DISSERTATION

β-LACTAM RESISTANCE MECHANISMS IN *Burkholderia pseudomallei* AND THE TOOLS USED FOR THEIR ELUCIDATION

Submitted by

Drew Anders Rholl

Department of Microbiology, Immunology and Pathology

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Doctoral Committee:

Advisor: Herbert P. Schweizer

Steven W. Dow
Richard A. Slayden
Laurie A. Stargell

ABSTRACT

β-LACTAM RESISTANCE MECHANISMS IN *Burkholderia pseudomallei* AND THE TOOLS USED FOR THEIR ELUCIDATION

A state of fear can arise from being unable to stop an impending threat. This can be the case of those infected with *Burkholderia pseudomallei,* the etiological agent of melioidosis. The 9-54% mortality rate, despite proper treatment, can be partially attributed to a combination of high levels of intrinsic and acquired antibiotic resistance causing treatment failure, unreliable/time-consuming diagnostics and a plethora of virulence factors. Even into the late 1980's, mortality was upwards of 80%. Its success as both a soil microbe and a highly antibiotic-resistant broad-host pathogen are in part due to its large genome and extensive metabolic capabilities. For these reasons and others, the Centers for Disease Control named *B. pseudomallei* a potential biothreat agent and a Category B select agent.

The above mentioned attributes of *B. pseudomallei* make the bacterium's study both challenging and necessary. CDC select agent guidelines complicate the use of many effective molecular tools, making the job of elucidating the attributes of specific genetic elements difficult. Thus, the construction of new systems for genetic manipulation was required. Chapters 3 and 6 and parts of chapter 5 describe novel tools that have since

been successfully used to genetically manipulate *B. pseudomallei* in an efficient and select-agent compliant fashion. Chapter 3 details construction of a transposon *Himar1*-based random mutagenesis system. Such systems have proven indispensable tools for the study of bacteria as they facilitate identification of metabolic pathways, virulence factors, antibiotic resistance mechanisms and other celluar processes. Chapter 6 describes improvements to existing tools, a new *Escherichia coli* mobilizer strain (RHO3) and an improved Tn*7* transposase expression vector (pTNS3). RHO3 is the most versatile *E. coli* mobilizer strain engineered to date. It combines the plasmid mobilization efficiency of the widely used SM10 mobilizer strain with engineered kanamycin susceptibility and metabolic counterselection on rich media. The pTNS3 helper plasmid was engineered to express the Tn*7* site-specific transposition pathway more efficiently by inclusion of the strong, broad-host-range *P1* integron promoter in addition to the *E. coli* lactose-tryptophan P_{tac} hybrid promoter. Chapter 5 describes the application of a combination of the mini-Tn*7*-based single-copy chromosomal integration and expression system with the site-specific Cre recombinase system for temporary expression of a rescue gene aiding in characterization of essential genes.

The work of defining novel resistance mechanisms is necessary to discover why recommended treatment regimens can fail. Use of ceftazidime in initial treatment of melioidosis halved the mortality rate. Because it is one of the few effective treatment options, resistance to this β-lactam is of great interest. Molecular definition of such mechanisms (chapters 4 and 5) could improve diagnostic capabilities, both clinically and in the case of a bioterrorism event. Chapter 4 describes the novel finding that the chromosomally encoded PenA β-lactamase is secreted via the twin arginine translocase

system and that *penA* neighboring regulatory genes are most likely not involved in the regulation of expression of this gene. The work described in this chapter also established that PenA is the major *B. pseudomallei* β-lactam resistance mechanism and defined several mutations leading to ceftazidime and amoxicillin + clavulanic acid resistance. These findings form the basis for development of diagnostic tools for the detection of mutations causing high level resistance to clinically significant antibiotics which will allow initiation (biodefense) or redirection (clinical melioidosis) of proper antibiotic therapy. Work described in Chapter 5 defines deletion of the penicillin-binding protein 3 BPSS1219 as a novel ceftazidime resistance mechanism observed in *B. pseudomallei* strains isolated from patients that failed ceftazidime therapy.

Through this body of work, I hope to have shed light on aspects of the biology of *B. pseudomallei*, novel genetic tools for its manipulation and novel mechanisms of β-lactam resistance.

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Any success I have had is mostly thanks to my advisor, Dr. Herbert P. Schweizer. Through a combination of providing guidance, trusting me to fill in the blanks and affording me fascinating projects, he was able to shape a novice admirer of microbiology into a (slightly) more capable researcher. To balance my growing confidence, he kept me grounded by showing me that our knowledge of even the simplest ideas is never complete, but is rather somewhat more accurate and precise than before. Likewise, I am grateful to my committee members, Drs. Steven Dow, Richard Slayden, and Laurie Stargell, who challenged me within a friendly environment. Their mentorship established in me a humble energy, pushing me to build upon the scientific foundation provided.

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Dedicated to

Laura, for supporting me through this entire process

(emotionally, psychologically and, at times, financially)

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LIST OF ABBREVIATIONS

AC	Amoxicillin
AM or Amp	Ampicillin
B.	*Burkholderia*
BAL	BAL30072
BLAST	Basic Local Alignment Search Tool
Bp	base pair(s)
BSL	biosafety level
C	Celsius
CB	Carbenicllin
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
Cef	Ceftazidime
CFU	colony forming units
Cm	Chloramphenicol
CWD	cell-wall deficient
DAP	diaminopimelic acid
Dox	Doxycycline
DNA	deoxyribonucleic acid
E.	*Escherichia*

e.g.	for example
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
EPI	efflux pump inhibitor
et al.	and the others
G	Gram
Gm	Gentamicin
H	Hour
HSL	Homoserine lactone
i.e.	id est- that is
IHA	Indirect hemagglutination assay
IP	Imipenem
IPTG	isopropyl-β-D-thiogalactopyranoside
Kb	Kilobase
Km	Kanamycin
L	Liter
LB	Luria-Bertani
LD	lethal dose
LDS	lithium dodecyl sulfate
LPS	lippopolysaccharide
Mb	Megabase
Mg	Milligrams
MNGC	Multinucleated giant cells

MHA	Mueller-Hinton agar
μm	micrometer(s)
μg	microgram(s)
μL	microliter(s)
MIC	minimum inhibitory concentration
mL	milliliter(s)
mM	millimolar
MP	Meropenem
NAG	*N*-acetylglucosamine
NAM	*N*-acetlymuramic acid
ND	not determined
Nm	Nanometer
OD_{600nm}	absorbance at 600 nm
OMP	outer membrane protein
P.	*Pseudomonas*
PAGE	polyacrylamide gel electrophoresis
PP	Piperacillin
PBP(s)	penicillin binding protein(s)
PCR	polymerase chain reaction
P_{s12}	*Burkholderia thailandensis* s12 gene promoter
P_{tac}	*E. coli* lactose-tryptophan operons hybrid promoter
qRT-PCR	quantitative real-time polymerase chain reaction
^{r}	Resistant

Rif	Rifampin
RNA	ribonucleic acid
RND	resistance-nodulation cell-division
rpm	Rotations per minute
^{s}	Sensitive or susceptible
SCV	small colony variants
SOEing PCR	splicing by overlapping extension polymerase chain reaction
Tmp or Tp	Trimethoprim
TMP-SMX	trimethoprim + sulfamethoxozole (co-trimoxazole)
T3SS	type three secretion system
T6SS	type six secretion system
TZ	Ceftazidime
x g	times gravity
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
XL	amoxicillin:clavulanic acid 2:1 (co-amoxiclav)
Zeo	Zeocin

LIST OF PUBLICATIONS

Portions of this dissertation have been published or written up in the following papers or submitted manuscripts.

D. A. Rholl and H. P. Schweizer. Molecular investigations of PenA-mediated β-lactam resistance in *Burkholderia pseudomallei*. Submitted to *Frontiers in Cellular and Infection Microbiology.*

Chantratita* N., **D. A. Rholl***, B Sim, V. Wuthiekanun, D. Limmathurotsakul, P. Amornchai, A. Thanwisai, H. H. Chua, W. F. Ooi, M. T.G. Holden, N. P. Day, P. Tan, H. P. Schweizer, S. J. Peacock. Genomic loss of penicillin-binding protein 3 in *Burkholderia pseudomallei* confers clinical resistance to ceftazidime. Submitted to Journal of Clinical Investigation.

Mima T., B.H. Kvitko, **D. A. Rholl**, M.G.P. Page, E. Desarbre and H. P. Schweizer. 2011. In vitro activity of BAL30072 against *Burkholderia pseudomallei*. International Journal of Antimicrobial Agents. In press.

López* C. M., **D. A. Rholl*,** L. A. Trunck, and H. P. Schweizer. 2009. Versatile dual-technology system for markerless allele replacement in *Burkholderia pseudomallei*. Appl Environ Microbiol 75:6496-6503.

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* Indicates shared first authorship.

CHAPTER 1:

INTRODUCTION TO *BURKHOLDERIA PSEUDOMALLEI*

1.1 History

Since its discovery, *Burkholderia pseudomallei* has had many names; *Pseudomonas pseudomallei*, *Bacillus pseudomallei*, *Malleomyces pseudomallei*, Whitmore's bacillus (20), *Loefflerella pseudomallei, Pfeiferella pseudomallei* (215), *Bacterium whitmori, Bacillus whitmori, Pfeifferella whitmori, Pfeifferella pseudomallei, Actinobacillus pseudomallei, Lofflerella whitmori,* and *Flavobacterium pseudomallei* (230), but never "*Burkholderia pseudonombrei*", although it seems appropriate based on the last sentence. It was first documented in 1913 by Alfred Whitmore, who differentiated it from *B. mallei* as the agent causing an outbreak in morphine addicts in Rangoon (in modern day Burma)(238, 239).

1.1.1 Use as a biological weapon

The first account of *Burkholderia* being used maliciously was a work of fiction, that is to say it was used as the murder weapon in Sir Arthur Conan Doyle's Sherlock Holmes novel, "The Adventure of the Dying Detective" (195). Unfortunately there are many non-fictional accounts of *Burkholderia* species used as a biowarfare agent (230). This includes use by the German army on Russian forces in World War I (103), in World War

II by Japanese forces (103) and by Soviet forces in Afghanistan in the early 1980's (4). For reasons discussed later in this chapter, *Burkholderia* requires weaponization before it can be efficiently used for terror attacks.

The Centers for Disease Control (CDC) has designated *B. pseudomallei* a Category B select agent due to its potential use as a bioterrorist agent (21), which is why work must be done in select agent approved biosafety level 3 (BSL-3) facilities (171). Category B is the second highest priority tier because *B. pseudomallei* are moderately easy to distribute and have low mortality and moderate morbidity rates (see section 1.3.2). These agents also require development of better diagnostic capabilities and increased safety measures when working with the bacteria (21). The stringent biosecurity of *B. pseudomallei* research laboratories in the United States is somewhat paradoxical since the bacteria are readily propagated from the environment in many tropical regions (216).

1.1.2 Manipulation of *B. pseudomallei*

The select agent classification has made genetic manipulation of *B. pseudomallei* difficult, because of the limited number of genetic markers permitted and what types of mutations are allowed to be created. The last decade has seen multiple select agent compliant genetic systems being published for gram negative bacterial research, in particular for *B. pseudomallei* (16, 43-45, 76, 95, 96, 118, 118, 148, 168)(Chapter 6). Recently a 1026b Δ*purM* strain, Bp82, was tested. The deletion caused the bacteria to become adenine and thymine auxotrophs. This resulting strain is completely attenuated, even when inoculating with $>10^4$ x LD_{50} (the number of bacteria required to be lethal in 50% of animals tested) in hyper-susceptible 129/SvEv mice, immune incompetent mice (INF-$\gamma^{-/-}$ and severe combined immune deficiency [SCID] breeds) and Syrian hamsters

(155). Due to this extreme level of attenuation, Bp82 has been granted exclusion from select agent regulations and can be manipulated at BSL2 conditions after review and approval by the respective Institutional Biosafety Committees. This has opened the door for testing mutations that may increase resistance and virulence, which is forbidden in wild-type *B. pseudomallei* per NIH and select agent regulations.

1.2 Evolution of *Burkholderia*

1.2.1 Phylogenetic relationships of *B. pseudomallei*, *B. thailandensis* and *B. mallei*.

The genus *Burkholderia* is very diverse, with many species of varying degrees of separation. *B. thailandensis* and *B. mallei* are closely related to *B. pseudomallei,* which is thought to be the progenitor species (145)(See Figure 1-1). Using a large sampling of these species with a MLST system based on a model for differentiating isolates within pathogenic populations (126), Pearson et al. showed the great diversity of MLST types for *B. pseudomallei*, illustrated in Figure 1-2 by eBurst, a program visually linking related types (63). There were ~600 MLST types for *B. pseudomallei* versus 2 for *B. mallei* and 20 for *B. thailandensis* at time of publication (152).

Although *B. pseudomallei* shows great molecular diversity, even within close geographic proximity (24), the species can also be defined by major branches. Australian strains can be distinguished from strains from other parts of the world by the presence of either a *B. thailandensis*-like flagellum and chemotaxis (BTFC) gene cluster or a *Yersinia*-like fimbrial (YLF) gene cluster, respectively, at the same genomic locus (223). The actin-polymerizing protein-encoding gene *bimA* also has two general sequence types,

one more prevalent in Australia. The Australian form is the type associated with *B. mallei*, suggesting that *B. mallei* came from an Australian strain of *B. pseudomallei* (191).

1.2.2 Hypermutability

In addition to being comparatively large, in the case of *B. thailandensis* and *B. pseudomallei* (~7.3 Mb)(81), *Burkholderia* genomes are also very plastic and recombination of large genomic segments within the same chromosome is frequent (120). *Burkholderia* species contain large numbers of simple sequence repeats (SSRs), which may increase the rate of homologous recombination (140). Even within a single patient over a 2 weeks course of infection, mutations of variable-number tandem repeats (VNTR) have been shown to yield up to 12 unique strains by multilocus VNTR analysis

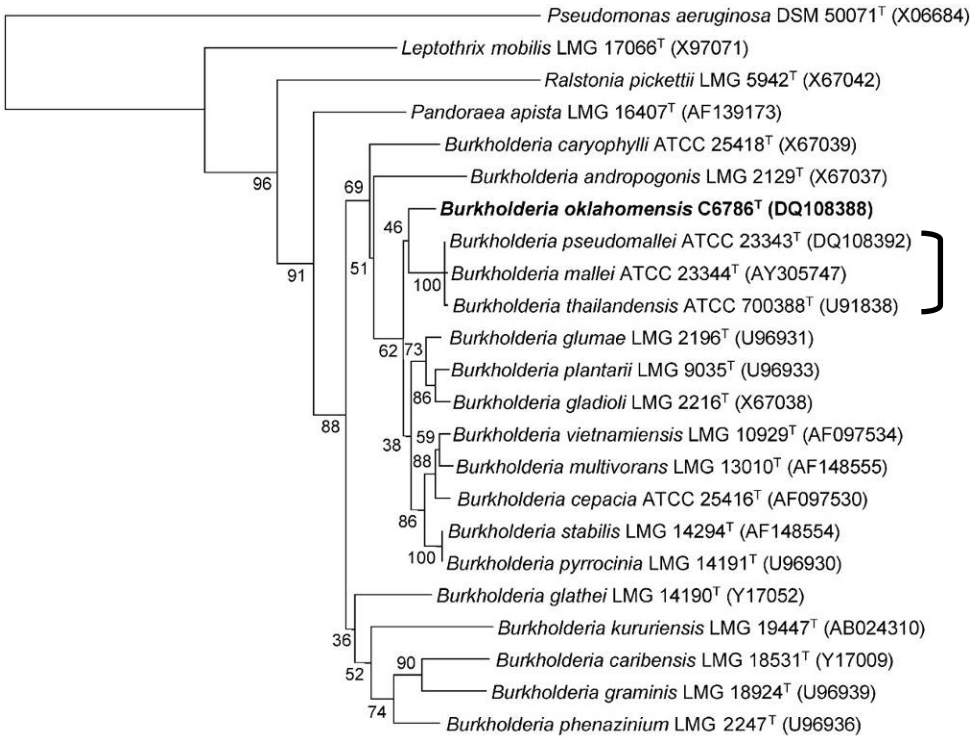

Figure 1-1. 16s rRNA sequence based phylogenic tree of *Burkholderia* species. Note that *B. mallei* is more closely related to *B. pseudomallei* than *B. thailandensis.* Modified from reference **(73)**.

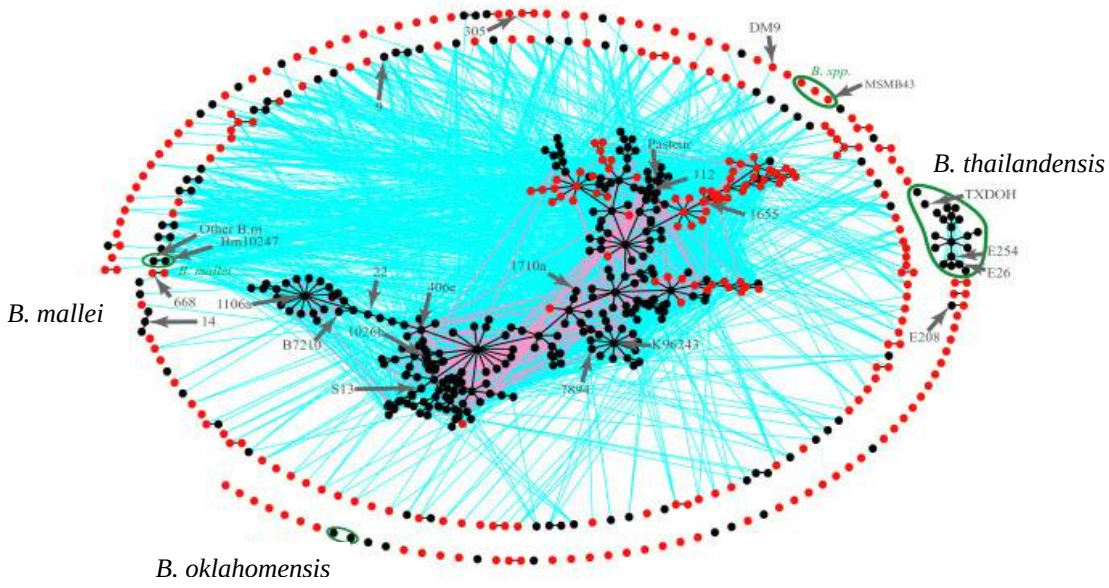

Figure 1-2. eBURST MLST distribution of *B. pseudomallei* and close relatives. This method uses MLST sequence typing to compare strains (modified from **(152)**). Red are Australian samples and black are from other locations. The lack of connectivity throughout the perimeter strains highlights the diversity of *B. pseudomallei*. Note the clusters of *B. mallei, B. thailandensis* and *B. oklahomensis*.

(MLVA) from a single progenitor type (154). This ability may facilitate adaptation to environmental and host niches. Although nucleotide substitutions occur at a much lower rate compared to recombination in *B. pseudomallei* (31), point mutations are found in clinical isolates with altered antibiotic resistance profiles (82, 175, 221).

In *B. mallei*, propagation of insertion sequences (ISs) has contributed to the high rates of genetic rearrangement, which lead to large scale deletions, genome reduction and the establishment of *B. mallei* as an obligate intracellular pathogen. *B. pseudomallei* has similar IS elements, although they do not cause rearrangement as prodigiously (197).

1.2.3 Large chromosomal deletion/addition

Large regions of chromosomally integrated DNA, known as genomic islands (GIs), are prevalent in *B. pseudomallei* (188, 224). Given their variation in GC content from the

rest of the genome, these regions are thought to have been transformed into the genome from environmental sources. Many GIs are also part of the accessory genome, meaning they are not found in all *B. pseudomallei* strains (81). As opposed to the core genome, which is universal throughout *B. pseudomallei* strains, certain GIs are found predominantly in clinical samples versus environmental samples, suggesting that they aid in virulence and pathogenesis (188). Based on the diversity of accessory genes within currently sequenced *B. pseudomallei,* the number of novel genes in future genome sequences is expected to hover between 25 and 50 (120). Additionally, virulence genes in *B. pseudomallei* may have been selected for by environmental factors rather than host interaction. This group may include Type IV pili and T3SS effector proteins (137).

High levels of recombination are accompanied by deletion events (145). Curiously, these large scale deletion events often do not compromise strain fitness. In a murine melioidosis model, strain 708a, which has a ~131 kb deletion (compared to strain K96243), is as virulent as wild-type *B. pseudomallei* strain 1026b (222).

The arabinose utilization operon has been lost by *B. pseudomallei* and arabinose utilization, or lack thereof, is a major difference between *B. thailandensis* and *B. pseudomallei*. Introduction of the arabinose assimilation operon from *B. thailandensis* into *B. pseudomallei* decreased virulence in hamsters, possibly due to the downregulation of genes within T3SS3 (to be discussed in section 1.5.2.1). This genomic loss may have unintentionally resulted in increased pathogenicity in *B. pseudomallei* (132).

1.2.4 Other *Burkholderia* species

The best studied members of the *Burkholderia* genus within the medical community are members of the *Burkholderia cepacia* complex (Bcc), which colonize the lungs of

people suffering from cystic fibrosis (CF). CF affects roughly 30,000 people in the United States and is caused by a mutation in a cellular chloride ion pump (cystic fibrosis transmembrane regulator [CFTR]). This results in lung epithelium laden with thick mucus, providing a good growth environment for opportunistic bacteria. While 80% of patients had *Pseudomonas aeurigonsa* in their lungs (149), there are a myriad of *Burkholderia* species associated with cases known as the *Burkholderia cepacia* complex (Bcc) (125). Bcc includes *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stablis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, *B. pyrrochinia* and *B. ubonensis* (listed by genomevars I to IX) although most infections are caused by *B. multivorans* and *B. cenocepacia* (48). *B. multivorans* was completely avirulent in a compromised mouse model, highlighting its opportunistic character (199).

Nearly all *Burkholderia* species are saprophytes and inhabit a wide variety of ecological niches. Because of their extensive metabolic repertoire, many species have been utilized for bioremediation and promotion of plant growth (48). *B. vietnamensis* has been shown to increase the yield of rice crops when interacting with the plants (144). *B. ubonensis* possesses antibiosis activity against a wide range of *B. pseudomallei*, meaning it can inhibit *B. pseudomallei* growth. This has been shown using controlled experiments on agar plates *in vitro* (127) and may be responsible for decreased levels of the pathogenic bacteria in soil samples, although this has not been proven (A. Baker, personal communication). Unfortunately, both of these bacteria exist in the *Burkholderia cepacia* complex, and as a precaution the use of such species for agricultural purposes is limited (149).

1.3 Epidemiology

1.3.1 Isolation from environment

B. pseudomallei is predominantly found in Southeast Asia and Northern Australia although it is found throughout the tropics (Figure 1-3)(32) in both agrarian (210) and urban environments (121, 129). Its range is also growing, as Taiwan emerged as the first location north of 20° N latitude with high prevalence of *B. pseudomallei* in soil (206). Changes in the landscape due to urbanization and agriculture can increase *B. pseudomallei* spread throughout the environment (93). Additionally, the presence of *B. pseudomallei* in the environment is likely to be underestimated. Even sampling within a particular location 5 times leaves up to 50% possibility the soil still contains the bacterium and to obtain a 99.5% confidence, one would need 1,000 samples (115).

1.3.2 Incidence of melioidosis

Annual melioidosis incidence rates vary greatly, even between geographically proximal locations (206), and may be reflective of the prevalence of *B. pseudomallei* in the environment (193) and/or contact with the bacteria (54). Average annual incidence rates range from 4/100,000 in parts of Thailand (36, 210) to 18/100,000 in Northern Australia. In the years with extreme weather events and outbreaks, the annual rate can increase up to 41/100,000 (54) and outbreaks can push it even higher (52/100,000 in a case of contaminated water)(129).

The rates in Northeast Thailand also appear to be increasing every year from 2000-2006 (8/100,000 to 21/100,000) and despite a decrease in mortality rate (48-41%), the larger number of cases allowed a near doubling of annual deaths due to melioidosis over the 7 year period (114). Still, the exact number of cases is probably much larger because

of under-diagnosis, death prior to conclusive microbiological testing and/or limited understanding (55, 248). Increased awareness may lead to increased diagnosis and reporting (94).

The relatively limited endemic region means that physicians in other parts of the world are often unaware of melioidosis and its symptoms, and therefore have even greater difficulty with accurate and timely diagnosis. A complete travel history to melioidosis hot spots is therefore essential in this process to avoid misdiagnosis and prolonged waiting perids before effective treatment is initiated (8, 9, 107, 169).

1.4 Environmental persistence

1.4.1 Persistence in soil

As a saprophyte, *B. pseudomallei* is a prominent member of the microbiome in the soil of endemic regions (115, 193). Its hardy nature allows it to persist in a laboratory

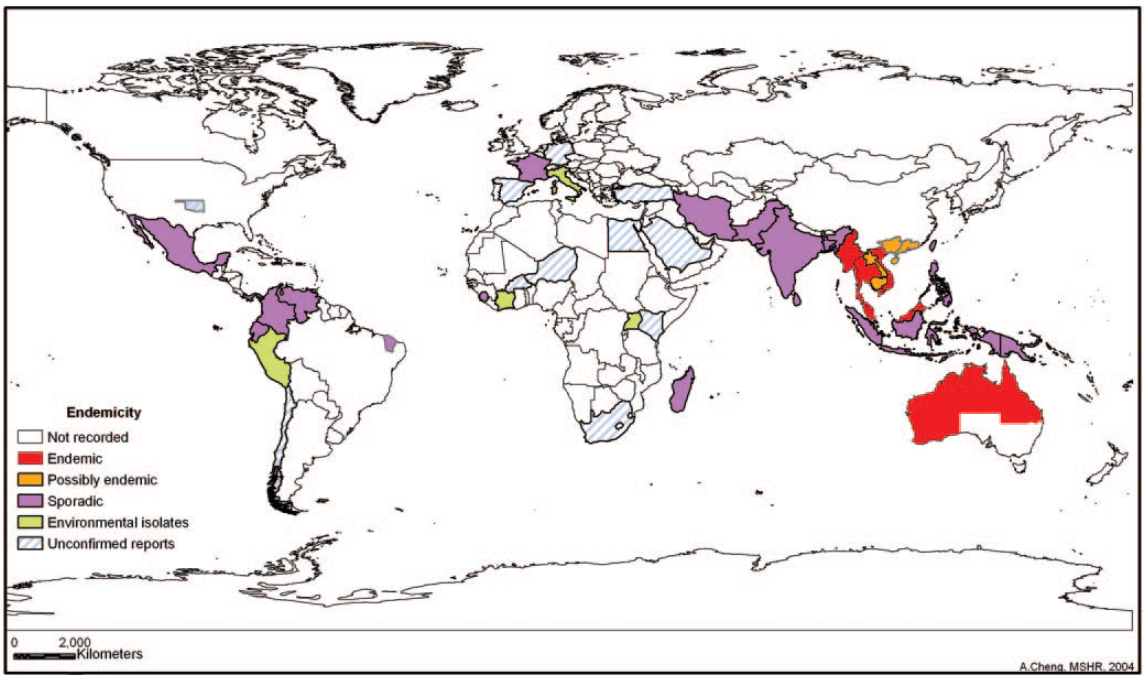

Figure 1-3. Distribution of melioidosis throughout the world circa 2004. Image from **(32)**. The "unconfirmed report" in the United States is actually a case caused by a new species, *B. oklahomensis* **(73)**.

setting at a wide range of pH levels (pH 4-9), providing the moisture content is ≥5% and temperature is ≥4°C (37). This hardiness most likely contributes the environmental ubiquity of *B. pseudomallei* in the warm and seasonally humid climates of Southeast Asia and Northern Australia. *B. pseudomallei* has even been isolated from flies in Kuala Lumpur, although nothing has come from the findings (207).

Field sampling in Thailand suggests soil with a pH of 5-6 and >10% moisture content is more likely to harbor *B. pseudomallei* (146). Greater numbers of the bacterium can be isolated from 25-45 cm below the soil's surface (216). This depth dependence is possibly due to the bacteria's high susceptibility to ultraviolet radiation (174) and its dependence on moist environments may stem from *B. pseudomallei*'s inability to form spores. Despite its hardiness in soil, *B. pseudomallei*'s reliance on moisture and its ultraviolet susceptibility decrease potential use as an aerosolized agent of bioterrorism.

Quicklime has been tested as a means of eradicating the bacteria from the soil, but was only found to be effective over an extended period of time (>6 weeks) when added to a final concentration of 40%. The requirement for such high concentrations makes this an unrealistic method of remediation (138).

1.4.2 Persistence in water

B. pseudomallei is capable of survival in water for >3 years (244), and the same group has shown continued viability at >19 years and counting (Vanaporn Wuthiekanun, personal communication). This finding lends support for the potential use of water as a dispersal mechanism (170). Part of this longevity is due to presence of the outer core of lipopolysaccharide (LPS) in its outer-membrane. This was demonstrated by decreased persistence in outer core deletion mutants (133). Contaminated water supplies have

already unintentionally served as infection sources (53, 89). Fortunately, chlorination is effective in the decontamination of water containing *B. pseudomallei* (83, 143).

1.5 Melioidosis

1.5.1 Manifestations

The multifaceted nature of melioidosis (Table 1-1) makes diagnosis and treatment difficult. The most deadly manifestation is septic shock, resulting in death 50% of the time. Death often occurs within the first 24 hours of hospitalization, which is why better prognosis comes with a speedy diagnosis. Additionally, mortality is three times higher when bacteremia is involved. Pneumonia is the second most prevalent cause of death, followed by illness with no clinical focus (54). Melioidosis can present acutely, with an average incubation time of 9 days, or chronically, for which mild manifestations can be present months before disease escalation (54). Acute melioidosis patients in Australia had 22% mortality, but no deaths occurred in a cohort of 30 chronic sufferers. However, chronic cases and latency may be exacerbated into becoming an acute infection by stressors, such as illness or injury (51, 124).

1.5.2 Pathogenesis and virulence factors

While *B. pseudomallei* can infect a wide-range of animals, including but not limited to human, ostrich, rabbit, goat, sheep, orangutan and pig (108), conventional laboratory animals are useful disease models. As can be expected from such a multifaceted disease, *B. pseudomallei* can be found throughout the body. An in-depth study into the course of infection in SWISS mice found *B. pseudomallei* in all organs tested, with largest numbers found in the spleen (69). Small animal models have been utilized to look at pathogenesis

Table 1-1. Different presentations and mortality rates of melioidosis. Data were compiled from multiple studies **(79)(160)(54)**.

	Percent of Patients	Percent Mortality in Group[a]
All cases	- [b]	19-65
Pneumonia or pleural effusion	45-58	20
Bacteremia	43-58	20
Septic shock	16-20	50
Genitourinary infection	8-14	7
Skin or soft tissue infection	13-17	0
Neurological	3-14	21
Bone or joint	4-12	2
Soft tissue abscesses	-	0
Spleen	2-4	-
Liver	2-7	-
Other intraabdominal	3-5	-
Prostatic (males only)	0.3-18	-
Parotid	0-2	-
No clinical focus	16-20	18

[a] Based on **(54)** or unknown

[b] "-" not computable or not applicable

and virulence for different scenarios; chronic infection, latent infection or high risk assessments (diabetic animals)(212, 219). Non-vertebrate models have also been used including *Caenorhabditis elegans* (68) and *Galleria mellonella* (233). Work with these models and mammalian cell culture has resulted in a rapid expansion of studies of *B. pseudomallei* pathogenesis and virulence (2). Microarray analyses of transcription levels and global gene regulation of *B. pseudomallei* during infection has given clues to potential virulence factors (225). Higher resolution of RNA-seq (*i.e.* a powerful genomic tool capable of accurately identifying millions of short reads from cDNA), which has been used to highlight different regulatory patterns of *B. cenocepacia* in environmental and clinical settings (249) has not yet been applied to studies with *B. pseudomallei*.

1.5.2.1 Type III Secretion Systems and Effector Molecules

T3SS use "needle complexes" to span both bacterial membranes and inject effector molecules into other cells (105). *B. pseudomallei* encodes three type III secretion systems (T3SS or TTSS)(234). Two T3SS operons (T3SS1 and T3SS2) have strong homology to the T3SS of the plant pathogen *Ralstonia solanacearum*. This suggests *B. pseudomallei* has a system for targeting animals and another for plant infection, further establishing *B. pseudomallei* as a diverse pathogen (13, 240). This is supported by the use of the tomato plant as a virulence model (109). There does appear to be a synergistic effect of the T3SS operons, as T3SS3 mutants with deletions in T3SS1 and T3SS2 operons are more attenuated than mutants containing either deletions in T3SS1 or T3SS2 (234).

The T3SS3 is encoded by the *Burkholderia* secretion apparatus cluster (BSA), which consists of ~30 genes similar to the T3SS in *Salmonella typhimurium*, which targets mammalian cells. The T3SS3 of *B. pseudomallei* allows evasion of endosomal degradation (204). Although not all T3SS3-related genes are required for full virulence (202), single mutations of various genes within the cluster (*bipD*, *bsaZ* and *bsaQ*) resulted in attenuation (134, 204). BipB, BipC and BipD are required to form a pore in the target cell membrane to translocate the effector molecules (135).

The functions of several effector molecules have been elucidated. BopA allows *B. pseudomallei* to escape autophagy, thereby increasing cellular persistence and propagation (50). BopE aids invasion of epithelial cells and induces actin filament rearrangements within the cell (201). Deletion of *bopE* did not significantly attenuate the bacteria (202). Other effector proteins may promote eukaryotic cell fusion (66). Cif_{Bp} is a cyclomodulin homolog capable of arresting the eukaryotic cell cycle (247). In what at

first glance appears to be an "avirulence" factor, the effector protein TssM decreases INF-β and inflammation. Deleting the *tssM* gene causes significantly higher levels of INF-β and inflammation, and it leads to more rapid death in acute animal infection models. Thus, TssM could assist in chronic infection and persistence (213).

1.5.2.2 Type VI Secretion Systems

Type VI secretion systems (T6SS) share homology with bacteriophage tail proteins and may have evolved from prophage insertions. T6SS may allow the direct injection of effectors into target cells by a mechanism similar to T3SS (110). *B. pseudomallei* has six type 6 secretion systems (T6SS), some of which are upregulated upon macrophage invasion. Additionally, the sheer space of genome taken up by these systems implies an evolutionary significance to the bacteria (184). One operon, *tss-5*, is required for efficient plaque formation *in vitro* in the PtK2 cell line (rat kangaroo kidney) and therefore is likely required for cell-to-cell spread (153).

1.5.2.3 Lipopolysaccharides (LPS)

Lipopolysaccharide (LPS) molecules are found in the outer membranes of Gram-negative bacteria, where in addition to providing structural stability, they interact with the host immune system. LPS mutants showed $>10^4$-fold attenuation when inoculated into BALB/c mice intraperitoneally compared to wild-type bacteria. They also had decreased survivability in macrophages, were 10^5-fold more susceptible to human serum and slightly more susceptible to reactive oxygen intermediates (241) This is presumably due to protective capabilities against the complement system. The LPS from *B. pseudomallei* activates macrophages *in vitro* less efficiently than that of *E. coli* (228), thereby slowing reaction time and decreasing the response level of the innate immune system (18).

Deletion of LPS clusters results in increased time to death, although the mutants are still lethal (177). LPS Type II O-PS is required for virulence (57), but the fact that *B. thailandensis* has similar LPS profiles suggests that it is not a major factor for virulence (6, 7). The release of LPS endotoxin into the host is not considered a factor in melioidosis pathophysiology since patients with high endotoxin levels (average 725 pg/mL) during treatment had similar rates of fever reduction, recovery and mortality than patients with low levels (average 146 pg/mL)(185).

1.5.2.4 Capsule

B. pseudomallei and *B. thailandensis* have multiple capsule polysaccharide (CPS) operons that are missing in *B. mallei*. Although *B. pseudomallei* is a potent inducer of the complement-cascade (60), the capsule may inhibit this protection mechanism by decreasing complement factor C3b association with bacteria (165). Deletion of the CPS III operon in *B. pseudomallei* had no effect on LD_{50}, so is thought to play a more prominent part in environmental survival as opposed to virulence (166). On the other hand, deletion of CPS I operon strongly affected virulence, with a 10^4-fold increase in bacteria required for lethality and inability to establish significant bacteremia (164). Acapsular mutants also have an increase in time to death when introduced intravenously and were cleared following intraperitoneal inoculation (12).

Further evidence of the capsule as a virulence factor was provided by Cuccui et al. (49). They pooled 892 transposon mutants and identified 39 (4.4%) as attenuated in signature-tagged mutagenesis competition studies in mice. It should, however, be noted that this only represented 25 unique genes due to several redundant gene inactivations. A

majority of these mutations were within a single operon of polysaccharide capsule biosynthesis (CPS IV)(49, 166).

1.5.2.5 Adhesion

In order to infect a cell the bacterium may need to attach itself first. Deletion of type IV pilin PilA showed decreased adhesion to multiple cell lines and significant attenuation in mice, although the mutant was still lethal in 33% of mice (n=6) after 18 days with inoculation of as few as 35 CFU. However, this is compared to 100% of death of mice at day 18 with 23 CFU of wild-type K96243 (61). Likewise, BoaB and BoaA adhesin mutants had significantly inhibited adherence to multiple respiratory cell-lines (14).

Flagella are also virulence factors, aiding in the invasion of non-phagocytic cells, although the efficiency of invading macrophage cells was also reduced in flagella-deficient mutants (46). Interestingly, *B. mallei* is non-motile (130), but does code for flagella (140) which, although apparently defective in terms of locomotion, may be required for cell adhesion.

Based on this data, we can say that adhesion is not necessary for, but may aid in, infection. It also appears that the route of infection may be significant when assessing adhesion mutants. Intranasal inoculation showed a marked attenuation of virulence, but in intraperitoneal inoculation experiments PilA deletion mutants and wild-type bacteria were equally patogenic (61).

1.5.2.6 Secreted molecules

Iron is essential for bacterial metabolism, and since free iron is scarce in mammalian hosts, *B. pseudomallei* produces siderophores for iron acquisition. The best characterized siderophore is malleobactin, which can acquire iron from erythrocytes within the

bloodstream (246). However, the fact that strains lacking the malleobactin synthesis operon are fully virulent likely means that *B. pseudomallei* has alternate means for iron sequestration (222). On the subject of erythrocytes, some *B. pseudomallei* strains can also carry genes for active hemolysins (11). Deletion of two genes encoding for different versions of Phospholipase C decrease the cytotoxicity of *B. pseudomallei* (102). This may be due to decreased degradation of eukaryotic membranes, particularly the phagosome, and/or by disrupting eukaryotic signal transduction pathways (163)

Gram-negative quorum-sensing (QS) uses secreted acyl-homoserine lactones (AHLs) to allow communication between bacteria. Recent studies showed that the multiple QS systems encoded by *B. pseudomallei* (LuxR and LuxI homologs) affect transcription of virulence factors (196, 226). Mutation of the quorum-sensing machinery can disable production of AHLs resulting in attenuation in animal models (226, 229). The fact that these systems are cell density-dependent means that virulence factors may be upregulated when *B. pseudomallei* is in close proximity, such as in a macrophage endosome or adhered to alveolar epithelium (100). While the association of BpeAB-OprB efflux-pump and quorum-sensing has been suggested (25), there is conflicting evidence regarding this relationship (131).

1.5.2.7 Cellular factors

Formation of multinucleated giant cells (MNGCs) is contingent upon escape of *B. pseudomallei* from lysosomes into the cytosol (77, 134). T3SS effectors BipB and RpoS are required for MNGC formation (208, 227). BimA binds monomeric cellular actin and polymerizes it into a "tail" (203). This is required for actin tail formation within the cell and *bimA* mutants cannot escape to infect adjacent cells (200). Unfortunately, more

seems to be known about the factors leading to MNGC formation than the actual function it serves bacteria, although it may simply be a way to evade humoral immunity (66).

B. pseudomallei is hardy within many types of cells including macrophages (91, 139). Phagosome-lysosome fusion was slower in macrophages recovered from melioidosis patients than in those of healthy subjects, suggesting an inhibition of macrophage function by an unknown mechanism (159). The bacteria can also induce eukaryotic apoptosis (98) or it can persist inactively within cells. Currently little is known about this latent state (67). The *katC* gene encodes a catalase-peroxidase, which provides protection from various oxidants (119).

1.5.3 Transmission

The accepted major routes of transmission are inhalation and cutaneous inoculation. Mice have been used to model these possibilities and other modes. Barnes et al. (15) compared the LD_{50} of intravenous, intraperitoneal, subcutaneous, intranasal and oral inoculation. Intravenous and intraperitoneal infection required less than 14 colony forming units (CFU) in BALB/c mice (15). This low dose could be why one of the first descriptions of the disease was in needle-sharing morphine addicts in Rangoon (238). However, today such routes are rarely reported in the literature.

It should be noted that the LD_{50} differs greatly not only between routes, but also between strains and animals models. Throughout this section, the reported LD_{50} for each route of infection in BALB/c mice using NCTC 13178 as recorded by Barnes et al. will be noted in the heading. LD_{50} for C57Bl/6 mice were significantly higher but had similar trends relative to infection route (15).

1.5.3.1 Intranasal (LD_{50} of 140 CFU) and inhalation inoculation

The intranasal route would mimic inhalation of aerosols and near-drowning events in contaminated water, and requires a very low dose (106, 157). Even more lethal is direct inhalation of aerosols (LD_{50} of 5 CFU)(111). This explains the link between outbreaks of melioidosis and natural disasters in endemic regions (*i.e.* floods, cyclones (34), and tsunamis (8, 40)), in which soil-bearing water is aerosolized. This also confirms aerosolized *B. pseudomallei* as a potential weapon of bioterrorism (173). A mouse model has been established for acute respiratory infection by inhalation of aerosols and it showed an extremely low median lethal dose (5 CFU)(111). This may be why helicopter pilots were a disproportionally large portion of American soldiers who were infected with *B. pseudomallei* during the Vietnam War despite having less direct contact with soil than ground troops (84).

1.5.3.2 Oral inoculation (LD_{50} of 7,200 CFU)

Oral inoculation can occur in humans by drinking contaminated tap water (53, 87, 88). 7,200 CFU may seem like large quantity, but within a 12 ounce glass of water that amount is visually undetectable. Outbreaks surrounding water systems are commonly due to lack of chlorination and/or leaking pipes. Oral inoculation may also account for the infection associated with near-drowning events, although based on the presentation of severe pulmonary melioidosis this is probably due to bacteria in the lungs (106, 157).

1.5.3.3 Cutaneous Inoculation (LD_{50} of 1,000 CFU)

Cutaneous inoculation is a common method among otherwise healthy individuals with no medical risk-factors (71). It will often result in a less severe manifestation. Only a small percent of the cases examined (n=58) had disseminated infection (7%) or became

septic (2%). Rice farmers are at higher risk for melioidosis (136, 206, 209, 210). This is due to increased exposure to contaminated soil or water, but more specifically may be due to exposure via multiple routes of inoculation (*e.g.* exposure of contaminated soil to bare feet or hands with broken skin (206), ingestion of dirty water or inhalation due to winds).

1.5.3.4 Human-to-human transmission

Even less is known about human-to-human transmission. While many of these putative cases cannot confidently define the source as another human, others leave little doubt that it can indeed occur, although it remains rare (58). Putative transmission has been documented several times in neonates. The suspected causes have been placental infection (1) and other environmental sources (123). When melioidosis causes mastitis, infected breast milk can contain enough *B. pseudomallei* to infect a nursing infant (123, 162). Evidence for horizontal transmission was also seen with an American Vietnam War veteran, whose wife developed hemagglutination titers of >640, which is a level indicative of infection. While his semen was negative, the man's prostatic secretions were culture positive for *B. pseudomallei* (128).

1.5.3.5 Laboratory transmission

Laboratory workers are at increased risk due to close contact with high concentrations of bacteria grown for clinical/diagnostic or research purposes. Cases of laboratory-acquired melioidosis include sonication of incorrectly identified *B. pseudomallei* (180) and a spill during centrifugation (75). Risk of infection/exposure with *Burkholderia* species can increase with poor laboratory practices, such as not wearing gloves (23) or sniffing culture plates when pathogens are suspected (22).

1.5.4 Diagnosis

Although the mortality rate was upwards of 80% 20 years ago (237), faster and more accurate diagnostic tests and advances in treatment options have reduced that number to under 10% in some places (54). There is a direct correlation between speed of diagnosis and prognosis (218). For this reason rapid diagnosis can be as important as antibiotic efficacy. Many studies have compared serological, molecular and biochemical tests for diagnosis (80, 122, 141, 181). Unfortunately, a major roadblock for development of new diagnostic tools is actually field testing them, and reported successes without widespread clinical use should be taken with a grain of salt (32, 150). It is one thing for a trained researcher to read a test in a well-resourced laboratory and another for a clinician in a remote town without adequate laboratory facilities to do the same.

1.5.4.1 Gold standard: Classical microbiology

Since its inception, culture on Ashdown agar has been the most trusted means of melioidosis diagnosis (10). The selectivity comes from using crystal violet, neutral red and gentamicin. Although other media have been used (65, 232), Ashdown media remains the gold standard (150, 243). The vast majority of *B. pseudomallei* isolates are resistant to aminoglycosides (187) due to expression of a multidrug efflux pump (45), thus allowing use of gentamicin for selection. However, a small number of isolates (roughly 1/1,000) are susceptible and therefore may not grow on Ashdown media (222). This number could be higher since Gm^s isolates may be missed in routine screening.

Isolation and culture of bacteria from a melioidosis patient is not always achievable, but this is the most reliable confirmatory test. Abscesses are relatively simple to sample from, and throat swabs and sputum are helpful in cases of respiratory melioidosis (35, 85,

245). Bacteria can also be cultured from blood samples, but prognosis becomes significantly worse when this is possible; the mortality rate more than doubles with >100 CFU mL^{-1} in the blood (231).

It should be noted that this "gold standard" is not perfect and that sensitivity is lacking. Statistical evidence suggests that up to 40% of melioidosis cases are missed when using culture alone as a guide (113). While the specificity and confidence of definitive diagnosis is much higher when culturing the bacteria than other tests, this trusted system requires a second thought. Furthermore, accepting a flawed standard as 100% accurate may have led to decreased efficacy of other diagnostic tests (113). For example, if a novel diagnostic tool were to identify a case of melioidosis that the gold standard missed, that result would be counted as a "false positives". This would unfairly lower the specificity value of the new test and shed a poor light on its utility.

If a culture is obtained, biochemical and classical microbiology tests beyond Ashdown agar can be used to differentiate *B. pseudomallei* from other pathogens. Multi-tests (*e.g.* API 20NE) and automated instruments (*e.g.* VITEK1 and VITEK2) have been used with varied success (56, 122, 235), but costs are much greater than Ashdown media. The API 20NE can identify *B. pseudomallei* with 99% sensitivity and 99% specificity, but can misidentify it as *Chromobacterium violaceum* and other less virulent/pathogenic bacteria (5). This was also seen during other studies (86) and may result in ineffective treatment plans.

1.5.4.2 Serology

Serological tests often use either an indirect hemagglutination assay (IHA), an immunochromatographic test or an enzyme-linked immunosorbent assay (ELISA)

(reviewed in (32)). Unfortunately, endemic regions see up to half of their populations having high background serological reactivity, probably from asymptomatic prior exposure or contact with soil containing related *Burkholderia* spp. (99). The rate is highest among youth, at 60-70% in Thailand (242). To further complicate things, it is suggested that melioidosis occurs only once for every 4,600 antibody-producing exposures, creating more possible false positives (36). In point of fact, an estimated 225,000 US Vietnam veterans had serology indicative of melioidosis infection (47), but only a handful of cases of melioidosis have been identified (17, 42, 101, 128). Although this could be partially attributed to limited awareness of melioidosis, this massive lack of parity may speak to the prevalence of false positive serology. One problem is balancing sensitivity and specificity, which seem to be mutually exclusive in antibody-based detection assays (189).

In an attempt to fix this, a massive protein microarray (~1,200 protein spots) was used to screen sera (n=747) from melioidosis patients and uninfected persons from Singapore and Thailand, where environmental exposure is prevalent, and elsewhere. Of the 170 spots significantly higher in positive sera, 10 proteins (mainly chaperonins and cell-surface proteins) were tested in a nitrocellulose-based assay for a rapid diagnostic test. This test, while more accurate than current serological tests, is still not 100% efficient (64), but again, the standard may be flawed.

1.5.4.3 Antigen detection

Cross-reactivity between *B. thailandensis* and *B. pseudomallei* may contribute to false positives in antigen detection (72, 158). However, there are antigenic differences that make *B. pseudomallei* stand out with immunofluorescence testing (190). Latex

agglutination targeting *B. pseudomallei* LPS had 100% specificity and 97% sensitivity (59). This system's diagnostic power has been confirmed independently, but it will not be readily available to the clinical community until production/commercial costs are lower (5).

1.5.4.4 Nucleic acid diagnostic systems

As early as 1994, DNA-based probes were used, but with limited success, partially because of the close relationship between *B. pseudomallei* and *B. thailandensis* (182). Single point mutations in the 16S rRNA region can differentiate *B. pseudomallei* and *B. mallei* (70), but both are responsible for morbid infections and have similar resistance profiles, so clinically this does not help (214). Fluorescent hybridization probes in real-time PCR machines is highly accurate, but it requires 300-3000 genome equivalents for detection (220) but because patients with >100 CFU/mL in the blood are nearly impossible to treat (96% mortality) and ~50% of melioidosis cases don't have bacteremia (231) enrichment is needed from blood or biopsy cultures. Microarray has been used to identify a specimen, as well as its virulence and resistance profiles in *Staphylococcus aureus*, but it also requires culturing to obtain enough bacterial DNA for testing (147). Microarray equipment and reagents are also expensive, and the technology is therefore not feasible for *B. pseudomallei* identification in most endemic areas.

Powerful bioinformatic techniques were employed to design the latest molecular test. By analyzing many sequenced genomes from *B. pseudomallei, B. mallei, B. oklahomensis* and other related bacteria, Bowers et al. selected a region that was well conserved in *B. pseudomallei* and *B. mallei* and designed real-time PCR primers. A product was only obtained from *B. pseudomallei* and *B. mallei*, and could be detected

reliably with as little as 100 genome equivalents. A Taqman probe can then be utilized to differentiate these two species from each other via a single nucleotide polymorphism with 100% accuracy (19). Despite the impressive increase in sensitivity and specificity, the need for expensive equipment and perishable reagents means limited utility in endemic areas, and like other nucleic acid-based tests, it may require culturing.

1.5.5 Risk factors

Large sets of patient histories from varying socio-economic backgrounds have allowed epidemiologists to define multiple risk factors based on health and lifestyle. Contact with soil, especially barefoot, is a risk factor (206, 209). Additionally, heavy drinkers, defined as those who have >5 (male) or >3 (females) alcoholic drinks daily, have increased risk of developing melioidosis (54).

Medical risk factors include diabetes (78), cystic fibrosis (142), renal disease, chemotherapy, malignancy and a history of tuberculosis (209). Being a male also tends to increase one's chances of getting melioidosis by ~2.4-fold (52, 209). Interestingly, acquired immunodeficiency syndrome (AIDS) was not a significant risk factor (41) although immunosuppressive therapy, such as after organ transplants, was a factor (54).

1.5.6 Treatment of melioidosis

There are two phases of treatment to effectively clear the bacteria; intensive therapy and eradication. Although there are several variations, current recommendations are typically set at more than 10 days of an intravenous β-lactam, often ceftazidime, followed by 12-20 weeks of oral trimethoprim-sulfamethoxazole with/without doxycycline, (52, 151, 236). (Note: Antibiotic resistance in *B. pseudomallei* will be discussed in depth in Chapter 2.)

1.5.6.1 Intensive phase

Until 1989, conventional primary treatment for melioidosis consisted of chloramphenicol, doxycycline and co-trimoxazole, which resulted in a mortality rate of roughly 80%. By switching to the cephalosporin ceftazidime, physicians were able to decrease the mortality rate to 43% (237). A similar study using the conventional treatment versus ceftazidime combined with co-trimoxazole showed an even greater change from the older therapy, especially in patients with disseminated septicemia (82% vs 31% mortality)(198). Later it was shown that co-trimoxazole did not have a significant effect in ceftazidime therapy (39), so the 31% vs. 43% may have been due to other factors. This is despite the presence of functional β-lactamases (82, 116).

Other β-lactams and combinations thereof have also been tested. In a comparative test between ceftazidime and co-amoxiclav (amoxicillin plus clavulanic acid) treatment, there was no difference in mortality, but the latter required a change in therapy more frequently (211). Carbapenems, such as imipenem and meropenem, have a faster time-kill compared to ceftazidime (194). While this better bactericidal activity may not affect survival (ceftazidime and imipenem treatment were equal), the advantage may show itself through a decreased failure rate for imipenem (186). Still, the prohibitive cost of imipenem and meropenem make ceftazidime the more frequent choice in most endemic regions (30, 32, 33). Due to the high mortality rate within 24 hours of arrival to the hospital (54), it has been suggested that intensive treatment start immediately in regions with high incidence rates until evidence points away from melioidosis (113).

1.5.6.2 Eradication phase

Because *B. pseudomallei* can establish latent infections (67), eradication therapy is essential for complete infection clearing. A multifaceted therapy of chloramphenicol, doxycycline and co-trimoxazole is more effective in eradication than co-amoxiclav (161) or doxycycline monotherapy (28). More recently, it was shown that doxycycline and co-trimoxazole eradication therapy was better than ciprofloxacin plus azithromycin (38) and does not benefit from the addition of chloramphenicol (27). When no advantage is gained from added antibiotics, they should not be used in order to avoid negative side-effects, extra costs and possible side effects.

1.5.6.3 Relapse

Relapse occurs in a significant portion of patients, 13% in a 1989-1999 Australian study with 2% having multiple relapses (51). More recently the rate has been cut to 5-9%, probably due to better eradication therapy (54, 112). Monotherapy of fluoroquinolones or doxycycline for eradication resulted in higher relapse rates (>20%) (29, 112), as did ciprofloxacin-azithromycin eradication therapy (38). Shorter eradication regimen (112) and/or poor compliance with eradication therapy also increase relapse rate (51, 161). Poor compliance is more frequent when adverse side-effects from the antibiotic regimen are common, as seen with doxycycline (28). Other risk factors for relapse include immunosuppression (51), diabetes (54) and having a multifocal disease, especially when bacteria are present in blood or urine (112). When relapse does occur, the strain can be more resistant than the initial infecting strain (90, 183).

1.5.6.4 Therapies of the future

Reinvigorating currently ineffective antibiotics can be accomplished by disabling the resistance determinant. Efflux pump inhibitors (EPIs) hold great promise for re-opening the physician's bag of antibiotics when multidrug efflux is involved (251). Inhibiting multidrug efflux pumps would increase susceptibility to many drug classes, as shown through *in vitro* testing of inhibition assays and pump deletion mutants (45, 131). However, many effective EPIs use disruption of the proton gradient as a mechanism of inhibition, making them toxic to eukaryotic cells and therefore clinically irrelevant (117, 167). Other EPIs act as clogs in the pump by attaching to the binding pocket and prohibiting drugs from associating with the pump (251). Within *B. pseudomallei*, various EPIs allowed for >1,000-fold decrease in MIC for aminoglycosides and macrolides (26). In a primate infection model, EPIs can improve treatment of *P. aeruginosa* by impairing the MexAB-OprM pump, which increases intracellular concentrations thus potentiating the antibiotics (250). Still, they are useful tools in describing the prevalence of multidrug efflux pumps and their effect on resistance of populations *in vitro* (104).

Adoption of new drug targets may increase our antibiotic arsenals. Latency is a clinical problem, often resulting in relapse (101). Isocitrate lyase is a persistence factor and as such, targeting the enzyme should induce an active metabolism. Unfortunately, this results in more active virulence and rapid death in small animal chronic infection models when not used in conjunction with other antibiotic (179). This result strongly supports continued use of traditional antibiotics in conjunction with novel classes and suggests that researchers must be wary of drugs that, while effective at inhibiting bacteria, may induce more intense pathogenicity (62).

Targeting virulence factors may also help treatment in combined therapy. Various compounds have been shown to inhibit the transfer of T3SS effector proteins from *P. aeruginosa* to mammalian cell lines *in vitro*, which could result in decreased virulence and evasion of macrophages (3). Oligosaccharides with moieties targeted by bacterial adhesion elements can inhibit attachment, and therefore virulence, in respiratory tract infections (217). Although these routes are not necessarily bactericidal, they could give the immune system a better chance to clear infections.

1.5.7 Prophylaxis

1.5.7.1 Vaccines

Currently there are no melioidosis vaccines. Attenuated live vaccine strains such as *B. pseudomallei* strains with auxotrophies have been tested (*ilvI, aroB* and *serC* single deletions) with some success, but studies were not designed uniformly and are difficult to compare (178, 219). These studies did highlight the difficulty in comparing animal models to human disease. A thorough review of melioidosis vaccines was recently published by Sarkar-Tyson and Titball (178). While they acknowledge that live attenuated vaccines showed the strongest protective immunity, they feared latency and incomplete clearance of the bacteria. They instead supported the testing of heat-inactivated immunogens, although they acknowledge that these *Burkholderia* sub-cellular antigens or inactivated whole cell vaccines have also been tested with limited success (178). The fear of non-clearance and resurgence may be less of an issue with the newly described avirulent Bp82 strain, described in section 1.1.1 (155).

1.5.7.2 Post-exposure prophylaxis

Of major interest for potentiating the effect of attack with biowarefare weapons is finding the most effective means of prophylaxis after exposure to the agent. Although differences in experimental procedures prohibit the direct comparison of many drugs tested, certain fluoroquinolones, macrolides and tetracyclines have also shown certain levels of prophylactic protection. This demonstrates that even some drugs that are normally less effective in treating full-blown infection may be employed to slow the bacteria's course of infection and improve clearance of the bacteria (97, 172, 205).

When testing clinically used drugs for prophylactic abilities in a murine melioidosis model, trimethoprim-sulfamethoxazole was found to be 100% protective when supplied either pre-exposure or up to 24 hours post-exposure. Doxycycline was effective to a lesser extent, while co-amoxiclav was all but ineffective (192).

Cationic liposome DNA complex (CLDC) protects 100% when introduced prior to exposure in a murine melioidosis model, and was 40% effective immediately upon exposure. However, the benefit of CLDC is lost if given 24 hours post-exposure (74). Of greater note is that CLDC can be combined with ceftazidime at sub-therapeutic levels to increase efficacy of both compounds, as can immunotherapy with recombinant INF-γ in a post-exposure capacity (156), which is in line with studies showing the importance of INF-γ in response to infection (176). As a side note, protection can be partially transferred when mice passively receive anti-*B. pseudomallei* monoclonal antibodies prior to infection, but the study did not test post-exposure utility of such a technique (92).

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CHAPTER 2:

ANTIBIOTICS AND RESISTANCE

2.1 Introduction to Antibiotic Compounds

2.1.1 A brief history of antimicrobials

Depending on one's definition, we could say that the use of pharmaceuticals began in prehistoric times with herbalist shaman, apothecaries and plants. The ancient Greeks applied mold to treat infected wounds (23). The 19th century produced a class of antimicrobial compounds known as "antiseptics". These compounds included hypochlorite and iodine tincture and were used to treat external wounds. However, these compounds could not be used systemically in patients. A different class of antimicrobials was needed (23).

The occurrence of "antibiosis", the process of one organism inhibiting the growth of another organism, was first described in 1877 by Louis Pasteur. This phenomenon was further illustrated in 1929 by Alexander Fleming when he characterized the presence of an anti-*Staphylococcus* substance produced by the fungus *Penicillium notatum* (111). The isolation of a natural, antibiotic compound with no effect on human health revolutionized healthcare.

However, before penicillin were the sulfonamides. Prontosil was the first systematic antibiotic to be regularly used medicinally, beginning in the early 1930's (80). The

compound was tested against bacterial infection because researchers believed that the dye's ability to bind proteins in wool would enable it to bind microorganisms as well. The antimicrobial mechanisms were fallible, but the result was still an effective drug (111). The treatment was so revolutionary, that many companies began producing and marketing variations of the drug without oversight or testing. In 1937 the industry received a tragic wake-up call in the form of 93 deaths due to use of a toxic compound, ethylene glycol, in one elixir formation. The need for a regulatory agency became clear (180).

It was years later that the term "antibiotics" was coined (179). Since then multiple classes of antibiotics have been found, modified or synthesized for use against a wide variety of antimicrobial drug targets. In Great Britain, use of early antibiotics is largely responsible for the almost doubling of life expectancy between 1960 and 1967 from 42 years to 70 years (23).

2.1.2 Antibiotic classes, targets and activity

Effective clinical antibiotics take advantage of evolutionary differences between target molecules and cellular machinery in pathogens and hosts. The most valuable compounds are those that do not target host processes while acting specifically against microorganisms. I will briefly touch on some targets and antibiotic mechanisms pertaining to bacteria.

2.1.2.1 Protein synthesis (translation)

The translation of mRNA to proteins by the ribosome is essential for most cellular functions. Fortunately for modern medicine, the bacterial ribosome is significantly

different from the eukaryotic ribosome, making it an excellent target for antibiotic therapy. Protein synthesis inhibitors are bacteriostatic agents, meaning they stop or slow growth and reproduction (111).

There are several classes of protein synthesis inhibiting antibiotics and several mechanisms by which they act. Aminoglycosides inhibit formation of the 30S ribosomal subunit by binding the A-site of 16S rRNA, thereby stopping translation before it starts (62, 115). Tetracyclines bind to two distinct regions of the 30S subunit and prevent translation by blocking tRNA from binding to the A-site or preventing binding of RF-1 and RF-2 during termination (20). Macrolides inhibit the transpeptidation reaction and translocation processes by binding the 50S ribosomal subunit (13). Chloramphenicol binds the peptidyltransferase center of the 50S subunit, thereby inhibiting elongation (152). Finally, translation can be disrupted by binding tRNA synthetases and depriving growing peptides of amino acids (82).

2.1.2.2 Tetrahydrofolic acid biosynthesis

Unlike animals, which code for tetrahydrofolic acid active-transport uptake proteins, bacteria need to synthesize their own tetrahydrofolic acid for amino acid and nucleic acid metabolism. Along the biosynthetic pathways, sulfonamides (*e.g.* sulfamethoxazole) inhibit tetrahydropteroic acid synthetase by mimicking *para*-aminobenzoic acid and binding dihydropteroate synthetase. Benzylpyrimidines (*e.g.* trimethoprim) inhibit dihydrofolate reductase by a similar mechanisms (175). By disrupting either of these two steps, nucleotides are eventually depleted and DNA replication stalls. Trimethoprim is often used with sulfamethoxazole (1:5 ratio) in a two pronged attack against nucleic acid synthesis. By doing so, multi-drug treatment would remain effective despite

development of spontaneous resistance to one compound (111). For example, tests in 1989 showed 97% resistance to trimethoprim and 25% resistance to sulfamethoxazole in *B. pseudomallei* (48). When in used in combination, Tmp-Smx resistance levels were recorded to be 18.6% (158). In the words of the Nobel laureate and pioneer in the field of chemotherapy Paul Ehrlich, "March apart but fight combined" (56).

While these drugs are typically considered bacteriostatic agents, trimethoprim can have a secondary killing mechanism. By starving the cell of thymine, the *mazEF* toxin-antitoxin system is activated, resulting in cell death (demonstrated by deletion of *mazEF* mutants having increased viability after exposure to trimethoprim). The discovery of this novel suicide system may even lead to new drug classes (148).

2.1.2.3 DNA/RNA machinery (replication and transcription)

Disruption of the DNA or RNA machinery can arrest cellular functions. Quinolones inhibit DNA replication by preventing the release of stress in super-coiled DNA during replication. They act by binding either the A subunit of DNA gyrase (topoisomerase II)(130) or topoisomerase IV (81) and drugs incorporating itself into the enzyme-DNA pocket. This disables the apparatus by forming hydrogen bonds with unpaired DNA bases. Eventually this can lead to double stranded breaks (111).

DNA-dependent RNA polymerase can be inhibited by rifampin or small peptides, effectively halting transcription. This acts in a similar fashion to translation inhibitors by disabling the bacteria's ability to produce new gene products, thereby halting cellular processes (3, 111).

2.1.2.4 Membrane permeability

Polymyxins B and E are toxic to many gram negative bacteria (163, 187). They consist of complex cationic surface-active compounds containing fatty acid and ammonium groups (111). The fatty acids interact with the hydrophobic portion of the outer membrane while ammonium groups interact with lipopolysaccharides. This displaces cations from membrane lipids resulting in disorder of the outer membrane and increased permeability. This effectively turns the once protective outer membrane into a sieve, disrupting cellular integrity. This allows periplasmic components to leak outside of the cell and potentially toxic compounds, including other antibiotics, to enter the cell more quickly. Of course the spectrum for activity is limited to species with an outer-membrane and polymyxins are therefore inactive against gram positive bacteria (24).

2.1.2.5 Cell wall synthesis

Most bacteria require structure to maintain cell shape and viability, especially in non-isotonic conditions. This structure comes from a cell wall composed of peptidoglycan; a lattice of peptides cross-linked to each other providing osmotic stability to bacteria (33). With the exception of *Mycoplasma*, a genus lacking a cell wall, when a bacterium is not able to generate a cell wall or that wall is destroyed, the result is a spheroplast. The cell is then held together only by fragile phospholipids bilayers. In this state, bacteria are very sensitive to osmotic gradients between the cytosol and extracellular space. In a hypertonic environment, the cell will lose water, contract and lyse. In a hypotonic medium, the cell will gain water, expand and rupture. For these reasons and the fact that animals do not have cell walls, peptidoglycan is a safe and effective target for antibiotics.

One class of drugs that targets cell wall synthesis consists of the β-lactams (Figure 2-1). These drugs bind to penicillin-binding-proteins (PBPs), named for their ability to bind penicillin (160). The exact bactericidal nature of β-lactams is still unknown, but it appears to be more complicated than a simple difference in osmotic pressure (15). It has been shown that inhibiting protein synthesis prior to treatment with β-lactams reduces killing effects, and that the disruption of bacterial autolysins is at least partially responsible for this diminished toxicity (145). Since then the regulation of metabolic PBPs and catalytic autolysins have been described, but the precise mechanisms for their bactericidal action remain unclear (15). Because they attack machinery responsible for cell wall construction and rearrangement, β-lactams require an actively growing cell to be bactericidal (168). β-lactams are described in more detail in section 2.3.1.

2.2 General resistance mechanisms

Resistance occurs when bacteria compromise inhibition by an antibiotic at a certain extracellular concentration, thereby allowing them to maintain cellular function. For as long as there have been naturally occurring antibiotics, there have been resistance determinants. β-lactamases have been in existence long before the use of β-lactams as therapeutics. This point was proven by the discovery of for penicillin-inactivating enzymes that fit into modern day classification systems found within sealed jars containing soil samples from 1689 (138). More evidence of this is the characterization of β-lactamase producing bacteria found deep in the Alaskan tundra, far from any penicillin-prescribing physician (5). Furthermore, *in silico* analysis of soil meta-genomes (genetic material from both culturable and unculturable organisms) provides evidence for even more antimicrobial resistance mechanisms (46).

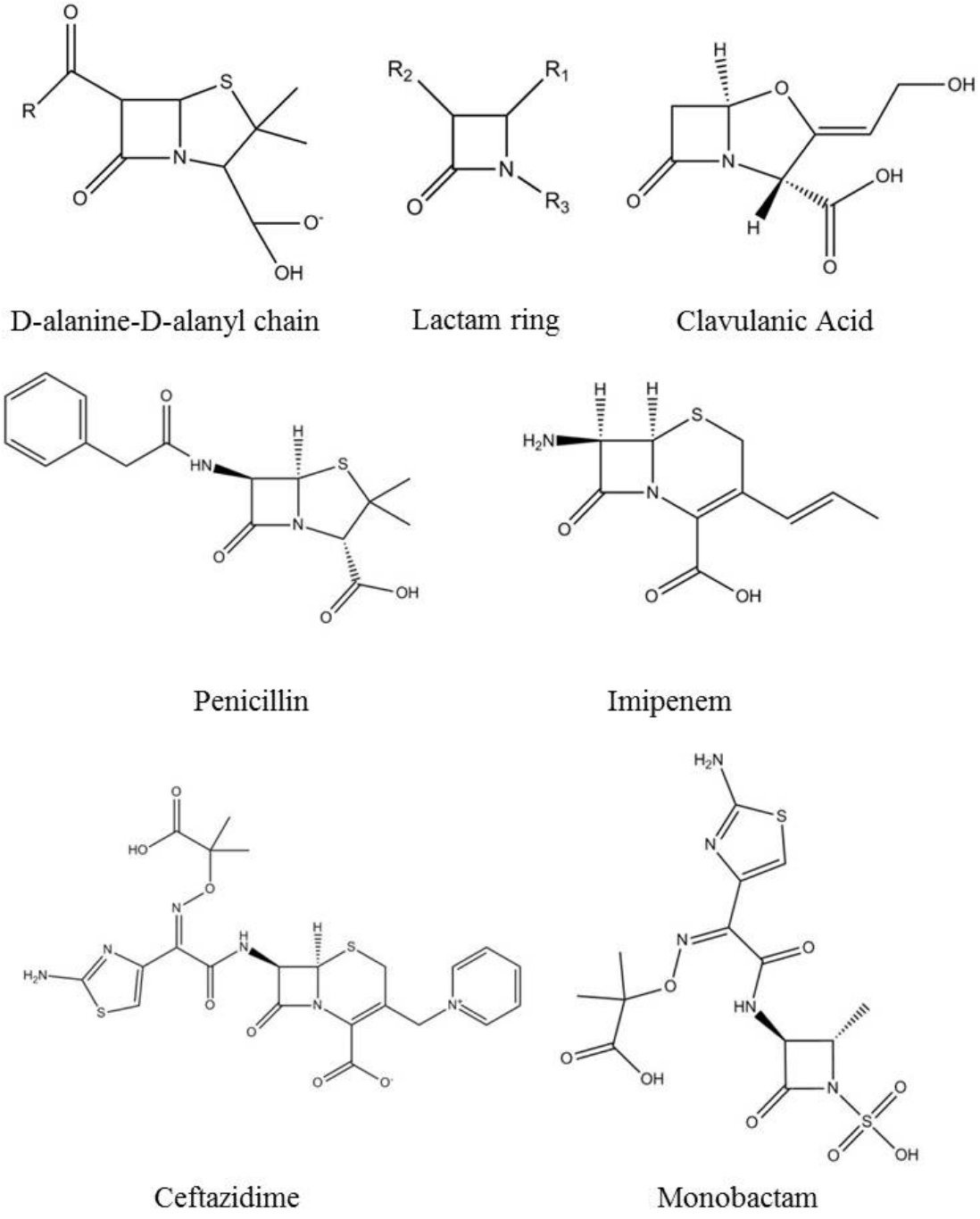


Figure 2-1. Chemical structures of various β-lactams and related molecules. Structures were drawn with Chemdraw (CambridgeSoft, Cambridge, MA). R, peptidyl side chain of *N*-acetylmuramic acid; R_1, R_2 and R_3, substitution groups.

Upon further reflection, the fact that these mechanisms are already so widespread should come as no surprise. At the turn of the 21st century roughly 80% of clinical antibiotics were either naturally-occurring compounds, or their semi-synthetic derivatives, made by saprophytic microorganisms (90). In order to accommodate production of a lethal compound, the producing bacteria must ensure that the antibiotic will not act against them. A fungus, such as *Penicillium,* may easily produce a β-lactam without any toxic effect because they lack the compound's target. However, a bacterium producing a bacterial ribosomal inhibitor needs a resistance mechanism for protecting itself from the antibiotic (16). What is more impressive and surprising is the resistance to purely synthetic compounds on their first encounter with the drugs (45).

Contrary to historical belief, resistance to antibiotics is born from random mutation whose presence and maintenance in the bacterial population is then selected for by the presence of antibiotics. Initially, it was thought that growing bacteria on media with antimicrobials produced spontaneous resistance. Lederberg et al. showed that streptomycin resistance occurred randomly without selection, meaning these mutations are not forced by outside pressure (98). In other words, use of these compounds clinically does not create the resistance determinants, but it does select for their propagation (134).

Bacteria may become resistant to antibiotics through several mechanisms. These mechanisms generally fall into one of three categories; modifying the antibiotic, decreasing the intracellular concentration of the antibiotic, and mutating the target molecular or its expression levels (63).

2.2.1 Modifying or destroying the threat

Bacteria that are resistant to a defined class of antibiotic compounds frequently encode an antibiotic-modifying enzyme. By binding a group or molecule to the drug, they may sterically disrupt the compound's inhibitory abilities. For example, chloramphenicol can be deactivated by addition of acetyl or phosphate groups by acetyltransferase (99) and phosphotransferase (84) activities, respectively. Similar mechanisms are also used for aminoglycoside inactivation (155). TetX provides resistance to multiple tetracycline-based antibiotics by adding a hydroxyl group to the drug and limiting its ability to bind the bacterial ribosome (191). *B. pseudomallei* encodes for at least one putative aminoglycoside acetyltransferase (78).

Antibiotic activity can also be eliminated by cleaving necessary bonds, especially within the pharmacophore (*i.e.* the functional unit of the antibiotic). β-lactamases cleave the lactam ring in β-lactams, and in doing so are one of the most clinically important antibiotic resistance mechanisms (29). This family of enzymes will be discussed in greater depth in section 2.3.2 as *B. pseudomallei* codes for seven putative β-lactamases; one Ambler class A, five class B and one class D (78).

2.2.2 Removing or excluding the threat

Multidrug efflux pumps can provide resistance to a wide array of potentially lethal compounds and antibiotics. Efflux substrates include, amongst others, aminoglycosides, β-lactams, bile salts, crystal violet, ethidium bromide, macrolides, fluoroquinolones, tetracyclines, sulphonamides, triclosan and trimethoprim (139). This covers a majority of the clinically relevant antimicrobials. For example, the *Pseudomonas aeruginosa*

MexAB-OprM efflux pump provides resistance to carbenicillin, penicillin G, cefoperazone, ceftriaxone, ciprofloxacin, erythromycin, tetracycline and chloramphenicol (105, 106). Although the "one-enzyme, one-substrate" rule does not apply to these pumps, most pumps have a finite spectrum of substrates for efflux, and so multiple pumps may be encoded by a bacterium in an effort to accommodate virtually all clinically significant antibiotic classes. *P. aeruginosa* chromosomally encodes at least seven documented efflux pumps (139). The substrate specificity is defined by the extramembrane loops of the inner membrane protein (*e.g.* MexB), as shown by substitutions/mutations to these loops (55). The clinical implication of efflux mediated resistance was shown in rabbit infection experiments with a *P. aeruginosa* strain over-expressing the MexAB-OprM pump. Even high concentrations of normally effective antibiotics (ticarcillin, piperacillin-tazocabtam and ceftazidime) resulted in treatment failure (19).

There are several families of efflux pumps in bacteria. Bacterial efflux pumps can be members of the ATP-binding cassette superfamily, multidrug and toxic compound extrusion family, major facilitator superfamily or small multidrug resistance family. For effective efflux in gram negative bacteria, a tripartite pump is optimal, spanning both membranes and the periplasm. This type of pump is exemplified by the resistance-nodulation-cell division (RND) superfamily, and is responsible for high levels of resistance in many clinically relevant bacteria (94). *B. pseudomallei* encodes for twelve putative RND pumps, three of which have been characterized (104, 107)

Removing the threat is made easier when a smaller amount of compound enters the cell in the first place. This can be affected by outer membrane permeability. The rate of

diffusion across the outer membrane may be based on compound hydrophobicity (193). Gram negative bacteria have the added benefit of lipopolysaccharides (LPS) on the outer membrane, which act as an effective permability barrier (94, 135). Charged LPS groups can exclude lipophilic drugs that would be able to pass through the phospholipid bilayer easily (111). By decreasing permeability and keeping harmful compounds out of the cell in the first place, the bacteria decrease the requirements for other detoxification mechanisms within the cell. This synergistic effect is a key attribute of many multi-drug resistant bacteria (154). The hardy LPS of *B. pseudomallei* has already been shown to provide higher levels of resistance to aminoglycosides and polymyxin B (26).

Another factor influencing permeability can be the number and type of membrane porins. Porins are responsible for allowing entry of numerous antibiotics, including chloramphenicol, fluoroquinolones and β-lactams, past the outer membrane (94). This effectively negates the protective effect of LPS. This is demonstrated by imipenem susceptibility in *P. aeruginosa*. Imipenem enters the cell by way of the porin OprD, but bacteria can become resistant by down-regulating expression of this porin, thereby decreasing the periplasmic imipenem concentrations (61).

The bacterial biofilm lifestyle is also contributing antibiotic resistance factor. Bacteria growing in a biofilm are often much more resistant than their planktonic counterparts, but the precise underlying mechanisms are not completely understood (8). Some have suggested that much of the resistance is due to the biofilm matrix itself. They propose that the thick exo-polysaccharide, DNA and protein matrix slow permeation of the threat, and therefore decreasing its contact with the cell, as reviewed by Anderson and O'Toole (8). However, studies have found that this purported slower antibiotic

penetration did not significantly inhibit antimicrobial activity (181). Others propose that instead of excluding antibiotics, biofilms can cause elevated concentrations of cell secreted inactivating enzymes. In support of this notion, biofilm matrices have been shown to retain β-lactamases in higher concentrations (149). Furthermore, biofilm and β-lactamase producing bacteria can protect other species in the vicinity *in vitro* and clinically by secreting these enzymes and decreasing the local concentration of functional β-lactams (21). A readily accepted antibiotic resistance feature of biofilms is its association with changes in gene expression. Among the genes upregulated in biofilms are multidrug efflux pumps (8). *B. pseudomallei* also produces biofilms under the proper conditions and biofilm growth has been associated with increased viability in the presence of up to 200-fold the MIC of planktonic cells for ceftazidime and trimethoprim-sulfamethoxazole (178).

2.2.3 Modifying the target

Antibiotic targets may possess decreased affinity for an inhibitory compound through amino acid or other substitutions in the target itself. These mutations may remove the antibiotic binding site, as seen in bacterial ribosomes with a point mutation in the 23S rRNA that causes resistance to ketolides (189). In *Burkholderia*, point mutations in DNA gyrase were implicated in fluoroquinolone resistance (176). Point mutations in PBPs can account for decreased affinity for antibiotics, and therefore increased resistance to β-lactam antibiotics (71, 95). However, these point mutations may come at the price of the target molecule having decreased efficiency, such as the case of β-lactam resistance caused by a mutated PBP2 with decreased enzymatic activity (195).

A target can also be modified by a secondary molecule. Secondary molecules (*i.e.* not the antibiotic target molecule) can bind to the target in a non-inhibitory way, but prevent antibiotic binding. The Tet(O) and Tet(M) proteins bind the bacterial ribosome in this way to either changing the conformation and preventing inhibition by tetracycline (43) or hiding the drug binding site (50).

2.2.4 Problems associated with antibiotic resistance

In clinical strains antibiotic resistance is often inadvertently selected for by prescription of ineffective antibiotics (41) or the premature termination of drug therapy by patients (86). Both situations involve incomplete eradication of a pathogen, thereby allowing the fittest organisms to proliferate and pass along their superior genetics. This makes "humans the world's greatest evolutionary force" for clinically relevant organisms according to the evolutionary biologist Stephen Palumbi (134). However, spontaneous antibiotic resistance has emerged during the course of treatment (48, 147, 172). It is therefore important to continuously examine susceptibility profiles during treatment in order to detect resistance and alter antibiotic regimens in a proactive manner (*i.e.* change treatment options before outward clinical presentations re-present themselves and patient's health deteriorates)(87).

Even before their widespread use in a clinical setting, the need for total sterilization was observed. In 1917, Akatsu and Noguchi proposed that barely sufficient levels of antimicrobials breed resistance in the causative agent of syphilis, *Treponema pallidum* (4). This was based on the observation that patients who were previously treated for syphilis were more difficult to cure. They showed this *in vitro* with arsenic, calling the progeny "arsenic-fast" strains. Seven "generations" of progressively increasing drug

concentrations produced an average of 5-fold greater "fastness". However, not all strains were able to increase resistance levels (4), indicating the necessary mutations did not occur in these samples.

2.2.5 Transfer of resistance mechanisms

Resistance mechanisms can be propagated by multiple mechanisms, although some methods are more efficient than others. Low level β-lactam resistance can be conferred by PBP mutations and these mutations can be spread between strains by natural transformation (*i.e.* the uptake and incorporation of naked DNA from the environment)(33). A more elegant proliferation strategy involves transfer of resistance determinants on mobile genetic elements, such as transposons, integrons and plasmids (155). These elements can contain either single or multiple resistance determinants to a wide range of antibiotics (44, 100), including but not limited to tetracycline (185), chloramphenicol (64) and macrolides (184). The speed at which new antibiotic resistance determinants are disseminated is alarming. This was seen by the emergence of plasmid-based cephalosporinases within two years of novel cephalosporin use in the clinic (91).

2.3 β-lactams and resistance mechanisms

β-lactams are extremely diverse in the size and nature of their side groups (170). Many different side chains change the interaction capabilities with both PBPs and β-lactamases, but the pharmacophore β-lactam ring remains the same. Resistance to β-lactams can arise from any of the mechanisms mentioned in section 2.2, although enzymatic inactivation by way of β-lactamases is most common (63).

2.3.1 β-lactams and their targets

The first studies of penicillin, the prototypical β-lactam, in humans concluded in 1941 (2). In the following years physicians began successfully producing the penicillin extracts themselves for clinical use (54). At the turn of the 21st century, β-lactams made up 60% of the global antibiotic market. Seven of the top ten antibiotics sold in the mid-1990's were β-lactams (51). These figures speak to the efficacy and diversity of β-lactams. β-lactams work by deactivating PBPs, so named for their tendency to bind penicillin. These proteins were named for a notable characteristic rather than their function.

2.3.1.1 Penicillin-binding protein (PBP) classes and functions

There are multiple classes of PBP, defined by their molecular weight and function. Although the function of each PBP is relatively conserved throughout the bacterial kingdom, some have varying roles and importance between species. Because PBPs have been most thoroughly studied in *E. coli*, I will illustrate their roles in this bacterium.

PBP1s are the largest molecules of the group and are the main players in cell elongation (159). PBP1b is the most active PBP in the cross-linking and elongation machinery (165, 167). PBP1a polymerizes glycans and cross-links puropeptides, but a PBP1a deficient mutant can still grow well (165). The same holds true for PBP1c, which has lost the transpeptidase activity, and can only transglycosylate (151). PBP2s play a role in cell division (177). PBP2 deficient mutants were highly mecillinam resistant (159).

PBP3 (*E. coli* FstI) is required for efficient cell division (162). Although PBP3 deficient bacteria are able to multiply, the generation time is drastically longer than for

wild-type cultures, and a shift back to permissive temperatures resulted in rapid cell division. Temperature-sensitive PBP3 mutations in *E. coli* had no apparent loss in other cellular functions (*e.g.* peptidoglycan synthesis, DNA synthesis, etc.) when grown at non-permissive temperatures (161). The only lacking function of PBP3 mutants is formation of the septa required for cell division. Thus, they produced long, filamentous cells that contain more than one chromosome (18, 182).

Low molecular weight PBPs (PBP4-7) are more prevalent in bacilli than cocci and are therefore thought to play a role in cell elongation (66). PBP4, PBP5 and PBP6 primarily have carboxypeptidase activity (83). PBP4 in *S. aureus* has both β-lactamase and carboxypeptidase activity (127).

2.3.1.2 β-lactams

β-lactams target transpeptidases and carboxypeptidases thereby inhibiting cross-linking in the peptidoglycan (9, 74). β-lactams are not only the oldest of modern biological therapeutics, but they are also one of the most diverse classes of semi-synthetic chemicals. In an effort to increase the drugs' spectra of activity, binding efficiencies and resistance to degradation, medicinal chemists have extensively substituted chemical groups at nearly every position in the β-lactam pharmacophore (170). There are numerous subclasses of β-lactam drugs used clinically, including penicillins, carbapenems, monobactams and cephalosporins (85). Just as PBPs have different characteristics, β-lactams have different affinities for each PBP class (122).

One key to the success of β-lactams is the affinity and tenacity with which they bind cellular enzymes, assuring neutralization (150). This is achieved through the creation of a covalent bond (92). The β-lactam pharmacophore resembles the D-alanine-D-alanine

side chain of *N*-acetylglucosamine (NAG), a major subunit of peptidoglycan along with *N*-acetlymuramic acid (NAM)(Figure 2-2 and Figure 2-3). During peptidoglycan synthesis, enzymes bind the polypeptide side chain and remove the terminal D-alanine. This prepares the site for later transpeptidase and ultimate cross-linking to an L-Lysine on an adjacent polypeptide chain (194). On a molecular level, these events are initiated by a hydroxyl group from a serine in the enzyme's active site. This group hydrolyzes the polypeptide, releasing the D-alanine. By mimicking the D-alanine-D-alanine side chain, β-lactams readily undergo this hydrolysis. The lactam ring is opened and an acyl-enzyme intermediate is formed. Unlike the acyl-intermediate of PBP and polypeptide chain, the acyl-enzyme intermediate is very stable. The result is a bound polypeptide chain, unable to be cross-linked to other chains (170).

2.3.2 Enzymatic inactivation

The most significant mechanism for β-lactam resistance is the β-lactamase (29). From day one of clinical penicillin use, hospitals have unwittingly selected for β-lactamase producing bacteria. The first good example of this is an English hospital which saw the percent of bacteria encoding β-lactamases grow from ≤8% to ~60% in just 5 years because of heavy penicillin use in World War II (14, 114). Although this dramatic increase may have been viewed as the *de novo* emergence of β-lactamases, evidence points to the contrary, as demonstrated by the afore-mentioned penicillinase from 1689 (138). Due to selective pressures coupled with close proximity of different β-

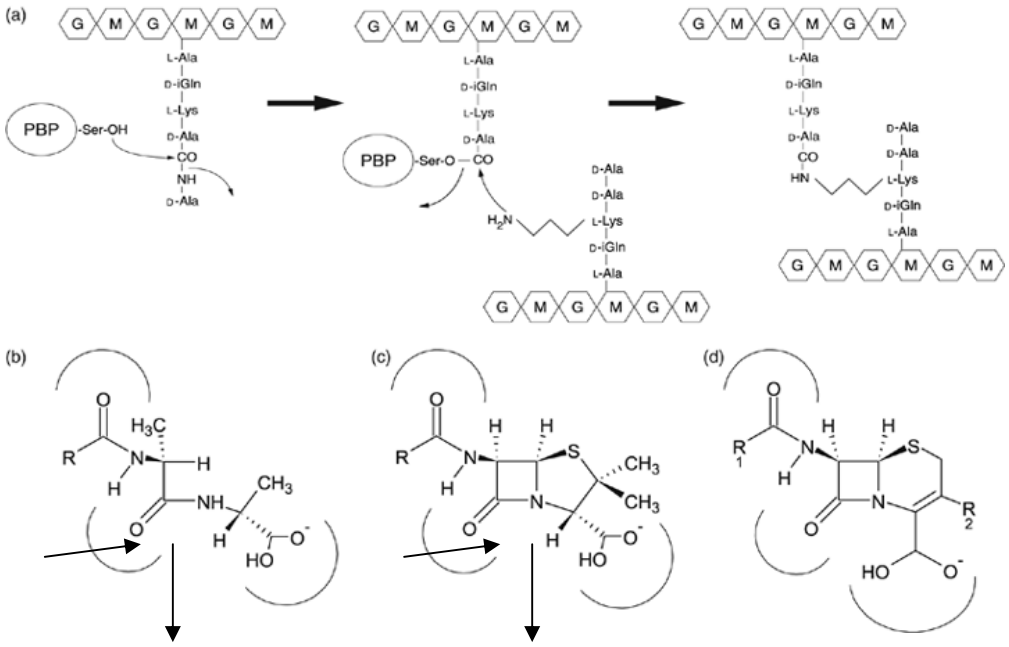


Figure 2-2. Transpeptidation of peptidoglycan in gram negative bacteria. (a) Peptidoglycan cross-linking (G = NAG; M = NAM); **(b)** *N*-Acyl-D-Alanyl-D-Alanine polypeptide; **(c)** Penicillin backbone; **(d)** Cephalosporin backbone. Arcs represent negatively charged regions. Image taken from reference **(194)**.

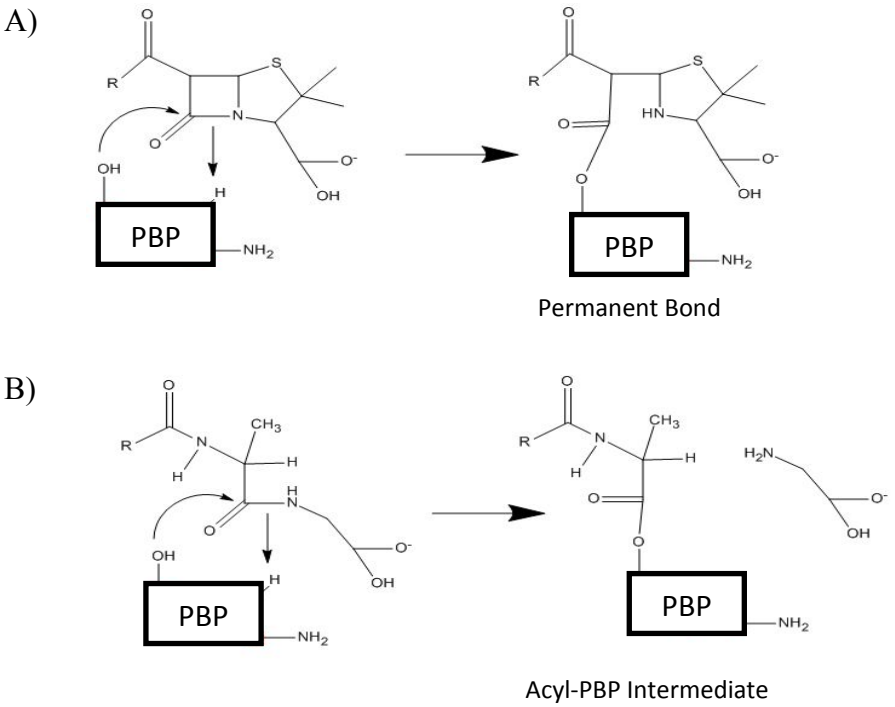


Figure 2-3. Enzymatic reactions of PBP and β-lactam or D-alanine chain. A) PBP attacking β-lactam core of penicillin-based β-lactam, forming a covalent, stable bond. **B)** PBP attacking D-alanyl-D-alanine chain, forming an acyl-PBP intermediate and releasing an alanine. (Figure drawn with Chemdraw.)

lactamase producers, especially in a hospital setting, clinical strains can carry upwards of five unique and functional β-lactamases, each with its own spectrum, creating bacterial strains resistant to virtually every class of β-lactam (59, 73, 173, 192). It is interesting to note that when β-lactamases were first observed clinically, their importance was not fully realized. Since the enzyme was found in a gram negative bacteria and penicillin was being used against gram positive bacteria, the problem was not considered to be significant (1, 28).

2.3.2.1 Mechanisms of β-lactamase activity

β-lactamases work by hydrating the amide bond within the lactam ring, effectively destroying the pharmacophore and inhibit the action of the β-lactam (67). This reaction is similar to the initial step of the inhibitory reaction of a β-lactam binding a target transpeptidase. By opening the lactam ring, an acyl-enzyme intermediate is formed, described in section 2.3.1.2 (170). The main difference between the β-lactam:PBP reaction and β-lactam:β-lactamase reaction is that the β-lactam:PBP intermediate does not allow progression to the catalytic step necessary for cross-linking and turn-over in peptidoglycan synthesis. Instead, the PBP stays acylated and is thus disfunctional. Conversely, when β-lactamases bind β-lactams, they allow efficient acylation, as with PBPs, but just as readily catalyze a second reaction to fully hydrolyze the β-lactam ring. At this point, the β-lactam is destroyed, the substrate released and the β-lactamase is ready to accept a new substrate molecule (112). Given the appropriate enzyme-substrate combination, β-lactamases can be extremely efficient enzymes, with up to 2,000 reactions per second (34).

A review of PBP and β-lactamases show highly conserved regions around the active sites (69). Although evolutionarily distant, functionally distinct and very different in amino acid sequence, a D-alanyl-D-alanine peptidase from *Streptomyces* and a β-lactamase from *Bacillus* shared great structural and active site similarities. This speaks to evolutionary power and the efficiency of β-lactam related mechanisms (89).

2.3.2.2 Classes of β-lactamases

Over the past few decades, the number of clinically relevant β-lactamases has increased substantially, roughly doubling in the past decade alone to almost 1,000 unique enzymes (29). Numerous classification systems have been established with various degrees of use (6, 27, 29, 30, 72, 118, 144). Some of these systems, particularly those that are functionally-based, seem to change every few years to accommodate novel enzymes that interact with new combinations of β-lactams (29). The most informative nomenclatures use functionality of the enzymes, such as in the Bush-Jacoby functional classification system. Each class has its own spectrum of substrate specificity, sensitivity to inhibitory compounds and amino acid sequence. First they are split by molecular subclass (Ambler class). Then they are divided by substrate specificity. β-lactam classes used for classification are cephalosporins (early generation), cephalosporins (extended-spectrum), penicillins, cloxacillin, carbenicillin and carbapenems. Finally, inhibitor resistance, to EDTA or clavulanate/tazobactum, is classified as susceptible. Combined, these divisions result in 16 classes (29).

On the other hand, the Ambler classification scheme is the simplest, using only amino acid sequence homology to categorize β-lactamases into 4 categories, up from 2 originally (6, 7, 72). Classes A, C and D have a serine at the catalytic site and class B

enzymes (metallo-β-lactamases) use Zn^{2+} as a catalyst (72). In the era of rapid sequencing capabilities, this system gives researchers a cheap and timely classification method. It also established several essential domains within the enzyme and assigned numbers to surrounding amino acids to create a universal nomenclature and allow comparison to related β-lactamases (7, 65). Still, classes A, C and D share structural homology, but little sequence homology (72).

As β-lactamases change substrate profiles by mutations, they change functional class but not Ambler class. This means that an Ambler class A β-lactamase that undergoes mutations that cause changes in substrate specificity (12, 147, 174) or increased resistance to amoxicillin-clavulanic acid (172) would remain Ambler class A, but change Bush-Jacoby classes.

Although each β-lactamase has a preferred variety of β-lactam substrates, even an inefficient substrate:β-lactamase pairing can overcome its inadequacy by hyper-expression (140). Up-regulation by promoter mutation has been shown to result in a 10-fold increase in MIC (96). Even if the enzyme does not properly inactivate the β-lactam by cleavage, the β-lactamase can serve a purpose by transiently interacting with or binding the antibiotic to effectively lower the free drug concentration, if only by a small amount (140).

2.3.3 Target mutagenesis and pathway/expression variation

Lab generated mutants showed that single amino acid substitutions in PBPs can produce low level resistance (roughly 10-fold)(95). This was later observed clinically, with the added complication that these low-level β-lactam resistance determinants were transferable (33, 116, 132). PBP3 deficient mutants had decreased inhibition by β-

lactams that preferentially bound PBP3 (furazlocillin and piperacillin)(153). In order to become resistant to multiple β-lactams, bacteria may need to acquire numerous point mutations in multiple PBP genes (52). However, PBP mutations that provide some level of resistance may come at a price. A mutant *Streptococcus pneumoniae* PBP with decreased β-lactam affinity also had decreased catalytic activity when tested *in vitro* (195), resistant *Staphylococcus aureus* with mutated PBP2a had a decreased growth rate (11), and a mutant PBP2 in *Escherichia coli* conferred increased susceptibility to osmotic shock (136).

Hakenbeck et al. suggest that bacteria are only as resistant as their most susceptible β-lactam target (71). While the weakest link theory may be true in many cases, acquisition of the non-essential PBP5 in *Enterococcus faecium* generated resistance to ampicillin because PBP5 has a low-affinity for ampicillin (143, 156). In another case, over-expressing PBP3 on a plasmid in *P. aeruginosa* conferred increased resistance to ceftazidime, which targets PBP3 (101). In these mutants over-expression or additional pathways led to increased resistance.

Bacteria may also thwart antibiotic inhibition by removing the target completely from an essential pathway. Serial passage of *Enterococcus faecium* in the presence of increasing concentrations of ampicillin increased the MIC >33,333-fold. This mutant became so resistant by altering the necessary cross-linking reaction of its PBPs from the traditional activity D-Ala(4) to D-Asx-Lys(3) transpeptidation to a novel L-Lys(3) to D-Asx-Lys(3) cross-link. Unlike cases cited in the above paragraphs, this mutant had no apparent changes in the affinities of traditional PBP to β-lactams. This suggested the emergence of a novel transpeptidase/carboxypeptidase. In this manner, the mutant

completely removed the ampicillin susceptible pathway from peptidoglycan synthesis (109).

2.3.4 Cell wall deficient forms

Cell wall deficient (CWD), or L-form, bacteria lack a peptidoglycan wall around their cytoplasmic membrane. As peptidoglycan synthesis is the target of β-lactam antibiotics, absence of a cell wall would negate any inhibitory effect the drugs provide. This has been documented with increased resistance to several β-lactams in the L-forms of *P. aeruginosa.* The same study also showed these cells to be more sensitive to tetracycline, chloramphenicol and others, presumably due to increased permeability and cell fragility (190). In line with this theory, CWD mutants are usually slow growers (113).

It has been shown that PBPs are expressed differentially through the stages of growth (*e.g.* stationary vs. mid-log) within a *Streptococcus* species. In this bacterium, PBP1 and PBP4 could not be detected during late stationary phase. It is therefore possible that β-lactams targeting these PBPs would have no effect on certain cell populations (164). This difference may account for the significant "inoculum effect" seen when testing MICs *in vitro*. When different concentrations of bacteria are used to inoculate MIC tests, a significant difference may be seen. These shifts can be in excess of differences that may be expected by a shift in bacteria to antibiotic ratio. Differences could mean an inaccurate MIC reading and improper treatment selection. This phenomenon is another reason to employ stringent and precise testing protocols (164).

CWD cells may account for another clinical observation that complicates treatment. In some cases, bacteria may not respond to the antibiotics *in vivo* despite showing

susceptibility to drugs when tested in/on media *in vitro*. In these situations, there are no mutations during treatment that result in a resistant population of bacteria, at least none that show resistance by *in vitro* testing. This was demonstrated by a case of endocarditis caused by *Staphylococcus aureus* that failed to respond to β-lactam treatment despite being sensitive *in vitro* (32).

2.3.5 Combating β-lactam resistance

2.3.5.1 Modifying the β-lactam

As previously discussed, β-lactams are very diverse. Side group substitutions are made in an effort to improve range and/or β-lactamase resistance by changing the steric interactions of β-lactams, β-lactamases and target PBPs (170). Nature already provided medicinal chemists with multiple templates and platforms on which to build new drugs.

There is currently no silver bullet for fighting bacteria. Newer compounds may have increased antibiotic activity against a particular bacterium or strain, but this improvement may come at a price. The 3rd generation cephalosporin ceftazidime has higher levels of β-lactamase resistance and killing power against gram negatives, but decreased efficacy against gram positives (170). Other β-lactams are less stable in human sera (*e.g.* meropenem (166)), oligomerize with themselves (*e.g.* imipenem (157)), or target each other for aminolysis and degradation (*e.g.* cephalosporins (125)). When compounds become more resistant to β-lactamases, they often also become less efficient at binding PBPs. This is because the ring's ability to be nucleophilically attacked not only determines hydrolysis, but also antimicrobial activity. This heralds back to the idea that the best way to interrupt a process is to closely mimic a substrate or enzyme in the pathway (170).

A compound's efficacy may be improved by increasing its pharmacokinetics, specifically its concentration within the cell. In some cases this can be done by changing the hydrophobicity of the compound. The hydrophobicity of a compound can affect the rate of permeability either through outer membrane porins or the phospholipid bilayer (128). Some ingenious semi-synthetic antibiotics are using the cells own uptake machinery against itself in a kind of "Trojan horse" scenario (25, 131). Such is the case with the new monobactam BAL30072, in which the side chain resembles a siderophore molecule. This moiety is recognized by iron-uptake systems in the bacteria and is actively imported into the cell, making it a very effective antibiotic *in vitro* (133). This compound has already shown remarkable efficacy against multi-drug resistant bacteria (e.g., *Acinetobacter baumannii* and *P. aeruginosa*)(123). A possible resistance mechanism may be to mutate/down-regulate the siderophore uptake system in an attempt to decrease uptake and intracellular concentration of the drug. However, this may be at the expense of losing iron scavenging capabilities and may decrease cellular fitness in a host.

2.3.5.2 Combinational therapy

The usefulness of otherwise ineffective β-lactam drugs can be restored by neutralizing β-lactamases with inhibitory compounds, such as clavulanic acid, tazobactam and sulbactam (119). Much like the way in which β-lactams mimic cell wall components to disrupt peptidoglycan formation, clavulanic acid inhibits β-lactamases by mimicking their target molecules; β-lactams (146). As reviewed by Drawz and Bonomo, the inhibitor molecules stick to β-lactamase groups, just as classes have specificity for certain categories of β-lactams (*i.e.* sulbactam is more effective than clavulanic acid for

certain class A enzymes, but less effective against some class D enzymes.). Inhibition is best achieved by mimicking the substrate of a target β-lactamase without being cleaved itself, thereby "distracting" the enzyme from inactivating β-lactams (53). There is evidence that carbapenems not only have β-lactam properties for disrupting PBPs, but also have β-lactamase inhibitory capabilities, much like clavulanic acid (53).

Clavulanic acid is a naturally occurring β-lactam that has little activity against bacteria by itself (58), but it is a strong inhibitor of β-lactamases and can therefore render many β-lactamase-producing bacteria sensitive to β-lactams when both compounds are administered simultaneously (Figure 2-4B)(22). Susceptibility of an enzyme to an inhibitory molecule, such as clavulanic acid, can vary widely as can the number of interactions before an inactivation complex is achieved. This turnover number (t_n) can be as low as 1 for a susceptible enzyme or >16,000 for a resistant one (31, 53). New inhibitory molecules need to undergo more tests but show great promise in potentiating modern day β-lactamases (53).

2.3.5.3 Detection of β-lactamases

A proactive approach to determining the efficacy of a β-lactam is more efficient than treating with a generic drug and observing patient response (169). β-lactamases can be detected *in vitro* by several means. Livermore et al. described colorimetric, acidimetric and iodometric tests. Nitrocefin is a common colorimetric indicator that turns red when cleaved by a β-lactamase and the test can be done within minutes of obtaining a culture. The downside is that these tests will not accurately differentiate between β-lactamase functional groups and will not indicate which antibiotics will be effective in treatment (102).

MIC testing by serial dilution, disc diffusion or Etest (bioMérieux, Marcy l'Etoile, France) provides a numerically based resistance profile that can be compared to clinical standards for an individual antibiotic (to be discussed in section 2.4.2)(186). In addition to showing the MIC for antibiotics, the disc diffusion technique allows clinicians to check for compounds and antibiotic interactions that may increase β-lactamase production *in vitro* (Figure 2-4A)(110). A chromosomal β-lactamase in *P. aeruginosa* may be induced by inactivation of PBP4 (121). Since PBP4 is a target of imipenem in *Enterococcus faecalis* (132), this induction may be an indirect result of PBP4 inhibition. Conversely, a PBP2a and non-functional β-lactamase in *S. aureus* share a regulatory pathway; induction by certain β-lactams resulted in increased expression of both PBP2a and the β-lactamase (70).

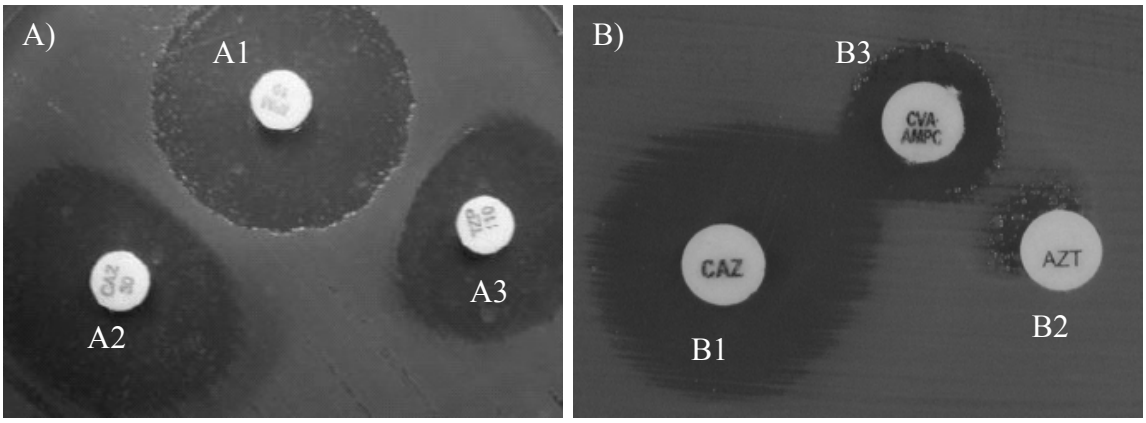


Figure 2-4. Interaction of β-lactams, β-lactamases and β-lactam inhibitors. A) Induction of *ampC* β-lactamase with imipenem (A1), demonstrated by increased ceftazidime (A2) and piperacillin-tazobactum (A3) resistance in *P. aeruginosa* **(110)**. **B)** Potentiation of ceftazidime (B1) and aztreonam (B2) by way of amoxicillin-clavulanic acid (B3) in *A. baumannii* **(124)**.

2.4 Resistance in *Burkholderia* species

B. pseudomallei has been shown to be intrinsically resistant to a broad range of antibiotics, including those from every class above (26, 47, 57, 117, 120). In a screen of environmental bacteria capable not only of resisting inhibitory effects of antibiotics, but actually subsisting on them as their sole carbon source, the order *Burkholderiales* accounted for 41% of the species isolated (49). This is a true testament to the metabolic capabilities and resistance mechanisms of these bacteria (42). This diversity may be the cause of melioidosis relapse and persistence (87).

Much of the resistance is due to low outer membrane permeability and numerous multidrug efflux pumps. Three of the twelve putative RND pumps encoded by *B. pseudomallei* have been characterized thus far (104, 107). The first pump to be identified in *B. pseudomallei* was AmrAB-OprA. This RND pump provides resistance to numerous aminoglycosides and macrolides (120). The BpeEF-OprC efflux pump can provide trimethoprim and chloramphenicol resistance when expressed in laboratory generated strains of *P. aeruginosa* and *B. thailandensis* (17, 93) and may be a cause of resistance in natural isolates as well (N. Podnecky and H. P. Schweizer, unpublished). BpeAB-OprB was initially thought to share many of the substrates effluxed by AmrAB-OprA (35), but this may not be accurate (117). This widespread resistance necessitated the testing and use of many different treatment regimens, with varied degrees of success (60).

2.4.1 β-lactam resistance in *B. pseudomallei*

Thus far, there has been no evidence for efflux-mediated β-lactam resistance in *B. pseudomallei*, leaving β-lactamases as the primary suspect for the broad β-lactam resistance (48, 68). Initial evidence for β-lactamase as a resistance determinant was

observed by the potentiation of β-lactams by adding clavulanic acid to *in vitro* MIC testing. Significant differences were seen for ampicillin, amoxicillin, and carbenicillin while ceftazidime and imipenem MICs dropped by 4-fold (103). The genome annotation of strain K96243 showed that *B. pseudomallei* potentially may encode as many as 7 putative β-lactamase genes of various classes (see Table 2-1)(78). This is a high number but is not unheard of. Additionally, most bacteria producing a metallo-β-lactamase (Ambler class B) have multiple β-lactamases (142). Despite the presence of multiple

Table 2-1. Putative β-lactamases encoded by *B. pseudomallei*. Adapted from **(78)** and according to annotated GenBank sequence for *B. pseudomallei* K96243 (accession numbers NC_006350 and NC_006351

Gene	Ambler Molecular Class[a]	Bush-Jacoby Functional Class[b]	Primary Substrate	Citation[d]
Chromosome 1				
BPSL0374	B	NC[c]	NC	-
BPSL1561	B	NC	NC	-
BPSL2708	B	NC	NC	-
Chromosome 2				
BPSS0946 (PenA)	A	2a/2b (2br or 2e)	Penicillins (Point mutations are unique)	(40, 79, 147, 172)
BPSS1915	B	NC	NC	-
BPSS1997 (OXA)	D	NC	Oxacillins	(88, 129)
BPSS2119	B	NC	NC	-

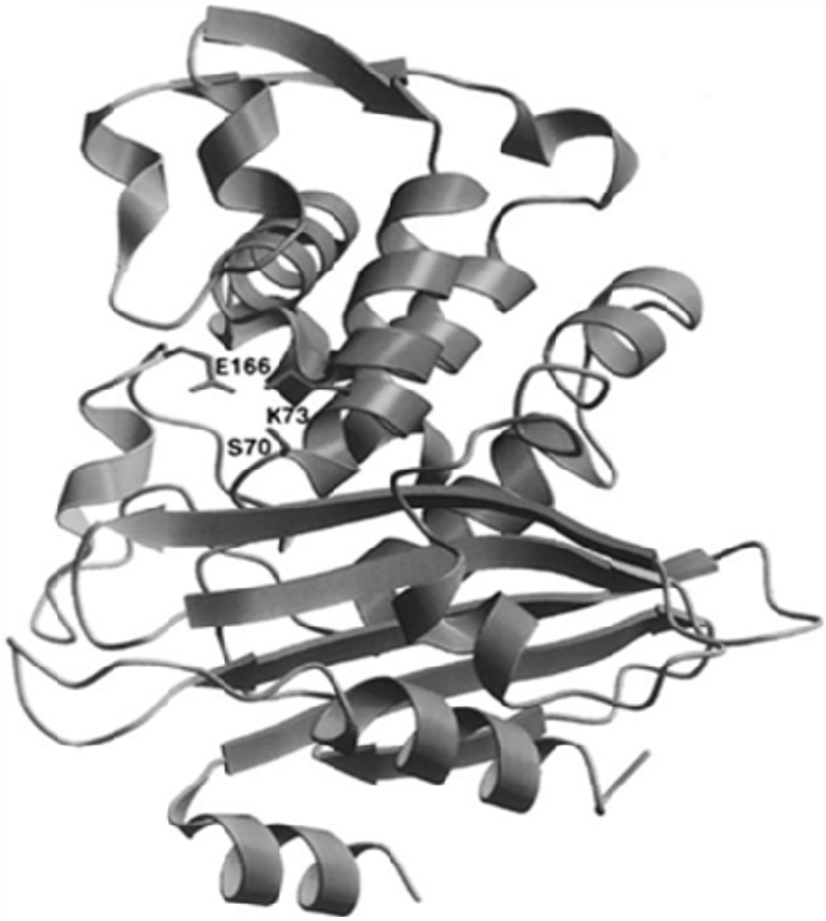
[a] Based on sequence only (78)
[b] Presumed class, based on criteria in reference (29)
[c] NC Not Characterized
[d] - No literature available

neutralizing enzymes, many β-lactams are still very effective at killing *B. pseudomallei* (10). A survey of strains from melioidosis patients in 1998 showed the following percentages of resistance to various β-lactams; 0% to imipenem, 0.9% to ceftazidime, 0.3% to piperacillin, and 1.5% to amoxi-clav (76).

2.4.1.1 Class A β-lactamase (BPSS0946)

The best characterized β-lactamase gene in *B. pseudomallei* is *penA,* originally refered to as bla_{BPS1}, which codes for an Ambler class A enzyme (40). When expressed from a plasmid in *E. coli*, PenA provided significant resistance to multiple β-lactams. This included antibiotics from various classes, including penicillins and many second generation cephalosporins (40). Point mutations within *penA* have been shown to alter β-lactamase substrate profiles significantly (79, 147, 172). A P167S (Ambler numbering) substitution in PenA also showed an increase in ceftazidime resistance both in a clinical isolate and a laboratory generated mutant (Figure 2-5)(79, 172). Another documented mutation that altered the PenA resistance profile is a S72F (Ambler numbering) substitution that increased resistance to clavulanic-acid (172). A single C69Y (Ambler numbering) amino acid substitution in PenA seems to be responsible for the change from ceftazidime susceptible to resistant. Within the same mutant, amoxicillin resistance changed to susceptibility. The fact that this occurred within a patient during treatment means that for at least a brief period there were subpopulations within a patient and therefore phenotypes (*i.e.* patients could have both Cef^r-Amx^s and Cef^s-Amx^r populations)(147).

A)



B)

```
Bp-PenA    GARIAHRGDERFPFCSTSKMMLCAAVLARSAGEPALLQRRIAYAKGDLIRYSPITEQHVG
Sa-PC1     GKEVKFNSDKRFAYASTSKAINSAILLEQVP--YNKLNKKVHINKDDIVAYSPILEKYVG
           * .: ...*:**.:.**** : .* :* : .     *::::   *.*:: **** *::**

Bp-PenA    AGMSVAELCAATLQYSDNTAANLLIALLGGPQTVTAYARSIGDATFRLDRREPELNTALP
Sa-PC1     KDITLKALIEASMTYSDNTANNKIIKEIGGIKKVKQRLKELGDKVTNPVRYEIELNYYSP
```

Figure 2-5. Structure and Sequence of a Class A β-lactamase. A) Ribbon diagram of *S. aureus* Ambler Class A β-lactamase PC1 (taken from **(12)**). Active residues are labeled. **B)** Amino acid sequence alignment of well-characterized PC1 from *S. auerus* and PenA from *B. pseudomallei* starting at Ambler residue 55 and ending at 174 (Locus BPSS0946 from NC_006351 and accession number P00807, respectively). Red highlights indicate active sites. Other colors indicate point mutations seen in PenA; yellow is C69Y, green is S72F, blue is P167S.

2.4.1.1 Class D β-lactamase (BPSS1997)

Only one other putative β-lactamase has been molecularly characterized; the oxacillinase BPSS1997. By serially passaging *B. pseudomallei* on ceftazidime at 4x MIC, Niumsup et al. were able to increase the specific activity of BPSS1997 for ceftazidime and imipenem by up to 32-fold and 52-fold, respectively. However, these dramatic increases in efficiency were tested *in vitro* with only enzyme and substrate present. When in *B. pseudomallei* they only translated to a minor change in ceftazidime and imipenem resistance, 2-fold to 8-fold and 0 to 4-fold respectively (8 μg/mL and 4 μg/mL maximum), in the strains. The differences are not documented specifically, so we can only speculate as to the exact levels. Additionally, none of these samples are resistant by clinical definitions. Cloning genes into plasmids in *E. coli* yielded no measureable differences in ceftazidime or imipenem MIC (129).

In a study of OXA-57 (Class D), the authors acknowledge the discrepancy between good *in vitro* activity of the cloned β-lactamase and the susceptibility of *B. pseudomallei* samples (88). One possible explanation for this is that the gene is simply not expressed in sufficient quantities to affect the pipericillin resistance profile.

2.4.1.2 Class B β-lactamases (multiple)

Functional class B enzymes, such as the putative metallo-β-lactamases BPSL0374, BPSL1561, BSL2708, BPSS1915 and BPSS2119, are less common but are efficient inactivators of carbapenems (170). Given the carbapenem susceptibility of *B. pseudomallei* (171) it is probable that these enzymes are not active in *B. pseudomallei*.

However, the contribution of a particular β-lactamase on the overall resistance profile can only be fully assessed *in vivo* within isogenic mutants of the species in

question. This is not only true of enzymes with proven function (*e.g.* PenA), but also for putative enzymes whose activity has yet to be demonstrated. For example, a β-lactam resistance in *Acinetobacter baumannii* was due to a synergy between a weak β-lactamase and over-expression of an efflux pump (77). It is possible that one of the many uncharacterized efflux:modification-enzyme combinations may be working in concert with each other for certain antibiotic classes.

2.4.2 Testing for resistance

The key to successful treatment is selection of effective antibiotics. More accurate tests will mean better selection of drugs, better patient prognosis and a slower journey toward increased multidrug resistant bacteria (169). Typical antimicrobial resistance testing consists of either diluting the antimicrobial to measure endpoint growth or by using agar to diffuse the antibiotic and measuring zone of clearance. The dilution method can be difficult to read endpoints consistently due to aggregation of cell debris and inhibited growth, but not total inhibition. This problem is even more pronounced with bacteriostatic agents, such as trimethoprim and sulfamethoxazole (N. Podnecky, personal communication).

Tests in 1989 showed 97% resistance to trimethoprim and 25% resistance to sulfamethoxazole in *B. pseudomallei* (48). Although this could be partially due to varied reading techniques, it does show the strong effect of synergy and multidrug therapy. When in used in combination, TMP-SMX resistance levels were recorded to be 18.6% by disk diffusion testing (158). However, bacteriostatic drugs are very difficult to read consistently. The Etest provides easy to read diffusion-based testing. Using this more consistent system, the reported TMP-SMX resistant population decreased significantly.

A large comparison study (1976 isolates) between Etest and disc diffusion measured resistance at 13% and 71%, respectively, with only 18% of strains that were deemed "resistant" by disc diffusion showing resistance by Etest (188). This means that in roughly one in five cases potentially effective treatment with TMP-SMX would not be used on account of false "resistant" classification.

Heine et al. showed great variation of MICs taken by broth dilution vs. Etest strips when testing *B. mallei*. Typically the Etest readings yielded a lower MIC. Clinically relevant drugs differed by as much as 21-fold for amoxicillin-clavulanic acid, 16-fold for ceftazidime and by 500-fold for co-trimoxazole (75). These significant differences in readings may drastically affect treatment selection, and thus clinical outcome, by incorrectly reporting resistant strains as sensitive and vice versa. Similar, albeit less dramatic, differences were also seen in other studies with *B. pseudomallei* (108, 137).

In 2004, the susceptibility of a large collection of *B. pseudomallei* (n=50) and *B. mallei* (n=15) strains to a panel of 35 antibiotics was tested (171). The tests confirmed the current treatment recommendations by showing carbapenems and third generation cephalosporins had the least resistant strains. However, these tests were done by agar dilution, which is not common practice in clinical labs and the level of *in vitro* antibiotic sensitivity can change with the method of measurement, as demonstrated above. Unfortunately, even the best made plans and perfect treatment selection can end in failure. Treatment failure has been associated with every treatment option tested (36-39, 141, 183), even when the best antibiotic available is selected and initial treatment is met with success (48).

2.5 Dual use research

Research into mechanisms of antibiotic resistance may fall under the "dual use" research category. This means that with the new knowledge comes the ability to create antibiotic resistance by malicious entities (126). During the cold war, Soviet researchers attempted to create multi-drug resistant *Burkholderia* strains for use as possible biowarfare agents (97). The best approach for potential dual use research is to focus on the positive uses of the new knowledge versus the potential negative uses when describing the findings. It is our hope that through elucidating resistance mechanisms we can improve selection of effective treatment options. This research is possible by adhering to Select Agent regulations and employing an exempt *B. pseudomallei* strain.

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2.7 Hypotheses and Aims

Before the start of this research, the presence of chromosomal β-lactamase genes (2) and putatively at least one functional β-lactamase (4) had been reported. However, the precise mechanisms for the high levels of intrinsic β-lactam resistance in *Burkholderia pseudomallei* (8) had not been empirically tested. Furthermore, the factors involved in emerging resistance to clinically relevant drugs (*e.g.* ceftazidime and amoxicillin-clavulanic acid) had not been elucidated. Clinical observations provide some evidence to the nature of these mechanisms (3, 7, 9), but without reconstructing the mutations in an isogenic background one cannot be certain that the observed mutations are the sole causes of the altered phenotypes. Due to the limited effective treatment options for melioidosis (6), it is important to know the reasons for emerging resistance to the preferred drugs. To this end I had the following hypotheses:

1) Design of a select agent compliant bacterial mutagenesis system for *Burkholderia pseudomallei* will allow construction of sequence-defined transposon mutant libraries for molecular genetic analyses of this bacterium.

2) Recreation of mutations from clinical and laboratory-generated strains in a defined genetic background will allow verification of causality on observed resistance as well as the implementation of molecular tests for rapid identification of resistance determinants.

In order to test these hypotheses, I worked toward the following specific aims:

1) Design of a select agent compliant bacterial mutagenesis system for *Burkholderia pseudomallei* (Chapter 3).
2) Define the role of chromosomally encoded PenA β-lactamase in resistance of *B. pseudomallei* to clinically significant β-lactams and characterize its secretion and regulation (Chapter 4).
3) Define a novel non-β-lactamase conferred β-lactam resistance mechanism observed in clinical isolates and laboratory generated mutants (Chapter 5).

During this work, additional tools were designed and constructed to aid in the genetic manipulation of *B. pseudomallei* in a select agent compliant manner. These include an improved helper plasmid (pTNS3) for the previously established mini-Tn*7* chromosomal integration system (1) and a novel *Escherichia coli* SM10(λ*pir*) (5) derivative (RHO3) for facile conjugation from *E. coli* into diverse bacteria, including *Burkholderia* species. This work is described in Chapter 6.

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CHAPTER 3:

IN VIVO *HIMAR1* TRANSPOSON MUTAGENESIS OF *BURKHOLDERIA PSEUDOMALLEI*

(Presented in Drew A. Rholl, Lily A. Trunck and Herbert P. Schweizer. 2008. *Applied and Environmental Microbiology*. 74(24):7529-35.)

The work presented in this paper introduced a *Himar1* mariner transposon system suitable for the creation of select-agent-compliant random mutagenic strains of *B. pseudomallei*. I constructed and tested the system in *B. pseudomallei* and *B. thailandensis*. L.A. Trunck assisted with Southern blot analysis.

3.1 Abstract

Burkholderia psedudomallei is the etiologic agent of melioidosis and the bacterium is listed as a potential agent of bioterrorism because of its low infectious dose, multiple infectious routes and intrinsic antibiotic resistance. To further accelerate research with this understudied bacterium, we developed a *Himar1*-based random mutagenesis system for *B. pseudomallei* (*HimarBP*). The transposons contain a Flp recombinase excisable, approved kanamycin resistance selection marker and an R6K origin of replication for transposon rescue. *In vivo* mutagenesis of virulent *B. pseudomallei* strain 1026b was highly efficient, with up to 44% of cells transformed with the delivery plasmid harboring

chromosomal *HimarBP* insertions. Southern analyses revealed single insertions with no evidence of delivery plasmid maintenance. Sequence analysis of rescued *HimarBP* insertions revealed random insertions on both chromosomes within open reading frames and intergenic regions, and that the orientation of insertions was largely unbiased. Auxotrophic mutants were obtained at a frequency of 0.72% and nutritional supplementation experiments supported the functional assignment of genes within the respective biosynthetic pathways. *HimarBP* insertions were stable in the absence of selection and could be readily transferred between naturally transformable strains. Experiments with *B. thailandensis* suggest that the newly developed *HimarBP* transposons can also be used for random mutagenesis of other *Burkholderia* spp., especially the closely related *B. mallei*. Our results demonstrate that comprehensive transposon libraries of *B. pseudomallei* can be generated, providing additional tools for the study of the biology, pathogenesis and antibiotic resistance of this pathogen.

3.2 Introduction

Burkholderia pseudomallei is the etiologic agent of melioidosis, a disease that is endemic to tropical and subtropical regions of the world (6, 30). Research with this bacterium has significantly increased with its listing as a priority pathogen by the U.S. National Institutes of Health and a Select Agent Pathogen by the Centers for Disease Control and Prevention and the United States Department of Agriculture. Despite the availability of complete annotated as well as draft genome sequences for several strains ((12) and several GenBank entries), efforts aimed at understanding *B. pseudomallei*'s biology and pathogenesis are still hampered by lack of genetic tools and the strict regulations that govern their use in the United States. Though many genetic tools have

previously been used to study *B. pseudomallei*'s biology and virulence (9, 10, 18, 25), most of them are not compliant with United States Select Agent regulations because they involve use of non-approved antibiotic selection markers. We recently published Select Agent compliant tools for allele replacement and single copy gene integration in *B. pseudomallei* which facilitate targeted gene mutations and complementation (7). What is still needed, however, is a Select Agent compliant method for efficient creation of random, transposon induced mutants. Availability of such a system would greatly facilitate low-throughput strategies such as identification of virulence or antibiotic resistance factors, as well as high-throughput strategies such as construction of ordered genome wide transposon mutant libraries. Though Tn*5*-based transposon mutagenesis systems were previously described for and successfully used in *B. pseudomallei* (9, 21), most of them use a tetracycline selection marker that cannot be used in the United States because it conflicts with potential use of doxycycline to treat *B. pseudomallei* infections in human and veterinary medicine. A previously described Tn*5*-based plasposon system with a kanamycin resistance marker (8) has to our knowledge not yet been tested in *B. pseudomallei*. Furthermore, the resistance marker residing on previously constructed transposons cannot be excised once inserted into the chromosome. In this study, we evaluated the use of *Himar1 mariner* transposon for random mutagenesis of *B. pseudomallei*. *Himar1* transposons have been used for random *in vitro* (1, 2, 20) and *in vivo* (3, 14, 22, 27, 31, 33) mutagenesis of numerous bacteria, including the Select Agent *Francisella tularensis* (16). *Mariner*-based transposons do not require host-specific factors and, other than preference for a TA dinucleotide target, do not display target site specificity. We describe development of an efficient *in vivo Himar* transposon

mutagenesis system for *B. pseudomallei* and demonstrate its use for isolation of auxotrophic and other mutants.

3.3 Materials and Methods

3.3.1 Bacterial strains, media and growth conditions.

Several *Burkholderia* strains were used in this study. Before working with *B. pseudomallei*, genetic constructs were routinely tested in *B. thailandensis* wild-type strain E264 (5). *B. thailandensis* is traditionally regarded as a naturally attenuated relative of *B. pseudomallei* (32) which can be handled at biosafety level 2 and is exempt from Select Agent guidelines. *B. pseudomallei* 1026b was used as wild-type strain (Table 3-1). *Escherichia coli* strains used for routine cloning experiments were DH5α (15), DH5α(λ*pir*) (laboratory strain), HPS1 (24) or S17-1 (26). All bacteria were routinely grown at 37°C. Strains containing temperature-sensitive plasmid derivatives were grown at 30°C (permissive temperature) or 37°C (non-permissive temperature). Low salt (5 g L^{-1} NaCl) Lennox LB broth (LSLB) and agar (MO BIO Laboratories, Carlsbad, CA) were used. M9 medium (17) with 10 mM glucose was used as the minimal medium. Nutritional supplements for auxotrophic mutants were added at the following concentrations: 20 μg mL^{-1} L-phenylalanine, L-tyrosine or L-tryptophan; 100 μg mL^{-1} L-aspartic acid or L-glutamine; and 40 μg mL^{-1} uracil. Unless otherwise noted, antibiotics were added at the following concentrations: 100 μg mL^{-1} ampicillin (Ap), 35 μg mL^{-1} kanamycin (Km) and 25 μg mL^{-1} zeocin (Zeo) for *E. coli*; 1,000 μg mL^{-1} Km and 2,000

Table 3-1. Strains, plasmids and primers used in this study.

Strain or plasmid	Relevant properties[a]	Reference or source
B. thailandensis		
E264	**Wild-type strain**	(9)
B. pseudomallei		
1026b	**Wild-type strain; clinical isolate**	(16)
Plasmids		
pFNLTP16 H1	Ap^r, Km^r; source of *tnp* and *nptII* genes and *Himar1* transposon with ori_{R6K}	(7)
pFLPe2[b]	Zeo^r; source of Flpe recombinase	(7)
pFKM2[b]	Ap^r, Km^r; source of *FRT-npt-II-FRT* cassette	(7)
pPS2163	Ap^r, Km^r; source of ColE1 *ori*, *oriT* and ori_{1600}-*rep*(Ts_{Bt})	(7)
pPS2413	Ap^r, Km^r; pCR2.1 (TA cloning kit, Invitrogen) with 1,456 bp PCR fragment amplified from pFKM2 with primers 596 & 1758	This study
pHBurk1	Km^r; ligation of fragment from pFNLTP16 H1 containing *Himar1* (with ori_{R6K}, *npt* gene, inverted repeats) and *tnp* gene and a pPS2163 fragment containing ColE1 *ori*, *oriT* and ori_{1600}-*rep*(Ts_{Bt})	This study
pHBurk-Link-2	Km^r; pHBurk1 with *Bgl*II-*Sma*I linker inserted at *Pvu*I site upstream of *tnp*	This study
pHBurk2	Km^r; pHBurk-Link-2 with *nptII* gene replaced by *FRT-nptII-FRT* containing PCR fragment from pFKM2; *nptII* gene oriented away from *tnp*	This study
pHBurk3	Km^r; like pHBurk2 but *nptII* facing toward *tnp*	This study
pHBurk4	Km^r; pHBurk2 with P_{lac}[b] downstream of *nptII* which is oriented away from *tnp*	This study
pHBurk5	Km^r; like pHBurk4 but *nptII* and P_{lac} facing toward *tnp*	This study
pHBurk6	Km^r; pHBurk-Link-2 with *B. thailandensis* P_{S12} promoter obtained by annealing oligos 1690 & 1691 and inserting the double stranded oligonucleotide fragment into *Pvu*I+*Sma*I digested pHBurk-Link-2	This study
Primers and other oligonucleotides		
1668[c]	5'-CGCTGACATCGAGATCTCTAACCCGGGAT	This study
1669[c]	5'-CCCGGGTTAGAGATCTCGATGTCAGCGAT	This study
596	5'-CGAATTAGCTTCAAAAGCGCTCTGA	This study
1758[d]	5'-CAC**AACATA**CGAGCCGGAAGCATAAAG**TGTAAA** GCCGAATTGGGGATCTTGAAGTACCT	This study
511	5'-ATTAACCGCTTGTCAGCCGTTAAGTGTTCCT	This study

512	5’-ATTACCGCGGCAGTTCAACCTGTTGATAGTAC	This study
1398	5’-GTCAGCACGTTGATCGAGAA	This study
1399	5’-CGCTGTGATGTTCCTCTTCA	This study
1768	5’-AGGCTTTACCAGTAAGAAGGAG	This study
1769	5’-GATTTCGACCTTCAAACGCTCC	This study
1670[e]	5’-TCGGGTATCGCTCTTGAAGGG	This study
1722	5’-GACTTGGTTGAGTACTCACCAG	This study
1690[f]	5’-ATG**TTGACT**CGCTTGGGATTTTCG**GAATATCAT**GCCGGT	This study
1691	5’-ACCCGGCATGATATTCCGAAAATCCCAAGCGAGTCAACA TAT	This study
1829[e]	5’-GCATTTAATACTAGCGACGCC	This study
1832	5’-GTTCCCTTCAAGAGCGATACC	This study
1833	5’-AACGCACTGAGAAGCCCTTAG	This study

[a] Abbreviations: Ap, ampicillin; Km, kanamycin; r, resistance; Zeo, zeocin

[b] P_{lac}, *E. coli lac* operon promoter

[c] *Bgl*II and *Sma*I recognition sequences are underlined

[d] *E. coli lac* promoter -10 (AACATA) and -35 (TGTAAA) reverse complement sequences are indicated in bold face letters

[e] 1670 and 1829 are DNA sequencing primers P1 and P2, respectively, used for determination of transposon-chromosome junction sequences

[f] *B. thailandensis* P_{S12} promoter predicted -10 (GAATATCAT) and -35 (TTGACT) sequences are indicated in bold face letters

μg mL^{-1} Zeo for wild-type *B. pseudomallei*; 200 μg mL^{-1} Zeo and 500 μg mL^{-1} Km for *B. thailandensis*. Antibiotics were either purchased from Sigma, St. Louis, MO (ampicillin and kanamycin) or Invitrogen, Carlsbad, CA (zeocin).

3.3.2 DNA methods and transformation.

Routine procedures were employed for manipulation of DNA (23). Plasmid DNAs were isolated from *E. coli* and *Burkholderia* spp. using the Fermentas GenJet Plasmid Miniprep Kit (Fermentas, Glen Burnie, MD). Bacterial chromosomal DNA fragments (20-30 kb) were isolated using the QIAamp DNA Mini Kit and the DNA was suspended in 200 μl of buffer AE (10 mM Tris-HCl, 0.5 M EDTA, pH 9). Plasmid DNA fragments were purified from agarose gels utilizing the Fermentas DNA Extraction Kit (Glen Burnie, MD). *E. coli* strains were transformed using chemically competent cells (23). Replicative plasmids were transformed into *B. thailandensis* and *B. pseudomallei* using a rapid electroporation procedure (7). Colony PCR with *Burkholderia* spp. was performed as previously described (7). Custom oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). DNA maps were constructed using Gene Construction Kit 2.5 (Textco, West Lebanon, NH) and exported to Microsoft Powerpoint for final annotation.

For determination of insertion sites, genomic *Burkholderia* spp. DNA was extracted from selected clones using initially the QiAmpDNA Mini Kit (Qiagen, Valencia, CA) but later the Gentra Puregene DNA purification kit (Qiagen) was used because of superior yield, and 1 μg was digested overnight with *Not*I. DNA was purified with Fermentas DNA Extraction Kit, treated with T4 DNA Ligase (Invitrogen) overnight at 14^{o}C, transformed into DH5α(λ*pir*), and Km^{r} transformants were selected. Plasmid

DNA was prepared and transposon-chromosomal junction sequences were determined by nucleotide sequencing with primers 1670 and 1829 (primers and other oligonucleotides are listed in Table 3-1).

HimarBP3 was transferred between chromosomes of transposon containing strains and strain 1026b utilizing DNA fragment transfer and naturally competent cells utilizing previously described methods (7, 28).

3.3.3 Southern blot analysis.

For genomic Southern analysis, genomic DNA was isolated utilizing the Centra Puregene DNA purification kit (Qiagen). DNA (4 μg) was digested with *Not*I overnight, electrophoresed on a 1% agarose gel, and transferred to positively charged nylon membranes (Roche Diagnostics Corp., Indianapolis, IN) by passive transfer as previously described (23). Following transfer and ultraviolet fixation, blots were probed with a PCR fragment biotinylated by random hexamer priming following the NEBlot Phototype labeling and detection kit protocols (New England Biolabs, Beverly, MA). The probe detected the *HimarBP* transposon with a 376 bp fragment recognizing the ori_{R6K} region.

3.3.4 Construction and transposition of *Himar1* derivatives.

All *Himar1* derivatives, as well as other plasmids used for their construction, are listed in Table 3-1**.** pHBurk1 (Figure 3-1) containing a temperature-sensitive *Burkholderia* spp. replicon was derived by combining a blunt-ended 2,974 bp *Bpm*I-*Nsi*I fragment from pPS2163 with a blunt-ended 3,971 bp *Not*I fragment from pFNLTP16 H1 containing the *Himar1* transposon and its transposase encoding *tnp* gene. Next, pHBurk-Link-2 was constructed by ligating a linker composed of oligos 1668 & 1669 containing a

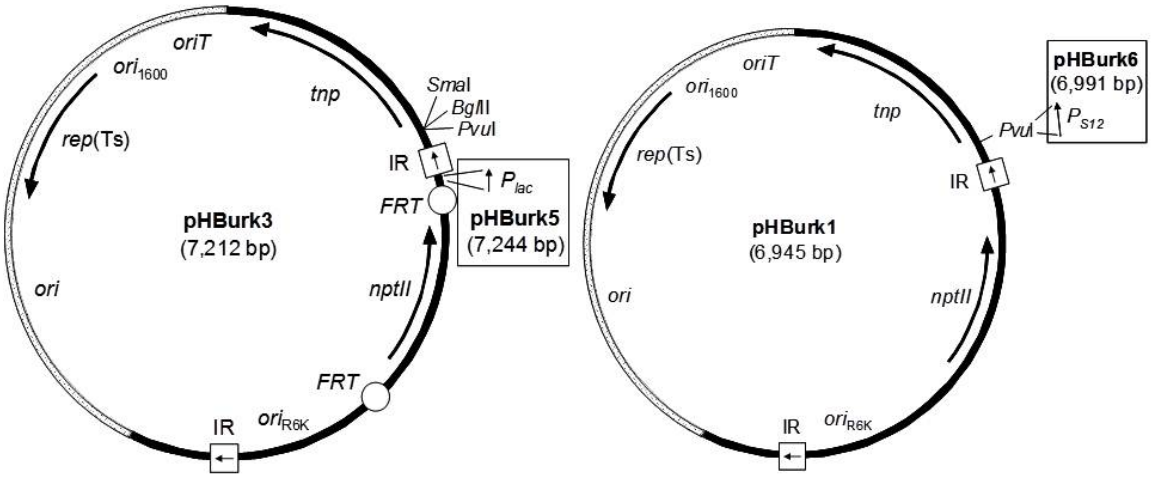

Figure 3-1. Maps of two representative *HimarBP* containing delivery plasmids. The plasmids contain the following shared features: IR, *Himar1* inverted repeat; *nptII*, neomycin phosphotransferase encoding gene; *ori*, *E. coli* ColE1 origin of replication; ori_{1600}, pRO1600 origin of replication requiring the rep(Ts)-encoded replication protein which confers a temperature-sensitive (Ts) phenotype in Burkholderia spp. at temperatures of 37°C and above; *oriT*, RK2 derived origin for conjugal plasmid transfer; ori_{R6K}, π-protein dependent R6K replication origin; *tnp*, transposase encoding gene. Plasmid pHBurk3 additionally contains Flp recombinase targets (*FRT*) and two unique restriction sites (*Pvu*I and *Sma*I) derived by insertion into the unique *Pvu*I site of pHBurk1. pHBurk5 has the same features as pHBurk3 but contains the *E. coli lac* operon promoter (P_{lac}) for transcription of genes adjacent to the promoter insertion site. Similarly, pHBurk6 is the same as pHBurk1, but tnp transcription is directed by the promoter for *B. thailandensis* ribosomal S12 protein-encoding gene (P_{s12}). The transposons harbored by the individual plasmids are named after plasmid numbers, e.g. pHBurk1 harbors *HimarBP1*, pHBurk3 *HimarBP3*, etc. Plasmids are not drawn to scale.

*Bgl*II and a *Sma*I site into the single *Pvu*I site located immediately upstream of the *tnp* gene of pHBurk1 such that a single *Pvu*I site was re-created at the linker insertion site. The *Sma*I and *Pvu*I sites were subsequently used to insert promoter containing linkers. Plasmids pHBurk2 and pHBurk3 (Figure 3-1) were derived from pHBurk-Link-2 by replacing a 1,206 bp blunt-ended *Mlu*I fragment containing the resident *nptII* gene with a *FRT-nptII-FRT* containing 1,444 bp *Sma*I fragment from pFKM2. Plasmids pHBurk2 and pHBurk3 differ in the orientation of the *nptII* gene. Next, pHBurk4 and pHBurk5

(Figure 3-1) were constructed by replacing the blunt-ended 1,206 bp *Mlu*I fragment of pHBurk-Link-2 containing the *npt* gene with a 1,476 bp blunt-ended *Eco*RI fragment from pPS2413 containing *FRT-nptII-FRT-*P_{lac}. Plasmids pHBurk4 and pHBurk5 differ in the orientation of the *nptII* gene and P_{lac}. Lastly, pHBurk6 was constructed by inserting a double-stranded oligonucleotide containing the *B. thailandensis* ribosomal S12 gene promoter (P_{S12}) between the *Pvu*I and *Sma*I sites of pHBurk-Link-2 such that *tnp* transcription was promoted by P_{S12}.

For *Himar1* transposition, the previously described one-step protocol (16) was followed with appropriate modifications. Briefly, 100 ng of each pHBurk plasmid was electroporated into freshly prepared electrocompetent *B. thailandensis* or *B. pseudomallei* cells. After incubation at 30°C in a shaker for 1 h, dilutions were plated on LB medium containing 1000 μg mL^{-1} kanamycin and incubated at 37°C to select for plasmid loss and chromosomal transposon integration. Dilutions were also plated on LB medium with and without kanamycin, and plates incubated at 30°C to determine total CFU transformed or total viable cells, respectively. Colonies grown at 37°C for 24-96 h were picked and patched on LB plates with kanamycin to recover individual clones containing *Himar1* in the chromosome. For auxotrophy screening, colonies were also patched on M9-glucose + kanamycin plates. Transposon presence in genomic DNA was assessed by PCR using primer pair 511 & 512 (specific for ori_{R6K}) and primer pair 1398 & 1399 (specific for the *amrB* efflux protein encoding gene) was used to amplify a positive control fragment. Delivery plasmid loss was verified by PCR using primer pair 1768 and 1769 specific for the *tnp* gene located on the plasmid backbone.

Flp recombinase-mediated Km^r marker excision was performed using pFLPe2 and a previously described protocol (7). The plasmid was cured by growing kanamycin susceptible colonies at 37°C, a non-permissive temperature for pFLPe2, resulting in markerless mutants.

3.3.5 Nucleotide sequence accession numbers.

The sequences for pHBurk3 and pHBurk5 were deposited in Genbank and assigned accession numbers EU919403 and EU919404, respectively.

3.4 Results and Discussion

3.4.1 Construction of *HimarBP* and transposition in *B. thailandensis* and *B. pseudomallei*.

Initial experiments using a non-replicative *Himar1* delivery plasmid, i.e. pFNLTP16 H1 (16), yielded Km^r transposants at frequencies that were between 11,000 (*B. thailandensis*) and 250 (*B. pseudomallei*) fold lower than those obtained with the same plasmid backbone in *F. tularensis* (not shown). For construction of a *Himar1* delivery system allowing efficient one-step transposon delivery and transposition we therefore made use of the previously isolated conditional broad-host-range ori_{1600}-*rep*(Ts) replicon (7). Plasmids containing this replicon can be efficiently electroporated into *B. thailandensis* and *B. pseudomallei* and maintained in single copy at permissive (30°C) but not at non-permissive (37°C or greater) temperature (7). The prototype plasmid pHBurk1 (Figure 3-1) contains a *Himar1* transposon named *HimarBP1* in which transcription of the *tnp* gene is initiated by a mycobacterial promoter and a *nptII* gene, approved for use in *B. pseudomallei*, transcribed from its endogenous promoter (16). The *nptII* gene specifying Km^r is the sole selection marker present in pHBurk1 and all of its derivatives.

Using a previously described one-step transposition protocol (16), *HimarBP1* transposed in *B. thailandensis* and *B. pseudomallei* with efficiencies of 8 x 10^{-6} and 1.2 x 10^{-4} to 4.7 x 10^{-5}, respectively. Similar efficiencies were observed with *HimarFT* in *F. tularensis* where *tnp* and *nptII* selection marker transcription were driven by *Francisella* specific promoters (16). Thus, there is no significant difference in transposition into the AT-rich *F. tularensis* genome versus the GC-rich *Burkholderia* spp. genomes.

The next generation pHBurk plasmids (pHBurk2, pHBurk3 and pHBurk6;Figure 3-1) were designed with two goals in mind: (i) utilization of excisable Km^r selection markers because of the paucity of approved selection markers for use in *B. pseudomallei* and (ii) increased *tnp* transcription which may result in increased transposition efficiencies. First, pHBurk1 was modified with a polylinker that would facilitate directed cloning of promoter-containing fragments (pHBurk-Link-2). Second, the resident *nptII* gene on pHBurk1 was replaced with a *FRT-nptII-FRT* cassette so that the *nptII* gene could be excised from transposon integrants with the help of Flp recombinase (pHBurk2 and pHBurk3, containing *HimarBP2* and *HimarBP3*, respectively). Third, pHBurk6 (*HimarBP6*) was constructed such that *tnp* transcription would be promoted by the *B. thailandensis* P_{S12} promoter which was previously used for driving gene expression in *B. thailandensis* (4) and *B. pseudomallei* (7). Using the one-step transposition protocol, pHBurk2, pHBurk3 and pHBurk6 (containing *HimarBP2*, *HimarBP3* and *HimarBP6*, respectively; *HimarBP6* was only studied in *B. thailandensis*) transposed in *B. thailandensis* and *B. pseudomallei* with similar efficiencies (1 x 10^{-5} to 1 x 10^{-7}), indicating that driving *tnp* transcription from the *B. thailandensis* P_{S12} promoter did not result in significantly increased transposition efficiencies. As a matter of fact, promoting

transcription from the strong P_{S12} promoter may be counter-productive as sequencing of the P_{S12} containing region of several pHBurk6 isolates with primer 1722 revealed a single base deletion in the -10 region. The data presented in Table 3-2 clearly indicate that transposition efficiencies were significantly higher with delivery vectors where the *nptII* and *tnp* genes are in the same orientation, e.g. pHBurk1, pHBurk3 and pHBurk5, versus those plasmids containing these genes in opposite orientation, e.g. pHBurk2 and pHBurk4. Transposition efficiencies were not significantly increased with pHBurk5 containing the *E. coli lac* operon promoter reading toward *tnp* in addition to the *nptII* promoter.

Because the highest transposition efficiencies were consistently obtained with pHBurk3, it was used for further studies.

3.4.2 *HimarBP3* transposition in *B. pseudomallei*.

Using the one-step delivery and transposition procedure, the efficiency of plating at the non-permissive temperature was approximately 35% of that observed at the permissive temperature (Table 3-2). Frequencies of transposition ranged from 1.81×10^{-4} to 3.22×10^{-5}. Chromosomal insertion versus plasmid maintenance was investigated by colony PCR using primer sets 511 & 512 and 1768 & 1769, respectively. These analyses revealed that the plasmid was lost in all investigated cases and that the Km^r phenotype was due to chromosomal *HimarBP3* insertion. Km^r colonies obtained after 24-72 h incubation at 37°C all contained *HimarBP3* insertions, as assessed by colony PCR with primer set 511 & 512 which was performed on 66 random colonies picked after 24, 48 and 72 h incubation time. The longer incubation times needed to obtain a significant

Table 3-2. Frequency of transposition of HimarBP derivatives into B. pseudomallei 1026b[a]

Himar1 Derivative	No. of transformants[b]	No. of insertions[c]	Avg. no. of insertions/avg. no. of transformants
pHBurk1	$1.86 \times 10^{4} \pm 1.35 \times 10^{-4}$	$8.15 \times 10^{-5} \pm 3.69 \times 10^{-5}$	0.44
pHBurk2	$2.45 \times 10^{-4} \pm 1.52 \times 10^{-4}$	$1.01 \times 10^{-5} \pm 1.18 \times 10^{-6}$	0.04
pHBurk3	$3.48 \times 10^{-4} \pm 2.43 \times 10^{-4}$	$1.2 \times 10^{-4} \pm 5.46 \times 10^{-5}$	0.35
pHBurk4	$1.46 \times 10^{-4} \pm 8.49 \times 10^{-5}$	$3.83 \times 10^{-6} \pm 2.61 + 10^{-6}$	0.03
pHBurk5	$2.78 \times 10^{-4} \pm 1.53 \times 10^{-4}$	$1.00 \times 10^{-4} \pm 4.69 \times 10^{-5}$	0.36

[a] Results shown are averages from two (pHBurk2), three (pHBurk1 and pHBurk4) or five (pHBurk3 and pHBurk5) separate experiments.

[b] Transformants are the average number of viable CFU mL^{-1} recovered after growth on selective medium at permissive temperature (30°C).

[c] Insertions are the average number of viable CFU mL^{-1} recovered after growth on selective medium at non-permissive temperature (37°C).

number of Km^r colonies are therefore not of concern. Similar observations were made with 62 colonies obtained with pHBurk5.

3.4.3 Verification of *HimarBP3* transposition and stability in *B. pseudomallei.*

The one-step transposition protocol with pHBurk3 was used to obtain Km^r colonies of strain 1026b which were picked and purified after a 48 h incubation at 37°C. Genomic DNA was isolated from 14 randomly selected Km^r colonies and four auxotrophic colonies (see below), digested with *Not*I and hybridized with a probe that recognized the ori_{R6K} sequences present on *HimarBP3*. Single bands of different sizes

were obtained in all cases, as shown in Figure 3-2 (lanes a) for five isolates, suggesting single and random insertion of *HimarBP3* into the *B. pseudomallei* genome. The same result was obtained when 15 Km^r *B. thailandensis* isolates mutagenized with *HimarBP1* were analyzed (data not shown), suggesting that *HimarBP* transposons are functional in and can be used for random mutagenesis strategies of other *Burkholderia* spp., especially the closely related Category B agent *B. mallei*, the etiologic agent of glanders (19, 29).

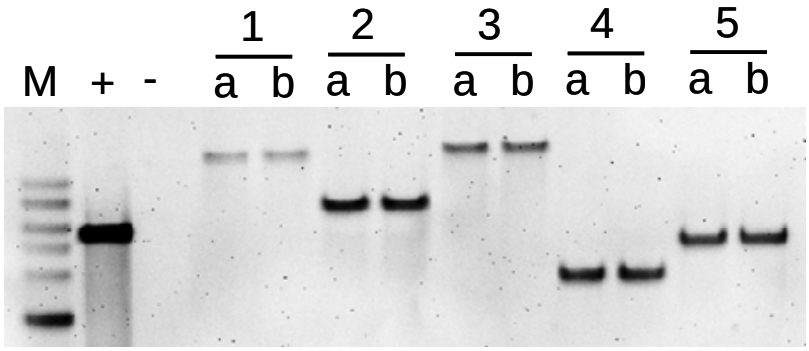

Figure 3-2. Transposition of HimarBP3 and stability in B. pseudomallei 1026b. Genomic DNA was prepared from mutants after initial isolation (a) or after ~100 generations in the absence of kanamycin selection (b), digested overnight with *Not*I and transferred to a nylon membrane. The membrane was hybridized with a probe that detected the ori_{R6K} present on *HimarBP3*. Isolates 1-5 are randomly selected Km^r colonies. Wild-type B. pseudomallei 1026b was included as negative control. The positive control was *Mlu*I digested pHBurk3. The 10, 8, 6, 5, 4 and 3 kb (top to bottom) fragments contained in the biotinylated 2-log DNA ladder (New England BioLabs) are shown in the column labeled M.

To assess the stability of *HimarBP3* insertions, five randomly selected Km^r mutants were grown for ~100 generations in the absence of kanamycin selection after which all five isolates recovered were still Km^r. Genomic DNA was extracted from the five original Km^r isolates and the five mutants that were grown in the absence of selection and Southern analysis was performed as described above. Identical bands were present in the original Km^r isolates and the bacteria grown in the absence of selection (Figure 3-2, lanes b). These results indicated that *HimarBP3* insertions in the *B. pseudomallei* genome are stable in the absence of continued antibiotic selection.

Since currently only kanamycin and zeocin markers are approved for genetic manipulation of wild-type bacteria *B. pseudomallei* (gentamicin is also approved but its use is confined to efflux pump deficient mutant derivatives), Km^r tagging of mutants severely impacts downstream genetic manipulations such as complementation, double-mutant isolation, reporter gene tagging, etc. This issue was overcome by equipping the *HimarBP* transposons with a Flp recombinase excisable Km^r marker. To assess Flp mediated Km^r marker excision, selected Km^r mutants were transformed with pFLPe2 containing a Zeo^r marker and Flp excision performed as previously described (7). As expected, kanamycin susceptible colonies were readily obtained with marker excision efficiencies ranging from 20-70%. Marker-free mutants were then obtained by growing kanamycin susceptible colonies at 37°C, a non-permissive temperature for pFLPe2. All marker-free mutants analyzed by sequence analysis of a 398 bp PCR fragment amplified with primers 1832 and 1833 had the expected physical structures, i.e. a single *FRT* site in place of the excised *FRT-nptII-FRT* cassette. Because *HimarBP3* mutants containing the Km^r selection marker were stable for ~100 generations in the absence of antibiotic selection, the isogenic marker-free mutants should also be stable.

3.4.4 Determination of *HimarBP3* insertion sites.

HimarBP3 insertion sites in *B. pseudomallei* strain 1026b were mapped by rescue of *HimarBP3* and sequence analysis of insertions. This was achieved by ligation of *Not*I digested DNA fragments and recovery of plasmid DNA from Km^r *E. coli* DH5α(λ*pir*) transformants. Both transposon-chromosomal DNA junction sequences were obtained by priming sequencing reactions with the transposon specific oligonucleotides 1670 and 1829.

Because the annotated sequence of strain 1026b is not yet available, insertions were mapped relative to the strain 1710b genome. This mapping revealed that insertions were randomly distributed on both chromosomes with no apparent regional bias (Figure 3-3A). The small number of insertions relative to the large genome allowed no conclusive predictions about the variety of insertions with respect to open reading frame (ORF) or transposon orientation, though there was slight tendency towards having transposons inserted in genes whose orientation was the same as the chromosome, irrespective of transposon orientation (Figure 3-3B). Of the 24 mapped insertions, four were in intergenic regions and 20 within predicted ORFs (Table 3-3). Transposon insertions were observed in genes involved in biosynthetic pathways, metabolic pathways, DNA repair, gene regulation and secretion. These observations are similar to those obtained during *Himar1* mutagenesis of *F. tularensis* (16).

The typical TA insertion site for *Himar1* transposons was observed in all 24 rescued and sequenced insertions. Deletions or duplications of flanking sequences were not detected in any of the mapped insertions.

3.4.5 Auxotrophic mutants obtained by *HimarBP3* transposition.

To test the utility of *HimarBP3* for mutant isolation, Km^r colonies were obtained after one-step transposition and 24-72 h incubation at 37°C. Km^r colonies were transferred to M9 minimal glucose kanamycin (MMGK) plates. From 2,781 Km^r colonies, 19 isolates were obtained that failed to grow on MMGK which corresponds to 0.68% recovery of auxotrophs. Analysis of 6,124 Km^r colonies generated with *HimarBP1*, *HimarBP2*, *HimarBP3*, *HimarBP4* and *HimarBP5* yielded 44 colonies which

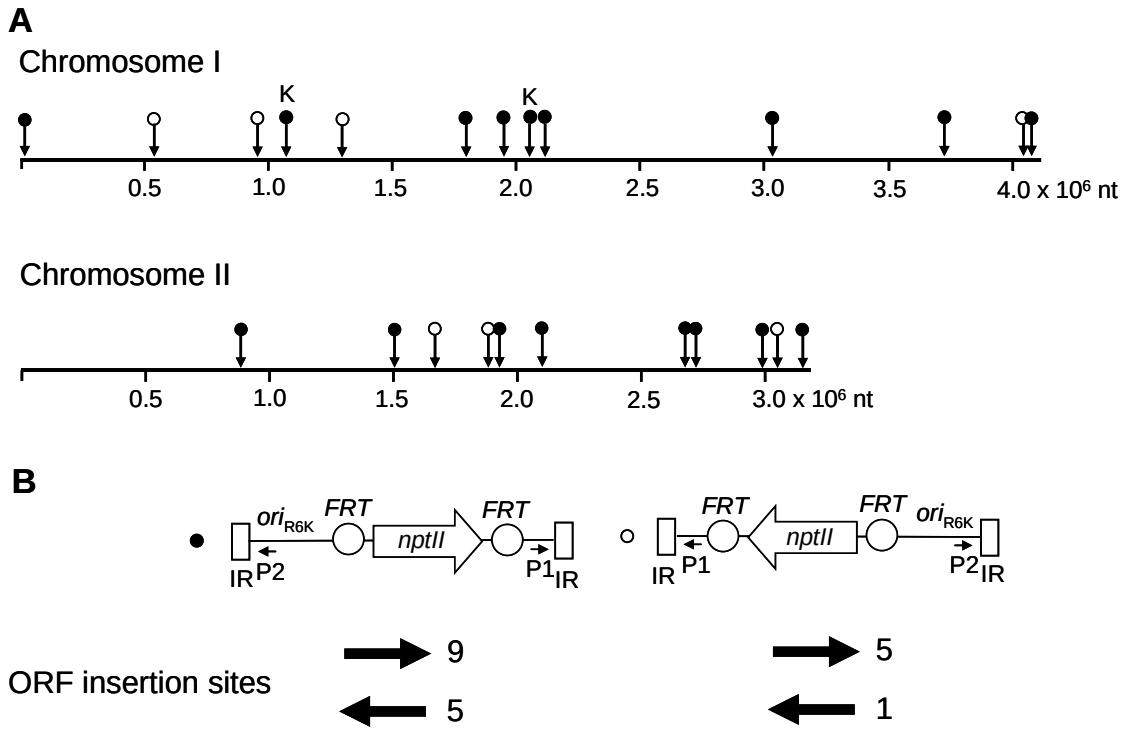

Figure 3-3. Mapping of *HimarBP3* insertions in the *B. pseudomallei* genome. **(A)** Transposon *HimarBP3* insertions were mapped to the chromosomes of 1710b, with the exception of two insertions (labeled K) that could only be mapped to the K96243 chromosome 1 (GenBank accession number NC006350). Filled and open circles denote insertions where *HimarBP3* is either inserted the same direction as or opposite to the chromosome. **(B)** Graphical representation of the orientation of *HimarBP3* insertion. Major features of *HimarBP3* are shown, including locations of the two sequencing primer (P1 and P2) binding sites and the orientations of sequence extensions from these primers are indicated by arrows. The transposon was found in both orientations in the *B. pseudomallei* chromosome with little bias to the orientation of the ORF (arrows) at the insertion site based on strain 1710b genome annotation. Numbers adjacent to each arrow denote isolates containing insertions into ORFs in the indicated orientations.

failed to grow on MMGK plates corresponding to 0.72% recovery of auxotrophs. This is comparable to the 1 to 2% recovery rate during isolation of auxotrophs in other bacteria (3, 13).

Four Km^r auxotrophs were selected for further characterization by genomic Southern analysis and mapping of genomic insertion sites. All mutants had single transposon

insertions in the genome (not shown). This was verified by insertion site mapping which showed that the four insertions were located in the *aroB*, *gltB*, *ppc* and *pyrC* genes, respectively, all of which are located on chromosome 1 (Table 3-3). These genes encode dehydroquinate synthase, glutamate synthase (large subunit), phosphoenolpyruvate carboxylase and dihyroorotase, respectively, which are involved in the phenylalanine, tyrosine and tryptophan, glutamine, oxaloacetate and pyrimidine biosynthetic pathways. The respective mutants are therefore phenylalanine, tyrosine and tryptophan (*aroB*), glutamine (*gltB*), aspartic acid (*ppc*) and pyrimidine (*pyrC*) auxotrophs. These auxotrophies were experimentally confirmed since growth of the *aroB*, *gltB*, *ppc* and *pyrC* mutants in MMGK medium was restored by addition of phenylalanine, tyrosine and tryptophan, glutamine, aspartic acid and uracil, respectively.

These results demonstrated the utility of *HimarBP3* for rapid mutant construction and characterization.

3.4.6 Transfer of *HimarBP3* insertions between *B. pseudomallei* chromosomes.

We previously showed that 20-30 kb linear chromosomal DNA fragments tagged with an antibiotic resistance marker could be readily transferred from strain 1026b derivatives back to strain 1026b and, to a lesser extent, strain 1710b (7). To test transfer of *HimarBP3* insertions, fragmented chromosomal DNA from the four Km^r *aroB*, *gltB*, *ppc* and *pyrC* mutants (Table 3-3) and a randomly selected Km^r prototroph were used to transform strain 1026b. Km^r 1026b transformants were obtained at a frequency of about 240 colonies per μg of DNA. All Km^r colonies obtained with DNA from the *aroB*, *gltB*, *ppc* and *pyrC* mutants were auxotrophs, whereas all of the Km^r colonies obtained with

Table 3-3. HimarBP insertions within open reading frames[a]

Location of Insertion	Chromosome	Gene name, putative function
BURPS1710b_3728	1	*aroB*, 3-dehydroquinate synthase
BURPS1710b_3718	1	*gltB*, glutamate synthase, large subunit
BURPS1710b_1228	1	*ppc*, phosphoenolpyruvate carboxylase
BURPS1710b_3425	1	*pyrC*, dihydroorotase, homodimeric type
BURPS1710b_A0679	2	*sctV*, type III secretion inner membrane protein SctV
BURPS1710b_A2481	2	*ribA*, GTP cyclohydrolase II
BURPS1710b_A1568	2	*uvrA*, excinuclease ABC, subunit A, form 2
BURPS1710b_A2192	2	sensor histidine kinase
BURPS1710b_1949	1	hypothetical protein (lipoprotein in strain K96243)
BURPS1710b_A2590	2	cytochrome c family protein
BURPS1710b_0018	1	indolepyruvate ferredoxin oxidoreductase
BURPS1710b_A2174	2	short chain dehydrogenase
BURPS1710b_A1577	2	*plcN*, phospholipase C
BURPS1710b_A1202	2	serine/threonine protein kinase
BURPS1710b_0528	1	*rbsR*, transcription regulator, LacI family
BURPS1710b_A1366	2	glutathione-dependent formaldehyde dehydrogenase
BURPS1710b_0918	1	*dgoA*, DgoA protein
BURPS1710b_2750	1	*prlC*, oligopeptidase A
BURPS1710b_1771	1	Rhs element Vgr protein subfamily, putative
BURPS1710b_1692	1	gp18

[a]Locations of *HimarBP* insertion in genes and putative functions according the *B. pseudomallei* strain 1710b genome sequence (GenBank accession numbers CP000124 and CP000125 for chromosomes 1 and 2, respectively).

DNA from the Km^r prototroph remained prototrophs. These data showed that *HimarBP3* induced mutations can readily be transferred between strain 1026b derivatives. In this context it should be noted, however, that not all *B. pseudomallei* strains are naturally transformable (28).

3.5 Conclusions

We have developed an efficient *HimarBP* mutagensis system for *B. pseudomallei* which continues to expand the arsenal of still fledgling Select Agent compliant tools that can be used with this bacterium. Its development takes advantage of previously constructed tools such as approved excisable selection markers and *in vivo* marker excision systems (7). The *HimarBP* elements are small (2,205 to 2,479 bp) and can thus be readily transferred between *B. pseudomallei* strains that are naturally transformable (7, 28), which facilitates double mutant construction and mutant sharing by virtue of sharing sterile exempt genomic DNA rather than non-exempt live strains. The basic *HimarBP* transposons were engineered with ease of use (e.g. rapid and simple transposon rescue and insertion site mapping) and versatility (e.g. they can be readily equipped with other genetic elements such as other approved selection markers, outward reading promoters, reporter genes, affinity tags, etc) in mind. Random mutagenesis strategies will greatly facilitate studies of the biology and pathogenesis of this and related understudied pathogens, and perhaps facilitate establishment of a comprehensive *B. pseudomallei* transposon mutant library. Such libraries have accelerated research with diverse other bacteria, including *F. tularensis* (11) and two *P. aeruginosa* prototype strains (13, 14).

3.6 Acknowledgments

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CHAPTER 4:

MOLECULAR INVESTIGATIONS OF PENA-MEDIATED β-LACTAM RESISTANCE IN *BURKHOLDERIA PSEUDOMALLEI*

(This work is presented in **Drew A. Rholl** and Herbert P. Schweizer. Molecular investigations of PenA-mediated β-lactam resistance in *Burkholderia pseudomallei*. *Frontiers in Cellular and Infection Microbiology.* Submitted.
A portion of this work is also presented in Takehiko Mima, Brian H. Kvitko, **Drew A. Rholl**, Malcolm G.P. Page, Eric Desarbre and Herbert P. Schweizer. 2011. *International Journal of Antimicrobial Agents*. In press.)

The work presented in this chapter describes further characterization of the *B. pseudomallei* Ambler class A β-lactamase PenA. By constructing directed mutants of the endogenous *penA* gene and putative regulatory and secretion genes, I was able to define the contribution of PenA to β-lactam resistance of *B. pseudomallei*. I also examined PenA processing and transcriptional regulation. I acknowledge Jeff Chandler and Drs. Darragh Heaslip and Marjorie Sutherland for their help with protein experiments.

4.1 Abstract

Burkholderia pseudomallei is the etiological agent of melioidosis. Because of the bacterium's intrinsic resistance and propensity to establish latent infections, melioidosis therapy is complicated and prolonged. Newer generation β-lactams, specifically ceftazidime, are used for acute phase therapy, but resistance to the drugs has been observed. The chromosomally-encoded *penA* gene encodes a putative twin arginine translocase (TAT)-secreted β-lactamase, and *penA* mutations have been implicated in ceftazidime resistance in clinical isolates. However, the role of PenA in resistance has not yet been systematically studied in isogenetic *B. pseudomallei* mutant backgrounds. We investigated the effects of *penA* deletion, point mutations, and up-regulation, as well as *tat* operon deletion and PenA TAT signal sequence mutations. These experiments were made possible by employing a *B. pseudomallei* strain that is excluded from Select Agent regulations. Deletion of *penA* significantly (>4-fold) reduced the susceptibility to six of the nine β-lactams tested and >16-fold for ampicillin, amoxicillin and carbenicillin. Over-expression of *penA* by single-copy, chromosomal expression of the gene under control of the inducible P_{tac} promoter, increased resistance levels for all β-lactams tested 2- to 10-fold. Recreation of the C69Y and P167S PenA amino acid substitutions previously observed in resistant clinical isolates increased resistance to ceftazidime by >64 and 5-fold, respectively. Similarly, a S72F substitution resulted in a 4-fold increase in resistance to amoxicillin + clavulanic acid. Susceptibility assays with PenA TAT signal sequence and Δ*tatABC* mutants, as well as Western blot analysis, confirmed that PenA is a TAT secreted enzyme and not periplasmic but associated with the spheroplastic

cell fraction. Lastly, we determined that two LysR-family regulators encoded by genes adjacent to *penA* do not play a role in transcriptional regulation of *penA* expression.

4.2 Introduction

Burkholderia pseudomallei, the etiological agent of melioidosis, is a saprophytic Gram negative bacterium endemic to many tropical and subtropical regions of the world although much of the disease and its investigation has historically been confined to Northern Australia and regions of SE Asia, notably NE Thailand, Singapore and Malaysia (Cheng and Currie, 2005; Wiersinga et al., 2006; Currie et al., 2008). Partially because of its large genome and diverse repertoire of metabolic functions *B. pseudomallei* can survive hostile conditions and is resilient to many antimicrobial agents, including antibiotics (Holden et al., 2004). This makes selecting and implementing effective therapeutic strategies difficult. Just over 30 years ago even the most effective treatment could not prevent a mortality rate of 74% (White et al., 1989). Clinical outcomes improved steadily with implementation of new therapies but the real breakthrough was achieved with the introduction of ceftazidime, a third generation cephalosporin, which halved the mortality rate compared to the traditional multidrug therapy of chloramphenicol, doxycycline and trimethoprim-sulfamethoxazole (White et al., 1989). Currently recommended melioidosis treatment involves acute phase therapy followed by a lengthy eradication therapy. Initial parenteral therapy involves ceftazidime or a carbapenem for a minimum of 10 to 14 days and longer (4 to 8 weeks) for deep-seated infection. This regimen may be supplemented with trimethoprim-sulfamethoxazole given orally for treatment of patients with neurologic, prostatic, bone,

or joint melioidosis. Oral eradication therapy is trimethoprim-sulfamethoxazole with or without doxycycline for at least 3 to 6 months (Peacock et al., 2008).

Because of the pivotal role that β-lactams play in the acute phase treatment of melioidosis emergence of resistance, though still considered rare, is of concern. It is believed that *B. pseudomallei*'s resistance to β-lactams is due to chromosomally-encoded β-lactamases (Livermore et al., 1987; Godfrey et al., 1991). These include a number of Ambler Class A, B and D β-lactamases that are encoded by the K96243 and other *B. pseudomallei* genomes (Holden et al., 2004). The *penA* gene (K96243 gene *BPSS0946* found on chromosome II; Figure 4-1) encodes a Class A β-lactamase (Cheung et al.,

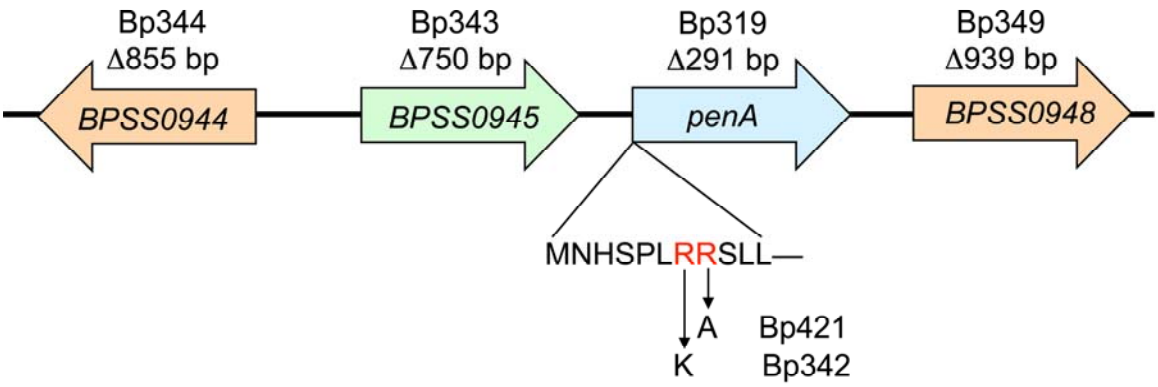

Figure 4-1. Genomic organization of the *B. pseudomallei penA* region. The genes and gene order are from sequenced strain K96243 (GenBank accession number NC_006351). The *penA* region encodes two LysR-type regulators (BPSS0944 and BPSS0948) and a putative peptidase (BPSS0945). The names of the mutants harboring gene deletion and extents of deleted sequences are shown above each gene. The putative PenA twin arginine translocase (TAT) signal sequence is shown below the *penA* gene with the two conserved arginine residues shown in red letters. Arrows indicate amino acid substitutions, R7K and R8A, in the TAT signal sequence and the names of the mutants are shown next to the respective amino acids replacing the original arginines.

Tribuddharat et al., 2003). This gene is present and expressed in prototype *B. pseudomallei* strains. PenA confers resistance to numerous β-lactam antibiotics when expressed in *Escherichia coli* (Cheung et al., 2002; Tribuddharat et al., 2003) and several reports described a role of this enzyme in acquired ceftazidime resistance in patients treated with this antibiotic (Godfrey et al., 1991; Tribuddharat et al., 2003; Sam et al., 2009). Mutations identified in clinical strains included a C69Y substitution leading to high-level ceftazidime resistance (Sam et al., 2009), a P167S substitution leading to medium-level ceftazidime resistance (Tribuddharat et al., 2003) and a S72F mutation that led to resistance to clavulanic acid (Tribuddharat et al., 2003). A Class D Oxa-57 β-lactamase has been studied *in vitro* but its role in clinically significant β-lactam resistance remains unclear (Keith et al., 2005).

While *B. pseudomallei* PenA β-lactamase has been studied in some detail, previously published reports suffered from until recently some unavoidable shortcomings. First, many mutations contributing to clinically significant β-lactam resistance were identified in genetically largely intractable clinical isolates. Thus, it remained unclear whether the mutations were solely responsible for causing the observed resistance. Second, because methods for genetic manipulation of *B. pseudomallei* were rather rudimentary until recently, most studies involved expression of putative β-lactamase enzymes in *E. coli*. Third, United States Select Agent and recombinant DNA regulations, as well as dual use concerns, do complicate studies of clinically significant antibiotic resistance mechanisms. To address shortcomings of previous studies, we employed state-of-the-art Select Agent-compliant genetic and biochemical methods and a defined genetic background of a Select Agent excluded *B. pseudomallei* strain, where applicable, to study the contribution of

PenA to *B. pseudomallei*'s resistance to clinically significant β-lactam antibiotics. The studies also revealed that PenA is secreted via the twin arginine translocase system and that its expression in prototype strains does not seem to be regulated by local transcriptional regulators.

4.3 Materials and methods

4.3.1 Bacterial strains and growth conditions

B. pseudomallei strains used in this study are listed in Table 4-1. *E.* coli strains DH5α (Liss, 1987) and MACH1 (Invitrogen, Carlsbad, CA) were used as general cloning strains, and DB3.1 (Invitrogen) for cloning with Gateway Vectors. RHO3 was used as a mobilizer strain for conjugation of plasmids from *E. coli* to *B. pseudomallei* (López et al., 2009). Bacterial strains were grown in Lennox LB (MO BIO Laboratories, Carlsbad, CA) or LB without salt (10 g/L tryptone and 5 g/L yeast extract) at 37°C. Antibiotics were used at the following concentrations: 100 μg/mL ampicillin (Amp), 35 μg/mL kanamycin (Km) and 15 μg/mL for *E. coli* and 1,000 μg/mL Km and 2,000 μg/mL Zeo for *B. pseudomallei*. Antibiotics were purchased from Sigma, (St. Louis, MO) except Zeo which was from Invitrogen. The Δ*purM* strain Bp82 was grown in media supplemented with 0.6 mM adenine to ensure growth rates comparable to strain 1026b. RHO3 was grown in media containing 400 μg/mL diaminopimelic acid (DAP; LL-, DD-, and meso-isomers; Sigma). Induction of gene expression from P_{tac} was achieved by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Gold Biotechnology, St. Louis, MO) to growth media.

Table 4-1. *B. pseudomallei* strains used in this study.

Strain	Description	Source
Strain 1026b based mutants		
1026b	Clinical isolate, wild-type	(DeShazer, *et al.*, 1997)
Bp319	1026b Δ*penA*	This study
Bp409	1026b Δ*tatABC*	This study
Bp420	1026b Δ*penA* Δ*tatABC*	This study
Bp343	1026b Δ*BPSS0945*	This study
Bp344	1026b Δ*BPSS0944*	This study
Bp349	1026b Δ*BPSS0948*	This study
Bp342	1026b PenA R7K	This study
Bp421	1026b PenA R8A	This study
Strain Bp82-based mutants		
Bp82	1026b Δ*purM*	(Propst, *et al.*, 2010)
Bp82.3	Bp82 PenA C69Y	This study
Bp82.4	Bp82 PenA S72F	This study
Bp82.5	Bp82 PenA P167S	This study
Bp82.11	Bp82 Δ*penA*	This study
Bp82.14	Km^r; Bp82:Tn*7*T-P_{s12}-FKM-*lox-BPSS0944*[a]	This study
Bp82.15	Km^r; Bp82:Tn*7*T-P_{s12}-FKM-*lox-BPSS0945*	This study
Bp82.16	Km^r; Bp82:Tn*7*T-P_{s12}-FKM-*lox-BPSS0948*	This study
Bp82.21	Km^r; Bp82:Tn*7*T-LAC-FKM-*penA*$^{+b}$	This study

[a]Cloned genes are transcribed from the constitutive *B. thailandensis* ribosomal s12 gene promoter

[b]Cloned *penA* gene is transcribed from the IPTG-inducible *E. coli* lactose operon/tryptophan hybrid promoter P_{tac}

4.3.2 Isolation of mutants containing chromosomal deletion or point mutations.

All deletion and allelic-exchange procedures were based on pEXKM5 (Lopez et al., 2009)(plasmids used in this study are listed in Table 4-2) and were performed using

previously described protocols. The desired chromosomal deletions were verified by colony PCR (Choi et al., 2008).

For construction of *penA* deletion mutants, PCR with primers 1687+1712 (PCR primers and mutagenic oligonucleotides are listed in Table 4-3) and *Taq* polymerase (New England BioLabs, Ipswich, MA) was used to amplify a 1,308 bp region containing the *BPSS0946* (*penA*) gene from 1026b chromosomal DNA. The gel-purified PCR fragment was cloned into the *Sma*I site of pUCP20 to yield pPS2370. This plasmid was then cleaved with *Nsi*I + *Pml*I, blunt-ended with T4 DNA polymerase, followed by re-ligation. This procedure deleted a 291 bp *Nsi*I-*Pml*I fragment from the *penA* gene and resulted in pPS2549. A 1,339 bp *Pvu*II Δ*penA* fragment was excised from this plasmid and ligated into the *Sma*I site of pEXKm5 to create pPS2550. This plasmid was used to create Bp82.11 and Bp319 by transferring the plasmid-borne deletion alleles to either Bp82 or 1026b, respectively, via conjugation from RHO3.

For deletion of *tatABC,* splicing by overlap extension (SOEing) PCR was employed for engineering of deletion constructs. SOEing reactions consisted of separately amplifying two fragments, one using an "internal" primer with overlapping sequence with the internal primer from the other fragment. These bands were gel purified and 50 ng of each product was added to a new PCR reaction where it underwent PCR for five cycles (95°C for 60 s, 54°C 30 s and 72°C for 60 s). At this point, the two non-overlapping primers were added and the reaction proceeded for another 30 cycles. Using in-house purified *Pfu* polymerase and primer sets 2018+2019 (amplifying a 537 bp *tatA* 5' fragment) and 2020+2021 (amplifying a 501 bp *tatC* 3' fragment), a 1,038 bp SOEing PCR product was generated to delete 1,527 bp from the *tatABC* gene cluster. This PCR

Table 4-2. Plasmids used in this study.

Designation	Description[a]	Source
Plasmids for *penA* deletion and over-expression		
pUCP20	Ap^r; Broad host-range cloning vector	(West, *et al.*, 1994)
pEXKm5	Km^r; Allelic exchange plasmid	(Lopez, *et al.*, 2009)
pTNS3	Ap^r; Tn*7* insertion helper plasmid	(Choi, *et al.*, 2008)
pUC18T-mini-Tn*7*T-Km-LAC	Ap^r Km^r; Tn*7* cassette vector with P_{tac} and *lacI*^{q} for regulated expression of cloned genes	This study
pPS2370	Ap^r; pUCP20 with a 1,308 bp *penA* fragment (amplified with primers 1687+1712) inserted into *Sma*I site	This study
pPS2549	Ap^r; pPS2370 with 291 bp *Nsi*I-*Pml*I fragment deleted from *penA*	This study
pPS2550	Km^r; 1,339 bp *Pvu*II fragment from pPS2549 was inserted into the *Sma*I site of pEXKm5	This study
pPS2605	Ap^r Km^r; pCR2.1 with 1,230 bp *penA* fragment (amplified with primers 2003+2005)	This study
pPS2608	Ap^r Km^r; pCR2.1 with the 1,261 bp *penA* fragment from pPS2605 (amplified with primers 2003+2004 to modify the 5' region)	This study
pPS2627	Ap^r Km^r; pUC18T-mini-Tn*7*T-Km-LAC with the 1,266 bp *penA* fragment from pPS2608	This study
Plasmid for *penA* putative regulatory gene deletion and over-expression		
pPS2609	Ap^r Km^r; 1,043 bp SOEing PCR product using primer sets 2014+2015 and 2016+2017 was ligated into pCR2.1	This study
pPS2610	Ap^r Km^r; 1,300 bp SOEing PCR product using primer sets 2010+2011 and 2012+2013 was ligated into pCR2.1	This study
pPS2611	Ap^r Km^r; 1,239 bp SOEing PCR product using primer sets 2006+2007 and 2008+2009 was ligated into pCR2.1	This study
pPS2614	Km^r; blunt ended 1,085 bp *Bst*XI fragment from pPS2609 ligated into *Sma*I site of pEXKm5	This study
pPS2615	Km^r; 1,316 bp *Eco*RI fragment from pPS2610 ligated into *Eco*RI site of pEXKm5	This study
pPS2616	Km^r; 1,255 bp *Eco*RI fragment from pPS2611 ligated into *Eco*RI site of pEXKm5	This study
pUC18-mini-Tn7T-Gm-Gateway	Ap^r Gm^r; mini-Tn*7*T-Gm with GATEWAY cassette. GenBank accession number AY737004.	(Choi, *et al.*, 2005)
pPS2735	Ap^r Km^r; pUC18T-mini-Tn*7*T-Km-*FRT* with P_{s12}	This study
pPS2737	Ap^r Km^r; Gateway-ready Tn*7* cassette vector with *s12* promoter toward insert (*Kpn*I-*Afe*I fragment containing 1,824 bp Gateway-cassette from pPS1612 ligated into pPS2735 between *Kpn*I-*Stu*I)	This study
pPS2745	Km^r; Nested PCR with primers 2015+2016, then 2155+2156 for	This study

	1,051 bp fragment, cloned into pENTR-SD-D-TOPO	
pPS2746	Km^r; Nested PCR with primers 2013+2010, then 2157+2158 for 1,056 bp fragment, cloned into pENTR-SD-D-TOPO	This study
pPS2747	Km^r; Nested PCR with primers 2009+2006, then 2159+2160 for 1,456 bp fragment, cloned into pENTR-SD-D-TOPO	This study
pPS2748	Ap^r Km^r; Gateway LR recombination reaction with pPS2737 + pPS2745	This study
pPS2749	Ap^r Km^r; Gateway LR recombination reaction with pPS2737 + pPS2746	This study
pPS2750	Ap^r Km^r; Gateway LR recombination reaction with pPS2737 + pPS2747	This study
Plasmids for engineering of TAT-signal sequence mutations		
pPS2674	Ap^r Km^r; 740 bp of the 5' region of *penA* gene amplified with primers 2010+2011 and cloned into pCR2.1	This study
pPS2613	Ap^r Km^r; Mutagenic primer 2022 substituted AAG for CGC at bases 19-21 of *penA* in pPS2674 to provide a R7K substitution	This study
pPS2618	Km^r; 736 bp *Eco*RI fragment from pPS2676 ligated into *Eco*RI of pEXKm5	This study
pPS2676	Ap^r Km^r; Mutagenic primer 2076 substituted GC for CG at bases 22-23 of *penA* in pPS2674 to provide a R8A substitution	This study
pPS2678	Km^r; 736 bp *Eco*RI fragment from pPS2676 ligated into *Eco*RI of pEXKm5	This study
Plasmids for engineering of *penA* point mutations		
pPS2675	Ap^r Km^r; Mutagenic primer 2075 mutated G to A at base 224 of *penA* to provide a C69Y substitution in pPS2674 sequence	This study
pPS2677	Km^r; 736 bp *Eco*RI fragment from pPS2675 ligated into *Eco*RI of pEXKm5	This study
pPS2712	Km^r; 1,094 bp *Nru*I-*Hinc*II fragment (entire *penA*) from pPS2370 ligated into the *Sma*I site of pEXKm5	This study
pPS2721	Km^r; Mutagenic primer 2136 mutated C to T at base 517 of *penA* to provide a P167S substitution using pPS2712 (pEXKm5-based)	This study
pPS2722	Km^r; Mutagenic primer 2137 C to T at base 233 of *penA* to provide a S72F mutation using pPS2712 (pEXKm5-based)	This study
Plasmids for *tatABC* deletion		
pPS2612	Ap^r; 1,038 bp Soeing PCR product using primer sets 2018+2019 and 2020+2021 ligated into pGem-T Easy (Promega, Madison, WI)	This study
pPS2617	Km^r; 1,058 bp *Eco*RI fragment from pPS2612 ligated into *Eco*RI of pEXKm5	This study

[a]Abbreviations: Ap, ampicillin; Km, kanamycin; Gm, gentamicin; *Ps12*, *B. thailandensis* ribosomal *s12* gene promoter.

Table 4-3. Primers used in this study

Primer	Sequence[a,b,c,d]	Source
Cloning/Deletion		
1687	5'-**GGATCC**GACGAGAGCTGATACGCTAG[a]	This study
1712	5'-**AAGCTT**ATACCGGCATCGTTTCGCTG	This study
2003	5'- GAATTCGATACCGGCATCGTTTCG	This study
2004	5'-GATATCAGCCGTTGACTTAGTTGGTATTTCCGGAATATCATG CTGGTTCCGAATAATTTTGTTTAACTTTAAGAAGGAGATATAC	This study
2005	5'-ACTTTAAGAAGGAGATATACATGAATCATTCTCCGTTGCGC	This study
2006	5'-CAATCTCGACGGAGCACG	This study
2007	5'-CTTGAATGCCCTGCAGATCTTGGCCGCTACAGATACGACAC[b]	This study
2008	5'-AAGATCTGCAGGGCATTCAAG	This study
2009	5'-GGTCATCGGGGACGAGTG	This study
2010	5'-CGAATAGCGGATGAGATCG	This study
2011	5'-GTTGTCTCGAGCATGAGCAAGGATTTTCTGACCGCTTACG	This study
2012	5'-TTGCTCATGCTCGAGACAAC	This study
2013	5'-AATGGGCGATACGGTAACAG	This study
2014	5'-ACGAGCTTCCGAAATACACG	This study
2015	5'-ATCGAGACGATTCGTTCAGC	This study
2016	5'-CGAGCATCTCAAAATTCATCC	This study
2017	5'-CGTGTATTTCGGAAGCTCGTTAATGGGCGATACGGTAACAG	This study
2018	5'- ATGAATCACGACCCGAACTG	This study
2019	5'- CTTGCTCTCGTCCTCTTCCTACGATCAGCAACACGATCAG	This study
2020	5'- AGGAAGAGGACGAGAGCAAG	This study
2021	5'- GACGAAGCTGCTGAACGTC	This study
2041	5'-AGATACGGCATCGGATTGAC	This study
2042	5'-GTCGCCGGCTGATTATTTC	This study
2043	5'-GCAACGCTTGTTTCAATACG	This study
2044	5'-GAAAGGCTCGGTCACGTTC	This study
2045	5'-AATTCGTCACACGAACATGC	This study
2046	5'-CGTCATTCCACCTTCCATTG	This study
2047	5'-AGGAGGTCTACCACCTGCAC	This study
2048	5'-TTTTGTTTGCCGCCATTC	This study
2187	5'-CGAGCTTTCGCTGTCCTATC	This study
2188	5'-CGTGATCTTCGTGTCCTTGAGTTGTGTCATTGCGCTTCTC	This study
2189	5'-TCAAGGACACGAAGATCACG	This study

2190	5’-CCGGCAATTGATCGAACTC	This study
2191	5’-CGATCAACGTGATCTTCGTG	This study
Mutagenic Primers		
2022	5’Phos/-GAATCATTCTCCGTTG*AAG*CGCTCGCTGCTCGTCGCAGC	This study
2075	5’Phos/-GCTTTCCCGTTCT*A*CAGCACATCCAAGATGATGC	This study
2076	5’Phos/-GAATCATTCTCCGTTGCGC*GC*CTCGCTGCTC GTCGCAGC	This study
2136	5’Phos/-GCGCCGTGTTCAGCTCAG *A* CTCGCGGCGATCGAGC	This study
2137	5’Phos/-AAAGCATCATCTTG *A* ATGTGCTGCAGAACTGG	This study
Real-time PCR Primers		
Bp23S-F	5’-GTAGACCCGAAACCAGGTGA	(Mima & Schweizer, 2010)
Bp23S-R	5’-CACCCCTATCCACAGCTCAT	(Mima & Schweizer, 2010)
2077	5’-GTTCTGCAGCACATCCAAGA	This study
2078	5’-CGGTGTTGTCGCTGTACTGA	This study
Cloning		
1687	5’-**GGATCC**GACGAGAGCTGATACGCTAG	This study
1712	5’-**AAGCTT**ATACCGGCATCGTTTCGCTG	This study
2010	5’-AGGCTGGCTGTACTTGAACG	This study
2011	5’-CGGGCGATATTCTGATGTC	This study
Tn*7* integration confirmation		
Tn*7*L	5’-ATTAGCTTACGACGCTACACCC	(Choi, *et al.*, 2005)
BPGLMS1	5’-GAGGAGTGGGCGTCGATCAAC	(Choi, *et al.*, 2008)
BPGLMS2	5’-ACACGACGCAAGAGCGGAATC	(Choi, *et al.*, 2008)
BPGLMS3	5’-CGGACAGGTTCGCGCCATGC	(Choi, *et al.*, 2008)

[a] Bold indicates a newly generated restriction enzyme cleavage site

[b] Underline indicates overlapping sequence for SOEing PCR; a double underline indicates a ribosome binding site

[c] Italics indicates introduced point mutations

[d] Phos, 5’ phosphorylated oligonucleotide

product was ligated into pGem-T Easy (Promega; Madison, WI) to create pPS2612. An *Eco*RI fragment was rescued from this plasmid and inserted into pEXKm5 to create pPS2617, which was used to create Bp409 and Bp420 by transferring the plasmid-borne deletions to either 1026b or Bp319 (1026b Δ*penA*), respectively. PCR using primers 2047+2048 was used to confirm the deletion.

Other genes located in the *penA* region of the chromosome were deleted using a SOEing PCR strategy and pCR2.1 (Invitrogen) as TA cloning vector. The *BPSS0944* deletion construct was created using primer sets 2014+2015 and 2016+2017 to generate pPS2609, from which a *Bst*XI fragment was excised and inserted into the *Sma*I site of pEXKm5 to yield pPS2614. The *BPSS0945* deletion construct was generated using primer sets 2010+2011 and 2012+2013 to create pPS2610, from which an *Eco*RI fragment was excised and inserted into the *Eco*RI site of pEXKm5 to yield pPS2615. The *BPSS0948* deletion construct was created using primer sets 2006+2007 and 2008+2009 to generate pPS2611, from which an *Eco*RI fragment was excised and inserted into the *Eco*RI site of pEXKm5 to yield pPS2616. The plasmid-borne deletion alleles were transferred to the *B. pseudomallei* 1026b genome which resulted in strains Bp343, Bp344 and Bp349, respectively. Deletions were verified by colony PCR using primer sets 2045+2446, 2041+2042 and 2043+2044, respectively.

Chromosomal *penA* point mutations were engineered using the QuikChange Multi Kit (Stratagene, La Jolla, CA), 5'-phosphorylated mutagenic oligonucleotides, and plasmid DNA templates. Mutagenic oligonucleotide 2075 was used with pPS2674 to create pPS2675 for the PenA C69Y mutation. A 736 bp *Eco*RI fragment from pPS2675 was then ligated into the *Eco*RI site of pEXKm5 to construct pPS2677. Plasmid

pPS2712 was created as a platform for other mutations by ligating the *Nru*I-*Hinc*II containing *penA* fragment from pPS2676 into the *Sma*I site of pEXKm5. Employing pPS2712 template DNA, mutagenic oligonucleotides 2136 and 2137 were used separately to create pPS2721 and pPS2722 carrying PenA P167S and PenA S72F substitutions, respectively. Allelic exchange was carried out by conjugal transfer of pPS2677 (C69Y), pPS2721 (P167S) and pPS2722 (S72F) from RHO3 into Bp82. Mutations were verified by PCR amplifying and sequencing the region containing the expected mutation. TAT signal sequence mutations were generated using a similar strategy. The R7K mutation was engineered using mutagenic oligonucleotide 2022 and pPS2674 to create pPS2613. The 736 bp *Eco*RI fragment from this plasmid was ligated into pEXKm5 to yield pPS2618. The R7K allele contained on this fragment was transferred to the 1026b genome which created Bp342. The mutagenic oligonucleotide 2076 was used with pPS2674 to engineer pPS2676 to create an R8A mutation. The 736 bp *Eco*RI fragment was excised from this plasmid and ligated into pEXKm5 to create pPS2678. The R8A allele contained on this fragment was transferred to the 1026b genome which created Bp421. The presence of the desired point mutations on plasmids and the genome was verified by DNA sequencing.

4.3.3 Gene complementation and overexpression using single-copy, chromosomally integrated mini-Tn*7* vectors

The mini-Tn*7* system was used for introducing site-specific, stable insertions into the *B. pseudomallei* genome for purposes of gene complementation or overexpression (Choi et al., 2008). Tn*7* transposition was achieved by tri-parental mating involving RHO3 harboring the mini-Tn*7* vector, RHO3 containing the helper plasmid pTNS3 and the *B. pseudomallei* recipient strain, as previously described (Choi et al., 2006). Integration

events were verified using primers Tn*7*L and either BPGLMS1, BPGLMS2 or BPGLMS3 (Choi et al., 2008). All Tn*7* mutants retained and used for further experimentation had insertions at the *glmS*2-associated Tn*7* insertion site.

For regulated *penA* expression and overproduction, the gene was PCR amplified from pPS2370 using primers 2003+2005 and *Pfu* polymerase and the 1,230 bp PCR product cloned into pCR2.1 (Invitrogen) to yield pPS2605. An optimized ribosome binding site (RBS) was introduced upstream of *penA* to create pPS2608 by PCR amplifying the *penA* region of pPS2605 with primers 2003+2004 and cloning the resulting 1,261 bp fragment into pCR2.1. (The amplicon was expected to be 1,295 bp but the 5' end was truncated by 34 bp which did not affect the integrity of the *penA* gene.) An expression construct where *penA* was transcribed from the inducible P_{tac} was obtained by cloning the 1,300 bp *Eco*RI fragment from pPS2608 into pUC18T-mini-Tn*7*T-Km-LAC to create pPS2627. The mini-Tn*7* expression cassette from pPS2627 was integrated into the genome of Bp82 at the *glmS*2-associated Tn*7* integration site to form Bp82.21.

Constitutive expression of genes was achieved from chromosomally-integrated mini-Tn*7* elements where the respective genes were transcribed from the *B. thailandensis s12* promoter (Choi et al., 2008). Nested PCR and *Pfu* polymerase was used to PCR amplify *BPSS0944* (primers 2015+2016 and 2155+2156), *BPSS0945* (primers 2010+2013 and 2157+2158) and *BPSS0948* (primers 2006+2009 and 2159+2160) from strain 1026b genomic DNA. Each PCR began with three cycles using only the outside set (listed first) then the inner set (listed second) was added for thirty more cycles. Inner primers were designed for use with the pENTR/SD/D-TOPO cloning vector (Invitrogen, Carlsbad, CA), which provides a RBS and directionality, and created pPS2745, pPS2746 and

pPS2747, respectively. These plasmids then underwent the Gateway LR recombination reaction (Invitrogen) with pPS2737, a mini-Tn*7* vector which enables constitutive expression from the *B. thailandensis s12* promoter (this promoter is directed toward the Gateway recombination cassette). To create pPS2737 the 1,824 bp Gateway-cassette-containing the *Kpn*I-*Afe*I fragment from pUC18-mini-Tn*7*T-Gm-Gateway was ligated into pPS2735 between the *Kpn*I and *Stu*I sites. This Gateway-compatible mini-Tn*7* element was used to create pPS2748, pPS2749 and pPS2750 for constitutive expression of *BPSS0944*, *BPSS0945* and *BPSS0948*, respectively. The mini-Tn*7* elements contained on these plasmids were individually inserted at the *glmS*2 site of Bp82 with the help of pTNS3 to create Bp82.14, Bp82.15 and Bp82.16.

4.3.4 MIC determinations

MICs were determined following general procedures recommended by the Clinical and Laboratory Standards Institute (2010). However, since Δ*tatABC* mutants do not grow well in the presence of salts LB without salt was substituted for Mueller-Hinton Broth. MICs for ampicillin, carbenicillin and BAL30072 (obtained from Basilea Pharmaceutica, Basel, Switzerland) were determined by the 2-fold broth microdilution technique. Etest strips were used to determine MICs for amoxicillin, amoxicillin-clavulanic acid, ceftazidime, imipenem, meropenem, and piperacillin according to manufacturer's instructions (AB BioMérieux, Marcy l'Etoile, France). When needed, IPTG was added to media at a final concentration of 1 mM. The MICs were recorded after incubation at 37°C for 18-24 h

4.3.5 Quantification of *penA* transcript levels

Overnight cultures were subcultured into LB medium, grown to an $OD_{600\ nm}$ of 0.5 and RNA was extracted with the RNeasy Protect Bacteria Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) and quantified using SYBR GreenER qPCR SuperMix for iCycler Instruments (Invitrogen) and the Bio-Rad iQ5 iCycler. The Bp23S-F+Bp23S-R primer set was used for the 23s rRNA housekeeping gene for data normalization and primers 2077+2078 were used to quantify the *penA* transcript. Data analyses were performed using the iCycler software. For induction studies with both wild-type and the mutants with constitutive regulatory gene expression or deletion several methods were employed. For salt stress testing, strains were grown in media with 150 mM NaCl or no NaCl according to Pumirat et al. (2009). For testing induction by β-lactams at sub-inhibitory levels, strains were subcultured into LB with 4-fold lower than MIC concentrations of either ceftazidime or carbenicillin until an OD_{600nm} of 0.5 was reached. Induction was also tested with 4 -fold higher than MIC concentrations of ceftazidime, carbenicillin, imipenem or penicillin-G (2,000 μg/mL for penicillin G) by growing in LB to an OD_{600nm} of 0.5, then adding β-lactams and shaking for an additional two hours prior to RNA extraction, according to Trépanier et al. (1997).

4.3.6 Protein Techniques

Cells were fractionated into periplasm and spheroplastic protein fractions (cytosol and membranes) using the PeriPreps™ Periplasting Kit (Epicentre Biotechnologies, Madison, WI) Cells were grown overnight and diluted 1:100 at 37°C in LB medium without NaCl until an optical density of 0.7 (600 nm) was reached. The kit was used

according to manufacturer's protocols, including extended incubation times and higher concentrations of lysozyme (25 μg/reaction), as recommended for hardier bacteria. For Western blots, protein samples were separated on NuPAGE® 4-12% Bis-Tris polyacrylamide gels (Invitrogen) alongside Precision Plus Protein Prestained Dual Color Standards (Bio-Rad, Hercules, CA). Western blots were performed using polyclonal rabbit anti-PenA antibodies which were provided by Dr. Robert Bonomo from Case Western Reserve University, Cleveland, OH. A goat anti-Rabbit IgG alkaline phosphatase-conjugated antibody (Sigma) was used as a secondary antibody and SIGMAFAST™ BCIP®/NBT tablets (Sigma) as a detection reagent according to the manufacturer's protocol.

4.4 Results

4.4.1 The role of of *penA* in β-lactam resistance

To assess whether Bp82 and its parent 1026b could interchangeably be used for PenA characterization experiments, the susceptibilities of these strains to various β-lactams were tested. There were no observable differences in the susceptibilities of these two strains for any of the β-lactams and clavulanic acid tested (Table 4-4), thereby validating the use of Bp82 in experiments otherwise not feasible under Select Agent regulations. Deletion of the *penA* gene from 1026b and Bp82 caused a significant (≥4-fold) decrease in the susceptibilities for six of nine β-lactams tested, and ≥16-fold for three of them (ampicillin, amoxicillin and carbenicillin). Likewise, up-regulation of *penA* by single-copy expression from the IPTG-inducible P_{tac} significantly increased the MIC for seven of the eight β-lactams tested with meropenem showing only slight change. (Amoxicillin could not be tested as the resistance level for the wild-type was already beyond

detection.) Quantitative real time PCR experiments showed that in the P_{tac}-*penA* strain (Bp82.21) *penA* transcript levels were 36-fold higher when compared to transcript levels observed in the wild-type strain (data not shown). This increase in transcript levels corresponds to the observed increases in resistance to all β-lactams. These experiments demonstrated that although PenA is a clinically significant β-lactam resistance mechanism, it affects some β-lactams more than others. While mutations in *penA* can significantly affect the utility of ceftazidime and amoxicillin + clavulanic acid, the enzyme has a lesser effect on the activity of carbapenems and novel experimental drugs such as BAL30072.

4.4.1 *penA* mutations are responsible for clinically significant ceftazidime and amoxicillin + clavulanic acid resistance

Previous studies identified several *penA* mutations in clinical and laboratory isolates that led to clinically significant ceftazidime or clavulanic acid resistance. Specifically, a C69Y substitution caused high-level ceftazidime resistance (Sam et al., 2009), a P167S substitution medium-level ceftazidime resistance (Tribuddharat et al., 2003), and a S72F mutation resistance to clavulanic acid (Tribuddharat et al., 2003). To assess whether these mutations alone were sufficient to cause the observed resistance phenotypes, they were engineered into the *penA* gene of strain Bp82 resulting in expression of a mutated PenA from the native *penA* promoter. Susceptibility studies revealed that the C69Y and P167S point mutations caused significant increases in ceftazidime resistance of >64- and 5-fold, respectively (Table 4-4). These mutations sensitized strains to other β-lactams such as amoxicillin, ampicillin, carbenicillin and imipenem but not amoxicillin + clavulanic acid, piperacillin, meropenem, and BAL30072, whose MICs were already at

Table 4-4. β –lactam susceptibilities for *B. pseudomallei* wild-type strains and mutants with *penA* point mutations.

		MIC (μg/mL)								
Strain[a]	**Mutation**	**Amoxillin**	**Amoxicillin + Clavulanic Acid**	**Ampicillin**	**Piperacillin**	**Carbenicillin**	**Ceftazidime**	**Imipenem**	**Meropenem**	**BAL30072**
1026b	None	>256[b]	3	256	8	1024	3	1.5	0.75	0.065
Bp82	None[c]	>256	3	ND	8	ND	3	1.5	0.75	0.031
Bp82.3	PenA C69Y	6	3	4-8	6-8	32-64	256	0.19	0.75	0.031
Bp82.4	PenA S72F	>256	12-16	64-128	8	512	3	1-2	0.75	0.031
Bp82.5	PenA P167S	6	3	4-8	4	32-64	16-24	0.19	0.5-0.75	0.031

[a] All mutants were derived from Bp82
[b] >256, the detection limit is 256 μg/mL
[c] no *penA* mutation but strain is a 1026b Δ*purM* derivative
ND, Not Determined

low levels. The S72F point mutation caused a 4-fold increase in resistance to amoxicillin+clavulanic acid and did not cause any changes in susceptibility to other β-lactams.

4.4.2 PenA is secreted via the TAT system

Analysis of the amino-terminal PenA amino acid sequence revealed the presence of a putative TAT signal sequence indicating that it may be a TAT secreted protein (Figure 4-1). To test this notion, the two signature arginine residues at positions 7 and 8 were changed to a lysine or alanine, respectively. MIC determinations revealed that disruption of the TAT signal sequence by an R7K mutation did not affect PenA activity (Table 4-5). This is in accordance with previous studies which have shown that mutation of the first arginine to a lysine can either have little effect or be completely inhibitory, depending on the rest of the signal sequence (Stanley et al., 2000). However, an R8A substitution completely abrogated PenA activity, consistent with PenA being a TAT secreted enzyme. This notion was further supported by the finding that a *tatABC* deletion mutant exhibited a susceptibility profile similar to those of the R8A substitution and Δ*penA* deletion strains. As expected then, a Δ*penA* Δ*tatABC* double mutant was most susceptible to PenA substrates.

4.4.1 Cellular localization of PenA

We next attempted to localize the PenA protein in the cell envelope using Western blot analysis and polyclonal α-PenA antibodies. Using this method, PenA could not be localized to the periplasmic fraction but rather only to the spheroplastic fraction which contains both cytosolic and membrane proteins. Multiple attempts at isolation of PenA from the periplasmic fraction employing other fractionation methods such as chloroform

Table 4-5. β-lactam susceptibilities of PenA deletion, TAT signal sequence and TAT secretion apparatus mutants.

		MIC (μg/mL)								
Strain[a]	**Mutation**	**Amoxicillin**	**Amoxicillin + Clavulanic Acid**	**Ampicillin**	**Piperacillin**	**Carbenicillin**	**Ceftazidime**	**Imipenem**	**Meropenem**	**BAL30072**
1026b	None	>256[b]	3	256	8	1024	3	1.5	0.75	0.065
Bp82	None[c]	>256	3	128	8	1024	3	1.5	0.75	0.031
Bp319	Δ*penA*	6	3	8-16	2	32	2	0.25	0.75	0.016
Bp82.11	Δ*penA*	ND	3	ND	ND	ND	ND	ND	ND	ND
Bp409	Δ*tatABC*	4-6	3	4-8	3	16-32	1.5	0.19	0.5	0.016
Bp420	Δ*penA* Δ*tatABC*	4	3	4		16	1	0.25	.05	0.016
Bp342	PenA R7K	>256	3	128		1024	3	2	0.75	0.031
Bp421	PenA R8A	8	3	8	2	32	1	0.25	0.5	0.031

[a] Bp319, Bp342, Bp409, Bp420 and Bp421 were derived from 1026b; Bp82.11 was derived from Bp82
[b] >256, the detection limit is 256 μg/mL
[c] no *penA* or *tat* mutation but strain is a 1026b Δ*purM* derivative
ND, Not Done

(Ames et al., 1984) or magnesium chloride (Imperi et al., 2009) extraction yielded the same results. Western blot analysis of the spheroplastic fraction (Figure 4-2) showed the mature 27 kDa PenA protein is seen only in an extract derived from wild-type 1026b (lane 5). In contrast, the unprocessed 31 kDa PenA protein was observed in extracts from the R8A (lane 1) and Δ*tatABC* (lane 2) mutants. A mixture of mature and unprocessed PenA was seen in the R7K mutant extract with the majority being the mature protein (lane 3). As expected, no PenA protein was observed in the extract from the Δ*penA* mutant (lane 4). This experiment provides biochemical evidence for PenA processing only in 1026b and the R7K mutants, both of which secrete active PenA via the TAT system as judged by β-lactam susceptibility assays. All other strains are susceptible to β-lactams.

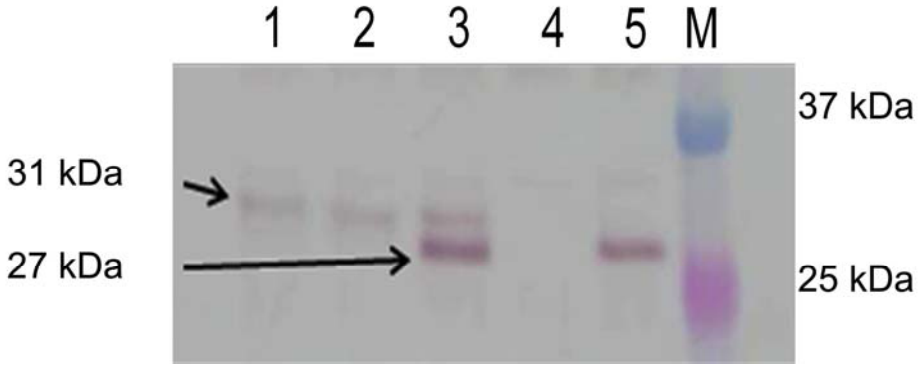

Figure 4-2. PenA is a TAT secreted protein. Spheroplastic proteins were analyzed by Western blot using anti-PenA polyclonal antibodies. The arrows point to the expected unprocessed (31 kDa) and processed (27 kDa) forms of PenA. Lanes: 1, R8A TAT signal sequence mutant; 2, Δ*tatABC* mutant; 3, R7K TAT signal sequence mutant; 4, Δ*penA* mutant; 5, wild-type 1026b; M, molecular weight markers (two proteins of the 10-250 kDa Precision Plus Protein Dual Color Standards from Bio-Rad, Hercules, CA).

4.4.2 Local regulators are not involved in regulation of *penA* gene expression

As shown in Figure 4-1, the *B. pseudomallei penA* region encodes two LysR-type transcriptional regulators and a putative peptidase. Since chromosomal β-lactamase gene expression was shown to be regulated by products of adjacent regulatory genes in several bacteria, including *B. cepacia* (Trepanier et al., 1997), the structural genes encoding for these regulators were either deleted or overexpressed and their effects on PenA transcription and activity assessed by either qRT-PCR or MIC determinations. Carbenicillin was used as a "sentry" β-lactam for assessing PenA activity by MIC experiments because it is one of the best PenA substrates (Table 4-4Table 4-5). MIC determinations showed that neither deletion of putative regulators (Bp343 and Bp349 or Bp82.30 and Bp82.32) nor constitutive expression of the regulators (Bp82.14 and Bp82.16) affected PenA activity (data not shown). The same observations were made when ceftazidime was used in susceptibility assays instead of carbenicillin. Likewise, deletion (Bp344 and Bp82.31) or over-expression (Bp82.15) of the putative peptidase gene up-stream of *penA* had no effect on PenA activity. Lastly, since bacterial β-lactamase gene expression can either be subject to substrate induction or influenced by environmental factors such as salts (Pumirat et al., 2009), MIC determinations were performed in the presence or absence of substrate and salt. However, presence of ceftazidime, carbenicillin, or high salt and the absence salt had no apparent effect on β-lactam susceptibilities. qRT-PCR assays supported the MIC data, with no change in *penA* expression levels observed between controls and strains treated with ceftazidime, carbenicillin, imipenem, penicillin G or high salt (data not shown).

4.5 Discussion

The data presented in this study employing isogenetic mutants in a defined genetic background confirm that PenA is a major β-lactam resistance factor in *B. pseudomallei*. Increased expression of *penA* conferred increased resistance levels to the majority of β-lactams tested. Conversely, *penA* deletion resulted in susceptibility to all β-lactams tested. Furthermore, clinically observed *penA* mutations were responsible for the altered β-lactam substrate spectrum of the enzyme and consequently the new resistance profile. Point mutations near the active site are the most common reason for substrate profile shifting because they accommodate different side chains of various β-lactams by either changing the active site steric properties or locations of the actual active residues (Drawz and Bonomo, 2010). Complete shifts in substrate profiles have been previously documented, such as with a mutant gram negative TEM-1 β-lactamase showing increased ceftazidime hydrolysis but decreased activity against ampicillin (Venkatachalam et al., 1994). The good news is that the *B. pseudomallei* C69Y PenA mutation sensitizes the cell to other β-lactams thus possibly enabling new therapeutic strategies. Additionally, our studies showed that of all β-lactams tested meropenem is the only β-lactam not affected by PenA mutations and overproduction, and thus the superior β-lactam antibiotic for melioidosis treatment with regard to potential emergence of PenA β-lactamase mutants.

The mutants created and analyzed in this study did not precisely mimic previously documented mutants. In all cases, our strains had an equal or lower resistance level. This may simply be due to variations in MIC methodologies, which can change the measurements significantly (Wuthiekanun et al., 2005), or *B. pseudomallei* strain

variability that may affect resistance patterns (Thibault et al., 2004). Using the S72F clinical mutant as an example, two groups determined the MIC of the parental strain 392a and its mutant, 392f. The first group showed a 16-fold increase in amoxicillin-clavulanic acid resistance (Godfrey et al., 1991) but the other group only showed a 2-fold increase (Tribuddharat et al., 2003). These numbers are significantly different from one another. The susceptibilities observed with our isogenetic Bp82 derivatives fell between the two previously reported set of numbers at 4- to 5- fold. Another potential cause for inconsistency was that some of the published experiments cloned the mutant *penA* gene and expressed it in an unrelated bacterium, *E. coli*, from a high-copy number plasmid (Ho et al., 2002; Tribuddharat et al., 2003). Our data showed that increased expression of *penA* from a single-copy, chromosomally inserted expression element with a strong inducible promoter can change the profile from susceptible to resistant. A high-copy number plasmid will obviously lead to higher gene expression and consequently high resistance levels. Additionally, other cellular factors, including outer membrane permeability, can affect the efficacy of resistance mechanisms. For the P167S mutation, Tribuddharat et al. (2003) showed a 16-fold increase in ceftazidime resistance in *B. pseudomallei*, but only a 2-fold change in an *E. coli* strain expressing recombinant *penA* genes.

Besides playing a crucial role in the export of virulence factors in many bacteria (De Buck et al., 2008), the TAT system has previously been shown to be required for the export of β-lactamases in *Mycobacterium smegmatis* (McDonough et al., 2005). Through deletion of the *tatABC* operon and mutation of a crucial arginine residue of the putative TAT-signal sequence of *B. pseudomallei* PenA, we showed that PenA is indeed a TAT

secreted enzyme. The exact cellular location of PenA β-lactamase could not be pinpointed. Various periplasmic extraction techniques failed to localize the enzyme to the periplasm but rather indicated that it is localized in the spheroplastic compartments of the cell which encompasses the cytosol and the membranes. Since a secreted enzyme is unlikely to be localized in the cytosol this experimental evidence points to the fact that PenA may be a membrane-associated enzyme. This is in agreement with earlier findings by Livermore et al. (1987) who demonstrated that after sonication and centrifugation, the vast majority of β-lactamase activity was present in the membrane fraction. Membrane association of β-lactamases is not common but has been documented. The first account of a membrane associated β-lactamase in Gram negative bacteria was from work with *Moraxella catarrhalis* (Bootsma et al., 1999). In this case it was hypothesized that the gene was a lipoprotein of Gram-positive origin, as membrane association in Gram positive bacteria had been previously observed. This is unlikely the case for PenA because *penA* has 70% GC content, comparable to the overall genome (68%). How *B. pseudomallei* PenA could become membrane-associated is unclear since there is no evidence of the enzyme being a lipoprotein.

Further evidence for TAT secretion was obtained by analyzing the PenA processing. Tullman-Ercek et al. (2007) describe the processing of TAT-signal sequences and the cleavage of their amino-termini upon passing through the inner membrane, using MdoD as an example. The authors describe a hydrophobic region before the processing site determined to be an AXA motif. A comparative analysis of the amino-termini of MdoD (MDRRRFKGSMAMAAVCGTSGASLFSQAAFA) and PenA (MNHSPLRRSLLVAAI-STPLIGACAOLRGQAKNVAAA) at http://expasy.org/tools/protscale.html using the

Kyte & Doolittle hydrophobicity algorithm revealed that the two sequences exhibited a similar hydrophobicity profile and comparable predicted processing sites (underlined AFA in MdoD and AAA in PenA)(Figure 4-3). Using this information, we calculated that the molecular mass of PenA changes from 31 kDa to 27 kDa after processing. Bands corresponding to these sizes were seen observed using Western blot analysis (Figure 4-2).

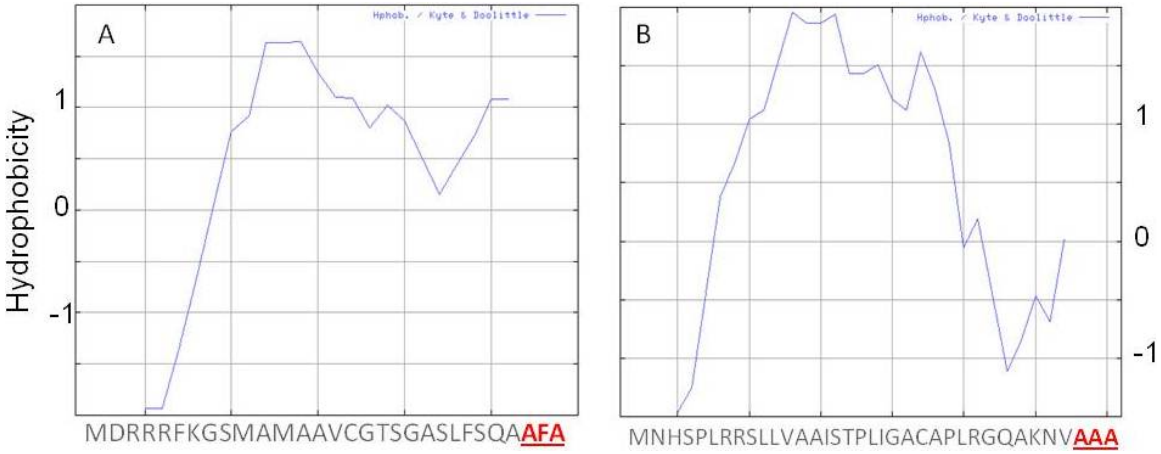

Figure 4-3. Hydrophobicity maps of A) MdoB and B) PenA.

Some β-lactamase genes require LysR regulatory factors for expression, with or without a β-lactam inducer. Trépanier et al. (1997) showed that the *penA* gene from *B. cepacia* was regulated by a LysR-family regulator encoded by the divergently transcribed *penR* gene when expressed from plasmids in *E. coli*. PenA β-lactamase gene expression was not only regulated by PenR but was also inducible in the presence of imipenem in this system. A gene, *BPSS0948*, homologous to *B. cepacia penR* is found in *B. pseudomallei* downstream of *penA* (Figure 4-1). An additional LysR-family regulator encoding gene, *BPSS0944*, is located upstream of *penA*, but it has less sequence

homology to *penR* and is separated from *penA* by a putative peptidase gene. Our analyses involving gene deletions, induction experiments, as well as growing cells under conditions such as salt stress (Pumirat*, et al.*, 2009) that may trigger β-lactamase induction showed that neither the LysR-type regulators BPSS0944 and BPSS0948 nor the putative peptidase encoded by *BPSS0945* are involved in regulation of *penA* expression in *B. pseudomallei* strain 1026b, at least not under the experimental conditions employed during these studies. Although many chromosomal β-lactamases are inducible, others are constitutively expressed (Neu and Chin, 1985; Jacoby and Bush, 2005). When multiple β-lactamases are present in a bacterial strain, they can be subject to complex regulation, including co-regulation with penicillin binding protein 2 (Hackbarth and Chambers, 1993; Naas et al., 1995). In this context it is noteworthy that by analysis of clinical strains obtained from patients that failed ceftazidime therapy and studies of recreated 1026b-based mutants we and others recently identified deletion of a *B. pseudomallei* PBP3 homolog as a mechanism causing high-level ceftazidime resistance (Chantratita et al., manuscript submitted).

Definition of the molecular basis of resistance mechanisms for clinically significant β-lactams forms the basis for design of diagnostic tools that allow rapid detection of emergence of resistance and thus redirection (clinical settings) or initiation (biodefense) of proper melioidosis therapy or prophylaxis.

4.6 Acknowledgements

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CHAPTER 5:

BPSS1219 AS A NOVEL CEFTAZIDIME RESISTANCE DETERMINANT IN *BURKHOLDERIA PSEUDOMALLEI*

(Presented in Chantratita* N., **D. A. Rholl***, B. Sim, V. Wuthiekanun, D. Limmathurotsakul, P. Amornchai, A. Thanwisai, H. H. Chua, W. F. Ooi, M. T.G. Holden, N. P. Day, P. Tan, H. P. Schweizer, S. J. Peacock. Genomic loss of penicillin-binding protein 3 in *Burkholderia pseudomallei* confers clinical resistance to ceftazidime. Submitted to *Journal of Clinical Investigation*)

The work presented in this chapter defines the deletion of the class 3 PBP BPSS1219 as a novel ceftazidime resistance determinant in *B. pseudomallei*. This deletion also confers glycerol dependence and filamentous cell morphology. I acknowledge Drs. Narisara Chantratita, Sharon Peacock and Patrick Tan Boon Ooi for providing strains and initial bioinformatic analyses.

5.1 Abstract

Melioidosis is a rare but serious tropical disease caused by the gram negative bacterium *Burkholderia pseudomallei*. Effective treatment options are limited due to the bacterium's intrinsic antibiotic resistance. The use of newer generation β-lactams,

specifically ceftazidime, in the initial treatment phase halved the mortality rate compared with previous combination therapies. Resistance to ceftazidime is rare but has been documented in several clinical cases. Each new case of resistance is of great concern as ceftazidime is the primary treatment option, especially in Southeast Asia where the incidence of melioidosis is high. Six culture-confirmed melioidosis patients failed ceftazidime therapy despite initial success. Secondary isolates were ceftazidime resistant and extremely slow growing on Ashdown media. Each resistant mutant had undergone large deletions on chromosome two around a recurrent region of 49 genes. Through genetic manipulation, I was able to identify *BPSS1219,* which codes for a class three penicillin binding protein (PBP3), as the gene responsible for the observed phenotype. *BPSS1219* deletion mutants derived from the genetically defined *B. pseudomallei* clinical isolate 1026b resembled the slow growth rate, glycerol dependence and ceftazidime resistance of the clinical large deletion mutants. Conversely, a wild-type *B. pseudomallei* phenotype could be restored in the clinical deletion mutants by complementing the *BPSS1219* gene into the chromosome using a mini-Tn*7* system. Until now, all characterized ceftazidime resistance mechanisms have dealt with mutations in the β-lactamase PenA. The difficulty faced during diagnosis via culturing on Ashdown agar demonstrates the possibility of undiagnosed cases caused by this difficult to culture mutant phenotype.

5.2 Introduction

B. pseudomallei is the etiological agent of melioidosis. Due to the high intrinsic resistance of the bacterium, there are limited effective treatment options (13, 16, 33). In order to avoid treatment failure and decrease the potential for relapse, melioidosis

patients received two phases of therapy; intensive and eradication. The intensive phase typically consists 10-14 days of intravenous later generation β-lactam (27). It is the rapid implementation and efficacy of the intensive phase that dictates patient outcome (11). The introduction of ceftazidime in melioidosis therapy halved the mortality rate to roughly 40% compared to treatment with amoxicillin and clavulanic acid (38). Currently imipenem and meropenem are often used in Northern Australia (6, 10) but their prohibitive costs maintain ceftazidime as the treatment of choice for most endemic regions (5).

Ceftazidime resistance has been documented in several clinical cases. Single amino acid substitutions within the Ambler class A β-lactamase PenA have increased the resistance of *B. pseudomallei* to ceftazidime by greater than 8- or 128-fold in the case of a C69Y mutation (Ambler identification method)(28, 35). Still, these cases are rare and ceftazidime therapy is successful more often than the "traditional" combination therapy of doxycycline, chloramphenicol and trimethoprim-sulfamethoxazole (29).

Six melioidosis patients in Thailand with ceftazidime sensitive isolates eventually failed ceftazidime treatment, despite favorable results initially. Secondary isolates were cultured and were shown to be extremely ceftazidime resistant (≥256 μg/mL). However, these isolates were slow to grow, required glycerol in culture media and had filamentous cell morphology. Sequence analysis of the *penA* gene showed no mutations however all secondary ceftazidime resistant strains had large deletions on chromosome 2, ranging from 146-310 kb. All deletions involved recurrent deletions of 49 specific genes (N. Chantratita et al, unpublished data). Because ceftazidime acts by inhibiting cell wall synthesis, I identified three genes as potential resistance determinants; *BPSS1219,*

BPSS1239 and *BPSS1240*, genes coding for a PBP3, a D-alanyl-D-alanine carboxypeptidase and an additional PBP3 homolog, respectively. The product of each of these genes is involved in peptidoglycan synthesis. Inactivation of PBP3 has been shown to increase β-lactam resistance in gram negative bacteria (30) and the activity of D-alanine carboxypeptidase increases prior to cell division which may be another reason for filamentation (2). My objective was to create mutants in these three genes in the 1026b wild-type background using the pEXKm5-based allele exchange system (23). Additionally, I had access to primary (ceftazidime susceptible) and secondary (ceftazidime resistant) isolates from three Thai patients. With 1these strains I performed complementation studies and were able to restore phenotypes similar to those observed in the primary isolates. This genetic approach allowed us to establish a direct link between ceftazidime resistance, growth-impaired phenotype and loss of function of one of the deleted PBP3-encoding genes, BPSS1219.

5.3 Methods

5.3.1 *B. pseudomallei* strains and media.

B. pseudomallei strains used in this study other than the clinical isolates described elsewhere in the text are listed in Table 5-1. Luria Bertani Lennox broth or agar (LB) (MO BIO Laboratories, Carlsbad, CA) was used for routine growth of all *E. coli* or *B. pseudomallei* strains during genetic manipulation experiments. Where applicable, *B. pseudomallei* strains were also grown in tryptic soy broth (TSB) or tryptic soy agar (TSA)(Difco [Difco, Detroit, MI]). Media were supplemented with 400 μg/mL diaminopimelic acid (DAP; LL-, DD-, and meso-isomers; Sigma, St. Louis, MO) for *E. coli* RHO3 (23). Mueller Hinton Broth (MHB) and Agar (MHA) were used for MIC

Table 5-1. Bacterial strains used in this study.

Strain	Relevant properties[1]	Reference
B. pseudomallei		
1026b	Clinical isolate and common laboratory strain	(14)
K96243	Clinical isolate and common laboratory strain	(22)
Bp276	1026b Δ*BPSS1239*	This study
Bp277	K96243 Δ*BPSS1239*	This study
Bp307	1026b Δ*BPSS1240*	This study
Bp308	1026b Δ*BPSS1239* Δ*BPSS1240*	This study
Bp309	K96243 Δ*BPSS1240*	This study
Bp310	K96243 Δ*BPSS1239* Δ*BPSS1240*	This study
Bp351	1026b::mini-Tn*7*T-*FRT*-LAC	
Bp417	1026b:Tn*7*-*FRT*-LAC-1219(2)	This study
Bp418	Km^r; 699d:Tn*7*-FKM-LAC-1219(2)	This study
Bp432	Km^r; 415e:Tn*7*-FKM-LAC-lox-1219(2)	This study
Bp430	1026b:Tn*7*-*FRT*-LAC-1219(2) d1219-*FRT*(2)	This study
Bp431	Km^r; 1026b:Tn*7*-*FRT*-LAC-loxP d1219-FKM(2)	This study
Bp433	1026b:Tn*7*-*FRT*-LAC-loxP d1219-*FRT*(2)	This study
Bp435	Km^r; 1142b:Tn*7*-Km-lac-lox-1219(2)	This study
Bp438	Km^r; 415e:Tn*7*-Km-lac-empty	This study
Bp439	Km^r; 699d:Tn*7*-Km-lac-empty	This study
Bp478	Km^r; 415e::mini-Tn*7*T-*FRT-nptII-FRT-Ps12-loxP-BPSS1219-loxP*[2] from pPS2752	This study
Bp479	Km^r; 415e-Tn*7*-s12-FKM-lox-BPSL3031	This study
Bp480	Km^r; 699d::mini-Tn*7*T-*FRT-nptII-FRT-Ps12-loxP-BPSS1219-loxP* from pPS2752	This study
Bp481	Km^r; 699d-Tn*7*-s12-FKM-lox-BPSL3031	This study
Bp482	Km^r; 1026b-Tn*7*-s12-FKM-lox-BPSL3031	This study
Bp483	1026b::mini-Tn*7*T-*FRT-Ps12-loxP-BPSS1219-loxP* from pPS2752	This study
Bp484	Km^r; 1142b::mini-Tn*7*T-*FRT-nptII-FRT-Ps12-loxP-BPSS1219-loxP* from pPS2752	This study
Bp485	Km^r; 1142b-Tn*7*-s12-FKM-lox-BPSL3031	This study
Bp504	Km^r; 415a::mini-Tn*7*T-*FRT-nptII-FRT-Ps12-loxP-BPSS1219-loxP* from pPS2752	This study
Bp505	Km^r; 415a-Tn*7*-s12-FKM-lox-BPSL3031	This study
Bp506	Km^r; 699c::mini-Tn*7*T-*FRT-nptII-Ps12-loxP-BPSS1219-loxP* from pPS2752	This study
Bp507	Km^r; 699c-Tn*7*-s12-FKM-lox-BPSL3031	This study
Bp508	Km^r; 1142a::mini-Tn*7*T-*FRT-nptII-FRT-Ps12-loxP-BPSS1219-loxP* from pPS2752	This study
Bp509	Km^r; 1142a-Tn7-s12-FKM-lox-BPSL3031	This study
Bp561	1026b::mini-Tn*7*T-*FRT-Ps12-loxP-BPSS1219-loxP* Δc*BPSS1219*[3]	This study
Bp560	1026b::mini-Tn*7*T-*FRT-Ps12-loxP*; Δc*BPSS1219*	This study
Bp562	Km^r; Bp560::mini-Tn*7*T-*FRT-nptII-FRT-Ps12-loxP-BPSS1219-*	This study

	loxP[4]	
E. coli		
DH5α	Cloning vector	(23)
RHO3	Mobilizing vector for conjugations	(23)

[1]Abbreviations: Ap, ampicillin; Km, kanamycin; Zeo, zeocin; *Ps12*, *B. thailandensis* ribosomal *s12* gene promoter.
[2]Unless otherwise noted all mini-Tn*7* elements are integrated at the *glmS2*-associated Tn*7* attachment site
[3]c*BPSS1219* denotes the chromosomal *BPSS1219* gene
[4]The mini-Tn*7*T-*FRT-nptII-FRT-Ps12-loxP-BPSS1219-loxP* is integrated at the *glmS3*-associated Tn*7* attachment site

determinations and prepared according to manufacturer's specifications (BD, Franklin Lakes, NJ). For screening of pEXKm5-carrying *B. pseudomallei* merodiploids, media were supplemented with 50 μg/mL 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc)(Gold Biotechnology, St. Louis, MO). YT agar (10 g/L yeast extract and 10 g/L tryptone [Fisher Scientific, Fairlawn, NJ]) with 15% sucrose was used for *sacB*-mediated *B. pseudomallei* merodiploid resolution. Where required, media were supplemented with 4% (v/v) glycerol. Ashdown agar (ASH) consisted of 10 g/L Tryptone Soy Broth, 4% glycerol, 0.005% crystal violet, 0.0005% neutral red, 15 g/L agar, and 5 mg/L gentamycin.

Antibiotics used with the *E. coli* cloning strains DH5α (22) and RHO3 were 100 μg/mL ampicillin (Ap), 15 μg/mL zeocin (Zeo) or 35 μg/mL kanamycin (Km). For *B. pseudomallei*, media were supplemented with1000 μg/mL Km or 2000 μg/mL Zeo. Antibiotics were purchased from Sigma, St. Louis, MO (Ap, Km) and Invitrogen, Carlsbad, CA (Zeo). Strains were routinely grown at 37°C except for manipulations such as merodiploid resolution on YT-sucrose medium and propagation of cells harboring temperature sensitive plasmids, when bacteria were grown at 30°C.

5.3.2 Bacterial growth curves.

Bacterial growth curves were measured using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT). Briefly, 100 μL of overnight *B. pseudomallei* cultures grown in LB + 4% glycerol was washed once in 1 mL saline and resuspended in 50 μL saline. Glycerol supplementation was required for growth of ΔBPSS1219 mutants. A 5 μL aliquot of this cell suspension was then added to 195 μL of LB medium in each well of a 96-well flat-bottom plate. Plates were incubated at 37°C with constant shaking at 200 rpm and the optical density at 600 nm was read every 30 min for 48 h.

5.3.3 Antimicrobial susceptibility testing.

MICs for the three initial *B. pseudomallei* isolates were determined for ceftazidime, imipenem, amoxicillin-clavulanic acid, trimethoprim-sulfamethoxazole and doxycycline using the Etest method (AB bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. The variant strains did not grow on MHA (the medium used for the Etest method), and a non-standard method was developed on ASH. *B. pseudomallei* was sub-cultured either on ASH and incubated for 2 d at 37°C in air or in TSB 4% glycerol. From these cultures a 0.5 MacFarland standard was made in saline for an approximate bacterial concentration of 1 x 10^8 cfu/mL. This was spread plated onto the agar surface using a large sterile cotton swab. An Etest strip was applied onto agar and the plate was incubated at 37°C in air for 18-24 h before the MIC values was read from the inhibition zone of bacteria that intersected the strip.

Table 5-2. Plasmids used in this study.

Plasmid	Relevant properties[1]	Reference
pCR2.1	Ap^r Km^r; TA cloning vector (Invitrogen; Carlsbad, CA)	Invitrogen
pGem-Teasy	Ap^r; TA cloning vector (Promega; Madison, WI)	Promega
pEXKm5	Km^r; gene replacement vector	(9)
pUC57-*loxP*-MCS	Ap^r; contains a multiple cloning site (MCS) flanked by *loxP* sites (synthesized by GenScript, Piscataway, NJ)	This study
pUC18T-mini-Tn*7*T-Km-*FRT*	Ap^r Km^r; mini-Tn*7* delivery vector	(9)
pUC18T-mini-Tn7T-Km-LAC	Ap^r Km^r; mini-Tn*7* delivery vector pUC18T-mini-Tn*7*T-Km-*FRT* with P_{tac} and $lacI^q$ for regulation control	L Trunck, unpublished
pLOX1	Ap^r; Vector containing cassette with *loxP* sites in same orientation	(9)
pFLPe2	Zeo^r; source of Flp recombinase	(9)
pCRE5	Km^r; source of Cre recombinase	(9)
pPS2619	Ap^r Km^r; pCR2.1 with the entire *BPSS1219* gene using primers 2027+2028	This study
pPS2679	Ap^r; pLOX1with *Eco*RI fragment from pPS2619 (*BPSS1219*)	This study
pPS2686	Ap^r Km^r; pUC18T-mini-Tn7T-Km-LAC with *Hin*dIII fragment from pPS2679 (*loxP::BPSS1219*)	This study
pPS2708	Ap^r; pGem-Teasy cloning vector (Promega; Madison, WI) with *BPSL3031* using primers 2127+2128.	This study
pPS2735	Ap^r Km^r; pUC18T-mini-Tn*7*T-Km-*FRT* with *Ps12*	This study
pPS2736	Ap^r Km^r; pUC18T-mini-Tn*7*T-Km-*FRT-Ps12-loxP*-MCS-*loxP*	This study
pPS2755	Ap^r Km^r; pPS2736 with the *Eco*RI fragment from pPS2708 containing *BPSL3031* under promotion of P_{s12}.	This study
pPS2619	Ap^r Km^r; pCR2.1 (Invitrogen, Carlsbad, CA) with 2,149-bp *BPSS1219* PCR fragment	This study
pPS2752	Ap^r Km^r; pPS2736 with 2,165-bp *Eco*RI fragment from pPS2619	This study
pPS2568	Ap^r Km^r; pCR2.1 with 1,381-bp *BPSS1239* PCR fragment	This study
pPS2569	Km^r; pEXKm5 with 1,397-bp *Eco*RI fragment from pPS2568	This study
pPS2584	Km^r; pPS2569 with 279-bp *Dra*III fragment deleted from *BPSS1239*	This study
pPS2586	Ap^r Km^r; pCR2.1 with 1,573-bp *BPSS1240* PCR fragment	This study
pPS2587	Ap^r Km^r; pPS2586 with 293-bp *Hin*cII fragment deleted from *BPSS1240*	This study
pPS2589	Km^r; pEXKm5 with 1,296-bp *Eco*RI fragment from pPS2587	This study
pPS2828	Km^r; pEXKm5 with 676-bp *Eco*RI fragment Δ*BPSS1219*	This study

[1]Abbreviations: Ap, ampicillin; Km, kanamycin; Zeo, zeocin; *Ps12*, *B. thailandensis* ribosomal *s12* gene promoter; ^{r}, resistant; ^{s}, sensitive

5.3.4 Construction and integration of PBP3 expression constructs

5.3.4.1 mini-Tn*7* vector with constitutive *BPSS1219* expression

A new mini-Tn*7* vector allowing constitutive expression of cloned genes was derived in two steps. First, pPS2735 (pUC18T-mini-Tn7T-FKM-s12)(Table 5-2) was created by replacing the 16 bp *Sma*I-*Sac*I fragment from the multiple cloning site (MCS) of pUC18T-mini-Tn*7*T-Km-*FRT* with a *s12* promoter-containing fragment. This fragment was obtained by linker tailing using primers 1586 and 2154 (Table 5-3). Next, pPS2735 was digested with *Sfi*I and *Eco*RI and the intervening sequences were replaced with a 147 bp *Sfi*I-*Mfe*I fragment from pUC57-*loxP*-MCS which introduces a MCS (*Sac*I-*Nru*I-*Age*I-*Kpn*I-*Sac*II-*Xho*I-*Eco*RI-*Apo*I-*Not*I-*Sma*I-*Hin*dIII) flanked by Cre recombinase target (*loxP*) sites. The resulting vector was named pPS2736 (pUC18T-mini-Tn7T-Km-s12-*lox*).

To create a BPSS1219 expression vector, the BPSS1219 gene was then cloned into pPS2736. Primers 2027 + 2028 and *Pfu* polymerase were used to PCR amplify a 2149 bp fragment containing *BPSS1219* from 1026b chromosomal DNA. The PCR fragment was adenylated with *Taq* polymerase, gel purified and ligated into pCR2.1 (Invitrogen, Carlsbad, CA) to yield pPS2619. The 2165 bp *Eco*RI fragment from pPS2619 was subsequently ligated into pPS2736 to create pPS2752. In this construct, expression of *BPSS1219* is driven by the *B. thailandensis s12* ribosomal promoter.

5.3.4.2 mini-Tn*7* vector with inducible *BPSS1219* expression

A new inducible expression mini-Tn*7* vector was derived from pUC18T-mini-Tn*7*T-LAC-Gm to allow for improved selection in *B. pseudomallei*. The 1,334 bp *Afe*I fragment from pFKM2 was used to replace the 968 bp *Afe*I fragment of pUC18T-

Table 5-3. Oligonucleotides used in this study.

Name	Relevant properties	Reference
1586	5'-GAGCTG*TTGACT*CGCTTGGGATTTTCG*GAAT ATCAT*GCCGGGTGGGCC[1]	(7)
2154	5'-GGCCCACCCGGC*ATGATATTC*CGAAAATCC CAAGCG*AGTCAA*CAGCTCAGCT[1]	This study
1879	5'-CGGCGATTTGCGCTTCG	This study
1882	5'-CGCGATCGGATCGCTGTC	This study
1884	5'-TGCTCGCGCTCCTCTGC	This study
1887	5'-ATGATCTCCGCCCATGCG	This study
2280	5'-**CATTCGTCAGATCTTTCAAC<u>GATATC</u>**GAAGCCCGCCGACGC GC[2]	This study
2279	5'-**CGTTGAAAGATCTGACGAATG**	This study
1986	5'-AGGCACGCATGTATCTGACG	This study
1993	5'-CAACATCGACAACGACAACGC	This study
1987	5'-CCTGTACCTGAAGCGCTGGAT	This study
1988	5'-GTATAGACGTCCGCGAGTTGC	This study
1979	5'-CGCTCGCCGTTTCACGCC	This study
2265	5'-GAGCGTGAAGTGCTTGTC	This study
2268	5'GTGGCGCGTAGAGTGACC	This study
Tn*7*L	5'-ATTAGCTTACGACGCTACACCC	(9)
BPGLMS1	5'-GAGGAGTGGGCGTCGATCAAC	(9)
BPGLMS2	5'-ACACGACGCAAGAGCGGAATC	(9)
BPGLMS3	5'-CGGACAGGTTCGCGCCATGC	(9)
2058	5'-CTGGGTGTAGCGTCGTAAGC	This study
2205	5'-GGGCTGCAGGAATTGATAAC	This study
2027	5'-CAGCCAATCCAGACGAAATC	This study
2028	5'-ATTGCGAGGGGTTCCTAAAG	This study
2127	5'-ATTACGCGCAGCTCCAGTATC	This study
2128	5'-GACGTCTTGCCGTTCGTG	This study

[1]Italicized letters indicate *B. thailandensis* ribosomal *s12* gene promoter sequences

[2]The underlined sequence represents an *Eco*RV restriction site and the bold sequences overlap with primer 2279

mini-Tn*7*T-LAC-Gm, swapping the gentamicin resistance marker with a kanamycin resistance marker that is still flanked by Flp recombinase target (*FRT*) sites. This created pPS2481 (pUC18T-mini-Tn*7*T-FKM-lac). The *BPSS1219 Eco*RI fragment was cloned from pPS2619 into pLOX1 to create pPS2625 (pLOX1-1219). This resulted in a *loxP*-flanked *BPSS1219* gene. The 2,406 bp *Hin*dIII fragment from pPS2625 was cloned into

the *Hin*dIII site of pPS2481 to yield pPS2626. In this construct the *BPSS1219* gene is expressed from the inducible *E. coli* lactose-tryptophan operon hybrid promoter (P_{tac}) promoter which is under lactose repressor ($lacI^q$) control. Expression can be induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG).

5.3.4.3 mini-Tn*7* vector with constitutive *BPSL3031* expression

A mini-Tn*7* vector expressing the PBP3 homolog *BPSL3031* was created to assess whether it could overcome the ceftazidime resistance and rescue the filamentous phenotype of clinical strains. Primers 2127+2128 were used to PCR-amplify a 2,402 bp region containing *BPSL3031* from strain 1026b chromosomal DNA. The fragment was then ligated into pGem-T Easy (Promega; Madison, WI) to create pPS2708. The *Eco*RI fragment from this plasmid was ligated into pPS2736 (pUC18T-mini-Tn*7*T-FKM-s12-*lox*) to create pPS2755 (pUC18T-mini-Tn*7*T-FKM-s12-*lox*-3031) for constitutive expression of *BPSL3031*.

5.3.4.4 Integration of mini-Tn*7* cassettes into *B. pseudomallei*

The mini-Tn*7* element contained on pPS2752 (P_{s12}-*BPSS1219*), pPS2686 (P_{tac}-*BPSS1219*) or pPS2755 (P_{s12}-*BPSL3031*) was inserted into the *B. pseudomallei* genome either by co-electroporation (1026b) or co-conjugation (all other strains) with helper plasmid pTNS3 (9) and mobilizer strain RHO3 (23). In co-electroporation experiments, 100 ng each of mini-Tn*7* and pTNS3 plasmids were electroporated into electrocompetent 1026b cells using previously described procedures (8). Transformation mixtures were plated on LB medium with 1000 μg/mL Km and 4% glycerol (LBKG). For co-conjugation experiments, 100 μL of the respective *B. pseudomallei* recipient strain (415a, 415e, 699c, 699d, 1142a or 1142b), 100 μL of RHO3/pTNS3 and 100 μL of

RHO3/pPS2752 were mixed and washed in 10 mM $MgSO_4$, the pellet resuspended in 30 μL of 10 mM $MgSO_4$ and spotted on prewarmed 0.45 μm cellulose acetate membranes as previously described (9) placed on DAP-supplemented LB. After overnight incubation at 37°C, filters were placed in 1.5 mL tubes with 1 mL LB, vortexed and conjugation mixtures were plated on LBKG plates. Km^r colonies were purified and checked for Tn*7* insertion at one of the three possible *glmS*-associated attachment sites using primer sets of Tn*7*L and either BPGLMS1, BPGLMS2, or BPGLMS3, as previously described (9). For expression of genes from mini-Tn*7* constructs where they were under P_{tac} regulation, plates were supplemented with 1 mM IPTG. For consistency, we used strains with a single mini-Tn*7* insertion at the *glmS2*-associated Tn*7* attachment site. An exception is strain Bp562 which contains two mini-Tn*7* insertions, one at *glmS2* and the second at *glmS3*. All strains are listed in Table 5-1.

5.3.5 Deletion of *BPSS1239* and *BPSS1240*.

Using *Taq* polymerase, PCR fragments and DNA from ceftazidime-susceptible strain K96243 (18), chromosomal fragments were amplified and used for engineering gene deletion constructs as follows. For BPSS1239, its coding sequence was amplified using primers 1879 + 1882. The resulting 1,381 bp DNA fragment was gel-purified and ligated into pCR2.1 (Invitrogen; Carlsbad, CA) to create pPS2568. A 1,397 bp *Eco*RI fragment from pPS2568 was then cloned into the *Eco*RI site of pEXKm5 to form pPS2569. Finally, pPS2569 DNA was digested with *Dra*III to delete 279 bp of the BPSS1239 coding sequence to create pPS2584. For BPSS1240, a 1,573 bp PCR amplicon obtained with primers 1884 + 1887 was ligated into pCR2.1 to create pPS2586. This plasmid was digested with *Hin*cII and self-ligated, deleting 293 bp from the BPSS1240 coding

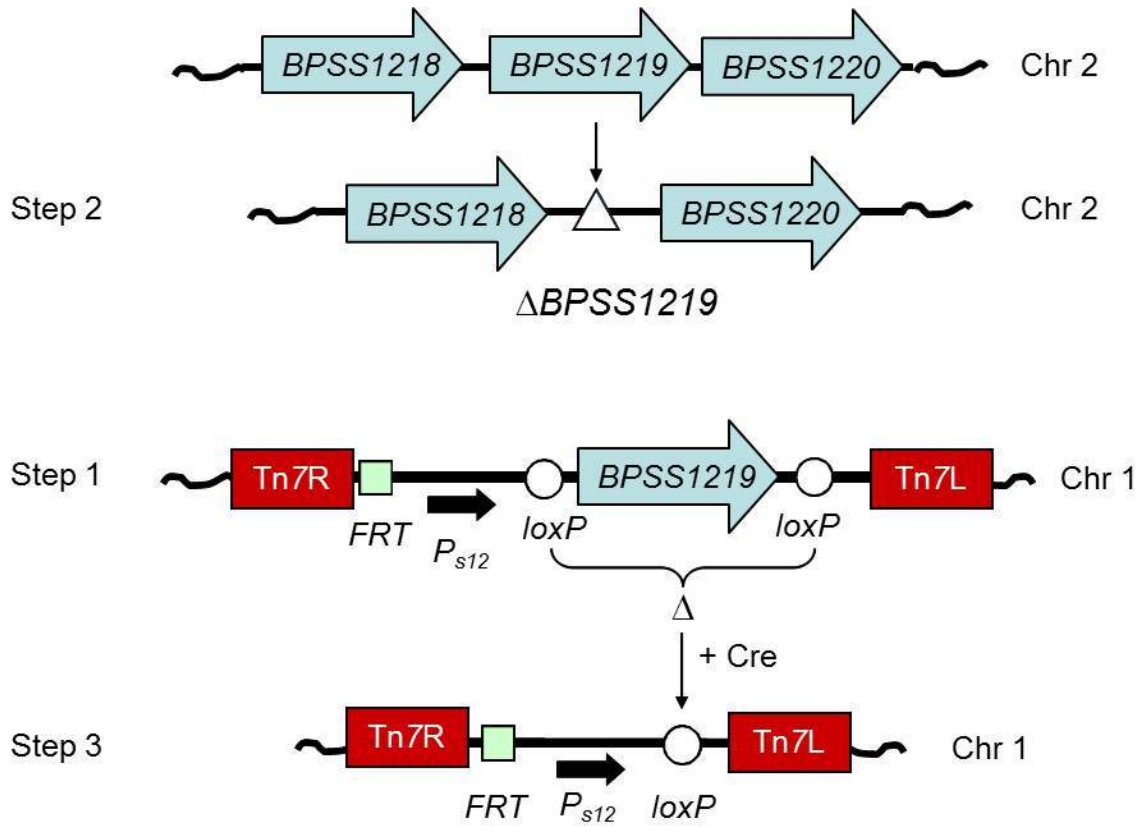

Figure 5-1. Deletion of BPSS1219 from *B. pseudomallei* strain 1026b. Deletion of the *BPSS1219* gene from chromosome 2 (Chr 2) was achieved in several steps. In step 1, a mini-Tn*7* element containing the *BPSS1219* gene flanked by *loxP* sites and expressed from a *B. thailandensis s12* promoter (P_{s12}) was inserted at the *glmS2 att*Tn*7* site on chromosome 1 (Chr 1) resulting in Bp483 (1026b::mini-Tn*7*-P_{s12}-*BPSS1219*$^+$ c*BPSS1219*$^+$). In step 2, the resident chromosomal BPSS1219 gene (c*BPSS1219*) was deleted from chromosome 2 using a gene replacement method yielding Bp561(1026b::mini-Tn*7*-P_{s12}-*BPSS1219*$^+$ Δ*cBPSS1219*). In step 3, the BPSS1219 rescue copy was deleted using Cre recombinase-mediated excision resulting in Bp560 (1026b::mini-Tn*7*-Δ*BPSS1219* Δc*BPSS1219*).

sequence and creating pPS2587. A 1,296 bp *Eco*RI fragment from pPS2587 was then ligated with *Eco*RI-digested pEXKm5 to yield pPS2589. After transformation of pPS2584 (BPSS1239) and pPS2589 (BPSS1240) into *E. coli* mobilizer strain RHO3, the respective plasmid-borne deletions were transferred to the 1026b genome using a previously described method (23). LBKG + X-Gluc was used for merodiploid isolation and YT + Sucrose + X-Gluc was used for resolution of these merodiploids. Using this method, ΔBPSS1239 (Bp276) and ΔBPSS1240 (Bp307) mutants could be obtained. Deletions in strains Bp276 + Bp307 were confirmed by colony PCR using primers 1986 + 1993 (BPSS1239) or 1987 + 1988 (BPSS1240). The ΔBPSS1239 ΔBPSS1240 double mutant Bp308 was obtained by transferring the BPSS1240 deletion allele contained on pPS2589 to the genome of Bp276.

5.3.6 Novel deletion method for *BPSS1219*

Since BPSS1219 could not be deleted from 1026b using the above described conventional gene replacement procedure (section 5.3.5), a rescue copy of BPSS1219 expressed from P_{s12} was introduced into chromosome one of 1026b. pFLPe2 (9) was used to remove the Km^r marker from this strain to create the unmarked Bp483 on LB + 4% glycerol + Zeo. A 656 bp PCR fragment where the entire 1,785 bp BPSS1219 coding region plus 93 bp of the upstream and 277 bp of the downstream intergenic regions were deleted was generated using splicing by overlap extension (SOEing) PCR which assembled two fragments of 351 bp (primer sets 2268 + 2279) and 326 bp (primer sets 2265 & 2280). This assembled fragment was cloned into pGEM-T Easy (Promega, Madison, WI) and the Δ*BPSS1219*-containing region was excised on a 676 bp *Eco*RI fragment which was cloned into the gene replacement vector pEXKm5 to form pPS2828.

This plasmid was conjugally transferred to Bp483 and merodiploids were selected and resolved as previously described (23). White colonies were checked for the deletion of the endogenous *BPSS1219* gene using primers 2265 + 1979. These strains maintained the Tn*7*-harbored copy of *BPSS1219*, as verified by using primers 2058 + 2205. Next, the Cre recombinase expression plasmid pCRE5 (9) was transformed into one retained Bp483 Δc*BPSS1219* mutant (Bp561) and plated on TSA + Km + glycerol with 0.2% rhamnose (for Cre recombinase induction) at 30^{o}C. Small Km^{r} colonies were picked and purified on the same medium at 30^{o}C. Excision of the *loxP*::*BPSS1219* cassette was verified using primers 2058 + 2205. Finally, mutants were grown at 37°C on TSA + glycerol to cure pCRE5 which resulted in strain Bp560.

5.4 Results and Discussion

5.4.1 Deletion of BPSS1239 and BPSS1240

Single deletion mutants defective in BPSS1239 (Bp276) or BPSS1240 (Bp307) were readily created in *B. pseudomallei* strain 1026b (a ceftazidime susceptible clinical strain originating from Thailand). These mutants did not show an altered growth phenotype or Gram stain appearance, and they remained susceptible to ceftazidime with no change in MIC value (data not shown). Similarly, the ΔBPSS1239 ΔBPSS1240 double mutant (Bp308) showed no change in phenotype when compared to wild-type 1026b. Based on this, we can infer that deletion of these two genes, individually or together, was not responsible for the clinically observed ceftazidime resistant, filamentous phenotype.

5.4.2 Deletion of BPSS1219

BPSS1219 could not be deleted using the pEXKm5 system despite numerous attempts and different selection parameters. This included selection of marked deletion

mutants on sucrose counter-selection media with kanamycin and glycerol. After several days of growth using this method we would obtain a number of small colonies, but all were still blue in the presence of X-Gluc, implying spontaneous sucrose resistance brought about by mutations in *sacB* (data not shown). The difficulty faced in deleting *BPSS1219* led us to believe it was an essential gene in certain conditions and necessary for cellular fitness.

To circumvent this roadblock, a strategy was developed to determine the essentiality of *BPSS1219* (Figure 5-1). A copy of BPSS1219 was cloned into the mini-Tn*7* vector pPS2736 where it was constitutively expressed from the *B. thailandensis* ribosomal *s12* gene promoter and flanked by *loxP* sites. Strain Bp483 was obtained by integration of this Tn*7* construct into chromosome 1 of strain 1026b at the *glmS2*-associated Tn*7* attachment site. The resident copy of BPSS1219 on chromosome 2 could then be readily deleted from Bp483 resulting in the BPSS1219 haploid strain Bp561. The rescue copy of BPSS1219 was then excised from Bp561 by Cre-mediated recombination to create the BPSS1219 null mutant Bp560.

Growth curves of Bp560 in LB medium revealed complete growth attenuation (Figure 5-2) and the cells exhibited a filamentous growth phenotype (Figure 5-3). This was not due to presence of a mini-Tn*7* cassette as Bp483 and Bp561 cells grew well in LB medium and looked normal in a Gram stain (Figure 5-3). Whereas 1026b was susceptible to ceftazidime (MIC = 3 μg/mL), Bp560 was highly ceftazidime resistant (MIC >256 μg/mL)(Table 5-4). Ceftazidime susceptibility was the same in strains 1026b and Bp562, the BPSS1219 null mutant Bp560 expressing BPSS1219 from a complementing mini-Tn*7* element integrated at *glmS3* (MIC = 3 μg/mL). The MICs for

other drugs, including amoxicillin-clavulanic acid, trimethoprim-sulfamethoxazole and doxycycline, were not substantially affected by deletion of and/or complementation with BPSS1219. A notable exception was the imipenem MIC which was not affected by constitutive BPSS1219 expression in 1026b (0.75 μg/mL) but dropped >10-fold in Bp560 (0.064 μg/mL) and was restored in Bp562 after complementation with BPSS1219 (MIC = 0.5 μg/mL).

Changes in expression of PBP's can affect the MICs of β-lactams targeting PBP3 in bacteria. PBP3 deficient *E. coli* mutants have decreased inhibition by β-lactams that preferentially bind PBP3 compared to wild-type strains (30). This was the precise phenomenon I observed. Conversely, over-expressing PBP3 on a plasmid in *P. aeruginosa* to an estimated 7-fold greater level was shown to increase resistance to the same type of β-lactams, presumably by titrating the drugs (21). Still, this change in resistance was only 8-fold (21), nowhere close to the greater than 100-fold change seen in the deletion mutants. When I overexpressed *BPSS1219* in *B. pseudomallei* ceftazidime susceptible mutants Bp417 and Bp483 (1026b with *BPSS1219* expression from P_{tac} and P_{s12}, respectively), I saw no difference in ceftazidime resistance.

Although all clinical mutants did not follow suit, deletion of *BPSS1219* in 1026b conferred a large decrease in MIC for imipenem. A similar situation was seen with the deletion of a PBP5 from *Enterococcus faecalis* (1). In this strain the MIC for the β-lactam ceftriaxone fell by >1000-fold (1). These observations are probably due to the ability of PBP's to compensate for each other in some manner. Deleting a "helper" PBP that compensates for inhibition of an enzyme specifically targeted by imipenem or

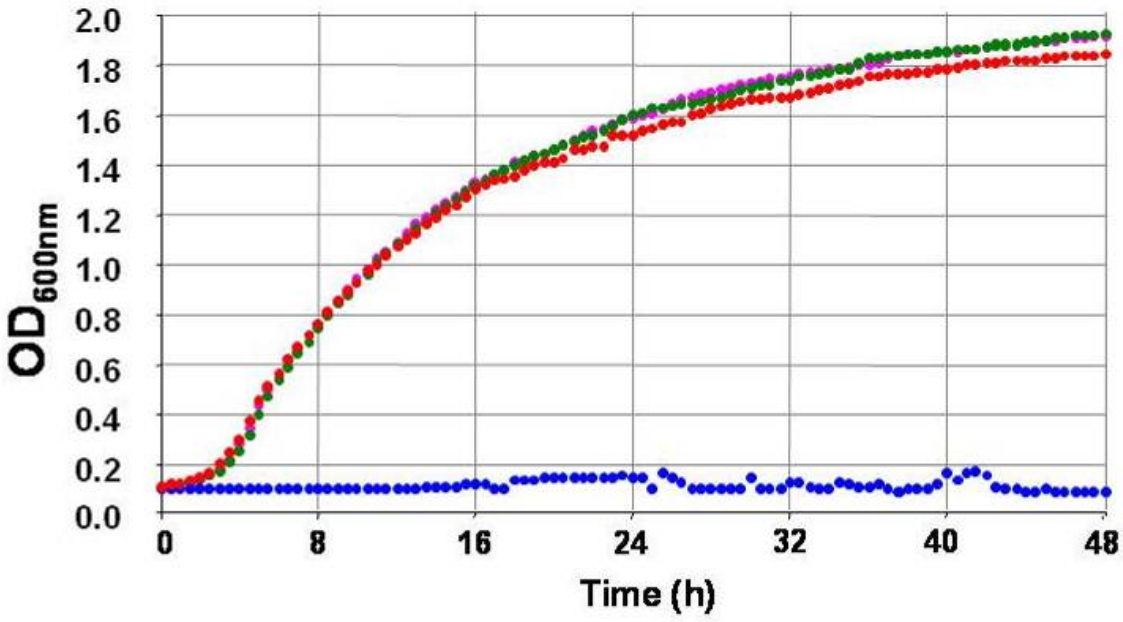

Figure 5-2. Growth curves of *B. pseudomallei* strain 1026b *BPSS1219* mutants. Growth curves of 1026b (red), Bp561 (magenta), Bp483 (green) and Bp560 (blue) of bacteria in LB medium.

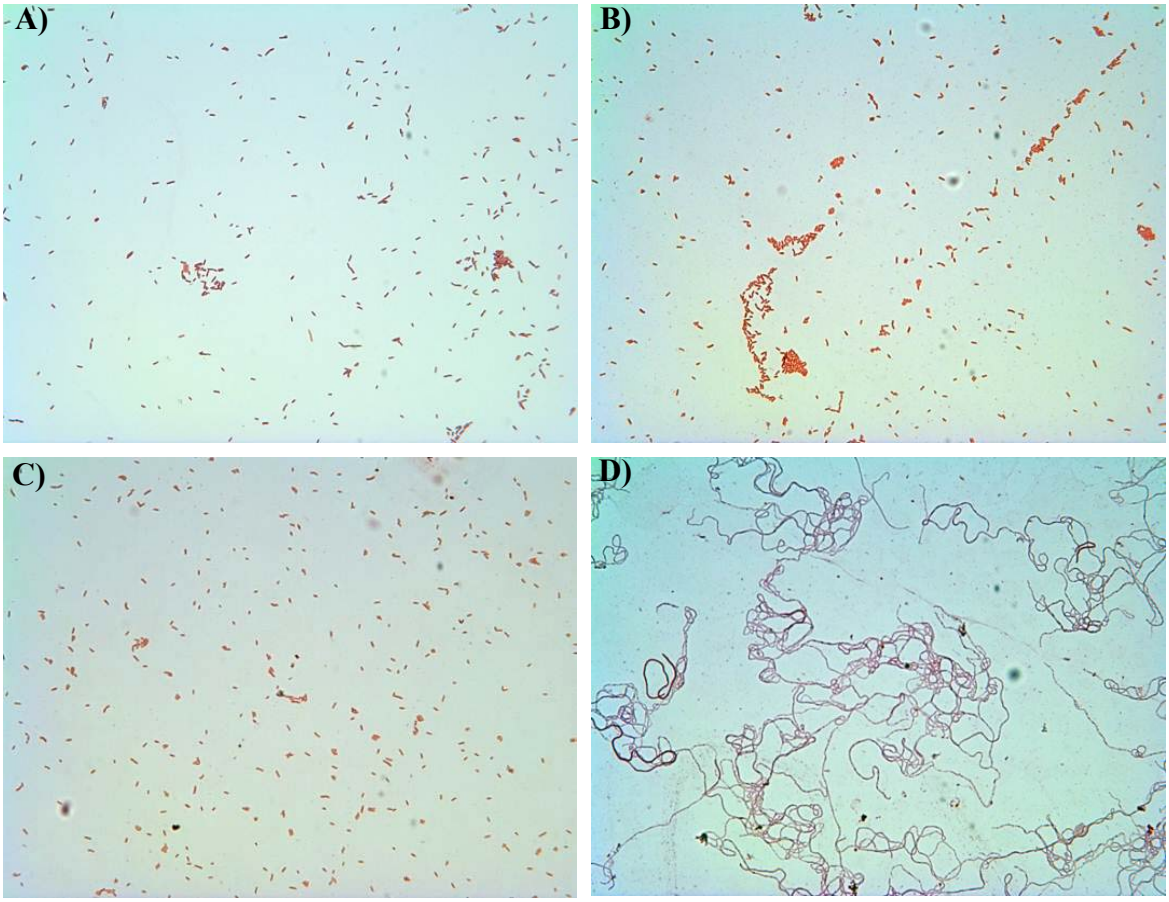

Figure 5-3. Cell morphology of strain 1026b and its *BPSS1219* derivatives. Strains were grown for 48 h in LB medium containing 4% glycerol. The cultures were washed with saline, fixed with 10% formalin and then Gram stained. The micrographs show cells photographed through a 40x objective. **A)** 1026b; **B)** Bp483 (1026b::mini-Tn*7-FRT-Ps12-BPSS1219*$^{+}$); **C)** Bp561 (1026b::mini-Tn*7-FRT-Ps12-BPSS1219*$^{+}$ Δ*cBPSS1219*); and **D)** Bp560 (1026b::mini-Tn*7-FRT-Ps12-loxP* Δc*BPSS1219*).

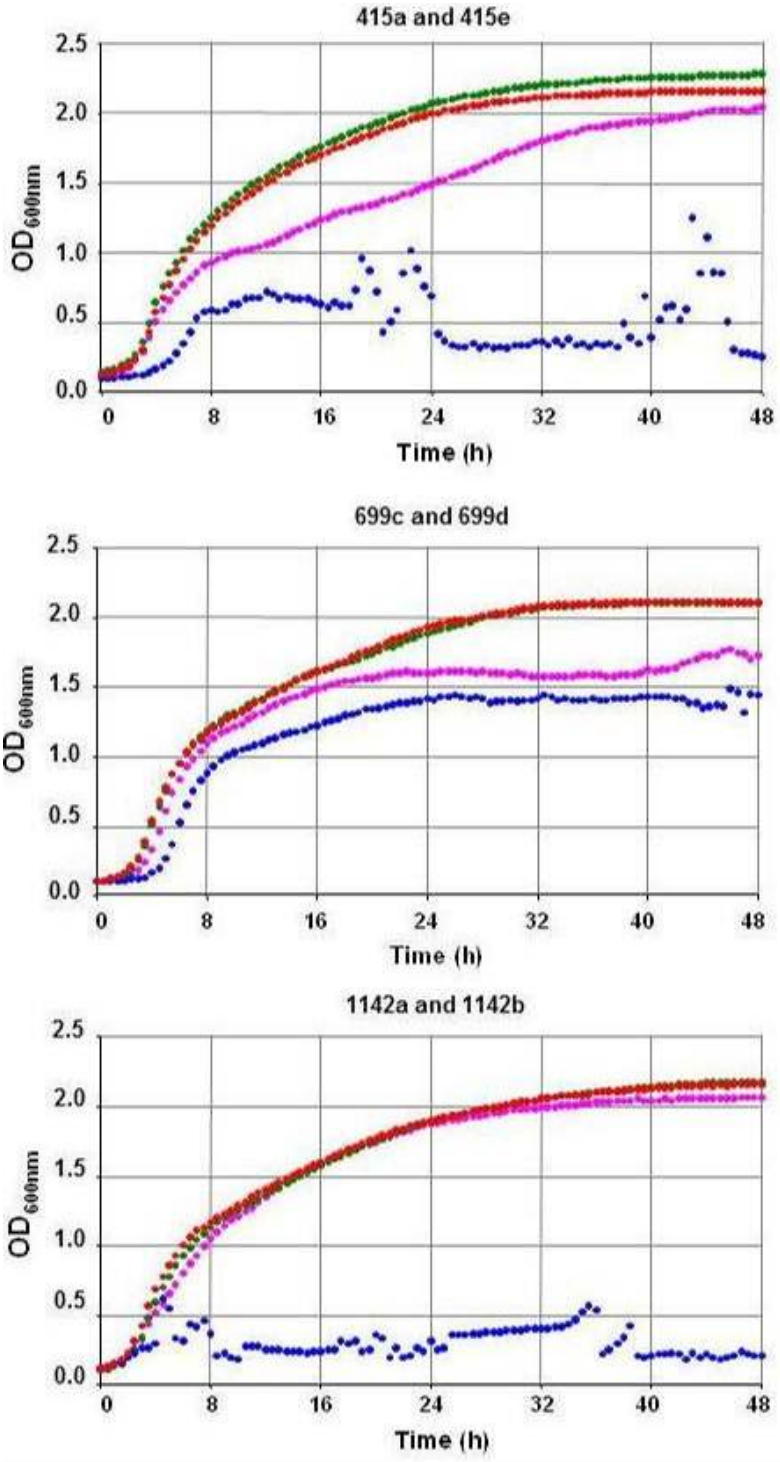

Figure 5-4. Growth curves of *B. pseudomallei* clinical and laboratory *BPSS1219* mutants. Primary isolate (red), ceftazidime resistant mutant (blue), primary isolate with Tn*7*-*BPSS1219* (green) and clinical mutant with Tn*7*-*BPSS1219* (magenta) are shown. All strains were grown overnight in LB medium containing 4% glycerol. The cultures were washed with saline and diluted 1:20 into fresh LB. In all strains, the mini-Tn*7* elements expressing *BPSS1219* from the *s12* promoter are integrated at the *glmS2*-associated Tn*7* attachment site.

Table 5-4. Etest MICs for clinical and laboratory *B. pseudomallei* strains with and without *BPSS1219* complementation.

		MIC (μg/mL)									
		Cef[1]		IP		XL		TMP-SMX		Dox	
Strain	**Complement**	**MHA[2]**	**ASH[3]**	**MHA**	**ASH**	**MHA**	**ASH**	**MHA**	**ASH**	**MHA**	**ASH**
415a	None	2	4	0.75	0.5	3	6	1.5	0.25	1	6
Bp504	415a::mini-Tn*7-Ps12-BPSS1219*	3	3	ND	ND	ND	ND	ND	ND	ND	ND
415e[4]	None	-[5]	>256	-	0.25	-	6	-	0.25	-	8
Bp478[4]	415e::mini-Tn*7-Ps12-BPSS1219*	1.5	8	0.38	1.5	2	6	1	0.5	0.75	8
699c	None	1.5	4	0.38	0.38	2	6	1	0.5	1.5	8
Bp506	699c::mini-Tn*7-Ps12-BPSS1219*	2	3	ND	0.5	ND	4	ND	ND	ND	ND
699d[4]	None	-	>256	-	0.094	-	4	-	1.5	-	12
Bp481[4]	699d::mini-Tn*7-Ps12-BPSS1219*	3	8	0.75	0.25	3	6	1.5	1.5	1	12
1142a	None	2	3	0.25	0.25	2	3	0.75	1.5	0.75	8
Bp508	1142a::mini-Tn*7-Ps12-BPSS1219*	2	3	ND	ND	ND	ND	ND	ND	ND	ND
1142b[4]	None	-	>256	-	0.125	-	4	-	0.25	-	8
Bp484[4]	1142b::mini-Tn*7-Ps12-BPSS1219*	2	8	0.5	1.5	3	8	0.75	0.25	0.75	12
1026b	None	1.5	3	0.5	0.75	3	4	0.125	0.125	0.5	4
Bp483	1026b::mini-Tn*7-Ps12-BPSS1219*	2	3	0.75	0.75	2	4	0.25	0.125	0.75	4
Bp560[4]	None	-	>256	-	0.064	-	3	-	0.125	-	4
Bp561[4]	1026b::mini-Tn*7-Ps12-BPSS1219*	2	3	0.75	0.75	2	4	0.25	0.125	0.38	4
Bp562[4]	Bp560::mini-Tn*7-Ps12-BPSS1219*	2	3	0.5	0.5	ND	ND	ND	ND	ND	ND

[1] Cef, ceftazidime; IP, imipenem; XL, co-amoiclav; TMP-SMX, co-trimoxazole; Dox, Doxycycline [2]MHA, Mueller-Hinton agar; [3]ASH, Ashdown agar; [4]Strains contain chromosomal BPSS1219 deletion; [5]-, no growth

ceftriaxone could remove that complementary pathway, thereby removing the enzymatic bypass of the antibiotic.

5.4.3 Complementation of clinical mutants

5.4.3.1 Growth phenotypes

Clinical ceftazidime resistant mutants 415e, 699d and 1142b had varying degrees of fitness and clumping when grown in LB medium. Expression of an exogenous *BPSS1219* cloned from strain 1026b using a chromosomally-integrated mini-Tn*7* element in these growth-impaired strains (making strains Bp418, Bp432 and Bp435) restored growth rates in mutants to levels more closely resembling those observed in the corresponding primary isolates 415a, 699c and 1142b (Figure 5-4). This showed that a major, if not the only, determinant for glycerol dependence in clinical strains was BPSS1219. No changes were seen in *BPSL3031* complemented strains for any growth phenotype or ceftazidime MIC.

5.4.3.2 Ceftazidime MIC

This near normal growth phenotype was accompanied by restoration of ceftazidime susceptibility (MIC = 1.5 to 3 μg/mL on MHA; Table 5-4). Interestingly, these BPSS1219-complemented mutants had consistently higher MIC levels when tested on Ashdown agar. However, the observed levels were still technically susceptible and were significantly lower than the ceftazidime resistant relatives (≥32-fold**).** There was also a large difference between the MIC results on Ashdown of strains complemented by *BPSS1219* expression promoted by P_{tac} versus P_{s12}. While the mutants with P_{s12} driving *BPSS1219* expression had clear zones of inhibition, those with P_{tac} regulation had a ring of inhibition was visible in all samples between 8 and 16 μg/mL accompanied by strong

growth within the areas of greatest ceftazidime concentration, even up to 256 μg/mL (Figure 5-5). The level of break-through resistance could be decreased by growing the bacteria in liquid media without glycerol and containing 1 mM IPTG prior to plating on Ashdown.

Still, the susceptibility of strains expressing *BPSS1219* from P_{tac} were similar to strains containing a P_{s12}-driven *BPSS1219* when plated on MHA. I may be able to account for this observation by heterogeneous expression from the P_{tac} vector. Plating on MHA would select against any bacteria without an active *BPSS1219* gene. When I combine this idea with the fact that the presence of BPSS1219 imparts ceftazidime susceptibility, one can see how there can be clean MIC definition on MHA. Meanwhile, Ashdown agar would allow bacteria lacking BPSS1219 to grow well, thereby allowing a ceftazidime resistant subpopulation to grow. Theoretically, the differences seen by subculturing bacteria with and without glycerol could change the requirement of BPSS1219. Similarly, subculturing with IPTG would increase BPSS1219 concentrations, thereby assuring the presence of the ceftazidime target molecular; PBP3. Both of these variables could affect cell populations. Growing bacteria in the presence of IPTG and without glycerol would not allow growth of BPSS1219 deficient cells and may therefore result in a more homogeneous ceftazidime susceptible population. This could explain the greater susceptibility to ceftazidime under such conditions.

5.4.3.3 Filamentation

The final confirmatory piece of the phenotypic puzzle would be a shift from long, filamentous mutants back to short rods after complementation with *BPSS1219*. Collaborating labs in Thailand showed filamentation in the clinical mutants, which I was

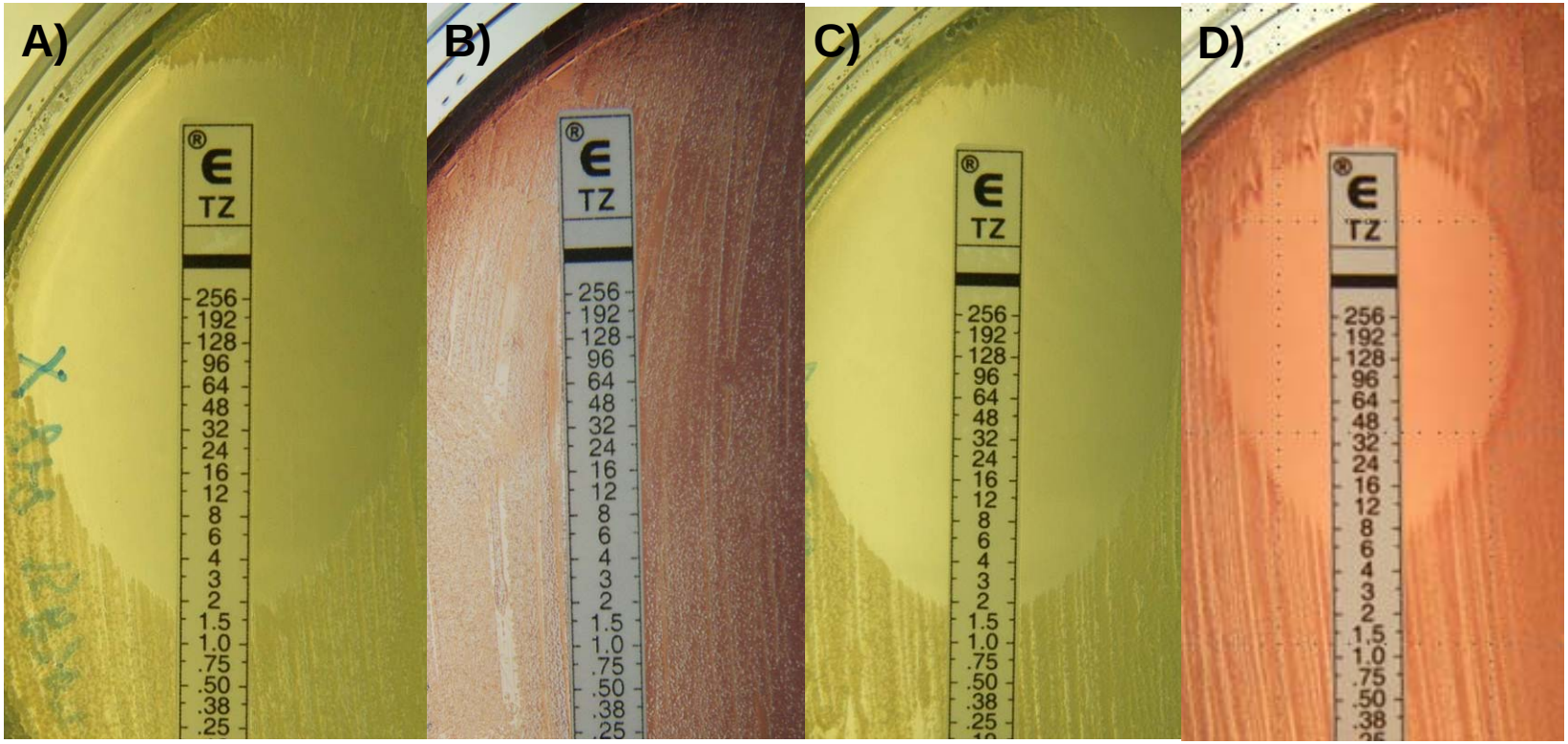

Figure 5-5. MIC determinations in complemented strains expressing BPSS1219 from P_{tac} vs. P_{s12}. **A)** Bp432 (415e::Tn*7*-P_{tac}-*BPSS1219*) on MHA + IPTG. **B)** Bp432 on ASH + IPTG. **C)** Bp478 (415e::Tn*7*-P_{s12}-*BPSS1219*) on MHA + IPTG. **D)** Bp478 on ASH + IPTG. Although it is difficult to see in the image, there is a region of lighter growth beginning around 24 µg/mL that is consistent well up to 256 µg/mL in panel B.

not able to reproduce. The clinical mutant strains may have undergone a significant degree of mutation prior to us receiving them. Thus, we were not able to see a difference in cellular structure by studying solely the clinical strains. However, the induction of filamentation by directed deletion of *BPSS1219* in 1026b is sufficient evidence to support this theory.

The phenomenon of filamentation induction by ceftazidime has been previously documented. Molecular inhibition of FtsI, a PBP3 homolog, in multiple bacterial species (*e.g. E. coli* and *Mycobacterium tuberculosis*) by β-lactams targeting PBP3 resulted in filamentation (31, 32, 37). Although concentric rings were formed along the elongated bacillus, cell division was incomplete (31). Ceftazidime has specifically been shown to induce filamentation, eventually leading to cell lysis (19). Other mechanisms of cell division have also been selected as novel drug targets (20), which would potentially allow multidrug therapy targeting actively growing cells. This could prove more effective, just as trimethoprim and sulfamethoxazole are synergistically more effective at targeting different steps of the folic acid synthesis process than each antibiotic by itself (24).

B. pseudomallei strain K96243 (GenBank accession numbers NC_006350 and NC_006351) codes for seven major classes of PBP genes (Table 5-5), including three PBP3 homologs; *BPSS1219*, *BPSS1240* and *BPSL3031*. The deletion of *BPSS1240* did not change the wild-type phenotypes. Additionally, introduction of a mini-Tn*7* with *BPSL3031* into clinical strains had no effect on ceftazidime resistance, filamentation or glycerol dependence (data not shown). This demonstrates that the PBP3 homologs BPSS1219 and BPSL3031 cannot substitute for each other under these conditions. This theory is supported by the lack of phenotypic change with the deletion of the third PBP3

Table 5-5. *B. pseudomallei* K96243 annotated PBPs. According to annotated GenBank sequence for *B. pseudomallei* K96243 (accession numbers NC_006350 and NC_006351). PBP3 homologs are in bold.

Locus	Name	PBP Type	Chromosome
BPSL2104		PBP1	1
BPSL3174	*mrcA*	PBP1a	1
BPSS2304		PBP1a	2
BPSS0238		PBP1b	2
BPSS0816		PBP1c	2
BPSL0183	*pbpA*	PBP2	1
BPSL3031 [a]	***pbpB***	**PBP3**	**1**
BPSS1219		**PBP3**	**2**
BPSS1240	***ftsI***	**PBP3**	**2**
BPSL0805		PBP4	1
BPSL0408		PBP6	1
BPSL1055	*pbpG*	PBP7	1
BPSL2297	*pbpG*	PBP7	1
BPSS0451		PBP7	2

[a] **Bold** indicates genes studied in this chapter.

homolog; BPSS1240. Despite their significant homology and possibly shared ancestry, these genes have been mutated sufficiently to disassociate from (possible) redundant pathways to other unknown functions. Though unlikely, it is also possible that the presence of three PBP3 genes allowed for two of the genes to mutate such that their gene products are no longer functioning in cell wall synthesis.

5.4.1 Summary

Based on the above data, we conclude that deletion of BPSS1219 was responsible for the severe growth defect and the ceftazidime resistance phenotype of the variant strains. The clinical mutants described above pose two problems clinically. Firstly, they represent an expansion of the already broad range of antibiotic resistance mechanisms in

B. pseudomallei (3, 12, 15, 25, 26). Secondly, they present a potential for evading conventional diagnostic measures (*e. g.* culturing on Ashdown agar).

Ceftazidime binds PBP3 in *Escherichia coli* (17). This explains decreased susceptibility to certain β-lactam in strains lacking a functional PBP3 (30). Cell wall deficient (CWD) bacteria, lacking the traditional peptidoglycan wall, have been associated with resistance to β-lactams (39). This is logical because a lack of pathway targeted by the drug, notably peptidoglycan synthesis, should result in resistance to such compounds. However, in those studies the CWD *P. aeruginosa* were also more sensitive to antibiotics not associated with cell wall synthesis, such as tetracycline and chloramphenicol (39). These changes in resistance patterns were purportedly due to increases in cell permeability and general cellular fragility (39). Our deletion mutant did not show changes in susceptibilities to non-β-lactam antibiotics (Table 5-4), but is also had an intact cell wall compared to true L-form bacteria, which lack the peptidoglycan lattice entirely.

The presence of CWD forms is very difficult to test for. However, in order to test the true antibiotic susceptibility of a cell *in vitro*, the testing conditions must mimic *in vivo* conditions as closely as possible. Only by inducing a potentially resistant form can clinicians accurately select the best therapeutic options. Failure to do this may result in treatment failure, despite *in vitro* susceptibility (4).

A second problem is in regard to the slow growing filamentous form. The inability to efficiently culture these slow growing mutants poses a problem similar to the identification of aminoglycoside susceptible strains. Approximately 1 in 1,000 *B. pseudomallei* isolates is susceptible to gentamicin, but because the Ashdown agar

culturing diagnostic test uses gentamicin as a means of selection these isolates are obviously missed. Thus, it is believed that the number of susceptible strains, and cases of melioidosis caused by them, are actually higher than the observed (36). This could very well be the case for our glycerol dependent mutants.

The loss or temporary absence of a PBP may not affect certain cellular functions, such as we witnessed with cellular elongation (Figure 5-3). It was shown in *Streptococcus* that certain PBP's are only present during certain growth stages (34). This means that drugs targeting such PBPs would only be effective during the stages which require activity/presence of the PBPs (34). This can be demonstrated by the MIC phenomenon known as the "inoculum effect". This effect is found when testing MICs *in vitro* in which the MIC dramatically changes based on the concentration of bacteria added to the test (34).

5.5 References

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CHAPTER 6:

IMPROVING TOOLS FOR GENETIC RESEARCH OF *BURKHOLDERIA PSEUDOMALLEI*

(Presented in Kyoung-Hee Choi, Takehiko Mima, Yveth Casart, Drew Rholl, Ayush Kumar, Ifor R. Beacham, and Herbert P. Schweizer. 2008. *Applied and Environmental Microbiology*. 74(4):1064-75.)

The work presented in this paper introduced a series of plasmids suitable for the manipulation of *B. pseudomallei* in a select-agent-compliant manner. It focused on mini-Tn*7* insertion and antibiotic marker recycling. I was responsible for constructing and testing pTNS3, an improved helper plasmid.

AND

(Presented in Carolina M. López*, Drew A. Rholl*, Lily A. Trunck, and Herbert P. Schweizer. 2009. Applied and Environmental Microbiology. 75(20):6496-6503.)

The work presented in this paper demonstrated a novel allele replacement system for use in *B. pseudomallei*. Dr. Carolina M. López and I contributed equally to the paper, so we were co-first authors (*). Dr. López created pEXKm4 and the pBADSce I-SceI homing endonuclease recombination method. I built pEXKm5 from pEXKm4,

constructed RHO3 to for efficient conjugation with *B. pseudomallei* and tested the new mobilizer strain.

6.1 Construction of an improved Tn*7* helper plasmid

6.1.1 Background on Tn*7* transposon elements

An efficient system for the stable insertion of foreign DNA is invaluable in the study of genetics. The Tn*7* system is one such system (9). It consists of two components, the transposase and the Tn*7* element. The Tn*7* transposase consists of several proteins, TnsA, B, C, D, and E. While TnsABC are required transposition proteins, TnsE catalyzes non-specific insertions and TnsD site-specific insertions at Tn*7* attachments (*att*Tn*7*) sequences (12, 13, 31). Most bacteria contain at least one *att*Tn*7* site which is usually located downstream of the essential *glmS* gene (8) and insertions into this site are generally neutral (*i.e.* insertion does not result in gene inactivation)(19, 26). Some bacteria, *e.g. Burkholderia* spp., contain multiple *glmS* genes and thus more than one *att*Tn*7* site (4) and others, e.g. *Proteus mirabilis*, contain *att*Tn*7* sites that are not associated with *glmS* genes and can be located within genes (7). The Tn*7* element is the second component of a Tn*7*-based transposition system. Since the elucidation of the necessary Tn*7*L and Tn*7*R sites required for site-specific insertion (22), plasmid-based mini-Tn*7* systems have been established (1, 27). In general, inserting a desired gene into a multiple cloning site flanked by Tn*7*L and Tn*7*R sites and introducing the construct into a bacterium along with the TnsABCD proteins allows for efficient insertion into the respective chromosomes. For more convenient cloning, the transposition machinery genes can be introduced *in trans* on a "helper" plasmid (1, 14). The system can be made

more efficient by increasing transcription of *tnsABCD* from the helper plasmid (5), which was my goal with pTNS3.

6.1.2 Construction of pTNS3

The Tn*7* helper plasmid pTNS2 expresses TnsABCD from an operon which is under control of the *E. coli tac* promoter (P_{tac}), allowing efficient site-specific insertion in bacteria such as *P. aeruginosa*. Transcription of *tnsABCD* from the *tac* promoter already significantly increased efficiency when compared to pTNS1, where the same genes where transcribed from the endogenous *tnsABCDE* promoter (5). Our goal was to increase transcription in *Burkholderia* spp. in order to increase transposition efficiencies. We used PCR primer set 1305+1548 (Table 6-1) to amplify the *P1* promoter and ribosomal binding site from p34E-Tp1, as well as introduce a *Sma*I site on the 3' end for easier cloning. The amplicon was placed into pCR2.1 to create pPS2225. From this plasmid, the *Eco*RI-*Sma*I fragment was removed and cloned into the corresponding sites in pTNS2, orienting the promoter region toward the *tnsABCD* genes (See Figure 6-1). The resulting plasmid was named pTNS3.

6.1.3 Testing of pTNS3

I compared transposition efficiencies of helper plasmids pTNS2 and pTNS3 when used with pUC18T-mini-Tn*7*T-Tp in *B. thailandensis* E264 and employing triparental mating experiments following previously described protocols (4). Briefly, I added 100 µl each of overnight SM10(λ*pir*) strains (helper and mini-Tn*7*) and recipient strain cultures to 700 µl of 10 mM $MgSO_4$. Cells were harvested by centrifugationat 7,000 x *g* for 2 min at room temperature. They were then washed in 1 mL of 10 mM $MgSO_4$ and

Table 6-1. Strains, plasmids and primers used for pTNS3 creation.

Strain or plasmid	Relevant properties[a]	Reference or source
E. coli		
DH5α(λ*pir*)	Cloning strain	Lab Strain
SM10(λ*pir*)	Km^r, Mobilizing Strain	(23)
B. thailandensis		
E264	Wild-type strain; environmental isolate	(3)
P879	Rif^r; E264	A. Kumar, unpublished
Plasmids		
p34ETp1	Ap^r Tp^r; cloning vector, source of *P1* promoter	(11)
pPS2225	Ap^r Km^r; pCR2.1 with 0.3-kb *P1* promoter PCR fragment from p34ETp1	(6)
pTNS2	Ap^r; plasmid expressing *tnsABCD* from P_{lac}	(5)
pTNS3	Ap^r; plasmid expressing *tnsABCD* from *P1* and P_{lac}	(6)
pUC18T-mini-Tn*7*T-Tp	Ap^r Tp^r; Tn*7* transposition vector	(6)
pUC18T-mini-Tn*7*T-Zeo-P1*::lux*	Ap^r Zeo^r; pUC18T-mini-Tn*7*T-Zeo with bacterial *luxCDABE* operon for bioluminescence	This study
Primers		
1305	5'-GAATTCACGAACCCAGTTGAC	(6)
1548[b]	5'-TACTTTGACCCGGGTCGAATC	(6)
Tn*7*L	5'-ATTAGCTTACGACGCTACACCC	(4)
PglmS1-DN	5'-GTTCGTCGTCCACTGGGATCA	(4)
PglmS1-UP	5'-AGATCGGATGGAATTCGTGGAG	(4)

[a] Abbreviations; Ap, ampicillin; Tp, trimethoprim; Km, kanamycin; Rif, rifampin; [r], resistant; [s], sensitive

[b] Underline signifies a SmaI site

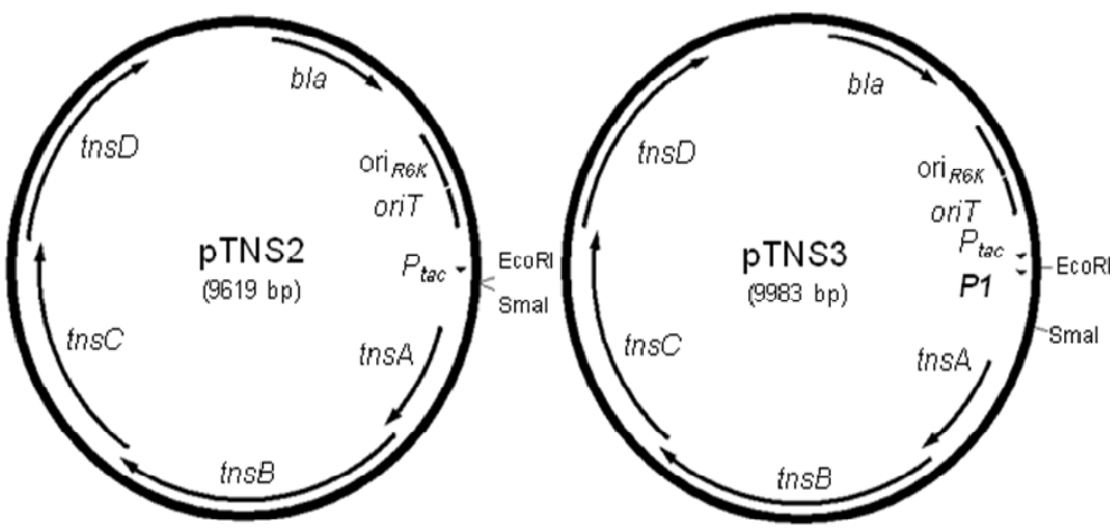

Figure 6-1. pTNS2 and pTNS3. The additional *P1* promoter is noted in bold on pTNS3. Also of note are the following features: *tnsABCD*, transposase machinery; *bla,* β-lactamase for Ampicillin selection; ori_{R6K}, narrow-host origin of replication requiring pir protein; P_{tac}, *E. coli tac* promoter; *oriT*, origin of transfer for conjugative transfer **(6)**.

resuspended in 30 µl of 10 mM $MgSO_4$. This suspension was applied to a 13 mm cellulose acetate membrane with 0.45 µm pore size (VWR # 22001-176) on a prewarmed LB plate, and the plate is incubated at 37°C overnight. The next day, filters were placed in 1.5 mL tubes with 400 µl of 0.9% NaCl and tubes were centrifuged at 7,000xg for 30 s to remove the cells from the filter. Cells remained suspended in the 0.9% NaCl and 100 µL aliquots were plated on LB Tp 100 µg mL^{-1} polymyxin B 15 µg mL^{-1} to select for transformants. Testing showed that pTNS3 yielded approximately 10-30 times more transposition events than pTNS2, upwards of $3x10^5$ Tp^r CFU per conjugation. Insertions were verified in several isolates by PCR using primers Tn*7*L and PglmS1-DN or PglmS1-UP (to test for insertions at *glmS1* and *glmS2*, respectively). All isolates had at least one insertion, with 3/10 isolates having insertions at both attachment sites. While typically

double insertions are not desirable, it does speak to the efficiency of the system and most of the mutants had single insertions.

Because our laboratory's experience with selection of marked mini-Tn*7* elements is that zeocin selection is problematic in *Burkholderia* spp., we also tested transposition of the mini-Tn7 element contained on pUC18T-mini-Tn*7*T-Zeo-*P1::lux*. Because zeocin selection and polymyxin B counter-selection do not work together, we needed to use a different method of selection against donor strains. To this end, we used a rifampin resistant *B. thailandensis* E264 derivative (strain P879) and plated conjugation mixtures on LB Zeo 200 μg mL^{-1} Rif 200 μg mL^{-1}. In these experiments transposition efficiencies with pTNS3 were >100-fold increased over pTNS2.

Mini-Tn*7* delivery via co-electroporation of helper and mini-Tn*7* delivery plasmid is significantly less efficient than co-conjugation, so increasing the efficiency by more than 1 log could mean the difference between successful transposition or failure of the experiment. To test electroporation efficiencies when using pTNS3 versus pTNS2, we washed 1 mL of overnight *B. thailandensis* culture in 300 mM sucrose three times, concentrated to 100 μl, then electroporated 500 ng of each plasmid (helpers pTNS2 or pTNS3 and pUC18T-mini-Tn*7*-Tp) into the cells according to the pre-set Bio-Rad GenePulserXcell™ *Pseudomonas aeruginosa* electroporation protocol (25 μF; 200 Ω; 2.5 kV) and recovering in 1 mL LB for 2 h before plating on LB Tp 100 μg mL^{-1}. In this experiment, pTNS3 yielded 10 Tp^r isolates per reaction while pTNS2 did not.

6.2 Construction of an improved *E. coli* mobilizer strain

6.2.1 Need for a new mobilizer strain

Select agent regulations limit the use of selectable markers to antibiotics not used in clinical or veterinary medicine. Given the broad spectrum of intrinsic antibiotic resistance of *B. pseudomallei* (30), our lab is mostly limited to kanamycin and zeocin for use as approved and reliable markers, although both must be used in extremely high concentrations (1000-2000 μg mL^{-1}) to prevent spontaneously resistant breakthrough colonies. The most commonly used *E. coli* mobilizer strain for conjugation in our lab has been SM10(λ*pir*). Unfortunately, SM10(λ*pir*) is kanamycin resistant, a result of inserting the mobilization genes into the chromosome, so kanamycin selection marker containing plasmids cannot be used in conjugation experiments with SM10(λ*pir*). This is unfortunate because kanamycin is the best antibiotic we are allowed to use with *B. pseudomallei*. As mentioned in the previous section, although polymyxin B is an excellent method of selecting against donor *E. coli* strains after conjugation, it does not work well with zeocin for unknown reasons (Schweizer Laboratory, unpublished observations). I and others experienced difficulties with the alternative mobilizing strain S17-1, mainly because conjugation efficiencies with this strain are significantly lower than those obtained with SM10(λ*pir*) (29)(Schweizer Laboratory, unpublished observations). Since many *B. pseudomallei* strains have poor electroporation competency, conjugation is the only efficient and reliable method of introducing genetic material. Thus, we perceived the need for a new mobilizer strain that was kanamycin susceptible and could be selected against easily.

6.2.2 Construction of RHO3

I sought to improve the SM10(λ*pir*) mobilizer strain in two ways; establishing a simple counter-selection technique against the strain and making the strain kanamycin susceptible. To accomplish the first goal I selected a gene whose deletion would make the strain an auxotroph on rich media, such as LB. The *asd* gene (*Escherichia coli* K12: accession #NC000913) codes for aspartate-semialdehyde dehydrogenase and is essential for synthesis of lysine, methionine, leucine and isoleucine, but most importantly diaminopimelic acid (DAP), which is essential for peptidoglycan synthesis and cell growth (28). Various groups have shown that *asd* mutants of multiple bacterial species are DAP auxotrophs (15, 17, 24) and DAP is not present (in sufficient quantities) in LB whose ingredients are derived from yeast and meat products. Derivation of a kanamycin susceptible strain will allow use of kanamycin as a selectable marker in conjugation experiments. All strains, plasmids and primers are listed in Table 6-2.

6.2.2.1 Deletion of *asd* from SM10(λ*pir*)

We initially used the deletion protocol described by Datsenko and Wanner (10) with primers 1692 and 1693 to amplify the *FRT*::*cat*::*FRT* cassette from plasmid pFCM1, yielding 35 bases of homology on either side with homology to nucleotides 230-264 and 1037-1002 of the *E. coli asd* gene which will eventually result in a 773-bp deletion. This linear PCR fragment was electroporated into SM10(λ*pir*) containing pKD46 (plasmid containing lambda RED recombinase proteins). Cells were prepared as follows: Overnight cultures were subcultured 1:100 in SOC media with 1 mM Arabinose at 30°C to induce RED recombinase production until reaching OD_{600nm} of 0.7, then 10 mL of culture was washed in 1 mL ice cold 10% glycerol three times and concentrated to a final

Table 6-2. Strains, plasmids and primers used for RHO3 creation

Strain or plasmid	Relevant properties[a]	Reference or source
E. coli		
DH5α	Cloning strain	(20)
SM10(λ*pir*)	Km^r; Mobilizer Strain	(23)
RHO1	Km^s; SM10(λ*pir*)Δ*aphA::FRT*	(21)
RHO2	Km^r, DAP-dependent SM10(λ*pir*)Δ*asd::FRT*	(21)
RHO3	Km^s, DAP-dependent SM10(λ*pir*)Δ*asd::FRT* Δ*aphA::FRT*	(21)
B. thailandensis		
E264	Wild-type strain; environmental isolate	(3)
P879	Rif^r; spontaneous rifampicin resistant E264 derivative	A. Kumar, unpublished
Plasmids		
pKD46	Ap^r; Lambda RED recombinase under P_{araB} regulation	(10)
pFCM1	Ap^r Cm^r; Source of *FRT::cat::FRT* cassette	(6)
pFLP2	Ap^r; source of Flp recombinase for removal of *FRT* cassettes and *Bacillus subtilissacB* gene for plasmid curing by sucrose counter-selection	(16)
pEX18Ap	Ap^r Km^r; Cloning vector	(16)
pPS2384	Ap^r Km^r; pCR2.1 (Invitrogen; Carlsbad, CA) with 1,672-bp *E. coli asd* gene amplicon from primers 32 + 37	(21)
pPS2385	Ap^r; pEX18Ap with *Eco*RI fragment with *asd* gene from pPS2384	(21)
pPS2404	Ap^r Cm^r; pPS2385with a 395-bp *Eco*RV *asd* gene fragment replaced with 1,143-bp SmaI *FRT::cat::FRT* fragment from pFCM1	(21)
pPS2416	Ap^r Km^r; pCR2.1 with a 1,546-bp *aph*A containing fragment amplified from from SM10(λ*pir*) chromosomal DNA using primers 1761+1760	(21)
pPS2417	Ap^r Cm^r Km^r; pPS2416 with a 743-bp *Hin*cII *aphA* gene fragment replaced with 1143-bp SmaI *FRT::cat::FRT* fragment from pFCM1	(21)
Primers		
32	5'-CCATAATCAGGATCAATAAAACTG	(21)
37	5'-CCGCAAAATGGCCTGCAATTA	(21)
1692[b]	5'-ATACCAACGAAATCTATCCAAAGCTTCGTGAAAGC**CGAATTAGCTTCAAAAGCGCTCTGA**	(21)
1693[b]	5'-GTAAAGGCTGACAGGAACTCTGGTCCCATATTCAGC**CGAATTGGGGATCTTGAAGTACCT**	(21)
1760[c]	5'-CAACTGAGTTATG*CCCGGG*TGTTGATCC	(21)
1761[d]	5'-<u>GATATC</u>CGGATCGGCAATGCCATATTGCGC	(21)

[a] Abbreviations; Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Rif, rifampin; [r], resistant; [s], sensitive

[b] **Bold** signifies sequence homologous to the *FRT::cat::FRT* cassette

[c] *Italics* signify a *Sma*I site

[d] <u>Underline</u> signifies an engineered *Eco*RV site

volume of 100 μL (10). Cell recovered in 1 mL of SOC + DAP 200 μg mL^{-1} by shaking at 37°C for 1 hand plating on LB DAP 200 μg mL^{-1} Cm 25 μg mL^{-1}. Despite numerous trials this attempt at Δ*asd* mutant construction failed. Attempts to recreate a *pstS* deletion shown in the original paper, using the exact primers and protocol, also failed (data not shown).

In an effort to increase efficiency, we decided to increase the area of homology available for recombination. To do this, using PCR and primers 32 and 37 we amplified a 1,672-bp DNA fragment containing the *asd* gene using SM10(λ*pir*) chromosomal DNA as the template. This fragment was gel-purified and ligated into the TA cloning vector pCR2.1 (Invitrogen) to create pPS2384. A 1,688-bp EcoRI fragment containing the asd gene was then excised from pPS2384 and ligated into the same site of pEX18Ap (16). Next, the 395 bp *Eco*RV fragment located within the *asd* gene was replaced with the 1,143-bp *Sma*I *FRT::cat:FRT* fragment from pFCM1 (6) to create pPS2404. A 1,980-bp SacII fragment (containing 369 and 470-bp of *asd* homology on either side of the *FRT::cat:FRT* fragment) was gel-purified. This *Sac*II fragment was electroporated into electrocompetent SM10(λ*pir*)/pKD46 (cells were prepared and transformed as described above). Half of the recovery culture was plated on LB DAP 200 μg mL^{-1} Cm 25 μg mL^{-1} and incubated at 37°C. The other half remained at room temperature for 24 h, and was subsequently subcultured into 5 mLof LB DAP 200 μg mL^{-1} Cm 25 μg mL^{-1}, incubatedovernight at 37°C and then plated on selective medium. Only the subcultured cells yielded the correct Cm^r and DAP-dependent colonies. The Cm^r marker was then removed from a tranformant. This was achieved by introducing the Flp recombinase source plasmid pFLP2 (16), selecting on LB DAP 200 μg mL^{-1} Ap100 μg mL^{-1} and then

streaking Cm^s colonies on LB DAP 200 μg mL^{-1} + 5% sucrose to cure pFLP2. The resulting strain was named RHO2.

6.2.2.2 Deletion of *aphA*

The same technique describe above (6.2.2.1) was employed to construct a kanamycin susceptible RHO2 derivative. Using the sequence for the Km^r determinant, *aphA* (Genbank accession number M20305)(25), from the RP4 construct used to create SM10 (23, 29), the *aphA* gene was PCR-amplified from SM10(λ*pir*) chromosomal DNA with primer set 1761+1760, The resulting 1,546-bp amplicon it was ligated into pCR2.1 to create pPS2416. Using *Hin*cII digestion, a743-bp segment was deleted from *aphA* and replaced by 1,143-bp *FRT::cat::FRT Sma*I fragment from pFCM1 to yield pPS2417. Using the *Sma*I and *Eco*RV sites engineered into the primers, a 1,927-bp fragment containing 376-bp and 408-bp of homology on either side of the *FRT* cassette was then gel purified. This fragment was electroporated into RHO2 cells containing pKD46, following the protocol for *asd* deletion described above (section 6.2.2.1). The resulting strain, RHO2 Δ*aphA::FRT*, was named RHO3. An additional strain, RHO1, was created by removing *aphA* in the same manner as described for RHO3, except that SM10(λ*pir*) was used as the starting strain instead of RHO2.

6.2.3 Testing of RHO mobilizer strains

To show that all three RHO strains were capable of mobilizing plasmids with an *oriT*, I tested conjugation efficiency as described in 6.1.3. The exception was that I used *B. thailandensis* E264 and did not use antibiotics for counter-selection for RHO2 and RHO3 (polymyxin B was used for RHO1 testing). Instead, after conjugation on LB DAP

200 μg mL^{-1}, I washed the cells to remove residual DAP and then plated on LB with only the antibiotic selecting for mini-Tn*7* insertions. The results showed no significant difference between SM10(λ*pir*) and RHO strains. Additionally, RHO2 and RHO3 counter-selection using LB without DAP is efficient and the strains can be used with any DAP prototroph recipient strain without the need for antibiotic counter-selection.

6.3 Conclusions

The helper plasmid pTNS3 is a significant improvement to the mini-Tn*7*system . Just 30 months after being published, the paper describing pTNS3, and associated genetic tools, has received 21 citations; a testament to the utility of the system. RHO3 is also a great asset, particularly to the select agent community or researchers working with intrinsically antibiotic resistant bacteria. It provides a simple, cost-effective mode of counter-selection on rich media without antibiotic counter-selection or the necessity of minimal media often used for metabolic counter-selection (2, 24).

6.4 References

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CHAPTER 7:

CONCLUDING REMARKS

Burkholderia is an extremely diverse genus and its members have widespread metabolic capabilities and occupy diverse environmental niches (7). *B. pseudomallei* has a large and plastic genome (10) and is capable of undergoing large deletions, frequent recombinations and point mutations that may alter its phenotype (18, 23, 24). The potential for its use as a weapon of bioterrorism (25) has made the bacterium the subject of research in many Western nations. However, because *B. pseudomallei* consistently and increasingly causes the fatal disease melioidosis in endemic regions of the world, notably Southeast Asia and Northern Australia (4, 12), research into its biology is pertinent to the everyday world. As I began to work on this fascinating species, many questions remained; Why is *B. pseudomallei* so resistant to β-lactams? How is resistance changed by chromosomal mutations? How can we develop genetic systems to further investigate this bacterium? In an attempt to obtain answers to some of these questions, the work described in this dissertation has established the following:

(i) A random mutagenesis system for *B. pseudomallei*.

Transposon-based random mutagenesis systems have been used in a wide range of bacterial strains, including two *P. aeruginosa* strains (13, 15), the

select agent *F. tularensis* (9) and others (1, 20, 22). I constructed a select-agent compliant transposon system based on the *mariner Himar1* transposon. Unlike other transposons, which have insertion "hot spots" (2), the *Himar1* transposon system requires only a TA sequence for insertion into target DNA (14). The newly developed system, pHBurk3, was shown to be well-suited for establishing stable, single, random insertions within *Burkholderia* bacteria. One potential problem with the pHBurk3 system is the high GC content of *B. pseudomallei* (10). When examining the K96243 genome (GC content of ~68%) I found that it has long stretches of sequence without a TA target site, some greater than 1 kb. This means transposon libraries may miss important DNA regions, including both open reading frames and non-coding regions of interest (*e.g.* promoters, ribosomal RNAs, etc.).

(ii) PenA is the major β-lactam resistance mechanism in *B. pseudomallei.*

β-lactam resistance has been recorded in *B. pseudomallei* since screening for it began (6, 8, 16). The presence of a β-lactamase has also been established (16), but which of the seven potential β-lactamases (10) play a role and the level of resistance they may afford *B. pseudomallei* has not been shown. By creating a clean deletion of the Ambler class A β-lactamase PenA in *B. pseudomallei* 1026b, I was able to show that the resulting strain was susceptible to a wide range of β-lactams to which wild-type *B. pseudomallei* is either intrinsically resistant or can acquire resistance. Similarly, the up-regulation of *penA* expression provided higher levels of resistance to β-lactams. These data clearly demonstrate that PenA is the bacterium's major β-

lactam resistance determinant. Clinical and laboratory-generated strains of *B. pseudomallei* with altered β-lactam resistance profiles often contain mutations within the *penA* gene (5, 11, 23). However, the contribution of these mutations to profile shifts has not been empirically established. By employing the pEXKm5 allele replacement system (17) and the select-agent-regulation excluded *B. pseudomallei* strain Bp82 (21), I recreated clinically documented point mutations in a defined *B. pseudomallei* genetic background. Susceptibility testing definitively supported findings of the previous reports and demonstrated that the previously identified *penA* mutations were the sole resistance determinants in the respective clinical strains (5, 11, 23). Molecularly defining the effects of *penA* point mutations on the β-lactamase substrate profile is a valuable asset. Our lab is currently optimizing a multi-plex PCR system that will potentially identify resistance determinants in a timely fashion. To further our understanding of the framework within which PenA functions, I examined putative regulators (BPSS0944 and BPSS0948) for their role in *penA* expression. Deletion and constitutive expression of these genes had no effect on *penA* transcription nor on β-lactam susceptibility profiles. Because regulation of *penA* transcription may be substrate inducible or subject to other environmental factors, multiple methods of induction were attempted, including variation of NaCl concentrations and induction by various β-lactams (*e.g.* ceftazidime, carbenicillin, penicillin G and imipenem). However, there was no significant change in *penA* transcription in cells grown under the various conditions. My findings suggest that under the conditions

tested *penA* is constitutively expressed. Finally, I studied the role of the twin-arginine translocation (TAT) system in PenA secretion. It was previously established that a chromosomally encoded β-lactamase in *Mycobacterium smegmatis* is secreted via the TAT system (19). Deletion of the TAT operon (*tatABC*) and mutation of the putative PenA TAT signal sequence resulted in β-lactam susceptibility (MIC's mirrored those of *penA* deletion strains.). Western blot analysis showed a non-processed PenA in these mutants. Attempts to localize wild-type and unprocessed PenA proteins found the protein in the spheroplast fraction after removal of the periplasm. This fraction contains total cytosolic and membrane proteins which could mean that PenA is a membrane-associated protein. Membrane-associated β-lactamases have been previously documented in gram negative bacteria (3). This work represents the first comprehensive study on the role of PenA in *B. pseudomallei*'s β-lactam resistance. It also establishes the first example of a TAT secreted β-lactamase in Gram-negative bacteria and shows that genetically linked transcriptional regulators do not seem to play a role in regulation of *penA* expression, at least not under the conditions employed in this study.

(iii) BPSS1219 is a novel ceftazidime resistance determinant in *B. pseudomallei*.

The cases of several clinical strains that became ceftazidime resistant after patients initially responded well to ceftazidime treatment were examined. Each mutant strain, which was also glycerol dependent and grew as a filamentous rod, had a large deletion in chromosome two with a recurrent 49

genes deleted in all strains. Using the pEXKm5 system (17) and a novel complementation strategy using a mini-Tn*7* expression vector, we were able to individually delete potential ceftazidime resistance determinants found in the deleted region within the background of 1026b. The deletion of *BPSS1219*, which codes for one of the three predicted class three penicillin-binding proteins (also *BPSS1240* and *BPSL3031*) conferred the same phenotype as the clinical mutant strains. Similarly, complementation of the large deletion strains with *BPSS1219* alone restored normal growth rates (with and without glycerol) and ceftazidime susceptibility. Since strains lacking *BPSS1219* failed to grow on Mueller-Hinton Agar (MHA), susceptibility testing was done on Ashdown agar. On this agar, strains with *BPSS1219* under P_{tac} regulation yielded non-definitive results with zones of partially inhibited growth surrounding areas of strong growth, even in the presence of higher concentrations of ceftazidime up to the limit of detection (256 μg/mL). However, growth on MHA, the standard medium used for MIC determinations, showed complete growth inhibition at a low level of ceftazidime (2 μg/mL). This drastic difference in resistance observed under the two different growth conditions raises questions about currently accepted methods for MIC testing. Based on the MHA test alone, a clinician would assume that the bacteria were ceftazidime susceptible, when clearly they are resistant under certain enabling circumstances. Because these large deletion strains were still causing disease in humans and these mutants failed to grow on MHA while growing on Ashdown agar, we can potentially say that at least

certain aspects of the Ashdown medium (*e.g.* osmolarity) may more closely resemble the host environment than MHA. Given the disparity of the strains' MIC and growth data between the two media, we should look into testing with a medium that more accurately mimics the host environment.

As more strains displaying antibiotic resistance phenotypes of unknown origin emerge, we must continue to elucidate the underlying mechanisms. Defining the precise mechanisms of resistance may provide clinicians with tools to more accurately diagnose and more effectively treat patients afflicted with such strains. This can be done retroactively by analyzing clinical mutants that resulted in failed therapy, as in the cases discussed in aims 2 and 3 (Chapters 4 and 5). This can also be done using random mutagenesis systems, such as the *Himar1* transposon-based pHBurk3 system (Chapter 3), to generate comprehensive mutant libraries and screen them for various phenotypes. Using powerful, modern sequencing technologies and a pool of the comprehensive library, we can test the gamut of strains grown under various conditions and observe both positive and negative selection (*i.e.* mutants missing from the pool indicate an insertion that disrupts a gene essential for a certain condition and those thriving represent a healthier phenotype for that environment.). The same can also be achieved using a perhaps less costly microarray platform, as done by Winterberg et al. with *E. coli* (26). A comprehensive, sequence-defined transposon mutant library represents a very powerful tool for screening for phenotypes of interest and is currently being constructed by our lab in collaboration with the Manoil lab at the University of Washington.

There is little doubt that *B. pseudomallei* will continue to evolve through both natural and clinical selection. The best we can hope for is to closely monitor these changes and try to adapt along with it.

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