THESIS

SUB-CELLULAR LOCALIZATION OF THE PENA β-LACTAMASE IN *BURKHOLDERIA PSEUDOMALLEI*

Submitted by

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ABSTRACT

SUB-CELLULAR LOCALIZATION OF THE PENA β-LACTAMASE IN *BURKHOLDERIA PSEUDOMALLEI*

Burkholderia pseudomallei, a Gram-negative soil bacterium found in tropical regions, is the etiologic agent of melioidosis. *B. pseudomallei* is intrinsically resistant to many antibiotics, and melioidosis treatment involves prolonged antibiotic therapy. PenA, a chromosomal β-lactamase in *B. pseudomallei*, confers resistance to many β-lactams. Point mutations in *penA* leading to PenA amino acid changes can cause resistance to ceftazidime and amoxicillin-clavulanate, which can result in treatment failure. Typically β-lactamase enzymes are found in the periplasm of Gram-negative bacteria. Previous studies have shown that PenA is secreted via the twin arginine translocase (Tat) system, but failed to demonstrate periplasmic localization.

The purpose of this study was to determine the sub-cellular localization of PenA in *B. pseudomallei*. Using alkaline phosphatase as a periplasmic marker, we optimized a method for extracting periplasmic proteins from *B. pseudomallei*. Through subcellular fractionations, immunoblotting, and colorimetric enzyme assays, we have shown that PenA does not localize to the periplasm. Rather, it is present in a detergent-soluble fraction of the cellular membranes. Further experiments including site-directed mutagenesis, metabolic labeling with ^{14}C-palmitate, globomycin treatment, and mass spectrometry indicate that PenA is likely a lipoprotein with post-translational lipid modification of the cysteine residue at position 23. This work implicates PenA as the first example of a β-lactamase that is a Tat-secreted lipoprotein, and provides a better physiological understanding of an important antibiotic resistance mechanism in *B. pseudomallei*.

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INTRODUCTION

1.1 *Burkholderia pseudomallei* and melioidosis

Burkholderia pseudomallei is a saprophytic Gram-negative rod. Considered to be endemic in Southeast Asia and Northern Australia, it is found throughout tropical regions of the world (Figure 1.1) (1). As the causative agent of melioidosis, *B. pseudomallei* has been designated a Tier 1 Select Agent by the United States Centers for Disease Control and Prevention, due to its low infectious dose, intrinsic antibiotic resistance, and relatively high morbidity and mortality (2, 3).

The genome of *B. pseudomallei* is very large and highly plastic, with two chromosomes of approximately 3.2 and 4.1 million base pairs encoding some 5,800 genes (4). With this large coding capacity, *B. pseudomallei* is able to survive and thrive in a diverse set of environments, from its natural reservoir in the soil to a wide variety of mammalian hosts (5). Virulence factors include three type III secretion systems (6), six type VI secretion systems (7), capsular polysaccharide (8), lipopolysaccharide (9), flagella (10), multiple quorum sensing systems (11), at least one cytotoxin (12), and a variety of other components that allow *B. pseudomallei* to infect host organisms, survive intracellularly, and cause disease.

B. pseudomallei infection can result from percutaneous inoculation, inhalation, or ingestion of contaminated food or water (13). Melioidosis has been referred to as "The Great Mimicker," reflecting the wide variety of clinical manifestations and their resemblance to other diseases. The most common of these manifestations are pneumonia, septicemia, skin abscesses, and genitourinary infection (14). Melioidosis treatment is lengthy and involves two phases of

antibiotic treatment. The first, intensive phase consists of at least 10-14 days of intravenous ceftazidime, meropenem, or imipenem. This is followed by 3-6 months of oral eradication therapy with co-trimoxazole or amoxicillin-clavulanic acid (13).

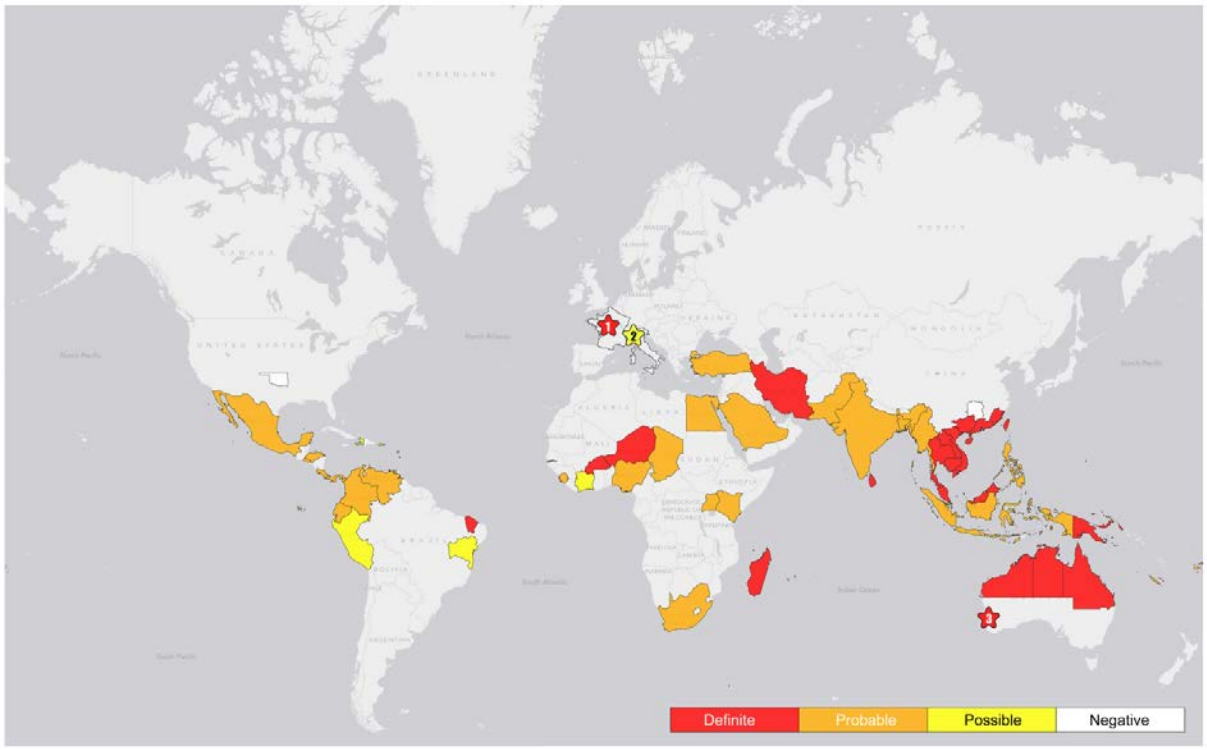

Figure 1.1 Geographic distribution of melioidosis. *B. pseudomallei* has been identified in environmental samples from tropical regions around the world, especially in Southeast Asia and Northern Australia. Adapted from Limmathurotakul *et. al.* (1).

1.2 Antibiotic resistance mechanisms

Bacteria possess a variety of antibiotic resistance mechanisms, as illustrated in Figure 1.2 (15). Drugs can be prevented from entering the cell by physical exclusion, or effluxed out of the cell by active transport. Within a bacterial cell, drugs can be sequestered to block their activity, or enzymatically inactivated by degradation or chemical modification. Bacteria can also avoid

the effects of antibiotics by overproducing, altering, or deleting the drug target, or by bypassing the susceptible enzyme or metabolic pathway with a resistant alternative. A majority of these antibiotic resistance mechanisms have been demonstrated in *B. pseudomallei*, and the extensive intrinsic resistance of this organism dictates the limited number of drugs available to treat melioidosis (15).

The very nature of the Gram-negative cell envelope provides a significant permeability barrier for drugs entering the cell. In addition, the lipid A moiety of lipopolysaccharide in *B. pseudomallei* has a 4-amino-4-deoxy-L-arabinose modification, which reduces the negative charge on the exterior of the cell, protecting the bacteria from cationic antimicrobial peptides such as polymyxin B (16).

Efflux pumps provide an important means of antibiotic resistance in *B. pseudomallei*, whose genome encodes up to ten resistance nodulation and cell division (RND) family multidrug efflux pumps (4), though only three have been characterized to date. Among these, the AmrAB-OprA pump has been shown to efflux aminoglycosides such as gentamycin, kanamycin, streptomycin, and spectinomycin, and macrolides such as erythromycin, clarithromycin, and clindamycin, providing high levels of intrinsic resistance to those drug families (17). Fluoroquinolones and tetracyclines are also effluxed by AmrAB-OprA, but to a much lesser extent (18). The BpeAB-OprB efflux pump provides low level intrinsic resistance to macrolides, fluoroquinolones, tetracyclines, and chloramphenicol (18). Finally, the BpeEF-OprC efflux pump is of clinical significance due to its ability to efflux chloramphenicol, trimethoprim, tetracyclines, and fluoroquinolones (19 and Podnecky, N. *et al.*, unpublished data).

While target deletion is a fairly unusual form of antibiotic target modification, the deletion of penicillin-binding protein 3 in *B. pseudomallei* has been shown to confer resistance to

ceftazidime (20). Although the resulting strains have severe growth defects in laboratory culture media, ceftazidime-resistant strains lacking PBP3 have been isolated from six Thai melioidosis patients following ceftazidime treatment (20). Thus, this example of target deletion is an important means of acquired ceftazidime resistance in *B. pseudomallei* which can result in melioidosis treatment failure.

Finally, another important β-lactam resistance mechanism in *B. pseudomallei* involves enzymatic inactivation by the PenA β-lactamase. PenA is responsible for both intrinsic resistance to older β-lactam antibiotics such as ampicillin and carbenicillin, and acquired resistance to ceftazidime and amoxicillin-clavulanate through amino acid changes or increased transcription of *penA* (21). Of particular clinical concern, there have been several documented cases of PenA-mediated treatment failure in melioidosis patients as a result of β-lactam antibiotic therapy (22-26).

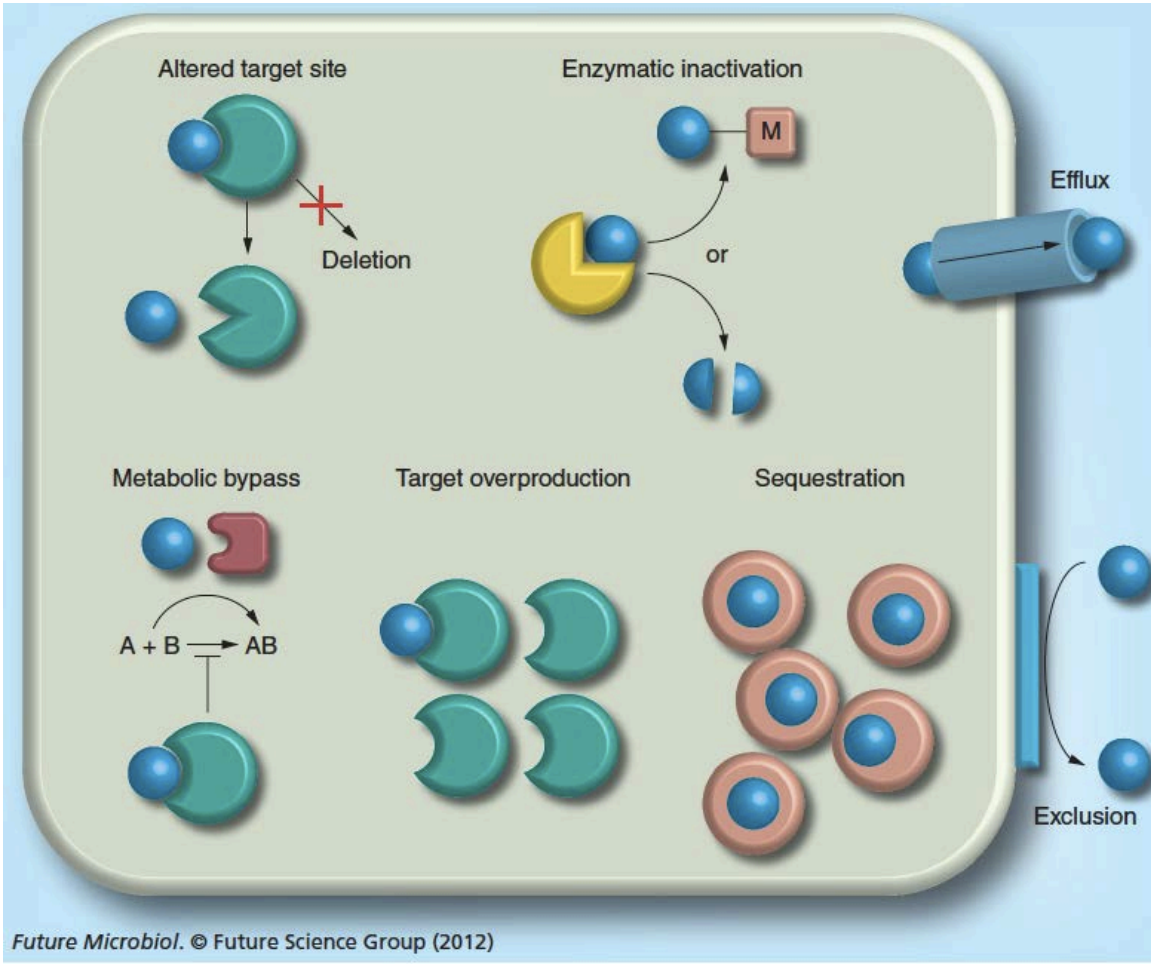

Figure 1.2 Bacterial antibiotic resistance mechanisms. Antibiotic resistance mechanisms include drug exclusion or efflux, sequestration or enzymatic inactivation of the drug, metabolic bypass of the drug target, or alteration or overproduction of the target. Adapted from Schweizer (15).

1.3 β-lactam antibiotics

Of the five antibiotics recommended for treatment of melioidosis (13), four are β-lactam compounds. Ceftazidime is a third generation cephalosporin (27), meropenem and imipenem are carbapenems (28, 29), and amoxicillin is a semisynthetic penicillin (30) which is administered in combination with the β-lactamase inhibitor clavulanic acid (31).

Penicillins, cephalosporins, and carbapenems comprise three major groups of β-lactam compounds. All contain a characteristic four-membered β-lactam ring which is fused with a five-membered sulfur-containing ring in penicillins, a six-membered ring in cephalosporins, or an unsaturated 5-membered ring in carbapenems (Figure 1.3).

β-lactam antibiotics bind and inhibit penicillin-binding proteins (DD-transpeptidases), which are responsible for cross-linking the peptidoglycan of bacterial cell walls. This weakening of the cell wall generally results in a bactericidal effect on actively growing cells (32).

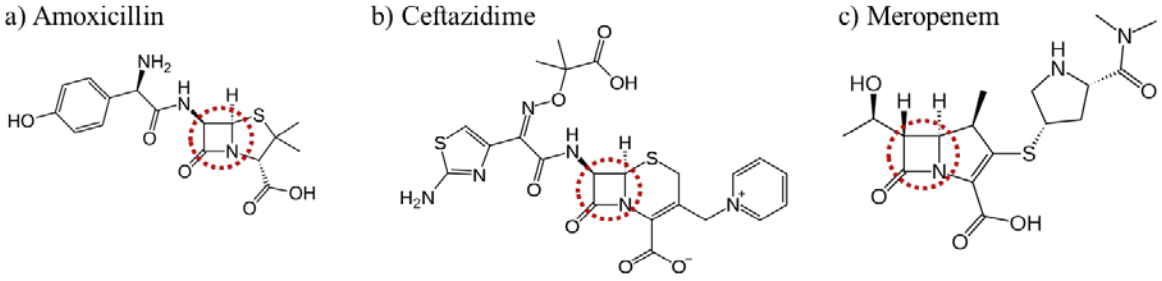

Figure 1.3 Examples of β-lactam structures. (a) Amoxicillin is a penicillin, (b) ceftazidime is a cephalosporin, and (c) meropenem is a carbapenem. β-lactam rings are circled in red.

1.4 β-lactamases

Just as antibiotic compounds such as β-lactams are natural products of microorganisms, so too are antibiotic-resistance mechanisms. β-lactamase enzymes provide a means for bacteria

to avoid the killing effects of β-lactam antibiotics by hydrolyzing the β-lactam ring, thereby inactivating the drug. More than 300 β-lactamases have been identified across bacterial genera (33).

The Ambler classification system divides β-lactamases into four groups based on sequence. Classes A, C, and D all have a serine residue in the active site, while the more distantly related Class B enzymes are metallo β-lactamases requiring zinc for hydrolytic activity (33). Different sub-groups of Class A enzymes can cleave penicillins and cephalosporins, and a few are able to hydrolyze carbapenems. They are generally susceptible to inhibitors such as clavulanic acid, although enzymes with amino acid changes making them resistant to inhibition are emerging (34). Class B β-lactamases are able to cleave most β-lactam structures, and while they are inhibited by metal chelators such as EDTA, they are resistant to traditional inhibitors such as clavulanic acid (33). Class C enzymes are cephalosporinases, which are resistant to clavulanic acid inhibition (34), and have only been identified in Gram-negative organisms (33). Class D β-lactamases, which can usually be inhibited by clavulanic acid, are characterized by their efficient hydrolysis of oxacillin (32) as well as other penicillins (34).

1.5 Sec and Tat secretion systems

In Gram-negative bacteria, β-lactamase enzymes are generally found in the periplasmic space between the inner and outer membranes. There, they are able to cleave β-lactam antibiotics, preventing them from interfering with peptidoglycan cross-linking. The TEM-1 β-lactamase from *E. coli* has been well-characterized as a Sec-secreted periplasmic protein, and its periplasmic activity has been exploited in a variety of gene-fusion reporter systems (35-40).

The majority of β-lactamases tested, including TEM-1, have been shown to be transported across the cytoplasmic membrane by the general secretary (Sec) system (41). However, there are several examples of β-lactamases that are secreted by the alternative twin arginine translocase (Tat) system, including PenA from *B. pseudomallei* (21, 42-44).

The Sec system transports unfolded proteins across the cytoplasmic membrane, while the Tat system generally transports pre-folded proteins, often in complex with cofactors (45). Pre-proteins in the cytoplasm are targeted for Sec or Tat transport by N-terminal signal sequences. Both Sec and Tat signal sequences are tripartite, comprised of a positively charged N-terminal region, a hydrophobic core, and a C-terminal region containing a cleavage site. Tat signals are longer and less hydrophobic than their Sec counterparts, and contain a characteristic pair of arginine residues in the N-terminal region, though some TAT signal sequences contain only one arginine (46).

Proteins destined for Sec secretion are either bound by the signal recognition particle (SRP) during translation, or post-translationally by the SecB chaperone. As is depicted in Figure 1.4, SRP or SecB targets the new polypeptide to the SecA-SecYEG complex in the cytoplasmic membrane, where the SecA motor hydrolyzes ATP to drive the protein through the channel formed by SecYEG (45). The Tat secretion system, illustrated in Figure 1.5, involves a larger channel comprised of the TatABC proteins (though some organisms lack TatA or TatB). The protein to be secreted is recognized by TatC, and transport is driven by the proton motive force. (45).

In the case of non-lipoproteins, Sec or Tat signal peptides are cleaved by signal peptidase (SPase) I upon reaching the periplasm to generate the mature proteins (46). Lipoproteins are also

transported across the cytoplasmic membrane by the Sec and Tat systems, and their signal peptides are cleaved by SPase II during the lipoprotein maturation process (47).

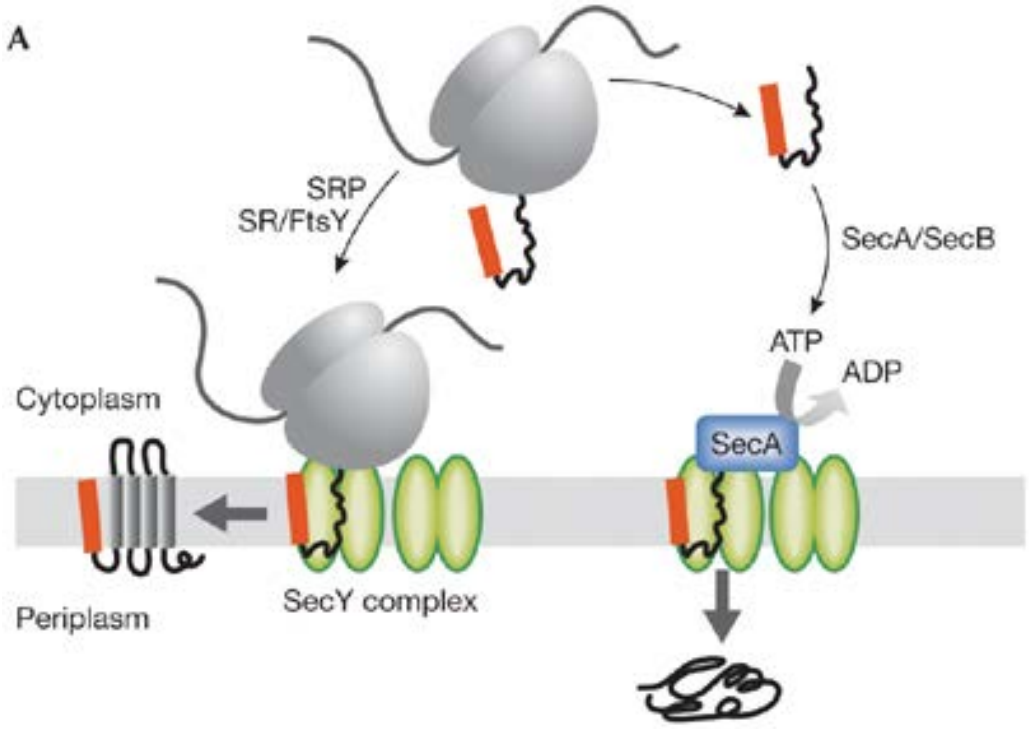

Figure 1.4 Sec secretion mechanism. N-terminal Sec secretion signals are recognized either during or following translation in the cytoplasm, and targeted to the SecYEG translocon for export across the cytoplasmic membrane. Following translocation, the signal sequence is removed by a signal peptidase. Adapted from Robson & Collinson (48).

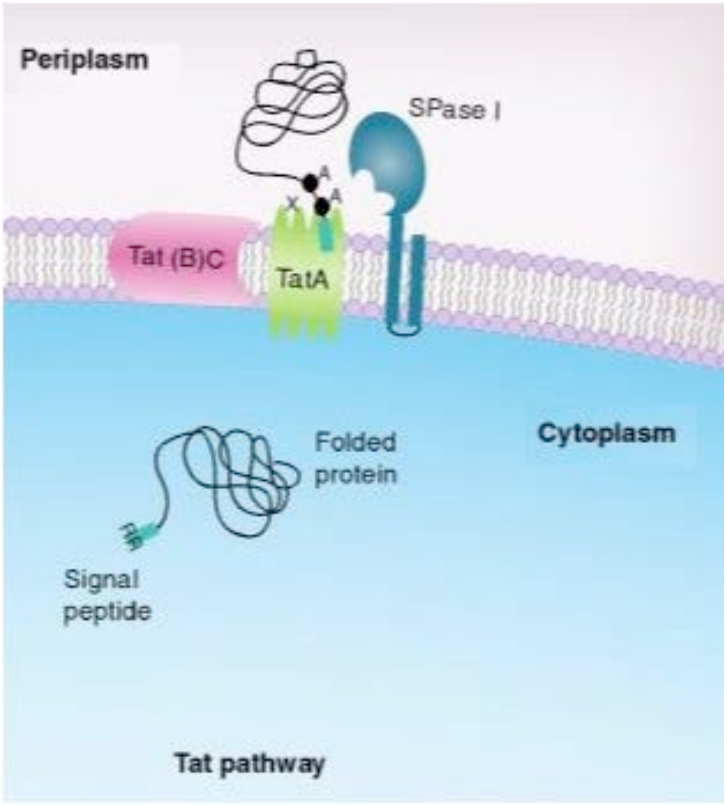

Figure 1.5 Tat secretion mechanism. Pre-folded polypeptides containing N-terminal Tat signal sequences are transported across the cytoplasmic membrane by the TatABC complex, and a signal peptidase subsequently removes the signal peptide. Adapted from Smitha Rao & Anné (49).

1.6 Lipoproteins

Gram-positive bacteria, lacking an enclosed periplasmic space, often have membrane-anchored lipoprotein versions of proteins that are soluble in the periplasmic space of Gram-negative organisms (50). This group includes several β-lactamase enzymes, in organisms such as *Bacillus licheniformis*, *Bacillus cereus*, and *Staphylococcus aureus* (51). Interestingly, the

BRO β-lactamase of the Gram-negative organism *Moraxella catarrhalis* has also been shown to be a lipoprotein, suspected to have been acquired from a Gram-positive source (52).

Lipoprotein precursors in both Gram-negative and Gram-positive organisms contain a characteristic amino acid motif, termed the lipobox, within a Sec or Tat signal sequence. The lipobox consensus is [LVI] [ASTVI] [GAS] C, where the cysteine residue is absolutely essential for lipidation (47). After the preprolipoprotein is translocated across the cytoplasmic membrane, the lipobox is recognized by the lipoprotein diacylglyceryl transferase enzyme (Lgt), which adds a diacylglycerol to the cysteine via a thioether bond, to generate a prolipoprotein (50). The lipoprotein signal peptidase (Lsp), also known as Spase II, then removes the signal sequence, leaving the acylated cysteine at the N-terminus. In most Gram-positive bacteria this product constitutes a mature lipoprotein, which remains anchored in the outer leaflet of the cytoplasmic membrane. In Gram-negative organisms, the lipoprotein N-acyl transferase (Lnt) enzyme adds a third fatty acid to the N-terminal cysteine via an amide linkage (47), forming the mature lipoprotein, which can then either remain in the outer leaflet of the inner membrane, or be transported to either leaflet of the outer membrane by the lipoprotein localization (Lol) pathway (50). The bacterial lipoprotein biosynthesis process is summarized in Figure 1.6.

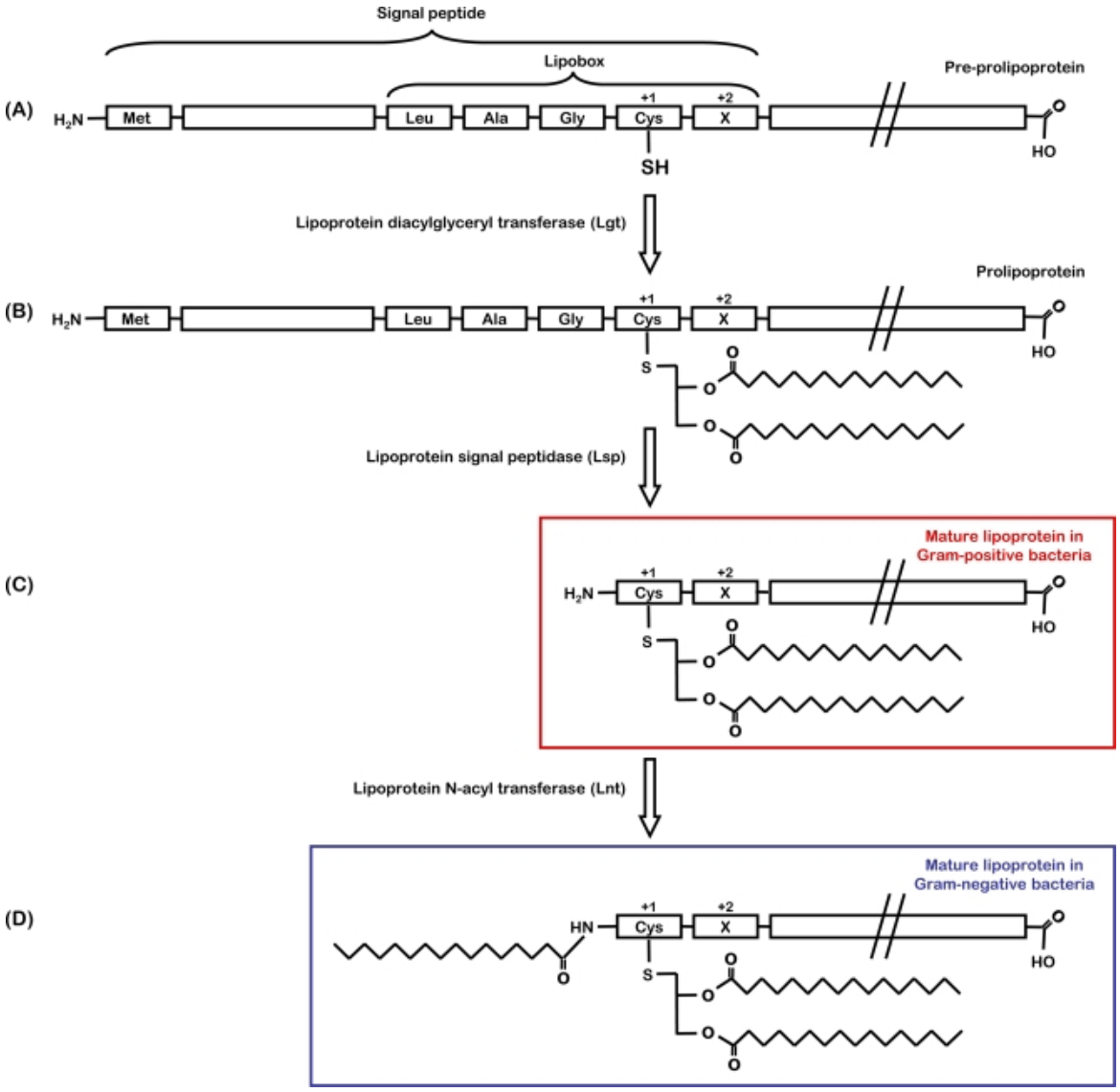

Figure 1.6 Prokaryotic lipoprotein biosynthesis. Bacterial pre-prolipoproteins contain a lipobox, including the critical cysteine residue, which is modified with a diacylglycerol. After diacylglycerol addition, the signal sequence is cleaved by lipoprotein signal peptidase. This reaction can be specifically inhibited by globomycin. In Gram-positive bacteria this cleavage results in mature lipoprotein. In Gram-negative bacteria mature lipoprotein formation requires addition of an additional N-linked fatty acid. Adapted from Kovacs-Simon *et. al.* (47).

1.7 The PenA β-lactamase

The *B. pseudomallei* genome encodes several β-lactamases (4, 53). While some research has examined a Class D oxacillinase (54, 55), the most studied β-lactamase from *B. pseudomallei* is PenA, which has been implicated in clinical resistance to ceftazidime and amoxicillin-clavulanate (22-26). The *penA* gene is highly conserved among *B. pseudomallei* strains, and has homologues across other species of *Burkholderia* (56). Wild-type PenA confers resistance to many older β-lactam antibiotics, including amoxicillin, ampicillin, and carbenicillin (21), but clinically significant ceftazidime resistance is only achieved through certain amino acid changes that modify the substrate specificity, or overexpression of the wild-type gene (21-26). Similarly, wild-type PenA is inhibited by clavulanic acid, allowing amoxicillin-clavulanate to be used for treatment of melioidosis, but a point mutation changing the serine at position 72 (Ambler numbering [57]) to a phenylalanine results in a clavulanic acid resistant enzyme (21, 23). While relatively rare, it is not surprising that these *penA* mutations conferring resistance to ceftazidime and amoxicillin-clavulanic acid have been found in clinical isolates, given the strong selection pressure and lengthy melioidosis treatment period.

Rholl *et. al*. demonstrated that PenA-mediated resistance requires secretion of PenA by the Tat secretion system. Both a strain lacking the *tatABC* genes, and one with a PenA R8A amino acid change had β-lactam susceptibility phenotypes similar to a strain completely lacking *penA* (21). Tat-secreted β-lactamases have also been identified in *Mycobacterium tuberculosis* (42), *Stenotrophomonas maltophilia* (41), *Xanthomonas campestris* (44), *Yersinia entercolitica*, and *Photorhabdus asymbiotica* (43). Interestingly, the BlaA β-lactamase from *Yersinia entercolitica* is the closest homologue to PenA outside of *Burkholderia* species.

It has been suggested that PenA is membrane-associated (21, 58). While an example of a membrane-bound lipoprotein β-lactamase has been shown in Gram-negative *Moraxella*

catarrhalis (52), such membrane-associated β-lactamases are generally found in Gram-positive organisms (51), while Gram-negative β-lactamases are usually found in the periplasmic space. Rholl *et. al.* failed to show PenA in periplasmic fractions from *B. pseudomallei*, but those experiments had no control to show if any periplasmic proteins were actually being extracted (21).

1.8 Hypothesis and Research Aims

The purpose of this study was to examine the sub-cellular localization of the PenA β-lactamase in *B. pseudomallei*. Our hypothesis was that in *B. pseudomallei*, PenA is a Tat-secreted periplasmic enzyme, whose cellular localization can be determined using appropriate methods and controls.

The specific aims of this study were:

1) To evaluate different methods for extracting periplasmic proteins from *B. pseudomallei* using a well-characterized periplasmic marker protein.
2) To determine the localization of PenA within defined fractions of the *B. pseudomallei* cell envelope.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

Bacterial strains are listed in Table 2.1. Bacteria were routinely cultured in Lennox LB (MO BIO Laboratories, Carlsbad, CA) at 37°C. Bp82-derived strains were grown in media supplemented with 80 μg/ml adenine. Antibiotics were used at the following concentrations: 100 μg/ml ampicillin (Ap) and 35 μg/ml kanamycin (Km) for *E. coli* and 1,000 μg/ml Km and 2,000 μg/ml zeocin (Zeo) for *B. pseudomallei*. YT media (65) with 15% sucrose and 50 μg/ml X-Gluc (Gold Biotechnology, Saint Louis, MO) was used for resolution of merodiploids during allelic exchange. For long-term storage, overnight cultures were mixed with sterile glycerol to a final concentration of 20% and frozen at -80°C.

Table 2.1 Bacterial strains used in this study

Strain	Description	Source
E. coli		
DH5α	Cloning strain	(59)
DH5α(λ*pir*$^+$)	Cloning strain	Schweizer lab collection
MC4100	Wild-type strain	(60)
B. pseudomallei		
Bp82	1026b Δ*purM*	(61)
Bp82.11	Bp82 Δ*penA*	(21)
Bp82.22	Bp82.11::mini-Tn*7*T-FKM-P_{tac}-*penA*	D. Rholl, unpublished
Bp82.75	Bp82::mini-Tn*7*T-FKM-P_{S12}-*phoA*	This study
Bp82.101	Bp82.75 Δ(*wcbR-A*)::Zeo	This study
Bp82.143	Bp82 PenA C23S	This study

2.2 DNA techniques

Plasmids and oligonucleotide primers are listed in Tables 2.2 and 2.3, respectively. Plasmid DNA was isolated from *E. coli* using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA). Nucleic acid samples were quantified using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). Genomic DNA to be used as a template for polymerase chain reaction (PCR) was extracted by boiling a single colony picked from an agar plate in 50 µl deionized water (diH_2O) for 10 min, and centrifuging for 2 min at 12,000 x g to precipitate cellular debris. 5 µl of supernatant was used as template in a 50 µl PCR reaction.

PCR was performed using either standard *Taq* DNA Polymerase (New England Biolabs, Ipswich, MA) for routine PCR, or Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) for cloning or sequencing, according to the manufacturer's instructions, using custom oligonucleotide primers purchased from Integrated DNA Technologies (Coralville, IA). PCR products were purified and visualized by agarose gel electrophoresis with ethidium bromide, and extracted using the Sigma GenElute Gel Extraction Kit (Sigma-Aldrich, Saint Louis, MO) when required.

The pGEM-T Easy Vector System (Promega, Madison, WI) was used to TA-clone PCR products, according to the manufacturer's instructions. All newly constructed plasmids were confirmed by restriction digest and DNA sequencing (performed at the Colorado State University Proteomics and Metabolomics Facility, Fort Collins, CO).

Restriction digests were performed using enzymes from New England Biolabs (Ipswich, MA) according to the manufacturer's instructions. After restriction enzyme digestion, DNA fragments were separated by agarose gel electrophoresis, visualized with ethidium bromide, and

fragments were extracted using the Sigma GenElute Gel Extraction Kit (Sigma-Aldrich, Saint Louis, MO). Ligations were performed using T4 DNA Ligase (Invitrogen, Carlsbad, CA).

Plasmids were transformed into chemically competent *E. coli,* as previously described (62). Plasmids were introduced into *B. pseudomallei* by electroporation (63).

Table 2.2 Plasmids used in this study

Plasmid	Description	Source
pGEM-T Easy	Ap^r; TA cloning vector	Promega
pTNS3	Ap^r; helper plasmid encoding the Tn*7* site-specific transposition pathway	(64)
pEXKm5	Km^r; allelic exchange plasmid	(65)
pPS2358	Ap^r; pGEM-T Easy-Δ(*wcbR-wcbA*)::Zeo	T. Mima, unpublished
pPS2669	Km^r; pUC18T-mini-Tn*7*T-FKM-P_{S12}	B. Kvitko, unpublished
pPS2712	Km^r; pEXKm5-*penA*; contains C218A SNP	(21)
pPS2921	Ap^r; pGEM-T Easy-*phoA*; pGEM-T Easy with 1,435 bp PCR product containing the *phoA* gene amplified from *E. coli* MC4100 genomic DNA using primers 2390 + 2391	This study
pPS2926	Km^r; pUC18T-miniTn7T-FKM-P_{S12}-*phoA*; a 1,525 bp *Zra*I x *Sac*I fragment from pPS2921 containing *phoA* was ligated into *Sma*I + *Sac*I-linearized pPS2669	This study
pPS3066	Km^r; pEXKm5-Δ(*wcbR-wcbA*)::Zeo; a 2,454 bp *Eco*RI fragment from pPS2358 containing the Δ(*wcbR-wcbA*)::Zeo cassette was ligated into the *Eco*RI site of pEXKm5	This study
pPS3092	Km^r; pEXKm5-*penA* C23S; mutagenic primer 2594 was used to change *penA* nucleotide 67 from T to A, providing a C23S substitution, and mutagenic primer 2482 was used to change *penA* nucleotide 218 from A to C using a pPS2712 DNA template.	This study

Abbreviations: Ap, ampicillin; Km, kanamycin, Zeo, zeocin; r, resistant.

Table 2.3 Oligonucleotide primers used in this study

Primer	Sequence	Source
Cloning		
1687	5'-GGATCCGACGAGAGCTGATACGCTAG[a]	(21)
1712	5'-AAGCTTATACCGGCATCGTTTCGCTG	(21)
2390	5'-CATGGAGAAAATAAAGTGAAACAAAGC	This study
2391	5'-GGTTTTATTTCAGCCCCAGAG	This study
Mutagenic primers		
2482	5'Phos/-GCGACGAGCGTTTCC**C**GTTCTGCAGCACATC[b]	This study
2594	5'Phos/-TGATCGGCGCC**A**GCGCGCCGCTG	This study
Capsule knockout screening		
536	5'- TCCGCTGCATAACCCTGCTTC	(66)
537	5'- CAGCCTCGCAGAGCAGGATTC	(66)
1664	5'- GTGTTCGCGTACAGCATGTC	T. Mima, unpublished
1716	5'- CTGCAGTACATCCGGCACTA	T. Mima, unpublished
1717	5'- GGGTGTATTCGGCATGAGTC	T. Mima, unpublished
1718	5'- CTTGCGACGGTTCAGAAAGT	T. Mima, unpublished
1719	5'- GAAAAGCCGCCAGTATGTTC	T. Mima, unpublished
1721	5'- AAGGTGTTTGACCAGTTCCG	T. Mima, unpublished
Tn*7* integration confirmation		
TN7L	5'-ATTAGCTTACGACGCTACACCC	(64)
BPGLMS1	5'-GAGGAGTGGGCGTCGATCAAC	(64)
BPGLMS2	5'-ACACGACGCAAGAGCGGAATC	(64)
BPGLMS3	5'-CGGACAGGTTCGCGCCATGC	(64)
RT-qPCR		
2077	5'-GTTCTGCAGCACATCCAAGA	(21)
2078	5'-CGGTGTTGTCGCTGTACTGA	(21)
Bp23S-F	5'-GTAGACCCGAAACCAGGTGA	(18)
Bp23S-R	5'- CACCCCTATCCACAGCTCAT	(18)

[a]Underline indicates a newly generated restriction enzyme cleavage site.
[b]**Bold** indicates introduced point mutations.

2.3 Strain construction

Mutant Bp82-based strains were constructed using the previously published mini-Tn*7* and pEXKm5 systems (64, 65). Briefly, the pEXKm5 system was used to generate allelic exchange mutants, and the mini-Tn*7* transposon was used to insert sequence at a known, non-disruptive site in the chromosome.

2.3.1 Construction of strains with mini-Tn*7* insertions

Stable, single-copy gene insertions at a non-disruptive site in the chromosome were achieved using the mini-Tn*7* system (64). The *E. coli phoA* gene was directionally cloned into pPS2669 (Figure 2.1), which contains, between Tn7L and Tn7R flanks, a *FRT*-flanked Km^r marker (FKM) and the promoter from the *B. thailandensis* ribosomal S12 protein-encoding gene (P_{S12}), so that *phoA* was expressed constitutively from P_{S12}. Specific plasmid construction details are found in Table 2.2.

These pUC18T-mini-Tn*7*T-based constructs were introduced into Bp82 and/or Bp82.11 using the helper plasmid pTNS3. The pUC18T-based plasmids contain a ColE1 origin of replication, and pTNS3 contains an R6K origin of replication, neither of which supports plasmid replication in *Burkholderia* spp (64, 65). pTNS3 supplies the *tnsABCD* genes encoding the Tn*7* site-specific transposition pathway, which inserts any sequence between Tn*7*L and Tn*7*R flanks into the bacterial chromosome at an *att*Tn7 site, usually located downstream of a *glmS* gene (67).

Bacteria containing Tn*7* insertions were selected for kanamycin resistance, since for the Km^r marker to be maintained it must have been chromosomally integrated. Because *B. pseudomallei* has three *glmS* genes, there are three possible Tn*7* insertion sites. Integrants were

screened by PCR using primer TN*7*L paired with BPGLMS1, BPGLMS2, and BPGLMS3, and clones with insertions at the *glmS2*-associated *att*Tn*7* were chosen for consistency.

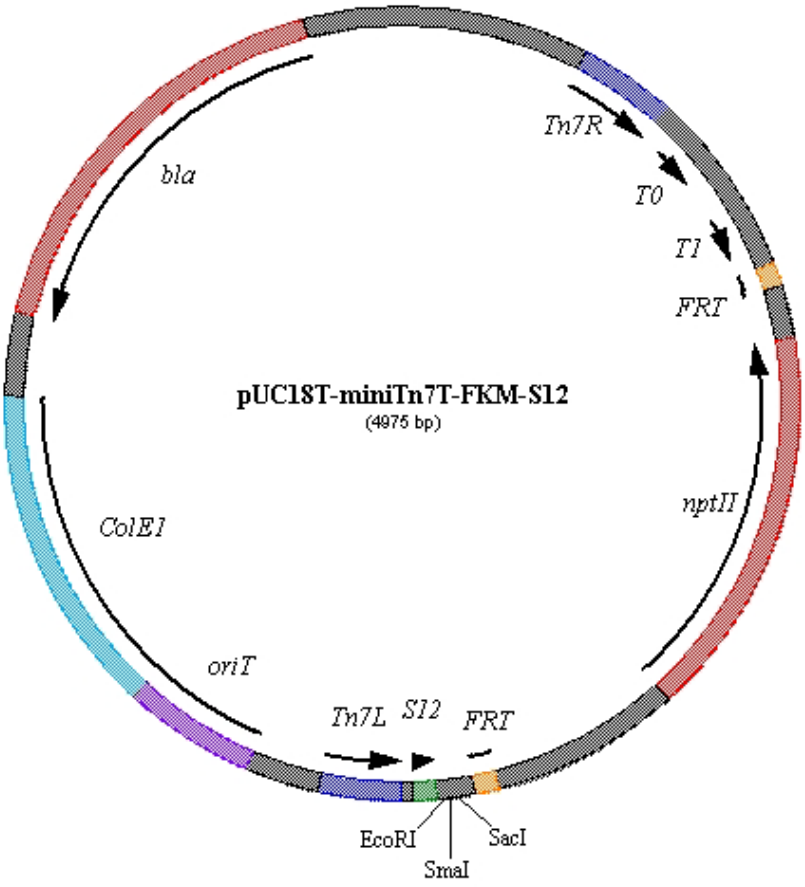

Figure 2.1: Map of mini-Tn*7* vector pPS2669. The sequence between the Tn*7*L and Tn*7*R flanks, which can be transposed into the *B. pseudomallei* genome, contains a *FRT*-flanked *nptII* gene conferring kanamycin resistance, T0 and T1 transcriptional terminators, and the P_{S12} promoter. Genes of interest can be cloned downstream of P_{S12} for constitutive expression using the unique *Eco*RI, *Sma*I, and *Sac*I restriction sites. The *ColE1* replicon allows replication in *E. coli*, but not *B. pseudomallei*, and *oriT* allows for conjugal plasmid transfer.

2.3.2 Construction of mutants by allelic exchange

For construction of Bp82.143, the PenA C23S point mutation was introduced into Bp82.11 using the pEXKm5 allelic exchange system (65). pPS3092 (Figure 2.2) was electroporated into Bp82.11 and the transformation mixture was plated on YT media containing adenine, kanamycin, and X-Gluc. The kanamycin selected for merodiploids with the plasmid integrated in the chromosome through homologous recombination. Merodiploids also produced the β-glucuronidase enzyme encoded by the *gusA* gene on pEXKm5, which cleaves the indicator X-Gluc, resulting in a blue colony appearance.

Merodiploids were then resolved by growth on YT media containing adenine, 15% sucrose, and X-Gluc. The sucrose prevented growth of merodiploids, due to the *sacB* gene on the pEXKm5 backbone, encoding levansucrase from *Bacillus subtilis*. However, bacteria that underwent a second homologous recombination event to excise the plasmid backbone (including *sacB*, *gusA*, and the Km^R marker), were sucrose resistant and white in the presence of X-Gluc. These resolved clones were confirmed to be kanamycin-susceptible, and screened by PCR and Sanger sequencing (with primers 1687 and 1712) to determine whether the desired *penA* allele had been introduced and replaced the corresponding wild-type sequence. A confirmed mutant strain (Bp82.143) was frozen for future use.

Strain Bp82.101 (Bp82.75Δ(*wcbR-A*)) was also created with a pEXKm5-based plasmid (pPS3066, Figure 2.3) but because the parental strain, Bp82.75, was already Km^r, and the Δ(*wcbR-A*) knockout cassette contained a Zeo^r marker, recombinants were selected with zeocin, rather than kanamycin. Following sucrose resolution, zeocin resistant clones were PCR screened for the absence of *wcbB* and *gmhA* genes from the deleted operon, presence of the Zeo^r marker,

and absence of *oriT* found on the pEXKm5 backbone using primer pairs 1716 + 1717, 1718 + 1719, 1664 + 1721, and 536 + 536, respectively.

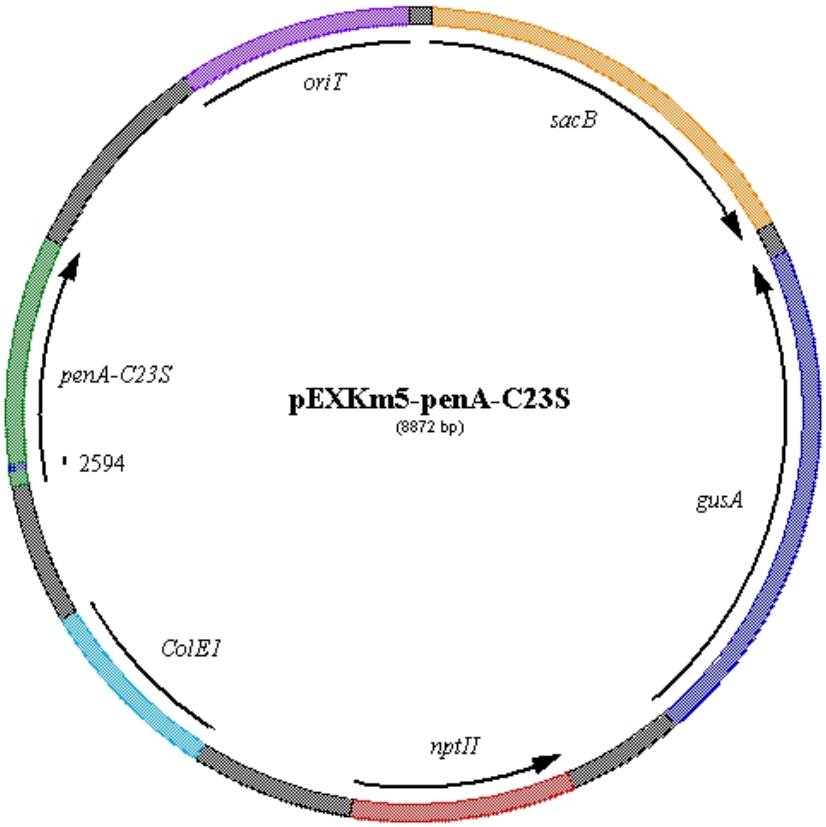

Figure 2.2 Map of the PenAC23S allele replacement plasmid pPS3092. The *nptII* gene, conferring kanamycin resistance allows for selection of merodiploids, the *Bacillus subtilis sacB* gene allows for counter-selection in the presence of sucrose, and *gusA*, encoding *E. coli* β-glucuronidase, allows colorimetric screening with X-Gluc. The *ColE1* replicon allows replication in *E. coli*, but not *B. pseudomallei*, and *oriT* allows for conjugal plasmid transfer. The *penA* gene on this plasmid contains a point mutation that changes amino acid 23 from a cysteine to a serine. This underlying nucleotide change was introduced with mutagenic primer 2594.

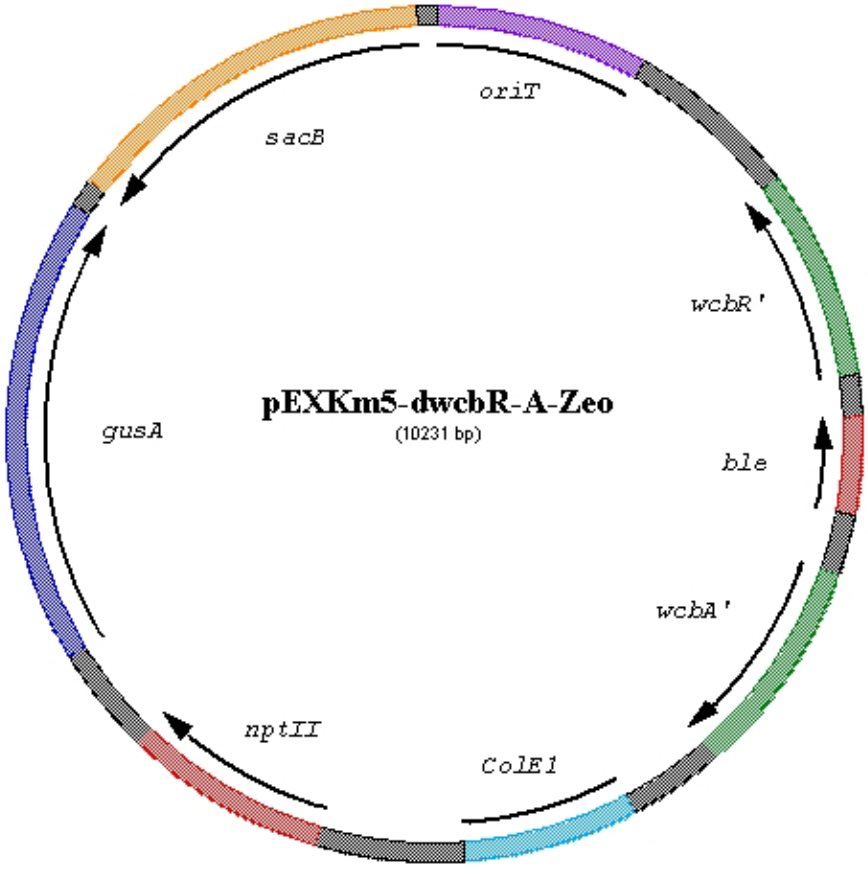

Figure 2.3 Map of Δ(*wcbR-A*) gene replacement vector pPS3066. The *nptII* gene, conferring kanamycin resistance allows for selection of merodiploids, *sacB* allows for counter-selection in the presence of sucrose, and *gusA* allows colorimetric screening with X-Gluc. The *ColE1* replicon allows replication in *E. coli*, but not *B. pseudomallei*, and *oriT* allows for conjugal plasmid transfer. The *ble* gene between truncated *wcbR* and *wcbA* gene fragments confers zeocin resistance.

2.4 Extraction of periplasmic proteins

The PeriPreps Periplasting Kit from EpiCentre Biotechnologies (Madison, WI) was used according to the manufacturer's instructions. Cells from a 1 ml bacterial culture grown overnight were harvested by centrifugation at room temperature for 30 sec at 14,000 x g, resuspended in 50 µl PeriPreps Periplasting Buffer, and incubated for 5 min at room temperature. 50 µl ice cold water was added and mixed by inversion. The suspension was incubated for 5 min on ice, and then centrifuged for 2 min at 14,000 x g at room temperature. Supernatants, containing periplasmic fractions, were removed to clean tubes. If required, spheroplastic proteins were prepared by resuspending the remaining pellets in 100 µl of PeriPreps Lysis Buffer with OmniCleave Endonuclease, and 1 µl of 1 M $MgCl_2$ was added, mixed by inversion, and incubated for 5 min at room temperature. Cell debris was collected by centrifugation at room temperature for 5 min at 14,000 x g. Supernatants, containing spheroplastic fractions, were transferred to new tubes, and 2 µl 500 mM EDTA was added.

Cold osmotic shock was performed according to the method of Higgins and Hardie (68). 0.5 ml of 0.5 M Tris pH 7.8 was added to 5 ml of overnight cultures and the mixture was incubated for 10 min at room temperature. Cells were collected by centrifugation at 2,500 x g for 10 min at room temperature. Pellets were resuspended in 0.8 ml of sucrose solution (30 mM Tris pH 7.8; 40% sucrose; 2 mM EDTA), incubated with shaking for 10 min at 30°C, and centrifuged for 30 sec at 14,000 x g at room temperature. Pellets were rapidly resuspended in 0.5 ml ice cold water, incubated on ice for 10 min, and the suspensions were centrifuged at 14,000 x g for 5 min at 4°C. Supernatants (periplasmic fractions) were removed and stored at -20°C.

Chloroform shock treatment was performed based on the method of Ames *et. al.* (69). Cells were collected from 2 ml of overnight cultures by centrifugation for 30 sec at 14,000 x g at

room temperature. Pellets were resuspended in approximately 20 µl of residual media, 20 µl of chloroform was added, and the cell suspensions were vortexed periodically while incubating at room temperature for 15 min. 200 µl of 10 mM Tris pH 8 was added, followed by centrifugation at 6,000 x g for 20 min at room temperature. The top, aqueous layers were removed as periplasmic fractions and stored at -20°C.

2.5 Membrane fractionation

Total membrane fractions were prepared from 10 ml of overnight cultures. Cells were collected by centrifugation at 2,500 x g for 10 min at 4°C, and resuspended in 5 ml diH_2O. They were then lysed by sonication on ice. A Sonics Vibracell VC750 sonicator (Newtown, CT) was used at 30% amplitude, using 4 cycles of 1 sec pulses for 1 min followed by a 30 sec pause. Cell debris was pelleted by centrifugation at 2,500 x g for 10 min at 4°C. Supernatants were transferred to new tubes and centrifugation was repeated. The resulting supernatants, containing cell lysates, were spun for 1 hr at 30,000 rpm at 4°C in a Sorvall WX Ultra 100 ultracentrifuge (Thermo Fisher Scientific, Waltham, MA), using a TH-641 swinging bucket rotor. Supernatants, containing cytoplasmic and periplasmic proteins, were removed to new tubes, and the membrane pellets were resuspended in 1 ml of diH_2O and frozen at -20°C.

2.6 Phase separation of membrane proteins using Triton X-114

Total membranes were prepared as described above, but instead of water, membrane pellets were resuspended in 150 µl 4% Triton X-114 (TX-114) in PBS, transferred to microcentrifuge tubes, rocked overnight at 4°C, and centrifuged for 1 hr at 25,000 x g, 4°C. Supernatants were moved to new tubes and stored at 4°C, while pellets were resuspended in 150

µl 4% TX-114 in PBS, rocked for 1 hr at 4°C, and centrifuged as above. Supernatants were combined with those obtained from the overnight extraction, incubated at 37°C for 1 hr with occasional rocking, and centrifuged for 1 hr at 25,000 x g, 37°C. Upper (aqueous) phases were removed, and an equal volume of ice cold PBS was added to return the TX-114 concentration to 4%. The 37°C incubation and centrifugation steps were repeated twice. 9 volumes of ice cold acetone were added to final detergent phases and mixtures were incubated at -20°C overnight. Acetone precipitates were centrifuged for 1 hr at 25,000 x g at 4°C, supernatants were decanted, pellets were resuspended in 150 µl ice cold acetone, and centrifugation was repeated. After acetone supernatants were decanted, pellets were air dried completely, resuspended in 200 µl diH_2O, and frozen at -20°C.

2.7 Detection of proteins by Western blot

Cellular fractions were diluted 1:1 in 2X Laemmli sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol, Bio-Rad Laboratories, Hercules, CA), boiled for 10 min, cooled, and separated on 12% polyacrylamide Tris-glycine SDS-PAGE gels for approximately 1.5 hr at 200 volts. Proteins were transferred to PVDF membranes using a Bio-Rad Trans-Blot SD semi-dry cell (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 4% BSA in TBS-T (20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5) rocking for one hour at room temperature, or overnight at 4°C. Blots were washed twice for 5 min each with water, then stained with primary antibody (see Table 2.4) diluted in blocking solution for 1 hr with rocking at room temperature. Blots were washed three times, 5 min each with TBS-T, then stained with secondary antibody for 30 min, rocking at room temperature. Blots were washed 3 times with TBS-T and twice with water (5 min per wash). 1

ml of Novex ECL chemiluminescent substrate (Life Technologies, Carlsbad, CA) was added, and rocked for 1 min. Blots were imaged on a ChemiDoc XRS (Bio-Rad Laboratories, Hercules, CA).

Table 2.4 Antibodies used for Western blot analyses

Antigen	Type	Dilution	Source
E. coli PhoA	Mouse monoclonal	1:1,000	Life Technologies, Carlsbad, CA
B. pseudomallei PenA	Rabbit polyclonal	1:1,000	(21)
E. coli RpoB	Mouse monoclonal	1:2,000	Neoclone, Madison, WI
B. pseudomallei Omp85	Mouse polyclonal	1:500	(70)
Mouse IgG	Goat polyclonal, HRP conjugate	1:2,500	Promega, Madison, WI
Rabbit IgG	Goat polyclonal, HRP conjugate	1:2,500	Promega, Madison, WI

2.8 Protein quantification

The protein content of samples was quantified using the Pierce BCA Protein Assay Reagent kit (Thermo Fisher Scientific, Waltham, MA). BSA standards were made by serially diluting the 2 mg/ml stock in the same diluent as samples (diH_2O or 10 mM Tris pH 8) from 320 µg/ml to 5 µg/ml. 25 µl of blanks (diluent only), standards and samples were aliquotted into a microtitre plate wells in duplicate or triplicate, and 200 µl of Working Reagent was added to each well. After incubating 30 min at 37°C and cooling to room temperature, the A_{570nm} of each well was read using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA). Protein concentrations were calculated using Revelation Data Processing software (Dynex Technologies, Chantilly, VA).

2.9 Enzyme assays

Alkaline phosphatase activity was detected using the colorimetric substrate *p*-nitrophenylphosphate (*p*NPP). In microtitre plates, 20 µl of cellular fractions were diluted in 180 µl 10 mM Tris pH 8.0, and 4 µl of 40 mM *p*NPP (Sigma-Aldrich, Saint Louis, MO) was added and mixed by pipette. Samples were incubated 30 min at 37°C, and the A_{410nm} was read on a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA). Samples were assayed in duplicate, and alkaline phosphatase activity was calculated as (average sample A_{410nm} - average blank A_{410nm})/(A_{600nm} starting culture x ml culture used). Each assay was repeated in three separate technical replicates.

β-lactamase enzymatic activity was detected using the colorimetric substrate nitrocefin. In microtitre plates, 200 µl of 50 µg/ml nitrocefin (TOKU-E, Bellingham, WA) in 100 mM sodium phosphate buffer (pH 7) was added to 20 µl of cellular fractions, mixed by pipette, and incubated 30 min at 37°C. A_{486nm} was read with a Multiskan Spectrum plate reader (ThermoFisher Scientific, Waltham, MA), and β-lactamase activity from duplicate sample wells was calculated as (average sample A_{486nm} - average blank A_{486nm})/µg protein. Each assay was repeated in three separate technical replicates.

2.10 Reverse transcriptase quantitative real time PCR (RT-qPCR)

Overnight cultures were sububcultured into LB + adenine, and upon reaching log phase (OD_{600} between 0.6 and 0.8), RNA was extracted from 1 ml of culture using the Qiagen RNeasy RNA Protect Mini Bacteria kit according to manufacturer's instructions (Qiagen, Germantown, MD). RNA was quantified with a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA), and 1 µg was treated with DNaseI (Thermo Fisher Scientific, Waltham, MA). cDNA was

synthesized using the Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA). qPCR was then performed on an iCycler iQ instrument (Bio-Rad Laboratories, Hercules, CA) with SYBR GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA) with primers 2077 and 2078 to amplify *penA* derived cDNA and Bp23S-F and Bp23S-R to amplify 23S ribosomal RNA derived cDNA. Each sample was assayed in technical triplicate, melt curve analysis showed clean amplification, and control reactions with no template, or template untreated with reverse transcriptase showed no amplification. *penA* transcript levels were normalized to the 23S rRNA gene, and expressed as fold change relative to wild-type Bp82.

2.11 ^{14}C-palmitic acid labeling

10 ml cultures were grown to mid-log phase in in M9 minimal medium (71) with 10 mM glucose, 80 μg/ml thiamine and 80 μg/ml adenine. Cells were harvested by centrifugation at 2,000 x g for 15 min and resuspended in 1 ml of fresh growth media. 5 μCi of ^{14}C-palmitic acid (American Radiolabeled Chemicals, Saint Louis, MO) was added and the cell suspension was incubated at 37°C for 2 hr. Labeled cells were harvested by centrifugation at 2,000 x g for 30 min, and proteins were extracted with three washes with 5 ml chloroform/methanol. The final pellet was dried under a N_2 stream, resuspended in 100 μl Laemmli electrophoresis buffer and boiled. Samples were analyzed by electrophoresis on a 4-15% gradient SDS-PAGE gel (Bio-Rad, Hercules, CA), the gel was dried, and labeled proteins were imaged by phosphorimaging.

2.12 Globomycin treatment

PenA expression was induced by adding IPTG to two 1 ml cultures of Bp82.22 at OD_{600} 0.6. One culture was treated with 250 μg globomycin (10 mg/ml in DMSO [Sigma-Aldrich,

Saint Louis, MO]), and the other with an equal volume of DMSO only, as a control. Cultures were incubated for one hour at 37°C. The OD_{600} was measured again, and cells from 0.5 ml of the cultures were pelleted by centrifugation and resuspended in Laemmli electrophoresis buffer. Samples were then separated by SDS-PAGE and examined by Western blot, as described above.

2.13 MALDI-TOF mass spectrometry

Membrane fractions were prepared as described above from cultures of Bp82.22 grown with 1mM IPTG to induce *penA* expression, and Bp82.11. After resuspension in 1 ml diH_2O, these membrane samples were washed once with 8 volumes of ice cold acetone, incubated at -20°C overnight, and pelleted by 27,000 x g for 1 hour at 4°C. Pellets were air-dried and resuspended in 50 µl 50% acetonitrile 0.1% trifluoroacetic acid. 1 µl of each sample was mixed with 1 µl 10 mg/ml sinapinic acid matrix on the MALDI-TOF target plate and allowed to air-dry overnight. The samples were then analyzed using a Bruker Ultraflex II MALDI-TOF/TOF instrument and Flex Analysis software (Bruker Daltonics Inc., Billerica, MA).

CHAPTER 3

RESULTS

3.1 Evaluation of periplasmic fractionation methods

In order to determine the sub-cellular localization of PenA, we developed methods for generating controlled subcellular fractions from *B. pseudomallei*. Specifically, a known periplasmic marker protein was necessary to test our hypothesis that PenA is a periplasmic protein.

3.1.1 Construction of a Bp82 reporter strain expressing *E. coli* PhoA

A control strain was generated that constitutively expresses the *E. coli phoA* gene from the *B. thailandensis* ribosomal S12 protein promoter (P_{S12}), for the purpose of comparing different methods of extracting periplasmic protein from *B. pseudomallei*.

E. coli alkaline phosphatase (PhoA) is a well-characterized periplasmic protein (72), whose activity in the periplasm has been utilized in various reporter systems (73-76). In addition, alkaline phosphatase activity can easily be detected and quantified using the colorimetric substrate *p*NPP.

The mini-Tn*7*T-FKM-P_{S12}-*phoA* construct was introduced into Bp82 as described above, to generate Bp82.75. PhoA expression was confirmed by enzyme assay (Figure 3.1) and Western blot (Figure 3.2). Both detection methods show increased expression of alkaline phosphatase activity as compared with wild-type Bp82. The low-level alkaline phosphatase activity in Bp82 is likely due to an endogenous alkaline phosphatase enzyme.

3.1.2 Construction of a Bp82 capsule-deficient strain

To examine the possibility that the *B. pseudomallei* polysaccharide capsule might inhibit periplasmic extraction, a derivative of Bp82.75 (Bp82 expressing *E. coli phoA*) was generated that lacks 23,098 bp of the *wcbR-A* capsule synthesis operon. The knockout cassette was derived from pPS2358, with truncated portions of the *wcbR* and *wcbA* genes surrounding a zeocin resistance marker, and was cloned into pEXKm5 for allelic exchange, as described in Section 2.3.2. In the *B. pseudomallei* genome, the *wcbQ, wcbP, wcbO, wcbN, wcbM, gmhA, wcbL, wcbK, wcbJ, wcbI, wcbH, wcbG, wcbF, wcbE, wzt2, wcm2, wcbD, manB, wcbC,* and *wcbB* genes of the capsular polysaccharide synthesis operon are found between *wcbR* and *wcbA* (4, 53). In the resulting mutant strain, Bp82.101, the *gmhA* and *wcbB* genes were not detected by PCR, while the presence of the Zeo^r marker was confirmed by both PCR and bacterial growth in the presence of zeocin.

3.1.3 Comparison of periplasmic fractionation methods

Bp82.75 (Bp82::mini-Tn*7*T-FKM-P_{S12}-*phoA*) and Bp82.101 (Bp82.75 Δ(*wcbR-A*)::Zeo) were used to compare several published methods for extracting periplasmic proteins from *E. coli*. Extraction efficiency was assessed by measuring alkaline phosphatase activity in each of the extracted fractions. Wild-type Bp82 was used as a negative control.

As shown in Figure 3.2, chloroform shock treatment extracted more alkaline phosphatase activity from both Bp82.75 and Bp82.101 than either the PeriPreps or cold osmotic shock methods, though none of the methods extracted all of the activity seen in whole cells, indicating that periplasmic protein extraction was not complete. In addition, no difference in alkaline

phosphatase extraction efficiency was seen between the strains possessing (Bp82.75) or lacking (Bp82.101) the *wcbR-A* capsule synthesis genes.

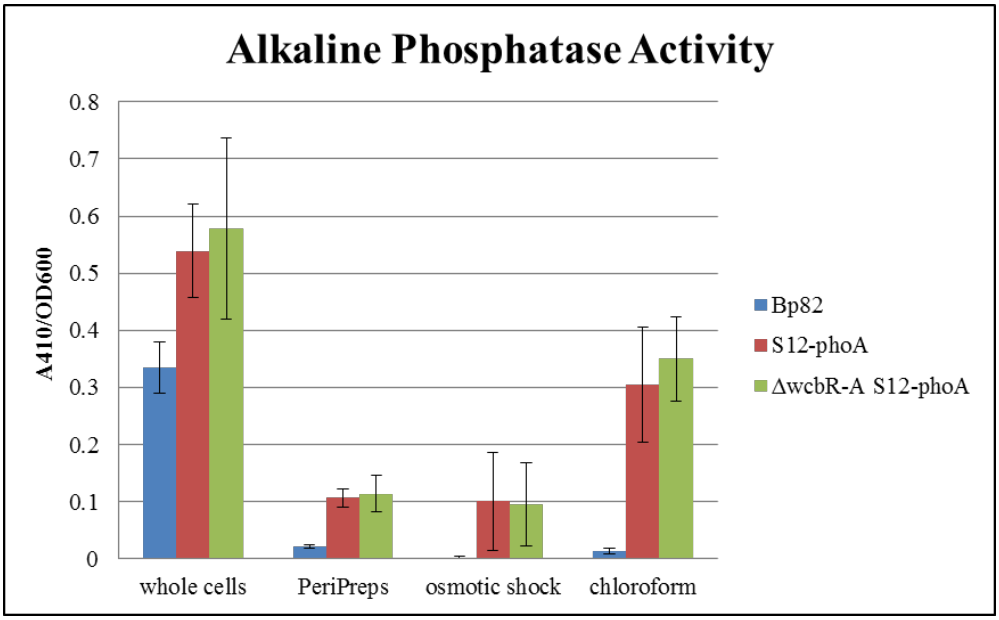

Figure 3.1 Alkaline phosphatase activity in Bp82, Bp82.75, and Bp82.101 whole cells and periplasmic fractions. Periplasmic fractions were prepared using the PeriPreps, cold osmotic shock, and chloroform shock methods. Alkaline phosphatase activities are expressed as (average sample A_{410nm} - average blank A_{410nm})/(A_{600nm} starting culture x ml culture used). Each assay was performed in duplicate, and values shown are the averages of two separate assays. Error bars represent one standard deviation from the mean.

3.2 Cellular PenA localization

Upon establishment of a successful periplasmic extraction method in *B. pseudomallei*, defined sub-cellular fractions were examined for the presence of PenA by Western blot to detect the protein, and by enzyme assay for β-lactamase activity.

3.2.1 PenA is not a periplasmic protein

Chloroform-extracted periplasmic protein samples were examined by Western blot for the presence of PenA and the alkaline phosphatase marker protein (Figure 3.2). PhoA (approximately 50 kD) is present in significant amounts in both whole cell and periplasmic fractions from Bp82.75, which is in agreement with alkaline phosphatase enzyme activity in both whole cells and periplasmic fractions (Figure 3.1). No PhoA protein is detectable in extracts derived from wild-type Bp82. The 85 kD outer membrane protein Omp85 and the 150 kD cytoplasmic RpoB protein are present in whole cell fractions from all three strains, but absent from any of the periplasmic fractions, indicating that periplasmic fractions are free of outer membrane and cytoplasmic contamination.

Whole cell fractions derived from both strains with the wild-type *penA* gene (Bp82 and Bp82.75 [Bp82 expressing *E. coli* PhoA]) contain a strong ~27 kD PenA band that is absent in Bp82Δ*penA*. However, periplasmic fractions from these strains contain very little PenA protein. Furthermore, while β-lactamase activity from PenA is present in wild-type Bp82 whole cell fractions (as compared with Bp82Δ*penA* [Bp82.11]), essentially no β-lactamase activity could be detected in chloroform extracted periplasmic fractions (Figure 3.3).

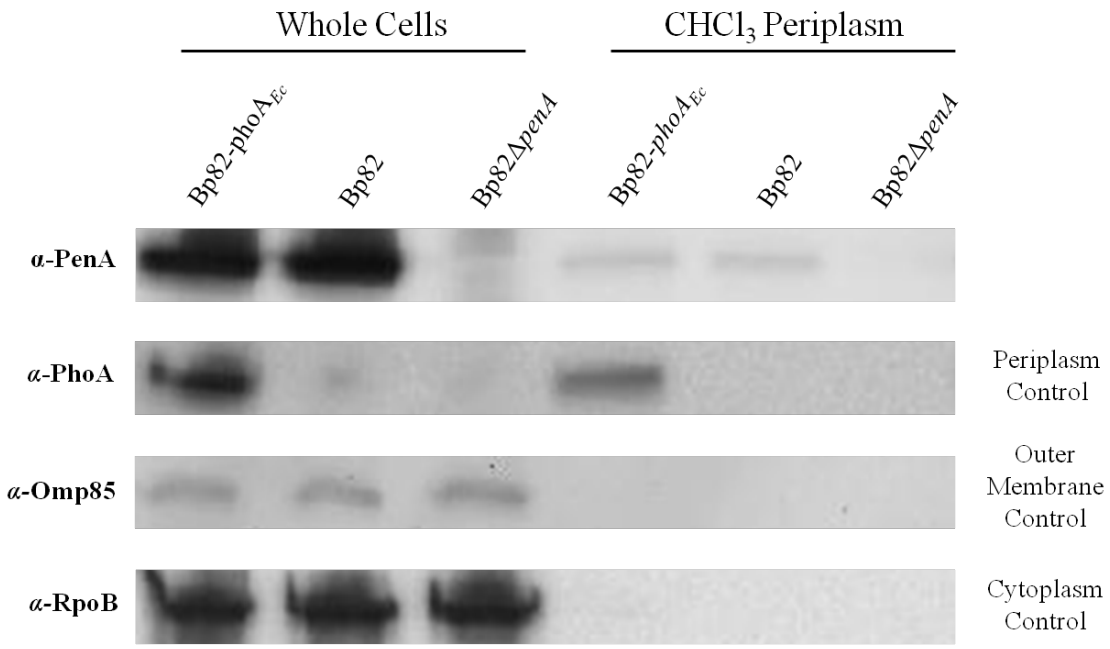

Figure 3.2 Cellular localization of PenA assessed by Western blot analysis. Whole cell and chloroform-extracted periplasmic fractions derived from Bp82 expressing *phoA* (Bp82-*phoA*$_{EC}$ or Bp82.75), wild-type Bp82, and Bp82Δ*penA* (Bp82.11) were separated by SDS-PAGE, transferred to PVDF membranes, and probed with antibodies against PenA, PhoA, Omp85, and RpoB. The panels shown represent four separate blots.

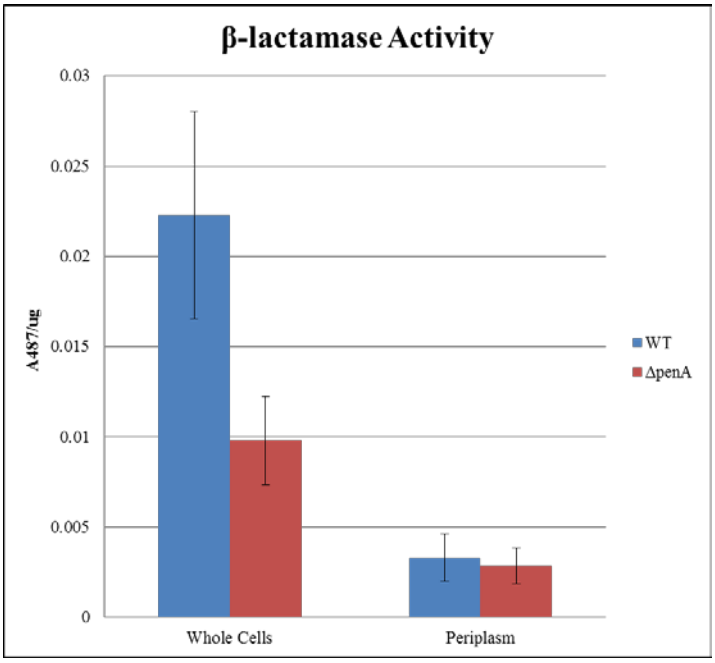

Figure 3.3 Cellular localization of PenA β-lactamase activity. β-lactamase activity was determined in Bp82 and Bp82.11 whole cell and chloroform-extracted periplasmic samples using nitrocefin as a substrate. Activity was calculated as (average sample A_{486nm} - average blank A_{486nm})/μg protein, with protein concentration calculated by BCA assay. Each assay was performed in duplicate, and the values shown are the averages from three separate assays, with error bars representing one standard deviation from the mean.

3.2.2 PenA is a membrane associated protein

After seeing less than convincing evidence of PenA being located in the periplasm, membrane fractions were prepared from wild-type Bp82 and Bp82Δ*penA*. The presence of PenA in these fractions was assessed by Western blot (Figure 3.4) and β-lactamase enzyme assay (Figure 3.5). Large quantities of PenA protein and its corresponding β-lactamase activity were detected in whole cell, total membrane, and detergent extracted membrane fractions. In fact, PenA β-lactamase activity was highly enriched in the latter two fractions when compared to the

whole cell fraction. The presence of Omp85 and absence of PhoA in membrane fractions was confirmed by Western blot (data not shown).

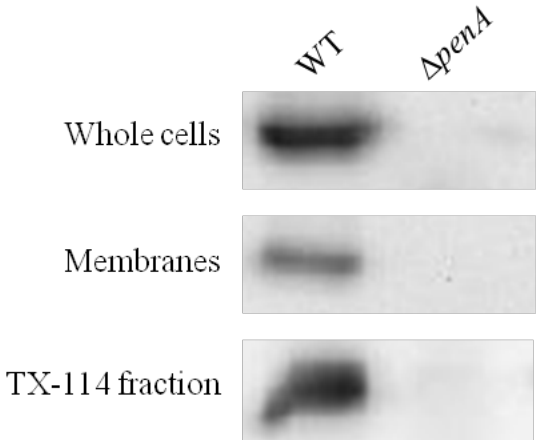

Figure 3.4 PenA membrane localization analyzed by Western blot. Wild-type Bp82 and Bp82Δ*penA* (Bp82.11) whole cell, total membrane and TX-114 extracted samples were electrophoresed by SDS-PAGE, transferred to a PVDF membrane, and blotted with α-PenA antibodies.

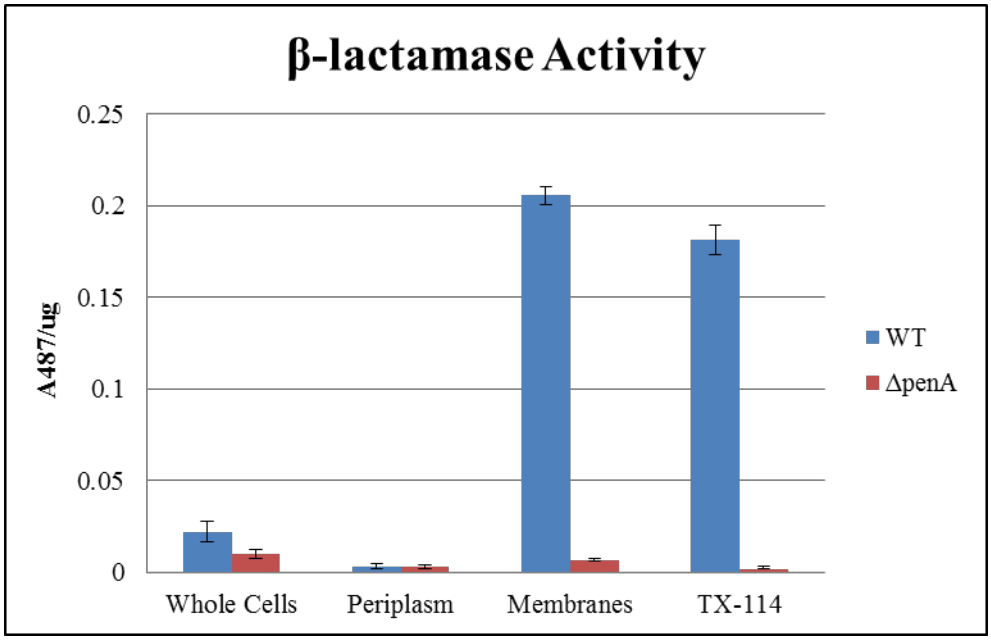

Figure 3.5 PenA β-lactamase activity is membrane associated. β-lactamase activity was determined in wild-type Bp82 and Bp82Δ*penA* (Bp82.11) whole cell, total membrane, and TX-114 extracted samples using nitrocefin as the substrate. Activity was calculated as (average sample A_{486nm} - average blank A_{486nm})/μg protein, with protein concentration determined by BCA assay. Each assay was performed in duplicate, and the values shown are the averages from three separate assays, with error bars representing one standard deviation from the mean.

3.3 PenA is a lipoprotein

The experiments described in section 3.2.2 showed that PenA protein and associated β-lactamase activity are membrane associated and can be extracted with Triton X-114 (TX-114). TX-114 is a nonionic detergent with a low cloud point (77), which has been used to enrich for lipoproteins from a variety of bacterial species (78-82). Following a temperature-based TX-114 phase separation, lipoproteins, as well as other lipophilic proteins are found in the detergent phase, while hydrophilic non-lipoproteins partition to the aqueous phase. While separation of a protein to the TX-114 detergent phase cannot definitively identify it as a lipoprotein, this hydrophobic behavior provided additional evidence supporting the model of PenA as membrane-bound lipoprotein. Attempts were therefore made to assess whether PenA membrane association is a result of post-translational lipid modification.

3.3.1 LipoP software prediction

Bacterial lipoproteins contain lipobox motifs that mediate lipid modification and signal peptide cleavage, allowing for anchoring to the inner or outer membrane (47). The online LipoP software predicts whether prokaryotic protein sequences contain lipoprotein signal peptides, and identifies any signal peptidase (SPase) II cleavage sites (83). According to LipoP, PenA is likely a lipoprotein, with SPase II cleaving between amino acids A_{22} and C_{23} (Figure 3.6). Bacterial lipoprotein biosynthesis requires a cysteine residue at the amino-terminal of the mature protein, to which a diacylglycerol molecule is covalently attached. Since the PenA amino terminus contains a potential non-canonical lipobox with a conserved cysteine (Cys_{23}), it is highly probable that PenA is a lipoprotein and that the post-translational lipid modification occurs at Cys_{23}.

Figure 3.6 The predicted PenA signal sequence. The 29 N-terminal amino acids of PenA are shown. The twin arginines directing PenA Tat secretion are shown in red letters. The predicted non-canonical lipobox is shown in blue letters. The critical cysteine required for post-translational lipid modification is marked with an asterisk.

3.3.2 Cys_{23} is required for PenA maturation

Based on current understanding of prokaryotic lipoprotein biosynthesis, replacement of Cys_{23} with a different amino acid should prevent the post-translational lipid modification and potentially PenA's membrane association.

An isogenic mutant was therefore constructed by allelic exchange that resulted in a single amino acid change from cysteine to serine at position 23 of PenA. The mutant allele was introduced from pPS3092 into Bp82.11 to generate Bp82.143, as described in Section 2.3.2. The single mutation to *penA* was confirmed by DNA sequencing.

Surprisingly, while the resulting strain, Bp82.142, had *penA* transcript levels indistinguishable from wild-type Bp82 by qRT-PCR (Figure 3.7), the $PenA_{C23S}$ mutant produced neither PenA protein nor β-lactamase activity, as assessed by Western blot analysis of whole cells or membranes and enzyme assay (Figure 3.8 and 3.9). Therefore, it appears that the $PenA_{C23S}$ protein is being degraded, indicating that Cys_{23} is essential for the processing and generation of the mature PenA protein.

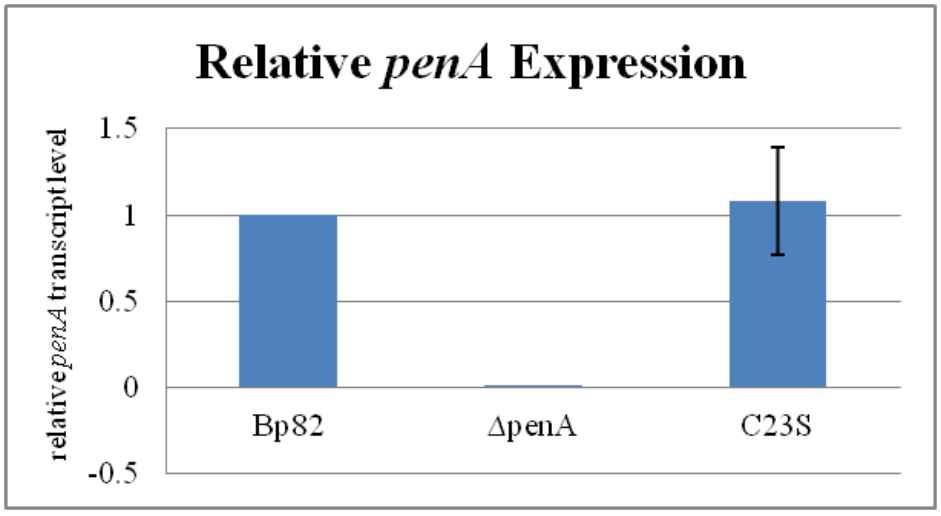

Figure 3.7 The *penA*$_{C23S}$ mutant gene is transcribed at wild-type levels. qRT-PCR was employed to determine *penA* transcripts levels in wild-type Bp82, Bp82Δ*penA* (Bp82.11), and Bp82 PenA$_{C23S}$ (Bp82.143). Data shown represent the average expression from three biological replicates, relative to wild-type Bp82. Error bars represent one standard deviation from the mean.

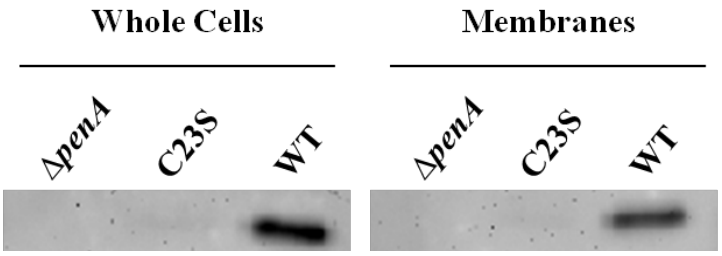

Figure 3.8 PenA lipidation is required for proper cellular localization. Western blots of whole cells and total membranes from Bp82Δ*penA* (Bp82.11), Bp82 PenA$_{C23S}$ (Bp82.143), and wild-type Bp82. Whole cells and membrane fractions were separated by SDS-PAGE, transferred to PVDF, and detected with α-PenA antibodies.

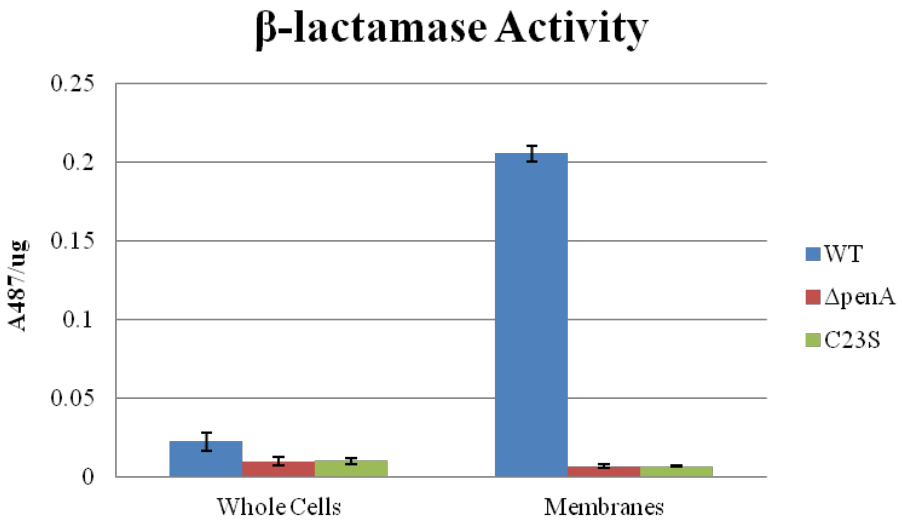

Figure 3.9 PenA lipidation is required for enzymatic activity. β-lactamase enzyme assays were performed with wild-type Bp82, Bp82Δ*penA* (Bp82.11), and Bp82 $PenA_{C23S}$ (Bp82.143) whole cells and total membrane fractions. Activity was calculated as (average sample A_{486nm} - average blank A_{486nm})/μg protein, with protein concentration determined by BCA assay. Each assay was performed in duplicate, and the values shown are the averages from three separate assays, with error bars representing one standard deviation from the mean.

3.3.3 PenA is likely modified by palmitic acid

Another means of demonstrating post-translational lipid modification of PenA involves incorporation of radioactive fatty acids. Wild-type Bp82 and Bp82Δ*penA* (Bp82.11) were metabolically labeled with ^{14}C-palmitic acid. Proteins were extracted with chloroform/methanol and separated by SDS-PAGE, and the gel was examined for differences in incorporation of the ^{14}C-palmitic acid between strains that were or were not expressing *penA*. The arrow in Figure 3.10 indicates a radioactive band of approximately 27 kD that appears to be present in Bp82 samples but not in the Δ*penA* strain Bp82.11, though high background makes it difficult to draw any definite conclusions.

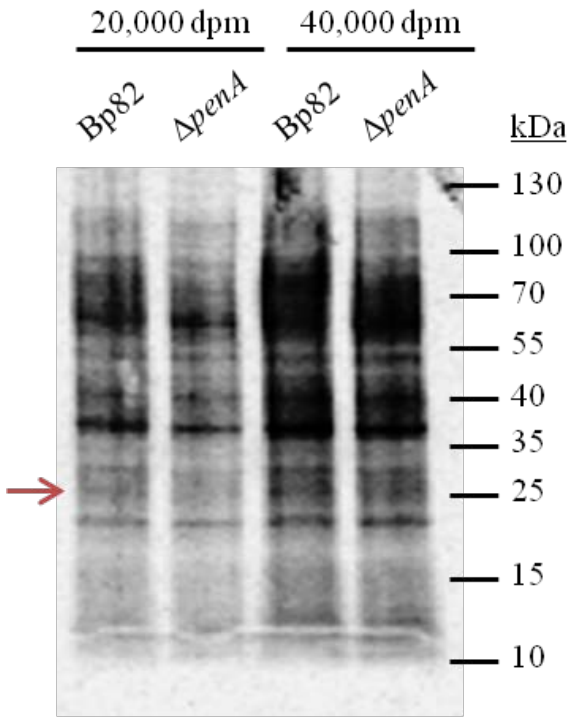

Figure 3.10 ^{14}C-palmitic acid labeling of *B. pseudomallei* proteins. Strains Bp82 and Bp82.11 were labeled for 2 hr using ^{14}C-palmitic acid. 20,000 dpm and 40,000 dpm of each sample were separated by SDS-PAGE and the dried gel was subjected to autoradiography. The red arrow indicates the position of the putative ^{14}C-palmitic acid-labeled PenA lipoprotein.

3.3.4 Globomycin treatment inhibits PenA lipoprotein processing

Further evidence that PenA is a lipoprotein was obtained by assessing whether inhibition of prolipoprotein processing would result in accumulation of PenA prolipoprotein. Globomycin is a peptide antibiotic that specifically inhibits SPase II, the enzyme that removes signal peptides from bacterial lipoproteins (84). Therefore, treatment of bacterial cells with sub-lethal levels of globomycin results in an accumulation of uncleaved prolipoproteins. A Bp82-based strain with *penA* under the control of the inducible P_{tac} promoter (Bp82.22) was treated with globomycin and IPTG, so that *penA* was only expressed during globomycin treatment. Following treatment,

samples were examined by α-PenA Western blot (Figure 3.11). While a single mature PenA band was detected in the untreated control, the globomycin-treated sample contained a slightly larger band, representing the PenA proliporotein, in addition to the smaller, mature protein. Based on the specific inhibition of SPaseII by globomycin, this provides convincing evidence that PenA is indeed a lipoprotein.

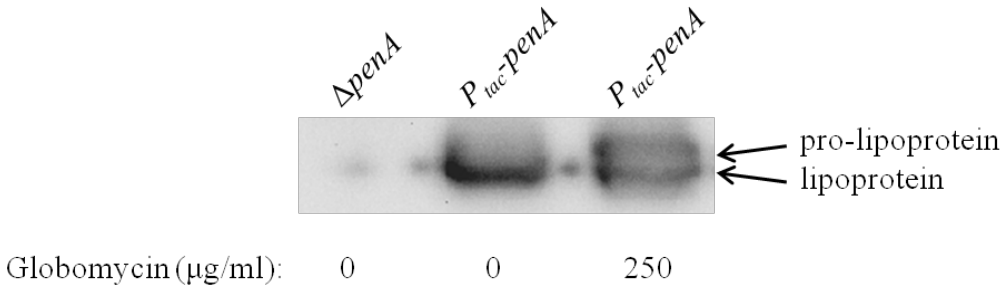

Figure 3.11 Globomycin treatment results in PenA prolipoprotein accumulation. Whole cell samples derived from cultures that were either left untreated or were treated with globomycin at the indicated concentration were separated by SDS-PAGE, transferred to a PVDF membrane, and stained with α-PenA antibodies.

3.3.5 Mass spectrometry indicates post-translational lipid modification of PenA

Total membrane samples from Bp82Δ*penA* (Bp82.11) and IPTG-induced Bp82.22 expressing *penA* from the P_{tac} promoter were compared using MALDI-TOF mass spectrometry. Shown in Figure 3.12, the peak corresponding to the mature PenA protein was identified by its presence in the induced sample and absence from Bp82Δ*penA*. All other peaks, representing other membrane proteins, were present in both samples. Based on the mass to charge ratio detected, the molecular weight of the mature PenA molecule is 29,796.547 Da. Using ExPASy online bioinformatics software (85), the molecular weight of the full-length PenA polypeptide was calculated to be 31,315.69 Da, while the amino acid sequence of our proposed mature PenA lipoprotein, beginning at Cys_{23}, should have a molecular weight of 29,015.93 Da. The addition of a diacylglycerol molecule of ~500 Da and a third, amide-linked fatty acid of ~200-300 Da would yield a mature triacylated PenA lipoprotein of ~29,700-29,800 Da, which is reflected in the molecular weight measured by mass spectrometry.

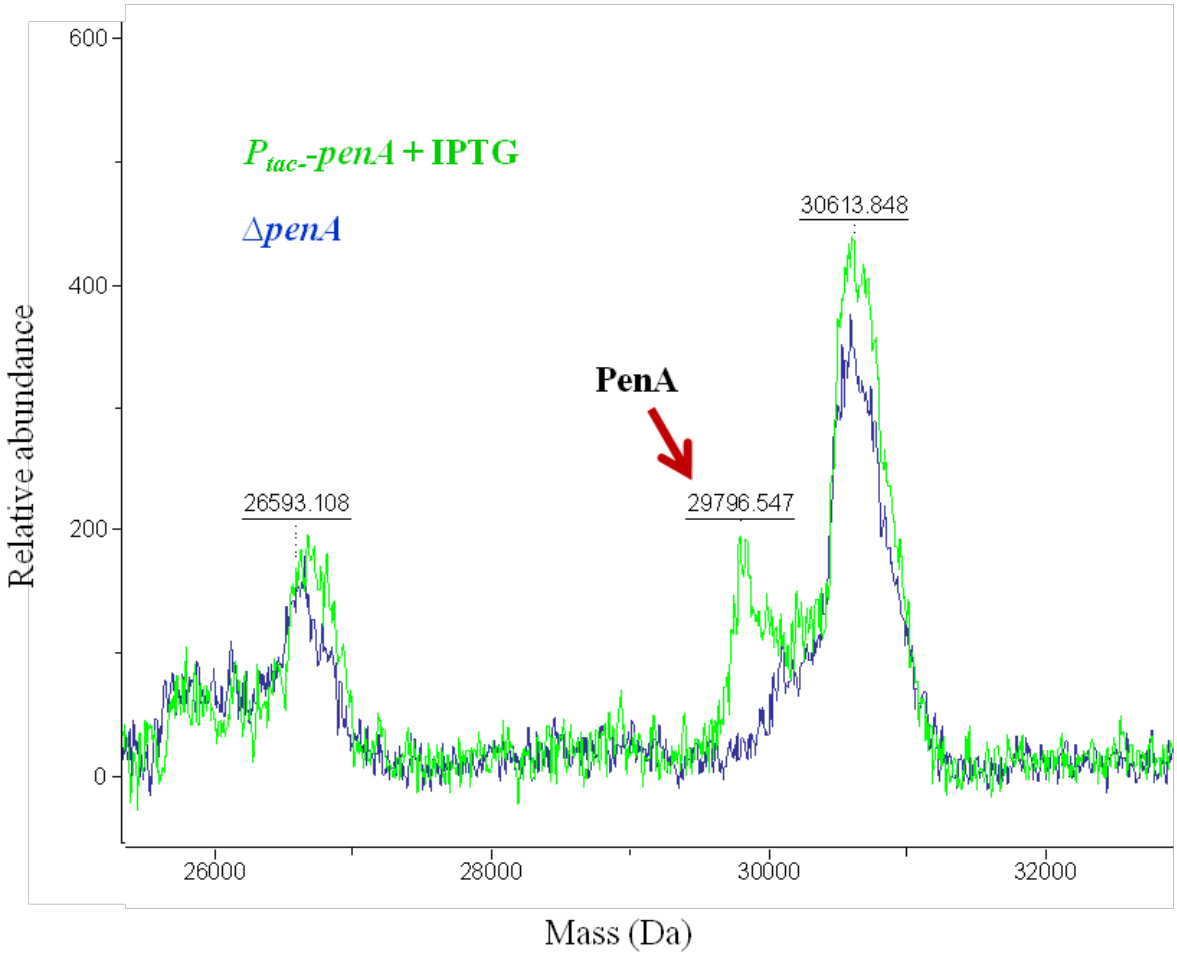

Figure 3.12 MALDI-TOF mass spectrometry identification of PenA from total membrane fractions. Total membrane samples from Bp82Δ*penA* (Bp82.11) (blue) and Bp82 expressing *penA* from the P_{tac} promoter induced with IPTG (green) were analyzed by MALDI-TOF mass spectrometry. The precise molecular weights of the three peaks are labeled, and the peak corresponding to the mature PenA protein is indicated by a red arrow.

CHAPTER 4

DISCUSSION

As the causative agent of melioidosis, *B. pseudomallei* is a public health concern in terms of natural infections in endemic regions of the world, as well as for its potential use as a biological weapon. In combination with its relatively high rates of morbidity and mortality and low infectious dose, the intrinsic antibiotic resistance of *B. pseudomallei* is particularly alarming. A variety of resistance mechanisms, including several chromosomal β-lactamases, an impermeable outer membrane, and a wide array of efflux pumps, make *B. pseudomallei* impervious to many of the drugs commonly used to treat bacterial infections. As a result, melioidosis treatment options are limited to a small group of antibiotics, which must be administered over an extended period of time. Given the lengthy treatment regimen and the resilient nature of *B. pseudomallei*, it is not altogether surprising that cases of treatment failure have been documented, where the organism has developed resistance to the drug used for treatment. Many of these instances of treatment failure have been attributed to mutations in the chromosomal β-lactamase PenA, conferring resistance to the front-line drug of choice, ceftazidime.

While the hydrolysis of ceftazidime by wild-type PenA is very limited and does not interfere with treatment, upregulation of the gene or certain amino acid changes that have been identified in clinical isolates can render the organism resistant. Similarly, wild-type PenA is susceptible to clavulanic acid inhibition, and therefore amoxicillin-clavulanate can be used as an alternative melioidosis treatment. However, an amino acid change in PenA, or overexpression of the β-lactamase confers resistance to amoxicillin-clavulanate.

In addition to definitively showing that these changes in PenA drastically alter the β-lactam susceptibility profile of *B. pseudomallei*, Rholl *et. al.* also demonstrated that PenA must be transported across the cytoplasmic membrane by the Tat secretion system in order to confer resistance (21). While not unprecedented, this example of a Tat-secreted β-lactamase was somewhat unusual. Furthermore, unlike classic examples of β-lactamase enzymes, the final sub-cellular destination of PenA was unclear, with varying predictions and reports suggesting it might be periplasmic, or could be membrane-associated. We hypothesized that PenA is indeed secreted by the Tat system to the periplasm, and that previous studies were unable to show periplasmic localization because the methods used were not suited to *B. pseudomallei*.

To test this hypothesis, strains were generated that expresses the known periplasmic protein, PhoA. Using these *B. pseudomallei* reporter strains, several methods for extracting periplasmic proteins were compared. While all of these methods have been used successfully with *E. coli* and other Gram-negative organisms, the PeriPreps and cold osmotic shock methods proved to be fairly ineffective at extracting periplasmic protein from *B. pseudomallei*. Fortunately, the chloroform treatment method yielded more efficient periplasmic extraction, and was chosen for future use. Periplasmic extraction by any of the methods tested was not enhanced by lack of capsular polysaccharide, indicating that the *B. pseudomallei* capsule is not responsible for the differences observed in extraction efficiency compared with other Gram-negative organisms.

However, these defined periplasmic protein samples did not contain the high levels of PenA that were expected. Instead, the vast majority of PenA was detected in total membrane fractions. Further experiments have shown that this membrane association is likely due to the post-translational addition of lipid molecules to the cysteine residue at position 23 (Cys_{23}) of

PenA. The degradation of a C23S mutant version of PenA suggests that Cys_{23} is required for proper processing of the protein, and TX-114 phase partitioning indicates that the mature PenA is lipophilic. Metabolic labeling of *B. pseudomallei* with ^{14}C-palmitic acid suggests that palmitic acid could be incorporated into PenA. More definitively, globomycin treatment, which interferes with SPase II cleavage of signal peptides from bacterial lipoproteins, resulted in the appearance of a larger form of PenA, presumed to be the uncleaved pro-lipoprotein, and the molecular weight of mature PenA determined by MALDI-TOF was consistent with a triacylated lipoprotein. This evidence suggests that PenA is a Tat-secreted lipoprotein.

Future work will include purifying PenA by immunoprecipitation or epitope tag purification in order to more definitively show specific ^{14}C-palmitic acid labeling. Alternative methods for confirming post-translational lipid modification of PenA include mass spectrometry and acylbiotin-exchange chemistry.

It is intriguing that replacement of Cys_{23} of PenA with a serine residue results in degradation of the protein, presumably because it is no longer recognized by the lipoprotein synthesis proteins. Replacement of Ile_{20} with an alanine would convert the signal sequence to one that should be recognized by SPaseI, potentially allowing this doubly mutated version of PenA to be processed by the cellular machinery involved in maturation of non-lipidated Tat-secreted proteins. Consequently the $I_{20}A$ amino acid change might rescue the $PenA_{C23S}$ protein. If so, the resulting protein would be expected to localize to the periplasm like a more typical β-lactamase, and it would be interesting to compare the β-lactam susceptibility profile of such a mutant with the wild-type strain.

Additionally, further research could be done to show specific inner or outer membrane localization of PenA, though a means of detecting a defined inner membrane marker protein in

B. pseudomallei must be developed first. Lipoproteins in Gram-negative bacteria are transported to the outer membrane by the lipoprotein localization (Lol) system or retained in inner membrane by so called "Lol-avoidance" signals. In *E. coli* the inner membrane retention signal involves an aspartate residue at position +2 of the mature protein, while in *P. aeruginosa* lipoproteins are retained in the inner membrane by lysine and serine at positions +3 and +4 (50). As PenA does not have either of these canonical signals following Cys_{23} (see Figure 3.6), it would be predicted to localize to the outer membrane. However, the variability of Lol-avoidance signals between species dictates experimental confirmation.

There are well-documented examples of both Tat-secreted β-lactamases (41-44) and lipoprotein β-lactamases (51, 52). Evidence of Tat-secreted lipoproteins is emerging as well (86-91), though many of these reports rely heavily on bioinformatic predictions and only a few examples have been confirmed experimentally. Therefore, as a Gram-negative Tat-secreted lipoprotein β-lactamase, PenA is highly unusual.

This new understanding of PenA processing and maturation could potentially open the door to new treatment options. The development of PenA-mediated ceftazidime or amoxicillin-clavulanate resistance during melioidosis treatment might be avoided by treating with an inhibitor of Tat secretion or lipoprotein biosynthesis in combination with the antibiotic. In addition, the efficacy of older β-lactams such as penicillin and amoxicillin might be restored against *B. pseudomallei* if used in combination with lipoprotein or Tat inhibitors. Considering that the limited number of antibiotics currently used during the acute phase of melioidosis therapy all require intravenous administration, new treatment options, especially ones that could potentially be delivered in a less invasive manner, for a shorter duration, or at a lower cost, would be extremely helpful.

Tat secretion systems have been identified in plants, bacteria, and archaea, but not in animal cells. Similarly, the bacterial lipoprotein biosynthesis pathway is unique to prokaryotic organisms. The lack of either of these systems in animals makes them potential targets for new antibiotics. Two natural products have been identified that inhibit the lipoprotein signal peptidase. Globomycin, which was used in this study, has been around for several decades (84), while the so called "TA" antibiotic produced by *Myxococcus xanthus* has been a more recent topic of study (92). Vasil *et. al*. recently described an *in vitro* screen for Tat inhibitors that yielded two possible hits (93). Even if these compounds do not turn out to be ideal antibiotics on their own, they might make excellent PenA inhibitors in combination with traditional β-lactam antibiotics.

These findings may also have implications outside the realm *B. pseudomallei* treatment. PenA is the second documented example of a lipoprotein β-lactamase in a Gram-negative organism, following the characterization of the BRO enzyme from *Moraxella catarrhalis* (50). As antibiotic resistance continues to emerge as a public health concern, research is likely to uncover more diversity among β-lactamase enzymes. PenA will serve as an example of one such β-lactamase whose secretion and processing differ from the classic TEM-1 model enzyme. In terms of more general bacterial physiology, this work demonstrates experimental characterization of a Tat-secreted lipoprotein, most descriptions of which thus far have been based primarily on bioinformatic predictions.

While these results effectively disprove the original hypothesis of PenA being a periplasmic enzyme, its classification as a Tat-secreted lipoprotein is novel among characterized β-lactamase enzymes. This provides insight into β-lactamase enzymes in general, as well as

giving us a better understanding of an important antibiotic resistance determinant in *B. pseudomallei*, specifically.

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LIST OF ABBREVIATIONS

°C	degrees Celcius
A_{410nm}	absorbance at 410 nm
A_{486nm}	absorbance at 486 nm
A_{570nm}	absorbance at 570 nm
A_{600nm}	absorbance at 600 nm
A	alanine
Ap	ampicillin
B.	*Burkholderia*
BCA	bicinchoninic acid
bp	base pair(s)
BSA	bovine serum albumin
C	cysteine
cDNA	complementary DNA
$CHCl_3$	chloroform
Cys	cysteine
diH_2O	deionized water
Da	daltons
dpm	disintegrations per minute
E.	*Escherichia*
EDTA	Ethylenediaminetetraacetic acid
FKM	*FRT*-flanked Km^r marker
FRT	Flp-recombinase target

g	Earth’s gravitational acceleration
G	glycine
hr	hour(s)
HRP	horseradish peroxidase
I	isoleucine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kD	kilodalton
Km	kanamycin
Km^r	kanamycin resistance marker
L	leucine
M	molar
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
mM	millimolar
N-	amino-
nm	nanometer(s)
P.	*Pseudomonas*
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
*p*NPP	*p*-nitrophenylphosphate

PVDF	polyvinylidene fluroide
R	arginine
RND	resistance nodulation and cell division
rpm	rotations per minute
RT-qPCR	reverse transcriptase real time PCR
S	serine
SDS	sodium dodecyl sulfate
sec	second(s)
SNP	single nucleotide polymorphism
SOE	splicing overlap extension
SPase	signal peptidase
spp.	species
SRP	signal recognition peptide
SS	signal sequence
T	threonine
Tat	twin arginine translocase
TBS	Tris-buffered saline
TBS-T	0.05% Tween 20 in TBS
TX-114	Triton X-114
µg	microgram(s)
µl	microliter(s)
V	valine
WT	wild-type

X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid
Zeo	zeocin