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Characterization of a genetic locus in *Burkholderia pseudomallei* encoding a putative biofilm-associated protein

by

William E. Grose

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biomedical Sciences

Dr. Eric R. Lafontaine, Committee Chair

Dr. R. Mark Wooten, Committee Member

Dr. Robert M. Blumenthal, Committee Member

Dr. Randall G. Worth, Committee Member

Dr. Z. Kevin Pan, Committee Member

Dr. Patricia R. Komuniecki, Dean

College of Graduate Studies

The University of Toledo

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An Abstract of

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We identified an open reading frame (ORF) in *Burkholderia pseudomallei* that resembles *Staphylococcus aureus* Biofilm-associated protein (Bap). We designated this *B. pseudomallei* ORF Bap-like protein (Blp). Biofilm-associated proteins are surface exposed molecules that are involved in adherence and biofilm formation. In order to study the function of *B. pseudomallei* Blp, we wanted to express the protein in a heterologous expression system. *B. thailandensis* is genetically similar to *B. pseudomallei*, but is relatively avirulent and does not possess the locus encoding Blp. A plasmid was created, designated pWGBtBlp, harboring blp and regions flanking the gene that may encode factors needed for secretion of Blp. *B. thailandensis* pWGBtBlp produces more biofilm and adheres to epithelial cells at higher levels than the vector control; however, a Tn insertion in the blp gene on the plasmid did not result in wild-type levels of biofilm production and adherence in *B. thailandensis*, suggesting Blp is not responsible for these processes. In order to determine the factor mediating these phenotypes, we generated plasmids containing portions of the blp locus and identified a small ORF with no known homologues that is able mediate biofilm production and adherence independently of any additional factors.
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Chapter 1

Introduction

1.1 Protein Secretion in Gram-Negative Bacteria

Eukaryote and prokaryote organisms have evolved mechanisms that allow them to best survive in their preferred environments. As interactions with other cells or environmental factors generally occur first through surface receptors, it is essential to have systems in place that allow the translocation of necessary proteins to the surface of these cells. In Gram-negative bacteria, this process is made more complex by the double membrane structure of the organisms. Proteins bound for the surface or which are secreted must cross the inner membrane, pass through the periplasm and peptidoglycan layer, and finally cross the outer membrane before reaching their final destination. Translocation of these molecules to the surface is performed by several export systems and these are diverse in their composition and function: secretion can be one step or two steps, specific recognition of the secretion substrates can occur through encoded ‘signal sequences’ or by specific protein-protein interactions, proteins can be secreted in their
folded or unfolded state, and the number of different proteins involved in the secretion machinery can range from a few (Type I secretion) to more than 15 which form large macromolecular complexes (Type VI secretion). Currently, there are six secretion systems that have been described in Gram-negative bacteria.

1.1.1 Type I Secretion

Type I secretion systems (T1SS) secrete toxins, proteases, and adhesion factors. The first type I secreted protein described was the hemolysin HlyA from uropathogenic Escherichia coli (105). HlyA is a 107 kDa protein containing 2 prominent domains, an N-terminal amphipathic domain and a C-terminal domain that binds calcium through its glycine-rich repeats (236). These repeated domains gave rise to the family of repeat toxins (RTX). The secreted HlyA integrates into the plasma membrane of host cells and causes leakage of the cytoplasmic contents via the formation of membrane pores (28). While it was initially believed that the N-terminal amphipathic region was solely responsible for the membrane interactions that led to porin formation, it has recently been shown that the C-terminal calcium-binding repeats are also important for this function (236).

HlyA is secreted from E. coli via the formation of a type I secretion system composed of HlyB, HlyD, and TolC. HlyB resides in the inner membrane and possesses an ATP-binding cassette (ABC transporter) which provides the energy required for translocation (302). ABC transporters are involved in a variety of processes including iron uptake (117), solute uptake (to counteract osmotic stress) (207), antibiotic efflux
Hydrolysis of ATP is required for the transport of the substrate. These systems are composed of a transmembrane domain (TMD) and a nucleotide binding domain (NBD) and are believed to function as homodimers. The TMD is embedded in the membrane and undergoes conformational changes to transport a substrate across the membrane. Overall, the sequence and structure of the TMD are more variable than the NBD. The nucleotide binding domain of the ABC transporter consists of 5 characterized motifs that collectively allow the machinery to bind ATP, hydrolyze the ATP, and alter the conformation of the TMD to allow substrate secretion. The Walker A motif is a phosphate binding loop while the Walker B motif appears to act as a magnesium-binding site. The H switch region contains a highly conserved histidine residue that is also important for the interaction of the NBD with ATP. Collectively, these three motifs are very important for ATP binding. One characteristic sequence found in all ABC subunits is LSGGQ, and is therefore described as the ‘signature motif’ and is responsible for maintaining extensive tertiary contacts throughout hydrolysis. Finally, the Q-loop motif is a flexible loop containing a glutamine residue that functions in bridging the NBD with the TMD. The Q-loop is believed to be specifically involved in the translation of ATP binding with the conformational changes in the TMD. While there are ABC transporters in prokaryotes to humans which can be involved in either import or export of substrates, ABC transporters involved in type I secretion are only 1 component of a system that actively exports a substrate across both membranes of a Gram-negative bacterium. While the exact domain for substrate recognition has not yet been clearly elucidated, it has been clearly shown that the ABC transporter is the molecule responsible
Therefore, HlyB is embedded in the inner membrane of *E. coli* where it binds and hydrolyzes ATP, alters conformation, and allows for the export of HlyA.

HlyD, the so-called membrane fusion protein (MFP), forms a homotrimeric structure and binds directly to both the inner membrane ABC transporter, HlyB, and the outer membrane transporter, TolC (244, 275). HlyD resides primarily in the periplasm but is associated with the inner membrane. The exact function of HlyD appears to be to exert a mechanical force on the outer membrane protein, TolC, to ‘open’ the substrate channel (325). HlyD contains a cytoplasmic N-terminal domain that has been shown to interact with the substrate, HlyA (275); however, this cytoplasmic domain is not found in most HlyD-like proteins (167). The process by which substrates are recognized by a T1SS and the specific component responsible for this recognition are not well understood.

TolC is responsible for exporting the substrate across the outer membrane of *E. coli* (153). The crystal structure of TolC reveals a membrane domain composed of four β–sheets and a periplasmic domain possessing four 10-nm α–helices (153). In the outer membrane, TolC assembles into a trimeric structure forming a twelve stranded β–barrel with an inner diameter of 3.5 nm (153). Interestingly, the periplasmic domains of the trimeric polyprotein taper and almost close (3.5 Å) at the distal end of the α–helices. It has been proposed that this constriction could be overcome by unwinding the helices, which would make the opening on the periplasmic side approximately 5 times larger (16 – 20 Å) than its ‘closed’ state (153). It appears as though HlyD is responsible for exerting a mechanical force on TolC that results in this unwinding (325). Therefore, TolC is an outer membrane structure with a large periplasmic domain that can be
structurally modified to allow for passage of specific substrates across the outer membrane.

A wide variety of type I secretion substrates have been studied. Overall, type I secretion substrates range in size from 78 to 8682 amino acids, have relatively low pH (~4), and contain few, if any, cysteine residues (79). Many of these proteins are directly involved in virulence such as the secreted toxin HlyA in *E. coli* (301), and the iron-binding Hemophore in *Serratia marcescens* (168), to the surface associated adhesin/biofilm-associated protein LapA of *Pseudomonas fluorescens* (121). It appears as though the C-terminal glycine-rich repeats in these proteins, like those found in HlyA, are characteristic of type I secretion substrates (17). This region is typically located in the C-terminus and is not cleaved during transport across the outer membrane. The consensus sequence of these regions has been determined (GGXGXDXXX) and has led to the identification of numerous putative substrates (79), many of which have unknown functions.

### 1.1.2 The Sec Pathway

Unlike translocation via the type I secretion system, which transports proteins directly across both membranes in a one-step process, other Gram-negative secretion systems transport substrates in a two-step process. In these instances, proteins are first exported across the inner membrane into the periplasm where they are then recognized by the secretion machinery and transported to the surface. The first step in this two-step process is performed by one of two mechanisms, the Sec pathway or the Tat pathway.
Proteins exported to the periplasm by the secretory (Sec) pathway contain a signal sequence in the N-terminal portion of the protein (213). Signal sequences have been extensively studied and contain a positively charged N terminus (n-region), followed by a core of hydrophobic amino acids (h-region) and a more polar C terminus (c-region) preceding the signal peptidase cleavage site (91). In *E. coli*, signal peptide bearing precursor proteins are initially bound in the cytoplasm by the chaperone, SecB, which appears to bind the preproteins without specificity for sequence or structure (220). Proteins exported by the Sec pathway are secreted as unfolded peptides as SecB binding restricts folding (324). SecB then binds to the inner membrane ATPase, SecA, and transfers the unfolded substrate to SecA (92). Upon interaction with the translocation machinery, SecA undergoes a conformational change which is thought to be responsible for providing the energy to push the preprotein through the translocation apparatus (83). The inner membrane translocation machinery is composed of the essential proteins SecY and SecE and the nonessential proteins SecD, SecF, YajC and SecG (195, 205). SecY and SecG appear to form part of the channel involved in secretion of substrates as these two proteins form ring structures in detergents and lipid bilayers and interact with SecA (181). SecD associates with SecF and YajC in a trimeric complex that stabilizes SecA in the inner membrane and may be involved in closing the translocation channel following substrate export (176). SecG appears to assist in SecA associations with the SecY/E structure and may even contribute to signal sequence recognition (32). Once the substrate has been exported by the Sec translocation channel, a signal peptidase often cleaves the signal sequence leaving the mature protein free in the periplasm (71).
Mechanisms for secretion of the mature protein across the outer membrane will be discussed in a later section.

An alternative mode of secretion for the signal bearing substrate involves the collective action of the proteins Ffh and 4.5S RNA (Ffs), which form the signal recognition particle (SRP). This alternate system is believed to be important primarily for targeting and assembly of inner membrane proteins (288). The SRP complex is believed to bind the signal-bearing peptide and stall ribosomal translation (209). Cotranslational translocation of the substrate initiates as the ribosome docks with the Sec translocation machinery at the inner membrane and the SRP binds its receptor FtsY (183). Therefore, the destination for both the general Sec pathway and the SRP pathway are the same.

1.1.3 The Tat Pathway

Although the general Sec system is the major pathway for transporting proteins into the periplasm (213), a Sec-independent mechanism has been increasingly studied since its recognition in the 1990s and possesses several characteristic features. One similarity of the twin-arginine translocation (Tat) and Sec pathways is that both transport proteins with very similar signal sequences; however the Tat signal sequence generally possesses two highly conserved arginine residues (238). These residues are a part of a larger signal sequence with the consensus (S/T)-R-R-x-F-L-K that occurs at the junction of the n-region/h-region (21). Although the twin arginine (RR) is the signature motif for the Tat pathway, it is important that at least one protein with a ‘RR’ containing signal
sequence, polyphenol oxidase, can be secreted by either the Sec or the Tat pathway (155). In *E. coli*, the periplasmic protein, leader peptidase, is normally secreted by the Sec pathway. If dual arginine residues are substituted into the signal sequence the protein is then transported by the Tat pathway. However, if this Tat pathway substrate was then altered such that the hydrophobicity of the h-region was increased, the protein is then secreted by the Sec pathway, even with the presence of the ‘RR’ (64). In addition, as most h-regions of potential Tat substrates are less hydrophobic than those of Sec substrates (227), it is likely that there is a significant role for the hydrophobicity of the h-region in determining Sec/Tat pathway specificity (22).

Perhaps the most definitive aspect of the Tat system that sets it apart from the Sec system is that it is able to transport completely folded proteins into the periplasm. Using Green Fluorescent Protein (GFP), it was found that transport via the Sec pathway resulted in an inactive GFP in the periplasm, due to improper folding (90). However, if the Tat-specific signal sequence was added to the protein, fully functional GFP was delivered into the periplasm (276). In fact, many of the putative Tat substrates appear to bind redox factors in the cytoplasm before translocation to the periplasm (307), indicating a folded state prior to transport. Other studies have even shown that the Tat pathway transports oligomeric structures (60, 229). If the Tat machinery transports folded proteins, and it appears this is the case, then the system must be extremely flexible with potential substrates ranging from the 9 kDa high-potential iron-sulfur protein (HiPIP) of *Chromatium vinosum* (with a predicted maximum diameter of 30 Å (49)), to the 142 kDa FdnGH subcomplex of *E. coli* formate dehydrogenase-N (with a predicted maximum diameter of 70 Å (20)).
The proteins that contribute to secretion by the Tat pathway are few in number and their functions are currently poorly defined. In *E. coli*, four proteins have been studied and found to be involved in secretion by the Tat pathway. Of these proteins, TatB and TatC appear to be essential (29, 307), while TatA and TatE may have similar roles as only the loss of both abrogates export via the Tat pathway (238). Interestingly, *tatA, tatB, and tatC* are the first 3 cistrons of the *tatABCD* operon (307), but it appears as though TatD is dispensable as mutations in this gene have no observable effects on secretion (22). TatA, TatB and TatE each appear to contain one transmembrane domain followed by a short cytoplasmic amphipathic helix (227). TatC appears to have six transmembrane domains with N- and C- terminal ends of the protein in the cytoplasm (238). Recent studies have shown that the purified Tat complex from *E. coli* contains only TatA, TatB, and TatC (30). This would suggest a very simple translocation system under some circumstances. However considering the size of some of the predicted folded proteins the Tat system must export, it has been speculated that this system must be able to form complexes containing 21-23 transmembrane helices (22). The energy requirements for substrate translocation are also unclear. Studies on the Tat system in plants, where it was initially characterized, reveal that translocation requires the proton motive force but not ATP hydrolysis (274). Although the mechanism is not yet clear, this suggests a novel mechanism for protein transport.

### 1.1.4 Type II Secretion
Type II secretion systems (T2SS) have been referred to as secretons, the secreton-dependent pathway, or the main terminal branch of the general Sec pathway and their components have been given a standard nomenclature (213). Proteins secreted by this pathway are first translocated across the inner membrane by the Sec or Tat pathway, as described previously, and then exported to the exterior of the bacteria by the outer membrane secretin (162, 299). Importantly, translocation across the outer membrane by the T2SS requires proper substrate folding, as secretion is blocked when molecules are not folded properly or are destabilized (122, 151). Studies of these systems in various species have established a solid understanding of the components required for this pathway. In the well-characterized *Klebsiella pneumoniae* T2SS, twelve different proteins make up the core of this translocon. Perhaps the most central component of the type II secretion system is the outer membrane secretin, SecD or T2S D. This protein oligomerizes into a dodecameric ring structure that is believed to act as the pore in the outer membrane for transport of polypeptides (196). Interestingly, homologs of T2S D play a critical role in many diverse secretion processes including type III secretion systems, the assembly pathway of filamentous phage, and the polymerization of type IV pili (233). In each of these systems, T2S D homologs form the outer membrane pore forming component of the secretion machinery (104). One protein of the type II secretion system (T2S E) is located in the cytoplasm and is believed to provide the energy required for this pathway by the hydrolysis of ATP (237). Ten other proteins are positioned in the inner-membrane. Three of these proteins, namely T2S F, T2S L, and T2S M appear to form a platform in the inner membrane along with the cytoplasm ATPase T2S E (217). T2S C possesses an inner membrane domain but largely resides in
the periplasm where it appears to be responsible for substrate recognition and/or secretin interactions and is essential for secretion of polypeptides (59). Another of the inner membrane proteins is T2S O which appears to be a specialized peptidase that when mutated, abolishes not only secretion by this system but also twitching motility of the bacteria that express a type IV pilus (95). The remaining five inner membrane proteins involved in type II secretion have sequence similarities to type IV pilins and have therefore been termed pseudopilins (95). Proper processing of the major (T2S G) and minor (T2S H, T2S I, T2S J, T2S K) pseudopilins by T2S O is necessary for secretion of substrates (16, 198). It has been suggested that the pseudopilins play a structural role in the formation of the T2SS (217), but this has not been proven. In addition to the 12 core components, there are some proteins that appear in some type II secretion systems, but not others. These include T2S N, which may be similar to T2S C, and three proteins, namely T2S A, T2S B, and T2S S, that may be involved in placement of the secretin (T2S D) in the outer membrane (59, 95). Therefore, the T2SS requires many components that secrete folded proteins across the outer-membrane.

Many proteins have been identified that are secreted by type II secretion systems. A lipoprotein, pullulanase (PulA) of *Klebsiella oxytoca*, is perhaps the most well-known substrate for this pathway (182). Expressing pullulanase without a functional type II secretion system results in an accumulation of the protein in the periplasm, while subsequent expression of the T2SS allows for PulA to be released into the extracellular milieu (208, 212). Recent work has highlighted the role of this system in the virulence of many Gram-negative bacteria in both plants and animals. The type II secreted protein *E. coli* K-12 OmpA is important for maintaining membrane stability and is involved in
serum resistance (308). *Vibrio cholerae* expresses cholera toxin subunits CtxA and CtxB which assemble into a pentameric ring structure in the periplasm before being secreted by a T2SS (122). Toxins are released in a similar manner by other organisms like *E. coli*, and *Shigella dysenteriae* (243, 272). Some organisms, like *L. pneumophila*, secrete as many as 11 virulence-related factors via type II secretion systems (*e.g.* phospholipase C, ProA/Msp metalloprotease) (231, 254), while others such as *Klebsiella oxytoca* only secrete one (*i.e.* Pullanase) (59). Therefore, the relative importance of this secretion system to the virulence of the organism appears to be species dependent. It is also interesting to note that some organisms (*e.g.* *Pseudomonas aeruginosa, Yersinia enterocolitica*) possess multiple T2SS each dedicated to its own set of substrates (15, 137).

1.1.5 Type III Secretion

One secretion system has received attention for its apparent ability to ‘inject’ effector proteins into host cells, thereby enabling the bacterium to manipulate certain cellular processes. *Yersinia* and *Salmonella* species are able to sense the ion concentration of target cells, which trigger expression of these complexes, called type III secretion systems (T3SS) (318). Both plant and animal pathogens possess type III secretion systems. It has been shown that animal pathogens use these systems to secrete effectors which promote entry into epithelial cells (*Salmonella typhimurium*) (102), survival within macrophages (*Salmonella typhimurium*) (282), and induction of apoptosis (*Shigella flexneri*) (294). Type III secretion systems in plant pathogens are necessary for
disease progression and for the elicitation of the hypersensitivity response (8).

Structurally, the T3SS resembles a hypodermic needle and the formation of proteins constituting this system is collectively referred to as the needle complex, which was originally identified in *Salmonella typhimurium* (156). Contrary to the idea of a needle, the T3SS does not actually puncture target cell membranes itself, but instead secretes pore-forming proteins that are believed to insert into the target cell membrane through which secreted effector proteins can transit (62, 110).

The construction of the type III secretion complex is sequential and begins with the formation of the base substructure (157). In *Salmonella typhimurium*, this structure is composed of the inner membrane proteins PrgH and PrgK, as well as the outer membrane secretin, InvG (154, 177), that is homologous to the secretin protein that forms the outer membrane pore in type II secretion systems (T2S D, see above). Assembly of PrgH, PrgK, and InvG into their base structure allows for the secretion of more components required for type III secretion (101). These molecules include a putative inner rod protein, PrgJ, the needle protein subunit, PrgI, and a protein required for proper assembly of the needle complex, InvJ (157, 177). Bacteria lacking InvJ assemble needle complexes that have abnormally long needles (61). Additional components of the needle complex include a putative ATPase, InvC, which would provide the energy required for translocation (4). Overall, the total number of proteins involved in T3SS numbers from around twenty to thirty (131). Once the T3SS is properly formed, substrate specificity changes from constituents of the secretion apparatus to effector substrates (61). This completed secretion system then has the ability to secrete a variety of substrates. In
Salmonella typhimurium, at least nine substrates have been identified and these effector proteins include both toxins and chaperones (120).

Effector proteins must possess a specific signal sequence as many bacteria contain more than one type of T3SS, which are often expressed at the same time (101). Additionally, evidence suggests that there is predetermined order in which different secreted proteins are engaged and then secreted (204). Analysis of many secreted proteins has identified a secretion signal within the first 20-30 amino acids (241, 256) that remains uncleaved after secretion. Interestingly, the secretion signal composition varies greatly between proteins and it is still unclear whether the actual signal is found in the RNA (5) or the peptide (171, 234). Complicating this issue is the fact that at least some type III secreted effectors are specifically targeted by cytosolic chaperones (306), thus adding an additional layer of complexity. Therefore, T3SS possess some mechanism of recognizing effector molecules, but whether there is a conserved signal remains unclear. Several well characterized effector proteins are important for virulence. In Yersinia pestis, the entire plasmid pYV specifies the proteins of the T3SS as well as the effectors. YopE has been characterized as a GTPase-activating protein that results in the loss of motility and a reduction in phagocytosis by host cells, thus allowing Y. pestis to survive outside of macrophages (23). Y. pseudotuberculosis YopH is also important for inhibiting phagocytosis and is indispensable for virulence due to its function as a tyrosine phosphatase. YopH very effectively dephosphorylates all phosphotyrosine proteins, causing severe perturbations in host cellular processes (26). Y. pseudotuberculosis YopB functions in a different manner by forming pores in the membranes of host cells and allowing an influx of other effector proteins (110). Therefore, effector proteins are
important for virulence and function in a variety of manners following secretion by the type III secretion systems.

1.1.6 Type IV Secretion

The type IV secretion system (T4SS) is perhaps the most functionally flexible of the secretion systems as it is able to transport both proteins and DNA. Components of this system were initially discovered as being necessary for tumor production in plants after infection with Agrobacterium tumefaciens (259). It was known that these tumors contained plant cells that had been transformed with bacterial DNA. Expression of the gene products encoded by the agrobacterial DNA results in the production of nutrients from the plant that are then metabolized by A. tumefaciens (180). Interestingly, analysis of the bacterial DNA responsible revealed homology to genes required for conjugation. Conjugation, or bacterial mating, requires an extracellular macromolecular structure that is necessary for connecting two bacterial cells together and for the subsequent transfer of DNA. This structure is called a pilus. Investigation into the genes responsible for the transfer of the A. tumefaciens tumor DNA (T-DNA) identified VirB2 as the major subunit of the T-pilus which polymerizes into a long filament of approximately 10 nm (98). Associated with this structure is the minor subunit VirB5, which appears to be required to assemble the structure (242). Several components form the T4SS channel through which substrates can pass. VirB6 – VirB10 are the main structural elements to this channel (56). Specifically, VirB7 and VirB9 form a heterodimer in the outer membrane and stabilize other VirB proteins during assembly of the transfer machine (94). In the inner
membrane, VirB6 is believed to span the membrane several times and is likely the channel-forming protein (56). Also in the inner membrane are a presumed muramidase, VirB8, and VirB10, which possesses a large periplasmic domain and is believed to link the inner and outer membrane VirB subunits (18). These subunits form a channel similar to the type III secretion system so that the DNA substrate can transverse both membranes in one step. One or more ATPases are also thought to be associated with the T4SS. Two of these proteins, VirB4 and VirB11 appear to reside, at least partially, in the bacterial inner membrane (74, 97), while a third, namely VirD4, appears to locate in the cytoplasm and there is some evidence that it may provide some plasmid substrate discrimination (185). Therefore, ATP hydrolysis provides the energy requirements for protein transport across the bacterial membranes.

T4SS are currently known to secrete three types of molecules: DNA with associated proteins as described above, the multishubunit pertussis toxin from *Bordetella pertussis*, and monomeric proteins such as CagA from *Helicobacter pylori* (57). Interestingly, these substrates get secreted in slightly different manners with each substrate requiring only some of the components of the T4SS. Although DNA gets secreted in one step, *B. pertussis* secretes pertussis toxin (PT) in a two step reaction. Initially PT subunits are secreted to the periplasm by the Sec pathway where the subunits assemble into the PT holotoxin. The Ptl type IV secretion system then secretes the active toxin across the outer membrane (309). In another example, type I strains of *H. pylori* carrying the *cag* pathogenicity island are known to secrete the kinase CagA into gastric epithelial cells by type IV secretion. CagA then tyrosine phosphorylates specific proteins in these cells near the site of bacterial attachment (12). One final example of the
virulence properties of T4SS is with *Brucella suis*. While the effector protein has yet to be identified, expression of the proteins that form the T4SS is required for survival in epithelial cells and macrophages (199). Therefore, T4SS secrete a wide variety of substrates that are involved in many aspects of pathogenesis.

### 1.1.7 Type V Secretion

While many of the secretion systems discussed involve multiple proteins that associate into macromolecular structures, the type V secretion system (T5SS) is, by comparison, relatively simple. All of the proteins involved in type V secretion are first transported across the inner membrane by the Sec pathway (119). The ability of the protein to be secreted across the outer membrane without any apparent energy requirements led to the description of these proteins as autotransporters. However, secretion across the outer membrane varies somewhat and has therefore been broken down into the three subtypes: Va, Vb, and Vc. All of these subtypes appear to be dedicated to the translocation of very large proteins in excess of 100 kDa (138). The first protein described to be secreted by subtype Va secretion was the 169 kDa IgA1 protease from *Neisseria gonorrhoeae*. Analysis of the precursor IgA1 protease identified three functional domains: an N-terminal leader domain containing the Sec signal sequence, the functional passenger domain which becomes the mature extracellular IgA1 protease, and a C-terminal β-domain possessing β-strands that is required for secretion across the outer membrane (206). Importantly, all of the functionally characterized passenger domains of type Va secreted proteins have been implicated in virulence (119). For example,
Bordetella pertussis Pertactin promotes adherence to epithelial cells (53), Moraxella catarrhalis UspA2 promotes serum resistance (2), and Pseudomonas aeruginosa EstA is an esterase which is required for rhamnolipid production, cell motility, and biofilm formation (315). As secretion across the inner membrane via the Sec pathway has already been discussed (see The Sec pathway), this aspect of type V secretion will not be described here other than to say that the N-terminal signal sequence directs the molecule to the proteins of the Sec pathway which export the autotransporter into the periplasm.

Many models exist for how the periplasmic intermediate gets secreted across the outer membrane. All of these models agree on the formation of a β-barrel in the outer membrane by the C-terminal domain of the autotransporter, mediated by Omp85 (300). The models differ around how the β-barrel exports the passenger domain. The initial hypothesis suggested that the passenger domain was secreted through the hydrophilic channel formed by a single β-barrel (147). As N-terminal deletions of the passenger domain are still transported across the outer membrane, it is most widely believed that this process is initiated with the C-terminus (292). Another proposed model involves the secretion of multiple passenger domains through an oligomeric ring-shaped structure composed of at least six β-barrels that would form a central hydrophilic pore of approximately 2 nm (291). One additional model, which forms the basis of the type Vc secretion system, hypothesizes that three autotransporter β-barrels form a ring in the outer membrane and allow for the secretion of the passenger domains (123). YadA, of Yersinia enterocolitica, and the Hia adhesin of Haemophilus influenzae are two well-known examples of this type of secretion (197, 257). Once across the outer membrane, the autotransporters are either secreted into the extracellular milieu (86) or attached to the β-
barrel at the surface of the bacteria (165). The free energy of folding at the cell surface has been hypothesized to be the driving force for translocation across the outer-membrane (147).

In type Vb secretion, also called the two-partner secretion (TPS) pathway, the preprotein still possesses a signal sequence that allows for secretion across the inner membrane by the Sec pathway (118). Once in the periplasm, the passenger domain also associates with a β-barrel for export across the outer membrane. At the surface of the bacteria, proteins secreted via the TPS pathways are folded (providing the energy for translocation) and may be processed (138). Clearly, many similarities exist between the type Va and the type Vb secretion pathways. The difference between the pathways is simple; in type Va secretion, the passenger domain and the β-domain reside on a single polypeptide, whereas, in type Vb secretion, the two domains are translated as two separate proteins, referred to as TpsA (passenger domain) and TpsB (β-domain) family members (138). Filamentous haemagglutinin (Fha), an adhesin of Bordetella spp., is the prototypical example of a protein secreted via the TPS pathway. FhaC forms a β-barrel in the outer-membrane of the bacteria through which Fha can transit. In comparison to type Va secretion in which the β-barrel is predicted to have 14 amphipathic β-strands, TpsB proteins, such as FhaC, are predicted to have 19 (109). One additional distinction between the two type V subtypes is that unlike type Va autotransporters, proteins secreted by the TPS pathway must have specific recognition between the passenger domain and the β-domain. In fact, a conserved TPS domain exists at the N-terminus of TpsA which interacts specifically with repeated POTRA domains in TpsB to initiate translocation across the outer-membrane (107). Other examples of proteins secreted by the TPS
pathway are MhaB1 and MhaB2 of *Moraxella catarrhalis* (13), and HMW1 and HMW2 of *H. influenzae* (258).

### 1.1.8 Type VI Secretion

The first description of a type VI secretion system (T6SS) was in the symbiotic plant bacterium, *Rhizobium leguminosarum* (230). Studies were conducted to understand how the organism was able to form nodules on plant roots and this led to the identification of a 33 kb region containing a putative operon of 14 genes named *impA-* *impN* (*impaired in nodulation*) (24). Most of the encoded proteins had unknown function but ImpK and ImpL had homology to the *Legionella pneumophila* type IV secretion components DotU and IcmF (246)), respectively. DotU and IcmF are inner membrane proteins and are important for the intracellular growth of *L. pneumophila* in macrophages (6). Orthologs of DotU and IcmF have been identified in many Gram-negative bacteria but the genes are often found apart from other type IV secretion components. Curiously, a different set of conserved genes were consistently found, originally identified as IcmF-associated homologous proteins (IAHP) (75). The presence of the IAHP in *R. leguminosarum* led to the discovery that in some *imp* mutants, several proteins were absent in culture supernatants (24). This relatively recent discovery led to the first characterization of the T6SS in Gram-negative bacteria.

Recent studies have clarified the core components of some T6SS and have characterized several effector proteins, though the overall knowledge is limited in comparison to what is known about other secretion systems. Studies of *Vibrio cholerae*
identified Haemolysin A co-regulated protein (Hcp) as a protein that was absent in culture supernatants from IAHP mutants which led to the hypothesis that Hcp was a type VI effector protein (316). Interestingly, other proteins missing in these supernatants, namely VgrG, were later shown to be secreted in an Hcp-dependant manner (215). Obviously, this observation was not expected. As Hcp appears to be secreted by the T6SS, it is interesting that Hcp is directly involved in the secretion of other molecules from this same secretion system. Additionally, the VgrG proteins have been shown to form homotrimeric or heterotrimeric complexes, forming E. coli bacteriophage T4-like tail-spike structures (214) and are therefore likely to also serve as a structural component of the T6SS (96). It has been suggested that this tail-spike-like structure may act to puncture host cells in order to deliver type VI secretion effectors (214). Therefore, both Hcp and VgrG appear to be secreted by a T6SS but also appear to be necessary for the secretion of additional factors.

The current model for type VI secretion centers around an ATPase, ClpV, that forms hexameric rings in the inner membrane and may provide energy-dependent coupled transport of polypeptides (322). As mentioned initially, the inner membrane proteins, DotU and IcmF are important components of type VI secretion. Little is known about the exact functions of these proteins but it is known that IcmF possesses a nucleotide binding domain and has been shown to interact with ClpV (174). As mentioned previously, Hcp appears to be secreted by this complex before re-associating with the type VI secretion machinery in order to provide a conduit for VgrG extension of the tail-spike-like structure directly into the host cell (96). In support of this model, Hcp and VgrG lack N-terminal signal sequences and are therefore exported in a Sec-
independent manner (215). This structure would then be able to secrete effector proteins necessary for bacterial virulence. Proteins that have been shown to be secreted by T6SS include TssB and TssM from *Burkholderia mallei*, which were shown to be important for virulence in a hamster model (240), and PigG in *Francisella tularensis*, (190), and SciK and VgrS in *Salmonella typhimurium*, which were shown to increase bacterial survival in macrophages (202).

1.2 *Burkholderia pseudomallei*

1.2.1 General Characteristics

In 1912, Whitmore and Krishnaswami first reported the discovery of a febrile illness, with disease states ranging from acute pneumonia or septicemia to chronic abscesses, affecting the people of what is now Yangon, Myanmar (312). The causative agent of this disease was shown to be, what is now called, *Burkholderia pseudomallei*. Initial studies identified this organism as an encapsulated, motile, Gram-negative bacillus that could often be isolated from wet soils and rice fields. Today, this disease, called melioidosis, is endemic to many of the tropical areas of the world between 20°N and 20°S of the equator but is most often reported in Southeast Asia and Northern Australia (72). Melioidosis results from inoculation into breaks in the skin or by inhalation of aerosolized organisms. The bacteria are able to quickly disseminate from the site of infection and spread to the major organs, causing significant pathology. Pneumonia is associated with half of all case reports of the disease, leading to fevers of long duration (mean = 9 days) and general malaise (55). In some parts of Thailand, melioidosis causes
20% of community-acquired infections (52), and at the Royal Darwin Hospital in Australia it is the most common cause of fatal community-acquired bacteremic pneumonia (68). One problem associated with melioidosis is that the disease initially produces symptoms that are similar to other general infections, which can lead to misdiagnosis and improper treatment. As *B. pseudomallei* is highly resistant to a variety of antibiotics (310) due to the presence of numerous antibiotic efflux pumps (51, 187), this often results in death before a correct diagnosis has been determined. Currently, diagnosis is dependent on culture which takes days to develop. With the rapid progression of melioidosis, this amount of time can be too long. There is no vaccine to protect against infection by *B. pseudomallei*. Overall, mortality rates for primary disease have been reported to be 50% in northeast Thailand and 20% in northern Australia (314). Animal models of infection have shown that inhalation of aerosolized bacteria leads to death in as few as 2 days following inoculation and as few as 10 organisms represent the LD$_{50}$ (140). Because of the ease of acquisition, the low infectious dose of aerosolized organisms, and the mortality rates associated with disease, *B. pseudomallei* has been categorized as a category B select agent. The first *B. pseudomallei* strain to be sequenced, annotated and published was K96243 (The Sanger Institute), which revealed the presence of 2 chromosomes of 4.07 and 3.17 Mb. There are currently 23 sequenced *B. pseudomallei* strains which have yielded a wealth of information regarding the genetic composition of the bacterium. Genomic analysis suggests that the larger chromosome contains many of the genes involved in housekeeping and metabolism while the smaller chromosome harbors many genes that encode proteins required for adaptation and survival in different environments (124).
1.2.2. Pathogenesis

1.2.2.1 Host Infection from the Environment

*B. pseudomallei* is a terrestrial bacterium that has adapted to survive in the environment primarily in rice paddies, stagnant waters, and moist tropical soils (38). Its persistence is demonstrated in that it is able to survive for prolonged periods of time in low-nutrient conditions (*i.e.* distilled water >10 years after inoculation) (320), antiseptic and detergent solutions (100), acidic environments (78), wide temperature ranges and dehydration (54). Some studies have shown that this organism may persist in the environment as a viable but non-culturable state (39). However, the virulence of environmental isolates is not significantly different from clinical isolates (295). People with occupational or recreational exposure to stagnant water have higher incidence of disease and during the rainy season when bacteria are leached from the soil there are more cases of melioidosis (52, 68). Therefore, the bacterium is able to quickly adapt from a terrestrial lifestyle to the mammalian environment.

Melioidosis can present with a wide variety of symptoms and *B. pseudomallei* has been called ‘the great mimicker’ (314). The most common presentation is pneumonia which is involved in about half of all cases (55). It has been hypothesized that bacterial infection in the lungs often results following hematogenous dissemination from distal sites. Studies with Vietnam helicopter crews suggested that inhalation was a major route of infection (128) and infection following inhalation of aerosolized organisms is also observed during the monsoon seasons when the total number of cases of disease increase.
dramatically (70). Alternatively, inoculation of *B. pseudomallei* into skin lesions is also a significant route of infection for this disease. During periods of planting or harvesting, rice farmers spend long hours in the wet soils where the organism is found in significant quantities, and commonly contract minor wounds to the feet and legs (52). Inoculation in skin breaks can be problematic, as presentations may progress rapidly in a manner similar to necrotizing fasciitis (303). Certainly, both inoculation into open wounds in the skin and inhalation of aerosolized bacteria are major routes of infection. Other modes of inoculation include ingestion (128) and person-to-person transmission (125), but the significance of these routes of infection is believed to be very low. Predisposing conditions are commonly reported for melioidosis patients. The most common is type II diabetes mellitus which is reported in 37% - 60% of melioidosis patients (69). Other factors contributing to acquisition of melioidosis include occupational exposure as described before, renal disease and thalassemia (271). Long-term infection without clinical symptoms has been observed (296), as well as latency periods of up to 62 years (192).

### 1.2.2.2 Attachment to Epithelial Cells

The major routes of infection by *B. pseudomallei* appear to be from inoculation in the skin and inhalation. During infection, it is certain that *B. pseudomallei* encounters epithelial barriers in the skin and/or in the lungs. To date, three structures, namely capsule, flagellum, and pilus, and two individual proteins, namely BoaA and BoaB, have been shown to mediate attachment of the bacterium to epithelial cells. This highly
pathogenic bacterium binds these cells at relatively low levels (3). The bacterial capsule adheres to pharyngeal cells, though it is not known what component of the capsule is responsible for this attachment (3). Another macromolecular structure implicated in binding to epithelial cells is the type IV pilus (88), though this process also remains poorly defined. The role of the flagellum in adherence was shown in an amoeba model, which revealed that the wild-type \textit{B. pseudomallei} 1026b was tethered to the surface of the amoeba by the flagella while the flagella mutant strain lacked this adherence (134). Studies on the regulation of the structural pilus component, \textit{pilA}, show that adherence mechanisms appear to vary between strains (27). In addition to large surface structures displaying attachment to epithelial cells, several single molecule adherence factors have recently been characterized including the autotransporters BoaA and BoaB (14). As with all autotransporters, BoaA and BoaB possess C-terminal $\beta$-barrels that insert into the outer membrane of the bacterium and are necessary for secretion of the passenger domains. The BoaA and BoaB proteins in \textit{B. pseudomallei} were shown to be important for binding to the respiratory epithelial cells lines, A549 (type II pneumocyte) and HEp2 (laryngeal). The \textit{boaA} and \textit{boaB} mutant strains were also found to have lower levels of adherence to a primary culture of normal human bronchial epithelium (14).

\subsection*{1.2.2.3 Invasion of Cells}

Following adherence to epithelial cells, \textit{B. pseudomallei} is able to invade, survive, and replicate (211). One molecule shown to be involved in the invasion of cells by the organism is BopE. This protein was shown to be secreted by the Bsa type III secretion
system (T3SS3) in *B. pseudomallei* and expression of BopE in eukaryotic cells led to dramatic rearrangements in the actin cytoskeleton and membrane ruffling. In agreement with this observation, purified BopE displayed guanine nucleotide exchange factor activity. *B. pseudomallei* bopE mutants were unable to invade epithelial cells at wild-type levels (262). Additionally, a *B. pseudomallei* bipD mutant, which lacks expression of the functional T3SS3, has an even greater defect in invasion than the bopE mutant alone, suggesting that more type III secretion effectors may also be involved in this process (262).

Two-component response regulators are composed of a receptor that senses a specific signal and then promotes expression of the regulator which differentially affects expression in a set of target genes. In *B. pseudomallei* 1026b, a Tn mutant strain was found to be deficient in its ability to invade A549 cells. The Tn was found to be contained in one of the genes (irlR) of a two-component response regulator, irlRS. In the RAW 264.7 murine macrophage-like cell line, the irlRS mutant had levels of invasion that were similar to the wild-type (142) and the irlRS mutant was still as virulent as the parental strain in several animal models of infection (142, 313). It is not known whether the observed invasion defects in the epithelial cell line was due solely to a delay in the invasion process or some other compensatory mechanism.

Following invasion of cell lines, *B. pseudomallei* is able to escape from the endocytic vacuoles into the cytoplasm (115). Subsequent studies in cell lines and in animal models have shown that once in the cytoplasm, the organism is capable of manipulating cellular process to survive. Specifically, the organism is able to bind and polymerize actin to move intercellularly while protected from extracellular immune
recognition (103). *B. pseudomallei* expresses three functional type III secretion systems. The T3SS3 (important for invasion of epithelial cells) was also shown to be important for the intracellular lifestyle of *B. pseudomallei* as mutants that were unable to form a functional T3SS3 demonstrated defects in the ability to escape from the vacuole, survive, and replicate within these cells (265).

### 1.2.2.4 Survival Within Cells

*B. pseudomallei* survives and replicates within a variety of professional phagocytes including monocytes, macrophages, and neutrophils (211). Studies have shown that the majority of internalized bacteria are able to avoid normal phagosome/lysosome fusion in macrophages by escaping into the cytoplasm, where the organisms multiply to high numbers and eventually rupture the cell (191). This damage can be limited, and increased numbers of *B. pseudomallei* are killed, if the macrophages are first activated by IFN-γ (184). Reactive oxygen intermediates (ROIs) appear to play a minor role in this killing while Reactive nitrogen intermediates (RNIs) seem to be primarily responsible. The induction of inducible nitric oxide synthase (iNOS) by IFN-γ appears to be essential for the clearance of the organism (184); however, *B. pseudomallei* is able to suppress iNOS production (84). *B. pseudomallei* appears to repress expression of iNOS by activating two negative regulators, suppressor of cytokine signaling 3, SOCS3, and cytokine-inducible src homology 2-containing protein, CIS (84). These two factors are activated in the presence of intracellular bacteria (85), but the exact mechanism has yet to be identified.
In Raw 264.7 murine macrophages, one of the six type VI secretion systems, namely T6SS1, was shown to be important for intracellular growth. A K96243 *hcp1* mutant, which lacks expression of the Hcp protein for the T6SS1, was shown to have significantly less numbers of intracellular bacteria compared to the wild-type *B. pseudomallei* strain K96243 (45). The effector protein involved in the intracellular growth has not been identified.

Expression of the autotransporter proteins, BoaA and BoaB, is important for the invasion of *B. pseudomallei* DD503 in J774A.1 murine macrophages. These molecules also appear to be important for the ability of the organism to survive and replicate. While DD503.*boaA* and DD503.*boaB* show only modest decreases in survival in these macrophages, the DD503.*boaA.boaB* double mutant showed a significant decrease in survival (14). The mechanism behind the survival in macrophages by these two autotransporters is not clear.

### 1.2.2.5 Intercellular Spread

While in the cytoplasm of host cells, *B. pseudomallei* is able to spread from cell-to-cell by membrane protrusions, propelled by actin-mediated motility (34). This type of intercellular spread allows the organism to move between host cells without exposure to soluble immune factors. BimA is an autotransporter protein that possesses an actin-binding domain necessary for binding host actin. *B. pseudomallei* expressing BimA is able to manipulate the actin inside host cells and create the characteristic actin-tail formation. This process appears to be independent of the host cell protein Arp 2/3.
complex which is known to polymerize actin (261). The mechanism allowing BimA to manipulate this process is not currently understood.

The spread of bacteria from cell-to-cell appears to cause host cell membrane fusion and the formation of multinucleated giant cells (MNGC) (146). One of the effector proteins secreted by the Bsa type III secretion system, namely, BipB, appears to be important for this process. The gene encoding BipB was found in the Bsa type III secretion system locus (T3SS3) and BipB was found to be very similar to *Salmonella* spp. proteins that integrate into membranes and are involved in the formation of liposomes (270). A $bipB$ mutant was constructed in the *B. pseudomallei* strain K96243 and then tested for its ability to form membrane fusions in infected macrophages. While the wild-type strain was able to cause a large amount of MNGC formation, the $bipB$ mutant produced significantly less numbers of these giant cells signifying the importance of BipB in this process (270). It has been hypothesized that MNGC formation may provide a niche for *B. pseudomallei* to survive outside of the influence of specific antibodies and immune cells. In addition to BipB, the T6SS1 also appears to be involved in the formation of the MNGC as a K96243 $hcp1$ mutant no longer formed these giant cells. It has not been determined whether this was simply a result of fewer bacterial numbers within the macrophage, as the $hcp1$ mutant has growth defects in macrophages, or if a T6SS1 effector protein is involved in the MNGC formation (45).

1.2.2.6 Cell Lysis
B. pseudomallei is able to induce cell death in host cells following infection. Macrophages infected with the organism have been shown to undergo oncosis (necrotic cell death) as well as pyroptosis (caspase-1 mediated cell death) depending on the relative multiplicity of infection (higher MOI leads to oncosis). Internalization is required for this cytotoxicity, as macrophages were viable when infected with an invasion-deficient strain of B. pseudomallei or when invasion was inhibited (269). The T6SS1 is also implicated in cell lysis as the K96243 hcp1 mutant caused significantly less damage to Raw 264.7 macrophages compared to the wild-type following intracellular growth (45). Autophagy is an additional type of cell death in which the host cell begins to consume itself using its own lysosomes. B. pseudomallei-infected macrophages were shown to colocalize with the autophagy marker, LC3, and artificial induction of autophagy in B. pseudomallei-infected macrophages led to an increase in colocalization with LC3 (67). Induction of autophagy in infected macrophages led to a decrease in bacterial survival, and an effector of the Bsa type III secretion system, BopA, appears to be involved in the evasion of bacterial killing by autophagy, as a bopA mutant was shown to be colocalized more often with autophagocytic vesicles and had decreased survival (67). It has been suggested that mechanical lysis of the macrophage may represent the escape mechanism for B. pseudomallei after sufficient numbers of bacteria have accumulated in the cell (1).

1.2.2.7 Dissemination

Acute melioidosis is characterized by inoculation in the lungs or in the skin and then a rapid dissemination of B. pseudomallei to secondary locations including the liver,
spleen, and even the brain (310). Following invasion of host cells, the organism replicates to high numbers and spreads cell-to-cell by actin polymerization, or escapes and disseminates hematogenously. *B. pseudomallei* is able to survive in serum and resist complement-mediated killing via production of a capsule. In fact, a strain failing to express WcbB, which is a critical capsule biosynthetic protein, was very attenuated in its ability to survive in the blood of infected hamsters and had significantly less numbers of bacteria in tissues compared to the wild-type *B. pseudomallei* strain (221). This suggests that the capsule is essential for the organism to disseminate throughout the host following infection. In addition to capsule, *B. pseudomallei* also produces LPS that was shown to provide protection from serum (82) and from cationic peptides (46), suggesting that LPS also is a factor contributing to the ability of *B. pseudomallei* to disseminate.

### 1.2.2.8 Latency

Treatment of melioidosis can be difficult, and recurrent disease is a problem. Approximately 6-13% of patients become sick again, and this is usually due to relapse rather than reinfection (175). Experiencing a relapse is common within a year of primary infection; however, many cases have been reported of prolonged latency including up to 62 years (192). Studies of patients in endemic regions who have recovered from melioidosis have shown that the body maintains high antibody titers for many years, which suggests continuous exposure (290). It is not known where the bacteria are able to reside during these periods of latency or how this is possible. One hypothesis is that *B.
*Pseudomallei* has the ability to enter a dormant state, possibly in an intracellular location, where it can avoid detection (1).

### 1.2.3 Virulence Determinants

*B. pseudomallei* is a virulent pathogen and very few organisms are needed to cause death in animal models. Upon inhalation of aerosolized bacteria, the organisms are able to bind to epithelial cells, invade, spread cell-to-cell, and then disseminate to other tissues. In the process, the organism is able to subvert host immune responses in professional phagocytes by disrupting phagosome/lysosome fusion events and escaping into the cytoplasm where it can replicate to large numbers. Additionally, *B. pseudomallei* is able to evade complement mediated killing. While many of the factors responsible for these processes have yet to be characterized, some have been described.

#### 1.2.3.1 Secretion Systems

Bacteria use secretion systems to express virulence factors on the surface of the bacterium or to direct virulence proteins onto or into host cells. In *B. pseudomallei*, some of these systems have been well defined while others have yet to be characterized. Currently, no type I secretion systems have been characterized in *B. pseudomallei*. These systems were first characterized in the context of secretion of hemolysin A (HlyA) in *E. coli*. Many putative hemolysin-like proteins are predicted based on sequence analysis of *B. pseudomallei* K96243 (124). In addition, an analysis of ABC transporters in *B.
*B. pseudomallei* revealed the presence of four ABC transporters that were in the same locus as a membrane fusion protein (MFP) and an outer membrane protein, suggesting the existence of four independent T1SS. The locus tag numbers and predicted substrates are listed in Table 1.1, which is adapted from Harland *et al.* (114). While these systems have not been studied, it is interesting that three of the T1SS are involved in secreting putative hemolysin-like proteins, and one is predicted to be involved in drug resistance. A putative T1SS was identified in *B. pseudomallei* strain KHW and has been characterized as an antibiotic efflux pump (50). It is not known whether the three genes in KHW are the same as BPSS 0623, BPSS 0625, and BPSS 0624 from *B. pseudomallei* K96243. More information is needed regarding the role of these systems in *B. pseudomallei*.

In contrast to the function of type I secretion systems in *B. pseudomallei*, the role of type II secretion systems (T2SS) is better understood. Studies have shown that *B. pseudomallei* secretes protease, lipase, and phospholipase C into the extracellular milieu (9). An additional study found that in a screen of transposon mutants, 29 individual mutants were unable to secrete these toxins. Analysis of the transposon mutants resulted

<table>
<thead>
<tr>
<th>Outer membrane transporter</th>
<th>Membrane fusion protein</th>
<th>ABC transporter</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  BSPL 1660</td>
<td>BPSL 1665</td>
<td>BPSL 1664</td>
<td>Hemolysin</td>
</tr>
<tr>
<td>2  BPSL 3094</td>
<td>BPSL 3092</td>
<td>BPSL 3093</td>
<td>Hemolysin</td>
</tr>
<tr>
<td>3  BPSS 1287</td>
<td>BPSS 1283</td>
<td>BPSS 1285</td>
<td>Hemolysin</td>
</tr>
<tr>
<td>4  BPSS 0623</td>
<td>BPSS 0625</td>
<td>BPSS 0624</td>
<td>Drug resistance</td>
</tr>
</tbody>
</table>

Table 1.1: Predicted type I secretion systems in *B. pseudomallei* K96243 and their predicted functions. Adapted from Harland *et al.* (114).
in the characterization of a locus encoding a T2SS (80). In order to determine if the T2SS, and the effector proteins, were important during animal infection, challenge studies were conducted with Syrian hamsters. The relative LD$_{50}$ values for the wild-type 1026b and the mutant strain lacking expression of a functional T2SS were $> 5$ and 13, respectively, indicating that the T2SS is not required for virulence in this animal model (80).

*B. pseudomallei* possesses three type III secretion system gene clusters (124). The T3SS1 and T3SS2 gene clusters share homology with similar systems in the plant pathogen, *Ralstonia solanacearum* (219). Even though *B. pseudomallei* is a well characterized animal pathogen, and *R. solanacearum* is a well characterized plant pathogen, the two organisms are both β-proteobacteria and possess many genetic similarities. In fact, a recent study has shown that *B. pseudomallei* is able to infect tomato plants (163). Bacterial strains with mutations in genes encoding T3SS1 or T3SS2 structural components were not altered for virulence in a hamster model (304). Therefore, it is possible that the primary function of these systems is for *B. pseudomallei*-plant interactions (11). In contrast, the T3SS3 was shown to be required for full virulence in a hamster model of infection. However, a triple mutant lacking function of all three of the secretion systems was even more attenuated in virulence than the T3SS3 mutant alone, suggesting that the T3SS1 and T3SS2 provide some benefit during animal infections (304).

The T3SS3 of *B. pseudomallei*, termed Bsa (*Burkholderia* secretion apparatus), is similar to those of *Salmonella* and *Shigella* sp. (11). Mutants in the genes encoding for structural components of the Bsa show reduced replication in macrophage cell lines, an
inability to escape from endosomes, and an inability to form MNGC and actin tails (265). The T3SS3 also harbors genes that encode for homologs of the *Salmonella* Sip translocator proteins, which form a pore in target cells allowing the entry of effector molecules (263). One of these homologs, designated BipB, was shown to mediate MNGC formation, cell-to-cell spreading, and apoptosis in a macrophage cell line. A *bipB* mutant was also shown to be attenuated in BALB/c mice proving it is also very important *in vivo* (270). Another Sip homolog, BipD, was shown to be involved in invasion of epithelial cells, escape from endocytic vesicles, intracellular replication and the formation of actin tails (265). A *bipD* mutant was also shown to be attenuated in BALB/c mice and had reduced numbers of bacteria in the liver and spleen (263).

Although there is clear evidence of the importance of the T3SS3 and of the proteins that are putatively involved in the translocation of the effector proteins into the host cell (*i.e.* BipB and BipD), the role of the individual effector proteins is uncertain. One effector protein, BopA, is homologous to *Shigella* spp. IscB, which is involved in cell-to-cell spread by lysing the membranes surrounding acting tails. A *B. pseudomallei bopA* mutant showed reduced replication in macrophages (67), but was not attenuated in different animal models (263, 304). Two other effector molecules, a predicted muramidase (BapC) and a putative phosphatase (BopB), were also shown to be dispensable in animal models (263, 304). As mentioned previously, BopE is an effector protein that has been shown to be important for invasion of epithelial cells (262); however, it does not appear to be needed for survival in macrophages (265) or for virulence in BALB/c mice (263) and Syrian hamsters (304). Many additional effector proteins
proteins have been identified and are currently being tested for their involvement in adherence (268).

For many bacterial pathogens, a type IV secretion system is used to inject molecules directly into host cells in a manner similar to a type III secretion system or a type VI secretion system (57). Genomic analysis of *B. pseudomallei* K96243 has not revealed the presence of a T4SS. It is possible this organism uses other methods of secretion (*e.g.* T3SS) to compensate for the lack of this system.

Nine ORFs have been identified that encode proteins resembling typical autotransporters (type V secretion system), specifically BPSL 1631, BPSL 1705, BPSL 2063, BPSS 0088, BPSS 0796, BPSS 0908, BPSS 1434, BPSL 1439, and BPSS 1492 (218). Three of these ORFs have been characterized. BPSL 1705 encodes BoaB, which is a *B. pseudomallei* protein involved in adherence to epithelial cells and in partnership with BPSS 0796, BoaA, are involved in the survival of *B. pseudomallei* in macrophages (14). The other characterized autotransporter, BPSS 1492, or BimA, has been show to be important for the actin based motility of *B. pseudomallei* which allows the organism to spread cell-to-cell (264).

*B. pseudomallei* possesses six type VI secretion system gene clusters (240, 247). Using *in vivo* expression technology, three genes were identified in one cluster (T6SS5) that were expressed during macrophage infection (*tssH*-5, *tssl*-5 and *tssM*-5). This cluster also contains mntH, encoding a natural resistance-associated macrophage protein (NRAMP)-like manganese ion transporter, and a heme acquisition gene, *bhuT*, which indicate this T6SS may express virulence factors (247). However, in BALB/c mice
infected with *B. pseudomallei* K96243 or six hcp mutant strains (one for each of the six T6SS), only the hcp1 mutant had a decreased LD50 compared to the wild-type (45). This result suggests that the T6SS1 appears to be the only T6SS important for virulence in mice.

### 1.2.3.2 Secreted Factors

Several eukaryotic cell types have been shown to be susceptible to the cytotoxic effects of cell-free *B. pseudomallei* supernatants (135), suggesting that secreted factors are mediating these effects. Several factors, namely protease, lipase, and phospholipase C, are secreted by a T2SS. A mutant strain lacking expression of a functional T2SS did not secrete these products. However, it was discovered that this mutant strain maintained high levels of virulence in hamsters suggesting these toxins are not required for virulence (80). *B. pseudomallei* is also reported to produce a rhamnolipid. Expression of this protein led to alterations in macrophages such as retraction, rounding up, and also detachment. These effects were due to reorganization of the F-actin network leading to impaired cell-cycle progression and impaired phagocytic activity (116). The bacterium is also known to secrete lecithinases, hemolysins, phosphatase, catalases, peroxidases, and superoxide dismutases, but the role of these individual toxins has not been determined (9, 293). An iron-sequestration protein, malleobactin, is secreted by *B. pseudomallei*, and is important for growth in the host where iron availability is scarce (321). As discussed above, secreted factors from the Bsa type III secretion system include the putative pore-forming proteins BipB and BipD, the putative membrane lysing protein BopA, the
predicted muramidase (BapC) and the putative phosphatase (BopB) (67, 263, 270, 304). Therefore, many secreted factors have been identified some of which, namely BipB and BipD, appear to play important roles in virulence. More work is needed to characterize surface exposed or secreted factors involved in the pathogenesis of this organism.

1.2.3.3 Capsule and Biofilm Formation

*B. pseudomallei* has been shown to produce four polysaccharide structures, but only three capsule biosynthesis loci have so far been identified. Electron microscopy analysis identified several capsule variants of the organism. One group of bacteria appeared to lack any capsule, while another group had capsules of approximately 0.086 µm thick, and finally another group appeared to have a very thick capsule of approximately 0.1-0.25 µm (216). Whether this difference was simply due to the amount of capsule produced or whether these two capsule-producing strains were producing different variants of capsule has not been determined. The first polysaccharide molecule characterized was a type I OPS capsular polysaccharide that was determined following the identification of the biosynthetic locus (222) and the absence of lipid A (136). The structure of this capsular polysaccharide is an unbranched polymer with repeats of the following: -3)-2-O-acetyl-6-deoxy-β-D-manno-heptopyranose-(1(type I capsule) (148, 203). Inactivation of several of the genes in the biosynthetic locus of the type I capsule led to reduced survival in mice (10) and Syrian hamsters (222). These mutants were also shown to be 1000 times more sensitive to the bactericidal effects of human serum, which could be reversed by the addition of purified capsule. Further analysis revealed that the
mutant lacking type I capsule had more complement deposition on the surface than the
wild-type strain, which agrees with the sensitivity of the mutant strain to human serum
(221). Additionally, type I capsule has been shown to be important in the context of
macrophage infections following opsonization with serum components as the *B. pseudomallei*
capsule mutant was internalized in greater numbers than the wild-type following incubation with human serum (221).

Two other capsular polysaccharides have been described, including a 1-4 linked
glucan (type III capsule) and an acidic polymer composed of galactose and 3-deoxy-D-
manno-octulosonic acid (type IV capsule) (144). *B. pseudomallei* strains lacking
expression of the type III capsule showed a delayed time of death in mice of 7.8 days
compared to the wild-type (3 days). The type IV capsule mutant also showed a delayed
time of death in mice (11.6 days) (239). A direct comparison between the three capsular
polysaccharides has not been performed.

*B. pseudomallei* has been observed by electron microscopy to be in a biofilm in
the infected lung tissue of a guinea pig as well as in a human patient (298). In fact, the
bacteria producing very thick capsules (0.1-0.25 µm, see above) were sometimes shown
to aggregate into a microcolony completely surrounded by a polysaccharide structure,
which may represent the beginning of biofilm production (298). Overall, there are
significant differences in the amount of biofilm produced by *B. pseudomallei* strains and
there is no correlation between biofilm formation and virulence in mice. Even strains
lacking biofilm production were still as virulent as wild-type bacteria (273). These
studies suggest biofilm production is dispensable during early stages of infection;
however, it is important that biofilm production during latent infection has not been
studied. In addition, the role of biofilm production in the environment has also not been studied. Overall, very little is known regarding the mediators of biofilm formation in *B. pseudomallei*.

1.2.3.4 Lipopolysaccharide

In comparison to enterobacteria, *B. pseudomallei* lipopolysaccharide (LPS) exhibits weaker pyrogenic activity in rodents (178). Isolated LPS also activates macrophages at much lower levels than LPS derived from *E. coli* (286). The structure of the LPS was identified as an unbranched polymer with the following disaccharide repeats: \(-3\)-\(\beta\)-\(\text{D-glucopyranose-(1-3)-}\text{6-deoxy-\(\alpha\)-L-talopyranose-(1-})\) (203). 2-O-methyl and 4-O-acetyl or 2-O-acetyl alone can decorate the talose residue on these repeats (203). Three types of LPS, specifically two smooth LPS serotypes A and B and a rough serotype, have been described (7). The genes responsible for LPS production have been studied and it has been shown that mutants in some of these genes resulted in *B. pseudomallei* strains that had increased sensitivity to polymyxin-B (46), increased sensitivity to the bactericidal effects of human serum, increased uptake by phagocytes, decreased intracellular survival in phagocytes, and attenuated virulence in animal models (82). Therefore, LPS is an important virulence determinant in *B. pseudomallei*.

1.2.3.5 Flagella and Pili
*B. pseudomallei* is a motile organism and electron microscopy has shown the presence of flagella and variable expression of pili (298). Flagella are structures that are composed of many proteins homologous to molecules involved in the formation of type III secretion systems (162). Flagella consist of a central shaft, anchored in both membranes by two ring structures connected to the flagellar filament by a bent hook. Rotation of the filament allows for the propulsion of the bacteria (162). In *B. pseudomallei*, two to four flagella are localized at one of the bacterial poles which confer motility (81). The role of this motility in virulence is unclear. One study demonstrated that a flagellar mutant of *B. pseudomallei* strain 1026b was not attenuated in diabetic rats or Syrian hamsters (81). However, this isogenic mutant strain was unable to adhere to the amoeba *Acanthamoeba astronyxis*, specifying a role in adherence for flagella (134). A separate study, using a different strain, *B. pseudomallei* KHW, with a mutation in the same flagella gene, revealed that the mutant was attenuated in BALB/c mice, regardless of the route of infection. While this strain was not tested for its ability to adhere to any cells, the mutant was not deficient in its ability to invade epithelial cells (58). Whether the discrepancies observed between these flagellar mutants was due to differences in strain (1026b vs KHW) or animal models is not known. Therefore, the exact role of the flagella in pathogenesis has yet to be elucidated.

Type IV pili are believed to contract in a coordinated manner that has been associated with a type of movement called twitching motility (33). Twitching motility has been observed for *B. pseudomallei* (173), but the role of the type IV pilus in this process has not been shown. The main outer membrane pore protein of the type IV pilus, T2S D, and many other proteins involved in type II secretion are also involved in the
formation of the pilus structure (162). The major subunit is PilA. In *P. aeruginosa*, the proteins that are involved in the biosynthesis of the type IV pilus are encoded in the regions flanking *pilA* in the chromosome. In *B. pseudomallei*, a PilA-like protein was coded for in a locus lacking any putative pilus biosynthetic genes (88). However, eight ORFs were identified in *B. pseudomallei* K96243 that encoded putative type IV pilus subunits. In order to test the function of the PilA protein in *B. pseudomallei* K96243, a *pilA* mutant was constructed. The virulence of the *B. pseudomallei* *pilA* mutant strain was less in mice infected via the intranasal route compared to the wild-type strain but was equally as virulent when infected via the intraperitoneal route (88). Therefore, the type IV pilus is involved in the virulence of *B. pseudomallei* but only under some conditions.

### 1.2.3.6 Quorum Sensing

Quorum sensing allows bacteria to coordinate gene expression based on the concentration of organisms in an area. Many bacteria express LuxI, which is responsible for *N*-acyl homoserine lactone (AHL) synthesis. AHLs are secreted by the bacteria and are also imported to collect information regarding cell density. As the concentrations of bacteria increase, AHLs promote expression of the transcriptional regulator, LuxR. The *B. pseudomallei* genome is predicted to encode three LuxI and five LuxR homologs and culture supernatants have been shown to contain many of the AHL signaling molecules (284). Individual mutant strains were created for each of the LuxIR homologs, and each of these strains showed reduced virulence in the Swiss mouse (287), reduced colonization
and virulence in BALB/c mice, and an increased LD$_{50}$ in Syrian hamsters (284). The impact of these systems during the formation of biofilm has not been described.

### 1.2.4 Vaccines and Therapeutics

#### 1.2.4.1 Melioidosis Therapy

Treatments for melioidosis have been limited as *B. pseudomallei* is resistant to many antibiotics including first- and second-generation cephalosporins, penicillins, macrolides, and aminoglycosides (73, 164). Misdiagnosis and administration of incorrect antibiotics can lead to bacterial dissemination to the blood, where the mortality rates are high (311). Currently, confirmation of infection requires bacterial culture (305) which is time prohibitive when proper treatment is imperative. Additionally, therapy for melioidosis is complicated by the fact that *B. pseudomallei* is able to enter into extended periods of latency where the organism is most likely in an immunologically privileged site. Therefore, the consensus for the most effective treatment is for the use of high-dose intravenous ceftazidime for 2 weeks, followed by oral eradication therapy of up to 20 weeks using trimethoprim-sulfamethoxazole with or without doxycycline (89).

#### 1.2.4.2 Vaccine Development

Melioidosis is difficult to treat as *B. pseudomallei* is intrinsically resistant to a variety of antibiotics, diagnosis of the disease is time prohibitive, and treatment requires the administration of antibiotics for prolonged periods of time. Therefore, the
development of a vaccine would be a great benefit to people living in or visiting endemic regions. This has led to many studies on the efficacy of vaccines for treating or preventing melioidosis. Most of these studies have resulted in only limited successes.

Many studies have attempted to define *B. pseudomallei* components that would protect animals from the infection. In one trial, individual monoclonal antibodies generated to capsule, LPS, and undetermined *B. pseudomallei* proteins were used to passively protect mice from challenge with *B. pseudomallei*. Upon low-dose challenge, all animals survived. However, upon challenge with a larger inoculum, all animals died, even following passive immunization using all seven mAbs (143). An additional study generated monoclonal antibodies to the flagella of *B. pseudomallei*. Monoclonal antibodies were delivered intraperitoneal or intravenous but both routes failed to completely protect all animals from challenge. Under the best conditions, passive protection via administration of monoclonal antibodies intravenously resulted in 80% survival (37).

Another strategy that has been employed to provide protection against *B. pseudomallei* is to conjugate immunoreactive molecules with antigens that can generate larger and more complete immunological responses than with a single antigen. One such study conjugated the O-polysaccharide of LPS to flagellin protein. This large antigenic molecule generated strong immune responses in animals and immunization with antibodies generated against this hybrid molecule protect diabetic rats from challenge following challenge with low inocula; however, larger inocula resulted in up to 90% mortality rates (35). Six Hcp proteins representing each of the six T6SS were used to immunize mice and it was found that Hcp2 provided the best protection (80%) (45).
attempt to immunize mice with more than one individual Hcp protein was not reported. Another immunization strategy has been to use whole bacterial preparations as a vaccine. One such study used the relatively avirulent Burkholderia species, *B. thailandensis*, as the vaccine. In this study, only 50% of the animals that were challenged were protected following immunization with *B. thailandensis* (133). Therefore, many methods have been utilized in order to generate immune responses in animals to protect against *B. pseudomallei* challenge; however, many challenges have been encountered and no vaccine candidate has passed all trials. As a vaccine would be greatly beneficial due to misdiagnosis, mistreatment, and the difficulties associated with treatment, more work is needed to identify candidate vaccine targets.

### 1.3 *Burkholderia thailandensis*

#### 1.3.1 General Characteristics

*B. thailandensis* is a relatively avirulent, Gram-negative, terrestrial organism that was regarded as an avirulent *B. pseudomallei* strain until 1998. The two organisms are highly similar in many respects and diagnostic tests are often unable to distinguish between the two (319). Comparisons of 16S rRNA sequences suggest that *E. coli* and *Salmonella typhimurium* diverged from *B. pseudomallei* 140 million years ago, while *B. thailandensis* diverged 47 million years ago (323). The main difference between these organisms is the relative levels of virulence. Even before *B. thailandensis* was considered a distinct species it was clear that there were some *B. pseudomallei*-like strains that were relatively avirulent and would only cause infection under very unusual...
circumstances such as in near drowning instances (179). In fact, infections of Syrian golden hamsters revealed the LD₅₀ of *B. pseudomallei* to be <12 CFU when the organisms were injected IP, whereas the LD₅₀ of *B. thailandensis* was observed to be 1.8 x 10⁶ CFU (36). A comparison between the two organisms has revealed that the genomes are relatively similar. *B. pseudomallei* K96243 possesses 2 chromosomes that are 4.07 Mb and 3.17 Mb in size while *B. thailandensis* E264 possesses 2 chromosomes of 3.81 Mb and 2.91 Mb (323). The total number of genes in *B. pseudomallei* is 5854 while *B. thailandensis* has 5645. Of these, 4994 genes are conserved between the two species. Overall, the %GC content, numbers of pseudogenes, and average gene length are very similar between the two *Burkholderia* species (323). Specific differences leading to the lack of virulence in *B. thailandensis* are discussed below.

### 1.3.2 Molecular Factors Affecting Virulence

A comparison between *B. pseudomallei* and *B. thailandensis* revealed that the two organisms have highly conserved genetic synteny and share a large collection of genes involved in core metabolism, accessory pathways, and virulence (323). Although these similarities exist, many differences have been characterized between the two organisms that may explain the virulence differences. One major virulence determinant that is absent in *B. thailandensis* is the type I capsule. As mentioned previously, this polysaccharide capsule in *B. pseudomallei* has been shown to be essential for virulence (222). In *B. pseudomallei*, the biosynthetic locus responsible for the production of type I OPS capsule actually replaced a pre-existing capsule biosynthetic locus that is present in
B. thailandensis (323). It has not been determined if the biosynthetic locus in B. thailandensis is capable of producing a polysaccharide. As capsule mutant strains of B. pseudomallei show increased opsonophagocytic uptake and decreased survival in serum and animals (221), B. thailandensis lacks a major virulence determinant for survival inside human and animal hosts.

B. pseudomallei is more effective than B. thailandensis at adhering to and invading host cells (145). One structure that often mediates binding of bacteria to surfaces and host cells is the short pilus structures called fimbriae. B. pseudomallei possesses six gene clusters that are potentially responsible for producing these structures, while B. thailandensis only possesses three of these gene clusters (323). The presence of these structures on the surface of the bacteria enables these macromolecules to be involved in interactions with host cells and thus contribute directly to the virulence of the organism.

B. thailandensis also lacks a cluster of genes which encode the components of one of the B. pseudomallei type III secretion systems (T3SS1) (219). Though this system was not essential for virulence like the T3SS3, it was speculated that the T3SS1 plays a minor role in infection along with the T3SS2 (219). As B. thailandensis possesses T3SS3, it has been suggested that this organism could be used as a model for the study of some aspects of B. pseudomallei type III secretion (112).

One of the earliest differences observed between these two bacteria is that only B. thailandensis can utilize arabinose as a carbon source (253). Arabinose exposure downregulates expression of other genes, including the bsa gene cluster responsible for
formation of the T3SS3 (186), which in turn is required for the full virulence of \textit{B. pseudomallei} in animal models (304). Due to this, the genes responsible for the arabinose utilization in \textit{B. thailandensis} have been called antivirulence genes (186).

### 1.3.3 The Use of \textit{Burkholderia thailandensis} to Model Pathogenesis

**Mechanisms of \textit{Burkholderia pseudomallei}**

While specific differences exist between \textit{B. pseudomallei} and \textit{B. thailandensis}, as described above, the overall similarity between the two species is high. In addition to the genetic similarities, the organisms also behave similarly \textit{in vitro} under many conditions. After internalization by macrophages, both bacteria are able to escape from the phagosome, replicate, cause multinucleated giant cell formation, and cause cell death (112, 115, 141). In fact, \textit{B. thailandensis} is capable of causing lethal infection in one model system using \textit{Caenorhabditis elegans} (201). The high levels of similarity between the two organisms and the fact that \textit{B. thailandensis} does not require a biosafety level 3 laboratory has led some to suggest that, in some circumstances, \textit{B. thailandensis} can be used as a model system for the study of \textit{B. pseudomallei} pathogenesis (323). In fact, one study has already used \textit{B. thailandensis} as a model system to study the \textit{bsa} type III secretion system. Mutants in the Bsa secretion system in \textit{B. thailandensis} were unable to escape from the phagosome, replicate intracellularly, form actin tails, and secrete effector molecules. In addition, unlike the wild-type strain, the \textit{bsa} mutant was unable to kill mice after aerosol infection (112). As these mechanisms are also a part of \textit{B.}
pseudomallei infection, it is possible that the study of the bsa system in B. thailandensis could lead to important discoveries for B. pseudomallei.

1.4 Biofilm-Associated Proteins

1.4.1 General Characteristics

Overall, very little is known regarding biofilm production in B. pseudomallei. Biofilm production is believed to be a necessity for bacteria to survive in many environmental conditions (63). A biofilm is composed of a community of bacteria adhered together and to surfaces that are enclosed in a mixture of self-produced DNA, protein, and polysaccharide, the composition of which varies depending on the bacteria present in the community. In addition to providing protection to the organisms, biofilms allow interactions between potentially diverse bacteria, and may serve as a significant site of gene exchange through conjugation and recombination events. A major family of molecules that have been shown to be important for biofilm formation in a variety of Gram-positive and Gram-negative bacterial species are the biofilm-associated proteins (Bap). The first characterized protein of this family was Bap of Staphylococcus aureus. The staphylococcal Bap protein is a 2276 amino acid surface-associated molecule that is required for biofilm formation (65). Orthologs of Bap have since been identified in a number of organisms. In general, these family members have been shown to be high-molecular weight proteins responsible for biofilm formation which possess many domains of peptide repeats (160). LapA is a very large protein of 8682 amino acids (~888 kDa) that is found loosely attached to the surface of Pseudomonas fluorescens and
is required for biofilm formation. Like other Bap members, LapA possesses two domains that are composed of long multiple repeats, which represent more than 75% of the total length of the protein (121). Interestingly, while most Bap members possess Sec-signal sequences and are secreted in a type II secretion manner, LapA is secreted by a type I secretion system. The components of this system are encoded immediately downstream from lapA in the chromosome of *P. fluorescens* (121). LapE is an outer membrane transporter that is encoded immediately downstream of lapA. Downstream of lapE is lapB which encodes the inner membrane ABC transporter and located downstream of lapB is lapC which encodes the membrane fusion protein. Many Bap members are contained in mobile elements. *S. aureus* Bap is in a pathogenicity island whose mobility appears to be dependent on a functional recombinase (281). Esp, of *Enterococcus faecalis*, is also flanked by insertion elements and the mobility of this region of DNA appears to occur at frequencies similar to those observed in the modulation of virulence of other organisms (161, 248).

### 1.4.2 Role in Pathogenesis

In *S. aureus*, Bap mediates attachment to abiotic surfaces and between individual bacteria (65). Bap is able to initiate biofilm formation independent of the normally produced exopolysaccharides of the biofilm matrix (278). Not all Bap members function in the exact same manner. In another system, *P. fluorescens* is able to transiently attach to many surfaces in the absence of the Bap member LapA. However, LapA is necessary for mediating irreversible adherence to abiotic surfaces and the environmentally relevant
substrate quartz sand, and lapA mutants do not produce biofilms on these surfaces (121). In another example, *Salmonella enteritidis* has a Bap homolog designated BapA which has been shown to be essential for biofilm production at the liquid-air interface of broth cultures. Additionally, overexpression of BapA resulted in a stronger and thicker biofilm, possibly by strengthening intercellular attachments (159). Some Bap members are involved in late stages of biofilm development. In *Burkholderia cepacia*, a Bap mutant was unable to form mature biofilms and instead had well-separated cell aggregates forming unusually porous biofilms (130). Therefore, although there are differences in the mechanisms, the Bap members all contribute to biofilm formation in their respective bacteria.

In addition to biofilm formation, many of the Bap members also play significant roles in pathogenesis in other ways. In many of the cases, the Bap members are involved in adherence of the organism to the substrate prior to biofilm production (160). As there is a strong component of adherence in biofilm formation, there also appears to be a significant role for biofilm-associated proteins in host colonization. In *P. putida*, colonization of seeds is abrogated in a mutant of a Bap-like protein, Mus-20 (87). Biofilm-associated proteins also are important factors for bacterial infections in animals. Wild-type *E. faecalis* can colonize murine bladders in significantly greater numbers than an Esp mutant strain (249). In addition to colonization, some studies suggest that Bap members are involved in prolonging the course of infection. In one study, mice infected with a lethal dose of a *S. enteriditis bapA* mutant strain survived longer than the wild-type (159). In another study, *S. aureus* isolates possessing a functional bap gene were better able to colonize and persist in bovine mammary glands than isolates that lacked this gene.
These studies highlight the ability of the biofilm-associated proteins to function in many capacities beyond simply forming biofilm. The presence of a biofilm-associated protein in *B. pseudomallei* has not been reported.

### 1.5 Outer-Membrane Protein A Family of Molecules

#### 1.5.1 General Characteristics

In an attempt to find new vaccine targets to protect against *B. pseudomallei* infection, a recent study identified 12 outer membrane proteins in *B. pseudomallei* as having outer membrane protein A (OmpA) domains (PF00691). *E. coli* OmpA is a heat-modifiable outer membrane protein that is one of the most well studied outer membrane proteins. OmpA-like proteins are very common among Gram-negative bacteria, as one study found that all organisms tested from 17 genera had at least one homolog (19). The structure of OmpA begins with a signal sequence, followed by an N-terminal domain consisting of eight β-sheets that form a β-barrel with four large surface exposed loops and four short periplasmic turns, and a periplasmic C-terminal domain (297). The presence of the signal sequence suggests that these molecules are secreted by the type II secretion system. The C-terminal periplasmic domain is believed to be important for structural integrity, as *ompA* mutants had a peptidoglycan layer that was unassociated with the outer membrane, and the bacteria were found to have a spherical morphology (255). In addition to membrane stability, OmpA-like molecules are involved in bacterial conjugation (149) and as receptors for bacteriophages (188). In *E. coli*, OmpA is the
major outer-membrane protein with over 100,000 copies per cell (150), and has been shown to be the major immunological antigen of \textit{S. Typhimurium} in mice (251).

1.5.2 Role in Pathogenesis

OmpA forms a pore in the outer-membrane of bacteria and has been shown to have some pore-like activity (31, 266), but it seems to have much more complex functions. OmpA-like proteins have been shown to play a role in numerous processes during infection. At the initial stage of infection, OmpA has been shown to be important for adherence to cell lines and for the production of biofilm (99, 279). An \textit{E. coli} \textit{ompA} mutant was also shown to have decreased invasion of brain microvascular endothelial cells (210), and OmpA was one of the molecules identified as being important for the ability of \textit{E. coli} to penetrate the blood-brain barrier (129). Immune cells are also affected by OmpA-like molecules. Purified OmpA from \textit{Klebsiella pneumoniae} was able to cause dendritic cells to migrate towards draining lymph nodes (139). \textit{E. coli} OmpA was shown to be essential for the prevention of apoptosis in macrophages and was therefore suggested to help \textit{E. coli} survive by providing a privileged site for replication (267). In addition to providing a reservoir for intracellular replication, OmpA also confers upon \textit{E. coli} the ability to survive the bactericidal effects of serum and an \textit{ompA} mutant was less virulent in neonatal rats (308). Another OmpA-like protein, Loa22, of \textit{Leptospira interrogans} was the first genetically defined virulence factor of the spirochete as mutants were attenuated for virulence in guinea pig and hamster models of leptospirosis (225). The ability of this pore-forming protein to be involved in so many
diverse functions is impressive. Although it is possible the large surface exposed loops of the protein are involved in all of these various aspects of pathogenesis, the role of this molecule in the stability of the outer-membrane and other outer-membrane proteins is likely to be equally important. The role of the twelve outer membrane protein A-like proteins in the pathogenesis of *B. pseudomallei* has not been studied.
Chapter 2

Results

2.1. Introduction

*Burkholderia pseudomallei* causes the potentially fatal disease melioidosis and is commonly isolated from the wet soils of southeast Asia and northern Australia. Currently, there is no vaccine to protect against infection by this organism. Surface exposed proteins involved in pathogenesis are attractive vaccine candidates due to the ability of these molecules to be easily recognized by the immune system, the necessity for expression of these molecules during infection, and the potential of antibodies to interfere with the function of these proteins. Characterization of the biological role of surface proteins will enable us to determine whether these molecules may be suitable for use in a vaccine to protect against infection by *B. pseudomallei.*
2.2 Identification of a Putative Biofilm-Associated Protein in \textit{Burkholderia pseudomallei}

Genomic analysis of the \textit{Burkholderia pseudomallei} strain K96243 identified an ORF with the locus tag BPSL1661, that was highly similar to the biofilm-associated protein (Bap) of \textit{Staphylococcus aureus} (NCBI blastp; e-value $2 \times 10^{-27}$). Biofilm-associated proteins are found in a variety of Gram-negative and Gram-positive species and where tested are surface-exposed molecules that are involved in many aspects of pathogenesis, including the production of biofilms, adherence, and host colonization (160). We designated the protein encoded by BPSL 1661 Bap-like protein (Blp). Searching the NCBI Genomic Blast database identified \textit{blp} in all 23 \textit{B. pseudomallei} strains currently sequenced.

Similar to other Bap molecules, Blp is predicted to be a very large protein of 3,229 amino acids (325 kDa) containing a number of repeated sequences as shown in Figure 2-1 (colored squares). A comparison of the predicted Blp proteins from the sequenced \textit{B. pseudomallei} strains revealed that many of these strains appear to lack a region of the protein corresponding to residues 668-985 of strain K96243 (Figure 2-1, section marked as 1). These residues are nearly identical to amino acids 986-1303. As can be seen in Figure 2-1, a third long repeat also appears to be present in K96243 Blp at amino acids 1304-1621, but the sequence in this region diverges slightly from the sequences of the first two. All of the Blp sequences possess the third long repeat. Using the multiple sequence alignment tool, ClustalW, it was found that the Blp molecules from
Figure 2-1: Structural characterization of Blp from *B. pseudomallei* K96243. Protein domains and repeated amino acid sequences are given below the diagram. Three sections of repeats are shown in diagram (The section marked 1 appears to be absent in some *B. pseudomallei* strains).

The sequenced *B. pseudomallei* strains are between 89-99% identical at the amino acid level. The amount of Blp identity between these strains is almost entirely dependent on whether the individual proteins possess all three of the long repeats. Genomic searches were also conducted on the closely related host-adapted organism *B. mallei* and the relatively avirulent *B. thailandensis*. Analysis of the 10 *B. mallei* and 5 *B. thailandensis* publicly available genomes using the NCBI Genomic Blast service revealed that neither organism possesses a *blp* gene. Therefore, Blp is a very highly conserved protein that is specific to *B. pseudomallei*. These properties make Blp an attractive target for the development of a *B. pseudomallei*-specific vaccine, therapeutic strategy, or diagnostic tool.
Many biofilm-associated proteins are encoded in mobile elements (158). In agreement with this observation, the genetic locus containing *blp* from *B. pseudomallei* K96243 is contained in a 92.3 kb genomic region, encompassing BPSL 1637 to BPSL 1709 that is one of 12 genomic islands on chromosome 1 of *B. pseudomallei* (124). Therefore, the region encoding Blp appears to have been acquired via horizontal transfer. As it was determined that all *B. pseudomallei* strains publicly available possess the genomic regions flanking *blp*, it is possible the acquisition of this potential mobile element took place early in the evolution of this organism.

Sequence analysis of Blp using Vector NTI® (Invitrogen™) identified an Arg-Gly-Asp (RGD) motif (residues 2610-2612) that has been defined as being directly involved in adherence for several well characterized proteins (232). The RGD motif has not been reported previously for any of the biofilm-associated proteins. It is possible that this motif enables Blp to function as an adhesin for *B. pseudomallei*. While most Bap molecules are secreted across the inner membrane of Gram-negative bacteria by the general Sec pathway and thus contain a typical Sec-signal sequence, analysis of Blp using SignalP determined that no obvious signal sequence was present at the N-terminus of the protein. This finding suggests that Blp is not secreted via the Sec system. However, it does appear as though the protein is surface exposed, as the sequence of Blp was analyzed by PSORTb and was predicted to encode an extracellular protein (score 9.98). Using TMpred, it was predicted that this protein may have up to 12 transmembrane domains at the following amino acids: 684-707 (score, 584), 758-774 (score, 612), 1002-1025 (score, 584), 1076-1092 (score, 612), 1320-1343 (score, 576), 1399-1417 (score, 584), 1502-1521 (score, 939), 1726-1743 (score, 827), 1833-1853 (score, 991), 2057-
2081 (score, 580), 2623-2646 (score, 988), and 2806-2824 (score, 513). It should be noted that all of these scores are low (<1000) (47). Further analysis of the Blp sequence using Prosite on the ExPASy proteomics server identified residues 3004-3022 as being a hemolysin-type calcium binding domain (PS00330). Many proteins which contain this domain also possess distinctive glycine rich regions with the consensus sequence GGXGXDXXX, that specifically bind calcium and, in most cases, specify secretion by a type I secretion system (79). Analysis of the sequence surrounding the hemolysin-type calcium binding domain in Blp using Vector NTI® identified tandem sequences of GGXGXDXXX at residues 2999-3007 and 3017-3025. The lack of a Sec-signal sequence and the presence of the glycine rich domains suggests that Blp may be transported to the surface by a type I secretion system. This evidence, along with the sequence similarities to several surface exposed biofilm-associated proteins, strongly suggests that this protein is transported to the outer-membrane of \emph{B. pseudomallei}. Based on these findings, we hypothesize that the highly-conserved Blp is a surface localized protein in \emph{B. pseudomallei} involved in biofilm formation and adherence to host cells.

\section*{2.3 Analysis of the Genetic Locus Encoding \textit{Burkholderia pseudomallei} Blp}

Type I secretion systems (T1SS) are composed of an inner membrane ABC transporter, a periplasmic membrane fusion protein (MFP), and an outer membrane transporter. These transport systems have been shown to secrete a variety of substrates (79); however, in \emph{Pseudomonas fluorescens}, a T1SS is responsible for the transport of a
large biofilm-associated protein, LapA. In fact, in *P. fluorescens*, the loss of any one of the T1SS proteins is sufficient to block LapA secretion to the surface which results in a loss of biofilm production by this organism (121). Located immediately downstream of *lapA* in the chromosome of *P. fluorescens* are three genes that encode the outer membrane transporter (LapE), the inner membrane ABC transporter (LapB), and the MFP (LapC, **Figure 2-2A**). Investigation into the regions immediately surrounding the *blp* sequence in *B. pseudomallei* K96243 revealed three genes that encode what appear to be the components of a type I secretion system. As shown in **Figure 2-2B**, a 1,797 bp ORF was found upstream of *blp* with locus tag BPSL 1660 that was predicted to encode a 65 kDa protein of 599 amino acids. Sequence analysis of the predicted protein using NCBI Genomic Blast revealed a TolC domain (COG1538; e-value 6.3e^{-20}) at amino acids 29-479. This analysis also revealed that the protein encoded by BPSL1660 was most similar to a TolC-like protein from *Methylophaga thiooxidans* (e-value 1e^{-08}). The localization of the protein was predicted to be the outer membrane using PSORTb (score, 9.92). This evidence led us to the hypothesis that BPSL1660 likely encodes a TolC-like protein in the outer-membrane of *B. pseudomallei*. As Blp is predicted to be secreted by a type I secretion system and the fact that BPSL1660 is highly similar to other outer membrane proteins, we designated the molecule Blp-secretion protein A (BspA). The *E. coli* TolC OM transporter is predicted to be composed of 3 monomers, each contributing a β-barrel membrane-spanning domain (153). We analyzed the BspA sequence using the TMpred server to determine if any transmembrane domains were part of the molecule. This algorithm predicted one transmembrane domain at amino acids 343-362 (score, 875). Most TolC-like proteins contain a Sec-signal sequence but analysis of BspA by
Figure 2-2: Comparison of the lap locus from *P. fluorescens* (A) with the genes in the *B. pseudomallei* blp locus (B). Below each gene is the predicted function of the encoded protein. The table at the bottom displays the size of the genes, the locus tag, the molecular mass and the putative function of the encoded proteins.

SignalP revealed no such sequence. In an attempt to identify an alternate signal sequence such as a Tat-signal sequence or a T1SS signal sequence, we analyzed the protein using...
TatP or Vector NTI®, respectively, but no such signals were identified. As all other evidence points towards BspA being an OM protein, the protein likely has some means to traverse the IM. One possible explanation is that BspA does not possess a typical Sec-signal sequence which causes it to be unrecognized by the SignalP algorithm. Overall, our results indicate that BspA possesses a high degree of similarity to TolC-like proteins and it is our hypothesis that this protein is an OM transporter for a type I secretion system.

Further analysis of the regions surrounding blp in the chromosome of B. pseudomallei identified a 2298 bp ORF with the locus tag BPSL 1664, located downstream of blp (Figure 2-2B). The encoded protein is predicted to be 766 amino acids and 83 kDa, and is highly similar to a type I toxin efflux ATP-binding protein in P. fluorescens WH6 (NCBI blastp, e-value 3e-117). This analysis also identified the ATP binding site domain (CD03245) at amino acids 519-740 (e-value 2.05e-75) that contained all of the attributes of such domains including the Walker A motif, the Walker B motif, the H switch region, the Q loop, and the signature motif. Moreover, a type I secretion ATPase domain (TIGR03375) was also identified from amino acids 45-751 (e-value 1.8e-148). As the protein encoded by BPSL 1664 appears to be an ABC transporter for a type I secretion system, we designated this ORF Blp secretion protein B (BspB). Using TMpred, it was determined that BspB has six potential membrane-spanning regions at amino acids 208-231 (score, 2070), 238-256 (score, 2013), 321-344 (score, 2258), 344-360 (score, 2275), 424-452 (score, 984), and 455-481 (score, 978). These data agree with structural analysis of functional ATPases that are comprised of two monomers with a total of twelve transmembrane domains. While sequence analysis of BspB determined
that the protein does not possess a Sec-signal sequence using SignalP, this finding is also true for the ABC-transporters MetN from *E. coli* and LapB from *P. fluorescens* which is the inner-membrane component of the type I secretion system responsible for LapA secretion (121). The PSORTb algorithm predicts that BspB is an inner-membrane protein (score, 9.82). Based on these observations, we hypothesize that BspB is an inner-membrane ABC transporter that associates with BspA and an additional protein to form a type I secretion system in *B. pseudomallei*.

Another ORF was identified downstream of *blp* that overlaps the 3’ portion of *bspB* by 9 bp (*Figure 2-2B*). This 1413 bp gene has the locus tag BPSL 1665 and was predicted to encode a 51 kDa protein of 471 amino acids. Sequence analysis of this molecule revealed a type I secretion system domain of the HlyD family (TIGR01843) at amino acids 65-471 (NCBI blastp, e-value 8.59e⁻⁸¹). In fact, the protein is very similar to the well characterized type I secretion system MFP in *P. fluorescens* Pf0-1, LapC (e-value 3e⁻⁷⁵), suggesting that BPSL 1665 likely encodes the third component of the secretion apparatus. Therefore, we designated this predicted protein BspC. HlyD-like proteins are membrane-fusion proteins that localize to the periplasm but often possess a transmembrane domain and a small cytoplasmic domain (244). We analyzed BspC using TMpred and found that the molecule contains two putative transmembrane domains at amino acids 6-24 (score, 505) and 61-80 (score, 2441). Analysis of *E. coli* HlyD revealed the presence of only one transmembrane domain corresponding to amino acids 59-78 (score, 2624). While this prediction software did identify two potential transmembrane domains in BspC, it is worth noting that while the putative transmembrane domain at amino acids 61-80 scored similarly to the transmembrane
domain in HlyD (BspC 2441 vs HlyD 2624), the BspC putative transmembrane domain at amino acids 6-24 had a low score (505). This suggests that this predicted domain at amino acids 6-24 is much less likely to encode an actual transmembrane domain. Based on the sequence similarity to \textit{E. coli} HlyD, we hypothesize that \textit{B. pseudomallei} BspC contains only one actual transmembrane domain at amino acids 61-80. Additional analysis of BspC using PSORTb revealed that BspC is predicted to localize to the inner-membrane (score, 9.82). These results are contrary to our hypothesis that BspC functions in the periplasm as part of a T1SS. The sequence of \textit{E. coli} HlyD was analyzed by PSORTb and was also predicted to be localized to the inner membrane. As this protein has been shown to localize to the periplasm, it appears that PSORTb does not accurately predict the location of MFPs similar to HlyD. It is likely, therefore, that \textit{B. pseudomallei} BspC localizes to the periplasm. Further analysis by SignalP predicts that BspC possesses a Sec-signal sequence with a cleavage site in between amino acids 17 and 18 (LA-DA, probability 0.608). Based on the results described above along with the strong similarity to HlyD, we hypothesize that BspC associates with BspA and BspB to form a functional type I secretion system in \textit{B. pseudomallei}.

As with \textit{blp}, all of the sequenced \textit{B. pseudomallei} strains possess the \textit{bspA}, \textit{bspB}, and \textit{bspC} genes, and the encoded proteins from these different strains are 98-100% identical to BspA, BspB, and BspC from strain K96243. We conclude that the proteins encoded by this genetic locus, especially the putative outer membrane protein, BspA, may be useful for the development of a \textit{B. pseudomallei}-specific vaccine, therapeutic treatment, or diagnostic tool.
In summary, *B. pseudomallei* possesses a genetic locus encoding a potential biofilm-associated protein which is flanked by genes that putatively encode the components of a T1SS. In *P. fluorescens*, the Bap-like protein, LapA, is dependent on the expression of specific proteins that form a T1SS in order for LapA to be surface associated and for biofilm formation. Additionally, the proteins that form this type I secretion system are encoded by genes immediately downstream of *lapA* in the chromosome of *P. fluorescens*. Therefore, it is our hypothesis that BspA, BspB, and BspC form the type I secretion system responsible for the export of Blp.

An additional 873 bp ORF from *B. pseudomallei* K96243 with the locus tag BPSL 1663 was located downstream of *blp* and upstream of *bspB*. This gene was found in all currently available *B. pseudomallei* genomes and the predicted proteins are 99-100% identical. The encoded protein from *B. pseudomallei* K96243 was predicted to be 291 amino acids and approximately 33 kDa and sequence analysis using the Pfam database revealed the presence of a sulfotransferase domain (PF00685) at amino acids 5-197 (e-value $2.7e^{-97}$). Additionally, significant similarity was found to a sulfotransferase from *P. fluorescens* Pf0-1 (NCBI blastp, e-value $2e^{-62}$). This *P. fluorescens* sulfotransferase is not encoded in the same locus as *lapA*. Sulfotransferase proteins have been well characterized in eukaryotic systems but have also been described in several prokaryotes. In *Mesorhizobium loti*, a sulfotransferase is involved in modification of capsular polysaccharide (280). In *Mycobacteria tuberculosis* a sulfotransferase is responsible for the modification of a trehalose glycolipid on the surface of the bacteria (226). Also, in uropathogenic *Escherichia coli*, a sulfotransferase is located in the periplasm with two other proteins that are collectively involved in disulfide bond formation (108). As BPSL
1663 is downstream of blp and upstream of genes that we hypothesize are important for Blp secretion to the surface, and as the protein appears to be a sulfotransferase, we designated this molecule Blp-modification protein (Bmp). Because some sulfotransferase enzymes appear to function outside of the inner-membrane, we performed a sequence analysis with SignalP but found no Sec-signal. Additionally, we used PSORTb to determine where Bmp localizes in the bacteria, but the results were unclear as there was low level prediction (score, 2) for all cellular fractions. Bmp was also analyzed by TMpred to determine if the protein may be membrane bound, and it was found that the putative sulfotransferase possesses 3 transmembrane domains from amino acids 3-22 (score, 558), 27-46 (score, 672), and 90-111 (score, 708). While these scores are not very strong (compare to proven transmembrane domain in E. coli HlyD; score, 2624), these results suggest that the protein may localize to the inner membrane to perform its function. Due to its proximity to blp in the chromosome of B. pseudomallei, it is our hypothesis that Bmp may localize to the inner-membrane where it functions to allow Blp to successfully transport and/or function in the outer-membrane.

2.4 Construction of a plasmid containing blp, bspA, bspB, bspC, and bmp

Blp, BspA, BspB, BspC, and Bmp were cloned and sequenced in their entirety from our laboratory strain, B. pseudomallei DD503. This specific strain is hypersensitive to aminoglycosides and macrolides due to a mutation in the AmrAB-OprA efflux pump of the parent strain, B. pseudomallei 1026b, which makes B. pseudomallei DD503 easier...
to manipulate genetically (187). Based on sequence data from *B. pseudomallei* K96243, primers P1 and P2 (Table 2.1) were designed to amplify a PCR product specifying a region located 1 kb upstream of *bspA* (Figure 2-3A). The 3.8 kb piece of DNA was amplified from *B. pseudomallei* DD503 chromosomal DNA using Platinum® Pfx DNA polymerase (Invitrogen™). This blunt-ended PCR product was cloned into the vector, pCC1™ (Epicentre® Biotechnologies), using the CopyControl™ PCR Cloning Kit (Epicentre® Biotechnologies) according to the manufacturer’s recommended instructions. The ligation mixture was electroporated into the *E. coli* cloning strain EPI300™ (Epicentre® Biotechnologies) and colonies were selected for resistance to chloramphenicol (cmR, specified by the vector, pCC1™). Clones containing the 3.8 kb insert from the chromosome of *B. pseudomallei* DD503 were identified by PCR with the cloning primers P1 and P2 (Table 2.1), yielding the plasmid pWG1.

A 0.45 kb zeocin™ (Invitrogen™) resistance (zeoR) cassette was PCR amplified from the plasmid pEM7/Zeo (Invitrogen™) using the primers P3 and P4 (Table 2.1). This PCR product was then introduced into a unique XhoI site located in an ORF (K96243 locus tag BPSL 1658a) near the middle of the insert of pWG1. The resulting construct, designated pWG2, was digested with BamHI and a 4.2 kb insert corresponding to the 3.8 kb DNA fragment upstream of the DD503 *bspA* gene interrupted by the zeoR cassette was end-repaired using the End-It™ DNA End-Repair Kit (Epicentre® Biotechnologies) and then subcloned into the EcoRV site of the suicide vector pKAS46. This plasmid contains the R6K origin of replication which functions only in bacteria.
Table 2.1: List of primers and selected characteristics

<table>
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<th>Primer Name</th>
<th>Corresponding gene</th>
<th>Primer</th>
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<th>Function</th>
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<td>Construction of 6X His-fusion proteins</td>
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<td>P33</td>
<td>bspA</td>
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<td>P34</td>
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<tr>
<td>P41</td>
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<td>NA</td>
<td>Subcloning in pWGZABlp</td>
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<tr>
<td>P42</td>
<td>pBHR1 cm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CATGACGTCGAGGGCATGAAGGCCGAG</td>
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<td>P43</td>
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<td>P44</td>
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<td></td>
</tr>
<tr>
<td>P45</td>
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<td>351–621</td>
<td></td>
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<tr>
<td>P46</td>
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<td>P47</td>
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<td>CATGACGTCGAGGGCATGAAGGCCGAG</td>
<td>351–621</td>
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</table>

expressing the replication protein n (252). Therefore, we can maintain pKAS46-based plasmids in the laboratory E. coli strain S17 but not in B. pseudomallei DD503. The
resulting plasmid, pWG3, was introduced into *E. coli* S17 by electroporation and subsequently transferred into *B. pseudomallei* DD503 by conjugation as reported previously (44). Upon conjugation, *B. pseudomallei* colonies were first selected for resistance to polymyxin B (prevents growth of *E. coli* S17) and zeocin™ (to select *B. pseudomallei* clones containing the DNA fragment upstream of the *bspA* gene interrupted by the zeo<sup>R</sup> cassette). These putative mutants were then tested for their sensitivity to
kanamycin, which identified strains that did not contain the suicide vector pKAS46 integrated into their genome. Lastly, these polymyxin $B^R$, zeocin$^R$, kanamycin$^S$ transconjugates were screened by PCR using primers P1 and P2 to identify the mutant strain DD503.5’junk (Figure 2-3A). PCR using these cloning primers yielded a DNA fragment of 3.8 kb in $B. pseudomallei$ DD503 and 4.2 kb in the mutant $B. pseudomallei$ DD503.5’junk, suggesting the zeo$^R$ cassette inserted in the intended location, which was verified by sequencing.

Chromosomal DNA was isolated from $B. pseudomallei$ DD503.5’junk and was used to construct a plasmid-based library of large DNA fragments using the CopyControl™ Fosmid Library Kit (Epicentre® Biotechnologies) under the recommended conditions. This library was introduced into the $E. coli$ cloning strain EPI300™ (Epicentre® Biotechnologies), and recombinant clones were selected for resistance to chloramphenicol (specified by the vector pCC1™) and zeocin™ (specified by DD503.5’junk DNA). zeo$^R$cm$^R$ colonies were screened by PCR using primers P5 and P6 for $bspA$, P7 and P8 for $blp$, P9 and P10 for $bmp$, P11 and P12 for $bspB$, and P13 and P14 for $bspC$ identifying the plasmid pWGEcBlp, which was found to contain a 41 kb fragment encompassing all five of the genes (Figure 2-3B).

Both strands of the locus containing $bspA$, $blp$, $bmp$, $bspB$, and $bspC$ were sequenced from pWGEcBlp. Results of multiple sequence alignments using ClustalW determined that BspA, Blp, and BspB from $B. pseudomallei$ DD503 are 99% identical at the amino acid level to their respective proteins in $B. pseudomallei$ K96243. Bmp and BspC were found to be 100% identical between the two strains. Therefore, we constructed a plasmid from our laboratory strain, $B. pseudomallei$ DD503, containing $blp$, $bmp$, $bspA$, $bspB$, and $bspC$. This plasmid, pWGEcBlp, was used to transform $E. coli$ DH5α for further experiments.
*bspA*, *bspB*, *bspC*, and *bmp* and the encoded proteins share very high similarity to their respective molecules in *B. pseudomallei* K96243.

Sequence analysis of the predicted proteins in *B. pseudomallei* DD503 revealed some differences in the predicted size compared to these molecules in *B. pseudomallei* K96243. Importantly, all of the structural and functional domains described for the K96243 proteins were present in the DD503 proteins. It was determined that the BspA protein in *B. pseudomallei* DD503 is 643 aa and 70 kDa. The Blp protein is the same size in both K96243 and DD503. The Bmp molecule was found to be 325 aa and approximately 37 kDa. Analysis of the predicted DD503 ORF by Vector NTI® Software (Invitrogen™) suggests that the 3’ end of *bmp* overlaps the 5’ end of *bspB* by 73 bp. The BspB protein was shown to be 800 amino acids (87 kDa) and the BspC protein was found to be 471 aa (51 kDa). Analysis of the predicted DD503 ORFs by Vector NTI® suggests that the 3’ end of *bspB* overlaps the 5’ end of *bspC* by 6 bp.

### 2.5 Production of Antibodies to Study Expression of *Burkholderia pseudomallei* Proteins

A 0.8 kb PCR product encoding amino acids 351-621 of *B. pseudomallei* DD503 BspA was amplified with primers P17 and P18 (Tables 2.1 and 2.2) and cloned into the *AatII* and *PacI* sites of the expression vector pETcoco™-1 (Novagen), which specifies an N-terminal 6x-histidine affinity tag. The resulting plasmid, designated pWG5, was
Table 2.2: Information relevant to creation of 6x Histidine-fusion proteins

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>PCR product size (kb)</th>
<th>Restriction enzymes used for cloning into pETcoco-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>bspA</td>
<td>P17 and P18</td>
<td>0.8</td>
<td>AatII, PacI</td>
</tr>
<tr>
<td>blp</td>
<td>P19 and P20</td>
<td>1.9</td>
<td>HindIII, PacI</td>
</tr>
<tr>
<td>bmp</td>
<td>P21 and P22</td>
<td>1</td>
<td>AatII, PacI</td>
</tr>
<tr>
<td>bspB</td>
<td>P23 and P24</td>
<td>0.9</td>
<td>AatII, PacI</td>
</tr>
<tr>
<td>bspC</td>
<td>P25 and P26</td>
<td>1</td>
<td>AatII, PacI</td>
</tr>
<tr>
<td>omp5</td>
<td>P27 and P28</td>
<td>0.9</td>
<td>NheI, PacI</td>
</tr>
</tbody>
</table>

sequenced to verify that BspA was properly joined to the six N-terminal histidine residues and that no unwanted mutations were introduced during PCR. The same region of *B. pseudomallei* DD503 bspA was amplified using primers P29 and P30 (Tables 2.1 and 2.3) and cloned into the *BamHI* and *NotI* sites of the expression vector pGEX4T-2 (GE Healthcare LifeSciences), which specifies an N-terminal Glutathione – S – Transferase (GST) affinity tag. The resulting plasmid, pWG6, was also sequenced to verify PCR fidelity and proper fusion of BspA to its GST tag. The plasmids were introduced into the *E. coli* strain TUNER (Novagen) for the purpose of overexpressing recombinant proteins per the manufacturer’s recommended procedure. The
overexpressed His-BspA protein was extracted from inclusion bodies using the Bugbuster® HT Protein Extraction Reagent (Novagen) and rLysozyme™ Solution (Novagen). The recombinant protein was purified under denaturing conditions with the His-Bind® Resin System (Novagen) per the manufacturer’s instructions. The composition of the refolding buffer was determined using the AthenaES™ QuickFold™ protein refolding kit (Athena Enzyme Systems™) and urea was gradually removed by dialyzing the purified recombinant protein preparations at 4°C as previously reported (170). The purified His-BspA protein was emulsified in Freund’s adjuvants and used to immunize BALB/c mice as previously reported (170). Immune serum was then tested by western blot using cell lysates of *E. coli* TUNER overexpressing the GST-BspA protein. This analysis revealed the presence of BspA-specific antibodies (data not shown).

A similar strategy was used to generate His- and GST-tagged proteins specifying Blp, Bmp, BspB, and BspC. Specific differences (*e.g.* primers used, restriction sites used for cloning) are listed in Tables 2.1, 2.2, and 2.3. We were able to generate polyclonal antibodies (pAbs) specifically recognizing the Blp, Bmp, and BspC proteins fused to the GST-tag (data not shown). Our attempts at generating antibodies directed against BspB using purified His-BspB proved unsuccessful as the antibodies showed no reactivity with purified GST-BspB. Another attempt to generate antibodies against BspB involved ordering a synthetic peptide from GenScript corresponding to residues 176-195 (CATGLCQYFEYRQDQVPTGVG). This region of the protein was chosen for peptide synthesis because it is likely a soluble peptide due to its hydrophilic nature. This peptide was conjugated to keyhole lymphocyte hemocyanin (KLH) or ovalbumin (OVA) with the Imject® Maleimide Activated Immunogen Conjugation Kit (Pierce) using the N-terminal
cysteine of the peptide for disulfide bond formation. The KLH-peptide conjugate was
then used to immunize mice but antibodies did not react against the OVA-peptide
conjugate or lysates of *E. coli* expressing GST-BspB. For an alternate approach, GST-
BspB was purified as inclusion bodies from *E. coli* TUNER cells harboring pWG14. The
proteins were then used to immunize mice along with a second synthetic peptide-
conjugate. This GenScript synthetic peptide was designed to a second hydrophilic region
of the protein corresponding to residues 396-415 (CMQSSVHRSSQLSMQLGQERH).
This peptide was then conjugated to OVA or KLH as described above. The KLH-peptide
conjugate was then mixed with GST-BspB, and injected into mice as previously
described. Antibodies were collected and tested against the OVA-peptide conjugate or
lysates of *E. coli* expressing GST-BspB, but we were unable to detect expression of
BspB. One study has been published suggesting that post-transcriptional expression of
ATPase proteins is limited to low levels by mRNA structures corresponding to membrane
domains within these proteins (25). It is important that the regions of BspB that were
used in the generation of anti-BspB antibodies were mostly from peptides not predicted
to be a part of any transmembrane domain. As the binding and hydrolysis of ATP is a
normal process in eukaryotic cells, it is also possible that many of the domains in BspB
including the ATP-binding/hydrolysis domains are epitopes that are not recognized by
immune serum due to negative selection of T-cell precursors during maturation.
Regardless of the reason, anti-BspB antibodies were not able to be produced by any of
the immunization strategies.

Initial analysis of anti-BspC pAbs showed high levels of cross-reactivity with *E.
coli* cell lysates which complicated efforts to detect GST-BspC (data not shown). In
order to maximize BspC specificity, non-specific antibodies were removed with successive incubations with PVDF membranes containing lysates of *E. coli* harboring pGEX4T-2 (*i.e.* expressing GST only), and these adsorbed antibodies showed greater specificity for GST-BspC (data not shown). When these antibodies were used to probe protein preparations from recombinant *E. coli* expressing the other proteins encoded on pWGEcBlp, BspC was undetectable. Unfortunately, a cross-reactive band appeared at the approximate molecular weight of BspC (~50 kDa). In an attempt to generate antibodies that would lack this cross-reactivity, a synthetic peptide was ordered from GenScript corresponding to residues 201-220 of BspC (CRAAKQAEAAADYRGRIPQYVN). This peptide was conjugated to KLH and injected into mice as described above for BspB. Antibodies were recovered from mice and probed against the OVA-peptide conjugate, lysates of recombinant *E. coli* pWGEcBlp, and lysates of *B. thailandensis* harboring the plasmid pWGBtBlp, which also harbors all 5 genes. While we were unable to detect expression of BspC with the OVA-peptide conjugate or lysates from *E. coli* pWGEcBlp, we were able to detect expression of a 50 kDa protein in *B. thailandensis* pWGBtBlp that corresponds to the predicted size of BspC (data not shown).

Through collaboration with Dr. Jeff Hogan’s laboratory at the University of Georgia, monoclonal Abs (mAbs) were raised against Blp. Specifically, these antibodies were generated by immunizing mice with a synthesized peptide from GenScript with sequence (CATHYDFNGDGRGDLLWADGGTNMGN). This peptide corresponds to residues 2600-2625 that contain the Arg-Gly-Asp motif predicted to be an important adherence domain (*Figure 2-1, blue oval*). Antibodies directed against this region may
have the added benefit of disrupting the ability of Blp to mediate adherence by blocking the interaction between Blp and specific host receptors. This peptide was conjugated to KLH as described above for KLH-BspB and used to immunize mice. Spleen cells from these mice were fused with myeloma cells to create hybridomas. Supernatants, which contain the antibodies produced by the hybridomas, were collected and screened by ELISA using the OVA-peptide conjugate as the target. However, the monoclonal antibodies directed against the KLH-peptide conjugate did not react specifically against the OVA-peptide conjugate.

In a second attempt to obtain mAbs against Blp, we purified the His-Blp fusion construct from inclusion bodies of *E. coli* TUNER harboring pWG7, which expresses the 6x His-tag fused to residues 222-842 of Blp. These experiments were performed as described above for the generation of His-BspA. This fusion construct was used to immunize mice and spleen cells were collected and fused with a myeloma cell line to create hybridomas-secreting monoclonal antibodies. Supernatants from these cell lines were collected and screened by ELISA using purified GST-Blp from *E. coli* TUNER harboring pWG12 as the target. The ELISA results suggested that there was specific reactivity of the mAbs from several hybridomas to Blp. As the amount of antibody needed to detect GST-Blp was high, we used Amicon Ultra-15 Centrifugation Filter Units (Millipore™) to concentrate 15 mLs of supernatants into ~500 uL. Western blot analysis was then performed using lysates of *E. coli* pWGEcBlp and the concentrated mAbs which revealed that these antibodies did react against a very large protein in between 268 and 460 kDa (data not shown). Therefore, we were able to generate several hybridomas which produce mAbs that react against Blp.
In summary, we were able to generate polyclonal antibodies against Blp, BspA, BspC, and Bmp. After many attempts, we were unable to obtain antibodies against BspB. While we were unable to generate mAbs against KLH conjugated to a portion of Blp containing the RGD motif, we were able to create hybridomas that produce monoclonal antibodies against Blp by using the 6x histidine tag fused to a portion of the N-terminal region of Blp. As Blp, BspA, BspC, and Bmp are found in strains of \textit{B. pseudomallei} but not \textit{B. thailandensis} or \textit{B. mallei}, we believe that these antibodies could be valuable as tools for the detection of \textit{B. pseudomallei} in environmental or clinical samples.

2.6 Characterization of Recombinant \textit{Escherichia coli}

Expressing \textit{Burkholderia pseudomallei} Proteins

It is our hypothesis that BspA, BspB, and BspC form a type I secretion system that, in partnership with Bmp, exports Blp to the bacterial surface in a functional state where it can play a role in biofilm formation and adherence. In order to test this hypothesis, we studied \textit{E. coli} EPI300™ harboring pWGEcBlp (see above), as this strain possesses all five genes from \textit{B. pseudomallei} cloned into the CopyControl™ vector, pCC1™ (Epicentre® Biotechnologies). One advantage of using this cloning vector is that its copy number can be easily amplified from 1 per cell to > 25 copies per cell by growing the \textit{E. coli} strains in the presence of CopyControl™ Induction Solution (Epicentre® Biotechnology), which causes \textit{E. coli} EPI300™ to initiate replication of the plasmid from its ori\text{V} high-copy origin of replication. Thus, growth of \textit{E. coli} in the
presence of the induction solution favors increased protein expression. This ability to
switch from low to high copy number facilitates the cloning and expression of otherwise
toxic proteins.

The polyclonal antibodies that were generated against BspA, Blp, Bmp, BspB and
BspC were used to study the expression of the *B. pseudomallei* proteins in *E. coli*. It is
our hypothesis that Blp is a surface-associated protein that is transported by BspA, BspB,
BspC. Based on sequence analysis, we also hypothesize that BspA is surface associated
while Bmp, BspB and BspC are associated with the inner membrane. In order to gain
more insight on the cellular location of these proteins, whole cell lysates, total membrane
and sarkosyl-insoluble outer-membrane proteins were prepared from *E. coli* harboring
pCC1.3 or pWGEcBlp grown in LB broth containing the CopyControl™ Induction
Solution (Epicentre® Biotechnologies). Whole cell lysates revealed that bacteria grown
in the presence of the induction solution express significantly more protein than bacteria
grown without the induction solution, and in some cases, such as with Blp, we were
unable to detect protein expression unless the bacteria were grown with the induction
solution (Figure 2-4). Total membrane and sarkosyl-insoluble outer membrane
preparations were prepared as described previously (48). Overnight cultures of bacteria
were collected, sonicated, and then subjected to low speed centrifugation (4,000 x g) to
remove all large membrane fragments or intact cells. The supernatant was then
centrifuged at 21,000 x g to pellet inner and outer membrane proteins. This total
membrane protein pellet was resuspended in a solution containing 2% sarkosyl and will
Figure 2-4: *E. coli* strains were grown with or without the induction solution before whole cell lysates were generated and analyzed by western blot using anti-Blp antibodies. be referred to as the total membrane fraction. This suspension was then centrifuged again at high speed to pellet the outer membrane proteins which are insoluble in the sarkosyl solution. The whole cell lysates, total membrane protein preparations, and sarkosyl-insoluble outer membrane protein preparations were then analyzed by western blot. We discovered that *E. coli* harboring pWGEcBlp expressed a 70 kDa outer membrane protein reactive with the anti-BspA antibodies (Figure 2-5A), which is of the correct size based on the predicted molecular weight (MW) of the *B. pseudomallei* DD503 *bspA* gene product (predicted MW=69.7 kDa). We also discovered that *E. coli* pWGEcBlp expresses a very large protein of between 268 and 460 kDa that is reactive with anti-Blp antibodies (Figure 2-5B), which also localizes in the outer membrane of recombinant *E. coli*. The predicted MW of the *B. pseudomallei* DD503 *blp* gene product is 325 kDa. As shown in Figure 2-5C, *E. coli* pWGEcBlp expresses a ~ 35 kDa protein that is reactive with anti-Bmp antibodies (predicted MW=36.8 kDa). The Bmp protein appears to localize to the inner-membrane of recombinant *E. coli* as it was detected in total-membrane fractions but not in the sarkosyl-insoluble outer-membrane preparations (Figure 2-5C). Western blot analysis of whole cell lysates, total membrane protein preparations, and sarkosyl-insoluble outer membrane preparations using antibodies
Figure 2-5: Whole cell lysates (WCL), total membrane associated protein fractions (TM), and outer membrane associated protein fractions (OM) were analyzed by western blot using antibodies directed against BspA (A), Blp (B), and Bmp (C).

directed against BspB and BspC did not result in the detection of bands specific to *E. coli* pWGEcBlp. Taken together, these results indicate that recombinant *E. coli* harboring pWGEcBlp expresses BspA and Blp in the outer membrane, and Bmp in the inner membrane of the bacteria. These results are consistent with our hypotheses about the predicted localization of BspA, Blp, and Bmp.

Immunofluorescence was also used to study surface expression of the *B. pseudomallei* gene products in *E. coli*. Cultures of *E. coli* EPI300™ harboring pCC1.3 or pWGEcBlp were heat fixed to the surface of a glass slide, treated with 4% paraformaldehyde, blocked with 10% goat serum (Sigma-Aldrich®), and then probed
with polyclonal antibodies directed against BspA, Blp, or Bmp. Dilutions of primary antibody ranged from 1:100 to 1:5000. These slides were then washed, incubated with AlexaFlour® 488 goat anti-mouse secondary antibody (Molecular Probes®), washed again, and then coverslips were added over SlowFade® Gold antifade reagent containing DAPI (Invitrogen™). The coverslips were then sealed and the slides observed by microscopy using a Zeiss LSM 510 Meta Confocal System. Using this approach, we were unable to detect a difference in the immunofluorescence of *E. coli* pCC1.3 compared to *E. coli* pWGEcBlp, suggesting that the anti-BspA, anti-Blp, and anti-Bmp polyclonal antibodies may not be amenable for detecting expression of these proteins on the surface by immunofluorescence or that the proteins are not surface exposed. Similar attempts to detect Blp expression on the surface of *E. coli* were performed using anti-Blp monoclonal antibodies; however we were unable to detect expression of surface exposed Blp in *E. coli* pWGEcBlp even when using antibodies at dilutions as low as 1:10. While we were unable to detect differences in expression of surface bound proteins in *E. coli* pWGEcBlp, we observed a distinct difference in the size of the bacteria on the slides. When visualizing the bacteria stained with DAPI (Figure 2-6A) or probed with antibody (data not shown), *E. coli* pCC1.3 appeared as rod-shaped bacteria of ~2 µm in length which is the normal size of *E. coli*. However, *E. coli* pWGEcBlp appeared as very long rod-shaped bacteria that appeared to be several times longer than *E. coli* pCC1.3.

An ELISA was also used to detect surface expression of the *B. pseudomallei* proteins in *E. coli*. EPI300™ cells harboring either pCC1.3 or pWGEcBlp were grown on agar plates containing the CopyControl™ Induction Solution (Epicentre® Biotechnologies), suspended to a Klett™ 230 (~10⁹ bacteria per mL) and inoculated into
Figure 2-6: *E. coli* harboring pCC1.3 or pWGEcBlp were examined by immunofluorescence (A). Images are representative images of bacteria stained with DAPI to illustrate the difference in size of *E. coli* pWGEcBlp compared to *E. coli* pCC1.3. The relative growth of the two strains was then assessed by performing a growth curve in LB containing the induction solution (B). * indicates that the difference between *E. coli* pWGEcBlp and *E. coli* pCC1.3 is statistically different (p < 0.05).

Individual wells of a 96 well tissue culture plate. After an overnight incubation to allow the bacterial suspension to dry on the plastic, the bacteria were blocked with 3% milk, probed with our collection of antibodies, and then incubated with goat anti-mouse Ig (H+L) conjugated to horseradish peroxidase (Southern Biotech). All wells were then treated with SureBlue™ TMB Microwell Peroxidase Substrate (KPL) and absorbance was measured using an ELISA plate reader at 630 nm. In wells containing *E. coli*
pWGEcBlp, there were modestly higher levels of absorbance compared to *E. coli* pCC1.3 when probed with anti-BspA, anti-Blp, or anti-Bmp antibodies (data not shown); however, the amount of cross-reactivity of the antibodies limited the conclusions that could be made from these results. Additionally, as we observed an increase in absorbance in wells probed with the anti-Bmp antibodies and this ELISA should only allow for detection of surface exposed proteins, these results would argue against the results of the sarkosyl-insoluble outer membrane preparations where the Bmp protein was not found to be associated with the outer membrane.

Another approach to detect expression of surface associated proteins was performed using a Proteinase K protection assay as described previously (42) with some modifications. *E. coli* pCC1.3 and pWGEcBlp were grown on agar plates containing the CopyControl™ Induction Solution (Epicentre® Biotechnologies) and suspended to a Klett™ 300 (~10^10 bacteria/mL) in PBSG. Five hundred µL of these suspensions were processed as normal whole cell lysates while another 500 µL was treated with 0.25 µg/mL Proteinase K (Roche) and incubated at 37°C. At the indicated times, 10 mM PMSF (Sigma-Aldrich) was added to the suspensions to inhibit further protein degradation. These suspensions were then centrifuged to pellet the bacteria and resuspended in SDS-buffer to be analyzed by western blot. In these experiments, proteins that are exposed on the surface are degraded by the Proteinase K, while proteins that are in the cytoplasm, inner membrane, and periplasm will be protected from degradation. It is possible that outer membrane pore-forming proteins may also be protected as very little of these proteins are needed to be exposed on the surface of the bacteria in order for them to function. As can be seen in Figure 2-7, Blp is the only
Figure 2-7: *E. coli* pCC1.3 or pWGEcBlp were treated with Proteinase K for the indicated times after which PMSF was added to stop protease action. Suspensions were then pelleted, resuspended in SDS-buffer and then analyzed by western blot using antibodies against BspA, Blp or Bmp.

protein that appears to be surface exposed as the protein is not detected in samples treated with the Proteinase K for any amount of time. Bmp, observed under a cross-reacting band, is detected in all samples suggesting that the Proteinase K is not gaining access to the interior of the cell even after 3 h (Figure 2-7). The monomeric form of BspA (64 kDa) was similarly unaffected by the action of the Proteinase K; however, the intensity of the oligomeric band (130 kDa) appears to decrease with increased exposure to the Proteinase K and a slighter smaller band appears to increase in intensity over the same time (Figure 2-7). It is worth noting that BspA is predicted to be an outer membrane protein, but if its structure is similar to *E. coli* TolC, then the vast majority of the protein is located in the periplasm and only several transmembrane domains anchor the protein to the outer membrane (153). The Proteinase K protection assay suggests that some degradation is occurring on a complex involving BspA, but the exact mechanism of
action is unknown as monomeric BspA remains unaffected in the presence of the Proteinase K.

In summary, sarkosyl-insoluble outer membrane protein preparations, immunofluorescence, an ELISA, and a Proteinase K protection assay were used to detect expression of surface bound proteins in \textit{E. coli}. It was shown that BspA (Figure 2-5A) and Blp (Figure 2-5B) associate with the outer membrane after performing sarkosyl-insoluble outer membrane protein preparations, and that Bmp appears to localize with the inner membrane as the putative sulfotransferase is detected in the total membrane protein fraction but not in the outer membrane protein fraction (Figure 2-5C). The Proteinase K protection assay confirmed that Blp appears to be surface associated but was unable to conclusively show that BspA was also surface exposed (Figure 2-7). ELISA results also suggested that BspA and Blp are on the surface of \textit{E. coli} as wells probed with antibodies against these proteins showed modest increases in the levels of absorbance (data not shown), however, wells probed with antibodies against Bmp also showed modest increases in absorbance (data not shown). The results of these ELISAs suggest that the antibodies against BspA, Blp, and Bmp are cross-reactive with the negative control, \textit{E. coli} pCC1.3, and that this assay may not able to discriminate between proteins localized to different cellular fractions. We also performed immunofluorescence, but the antibodies were too cross-reactive which restricted any interpretations that could be made about the surface localization of BspA, Blp, or Bmp. Visualization of the bacteria under the microscope, however, did allow us to observe that \textit{E. coli} pWGEcBlp appear larger in size compared to \textit{E. coli} pCC1.3 (Figure 2-6A) suggesting that expression of the proteins encoded by pWGEcBlp may have a negative effect on \textit{E. coli} physiology.
2.7 Characterizing the Biological Functions of *Escherichia coli* Expressing the *Burkholderia pseudomallei* Proteins

It is our hypothesis that the *B. pseudomallei* Blp protein is involved in biofilm formation and adherence. This is based on the fact that the biofilm-associated proteins of other bacterial have been shown to mediate these functions for their respective organisms. Furthermore, the Blp protein of *B. pseudomallei* was found to be the only Bap-like protein to contain an Arg-Gly-Asp motif, which has been shown to be an important integrin-binding domain for several characterized adhesins (166, 224). In order to test the hypothesis that Blp is involved in adherence, we first wanted to test the ability of *E. coli* harboring pCC1.3 or pWGEcBlp to bind to the human extracellular matrix proteins type I collagen (COL1A2), type IV collagen (COL4A1), laminin (LAMA1) and fibronectin (FN1). Bacteria grown on agar plates supplemented with the CopyControl™ Induction Solution (Epicentre® Biotechnologies) were suspended to an optical density of 230 Klett™ units, and 25 μL of these suspensions (~10^7 bacteria) was added to duplicate wells of 24-well TC plates coated with either type I collagen, type IV collagen, laminin or fibronectin (BD Bioware™). Dilutions of each bacterial suspension were spread on agar plates and grown overnight to enumerate the number of colony-forming units (CFU) in the inoculum. The tissue culture plates were centrifuged at 165 x g for 5 min and placed in a 37°C incubator with 7.5% CO₂ for 3 h. To remove unbound bacteria, each infected well was washed 4 times with phosphate-buffered saline supplemented with gelatin (PBSG), fixed with methanol and stained with 5% Giemsa. Four individual fields from each well were visualized under 100X magnification using a
Zeiss microscope. Bacteria were counted by eye, and then normalized to the inoculum.

As shown in Figure 2-8C, there was significantly more \emph{E. coli} pWGEcBlp that adhered to the fibronectin-coated wells compared to \emph{E. coli} pCC1.3. This suggests that Blp is involved in attachment to fibronectin. As when we observed these bacteria by immunofluorescence, there was a clear difference in the size of the bacteria that we could observe by microscopy following staining with Giemsa. \emph{E. coli} cells harboring pCC1.3 appear normal (Figure 2-8A); however \emph{E. coli} harboring pWGEcBlp appear as very long rods (Figure 2-8B). We did not measure an increase in binding to type I collagen, type IV collagen or laminin (data not shown).
As *B. pseudomallei* causes severe disease following inhalation of aerosolized bacteria, we wanted to assess the ability of *E. coli* expressing BspA, Blp, Bmp, BspB, and BspC to adhere to human respiratory epithelial cells. Bacteria were grown on agar plates containing the induction solution and prepared as described above for the adherence assays to the extracellular matrix proteins. *E. coli* harboring pCC1.3 or pWGEcBlp were then inoculated into wells containing monolayers of A549 cells (human type II pneumocytes). The number of viable bacteria adhering to the A549 cells was measured by washing the monolayers 4 times with PBSG and lysing the epithelial cells with a solution containing 0.005% saponin. These suspensions were then diluted and spread onto agar plates to enumerate the number of attached bacteria, which was then normalized by the inoculum. As shown in Figure 2-9, *E. coli* pWGEcBlp had significantly increased levels of adherence to A549 cells compared to *E. coli* pCC1.3, which supports our hypothesis that the *B. pseudomallei* gene products are involved in adherence. As a positive control in these experiments, we used an *E. coli* strain (S62N) that expresses the *Moraxella catarrhalis* adhesin, McaP, that mediates attachment to epithelial cells (170).

![Figure 2-9: Analysis of *E. coli* binding to A549 pneumocytes. * indicates that the difference between *E. coli* pWGEcBlp and pCC1.3 is statistically different (p < 0.05).](image-url)
While performing these experiments, we noticed discrepancies between *E. coli* pCC1.3 and *E. coli* pWGEcBlp in the number of viable organisms that could be enumerated from cultures of the same optical densities. These observations led us to measure the growth of *E. coli* EPI300™ harboring pCC1.3 or pWGEcBlp in the presence of the CopyControl™ Induction Solution (Epicentre® Biotechnologies). These bacteria were grown overnight on agar plates and then resuspended to 50 Klett™ units in LB containing the induction solution. The two strains were grown at 37°C for 6 h at 200 rpm. At each hour, the growth of the bacteria was assessed by measuring the Klett™ value of the culture. As can be seen in Figure 2-6B, the growth of *E. coli* pWGEcBlp is retarded compared to *E. coli* pCC1.3 and this difference is statistically different beginning at just 2 h of growth. Alternatively, these bacteria were grown overnight on agar plates supplemented with 0, 3, 10, 30 (normal amount added), or 120 μL of the induction solution and then resuspended to 230 Klett™ units. Dilutions of these bacteria were then spread on agar plates to determine the number of viable bacteria present in the suspension. The results of these experiments indicated that *E. coli* harboring pWGEcBlp grown in the presence of any amount of induction solution had at least 5 times fewer viable bacteria as did *E. coli* EPI300™ harboring pCC1.3 (data not shown). These results, in addition to the microscopic observations of the *E. coli* cells (see *E. coli* expression studies above), suggests that expression of one or more of the proteins encoded on pWGEcBlp causes severe disturbances to the normal physiology of the bacteria. This finding complicates the interpretation of the results of the viable adherence assays shown in Figure 2-9. It is possible that higher levels of adherence would be observed for *E. coli* pWGEcBlp when compared to *E. coli* pCC1.3 if the bacteria were dividing properly.
Studies were also conducted to investigate whether the *B. pseudomallei* gene products enable *E. coli* to produce a biofilm. *E. coli* harboring either pWGEcBlp or pCC1.3 were grown statically in LB in a 24 well tissue culture plate (Cellstar) with and without induction solution for 24, 48, and 72 h and biofilm production was assessed by crystal violet staining. Compared to our positive control, pSLBp5, which forms biofilm in *E. coli* by expressing the *B. pseudomallei* adhesin, BoaC (Dr. Eric Lafontaine, unpublished data), *E. coli* pWGEcBlp produced significantly less biofilm, comparable to the biofilm production of the negative control, *E. coli* pCC1.3 (Figure 2-10). It is possible that biofilm is not produced in *E. coli* pWGEcBlp because of the abnormal growth discussed above for this strain.

![Figure 2-10: Analysis of biofilm formation in *E. coli*. Biofilm was stained with crystal violet which was extracted with ethanol and the absorbance was determined using a plate reader at 570 nm. * indicates that the difference between *E. coli* pWGEcBlp and pCC1.3 is statistically different (p < 0.05).](image)

At the C-terminal region of *B. pseudomallei* Blp is a hemolysin-type calcium binding domain (PS00330) that was identified by Prosite at amino acid residues 3004-3022. Several proteins that contain this domain have been shown to be involved in hemolysis including *E. coli* HlyA (105) and *Pasteurella haemolytica* leukotoxin (189).
To test the hypothesis that Blp is involved in hemolysis, *E. coli* harboring pWGEcBlp or pCC1.3 were grown on blood agar plates (BD – Diagnostic Systems) with and without induction solution. As a positive control, we also grew *Streptococcus agalactiae* on blood agar plates. While the Group B *Streptococcus* strain produced β-hemolysis, *E. coli* harboring pWGEcBlp, grown with or without the induction solution, produced neither alpha or beta-hemolysis (data not shown).

Overall, the results of the adherence assays, biofilm assays, and hemolysis assays suggest that *E. coli* appears to be a poor heterologous expression system for the study of these *B. pseudomallei* gene products. It is evident that overexpression of the proteins encoded on pWGEcBlp leads to a significant defect in growth (Figure 2-6B) which makes the interpretation of the biological assays difficult. It should be noted, however, that even in this imperfect system, expression of BspA, Blp, Bmp, BspB, and BspC still leads to an increase in adherence to epithelial cells and extracellular matrix proteins. While a recombinant system may be very useful for the study of the *B. pseudomallei* proteins, *E. coli* does not appear to be an appropriate organism in which to perform these types of studies.

### 2.8 Construction of *Burkholderia pseudomallei* Mutants

In order to study the function of *Burkholderia pseudomallei* BspA, Blp, Bmp, BspB, and BspC, we created mutant strains of *B. pseudomallei* DD503 containing a zeoR cassette in each of the genes. For *bspA*, primers P5 and P6 (Table 2.1) were used to amplify a 2.5 kb PCR product from *B. pseudomallei* DD503. This PCR product was
cloned into the vector pCC1™ using the CopyControl™ PCR cloning kit (Epicentre® Biotechnologies) as described above for the construction of plasmid pWG1. The zeoR cassette was introduced into a unique Bsu36I site located near the middle of the bspA ORF, yielding the plasmid pWG18 which was sequenced to verify insertion of the zeoR cassette in the intended position. This plasmid was digested with BamHI and a 2.9 kB fragment, corresponding to bspA interrupted by the zeoR cassette, was gel-purified using the High Pure PCR Product Purification Kit (Roche), end-repaired using the End-It™ DNA End-Repair Kit (Epicentre® Biotechnologies), and subcloned in the suicide vector pKAS46 as described above for the plasmid pWG3. The resulting plasmid, which we designated pWG19, was introduced into B. pseudomallei DD503 by conjugation and the mutant strain DD503.bspA was identified as described above for the construction of strain DD503.5’junk. Proper allelic exchange was verified by PCR (data not shown) and Southern blot analysis with probes against bspA (Figure 2-11) as well as the zeoR cassette (data not shown).

Figure 2-11: Southern blot analysis was performed on B. pseudomallei chromosomal preps using biotinylated probes that were generated using primers P5-P14 in Table 2.1. This is a representative Southern blot targeting bspA. The plasmid, pWG18, was used as a reactivity control.
Mutations were also created in the \textit{blp}, \textit{bmp}, \textit{bspB}, and \textit{bspC} genes of \textit{B. pseudomallei} DD503. The process for constructing these mutants followed that given for \textit{bspA}. Specific differences (e.g. primers used to amplify gene-specific fragments, restriction sites in which the \textit{zeo}^R cassette was introduced) are listed in \textbf{Tables 2.1 and 2.4}. Southern blot analysis and PCR were used to confirm inactivation of the intended gene in all mutant strains (data not shown).

Table 2.4: Information relevant to creation of the \textit{B. pseudomallei} mutants

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>PCR product size (kb)</th>
<th>Unique restriction enzyme used for zeo\textsuperscript{R} cassette insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{bspA}</td>
<td>P5 and P6</td>
<td>2.5</td>
<td>\textit{Bsu36I}</td>
</tr>
<tr>
<td>\textit{blp}</td>
<td>P7 and P8</td>
<td>1.8</td>
<td>\textit{XhoI}</td>
</tr>
<tr>
<td>\textit{bmp}</td>
<td>P9 and P10</td>
<td>2.8</td>
<td>\textit{EcoRI}</td>
</tr>
<tr>
<td>\textit{bspB}</td>
<td>P11 and P12</td>
<td>3.7</td>
<td>\textit{AatII}</td>
</tr>
<tr>
<td>\textit{bspC}</td>
<td>P13 and P14</td>
<td>2.8</td>
<td>\textit{XhoI}</td>
</tr>
<tr>
<td>\textit{omp5}</td>
<td>P15 and P16</td>
<td>1.7</td>
<td>\textit{EcoRI}</td>
</tr>
</tbody>
</table>

2.9 Characterizing the Biological Functions of the \textit{Burkholderia pseudomallei} Mutants

It is our hypothesis that Blp is involved in biological functions relevant to pathogenesis by \textit{B. pseudomallei} (\textit{i.e.} adherence, biofilm formation). We also believe that the BspA, BspB, BspC, and Bmp proteins are involved in the surface display and function of Blp. It is our hypothesis that in the absence of BspA, BspB, BspC or Bmp, Blp is unable to function at the surface of the bacteria. The first studies that we conducted were with \textit{B. pseudomallei} DD503 and the \textit{blp} mutant. It is our hypothesis...
that compared to the wild-type strain the *blp* mutant will produce significantly less biofilm and adhere to respiratory epithelial cells at significantly lower levels. Additional functions relevant to pathogenesis such as hemolysis and survival within macrophages will also be tested to fully characterize the function of the Blp protein in *B. pseudomallei*.

After characterizing *B. pseudomallei* Blp, we tested our hypothesis that the accessory proteins (*i.e.* BspA, BspB, BspC, and Bmp) are required for Blp to be transported and functional in the outer membrane of *B. pseudomallei*.

Biofilm-associated proteins have many similarities, but biofilm production is, as the name implies, their predominant attribute. In order to test the hypothesis that Blp is involved in biofilm formation for *B. pseudomallei*, we conducted biofilm assays with DD503 and the *blp* mutant grown in a variety of media shown to enhance biofilm production for other biofilm-associated proteins. Wells containing M9/0.5% casamino acids (CAA)/0.2% glucose, M9/0.4% citrate, or FAB media (15 mM (NH₄)SO₄, 33 mM (Na₂)HPO₄, 20 mM KH₂PO₄, 50 mM NaCl, 0.5% CAA, and 10 mM citrate) (77) supplemented with 1 mM MgCl₂ and/or 10 mM FeCl₃ were inoculated with either DD503 or the *blp* mutant and grown statically for 24, 48, or 72 h at either 37°C or 23°C. At the end of the incubation, culture supernatants were removed and crystal violet was added. After 20 min, the crystal violet was removed, the wells were rinsed with water and the crystal-violet stain was extracted with ethanol. Photographs were taken using a Cohu 4922 8 bit camera and then analyzed by densitometry software (TotalLab TL100, TotalLab). With this analysis, the higher the densitometry score, the more crystal violet that was extracted. In most conditions there was no appreciable difference in the amount of crystal violet extracted in wells inoculated with DD503 or the *blp* mutant strain;
however, the *blp* mutant showed increased biofilm formation when incubated in M9/0.5% CAA/0.2% glucose at 23°C (data not shown) and also in M9/0.4% citrate at 37°C (**Figure 2-12**). While we did not observe a defect in the ability of the DD503.blp strain to form a biofilm, we decided to determine whether any of the other DD503 mutant strains were deficient in their ability to produce biofilm. Experiments were performed as described above for the *blp* mutant strain. As with DD503.blp, most conditions yielded no defects in the mutant strains abilities to for biofilm. In addition, biofilm production in M9/0.5% CAA/0.2% glucose at 23°C and also in M9/0.4% citrate at 37°C revealed an increase in the amount of biofilm produced by DD503.bmp. Growth under these specific conditions did not yield an increase or decrease in biofilm formation in DD503.bspA, DD503.bspB, or DD503.bspC (**Figure 2-12**).

![Figure 2-12: Biofilm production in *B. pseudomallei* DD503 and the DD503 mutant strains were assessed by growing the bacteria statically in M9 broth supplemented with 0.4% citrate and then staining the biofilm with crystal violet. * indicates that the difference between the indicated mutant strain and DD503 is statistically different (p < 0.05).](image)

Studies have shown that many biofilm-associated proteins such as R28 of *Streptococcus pyogenes* (260) and Esp of *Enterococcus faecium* (289) are directly
involved in adherence to various surfaces. Additionally, *B. pseudomallei* Blp contains an Arg-Gly-Asp domain in its C-terminus that has been shown to be an important integrin-binding domain for many adhesins (232). Therefore, it is our hypothesis that Blp mediates adherence to human cells and extracellular matrix proteins. The ability of the *B. pseudomallei* DD503.blp strain to bind to A549 cells was tested using viable adherence assays. The *B. pseudomallei* strains were grown on Tryptic Soy Agar (TSA) plates overnight and were used to inoculate monolayers of A549 cells as described above for recombinant *E. coli*. Bacteria were inoculated with the epithelial cells for 3 h prior to washing off unbound bacteria and plating dilutions of well contents to enumerate viable *Burkholderia* cells that had been bound to the A549 pneumocytes. No differences were observed between the adherence of wild-type DD503 and the DD503.blp mutant strain (Figure 2-13). One possible explanation for this lack of phenotype is that there were too many bacteria inoculated onto the A549 cells, so we lowered the MOI from 100:1 to 10:1 and 1:1 but this also resulted in similar levels of adherence between the wild-type and the blp mutant strain (data not shown). Previous studies with *Pseudomonas* and *Burkholderia* spp. grown under iron-limiting conditions, such as with dialyzed chelated...
tryptic soy broth (TSBDC), have shown an upregulation of many virulence factors (169, 200). These growth conditions emulate infection in a host where iron availability is restricted. Therefore, we grew *B. pseudomallei* DD503 and the *blp* mutant in TSBDC and on TSBDC plates and performed adherence assays as described above. The results of these assays were the same as before; DD503 and DD503.blp adhered to the A549 monolayers at similar levels (data not shown). In order to determine whether BspA, Bmp, BspB, or BspC may be involved in adherence to host cells, we also performed adherence assays with the DD503 mutants but found that there were similar levels of adherence in all strains tested compared to the wild-type DD503 (data not shown).

Analysis of the C-terminal region of Blp using Prosite identified the presence of a hemolysin-type calcium binding domain (PS00330). To test the hypothesis that Blp is involved in hemolysis, *B. pseudomallei* DD503 and DD503.blp were grown on blood agar plates (BD – Diagnostic Systems). After 24 or 48 h of growth at 37°C, we were able to detect α-hemolysis on plates containing DD503 and DD503.blp, suggesting that there is no difference in the ability of these strains to lyse red blood cells (data not shown).

*B. pseudomallei* is able to invade, survive, replicate, and disseminate from a variety of cell types including immune cells such as macrophages. In order to test whether Blp is a virulence determinant in macrophages, kanamycin protection assays were performed (146, 264). *B. pseudomallei* DD503 and the *blp* mutant were grown on TSA overnight and used to inoculate wells containing J774A.1 macrophage-like cells at an MOI of 1. After a quick, low-speed spin to facilitate contact between bacteria and macrophages, tissue culture plates were incubated for 1 h to allow for the uptake of the bacteria by the macrophages. Culture supernatants were then removed and fresh media
containing 300 μg/mL kanamycin was added to kill all extracellular bacteria. These 24-well plates were then incubated for 2 h after which point the media was removed and the wells were rinsed with PBSG. One set of infected macrophages were then lysed with saponin to collect internalized bacteria (3 h), while a parallel set received fresh media without antibiotics. After 5 additional hours of incubation at 37°C, these wells were again rinsed with PBSG and the surviving intracellular bacteria were recovered by lysing the macrophages with saponin (8 h). Internalized bacteria at 3 h are expressed as the percent of bacteria internalized normalized by the number of bacteria inoculated into the well. Survival is expressed as the number of bacteria surviving at 8 h divided by the number present at 3 h. This ratio reflects the relative rate of growth vs. killing. As seen in **Figure 2-14**, *B. pseudomallei* DD503 and the *blp* mutant have similar levels of uptake.

**Figure 2-14**: Kanamycin protection assays were conducted using the J774A.1 macrophage-like cell line. **Panel A** is displaying levels of bacterial uptake in the macrophages. **Panel B** is showing relative levels of survival within the macrophages. (~1-2%) and survival (~2-4 fold increase) within the macrophages. In order to test whether any of the other DD503 mutant strains had defects in their ability to survive in macrophages, similar assays were conducted with DD503.bspA, DD503.bmp,
DD503.bspB, and DD503.bspC. Results from these experiments show that none of the mutants have defects in their ability to invade or survive within these murine macrophages (Figure 2-14).

2.10 Expression of BspA, Blp, Bmp, BspB, and BspC in *Burkholderia pseudomallei* DD503

The biological assay results described above suggested that the Blp protein is not involved in adherence to epithelial cells, biofilm production, hemolysis, or survival within macrophages. The results also suggested that BspA, Bmp, BspB, and BspC are not involved in biofilm formation and adherence. To confirm that the *B. pseudomallei* blp mutant strain lacks expression of Blp we examined whole cell lysates of bacteria grown on LB plates by western blot using antibodies against Blp. As expected, we did not detect Blp expression in the blp mutant; however, we were surprised to discover that we could also not detect expression of the Blp protein from wild-type *B. pseudomallei* DD503 or any of the other mutant strains (Figure 2-15). Western blot analysis using anti-BspA antibodies revealed a band at ~ 120 kDa in DD503 which was absent in the bspA mutant (Figure 2-15). While no band was detected in DD503 at the predicted MW of 70 kDa, it is possible the high molecular weight band may correspond to an oligomeric structure of BspA. The 120 kDa band was also observed in DD503.blp, DD503.bmp, DD503.bspB, and DD503.bspC. Western blots probed with anti-Bmp or anti-BspC
Figure 2-15: Whole cell lysates were obtained from \textit{B. pseudomallei} DD503, DD503.bspA, DD503.blp, DD503.bmp, DD503.bspB, DD503.bspC, and \textit{B. thailandensis} pWGBtBlp (positive control) grown on LB plates and analyzed by western blot. Antibodies revealed a lack of expression in DD503 and DD503.bmp or DD503.bspC, respectively (Figure 2-15). However, we were surprised to see expression of Bmp in DD503.bspA and BspC in DD503.bspA and DD503.bspB. It is possible that in these specific mutant strains the promoter for the zeo\textsuperscript{R} cassette is driving expression of Bmp and BspC. In summary, we were only able to observe protein expression in DD503 for BspA which appears to migrate at a much higher molecular weight than what is predicted. In an effort to find conditions that favored protein expression for Blp, Bmp, and BspC, \textit{B. pseudomallei} DD503 was grown at either 23°C or 37°C in or on TSA plates, TSBDC plates, TSBDC broth, M9/0.5% CAA/0.2% glucose, M9/0.4% citrate, or FAB media supplemented with 1 mM MgCl\textsubscript{2} and/or 10 mM FeCl\textsubscript{3}. These bacteria were then used to generate whole cell lysates which were analyzed as described above. We did not detect expression of any of the proteins from \textit{B. pseudomallei} grown in these conditions (data not shown). As we did not detect Blp expression in \textit{B. pseudomallei}
DD503, the lack of a decrease in function in any of the biological assay examined for the
blp mutant is understandable. Thus, until conditions are found in which B. pseudomallei
DD503 expresses BspA, Blp, Bmp, BspB, and BspC in vitro, the mutant strains will not
be useful for the characterization of these proteins. It should be noted that these proteins
do appear to be expressed in vivo which suggests a potential role in virulence for these
molecules and also argues against any of these ORFs being pseudogenes. Felgner et al.
used a protein microarray to show that pooled melioidosis patient sera reacted strongly
against 9 polypeptides encoded in the genetic locus containing blp, including Blp itself
(93).

2.11 A New Approach for the Characterization of

Burkholderia pseudomallei BspA, Blp, Bmp, BspB, and BspC

As B. pseudomallei does not express Blp, Bmp, BspB, and BspC at detectable
levels under any of the growth conditions tested, the mutant strains were not very useful
for providing insight into the function of the individual proteins. In addition, the study of
these molecules in the heterologous genetic background of E. coli demonstrated that
expression of these B. pseudomallei proteins led to an increase in adherence compared to
the negative control strain harboring an empty vector. While there were clear problems
associated with expressing these proteins in E. coli, the observation that we could detect
an increase in adherence in a heterologous expression system led us to the conclusion that
we should express these proteins in a bacterium more genetically similar to B.
pseudomallei. Previous studies with Burkholderia spp. have suggested that the relatively
avirulent *B. thailandensis* may be useful as a model system for the study of some aspects of *B. pseudomallei* pathogenicity (112) (323). Importantly, genomic searches of *B. thailandensis* using NCBI genomic blast did not locate any orthologues of *bspA, blp, bmp, bspB,* or *bspC*. As *B. thailandensis* is more genetically similar to *B. pseudomallei* than *E. coli* and possesses many similar characteristics (*e.g.* relative GC content, expression of similar virulence factors/determinants) (323), it was our hypothesis that *B. thailandensis* would allow expression of BspA, Blp, Bmp, BspB, and BspC without suffering the same problems as *E. coli*.

### 2.12 Construction of a Plasmid Encoding *Burkholderia pseudomallei* BspA, Blp, Bmp, BspB, and BspC

In order to study the function of BspA, Blp, Bmp, BspB, and BspC in *Burkholderia thailandensis*, the genetic locus encoding Blp needed to be subcloned from pWGEcBlp into a new vector as pWGEcBlp will not replicate in *Burkholderia* spp. A relatively small (5.3 kb), broad-host-range plasmid, namely pBHR1 (MoBiTec) has been shown to replicate in *Burkholderia* spp in many studies (285, 317), (283). This plasmid has a chloramphenicol<sup>R</sup> gene and a kanamycin<sup>R</sup> gene which is useful for both positive and negative selection. To subclone the *B. pseudomallei* genes from pWGEcBlp to pBHR1, we first digested pWGEcBlp with *Dra*I and *Ssp*I and gel-purified a 15.5 kb DNA fragment using the High Pure PCR Product Purification Kit (Roche) which contained the *zeo<sup>R</sup>* cassette, *bspA, blp,* and the first 730 of 975 nt of *bmp*. This DNA fragment was ligated into the middle of the chloramphenicol<sup>R</sup> gene following digestion of pBHR1 with
This ligation was introduced into the *E. coli* cloning strain EPI300™ (Epicentre® Biotechnologies), and recombinant clones were selected for sensitivity to chloramphenicol (*cm*<sup>R</sup> gene disrupted by DNA insert), resistance to zeocin™ (specified by DNA insert), and resistance to kanamycin (specified by pBHR1). *kan*<sup>R</sup>*zeo*<sup>R</sup>*cm*<sup>S</sup> colonies were screened by PCR using primers listed in Table 2.1 to verify the presence of *bspA* and *blp* which identified the plasmid, pWGZABlp (Figure 2-16A). Proper insertion of

Figure 2-16: In order to study the function of Blp in *B. thailandensis*, the genetic locus encoding Blp needed to be subcloned into pBHR1. pWGZABlp (A) and pWGBmpBC (B) were used in the construction of pWGBtBlp (C). The DNA fragment into pBHR1 was confirmed by sequencing into the insert from pBHR1 using the primers P41 and P42 (Table 2.1), which flank the *DraI* cut sites in the *cm*<sup>R</sup> marker.
In order to subclone the remaining *B. pseudomallei* genes into pBHR1, we digested the plasmid pWGZABlp with *ScaI* and *AclI* which resulted in the production of a 14 kb DNA fragment that was purified from an agarose gel using the High Pure PCR Product Purification Kit (Roche). This DNA fragment contains the zeo<sup>R</sup> cassette, *bspA*, and the first 9434 nucleotides of *blp* and was designated Z.A.Blp. At the same time pWGEcBlp was digested with *AscI* and *BbvCI* and the 7.2 kb DNA fragment was gel extracted and purified using the High Pure PCR Product Purification Kit (Roche), end-repaired using the End-It™ DNA End-Repair Kit (Epicentre® Biotechnologies), and then ligated into the cm<sup>R</sup> marker of pBHR1 as described above for the creation of pWGZABlp. This ligation was introduced into the *E. coli* cloning strain EPI300™ (Epicentre® Biotechnologies), and recombinant clones were selected for sensitivity to chloramphenicol (cm<sup>R</sup> gene disrupted by DNA insert) and resistance to kanamycin (specified by pBHR1). kan<sup>R</sup>cm<sup>S</sup> colonies were screened by PCR using primers listed in Table 2.1 to verify the presence of *bmp*, *bspB*, and *bspC* which identified the plasmid, pWGBmpBC (Figure 2-16B). This plasmid was then digested with *ScaI* and *AclI* to remove the 3’ end of the cm<sup>R</sup> marker from pBHR1 and leave the *AclI* site corresponding to nucleotide 9434 of *blp* available for sticky end ligation. The DNA fragment, Z.A.Blp, was ligated into pWGBmpBC and transformed into *E. coli* EPI300™ (Epicentre® Biotechnologies). Putative clones were selected for sensitivity to chloramphenicol (cm<sup>R</sup> gene disrupted by DNA insert), resistance to zeocin™ (specified by Z.A.Blp DNA insert), and resistance to kanamycin (specified by pBHR1). kan<sup>R</sup>zeo<sup>R</sup>cm<sup>S</sup> colonies were screened by PCR using primers listed in Table 2.1 to verify the presence of *bspA*, *blp*, *bmp*, *bspB*, and *bspC* which identified the plasmid, pWGBtBlp (Figure 2-16C). Proper insertion of
the DNA fragment into pBHR1 was confirmed by sequencing using the primers P43 and P44 (Table 2.1), which flank the AscI and AclI digest sites in the blp gene.

In order to detect expression of the bspA, blp, bmp, bspB, and bspC gene products by E. coli harboring pWGBtBlp or bspA and blp by E. coli harboring pWGZABlp, we performed western blots of whole cell lysates using antibodies directed against the B. pseudomallei proteins and revealed that only BspA was expressed (data not shown). Compared to pCC1, which was induced to high copy number in E. coli, pBHR1 is a medium copy plasmid so it is not remarkable that we did not detect expression of Blp, Bmp, BspB, or BspC in E. coli. As all of the PCR results suggested the genes were present on pWGBtBlp, and because the sequencing results confirmed that the subcloning process introduced no unwanted gaps or deletions in the blp gene, we decided to proceed with transforming pWGBtBlp into B. thailandensis.

2.13 Expression of Burkholderia pseudomallei BspA, Blp, Bmp, BspB, and BspC in Burkholderia thailandensis

Our laboratory strain of B. thailandensis is DW503, a mutant strain of the sequenced B. thailandensis E264 strain which is sensitive to aminoglycosides and macrolides due to the absence of the amrR-oprA efflux pump (44). The plasmid, pWGBtBlp, was electroporated into B. thailandensis strain DW503 and those potential clones resistant to kanamycin and zeocin™ were confirmed by PCR using primers P5-P14 (Table 2.1) to detect the presence of bspA, blp, bmp, bspB, and bspC. Expression of the
individual *B. pseudomallei* gene products in *B. thailandensis* was then determined by western blot analysis of whole cell lysates under reducing conditions. As seen in Figure 2-17, we detected a band of ~64 kDa in lysates probed with anti-BspA antibodies.

![Western blot analysis of whole cell lysates from B. thailandensis harboring pBHR1 or pWGBTBlp using antibodies against BspA, Blp, Bmp, or BspC.](image)

Western blots performed on whole cell lysates without the presence of a reducing agent yielded bands at ~130 kDa and ~180 kDa (data not shown). These results are consistent with our hypothesis that BspA forms the outer membrane transporter of a type I secretion system, as these proteins are known to form trimeric complexes in the outer membrane (153). It should be noted that in DD503, an oligomeric band was detected for BspA at ~120 kDa. It is interesting that in *B. thailandensis*, this higher molecular weight band appears at 130 kDa and in *B. pseudomallei* this band appears at 120 kDa. The reason for this difference has not been investigated. The *B. thailandensis* whole cell lysates were also probed with antibodies against Blp which revealed the presence of a band in between 268 kDa and 460 kDa (Figure 2-17). We were also able to detect expression of a ~35 kDa band when these whole cell lysates were probed with anti-Bmp antibodies and a ~50 kDa band when probed with anti-BspC antibodies (Figure 2-17), which were very
similar to the expected sizes of these proteins based on their predicted molecular weights. Western blot analysis of these samples with anti-BspB antibodies did not result in the detection of BspB.

2.14 Generation of Additional Plasmids Necessary for the Study of the Individual BspA, Blp, Bmp, BspB, and BspC Proteins in *Burkholderia thailandensis*

Although much of our work is focused on the functions of Blp, we are also interested in whether the *bsp* and *bmp* gene products are involved in the secretion and/or function of Blp. Our hypothesis is the BspA, BspB, and BspC form a type I secretion system necessary for the secretion of Blp. To test this hypothesis, we decided to pursue two independent strategies. The first strategy was to create a *B. thailandensis* strain harboring the previously described pWGZABlp (*Figure 2-16A*). It is our hypothesis that this strain expresses Blp, but that the organism is unable to export Blp to the surface because of the lack of Bmp, BspB and BspC in this strain. If the bacterium is able to express the protein, but not able to export Blp to the surface, it is unlikely that Blp will be able to mediate attachment to epithelial cells and produce biofilm in this organism. The second strategy was to mutagenize the plasmid pWGBtBlp with a transposon (Tn) and identify mutated plasmids containing Tn insertions in the *bspA, blp, bmp, bspB,* and *bspC* genes. The EZ::Tn5™ <DHFR> insertion kit (Epicentre® Biotechnologies) was used to mutagenize pWGBtBlp under the manufacturer’s recommended conditions and portions
of these reactions were electroporated into \textit{B. thailandensis} DW503. Trimethoprim-resistant colonies were then screened by PCR using the gene-specific primers listed in \textbf{Table 2.1} to identify mutated plasmids containing a Tn in \textit{bspA}, \textit{blp}, \textit{bmp}, \textit{bspB}, and \textit{bspC}. These plasmids were sequenced using the Tn-specific primers P45 and P46 (\textbf{Table 2.1}) to identify the site of the Tn insertion in these plasmids which are listed in \textbf{Table 2.5}.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Plasmid} & \textbf{Tn insertion} & \textbf{Site of Tn insertion (Total gene length)} \\
\hline
pWGbspATn & \textit{bspA} & 1130 (1929) \\
pWGblpTn & \textit{blp} & 762 (9687) \\
pWGbmpTn & \textit{bmp} & 538 (975) \\
pWGbspBTn & \textit{bspB} & 1427 (2400) \\
pWGbspCTn & \textit{bspC} & 1244 (1413) \\
pWGomp5Tn* & \textit{omp5} & 363 (897) \\
\hline
\end{tabular}
\caption{Location of Tn insertion in each of the genes in pWGBtBlp. *Alternatively, in pWGomp5Tn, a Tn was inserted in pWGZABlp.}
\end{table}

As with \textit{B. thailandensis} pWGZABlp, it is our hypothesis that the Tn mutants will have deficiencies in biological assays compared to \textit{B. thailandensis} pWGBtBlp because the Tn mutants will either lack expression of \textit{blp} (\textit{i.e.} pWGblpTn) or will be unable to export Blp to the surface where it can function.

\subsection*{2.15 Expression of \textit{Burkholderia pseudomallei} Proteins in \textit{Burkholderia thailandensis} Harboring Various Plasmids is Necessary for Determining the Function of BspA, Blp, Bmp, BspB, and BspC}
It is our hypothesis that Blp is required to be located on the surface of the bacteria in order for the protein to function in biofilm formation and adherence to epithelial cells. In order to test this hypothesis, we first needed to characterize the expression of BspA, Blp, Bmp, BspB, and BspC in the various *B. thailandensis* strains. Whole cell lysates were prepared for *B. thailandensis* harboring pBHR1, pWGZABlp, pWGBlpBlp, or the five Tn mutants and analyzed by western blot using antibodies against BspA, Blp, Bmp, and BspC. As expected, each of the Tn mutant strains lacks expression of their respective protein (Figure 2-18). Additionally, while *B. thailandensis* pWGZABlp only expresses BspA and Blp, *B. thailandensis* pWGBlpBlp expresses BspA, Blp, Bmp, and BspC as shown previously. Interestingly, the Tn insertion in *blp* not only eliminated expression of Blp but also abolished expression of Bmp (Figure 2-18). It is possible that *blp* and *bmp* are co-transcribed and that the Tn insertion in Blp has polar effects on Bmp expression. We also observed that the *blp* mutant (pWGBlpTn) lacked expression of BspC (Figure 2-18). As BspC is expressed in the *Bmp* mutant (pWGbmpTn), it does not
seem like Blp, Bmp, BspB and BspC are part of an operon but this has not yet been
investigated. It should also be noted that in the *bspB* mutant (pWGbspBTn), BspC is not
expressed. As it appears as though these genes overlap by 6 nt, it is possible that these
genes are co-transcribed and that the *bspB* mutant has polar effects on the expression of BspC.

In order to test the hypothesis that a type I secretion system is responsible for
secretion of Blp, we would like to conclusively demonstrate surface association or
secretion of Blp when the three *bsp* gene products are expressed, and a lack of surface
expression or secretion in the absence of one or more of the Bsp proteins. If possible, we
would also like to determine the subcellular compartment (*i.e.* cytosol, inner-membrane,
periplasm, and outer-membrane) of each of the Bsp proteins. An initial attempt to test
these hypotheses was conducted by using the ReadyPrep™ Sequential Extraction kit (Bio-
Rad) which is reported to separate proteins from bacteria based on solubility and has been
used previously for the isolation of *B. pseudomallei* surface proteins (113). Fifty mL
cultures of *B. thailandensis* harboring pBHR1 or pWGBtBlp were grown in LB broth
overnight and then pelleted, resuspended in 1 mL Solution 1, and disrupted by incubation
with 1µL rLysozyme™ (Novagen) for 1 h at 23°C and three sequential rounds of freeze-
thaws in a dry ice bath. After treatment with 10 µL of RNase cocktail™ (Ambion, Inc)
and 5 µL of DNase I (Roche) for 1 h, this suspension was centrifuged at 21,000 x g for 20
min, and the supernatant containing cytosolic proteins was collected. The pellet was then
resuspended in 0.5 mL Solution 2, centrifuged as described above, and the supernatant
containing inner-membrane proteins collected. Finally, the pellet was resuspended in
0.5mL Solution 3 and centrifuged resulting in a supernatant containing outer-membrane
proteins. These fractions were then assessed for protein expression by western blot using antibodies against BspA, Blp, Bmp, and BspC. Samples probed with anti-BspA antibodies, the anti-Bmp antibodies, and the anti-BspC antibodies had bands present in all of the 3 fractions and proteins preparations probed with anti-Blp antibodies showed Blp was present in the inner membrane and outer membrane fractions (data not shown). As it is unlikely that these proteins would all localize to multiple fractions normally, it is possible that this kit is unable to fully separate fractions based on solubility. Additionally, the fact that BspA, Blp, Bmp, BspB, and BspC are overexpressed and potentially complex to form large membrane-spanning structures may complicate this fractionation process. Alternative approaches to study surface localization are required.

One well-established method for isolating different cellular fractions is by sucrose gradient centrifugation. This procedure has been used before to isolate different membrane fractions of *B. pseudomallei* (106). After collecting a large pellet (6 g) from an overnight culture, bacteria are resuspended and disrupted by sonication. This material is then subjected to a low-speed centrifugation (10,000 x g) to remove any unbroken bacteria and large pieces of membrane debris. The remaining suspension is layered onto a two-step sucrose gradient of 15% and 70% sucrose and centrifuged at ~ 100,000 x g for 3 h. Two distinct bands located between the two gradients are then removed and placed on top of a six-step gradient of 30%, 40%, 48%, 52%, 58%, and 70% sucrose and centrifuged at ~ 100,000 x g for 15 h. At this stage, four distinct bands should be present, corresponding to an inner membrane protein fraction between 30% and 40% sucrose, an intermediate protein fraction between 40% and 48% sucrose, an outer membrane protein fraction in between 52% and 58% sucrose, and a second outer membrane protein fraction
in between 58% and 70% sucrose. We used this protocol to process our *B. thailandensis* strains harboring either pBHR1 or pWGBtBlp and encountered a few obstacles. The first problem was that at the stage of sonication, there appeared to be differences in the clarity of the suspensions between the two strains. As sonication progresses there is typically a noticeable color change that indicates that the bacteria are being broken into very small pieces and it was apparent that *B. thailandensis* harboring pWGBtBlp was not disrupted as well as *B. thailandensis* pBHR1. At any given point during sonication, suspensions of *B. thailandensis* pBHR1 will appear clearer than *B. thailandensis* pWGBtBlp which results in much less protein recovery post-sonication for *B. thailandensis* pWGBtBlp as all the large unbroken membrane sections are removed by the low-speed centrifugation. These results suggest that proteins encoded on pWGBtBlp make *B. thailandensis* significantly more difficult to break apart into small pieces. Another obstacle that we encountered was that after the six-step gradient centrifugation, we were unable to observe four distinct bands. Four bands could often be observed but were often merged with each other or very diffuse. In fact, modifications to this protocol (*e.g.* changing sonication parameters, increasing/decreasing starting material, and changing sucrose gradient layering techniques) in order to clarify these bands were unsuccessful. At most, we were able to obtain two semi-distinct bands that resembled those described by Gotoh *et al.*, but western blot analysis of these fractions using antibodies to BspA and Bmp revealed no differential separation between the fractions (data not shown), similar to results seen before with these strains (ReadyPrep™ Sequential Extraction, Bio-Rad). As the observations during sonication suggest, *B. thailandensis* harboring pWGBtBlp appears to be very difficult to disrupt and this appears to be caused by expression of the proteins
encoded on the plasmid as *B. thailandensis* harboring pBHR1 were easier to disrupt. This raises the possibility that the expression of the proteins that putatively interact to form a T1SS may be causing structural changes to *B. thailandensis* that make it difficult to isolate any membrane fraction.

In an attempt to study the surface localization of proteins in *B. thailandensis* harboring pWGBtBlp, we performed immunofluorescence microscopy as described for *E. coli* pWGEcBlp. We were pleased to notice that *B. thailandensis* harboring pWGBtBlp looked identical in size to *B. thailandensis* pBHR1, suggesting that the problems associated with expressing BspA, Blp, Bmp, BspB, and BspC in *E. coli* were not an issue in *B. thailandensis*. However, bacteria probed with antibodies directed against BspA, Blp, Bmp, or BspC did not result in increased immunofluorescence of *B. thailandensis* pWGBtBlp compared to *B. thailandensis* pBHR1 (data not shown).

An ELISA was used to detect surface bound proteins in *E. coli*. This assay was performed by suspending the different bacterial strains to a Klett™ 230 (~ 10^9 bacteria per mL) and then aliquoting these suspensions into individual wells of a 96 well tissue culture plate. After an overnight incubation to allow the bacterial suspension to dry on the plastic, the bacteria were blocked with 3% milk, probed with our collection of antibodies, and then incubated with goat anti-mouse Ig (H+L) conjugated to horseradish peroxidase (Southern Biotech). All wells were then treated with SureBlue™ TMB Microwell Peroxidase Substrate (KPL) and absorbance was measured using an ELISA plate reader at 630 nm. We were able to observe modest increases in the absorbance of wells containing *E. coli* pWGEcBlp compared to *E. coli* pCC1.3 and this suggested that antibodies were binding to surface bound proteins. As we saw limited success with this
assay, we wanted to test whether wells containing *B. thailandensis* pWGBtBlp would have increased absorbance compared to the vector control strain. Wells were treated as described above for *E. coli* and the absorbance was read at 630 nm. While we were able to detect a change in color in wells containing our control *E. coli* strain (pWGEcBlp), we were unable to detect a change in absorbance readings in wells containing *B. thailandensis* pWGBtBlp or pBHR1 (data not shown). This data suggests that the proteins are either expressed at lower levels in *B. thailandensis* (pBHR1 is medium copy compared to the high copy plasmid pCC1) or that the proteins are more difficult for the antibodies to access. Regardless, the ELISA was not an approach that allowed us to detect surface bound proteins in *B. thailandensis*.

Efforts to evaluate surface localization of proteins in *B. thailandensis* have proven to be difficult. As fractionation techniques do not appear to provide pure fractions for *B. thailandensis* pWGBtBlp and surface bound proteins cannot be visualized by immunofluorescence microscopy or an ELISA using our collection of antibodies, we began to search for alternative methods for the identification of surface proteins. This led us to conduct Proteinase K protection assays as described previously for *E. coli* strains. *B. thailandensis* pBHR1 and pWGBtBlp were grown on agar plates and suspended to a Klett™ 300 (~10^10 bacteria/mL) in PBSG. Five hundred µL of these suspensions was processed as a normal whole cell lysate while another 500 µL was treated with 0.25 µg/mL Proteinase K (Roche) and incubated at 37°C. At the designated times, 10 mM PMSF (Sigma-Aldrich) was added to the suspensions to inhibit further protein degradation. These suspensions were then centrifuged to pellet the bacteria and
resuspended in SDS-buffer to be analyzed by western blot. As can be seen in Figure 2-19, none of the proteins were degraded by the Proteinase K even after treatment for 3 h.

Figure 2-19: *B. thailandensis* pBHR1 or pWGBtBlp were treated with Proteinase K for the indicated times after which PMSF was added to stop protease action. Suspensions were then pelleted, resuspended in SDS-buffer and then analyzed by western blot using antibodies against BspA, Blp, Bmp, and BspC.

In comparison, Blp was noticeably degraded after just 3 minutes in *E. coli* pWGEcBlp. Therefore, the Proteinase K protection assay did not show that Blp or any other protein is surface localized in *B. thailandensis* pWGBtBlp.

In summary, the ReadyPrep™ Sequential Extraction kit (Bio-Rad), immunofluorescence, an ELISA, sucrose density gradient centrifugation and a Proteinase K protection assay were used to detect expression of surface bound proteins in *B. thailandensis*. The Bio-Rad kit was used to extract three fractions of proteins based on solubility. However, western blots performed on these samples showed that the proteins were found in all three fractions. Analysis of protein localization was also determined by sucrose density gradient centrifugation. At the end of the protocol, we expected to obtain four distinct bands corresponding to different cellular fractions but at best we were only
able to collect two fractions, both of which contained BspA and Bmp when analyzed by western blot. This suggests that we were unable to separate clean fractions using this protocol. We also performed immunofluorescence with the *B. thailandensis* strains but observed the same cross-reactivity with our antibodies that restricted analysis of our *E. coli* strains. Unlike *E. coli* pWGEcBlp, an ELISA was unable to detect protein expression on the surface of the bacteria with *B. thailandensis* pWGBtBlp even at the highest antibody concentrations. Perhaps the most beneficial experiment was the Proteinase K protection assay (Figure 2-19). In *E. coli*, treatment with Proteinase K resulted in Blp degradation after only 3 minutes which suggests Blp expression on the surface of the bacteria. However, in *B. thailandensis*, Blp was unaffected by the Proteinase K even after 3 h of treatment. This result suggests that either Blp is not making it to the surface in *B. thailandensis* or that some capsule-independent (*B. thailandensis* does not express a capsule) factor is protecting surface proteins from Proteinase K degradation. While it is not difficult to understand why we were unable to observe surface protein expression with immunofluorescence (antibodies are cross-reactive) or ELISA (protein expression too low), it is more difficult to understand the troubles associated with obtaining clean protein fractions using the Bio-Rad kit and the sucrose density gradient centrifugation as well as with the lack of degradation with the Proteinase K protection assay. During sonication for the sucrose density gradient centrifugation protocol, it was very evident that *B. thailandensis* harboring pWGBtBlp was much more difficult to disrupt than the vector control. This result suggests that proteins encoded on pWGBtBlp may provide a certain amount of strength to the membrane structure of the bacteria and may partially explain why the proteins appeared
to localize to all the protein fractions during these many experiments. Type I secretion systems are composed of only three proteins which form a structure spanning both the inner and outer membranes. If *B. thailandensis* is being forced to overexpress this system composed of BspA, BspB, and BspC, it is possible that these structures make the inner and outer membranes much more difficult to separate. If this is the case, it does not explain why Proteinase K is unable to degrade surface proteins as it was able to in *E. coli*. Neither Blp nor BspA were degraded following Proteinase K treatment in *B. thailandensis*. It is unclear whether these results suggest that these proteins do not localize to the outer membrane or if there are factors expressed by the bacterium that protect it from Proteinase K induced protein degradation. Further characterization of *B. thailandensis* pWGBtBlp is required to understand the function of the *B. pseudomallei* proteins encoded on the plasmid.

### 2.16 Biological Function of *Burkholderia pseudomallei* BspA, Blp, Bmp, BspB, and BspC Expressed in *Burkholderia thailandensis*

Our studies demonstrate that *B. thailandensis* expresses the *B. pseudomallei* proteins encoded on pWGBtBlp. It is our hypothesis that surface bound Blp is involved in biofilm formation and adherence. In *E. coli* pWGEcBlp, we saw that expression of the protein did lead to an increase in adherence to fibronectin and A549 pneumocytes. However, we also observed that expression of the proteins encoded on pWGEcBlp
perturbed the normal physiology of the bacterium, as the growth of *E. coli* pWGEcBlp was significantly altered compared to *E. coli* pCC1.3. We also observed that in cultures of the same optical density, there were significantly lower numbers of viable *E. coli* pWGEcBlp that could be recovered compared to *E. coli* pCC1.3. In order to test our hypotheses regarding Blp in *B. thailandensis*, we first wanted to ensure that the bacteria expressing the proteins encoded on pWGBtBlp were able to grow similarly to *B. thailandensis* harboring the empty vector pBHR1. We prepared suspensions of *B. thailandensis* harboring pBHR1 or pWGBtBlp in LB corresponding to a Klett™ value of 50 and then began growing these cultures at 37°C with shaking at 200 rpm. We then took the Klett™ reading of the cultures every hour for 6 h to monitor the growth rate of the organisms. As seen in Figure 2-20, while *B. thailandensis* pWGBtBlp grew at a

![Growth Curves](image)

Figure 2-20: *B. thailandensis* pBHR1 or pWGBtBlp were grown in LB and at each hour were assessed for growth as measured by their Klett™ value. * indicates that the difference between *B. thailandensis* strains is statistically different (p < 0.05).

slightly slower rate compared to *B. thailandensis* pBHR1 (compare values at 3 and 4 h), the growth of the two strains was more similar than with the *E. coli* strains. Additionally, we plated serial dilutions of these cultures at each hour and noticed no significant
differences in the numbers of viable bacteria that we obtained (data not shown). These results, along with the microscopic observations that the two strains appeared to be of similar size, suggest that *B. thailandensis* may be a better heterologous expression system for the study of these *B. pseudomallei* proteins compared to *E. coli* EPI300™ (Epicentre® Biotechnologies). These results gave us confidence that we would be able to study the function of BspA, Blp, Bmp, BspB, and BspC in *B. thailandensis*.

We first wanted to test whether the putative biofilm-associated protein has a role in the formation of biofilm in *B. thailandensis*. To study whether Blp plays a role in biofilm formation, all the *B. thailandensis* strains were suspended in LB to a Klett™ 100 and then 1 mL of these cultures were inoculated into 24-well TC plates to incubate statically overnight at 37°C. The following day, wells were emptied of the bacterial suspensions, stained with crystal violet, rinsed, and then assessed for the presence of a dark purple ring at the air-liquid interface around the ring of the well. Crystal violet was then extracted from the biofilm and then measured in a plate reader at 570 nm. As can be seen in Figure 2-21, *B. thailandensis* pWGBtBlp produced a significant amount more biofilm than *B. thailandensis* pBHR1 as shown by the 3-4 fold increase in absorbance. Altering growth conditions (*e.g.* temperature, growth medium) did not result in increased amount of biofilm production by *B. thailandensis* pWGBtBlp compared to the conditions described above (data not shown). In order to test the hypothesis that Blp expression and surface localization are required for this observed biofilm formation, we also tested the ability of the other *B. thailandensis* strains to produce biofilm under the same conditions as described above. Interestingly, we are able to observe similar levels of biofilm
Figure 2-21: Biofilm production was analyzed in the *B. thailandensis* strains using a crystal violet extraction assay. * indicates that the difference between *B. thailandensis* strains and the negative control is statistically different (p < 0.05).

Production in all of the *B. thailandensis* Tn mutants and *B. thailandensis* pWGZABlp compared to *B. thailandensis* pWGBtBlp (Figure 2-21). These results are true even for *B. thailandensis* pWGBlpTn, which lacks expression of the putative biofilm-associated protein. One possible explanation for these results is that there is an additional factor(s) that is mediating the biofilm production that we are observing under these conditions and that this may mask any biofilm production by the Blp protein.

Biofilm-associated proteins have been shown to be involved in many aspects of pathogenesis, including attachment to a variety of surfaces. We decided to test the ability of our strains to bind to matrix-coated tissue-culture (TC) plates as described previously for the *E. coli* strains. Similar to what was described previously with *E. coli* pWGEcBlp, we visually observed significantly more binding to the fibronectin-coated TC plates with *B. thailandensis* pWGBtBlp than *B. thailandensis* pBHR1 after fixing the attached bacteria with methanol and then staining with 5% Giemsa (data not shown). These assays were performed with an inoculum of ~10^6 bacteria per well. We then analyzed the
adherence of these two *B. thailandensis* strains to plastic, laminin, and type IV collagen. While we were unable to visually detect any binding to plastic or laminin, *B. thailandensis* pWGBtBlp adhered to type IV collagen at greater levels than *B. thailandensis* pBHR1 (data not shown). In order to determine more specific numbers of bacteria bound to the matrix-coated TC plates we performed quantitative viable colony count assays as described above for *E. coli*. In agreement with our visual assay results, *B. thailandensis* pWGBtBlp binds to fibronectin (Fn1) and type IV collagen (COL4A1) at significantly greater levels than *B. thailandensis* pBHR1 ([Figure 2-22A, B](#)) following a 7 h adherence assay. It was also shown in these assays that the *B. thailandensis* strains predicted to lack surface display of Blp (e.g. pWGZABlp) and the strain lacking expression of the Blp protein (*i.e.* pWGblpTn) all showed increased levels of binding to the extracellular matrix proteins ([Figure 2-22A, B](#)).

Adherence assays were then conducted under the same conditions using human respiratory epithelial cell lines, specifically A549 (type II pneumocytes), HEP2 (laryngeal), and NCIH292 (mucoepidermoid). These assays were performed using ~10^6 bacteria per well for multiplicity of infection (MOI) of 10:1 and were conducted over 7 h. Although we noticed increased binding of *E. coli* pWGEcBlp to A549 cells compared to the negative control, we did not observe an increase in adherence of *B. thailandensis* pWGBtBlp compared to the negative control (data not shown). However, when we compared the binding of *B. thailandensis* pWGBtBlp to *B. thailandensis* pBHR1 with HEP2 cells and NCIH292 cells, we observed a significant increase in the amount of
Figure 2-22: Adherence of the indicated strains was measured to the extracellular matrix proteins, Type IV Collagen (A) and Fibronectin (B), or to the human respiratory epithelial cells, HEp2 (C) and NCIH292 (D). Invasion assays were also performed with
the HEp2 cells (E) and NCIH292 (F) cells. * indicates that the difference between *B. thailandensis* strains and the negative control is statistically different (p < 0.05). # indicates that the difference between *B. thailandensis* pWGBtBlp and the indicated Tn mutant strain is statistically different (p < 0.05).

adherence for *B. thailandensis* harboring pWGBtBlp, corresponding to a 6- fold increase in HEp2 cells and a 7-fold increase in NCIH292 (Figure 2-22C, D). As with the extracellular matrix protein binding experiments, we also noticed a 3-fold to 10-fold increase in adherence of *B. thailandensis* pWGblpTn and in all strains that we predicted that Blp would not be surface exposed. It should be noted that *B. thailandensis* pWGbmpTn had significantly higher levels of adherence to HEp2 cells compared to *B. thailandensis* pWGBtBlp. This strain also had the highest level of adherence to NCIH292 cells but the difference compared to *B. thailandensis* pWGBtBlp was not statistically different. Whether these results suggest that Bmp is an anti-adherence factor has not been explored. Overall, these adherence assay results, and the conclusions that can be made, share similarity with the results and conclusions following the biofilm assays. In both cases, our hypotheses about the role of Blp in biofilm production and adherence appear to be incorrect. However, it is possible that another protein is masking any potential role in adherence (or biofilm formation) that Blp may possess. If this is the case, then this other factor is not BspA, Bmp, BspB, or BspC as all of the *B. thailandensis* Tn mutant strains had increased levels of biofilm formation and adherence to HEp2 cells and NCIH292 cells.

*B. thailandensis* harboring pWGBtBlp adheres to human epithelial cells at higher levels than the negative control. As *B. pseudomallei* has been shown to invade and replicate with a variety of cell types (211, 262), it is possible that the proteins encoded on pWGBtBlp are involved in the invasion of epithelial cells and so we wanted to test
whether *B. thailandensis* pWGBtBlp also is able to invade and replicate to higher levels in epithelial cells than *B. thailandensis* pBHR1. Bacterial suspensions were prepared at a Klett™ 230 and ~10^7 bacteria were added to the monolayers of epithelial cells. Following 4 h of incubation at 37°C, extracellular bacteria were removed after the addition of 50 µg/mL of gentamicin. After a total of 6 h of infection, the monolayers were lysed with a solution containing saponin, and the intracellular bacteria were enumerated by plating serial dilutions of the suspensions. Following 6 h infection, the numbers of intracellular *B. thailandensis* pWGBtBlp are clearly higher than negative control (Figure 2-22E, F). However, interpretation of these results is difficult. While there is clearly an increase in the numbers of *B. thailandensis* pWGBtBlp inside the epithelial cells compared to the negative control, it is very possible that this increase is just a reflection of increased numbers of bacteria that were bound to the epithelial cell prior to internalization. In fact, if a protein is directly involved in the invasion process, then we should observe a significant increase in the relative numbers of intracellular bacteria compared to the numbers of bacteria that were just bound to the epithelial cells. As can be seen in Figure 2-22E, F, the fold increase in invasion of *B. thailandensis* pWGBtBlp compared to the negative control is actually lower than what was observed for adherence (e.g. HEp2: Increase in adherence – 6-fold, Increase in invasion – 2-fold). Therefore, it is very likely that the increases in invasion are more a reflection of the increased numbers of bacteria bound to the epithelial cells rather than an active process by one of the proteins encoded on pWGBtBlp.

Although it does not appear that any of the proteins encoded on pWGBtBlp actively mediate invasion of epithelial cells, it is possible that these molecules would help
*B. thailandensis* survive and replicate within the epithelial cells. In order to test the hypothesis that proteins encoded on pWGBtBlp benefit *B. thailandensis* inside epithelial cells, we performed standard gentamicin protection assays. HEp2 cells and NCIH292 cells were infected and processed as described above for the invasion assays, but at 6 h post-infection some wells were processed for levels of bacterial internalization (invasion) while another set of wells were rinsed, thus removing the antibiotics, and allowed to incubate for another 6 h. At 12 h post-infection, these wells were treated with a solution containing saponin to lyse the epithelial cells allowing us to recover intracellular bacteria. The number of bacteria recovered at 12 h post-infection is then normalized by the number of bacteria that were recovered at 6 h post-infection. If this ratio is below 1 then the bacteria are dying while a number above 1 suggests that the bacteria are replicating. This ratio allows us to compare the intracellular survival of organisms that invade in different numbers. *B. thailandensis* pBHR1 and pWGBtBlp were assessed for their ability to survive and replicate within these cells and we observed that for the negative control the survival ratio was 1.9 +/- 0.27 and for *B. thailandensis* harboring pWGBtBlp the survival ratio was 1.8 +/- 0.23. Therefore, after being internalized by the epithelial cells, expression of the proteins encoded on pWGBtBlp does not increase the ability of *B. thailandensis* to survive.

Invasion and intracellular replication within HEp2 cells and NCIH292 cells did not appear to require the function of proteins encoded on pWGBtBlp. However, the intracellular environment of epithelial cells is not as stressing to bacteria as the intracellular environment of a macrophage. Therefore, in order to test whether Blp contributes to the interaction with these cells, adherence assays and a gentamicin-
A protection assay was performed using *B. thailandensis* harboring pBHR1 or pWGBtBlp. Monolayers of the murine macrophage-like cell line J774A.1 were infected with a MOI of 10, spun down at 165 x g for 5 min to synchronize infection, and then incubated at 37°C for 1 h. After bacterial internalization (1 h), extracellular bacteria were killed by incubation with 50 µg/mL gentamicin for 2 h. At this time (3 h), some of the infected monolayers were lysed, and intracellular bacteria were enumerated by spreading dilutions onto agar plates. The remaining monolayers were washed, fresh media without antibiotics was added, and the TC plates were then incubated for 5 additional h at 37°C to allow for intracellular growth (8 h). The macrophages were then lysed by a solution containing saponin, and intracellular bacteria were enumerated by spreading dilutions of these suspensions on agar plates. As can be seen in Table 2.6, there was no difference in

Table 2.6: Interaction of *B. thailandensis* strains with J774A.1 macrophages. Data in table correspond to mean and standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>% Adherence</th>
<th>% Uptake</th>
<th>Survival Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW503 pBHR1</td>
<td>7.0 (2.0)</td>
<td>4.5 (0.4)</td>
<td>1.6 (0.5)</td>
</tr>
<tr>
<td>DW503 pWGBtBlp</td>
<td>6.2 (0.7)</td>
<td>4.1 (0.8)</td>
<td>1.5 (0.9)</td>
</tr>
<tr>
<td>DW503 pWGZABlp</td>
<td>8.1 (0.7)</td>
<td>5.8 (0.6)</td>
<td>1.5 (0.4)</td>
</tr>
</tbody>
</table>

the ability of *B. thailandensis* harboring pWGBtBlp to adhere, invade, or survive within the J774A.1 macrophages compared to the negative control or *B. thailandensis* harboring pWGZABlp. Therefore, proteins encoded on pWGBtBlp do not enable *B. thailandensis* to survive at higher levels in the context of macrophage interactions.

Proteins encoded by pWGBtBlp enable *B. thailandensis* to produce more biofilm and adhere to epithelial cells at higher levels than *B. thailandensis* harboring an empty
vector. While we did observe increased numbers of intracellular *B. thailandensis* pWGBtBlp in epithelial cells, this is very likely due to the increased numbers of bacteria bound to the epithelial cells and not due to a direct role of a particular protein expressed by *B. thailandensis*. Interestingly, *B. thailandensis* strains lacking expression of Blp (pWGblpTn) or strains that are predicted to express Blp but not on the surface (pWGPABlp and the other Tn mutant strains), are equally as able to produce biofilm and adhere to respiratory epithelial cells. It was our initial belief that Blp mediates biofilm formation and adherence and that expression of Blp on the surface of *B. thailandensis* would enable us to prove this hypothesis. While we have not ruled out the possibility that the Blp protein does mediate attachment to epithelial cells and biofilm formation, it is clear that in *B. thailandensis* pWGBtBlp, Blp is not the dominant factor in these processes. A more comprehensive evaluation of the proteins encoded by pWGBtBlp will enable us to determine what factor is responsible for the increased biofilm production and adherence to human respiratory epithelial cells in *Burkholderia thailandensis*.

2.17 Analysis of pWGBtBlp to Identify Uncharacterized Open Reading Frames Located in the Genetic Locus Encoding *Burkholderia pseudomallei* Blp

In an attempt to study the function of *B. pseudomallei* Blp in a heterologous expression system, additional genes were possibly present first on pWGEcBlp and then on pWGBtBlp that may have interfered with our interpretation of the function of the Blp
protein. The initial pWGEcBlp plasmid contained a 41 kb DNA insert specific to \textit{B. pseudomallei}, and of that approximately 10 kb was upstream of the zeo\textsuperscript{R} cassette and approximately 10 kb was downstream of bspC. In the process of subcloning the locus encoding \textit{blp} into pBHR1 we removed almost all of the excess \textit{B. pseudomallei} specific DNA such that only 0.45 kb upstream of the zeo\textsuperscript{R} cassette and 1 kb downstream of the \textit{bspC} gene remain of \textit{B. pseudomallei} specific DNA. Therefore the total amount of DNA specific to \textit{B. pseudomallei} in pWGBtBlp is 20.3 kb which includes the zeo\textsuperscript{R} cassette. Two previously uncharacterized ORFs were identified in this genetic locus as shown in \textbf{Figure 2-23}. We identified a 900 bp ORF located upstream of \textit{bspA} that overlaps \textit{bspA} by 1 bp. This ORF has the \textit{B. pseudomallei} K96243 locus tag BPSL 1659 and is predicted to encode a 32 kDa protein that has an OmpA domain (PRK10808) at amino acids 160-297 (NCBI blastp, e-value 4.51e\textsuperscript{-25}), which designates this protein as having domains that interact with peptidoglycan similar to the C-terminal domain of \textit{E. coli}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|l|}
\hline
\textbf{} & \textbf{Size (bp)} & \textbf{Annotation/Description} \\
\hline
BPSL 1659 & 900 & Hypothetical protein; OmpA domain (PRK10808 e-value 4.51e\textsuperscript{-25}) at amino acids 160-297 \\
BPSL 1662 & 510 & Hypothetical protein; no significant homologues \\
\hline
\end{tabular}
\end{table}

\textbf{Figure 2-23:} The genetic locus encoding Blp was analyzed for additional factors that may be expressed in \textit{B. thailandensis} which may interfere with our interpretations regarding the function of Blp. Two ORFs were identified in pWGBtBlp (A) and descriptions are given for each (B).
OmpA. While the *B. pseudomallei* predicted protein does not possess a Sec-signal sequence (SignalP), the molecule is predicted to localize to the outer membrane (PSORTb, score 9.92). *E. coli* OmpA possesses 8 transmembrane domains that form a β-barrel in the outer membrane. Interestingly, *B. pseudomallei* BPSL 1659 is predicted to only possess 1 transmembrane domain at amino acids 15-34 (TMpred, score 2270) and this prediction specifies that the majority of the protein would be in the periplasm as the transmembrane domain is predicted to be outside → inside. It should be noted that alternatively TMpred also predicts that the C-terminal domain is extracellular (inside → outside) and this score is not much lower (score 1920). Therefore, the exact orientation and configuration of this protein in the outer membrane is not known. With only 1 predicted transmembrane domain, it does seem unlikely that the protein would form an *E. coli* OmpA-like β-barrel in the outer membrane of *B. pseudomallei*. OmpA-like proteins are the major surface antigen in a variety of bacteria and appear to provide structural integrity to the outer membrane (19, 245). As it appears to provide some structural strength to the outer membrane, it is tempting to speculate that overexpression of the OmpA-like protein (encoded on pWGBtBlp) in *B. thailandensis* may be the source of the problems associated with disrupting the bacteria (*e.g.* sonication discrepancies between strains) as described above. If this function were to be proven, it should be said that it may or may not have any relevance on the functions we are interested in characterizing here. Isogenic *ompA* mutants have shown a decreased ability to form biofilms, a decreased ability to adhere to epithelial cells, and are less virulent than wild-type bacteria in a variety of organisms (99, 225). Using NCBI blastn, we searched the genomes of all sequenced *B. pseudomallei* strains and found a BPSL 1659-like ORF in all strains and the
predicted proteins were found to be 99-100% identical. It was also observed that this ORF is *B. pseudomallei*-specific; the gene was not found in the genomes of *B. mallei* or *B. thailandensis*. A recent study has shown that this protein, designated Omp5, elicits antibody production in mice and human melioidosis patients infected with *B. pseudomallei* (111).

The second ORF that was identified in pWGBtBlp is 510 bp with the K96243 locus tag BPSL 1662 and is located 99 bp downstream of *blp*. The encoded protein is predicted to be 18.8 kDa and was annotated as a hypothetical protein (The Sanger Institute). There were no homologs of BPSL 1662 found using blastp (NCBI). Additional analysis was performed using PSORTb which was unable to predict any cellular fraction for this protein to localize within. Furthermore, no Sec-signal was identified (SignalP) and there were no predicted transmembrane domains. Therefore, the exact nature of this putative protein is uncertain.

### 2.18 Generation of Additional Plasmids Necessary for the Identification of the Factor Responsible for the Increased Biofilm Formation and Adherence to Epithelial Cells in *Burkholderia thailandensis*

Based on sequence analysis of these ORFs, it was our hypothesis that Omp5 expression in *B. thailandensis* was mediating biofilm formation and adherence to epithelial cells. This hypothesis was formed around the fact that OmpA-like proteins are
outer membrane proteins that have been shown to be involved in biofilm formation and adherence for other organisms (99, 279). In order to test this hypothesis, we generated two additional plasmids that would enable us to determine if Omp5 was the protein responsible for the phenotypes we observed in *B. thailandensis*. We began these pursuits with the observation that even *B. thailandensis* harboring pWGZABlp still had elevated amount of biofilm production and adherence to epithelial cells. Therefore, we decided to use the pWGZABlp plasmid to study the function of Omp5 (Figure 2-24A). Plasmid

Figure 2-24: pWGZABlp (A) was found to contain two ORFs that may be interfering with our interpretation of Blp function. Additional plasmids were created to study the function of these ORFs in the absence of *bspA, blp, bmp, bspB,* and *bspC* including a truncated version of pWGZABlp, pWGDel (B) a plasmid harboring only *omp5* (C), and a plasmid harboring only the 3’ portion of *blp* and BPSL 1662 (D).
DNA was obtained of pWGZABlp using the HiSpeed Plasmid Midiprep Kit (Qiagen) and then digested with *AflII*, which digests *bspA* at nucleotide 979, and *AclI*, which digests *bspB* at nucleotide 9435. DNA was then isolated using a standard sodium acetate/ethanol precipitation, ligated, and then electroporated into *E. coli* EPI300™ (Epicentre® Biotechnologies). \(\text{kan}^R\text{zeo}^R\) colonies were screened by PCR using primers P5-P8 to verify the absence of *bspA* and *bspB* and primers P47 and P48 (*Table 2.1*) to verify the presence of *omp5* which identified an *E. coli* strain harboring the plasmid, pWGDel (*Figure 2-24B*). This plasmid was then electroporated into *B. thailandensis* DW503 and proper transformation was confirmed by PCR.

The second approach was to PCR amplify a 1.7 kb DNA fragment encompassing the *omp5* gene from the plasmid pWGBtBlp using Platinum® *Pfx* DNA polymerase (Invitrogen™) and primers P47 and P48 (*Table 2.1*). This DNA fragment was cloned into the cm\(^R\) gene of pBHR1 using the restriction sites *EcoRI* and *NcoI*. This plasmid was then electroporated into *E. coli* EPI300™ (Epicentre® Biotechnologies) and potential clones that were resistant to kanamycin (specified by pBHR1) and sensitive to chloramphenicol (disrupted by insert of *omp5* PCR product) were screened by PCR using the cloning primers P47 and P48 listed in *Table 2.1*. Sequencing was also performed on potential clones to ensure that no unwanted substitutions occurred during PCR. This identified the plasmid pWGOmp5 (*Figure 2-24C*). This plasmid was then electroporated into *B. thailandensis* and proper transformation was confirmed by PCR using primers P47 and P48 (*Table 2.1*).

In order to generate a plasmid containing only BPSL 1662, we digested pWGZABlp with *DraI* and *AclI*, which removed all *B. pseudomallei* DNA from ~500 bp
upstream of the zeo\textsuperscript{R} cassette to 351 bp prior to the start of BPSL 1662, leaving only 252 bp of the 5' portion of \textit{blp} and BPSL 1662. This 6.4 kb DNA fragment was gel purified using the High Pure PCR Product Purification Kit (Roche), end-repaired using the End-It\textsuperscript{™} DNA End-Repair Kit (Epicentre\textsuperscript{®} Biotechnologies) and then ligated using T4 DNA Ligase (New England Biolabs) to form the plasmid pWG1662 (Figure 2-24D), which was sequenced using P41 and P42 (Table 2.1).

**2.19 Production of Omp5-Specific Antibodies**

A 0.8 kb PCR product encoding amino acids 45-299 of \textit{B. pseudomallei} DD503 Omp5 was amplified with primers P27 and P28 (Tables 2.1 and 2.2) and cloned into the \textit{NheI} and \textit{PacI} sites of the expression vector pETcoco\textsuperscript{™}-1 (Novagen), which specifies an N-terminal 6x histidine affinity tag. The resulting plasmid, designated pWG42, was sequenced to verify that Omp5 was properly joined to the six N-terminal histidine residues and that no unwanted mutations were introduced during PCR. The same region of \textit{B. pseudomallei} DD503 \textit{omp5} was amplified using primers P39 and P40 (Tables 2.1 and 2.3) and cloned into the \textit{BamHI} and \textit{XhoI} sites of the expression vector pGEX4T-2 (GE Healthcare LifeSciences), which specifies an GST affinity tag. The resulting plasmid, pWG43, was also sequenced to verify PCR fidelity and proper fusion of Omp5 to its GST partner. These plasmids were introduced into the \textit{E. coli} strain TUNER\textsuperscript{™} (Novagen) for the purpose of overexpressing recombinant proteins per the manufacturer’s recommended procedure. The overexpressed His-Omp5 protein was extracted from inclusion bodies using the Bugbuster\textsuperscript{®} HT Protein Extraction Reagent (Novagen) and
rLysozyme™ (Novagen). The recombinant protein was purified under denaturing conditions with the His-Bind® Resin System (Novagen) per the manufacturer’s instructions. The composition of the refolding buffer was determined using the AthenaES™ QuickFold™ protein refolding kit (Athena Enzyme Systems™) and urea was gradually removed by dialyzing the purified recombinant protein preparations at 4°C as previously reported (170). The purified His-Omp5 protein was emulsified in Freund’s adjuvants and used to immunize BALB/c mice as previously described (170). Pooled immune sera was then tested by western blot using cell lysates of E. coli TUNER overexpressing the GST-Omp5 protein (see above). This analysis revealed the presence of Omp5-specific antibodies (data not shown).

2.20 Expression of Omp5 in Burkholderia thailandensis Strains

It is our hypothesis that Omp5 directly contributes to the biofilm production and adherence to respiratory epithelial cells that we observed in B. thailandensis. In order to test this hypothesis, we first wanted to confirm that Omp5 was actually expressed in all strains that formed biofilms and adhered to epithelial cells. Whole cell lysates were generated from all B. thailandensis strains and analyzed by western blot using anti-Omp5 antibodies. As can been seen in Figure 2-25A, we detected a ~32 kDa band in all B. thailandensis strains that displayed an increased ability to produce biofilm and to adhere to epithelial cells compared to the vector control strain. Interestingly, we also detected a band at ~130 kDa in all strains except for B. thailandensis pWGbspATn which showed a major band at ~90 kDa and others at ~60 kDa and ~120 kDa (data not shown). In B.
Figure 2-25: Western blot analysis was performed on whole cell lysates of *B. thailandensis* strains using antibodies against Omp5 (A). Omp5 and BspA appear to form oligomers in *B. thailandensis* (B). Omp5 expression in other *B. thailandensis* strains (C), and affect of Proteinase K treatment on expression of Omp5 (D). *thailandensis* pWGbspATn, this banding pattern suggests that the Omp5 protein may form homooligomeric structures. The fact that the normal banding pattern in all other strains includes a ~130 kDa band that is also observed in western blots probed with anti-BspA antibodies suggests that Omp5 and BspA may form a heterotrimeric structure of
2xOmp5:1xBspA. In order to test this hypothesis, a Tn was inserted into omp5 in pWGZABlp as described above for the creation of the transposon mutants in pWGBtBlp. Western blot analysis was performed on *B. thailandensis* harboring pBHR1, pWGBtBlp, pWGbspATn, pWGZABlp, and pWGomp5Tn using antibodies against BspA or Omp5. Amazingly, it does appear as though BspA and Omp5 form oligomers as when both proteins are expressed a major band is detected at ~ 130 kDa. However, if you perform these experiments under reducing conditions (data not shown) or if one of the proteins is not expressed, as with the Tn mutants, the 130 kDa band is absent and the major band detected is of the monomers (Figure 2-25B). It is currently unclear whether this structure is functional in the outer membrane or if it is an affect of overexpressing these proteins outside of their natural host.

We also performed western blot analysis on the relevant new *B. thailandensis* strains using anti-Omp5 antibodies. The results of these experiments are shown in Figure 2-25C and show that Omp5 is expressed in *B. thailandensis* harboring pWGDel and pWGOmp5. It also appears as though Omp5 expression is elevated in pWGDel but this has not been investigated. A Proteinase K protection assay was also performed to determine if Omp5 was surface exposed. Like all other proteins in *B. thailandensis*, we did not detect a difference Omp5 expression following treatment with Proteinase K (Figure 2-25D). Further work is needed to determine if this factor is surface exposed. In conclusion, we were able to generate antibodies specific to Omp5 that show that the protein is expressed in all *B. thailandensis* strains that displayed an increase in adherence to epithelial cells and an increase in biofilm production, thus supporting our hypothesis that Omp5 may be the factor mediating these phenotypes in *B. thailandensis*. 
2.21 Functional Characterization of *Burkholderia thailandensis* Harboring pWGDel and pWGOmp5

OmpA-like proteins have been shown to be involved in many aspects of pathogenesis including biofilm formation and adherence to epithelial cells (99, 225). In order to determine if Omp5 specifically mediates biofilm production and adherence to epithelial cells in *B. thailandensis*, we performed assays with the new *B. thailandensis* strains that were shown to express Omp5 independently (pWGOmp5) or in addition to another factor, BPSL 1662 (pWGDel). Adherence assays were performed as described before. Plate-grown *B. thailandensis*, harboring pBHR1, pWGBtBlp, pWGDel, or pWGOmp5 were suspended to a Klett™ 230, and portions of these suspensions were added to monolayers of HEp2 or NCIH292 cells. Adherence was measured at 7 h post-infection by washing away unbound bacteria, and enumerating attached bacteria after lysing the epithelial cells. Our results indicate that *B. thailandensis* pWGBtBlp and *B. thailandensis* pWGDel were able to bind to the HEp2 (Figure 2-26A) and NCIH292 (Figure 2-26B) cell lines at much higher levels than *B. thailandensis* harboring the vector alone. Surprisingly, *B. thailandensis* pWGOmp5 was shown to adhere to both cell lines at levels that were similar to the negative control. Therefore, Omp5 expression alone does not appear to mediate adherence to HEp2 cells and NCIH292 cells.
Newly generated *B. thailandensis* strains were tested for their ability to adhere to HEp2 (A) or NCIH292 (B) epithelial cells. * indicates that the difference between *B. thailandensis* strains and the negative control is statistically different (p < 0.05).

We also wanted to determine if Omp5 expression alone was sufficient for *B. thailandensis* to form biofilm. *B. thailandensis* harboring pBHR1, pWGBtBlp, pWGDel, and pWGOmp5 were suspended to a Klett™ 100 and 1 mL was inoculated into wells of a 24-well TC plate. After 24 h incubation at 37°C, the wells were emptied, washed, and stained with a crystal violet solution as described previously. *B. thailandensis* harboring pWGDel was found to be as capable of forming a biofilm as *B. thailandensis* pWGBtBlp, as the wells from both strains had very distinct rings at the air-liquid interface unlike the negative control and the amount of crystal violet that was extracted from these wells was nearly identical (Figure 2-27). Surprisingly, we did not observe a distinct ring at the air-liquid interface in wells inoculated with *B. thailandensis* pWGOmp5 and the amount of crystal violet extracted from these wells was comparable to the negative control (Figure 2-27). Therefore, Omp5 expression alone does not mediate biofilm formation in *B. thailandensis*.
Figure 2-27: Newly generated *B. thailandensis* strains were tested for their ability to produce biofilm. * indicates that the difference between *B. thailandensis* strains and the negative control is statistically different (p < 0.05).

### 2.22 Characterization of *Burkholderia thailandensis* pWG1662

*B. thailandensis* pWGomp5 was unable to produce the same levels of biofilm or adhere to human epithelial cells at the same levels as *B. thailandensis* pWGBtBlp or pWGDel. These results argue that another factor encoded on pWGBtBlp and pWGDel is mediating these phenotypes in *B. thailandensis*. Therefore, it is our hypothesis that the protein encoded by BPSL 1662 is necessary for biofilm production and adherence in *B. thailandensis*. In order to determine if BPSL 1662 may be directly involved in adherence in *B. thailandensis*, we transformed DW503 with pWG1662 and conducted adherence assays with HEp2 cells as previously described. While the vector control maintained low
levels of adherence (Figure 28), B. thailandensis pWG1662 had significantly higher

![Bar graph showing adherence levels](image)

Figure 2-28: The indicated B. thailandensis strains were tested for their ability to adhere to HEp2 epithelial cells. * indicates that the difference between B. thailandensis strains and the negative control is statistically different (p < 0.05).

levels of adherence that was comparable to the positive control, B. thailandensis pWGBtBlp. It is currently unknown whether B. thailandensis pWG1662 also mediates adherence to extracellular matrix proteins or NCIH292 epithelial cells. However, these results suggest that the relatively small (~19 kDa) protein encoded by BPSL 1662 may be responsible for the adherence of B. thailandensis strains to epithelial cells.

In order to determine whether BPSL 1662 also mediates biofilm formation, we conducted biofilm assays as described previously. B. thailandensis pWG1662 was able to produce as much biofilm as B. thailandensis pWGBtBlp (Figure 2-29), which was significantly more biofilm production than from the vector control. These results suggest that the protein encoded by BPSL 1662 mediates biofilm formation when expressed in B. thailandensis. Confirmation of expression of this small protein will strengthen this argument.
Figure 2-29: The indicated *B. thailandensis* strains were tested for their ability to produce biofilm. * indicates that the difference between *B. thailandensis* strains and the negative control is statistically different (p < 0.05).
Chapter 3

Discussion

3.1. Conclusions Regarding the Proteins Encoded in the Same Genetic Locus as *Burkholderia pseudomallei* Blp

*B. pseudomallei* harbors an ORF that is highly similar to the biofilm-associated protein, Bap, in *Staphylococcus aureus*. Therefore, we named this putative protein, Bap-like protein (Blp). As this protein contains an integrin-binding domain in its C-terminus that is common in many adhesins (*Figure 2-1*), it was our hypothesis that Blp is involved in biofilm formation and adherence. Immediately surrounding this ORF in the chromosome of *B. pseudomallei* are ORFs that bear similarity to genes that encode proteins that comprise type I secretion systems. As Blp contains a type I secretion system signal sequence in its C-terminus, we hypothesize that Blp is secreted to the surface of *B. pseudomallei* by this specific secretion system. We designated these genes Blp-secretion protein A (BspA), BspB, and BspC. It is our hypothesis that these factors are required for Blp to be secreted to the surface of *B. pseudomallei* in order to form biofilm and
adhere to various surfaces. An additional factor was found to be encoded in between \textit{blp} and \textit{bspB} and shares homology with sulfotransferase enzymes. It is our hypothesis that this protein may be required for Blp to function.

In order to study the function of Blp, we initiated two independent projects. The first was to create mutations in each of the genes in \textit{B. pseudomallei} and demonstrate a loss of function in each of the mutants. Biofilm assays were conducted which showed that none of the mutants were deficient in their ability to form biofilm (\textbf{Figure 2-12}). Adherence assays were conducted which revealed that the \textit{blp} mutant was equally able to adhere to A549 epithelial cells as the wild-type strain (\textbf{Figure 2-13}). Similarly, all strains showed an inability to lyse red blood cells and were equally able to invade and survive within macrophages (\textbf{Figure 2-14}). In order to confirm that the mutants were truly lacking expression of their respective proteins, antibodies were generated and western blots were performed on whole cell lysates. While none of the mutants were expressing their respective proteins, the wild-type strain was only found to express BspA at detectable levels (\textbf{Figure 2-15}), which may explain why no differences were observed between the DD503 mutants and the wild-type in the assays that were conducted. As no condition was discovered that favored expression of all the proteins \textit{in vitro}, a new approach was needed to study the function of Blp.

The second independent project then became the primary approach to studying the function of Blp. It was our hypothesis that if we could express Blp in a heterologous background, such as in \textit{E. coli}, we could study a gain of function in this heterologous expression system. In order to study the function of Blp outside of \textit{B. pseudomallei}, and to ensure that the protein is expressed and functioning on the surface, we hypothesized
that we would need to express not just Blp but also BspA, Bmp, BspB, and BspC. Therefore, using a cosmid library approach we generated an \textit{E. coli} strain that harbored a plasmid, pWGEcBlp containing this genetic locus (\textbf{Figure 2-3B}). This plasmid was sequenced and expression of BspA, Blp, and Bmp was verified by western blot (\textbf{Figure 2-5}). Further analysis of protein expression of BspB and BspC by reverse-transcriptase PCR (RT-PCR) would be useful to at least show that RNA is being produced for these proteins. Blp also was shown to be surface localized in this \textit{E. coli} strain (\textbf{Figure 2-5B, 7}). In order to characterize the function of Blp, we performed adherence assays and observed an increased ability to bind extracellular matrix proteins and epithelial cells (\textbf{Figures 2-8 and 2-9}). We also conducted biofilm assays but did not detect an increased ability to form biofilm in \textit{E. coli} (\textbf{Figure 2-10}). Interestingly, upon examination of the bacteria by immunofluorescence, it was observed that \textit{E. coli} harboring pWGEcBlp appeared very long in comparison to \textit{E. coli} harboring an empty vector (\textbf{Figure 2-6A}) and growth curves showed that \textit{E. coli} expressing the proteins encoded on pWGEcBlp were not normal. Even after just 2 h, \textit{E. coli} pWGEcBlp was retarded for growth compared to \textit{E. coli} pCC1.3 (\textbf{Figure 2-6B}). This growth defect was further confirmed by plating serial dilutions of bacterial cultures at equal Klett™ values, which showed that the number of \textit{E. coli} pWGEcBlp was much lower (~5 fold) than \textit{E. coli} pCC1.3 (data not shown). As we were able to observe an increase in \textit{E. coli} pWGEcBlp binding to extracellular matrix proteins and epithelial cells, we decided to continue studying the function of these proteins in a heterologous genetic background more similar to \textit{B. pseudomallei}. 

\textit{pseudomallei}.
*B. thailandensis* is a relatively avirulent *B. pseudomallei*-like organism that does not contain the *blp* locus. As pWGEcBlp does not replicate in *B. thailandensis*, we subcloned the *blp* locus in 2 pieces into the medium-copy broad-host range plasmid, pBHR1, to create the plasmid pWGBtBlp (Figure 2-16). This plasmid was transformed into *B. thailandensis* and expression of the proteins encoded on pWGBtBlp was confirmed by western blot (Figure 2-17). While many strategies were employed to detect surface expression of proteins in *B. thailandensis* pWGBtBlp, we were unable to confirm what we observed in *E. coli* (Figure 2-7). While Blp was quickly degraded by the Proteinase K treatment in *E. coli* pWGEcBlp, Blp was still present in *B. thailandensis* pWGBtBlp, even after 3 h of treatment (Figure 2-19), suggesting that the protein is either not surface associated or that there are additional factors that interfere with the ability of the Proteinase K to degrade surface molecules. One possible way to gain more insight into the cellular localization of each of these proteins would be to clone each gene individually on pBHR1 and then overexpress these proteins in *B. thailandensis* or even in their native host, *B. pseudomallei*. As one of the obstacles associated with determining the localization of the molecules was that the type I secretion system may act as a clamp between the inner and outer membranes, overexpression of only one protein may allow for a better analysis of the location of the protein using sucrose density gradient centrifugation or sarkosyl-insoluble outer membrane preparations followed by western blot.

Unlike *E. coli* pWGEcBlp, growth curves suggest that *B. thailandensis* pWGBtBlp tolerates expression of the proteins encoded in the *blp* locus better as the relative growth is much more similar to the vector control, *B. thailandensis* pWGEcBlp.
In order to study the function of the individual proteins encoded on pWGBtBlp, we also generated additional plasmids that possessed a Tn in bspA (pWGbspATn), blp (pWGblpTn), bmp (pWGbmpTn), bspB (pWGbspBTn), and bspC (pWGbspCTn). It was our hypothesis that in order for *B. thailandensis* to form biofilm and adhere to epithelial cells Blp must be expressed and must be functional on the surface of the bacterium. Western blot analysis of these strains revealed that the Tn did eliminate expression of the intended protein, but RT-PCR would be useful for obtaining a more complete picture of the regulation involved in this locus. Biofilm assays were conducted to test this hypothesis and what we observed was that *B. thailandensis* harboring pWGBtBlp did have increased levels of biofilm production but surprisingly all of the Tn mutants also produced elevated amounts of biofilm (*Figure 2-21*). Additionally, adherence assays were conducted with extracellular matrix proteins or epithelial cells. *Figure 2-22* clearly demonstrates that while *B. thailandensis* pWGBtBlp does have increased binding to these surfaces, all of the Tn mutants also show an increased level of adherence. Therefore, proteins encoded on pWGBtBlp do mediate biofilm production and adherence in *B. thailandensis*, but it is not primarily due to BspA, Blp, Bmp, BspB, or BspC as strains lacking expression of these proteins still possess the ability to form biofilm and adhere to surfaces.

We identified two additional ORFs in pWGBtBlp that may encode proteins that mediate biofilm formation and adherence in *B. thailandensis* (*Figure 2-23*). One of these ORFs (BPSL 1659) encodes a protein that possesses an OmpA domain and shares homology with outer membrane proteins important in biofilm production, adherence, and virulence. BPSL 1659 was shown to be expressed *in vivo* as pooled patient sera reacted
against this protein which was designated Omp5 (111). The other ORF that was identified (BPSL 1662) encodes a hypothetical protein with no known homologs. It was our hypothesis that Omp5 is responsible for the increased biofilm formation and adherence in *B. thailandensis* due to sequence similarities with proteins involved in these functions.

In order to determine whether Omp5 mediates biofilm production and adherence in *B. thailandensis*, we cloned Omp5 independently into pBHR1 and transformed this plasmid, pWGOMP5, into *B. thailandensis*. We also removed most of *bspA* and *blp* from pWGZABlp to create a plasmid, pWGDel that only encodes the two ORFs discussed above and then transferred this plasmid to *B. thailandensis* (**Figure 2-24**). Antibodies were generated against Omp5 and western blots were performed to confirm that Omp5 was expressed in all *B. thailandensis* strains producing biofilm and adhering to surfaces. The results of this analysis confirmed that Omp5 was expressed which supports our hypothesis regarding Omp5 function (**Figure 2-25A**). Additionally, we were able show that Omp5 is also expressed in *B. thailandensis* harboring pWGDel and pWGOMP5 (**Figure 2-25C**). Attempts to show that Omp5 associates with the outer membrane were not successful (**Figure 2-25D**). In order to determine whether Omp5 is directly mediating biofilm production and adherence in *B. thailandensis*, we performed adherence assays and demonstrated that Omp5 expression alone is not sufficient for adherence to epithelial cells (**Figure 2-26**). We were also able to observe that *B. thailandensis* expressing Omp5 and potentially one additional factor (pWGDel) did still adhere to these cells, suggesting that the other ORF may be responsible for the phenotypes we observed. We also performed biofilm assays and saw similar results; Omp5 expression alone in *B.
*thailandensis* is not sufficient for biofilm production (Figure 2-27). However, *B. thailandensis* harboring pWGDel produced similar levels of biofilm as the positive control. Therefore, it appears as though Omp5 is not the primary factor governing the biofilm production and adherence.

As *B. thailandensis* pWGDel adheres to epithelial cells and forms biofilm at levels equal to *B. thailandensis* pWGBtBlp and as we were able to show that Omp5 expression alone is not responsible for these phenotypes, our new hypothesis was that these functions are directly associated with expression of the protein encoded by BPSL 1662. In order to determine whether this molecule is the factor mediating adherence and biofilm formation in the *B. thailandensis* strains, we transferred pWG1662 (Figure 2-24D) to *B. thailandensis* DW503. Adherence assays were conducted which demonstrated that BPSL 1662 alone is sufficient for *B. thailandensis* to adhere to HEP2 cells, as *B. thailandensis* pWG1662 adhered to these epithelial cells at the same level as *B. thailandensis* pWGBtBlp (Figure 2-28). Further analysis on the ability of *B. thailandensis* pWG1662 to adhere to other epithelial cells and extracellular matrix proteins is certainly warranted. Biofilm assays were also performed which showed that *B. thailandensis* pWG1662 and pWGBtBlp were able to form more biofilm compared to the vector control (Figure 2-29). The data strongly support the hypothesis that the protein encoded by BPSL 1662 is responsible for the increase in biofilm formation and adherence to epithelial cells in *B. thailandensis*. Confirmation of expression of this protein will greatly strengthen this hypothesis. Analysis of a BPSL 1662 mutant strain in *B. pseudomallei* DD503 will also be useful in deconstructing the function of this small (19 kDa) protein.
Chapter 4

Materials and Methods

4.1 Bacterial Strains and Plasmids

The strains and plasmids used in this study are listed in Table 4.1. *Burkholderia pseudomallei* DD503 was grown as indicated on LB, TSB, or TSB-DC agar plates or in LB, TSB, TSBDC, M9 supplemented with 0.5% casamino acids and 0.2% glucose, M9 supplemented 0.4% citrate, or FAB supplemented with 1 mM MgCl₂ and/or 10 mM FeCl₃ medium. Antibiotics were added where indicated at the following concentrations: 100 µg/mL polymyxin B (pmB), 100 µg/mL zeocin™ (Invitrogen™, zeo), or 100 µg/mL kanamycin (kan). *B. thailandensis* DW503 was grown on LB agar plates or in LB media. Antibiotics were added where indicated at the following concentrations: 100 µg/mL polymyxin B (pmB), 100 µg/mL zeocin™ (Invitrogen™, zeo), 100 µg/mL kanamycin (kan), or 50 µg/mL trimethoprim (tm). *Escherichia coli* strains were grown on LB agar
Table 4.1: Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
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<tr>
<td><strong>B. pseudomallei</strong></td>
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<tr>
<td>DDS03</td>
<td>Parental strain; pmB(^r) zeo(^r) kan(^r)</td>
<td>(187)</td>
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<td>DDS03.5'junk</td>
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<td>DDS03.bspA</td>
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<td>This study</td>
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<td>This study</td>
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<td>DW503</td>
<td>Parent strain; pmB(^r) zeo(^r) kan(^r)</td>
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<td>EPICENTRE (^TM) Biotechnologies</td>
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<td>Strain used for the overexpression of His- and GST-fusion proteins</td>
<td>Novagen</td>
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<td>S17</td>
<td>Strain used for conjugal transfer of suicide plasmids from E. coli to B. pseudomallei</td>
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<td>Cloning vector; cm(^r)</td>
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<td>This study</td>
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plates or in LB media supplemented with the following antibiotics where indicated at the following concentrations: 100 µg/mL polymyxin B (pmB), 100 µg/mL zeocin™ (Invitrogen™, zeo), 100 µg/mL kanamycin (kan), or 15 µg/mL chloramphenicol (cm).

Recombinant *E. coli* strains harboring pCC1™ (Epicentre® Biotechnologies)-based plasmids were also grown with the CopyControl™ Induction Solution (Epicentre® Biotechnologies) as described by Holm *et al.* (126).
4.2 Bioinformatic Analyses

Sequence analyses were performed using Vector NTI® (Invitrogen™) and the various online tools available through the ExPASy Proteomics Server (http://au.expasy.org/). Signal sequence cleavage sites were determined using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). Sequence searches were performed using the NCBI BLAST service using either blastn or blastp programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Subcellular localization prediction was performed by PSORTb (http://www.psort.org/psortb/). Prediction of transmembrane domains was performed using TMPred (http://www.ch.embnet.org/software/TMPRED_form.html).

4.3 PCR and Recombinant DNA Techniques

Standard molecular biology techniques were performed as described elsewhere (235). *B. pseudomallei* chromosomal DNA was isolated using the Easy-DNA™ Kit (Invitrogen™) using the manufacturer’s recommended protocol. DNA was extracted from agarose gels using the High Pure PCR Product Purification Kit (Roche) according to the manufacturer’s recommended conditions. Plasmid DNA was obtained using either the HiSpeed Plasmid Midi Kit (Qiagen) or the QIAnprep Spin Miniprep Kit (Qiagen), as indicted, according to the manufacturer’s instructions. All PCR reactions were
performed using the Platinum® Pfx DNA Polymerase (Invitrogen™). Primers were used as specified in the text and are shown in Table 2.1. Cloning PCR fragments into pCC1™ (Epicentre® Biotechnologies) was performed using the CopyControl™ Cloning Kit (Epicentre® Biotechnologies) using the manufacturer’s recommended protocol. All restriction enzymes were purchased from New England Biolabs. End-repair was performed using the End-It™ DNA End-Repair Kit (Epicentre® Biotechnologies) and ligations were performed using T4 DNA Ligase (New England Biolabs). Generation of pWGEcBlp was achieved using a cosmid-library approach with the CopyControl™ Fosmid Library Kit (Epicentre® Biotechnologies) under the recommended conditions.

### 4.4 *Burkholderia pseudomallei* Mutants and Transposon Mutagenesis

A zeo<sup>R</sup> cassette was inserted in the middle of the designated ORF on the pCC1™ (Epicentre® Biotechnologies)-based plasmid as indicated in the text. The ORF, disrupted by the zeo<sup>R</sup> cassette, was subcloned into pKAS46 and transferred to *E. coli* S17. The pKAS46-based plasmid was then delivered to *B. pseudomallei* DD503 by conjugation as previously reported (44). *B. pseudomallei* strains were first selected for pmB<sup>R</sup> and zeo<sup>R</sup> and then counterselected for kan<sup>S</sup>. Mutant strains were then identified by PCR and sequencing.
Transposon mutagenesis of pWGBtBlp was performed using the EZ::Tn5™ <DHFR> insertion kit (Epicentre® Biotechnologies) according to the manufacturer’s recommended conditions. The DHFR transposon specifies resistance to trimethoprim.

4.5 Sequence Analysis

Plasmids were sequenced at the University of Michigan Sequencing Core. Chromatograms were assembled and analyzed using Sequencher® 4.9 (Gene Codes Corporation). Additional sequence analysis was performed using Vector NTI® Software (Invitrogen™).

4.6 Production of Antibodies

His-tagged fusion proteins, specific to BspA, Blp, Bmp, BspB, BspC and Omp5 were generated by cloning a portion of the protein into the expression vector, pETcoco™-1 (Novagen), as indicated in the text. The same portion of each protein was also introduced into the vector pGEX4T-2 (GE Healthcare LifeSciences), which specifies an N-terminal Glutathione – S – Transferase (GST) affinity tag. These constructs were sequenced, and transferred to E. coli TUNER (Novagen). The overexpressed His-tagged protein was extracted from inclusion bodies using the Bugbuster® HT Protein Extraction Reagent (Novagen) and rLysozyme™ Solution (Novagen). The recombinant protein was purified under denaturing conditions with the His-Bind® Resin System (Novagen) per the
manufacturer’s instructions. The composition of the refolding buffer was determined using the AthenaES™ QuickFold™ protein refolding kit (Athena Enzyme Systems™) and urea was gradually removed by dialyzing the purified recombinant protein preparations at 4°C as previously reported (170). The purified His-tagged protein was emulsified in Freund’s adjuvants and used to immunize BALB/c mice as previously described (170). Immune serum was then tested by western blot using cell lysates of *E. coli* TUNER overexpressing the GST-tagged protein.

Alternatively, antibodies were also generated by ordering a synthetic peptide from GenScript corresponding to portions of the proteins described in the text. The peptides were conjugated to keyhole lymphocyte hemocyanin (KLH) or ovalbumin (OVA) with the Imject® Maleimide Activated Immunogen Conjugation Kit (Pierce) using the N-terminal cysteine of the peptides for disulfide bond formation. The KLH-peptide conjugate was then used to immunize mice and antibodies were tested against the OVA-peptide conjugate or lysates of *E. coli* expressing GST-BspB to confirm the production of protein-specific antibodies.

Monoclonal antibodies specific to Blp were generated, as described previously for His-Blp, with the following additions. The His-tagged fusion construct was used to immunize mice and spleen cells were collected and fused with a myeloma cell line to create hybridomas secreting monoclonal antibodies. Supernatants from these cell lines were collected and screened by ELISA using purified GST-Blp.

### 4.7 Western Blot Analysis of Protein Preparations
Whole cell lysates were generated from equal numbers of bacteria resuspended in a buffer containing sodium dodecyl sulfate (SDS) and in the presence of the NuPAGE® Reducing Agent (Invitrogen™), as indicated. Total membrane and sarkosyl-insoluble outer membrane preparations were prepared as described previously (48). Western blots were performed as described by Bullard et al (43). The preparations were resolved by SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide gels and transferred to polyvinylidenedifluoride (PVDF) membranes or using the NuPAGE® Large Protein Blotting Kit (Invitrogen™). Membranes were blotted as described in the text at the following concentrations: anti-BspA polyclonal antibodies (1:5000), anti-Blp polyclonal antibodies (1:1000), anti-Blp monoclonal antibodies (1:1000), anti-Bmp polyclonal antibodies (1:1000), anti-BspB polyclonal antibodies (1:500), anti-BspC polyclonal antibodies (1:1000), and anti-Omp5 polyclonal antibodies (1:5000). Protein bands were detected by chemiluminescence.

Immunofluorescence was conducted using equal numbers of bacteria that were heat fixed to the surface of a glass slide, treated with 4% paraformaldehyde, blocked with 10% goat serum (Sigma-Aldrich®), and then probed with polyclonal antibodies at concentrations ranging from 1:100 to 1:5000. Anti-Blp monoclonal antibodies were used at concentrations as low as 1:10. These slides were then washed, incubated with AlexaFlour® 488 goat anti-mouse secondary antibody (Molecular Probes®), washed again, and then coverslips were added over SlowFade® Gold antifade reagent containing DAPI (Invitrogen™). The coverslips were then sealed and the slides observed by microscopy using a Zeiss LSM 510 Meta Confocal System.
An ELISA was performed using bacteria grown as indicated in the text, suspended to a Klett™ 230 (~ 10^9 bacteria per mL) and inoculated into individual wells of a 96 well tissue culture plate. After an overnight incubation to allow the bacterial suspension to dry on the plastic, the bacteria were blocked with 3% milk, probed with the antibodies (concentrations same as for immunofluorescence), and then incubated with goat anti-mouse Ig (H+L) conjugated to horseradish peroxidase (Southern Biotech). All wells were then treated with SureBlue™ TMB Microwell Peroxidase Substrate (KPL) and absorbance was measured using an ELISA plate reader at 630 nm.

A Proteinase K protection assay was also performed as described previously (42) with some modifications. Bacteria were grown as described in the text and suspended to a Klett™ 300 (~10^{10} bacteria/mL) in PBSG. Five hundred µL of these suspensions was processed as a normal whole cell lysate while another 500 µL was treated with 0.25 µg/mL proteinase K (Roche) and incubated at 37°C. At the indicated times, 10 mM PMSF (Sigma-Aldrich) was added to the suspensions to inhibit further protein degradation. These suspensions were then centrifuged to pellet the bacteria and resuspended in SDS-buffer to be analyzed by western blot.

Protein fractions were also obtained using the ReadyPrep™ Sequential Extraction kit (Bio-Rad) with the following modifications. Fifty mL cultures were grown in LB broth overnight and then pelleted, resuspended in 1 mL Solution 1, and disrupted by incubation with 1µL rLysozyme™ (Novagen) for 1 h at 23°C and three sequential rounds of freeze-thaws in a dry ice bath. After treatment with 10 µL of RNase cocktail™ (Ambion, Inc) and 5 µL of DNase I (Roche) for 1 h, the suspension is centrifuged at 21,000 x g for 20 min, and the supernatant containing cytosolic proteins is collected. The
pellet is then resuspended in 0.5 mL Solution 2, centrifuged as described above, and the supernatant containing inner-membrane proteins is collected. Finally, the pellet is resuspended in 0.5mL Solution 3 and centrifuged resulting in a supernatant containing outer-membrane proteins. These fractions were then assessed for protein expression by western blot.

Sucrose density gradient centrifugation was also performed as previously reported (106). After collecting a large pellet (6 g) from an overnight culture, bacteria are resuspended and disrupted by sonication. This material is then subjected to a low-speed centrifugation (10,000 x g) to remove any unbroken bacteria and large pieces of membrane debris. The remaining suspension is layered onto a two-step sucrose gradient of 15% and 70% sucrose and centrifuged at ~ 100,000 x g for 3 h. Two distinct bands located between the two gradients are removed and placed on top of a six-step gradient of 30%, 40%, 48%, 52%, 58%, and 70% sucrose and centrifuged at ~ 100,000 x g for 15 h. At this stage, four distinct bands should be present corresponding to an inner membrane protein fraction between 30% and 40% sucrose, an intermediate protein fraction between 40% and 48% sucrose, an outer membrane protein fraction in between 52% and 58% sucrose, and a second outer membrane protein fraction in between 58% and 70% sucrose. These protein samples were then analyzed by western blot.

4.8 Eukaryotic Cell Lines

The human epithelial cell lines A549 (type II alveolar lung epithelium; ATCC® number CCL-185™), HEp-2 (laryngeal epithelium; ATCC® number CCL-23™) and NCIH292 (lung mucoepidermoid; ATCC® number CCL-1848™) were cultured as
reported previously (277). The murine macrophage-like cell line J774A.1 (ATCC® number TIB-67™) were maintained in Dulbecco's Modified Eagle's Medium (Cellgro) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (GIBCO) at a temperature of 37°C and 7.5% CO₂.

4.9 Adherence Assays

Visual adherence assays were conducted as described previously (42) with slight modifications. Bacteria were suspended to an optical density of 230 Klett™ units, and 25 μL of these suspensions (~10⁷ bacteria) was added to duplicate wells of 24-well TC plates coated with either type I collagen, type IV collagen, laminin or fibronectin (BD Bioware™). Dilutions of each bacterial suspension were spread on agar plates and grown overnight to enumerate the number of colony-forming units (CFU) in the inoculum. The tissue culture plates were centrifuged at 165 x g for 5 min and placed in a 37°C incubator with 7.5% CO₂ for the indicated time. To remove unbound bacteria, each infected well was washed 4 times with phosphate-buffered saline supplemented with gelatin (PBSG), fixed with methanol and stained with 5% Giemsa. Four individual fields from each well were visualized under 100X magnification using a Zeiss microscope. Bacteria were counted by eye, and then normalized to the inoculum. Assays were performed in duplicate on at least three occasions.

Viable colony count adherence assays were performed as described by Holm et al. (127) with slight modifications. Bacteria were grown on agar plates and prepared as described above for the visual adherence assays. Suspensions of bacteria were inoculated
into wells containing monolayers of the indicated epithelial cells seeded at a density of ~ \(10^5\) cells per well. The number of viable bacteria adhering to the epithelial cells was measured by washing the monolayers with PBSG and lysing the epithelial cells with a solution containing 0.005% saponin. These suspensions were then diluted and spread on agar plates to enumerate the number of attached bacteria, which is then normalized by the inoculum. Assays were performed in duplicate or triplicate on at least three occasions.

4.10 Growth Curve

Bacteria were grown on agar plates overnight before resuspension in 25 mL LB in a nephelo culture flask at equal densities (Klett\(^\text{TM}\) 50). These cultures were then grown at 37\(^\circ\) C with shaking at 200 rpm for 6 h. At each hour, the density of the culture was assessed by taking the Klett\(^\text{TM}\) reading using a Klett\(^\text{TM}\) – Summerson colorimeter (Taylor Scientific). Growth curves were performed on at least 3 occasions.

4.11 Biofilm Formation

Bacteria were grown under the indicated conditions in the text, resuspended to a Klett\(^\text{TM}\) 100 density, and then 1 mL aliquots were inoculated into untreated 24 well tissue culture plates. The bacteria were incubated statically at 37\(^\circ\) C or at 23\(^\circ\) C for the designated times before the culture was removed and the well was washed gently with water, followed by incubation in a crystal violet solution. Following a 20 minute incubation, the crystal violet was removed and the well was washed gently with water.
After allowing the plate to dry, 2 mL of 95% ethanol was added to each well and then the plate was incubated for 15 minutes on a shaking platform. Photographs were taken using a Cohu 4922 8 bit camera and then analyzed by densitometry software (Totallab TL100, TotalLab) or the extracted crystal violet was then measured on a µQuant (BioTek) at 570 nm. Assays were performed in duplicate or triplicate on at least three occasions.

4.12 Hemolysis

Bacteria were grown on Trypticase™ Soy Agar plates with 5% sheep red blood (BD – Diagnostic Systems) for the indicated times at 37°C. α-hemolysis was recognized from the green color of the plate under the bacteria while β-hemolysis was observed as a transparent zone under the bacteria on the plate. Assays were conducted on three occasions.

4.13 Macrophage Infection

Antibiotic protection assays were performed essentially as described before (146)(264). Bacteria were grown as indicated in the text and used to inoculate wells containing ~ 10^5 J774A.1 (ATCC® number TIB-67™) macrophage-like cells at an MOI of 1. After a quick, low-speed spin synchronize infection, tissue culture plates were incubated for 1 h to allow for the uptake of the bacteria by the macrophages. Culture supernatants were then removed and fresh media containing the indicated amount of antibiotic was added to kill all extracellular bacteria. These 24-well plates were then
incubated for 2 h after which point the media was removed and the wells were rinsed with PBSG. One set of infected macrophages were then lysed with 0.005% saponin to collect internalized bacteria (3 h), while a parallel set received fresh media without antibiotics. After the indicated time of incubation at 37°C, these wells were again rinsed with PBSG and the surviving intracellular bacteria were recovered by lysing the macrophages with 0.005% saponin. Internalized bacteria at 3 h are expressed as the percent of bacteria internalized normalized by the number of bacteria inoculated into the well. Survival is expressed as the number of bacteria surviving over the course of the experiment divided by the number present at 3 h. Experiments were performed in duplicate on at least three occasions.

4.14 Statistical Analysis

Statistical analyses were performed with the Graph-Pad Prism software using the nonparametric Mann-Whitney test; \( P \) values of < 0.05 were reported as statistically significant.


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