genes was undertaken. From overnight cultures, 0.5 ml was inoculated into 10 ml LB broth, and OD of the culture measured hourly. Integration of the suicide vector was shown to have no effect on growth rate when compared to the parental strain ($p>0.05$) (Figure 4.8).

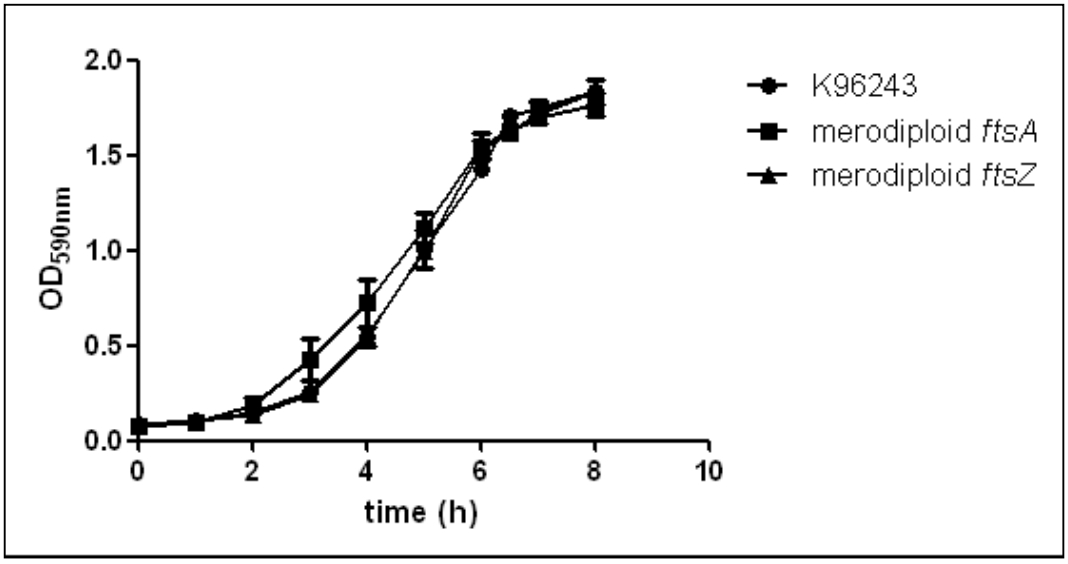

Figure 4.8 Growth of *B. pseudomallei* strains K96243, merodiploid *ftsA* and merodiploid *ftsZ* in LB broth at 37°C over 8 h. Growth was measured hourly by optical density at 590nm. Values are the means from biological triplicate experiments ± standard error.

4.3.2.2 Morphology of merodiploid strains *ftsA* and *ftsZ*

In order to determine whether integration of the suicide vector into the flank regions of *ftsA* and *ftsZ* genes in *B. pseudomallei* K96243 affects morphology of the bacteria, electron microscopy to visualize the cells was undertaken as described in section 2.6. Representative bacteria of each sample are visualized in Figure 4.9. No difference in gross morphology of cells was seen between strains.

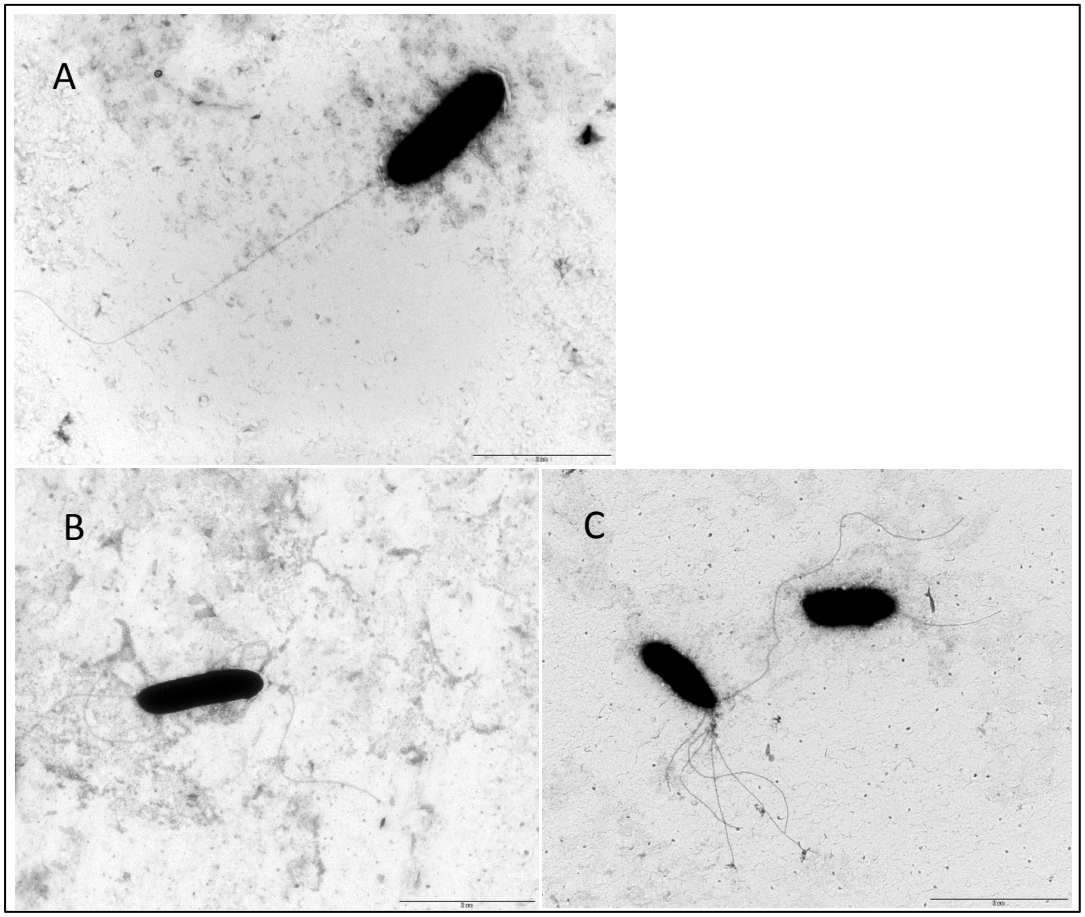

Figure 4.9 Electron microscopy of representative bacteria of *B. pseudomallei strains* K96243 (A), merodiploid *ftsZ* (B) and merodiploid *ftsA* (C)

4.3.2.3 *ftsA* and *ftsZ* sucrose selection

To isolate clones that had undergone a second crossover event, sucrose selection of *ftsA* and *ftsZ* merodiploid strains was carried out by plating dilutions of the broth cultured strains onto LB agar in the absence of NaCl with additional sucrose at 10%, to select for strains which had excised out the vector backbone by homologous recombination. Two rounds of sucrose selection were completed on both *ftsA* and *ftsZ* merodiploid strains. Seventy five colonies of *B. pseudomallei ftsA* sucrose selected clones were screened for chloramphenicol sensitivity, of which seven were sensitive. All of the seven clones were shown to be parental strain revertants by PCR, using primers ftsA LFF and ftsA LFR. Eighty five *B. pseudomallei ftsZ* merodiploid derived sucrose selected clones were screened for chloramphenicol sensitivity of which nine were sensitive. All but one of these clones were shown to be parental strain revertant clones as they amplified a DNA fragment of the same size (1209 bp) as the *B. pseudomallei* K96243 following a second crossover event, by PCR using primers ftsZ F and ftsZ R. A single clone amplified a fragment of the expected size for a *ftsZ* deletion mutant, 135 bp (Figure 4.10). Frequency of screened colonies and outcomes are summarised in Table 3.5.

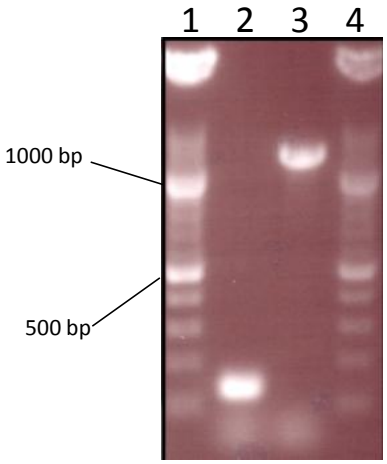

Figure 4.10 PCR to amplify the *ftsZ* gene region from genomic DNA of *B. pseudomallei* K96243 and *B. pseudomallei* putative *ftsZ* mutant using primers ftsZ LFF and ftsZ LFR.

Lane 1 - 100 bp DNA marker;
lane 2 - *B. pseudomallei* putative ftsZ mutant gDNA;
lane 3 - *B. pseudomallei* K96243 gDNA;
lane 4 - 100 bp DNA marker.

4.3.2.4 Putative *ftsZ* mutant

A single clone was obtained from sucrose selection of *B. pseudomallei ftsZ* merodiploid strain which was chloramphenicol sensitive and from which a 135 bp fragment was amplified by PCR using primers ftsZ F and ftsZ R which would be expected from a *B. pseudomallei ΔftsZ* clone (Figure 4.10). Further, no product was amplified by PCR using primers camr F and camr R, as well as sacB F and sacB R which amplify pDM4 borne *cam*^{r} and *sacB* genes respectively (Figure 4.11). This indicates a second recombination event had occurred in which to excision of the vector backbone containing *cam*^{r} and *sacB* genes produced a *B. pseudomallei* deletion mutant of *ftsZ*.

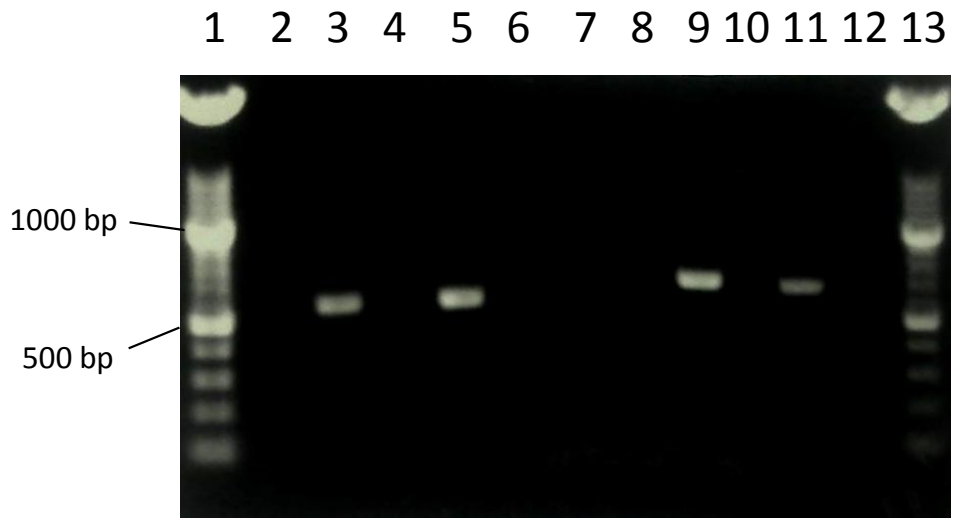

Figure 4.11 PCR amplification of *cam*^{r} (lanes 2 - 6) and *sacB* (lanes 8 - 12) genes from putative *B. pseudomallei ΔftsZ.*

Lane 1 - 100 bp DNA marker;
lane 2 - putative *B. pseudomallei* K96243 ftsZ mutant gDNA;
lane 3 - *B. pseudomallei* K96243 merodiploid ftsZ gDNA;
lane 4 - *B. pseudomallei* K96243 gDNA;
lane 5 - pDM4 plasmid DNA;
lane 6 - no DNA;
lane 7 - blank;
lane 8 - putative *B. pseudomallei* ftsZ mutant gDNA;
lane 9 - *B. pseudomallei* merodiploid ftsZ gDNA;
lane 10 - *B. pseudomallei* K96243 gDNA;
lane 11 - pDM4 plasmid DNA;
lane 12 - no DNA;
lane 13 - 100 bp DNA marker.

To establish whether *ftsZ* had been deleted, genomic DNA from the clone was restriction digested and probed for the left flank of *ftsZ* by Southern hybridisation as described in section 2.3.10. Two separate restriction digests were carried out, first using the enzyme *Sph*I and another with *Pst*I. Predicted fragment sizes which would be hybridised by an *ftsZ* left flank probe for *B. pseudomallei* K96243 genomic DNA were calculated as 2722 bp when digested with *Sph*I and 1920 bp when digested with *Pst*I (Figure 4.12.A.i). Predicted fragment sizes for *ΔftsZ* were 1642 bp when digested with *Sph*I and 2044 bp when digested with *Pst*I (Figure 4.12.A.ii). The left flank DIG labelled DNA probe was produced by PCR amplification using primers ftsZ LFF and ftsZ LFR. The probe hybridised to two DNA fragments in both *Sph*I and *Pst*I digested *B. pseudomallei ΔftsZ* samples. Both digests hybridised a fragment at the same size as similarly restricted parental strain, *B. pseudomallei* K96243 genomic DNA (Figure 4.12.B). Additionally, in the *Pst*I digested DNA, a larger fragment, of the approximate size expected of a deletion mutant (2044 bp) was hybridised. This fragment was expected to be larger because truncation of the gene by deletion of the middle portion removed a *Pst*I site present in the parental *B. pseudomallei* strain. For the *Sph*I digested DNA, a second band that was much smaller than expected for a deletion mutant (1642 bp) also hybridised.

To determine whether regions other than *cam*^{r} and *sacB* genes of the pDM4 suicide vector were present in any form within the apparent *B. pseudomallei ΔftsZ* clone, a series of primers which amplify regions of the pDM4 suicide were obtained (Figure 3.12.A) and used in a series of PCRs using the putative *B. pseudomallei ΔftsZ* genomic DNA as the template. All reactions using *B.*

pseudomallei ΔftsZ genomic DNA were negative, whilst positive controls using *ftsZ* merodiploid strain genomic DNA or purified *ftsZ* pDM4 plasmid amplified DNA fragments at the expected size (Figure 4.13.B).

Taken together these data suggest both a full size and truncated *ftsZ* exist in this clone, whilst portions of the pDM4 vector backbone have been excised.

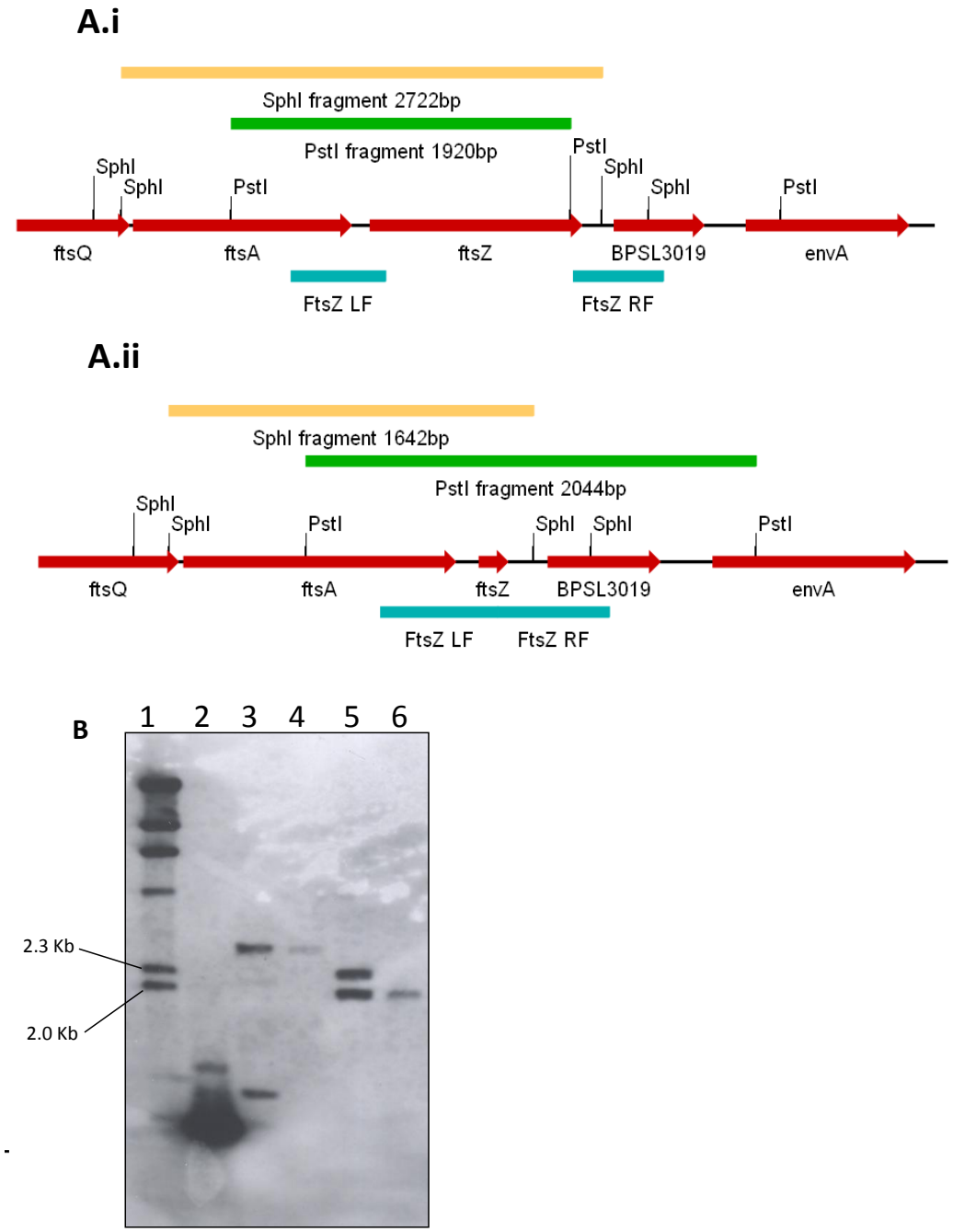

Figure 4.12 Southern hybridisation of *B. pseudomallei* K96243 and *B. pseudomallei* putative *ΔftsZ* mutant.

A – The gene region around *ftsZ* in i. - *B. pseudomallei* K96243 and ii. a putative *B. pseudomallei ΔftsZ* strain, and expected hybridised fragments following restriction digest with *Sph*I (orange) or *Pst*I (green).
B – Southern hybridisation of *B. pseudomallei* K96243 and *B. pseudomallei* putative *ΔftsZ* mutant. Digested genomic DNA was probed with DIG labelled left flank.

Lane 1 - DIG labelled DNA marker;
lane 2 - gel extracted left flank DNA fragment;
lane 3 - *B. pseudomallei* putative *ΔftsZ* gDNA digested with *Sph*I;
lane 4 - *B. pseudomallei* K96243 gDNA digested with *Sph*I;
lane 5 - *B. pseudomallei* putative *ΔftsZ* gDNA digested with *Pst*I;
lane 6 - *B. pseudomallei* K96243 gDNA digested with *Pst*I.

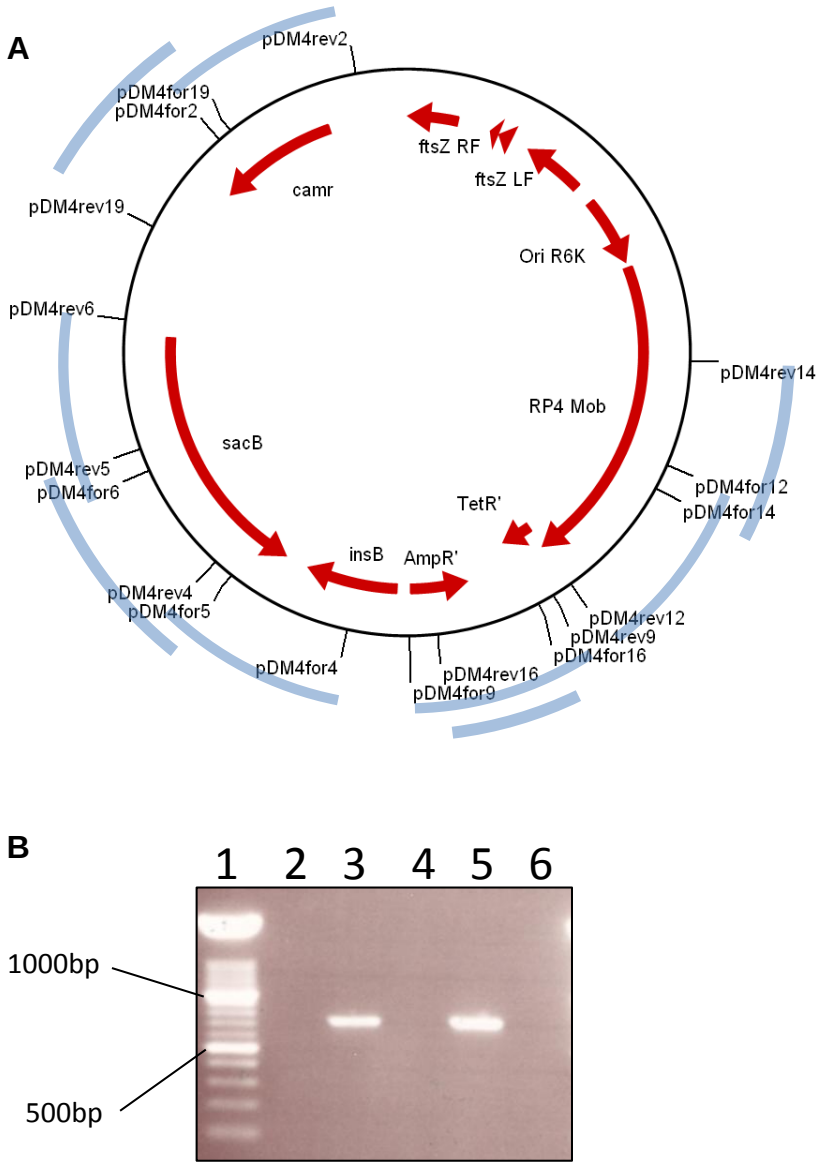

Figure 4.13 Investigation of regions of pDM4 present in a putative *ftsZ* mutant.

A - The suicide vector pDM4. Red arrows are genes encoded within the plasmid. Primer sites for screening of pDM4 by PCR are indicated. Blue bars represent PCR amplification products from each pair of primers.
B - A representative PCR of sections of the pDM4 suicide vector, in this example using primers pDM4 F 6 and pDM4 R 6, expected amplification product size 709bp. Similar results were obtained by amplification using primers pDM4 F and R 2, 4, 5, 9, 12, 14, 16 and 19.

Lane 1 - 100 bp DNA marker;
lane 2 - *B. pseudomallei* putative *ΔftsZ* mutant gDNA;
lane 3 - *B. pseudomallei ftsZ* merodiploid strain gDNA;
lane 4 - *B. pseudomallei* K96243 gDNA;
lane 5 - pDM4 plasmid DNA;
lane 6 - no DNA.

4.3.3 *mraW* mutant construction

4.3.3.1 *ΔmraW* mutant construction

A second recombination event of by *B. pseudomallei mraW* merodiploid strain was selected for by incubation of the strain on LB agar with 10% sucrose and no NaCl at 25°C. Following a single sucrose selection, one hundred colonies were screened after three days incubation for chloramphenicol sensitivity and no colonies were sensitive. Twenty five further colonies were screened after five days incubation, revealing seven chloramphenicol sensitive clones, all of which were screened for *mraW* gene size by PCR with primers mraW screen F and mraW screen R. Two clones amplified reduced *mraW* gene fragments by PCR and one of these mutants was confirmed as an *mraW* deletion mutant by Southern hybridisation (Figure 4.14). Frequencies of screened colonies and outcomes are summarised in Table 4.5.

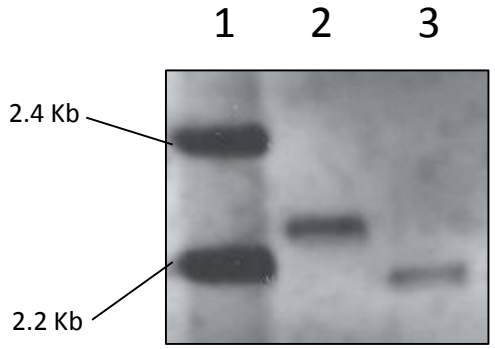

Figure 4.14 Confirmation of *B. pseudomallei ΔmraW* by Southern hybridisation. *Bgl*II digested genomic DNA was probed with DIG labelled *mraW* left flank.

Lane 1 – DIG labelled DNA marker;
lane 2 - *B. pseudomallei* K96243 gDNA;
lane 3 - *B. pseudomallei ΔmraW* gDNA.

4.3.4 *tonB* mutant construction

Sucrose selection as described in section 2.4.2 was undertaken to select for a second crossover event, in three separate confirmed *tonB* merodiploid strains was undertaken three times each. A total of 263 colonies were tested for chloramphenicol sensitivity and 181 resulting sensitive clones were screened by PCR for *tonB* gene size using primers tonB screen F and tonB screen R and all were found to be wildtype revertants (summarised Table 4.5). Construction of a *tonB* deletion mutant in *P. aeruginosa* required addition of $FeSO_4$ to selection media to support bacterial growth following interruption of *tonB* (Takase *et al*., 2000). Following selection of two *tonB* merodiploid strains on LB agar without NaCl, with 10% sucrose and 40 mM $FeSO_4$, twenty five colonies derived from each merodiploid strain. These strains were tested for chloramphenicol sensitivity, all of which remained resistant. Following six days incubation, twenty five further colonies from each merodiploid strain were screened for chloramphenicol sensitivity of which a total of seventeen colonies were identified to be resistant. Four of seventeen chloramphenicol sensitive clones were also dependent on additional 40mM $FeSO_4$ for growth on LB agar. PCR of these four iron dependent, chloramphenicol sensitive clones with primers tonB screen F and tonB screen R amplified a reduced *tonB* gene fragment in all four strains. Two of these clones were confirmed for deletion of *tonB* by Southern blot (Figure 4.15).

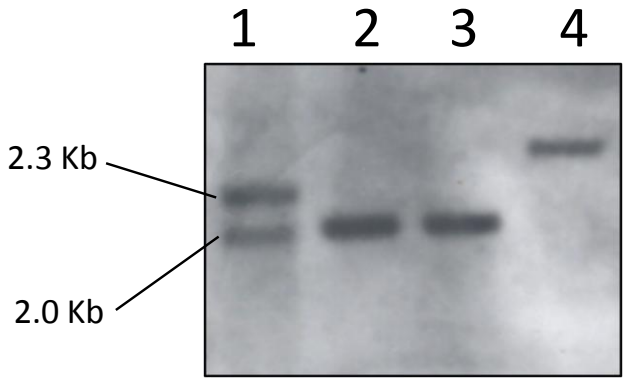

Figure 4.15 Confirmation of *B. pseudomallei ΔtonB* by Southern hybridisation. *Sph*I and *Cla*I digested genomic DNA was probed with DIG labelled *tonB* left flank.

Lane 1 – DIG labelled DNA marker;
lane 2 - *B. pseudomallei ΔtonB* clone 1 gDNA;
lane 3 - *B. pseudomallei ΔtonB* clone 2 gDNA;
lane 4 - *B. pseudomallei* K96243 gDNA.

4.4 Summary of mutant making

A comparison of frequencies of mutagenesis events at each stage of mutant making is presented in Table 4.5

Gene	Number of integrants selected	Rounds of sucrose selection	Clones screened for chloramphenicol sensitivity	Chloramphenicol sensitive clones	Deletion mutants identified
ftsA	2	2	75	7	0
ftsZ	1	2	85	9	1
mraW	1	1	125 (100 early, 25 late)	7 – all late	2
tonB	3	3	263	181	0
tonB + $FeSO_4$	1	1	75 (50 early, 25 late)	17 – all late	4

Table 4.5 Frequency of mutant making events for each antimicrobial target. Early clones were those colonies which appeared within 4 days of incubation at 25°C. Late colonies were defined as those which appeared after 4 days of incubation.

4.5 Discussion

Bioinformatic analysis of *B. pseudomallei* FtsA and FtsZ amino acid sequences revealed a high degree of identity and similarity of primary protein sequences to *B. thailandensis* and *B. mallei,* and to distantly related bacterial species. Conservation of many regions and residues of *B. pseudomallei* FtsA and FtsZ which have been shown to be important for interaction with other cell division proteins, as well as for the enzymatic activity of these proteins, support the burgeoning hypothesis that these proteins are potential targets for antibiotic therapy. Determination of the structures of FtsA and FtsZ from *B. pseudomallei,* as well as from other pathogenic bacteria, would confirm common structural regions to target novel inhibitors and aid in structure based drug design.

No *ftsA* or *ftsZ* deletion mutants were isolated in this study. This suggests that as in many other bacteria, *ftsA* and *ftsZ* are probably essential in *B. pseudomallei*. This adds to a body of evidence that FtsA and FtsZ are viable broad spectrum candidates for inhibition by novel antimicrobials. To confirm the essentiality of these genes, a conditional complementation approach could be adopted in which the gene of interest would be placed under the control of an inducible promoter and introduced into the bacteria. The genomic copy of the gene would then be deleted, and viability of the bacteria maintained by induction of the plasmid borne copy of the gene. If the gene is essential, when expression of the plasmid borne gene is stopped by removal of the induction, cells would cease to grow and stop dividing.

In *E. coli* the regulation of the dcw cluster has been shown to be important for maintenance of the cell division process, and likely occurs in a complex manner from the 3' end of the cluster (de la Fuente *et al*., 2001). During construction of *B. pseudomallei* ftsA and ftsZ mutants in this study, integration of the pDM4 suicide vector into the *B. pseudomallei* genome was expected to disrupt the dcw cluster. Whilst *ftsA* and *ftsZ* lie at the distal end of the cluster, and whole intact copies of the genes themselves will still be present in the merodiploid strain, integration of the suicide plasmid into the cluster might be expected to have an effect on expression of the genes within it, if expression of the genes in *B. pseudomallei* is regulated in a similar manner to that in *E. coli*. However, no gross deleterious effect on growth or morphology was seen in *ftsA* and *ftsZ* merodiploid strains, indicating their regulation in *B. pseudomallei* may be more relaxed than in *E. coli*.

The construction of an apparent viable ***ΔftsZ*** mutant was unexpected, as it was considered likely this gene was essential in *B. pseudomallei*. By PCR the mutant demonstrated all of the genotypes expected of an *ftsZ* merodiploid derived second crossover clone, including reduced *ftsZ* gene length and no amplification of *cam*^{r} and *sacB* genes by PCR. Additionally, the chloramphenicol sensitivity of this clone phenotypically confirms excision of *cam*^{r}. However, by Southern hybridisation, two fragments were identified in the restricted ***ΔftsZ*** genome, one of which matched the size of the similarly restricted parental strain *B. pseudomallei* K96243 genomic DNA, indicating a full size *ftsZ* gene is present in this clone. The hybridised second bands indicated the truncated *ftsZ* introduced by the suicide vector still exists in the

clone. Additionally, PCR to amplify various regions of the pDM4 vector from genomic DNA of the apparent *ftsZ* mutant were also all negative. The data regarding this clone indicates large portions of the integrated pDM4 suicide vector, including *cam*^{r} and *sacB* genes have been excised from the merodiploid strain, whilst both the complete *ftsZ* gene and smaller truncated version have been retained by the bacteria.

The production of a mutant which has excised portions of the pDM4 suicide vector backbone, but retained both a mutant and wildtype copy of the gene is further evidence that the bacteria requires *ftsZ* for viability. It could be hypothesised that the bacteria has excised the vector backbone under selection pressure and subsequently produced an unusual genotype by which the bacteria can remain viable. Sequencing of this clone would confirm the genotype of this mutant.

Isolation of this clone also underlines the importance of fully confirming mutant genotypes and the phenotypes resulting from this manipulation, are due specifically to deletion of the gene under investigation. Complementation should be undertaken to ensure any deletion mutant phenotypes observed are due to deletion of the specific gene and not polar effects as a result of the mutation, even when, as in this case, the mutants constructed are intended to be in-frame and un-marked. For this clone, to conclusively determine the genotype of this apparent *B. pseudomallei ΔftsZ* clone, full sequencing of the clone could be undertaken.

Conservation of the genes *ftsA* and *ftsZ* in *B. pseudomallei,* as well as highly conserved primary protein sequence with other Gram negative bacteria, including identified key functionally important residues, and probable essentiality of *ftsA* and *ftsZ* in *B. pseudomallei,* indicate the gene products of *ftsA* and *ftsZ* are potential broad spectrum antimicrobial drug targets. Several studies have identified molecules which inhibit FtsZ function both *in vitro* and *in vivo* (Margalit *et al.,* 2004; Lappchen *et al.,* 2005; Stokes *et al.,* 2005, Beuria *et al.,* 2005; Domadia *et al.,* 2007; Czaplewski *et al*., 2009). The most successful of these studies demonstrated use of a small synthetic molecule PC190723. This molecule directly inhibits GTPase activity of FtsZ. Importantly PC190723 does not affect host tubulin polymerisation. This inhibitor was shown to protect mice in a murine model of staphylococcal septicaemia in a dose dependent manner and is a promising novel antimicrobial (Haydon *et al*., 2008). FtsA has also been implicated as a potential target for novel antimicrobial inhibitors. Inhibition of *P. aeruginosa* FtsA ATPase activity was demonstrated using peptides identified by phage display *in vitro* (Paradis-bleau *et al*., 2005).

To date, the majority of FtsA and FtsZ inhibitors investigated as potential antimicrobial agents have targeted Gram positive bacteria. Determination of *B. pseudomallei* FtsA and FtsZ enzymatic activity and testing of potential inhibitory compounds against this Gram negative target would further elucidate the validity of these targets in *B. pseudomallei*.

Construction of *B. pseudomallei ΔmraW* demonstrates *mraW* is a readily disrupted gene in this bacterium, which demonstrates this gene is not essential in this species. In addition, whilst recombination frequency to produce chloramphenicol derivatives of the *B. pseudomallei tonB* merodiploid strain was relatively high, isolation of a *tonB* deletion mutant only occurred under permissive conditions of high $FeSO_4$ concentration in the selection media. This demonstrates that when attempting to construct a mutant in the absence of any information concerning a gene's likely role, for example for hypothetical genes, a gene may appear to be essential when deletion of that gene simply leads to auxotrophy, and under more favourable conditions, that gene would not be essential. Ideally, for a novel antibiotic target, true essentiality, in which a gene is required for cell viability when under the most favourable conditions and in the absence of any stress on the cell (Koonin, 2000), is most advantageous.

Furthermore, *mraW* and *tonB* mutants constructed in this study were all isolated from clones screened following extended incubation under selective conditions, whereas all clones screened for chloramphenicol sensitivity early during the incubation period had not undergone a second crossover event. Methodologically, this information is useful in construction of mutants in *B. pseudomallei* using the pDM4 suicide vector strategy.

B. pseudomallei ΔmraW and *B. pseudomallei ΔtonB* will be characterised for their *in vitro* and *in vivo* phenotypes to determine if they play roles in disease and therefore their potential for targeting with novel antimicrobials.

Chapter 5. Characterisation of *B. pseudomallei ΔmraW*

5.1 Introduction

In most bacteria*, mraW* is a gene which resides at the 5' end of the division and cell wall (dcw) cluster and is highly conserved among bacterial species, whilst being absent from archaea (Vicente *et al*., 1998). The dcw cluster contains genes involved in cell division, including *ftsA* and *ftsZ*, as well as genes involved in peptidoglycan synthesis, known as mur genes, in those bacteria which possess a cell wall (Vicente *et al*., 1998). *mraW* was hence named considering its position in murein region a (Carrion *et al*., 1999). Until recently, whilst the majority of the genes in the dcw cluster had been shown to play a role in either cell division or peptidoglycan synthesis, gene products for *mraW* and *mraZ* had not been ascribed a function. In 1999, Carrion *et al.* demonstrated methyltransferase activity of recombinant MraW using membrane cell extract as a substrate. Further, they described *mraW* as an essential gene in *E. coli* after finding it was only possible to isolate insertional mutants of this gene when a copy of the gene in trans was also present. Conversely, Daniels *et al.* had previously described the homologous gene *yllC* in *Bacillus subtilis* to be non-essential.

More recently in 2010, Kimura and Suzuki identified MraW as a gene product involved in methylation of 16S RNA in the ribosome and therefore renamed the gene encoding this protein *rsmH*. Their experiments demonstrated that loss of methylation at two positions by two gene products: MraW and YraL, at the P-site of 16S rRNA within the ribosome, led to reduced fidelity of translation of genes. This work was undertaken in *E. coli* and involved

construction of a deletion mutant of *mraW*, indicating in contrast to Carrion *et al.* that *mraW* is not essential in *E. coli* (Carrion et al., 1999).

Targeting MraW for broad spectrum antibacterial inhibition is an attractive strategy, as it is a well conserved enzyme. Whilst this methyltransferase has not been previously investigated as an antimicrobial target, other methyltransferases have been investigated as anti-infective targets. For example, flavivirus RNA methylases have been targeted in recent times with antivirals (Podvinec *et al.,* 2010). Furthermore, chemical libraries have been screened for *in vitro* inhibition of the methyltransferase activity of *E. coli* DNA adenine methyltransferases (DAM) or *Caulobacter crescentus* cell cycle regulated methyltransferase (CcrM) (Mashhoon *et al*., 2006). This study identified small molecules which inhibited the methyltransferase activity, some of which also demonstrated antibacterial properties against *C. crescentus* (Mashhoon *et al*., 2006). Some of the inhibitors preferentially targeted the bacterial enzymes over host mammalian cytosine methyltransferase Dnmt1, demonstrating avoidance of potential host toxicity (Mashhoon *et al*., 2006).

Furthermore, targeting processes within the ribosome accounts for a large proportion of currently licensed antibiotics, and therefore the recently discovered role for MraW to methylate rRNA in the ribosome provides a different avenue through which to target ribosomal activity. However, there is currently no data to determine whether MraW is important during pathogenesis. Therefore potential for antimicrobial inhibition of MraW must be practically validated.

The validity of MraW as an antimicrobial target has not previously been studied in any bacteria. In order to investigate the role of MraW in the bacterium *B. pseudomallei*, an in-frame unmarked deletion mutant of *mraW* was constructed in Chapter 4, and characterised for growth to determine whether deletion of this gene has any effect on cell viability here. Additionally, *B. pseudomallei ΔmraW* was assessed for virulence both *in vitro* and *in vivo*. Together, these characterisations clarify whether MraW is a valid target for novel antimicrobial inhibitors.

5.2 Results

5.2.1 Complementation of *ΔmraW*

To confirm the phenotypes demonstrated by *B. pseudomallei ΔmraW* were due specifically to deletion of mraW, complementation of this strain was undertaken. First, in order to identify the promoter of *mraW* to appropriately regulate expression of *mraW* from the complementation plasmid, BPROM software was used to predict bacterial promoters in the upstream region of *mraW*. No promoter sequences were predicted in the 200 bp upstream region of the *mraW* open reading frame (ORF) (Figure 5.1). The nearest promoter for control of expression of *mraW* was identified upstream of the adjacent gene to *mraW*, *mraZ* indicating these genes may be co-transcribed. Therefore the *mraW* ORF only was amplified by PCR from *B. pseudomallei* K96243 genomic DNA using primers mraW F and mraW R and cloned into the pBHR4 plasmid under the control of the *B. pseudomallei* chaperone groES promoter which is constitutively expressed. This plasmid was conjugated into *B. pseudomallei ΔmraW,* and its presence confirmed by PCR of the kanamycin cassette encoded on the pBHR4 plasmid. Expression of *mraW* mRNA transcripts from the plasmid in *B. pseudomallei ΔmraW* was confirmed by RT PCR of RNA isolated using the RNeasy kit (Qiagen) from mid-log cultures of each strain, using primers mraW RT F and mraW RT R as described in sections 2.3.11 and 2.3.12 (Figure 5.2). Expression of *mraW* mRNA in the complemented mutant was shown to be at a higher level than in the parental strain K96243, as the band of amplified *mraW* fragment from an equivalent amount of RNA is of greater intensity for the complemented strain than the parental strain.

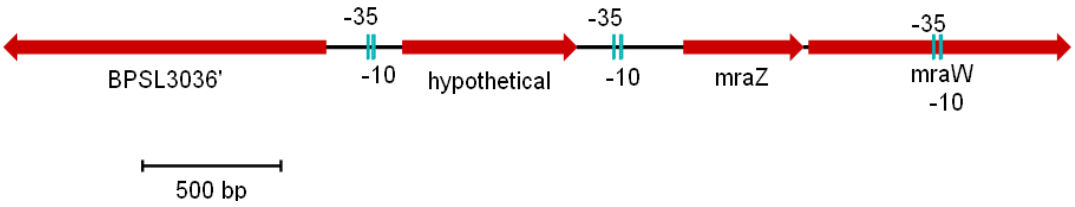

Figure 5.1 Promoters predicted by BPROM software in the upstream region from *mraW* (BPSL3033) in *B. pseudomallei* K96243. Blue bars represent predicted -35 and -10 promoter boxes.

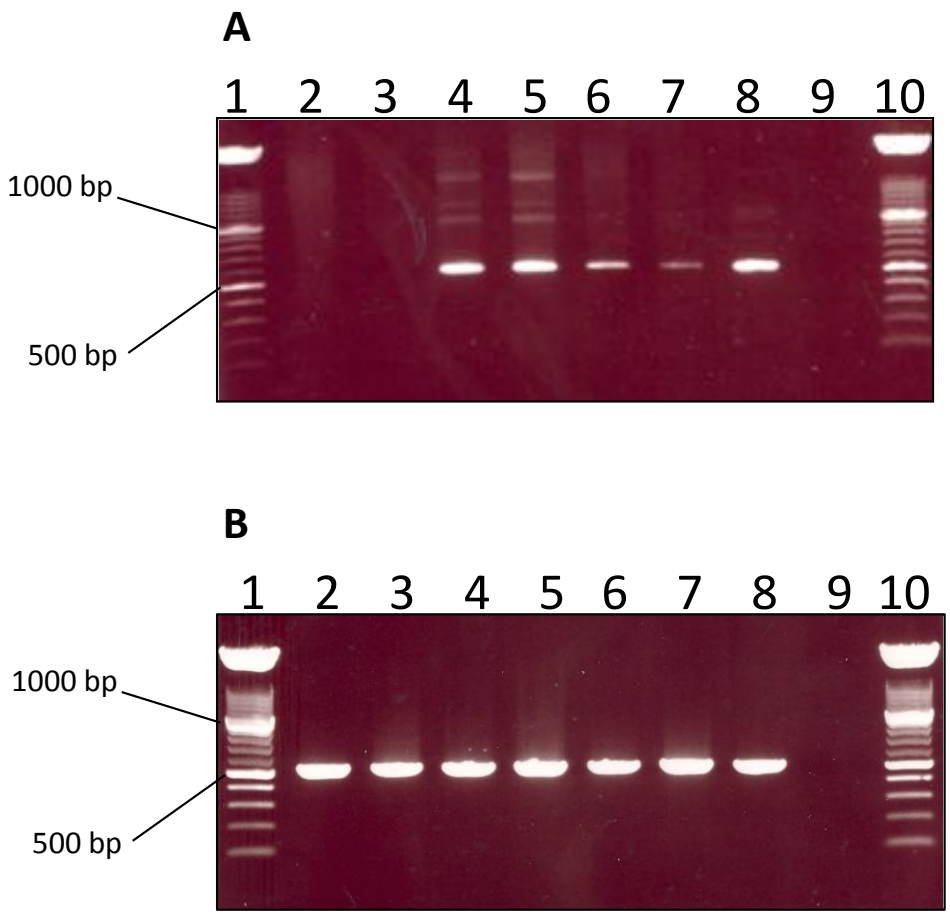

Figure 5.2 RT PCR to detect *mraW mRNA* transcripts in *B. pseudomallei* strains K96243, *ΔmraW* and *ΔmraW* + pBHR4 by RT PCR.

A - RT-PCR for 521bp region within mraW using primers mraW RT F and mraW RT R.
B – RT-PCR for *B. pseudomallei* 16S RNA using primers

Lane 1 – 100bp DNA marker;
lane 2 - *ΔmraW* sample 1*;*
lane 3 - *ΔmraW* sample 2*;*
lane 4 - *ΔmraW* + pBHR4 *mraw* sample 1*;*
lane 5 - *ΔmraW* + pBHR4 *mraw* sample 2;
lane 6 - K96243 sample 1;
lane 7 – K96243 sample 2;
lane 8 – positive control using K96243 genomic DNA;
lane 9 – negative control no DNA;
lane 10 – 100bp DNA marker.

5.3 Characterisation of *B. pseudomallei ΔmraW*

5.3.1 Growth in media

Growth of *B. pseudomallei ΔmraW* and *B. pseudomallei ΔmraW* + pBHR4 *mraW* in LB broth was measured and compared to the parental strain in order to determine if deletion of *mraW* has an effect on growth. From overnight cultures, 2 ml was inoculated into 100 ml LB broth and OD_{600} measured hourly. A small decrease in growth rate was seen by both the mutant and complemented strain. This difference was not significant ($p>0.05$) (Figure 5.3).

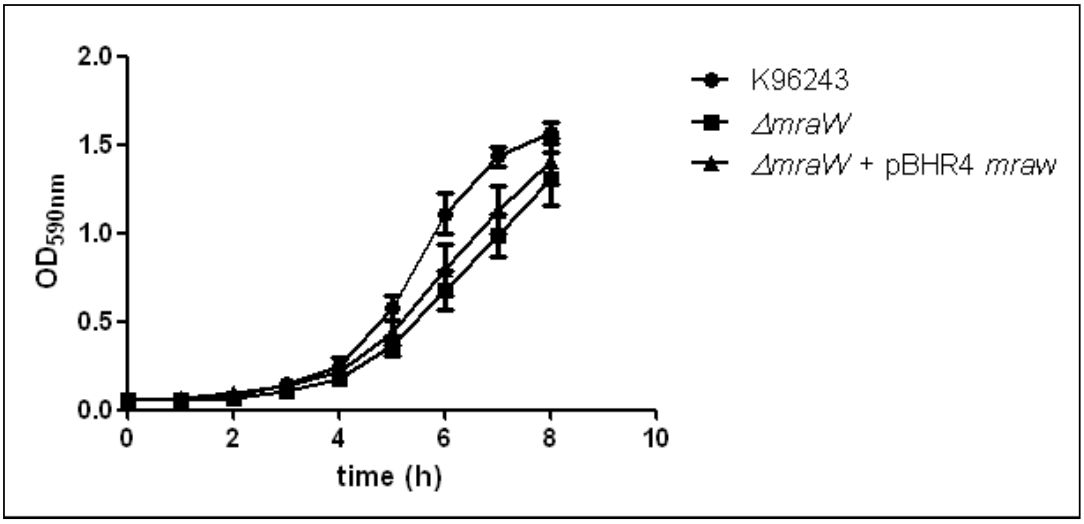

Figure 5.3 Growth of *B. pseudomallei* strains K96243, *ΔmraW* and *ΔmraW* + pBHR4 *mraw* in LB broth at 37°C over 8 h. Growth was measured hourly by optical density at 590nm. Values are the means from triplicate experiments ± standard error.

5.3.2 Measurement of swarming motility

It has recently been shown in *E. coli* that MraW plays a role in maintenance of fidelity of translation of mRNA transcripts and is therefore likely to affect a range of bacterial proteins including virulence factors (Kimura and Suzuki, 2010). Flagellin mediated motility has been shown to play an important role in virulence in a number of bacterial pathogens (Carsiotis *et al*., 1984; Gardel and Mekalanos, 1996), and aflagellate *B. pseudomallei* has been shown to be attenuated *in vivo* (Chua *et al*., 2003). Flagella are composed of a large number of flagellin monomers and lack of fidelity of translation of a proportion of these gene products might be expected to affect flagella integrity. To determine if MraW plays a role in motility, *B. pseudomallei* strains K96243, *ΔmraW*, and *ΔmraW* + pBHR4 *groES mraW* were inoculated into 3% LB agar to measure motility as section 2.5. *ΔmraW* was significantly less motile than parental strain K96243. Complementation in trans under the control of the *B. pseudomallei groES* promoter did not restore the motility defect of the deletion mutant (Figure 5.4).

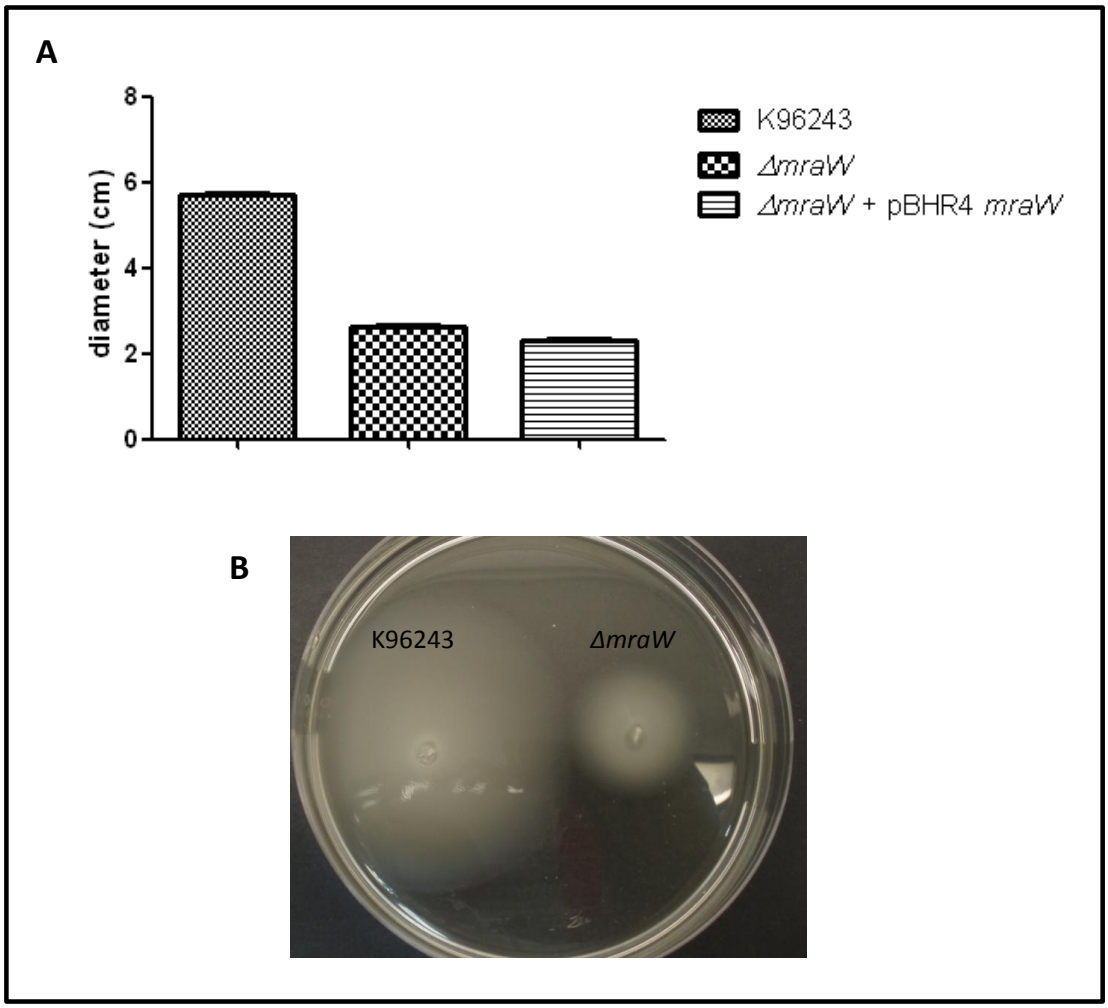

Figure 5.4 Determination of motility *of B. pseudomallei ΔmraW* and *ΔmraW* + pBHR4 *mraW* strains. Bacteria were inoculated into 3% LB agar and incubated at 37°C for 24 h. Values are the means from triplicate experiments ± standard error.

A – Measured zones of growth of *B. pseudomallei* strains K96243, *ΔmraW* and *ΔmraW* + pBHR4 *mraW*
B – Photograph of swarming motility of *B. pseudomallei* strains K96243 and *ΔmraW*

5.3.3 Electron microscopy

B. pseudomallei ΔmraW was shown to be non-motile. Therefore to determine whether *B. pseudomallei* produce flagella, this strain was fixed, stained and visualised under and electron microscope as section 2.6 (Figure 5.5). Bacteria were shown to possess flagella attached to the bacterial surface.

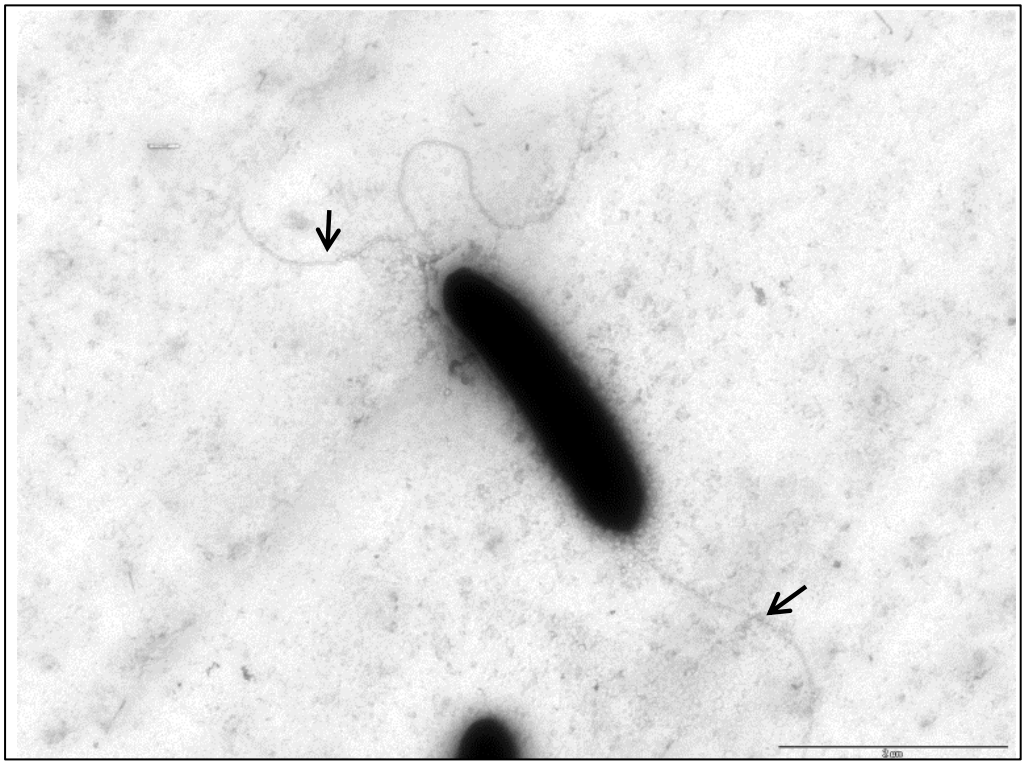

Figure 5.5 Electron microscope image of a representative *B. pseudomallei ΔmraW* bacterium in possession of flagella (arrows)

5.3.4 Growth in J774A.1 macrophages

B. pseudomallei is known to be a facultative intracellular pathogen which is able to invade and replicate within many different cell types (Jones, 1996). To determine if *mraW* plays a role during invasion of, or replication within murine macrophages, J774A.1 macrophages were infected with *B. pseudomallei* strains K96243 or *ΔmraW* as described in section 2.8.1. Similar numbers of intracellular bacteria of each strain were enumerated immediately following extracellular killing, indicating *mraW* does not play a role in invasion of cells. However, up to 3 h post infection, *B. pseudomallei ΔmraW* had a reduced growth rate compared to the parental strain, leading to an approximately ten fold lower quantity of intracellular *ΔmraW* bacteria compared to the parental strain at 3 h post extracellular killing, although this is not statistically significant at this time point. *ΔmraW* continued to replicate at a similar rate to strain K96243 post 3 h following extracellular killing, for up to 10 h. Over the total 10 h time course, intracellular replication was significantly decreased as a result of deletion of *mraW* from *B. pseudomallei* ($p<0.005$) (Figure 5.6).

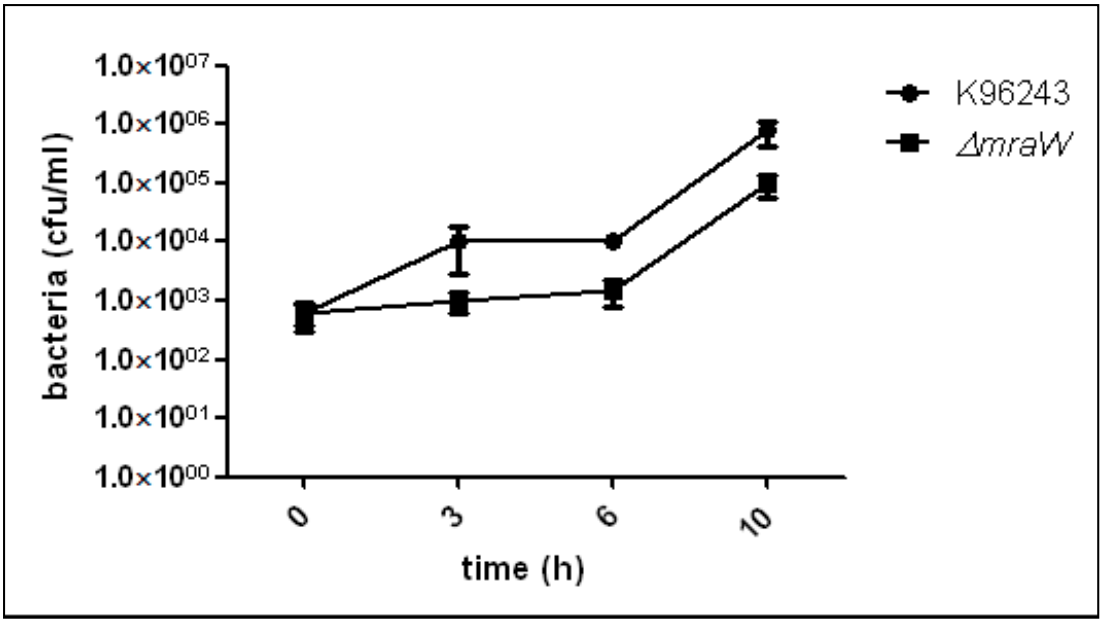

Figure 5.6 Survival of *B. pseudomallei ΔmraW* in J774A.1 macrophages over 10 h. Cells were infected with bacteria at an MOI of 1 and allowed to infect for 30 min. Extracellular bacteria were killed using 1 mg/ml kanamycin for 1 h. Intracellular bacteria were enumerated at 0, 3, 6 and 10 h post extracellular killing. Values are means of triplicate experiments ± standard error.

5.3.5 Virulence of *ΔmraW* strains *in vivo*

5.3.5.1 *G. mellonella* challenge

G. mellonella are a species of wax moth whose larvae have been used to study the pathogenesis of many bacterial pathogens, including *F. tularensis, P. aeruginosa* and *Cryptococcus neoformans* (Aperis 2007 *et al*., Miyata 2003 *et al*., Mylonakis *et al*., 2005). This model of infection has also been characterised for infection with *B. pseudomallei* and infection outcome has been shown to correlate well to that seen in mice (Wand, 2011). Approximately 100 cfu *B. pseudomallei ΔmraW*, *B. pseudomallei ΔmraW* + pBHR4 *groES mraW or B. pseudomallei* K96243 were injected into the first proleg of each *G. mellonella* and the larvae were monitored for signs of disease and scored for time to death as described in section 2.9.1. No significant difference was observed between time to death for *ΔmraW* and the parental strain K96243. *B. pseudomallei ΔmraW* carrying the intended complementation vector pBHR4 *mraW* was significantly attenuated compared to both parental strains ($p<0.0001$) (Figure 5.7).

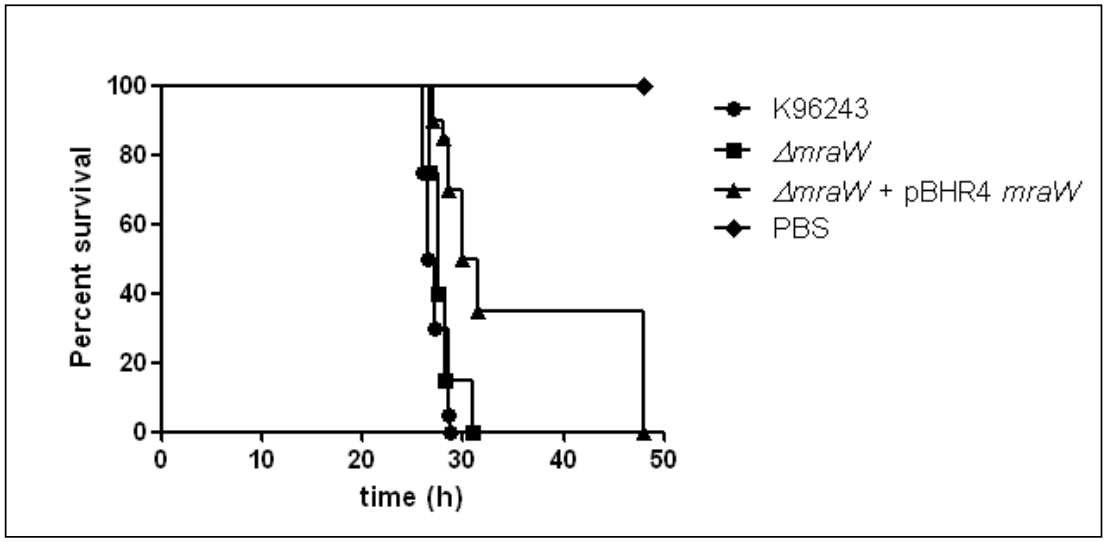

Figure 5.7 Virulence of *B. pseudomallei* strains K96243, *ΔmraW and complemented ΔmraW* in *G. mellonella.* Groups of 10 *G. mellonella* were injected with approximately 100 bacteria and monitored for signs of disease over 54 hours after which surviving larvae were culled. Percentage survival calculated from 20 *G. mellonella*, pooled from 2 biological replicates.

5.3.5.2 Mouse challenge

To determine if MraW plays a role in *B. pseudomallei* infection of a mammalian model of melioidosis, groups of six Balb/C mice were infected with 4.8 x 10^4 *B. pseudomallei* K96343 or 4.9 x 10^4 *B. pseudomallei ΔmraW* via the intra-peritoneal route as per section 2.9.2. All mice challenged with *B. pseudomallei ΔmraW* survived the 35 day course of the experiment, whereas all mice challenged with the parental strain *B. pseudomallei* K96243 had succumbed to infection by day 15. *B. pseudomallei ΔmraW* is therefore attenuated in the Balb/C mouse model of melioidosis ($p<0.001$) (Figure 5.8). Surviving mice were culled at day 35 and lungs, livers and spleens aseptically removed to enumerate bacteria in the organs. All organs were colonised with bacteria exhibiting typical *B. pseudomallei* colony morphology.

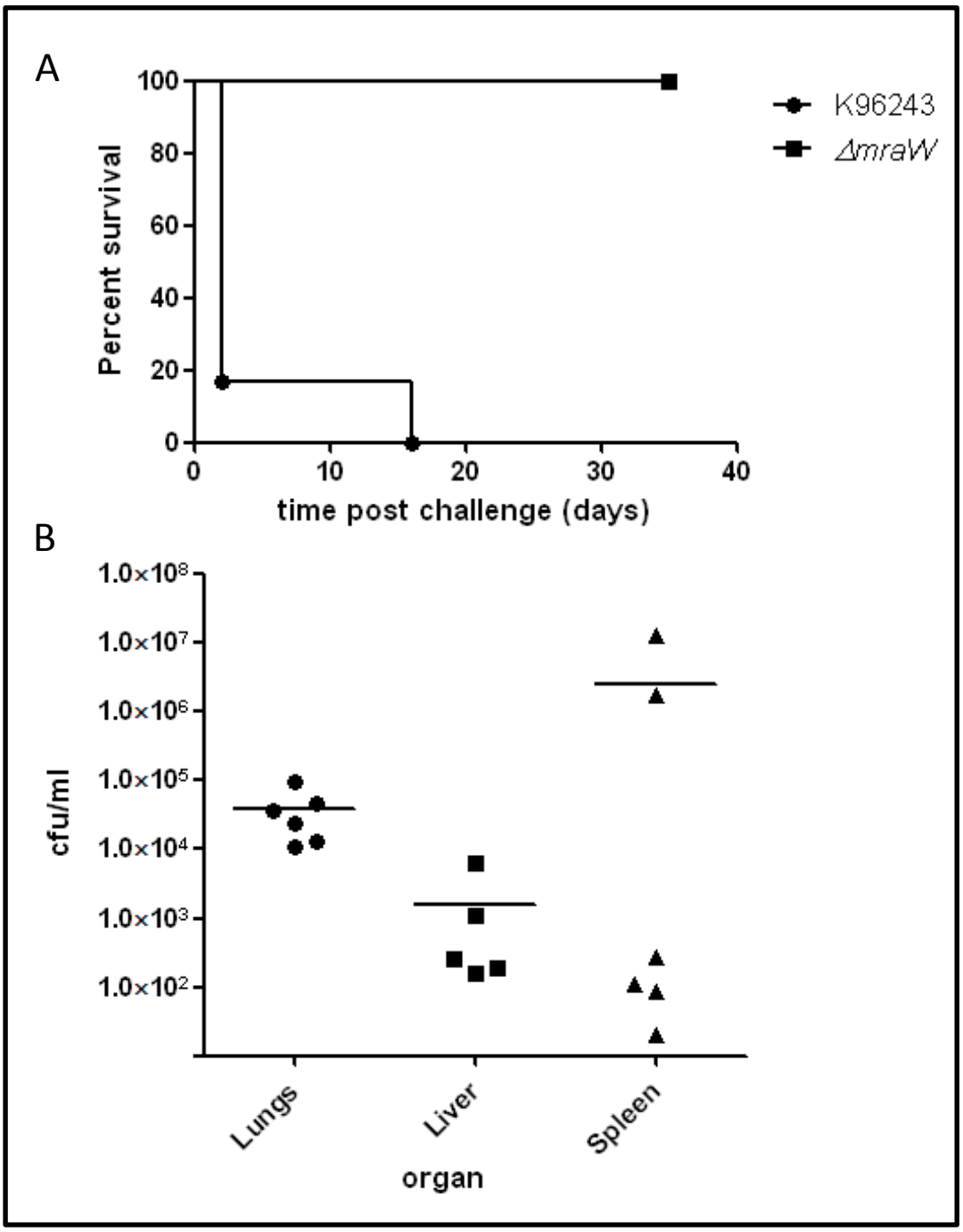

Figure 5.8 Virulence of *B. pseudomallei ΔmraW* in Balb/C mice. Groups of 6 age matched mice were challenged with 4.8 x 10^4 *B. pseudomallei* K96343 or 4.9 x 10^4 *B. pseudomallei ΔmraW*

A - Mice were monitored for survival over 35 days, after which surviving mice were culled.
B - Lungs, livers and spleens of surviving mice were homogenised and plated onto L. agar to determine bacterial load within each organ

5.4 Discussion

The gene *mraW* is well conserved gene in eubacteria, though absent in the archaea. The almost complete conservation of this gene among bacterial species, including as demonstrated here, in pathogenic *Burkholderia* species, alludes to an important role for *mraW* in the bacterial species in which it is present. The relative ease of isolation of an unmarked in-frame deletion mutant of *B. pseudomallei mraW* indicates the gene is non-essential in this species and a growth rate which is slightly reduced compared to the parental strain indicates the gene is not required for basic cellular processes. This is similar to the *E. coli mraW* mutant whose growth rate is slightly decreased on deletion of *mraW* (rsmH) (Carrion et al., 1999).

Flagella have been shown to be an important virulence determinant in the Balb/C mouse model of infection of *B. pseudomallei* (Chua *et al.,* 2003). In that study, mutation in *B. pseudomallei* of the gene encoding the repetitive subunit of flagella, *fliC,* had no effect during infection of human lung epithelial cells as well as infection of the invertebrate model *C. elegans* (Chua *et al*., 2003). These phenotypes are similar to those seen on deletion of *mraW* from *B. pseudomallei*. Firstly, motility of *ΔmraW* in low density agar was significantly reduced compared to the parental strain, indicating construction or function of flagella has been affected by deletion of *mraW.* Secondly, no significant difference was seen in invasion of J774A.1 macrophages between the parental strain and *ΔmraW*. For both A549 epithelial cell infection by *ΔfliC* by Chua *et al*. and J774A.1 infection by *ΔmraW* in this study*,* gravity was presumed to ensure contact between bacteria and the macrophage cell monolayer in these culture models (Chua *et al*., 2003). Further, in the

invertebrate model in this study, *B. pseudomallei ΔmraW* were injected directly into *G. mellonella* haemolymph ensuring bacterial access to all of the insects' tissues. Lastly, a complete attenuation of virulence in Balb/C mice was apparent for *B. pseudomallei ΔmraW* in a similar manner to *ΔfliC. B. pseudomallei ΔmraW* were injected into the mouse peritoneal cavity in both studies, and Chua *et al.* suggest that flagella are required for uptake of *B. pseudomallei* by monocytes and polymorphonuclear cells in this cavity and therefore spread of the bacterium (Chua *et al*., 2003). Additional work infecting other types of cells, such as primary cells from in the peritoneal cavity, as well as epithelial cell lines would contribute further information to understand the role played by *mraW* during infection of mammals.

Flagella are composed of approximately 20,000 copies of FliC (Yonekura *et al.,* 2002), therefore when considering an assumed role for *mraW*/*rsmH* in rRNA methylation and maintenance of fidelity of translation of genes, deletion of *mraW/rsmH,* might be expected to lead to ineffective construction of the flagella or motor, and therefore a *ΔfliC* like phenotype. Electron microscopy of mid-log *B. pseudomallei ΔmraW* demonstrated this strain does possess flagella. Therefore the analogous phenotypes between *ΔfliC* and *ΔmraW* could be ascribed to non-functional flagella, as a result of deletion of *mraW*.

Effective complementation of the deletion of *mraW* is required to demonstrate that the phenotypes observed here are not due to polar effects as a result of the mutation, despite deletion of *mraW* being in-frame and unmarked. Downstream polar effects due to the deletion of *mraW* may be expected to be

more accentuated when considering the positioning of this gene at the 5' end of the dcw cluster, which contains genes involved in important cellular processes including cell division and cell wall synthesis. Pertinently, *ftsA* and *ftsZ* lie at the distal 3' end of this cluster, shown earlier in this study to probably be essential in *B. pseudomallei.* Disruption of translation of these genes would be expected to have a significant effect on cell viability, which is not observed following deletion of *mraW*.

Although the attempted complementation of *mraW* did not restore any of the phenotypes observed to that of the parental strain, this may be due to the method of complementation rather than polar effects of the mutation. Complementation under the control of the *groES* promoter leads to constitutive expression of *mraW* to higher levels than in the wildtype strain, as demonstrated by RT-PCR. This may result in the accentuated phenotypes seen in the mutant, such as reduced motility. Further, *ΔmraW* + pBHR4 *mraW* was attenuated in the *G. mellonella* model of infection, when *ΔmraW* was not. Further attenuation on over-expression of *mraW* indicates regulation of this gene is important for its function and this must therefore be considered when approaching an alternative method of complementation. Use of the native promoter, likely the promoter identified upstream of *mraZ*, as well as ensuring an appropriate copy number of the gene, for example by *in cis* complementation would ensure appropriate expression levels of *mraW*. Additionally, deleterious effects due to over-expression of this methyltransferase may suggest that MraW is a more promiscuous methylator than specifically of the P-site of 16S rRNA alone, since this single site cannot

be ‘over-methylated’. Further work to confirm MraW plays an equivalent role in methylation of rRNA in *B. pseudomallei* as demonstrated for *E. coli,* as well as to determine other roles for this protein, is warranted.

To conclude, *mraW* was considered an important gene which could be targeted for inhibition by novel small molecules due to its conservation among bacterial species and its assumed role in maintenance of fidelity of translation as shown in *E. coli*. This study has demonstrated that *mraW* is not essential in *B. pseudomallei* and its deletion has no gross deleterious effect on the bacterium. Further in the absence of *mraW*, *B. pseudomallei* could still invade and replicate within murine macrophages, and infect *G. mellonella.* Whilst a complete attenuation of infection in Balb/C mice was demonstrated, this is thought to be due to production of non-functional flagella, or flagellar motors in the absence of MraW and therefore direct targeting of flagellin may be a more appropriate antibiotic strategy. Additionally, accentuated phenotypes on over-expression of *mraW* in *B. pseudomallei* suggests further roles for this gene product than only methylation of a specific position in 16S rRNA as demonstrated for *E. coli*.

Chapter 6. Characterisation of *B. pseudomallei ΔtonB*

6.1 Introduction

The bioavailability of iron is limited by its relative insolubility at neutral pH and in oxygenated environments. Consequently bacteria have evolved systems for high affinity uptake of both ferrous and ferric iron for acquisition of iron from their environment. TonB is a protein encoded by the majority of Gram negative bacteria, which plays a key role in an elaborate system for scavenging of iron (Krewulak and Vogel, 2011). Gram negative bacteria synthesize and secrete small molecules known as siderophores, which bind iron with high affinity. TonB subsequently functions to transduce energy gathered from the pmf at the cell membrane, across the periplasm, to power energy dependent uptake of siderophore complexed iron across the outer membrane (Miethke and Marahiel, 2007). Some TonB dependent transporters also transport other molecules which require active uptake including vitamin B12 and copper (Schauer *et al*., 2008). Additionally, TonB is increasingly being recognised as playing a role in sensing of extracellular iron concentration by bacteria and transducing the signal via sigma/anti sigma factor signalling cascade to influence gene expression in response to iron (Buchanan, 2005).

The TonB dependent uptake of substrates has been shown to play a particularly important role in pathogenic Gram negative bacteria, since part of the human innate immune response is to sequester iron in a form unavailable to invading pathogens in response to infection (Wang and Cherayil, 2009).

TonB dependent iron uptake systems have been shown to be particularly pertinent in obtaining iron from the host during infection and have been shown to be important for virulence in many pathogens, including uropathogenic *E. coli*, *Shigella dysenteriae* and *Bordetella pertussis* (Nicholson and Beall, 1999; Reeves *et al*., 2000; Torres *et al*., 2001). Direct inhibition of iron uptake by pathogens by blocking TBDTs using monoclonal antibodies has been shown to be bactericidal in *Acinetobacter baumannii* (Goel and Kapil, 2001) whilst TonB function has been inhibited using TonB box mimicking peptides in *E. coli* (Tuckman and Osburne 1991), indicating inhibition of this system is viable.

Whilst several studies have investigated the siderophore synthesis, transport and uptake systems in *B. pseudomallei* (Yang *et al*., 1991; Yang *et al*., 1993; Alice *et al*., 2006) the role played by iron uptake during infection has not previously been described. To determine whether iron uptake and specifically TonB is a viable target for novel antimicrobials in *B. pseudomallei,* a bioinformatic study characterising both the ferrous and ferric iron uptake systems encoded by *B. pseudomallei* was first undertaken. Additionally, the role of TonB dependent ferric iron uptake in *B. pseudomallei* growth and *in vitro* and *in vivo* models of melioidosis was determined by construction of a *B. pseudomallei ΔtonB* mutant.

6.2 Results

6.2.1 Identification of iron uptake systems encoded by *B. pseudomallei*

The iron uptake systems encoded by *B. pseudomallei* were investigated using bioinformatic methods. In *B. pseudomallei* K96243 BPSS0368 is annotated as a 'putative TonB-like transport protein'. Four TonB family domains are assigned to BPSS0368 on NCBI. The closest amino acid identity of BPSS0368 to other bacterial TonB sequences is that of *R. solanacearum* at 38%, as shown in Chapter 4 (Table 4.4). Two proteins, ExbB and ExbD, form a complex with TonB (Figure 6.1). This complex harnesses energy from the pmf at the cytoplasmic membrane, transduces it across the periplasm and interacts with TonB dependent transporters at the cytoplasmic membrane. This interaction energizes TonB dependent transporters (TBDTs) at the OM to actively take up substrates which require active uptake (Krewulak and Vogel, 2011). In *B. pseudomallei exbB* and *exbD* homologs lie downstream of BPSS0368 (Figure 6.1). BPSS0367 has 14 domains assigned to it by NCBI which all fall into a MotA_ExbB superfamily. BPSS0366 has 7 conserved domains assigned to it by NCBI which all fall in the ExbD superfamily. Upstream of BPSS0368, BPSS0369 contains conserved domains for Bacterioferritin-associated ferredoxin (BFD). A search for promoters in this region using BPROM software identifies a promoter upstream of BPSS0369 which has an associated Fur box. A second promoter lies 3' of BPSS0368 and upstream BPSS0367. A final promoter lies within the BPSS0368 locus. This suggests *tonB* may be transcribed under Fur regulation. Together, these genes form a cluster likely to encode the TonB complex in *B. pseudomallei* K96243. Therefore BPSS0368 was assumed to encode *tonB* and

mutagenised in Chapter 4 in order to confirm and investigate the role of *tonB* in *B. pseudomallei.*

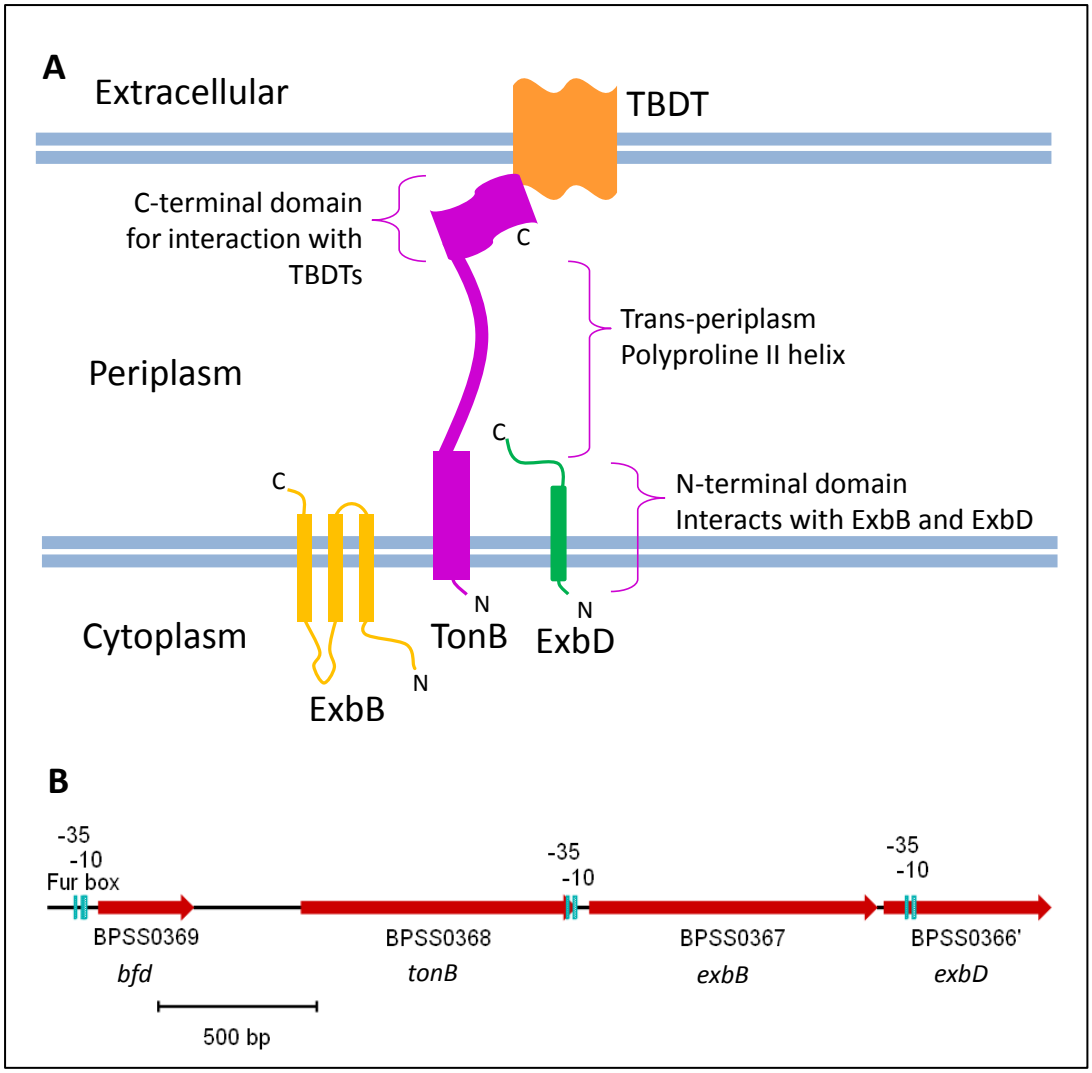

Figure 6.1 The TonB complex.

A - Structural overview and interactions. ExbB (yellow) (Kampfenkel and Braun, 1993) and ExbD (green) (Kampfenkel and Braun, 1992) both interact with TonB (pink), to harness pmf at the cytoplasmic membrane. TonB transduces the gathered pmf across the periplasm to energize transport of substrates through TBDTs (orange) at the outer membrane.
B – Genes encoding the TonB complex in *B. pseudomallei* K96243. Genes are indicated in red. Promoters identified using BPROM software are highlighted in blue.

6.2.1.1 TBDTs in *B. pseudomallei*

TBDTs have a remarkably similar overall architecture, despite varying degrees of sequence identity (Noinaj *et al*., 2010). These transporters are formed of a 22 stranded β-barrel with a periplasmic plug domain. Ten TBDTs were identified in the *B. pseudomallei* K96243 genome by searching for annotated genes which contained the conserved TBDT Pfam domains for the 22 stranded β barrel domain (PF00593), and tonB plug-domain (PF07715) (Figure 6.1). Two of these transporters, BPSL1775 and BPSS0591 are previously described to lie in siderophore synthesis and transport gene clusters for malleobactin and pyochelin respectively (Alice *et al.,* 2006). Another of the TBDTs, BPSS1029, was shown to contain an N-terminal extension domain associated with TonB transducers (Koebnik *et al*., 2005). In more detail: BPSS1029 lies next to an operon containing both a *FecR* like anti-sigma factor and an ECF sigma factor. This arrangement of genes indicates these gene products play a role in detection of bound ferri-siderophore and the transduction of this signal across two membranes, into the cytoplasm, leading to transcriptional activation of target genes (Koebnik *et al*., 2005). Another gene encoding a predicted TBDT, BPSS1742, contains a conserved domain for TonB dependent copper uptake as characterised for two *Pseudomonas* spp (Lee *et al*., 1991; Yoneyama and Nakae, 1996). BPSS0244, BPSS1850 and BPSL2553 contain conserved domains for TonB-dependent haemoglobin/transferrin/lactoferrin receptor family proteins, which likely play a role in iron acquisition from host proteins during infection. Further, a conserved domain for vitamin B12 uptake is contained within BPSL0976. Finally BPSL2724 and BPSS1204 are described as falling within the more general model for TonB dependent siderophore transporters.

Ferrous iron predominates in anaerobic environments and many bacteria also encode systems for high affinity uptake of this species of iron. The Feo system is recognised to as one of the major routes for ferrous iron uptake in bacteria (Cartron *et al.*, 2006). However, interrogation of the *B. pseudomallei* genome by BLAST and conserved domain search reveals an absence of the Feo system in this species. In fact, this system is unidentifiable in all *Burkholderia* species except *Burkholderia glumae*, a pathogen of rice. A more recently described iron uptake system: the EfeU system was identified in *E. coli* as a homolog of the yeast iron permease Ftr1p (Grosse *et al*., 2006). Two ORFs, BPSS1999 and BPSS0359 encode homologs of Ftr1p in *B. pseudomallei* and both are found in all four fully sequenced strains.

Gene	Additional information	Identity		
		1710b	1106a	668
BPSS0244	haem receptor protein haemoglobin/ transferrin/ lactoferrin	94%	94%	95%
BPSS0591	pyochelin receptor precursor (Alice *et al*., 2006)	95%	96%	95%
BPSS1029	TonB dependent transducer (Koebnik *et al*., 2005)	87%	87%	87%
BPSS1204	siderophore uptake	92%	92%	91%
BPSS1742	copper receptor	94%	93%	93%
BPSS1850	haemoglobin/ transferrin/lactoferrin	91%	91%	91%
BPSL0976	vitamin B12 uptake	85%	85%	85%
BPSL1775	*fmtA* for malleobactin uptake (Alice *et al*., 2006)	95%	95%	95%
BPSL2553	haemoglobin/ transferrin/ lactoferrin	92%	92%	92%
BPSL2724	siderophore uptake	95%	95%	95%

Table 6.1 TonB dependent transporters encoded by *B. pseudomallei* K96243 and their identity values in *B. pseudomallei* strains 1710b, 1106a and 668.

6.2.2 Complementation of *B. pseudomallei ΔtonB*

B. pseudomallei ΔtonB was complemented in order to confirm that any phenotype observed in this strain was specifically a result of inactivation of BPSS0368 and not due to polar effects caused by the inactivation. The complementation plasmid was constructed by PCR amplification of *tonB* using primers tonB F and tonB R which was subsequently ligated into the pBHR4 plasmid following sub-cloning. This cloning strategy placed *tonB* under the control of the *groES* promoter so that it is constitutively expressed by the bacterium harbouring it. Complemented mutants were selected for using chloramphenicol but were also readily identified for their ability to grow on LB agar in the absence of additional $FeSO_4$ as required by *B. pseudomallei ΔtonB.*

6.2.3 Characterisation of *B. pseudomallei ΔtonB*

6.2.3.1 Growth in media

B. pseudomallei ΔtonB had been shown to require additional $FeSO_4$ for growth on solid LB media during the mutant selection process. In order to determine if inactivation of *tonB* also affected growth of *B. pseudomallei ΔtonB* in liquid LB media, *B. pseudomallei* K96243*, B. pseudomallei ΔtonB* and *B. pseudomallei ΔtonB* + pBHR4 *tonB* were grown in LB and growth was measured by optical density over 24 h. *B. pseudomallei ΔtonB* grew significantly slower than the parental strain and complementation fully restored growth rate to that of the parental strain. In addition, growth rate of *B. pseudomallei ΔtonB* was fully restored to that of the parental strain by addition of 40 mM $FeSO_4$ to LB media (Figure 6.2).

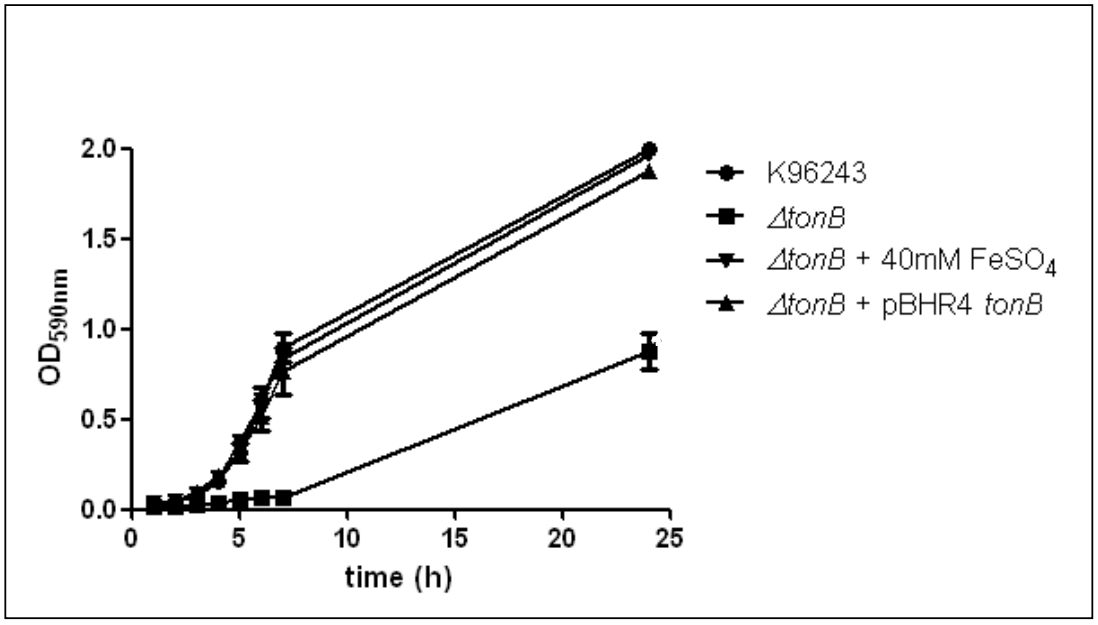

Figure 6.2 Growth of *B. pseudomallei* strains K96243, *ΔtonB* and *ΔtonB*+ pBHR4 *tonB.* Strains were grown in LB broth, or LB broth supplemented with 40 μM $FeSO_4$ at 37°C. Growth was monitored hourly by optical density at 590nm.

6.2.3.2 Virulence of *ΔtonB* strains *in vitro*

6.2.3.2.1 Growth in J774A.1 macrophages

To determine if *tonB* plays a role in invasion of or replication within murine macrophages, J774A.1 macrophages were infected with *B. pseudomallei* K96243*, B. pseudomallei ΔtonB* or *B. pseudomallei ΔtonB* + pBHR4 *tonB* at an MOI of 1 as described in section 2.8.1. Whilst complemented and parental strains were found in equal numbers intracellularly immediately following extracellular killing, a significant, eight fold more *B. pseudomallei ΔtonB* were enumerated ($p<0.01$) (Figure 5.3). Furthermore*, B. pseudomallei ΔtonB* replicates at a similar rate to complemented and parental strains over 24 hours post infection, indicating *tonB* is not required for acquisition of iron within macrophages (Figure 6.3).

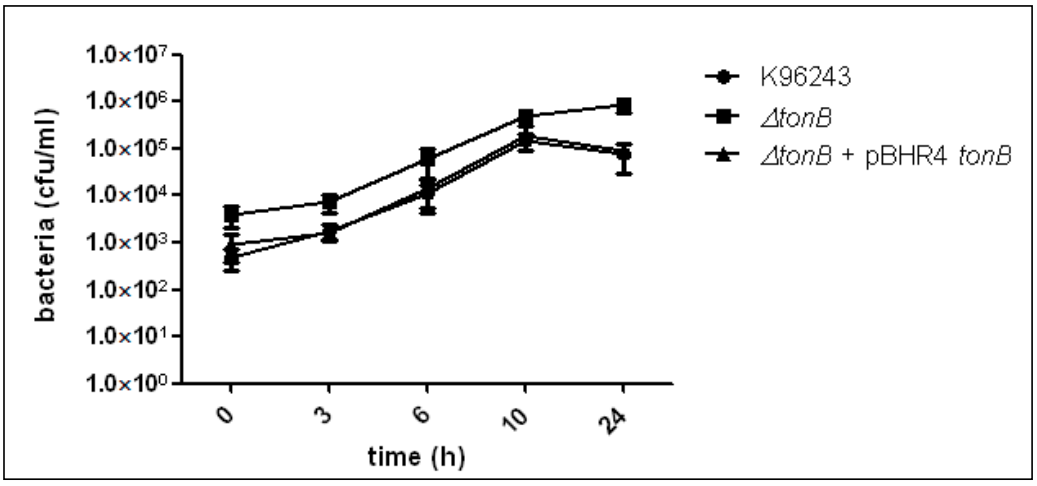

Figure 6.3 Invasion and survival of *B. pseudomallei* strains K96243, *ΔtonB* and *ΔtonB* + pBHR4 *tonB* in J774A.1 macrophages. Cells were infected with bacteria at an MOI of 1 and allowed to infect for 30 min. Extracellular bacteria were killed using 1 mg/ml kanamycin for 1 h. Intracellular bacteria were enumerated at 0, 3, 6, 10 and 24 h post extracellular killing. Values are means of triplicate experiments ± standard error.

6.2.3.2.2 Adherence to J774A.1 macrophages

To determine if the enhanced ability to invade macrophages was due to improved adhesion by *B. pseudomallei ΔtonB* to the macrophage membrane, cytochalasin-D was used to block actin mediated uptake of bacteria as described in section 2.8.2.

Bacteria were applied to macrophages at an MOI of 1 for 30 mins following the same method as for the macrophage invasion assay. After 30 mins bacteria which were not adhered to the cell surface were either removed by gentle washing of the monolayer three times with PBS (Figure 6.4 B) or media was replaced with L15 media containing 1mg/ml kanamycin (Figure 6.4 A). Infected monolayers washed with PBS allowed enumeration of bacteria which had both been internalised by the macrophages and were adhered to the macrophage surface. Separate monolayers were also pre-treated with cytochalasin-D for 30 mins, prior to infection with *B. pseudomallei* and during the 30 min infection with bacteria. Following PBS washing of these cytochalasin-D treated monolayers to remove non-adhered bacteria, adhered bacteria alone could be enumerated as uptake of bacteria into the cells had been blocked.

To confirm actin mediated uptake had been blocked by addition of cytochalasin-D, kanamycin was added to infected cell monolayers in order to kill extracellular bacteria. A similar number of each strain of bacteria, to that recovered at 0 h following extracellular killing during the macrophage invasion assay (Figure 6.3), were recovered when only kanamycin was applied to monolayers. This indicates bacteria were being internalised by the

macrophages as seen in the invasion assays. However, no bacteria were recovered when cells were treated with both cytochalasin-D and kanamycin, indicating internalisation of bacteria into the macrophages had been blocked and were therefore killed by the kanamycin present in the extracellular media.

As cytochalasin-D was required to be solubilised in DMSO, monolayers were also treated with and without DMSO in all cases as a control, to determine a cytochalasin-D only effect where cytochalasin-D in DMSO had been applied.

No significant difference was seen between numbers of *B. pseudomallei* K96243, *ΔtonB* or complemented *ΔtonB* bacteria adhered to macrophages. This indicated adhesion to the macrophage cell surface does not contribute to increased numbers of intracellular *B. pseudomallei ΔtonB* seen within macrophages immediately following extracellular killing (Figure 6.4).

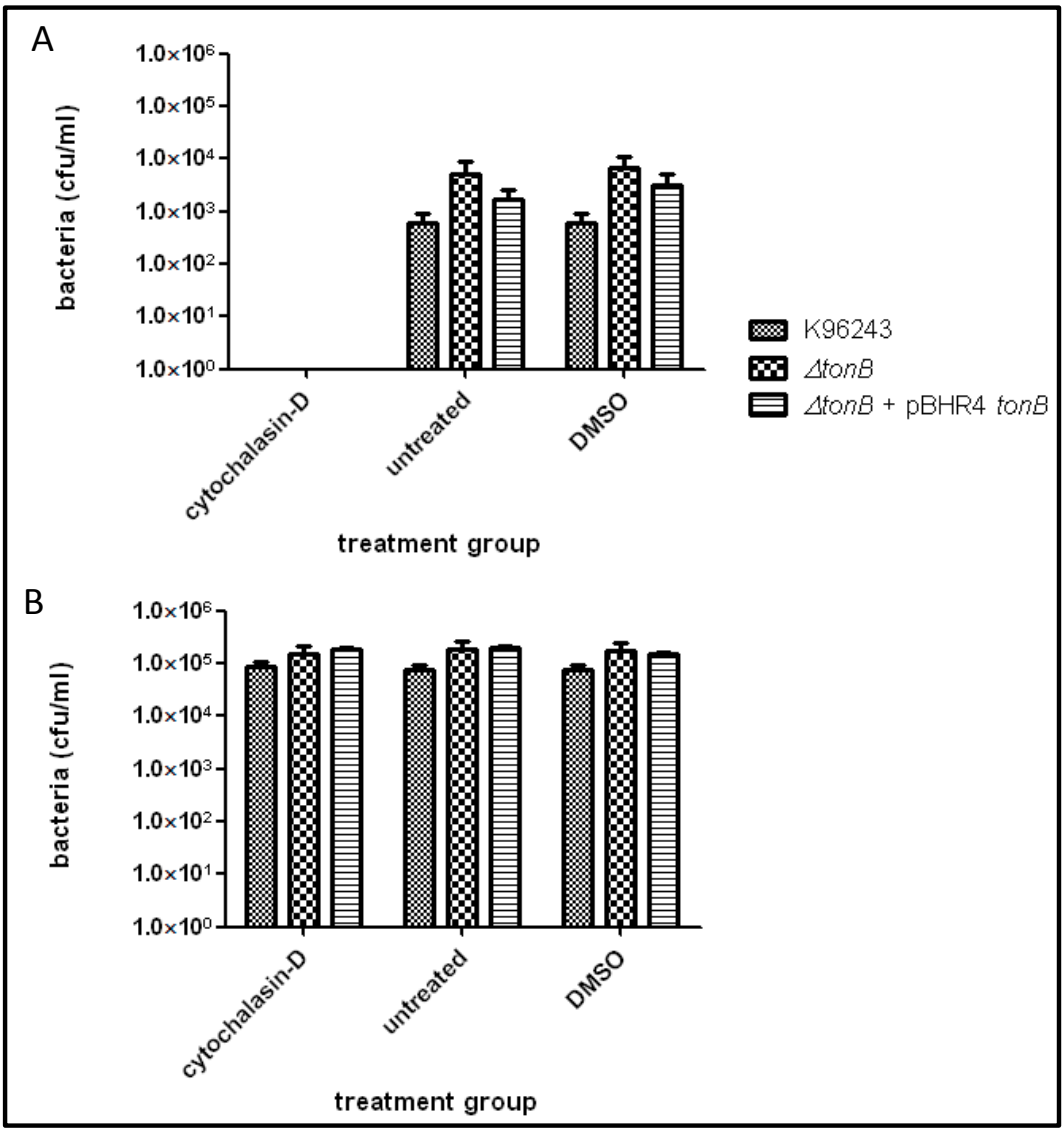

Figure 6.4 Adhesion to J774A.1 macrophages. Cells were infected at an MOI of 1 with *B. pseudomallei* strains K96243, *ΔtonB* or complemented *ΔtonB*. Cells were treated with or without cytochalasin-D to block actin based uptake and with (A) or without (B) 1mg/ml kanamycin following infection with bacteria in order to kill non-adhered extracellular bacteria.

Data of three biological replicates is presented with standard error of the means.

6.2.3.3 Virulence of *B. pseudomallei ΔtonB in vivo*

6.2.3.3.1 *G. mellonella* challenge

To determine if *tonB* is required for virulence in the wax moth larvae *G. mellonella*, groups of ten larvae were challenged with approximately 100 cfu *B. pseudomallei* K96243*, B. pseudomallei ΔtonB* or *B. pseudomallei ΔtonB* + pBHR4 *tonB* and monitored for time to death as described in section 2.9.1. The median survival time of larvae challenged with *B. pseudomallei ΔtonB* was 49.25 h, which is significantly longer than challenge with both *B. pseudomallei* K96243 at 27.38 h ($p<0.0001$) and *B. pseudomallei ΔtonB* + pBHR4 *tonB* at 45.50 h ($p<0.0001$) (Figure 6.5.A). The difference in survival between *B. pseudomallei* K96243 and *B. pseudomallei ΔtonB* + pBHR4 *tonB* was also significantly different ($p<0.0001$). All larvae administered PBS only survived. This indicates *B. pseudomallei ΔtonB* is attenuated in this model of infection, whilst introduction of pBHR4 *tonB* into *B. pseudomallei ΔtonB* partially restores the virulence phenotype.

It was subsequently hypothesised that since additional free iron restores growth of *B. pseudomallei ΔtonB* during growth in broth, injection of *G. mellonella* with iron salts, prior to bacterial challenge may restore virulence of *B. pseudomallei ΔtonB* in *G. mellonella.* This hypothesis was supported by the report of pre-injection of $FeCl_3$ rescuing virulence of an *exbD Photorhabdus luminescens* mutant during infection of the invertebrate *Manduca sexta* (Watson *et al*., 2010). Therefore four further groups *G. mellonella* were challenged with approximately 100 cfu *B. pseudomallei* K96243*, B. pseudomallei ΔtonB, B. pseudomallei ΔtonB* + pBHR4 *tonB* or PBS only, 30 minutes after injection with 10µl 5 mM $FeCl_3$. Pre-injection with $FeCl_3$

significantly accelerated time to death of *B. pseudomallei* K96243 and *B. pseudomallei ΔtonB* + pBHR4 *tonB* to 23.25 h and 22.88 h respectively ($p < 0.05$ in both cases), fully restoring virulence to that of the *B. pseudomallei* K96243 of the complemented strain. However, virulence of *B. pseudomallei ΔtonB* was not restored, with a median survival time of 46.00 h which was not significantly different to challenge without pre-injection of $FeCl_3$ (Figure 6.5.B). Whilst *B. pseudomallei ΔtonB* remained significantly more attenuated than both *B. pseudomallei* K96243 and *B. pseudomallei ΔtonB* + pBHR4 *tonB* ($p<0.0001$ and $p<0.001$ respectively), there was no significant difference between *B. pseudomallei* K96243 and *B. pseudomallei ΔtonB* + pBHR4 *tonB* survival.

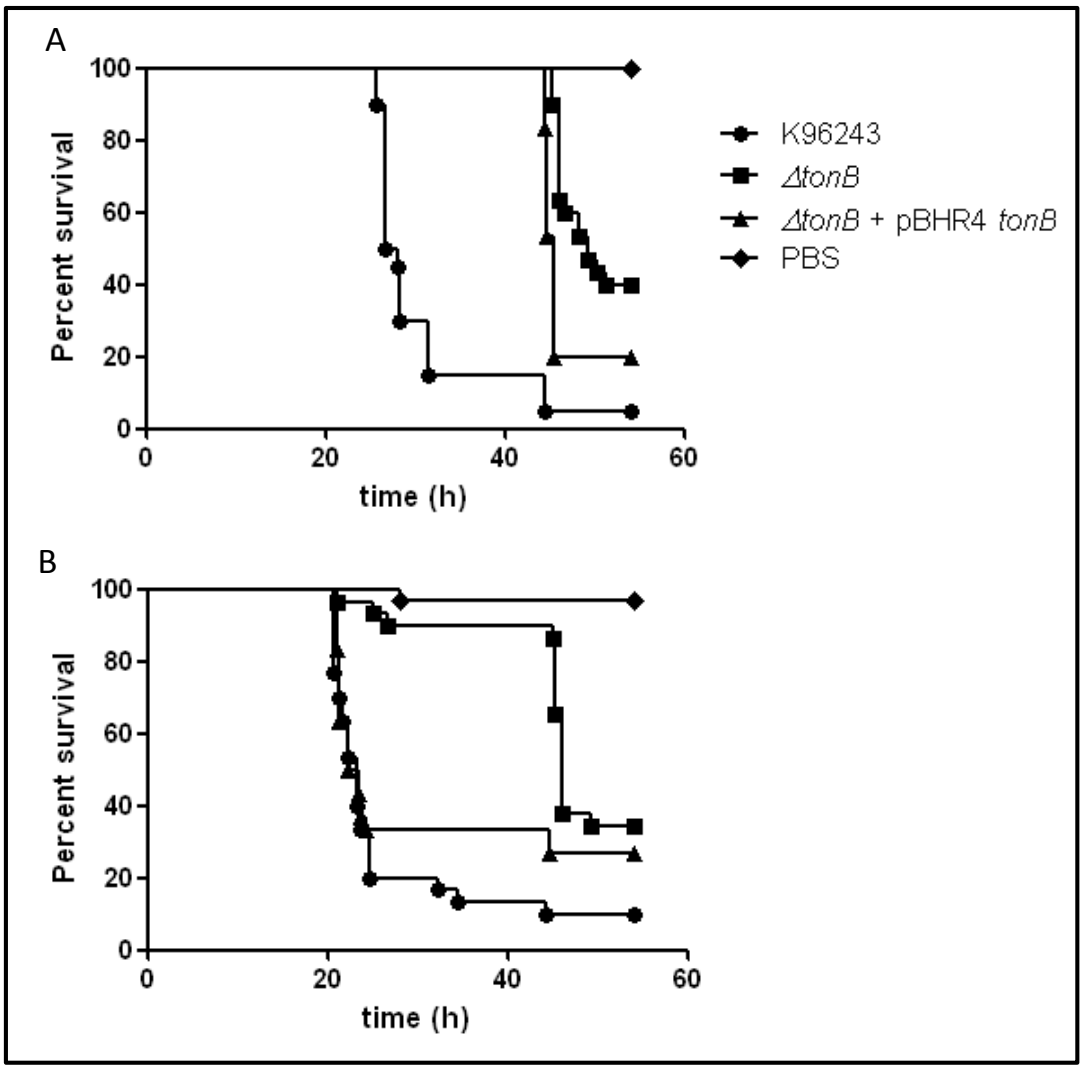

Figure 6.5 Virulence of *B. pseudomallei* strains K96243, *ΔtonB* and complemented *ΔtonB* in *G. mellonella* larvae. Groups of 10 larvae were injected with approximately 100 cfu bacteria or PBS and monitored for 54 hours after which surviving larvae were culled.

A – Bacterial challenge only
B – *G. mellonella* injected with 10 µl 5 mM $FeCl_3$ 30 minutes prior to bacterial challenge

6.2.3.3.2 Mouse challenge

To determine if *tonB* is required by *B. pseudomallei* to cause disease in mice, mice were challenged via the intra-peritoneal route with 1.50 x 10^4 *B. pseudomallei* K96243 or 1.01 x 10^4 *B. pseudomallei ΔtonB* and monitored as described in section 2.9.2. All mice challenged with *B. pseudomallei ΔtonB* survived the 35 day course of the experiment, whilst only one mouse of six challenged with the parental strain survived (Figure 6.6.A). Deletion of *tonB* from *B. pseudomallei* is therefore demonstrated to be significantly attenuating for infection of Balb/C mice ($p<0.005$). *B. pseudomallei ΔtonB* was recovered from homogenised lungs, liver and spleen of all surviving animals 35 days post infection (Figure 6.6.B), indicating *tonB* is not required for survival within the mouse.

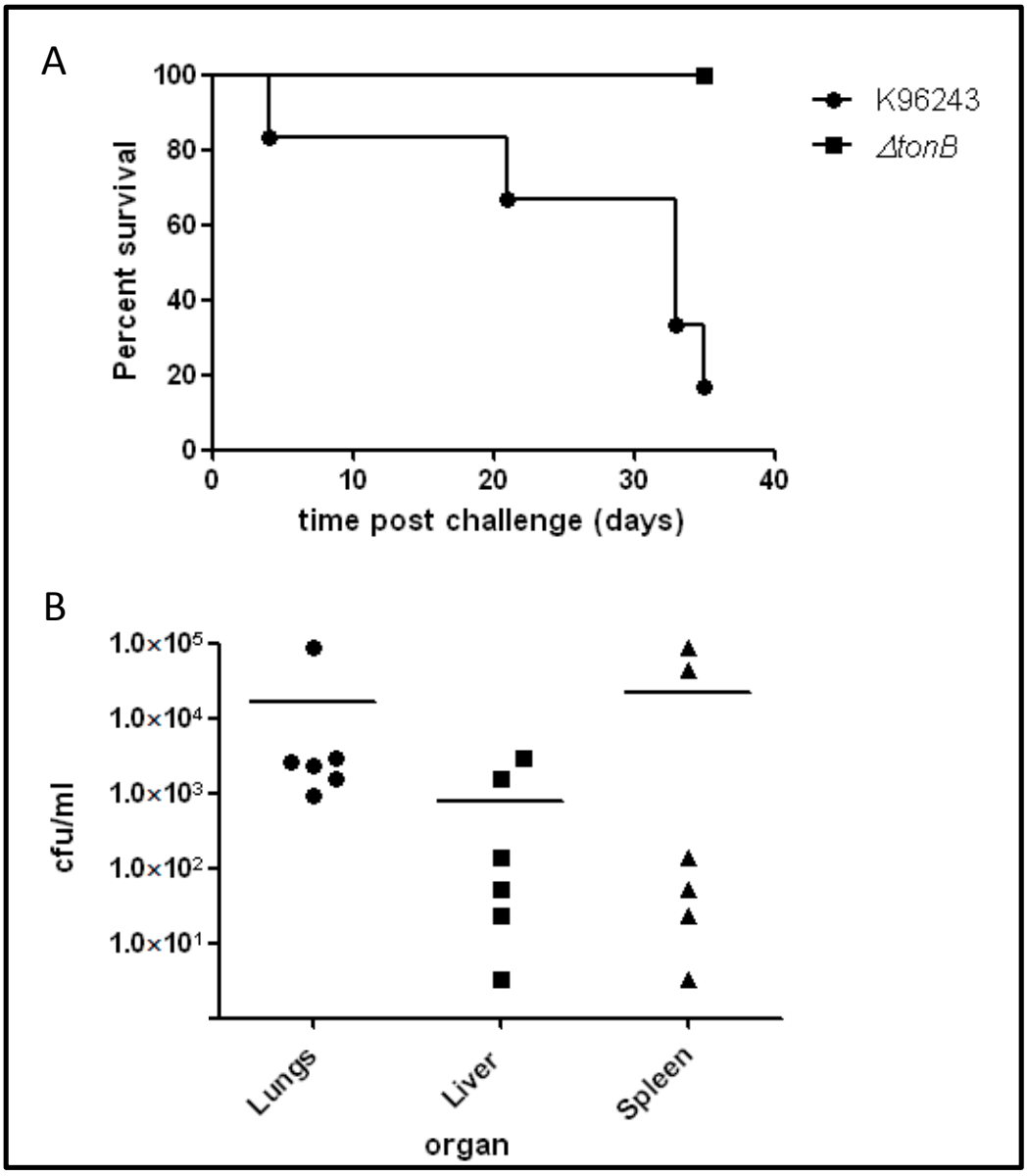

Figure 6.6 Virulence of *B. pseudomallei* K96253 and *B. pseudomallei ΔtonB* in Balb/C mice. Groups of six Balb/C mice were challenged with 1.50 x 10^4 *B. pseudomallei* K96243 or 1.01 x 10^4 *B. pseudomallei ΔtonB.*

A - Mice were monitored for survival over 35 days, after which surviving mice were culled.
B - Lungs, livers and spleens of surviving mice were homogenised and plated onto L. agar to determine bacterial load within each organ.

6.3 Discussion

TonB dependent high affinity ferric iron uptake is a well described mechanism to obtain iron from environments where this element is limited. Here it has been demonstrated that deletion of *tonB* from *B. pseudomallei* K96243 reduces the bacteria's ability to grow *in vitro*. Restoration of growth rate on addition of $FeSO_4$ to media demonstrated the growth defect is due specifically to the increased demand for free iron in the absence of *tonB*.

Ferric iron uptake powered by TonB has been shown to play a role in virulence in many Gram negative bacterial pathogens as summarised in Table 6.1. In these bacteria, deletion of *tonB* leads to reduced colonisation and virulence in the appropriate *in vivo* models of infection. The current study demonstrated that *tonB* is also required for virulence of *B. pseudomallei* in both Balb/C mice and *G. mellonella.* However, data regarding both of these models suggest iron uptake is not the sole reason for attenuation in these models. First, virulence of *B. pseudomallei ΔtonB* in *G. mellonella* was not restored by co-injection of high concentrations of free iron, suggesting iron availability is not the reason for attenuation in this case. Acceleration of time to death of *G. mellonella* larvae which have been pre-injected with ferric chloride and subsequently challenged with *B. pseudomallei* K96243 and complemented *B. pseudomallei ΔtonB* does indicate that iron restriction by the larvae plays a role in limiting infection by *B. pseudomallei* K96243 in this model. However it does not necessarily indicate that iron restriction is the cause of attenuation of *B. pseudomallei ΔtonB.* Further, whilst inactivation of *tonB* attenuates virulence mice infected with this strain, the presence of this strain in multiple organs within the Balb/C mouse host 35 days following

Species	Inactivation	Phenotype	Virulence	Reference
A. pleuropneumoniae	*ΔtonB1* transposon	Unable to utilise transferrin		Beddek *et al*., 2004
	ΔtonB2 transposon	Reduced growth in low iron Unable to utilise transferrin, haemoglobin or hemin	Attenuated in acute porcine infection	Beddek *et al*., 2004
Bordetella bronchiseptica	*ΔtonB* insertional inactivation	Albomycin resistant Unable to utilise alcaligin, enterobactin, ferrichrome, desferoxamine B or haemin for growth		Nicholson and Beall, 1999
Bordetella pertussis	*ΔtonB* insertional inactivation	Albomycin resistant Unable to utilise alcaligin, enterobactin, ferrichrome, desferoxamine B or haemin for growth		Nicholson and Beall, 1999
	ΔtonB deletion	Reduced growth in low iron Albomycin resistant Unable to utilise ferrichrome, desferoxamine B or haemin for growth	Attenuated in murine respiratory colonisation	Pradel *et al*., 2000
Avian pathogenic *E. coli*	Partial *ΔtonB* deletion	Streptonigrin resistant, increased tolerance to H_2O_2	Attenuated in chickens	Holden *et al*., 2012
Uropathogenic *E. coli*	Presumed *ΔtonB* isolated using pirazmonam	Reduced growth in low iron	Attenuated murine UTI infection	Torres *et al*., 2001
Flavobacterium psychrophilum	*exbD transposon*	Reduced growth in low iron	Attenuated in rainbow trout fry	Alvarez *et al*., 2008
Klebsiella pneumoniae	*ΔtonB* deletion	Reduced growth in low iron	Attenuated in mice by i.p. route	Hsieh *et al*., 2007
Photorhabdus luminescens	*ΔexbD* deletion	Reduced growth in low iron	Attenuated in *G. mellonella* and *M. sexta*	Watson *et al*., 2010
Photorhabdus termperata	*ΔexbD* transposon	Reduced growth in low iron Increased siderophore production	Attenuated in *G. mellonella*	Watson *et al*., 2005
P. aeruginosa	*ΔtonB1* deletion	Unable to utilise pyoverdine, pyochelin,	Attenuated in mice via i.m route	Takase *et al*., 2000

		haemoglobin or hemin for growth Hyper-susceptible to a range of antibiotics		
S. enterica serovar Typhi	*ΔexbD* transposon	Reduced growth in low iron	Reduced growth in Mono Mac 6 monocytes Attenuated in mice via the i.p. route	Furman *et al*., 1994; Gorbacheva *et al*., 2001
S. typhimurium	*ΔtonB* insertional inactivation	Reduced growth in low iron Unable to utilise ferrioxamine for growth	Attenuated in mice by i.g. route	Tsolis *et al*., 1996
S. dysenteriae	Presumed *ΔtonB* isolated using pirazmonam	Unable to utilise hemin, enterobactin or ferrichrome	No replication in Henle cells	Reeves *et al*., 2000
V. cholerae	*ΔtonB1*	Unable to utilise haemin at increased NaCl concentrations	Attenuated in suckling mouse	Seliger *et al*., 2001
	ΔtonB2		Attenuated in suckling mouse	Seliger *et al*., 2001
Yersinia pestis	*ΔtonB* insertional inactivation	Unable to utilise hemin, haemoglobin, myoglobin or hemopexin for growth		Perry *et al*., 2003

Table 6.2 Bacterial *ΔtonB* mutants and their phenotypes

infection, suggests that high affinity iron uptake utilising TonB is not absolutely required for survival and replication within the mammalian host.

Paradoxically to the attenuation of virulence by *B. pseudomallei ΔtonB in vivo*, and in contrast to previously reported infection of cells *in vitro* with other *tonB* mutants (Gorbacheva *et al*., 2001; Reeves *et al*., 2000), *B. pseudomallei ΔtonB* invaded macrophages more efficiently when compared to the parental strain. The increased efficiency of *B. pseudomallei ΔtonB* was shown not to be a result of increased adherence to the macrophage surface. Therefore some other, likely bacterial surface located and possibly iron regulated factor, may allow more efficient invasion of macrophages by this mutant. This strain also replicated at a similar rate to the parental strain over 24 hours. Replication of this mutant within the highly iron limited macrophage suggests an alternative mode of iron uptake plays a role during the intracellular portion of this pathogens lifestyle.

Whilst TonB is described as the primary mechanism for high affinity uptake of ferric iron by Gram negative bacteria, there are bacterial species which do not encode an identifiable *tonB* gene. For example the *Francisella spp.*, do require the pmf for uptake of the siderophore rhizoferritin which they produce, however they do not encode a recognisable *tonB* gene (Crosa *et al.*, 2009). Additionally, *Legionella pneumophila* has been shown to encode a peptide transporter which plays a role in iron uptake and virulence in

both macrophages and guinea pigs (Pope *et al*., 1996; Viswanathan *et al*., 2000), a homolog of which is encoded by BPSS2143 in *B. pseudomallei* K96243. Furthermore, a transporter of the siderophore legiobactin in *Legionella pneumophila* was recently shown to promote utilisation of this siderophore independently of TonB (Chatfield *et al.*, 2011), although no homolog of this transporter was found in any of the *B. pseudomallei* strains. These reports demonstrate the possibility for previously unidentified alternative high affinity ferric iron uptake systems in Gram negative bacteria, and therefore also in *B. pseudomallei.* The ability to survive within the iron limited macrophage, even in the absence of *tonB*, may shed light on the ability of this bacteria to evade the immune system and remain undetected for extended periods within macrophages, causing latent infection a considerable time after initial exposure to the bacterium.

The iron salt supplemented media required to culture this mutant must be noted when examining the results presented here, since excess iron in the experimental system may allow for replication in the absence of *de novo* uptake of iron. However, the amount of iron supplemented to media for growth of *B. pseudomallei* prior to each assay was determined as the minimum concentration of iron required to match growth of the parental strain. The growth of *B. pseudomallei* K96243 remained unaltered despite iron salt supplementation of media.

Gene *tonB* has been shown to be important for virulence of *B. pseudomallei in vivo* and that *in vitro* the growth defect on inactivation of *tonB* can be restored

formation and quorum sensing by *P. aeruginosa* (Ochsner *et al*., 1995), hemolysin production in *Vibrio cholerae (*Stoebner and Payne, 1988), outer membrane protein expression by *Neisseria gonorrhoeae (*Sebastian *et al.* 2002) and Shiga toxins and hemolysin production in *Neisseria meningitidis* (Grifantini *et al.* 2003). In *B. pseudomallei* a *fur* inactivated mutant has been constructed (Loprasert *et al*. 2000). This mutant was shown to have reduced SOD and peroxidise activity.

Secondly, a more recently discovered role for a subset of the TBDTs is as a transducer of extracellular signals across the both OM and CM to induce transcription of genes. This system was first described in *E. coli* K12 and was shown to signal through binding of ferric citrate to FecA, a TBDT which has an N terminal extension (Buchanan, 2005). This extension interacts with a cytoplasmic membrane bound anti sigma factor known as FecR, (Buchanan, 2005). In turn FecR interacts with an extracytoplasmic sigma factor FecI which interacts with RNA polymerase to initiate transcription of *fec* transport genes (Buchanan, 2005). *B. pseudomallei* encodes a TonB dependent transducer BPSS1029 and deletion of *ΔtonB* would be expected to prevent any transduction of extracellular signal via this pathway.

Dysregulation of iron responsive gene regulation via either of these mechanisms following deletion of *tonB* may lead to phenotypes such as an increased ability of *B. pseudomallei ΔtonB* to enter macrophages.

Whilst attenuation of this mutant might suggest TonB is a promising target for novel antimicrobials, the role of TonB in iron responsive gene regulation and the resulting modulation of virulence must not be underestimated. In the case of either a direct TonB targeting antimicrobial strategy, or a more general iron chelation therapy to treat infection, bacteria would be expected to respond to the iron cue, either increasing or decreasing virulence, possibly in an unpredictable manner. The iron response should therefore be far better understood before the strategy to target TonB dependent iron uptake is taken forward. In addition, the investigation of alternative iron uptake systems which play a role during infection by this bacterial species is also warranted.

Chapter 7. General discussion and future work

B. pseudomallei is a leading cause of death in endemic regions as well as being listed as a category B bioterrorism agent by the USA Centres for Disease Control (CDC). The treatment of melioidosis, the disease caused by this bacterium, is intensive and prolonged (White, 2003). Effective treatment could be significantly improved and compliance enhanced if an antibiotic with improved activity was identified.

B. pseudomallei is intrinsically resistant to a range of antibiotics. An extensive array of resistance mechanisms are encoded in the *B. pseudomallei* K96243 genome (Holden *et al*., 2004). Gene clusters for six efflux pumps, including one demonstrated to be responsible for resistance to aminoglycosides and macrolides (Moore *et al*., 1999) are present in the *B. pseudomallei* K96243 genome. In addition, genes predicted to encode seven Ambler class A, B and D β-lactamases have been identified in the genome (Holden *et al*., 2004). The most significant of these, PenA, has been shown to be responsible for resistance to β-lactams including ampicillin, amoxicillin and carbenicillin (Rholl *et al*., 2011). PenA is readily inhibited by clavulanic acid, however, resistance to clavulanic acid by modification of PenA has also been documented in *B. pseudomallei* (Godfrey *et al*., 1991). Evolution of antibiotic resistance to currently utilised antibiotics during therapy has also been described (Tribuddharat *et al*., 2003). Therefore the purpose of this study was to identify and investigate targets for novel antimicrobials to which resistance has not already developed, to improve treatment of infection with *B. pseudomallei.*

Four *B. pseudomallei* genes were selected for investigation as antibiotic targets due to their hypothetical potential for inhibition. First, *ftsZ* and *ftsA* encode early effectors of cell division and have been shown to be essential in other bacteria (Errington *et al*., 2003). GTP binding and hydrolysis by FtsZ has been demonstrated by the diverse bacterial species *T. maritima, Azotobacter vinelandii* and *E. coli,* indicating this activity is a general feature of the protein (Lu *et al*., 1998). FtsZ monomers have been shown to assemble in a head-to-tail fashion with the synergy loop in the CTD of one monomer interacting with the nucleotide binding site in the NTD of the next, leading to interaction the catalytic residues allowing for nucleotide hydrolysis (Oliva *et al.*, 2004). The GTP activity of FtsZ has been inhibited *in vitro* by several small molecules, primarily GTP analogues, as a proof of principle for inhibition of FtsZ as a novel antimicrobial strategy (Margalit *et al*., 2004; Stokes *et al*., 2005; Paradis-Bleau *et al*., 2005; Huang *et al*., 2006). The most promising of these, PC190723, was synthesized as an improvement of 3-Methoxybenzamide (MBA), which was initially identified as an inhibitor of *B. subtilis* cell division (Ohashi *et al*., 1999). PC190723 has been shown to possess potent anti-staphylococcal activity in mice (Haydon *et al*., 2008). The molecule did inhibit GTPase activity of FtsZ, though was not predicted to bind in the nucleotide binding site of the molecule. By *in silico* modelling, the highest docking score for PC190723 and FtsZ occurred in the cleft between the helix joining the CTD and NTD, and the CTD. The inhibitor was therefore proposed to alter the inter-domain orientation of FtsZ, preventing GTP hydolysis (Figure 7.1 Haydon *et al*., 2008).

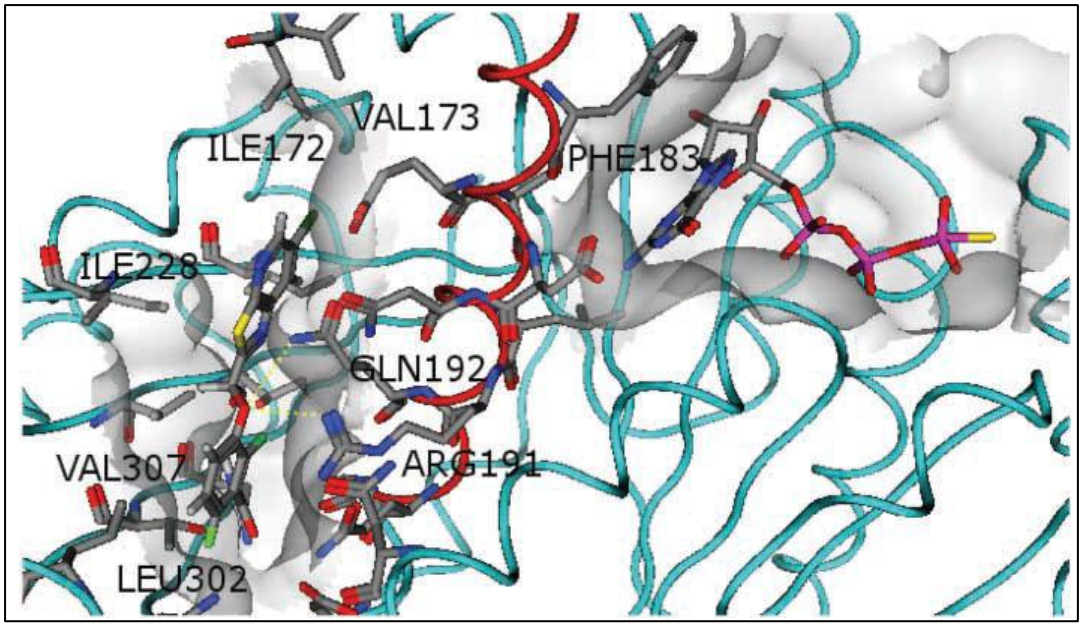

Figure 7.1 Proposed binding of the FtsZ inhibitor PC190723 to FtsZ. The side chains of residues forming the putative binding pocket are labelled and are shown in ball and stick representation. The protein backbone is shown as a light blue ribbon. The core helix 7 is highlighted in red. In this model, hydrogen bonds are formed between the phenoxy ether of PC190723 and R191 and Q192 (dashed yellow lines). Blue, nitrogen; yellow, sulphur; gray, carbon; red, oxygen (Haydon *et al*., 2008)

FtsA has also been implicated as a potential target for novel antimicrobial inhibitors although to a much lesser extent than FtsZ. FtsA is formed of two sub-domains (van den Ent and Lowe, 2000). The domains interact by folding of the protein, at which position a nucleotide binding site is formed. The *T. maritima* crystal structure contained an Mg-ATP molecule demonstrating the nucleotide binding capacity of this protein (van den Ent and Lowe, 2000). *B. subtilis* FtsA has been shown to behave as a dimer which can bind ATP and has ATPase activity in a similar manner to its structural relative actin (Feucht *et al.*, 2001). Inhibition of *P. aeruginosa* FtsA ATPase activity was demonstrated using peptides identified by phage display *in vitro* (Paradis-bleau *et al*. 2005). The specific mechanism of inhibition of these peptides has not been described.

In this study, an inability to construct in-frame deletion mutants of *ftsA* and *ftsZ* in *B. pseudomallei*, suggests essentiality of these targets in this species, as in many bacterial species, therefore making them attractive novel targets for antimicrobials. To further determine the feasibility of targeting *B. pseudomallei* FtsA and FtsZ as a strategy, expression and purification of FtsA and FtsZ to establish whether they possess ATPase or GTPase activity could be undertaken. In addition, solved structures of *B. pseudomallei* FtsA and FtsZ to compare to other bacterial structures would be extremely useful in the development of broad spectrum cell division inhibitors.

Those inhibitors currently described to target FtsZ and FtsA in other pathogenic bacteria have generally demonstrated activity against Gram

positive species (Margalit *et al*., 2004; Stokes *et al*., 2005; Paradis-Bleau *et al*., 2005; Huang *et al*., 2006). Herein, *B. pseudomallei* FtsA and FtsZ were both shown to have high degrees of amino acid identity to their Gram positive, *B. subtilis* protein counterparts. Cross reactivity of existing FtsA and FtsZ inhibitors, against *B. pseudomallei* FtsA and FtsZ, is therefore a possibility and could be tested *in vitro*. Penetration of the *B. pseudomallei* Gram negative cell wall by any inhibitor would be expected to be less efficient than into Gram positive bacteria. Therefore, methods of delivery of the inhibitors, and chemical modification to improve their uptake by Gram negatives could be explored. Ultimately, any successful inhibitors *in vitro* would be tested for antimicrobial activity *in vivo*.

Whilst other methods of mutagenesis could have been employed in order to disrupt or inactivate *ftsA and ftsZ,* in-frame deletion was considered least likely to cause polar effects. This was particularly important in the case of *ftsZ* and *ftsA*, as well as the third target *mraW*, since they lie in a large cluster of cell division and cell wall synthesis genes. To confirm essentiality of *ftsA* and *ftsZ*, a conditional complementation methodology could be employed.

Construction of an *ftsZ* mutant derivative with an unexpected genotype, in which both truncated and full size genes were retained, highlights the need for complete confirmation of mutants by additional methods to PCR alone. Confirmatory methods such as Southern hybridisation and complementation should be employed. Development of tools for different types of mutant

complementation in *B. pseudomallei* should be a priority when investigating the roles of gene products in this bacterium.

MraW was the third gene product to be investigated for potential as a novel antimicrobial target in *B. pseudomallei,* due to its highly conserved nature and *in vitro* enzyme activity. During this study, research published by Kimura and Suzuki (2010), identified methylation of a specific base of 16S rRNA during ribosome assembly in *E. coli* by MraW. Therapeutic targeting of methyltransferases is not a new concept. Ineffective epigenetic control of DNA expression by aberrant methylation of DNA has been targeted as a strategy for anti-cancer drugs (Brueckner *et al*., 2007) and flavivirus RNA methylases have been targeted in recent times as antivirals (Podvinec *et al.,*. 2010). Furthermore, several studies have investigated inhibition of bacterial DNA methyltransferases as an antimicrobial strategy. As described previously, small molecules which inhibit the methyltransferase activity of bacterial CcrM and DAM have been identified and some of these molecules also demonstrated antimicrobial activity against *C. crescentus* (Mashhoon *et al*., 2006). In addition, inhibitors of rRNA methylators such as the Erm proteins, to prevent methylation of rRNA which confers antibiotic resistance, have been investigated (Hajduk *et al*., 1999). These inhibitors reverse resistance to MLS antibiotics, and therefore an Erm inhibitor would be administered alongside the most appropriate of the MLS drugs, in a similar manner to β-lactam/β-lactamase inhibitor combination therapy. Triazine containing compounds had been identified as weak binders of the ErmAM active site (Hajduk *et al*.,

1999). Their structure was subsequently optimised to improve inhibition of the ErmAM methyltransferase activity (Hajduk *et al*., 1999).

The requirement of MraW for infection of a mammalian model of melioidosis demonstrates this protein also has potential as a novel antimicrobial target for *B. pseudomallei*. Purification of *B. pseudomallei* MraW and its structural and functional determination would aid in MraW targeted, inhibitor discovery. An MraW *in vitro* enzyme assay would be useful for screening of chemical libraries for inhibitory compounds. Testing of inhibitors such as those which bind Erm- proteins, against MraW activity, would begin to determine how specific the rRNA methylation inhibitors are.

The role of MraW during infection has not previously been investigated. *B. pseudomallei ΔmraW* was significantly attenuated in Balb/C mice, although not in the invertebrate model of infection *G. mellonella,* or during infection of macrophage monolayers. In addition, *B. pseudomallei ΔmraW* was immotile compared to the parental strain, though shown to possess flagella by electron microscopy. These data suggests flagella are present but non-functional in the absence of *mraW*, rendering the bacteria immotile and unable to cause disease in the mammalian host. However the presence of non-functional flagella is demonstrated to be sufficient to infect *G. mellonella* and macrophage monolayers. The proposed role in *E. coli* as a methylator of 16S rRNA and resultingly, fidelity of translation by bacteria, suggests it may have a pleiotropic effect on many pathways due to the production of altered protein structures. Here, the non-functioning of flagella in the absence of *mraW* might

suggest reduction in translation fidelity leads to faulty flagella or motor construction and resultantly, a loss of motility.

Confirmation that observed *B. pseudomallei ΔmraW* phenotypes were as a result of inactivation of *mraW* alone is required. Complementation ensuring a native copy number of *mraW* transcripts, or quantitative RT PCR of transcripts from around the *mraW* locus could also rule out polar effects due to the mutation. To re-iterate, development of tools for effective complementation of *B. pseudomallei* mutant strains would also be of benefit in this case.

The final antibiotic target in this study was TonB, a protein which energizes transport of large molecules across the outer membrane in Gram negative bacteria (Postle and Kadner, 2003). Like many other bacterial *tonB* mutants, *B. pseudomallei ΔtonB* required free iron for growth and was attenuated in two *in vivo* models of infection. Unexpectedly, in tissue culture, *B. pseudomallei ΔtonB* was able to enter macrophages more efficiently than the parental strain and was able to replicate within the cells at a similar rate to the parental strain. This phenotype may be an artefact of this macrophage culture model and may not be replicated in other types of cells, for example in epithelial cells. Alternative cell culture models could therefore be investigated to examine this. A more *in vivo*-like cell culture model such as normal human bronchial epithelium described by Balder *et al*. in 2010 would be more appropriate still. However, the intracellular macrophage environment and tissue culture media would still be expected to be severely restricted for free iron.

Whilst *B. pseudomallei ΔtonB* was attenuated in a Balb/C mouse model of infection, the mutant had colonised lungs, livers and spleens of surviving mice, suggesting this strain was able to replicate *in vivo*. This suggests an as yet unidentified redundant mechanism in *B. pseudomallei* may be used for uptake of iron in environments where this element is restricted. This is supported by recent work showing that the known siderophore synthesis systems in *B. pseudomallei* are dispensable in a murine model of melioidosis (Kvitko *et al*., 2012). Therefore, the importance during infection of the *efeU* system identified here by bioinformatic methods, as well as other possible unidentified iron uptake systems for high affinity iron uptake by *B. pseudomallei,* are worthy of future study.

The increased ability of *B. pseudomallei ΔtonB* to invade macrophages was shown not to be due to increased ability to attach to the cells and therefore must be due to some other factor mediating uptake. A dysregulated surface factor might be expected to be responsible for this difference. Altered regulation of surface factors could be due the absence of a TonB dependent transducer signal from the extracellular environment. Alternatively, gene regulation may have been disrupted due to altered intracellular iron concentration, in the absence of TonB dependent ferric iron uptake. Comparing intracellular iron levels of *B. pseudomallei ΔtonB* and the parental strain could answer this question. In addition, looking for differentially expressed mRNA transcripts by microarray or transcriptome sequencing would identify any differentially regulated genes between each strain.

Incomplete clearance of *B. pseudomallei ΔtonB* by the mice at 35 days post infection also suggests therapeutically targeting this system alone may not be appropriate. Alternative animal models of disease could be used to further investigate persistence of *B. pseudomallei* in the host. Extending the time which infected mice were monitored for and measuring clearance of the bacteria over time would provide additional evidence for or against therapeutically targeting iron uptake. The attenuation does suggest restriction of iron to the bacteria may limit the disease causing ability of the bacteria. Therefore, iron restriction with a chelator, used in combination with traditionally used antibiotics, may improve antibiotic efficacy. This strategy has been explored for the periodontal pathogen *Porphyromonas gingivalis* (Moon *et al*., 2011). However, the host requirement for iron should be prioritised when considering this strategy.

Inhibition of TonB function to energise TBDTs to transport substrates across the OM has been demonstrated *in vitro* in *E. coli* using a TonB box-like pentapeptide. The TonB box is a conserved sequence found at the amino end of TBDTs, thought to be the region at which TonB interacts with the transporters, conferring transduced energy for transport of substrates (Larsen *et al*., 1997). Application of the inhibitory TonB box like pentapeptide to *E. coli* replicated a *ΔtonB* phenotype. This was presumed to occur by preventing TonB dependent functions at the outer membrane by blocking interaction of TonB with TBDTs. This strategy could be investigated for *B. pseudomallei*.

Another route to utilise TonB dependent uptake by *B. pseudomallei* to improve penetration of antimicrobials, is to chemically couple siderophore moieties to antimicrobial molecules to accelerate their uptake. For example, cephalosporins with catechol substitutions have increased activity against Gram negative bacteria, due to their active uptake by a TonB dependent mechanism (reviewed Braun, 1999). In a more recent study a novel monobactam with a siderophore sidechain was potently inhibitory *in vitro* to the Gram-negative *Acinetobacter* spp,, presumed to be due to its accelerated transport across the OM (Russo *et al*., 2010). This chemical coupling of antimicrobials to siderophores could be considered for any novel *B. pseudomallei* antimicrobial agent, such as FtsA or FtsZ inhibitors.

It is important to keep in mind the regulatory role iron plays in virulence when targeting bacterial iron uptake as a therapeutic strategy. Data from this study suggests TonB plays additional roles during infection than simply iron scavenging and uptake. An extended understanding of the iron uptake systems and the role of iron signalling during infection by *B. pseudomallei* is required before therapeutic targeting of this system is investigated further.

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