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Subversion of host cellular processes by the melioidosis pathogen, \textit{Burkholderia pseudomallei}

Charles W. Vander Broek
Lay Summary

*Burkholderia pseudomallei* is a bacterium that causes severe disease in humans. The disease often results in death and is very difficult to treat which is why *Burkholderia pseudomallei* is classified as a bioterrorism agent. The bacteria is able to get inside of your cells where it can multiply and avoid detection by the immune system. To enter and survive inside your cells, *Burkholderia pseudomallei* assembles a complex molecular machine on its surface, which works similarly to a syringe. The syringe injects proteins into your cells, reprogramming them help with the bacteria’s life cycle. Using the proteomics technique iTRAQ, we identified two new proteins, called effectors, that the *Burkholderia pseudomallei* syringe injects into your cells. We then used molecular biology techniques to explore which human proteins these new effectors interact with and what these interactions may do to human cells. The reason that this research is important is that it is possible that in the future we can use the identified proteins to design vaccines or design drugs to prevent the reprogramming of your cells, lowering the burden of disease.
Abstract

*Burkholderia pseudomallei* is an intracellular pathogen and the causative agent of melioidosis, a severe disease of humans and animals. One of the virulence factors critical for early stages of infection is the *Burkholderia* secretion apparatus (Bsa) Type 3 Secretion System (T3SS), a molecular syringe that injects bacterial proteins, called effectors, into eukaryotic cells where they subvert cellular functions to the benefit of the bacteria. Although the Bsa T3SS itself is known to be important for host cell invasion, intracellular replication, and virulence, only a few genuine effector proteins have been identified and the complete repertoire of proteins secreted by the system has not yet been fully characterized.

The aims of this study are twofold. The first is to expand the repertoire of known effector proteins using modern proteomics techniques. The second is to explore the function of a subset of effector proteins to better understand their interaction with host cells.

Isobaric Tags for Relative and Absolute Quantification (iTRAQ), a gel-free quantitative proteomics technique, was used to compare the secreted protein profiles of the Bsa T3SS hyper-secreting mutants of *B. pseudomallei* with the isogenic parent strain as well as a mutant incapable of effector protein secretion. This study provides one of the most comprehensive core secretomes of *B. pseudomallei* described to date and identified 26 putative Bsa-dependent secreted proteins that may be considered candidate effectors. Two of these proteins, BprD and BapA, were validated as novel effector proteins secreted by the Bsa T3SS of *B. pseudomallei*. To determine the possible function of two effector proteins, BipC and BapA, a yeast two-hybrid system was used to identify host cell proteins the effectors interact with. The proteins were screened against a library of human proteins for interactions. BapA interacted with 2 proteins while BipC interacted with 14. Both BapA and BipC were shown to interact with human C1QBP, a mitochondrial protein involved in inflammation, immunity and autophagy.
Finally, the Bsa T3SS protein BipC was characterised in its ability to interact with actin. This study is the first evidence that BipC has the ability to bind to filamentous actin, but not monomeric actin. This binding is direct and no intermediate proteins are required for the interaction. Ectopic expression of BipC in eukaryotic cells caused cytoskeletal rearrangements consistent with an actin-binding protein.

The core secretome represents a substantial resource of targets that will be mined for improved diagnostic assays and vaccines. Diagnostics that will detect early stages of disease to allow for more effective antimicrobial intervention are currently lacking. Furthermore, there is scope to design diagnostic assays with dual use such as to detect both melioidosis and infection of cystic fibrosis patients with the closely related opportunistic pathogen *B. cepacia*. The description of novel T3SS effector proteins is also of considerable value since T3SS proteins are often potent B- and T-cell antigens representing promising components of sub-unit vaccines. Such effector proteins commonly modulate cellular processes such as phagocytosis, inflammasome activation and cell cycle progression, hence the function of the predicted T3SS effectors will provide a series of future research opportunities.
Declarations

I, Charles W. Vander Broek, have read and understood The University of Edinburgh guidelines on plagiarism and declare that the work presented in this thesis is the result of my own original research, except where I indicate otherwise by proper use of quotes and references.

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Chapter 1

Introduction

1.1 Melioidosis

1.1.1 Epidemiology

Originally described in drug addicts in Rangoon in the early 20th century by Alfred Whitmore (Whitmore, 1913), *Burkholderia pseudomallei*, formerly *Pseudomonas pseudomallei* (Yabuuchi et al., 1992), is a facultative intracellular pathogen (Pruksachartvuthi et al., 1990) that is the causative agent of melioidosis, or Whitmore’s disease. It is a rod shaped bacterium that is motile, oxidase-positive and Gram-negative, but with a staining pattern that resembles a safety pin in appearance (Dance et al., 1989). Melioidosis is not host specific, causing disease in humans, a wide range of other mammals, as well as birds and reptiles (Reviewed in (Titball et al., 2008)). Despite the fact that *B. pseudomallei* is an important human pathogen, it is considered a saprophyte and is found in the soil and water in Northern Australia and Southeast Asia (Brook et al., 1997, Draper et al., 2010). Clinical cases of melioidosis have also been reported in Northeastern Brazil, on the Indian sub-continent, Africa, China, the Caribbean and Mexico (Rolim et al., 2005, Vidyalakshmi et al., 2007), (Reviewed in Currie et al. (2008), Birnie et al. (2015)).

Infection with *B. pseudomallei* is usually associated with environmental exposure and can occur through breaks in the skin, inhalation or ingestion (Reviewed in (Cheng and Currie, 2005)). Up to 85% of cases of *B. pseudomallei* infection in Thailand occur during the rainy season (Currie and Jacups, 2003). Infection with the bacteria has been linked to extreme weather events such as cyclones, and due to an increase in *B. pseudomallei*-related pneumonia after such events, it is thought that this may be caused by bacteria being aerosolized
from surface water and soil and then inhaled (Currie and Jacups, 2003). It is also thought that melioidosis can be caused by ingestion. In a mouse model of infection the bacteria is able to colonize the stomach after oral inoculation (Goodyear et al., 2012). *B. pseudomallei* is the leading cause of community-acquired bacteraemia in Thailand with an incidence of melioidosis reported to be 12.7 cases per 100,000 people (Limmathurotsakul et al., 2010). In Northern Australia, *B. pseudomallei* causes 32% of septicaemia-related pneumonia, more than any other community-acquired bacterial infection (Douglas et al., 2004).

### 1.1.2 Clinical Symptoms and Treatment

In the majority of cases, the incubation period for melioidosis is between 1 and 21 days (Ngauy et al., 2005). About 50% of melioidosis cases affect people with diabetes and other important risk factors include lung disease, cystic fibrosis and excessive alcohol consumption (Currie et al., 2010). There are varied clinical presentations of *B. pseudomallei* infection ranging from skin infections to pneumonia and septic shock (Figure 1.1) (Currie et al., 2010). In one study of melioidosis in Northern Australia, the most common presentations were pneumonia, genitourinary tract infection and skin infection (Currie et al., 2010). In the same study of 540 cases of melioidosis, 298 of the patients developed bacteraemia of which 58 died (Currie et al., 2010). Even with modern medical care the mortality of patients diagnosed with melioidosis remains high with a mortality rate of over 40% in Northeast Thailand and 14% in Northern Australia (Currie et al., 2010, Limmathurotsakul et al., 2010).

One interesting aspect of melioidosis infection is its ability to reactivate after remaining latent for years following a primary infection. The longest reported period between infection and reactivation occurred in a World War II veteran who manifested symptoms 62 years after exposure (Ngauy et al., 2005). Patients who survive an initial infection with *B. pseudomallei* have a 13% chance of recurrence over the following 10 years (Maharjan et al., 2005). Of recurrent cases, 75% are caused by the same strain indicating a relapse (Maharjan et al., 2005). In one patient with chronic asymptomatic melioidosis, the genome of the single infecting strain of *B. pseudomallei* was sequenced twelve years apart (Price et al., 2013). The sequencing indicated that over the twelve year period the *B. pseudomallei* strain underwent host adaptation resulting in the loss of over 221 genes, including genes involved in lipopolysaccharide biosynthesis (Price et al., 2013). It is not uncommon for melioidosis patients to be infected
Figure 1.1: Transmission and outcome of infection with *B. pseudomallei*. The routes of infection for *B. pseudomallei* are through cuts or breaks in the skin, inhalation or ingestion (indicated by the blue boxes). Clinical presentations of melioidosis are varied and range from mild skin infections, genito-urinary infections, organ abscesses and pneumonia. Figure taken from Wiersinga et al. (2012).
by more than one strain of *B. pseudomallei* concurrently with 5 out of 18 patients having 2 or more strains upon hospitalization (Pitt et al., 2007).

Isolation of *B. pseudomallei* from patients by culture is still the primary method of diagnosis which is usually carried out using Ashdown’s selective agar (Peacock, 2006). Because bacterial culture methods with *B. pseudomallei* may not be available in all locations, it is likely that the incidence of melioidosis may be under-reported (Limmathurotsakul et al., 2010).

Treatment of melioidosis often requires supportive therapy as well as administration of antibiotics as there is currently no approved vaccine for melioidosis (Reviewed in Cheng and Currie (2005)). *B. pseudomallei* is highly resistant to many antibiotics including cephalosporins, penicillins, rifamycins, aminoglycosides, quinolones and macrolides making it very difficult to treat (Reviewed in Cheng and Currie (2005)). Recommended antibiotic treatment includes ceftazidime, imipenem or meropenem with co-trimoxazole (Reviewed in Cheng and Currie (2005)). Because of the high mortality rate, the resistance to a large number of antibiotics, the ability to be cultured easily as well as disseminated as an aerosol and spread into the environment, *B. pseudomallei* has been classified as a schedule 5 organism in the UK and as a class B bioterrorism agent by the US Centres for Disease Control and Prevention (Reviewed in Cheng and Currie (2005), (Rotz et al., 2002)).

### 1.1.3 Related Species of Importance

The genus *Burkholderia*, originally defined by Yabuuchi et al. (1992), now contains over 40 species of bacteria including the human pathogens *B. pseudomallei*, *Burkholderia mallei* and the *Burkholderia cepacia* complex of species (Reviewed in (Compant et al., 2008)). Members of the genus *Burkholderia* are found in a range of environments from soil and water to animals and humans, and species are used for their ability to assist with bioremediation and plant growth promotion (Reviewed in (Coenye and Vandamme, 2003) and Compant et al. (2008)). *Burkholderia* are primarily pathogens of plants, but some species are animal and human pathogens, with two clinically relevant species being *B. pseudomallei* and *B. mallei* (Reviewed in (Compant et al., 2008)). In addition, the *B. cepacia* complex is known for causing severe opportunistic infections in cystic fibrosis patients (Reviewed in (Drevinek and Mahenthiralingam, 2010)).

*B. mallei*, the causative agent of glanders, is an obligate pathogen unable to survive outside its host with restricted host range (Yabuuchi et al., 1992, Srinivasan et al., 2001). Glanders is an infection of equids which can also be trans-
mitted to humans (Srinivasan et al., 2001). Symptoms of glanders in equids include nasal discharge, enlargement of lymph nodes and the presence of nodules and ulcers on the extremities, as well as fever and emaciation (Srinivasan et al., 2001, Khan et al., 2013). Glanders was a major disease in horses in the past, but has been largely eradicated in the West, though there are signs it may be re-emerging (Srinivasan et al., 2001, Khan et al., 2013). In humans, B. mallei causes a disease similar to melioidosis and because of its similarities to B. pseudomallei, B. mallei is also listed as a schedule 5 organism in the UK and a class B bioterrorism agent by the US CDC (Van Zandt et al., 2013, Rotz et al., 2002).

*Burkholderia thailandensis* is a bacterium that is considered non-pathogenic to humans and has been used as an alternative model system for *B. pseudomallei* and *B. mallei* in some studies as it contains many homologues of virulence factors from these pathogenic species (Brett et al., 1998, Haraga et al., 2008, Moore et al., 2004, Yu et al., 2006). *B. thailandensis* was originally differentiated from *B. pseudomallei* in soil samples by its ability to assimilate L-arabinose, a function which *B. pseudomallei* lacks (Wuthiekanun et al., 1996). It was shown that clinical samples from melioidosis patients never contained bacteria with the ability to assimilate L-arabinose (*B. thailandensis*) (Wuthiekanun et al., 1996). However, there has been a reported case of non-fatal *B. thailandensis* infection in Thailand in a 16-year-old man that sustained injury causing heavy inoculation of the bacteria as the result of a motorcycle accident (Lertpatanasuwan et al., 1999). Another reported case of non-fatal *B. thailandensis* infection was in a 2 year old male in the United States after a car accident left the child submerged under water for 2 minutes allowing bacteria to enter his lungs (Glass et al., 2006a). Importantly, while it has been shown that *B. thailandensis* can be used as a model system to study the virulence associated T3SS of *B. pseudomallei*, because *B. thailandensis* is less pathogenic to humans, study of the Bsa T3SS in the native organism is still warranted (Haraga et al., 2008).

Other similar species include *Burkholderia humptydooensis* which is also able to assimilate L-arabinose and is found in the waters of Northeastern Australia (Gee et al., 2008, 2014) and *Burkholderia oklahomensis*, which is a pathogenic *B. pseudomallei*-like strain found in soil in the United States. It was initially isolated from a leg wound of a farmer in Oklahoma and also having caused disease in an automobile accident victim in Georgia (Glass et al., 2006b, McCormick et al., 1977, Nussbaum et al., 1980).
Figure 1.2: The two chromosomes of *B. pseudomallei* K96243. Chromosome 1 is comprised of 4.07 Mb and contains many housekeeping genes while chromosome 2 is comprised of 3.17 Mb and contains genes that are associated with adaptation to changing environments. The genome contains 16 genomic islands that are thought to be the product of horizontal gene transfer. The outside ring depicts the locations of the 16 genomic islands in red. The second ring in shows a scale bar in Mb. The 3rd ring shows annotated coding sequences colour coded as follows: dark blue, pathogenicity/adaptation; black, energy metabolism; red, information transfer; dark green, surface-associated; cyan, degradation of large molecules; magenta, degradation of small molecules; yellow, central/intermediary metabolism; pale green, unknown; pale blue, regulators; orange, conserved hypothetical; brown, pseudogenes; pink, phage plus IS elements; gray, miscellaneous. The 4th, 5th and 6th rings depict: additional coding sequences compared to the sequenced *B. mallei* strain ATCC 2944; the percentage of G + C content plot; (G–C)/(G + C) deviation plot (>0%, olive; <0%, purple). Figure taken from Holden *et al.* (2004).
1.1.4 Genomics

The genome of the *B. pseudomallei* reference strain K96243 is large for a bacterium (7.2 Mb) and consists of two circular chromosomes (Figure 1.2) (Holden et al., 2004). Chromosome one (4.07 Mb) encodes mostly housekeeping genes, while chromosome two (3.17 Mb) encodes largely genes that are expressed in response to different environments the bacteria may encounter, including many of the known virulence factors (Holden et al., 2004). Full genome comparisons of almost 100 strains of *B. pseudomallei* identified 86% of genes as being conserved and present in all strains (Sim et al., 2008). The other 14% of genes were considered accessory genes, and disproportionately represented genes present in strain K96243 genomic islands (GIs) (14 of 16 GIs were represented by accessory genes) and were associated with clinical isolates (Sim et al., 2008). The GIs in *B. pseudomallei* are highly variable between strains. A study of five clinical *B. pseudomallei* strains identified a total of 71 GIs distributed between the strains, with at least half being unique to the strain in which they were identified (Tuanyok et al., 2008). The variability in these regions is largely due to horizontal gene transfer and *B. pseudomallei* has one of the highest rates of lateral gene transfer as compared to the mutation rates of any other bacterial species measured (Pearson et al., 2009). Interestingly, large differences in the genomes of *B. pseudomallei* can be correlated with the geographic distribution of the strains in the endemic regions of Northern Australia and Northeast Thailand (Vesaratchavest et al., 2006).

1.1.5 Pathogenesis

*B. pseudomallei* has many factors associated with its ability to cause disease including the production of surface polysaccharides, quorum sensing, flagella, pili, intracellular motility (BimA), as well as Type 2, 3 and 6 secretion systems (Reviewed in Galyov et al. (2010)).

*B. pseudomallei* produces four types of surface polysaccharides identified as types I-IV O-PS. Type I O-PS produces an –3)–2-O-acetyl–6-deoxy-β-D-manno-heptopyranose-(1- capsule that is similar to that of *Neisseria meningitides* and *Haemophilus influenza* (Reckseidler et al., 2001). The capsule has been shown to provide resistance to complement (Reckseidler-Zenteno et al., 2005). It was shown that the LD50 for a capsular mutant was over 1000 fold higher than that of wild type *B. pseudomallei* in an animal model of infection (Reckseidler et al., 2001). Type II O-PS forms the *B. pseudomallei* lipopolysaccharide which
is required for full virulence in hamster, guinea pig and rat models of melioidosis (DeShazer et al., 1998). The importance of type I and II O-PS in virulence is further demonstrated by the ability of antibodies to the two types of O-PS to provide passive protection to mice infected intraperitoneally with $10^4$ CFU of B. pseudomallei (Jones et al., 2002). Type III and IV O-PS are less well characterised, but of the two only type IV O-PS is required for full virulence in a murine model of melioidosis (Sarkar-Tyson et al., 2007).

The intracellular life of B. pseudomallei is shown in Figure 1.3. B. pseudomallei is a facultative intracellular pathogen capable of survival in both phagocytic and non-phagocytic cell lines (Jones et al., 1996). Internalization of B. pseudomallei can be inhibited by cytochalasin D, which is an actin polymerization inhibitor (Jones et al., 1996). This indicates the importance of actin cytoskeletal rearrangement in uptake of B. pseudomallei. Once in the cytosol a bacterium needs to be able to survive the antimicrobial peptides produced by the host cell. B. pseudomallei was shown to be resistant to antimicrobial peptides, showing a statistically significant increase in growth rate when exposed to one antimicrobial peptide, the defensin human neutrophil peptide 1 (HNP–1), which is commonly found in the granules of neutrophils (Jones et al., 1996). B. pseudomallei also appears to interfere with host cell synthesis of inducible nitric-oxide synthase preventing the production of reactive nitrogen species, which likely helps the bacteria to survive the intracellular environment of the macrophage (Utaisincharoen et al., 2006).

The mechanisms by which B. pseudomallei escape the host cell are still unclear, but it has been suggested it may be related to intracellular motility and the fusion of host cells into multinucleated giant cells (MNGC) (Kespichayawattana et al., 2000). B. pseudomallei has been shown to induce MNGC formation when internalized in macrophages as well as in non-phagocytic cell lines (Kespichayawattana et al., 2000). MNGCs are formed by the fusion of macrophages or monocytes and have been associated with infections by pathogens such as Mycobacterium tuberculosis and HIV (Brodbeck and Anderson, 2009). Intracellular motility is required for the formation of MNGCs by B. pseudomallei (French et al., 2011). B. pseudomallei has the ability to polymerize host cell actin at one pole to create actin-based motility through a mechanism involving the bacterial protein BimA (Stevens et al., 2005). Other intracellular bacteria including Shigella flexneri and Listeria monocytogenes are known to polymerize actin at one pole in order to spread directly from cell to cell, but these bacteria do not induce MNGC formation (Kespichayawattana et al.,
Figure 1.3: The intracellular life of *B. pseudomallei*. Upon contact with a host cell, *B. pseudomallei* become internalized (a), escape the endocytic vacuole (b), polymerize actin at one pole creating actin-based motility (c) and cause membrane fusion forming multinucleated giant cells (MNGC). Figure taken from Wiersinga *et al.* (2006).
Another bacterial factor involved in MNGC formation is LfpA, which drives infected RAW 264.7 cells towards an osteoclast-like phenotype (Boddey et al., 2007). MNGCs are important in vivo, and are not just an in vitro experimental artefact, because they can be found in the tissues of infected humans and animals (Wong et al., 1995). It is speculated that the fusion of host cells in this way may be a mechanism for cell-to-cell spreading while evading the host immune system (Kespichayawattana et al., 2000).

Secretion systems play a role in almost all steps of the B. pseudomallei infection cycle and allow the transport of bacterial proteins across the inner and outer bacterial membranes. The Type 2 secretion system (T2SS) allows transport of proteins into the surrounding environment, while the Type 3 (T3SS) and Type 6 secretion systems (T6SS) inject proteins directly into the cytoplasm target host cells (Reviewed in Costa et al. (2015)). The B. pseudomallei T2SS is responsible for the secretion of 48 proteins including 12 proteases and the deubiquitinase TssM (Burtnick et al., 2014). TssM inhibits the activation of NF-κB and TssM mutants are attenuated in a murine model of inhalation melioidosis (Tan et al., 2010). Yet T2SS mutants display no attenuation of virulence in mice inoculated intraperitoneally indicating further work is needed for a full understanding of the importance of the T2SS in B. pseudomallei (Burtnick et al., 2014).

The ability of B. pseudomallei to invade cells has also been linked to the Burkholderia Secretion Apparatus (Bsa) Type 3 Secretion System (T3SS) (Stevens et al., 2003). The Bsa T3SS is also required for efficient escape of the bacterium from the endocytic vesicle following uptake (Stevens et al., 2002). The Bsa T3SS is similar to that of the Inv/Mxi-Spa T3SS found in Salmonella Spp. and S. flexneri which is required in these bacteria for invasion and escape from the endocytic vacuole into the cytosol of the host cell (Stevens et al., 2002). In B. pseudomallei, it has also been shown that escape from the vacuole is required for MNGC formation which is further supported by a loss of MNGC formation when a deletion mutation is made deactivating the Bsa T3SS (Burtnick et al., 2008). The Bsa T3SS is discussed in more depth in the next section.

The T6SS is a mechanism Gram-negative bacteria use to transport a protein across the cell envelope and into a target eukaryotic or bacterial cell (Bingle et al., 2008). B. pseudomallei K96243 encodes six T6SSs in its genome (Burtnick et al., 2011). The bacterial T6SS resembles the T4 bacteriophage tail in structure with the hexameric Hcp protein polymerizing to form the tube-like structure and VgrG proteins forming the cell-puncturing device at the tip of the secre-
A recent study showed that a mutation in the hcp1 gene of the T6SS–5 of B. pseudomallei K96243 ablated the ability of the bacteria to induce MNGCs whilst maintaining the ability to enter cells, escape into the cytosol and display actin-based motility in RAW 264.7 macrophages (Burtnick et al., 2011). The same study also demonstrated that the hcp1 mutant had lowered virulence in a Syrian hamster model of melioidosis indicating its importance in vivo (Burtnick et al., 2011). It has also been shown that VgrG-5 is required for formation of MNGCs (Toesca et al., 2014, Schwarz et al., 2014). The regulation of the T6SS–5 of B. pseudomallei is itself regulated by BsaN, which also regulates effector protein secretion in the Bsa T3SS (Chen et al., 2011).

1.1.6 Animal Models of Infection

In order to better investigate the pathogenesis of B. pseudomallei in vivo, a number of animal models of infection have been developed. The most frequently used animal model of melioidosis is the murine model of infection using BALB/c or C57BL/6 (Reviewed in Titball et al. (2008), Warawa (2010)). BALB/c mice are more susceptible to B. pseudomallei and represent an acute model of infection, while C57BL/6 mice are comparatively more resistant and represent a chronic model of disease (Leakey et al., 1998). Both the strain of B. pseudomallei used and the route of infection determine the median lethal dose, which ranges from <10 to 10^6 CFU over a period from 10 to 65 days (Reviewed in Titball et al. (2008), Warawa (2010)). Large numbers of bacteria are recovered from the liver and spleen of infected animals, which reflects human septicaemic disease (Reviewed in Titball et al. (2008)).

While mice are the primary surrogate model used for melioidosis, there are a range of other models used less frequently. Syrian hamsters have been used as an infection model of B. pseudomallei and are extremely sensitive to the bacterium, with a median lethal dose at 48 hours of <10 CFU (Brett et al., 1997). In order to conduct research in a model that is more physiologically relevant, models of melioidosis in non-human primates, generally rhesus macaques (Macaca mulatta), have also been described (Yingst et al., 2014).

There are also well described models of melioidosis in invertebrates. For example, the wax moth larvae (Galleria mellonella) infection model has been shown to be sensitive enough to differentiate between B. pseudomallei strains shown to be virulent or avirulent in mice (Wand et al., 2011). B. pseudomallei has been shown to be intracellular in the haemocytes of infected wax moths (Wand et al., 2011). Invertebrate models such as the wax moth larvae may help
to reduce the use of animals in early stages of pathogenesis research.

1.2 Type 3 Secretion System

Type 3 secretion systems (T3SSs) span the bacterial inner and outer membranes forming a "molecular syringe" which allows Gram-negative bacteria to export proteins called effectors from the bacterial cytoplasm into a target eukaryotic cell (Reviewed in (Galán et al., 2014)). T3SSs have been shown to be important for virulence in many bacteria including pathogenic Escherichia coli, Salmonella, Shigella, Yersinia and Burkholderia (Galan and Curtiss, 1989, Maurelli et al., 1985, Gemski et al., 1980, Jarvis et al., 1995, Stevens et al., 2002). The B. pseudomallei genome encodes three T3SSs. T3SSs-1 and -2 share homology with Hrp2 family of T3SSs found in plant pathogens (Winstanley et al., 1999, Rainbow et al., 2002). Indeed, B. pseudomallei T3SS-1 and -2 mutants are attenuated in a tomato plant model of infection (Lee et al., 2010). Though T3SSs-1 and -2 were shown to be dispensable in a Syrian hamster model of infection (Warawa and Woods, 2005), a more recent study found that T3SS-1 was required for full virulence in a respiratory murine model of melioidosis (D’Cruze et al., 2011a). T3SS-3, also known as the Burkholderia Secretion Apparatus (Bsa) T3SS, is similar to the Inv-Spa (Salmonella pathogenicity island (SPI)-1) and Mxi-Spa T3SSs of the animal pathogens Salmonella spp. and S. flexneri, respectively (Stevens et al., 2002). The Bsa T3SS is required for full virulence in both murine and Syrian hamster models of infection (Stevens et al., 2004, Warawa and Woods, 2005, Gutierrez et al., 2015). T3SSs-2 and -3 are present in the genomes of B. mallei and B. thailandensis, whereas T3SS-1 is absent from both (Rainbow et al., 2002).

1.2.1 Structure

The core structure of the T3SS is well conserved and is similar to that of the bacterial flagella system (Figure 1.4:A-B)(Young et al., 1999, Kubori et al., 1998, Gophna et al., 2003). It is thought that the flagella and the T3SS evolved from a similar ancestor, but the T3SS has been the product of a large amount of horizontal gene transfer (Gophna et al., 2003). T3SSs are separated into seven different families named after the archetype system in each family, each with slight differences in structure and host cell target, with multiple types of T3SS present in some bacterial species (Figure 1.4:C-D)(e.g. plant or animal) (Reviewed in Büttner (2012)).
Figure 1.4: Overview of T3SSs from animal- and plant-pathogenic bacteria. (A) The translocation-associated T3SS functions similar to a molecular syringe, allowing injection of bacterial proteins into the cytoplasm of a target host cell. (B) The flagellar T3SS has an extracellular hook and is involved in bacterial motility. (C) A list of the different families of translocation-associated T3SSs from bacterial pathogens of plants or animals. (D) Examples of bacterial species and their corresponding translocation-associated T3SSs (note some species contain multiple systems). Figure taken from Büttner (2012).
Figure 1.5: The 3-D structure of the Salmonella T3SS as determined by cryo-EM. (a) Surface views of the T3SS showing base comprised of the membrane spanning inner and outer membrane rings connected by a neck, and the filament forming the extracellular “needle”. (b) Section view showing the path an unfolded effector protein (substrate) takes through the needle complex. (c) Component view of the T3SS. Figure taken from Galán et al. (2014).
In recent years the structure of the *Salmonella* T3SS has been solved using cryo-EM (Schraidt and Marlovits, 2011). The structure of the *Salmonella* T3SS (Reviewed by Galán *et al.* (2014)) consists of inner and outer membrane rings connected by a rod, the extracellular needle and a secreted translocation pore which spans the target cell membrane (Figure 1.5). Assembly of the T3SS appears to be hierarchical and is the subject of multiple recent reviews (Büttner, 2012, Diepold and Wagner, 2014). Based on evidence from *Yersinia* and *Salmonella*, it appears construction of the T3SS begins with the formation of the outer-membrane/structural ring (Secretin) and independently the inner-membrane export machinery, which are then linked by YscJ (*Yersinia*) or PrgK (*Salmonella*), followed by assembly of the ATPase/C-ring and the formation of the mature needle complex (Figure 1.6) (Diepold *et al.*, 2010, 2011, Wagner *et al.*, 2010).

Only a few structural proteins in the *B. pseudomallei* Bsa T3SS have been studied. Figure 1.7 shows the predicted location of each of the structural components of the Bsa T3SS. BsaZ(BPSS1534) is a structural component with homology to SpaS of *Salmonella* (Stevens *et al.*, 2002). SpaS is a component of the inner membrane complex and has been shown to undergo autocleavage causing a switch between secretion of structural components to secretion of the needle complex and translocator proteins (Zarivach *et al.*, 2008). Mutations in *bsaZ* caused delays in escape from the phagosome and reduced intracellular survival in J774.2 cells (Stevens *et al.*, 2002, Burtnick *et al.*, 2008). *bsaZ* mutants have also been shown to be attenuated in murine (Stevens *et al.*, 2004, Burtnick *et al.*, 2008) and Syrian hamster (Warawa and Woods, 2005) models of melioidosis. The *bsaZ* mutant phenotype is thought to be due to its inability to secrete effector and translocator proteins such as BopE and BopC (Stevens *et al.*, 2003, Muangman *et al.*, 2011).

Another component of the inner membrane ring of the *B. pseudomallei* Bsa T3SS that has been described is BsaQ (BPSS1543), which is homologous to InvA from *Salmonella* (Sun *et al.*, 2005). InvA is required for the formation of the mature T3SS in *Salmonella* as well as secretion of effector proteins (Kubori *et al.*, 2000). *bsaQ* mutants displayed a similar phenotype to *bsaZ* mutants in that they are delayed in phagosome escape resulting in reduced intracellular survival (Muangsombut *et al.*, 2008). The *bsaQ* mutants are unable to secrete BopE or BipD into the supernatants and showed defects in cell invasion (Muangsombut *et al.*, 2008).

A third inner membrane component, BsaM (BPSS1547), is homologous to
Figure 1.6: The assembly of the T3SS in *Yersinia*. Assembly of the outer membrane/structural rings (A) and the inner membrane export machinery (B) appear to happen simultaneously in two separate branches. The recruitment of YscJ by both complexes allows the two structures to merge (C). Finally, the ATPase-C ring is added to the base of the injectisome (D), followed by secretion and assembly of the needle (E). Figure taken from Diepold *et al.* (2011).
Figure 1.7: Predicted structure of the *B. pseudomallei* Bsa T3SS. Each of the proteins of the Bsa T3SS are mapped onto their predicted location corresponding to homologous proteins within *Salmonella*. Figure taken from Sun and Gan (2010).
Salmonella PrgH, which forms the inner membrane ring of the Salmonella T3SS and is required for secretion of effector proteins (Kubori et al., 2000, Marlovits et al., 2004). A B. pseudomallei bsaM mutant was shown to induce lower levels of NF-κB in HEK 293T cells when compared to WT, but similar levels to a bsaS mutant (Teh et al., 2014). The bsaM mutant was shown to activate NF-κB at time points corresponding to a delayed escape from the phagosome suggesting it has similar effects to the other bsa T3SS structural knockouts (Teh et al., 2014).

BsaU (BPSS1539) is homologous to InvJ from Salmonella which is the "molecular ruler" which determines the length of the T3SS needle (Kubori et al., 2000). Mutation of bsaU in B. pseudomallei resulted in a delay in phagosome escape and reduced virulence in a BALB/c intranasal mouse infection model (Pilatz et al., 2006). The mutant was also deficient in its ability to secrete BopE and BipD into the supernatant (Pilatz et al., 2006).

The protein BsaL (BPSS1548) has had its crystal structure solved and was shown to have a similar structure to MxiH (Shigella) and PrgI (Salmonella), which form the external needle structure (major needle subunit) of the T3SS (Zhang et al., 2006, Wang et al., 2007, Barrett et al., 2008). The Nod-like receptor NLRC4 recognises T3SS minor needle component proteins from a range of Gram-negative bacteria, including BsaK (BPSS1549) from B. pseudomallei, activating an innate immune response through casapase-1 (Miao et al., 2010, Zhao et al., 2011, Bast et al., 2014). A B. pseudomallei bsaK mutant was highly attenuated in an intranasal murine model of melioidosis (Bast et al., 2014).

1.2.2 Energising Secretion

Proteins are secreted in an unfolded state due to the narrow width of the needle channel which is around 2–3 nm in S. flexneri (Blocker et al., 2001, Radics et al., 2014). This was proven by cryo-EM imaging of a substrate which was engineered to become trapped in the secretion channel (Radics et al., 2014). The ATPase at the base of the secretion system has been shown to dissociate proteins from their chaperones and also is thought to be involved in unfolding of the protein (Akeda and Galán, 2005). The source of energy for exporting proteins is the subject of some debate. It has been shown in the flagellar T3SS of Yersinia and Salmonella that secretion can take place in the absence of the ATPase (Wilharm et al., 2004, Erhardt et al., 2014). This evidence, combined with a study which showed flagellar T3S was halted in the absence of a proton gradient, has led to the hypothesis that the ATPase is primarily for unfolding and proton motive force energises secretion of the unfolded substrate (Paul et al.,
A. pseudomallei bsaS (BPSS1541) mutant was shown to be unable to secrete the known effector protein BopE (Gong et al., 2015). The mutant also showed a reduction of intracellular survival in RAW 264.7 cells and was attenuated in BALB/c mice infected intranasaly, demonstrating that the ATPase is required for Bsa T3SS function (Gong et al., 2015).

### 1.2.3 Needle Tip and Translocators - Sensing Host Contact

Upon host cell contact, the translocators of the T3SS are inserted into the host cell membrane forming a pore through which effector proteins may pass. *Shigella* was shown to lyse red blood cells (RBC) by inserting the translocators IpaB and IpaC into the RBC membrane forming a 25 angstrom pore (Blocker et al., 1999). Secretion of the translocator proteins is regulated by the needle tip protein. A *Shigella* tip protein mutant, *ipaD*, constitutively secreted the translocators, IpaB and IpaC (Picking et al., 2005). Immunofluorescence staining for IpaD of *Shigella*, demonstrates the presence of the protein on the surface of the bacteria in the absence of a host cell membrane, which was further confirmed by electron microscopy (Espina et al., 2006). The needle tip protein probably acts to sense host cell contact, stopping early secretion of translocators (Espina et al., 2006). In the same study it was shown that antibodies to the tip protein disrupted the haemolysis of sheep erythrocytes indicating IpaD’s functional importance in the insertion of the translocator complex into eukaryotic cell membranes as well as regulating effector secretion (Espina et al., 2006).

Continuing with *Shigella* as an example, growth in the bile salt deoxycholate (DOC) causes IpaB to be present at the tip of the needle in a manner which correlates to DOC binding IpaD (Olive et al., 2007, Stensrud et al., 2008). The authors suggest this may correspond to *Shigella* sensing bile salts as it passes through the human GI tract and is an initial step in “priming” the T3SS (Stensrud et al., 2008). The final step in secretion of the translocation pore was shown to be dependent on *Shigella* interacting with liposomes to induce IpaC secretion (Epler et al., 2009). When cultured in the presence of liposomes, IpaC localised to the bacterial surface and was secreted into the supernatant as shown by electron microscopy and Western blot (Epler et al., 2009). This agrees with earlier evidence that cholesterol is bound by translocation components (*Salmonella* SipB and *Shigella* IpaB), is important for entry into host cells and is required for efficient translocation of effector proteins into host cells (Lafont et al., 2002, Hayward et al., 2005).
BipD (BPSS1529)

BipD (BPSS1529) is the needle tip protein of the Bsa T3SS. It is homologous to SipD (Salmonella), IpaD (Shigella) and LcrV (Yersinia). The crystal structure of BipD was solved (Erskine et al., 2006, Knight et al., 2006, Roversi et al., 2006, Johnson et al., 2007, Pal et al., 2010) and the 3-dimensional structure is highly similar to IpaD of Shigella (Johnson et al., 2007) and Salmonella SipD (Espina et al., 2007). It was demonstrated that the structure of BipD, as well as IpaD and SipD, is dependant on pH changes (Markham et al., 2008). Interestingly when cultured in a more acid pH of 4.5, B. thailandensis secretes larger amounts of BipD as well as BopE (Jitprasutwit et al., 2010). The same study also described distinct forms of BipD in B. pseudomallei and B. thailandensis, but one B. thailandensis strain carried a B. pseudomallei-like BipD, possibly due to a shared environmental reservoir (Jitprasutwit et al., 2010).

Needle tip proteins are of interest because of their potential as subunit vaccines. Most notably the needle tip protein LcrV (V antigen) from Yersinia pestis, especially when combined with the Fraction 1 (F1) protein, is an effective vaccine that has been tested in human clinical trials (Reviewed by Williamson (2009)). Vaccination of mice with the Shigella needle tip protein IpaD, along with the translocator IpaB, induces high levels of protection upon subsequent challenge (Martinez-Becerra et al., 2012, Heine et al., 2013, Martinez-Becerra et al., 2013).

In B. pseudomallei, it was initially described that convalescent serum from a melioidosis patient reacted specifically with recombinant GST-BipD (Stevens et al., 2002). CD4+ T cells taken from mice infected with an attenuated strain of B. pseudomallei showed specificity for BipD (Haque et al., 2006). Similarly, human monocyte-derived dendritic cells from healthy B. pseudomallei seropositive donors were pulsed with purified BipD after which CD4+ T cells were able to recognise the recombinant protein (Tippayawat et al., 2011). BipD mutants in B. pseudomallei strains 10276 and 576 were significantly attenuated in a BALB/c intranasal murine infection model when compared to WT, demonstrating the importance of BipD in vivo (Stevens et al., 2004). Multiple attempts have been made to use BipD as a recombinant subunit vaccine in a BALB/c murine intraperitoneal model of infection, but in both studies the vaccine showed no protection when the mice were challenged (Stevens et al., 2004, Druar et al., 2008).
**BipB (BPSS1532)**

BipB (BPSS1532) shares 46% amino acid identity with the translocator protein SipB from *Salmonella*. A *B. pseudomallei* K96243 *bipB* insertion mutant showed reduced invasion and cell-to-cell spread in HeLa cells and a reduced ability to form MNGCs in J774A.1 cells. *In vivo* in BALB/c mice infected intranasally, the *bipB* mutant was greatly attenuated and showed a phenotype similar to that of the BipD mutant (Suparak *et al.*, 2005, Stevens *et al.*, 2004). This is likely due to the inability of the T3SS to function correctly without the formation of the translocation pore. The N-terminal region of BipB was tested as a protective antigen for *B. pseudomallei* infection in mice, but showed no protection (Druar *et al.*, 2008).

**BipC (BPSS1521)**

BipC (BPSS1531) is a homologue of the translocators SipC from *Salmonella* and IpaC from *Shigella*. SipC has been shown to interact with SipB to form the translocon pore (Myeni *et al.*, 2013). Beyond their role as a translocator protein, SipC and IpaC also function as effector proteins within the eukaryotic cell. SipC has actin nucleation activity and bundles F-actin (Hayward and Koronakis, 1999). The ability of SipC to nucleate and bundle F-actin as well as form the translocation pore, are all dependant on the C-terminal 209 amino acids of the 409 amino acid protein (Hayward and Koronakis, 1999, Chang *et al.*, 2005, Myeni and Zhou, 2010). Actin bundling appears to be important for cell invasion, as *Salmonella* containing a mutated form of *sipC* that is unable to bundle actin, but still able to act as a translocon, was less invasive than WT *Salmonella* (Myeni and Zhou, 2010). SipC has also been shown to bind to host Syntaxin6 and thereby recruit LAMP1 to the *Salmonella* containing vacuole (SCV), helping to stabilise its membrane (Madan *et al.*, 2012). Interestingly, the protein SipA enhances SipC’s ability to nucleate and bundle F-actin (McGhie *et al.*, 2001), but a homologue of SipA is not present in *B. pseudomallei*.

The C-terminal and N-terminal regions of BipC were separately tested as a protective antigen for *B. pseudomallei* infection in mice, but neither antigen showed any protection (Druar *et al.*, 2008). These results agreed with previously reported unpublished results from other studies (J. Hill, M. W. Wood & E. E. Galyov, unpublished observations). In another study, a *B. pseudomallei* K96243 *bipC* insertion mutant showed reduced cell adhesion and invasion of A549 cells when compared to the WT strain (Kang *et al.*, 2015). This *bipC*
mutant also showed a delay in escape from the phagosome leading to a delay in formation of actin tails and intracellular replication (Kang et al., 2015). The mutant was also attenuated in BALB/c mice infected intraperitoneally when compared to WT (Kang et al., 2015). While this shows the importance of BipC in virulence, both in vivo and in vitro, it does not separate BipC’s role as a translocator necessary for a functioning T3SS, from roles it may have as an effector protein. More work is needed to determine whether BipC, similar to SipC and IpaC, may play a secondary role.

1.2.4 Regulation of the T3SS

The regulation of the bsa T3SS has been elucidated by Sun et al. (2010) (Figure 1.8). At the top of the regulatory hierarchy is the gene bspR (BPSL1105), which, when disrupted caused a greatly reduced level of expression in genes in the bsa T3SS locus as shown by microarray and real time PCR (Sun et al., 2010). bspR signals through the membrane bound regulator bprP (BPSS1553) which controls expression levels of both structural and secreted components of the bsa T3SS (Sun et al., 2010). bprP further signals through bsaN (BPSS1546) and it’s co-activator, the chaperone bicA (BPSS1533), controlling transcription of the known effectors bopE and bopA, as well as the genes bicP (BPSS1523) and bprB-D (BPSS1520-22) (Sun et al., 2010, Chen et al., 2014). bsaN also relays the regulation signal to the virulence-associated Type 6 secretion system and virulence factors such as bimA and virAG (Sun et al., 2010, Chen et al., 2014).

The presence of genes allowing for the ability to assimilate arabinose was noticed to be one of the differences between virulent B. pseudomallei and avirulent B. thailandensis (Smith et al., 1997, Moore et al., 2004). It was later shown that by expressing the B. thailandensis arabinose assimilation operon in B. pseudomallei, virulence in a Syrian hamster model was reduced (Moore et al., 2004). There was also a down-regulation of bsa T3SS genes, notably the T3SS regulator bsaN (Moore et al., 2004). Interestingly bprP, the regulator of bsaN and the structural components of the bsa T3SS, was not repressed in the presence of the arabinose assimilation operon (Moore et al., 2004).

1.2.5 T3SS Signals and Chaperones

Type 3 secretion signals are located at the N-terminus of a protein, are not cleaved and do not share sequence homology with each other (Michiels and Cornelis, 1991, Schesser et al., 1996). In Yersinia, it was shown that as little
Figure 1.8: The *B. pseudomallei* K96243 *bsa* T3SS gene locus. The locus ranges from *bopC* (BPSS1516) to *bprQ* (BPSS1554). The Bsa T3SS comprises of regulators (red), structural components (blue), translocators (purple), chaperones (green), effectors (yellow) and genes with unknown function (grey). Figure taken from Chen *et al.* (2014).
as 15 amino acids of the N-terminus of YopE fused to calmodulin-dependant adenylate cyclase were enough for secretion into the supernatant (Sory et al., 1995). Interestingly, substituting the alanines at position 2 and 15 in the YopE secretion signal with glutamic acid, did not affect secretion (Anderson and Schneewind, 1997). Even shifting the reading frame did not prevent the secretion of YopE, which the authors suggested may indicate a secretion signal located in the mRNA of the effector protein (Anderson and Schneewind, 1997). A second important signal in the N-terminal region of T3S effector proteins is the chaperone binding domain (CBD) which is required for the specific secretion of effector proteins (Lee and Galán, 2003, Ehrbar et al., 2003, Lee and Galán, 2004, Ehrbar et al., 2006, Abe et al., 1999) as well as for their stability in the bacterial cytoplasm (Frithz-Lindsten et al., 1995, Abe et al., 1999). By determining the crystal structure of an effector protein bound to its chaperone, it is known that T3S chaperones in Yersinia and Salmonella bind the N-terminal amino acids of their effector protein, just after the T3S signal, but before any functional domains, and maintain the bound effector protein in a partially unfolded state that may be more competent for secretion (Stebbins and Galán, 2001, Birtalan and Ghosh, 2001).

The T3S signal also appears to be promiscuous between systems, allowing secretion of effector proteins from bacteria in an unrelated host bacterium containing a T3SS (Rossier et al., 1999, Hovis et al., 2013). This is even true of less similar T3SSs, as shown by the ability of the Hrp Plant T3SS of Xanthomonas to secrete the mammalian effector protein YopE of Yersinia (Rossier et al., 1999). Maybe because of the similarity between the two, virulence associated T3SS effectors can be secreted by the bacterial flagellar T3SS system in Yersinia and Salmonella (Young and Young, 2002, Warren and Young, 2005, Lee and Galán, 2004, Ehrbar et al., 2006). It is only through interaction with the appropriate chaperone that specific secretion through one system is achieved. Removal of the ability of SopE in Salmonella to bind it’s chaperone InvB, caused secretion through both the flagellar and SPI-1 virulence associated T3SS (Lee and Galán, 2004, Ehrbar et al., 2006).

B. pseudomallei has 4 putative Bsa T3SS chaperones, though little work concerning their function has been performed. The protein encoded by the gene BPSS1517 is a putative chaperone and has been shown to interact with the downstream effector protein BopC (BPSS1516) (Muangman et al., 2011). Another putative chaperone, BicA (BPSS1533) has been shown to be required for the secretion of the known effector protein BopE (Sun et al., 2010). Two hy-
pothetical chaperones, BicP (BPSS1523) and BsaR (BPSS1542) have not been studied to date.

1.3 Bsa T3SS Effector Proteins

The role of the T3SS is to deliver an array of effector proteins into the target cell to subvert host cell functions. The function of different effector proteins is extremely varied, ranging from blocking apoptosis (E. coli NleH), prevention of phagocytosis (Yersinia YopH), cytotoxic activity (Pseudomonas aeruginosa ExoU) and disruption of the actin cytoskeleton (Salmonella SopE) (Hemrajani et al., 2010, Persson et al., 1997, Sato et al., 2003, Hardt et al., 1998). The location of the genes encoding effector proteins within bsa T3SS locus are depicted in Figure 1.8. B. pseudomallei encodes three effector proteins known to be secreted by the Bsa T3SS (CHBP, BopC and BopE) as well as six hypothetical T3SS effectors (BopA, BapA, BapB, BapC, BprD and BopB) (Reviewed in Sun and Gan (2010)).

1.3.1 CHBP/Cif (BPSS1385) - Confirmed Bsa Effector

CHBP (BPSS1385) is a homologue of cell cycle inhibiting factor (Cif) in E. coli. Cif and CHBP are able to deamidate NEDD8 causing cell cycle arrest (Nougayrède et al., 2001, Cui et al., 2010). CHBP has been shown to cause cell cycle arrest when expressed in B. thailandensis, but until recently it had yet to be demonstrated that CHBP is secreted in a Bsa-dependent manner by B. pseudomallei (Cui et al., 2010).

In a recent study, we determined that CHBP is present in 76.7% (33/43) of the available B. pseudomallei genomes (Pumirat et al., 2014). A Western blot assay used to probe for the presence of the CHBP protein in B. pseudomallei clinical isolates from the endemic region detected CHBP in 46.6% (7/15) isolates (Pumirat et al., 2014). It was also shown that CHBP is not secreted under standard growth conditions, but wild type (WT) B. pseudomallei was able to secrete CHBP into U937 cells, whereas the bsaQ mutant was unable to (Pumirat et al., 2014). A B. pseudomallei K96243 chbP insertion mutant showed a reduction in the ability to form plaques in HeLa cells at 24 hours and lower cytotoxicity at 6 hours as assessed by LDH release assays (Pumirat et al., 2014). Both of these phenotypes were complemented when chbP was expressed on a plasmid indicating the phenotype was due to disruption of the chbP gene and not due to
unexpected polar effects of the insertion mutation (Pumirat et al., 2014).

### 1.3.2 BopB/FolE (BPSS1514) - Hypothetical Bsa Effector

BopB (BPSS1514) or FolE, is annotated to be a GTP cyclohydrolase I and was thought to be a candidate effector protein (Stevens et al., 2004). Yet a bopB mutant did not display a significantly reduced time to death when compared to WT *B. pseudomallei* in a BALB/c intraperitoneal infection (Stevens et al., 2004). Similarly, mutation of bopB did not affect invasion and intracellular replication within cells (Chen et al., 2014). Expression of bopB is co-regulated with the other effectors of the *bsa* T3SS by BsaN, but the role BopB plays in infection is still unknown (Chen et al., 2014).

### 1.3.3 BopC (BPSS1516) - Confirmed Bsa Effector

BopC (BPSS1516) is a 509 amino acid protein with no high sequence homology to proteins from other species besides *B. mallei*. It is encoded just before the T3SS locus along with BPSS1517, which has been shown to be a chaperone for BopC (Muangman et al., 2011). BopC was detected in the supernatants of WT *B. pseudomallei* 10276, but not in supernatants of a *bsaZ* insertion mutant, indicating that BopC is secreted by the Bsa T3SS (Muangman et al., 2011). The first 20 amino acids of BopC fused to the beta-lactamase gene TEM1, was also shown to be sufficient for translocation into HeLa cells in a T3SS-dependant manner (Muangman et al., 2011).

A *B. pseudomallei* K96243 bopC mutant was hindered in its ability to invade A549 cells (Muangman et al., 2011). Another study demonstrated that the mutation in bopC reduced levels of intracellular survival when compared to WT bacteria (Srinon et al., 2013). It was also shown by staining for co-localisation with LAMP–1 that the bopC mutant showed a delayed escape from the phagosome in J774A.1 cells compared to WT, which likely explains the difference in intracellular survival (Srinon et al., 2013).

### 1.3.4 BprD (BPSS1521) - Hypothetical Bsa Effector

BprD (BPSS1521) has no known homology to proteins outside of *B. pseudomallei* and the closely related *Burkholderia* species. It is labelled as a putative regulator of the *bsa* T3SS, though a knockout of the bpr operon (*bprB,C,D*) showed no effect on the expression of genes in the *bsa* T3SS (Sun et al., 2010). Also,
it is regulated by BsaN along with the known effector proteins BopA, BopC and BopE (Sun et al., 2010). The gene expression of \( B. pseudomallei \) was analysed from the lungs, livers and spleens of BALB/c and C57BL/6J mice by microarray and \( bprD \) emerged as being highly over expressed in all tissues in both strains of mice (Chirakul et al., 2014). A mutation in \( bprD \) increased the expression of the downstream gene in the same operon, \( bprC \), which regulates expression of the T6SS-1 (Chirakul et al., 2014). BALB/c mice infected intraperitoneally with the \( bprD \) mutant had a shorter time to death when compared to WT \( B. pseudomallei \), which the authors point out may be due to the up-regulation of the T6SS-1 (Chirakul et al., 2014).

1.3.5 BopA (BPSS1524) - Hypothetical Bsa Effector

BopA (BPSS1524) shares 23% homology with IcsB from \( \text{Shigella} \) (Cullinane et al., 2008). It has been predicted to contain a Rho GTPase inactivation domain (RID) similar to that found in VcRtxA (\( \text{Vibrio cholera} \)) and other MARTX toxins which indirectly inactivate Rho GTPases (Pei and Grishin, 2009). IcsB, along with its chaperone IpgA, are important for \( \text{Shigella} \)'s ability to escape LC3 positive phagosomes once inside the host cell, and this activity is dependent on IcsBs cholesterol binding domain (Kayath et al., 2010, Campbell-Valois et al., 2015). BopA also contains a functional cholesterol-binding domain (Kayath et al., 2010). The \( B. pseudomallei \) homologue of IpgA is BicP, which co-purifies with BopA and helps to prevent its degradation, indicating that it is the chaperone for BopA (Kayath et al., 2010).

While BopA has not been proven to be secreted by the Bsa T3SS in \( B. pseudomallei \), BopA from \( B. mallei \) has been shown to be secreted in a surrogate \( E. coli \) host. By fusing the first 58 amino acids of BopA from \( B. mallei \) ATCC 23344, which contain the T3S signal, to the \( \text{Yersinia enterolitica} \) phospholipase YplA, the fusion protein was secreted by EPEC E2348/69, but not its isogenic \( escN \) mutant, which contains a non-functional secretion system (Whitlock et al., 2008).

BopA is important to the intracellular survival of \( B. pseudomallei \) in phagocytic cells (Cullinane et al., 2008). A \( bopA \) mutant in \( B. pseudomallei \) K96243 displayed reduced intracellular survival and an increased localisation with GFP-LC3, an indicator of autophagy stimulation, in RAW 264.7 cells (Cullinane et al., 2008). This reduction in intracellular survival was eliminated when cells were treated with wortmannin, indicating the reduction was due to autophagy (Cullinane et al., 2008). Another study demonstrated that BopA is
important for escape from the phagosome and the increased co-localisation of LC3 with bacteria was due to the inability of the bacteria to escape the phagosome allowing for recruitment of LC3 (Gong et al., 2011).

A B. mallei ATCC 23344 bopA mutant had a decrease in intracellular survival in J774A.1 cells (Whitlock et al., 2008). Interestingly, in the murine alveolar macrophage cell line MH-S, the same B. mallei bopA mutant exhibited increased intracellular survival when compared to the WT strain, indicating that different cell types may rely on different mechanisms to clear B. mallei (Whitlock et al., 2009).

BALB/c mice infected intraperitoneally with a B. pseudomallei 576 bopA insertion mutant were significantly delayed in time to death when compared to the WT strain (median = 32 days vs. 20 days respectively) (Stevens et al., 2004). BALB/c mice infected intranasally with a B. mallei ATCC 23344 bopA insertion mutant showed a delayed time to death compared to the WT strain (median = 5 days vs. 7.5 days respectively). No bacteria were recovered from the lung tissue of animals infected with the bopA mutant while $10^8$ bacteria were recovered from the lungs of animals infected with WT B. mallei (Whitlock et al., 2009). In mice immunized with recombinant BopA and challenged intranasally with B. mallei ATCC23344 and B. pseudomallei 1026b, the BopA vaccine protected 100% and 60% respectively of animals 21 days post infection (Whitlock et al., 2010).

1.3.6 BopE (BPSS1525) - Confirmed Bsa Effector

BopE (BPSS1525) is the best characterised of the B. pseudomallei effector proteins. BopE shares 16% homology to the guanine nucleotide exchange factor (GEF) SopE from Salmonella. BopE was shown to be secreted by the Bsa T3SS in B. pseudomallei 10276 in a manner dependent on the structural protein BsaZ (Stevens et al., 2003). Similarly, deletion of another structural component bsaQ in B. pseudomallei K96243, was shown to inhibit secretion of BopE into the supernatant (Muangsombut et al., 2008). BopE is important for invasion of non-phagocytic cells, as a B. pseudomallei bopE insertion mutant was impaired in its ability to invade HeLa cells (Stevens et al., 2003). Also, expression of BopE on a plasmid in HeLa cells caused the formation of actin rearrangements (Stevens et al., 2003). Using fluorescence spectrometry, it was demonstrated that purified BopE, similar to SopE, is a functional guanine nucleotide exchange factor for both Rac1 and Cdc42, but with about 10 fold lower activity than SopE (Stevens et al., 2003). This lower activity may be explained by differences in the catalytic domains, as the SopE catalytic domain stays in an open confor-
mation, while BopE adopts a closed conformation that must open upon interaction with Cdc42 to allow for GEF activity (Upadhyay et al., 2008).

HEK 293T cells transfected with plasmids expressing BopE and casapase–1, resulted in the increased activation of casapase–1 and –7 (Bast et al., 2014). When the active site of the transfected BopE was mutated, levels of activation of casapase–1 and –7 returned to basal levels indicating BopE’s GEF activity is required for the activation of casapase–1 and –7 (Bast et al., 2014). However, when \textit{B. pseudomallei} E8 was used to infect murine macrophages, a mutation in BopE caused only slight differences in casapase–1 processing and does not seem to play a major role in infection (Bast et al., 2014). This may be due to redundancy in other effector proteins.

In a murine model of melioidosis, BALB/c mice infected intraperitoneally with \textit{B. pseudomallei} 10276 containing an insertion mutation in \textit{bopE} did not show a significant increase in median time to death when compared to WT (21 days vs. 20 days respectively) (Stevens et al., 2004). Immunisation of mice with a live attenuated mutant of \textit{B. pseudomallei} induced CD4$^+$ and CD8$^+$ T cells specific for BopE (Haque et al., 2006). Using the sera from healthy patients that are seropositive for \textit{B. pseudomallei}, CD4$^+$ T cells were able to recognize BopE (Tippayawat et al., 2011).

### 1.3.7 BapB-C (BPSS1526-7) - Hypothetical Bsa Effectors

Little work has been done on the putative effector proteins BapB (BPSS1527) and BapC (BPSS1526). BapC is homologous to IagB from \textit{Salmonella} (Stevens et al., 2002). IagB is thought to be a lytic transglycosylase involved in the breakdown of the bacterial peptidoglycan layer, allowing connection of the inner and outer membrane components of the secretion system, though this function has not formally shown (Zahrl et al., 2005). A \textit{bapC} mutant showed no significant attenuation in a Syrian hamster model of melioidosis (Warawa and Woods, 2005). BapB is homologous to IacP from \textit{Salmonella} (Stevens et al., 2002). IacP has been shown to be important for \textit{Salmonella} invasion into host cells by playing a role in regulating SopA, SopB and SopD secretion (Kim et al., 2011). The role of BapB in \textit{B. pseudomallei} has not been explored.

### 1.3.8 BapA (BPSS1528) - Hypothetical Bsa Effector

BapA (BPSS1528) has no known homology to proteins outside of \textit{B. pseudomallei} and the closely related \textit{Burkholderia} species. A \textit{bapA} mutant in \textit{B. pseu-
domallei 1026b showed no attenuation in a Syrian hamster model of infection (Warawa and Woods, 2005). There has been no other published work on this protein.

1.3.9 Unidentified Effector Proteins

It can be hypothesized that there are more Bsa T3SS effectors to be characterized. There are at least 21 effectors secreted by the T3SS of Enteropathogenic E. coli (EPEC) (Reviewed in (Dean and Kenny, 2009)) and 25–30 effectors secreted by S. flexneri (Reviewed in Parsot (2009)), and some of these effector proteins are encoded outside of the T3SS genetic locus. With so many uncharacterised and hypothetical effector proteins associated with the Bsa T3SS, it is clear that more research is required to understand importance these proteins may play in B. pseudomallei infection.
1.4 Aims and Objectives

Aims

The aim of this research is to identify novel effector proteins secreted by the *B. pseudomallei* Bsa T3SS and choose one or two effector proteins for further characterisation of the role of these effector proteins in pathogenesis.

Objectives

The following objectives have been set out in order to accomplish the aims of this research;

1. To dysregulate the *B. pseudomallei* Bsa T3SS creating mutants which hyper-secrete effector proteins under standard laboratory conditions.

2. To use quantitative proteomics to identify Bsa T3SS effector proteins secreted by *B. pseudomallei* WT, a mutant lacking a functional T3SS and hyper-secreting mutants.

3. To choose a subset of novel effector proteins identified by the study and use a yeast two-hybrid system to identify human proteins with which they interact.

4. To use relevant biochemical assays and confocal microscopy to further characterise the function of effector proteins.
Chapter 2

Materials and Methods

2.1 Bacterial Strains, Plasmids and Culture Media

Bacterial strains and plasmids are listed in Table 7.1. Bacteria were routinely cultured at 37°C on LB agar (Miller) or LB broth (Lennox), except for during sucrose counter selection when 15% sucrose LB agar plates (10 g/L tryptone, 5 g/L yeast extract, 150 g/L sucrose, 15 g/L agar, water to 1 L, pH 7.4) were incubated at 28°C. Antibiotic selection was performed at the following concentrations unless otherwise stated: ampicillin (Amp) 100 µg/ml, kanamycin (Kan) 50 µg/ml, chloramphenicol (Cm) 34 µg/ml, and tetracycline (Tet) 15 µg/ml. Isopropyl-β-D-thiogalactopyranosid (IPTG) was used at a final concentration of 0.5 mM where appropriate. Unless otherwise stated all chemicals were purchased from Sigma Aldrich (Dorset, UK). Eukaryotic tissue culture was performed at 37°C in the presence of 5% CO₂. HeLa cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin.

2.2 Molecular Methods

2.2.1 PCR and Ligations

All gel electrophoresis was performed using 1% tris-acetate-EDTA (TAE) agarose gels. Gel extraction kits (Qiagen), polymerase chain reaction (PCR) purification kits (Qiagen), and Miniprep plasmid purification kits (Qiagen) were used according to the manufacturer’s instructions. Primers, purchased from Sigma Aldrich (Dorset, UK), are listed in Table 7.2. Unless otherwise stated,
the standard PCR was composed of: 0.4 U of Phusion polymerase (Thermo Scientific), 1x Phusion GC buffer, 1 µM of each primer, 2.5% dimethyl sulfoxide (DMSO), 200 µM deoxynucleotides (dNTPs), and 10–500 ng of template DNA in a total volume of 20 µl. The reaction conditions used were 98°C for 2 minutes, followed by 30 cycles of (98°C for 10 seconds, 72°C for 1–3 minutes) and one cycle of 72°C for 10 minutes.

PFU polymerase (0.8 U, Promega) was used to amplify regions flanking the bsaP gene with 1x PFU buffer, 1 µM of primers bsaP1/bsaP2 or bsaP3/bsaP4, 2.5% DMSO, 200 µM dNTPs, and B. pseudomallei 10276 genomic DNA in a total volume of 20 µl. The reaction conditions used were 97°C for 3 minutes, followed by 30 cycles of (97°C for 30 seconds, 68°C for 2.5 minutes) and one cycle of 68°C for 30 minutes. PCR products were then purified using gel electrophoresis and a gel extraction kit. To amplify the ligated bsaP1/bsaP2 and bsaP3/bsaP4 fragments, 0.8 U of PFU polymerase (Promega) was used with 1x PFU buffer, 1 µM of primers bsaP1 and bsaP4, 2.5% DMSO, 200 µM dNTPs, and the ligated product of bsaP1/bsaP2 and bsaP3/bsaP4. The reaction conditions used were 97°C for 3 minutes, followed by 30 cycles of (97°C for 30 seconds, 68°C for 3.5 minutes) and one cycle of 68°C for 30 minutes. PCR products were then purified using gel electrophoresis and a gel extraction kit.

Ligations were performed using 1 U of T4 DNA ligase (Promega), 1x ligation buffer, and 100–300 ng of each of the fragments of DNA to be ligated. Reactions were incubated for 30 minutes at room temperature or at 4°C overnight.

All sequencing of plasmids and PCR products was carried out by Sanger sequencing performed by Source BioScience (Lanarkshire, UK).

2.2.2 Electrocompetent Cells and Transformations

To prepare electrocompetent E. coli S17–1 λpir, overnight cultures were grown to an OD_{600} of 0.4±0.05 and then chilled on ice for 20 minutes. The cells were centrifuged four times at 3220 relative centrifugal force (RCF) for 10 minutes at 4°C and after each spin the supernatant was discarded and the cells were washed with either sterile water (washes 1 and 2) or 10% glycerol (washes 3 and 4) pre-chilled to 4°C. Individual aliquots were placed in a dry ice-ethanol bath for snap freezing. The cells were stored at –80°C until use.

Transformations of XL1-Blue (Agilent) and One Shot PIR1 (Invitrogen) E. coli were performed according to manufacturer’s instructions. S17–1 λpir E. coli were electroporated with pDM4 constructs using a 0.2 cm cuvette at 2.5 kV, 25
µF, and 200 Ω. Bacteria were recovered in SOC media (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) shaking at 200 revolutions per minute (RPM) for 2 hours at 37°C followed by inoculation and overnight growth on LB plates containing the appropriate antibiotics. The resulting resistant colonies were confirmed by re-streaking onto LB plates containing the appropriate antibiotic, overnight growth, and colony PCR. Colonies containing the correct size insert were stored as glycerol stocks at -80°C.

Transformations of B. pseudomallei were performed using a method that requires no chilling as previously described (Choi et al., 2006). Overnight cultures were centrifuged three times at 16,000 RCF for 2 minutes in a microcentrifuge and after each spin the bacteria were washed with sterile 300 mM sucrose. To 50 µl of bacteria, 2 µl of intact plasmid (about 1.5 µg) was added. Using a 0.1cm cuvette an electroporation was performed at 1.8 kV, 25 µF, and 200 Ω. Bacteria were recovered in SOC media shaking at 200 RPM for 2 hours at 37°C followed by inoculation and overnight growth on LA plates containing the relevant selective antibiotics.

2.2.3 B. pseudomallei Mutagenesis

The 10276 ΔbsaP in-frame deletion mutant was made using allelic exchange and the sacB containing positive-selection suicide vector pDM4 essentially as previously described (Logue et al., 2009). DNA fragments upstream and downstream of bsaP (BPSS1544) were amplified by PCR from B. pseudomallei 10276 genomic DNA using primer pairs bsaP-del-1/bsaP-del-2 and bsaP-del-3/bsaP-del-4 which contain a SpeI and BglII restriction site respectively. The resulting DNA fragments were separated by electrophoresis on a 1% agarose gel, gel purified, and combined using PCR-ligation-PCR (Ali and Steinkasserer, 1995). In brief, the two fragments were phosphorylated to combine the bsaP1/bsaP2 and bsaP3/bsaP4 fragments using 20 U of T4 polynucleotide kinase (Promega), with 1x kinase buffer, 1 mM ATP, and 150 ng of each of the bsaP1/bsaP2 and bsaP3/bsaP4 fragments. The reaction was incubated at 37°C for 30 minutes followed by a 5 minute deactivation at 70°C. The two fragments were ligated together and amplified by PCR. The product was run on a 1% agarose gel and a band of the correct size was cut out and gel purified.

A-tailing was performed to add an adenosine to the blunt-ended PCR product using 3.5 U of Taq polymerase (Invitrogen), 1x Taq buffer, 1.5 mM MgCl₂, 1 mM ATP, and 50–100 ng of blunt ended DNA. The reaction was in-
cubated at 70°C for 30 minutes then purified with a PCR purification kit. The fragment was then ligated into pGEM-T resulting in the plasmid pGEM-T-ΔbsaP. The plasmid was transformed into XL1-Blue E. coli according to the manufacturer's instructions and the bacteria were plated onto LB plates containing Amp. pGEM-T-ΔbsaP was isolated from the Amp resistant colonies using a miniprep kit and the insert was sent for Sanger sequencing (Source Bioscience) using T7-Forward and SP6 primers. A restriction digest was performed with SpeI and BglII on pGEM-T-ΔbsaP and the SpeI and BglII digested fragment containing the 10276 bsaP deletion was ligated into similarly digested pDM4, resulting in pDM4-ΔbsaP. To propagate larger amounts of pDM4-ΔbsaP, the plasmid was transformed into TOP 10 PIR1 cells (Life Technologies, Paisley, UK), a higher copy number strain, according to manufacturer’s instructions, and then plated onto LB plates containing Cm. The pDM4-ΔbsaP plasmid was isolated with a miniprep kit and transformed into E. coli S17–1 λpir conjugal donor with selection for Cm resistance. pDM4-ΔbsaP was introduced into B. pseudomallei strain 10276 by conjugation with E. coli S1–17 λpir. Conjugation between B. pseudomallei strain 10276 and E. coli S1–17 λpir was performed as previously described (Stevens et al., 2002). In brief overnight cultures were grown of E. coli S1–17 λpir containing pDM4-ΔbsaP and B. pseudomallei strain 10276. The following day, the OD$_{600}$ was measured and cultures were used if their absorbance was between 0.6 and 0.8. If the absorbance was too high, the cultures were sub-cultured into fresh media and grown for a further 2–3 hours until the OD$_{600}$ was in the correct range. The following mixtures were then set up in universals containing 5 ml of filter sterilised 10 mM MgSO$_4$: a) 500 µl S1–17 λpir pDM4-ΔbsaP, b) 500 µl S1–17 λpir pDM4-ΔbsaP + 500 µl B. pseudomallei 10276 WT. The mixtures were gently mixed and run through sterile 0.22 µm nylon filters. The filters were removed and placed on a LB plate, bacteria side up, and incubated overnight at 37°C. The filter was then placed in 500 µl of sterile LB broth, vortexed for 15 seconds, and dilutions of the turbid solution were plated onto LB plates with Cm (50 µg/ml) and Kan (50 µg/ml) to select for B. pseudomallei with pDM4-ΔbsaP integrated into the chromosome by homologous recombination. Colonies were re-patched onto LA plates containing Cm (50 µg/ml) and Kan (50 µg/ml) to confirm resistance. Overnight cultures of the Cm resistant (Cm$^r$) colonies were cultured in LB broth without selection to allow for a second round of homologous recombination to take place, either restoring the full length bsaP gene, or resulting in bsaP deletion. To select for double
recombinants lacking pDM4-\(\Delta bsaP\), the overnight cultures were plated, 100 \(\mu l\) of neat, \(10^{-1}\), and \(10^{-2}\) dilutions, onto 15% sucrose plates lacking NaCl and cultured at 28\(^{\circ}\)C for three days. Chromosomal deletion of \(bsaP\) was confirmed in sucrose resistant/Cm\(^{s}\) colonies by colony PCR and the resulting strain designated 10276 \(\Delta bsaP\), where the \(bsaP\) gene has been replaced by an in-frame start-stop (atg-taa) cassette.

### 2.2.4 Complementation of the \(\Delta bsaP\) Mutant

For complementation of the \(\Delta bsaP\) mutant the 10276 \(bsaP\) sequence was amplified by PCR using the forward primer \(bsaP\)-exp-F containing a ClaI restriction site and a ribosome-binding site and the reverse primer \(bsaP\)-exp-R containing a HindIII restriction site. The PCR fragment was cloned into pBHR1 (MoBiTec, 2B Scientific Ltd., Oxford, UK) to create pBHR1-\(bsaP\). The plasmid was purified and transformed into \(B.\ pseudomallei\) 10276 \(\Delta bsaP\) using the fast sucrose method of electroporation, creating strain 10276 \(\Delta bsaP\)-p\(bsaP\).

### 2.2.5 Cloning of Candidate Effectors to Express c-Myc Epitope Fusion Proteins

The coding region for candidate effectors based on the \(B.\ pseudomallei\) K96243 genome sequence were amplified from \(B.\ pseudomallei\) 10276 genomic DNA by PCR with a 5’ forward primer containing a ribosome-binding site and a 3’ reverse primer encoding an in-frame c-Myc tag followed by a stop codon. Each primer also contained a restriction site suitable for directional insertion into the vector pME6032. Plasmids were transformed into \(E. coli\) and the inserts sequenced. Purified plasmids were then transformed into \(B.\ pseudomallei\) 10276, 10276 \(bsaZ::pDM4\), 10276 \(bipD::pDM4\) and 10276 \(\Delta bsaP\).

### 2.3 General Protein Methods

#### 2.3.1 Preparation of Bacterial Supernatants

Preparation of \(B.\ pseudomallei\) supernatants was performed by centrifuging overnight cultures of \(B.\ pseudomallei\) or the appropriate derivatives at 3220 RCF for 10 minutes at 4\(^{\circ}\)C, followed by disposal of the supernatant and resuspension of the bacteria in fresh LB to an \(OD_{600}\) of 1.0. Of the bacterial suspension, 1 ml was added to a new culture of 10 ml of fresh LB with the
appropriate antibiotics and IPTG, if needed, or the appropriate media. If required a sample was removed from the culture for serial dilutions and growth on LA plates to determine the starting bacterial count in a sample. The culture was incubated for 6 hours shaking at 225 RPM at 37°C. At the end of the 6 hours, if required, another sample was removed for dilutions and grown on LA plates for a final bacterial count. The sample was chilled on ice for 15 minutes and then centrifuged at 3220 RCF for 20 minutes at 4°C. The supernatant was collected and centrifuged a second time at 3220 RCF for 20 minutes at 4°C. The supernatant was passed through a 0.2 µm low protein binding cellulose acetate membrane filter (Millipore, Watford, UK) and stored at –20°C until use. For the investigation of induction conditions the bacteria were incubated in one of the following media: Lennox LB, LB with 0 mM NaCl, LB with 320 mM NaCl, trypticase soy broth (17 g/L enzymatic digest of casein, 3 g/L enzymatic digest of soybean meal, 5 g/L NaCl, 2.5 g/L K2HPO4, 2.5 g/L dextrose, pH 7.3), and LB containing 10 mM EGTA.

To prepare *S. flexneri* supernatants, overnight cultures of *S. flexneri* were centrifuged at 3220 RCF for 10 minutes at 4°C followed by disposal of the supernatant and re-suspension of the bacteria in fresh LB to an OD600 of 1.0. Of the bacterial suspension, 3 ml was added to a new culture of 20 ml of fresh LB. The culture was incubated for 4 hours shaking at 225 RPM at 37°C. The sample was chilled on ice for 15 minutes and then centrifuged at 3220 RCF for 20 minutes at 4°C. The supernatant was passed through a 0.2 µm low protein binding membrane filter cellulose acetate membrane filter and stored at –20°C until use.

### 2.3.2 Protein Precipitation

Proteins precipitation using a pyrogallol red-molybdate methanol (PRMM) solution was performed as previously described (Caldwell and Lattemann, 2004). In brief, equal volumes of bacterial culture supernatant and PRMM solution (0.5 mM pyrogallol red (Alfa Aesar), 0.16 mM sodium molybdate, 1.0 mM sodium oxalate, 50 mM succinic acid, 20% MeOH, pH to 2.0 w/HCl) were thoroughly mixed and the pH was adjusted to 2.8±0.1 with HCl. The samples were then precipitated at room temperature for one hour followed by overnight at 4°C. The resulting purple-blue precipitate was centrifuged at 3220 RCF, 4°C for 1 hour and the supernatant was discarded. The pellet was washed twice with acetone pre-chilled to –20°C and allowed to air dry. The protein pellet was then solubilised in freshly made 50 mM ammonium bicar-
bonate. Proteins were quantified using a Direct Detect Infrared Spectrometer (Millipore, Watford, UK) or with a bicinchoninic acid (BCA) protein assay kit (Pierce) using Quick Start bovine serum albumin (BSA, BioRad) to prepare a standard curve. Protein samples were stored at −20°C until use.

Trichloroacetic acid (TCA) precipitation was performed with and without deoxycholate (DOC). If DOC was used in the precipitation, DOC was added to the bacterial culture supernatant to a final concentration of 0.02% (w/v) and incubated at 4°C for 30 minutes. TCA precipitation was carried out by adding 100% TCA to bacterial culture supernatant to a final concentration of 10–15% followed by overnight incubation at 4°C. The samples were then centrifuged four times at 3220 RCF for 15 minutes at 4°C and the first three spins were followed by a wash with acetone pre-chilled to −20°C. After the final spin the supernatant was removed and the pellet was allowed to air dry, and then suspended in an appropriate volume of Laemmli buffer (0.2% bromophenol blue, 20% glycerol, 100 mM Tris pH 8.8, 4% SDS, 2% β-mercaptoethanol). Proteins were stored at −20°C until use.

Protein purification using Stratagene beads was performed by adding Stratagene beads at a ratio of 1:1000 v/v to bacterial culture supernatant and then mixed and incubated for 15 minutes with shaking at room temperature followed by centrifugation at 3220 RCF for 5 minutes. The beads were washed with phosphate-buffered saline (PBS), followed by another centrifugation to remove the PBS. The beads were then suspended in Laemmli buffer and heated to 90°C for 5 minutes followed by storage at −20°C until use.

2.3.3 SDS-PAGE and Immunoblotting

Proteins were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Equal volumes of protein samples and Laemmli buffer were mixed and heated to 90°C for 5 minutes. Samples were separated on Any kD™ Mini-PROTEAN TGX gels (Bio-Rad, Hemel Hempstead, UK) or 4-12% TGX gels (Novex) in Tris-Glycine running buffer. Proteins were visualised by Coomassie staining. To visualize proteins by Coomassie staining, gels were submerged in Coomassie staining solution (0.25 g/L Coomassie Blue G–250, 45% MeOH, 10% acetic acid) for 1 hour, washed twice for 15 minutes in destaining solution (45% MeOH, 10% acetic acid), followed by further destaining in deionised H₂O overnight.

Western blots were used to visualise proteins of interest. Proteins were transferred onto a nitrocellulose or PVDF membrane using the Trans-Blot...
Turbo semi-dry transfer system (Bio-Rad, Hemel Hempstead, UK) or a Trans-blot SD semi-dry transfer system (Bio-Rad). A list of all antibodies used in this study are listed in Table 7.3. α-BopE (Stevens et al., 2003), α-BipD (Stevens et al., 2004), or α-cMyc (Insight Biotechnology, Wembley, UK) rabbit polyclonal antibodies were used at 1 µg/ml. For detection by infrared fluorescence, goat-α-rabbit IgG (H+L) (DyLight™ 800 Conjugate) was used at a 1:10,000 dilution (Cell Signalling Technology, Hitchin, UK). Blots were visualised using an Odyssey Infrared Imaging System (LI-COR Biosciences, Cambridge, UK) according to the manufacturer’s instructions. Images were captured and analysed using Image Studio Lite (LI-COR Biosciences, Cambridge, UK). The membrane was then blocked for 1 hour in tris-buffered saline tween (TBS-T, 0.1% tween 20) with 5% BSA followed by three 15 minute washes in TBS-T. The membrane was then incubated with 1 µg/ml of primary antibodies, α-BopE (Stevens et al., 2003) or α-BipD (Stevens et al., 2004) rabbit polyclonal antibodies for 1 hour. The membrane was washed once for 15 minutes in TBS-T followed by two 5 minute washes in TBS-T and then incubated for 1 hour with horseradish peroxidase (HRP) linked goat α-rabbit IgG used at a 1:10,000 dilution (Invitrogen). The membrane was then washed one time for 15 minutes in TBS-T and then four times for 5 minutes in TBS-T. Enhanced chemiluminescence (ECL) reagent (GE Healthcare) was used according to manufacturer’s instructions and the blot was visualized using the G-Box imaging system (Syngene) or following exposure to film (GE Healthcare).

2.3.4 Preparation of Glutathione S-Transferase-Fusion Proteins

To prepare the glutathione (GSH)-linked sepharose beads, 400 µl of GSH-linked sepharose 4B beads (GE healthcare) 50% v/v in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) were centrifuged at 16,000 RCF in a microcentrifuge for 1 minute, the supernatant was removed, and the beads were re-suspended in 1 ml of sterile PBS. The wash was repeated 4 times and after the final spin a re-suspension volume of 200 µl was used. The washed GSH-linked sepharose beads were stored at 4°C for up to two weeks until use.

To create proteins with N-terminal glutathione S-transferase (GST)-fusions, genes encoding proteins of interest were cloned into the vector pGEX-4T-1. The vector was transformed into E. coli Rosetta BL21 (Novagen). XL1-Blue E. coli containing pGEX-BipC, pGEX BapA, pGEX-SipC, pGEX-BimA, pGEX-
BopE or empty pGEX were inoculated into 5 ml of LB containing Amp and grown overnight shaking at 37°C. The following day 5 ml of the bacterial culture was used to inoculate 250 ml of LB containing Amp which was then incubated for 3 hours shaking at 37°C. The culture was then removed from the incubator and allowed to cool for 20 minutes to room temperature and IPTG was added to a final concentration of 0.5 mM followed by incubation for 3 hours at 37°C. Due to solubility concerns, for GST-BapA 2 mM IPTG was used and the culture was induced overnight shaking at 16°C. The bacteria were collected by centrifuging the culture at 3,220 RCF and removing the supernatant. The remaining bacterial pellet was lysed at 4°C with 5 ml of Bug Buster master mix (Merck Millipore) rocking for 1 hour or until the solution became clear. The insoluble fraction was removed by centrifuging at 10,000 RCF for 10 minutes at 4°C and discarding the pellet. Washed GSH-linked sepharose beads (200 µl 50% v/v in PBS) were added to the bacterial lysate which was then incubated at 4°C for 1 hour with agitation. The beads were centrifuged at 3,220 RCF for 5 minutes at 4°C, the supernatant was discarded and the beads were transferred to a microcentrifuge tube. The beads were then washed 8 times with 1 ml of ice cold NP–40 lysis buffer using a microcentrifuge at 16,000 RCF for 1 minute and discarding the supernatant after each wash. The beads were then re-suspended in an equal volume of cold sterile PBS (about 100 µl) and either used immediately for a pull-down or were stored at 4°C for up to 2 weeks before use.

When necessary, to elute the GST-fusion proteins from the GSH-linked sepharose beads, the beads were re-suspended in 200 µl of elution buffer (20 mM reduced GSH, 50 mM Tris, pH 8.0) in a microcentrifuge tube and incubated for 1 hour at room temperature with agitation. To remove the sepharose beads, the microcentrifuge tube was centrifuged at 16,000 RCF and the supernatant containing the eluted protein was transferred to a new microcentrifuge tube. The elution was performed 2 more times and the three sets of supernatants were combined. The solution was dialysed three times in 1000 volumes of PBS for a minimum of 2 hours each time to remove the detergents and salts from the eluted protein. The dialysed protein was concentrated using a 10 kD cut-off spin column centrifuged at 16,000 RCF for 30 minutes or until the final volume was about 200 µl. The protein concentrations were determined using the Bradford assay (Pierce Scientific). Proteins were aliquoted and stored at –80°C until use.

To bind proteins eluted from GSH-linked sepharose beads to UltraLink Bio-
support resin (Thermo Scientific), 0.02 g of UltraLink resin and 40 µl of 3 M sodium citrate was added to 200 µl of eluted GST-fusion protein. After incubation for 1 hour at room temperature with agitation, ten bead volumes of quench solution (3 M ethanolamine, pH 9.0) was added followed by an incubation for 2.5 hours with agitation at room temperature. The mixture was centrifuged for 10 minutes at 3,220 RCF, supernatants were discarded and 10 bead volumes of PBS was added followed by another 15 minute incubation at room temperature with agitation. The mixture was centrifuged for 10 minutes at 3,220 RCF, supernatants were discarded, and 1 ml of 1 M NaCl was added. After 15 minutes incubation with agitation at room temperature, the mixture was centrifuged for 10 minutes at 3,220 RCF, supernatants were discarded and 10 bead volumes of PBS was added followed by another 15 minute incubation at room temperature with agitation. This wash step was repeated one more time after which the beads were stored at 4°C until use.

2.3.5 Pull-Down Assays With Eukaryotic Cell Lysates

HeLa Cell Lysate Pull-Downs

To confirm interactions seen in the yeast two-hybrid (Y2H) system with the proteins of interest (BipC and BapA) as well as to test BipC-actin interactions in the absence of polymerisation buffer, pull-downs were performed with GST-fusion proteins and HeLa cell lysates. HeLa cells were grown until confluent in a 150 mm flask. The flask was immediately placed on ice and the culture media was remove and, the cells were washed with ice cold sterile PBS. Cells were then lysed in 1.5 ml of ice cold sterile NP–40 lysis buffer (20mM Tris, pH 8; 150 mM NaCl, 20 µM leupeptin, 1 mM PMSE, 1 mM NaF, 1 mM β-glycerophosphate, 2 mM sodium orthovanadate, 2 mM EDTA, 10% glycerol, 1% NP–40) and a sterile cell scraper was used to disrupt the adherent cells. After 10 minutes of lysis, the liquid in the culture flask was removed and placed into a pre-chilled sterile 1.5 ml microcentrifuge tube. The cell lysates were incubated at 4°C for a further 30 minutes with agitation followed by centrifugation at 16,000 RCF for 20 minutes in a microcentrifuge pre-chilled to 4°C. The pellet was discarded and to clear the lysates, 50 µl of washed sepharose beads 50% v/v in PBS chilled to 4°C were added to the supernatant and incubated for 30 minutes at 4°C rotating. The lysate bead mixture centrifuged for 1 minute at 16,000 RCF in a microcentrifuge and the supernatants were collected. The supernatants were either used immediately for a pull-down, or were stored at
To perform the pull down experiment, 100 \( \mu l \) of beads bound to bacterial proteins 50% \( v/v \) in PBS was added to 1 ml of cleared HeLa cell lysates in a microcentrifuge tube which was incubated overnight at 4\( ^\circ \)C rotating. The beads were then collected by spinning at 16,000 RCF in a microcentrifuge and were washed 8 times with ice cold NP–40 buffer in a similar fashion. One final spin was performed, the supernatant was removed, and 50 \( \mu l \) of 2x Laemml buffer was added. The beads were then boiled at 95\( ^\circ \)C for 5 minutes to denature the proteins, and then stored at -20\( ^\circ \)C until use in downstream applications. To probe the pull downs for the proteins of interest, PAWR and c1QBP, 5–10 \( \mu l \) of the bead and Laemmli buffer mixture was loaded onto a 4–15% TGX gel for SDS-PAGE in duplicate. One gel was stained using Coomassie brilliant blue to visualise all proteins. From the other gel, proteins were transferred onto a nitrocellulose membrane, and a Western blot was performed with rabbit \( \alpha \)-PAWR and rabbit \( \alpha \)-C1QBP primary antibodies and goat \( \alpha \)-rabbit 800 DyLight secondary antibody. The Western blots were visualised using the Odyssey imaging system.

A549 Cell Lysate Pull-Downs

To prepare lysates of A549 cells, 75 mm flasks containing confluent A549 cells were washed with PBS and then treated with trypsin for 3 minutes to detach the adherent cells. Cells were collected in a 15 ml falcon tube and washed once with PBS followed by incubation in 5 ml of 2x NP–40 at room temperature for 5 minutes. Insoluble material was removed by centrifuging for 10 minutes at 3,220 RCF.

Lysates were incubated with GST-BopE, GST-BipC and GST bound to GSH-linked sepharose beads for 1 hour at room temperature with agitation followed by five washes in ice cold PBS after which 50 \( \mu l \) of 2x Laemml buffer was added. The beads were then boiled at 95\( ^\circ \)C for 5 minutes to denature the proteins, and then stored at -20\( ^\circ \)C until use in downstream applications. Proteins were visualised by SDS-PAGE using a 4–15% TGX gel followed by Coomassie or silver staining (Pierce Scientific).

Murine Splenic Lysate Pull-Downs

Pull-downs with murine splenic lysates (MSLs) were performed using dilutions of 10 x polymerisation buffer (G-Mg buffer supplemented with 1 x
K50MEI buffer). G-Mg buffer (2 mM Tris – pH 8, 0.5 mM DTT, 0.2 mM ATP, 0.1 mM MgCl₂, 0.01% NaN₃) and K50MEI buffer (500 mM KCl, 10 mM MgCl₂, 10 mM EGTA, 100 mM imidazole – pH 7.0) were pre-prepared and stored at -20°C until use. Spleens from BALB/c or C57BL/6J mice were stored at -80°C until use. To prepare MSLs, the spleens were thawed on ice and then rinsed briefly in ice-cold 1 x polymerisation buffer. Up to four spleens were placed in a pre-chilled glass universal or beaker. To the spleens, 1 ml of ice-cold 1 x polymerisation buffer with protease inhibitors (1 mM PMSF, 1 µg/ml pepstatin A, 10 µg/ml leupeptin, 1 µg/ml aprotinin) was added per spleen. The spleens were homogenized using a tissue homogenizer ensuring the resulting mixture did not heat up. The homogenate was transferred to a plastic falcon tube and was centrifuged at 3,220 RCF for 15 minutes at 4°C to remove the large insoluble material. The remaining supernatant was transferred to an ultracentrifuge tube and centrifuged at 100,000 RCF at 4°C for 2 hours to remove remaining the insoluble material. The supernatant was collected and aliquoted into chilled microcentrifuge tubes and stored at -80°C until use.

To perform pull downs with the MSLs, a 500 µl aliquot of MSLs was defrosted on ice. It was diluted to a final volume of 2 ml with 1 x polymerisation buffer and CaCl₂ was added to a final concentration of 100 µM followed by thorough mixing. The GST-fusion proteins of interest (BopE, BimA, BipC, SipC and GST) bound to GSH-linked sepharose beads were added to microcentrifuge tubes containing 200 µl of MSL in polymerisation buffer followed by incubation for 1 hour at room temperature with agitation. The sepharose beads were then washed 5 times with ice-cold PBS by centrifuging at 16,000 RCF and removing the supernatant. To the beads, 10 µl of 2x Laemelli buffer was added, the samples were boiled for 5 minutes at 95°C and stored at -20°C until use. Proteins were analysed by SDS-PAGE using a 4-12% TGX gel. The proteins were then visualised by Coomassie blue staining of Silver staining. For immunoblotting, proteins were transferred to PVDF membranes and visualised using rabbit α-Cdc42, rabbit α-Rac1 and goat α-actin primary antibodies followed by goat α-rabbit or rabbit α-goat HRP-linked secondary antibodies. The antibodies were detected using ECL followed by film exposure.
2.4 iTRAQ Quantitative Proteomics

2.4.1 iTRAQ Experimental Design

Bacterial culture supernatants of B. pseudomallei 10276, 10276 bsaZ::pDM4, 10276 bipD::pDM4, and 10276 ΔbsaP were generated in three independent experiments. Equal amounts of protein from each replicate were combined creating a single pooled sample for each strain. These samples were sent to Dundee Cell Products where they were enzymatically digested, peptides were labelled using isobaric tag for relative and absolute quantitation (iTRAQ) reagents, and then pooled and submitted to liquid chromatography and tandem mass spectrometry (LC MS/MS) in three technical replicates as follows.

2.4.2 Protein Digestion and Peptide Fractionation

Protein samples were prepared for MS analysis using a protocol adapted from Boisvert et al. (2010). Following protein quantification, samples were reduced in 10 mM dithiothreitol (DTT) and alkylated in 50 mM iodoacetamide prior to boiling in loading buffer, and then separated by one-dimensional SDS-PAGE (4–12% Bis-Tris Novex mini-gel, Invitrogen) and visualized by colloidal Coomassie staining (Novex, Invitrogen). The entire protein gel lanes were excised and cut into 10 slices each. Every gel slice was subjected to in-gel digestion with trypsin overnight at 37°C (Boisvert et al., 2010). The resulting tryptic peptides were extracted using formic acid (FA, 1%) and acetonitile (ACN), lyophilized in a speedvac and resuspended in 1% formic acid.

2.4.3 Peptide Concentration

To determine the concentration of the peptides, the dried samples were re-suspended in 100 mM triethylammonium bicarbonate (TEAB) 1 M ACN. To determine the volume of sample needed for an even protein load across all samples, 1 µl was injected onto an LTQ Velos Mass Spectrometer (Thermo Fisher Scientific, MA, USA) and the total ion current (TIC) trace was used.

2.4.4 iTRAQ Labelling

Samples were labelled according to the manufacturer’s instructions using an iTRAQ Reagents Multiplex kit - 4 plex (AB Sciex UK Ltd., Warrington, UK). To each vial of labelling reagent (labels- 114, 115, 116 & 117), 70 µl ethanol was
added, after which they were vortexed and centrifuged. A single vial of label was added to a single vial of sample and mixed. The samples used with each label were: 10276 bsaZ::pDM4–114, 10276 WT–115, 10276 bipD::pDM4–116, and 10276 ΔbsaP–117. Samples were incubated with shaking at room temperature for 1 hour, after which 100 µl of water was added and incubated for a further hour. The 4 vials of labels/samples were pooled into one vial and then dried under vacuum. Samples were desalted using a standard method.

2.4.5 SCX Fractionation and Salt Cuts

To a Millipore (Watford, UK) C18 ziptip, 10 µl Poros HS resin (Life Technologies, Paisley, UK) was added and washed with 3 x 25 µl Buffer A (35% ACN, 0.1% FA). Samples were re-suspended in Buffer A and loaded onto the resin, washed with 3 x 25 µl Buffer A, and eluted with 2 x 25 µl of each of 11 salt cuts ranging from 5 mM to 800 mM NaCl. A final elution was performed using 2 x 25 µl 50% isopropanol, 0.4% ammonium hydroxide. Samples were centrifuged and speed vacuumed until dry. Samples were re-suspended in 1% FA and pooled as six samples containing the salt cuts as follows: JS–1 (5 mM, 10 mM, 20 mM), JS–2 (50 mM), JS–3 (100 mM), JS–4 (150 mM), JS–5 (200 mM), JS–6 (250 mM, 300 mM, 400 mM, 80 mM).

2.4.6 Mass Spectrometry

Peptides were separated using an Ultimate 3000 RSLC nanoflow LC system (Thermo Fisher Scientific, MA, USA). With a constant flow of 5 µl/minute, 15 µl of sample was loaded onto an Acclaim PepMap100 nanoViper C18 trap column (100 µm inner-diameter, 2 cm) (Thermo Fisher Scientific, MA, USA). After trap enrichment, peptides were eluted onto an Acclaim PepMap RSLC nanoViper, C18 column (75 µm, 15 cm) (Thermo Fisher Scientific, MA, USA) with a linear gradient of 2–45% solvent B (80% ACN with 0.08% FA) over 125 minutes with a constant flow of 300 nl/minute. The high-performance liquid chromatography (HPLC) system was coupled to a linear ion trap Orbitrap hybrid mass spectrometer (LTQ-Orbitrap Velos) (Thermo Fisher Scientific, MA, USA) via a nanoelectrospray ion source (Thermo Fisher Scientific, MA, USA). The spray voltage was set to 1.2 kV and the temperature of the heated capillary was set to 250°C. Full-scan MS survey spectra (m/z 335–1800) in profile mode were acquired in the Orbitrap with a resolution of 60,000 after accumulation of 1,000,000 ions. The ten most intense peptide ions from the preview scan
in the Orbitrap were fragmented sequentially by higher-energy collisional disassociation (HCD) (two-step (6%) normalised collision energy (NCE), 40%; and activation time, 0.1 ms) prior to analysis in the Orbitrap after accumulation of 50,000 ions. Maximal filling times were 500 ms for the full scans and 200 ms for the MS/MS scans. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged species were rejected. The lock mass option was enabled for survey scans to improve mass accuracy (Olsen et al., 2005). Data were acquired using the Xcalibur software (Thermo Fisher Scientific, MA, USA).

2.4.7 Quantification and Bioinformatic Analysis

The raw mass spectrometric data files obtained for each experiment were collated into a single dataset using Proteome Discoverer (Thermo Fisher Scientific, MA, USA) and the Mascot search engine (www.matrixscience.com) using a translated B. pseudomallei K96243 genome as a reference for protein identification. Enzyme specificity was set to that of trypsin, allowing for cleavage N-terminal to proline residues and between aspartic acid and proline residues. Other parameters used were: (i) variable modifications, methionine oxidation, protein N-acetylation, gln pyro-glu; (ii) fixed modifications, cysteine carbamidomethylation; (iii) database: B. pseudomallei K96243_20120601; (iv) iTRAQ labels: standard iTRAQ 4-plex quant method with labels 114–117; (v) MS/MS tolerance: FTMS- 10 ppm, FTMS/MS- 0.06 Da; (vi) minimum peptide length, 6; (vii) maximum missed cleavages, 2; (viii) false discovery rate, 1%. Peptide ratios were calculated for samples using the 10276 bsaZ::pDM4–114 label as the denominator. Protein ratios were normalised using the overall median ratio for all quantified proteins in each sample based on the assumption the abundance of a majority of secreted proteins remain unchanged in each condition. Proteins were selected as potential T3SS effector protein candidates if they had at least 2 unique peptides used in quantification with a MASCOT peptide score of over 21, which corresponds to the threshold value for a 95% confidence level (a 1 in 20 chance that the match is random), and a “high” iTRAQ ratio in at least one strain, which we defined as a value greater than 1.5 X inner quartile range (IQR). When n = complete set of ratios over 10276 bsaZ::pDM4 for all quantified proteins in a given sample, IQR is defined as:

\[ IQR = Q_3 - Q_1 \]
where

\[ Q_3 = \left(\frac{3(n + 1)}{4}\right)^{th\ term} \]

and

\[ Q_1 = \left(\frac{(n + 1)}{4}\right)^{th\ term} \]

### 2.5 *Saccharomyces cerevisiae* Strains

*Saccharomyces cerevisiae* strains were grown at 30°C on either yeast extract peptone dextrose (YPD) agar, YPD broth (20 g/L peptone (oxoid), 10 g/L yeast extract, glucose at 2% final concentration, water to 1 L, pH 6.5). If selection was required, yeast strains were instead grown in either SD agar (6.7 g/L yeast nitrogen base without amino acids, 20 g/L agar (Invitrogen), glucose at 2% final concentration, amino acid supplements (Clontech), water to 1 L, pH 5.8) or synthetic defined (SD) broth (6.7 g/L yeast nitrogen base without amino acids, glucose at 2% final concentration, amino acid supplements (Clontech), water to 1 L, pH 5.8). The amino acids were added in the following concentrations: L-adenine hemisulfate salt 20 mg/L, L-arginine HCl 20 mg/L, L-histidine HCl monohydrate 20 mg/L, L-isoleucine 30 mg/L, L-leucine 100 mg/L, L-lysine HCl 30 mg/L, L-methionine 20 mg/L, L-phenylalanine 50 mg/L, L-threonine 200 mg/L, L-tryptophan 20 mg/L, L-tyrosine 30 mg/L, L-uracil 20 mg/L, L-valine 150 mg/L. The amino acids Trp, Leu, Ade and His were excluded from SD media where appropriate to select for plasmids encoding the amino acids. The nomenclature for SD media lacking an amino acid is SD/- (amino acid). For example, SD media lacking Trp, Leu, Ade and His is written SD/-Trp/-Leu/-Ade/-His. Kan was used at a final concentration of 50 µg/ml in all yeast media. X-α-Gal (Clontech) was dissolved at 20 mg/ml in DMF and stored at −20°C in the dark. For blue/white colony screening 200 µl of X-α-Gal was spread onto 10 cm SD plates and allowed to dry before streaking of yeast strains.


2.6  *S. cerevisiae* Molecular Methods

2.6.1  *S. cerevisiae* DNA and Protein Extraction

To extract DNA from yeast, 20 µl of 0.02 M NaOH was added to a PCR tube along with a partial colony of *S. cerevisiae* (until cloudy). The yeast suspension was then boiled in a PCR cycler at 99°C for 10 minutes to lyse the yeast. The resulting yeast lysates were stored at –20°C until use as a template at a 1:20 final concentration in PCR reactions.

Extraction of plasmids from yeast grown in liquid or on solid media was performed using the Zymoprep yeast plasmid miniprep II kit (Zymo Research) according to the manufacturers instructions. The kit uses zymolase to lyse the yeast and plasmids were eluted from the spin columns with DNAase free water.

Yeast glycerol stocks were made by suspending a colony of yeast in 200 µl of sterile water, vortexing thoroughly, and then adding 200 µl of sterile 50% PBS/glycerol. Glycerol stocks were stored at –80°C until later use.

To extract protein from yeast, 5 ml cultures were grown shaking overnight at 30°C. The cultures were then centrifuged at 1,000 RCF, the supernatant was discarded, and the pellet was resuspended in 2 ml of YeastBuster protein extraction reagent containing 1 x tris(hydroxypropyl)phosphine (THP) and 25 U/ml Benzonase Nuclease (Merck Millipore). After a 30 minute incubation, or until the solution was clear, an equal volume of 2x RSTB was added to the lysates and the mixture was heated at 95°C for 10 minutes. Protein extracts were stored at –20°C until their use in downstream applications.

2.6.2  *S. cerevisiae* Transformations

Plasmids were transformed into *S. cerevisiae* AH109 using a protocol similar to that described in Gietz and Woods (2002). A single colony about 2 mm in size of the *S. cerevisiae* AH109 strain to be transformed was inoculated into 5 ml of YPD broth, or the appropriate dropout media, and was grown overnight shaking at 30°C . The following day, the yeast culture was centrifuged at 1,000 RCF, the supernatant was discarded, and the pellet was resuspended in 1 ml of sterile water and added to a 1.5 ml microcentrifuge tube. The yeast was centrifuged again at full speed in a microcentrifuge (16,000 RCF) for 30 seconds and the supernatant was discarded. Single stranded herring sperm carrier DNA (10 mg/ml, Clontech) was pre-boiled at 99°C for 5 minutes then
immediately put into an ice bath. Reagents were added to the pellet in the following order with mixing after each addition: 240 µl of filter sterilized PEG BioUltra 3,500 (Sigma) 50% w/v in water, 36 µl 1.0 M LiAc, 10 µl chilled herring sperm carrier DNA, 1 µg plasmid DNA (containing the insert of interest), sterile water to a total of 360 µl of liquid added to the yeast pellet (about 60–70 µl depending on plasmid concentration). The transformation mixture was then incubated in a 42°C water bath for 3 hours after which it was pelleted in a microcentrifuge at 16,000 RCF for 30 seconds. The supernatant was discarded and the pellet was resuspended in 1 ml of sterile water by disrupting with the pipette tip and vortexing thoroughly. The re-suspended yeast was spread onto SD plates lacking the appropriate amino acids using 100 µl of neat and a 10⁻¹ dilution and then incubated overnight at 30°C. Resulting colonies were re-streaked onto SD plates lacking the appropriate amino acids and incubated overnight at 30°C. Colonies were confirmed to contain the correct plasmid by colony PCR and glycerol stocks were made.

2.6.3 Cloning of Yeast Two-Hybrid Bait Proteins

Primers were designed to amplify each gene of interest, BprD (BPSS1521), BapA(BPSS1528), and BipC(BPSS1531), in their full length adding an NdeI restriction site using the forward primer and a BamHI restriction site using the reverse primer. Genes were amplified from B. pseudomallei 10276 genomic DNA. The standard PCR cycle conditions were used and the product was run on a 1% agarose gel, imaged, and the band corresponding to the correct size was cut out and purified. The DNA fragments were then quantified and then both the fragments and the plasmid pGBK7 were submitted to a double digest using NdeI and BamHI at 27°C for 1 hour. The digested products were run on a 1% agarose gel, the correct sized bands were cut out, and gel purified. A ligation was performed with T4 DNA ligase overnight at 4°C and the ligation product was transformed into XL1-Blue E. coli. After 2 hours of recovery shaking at 37°C, the E. coli was plated onto LA plates containing Kan and grown overnight at 37°C. Colonies were selected and re-streaked onto Kan containing LA plates to confirm resistance to the antibiotic. The resulting colonies were grown overnight shaking at 37°C in LB containing Kan. Plasmids for each of the proteins of interest (pGBK7-bprD, pGBK7-bapA, pGBK7-bipC) were extracted from the overnight cultures using a mini-prep kit and then sent for Sanger sequencing using the T7 Forward and 3’ DNA-BD. Sequences were compared to that of B. pseudomallei K96243 using Blast P and plasmids which
showed no amino acid differences to K96243, or other strains of \textit{B. pseudomallei}, were chosen for use in the Y2H testing and the corresponding strains of XL1-Blue \textit{E. coli} were stored at \(-80^\circ\text{C}\) as glycerol stocks.

The pGBK7 plasmids (pGBK7-\textit{bprD}, pGBK7-\textit{bapA}, pGBK7-\textit{bipC}) were transformed into \textit{S. cerevisiae} AH109 and colonies containing the plasmids were selected by inoculation onto SD/-Trp plates. Colonies were confirmed to contain the correct plasmid by colony PCR. To ensure the proteins of interest were being expressed in \textit{S. cerevisiae} AH109, protein extracts were made and were run on a 4–15\% TGX gel by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and visualized by Western blot using a rabbit \(\alpha\)-c-Myc primary antibody and a goat \(\alpha\)-rabbit secondary antibody. Colonies showing bands at the correct size were stored at \(-80^\circ\text{C}\) as glycerol stocks until use in Y2H testing.

In order to test for auto activation of the Y2H reporter genes by the bait proteins, the \textit{S. cerevisiae} AH109 strains containing pGBK7-\textit{bprD}, pGBK7-\textit{bapA}, pGBK7-\textit{bipC} were plated onto both SD/-Trp/\(X\alpha\text{-Gal}\) and SD/-Trp/\(-\text{Ade}/-\text{His}/X\alpha\text{-Gal}\) and grown for 2–3 days at 30\(^\circ\)C. Colonies were compared to controls and checked for growth on each media as well for presence or absence of blue colony color. The size of colonies on SD/-Trp/\(X\alpha\text{-Gal}\) were also compared to colonies of \textit{S. cerevisiae} AH109 pGBK7 that were plated simultaneously to test for toxicity of the bait protein to the yeast.

### 2.7 Yeast Two-Hybrid

#### 2.7.1 Yeast Two-Hybrid Media and Controls

The three putative proteins of interest submitted to Y2H screening were BprD (BPSS1521), BapA(BPSS1528), and BipC(BPSS1531). Yeast two hybrid was performed using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech) essentially as the manufacturer described.

In order to confirm batches of SD amino acid drop out media used in Y2H screening were stringent and to ensure the Y2H system was performing as expected, control strains of yeast were made and tested. The control plasmids pGBK7–53 and pGBK7-Lam were transformed into \textit{S. cerevisiae} AH109 and colonies containing the plasmids were selected by inoculation onto SD/-Trp plates. The control plasmid pGADT7-T was provided by the manufacturer (Clontech) and was plated onto SD/-Leu plates. Control strains were pro-
duced by mating the following combinations of plasmid containing strains using the small scale mating procedure according to the manufacturer’s instructions (Clonetech): Positive control: *S. cerevisiae* AH109 pGBKT7–53 and *S. cerevisiae* Y187 pGADT7-T. Negative control: *S. cerevisiae* AH109 pGBKT7-Lam and *S. cerevisiae* Y187 pGADT7-T. Colonies containing both plasmids were selected by inoculation onto SD/-Trp/-Leu plates and glycerol stocks were made for later use. The control strains were then used to test the stringency of the Y2H SD drop out media by looking for growth and blue colony colour on SD/-Trp/-Leu/X-α-Gal and SD/-Trp/-Leu/-Ade/-His/X-α-Gal.

### 2.7.2 Yeast Two-Hybrid Bait–Library Mating

The library used in the Y2H screen was a Mate and Plate universal human normalised library in the yeast strain *S. cerevisiae* Y187 (Clontech, Catalogue number 630480, Lot number 1312192A). The manufacturer states that the library represents genes transcribed in a range of human tissues designed to offer nearly complete human gene coverage. Genes are amplified as cDNA from which high abundance transcripts are normalised using a duplex-specific nuclease to increase the relative quantity of lower abundance transcripts. The normalised cDNA is then cloned into pGADT7 for use in library screening. Quality control data pertaining to the library include: titer: $\geq 5 \times 10^7$ cfu/ml, number of independent clones: $1.84 \times 10^7$, average cDNA size: 1.43 kb, cDNA size range: 0.5–2.9 kb. The *S. cerevisiae* AH109 strains containing the genes of interest were mated with the library according to the manufacturers instructions. In brief, overnight cultures of the *S. cerevisiae* AH109 strains were mixed and incubated, each with a single aliquot of library shaking at 45 RPM at 30°C. After 24 hours the culture was checked for the formation of zygotes using a light microscope (40X). If mating occurred, the yeast cultures were pelleted at 1000 RCF for 10 minutes, washed once with sterile water, and resuspended in 10 ml of sterile water. A small amount of culture was plated onto SD/-Trp, SD/-Leu, and SD/-Trp/-Leu as a control for the number of colonies screened and the remaining culture was plated on SD/-Trp/-Leu/-Ade/-His plates (100 µl per plate). Colonies were incubated at 30°C for 10 days. The plates were checked at days 5 and 10 and any resulting colonies were re-streaked onto SD/-Trp/-Leu/-Ade/-His plates which were grown for 3 days at 30°C. In order to separate plasmids colonies were re-streaked on SD/-Trp/-Leu/-Ade/-His plates and grown twice more. Colonies were then streaked onto SD/-Trp/-Leu/-Ade/-His/X-α-Gal plates to check for a blue colour change and
glycerol stocks were made for each colony.

### 2.7.3 Identification of Interacting Library Plasmids

In order to determine what library plasmid each of the colonies contained a colony PCR was performed using the T7 Forward and 3' DNA-AD primers which are specific for the library plasmid. The resulting product was run on a 1% agarose gel and inserts of different sizes were cut out, gel purified, and sent for Sanger sequencing (Source BioScience) using the T7 Forward primer. To confirm the interactions were not due to secondary library plasmids in the yeast strains, plasmids were extracted from the mated yeast strain using a mini prep kit and were transformed into *S. cerevisiae* AH109 containing the pGBKT7-bait protein. The strains were plated onto SD/-Trp/-Leu/-Ade/-His/X-α-Gal and SD/-Trp/-Leu/X-α-Gal plates. The library plasmids of interest were also checked for auto activation by transforming them into *S. cerevisiae* AH109 and inoculating the transformed strain onto SD/-Leu/X-α-Gal and SD/-Leu/-Ade/-His/X-α-Gal.

### 2.8 BipC Methods

#### 2.8.1 Actin Binding Using GSH-linked Sepharose Beads

To investigate the ability of proteins of interest to directly bind actin, GSH-linked sepharose beads bound to GST, GST-BimA, GST-SipC, or GST-BipC were combined with 1 x polymerisation buffer containing 1 μM rhodamine-labelled actin on a glass microscope slide. The beads were immediately visualised using a Zeiss LSM 710 Scanning Laser Confocal Microscope using an excitation of 535 nm and an emission of 585 nm. If a protein is able to bind actin, it creates a high concentration of the rhodamine-labelled actin at the surface of the sepharose bead, which appears as a red “halo” around the clear bead in microscope images.

#### 2.8.2 Pyrene Actin Polymerisation

A 1 mg aliquot of pyrene-labelled actin was suspended in 50 μl of deionised water. A wide-bore tip was used to transfer the actin to a chilled bijou containing 2.495 ml G-buffer (2 mM Tris - pH 8, 0.5 mM DTT, 0.2 mM ATP, 0.1 mM CaCl₂, and 0.01% NaN₃), the mixture was briefly vortexed, and the actin was
incubated on ice in the dark for 1 hour to de-polymerise. The actin was then transferred to a polycarbonate ultracentrifuge tube and was ultracentrifuged at 100,000 RFC for 2 hours at 4°C to pellet any nucleation centres. The top 80% of the supernatant was transferred to a chilled bijou and stored on ice in the dark until use. The concentration of actin was determined using a Bradford assay (Pierce Scientific) and the molar concentration was determined. Monomeric actin used in polymerisation assays at a final concentration of 2-3 µM.

All assays were run in black opaque 96-well plates and run with a kinetic cycle where data was collected every 30 seconds for up to 1 hour using a FLU-Ostar Omega plate reader (BMG Labtech). Samples were excited with a wavelength of 365 nm and emission was data was collected at a wavelength of 407 nm. For each preparation of pyrene-labelled actin, the polymerisation capabilities were first determined and a gain was assigned and use in all subsequent reactions. To set the gain, reactions were set up in a 96 well plate as listed in 2.1 below. The reactions were allowed to polymerise in the dark for 1 hour, before measuring and using the value from Well 3 as the gain.

Table 2.1: Pyrene actin polymerisation assay control reactions used to set gain.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Well 1</th>
<th>Well 2</th>
<th>Well 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris pH 7.5</td>
<td>100 µl</td>
<td>90 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>20 µM pyrene actin</td>
<td>0 µl</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>10x polymerisation buffer</td>
<td>0 µl</td>
<td>0 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

The actin polymerisation assay was performed using recombinant proteins as controls, including GST-WASP-VCA (Cytoskeleton, VCG03) and bovine Arp2/3 (Cytoskeleton, RP01). GST-WASP-VCA was suspended in 500 µl of water at a final concentration of 1 mg/ml (Rmw is 43 kDa, so final concentration was 23 µM). The GST-WASP-VCA diluted to 10 µM, aliquoted in 10 µl aliquots, and was stored at -80°C until use. The working concentration for GST-WASP-VCA in the actin polymerisation assay was 100 nM.

The Arp2/3 complex was prepared by suspending in 10 µl of water and 10 µl of Arp2/3 buffer (20 mM Tris pH 7.5, 25 mM KCl, 1 mM MgCl2, 0.1 mM ATP and 1 mM DTT) The resulting solution had a concentration of 2.5 mg/ml (Rmw is 224 kDa, so final concentration was 11 µM) and was stored at -80°C.
Table 2.2: Pyrene actin polymerisation assay example.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Well 1</th>
<th>Well 2</th>
<th>Well 3</th>
<th>Well 4</th>
<th>Well 5</th>
<th>Well 6</th>
<th>Well 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris pH 7.5</td>
<td>90 µl</td>
<td>80 µl</td>
<td>79 µl</td>
<td>79 µl</td>
<td>78 µl</td>
<td>70 µl</td>
<td>70 µl</td>
</tr>
<tr>
<td>10 µM VCA</td>
<td>– – –</td>
<td>– – –</td>
<td>1 µl</td>
<td>– – –</td>
<td>1 µl</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>1.3 µM Arp2/3</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>1 µl</td>
<td>1 µl</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>1 um GST</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>10 µl</td>
<td>– – –</td>
<td></td>
</tr>
<tr>
<td>GST-X</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>10 µl</td>
</tr>
<tr>
<td>20 µM pyrene actin</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>10x polymerisation buffer</td>
<td>– – –</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

in 2 µl aliquots. The Arp2/3 complex was used at 13 nM in polymerisation assays. Assays were set up as in Table 2.2. The 10x polymerisation buffer was added last using a multichannel pipette so that all wells received the buffer at the same time. The plate was immediately loaded into the plate reader and after and initial shaking sequence, readings were taken every 30 seconds for 1 hour. The assay was performed in three independent replicates with three measurements per replicate. Data was plotted in Excel. The rates of polymerization were calculated as the rise in fluorescence units per second during the linear phase of polymerization. The mean rates of polymerization for each protein were analysed by pairwise Student t tests using R software and P values of ≤0.05 were taken as significant.

### 2.8.3 Actin Depolymerisation

To assess the ability of BipC to stabilise actin filaments, an actin depolymerisation assay was performed. An aliquot of pyrene actin was thawed and converted to Mg-Actin. This conversion was done by incubating the actin suspended in G-buffer at 1 mg/ml with 125 µM EGTA and 50 µM MgCl₂ for 10 minutes at room temperature. The Mg-actin was then polymerised by adding
500 µl of G-Mg buffer and 110 µl of 10 x polymerisation buffer and incubating at room temperature in the dark for 2-4 hours. The depolymerisation assay was set up in a 96 well black opaque plate with each well containing 10 µl of F-actin (final concentration 2.3 µM) and 90 µl G-Mg buffer, plus or minus 2.5 µM GST-X protein. As controls, GST, phalloidin and MeOH were used and the gain was set using a well containing 10 µl of F-actin, 16 µl of H2O and 74 µl of G-Mg buffer. The assay was set up as in Table 2.3. All assays were run in black opaque 96-well plates and run with a kinetic cycle where data was collected every 30 seconds for up to 1 hour using a FLUOstar Omega plate reader (BMG Labtech). Samples were excited with a wavelength of 365 nm and emission was data was collected at a wavelength of 407 nm. The assay was performed in three independent replicates with three measurements per replicate. Data was plotted in Excel.

Table 2.3: Pyrene actin depolymerisation assay example.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Well 1</th>
<th>Well 2</th>
<th>Well 3</th>
<th>Well 4</th>
<th>Well 5</th>
<th>Well 6</th>
<th>Well 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-actin</td>
<td>– – –</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
<td>– – –</td>
</tr>
<tr>
<td>G-Mg buffer</td>
<td>100 µl</td>
<td>74 µl</td>
<td>74 µl</td>
<td>74 µl</td>
<td>74 µl</td>
<td>74 µl</td>
<td>74 µl</td>
</tr>
<tr>
<td>GST</td>
<td>– – –</td>
<td>– – –</td>
<td>10 µl</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>GST-X</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>10 µl</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>H2O</td>
<td>– – –</td>
<td>16 µl</td>
<td>6 µl</td>
<td>6 µl</td>
<td>0.85 µl</td>
<td>0.85 µl</td>
<td>36 µl</td>
</tr>
<tr>
<td>phalloidin633</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>15.15 µl</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>MeOH</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>15.15 µl</td>
<td>– – –</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

2.8.4 Actin Sedimentation

To perform an actin sedimentation assay, rhodamine actin was first polymerised. In a microcentrifuge tube, 180 µl of sterile water containing 4 µg/µl rhodamine actin, 0.05 µM MgCl2, and 0.125 µM EGTA was allowed to incubate for 10 minutes at room temperature in the dark to form Mg-actin. To the
mixture, 20 µl of 10x polymerisation buffer was added and was incubated for 2 hours at room temperature in the dark to polymerise the G-actin into F-actin. F-actin was used at a final concentration of 0.4 ug/µl in reaction mixtures listed in Table 2.4.

**Table 2.4: Actin sedimentation assay reactions.**

<table>
<thead>
<tr>
<th>Negative Control</th>
<th>GST</th>
<th>BimA</th>
<th>SipC</th>
<th>BipC</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ug F-actin</td>
<td>10 ug F-actin</td>
<td>10 ug F-actin</td>
<td>10 ug F-actin</td>
<td>10 ug F-actin</td>
</tr>
<tr>
<td>– – – – –</td>
<td>1 µM protein</td>
<td>1 µM protein</td>
<td>1 µM protein</td>
<td>1 µM protein</td>
</tr>
<tr>
<td>59 µl G-Mg buffer</td>
<td>59 µl G-Mg buffer</td>
<td>59 µl G-Mg buffer</td>
<td>59 µl G-Mg buffer</td>
<td>59 µl G-Mg buffer</td>
</tr>
<tr>
<td>dH₂O to 100 µl</td>
<td>dH₂O to 100 µl</td>
<td>dH₂O to 100 µl</td>
<td>dH₂O to 100 µl</td>
<td>dH₂O to 100 µl</td>
</tr>
</tbody>
</table>

The reaction was incubated at room temperature for 30 minutes in the dark and then the F-actin was centrifuged at 100,000 RCF for 1 hour at 20°C to sediment the F-actin (Beckman Coulter TLA100.1 rotor was used at 53,000 RPM). The soluble fraction (monomeric G-actin fraction) was carefully removed into a fresh microcentrifuge tube and a suitable volume of 6 x Laemelli buffer was added. The pellet (filamentous F-actin fraction) was re-suspended in a suitable volume of 6x Laemelli buffer and transferred to a fresh microcentrifuge tube. The F-actin and G-actin were then both heated for 5 minutes at 95°C and the proteins were separated by 10% SDS-PAGE followed by visualisation using Silver staining (Pierce Scientific).

### 2.8.5 Transfections and Immunofluorescence

In order to investigate the effects of eptopically expressed BipC in eukaryotic cells, HeLa cells were transfected with the constitutive eukaryotic expression vector pRK5-myc-BipC, which adds an in-frame N-terminal Myc tag to the protein. The plasmid pEGFP which encodes enhanced green fluorescent protein (EGFP) was used as a control. HeLa cells were seeded at a density of 2 x 10^5 cells per well into 6-well plates containing glass cover slips and were grown overnight in DMEM at 37°C in the presence of 5% CO₂. Transfections were using Lipofectamine 2000 (Invitrogen) according to the manufacturers protocol.
and 4.0 μg of plasmid per well. The transfected HeLa cells were grown for 48 hours in DMEM at 37°C in the presence of 5% CO₂ after.

To visualise the Myc-tagged BipC, the transfected cells were fixed in 4% paraformaldehyde dissolved in PBS (pH 7.4) for 1 hour at room temperature. The cells were washed twice with PBS and permeabilised using 1 ml of 0.5% Triton X-100 dissolved in PBS for 15 minutes at room temperature. Cells were washed twice with PBS and then blocked for 30 minutes at room temperature with blocking buffer (PBS containing 0.5% bovine serum albumin w/v, 0.02% NaN₃ w/v, filtered using a 0.2 µm syringe filter). In order to detect the Myc-BipC, cover slips were incubated with 500 µl of rabbit α-c-Myc primary antibody (0.5 µl/ml, Santa Cruz) at 37°C for 1 hour. The cover slip was washed 3 times with PBS followed by incubation with 500 µl of goat α-rabbit 488 secondary antibody (2 mg/ml, Molecular Probes) diluted in blocking buffer (1:200) for 1 hour. The cover slips were washed 3 times with PBS and incubated with 500 µl of phalloidin 568 (2 mg/ml, Invitrogen) for 15 minutes. The cover slip was washed six times with PBS, one time with water and was mounted on a glass slide using Prolong Gold (5 µl, Invitrogen). The slides were visualised using a Zeiss LSM 710 Scanning Laser Confocal Microscope.

2.9 Statistical Analysis

Statistical analysis is also described in the methods sections in which it was used. For the iTRAQ experiment, proteins were selected as potential T3SS effector protein candidates if they had at least 2 unique peptides used in quantification with a MASCOT peptide score of over 21, which corresponds to the threshold value for a 95% confidence level (a 1 in 20 chance that the match is random, P value of =0.05), and a “high” iTRAQ ratio in at least one strain, which we defined as a value greater than 1.5 X inner quartile range.

To analyse actin polymerisation data, the fluorescence data was first plotted in Excel. The rates of polymerization were calculated as the rise in fluorescence units per second during the linear phase of polymerization. A pairwise Student t tests was performed using R software and P values of ≤0.05 were taken as significant.
2.10 General Software Used in This Study

Management of data sets as well as graphing of BipC-Actin polymerisation was performed in Excel (Microsoft). The following open source software was used throughout the research. For calculation of cut off values, statistical analysis the R software ((R Core Team, 2015)) was used. The thesis was written using the \LaTeX software (https://latex-project.org/). For image management the GNU Image Manipulation Program (GIMP) 2.0 (https://www.gimp.org/) was used. Inkscape (https://inkscape.org/en/) was used to create vector based graphic diagrams. Database searches for nucleotide and protein similarity was performed using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Madden, 2013).
Chapter 3

Quantitative Proteomic Analysis of Bsa T3SS Effector Proteins Using iTRAQ

3.1 Introduction

The genome of *Burkholderia pseudomallei* encodes three Type III Secretion Systems (T3SS). T3SS-1 and T3SS-2 are more similar to plant pathogen-like secretion systems (Rainbow *et al*., 2002) and are not required for virulence in a Syrian hamster model of infection (Warawa and Woods, 2005). One more recent study shows that T3SS-1 may be more important than previously thought, as a system knockout is attenuated in a murine respiratory model of melioidosis (D’Cruze *et al*., 2011b). The T3SS-3, known as the *Burkholderia* Secretion Apparatus (Bsa) T3SS, has been shown to be important for non-phagocytic cell entry (Stevens *et al*., 2003), escape of the endosome (Stevens *et al*., 2002), and virulence in murine (Stevens *et al*., 2004, Gutierrez *et al*., 2015) and Syrian hamster models of infection (Warawa and Woods, 2005). There are only two effectors known to be secreted by the Bsa T3SS; BopE, a guanine nucleotide exchange factor (Stevens *et al*., 2003), and BopC, a protein of unknown function involved in intracellular survival (Muangman *et al*., 2011, Srinon *et al*., 2013). By comparison to other T3SSs it can be hypothesized that with only three known secreted effector proteins in the *B. pseudomallei* Bsa T3SS, there are many more effectors to be characterized. For example there are at least 21 effectors secreted by the T3SS of Enteropathogenic *E. coli* (EPEC) (Reviewed in (Dean and Kenny, 2009)) and 25–30 effectors secreted by the T3SS of *Shigella flexneri* (Reviewed in Parsot (2009)).
CHAPTER 3. QUANTITATIVE PROTEOMIC ANALYSIS OF BSA T3SS EFFECTOR PROTEINS USING ITRAQ

One of the problems facing T3SS effector protein discovery is the tight regulation of the T3SS under laboratory conditions, with different T3SSs responding to very different environmental cues such as temperature and calcium ion concentration (*Yersinia*) (Forsberg and Wolf-Watz, 1988), the dye Congo Red (*Shigella*) (Parsot et al., 1995), extracellular amino acids and serum proteins (*Yersinia*) (Lee et al., 2001), bile salts (*Shigella*) (Olive et al., 2007) and calcium restriction/depletion (*Chlamydia*) (Jamison and Hackstadt, 2008). The *B. pseudomallei* Bsa T3SS has been shown to be stimulated by salt stress (Pumirat et al., 2009, 2010), and in the closely related species *B. thailandensis*, pH stimulated the Bsa T3SS (Jitprasutwit et al., 2010). Yet, stimulating the T3SS system in such a way may not result in sufficient levels of effector secretion for less sensitive detection methods, illustrated by detection of BopE by western blot (Pumirat et al., 2010), but not by 2D SDS-PAGE (Pumirat et al., 2009).

To override the tight control of the T3SS, one solution employed in the study of other Gram-negative bacteria (for example *Citrobacter rodentium* and EPEC) was to generate mutations of T3SS components that dysregulate the system, thereby creating a hyper-secretion phenotype (Deng et al., 2010, 2012). Although some genes involved in transcriptional control of the Bsa T3SS were recently identified (Sun et al., 2010), little is known about the post-translational regulation of the system and what environmental signals may activate it. In one study the gene encoding the Bsa T3SS needle cap protein of *B. pseudomallei*, *bipD*, was interrupted and showed increased secretion of the effector BopE by western blot analysis (Stevens et al., 2003).

One group of T3SS proteins that have been shown in many systems to be involved in the control of effector and translocator secretion are the so-called ‘gatekeeper’ family of proteins, InvE/MxiC/SepL/YopN-TyeA of *Salmonella/Shigella/E. coli/Yersinia*, respectively (Reviewed in Büttner (2012)). Investigation of these proteins has given insight into the temporal regulation of their respective T3SSs. Although deletion of all of these proteins causes an increase in levels of secreted effectors there are also differences between them. Deletion of *invE* (Kubori and Galán, 2002) and *sepL* (Kresse et al., 2000, Deng et al., 2004, 2005) cause a reduction in secreted levels of translocators, deletion of *mxic* (Botteaux et al., 2009) has no effect on their levels, and deletion of *yopN* (Forsberg et al., 1991, Iriarte et al., 1998) increases levels of secreted translocators. The closest homologue to this family of proteins in *B. pseudomallei* is the uncharacterised protein BsaP (BPSS1544), but its function has not been determined experimentally.
Upon generation of bacterial supernatants containing potential T3S effector proteins, there is still the task of identifying the T3SS proteins in a complex mixture with a large number of other non-Type 3 secreted proteins. In the past methods such as 2D gel electrophoresis have been used (Pumirat et al., 2009), but suffer from low resolution in complex protein samples and a tendency to pick out only the most highly abundant proteins (reviewed in Rabilloud and Lelong (2011)). A recent unpublished study used 2D gel proteomics to attempt to identify \textit{B. pseudomallei} Bsa T3SS effector proteins (M. Stevens and K. Brown, personal communication). \textit{B. pseudomallei} K96243 and 10276 supernatants were prepared by culturing bacteria in LB to stationary phase at room temperature, subculturing into pre-warmed LB (37$^\circ$C), and then incubating for 6 hours shaking at 37$^\circ$C. Secreted proteins were precipitated by trichloroacetic acid (TCA) precipitation and analysed using 2D gel electrophoresis. Spots were cut out of the gel and analysed by trypsin digest and mass spectrometry (MS). The study failed to identify BopE in the bacterial supernatants. The study did however identify BopC and BipD in the supernatants. Because the effector protein BopE is secreted by WT \textit{B. pseudomallei} at very low levels under standard laboratory culture conditions, the inability to detect BopE could be due to sub-optimal precipitation of the secreted proteins or could be due to the gel-based proteomics method used.

Because of the lack of sensitivity, depth and reproducibility, 2D gel proteomics is being replaced by gel-free MS methods (Reviewed in Wasinger et al. (2013)). Indeed, recent secretomics studies have used gel-free relative quantitative proteomic techniques as a sensitive way to determine proteins that are secreted in a T3SS-dependent manner (Sikora et al., 2011, Deng et al., 2010, 2012). Two common methods of gel-free quantitative proteomics are Stable Isotope Labelling of Amino Acids (SILAC) and Isobaric Tags for Relative and Absolute Quantification (iTRAQ) (Reviewed in Elliott et al. (2009)). SILAC uses a medium containing isotopically labelled amino acids which are incorporated into proteins as they are translated \textit{in situ}, which can then be differentiated using MS (Ong et al., 2002). In order to use SILAC, bacterial mutants that lack the ability to synthesise the chosen labelled amino acids need to be generated (Ong et al., 2002). The alternative and more tractable method iTRAQ consists of binding isobaric tags of different molecular weights to the processed peptides immediately prior to analysis by MS (Wiese et al., 2007). iTRAQ also allows for multiplexing of up to eight samples to be compared simultaneously (Reviewed in Elliott et al. (2009)).
3.2 Results

3.2.1 Bioinformatic Prediction of Bsa T3SS Effector Proteins

Recently there has been a focus on the possibility of predicting effector proteins using a bioinformatics approach. There have been a number of publications in close proximity using different algorithms to try to detect effector proteins (Arnold et al., 2009, Lower and Schneider, 2009, Samudrala et al., 2009, Yang et al., 2010, Wang et al., 2011). These studies use features from experimentally proven effector proteins such as N-terminal secretion sequence (T3S signal) to try to inform predictions (Reviewed in McDermott et al. (2011)). When used to predict effectors in *B. pseudomallei*, the programs show inconsistency and predict candidate effector proteins that are known to be secreted by alternative secretion systems or fail to identify known effector proteins. For example, the validated Bsa T3SS effector proteins BopE and BopC were subjected to the 3 different web portals for prediction. BopE or BopC were predicted as not being secreted by the T3SS using Effective (www.effectors.org). Similarly, SIEVE (http://cbb.pnnl.gov/portal/tools/sieve.html) predicted neither protein, BopE or BopC, was secreted by the T3SS. Modlab (http://gecco.org.chemie.uni-frankfurt.de/T3SS_prediction/T3SS_prediction.html) predicted BopE was secreted by the T3SS, BopC was not. While the ability to predict T3SS effectors using bioinformatics has been effective for identifying known effector proteins in model organisms (Reviewed in McDermott et al. (2011)), the most recent programs are still lacking in their ability to correctly identify effectors in *B. pseudomallei* and a better understanding of T3SS signals may still needed for comprehensive and accurate prediction. Because of the current inconsistencies of a bioinformatics approach, effector discovery and especially validation, still requires a lab based approach.

3.2.2 Optimisation of Sample Precipitation Methods

Before proceeding with the generation of bacterial supernatants for analysis by proteomics methods, it was important to optimise methods for generation and precipitation of the proteins in the bacterial culture supernatants. The method was first optimised in a containment level 2 laboratory using *Shigella flexneri* as a model organism, as it is known the *S. flexneri* T3SS is induced by addition of the dye Congo red (Parsot et al., 1995). Cultures of *S. flexneri* were incubated overnight with shaking at 37°C. The overnight cultures were
centrifuged and re-suspended to an OD\textsubscript{600} of 0.5 in fresh LB media containing 20 $\mu$M Congo Red. From the adjusted culture, 1 ml was added to 9 ml of LB media containing 20 $\mu$M Congo Red which was then incubated at 37$^\circ$C for 4 hours with agitation. The supernatants were collected by centrifugation and filtered through a 0.2 $\mu$m low protein binding cellulose acetate syringe filter.

Proteins were first precipitated using TCA and Strataclean beads (hydroxylated silica particles which bind protein, Agilent Technologies). Of the precipitated proteins, half the total volume was analysed by SDS-PAGE and visualised by staining with Coomassie blue. Interestingly, there was little noticeable difference between the supernatant proteins cultured in the presence or absence of Congo red when precipitated with Strataclean Beads, while a difference was apparent in the lanes containing protein precipitated using TCA (Figure 3.1). Congo Red had no noticeable effect on the bacterial whole cell lysates. The lane containing bacterial supernatants precipitated using TCA had considerably more overall protein than the Strataclean bead lanes.

To test whether precipitation could be further improved, proteins were next precipitated from \textit{Shigella} supernatants using one of three methods: Pyrogallol Red-Molybdate-Methanol (PRMM), TCA or TCA plus 2% sodium deoxycholate (TCA+DOC) as described in Chapter 2 (Caldwell and Lattemann, 2004, Bensadoun and Weinstein, 1976). Of the total volume of each precipitation, 1/50\textsuperscript{th} was analysed by SDS-PAGE and visualised by staining with Coomassie blue. When comparing the different secretion methods, the amount of protein present in the PRMM- and TCA-DOC-treated lanes were greater than that in the lane containing the TCA alone-treated sample (Figure 3.2). A blank LB sample was also precipitated and analysed to test for background proteins/peptides in the yeast extract used to prepare the media. No proteins were present in the LB sample above the dye front on the gel. PRMM was chosen as the precipitation method for all further experiments. This was advantageous because it does not contain detergents making it more compatible with downstream applications such as MS.

### 3.2.3 Chemical Induction of the Bsa T3SS

In order to generate high levels of Bsa T3SS effector proteins in the supernatants, initial studies sought to chemically induce the system by changing the composition of the culture media. Because previous reports have identified salt stress in \textit{B. pseudomallei} as inducing the Bsa T3SS (Pumirat \textit{et al.}, 2009, 2010), this condition, as well as calcium limited (EGTA) media, were tested
Figure 3.1: Comparison of the Use of Strataclean Beads and TCA for precipitating proteins from *S. flexneri* bacterial supernatants. Supernatants were collected from *S. flexneri* after 4 hours incubation at 37°C with (CR +ve) or without (CR -ve) the addition of 20 µM Congo Red. Proteins were precipitated with Strataclean beads or TCA. Half the total volume of precipitated proteins were separated by reducing SDS-PAGE and visualised by Coomassie Blue staining.
Figure 3.2: Comparison of PRMM, TCA and TCA with DOC for precipitating proteins from *S. flexneri* bacterial supernatants. Supernatants were collected from *S. flexneri* after 4 hours incubation at 37°C with the addition of 20 µM Congo Red. Proteins were precipitated by PRMM, TCA+DOC or TCA methods as described in Chapter 2. Equal proportions of the precipitated proteins were separated by reducing SDS-PAGE and visualised by Coomassie Blue staining. Lane 2 is LB media precipitated with PRMM (LB).
as possible means to increase the output of the known effector protein BopE. As controls, the standard laboratory media LB and TSB were used. A single colony of \textit{B. pseudomallei} 10276 and a \textit{bsa} T3SS-null mutant, \textit{bsaZ::pDM4}, were used to inoculate 10 ml of LB which was then incubated at 37°C overnight with shaking. The following day the bacteria were centrifuged and the supernatant was replaced with fresh LB to an OD$_{600}$ of 1.0. Of the adjusted culture, 1 ml was used to inoculate 9 ml of each of the different media: LB, TSB, LB without NaCl, LB containing 320 mM NaCl, and LB containing 10 mM EGTA. Bacterial numbers were determined by plating dilutions and all samples had similar starting numbers of about 10$^5$ CFU/ml. The cultures were incubated at 37°C for 6 hours. The bacterial numbers after the incubation were determined by plating of samples and all cultures again had a similar number of CFUs at about 10$^7$ /ml, except for \textit{B. pseudomallei} grown in the presence of 10 mM EGTA which showed a 10 fold reduction in bacterial counts (10$^6$ CFU/ml). The supernatant was collected by centrifugation and filtered through a 0.2 µm low protein binding cellulose acetate syringe filter, and the proteins were precipitated using the PRMM method. Equal volumes of the precipitated proteins were analysed by SDS-PAGE and visualised by Coomassie staining and by Western blot using a rabbit α-BopE antibody (Stevens \textit{et al.}, 2003). When visualised by Coomassie staining, it was apparent that \textit{B. pseudomallei} secretes a complex set of proteins into the supernatant and it was not clear which proteins may be specifically Type 3 secreted when compared to the \textit{bsaZ} mutant samples (Figure 3.3). Interestingly, there was not an increase in the amount of BopE in the supernatant of the WT \textit{B. pseudomallei} under any of the conditions tested, when compared to the standard LB and TSB media by Western blot (Figure 3.3). Another interesting result is the presence of an unknown protein at about 50 kDa in the \textit{bsaZ::pDM4} supernatant. It is unlikely that this protein is a T3S protein, as the system is mutated in this strain, but may be a protein secreted in another manner, perhaps a metal ion acquisition protein, due to low abundance of calcium ions in the EGTA medium. It was determined that standard LB would be used for the bacterial secretion preparations for all other experiments as it is shows a similar profile to other media and had been used extensively in \textit{B. pseudomallei} culture. The supernatants of the \textit{B. pseudomallei} pDM4::\textit{bsaZ} samples contained little or no BopE indicating that minimal cell lysis had occurred. The culture and precipitation methods yielded very consistent results over the course of this study with similar bacterial growth rates, protein yields, and Western blot patterns each time the method was performed.
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Figure 3.3: Attempts at chemical induction of the *B. pseudomallei* 10276 Bsa T3SS. Supernatants were collected from *B. pseudomallei* strains 10276 and 10276 bsaZ::pDM4 after culture for 6 hours at 37°C in LB, TSB, LB w/ 0 mM NaCl, LB w/ 320 mM NaCl or LB+10 mM EGTA. Equal volumes of protein were separated by reducing SDS-PAGE and visualised by Coomassie blue staining or transferred to a PVDF membrane for protein detection using polyclonal rabbit α-BopE (Stevens *et al.*, 2003).
3.2.4 Construction and Characterization of Hyper-secreting Bsa Mutants

As chemical induction failed to induce secretion of larger amounts of BopE into the supernatant when compared to standard growth conditions (LB), methods of genetic dysregulation of the Bsa T3SS were explored. It was hypothesized that BsaP may control the timing and magnitude of effector secretion based on homology to ‘gatekeeper’ proteins from the T3SSs of other Gram-negative bacterial pathogens. BLAST analysis of available genomes indicated that BsaP shares 33% identity over 92% coverage (E-value 1e^{-41}) with the Shigella MxiC gatekeeper protein and 34% identity over 88% coverage (E-value 4e^{-32}) with the Salmonella InvE gatekeeper protein (Figure 3.4). A non-polar bsaP deletion mutant was therefore constructed in B. pseudomallei strain 10276 by double homologous recombination (Logue et al., 2009).

In order to make the deletion of bsaP (BPSS1544), a deletion allele containing ‘start-stop’ codons was created for eventual homologous recombination into the chromosome (Figure 3.5 A). Upstream and downstream fragments of DNA were chosen that were outside of the coding region of bsaP and unique within the B. pseudomallei K96243 chromosome to avoid recombination with non-target sequences. The upstream fragment was amplified by PCR using the primers bsaP1/bsaP2 to amplify a region of 540 bp of intergenic sequence while the downstream fragment was amplified by PCR using the primers bsaP3/bsaP4 to amplify a region of 575 bp of intergenic sequence. To form the start-stop deletion allele, PCR-ligation-PCR was used (Ali and Steinkasserer, 1995). The two PCR products were phosphorylated and then ligated together followed by PCR amplification using bsaP1/bsaP4 to yield a 1115 bp product. The deleted allele was then ligated into the p-GemT plasmid and propagated in E. coli XL1-Blue. The insert sequence was verified by sequencing before it was transferred into the suicide vector pDM4 in E. coli PIR1. pDM4 is a suicide vector containing the R6Kγ origin which requires the pi initiation protein, encoded by the pir gene to replicate. pDM4 can be maintained in pir+ hosts such as E. coli PIR1 and S17-1, but not in B. pseudomallei (Pennfold and Pemberton, 1992). The pDM4-ΔbsaP plasmid was transformed into E. coli S17-1 from which it was introduced into B. pseudomallei by conjugation. B. pseudomallei cannot replicate the pDM4 plasmid, therefore selection for Cm resistance, selects for bacteria that have undergone a homologous recombination event, inserting the pDM4-ΔbsaP sequence into the chromosome (Figure 3.5 B). A second homologous recombination was selected by growth on me-
Figure 3.4: Amino acid sequence alignment of *B. pseudomallei* K96243 BsaP with Salmonella InvE and Shigella MxiC ‘gatekeeper’ proteins. Amino acid sequences (BsaP, YP_111550.1; InvE, NP_461818.1; MxiC, NP_085309.1) were aligned using Clustal Omega software (Sievers et al., 2011). BsaP shares 33% identity over 92% coverage (E-value $1 \times 10^{-41}$) with the Shigella MxiC gatekeeper protein and 34% identity over 88% coverage (E-value $4 \times 10^{-32}$) with the Salmonella InvE gatekeeper protein. Amino acid conservation is indicated as fully conserved (*), or having strongly similar (: , scoring $>0.5$ in the Gonnet PAM 250 matrix), or weakly similar properties (. , scoring $\leq 0.5$ in the Gonnet PAM 250 matrix).
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Figure 3.5: Mutagenesis of the bacterial chromosome by double homologous recombination using the suicide vector pDM4. (A) A PCR product with homologous sequence to upstream and downstream regions of the gene to be deleted is cloned into the vector pDM4. (B) pDM4 is introduced into *B. pseudomallei* by conjugation, and due to the inability of the bacteria to replicate the plasmid, homologous recombination into the chromosome at the sites flanking the gene of interest is selected using the antibiotic Cm. (C) The inserted plasmid is excised from the chromosome by negative selection with sucrose due to the presence of products of the plasmid encoded *sacBR* genes metabolising the sugar into toxic end products. Resulting colonies are screened for Cm sensitivity and sucrose resistance, followed by PCR and sequencing across the target region. Figure taken from Logue *et al.* (2009).
dia containing sucrose (Figure 3.5 C). The pDM4 vector contains the sacB gene which codes for the enzyme levansucrase which catalyses the synthesis of levans (Reviewed by Ried and Collmer (1987)). It is thought that the build-up of high molecular weight levans is fatal to the cell resulting in a sucrose-sensitive phenotype. Only bacteria that have lost the sacB gene by excision of the plasmid can survive, resulting in either WT or mutant strains. Rarely, a third outcome of escape due to inactivation of the sacB gene can occur, which can lead to false positive mutations by PCR (Hmelo et al., 2015). These are detectable by screening for loss of Cm resistance and screening for protein production by Western blot. Of the resulting colonies, 15 were screened for the bsap chromosomal deletion by amplifying across the bsap adjoining regions by PCR, of which 2 colonies had mutations. As expected the resulting product was about 1100 bp smaller than that of the WT allele (Figure 3.6) and one colony was taken forward for all further assays.

Since bsap is predicted to reside in the middle of an operon, with seven genes upstream and two downstream, the deletion of bsap was complemented using a constitutive expression vector encoding bsap to confirm any phenotypes were not caused by polar effects. bsap was cloned into the vector pBHR1 under the control of the kanamycin resistance gene promoter. The 10276 bipD::pDM4 lacking the BipD needle tip protein that has been previously reported to secrete elevated levels of the effector protein BopE was analysed alongside the bsap mutant (Stevens et al., 2003).

Figure 3.7 is a diagram showing the location and function of each Bsa T3SS mutant used in this study. Bacteria-free culture supernatants were prepared by growing an overnight culture in LB at 37°C from a single colony of B. pseudomallei 10276, 10276 bsaz::pDM4, 10276 bipD::pDM4, 10276 Δbsap, and 10276 Δbsap(pbsap). The cultures were centrifuged and the media was replaced with fresh LB to an OD of 1.0, 1 ml was taken from the adjusted culture and added to 10 ml of fresh LB, and the cultures were incubated for 6 hours shaking at 37°C. Bacterial counts taken after the secretion preparations were comparable (10^7 CFU/ml) in all strains indicating there were no significant differences in growth rates. The supernatants were collected by centrifugation and were precipitated overnight using PRMM. The secreted proteins were analysed by SDS-PAGE followed by Coomassie Blue staining. The secreted protein profile of B. pseudomallei LB-culture supernatant is highly complex with few obvious differences between the strains (Figure 3.8). The bacterial supernatants and whole cell lysates were also analysed by Western blot using antibodies to de-
Figure 3.6: PCR confirmation of deletion of the bsaP gene in B. pseudo-
mallei 10276ΔbsaP strain. The region flanking the bsaP gene in B. pseudo-
mallei 10276 was amplified in the ΔbsaP deletion mutant using the primers bsaPSeqF/bsaPSeqR and was visualised on a 1% TAE gel. The WT allele produced a PCR product of about 2300 bp while the ΔbsaP deletion mutant produced a PCR product of around 1200 bp, which corresponds to the expected deleted allele of 1104 bp.
Figure 3.7: *B. pseudomallei* 10276 Bsa T3SS mutants used in this study. The *B. pseudomallei* Bsa T3SS facilitates secretion of effector proteins from the bacterial cytoplasm into the cytoplasm of a target eukaryotic cell. *B. pseudomallei* strain 10276 *bsaZ::pDM4* is an insertion mutant where the gene encoding an integral structural component of the inner membrane (*bsaZ*) has been disrupted by integration of the pDM4 suicide vector. *B. pseudomallei* strain 10276 *bipD::pDM4* is also an insertion mutant where the pDM4 suicide vector has integrated into the *bipD* gene, thereby abrogating expression of the needle tip protein. *B. pseudomallei* strain 10276*ΔbsaP* is a deletion mutant where the gene encoding BsaP, a predicted ‘gatekeeper’ family protein which plays a role in T3SS substrate specificity, has been removed.
Figure 3.8: *B. pseudomallei* 10276 and mutant strain secreted protein profiles. Supernatants were collected from *B. pseudomallei* strains 10276, 10276 bsaZ::pDM4, 10276 bipD::pDM4, 10276ΔbsaP and 10276ΔbsaP (pbsaP) after culture for 6 hours at 37°C. Equal amounts of protein were separated by reducing SDS-PAGE and visualised by Coomassie Blue staining.
Figure 3.9: Western blot analysis of the expression and secretion of BopE and BipD proteins by *B. pseudomallei* 10276 and mutant strains. 10276, 10276 bsaZ::pDM4, 10276 bipD::pDM4, 10276ΔbsaP, and 10276ΔbsaP p-bsaP were cultured for 6 hours at 37°C. Equal quantities of protein from the whole cell lysates or cell-free supernatants were separated by denaturing SDS-PAGE and proteins transferred to a nitrocellulose membrane for protein detection using polyclonal rabbit α-BopE (Stevens *et al.*, 2003) or α-BipD (Stevens *et al.*, 2004).
tect the BopE effector protein and the BipD translocon protein (Stevens et al., 2004) (Figure 3.9). The absence of both BopE and BipD in the supernatants of the 10276 bsaz::pDM4 insertion mutant indicates that there was no detectable cell lysis at the time when the secretome was sampled. When compared with the WT strain, both 10276 bipD::pDM4 and 10276 ΔbsaP strains demonstrated increased secretion of BopE. Interestingly, the levels of BipD secreted into the supernatant was reduced in 10276 ΔbsaP compared with WT. This pattern of increased levels of the effector BopE and reduced secretion of the translocator BipD provides the first evidence of a role for BsaP as a “gatekeeper” protein, controlling a switch between the secretion of translocators and effectors. In the case of both BopE and BipD, the phenotype of 10276 ΔbsaP was partially restored by plasmid-mediated complementation, indicating the effects are unlikely to be caused by polar or secondary defects.

3.2.5 Characterization of the Core B. pseudomallei Secretome Under Standard Laboratory Conditions

Having identified two bsaz mutant strains with dysregulated BopE secretion profiles in bacterial culture supernatants, the next step was to identify the total secreted protein profile from these two strains in comparison to the isogenic parent strain (10276) and a bsaz-null mutant strain. To allow for relative quantitative comparisons of protein levels in the samples, iTRAQ was chosen as the proteomics method to use. iTRAQ offers a few advantages over SILAC in this case. iTRAQ has the ability to multiplex 4 differentially labelled samples simultaneously allowing WT, bsaz::pDM4, bipD::pDM4, and ΔbsaP B. pseudomallei 10276 to be pooled and analysed in a single MS analysis. iTRAQ also does not require generation of amino acid synthesis mutants. The iTRAQ work flow used in this study consisted of generating 3 biological replicates of bacterial secreted proteins which were then pooled, digested, labelled with the iTRAQ reagent, and analysed by MS/MS in 3 technical replicates (Figure 3.10). Given the intrinsic variability introduced by the complexity of the process involved in LC-MS/MS analysis, it was decided that at least 3 technical replicates would need to be performed. While three independent biological replicates each with separate technical replicates would have been ideal, the cost of this experimental design was prohibitive, hence the decision to pool the biological replicates.

In three independent experiments, culture supernatants were prepared from the four test strains B. pseudomallei 10276, 10276 bsaz::pDM4, 10276
Figure 3.10: Work flow for analysis of *B. pseudomallei* secreted proteins by iTRAQ. **A**, Bacterial supernatants were generated in 3 independent biological replicates. **B**, Proteins were precipitated from the bacterial supernatants. Samples were checked for lysis and sample quality by immunoblotting for BopE (Figure 3.11). **C**, Equal volumes of protein from each sample were pooled, digested into peptides with trypsin and labelled with isobaric tags. **D**, The labelled peptides were pooled and submitted to 3 technical replicates of MS/MS. **E**, The data was combined, proteins were identified using MASCOT and quantitation was performed relative to the *B. pseudomallei* 10276::bsaZ sample.
bipD::pDM4, and 10276 ΔbsaP. Bacterial strains were cultured overnight shaking in LB at 37°C, washed in fresh LB, adjusted to an OD_{600} of 1.0, and 1 ml of the adjusted culture was used to inoculate 10 ml of fresh LB. The culture was incubated with shaking for 6 hours at 37°C after which the bacterial supernatants were collected by centrifugation and sterilised using a 0.2 μm low protein binding cellulose acetate syringe filter. Bacterial counts were assessed before and after the 6 hour incubation and all strains demonstrated similar bacterial numbers and growth rates (10^5 CFU/ml at 0 hours and 10^7 CFU/ml at 6 hours). Proteins were precipitated overnight using PRMM. Prior to pooling the biological replicates, the individual samples were all assessed individually for a lack of cell lysis and suitability by analysing equal amounts of protein by SDS-PAGE and immunoblotting for BopE. All 10276 bsaZ::pDM4 samples lacked BopE, indicating the samples were suitable for analysis (Figure 3.11 A). After mixing equimolar amounts of each of the three biological replicates, equal volumes of the mixed samples were visualised by SDS-PAGE and Coomassie Blue staining to ensure similar amounts of protein appeared in each lane and ensuring no extensive protein degradation had occurred during the stages of sample assessment and pooling (Figure 3.11 B). The protein profiles of each strain are similar due to the large amount of proteins secreted by B. pseudomallei and the relatively low abundance of T3S effectors when compared to other secreted proteins. As a final step, the pooled samples were checked for suitability by immunoblotting for BopE, which showed a similar profile to the samples before pooling had taken place (Figure 3.11 B).

The samples were subjected to iTRAQ labelling and three technical replicates of MS/MS analysis at Dundee Cell Products as described under “Experimental Procedures.” iTRAQ allowed the simultaneous identification and relative quantification of peptide fragments in each of the four samples, resulting in the positive identification of a total of 475 proteins present in all samples. The data was expressed as ratios of relative protein abundance either in the 10276, 10276 bipD::pDM4, or 10276 ΔbsaP samples over the 10276 bsaZ::pDM4 sample as a denominator (i.e. 10276 WT/10276 bsaZ::pDM4, 10276 bipD::pDM4/10276 bsaZ::pDM4, and 10276 ΔbsaP/10276 bsaZ::pDM4). The ratios were normalized to the median for each strain and a density plot showed that the majority of protein ratios cluster around the median of one, indicating that the mutations made did not radically change the secretion profile of the bulk of proteins (Figure 3.12). This analysis identified a core set of 426 proteins which were secreted at similar levels in all strains (Figure 3.13), out of the pre-
Figure 3.11: Western blots of 3 biological replicates before and after pooling for iTRAQ labelling. (A) In three independent experiments, supernatants were collected from *B. pseudomallei* 10276, 10276 *bsaZ::pDM4*, 10276 *bipD::pDM4*, and 10276Δ*bsaP* after culture for 6 hours at 37°C. Equal quantities of protein from the cell-free supernatants were separated by denaturing SDS-PAGE and transferred to a nitrocellulose membrane for protein detection using polyclonal rabbit α-BopE (Stevens *et al.*, 2003). (B) The supernatants from the three independent biological replicates where pooled in equimolar ratios. Equal quantities of protein were separated by denaturing SDS-PAGE and visualised by Coomassie Blue staining or transferred to a nitrocellulose membrane for protein detection using polyclonal rabbit α-BopE (Stevens *et al.*, 2003).
dicted total proteome of \textit{B. pseudomallei} K96243 of about 5900 proteins (Holden \textit{et al.}, 2004). Strikingly, the majority of these proteins (about 80\%) are encoded by Chromosome 1.

Of the proteins identified in the core secretome, 54 are annotated with reference to the K96243 genome as hypothetical (uncharacterised) proteins, several more are annotated with metabolic functions. The core secretome also consisted of several elongation factors (Ts, G, P, Tu) and heat shock/chaperone proteins (GroES, GroEL), many of which have previously been reported to be present on the surface of \textit{B. pseudomallei} or part of the outer membrane proteome and recognized by the sera of recovering melioidosis patients (Felgner \textit{et al.}, 2009, Harding \textit{et al.}, 2007, Su \textit{et al.}, 2008, Schell \textit{et al.}, 2011). In addition to the presence of a collagenase, chitobiase, bacterioferritin, and Sec secretory pathway constituents, the secretome also harboured several proteases and lipoproteins. Several phage components, Type I fimbriae and flagellar subunits were also present, although there was a notable lack of any of the predicted T6SS structural or effector proteins or the Type V family of autotransporters. This is in agreement with previous studies which found low expression of the \textit{virAG} virulence regulator, as well as both the T6SS and the Type V auto transporter \textit{bimA}, when \textit{B. pseudomallei} was cultured in rich media (Chen \textit{et al.}, 2011, Burtnick \textit{et al.}, 2011, Burtnick and Brett, 2013).

### 3.2.6 Identification of the Potential Bsa Effector Repertoire

Putative T3SS effector proteins were identified in the data sets primarily on the calculated iTRAQ protein abundance ratios. The protein abundances obtained in each of the WT or mutant strain samples were each expressed as a ratio of the 10276 \textit{bsaZ::pDM4} sample and proteins with an iTRAQ ratio higher than the $1.5 \times$ interquartile range (IQR), together with at least two unique peptides with a MASCOT peptide score $\geq 21$, were taken to be T3SS effector protein candidates.

The $1.5 \times$ IQR values were calculated using the R software and the cutoffs set at 1.56 for the 10276 WT/10276 \textit{bsaZ::pDM4} data set, 1.79 for the 10276 \textit{bipD::pDM4/10276 bsaZ::pDM4} data set and 1.46 for the 10276 $\Delta$\textit{bsaP}/10276 \textit{bsaZ::pDM4} data set, based on the criteria described above. When the data was plotted as a boxplot, the data showed a trend toward more proteins with larger ratios in the 10276 \textit{bipD::pDM4/10276 bsaZ::pDM4} data sets when compared with the 10276 WT/10276 \textit{bsaZ::pDM4} data set (Figure 3.14A). A total of 26 proteins met the selection
Figure 3.12: Density plot constructed with R software (R Core Team, 2015) illustrating the distribution of the median normalised iTRAQ ratios. This density plot displays proteins with a ratio between 0 and 2.5 in each of the three datasets, where protein ratios were expressed as either 10276 (WT), 10276 bipD::pDM4 (bipD) or 10276 ΔbsaP (bsaP) samples compared to the 10276 bsaZ::pDM4 (bsaZ) sample. Ratios close to 1 represent proteins that were present in similar quantities in the supernatants of a given strain when compared to the bsa-null mutant.
Figure 3.13: Venn diagram of the core secretome of \textit{B. pseudomallei}. Proteins present in the secretome of 10276, 10276 \textit{bipD::pDM4} and 10276 \textit{ΔbsaP} compared to the 10276 \textit{bsaZ::pDM4} sample under standard laboratory conditions were used to construct the Venn diagram. Each circle represents the set of proteins for each strain which had iTRAQ ratios within the cut off limit of 1.5 x IQR, indicating there was no change compared to the 10276 \textit{bsaZ::pDM4} sample. A total of 426 proteins had ratios within the cut off limit in all strains and can be considered part of the core secretome of \textit{B. pseudomallei} 10276.
Figure 3.14: Proteins with high iTRAQ protein abundance ratios are enriched in hyper-secreting *B. pseudomallei* strains and encoded within the Bsa T3SS locus. **A**, Boxplot generated with R software (R Core Team, 2015) showing the distribution and median values of the ratios of the protein abundances in the 10276 (WT), 10276 bipD::pDM4 (bipD), or 10276 ΔbsaP (bsaP) samples compared with the 10276 bsaZ::pDM4 (bsaZ) sample. The cut-off values (calculated using R software) are shown by the dotted lines. Points shown above the cut-off value are candidate T3SS effectors.  

**B**, A Venn diagram depicting the number of strain-specific and shared candidate T3SS effector proteins identified in each of the strains 10276 (WT), 10276 bipD::pDM4 (bipD), and 10276 ΔbsaP (bsaP).  

**C**, Distribution of the candidate T3SS effector proteins plotted according to *B. pseudomallei* gene number across both chromosomes and their ratios. Gene numbers refer to the *B. pseudomallei* K96243 reference strain. The Bsa T3SS locus is indicated by the red box.
criteria and can be considered putative effectors (Table 3.1). Of the 26 proteins there were strain-specific subsets, with eight proteins with high ratios in two of the data sets and three proteins with high ratios in all three (Figure 3.14B). The three proteins that displayed high ratios in all three data sets, BopC, BopA, and BopE, are all known B. pseudomallei Type III secreted (T3S) effector proteins or predicted to be T3S proteins based on their T3SS-dependent secretion in a surrogate host system (Muangman et al., 2011, Stevens et al., 2003, Whitlock et al., 2008) (Figure 3.14B). Of the 26 proteins, 20 were only found in the hyper-secreting mutant strain samples and would not have been identified had this study focused solely on the secreted proteins of the 10276 WT strain (Figure 3.14B). Plotting the iTRAQ ratios of the 26 putative effector proteins against their B. pseudomallei K96243 genetic locus number, it is clear that there is a concentration of eight proteins with especially high ratios encoded within the annotated Bsa T3SS locus on Chromosome 2 compared with that of the rest of the genome (Table 3.1, Figure 3.14C).

As expected, the Bsa T3SS-dependent hyper-secretion of BopE in the 10726 bipD::pDM4 and 10276ΔbsaP strains was observed, in full agreement with Western blot data (Figure 3.9). BopC and BopA, the latter of which has only been proven to be secreted in a T3SS-dependent manner in Enteropathogenic E. coli (EPEC), followed a similar pattern of secretion to BopE. Although both of the two putative translocator proteins, BipB and BipC, were secreted at higher levels in 10276 WT than 10276bsaZ::pDM4, only BipB was secreted at levels above the cut-off ratio of 1.56. Similar to the effect of Shigella ipaD mutation, the mutation of bipD in B. pseudomallei 10276 caused an increase in secretion of both BipB and BipC into the supernatant compared with WT. Where 10276 bipD::pDM4 had increased ratios of the translocators, 10276ΔbsaP showed a decrease in ratios below that of WT in agreement with Western blot data (Figure 3.9). This is further evidence that BsaP plays a similar role in B. pseudomallei to its homologue InvE in Salmonella as a gatekeeper protein. The only proven effector missing from this screen was CHBP, however this is not surprising as we have recently reported that the gene encoding CHBP, bpss1385, is absent from the genome of the B. pseudomallei 10276 strain used here (Pumirat et al., 2014).

Besides the needle tip protein BipD, both putative translocators BipB and BipC, BopA, BopC, and BopE, there are also two hypothetical proteins encoded within the bsa T3SS locus identified by the screen that have high iTRAQ ratios. The first, BapA (BPSS1528), is a predicted 880 amino acid protein with
Table 3.1: Candidate Bsa T3SS effector proteins identified by iTRAQ. The table lists proteins in either the 10276, 10276 bipD::pDM4 or 10276 ΔbsaP culture supernatant samples that displayed protein abundance ratios (compared to the 10276 bsaZ::pDM4 mutant) higher than the calculated cut-off values for each comparison. The cut-off values were set at 1.56 for the 10276 WT/10276 bsaZ::pDM4 dataset, 1.79 for the 10276 bipD::pDM4/10276 bsaZ::pDM4 data set, and 1.46 for the 10276 ΔbsaP/10276 bsaZ::pDM4 data set, as described in “Experimental Procedures.” Additional criteria for protein identification included the presence of at least two unique peptides with a MASCOT peptide score of ±21 corresponding to a 95% confidence level. The proteins are listed in order of gene number with genes from chromosome one listed first. Those proteins fulfilling the criteria for selection as candidate T3SS effector proteins are highlighted in the coloured boxes, each colour representing a different strain. Protein score refers to the adjusted sum of scores of the individual peptide scores as calculated by MASCOT. Number of unique peptides refers to the number of significant peptides specific to a protein.
no conserved domains and homologues only in the closely related species *B. mallei* and *B. thailandensis*. Unlike the known effector proteins in the iTRAQ screen, although secretion of BapA appears to be Bsa-dependent in 10276 WT and the ratio increases in the 10276 ΔbsaP data set, it does not appear to be secreted abundantly by the 10276bipD::pDM4 mutant. Interestingly, under the conditions used there was no detection of BapB or BapC, which are encoded downstream of BapA in the same operon and predicted to be substrates of the T3SS (Stevens *et al.*, 2002).

The other candidate effector protein encoded in the *bsa* T3SS locus is BprD (BPSS1521), which is annotated as a hypothetical regulator (Sun *et al.*, 2010). Predicted to be much smaller than BapA at 147aa, BprD is another protein with no known conserved domains and homologues that exist only within closely related *Burkholderia* species. The iTRAQ ratios suggest that it is not secreted at detectable levels by 10276 WT *B. pseudomallei*, but secreted by both 10276 bipD::pDM4 and 10276ΔbsaP, with the ratio of 13.4 in the 10276 ΔbsaP/10276 bsaZ::pDM4 data set being the highest of any protein described in this iTRAQ study.

Outside of the Bsa T3SS locus the remaining 18 proteins with high ratios are distributed across both *B. pseudomallei* chromosomes. One of the proteins encoded by a gene closely flanking the *bsa* locus is the virulence factor TssM (BPSS1512), which is known to be co-regulated with the Bsa T3SS as well as secreted in a Type II-dependent manner (Burtnick *et al.*, 2014).

### 3.2.7 Validation of iTRAQ Data by Analysis of the Secretion of Epitope-Tagged Candidate Effector Proteins

To confirm a number of targets are secreted in a Bsa-dependent manner, a number of candidates were selected to be expressed as in-frame C-terminal c-Myc tagged fusion proteins. The targets chosen were BprD, BapA, BPSS0860, BPSS1512, BopE, and BopA, as well as BPSS1916, a protein with a very high iTRAQ ratio in all strains that did not meet the initial selection criteria due to being identified with only a single unique peptide. Targets were chosen to represent proteins with both high and low protein scores, high and low numbers of peptides, and to be encoded inside and outside of the *bsa* T3SS locus. All of the proteins selected had high ratios in both the 10276 bipD::pDM4 and 10276 ΔbsaP/10276 strains. Proteins known to be ribosomal subunits were excluded from the selection. Initially the target effector proteins were cloned as in-frame
C-terminal c-Myc fusions in the shuttle vector pBHRT under a constitutive kanamycin resistance gene promoter. The plasmids were transformed into strains 10276, 10276\textit{bsaZ}::pDM4, 10276\textit{bipD}::pDM4 and 10276\textit{ΔbsaP}. Control strains containing pBHRT\textit{-bopE} indicated that BopE-c-Myc was being secreted in a BsaZ-independent manner. This was surprising, as endogenous BopE is not secreted by the \textit{bsaZ} mutant (Stevens \textit{et al.}, 2003) (Figure 3.9). To ensure this was not due to the c-Myc antibody interacting non-specifically with other \textit{B. pseudomallei} proteins, the samples were probed again using the α-BopE antibody. The western blots confirmed that BopE-c-Myc was secreted in a BsaZ-independent manner, possibly owing to the high level of expression (Figure 3.15). To investigate the possibility that the presence of BopE in the \textit{bsaZ} supernatants was due to lysis of the bacterial cell, supernatants and bacterial cell lysates were probed using an α-GAPDH antibody. One would expect to only find GAPDH in the bacterial whole cell lysates, unless lysis of bacterial cells occurred, releasing GAPDH into the supernatants. GAPDH was found in the cell lysates of all samples, but none of the supernatant samples, suggesting BsaZ-independent secretion BopE-c-Myc was occurring in a cell lysis-independent manner, likely as a result of abnormal expression levels (Figure 3.16). In samples containing an empty pBHRT vector, BopE was secreted in a similar manner to the corresponding untransformed \textit{B. pseudomallei} strains, ruling out cell lysis or dysregulation caused by the pBHRT vector itself. Upon overexpression it is possible that BopE secreted through one of the other T3SSs or the flagellar secretion system.

It was therefore elected to clone effector-c-Myc fusions under an IPTG-inducible \textit{lacI} promoter in the plasmid pME6032 (Heeb \textit{et al.}, 2002). The plasmids were transformed into strains 10276, 10276\textit{bsaZ}::pDM4, 10276\textit{bipD}::pDM4 and 10276\textit{ΔbsaP}. Three concentrations of IPTG were tested (0 mM, 0.5 mM and 2.0 mM) and 0.5 mM was selected as the lowest level of inducer at which BsaZ-dependent BopE-c-Myc secretion was observed (Figure 3.17). BopA-c-Myc was absent in the supernatant of the 10276\textit{bsaZ}::pDM4 strain, but secreted by the parent strain and secreted at elevated levels by 10276\textit{ΔbsaP} (Figure 3.18), confirming for the first time in \textit{B. pseudomallei} that it is a substrate of the Bsa T3SS, not just the locus of enterocyte effacement-encoded T3SS in EPEC (Whitlock \textit{et al.}, 2008). Furthermore, both of the putative effector proteins located within the \textit{bsa} locus, BprD and BapA, were secreted in a BsaZ-dependent manner (Figure 3.18), confirming that these are novel effectors of the Bsa apparatus.
Figure 3.15: Constitutive expression of the effector protein BopE by the plasmid pBHRT causes T3SS-independent secretion of BopE in *B. pseudomallei* 10276. The coding region of *bopE* was expressed with a c-terminal c-Myc tag in the constitutive expression vector pBHRT in *B. pseudomallei* 10276 *bsaZ::pDM4*. The controls for BopE secretion were empty pBHRT in *B. pseudomallei* strains 10276, 10276 *bsaZ::pDM4*, 10276 *bipD::pDM4* or 10276 Δ*bsaP*. Supernatant were collected after 6 hours of incubation at 37°C. Equal quantities of protein were separated by reducing SDS-PAGE, blotted onto PVDF membranes and probed using polyclonal rabbit α-BopE (Stevens *et al.*, 2003).
Figure 3.16: Leaky secretion of constitutively expressed BopE in \textit{B. pseudomallei} 10276 \textit{bsaZ::pDM4} is not caused by bacterial cell lysis. BopE was expressed with a c-terminal c-Myc tag in the constitutive expression vector pBHRT in \textit{B. pseudomallei} 10276 \textit{bsaZ::pDM4} and compared to empty pBHRT in \textit{B. pseudomallei} strains 10276 and 10276 \textit{bsaZ::pDM4}. Supernatant and whole cell fractions were collected after 6 hours of incubation at 37°C. Equal quantities of protein were separated by reducing SDS-PAGE, blotted onto PVDF membranes and probed using HRP linked polyclonal rabbit \(\alpha\)-GAPDH antibody.
Figure 3.17: Western blot analysis of the effect of IPTG on expression and secretion of the known B. pseudomallei Bsa T3SS effector protein BopE expressed as c-Myc tagged fusion protein. BopE was expressed with a c-terminal c-Myc tag by the IPTG-inducible expression vector pME6032 in B. pseudomallei strains 10276 and 10276 bsaz::pDM4. Supernatant and whole cell fractions were collected after 6 hours of incubation at 37°C in the presence of 0 mM, 0.5 mM or 2.0 mM IPTG. Equal volumes of protein were separated by reducing SDS-PAGE, blotted onto nitrocellulose membranes and probed using polyclonal rabbit α-c-Myc antibody.
CHAPTER 3. QUANTITATIVE PROTEOMIC ANALYSIS OF BSA T3SS EFFCTOR PROTEINS USING ITraq

Figure 3.18: Western blot analysis of the expression and secretion of putative *B. pseudomallei* Bsa T3SS effector proteins expressed as c-Myc tagged fusion proteins. The coding region of *bopE*, *bopA*, BPSS0860, BPSS1512, BPSS1916, *bprD*, and *bapA* were expressed with a c-terminal c-Myc tag in the IPTG-inducible expression vector pME6032 in *B. pseudomallei* strains 10276, 10276 *bsaZ::pDM4*, 10276 *bipD::pDM4* or 10276 Δ*bsaP*. Supernatant and whole cell fractions were collected after 6 hours of incubation at 37°C in the presence of 0.5 mM IPTG. Equal quantities of protein were separated by reducing SDS-PAGE, blotted onto nitrocellulose membranes and probed using polyclonal rabbit α-c-Myc antibody.
Interestingly, Bsa-dependent secretion of proteins encoded outside of the Bsa T3SS locus could not be validated. BPSS0860 and BPSS1512 appeared in the supernatants of all strains, including 10276 bsaZ::pDM4, indicating that although they are secreted, the Bsa system is not required for their secretion (Figure 3.18). Secretion of the protein BPSS1916 could not be detected in any of the strains tested (Figure 3.18). These examples highlight the need for validation of such data sets.

3.3 Discussion

One of the known virulence factors that is important for *B. pseudomallei* invasion, intracellular net replication and virulence in mice is the Bsa T3SS (Stevens et al., 2002, 2004, Warawa and Woods, 2005, Burtnick et al., 2008), but the repertoire of effector proteins secreted by the system has not yet been fully characterized. This study describes an effective way to reliably precipitate *B. pseudomallei* supernatants in small volumes using a protocol adapted from Caldwell and Lattemann (2004). To establish a better understanding of the Bsa T3SS, mutations were made in key structural components shown to dysregulate other T3SSs. It is demonstrated that these mutations lead to hyper-secretion of known effector proteins. Using these mutants, a quantitative proteomic analysis of the total secretome of the Bsa T3SS system was performed. This iTRAQ analysis identified all known effector proteins and translocators present in *B. pseudomallei* 10276, as well as identifying a number of novel putative effector proteins, two of which were independently validated. Also, examining the changes in the abundance of effectors and translocators secreted by the different mutants provides insight into the role the proteins likely play in the temporal regulation of the Bsa T3SS compared with other T3SSs of other Gram-negative bacteria.

The inability of previous proteomics studies to identify known Bsa T3SS effectors highlights the need for the use of more sensitive techniques such as iTRAQ in combination with genetic dysregulation. The *B. pseudomallei* secretome is very complex (Figure 3.8). This is not surprising when taking into account the large 7.2 Mb genome (Holden et al., 2004), which encodes about 5900 proteins, of which over 55% are expressed in LB (Ooi et al., 2013). This study produced one of the most comprehensive secretomes of *B. pseudomallei* to date, identifying a core secretome of 426 proteins secreted under standard laboratory conditions.
Although known effectors were absent from the secreted proteome of the bsaZ mutant by Western blotting (Figure 3.9) and largely undetectable by iTRAQ (Table 3.1) implying a lack of cell lysis, a number of ribosomal proteins that are enriched in the supernatant when compared with the supernatant harvested from the 10276 bsaZ::pDM4 strain were observed. This observation has been made in several other proteomic screens of T3SS effector repertoires (Deng et al., 2012, Schumacher et al., 2014, Kurushima et al., 2012, Bergh et al., 2013) and may be because of a combination of the high abundance of ribosomal proteins in the bacterial cell coupled with the sensitivity of iTRAQ.

Concurrent to this study, a proteome analysis investigating Type II secreted proteins of B. pseudomallei strain MSHR688 was published (Burtnick et al., 2014). From our screen, 27 of the 38 proteins identified as being substrates of Type II secretion in LB by Burtnick et al. (2014) were also identified in our B. pseudomallei 10276 culture supernatants. Whether all 38 Type II secreted proteins identified in B. pseudomallei MSHR688 are encoded by B. pseudomallei 10276 is unknown because the genome sequence for B. pseudomallei 10726 is not yet available. Interestingly, three of the Type II secreted proteins were enriched in the supernatants of the hyper-secreting mutants (BPSL0707, BPSS1512, BPSS1555) and two of the proteins are encoded by regions flanking the Bsa T3SS locus, BPSS1512 and BPSS1555. The deubiquitinase BPSS1512 (TssM) has been shown to be secreted inside host cells where it suppresses the host innate immune response (Shanks et al., 2009, Tan et al., 2010). Although TssM is secreted independently of the Bsa T3SS, its expression appears to be co-regulated with the Bsa T3SS and T6SS-5 (Tan et al., 2010, Burtnick et al., 2011). This co-regulation may explain the increased levels of TssM in the supernatants of the hyper-secreting mutants in this study and again highlights the importance of validating candidate effector proteins.

The profile of secreted proteins encoded within the Bsa T3SS locus provides a comprehensive picture of the secreted levels of the three known effector proteins. The profiles of both BopE and BopC in the iTRAQ screen correspond with previous studies in which they were shown to be secreted in a Bsa-dependent manner (Muangman et al., 2011, Stevens et al., 2003). Although BopA from B. mallei has been shown to be Type 3 secreted in a surrogate host (Whitlock et al., 2008), this study has confirmed its secretion to be dependent on the Bsa T3SS in B. pseudomallei and not one of the other T3SSs.

Two of the putative Bsa T3SS substrates encoded by the Bsa T3SS locus (BapA and BprD) were validated as Bsa-dependent by Western blotting to
detect C-terminal c-Myc tagged proteins expressed extra-chromosomally. Although the function of BapA is currently unknown, a bapA insertion mutation was not attenuated in a Syrian hamster model of acute melioidosis (Warawa and Woods, 2005). BprD was previously annotated as a transcriptional regulator (Sun et al., 2010). In a previous study, when bprD was deleted along with bprB and bprC, there was no change in levels of transcription of other Bsa T3SS genes leaving the role of BprD as a regulator in doubt (Sun et al., 2010). More recently, a bprD mutant of B. pseudomallei K96243 was shown to have increased expression of bprC (Chirakul et al., 2014), a downstream gene in the same operon shown to be involved in regulation of the virulence-associated T6SS (Chen et al., 2011). The bprD mutant also showed decreased time to death in intraperitoneally inoculated BALB/c mice demonstrating its importance in vivo and indicating its possible role as a negative regulator of virulence (Chirakul et al., 2014). It is not without precedent that a regulator of the T3SS is also a substrate for secretion, for example, LcrQ of Yersinia (Cambronne et al., 2000).

Another candidate protein identified in this study, BPSS0860, appears to be a fliD homologue. There is a second copy of fliD (BPSL3320) located within the flagellar locus on Chromosome 1, which had protein abundance ratios that were similar between all strains. Interestingly, the expression of BPSL3320 was up-regulated, whereas the expression of BPSS0860 was down-regulated in the organs of hamsters infected with B. pseudomallei when compared with bacteria cultured in vitro (Tuanyok et al., 2006). Using a c-Myc expression system, Bsa-dependent secretion of BPSS0860 was not established; however, it is possible it may be co-regulated with the Bsa T3SS in a similar manner to BPSS1512. One recent study has shown an effector protein may be secreted by more than one secretion system, thereby masking its secretion by the T3SS (Neeld et al., 2014). This could also be the case for BPSS0860. It is also possible these proteins may be false positive results. Because this study involved a set of pooled biological replicates, a higher resolution would be obtained by using three separate biological replicates which may help to narrow down the list of putative effector proteins, as well as identify any that may not have been detected.

At the time of writing, little is known about the post-translational control of the Bsa T3SS. Generally it is accepted that T3SSs are under tight temporal regulation to ensure substrates are secreted in the correct order (reviewed in Büttner (2012)). This starts with assembly of the membrane-associated structural components, assembly of the needle with needle tip, secretion of translocators to form a pore in the host cell membrane, followed by secretion of ef-
factor proteins. Changes in the patterns of secreted translocators and effector proteins in different mutant strains can inform us of the possible role these proteins play in the hierarchy of secretion.

Inactivation of the needle tip protein of *B. pseudomallei*, BipD, shows a large increase in levels of both translocators and effectors in the culture supernatant, in agreement with data published for the homologous *Shigella* protein IpaD (Parsot *et al.*, 1995, Picking *et al.*, 2005). IpaD is the needle cap protein, acting to block secretion of effector proteins until host cell contact has taken place (Roehrich *et al.*, 2013). Because of its high similarity in both sequence and structure (Erskine *et al.*, 2006) it is perhaps unsurprising that BipD would have a similar effect on the levels of substrates secreted by the Bsa T3SS. Interestingly there was a high ratio of BipB, but not BipC in the WT *B. pseudomallei* 10276 strain culture supernatant compared with 10276 bsaZ::pDM4 (Table 3.1). In *Shigella*, IpaB is present in the needle tip in complex with IpaD, whereas IpaC is secreted later, only upon host cell contact or activation (Epler *et al.*, 2009). With this in mind, it is possible that the stimuli required for BipC secretion in WT *B. pseudomallei* 10276 is lacking when cultured in LB.

Here, evidence is presented that BsaP functions as a gatekeeper protein for effectors in a manner similar to the homologous T3SS proteins InvE/ SepL/ MxiC/ YopN-TyeA (Kubori and Galán, 2002, Kresse *et al.*, 2000, Deng *et al.*, 2004, 2005, Botteaux *et al.*, 2009, Forsberg *et al.*, 1991, Iriarte *et al.*, 1998). Members of this family of proteins function differently from each other, but are all thought to be important in allowing effectors to be secreted by the mature T3SS. Here, it is shown that deletion of *bsaP* creates a phenotype in which effector proteins are hyper-secreted and levels of translocators decrease. To fully understand BsaP in the context of the other members of the gatekeeper family of proteins, more work would be required to determine the molecular interactions of BsaP with other components of the Bsa T3SS.

This study is another example of the power of a combination of classic targeted mutagenesis and a gel-free quantitative proteomics approach, such as iTRAQ, to study a complex secretion system, providing insights into the involvement of certain proteins on the regulation of the T3SS itself. Two new substrates of the Bsa T3SS were revealed by the study, confirming the effector repertoire is more complex than reported so far.

Note: Much of the data from this chapter has been published in Molecular and Cellular Proteomics in 2015. A copy of the publication is included as an appendix.
Chapter 4

Identification of Effector Protein Interacting Partners

4.1 Introduction

From the putative effector proteins identified during the iTRAQ screen (Chapter 3), three were chosen for further characterisation; BipC, BprD and BapA. BipC is homologous to SipC from *Salmonella* (Stevens *et al.*, 2002), a protein known to have a dual function as a translocator and an effector which can bind and polymerise host cell actin (Hayward and Koronakis, 1999). The molecular function of BipC has not been described thus far and its study may provide important insights as to whether it has a role beyond that of being a translocator protein. BapA and BprD, share no homology with any protein outside of the closely related *Burkholderia* species and contain no known conserved protein domains. This makes BapA and BprD exciting candidates for interesting and novel biology, but also makes determining the function of these proteins difficult, as homology is often used to inform potentially useful functional assays.

One method of discovering the function of an unknown protein is to identify any other proteins it may interact with. There are many methods for investigating protein-protein interactions which can be broadly split into two categories; low-throughput such as X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) for studying single known protein-protein interactions and high-throughput methods such as yeast two-hybrid (Y2H) and antibody or tag based methods for interrogating vast numbers of interactions simultaneously (Reviewed in Williamson and Sutcliffe (2010)). The most prevalent methods used to detect protein-protein interactions are Y2H and affinity purification (Reviewed in Brückner *et al.* (2009)). Each method has dis-
tinct advantages and disadvantages. Important differences between the two systems are that Y2H is \textit{in vivo} and identifies binary (direct) protein-protein interactions while affinity purification is \textit{in vitro} and identifies entire interacting complexes (Reviewed in Brückner \textit{et al.} (2009)). Some other advantages of Y2H are that it is relatively less expensive than affinity purification and does not require access to advanced MS equipment (Reviewed in Brückner \textit{et al.} (2009)).

Y2H was originally described by Fields and Song (1989) as a genetic system using the GAL4 protein in \textit{Saccharomyces cerevisiae} to identify protein-protein interactions. GAL4 is a transcriptional activator required for the activation of genes responsible for galactose metabolism and has separate domains for DNA activation and DNA binding (Reviewed in Traven \textit{et al.} (2006)). The Y2H system exploits modular characteristics of the \textit{S. cerevisiae} GAL4 gene by separating the two domains and fusing them to proteins to be screened for interaction (Fields and Song, 1989). These are expressed in a reporter yeast strain where the specific DNA sequences that the GAL4 DNA binding domain recognises are placed upstream of the \textit{GAL1} promoter sequence fused to the reporter gene \textit{lacZ} (Fields and Song, 1989). If proteins interact, bringing the modular GAL4 DNA activation domain in close proximity to the DNA activation domain, \textit{lacZ} transcription occurs resulting in \(\beta\)-galactosidase production, which can be measured enzymatically (Figure 4.1) (Fields and Song, 1989). Later, greater specificity was added to the system by adding a larger number of reporter genes, notably genes required for \textit{S. cerevisiae} growth on media lacking adenine and histidine and the creation of library systems to screen large numbers of proteins simultaneously (James \textit{et al.}, 1996). While a very effective method for the discovery of protein-protein interactions, Y2H screening has some limitations. The classical Y2H system can only detect interactions that take place inside the nucleus of \textit{S. cerevisiae} and proteins toxic to the yeast can not be studied, hydrophobic membrane proteins function poorly in the screen and post-translational modifications required for interactions may not take place (Reviewed in Koegl and Uetz (2007)). The screen also suffers from false-negative and false-positive results, the latter of which is addressed by confirming interactions detected by Y2H using independent methods (Reviewed in Koegl and Uetz (2007)).

Y2H has been used successfully in the past to identify the interacting partners and characterise T3SS effector proteins in other Gram negative bacteria such as: \textit{E. coli} EspF (Viswanathan \textit{et al.}, 2004), NleA (Lee \textit{et al.}, 2008), NleH
Figure 4.1: The yeast two-hybrid system for identifying protein-protein interactions. In the yeast two-hybrid system, the modular GAL4 DNA activating and binding domains are fused to bait (X) and prey (Y) proteins. (A) The GAL4 DNA binding domain (BD) fused to protein X binds the upstream activating sequence (UAS), but can not activate the reporter gene alone. (B) The GAL4 activating domain (AD) fused to protein Y in the absence of the GAL4 BD can not bind to UAS so the reporter gene is not activated. (C) When proteins X and Y interact, they bring the GAL4 BD and AD into close proximity leading to activation of the reporter gene. Figure taken from Ferro and Trabalzini (2013).
(Hemrajani et al., 2010); Shigella flexneri IpaH4.5 (Wang et al., 2013); and Pseudomonas aeruginosa ExoS (Okuda et al., 2014). In this study, three Bsa T3SS effector proteins, BprD (BPSS1521), BapA (BPSS1528), and BipC (BPSS1531), were selected for a Y2H screen using a universal library of human proteins. From the screen, two human proteins were identified as interacting partners for BapA and 14 human proteins were identified as interacting partners for BipC. Interestingly, no interacting partners were identified for BprD. One protein shown to interact with both BipC and BapA, Complement component 1 Q subcomponent-binding protein (C1QBP), was confirmed using protein pull-downs. This allows informed design for future studies to determine a possible role for both BipC and BapA in B. pseudomallei pathogenesis.

4.2 Results

4.2.1 Investigation of BipC-Protein Interactions Using Pull-Downs and Mass Spectrometry

To explore the possible function of the Bsa T3SS effectors of interest, a decision was made to investigate possible protein-protein interactions with eukaryotic host cell proteins. Under my supervision, an honours student in the lab, Stefanie Küchler, performed pull-downs using cell lysates followed by MS analysis using glutathione S-transferase (GST)-BipC fusion proteins. A GST fusion of another B. pseudomallei effector protein, BopE, was prepared as a control. GST was chosen as an affinity tag because it produces high yields of fusion protein when expressed in E. coli and requires mild elution conditions and cell lysates were used because they allow for a high concentration of host cell protein (Reviewed in Waugh (2005)). BopE has been shown be a guanine nucleotide exchange factor for Cdc42 and Rac1 (Stevens et al., 2003). This provides known targets to assess the quality of the pull-down and MS analysis. The full length proteins were expressed in E. coli Rosetta BL21 (Novagen) using the vector pGEX-4T-1 which adds an in-frame N-terminal GST to the protein of interest. Protein expression was induced in culture using IPTG, bacteria were collected, lysed with bugbuster, and the bacterial cell lysates were incubated for 1 hour at room temperature with GSH linked sepharose beads. The beads were washed and then incubated for 1 hour at room temperature with A549 cell lysates. A549 cells were selected as they are a well studied in vitro model of B. pseudomallei infection (Reviewed in Allwood et al. (2011)). The
beads were washed and proteins were visualised by SDS-PAGE followed by silver staining.

Pull-downs using A549 cells resulted in protein levels that were too low for downstream MS analysis when visualised by silver staining (Figure 4.2). It was hypothesised that premature elution of proteins from the GSH-linked sepharose beads or low protein concentration in the A549 cell lysates may have caused the lack of protein in the final sample. The presence of the GST protein at 23 kDa in the BopE sample was also seen, possibly due to early termination of transcription or translation of the fusion protein. To address this, GST-BopE was produced again using the conditions above and the GST-fusion proteins were eluted from the sepharose beads and covalently linked to the UltraLink Biosupport beads, which prevents loss of the fusion proteins. The GST proteins bound to UltraLink beads were then incubated for 1 hour at room temperature with murine splenic lysates (MSL), which had a higher protein concentration than the A549 cell lysates (MSL = 6.357 mg/ml; A549 = 0.48 mg/ml). The beads were washed and proteins were visualised by SDS-PAGE followed by silver staining or Western blotting with rabbit α-Cdc42 or rabbit α-Rac1 antibodies. Rabbit α-actin antibodies were also used as it was hypothesised that due to its homology (Stevens et al., 2002) to the actin binding protein SipC (Salmonella) (Hayward and Koronakis, 1999), BipC may also bind actin.

The UltraLink beads and MSLs yielded more protein than GSH-linked sepharose beads and A549 cell lysates, with different banding patterns being seen between the GST and GST-BipC lanes by silver staining (Figure 4.3). The GST-BopE lane had less protein than the other two lanes. When immunoblotting with an α-actin antibody was performed, actin could be seen in all lanes including the GST only control lane, though at lower levels than the GST BipC lane (Figure 4.4). As GST should not bind actin (Stevens et al., 2005), this may have been due to the high concentration of protein in the MSL or improper washing. Yet, Cdc42 and Rac1 were not seen in the GST-BopE samples by Western blot (data not shown). To better understand what proteins may be bound to the different fusion proteins, 14 bands were excised from the silverstained gel and submitted to MS analysis using the in-house proteomics facility at the Roslin Institute (Figure 4.3). Proteins appearing in the GST only sample were removed as background proteins and the web-based database CRAPome (http://www.crapome.org/) was used to remove a set of proteins commonly found as false-positives in pull-down experiments.

The resulting list of interacting proteins for GST-BipC contained 27 unique
CHAPTER 4. IDENTIFICATION OF EFFECTOR PROTEIN INTERACTING PARTNERS

Figure 4.2: Pull-downs using A549 cell lysates and GST-fusion proteins bound to GSH-linked sepharose beads visualised by silver staining. GST, GST-BopE and GST-BipC bound to GSH-linked sepharose beads were incubated with A549 cell lysates or PBS for 1 hour. Proteins remaining bound to the beads following washing were analysed by SDS-PAGE followed by silver staining.
Figure 4.3: Pull-downs using murine splenic lysates and GST-fusion proteins bound to UltraLink Biosupport beads visualised by silver staining. GST, GST-BopE and GST-BipC covalently bound to UltraLink Biosupport beads were incubated with murine splenic lysates for 1 hour. Proteins remaining bound to the beads following washing were analysed by SDS-PAGE followed by silver staining.
Figure 4.4: $\alpha$-actin immunoblot of proteins pulled-down from murine splenic lysates by GST-BipC and GST-BopE. GST, GST-BopE and GST-BipC covalently bound to UltraLink Biosupport beads were incubated with murine splenic lysates for 1 hour. Proteins remaining bound to the beads following washing were analysed by SDS-PAGE followed by Western blotting using a rabbit $\alpha$-actin antibody.
identifications which were investigated using the Uniprot database, while there were no proteins identified in the BopE sample (Table 4.1). Neither Cdc42 or Rac1 was present in the GST-BopE sample. One hypothesis is that the interaction of BopE with these proteins is transient or that the pull-down conditions were not appropriate for the interaction. The list of proteins present in the GST-BipC sample included proteins involved in multiple metabolic processes, apoptosis, cell proliferation, endocytosis as well as cytoskeletal components, making it difficult to infer a specific pathway that BipC may be interacting with.
Table 4.1: Proteins identified as possible BipC-interacting partners using pull-downs and mass spectrometry analysis. The table contains the Protein name and the cellular location of identified proteins as found in the Uniprot database.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Location in Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Actin</td>
<td>cytoskeleton</td>
</tr>
<tr>
<td>β-Actin</td>
<td>cytoskeleton</td>
</tr>
<tr>
<td>γ-Actin</td>
<td>cytoskeleton</td>
</tr>
<tr>
<td>Actin-related protein 2</td>
<td>Arp2/3 complex, actin cap, cytoskeleton, cell cortex, cell projection</td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase B</td>
<td>centriolar satellites, microtubule organising centre, cytoskeleton</td>
</tr>
<tr>
<td>Asialoglycoprotein receptor 1</td>
<td>plasma membrane, extracellular</td>
</tr>
<tr>
<td>Formin-like protein 1</td>
<td>cytoplasm, phagocytic vesicles, plasma membrane</td>
</tr>
<tr>
<td>Formimidoyltransferase-cyclodeaminase</td>
<td>golgi apparatus, cytoskeleton</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>cell body, cytoplasm</td>
</tr>
<tr>
<td>Prolow-density lipoprotein receptor-related protein 1</td>
<td>cytoplasm, clathrin coated vesicles, endosome, plasma membrane, lysosomal membrane, neuronal cell body, nucleus, receptor complex</td>
</tr>
<tr>
<td>Acetyl-CoA acetyltransferase</td>
<td>cytoplasm, nucleus, mitochondria</td>
</tr>
<tr>
<td>Alcohol dehydrogenase 1</td>
<td>cytoplasm, plasma membrane</td>
</tr>
<tr>
<td>Alcohol dehydrogenase [NADP(+)]</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>Estradiol 17 beta-dehydrogenase 5</td>
<td>cytoplasm, nucleus</td>
</tr>
<tr>
<td>Delta-aminolevulinic acid dehydratase</td>
<td>cytoplasm, extracellular space</td>
</tr>
<tr>
<td>Cytosolic formyltetrahydrofolate dehydrogenase</td>
<td>cytoplasm, mitochondria</td>
</tr>
<tr>
<td>Argininosuccinate synthase</td>
<td>cell body fibre, cytoplasm, ER, lysosome, mitochondria, nucleus</td>
</tr>
<tr>
<td>Carbonic anhydrase 3</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>L-xylulose reductase</td>
<td>brush border, plasma membrane</td>
</tr>
<tr>
<td>Fructose-1,6-bisphosphatase 1</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>Galactokinase</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>Glycylpeptide N-tetradecanoyltransferase 2</td>
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</tr>
<tr>
<td>Ornithine carbamoyltransferase</td>
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</tr>
<tr>
<td>Selenium-binding protein 2</td>
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</tr>
<tr>
<td>Sepiapterin reductase</td>
<td>cytoplasm, mitochondria, nucleus</td>
</tr>
<tr>
<td>14-3-3 protein theta</td>
<td>cytoplasm</td>
</tr>
</tbody>
</table>
4.2.2 Generation of Strains and Validation of the Yeast Two-Hybrid System

Because of the presence of actin in the negative control lane in Figure 4.4 and the lack of Cdc42 and Rac1 in the BopE control by Western blot as well as a lack of detection of BopE interacting partners by MS, it was decided that direct protein-protein interactions would be explored using a Y2H approach. The Clontech Matchmaker GAL4 Two-Hybrid System 3, which reports protein-protein interactions by activating transcription of the reporter genes ADE2, HIS3, lacZ as well as MEL1 was utilised. Activation of the reporter genes allow growth on media lacking adenine and histidine. Reporter gene activation can also be measured by the production of $\beta$-galactosidase and $\alpha$-galactosidase, facilitating blue/white screening.

The Y2H system uses two plasmids; the bait plasmid pGBK7 which encodes the GAL4 DNA binding domain and the prey plasmid pGADT7 which encodes the GAL4 DNA activating domain. The three effector proteins of interest selected for this analysis were BipC, BapA, and BprD. The full length coding sequences of BipC, BprD, and BapA from B. pseudomallei strain 10276 were cloned into the vector pGBK7, which encodes an in-frame N-terminal fusion of the first 147 amino acids of the GAL4 DNA binding domain and an N-terminal c-Myc tag. The plasmid also contains the gene TRP1, allowing the S. cerevisiae strains to replicate in the absence of tryptophan. The plasmids were confirmed to contain the correct in-frame sequence by sequencing using the T7 Forward and DNA-BD primers (Figures 4.5, 4.6, 4.7, 4.8). Because the sequence of B. pseudomallei 10276 is not available, the amino acid sequences of the proteins were compared to that of B. pseudomallei K96243, and differences in the amino acid sequence were checked against other B. pseudomallei strains to confirm they are natural and not due to mutations introduced during cloning.

The BipC sequence contained one difference at amino acid A63G that was present in other B. pseudomallei strains (Figure 4.5). Due to its length, the BapA sequence was sequenced in four sections which were combined. There are 14 amino acids that are not present in pGBK7-BapA that are present in the B. pseudomallei K96243 reference sequence (Figure 4.6). However, the B. pseudomallei 10276 strain is not sequenced so it is not known whether this amino acid deletion is naturally occurring. To address this issue, the cloned pGBK7-BapA was searched against all sequenced WT strains of B. pseudomallei to identify whether it represented a natural variant of the BapA protein. The same 14 amino acid deletion is present in the sequenced strain MSHR511, indicating
Figure 4.5: Comparison of the amino acid sequence of BipC expressed by pGBK7. The entire coding sequence of the bipC gene (BPSS1531) from B. pseudomallei 10276 was cloned into the Y2H vector pGBK7. The insert was sequenced and Clustal Omega (Sievers et al., 2011) was used to compare the translated pGBK7-bipC DNA sequence with the amino acid sequence of BipC from B. pseudomallei K96243 (YP_111537.1) and B. pseudomallei Pakistan 9 (EEH27955.1).
Figure 4.6: Comparison of the amino acid sequence of BapA expressed by pGBK7. The entire coding sequence of the bapA gene (BPSS1528) from B. pseudomallei 10276 was cloned into the Y2H vector pGBK7. The insert was sequenced and Clustal Omega (Sievers et al., 2011) was used to compare the translated pGBK7-bapA DNA sequence with the amino acid sequence of BapA from B. pseudomallei K96243 (YP111534.1) and B. pseudomallei MSHR511 (AHG37918.1).
Figure 4.7: Comparison of the amino acid sequence of the C-terminus of BapA expressed by pGBK7. Clustal Omega (Sievers et al., 2011) was used to compare the C-terminal 304 amino acids of the translated pGBK7-bapA DNA sequence with the amino acid sequence of BapA from B. pseudomallei strains K96243 (YP_111534.1), MSHR_2451 (KGW34630.1), NTCT_13179 (AGZ32639.1), and 576 (EEC31748.1).
**Figure 4.8: Comparison of the amino acid sequence of BprD expressed by pGBK7.** The entire coding sequence of the \textit{bprD} gene (BPSS1521) from \textit{B. pseudomallei} K96243 was cloned into the Y2H vector pGBK7. The insert was sequenced and Clustal Omega (Sievers et al., 2011) was used to compare the translated pGBK7-\textit{bprD} DNA sequence with the amino acid sequence of BprD from \textit{B. pseudomallei} K96243 (YP_111527.1).

<table>
<thead>
<tr>
<th>pGBK7-\textit{bprD}</th>
<th>MKINGMTAQCAQRHGAIERHAPGKDGRRTAGVADERGRTMKGEIKAIHMDVLKEIRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>K96243</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>MKINGMTAQCAQRHGAIERHAPGKDGRRTAGVADERGRTMKGEIKAIHMDVLKEIRF</td>
</tr>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td>pGBK7-\textit{bprD}</td>
<td>NSAVSTPVLAKRHIAANIEVSEKIDRGFLGFMFAQKVCQVRGLYYDRLALKLD</td>
</tr>
<tr>
<td>K96243</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>NSAVSTPVLAKRHIAANIEVSEKIDRGFLGFMFAQKVCQVRGLYYDRLALKLD</td>
</tr>
<tr>
<td></td>
<td>120</td>
</tr>
<tr>
<td>pGBK7-\textit{bprD}</td>
<td>FVVRHIGDRPEDRKFVLPTRAQRPAF</td>
</tr>
<tr>
<td>K96243</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>FVVRHIGDRPEDRKFVLPTRAQRPAF</td>
</tr>
<tr>
<td></td>
<td>147</td>
</tr>
</tbody>
</table>
it is present in WT \textit{B. pseudomallei}. The BapA protein does appear to show variability in sequence, especially towards the C-terminus, and all differences identified between the cloned C-terminal BapA sequence and the \textit{B. pseudomallei} K96243 reference sequence were also identified as naturally occurring in other \textit{B. pseudomallei} strains (Figure 4.7). Interestingly, one difference identified in the pGBK7-BapA construct, as well as other \textit{B. pseudomallei} strains, is a variation in four amino acid repeats, spanning amino acids 735–770 with reference to the \textit{B. pseudomallei} K96243 BapA protein. The repeats consist of the amino acids V[A/T]S[N/G]. Another difference is in the number of D-A-G repeats spanning amino acids 238–243 with reference to the \textit{B. pseudomallei} K96243 BapA protein. The BprD sequence contained no amino acid differences when compared to the \textit{B. pseudomallei} K96243 reference strain (Figure 4.8).

The sequenced pGBK7-BipC, pGBK7-BprD and pGBK7-BapA plasmids were transformed into \textit{S. cerevisiae} strain AH109. In order to confirm proper expression of the fusion proteins, two colonies from each of the \textit{S. cerevisiae} strains were cultured at 30°C overnight with agitation in SD/-Trp broth. Cells were collected by centrifugation and lysed using Yeast Buster. Cell lysates were visualised by SDS-PAGE followed by Western blotting using a rabbit α-c-Myc antibody to detect the myc-tagged proteins. c-Myc-reactive proteins were detected at the expected size for the effector proteins: GAL4 DNA binding domain fusions in \textit{S. cerevisiae} containing pGBK7-BipC (∼45 kDa) or pGBK7-BapA (∼130 kDa), (Figure 4.9). As the high molecular weight band seen in the BapA samples was not present in the other samples, it was hypothesised that this was indeed due to the production of BapA by the yeast. The BprD expressing yeast strain showed a c-Myc-reactive band of ∼60 kDa, which is four times the expected molecular weight (∼15 kDa). At the present time it is not clear why this occurs, but could be due to the formation of multimers by BprD.

Two of the known issues that can affect Y2H screening are toxicity of the expressed bait protein and auto-activation of the reporter genes by the bait protein in the absence of the GAL4 activation domain. The strains were checked for toxicity by assessing growth rate and colony size when grown at 30°C for 3 days on SD/-Trp plates. When compared to WT \textit{S. cerevisiae}, the strains expressing the proteins of interest did not show any notable growth defect, showing formation of colonies of about 3 mM after 3 days growth, indicating they were suitable for Y2H screening. To test for auto-activation, the \textit{S. cerevisiae} strains expressing the proteins of interest were streaked onto both SD/-Trp
Figure 4.9: Western blot showing expression of BapA, BprD and BipC fusion proteins in *S. cerevisiae* AH109. Two colonies from each of the *S. cerevisiae* AH109 strains containing pGBKT7-BipC, pGBKT7-BprD or pGBKT7-BapA were cultured overnight at 30°C with agitation. The yeast was collected by centrifugation, lysed with Yeast Buster, and of the resulting lysates, equal amounts were visualised by SDS-PAGE and probed by Western blot using a rabbit α-c-Myc antibody. Arrows indicate the c-Myc-reactive fusion proteins.
plates, to select for the pGBK7 plasmid, and SD/-Trp/-Ade/-His, to test for activation of the Ade2 and His3 reporter genes. The plates were incubated at 30°C for 5 days. While all three strains showed growth on the control media (SD/-Trp), none of the strains grew on the SD/-Trp/-Ade/-His plates, indicating the strains were suitable for further testing (Figures 4.10 and 4.11).

In Y2H testing, it is important to test the stringency of the media, as growth of *S. cerevisiae* on selective media in the absence of protein-protein interactions would produce false-positive results. Positive (*S. cerevisiae* AH109 pGBK7-53 pGADT7-T) and negative (*S. cerevisiae* AH109- pGBK7-LAM pGADT7-T) control strains were produced by performing a double transformation using plasmids supplied by the manufacturer. The plasmids in the positive control strain encode the proteins murine p53 and simian vacuolating virus 40 (SV40) large T-antigen which are known to interact in the Y2H system. The plasmids in the negative control strain encode the proteins human lamin C and SV40 large T-antigen which do not interact in the Y2H system. The plasmid pGADT7 contains the gene *LEU2* allowing *S. cerevisiae* strains to grow on media lacking leucine. The strains were plated onto SD/-Trp/-Leu and SD/-Trp/-Leu/-Ade/-His plates to ensure selectivity of the media. In a working Y2H system both the positive and negative strains would grow on the SD/-Trp/-Leu media, but only the positive control strain in which the GAL4 DNA binding and activating domains are brought into close proximity through a bait-prey protein-protein interaction should grow on the SD/-Trp/-Leu/-Ade/-His media. The media proved to be very stringent, as 5 days of growth at 30°C yielded no colonies on the SD/-Trp/-Leu/-Ade/-His plates for the negative control strain, while the positive control formed colonies on all plates (Figure 4.12).

### 4.2.3 Yeast Two-Hybrid Screen of BipC, BprD and BapA

To perform the Y2H screen, the *S. cerevisiae* AH109 strains containing pGBK7-BipC, pGBK7-BprD and pGBK7-BapA were mated with *S. cerevisiae* Y187 containing a universal human cDNA library that had been normalised to reduce high abundance transcripts. The library genes were cloned into the plasmid pGADT7. Quality control data pertaining to the library include: titer: $\pm 5 \times 10^7$ CFU/ml, number of independent clones: $1.84 \times 10^7$, average cDNA size: 1.43 kb, cDNA size range: 0.5–2.9 kb. The total number of cells in each 1 ml aliquot of library was estimated to be between 0.7–2.25x10^9 cells/ml using a cell counter. Overnight cultures of *S. cerevisiae* AH109 strains containing
Figure 4.10: Auto-activation of *S. cerevisiae* containing pGBK7-BapA or pGBK7-BipC. *S. cerevisiae* AH109 containing pGBK7-BapA or pGBK7-BipC were streaked onto SD/-Trp and SD/-Trp/-Ade/-His plates followed by incubation at 30°C for 3 days. Growth on SD/-Trp/-Ade/-His plates indicates auto-activation of the *Ade2* and *His3* reporter genes.
Figure 4.11: Auto-activation of *S. cerevisiae* containing pGBK7-BprD. *S. cerevisiae* AH109 containing pGBK7-BprD was streaked onto SD/-Trp and SD/-Trp/-Ade/-His plates followed by incubation at 30°C for 3 days. Growth on SD/-Trp/-Ade/-His plates indicates auto-activation of the *Ade2* and *His3* reporter genes.
Figure 4.12: Validation of Y2H media stringency. Positive (*S. cerevisiae* AH109 pGBK7-53 pGAD77-T) and negative (*S. cerevisiae* pGBK7-LAM pGAD77-T) control strains were plated onto SD/-Trp/-Leu and SD/-Trp/-Leu/-Ade/-His plates followed by incubation at 30°C for 5 days. Stringent media will allow for growth of the positive control, but not the negative control on SD/-Trp/-Leu/-Ade/-His plates.
pGBK7-BipC, pGBK7-BprD or pGBK7-BapA were mixed with individual aliquots of *S. cerevisiae* Y187 containing the universal human cDNA library according to the manufacturers protocol. Yeast mating was allowed to proceed for 24 hours in 50 ml of 2×YPDA media at 30°C, shaking at 45 RPM, to ensure the cells did not sediment. Before plating onto selective media, the culture was examined using a light microscope at 40X magnification for the formation of zygotes in order to confirm mating had taken place. Figure 4.13 shows representative images showing the lobed appearance of the zygotes of *S. cerevisiae*, indicating that the yeast had undergone mating. The culture was washed and suspended in 10 ml of de-ionised water. From the culture, 100 µl was removed and dilutions were plated onto SD/-Trp/Leu media to determine the number of colonies screened. The remainder of the mated culture was plated onto SD/-Trp/-Leu/-Ade/-His plates to select for activation of the reporter genes. The plates were incubated at 30°C for 10 days. Resulting colonies were re-streaked onto SD/-Trp/-Leu/-Ade/-His plates and incubated a further 3 days at 30°C. The number of independent clones screened for each Y2H experiment were: BipC 42 million, BprD 4.2 million, and BapA 17 million; all above the recommended minimum of 1 million independent clones suggested by the manufacturer for a Y2H experiment using a normalised library. The colonies showing growth on the SD/-Trp/-Leu/-Ade/-His plates were counted. From the *S. cerevisiae* pGBK7-BapA library mating, 11 colonies were obtained (Figure 4.14) and from the *S. cerevisiae* pGBK7-BipC library mating, 212 colonies were obtained (Figure 4.15). The *S. cerevisiae* pGBK7-BprD library mating yielded no colonies, possibly due to a problem with BprD production within the yeast as was seen by Western blot (Figure 4.9).

As yeast has the ability to maintain multiple plasmids conferring the same resistance, colonies were streaked multiple times onto SD/-Trp/-Leu/-Ade/-His plates and grown at 30°C for 3 days to segregate any plasmids. As there were only 11 colonies for BapA, plasmids were extracted from all 11 *S. cerevisiae* colonies and then transformed into *E. coli*, selecting for the library plasmid pGADT7 using Amp. The plasmids were propagated in the bacteria, purified, and sequenced using the primers T7 Forward and DNA-AD. For BipC, instead of purifying the plasmids from the yeast, colony PCR was performed on the 212 yeast colonies using the primers T7 Forward and DNA-AD after which the product was run on an agarose gel to visualize the PCR product size differences (Figure 4.16) (Supplemental figure 7.2). From the colony PCR, 65 colonies (∼30%) were selected for sequencing representing a range of band sizes. PCRs
were performed on the 65 selected colonies and the PCR product was purified and sent for sequencing using the primers T7 Forward and DNA-AD. The sequencing results were searched against the UniProtKB/Swiss-Prot database of human proteins using Blastx, resulting in 10 identifications for BapA and 52 identifications for BipC. Several colonies (BipC: 13, BapA: 1) yielded PCR products or plasmids which returned poor quality sequences, even after repeated attempts to re-isolate DNA from these colonies. Reasons for this are unknown.

The names of the human proteins identified as well as the number of independent identifications made for each of the proteins are listed in Table 4.2. Of the 10 identified proteins from the *S. cerevisiae* pGBKT7-BapA library mating, 2 unique proteins were identified. Five colonies contained a plasmid encoding PRKC apoptosis WT1 regulator protein (PAWR) and five colonies contained a plasmid encoding Complement component 1 Q subcomponent-binding protein (C1QBP). Of the 52 identified proteins from *S. cerevisiae* containing pGBKT7-BipC, a total of 14 unique proteins were identified. The most frequent identifications were C1QBP, Staufen homologue 2 (STAU2), and PAWR (Table 4.2).
Figure 4.13: Microscope images of *S. cerevisiae* AH109 zygotes. Examples of microscope images taken 24 hours after *S. cerevisiae* AH109 cultures containing either pGBK7-BipC or pGBK7-BapA were mixed with *S. cerevisiae* Y187 containing a universal human cDNA library to allow mating to occur. The mixed culture was incubated at 30°C with shaking at 45 RPM after which a 10 µl sample was imaged using a light microscope at 40X magnification. The lobed structure indicates the formation of *S. cerevisiae* zygotes and successful mating of the yeast strains.
Figure 4.14: Positive *S. cerevisiae* colonies resulting from the BapA Y2H screen. After mating of *S. cerevisiae* AH109 containing pGBK7-BapA with the universal human library, the culture was plated onto SD/-Trp/-Leu/-Ade/-His media to select for protein-protein interactions. The 11 resulting colonies were re-streaked onto SD/-Trp/-Leu/-Ade/-His/X-α-Gal plates. The blue colour indicates the activation of the α-galactosidase gene.
CHAPTER 4. IDENTIFICATION OF EFFECTOR PROTEIN INTERACTING PARTNERS

Figure 4.15: Positive *S. cerevisiae* colonies resulting from the BipC Y2H screen. After mating of *S. cerevisiae* AH109 containing pGBK7-BipC with the universal human library, the culture was plated onto SD/-Trp/-Leu/-Ade/-His media to select for protein-protein interactions. The 212 resulting colonies were re-streaked onto SD/-Trp/-Leu/-Ade/-His plates. This image shows the first 42 colonies from the BipC Y2H screen.
Figure 4.16: Example of a PCR screen from *S. cerevisiae* colonies containing pGBK7-BipC after mating. *S. cerevisiae* pGBK7-BipC was mated with *S. cerevisiae* containing a library of human genes cloned into the plasmid pGADT7. PCR was performed to amplify the human DNA insert in the bait plasmid from resulting colonies and shows the range of insert sizes obtained. Pictured is gel #6.
Table 4.2: Human proteins identified as interacting partners for BapA and BipC by Y2H screening. The table contains the Uniprot gene name of each protein identified from a universal human library as an interacting partner with BapA or BipC in a Y2H screen as well as the Entrez gene identifier and the number of independent identifications made for each protein.

<table>
<thead>
<tr>
<th>Name of Interacting Protein (Entrez Identifier)</th>
<th>Number of Identifications With BipC</th>
<th>Number of Identifications With BapA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1QBP (708)</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>PAWR (5074)</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>STAU2 (27067)</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>PNISR (25957)</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>SLC7A13 (157724)</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>TMED7 (51014)</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>SCEL (8796)</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>COMMD1 (150684)</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>DMRTA (63951)</td>
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<td>—</td>
</tr>
<tr>
<td>FAIM1 (55179)</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>CMPK1 (51727)</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>MARCH7 (64844)</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>MMAD (27249)</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>RNF2 (6045)</td>
<td>1</td>
<td>—</td>
</tr>
</tbody>
</table>
4.2.4 Confirmation of Yeast-Two Hybrid Results

The Y2H screens of both BipC and BapA resulted in the identification of C1QBP and PAWR as potential interacting partners. These two proteins were therefore chosen for further confirmation and validation. It is important to address the possibility of the prey proteins causing auto-activation of the reporter genes, independent of interactions with the bait protein. To confirm C1QBP and PAWR did not appear in the screen due to auto-activation of the reporter genes, the pGADT7 plasmids encoding C1QBP and PAWR were transformed into *S. cerevisiae* AH109 with a clean background and plated onto SD/-Leu and SD/-Leu/-Ade/-His plates. The plates were incubated at 30°C for 5 days. *S. cerevisiae* AH109 containing either pGADT7-C1QBP or pGADT7-PAWR formed colonies on SD/-Leu plates, but neither of the strains formed colonies on SD/-Leu/-Ade/-His plates indicating that the prey proteins were not auto-activating the reporters *His* and *Ade* (Figure 4.17). Because it is possible for yeast to contain multiple plasmids with the same selectable marker, pGADT7-PAWR and pGADT7-C1QBP were transformed into a clean background of *S. cerevisiae* AH109 containing either pGBK7-BapA or pGBK7-BipC. Colonies were streaked onto SD/-Trp/-Leu and SD/-Trp/-Leu/-Ade/-His plates and incubated at 30°C for 5 days. The yeast expressing GAL4-DNA BD-BapA or -BipC grew on both SD/-Trp/-Leu and SD/-Trp/-Leu/-Ade/-His plates when GAL4-DNA AD-C1QBP or -PAWR were also expressed, indicating a activation of the reporter genes was not caused by a contaminating plasmid (Figures 4.18, 4.19).
Figure 4.17: Testing the bait proteins PAWR and C1QBP for auto-activation. pGADT7-PAWR or pGADT7-C1QBP were transformed into S. cerevisiae AH109 with a clean background and plated onto SD/-Leu and SD/-Leu/-Ade/-His plates followed by incubation at 30°C for 5 days. Growth on SD/-Leu/-Ade/-His plates indicates auto-activation of the reporter genes.
Figure 4.18: Testing BapA interaction with PAWR and C1QBP in a clean S. cerevisiae AH109 background. pGBK7T7-BapA and pGADT7-PAWR or pGADT7-C1QBP were transformed into S. cerevisiae AH109 with a clean background and streaked onto SD/-Trp/-Leu and SD/-Trp/-Leu/-Ade/-His plates followed by incubation at 37°C for 5 days. This was performed to confirm that interactions between the proteins were not due to other plasmids that may be in the mated strains. S. cerevisiae AH109 containing both pGBK7T7-53 and pGADT7-T was used as a positive control and S. cerevisiae AH109 containing both pGBK7T7-Lam and pGADT7-T was used as a negative control for growth on SD/-Trp/-Leu/-Ade/-His plates.
Figure 4.19: Testing BipC interaction with PAWR and C1QBP in a clean \textit{S. cerevisiae} AH109 background. pGBK7-T7-BipC and pGAD7-T7-PAWR or pGAD7-T7-C1QBP were transformed into \textit{S. cerevisiae} AH109 with a clean background and streaked onto SD/-Trp/-Leu and SD/-Trp/-Leu/-Ade/-His plates followed by incubation at 37°C for 5 days. This was performed to confirm that interactions between the proteins were not due to other plasmids that may be in the mated strains. \textit{S. cerevisiae} AH109 containing both pGBK7-T7-53 and pGAD7-T7-T was used as a positive control and \textit{S. cerevisiae} AH109 containing both pGBK7-Lam and pGAD7-T7-T was used as a negative control for growth on SD/-Trp/-Leu/-Ade/-His plates.
4.2.5 Independent Validation of Protein Interactions

With screens such as Y2H, that potentially generate some false-positives, it is important to validate the results using an independent method (Reviewed in (Brückner et al., 2009)). The method chosen to validate the interactions of BipC and BapA with PAWR and C1QBP, was protein pull-downs. BipC and BapA were expressed in *E. coli* Rosetta BL21 as N-terminal GST-fusion proteins in the plasmid pGEX-4T-1. Protein expression was induced in culture using IPTG, bacteria were collected, lysed with Bugbuster, and the bacterial cell lysates were incubated on ice for 1 hour with GSH-linked sepharose beads. The coated sepharose beads were washed thoroughly with PBS. At the same time HeLa cell lysates were prepared by lysing $10^7$ cells in 1 ml of NP-40 lysis buffer containing protease inhibitors and 2 mM EDTA. The GST proteins bound to the GSH-linked sepharose beads were incubated overnight at 4°C with the HeLa cell lysates, washed thoroughly, and then visualised by SDS-PAGE and Western blot with rabbit α-C1QBP and rabbit α-PAWR antibodies. Figure 4.20 shows the GST-fusion protein input into the pull-down assay analysed by SDS-PAGE and Coomassie staining, demonstrating the presence of full length fusion proteins at the expected sizes (GST: 26 kDa, GST-BipC: 70 kDa, GST-BapA: 150 kDa) and in similar quantities. The Western blots confirmed the interaction of C1QBP with both GST-BipC (Figure 4.21) and GST-BapA (Figure 4.23), but no interaction was detected between PAWR and the two effector proteins (Figures 4.22 and 4.24). Also, it would be advantageous in the future to use GST fused to a scrambled protein as a further control for non-specific binding.
Figure 4.20: Coomassie stained gel of BipC-GST and BapA-GST fusion proteins used in pull-down assays. BipC and BapA were expressed as in-frame N-terminal GST-tagged fusion proteins. The proteins were bound to GSH-linked sepharose beads and were analysed by SDS-PAGE and Coomassie staining.
Figure 4.21: Validation of BipC-C1QBP interaction using protein pull-downs. GST and GST-BipC sepharose beads were incubated with HeLa cell lysates overnight at 4°C. The beads were washed and visualised by SDS-PAGE followed by Western blotting using rabbit α-C1QBP antibodies.
Figure 4.22: Validation of BipC-PAWR interaction using protein pull-downs. GST and GST-BipC sepharose beads were incubated with HeLa cell lysates overnight at 4°C. The beads were washed and visualised by SDS-PAGE followed by Western blotting using rabbit α-PAWR antibodies.
Figure 4.23: Validation of BapA-C1QBP interaction using protein pull-downs. GST and GST-BapA sepharose beads were incubated with HeLa cell lysates overnight at 4°C. The beads were washed and visualised by SDS-PAGE followed by Western blotting using rabbit α-C1QBP antibodies.
Figure 4.24: Validation of BapA-PAWR interaction using protein pull-downs. GST and GST-BapA sepharose beads were incubated with HeLa cell lysates overnight at 4°C. The beads were washed and visualised by SDS-PAGE followed by Western blotting using rabbit α-PAWR antibodies.
4.3 Discussion

In the previous chapter (Chapter 3), the effector repertoire of the *B. pseudomallei* Bsa T3SS was described, including the identification of two novel effector proteins, BprD and BapA. Yet, the functions of many of the effector proteins in the T3SS locus are poorly understood. One method commonly used to inform the investigation of protein function is to identify protein-protein interactions. Initial work was performed using GST-fusion protein pull-downs followed by MS analysis. GST-BipC bound to GSH-linked sepharose beads were incubated with murine splenic lysates. When visualised by Western blotting using a rabbit α-actin antibody, actin was present in a GST-alone sample. This may be due to the high concentrations of protein in the murine splenic lysates. It is also known that buffer conditions can have a large impact on the outcome of pull-down experiments (Vikis and Guan, 2004). Bands were excised and analysed by MS analysis yielding a large numbers of proteins that were members of broad cellular pathways. A feature of pull-downs is the ability to identify protein complexes, which explains the large number of proteins identified by MS. Yet, for the purpose of identifying protein function, knowing which proteins BipC directly interacts with may be more helpful in forming a hypothesis. For this reason, it was decided a Y2H system would be used to detect direct protein-protein interactions. BprD (BPSS1521), BapA (BPSS1528), and BipC (BPSS1531) were analysed in a Y2H screen against a universal human library. The system resulted in the identification of 2 human proteins which interact with BapA, and 14 human proteins which interact with BipC. Interestingly, the two human proteins C1QBP and PAWR interacted with both BapA and BipC.

One of the important considerations in Y2H screening is missing true interactions (false-negatives), and incorrectly identifying interactions (false-positives). False-negatives may be caused by a number of factors including a lack of post-translational modifications, steric hindrance caused by fusion of the GAL4 domains, or missing co-factors in the yeast nuclear environment (Reviewed in (Brückner et al., 2009)). Another class of interactions that are often not identified are membrane proteins (Reviewed in Brückner et al. (2009)). This may be hinder detection of protein interactions with T3SS effectors, as the system is often involved in bacterial entry through the host cell membrane (Stevens et al., 2003), as well as escape from the endosome (Stevens et al., 2002). Both of these processes are likely to involve membrane proteins. False-positives are arguably more important than false-negatives in this setting due to the potential for misinforming future research efforts. In an investigation of
false-positives in the Y2H system, Serebriiskii et al. (2000) state that with appropriate bait proteins, the Y2H system reports around 70% true interactions. Yet, this still means at least some of the identified proteins are likely to be false-positives. Classes of commonly found false-positives include heat-shock proteins, ribosomal proteins and mitochondrial proteins (Serebriiskii et al., 2000). A list of common protein types that yield false-positives in Y2H screens can be found at http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html. Because of the possibility of false-positives, the interactions identified by Y2H screens should always be validated by independent methods such as co-immunoprecipitation or pull-downs (Reviewed in (Brückner et al., 2009)).

Though Y2H has not been performed on *B. pseudomallei* Bsa T3SS effector proteins, a previous study has screened T3SS effector protein interactions in the closely related species *B. mallei*. Memišević et al. (2013) screened a list of *B. mallei* ATCC 23344 proteins predicted to be involved in pathogenesis against whole human and whole murine libraries in a Y2H system. Among the proteins they screened were the homologues of the *B. pseudomallei* K96243 proteins BopA, BopE, BipB, BipC and BipD (Memišević et al., 2013). BapA and BprD were not tested in the *B. mallei* screen. The only interaction identified when *B. mallei* BipC was screened against the human library was C1QBP, agreeing with this study and lending confidence to the identification, although this does not eliminate the need to independently validate this data. Protein pull-downs performed with GST-BipC incubated with HeLa cell lysates successfully identified C1QBP as a binding partner of BipC. BapA was also confirmed to bind C1QBP by protein pull-down.

The possible importance of the interaction between BipC/BapA and C1QBP is difficult to predict. C1QBP (also known as gC1q-R, HABP1, p32, and p33) is a protein involved in inflammation, immunity and autophagy (Reviewed in Ghebrehiwet and Peerschke (2004)). There are conflicting reports as to the sub-cellular localisation of C1QBP, with it being reported as primarily mitochondrial (MengJie et al., 2013), but also being reported as being localised at the cell surface (Peerschke et al., 2003). C1QBP is a receptor for the first component of complement C1q involved in the Bradykinin proinflammatory pathway (Ghebrehiwet et al., 2014). C1QBP is also required for the morphology and function of mitochondria and the endoplasmic reticulum (MengJie et al., 2013) and has also been shown to be involved in TLR4-induced production of IL-12 through the PI 3-kinase pathway (Waggoner et al., 2005). C1QBP has been reported as an important target for viruses and bacteria in the human
cell. In HIV-1 the viral protein gp41 has been shown to interact with C1QBP, causing production of reactive oxygen species and expression of the natural cytotoxicity receptor NKp44L on the surface of infected CD4+ T cells, targeting them for lysis by NK cells (Fausther-Bovendo et al., 2010). The *Coxiella burnetii* type IV secretion system effector protein AnkG has been shown to interact with C1QBP (Lührmann et al., 2010). When injected into host cells by *Legionella pneumophila*, AnkG prevents cellular apoptosis allowing intracellular replication of the *L. pneumophila* (Lührmann et al., 2010). The *Listeria monocytogenes* protein InlB interacts with C1QBP on the cell surface, causing the recruitment and activation of PI 3-kinase and mediating bacterial entry into the cell (Braun et al., 2000). Hepatitis C virus core proteins bind to C1QBP, inhibiting T cell proliferation through cell cycle arrest (Yao et al., 2003) as well as inhibiting gamma interferon production (Yao et al., 2005). C1QBP has also shown to be important in gamma herpes virus infections for down-regulation of host immunity (Liang et al., 2004) and nuclear egress (Wang et al., 2014). C1QBP has also been shown to inhibit RIG-I and MDA-5 antiviral signalling through MAVS (Xu et al., 2009). Such a precedent for pathogens interacting with C1QBP to exploit host cell function is encouraging. It is possible that by interacting with C1QBP, BipC and BapA may interfere with the cells ability to recognise intracellular double stranded DNA through MAVS increasing bacterial survival. This could be tested using C1QBP knockout cell lines along with bacterial BipC and BapA to investigate the importance of this interaction in *in vitro* infection. Ideally, it would also be useful to express fragments of both C1QBP and BipC/BapA to map what portions of the proteins are involved in the interaction.

The other protein identified as an interacting partner for both BipC and BapA by the Y2H screen was PAWR. PAWR (also known as par-4) is a proapoptotic protein which traffics Fas/FasL to the cellular membrane and has also been shown to inhibit the transcription of NF-κB (Reviewed in Hebbar et al. (2012)). Unlike C1QBP, independent validation of the interaction of PAWR with BipC and BapA did not occur, possibly due to inappropriate pull-down conditions, the interaction being transient, or a false-positive interaction. This highlights the need for independent validation, and while it does not rule out BipC/BapA and PAWR interacting, more work would need to be done to explore this hypothesis. It is possible that under different, more physiological conditions, such as inside cellular cytoplasm BipC/BapA and PAWR may interact.
A number of other putative BipC interacting partners were identified by Y2H that were not taken forward for validation. When considering proteins with 3 or more independent identifications, the most interesting of these hits is Transmembrane Emp24 Protein Transport Domain Containing 7 (TMED7). TMED7 is thought to be involved in trafficking of TLR4, though two recent reports are conflicting as to whether TMED7 inhibits or enhances the activity of TLR4 (Doyle et al., 2012, Liaunardy-Jopeace et al., 2014). Another of the proteins identified, Staufen Double-Stranded RNA Binding Protein 2 (STAU2) is a double stranded RNA binding protein involved in Staufen-mediated mRNA decay (Park and Maquat, 2013). PNN-interacting serine/arginine-rich protein (PNISR or SFRS18) is a poorly characterised mRNA splicing factor that has been identified in another Y2H screen as an interacting partner of a Vesicular Stomatitis Virus protein, but this interaction was not validated (Moerdyk-Schauwecker et al., 2011). Solute carrier family 7 member 13 (SLC7A13 or S7A13) is an amino acid transporter only characterised by its homology to other transporters of the same family (Blondeau, 2002).

This study provides the first evidence that the Bsa T3SS effector proteins BapA and BipC interact with human proteins. One protein shown to interact with BipC and BapA, C1QBP, was confirmed using protein pull-downs. Due to C1QBP’s role in innate immunity as well as the numerous examples of other pathogens exploiting the protein for their benefit, the effect of the interaction between BipC/BapA and C1QBP on the host cell is an interesting area for further investigation.
Chapter 5

Functional Characterisation of the Bsa T3SS Translocon Protein BipC

5.1 Introduction

*Burkholderia pseudomallei* is a facultative intracellular pathogen capable of invasion of both phagocytic and non-phagocytic cells (Reviewed in Wiersinga et al. (2006)). The mechanisms behind *B. pseudomallei* cellular invasion are not well characterised. One common theme in bacterial entry into host cells is the exploitation of host cell actin to assist invasion (Reviewed in Pizarro-Cerda and Cossart (2006)). This can be accomplished by different mechanisms such as direct manipulation of the host cell protein actin or transmission of a signal to host cell machinery disrupting the actin cytoskeleton (Reviewed in Pizarro-Cerda and Cossart (2006)). Within important pathogenic Gram negative bacteria, such as *Yersinia, Salmonella* and *Shigella*, the Type 3 secretion system (T3SS) delivers bacterial effector proteins which subvert host actin (Reviewed in Büttner (2012)). The T3SS functions similarly to a molecular syringe, allowing delivery of proteins from the bacterial cytosol directly into the cytosol of a target host cell (Reviewed in Wagner et al. (2010)). Figure 5.1 shows two major intracellular pathogens, *Shigella* and *Salmonella*, and the use of their T3SSs to subvert host cell actin and induce uptake (Reviewed in Pizarro-Cerda and Cossart (2006)). *Salmonella* injects SipC and SipA causing actin polymerisation and bundling, while SopE and SopB activate Rho GTPases causing further host cell actin polymerisation leading to membrane ruffling and bacterial uptake. SptP has an inverse effect, inactivating the Rho GTPases to restore the cellular surface after *Salmonella* entry. *Shigella* injects IpaC which polymerises actin, VirA which activates Rho GTPases, and IpgD which disrupts the actin
host cytoskeleton inducing membrane ruffle formation. Similarly to SptP, IpaA causes actin depolymerisation leading to recovery of the actin cytoskeleton at the site of bacterial entry.

The *B. pseudomallei* Bsa T3SS is homologous to the Inv-Spa (*Salmonella* pathogenicity island (SPI)-1) and Mxi-Spa T3SSs of *Salmonella* spp. and *Shigella flexneri*, respectively (Stevens et al., 2002) and has also been shown to be involved in bacterial invasion of non-phagocytic host cells by injecting the effector protein BopE, a guanine nucleotide exchange factor homologous to SopE (*Salmonella*) (Stevens et al., 2003). Yet, compared to *Salmonella* and *Shigella*, the invasion process of *B. pseudomallei* is poorly characterised. The *B. pseudomallei* effector protein BopE, activates the Rho GTPases Cdc42 and Rac1 inducing host actin cytoskeleton rearrangements (Stevens et al., 2003). The less characterised proteins BopC and BipC are also required for full invasion efficiency (Muangman et al., 2011, Kang et al., 2015). Of particular interest is BipC, a homologue of the translocators SipC from *Salmonella* and IpaC from *Shigella* (Stevens et al., 2002). T3SS translocator proteins are responsible for forming a pore in the eukaryotic cell membrane allowing the injection of effector proteins into the cytoplasm (Reviewed in Diepold and Wagner (2014)). Though SipC and IpaC are both bacterial translocators, they also have effector functions with the ability to directly bind and polymerise host cell actin (Hayward and Koronakis, 1999, Terry et al., 2008).

SipC was first shown to directly interact with actin by Hayward and Koronakis (1999). It was demonstrated that SipC has the ability to polymerise actin as well as bundle F-actin in the absence of host cell proteins (Hayward and Koronakis, 1999, McGhie et al., 2001). The ability of SipC to polymerise and bundle F-actin, as well as form the translocation pore, are all dependant on the C-terminal 209 amino acids of the 409 amino acid protein (Hayward and Koronakis, 1999, Chang et al., 2005, Myeni and Zhou, 2010). Yet, the ability to polymerise actin is distinct from the translocation function and important for invasion. *Salmonella* containing a mutated form of *sipC* that is unable to bundle actin, but still able to act as a translocon, was less invasive than WT *Salmonella* (Myeni and Zhou, 2010). In order to efficiently polymerise actin SipC forms either dimers or multimers (Chang et al., 2007), and another actin binding protein, SipA, stabilises actin filaments enhancing the polymerisation function of SipC (McGhie et al., 2001). SipC also interacts with host Syntaxin 6 and thereby recruit LAMP1 to the Salmonella-containing vacuole (SCV), helping to stabilise its membrane (Madan et al., 2012). It is clear that SipC is a multi-functional pro-
Figure 5.1: Entry of host cells by *Salmonella* and *Shigella* via T3SS induced actin rearrangement. Upon host cell contact, effector proteins are injected into the host cell by the Inv-Spa (*Salmonella* spp.) and Mxi-Spa T3SS (*Shigella flexneri*) where they alter host actin networks to induce uptake. (A) *Salmonella* injects SipC and SipA causing actin polymerisation and bundling, while SopE and SopB activate Rho GTPases causing further host cell actin polymerisation leading to membrane ruffling and bacterial uptake. SptP has an inverse effect, inactivating the Rho GTPases helping to restore the cellular surface after *Salmonella* entry. (B) *Shigella* injects IpaC which polymerises actin, VirA which activates Rho GTPases, and IpgD which disrupts the actin host cytoskeleton inducing membrane ruffle formation. Similar to SptP, IpaA causes actin depolymerisation leading to recovery of the actin cytoskeleton at the site of bacterial entry. Figure adapted from Pizarro-Cerda and Cossart (2006).
tein leading to the hypothesis that BipC may also have roles beyond that of a translocator protein.

Another homologue of BipC is IpaC from *Shigella* (Osiecki *et al.*, 2001). IpaC also causes actin polymerisation when expressed or microinjected into cells (Van Nhieu *et al.*, 1999) and the C-terminus of the protein nucleates actin (Terry *et al.*, 2008). IpaC can recruit Src kinase which causes actin polymerisation followed by membrane ruffling which is thought to facilitate bacterial entry (Mounier *et al.*, 2009). While IpaC and SipC are similar, they also have their differences. Interestingly, when expressed on a plasmid, SipC and IpaC are able to complement a *Salmonella sipC* mutant, but only IpaC can complement a *Shigella ipaC* mutant (Osiecki *et al.*, 2001). This indicates that the proteins have some differences in their biochemical function. The authors suggest the different functions may be due to the different intracellular lifestyles of the pathogens, with *Salmonella* residing in the vacuole and *Shigella* escaping into the cytosol (Osiecki *et al.*, 2001). It is therefore possible BipC may also have distinct functions compared to the other homologues.

Research focused on BipC has been limited. A *B. pseudomallei* K96243 *bipC* insertion mutant showed reduced cell adhesion and invasion of A549 cells when compared to the WT strain (Kang *et al.*, 2015). This BipC mutant also showed a delay in escape from the phagosome leading to a delay in formation of actin tails and intracellular growth (Kang *et al.*, 2015). The mutant was also attenuated in BALB/c mice infected intraperitoneally when compared to WT (Kang *et al.*, 2015). While this shows a clear role of the importance of BipC in virulence both *in vivo* and *in vitro*, it does not separate BipC’s role as a translocator necessary for a functional T3SS from roles it may have as an effector protein. Particularly, there has been no evidence that BipC is capable of polymerising actin.

In this study, the first evidence of an interaction between BipC and actin is reported. It is shown that this interaction does not require the presence of other host cell proteins and that BipC has the ability to polymerise actin *in vitro*. BipC preferentially binds F-actin, but does not stabilize F-actin under depolymerising conditions. HeLa cells expressing N-terminal Myc-tagged BipC show disruption of the actin cytoskeleton and pseudopodia formation suggesting involvement in actin dynamics *in vivo*. A surrogate host system in EPEC was established for the downstream study of BipC translocation into cells. This study furthers the understanding of the role BipC may play beyond its role as a translocator.
5.2 Results

5.2.1 BipC Directly Binds Actin

Due the similarity of BipC to SipC (*Salmonella*, 58% coverage, 24% identity) and IpaC (*Shigella*, 58% coverage, 22% identity), we hypothesised that BipC would be an actin-binding protein and may possess the ability to bundle F-actin. Figure 5.2 shows an amino acid alignment of BipC with SipC and IpaC. To investigate the ability of BipC to bind actin, a pull-down experiment was performed using N-terminal GST fusion proteins and murine splenic lysates under actin-polymerising conditions. BipC was expressed in *E. coli* Rosetta BL21 as an N-terminal GST-fusion protein using the plasmid pGEX-4T-1-BipC. GST-SipC *Salmonella* and GST-BimA, the known *B. pseudomallei* actin-binding and polymerising protein (Stevens et al., 2005), were used as positive controls. As a negative control GST alone was prepared. Protein expression was induced in culture using IPTG, bacteria were collected, lysed with bugbuster, and the bacterial cell lysates were incubated at room temperature for 1 hour with GSH-linked sepharose beads. The GST-fusion proteins bound to the sepharose beads were analysed by SDS-PAGE and visualised by Coomassie staining demonstrating that each protein was expressed at the expected molecular weight and there was relatively similar amounts of protein present in all pull-down samples (Figure 5.3). The coated sepharose beads were washed thoroughly with PBS and incubated for 30 minutes at room temperature with murine splenic lysates (MSL) suspended in polymerisation buffer supplemented with 100 µM CaCl₂. The beads were washed thoroughly with PBS and then visualised by SDS-PAGE and by Western blot with a rabbit α-actin antibody. When probed with the α-actin antibody, actin is present in all three test lanes of GST-BimA, GST-SipC, and GST-BipC (Figure 5.3). The negative control lacks actin, indicating the interaction is not due to the N-terminal GST fusion or due to non-specific binding of actin to the sepharose beads (Figure 5.3).

Because pull-downs may identify interactions that are indirect through large protein complexes (Reviewed in Brückner et al. (2009)), it must be determined if BipC has the ability to bind actin directly, or if the binding is indirect and requires other proteins for the binding of the actin molecules. To test this, GSH-linked sepharose beads were coated with GST, GST-BimA, GST-SipC, or GST-BipC. Polymerisation buffer containing 1 µM rhodamine-labelled actin was added to the beads on a microscope slide. The beads were immediately
### Figure 5.2: Amino acid alignment of BipC (B. pseudomallei) with SipC (Salmonella) and IpaC (Shigella).

The alignment was performed using Clustal Omega (Sievers et al., 2011). In the alignment, a (*) indicates conserved residues, a (:) indicates highly similar amino acids, and a (.) indicates weakly similar amino acids. Accession numbers for the proteins are; BipC (YP_111537.1), SipC (EHY68440.1) and IpaC (EGK31204.1)
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Figure 5.3: Pull-down assay using GST-BipC and murine splenic lysates. GST, GST-BimA, GST-SipC and GST-BimA bound to GSH-linked sepharose beads were incubated with murine splenic lysates (MSL) in polymerisation buffer for 30 minutes at room temperature. The beads were washed and analysed by SDS-PAGE. **A.** The Coomassie stained gel shows the proteins input into the pull-down assay demonstrating each protein was present in relatively similar amounts. **B.** The α-actin Western blot indicates actin binding to the fusion proteins.
visualised using a confocal microscope. If a protein is able to bind actin, it creates a high concentration of the rhodamine-labelled actin at the surface of the sepharose bead, which appears as a red ‘halo’ around the clear bead in microscope images. The results show a red halo around both the GST-BimA and GST-BipC coated beads (Figure 5.4). The beads coated with GST alone show no ring, while surprisingly the GST-SipC beads show only a faint red halo (Figure 5.4). This may be due to a relatively low concentration of GST-SipC on the beads. The results demonstrate that BipC is able to bind actin directly, in the absence of other proteins. This is the first evidence that BipC is an actin-binding protein.

With evidence that BipC has the ability to bind actin directly, it was surprising that actin was not identified as an interacting partner for BipC in the Y2H screen (Chapter 4). Because this could have been missed in the screen as a false-negative result, this interaction was tested independently in a clean S. cerevisiae AH109 background. pGBK77-BipC and pGADT7 containing the coding sequence of β actin (pGADT7-Actin) were transformed into S. cerevisiae AH109 and cultured on SD/-Trp/-Leu and SD/-Trp/-Leu/-Ade/-His plates for 5 days at 30°C to test for activation of the reporter genes. S. cerevisiae containing pGBK77-BipC and pGADT7-Actin did not form colonies on the SD/-Trp/-Leu/-Ade/-His media, but did form colonies on the control media containing SD/-Trp/-Leu, confirming that BipC and Actin do not interact in the Y2H system (Figure 5.5). This may be due to the yeast nucleus not supporting actin polymerising conditions which may be required for interactions with BipC.

It was hypothesised that one reason actin was not identified in the Y2H screen may be due to BipC binding filamentous actin (F-actin) preferentially. To investigate this, an actin co-sedimentation assay was performed, similar to that of Stevens et al. (2005). Purified GST, GST-BipC, GST-SipC, and GST-BimA (1 µM) were incubated with 10 µg of pre-polymerised actin in G-Mg buffer for 30 minutes at room temperature. The mixtures were then separated by ultra-centrifugation, which results in the co-sedimentation of F-actin and any protein bound to the F-actin. Monomeric G-actin and proteins bound to G-actin, or proteins that are unbound, remain in the supernatant. The supernatants and pellets were then analysed by SDS-PAGE and visualised by silver staining. The GST protein is only present in the supernatant since it does not bind F-actin (Figure 5.6). GST-BimA is present in both the pellet and supernatant, but in higher amounts in the supernatants indicating it preferentially
Figure 5.4: Direct binding of rhodamine-labelled actin by GST-BipC. Sepharose beads coated with either GST, GST-BimA, GST-SipC or GST-BipC were mixed with rhodamine-labelled actin and immediately imaged using a confocal microscope. The formation of a red “halo” around the bead indicates binding and a high concentration of the labelled actin at the bead surface. Scale bar = 50 μm.
Figure 5.5: Testing BipC interaction with Actin in a clean *S. cerevisiae* AH109 background. pGBK7-BipC and pGADT7-Actin were transformed into *S. cerevisiae* AH109 with a clean background and streaked onto SD/-Trp/-Leu and SD/-Trp/-Leu/-Ade/-His plates followed by incubation at 37°C for 5 days. *S. cerevisiae* AH109 containing pGBK7-53 and pGADT7-T was used as a positive control and *S. cerevisiae* AH109 containing pGBK7-Lam and pGADT7-T was used as a negative control for growth on SD/-Trp/-Leu/-Ade/-His plates.
binds G-actin (Figure 5.6), as previously demonstrated (Stevens et al., 2005). GST-SipC shows larger amounts of protein present in the pellet demonstrating its preferential binding to F-actin in agreement with Hayward and Koronakis (1999) (Figure 5.6). Interestingly, GST-BipC is almost exclusively found in the pellet indicating that it preferentially binds F-actin under the conditions used in the sedimentation assay (Figure 5.6).

To further test the hypothesis that BipC preferentially binds to actin polymers, a pull-down assay was performed, but instead of polymerisation buffer, NP-40 lysis buffer was used. GST-BipC bound to GSH-linked sepharose beads was incubated with HeLa cell lysates prepared in NP-40 lysis buffer containing protease inhibitors and EDTA. The sepharose beads were incubated overnight at 4°C with the HeLa cell lysates, washed thoroughly, and then visualised by SDS-PAGE and Western blot with rabbit α-actin antibodies. A band at the size corresponding to actin (about 43 kDa) was present in the HeLa cell lysate control, but not in the GST or GST-BipC samples (Figure 5.7). This is in contrast to the earlier pull-down in which polymerisation buffer was used (Figure 5.3). This suggests that BipC may only interact with actin under conditions in which actin polymerisation is feasible.

5.2.2 BipC Polymerises Actin and Co-localises With the Actin Cytoskeleton in HeLa Cells

SipC has the ability to bind and bundle F-actin, but also to polymerise actin (Hayward and Koronakis, 1999). To determine if BipC is also able to polymerise actin, a polymerisation assay was performed with pyrene-labelled actin which emits fluorescence as it polymerises (Stevens et al., 2005). An increase in the fluorescence intensity indicates an increase in actin polymerisation. GST, GST-BimA, GST-SipC, and GST-BipC (1 µM) were incubated with 2.3 µM of pyrene actin in polymerisation buffer and compared in their ability to polymerise actin over a 1 hour period. SipC showed a statistically significant increased rate of polymerisation (average rate of increase in fluorescence intensity = 14.656±5.01 U/s) when compared to the GST control (average rate of increase in fluorescence intensity = 7.453±3.65 U/s) which mimics the rate of intrinsic actin polymerisation in the absence of a polymerising factor (Figure 5.8). BipC also showed a statistically significant increase in the rate of actin polymerisation (average rate of increase in fluorescence intensity = 9.876±3.87 U/s) when compared to the GST control indicating an intrinsic actin poly-
Figure 5.6: Co-sedimentation assay of GST-BipC with actin. Mg-actin was allowed to polymerise at room temperature for 2 hours before being mixed with GST, GST-BimA, GST-SipC or GST-BipC followed by a 30 minute incubation. The mixtures were submitted to a high speed centrifugation at 100,000 RCF for 1 hour to separate the monomeric actin (Supernatant) and the filamentous actin (pellet). The supernatant and pellet were carefully separated and suspended and denatured in Laemelli buffer. The samples were separated by SDS-PAGE and visualised using silver staining.
Figure 5.7: Investigation of BipC-Actin interaction using protein pull-downs. GST and GST-BipC sepharose beads were incubated with HeLa cell lysates overnight at 4°C. The beads were washed and visualised by SDS-PAGE followed by Western blotting using rabbit α-Actin antibodies.
merising activity (Figure 5.8).

In *Salmonella*, the ability of SipC to polymerise and bundle F-actin is enhanced by another T3SS effector protein SipA (McGhie et al., 2001). Due to their F-actin bundling ability, SipC and SipA inhibit the depolymerisation of F-actin. However, there is no homologue of SipA in *B. pseudomallei*. To investigate whether BipC may have the ability to stabilise F-actin on its own, a depolymerisation assay was performed. The inverse of the polymerisation assay, the depolymerisation assay measures a reduction in fluorescence indicating conversion of actin filaments (F-actin) to actin monomers (G-actin) over time. GST, GST-BimA, GST-SipC, or GST-BipC (1 \( \mu \)M) were incubated with 2.3 \( \mu \)M of pre-polymerised pyrene-actin for 1 hour in G-Mg buffer (depolymerising conditions). Both GST and BipC showed similar levels of depolymerisation to that of the actin only control and did not appear to stabilise F-actin (Figure 5.9). F-actin incubated with SipC was stabilised and showed slower rates of depolymerisation. Surprisingly, actin filaments incubated with GST-BimA underwent rapid depolymerisation.

With the establishment of BipC’s ability to bind and polymerise actin *in vitro*, it is important to investigate whether BipC retains this ability *in vivo*. The full length BipC gene into the eukaryotic expression vector pRK5-Myc, which adds an in-frame N-terminal Myc tag to a protein of interest. HeLa cells were transfected with pRK5-Myc-BipC, or pEGFP as a control, using lipofectamine 2000. After 48 hours the HeLa cells were fixed in paraformaldehyde and probed using phalloidin to label F-actin and rabbit \( \alpha \)-Myc antibodies to label BipC. Cells were imaged using a confocal microscope. Figure 5.10 is a representative image where the actin cytoskeleton (red) of the cells transfected with pRK5-Myc-BipC (green) appears irregular when compared to untransfected HeLa cells and HeLa cells transfected with pEGFP. BipC-expressing cells clearly demonstrate an altered morphology and evidence of perturbation of actin dynamics, especially at the plasma membrane. The Myc tagged BipC appeared to localise to the cellular membrane at membrane protrusions.

5.2.3 BipC is Secreted Independently of the Bsa T3SS

While BipC has been assumed to be secreted by the Bsa T3SS due to its position within the *bsa* T3SS locus and its close homology to SipC from *Salmonella*, this has not been formally shown. To demonstrate BipC is secreted in a manner dependant on the Bsa T3SS, the *bipC* gene was cloned into the inducible
Figure 5.8: Polymerisation of pyrene-actin by *B. pseudomallei* GST-BipC. GST, GST-SipC and GST-BipC were mixed with monomeric pyrene-actin in actin polymerisation buffer. The polymerisation assay was monitored every 30 seconds for 40 minutes using a plate reader with an excitation wavelength of 365 nm and an emission wavelength of 407 nm. Both GST-SipC and GST-BipC show statistically faster rates of increase in fluorescence intensity than the GST control. The graph represents the average of 3 independent replicates each containing 3 technical replicates.
Figure 5.9: F-actin depolymerisation in the presence of GST-BipC. GST, GST-BipC, GST-SipC and GST-BimA were added to pyrene labelled F-actin. Fluorescence (excitation wavelength 365 nm; emission wavelength 407 nm) was measured for 40 minutes in 30 second intervals. The graph represents the average of 3 independent replicates each containing 3 technical replicates.
Figure 5.10: Confocal microscope images of HeLa cells eptopically expressing Myc tagged BipC. HeLa cells were transfected with pRK5-Myc-BipC using lipofectamine 2000. The HeLa cells were fixed in paraformaldehyde 48 hours post-transfection, probed using phalloidin and rabbit α-Myc antibodies, then imaged using a confocal microscope. Untransfected HeLa cells or HeLa cells transfected with pEGFP were also imaged. In the representative images, the actin cytoskeleton stained with (phalloidin) appears red and Myc-BipC or pEGFP appear green.
expression vector pME6032 (Heeb et al., 2002) and expressed as an in-frame C-terminal c-Myc tagged fusion protein. pME6032-bipC was transformed into B. pseudomallei 10276 WT, 10276bsaZ::pDM4, 10276bipD::pDM4 and 10276ΔbsaP. The strains were cultured overnight shaking at 37°C in LB. The following day the overnight cultured was centrifuged and the supernatant was replaced with fresh LB containing 0.5 mM IPTG to an OD₆₀₀ of 1.0. Of the adjusted culture 1 ml was added to 10 ml of LB containing 0.5 mM IPTG which was then incubated for 6 hours shaking at 37°C. WT B. pseudomallei grown in the absence of IPTG was used as a control. Bacterial counts were assessed before and after the 6 hour incubation period, with all strains showing similar bacterial numbers. The bacterial supernatants were collected and the proteins were precipitated using PRMM. Equal volumes of protein were analysed by SDS-PAGE and assessed by Western blot for the presence of the myc-tagged BipC. c-Myc tagged BipC (molecular weight approximately 45 kDa) was detected in all of the bacterial cell lysates that had been induced with 0.5 mM IPTG (Figure 5.11). There was also a small non-specific band detected in the lysates of all strains, but it did not interfere with detection of myc-tagged BipC. Surprisingly, BipC-c-Myc was also present in equivalent levels in all supernatant samples induced by 0.5 mM IPTG, including the 10276bsaZ::pDM4 strain which is unable to secrete the known Bsa T3SS effector, BopE.

It was hypothesised that the Bsa T3SS-independent secretion of the tagged BipC proteins may be due to the presence of multiple T3SSs as well as a flagellar system in B. pseudomallei (Reviewed in Wiersinga et al. (2006)). It was also of interest to formally show that BipC is translocated into host cells. As B. pseudomallei is an intracellular pathogen, it is difficult to separate protein that may be translocated by the T3SS from protein that may be exported by other means once the bacterium is in the cytosol. Two possible solutions to this problem are to prevent the B. pseudomallei from invading the cell, or to use a surrogate host containing a functional T3SS that remains extracellular. Enteropathogenic E. coli (EPEC) is a major cause of foodborne illness which, upon making contact with host cells, forms a pedestal on the surface of the host cell using its T3SS where it can then continue to inject a series of T3S effector proteins (Reviewed in Wong et al. (2011)). EPEC encodes only one non-flagellar T3SS (Reviewed in Dean and Kenny (2009)). This feature makes EPEC an ideal surrogate host for studying T3SS translocation and it has been used with success in previous studies of the B. pseudomallei effector protein BopC (Muangman et al., 2011) and the B. mallei effector protein BapA (Whitlock et al., 2008).
Figure 5.11: Western blot analysis of the \textit{B. pseudomallei} Bsa T3SS effector/translocator protein BipC expressed as a c-Myc tagged fusion protein. The coding region of \textit{bipC} was expressed with a C-terminal c-Myc tag in the IPTG-inducible expression vector pME6032 in \textit{B. pseudomallei} strains 10276 WT, 10276 \textit{bsaZ::pDM4}, 10276 \textit{bipD::pDM4} or 10276 \Delta\textit{bsaP}. Supernatant and whole cell fractions were collected after 6 hours of incubation at 37°C in the presence of 0.5 mM IPTG. Equal quantities of protein were separated by reducing SDS-PAGE, blotted onto nitrocellulose membranes and probed using polyclonal rabbit α-c-Myc antibody.
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Figure 5.12: Western blot of BipC secretion using EPEC as a surrogate host. The full length coding region of bipC was expressed with a C-terminal c-Myc tag in the constitutive expression vector pBHRT. EPEC strain E2348/69 WT and ΔescN containing pBHRT-BipC were cultured for 4 hours in DMEM containing 25 mM HEPES shaking at 37°C. WT EPEC E2348/69 containing empty pBHRT was used as a control. The bacterial supernatants were collected and the proteins were precipitated using PRMM. Equal volumes of protein were separated by SDS-PAGE and assessed by Western blot using a c-Myc tag-specific polyclonal antibody.
BipC was cloned as a c-Myc fusion in the shuttle vector pBHRT under a constitutive kanamycin resistance gene promoter. The plasmid was transformed into EPEC O127:H6 strain E2348/69 WT and an isogenic \( \Delta \text{escN} \) mutant (Marchés et al., 2005), which is unable to secrete proteins via the T3SS. WT EPEC E2348/69 containing empty pBHRT was used as a negative control. The strains were cultured overnight shaking at 37°C in LB. The following day the overnight cultured was centrifuged and the supernatant was replaced with DMEM containing 25 mM HEPES to an OD\(_{600}\) of 1.0. Of the adjusted culture 1 ml was added to 10 ml of DMEM containing 25 mM HEPES which was then incubated for 4 hours shaking at 37°C. The bacterial supernatants were collected and the proteins were precipitated using PRMM. Equal volumes of protein were analysed by SDS-PAGE and assessed by Western blot for the presence of the tagged BipC using a c-Myc tag-specific polyclonal antibody. The bacterial cell lysates of EPEC WT and \( \Delta \text{escN} \) express BipC-c-Myc (45 kDa) indicating the tagged protein was being produced in the strains (Figure 5.12). In the bacterial supernatants, BipC-c-Myc was secreted in a T3SS-dependant manner and was only found in the WT sample. This is evidence that EPEC can be used as a surrogate host for the study of translocation of \( \text{B. pseudomallei} \) BipC. Interestingly, BipC was seen as a doublet in the bacterial lysates, possible due to degradation by cytoplasmic proteases. It would be useful in the future to run the lysates next to the supernatant on a SDS-PAGE gel to determine which of the doublet bands correspond to the secreted form of BipC in EPEC.

### 5.3 Discussion

The \( \text{B. pseudomallei} \) translocon protein BipC is homologous to the T3SS proteins SipC from \( \text{Salmonella} \) and IpaC from \( \text{Shigella} \) (Stevens et al., 2002). Both SipC and IpaC bind and nucleate actin (Hayward and Koronakis, 1999, Terry et al., 2008), facilitating bacterial invasion of host cells (Mounier et al., 2009, Myeni and Zhou, 2010), a function that has not previously been demonstrated for BipC. In order to investigate this, pull-down experiments were performed using a GST-BipC fusion protein and murine splenic lysates in polymerisation buffer which resulted in the first evidence that BipC is an actin-binding protein. It was also demonstrated that GST-BipC has the ability to bind to actin directly, in the absence of other proteins.

The interaction between BipC and actin was further characterised using a co-sedimentation assay in which GST-BipC primarily co-sedimented with F-
actin. This suggests that BipC preferentially binds F-actin. This is similar to SipC, which has been shown to co-sediment primarily with F-actin (Myeni and Zhou, 2010). Further, actin was not found in GST-BipC samples when protein pull-downs were performed under non-polymerising conditions. Beyond its ability to directly bind actin, it was further demonstrated that BipC has the ability to polymerise actin \textit{in vitro}. The activity appears to be lower than that of the SipC control. It has been shown that the activity of SipC is increased by another \textit{Salmonella} T3SS effector protein SipA (McGhie \textit{et al.}, 2001). It is possible that other \textit{B. pseudomallei} T3SS effector proteins may work synergistically to polymerise actin with BipC. BipC did not appear to stabilize F-actin, suggesting that its role in actin dynamics may differ from that of SipC.

These findings may explain why actin was not identified as a binding partner for BipC in the Y2H screen described in Chapter 4. In agreement, the study by Memišević \textit{et al.} (2013) did not identify actin as an interacting partner for \textit{B. mallei} BipC. Another study using a Y2H system to investigate SipC protein-protein interactions resulted in the identification of cytokeratins as interacting partners, but actin identified by the screen (Carlson \textit{et al.}, 2002). Due to the possibility of the interaction being missed in a high-throughput library screen, the ability of BipC and actin to interact was tested in a clean \textit{S. cerevisiae} background using the plasmids pGBK7-BipC and pGADT7-actin, which again resulted in no activation of the Y2H reporter genes and lack of growth on selective media. It is possible that the environment of the yeast nucleus is not conducive of BipC-F-actin binding. When expressed as an N-terminal Myc tagged protein in HeLa cells, BipC appears to cause the formation of pseudopodia and membrane protrusions at the cell membrane. The Myc-tagged BipC localised to the cell membrane with F-actin and the pseudopodia. These areas of actin disruption on the cell periphery are similar to what has been described as “membrane ruffles”, seen in an \textit{ipaC}-dependant manner when Swiss 3T3 cells were permeabilised in the presence of \textit{Shigella} supernatants (Van Nhieu \textit{et al.}, 1999). Further work will need to be done to explore the mechanisms by which BipC binds and nucleates actin, as well the role this may play in \textit{B. pseudomallei} pathogenesis.

It is tempting to speculate that the ability of BipC to polymerise actin is involved in the invasion of host cells. Though it was previously demonstrated that a \textit{bipC} mutant is deficient in its ability to invade A549 cells, this function was not separated from BipC’s role as a translocator (Kang \textit{et al.}, 2015). A mutation in another \textit{B. pseudomallei} Bsa T3SS translocator \textit{bipB} also showed an
invasion defect in HeLa cells (Suparak et al., 2005). Because the insertion of the translocon into the host cell membrane is necessary for the subsequent injection of effector proteins (Reviewed in Büttner (2012)), studies of secondary functions of translocator proteins must be carefully controlled in order to draw conclusions.

Interestingly, BipC expressed as a C-terminal c-Myc tagged protein by an inducible expression vector was secreted independently of the Bsa T3SS under the conditions used in this study. There are a number of possible explanations for this. One possibility is that the bacterial cell may have been overwhelmed with levels of BipC-c-Myc, similar to what was hypothesized when BopE-c-Myc was expressed constitutively (Chapter 2, Figure 3.15). However, in the case of BipC, an inducible expression vector was used with levels of inducer that were appropriate for other effector proteins to be secreted in a Bsa-dependent manner. Another possibility is that BipC is being promiscuously secreted by one of the other two plant-like T3SSs or the flagellar T3SS of B. pseudomallei. This phenomenon is not without precedent. The Hrp Plant T3SS of Xanthomonas can secrete the effector protein YopE of Yersinia (Rossier et al., 1999). Removal of the ability of SopE in Salmonella to bind its chaperone InvB, caused secretion through both the flagellar and SPI-1 virulence associated T3SS (Lee and Galán, 2004, Ehrbar et al., 2006). Virulence associated T3SS effectors can be secreted by the bacterial flagellar T3SS in Yersinia (Young and Young, 2002, Warren and Young, 2005). Full length BipC-c-Myc was also secreted by EPEC, which only contains one T3SS (Reviewed in Dean and Kenny (2009)), in a T3SS-dependant manner. This suggests BipC may be more promiscuous in which T3SSs it can be secreted from. More work needs to be performed, such as expression of BipC without the N-terminal T3SS signal, to determine whether BipC-c-Myc secretion is dependant on any T3SS or may be through an independent mechanism. As an added benefit, this study established EPEC as an acceptable host for study of translocation of BipC-c-Myc, which may be useful in downstream studies.
Chapter 6

General Discussion

6.1 Impact of This Study

The primary aim of this study was to identify the repertoire of effector proteins secreted by the *B. pseudomallei* Bsa T3SS. It was hypothesised that under standard laboratory conditions this system was under tight regulation and would need to be dysregulated. To this end *bsaP*, which is predicted to encode a structural component of the T3SS, was deleted from the chromosome. It was shown that BsaP functions similarly to T3SS gatekeeper proteins in other Gram-negative bacteria that are involved in controlling a switch from secretion of translocator proteins to effector proteins (Reviewed in Büttner (2012)).

The identification of proteins with homologous function within the T3SS may allow for the informed design or use of previously existing T3SS inhibitors as a method of treatment. For example, a small molecule ATPase inhibitor effective in *Yersinia pestis* prevented the secretion of BopE in *B. pseudomallei* and treatment of infected RAW 264.7 cells with the inhibitor reduced bacterial survival (Gong *et al.*, 2015). There are a range of other small molecule inhibitors of the T3SS effective in other Gram negative bacteria not yet tested in *B. pseudomallei* (Reviewed in Gu *et al.* (2015)).

Another possible outcome of dysregulating the Bsa T3SS is the potential for use as a live attenuated vaccines. A *B. pseudomallei bsaZ* mutant lacking a functional Bsa T3SS is highly attenuated in a murine melioidosis model, although still lethal to the mice over time (Stevens *et al.*, 2004). It is possible that a dysregulated T3SS (*bsaP* mutant) may be similarly attenuated due to a lack of translocator proteins, but create a stronger immune response due to the presence of high levels of bacterial effector proteins.

In this thesis bacterial supernatants were generated from WT, a Bsa T3SS
null mutant (bsaZ) and two structural mutants that hyper-secrete the known effector BopE (bipD and bsaP). Proteins precipitated from the supernatants were digested, labelled with mass tags and analysed by mass spectrometry using a method known as iTRAQ. The resulting data set is the most complete set of *B. pseudomallei* secreted proteins so far produced and is published in Molecular and Cellular Proteomics (Vander Broek et al., 2015). This data set will prove important to the wider community as a reference for selecting secreted proteins for further study. The list contains some proteins that were annotated as hypothetical which are know known to be translated. The community could use the resource to produce a list of possible vaccine candidates by looking for extracellular proteins which may be readily visible to the host immune system. It also allows for scanning of different factors that may be shared with other bacteria useful in informing antigen choice for vaccines that protect against multiple pathogenic bacterial species. One study has shown CD4 T cells that recognise *B. pseudomallei* FliC epitopes cross-react with FliC from *B. cenocepacia* and *B. multivorans*, species often involved in infection in cystic fibrosis patients (Musson et al., 2014).

Beyond the core secretome of *B. pseudomallei*, BapA and BprD were identified as novel Bsa T3SS effector proteins and BipC and BapA were shown to be secreted in a Bsa T3SS-dependent manner for the first time. The first steps towards understanding the role of BipC and BapA were taken in the form of yeast two-hybrid screening. The human protein C1QBP was identified and confirmed as an interacting partner for both BipC and BapA. As well as helping to understand the disease process, the subtle effects of T3SS effector proteins have on host cell processes can provide important tools to help interrogate host cell biology (Reviewed by Lee et al. (2013)). C1QBP plays a role in cancers and is involved in innate immunity, pathogen binding and major inflammation pathways (Reviewed in Peerschke and Ghebrehiwet (2014) and Peerschke and Ghebrehiwet (2007)). This makes C1QBP an attractive target for further study and potential therapies.

In this study it was shown that BipC, similar to the homologous proteins SipC (*Salmonella*) and IpaC (*Shigella*), is able to directly bind and polymerise actin (Hayward and Koronakis, 1999, Terry et al., 2008). The actin polymerisation function of SipC has been shown to be involved in the entry of *Salmonella* into host cells (Myeni and Zhou, 2010) and it is tempting to hypothesis BipC may function similarly in *B. pseudomallei*. Blocking the entry into host cells may be an effective way to treat *B. pseudomallei* infection.
6.2 Future Work

6.2.1 iTRAQ Analysis of the Bsa T3SS Secretome

The work presented in this thesis forms the basis of several potential future studies. In particular, the iTRAQ screen resulted in a large dataset that warrants further investigation. While the most obvious candidates from the iTRAQ screen were validated, it would be interesting to validate other targets that were close to the selected cut-offs. It is possible that that some of these "borderline" proteins may indeed be effector proteins, and simply are secreted at low levels under the culture conditions used in this study. As the effector protein CHBP was only secreted in infected cells Pumirat et al. (2014), it would be interesting to analyse Bsa T3SS-dependent proteins secreted into infected cells using iTRAQ. Due to the drastically different environment within a host cell it is certainly possible that a different set of proteins may be present in a BsaZ dependant manner than were present in LB media. This would not only give further insight into the full Bsa T3SS effector repertoire, but would also inform global changes in the general B. pseudomallei secretome during infection.

One interesting aspect of T3SS effector proteins is different repertoires can exist between different bacterial strains of the same species, often due to horizontal gene transfer (Rohmer et al., 2004). This is highlighted in B. pseudomallei by the known Bsa T3SS effector protein CHBP which was present in 76.7% of B. pseudomallei strains surveyed by Pumirat et al. (2014). This effector protein was absent from the strain used in this study, 10276. Also, B. pseudomallei is known to have large genomic variability. A recent study of 37 B. pseudomallei isolates found that on average, there were 152 strain-specific genes per isolate (Spring-Pearson et al., 2015). This suggests there is the possibility of other effector proteins in other B. pseudomallei strains. It would be interesting to use comparative proteomics to compare the repertoire of proteins secreted in a Bsa T3SS-dependent manner between different isolates of B. pseudomallei such as those from Australia, Thailand, Africa and the Americas, or from strains with defined differences in virulence.

In this study BrpD and BapA were identified as novel effector proteins secreted in a Bsa T3SS-dependent manner, but it has not yet been demonstrated that the proteins are translocated into host cells by the T3SS. This is more difficult to investigate in B. pseudomallei because it is an intracellular pathogen. It is necessary to separate proteins injected into a host cell from the outside of the
cell by the T3SS from proteins that are secreted for example through the vacuolar membrane into the cytoplasm. One solution is to ensure the \textit{B. pseudomallei} cannot enter the host cell following treatment with cytochalasin D. Cytochalasin D inhibits actin polymerisation, stopping microfilament function and preventing actin mediated bacterial entry into the host cell (Reviewed by Rosen-shine \textit{et al.} (1994)). By preventing bacterial entry into the host cell, proteins injected into the host cell can be evaluated by selectively lysing the eukaryotic cells and testing for the presence of eptopically expressed myc-tagged effector proteins using SDS-PAGE and Western blot. As demonstrated in Chapter 5, the use of an extracellular surrogate host, such as EPEC, is another effective way to study the function of an effector protein such as BipC, without the actin disrupting effects of cytochalasin D. The EPEC host system could be used to investigate other effector proteins in the future.

With the creation of the \textit{bsaP} mutant as a tool to study the Bsa T3SS, it would also be possible to test the outcome of this mutation in infection models. During this study, the \textit{Galleria mellonella} (Wax moth) infection model (Wand \textit{et al.}, 2011) was adapted for screening of the \textit{B. pseudomallei} T3SS (Figure 7.1, more information included in the appendix). This system could be used to compare the attenuation of the \textit{bsaP} mutant to other structural mutations in the Bsa T3SS in order to justify later experiments in more complex models, such as the murine model of melioidosis.

\subsection*{6.2.2 Yeast-Two Hybrid Screen of BapA, BipC and BprD}

It was surprising that no human proteins were found to interact with BprD. Previous studies state that BprD is a bacterial regulator which may explain why it does not interact with human proteins (Chirakul \textit{et al.}, 2014). The number of independent library clones screened in the Y2H analysis of BprD was 10 fold and 4 fold lower than that of BipC and BapA respectively. There may be value in repeating the screen to survey a larger number of independent clones. Also, a lower stringency could be used to attempt to detect lower affinity interactions.

One of the studies that interests me the most is to explore which domains or regions of BapA and BipC bind to specific regions of C1QBP. Both effector proteins binding the same human protein raise the question as to whether BapA and BipC may share sequence homology that allows binding of C1QBP at a specific site. It is also possible that the two proteins bind C1QBP at different sites and may have distinct effects on C1QBP. It is not unheard of that two ef-
factor proteins bind to similar targets causing different outcomes. This is highlighted by *Salmonella* SopE and SptP which activate and deactivate Cdc42 and Rac1 respectively, modulating host cell actin, but maintaining balance within the cell (Kubori and Galán, 2003).

Though two proteins may interact in an artificial environment, *in vivo* the proteins may never come into proximity with each other and the interaction may not be relevant to infection. To investigate this, confocal microscopy could be used to analyse co-localisation of either BapA or BipC with C1QBP. In addition, cells in which C1QBP is knocked down by either siRNA or the CRISPR/Cas9 system could be used to determine the importance of C1QBP on the intracellular life of *B. pseudomallei*. Unfortunately, C1QBP knockout mice are embryonic lethal prohibiting investigation of the role of C1QBP in a murine model of melioidosis (Yagi *et al.*, 2012).

### 6.2.3 Investigation of the Interaction Between BipC and Actin

One important study to complete is to identify which BipC domain is responsible for binding/polymerisation of actin. *B. pseudomallei* bipC mutants could be complemented with bipC retaining translocator function, but lacking the ability to polymerise actin. This would allow the study of the role BipC plays in bacterial entry to be separated from its function as a translocator.

One very interesting observation made during this study was the Bsa T3SS-independent secretion of BipC. This is in contrast to the other proteins within the Bsa T3SS locus. One hypothesis is that BipC may be promiscuously secreted by another T3SS or the flagellar system. One way this could be confirmed is by creating a series of structural mutations in each secretion system and determining its effect on BipC secretion by SDS-PAGE and Western blot.

### 6.2.4 Closing Statement

*Burkholderia pseudomallei* has attracted much attention in the UK and the USA following its classification as a Bioterrorism agent with high mortality rates, no available vaccine and resistance to many antibiotics (Reviewed in Gilad *et al.* (2007)). Yet despite this growing body of research, in endemic Thailand, *B. pseudomallei* is the leading cause of community-acquired bacteraemia and the third most common cause of death from infectious disease, after HIV and tuberculosis (Limmmathurotsakul *et al.*, 2010). *B. pseudomallei* is an emerging disease and with improved surveillance the bacteria is now found outside of
the endemic region in sub-tropical soils in Africa, the Americas and the Indian Sub-continent (Reviewed in Currie et al. (2008)). For these reasons, the continued investigation of both basic biology and clinical aspects of this neglected tropical pathogen are vital in order to improve control and treatment in the future.
Bibliography


Persson, C., N. Carballeira, H. Wolf-Watz, and M. Fällman (1997). The PTPase YopH inhibits uptake of *Yersinia*, tyrosine phosphorylation of p130Cas and
FAK, and the associated accumulation of these proteins in peripheral focal adhesions. *The EMBO journal*, 16(9):2307–2318.


Schesser, K., E. Frithz-Lindsten, and H. Wolf-Watz (1996). Delineation and mutational analysis of the *Yersinia pseudotuberculosis* YopE domains which


Chapter 7

Supplemental

7.1 Galleria mellonella Infections

The Galleria mellonella (Wax moth) larvae infection model was investigated for its ability to detect differences in virulence between WT B. pseudomallei and bsa T3SS mutants. G. mellonella larvae were sourced fresh from the supplier (Live-food UK Ltd., Somerset, UK) and were stored at 15°C for no more than 1 week before use. B. pseudomallei 10276, 10276 bsaZ::pDM4, 10276 bipD::pDM4 and 10276 bopE::pDM4 were grown overnight in 10 ml of LB containing the appropriate antibiotics at 37°C with agitation. The cultures were then centrifuged at 4,000 RPM for 10 minutes and the supernatant was removed, replaced with PBS and the OD$_{600}$ was adjusted to 0.5. Groups of 10 G. mellonella larvae were injected with 10 µl of either PBS or 2x10$^2$ CFU B. pseudomallei. The larvae were incubated at 37°C for 50 hours and checked periodically for death. GraphPad Prism software was used to graph and analyse the data using a Log-rank (Mantel-Cox) test.

All bsa T3SS mutants show a significantly delayed time to death when compared to WT B. pseudomallei (Figure 7.1). Surprisingly, the bipD mutant shows a larger attenuation in virulence than the bsaZ structural mutant. This study indicates the G. mellonella larvae infection model is sensitive enough to assess other bsa T3SS mutants in the future, though with only two repeats, more work will need to be done to confirm this in the future.
Figure 7.1: Infection of *Galleria mellonella* larvae using *B. pseudomallei* and *bsa* T3SS mutants. *B. pseudomallei* 10276, 10276 *bsaZ::pDM4*, 10276 *bipD::pDM4* and 10276 *bopE::pDM4* were injected into *G. mellonella* larvae with a dose of $2 \times 10^2$ CFU. The larvae were incubated at 37°C for 50 hours and checked periodically for death. GraphPad Prism software was used to graph and analyse the data using a Log-rank (Mantel-Cox) test.
7.2 Y2H Colony PCRs

Figure 7.2: Colony PCR screen of *S. cerevisiae* containing pGBKT7-BipC after mating (gels 1-5).
7.3 Strains, Plasmids, Primers and Antibodies

Table 7.1: Strains and plasmids used in this study.

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<td>Cm&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>B. pseudomallei Strains</td>
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<td>10276 WT</td>
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<td>10276 bsaZ::pDM4</td>
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### Chapter 7. Supplemental

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### Salmonella and Pathogenic E. coli Strains

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<td>Enteropathogenic E. coli (EPEC)</td>
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<td>EPEC O127:H6 ΔescN</td>
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### S. cerevisiae Strains

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### Plasmids

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Continuation of Table 7.1

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<td>pGADT7-RecAB containing C1QBP with an in frame N-terminal GAL4 DNA activating domain and HA tag, isolated from library mating, Leu&lt;sup&gt;+&lt;/sup&gt;, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This Study</td>
</tr>
<tr>
<td>pGADT7-RecAB-PAWR</td>
<td>pGADT7-RecAB containing PAWR with an in frame N-terminal GAL4 DNA activating domain and HA tag, isolated from library mating, Leu&lt;sup&gt;+&lt;/sup&gt;, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This Study</td>
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End of Table
### Table 7.2: Primers used in this study.

<table>
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<tr>
<th>Primer Name</th>
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<th>Purpose</th>
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<tbody>
<tr>
<td>T7 For-</td>
<td>TAATACGACTCACTATAGGGC</td>
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</tr>
<tr>
<td>ward</td>
<td></td>
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<td>DNA-BD</td>
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<td>Sequencing of pGBKT7</td>
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<td>DNA-AD</td>
<td>AGATGGTGACACGATGCACAG</td>
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<tr>
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<td>ATATATATCCGCATAAGCTCAATGGAAATGCA</td>
<td>Cloning of bprD into pGBK7</td>
</tr>
<tr>
<td>bprD-Y2H-F</td>
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<td>Cloning of bprD into pGBK7</td>
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<tr>
<td>bprD-Y2H-R</td>
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Continuation of Table 7.2

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<td>bsaP-chk-2</td>
<td>CATCGGCCCTTGATGAACTTCATC</td>
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<td>bsaP-exp-F</td>
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<td>Expression of c-Myc tagged BapA</td>
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<td>bopE-F</td>
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<td>bopE-myc-R</td>
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<td>bprD-F</td>
<td>ATATATCCATGGAGGTGGCCA TCATGAGCTCAATGGAATGACCG CCGC</td>
<td>Expression of c-Myc tagged BprD</td>
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<tr>
<td>bprD-myc-R</td>
<td>ATATAGATCTTCACAGGTCTCTCT CGGAGATCAGCTTCTGCTCAGC CGGAGAACC CGGAGAA</td>
<td>Expression of c-Myc tagged BprD</td>
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<td>BPSS0860-F</td>
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<td>bopA-F</td>
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<td>bopA-myc-R</td>
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<td>BPSS1916-F</td>
<td>ATATATCCATGGAGGTGGCCA TCATGCAAGCAAAACCGTGGCA TT</td>
<td>Expression of c-Myc tagged BPSS1916</td>
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<td>BPSS1916-myc-R</td>
<td>ATATA<strong>GATC</strong>TTTCACAGGTCTCTCTC&lt;br&gt;GGAGATCAGCTTCTGTCTCGGACAT&lt;br&gt;GTGCATCCCGCCGTTGA</td>
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<td>BPSS1512-myc-R</td>
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<td>Expression of c-Myc tagged BPSS1512</td>
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Table 7.3: Antibodies used in this study. Abbreviations in table include: Western blot (WB) and immunofluorescence (IF).

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<tr>
<th>Antibody (species) Specificity Application Reference/Source</th>
<th>Dilution/concentration used</th>
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<th>Goat IgG</th>
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<td>α-BopE (rabbit polyclonal) B. pseudomallei WB (0.5 µg/ml) (Stevens et al., 2003)</td>
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<tr>
<td>α-BipD (rabbit polyclonal) B. pseudomallei WB (0.5 µg/ml) (Stevens et al., 2003)</td>
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<tr>
<td>α-c-Myc (rabbit polyclonal) Human, Monkey WB (1.0 µg/ml), IF (1:500) Santa Cruz Biotechnology (sc-789)</td>
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<td>α-Actin (rabbit polyclonal) Human, Mouse, Rat WB (1.0 µg/ml) Santa Cruz Biotechnology (sc-1615)</td>
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<td>α-PAWR (rabbit monoclonal) Human, Mouse WB (1:10,000) Origene (TA307434)</td>
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<td>α-C1QBP (rabbit monoclonal) Human, Mouse, Rat WB (1:10,000) Abcam (ab134926)</td>
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<td>α-CDC42 (rabbit polyclonal) Human, Mouse, Rat WB (1.0 µg/ml) Santa Cruz Biotechnology (sc-87)</td>
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<td>α-Rac1 (rabbit polyclonal) Human, Mouse, Rat WB (1.0 µg/ml) Santa Cruz Biotechnology (sc-217)</td>
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<td>α-GAPDH HRP (rabbit polyclonal) Human, Mouse, Rat E. coli WB (1:5000) Abcam (ab85760)</td>
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<td>α-rabbit HRP (goat) Rabbit IgG WB (1:15,000) Life Technologies (G-21234)</td>
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<td>α-rabbit DyLight 800 (goat) Rabbit IgG WB (1:10,000) Cell Signaling (5257)</td>
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<td>α-rabbit 488 (goat) Rabbit IgG IF (1:200) Molecular Probes (A-11008)</td>
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Abbreviations in table include: Western blot (WB) and immunofluorescence (IF).
7.4 Publications


* Publication attached below.
Quantitative Proteomic Analysis of *Burkholderia pseudomallei* Bsa Type III Secretion System Effectors Using Hypersecreting Mutants

Charles W. Vander Broek†, Kevin J. Chalmers§, Mark P. Stevens‡, and Joanne M. Stevens‡¶

*Burkholderia pseudomallei* is an intracellular pathogen and the causative agent of melioidosis, a severe disease of humans and animals. One of the virulence factors critical for early stages of infection is the *Burkholderia* secretion apparatus (Bsa) Type 3 Secretion System (T3SS), a molecular syringe that injects bacterial proteins, called effectors, into eukaryotic cells where they subvert cellular functions to the benefit of the bacteria. Although the Bsa T3SS itself is known to be important for invasion, intracellular replication, and virulence, only a few genuine effector proteins have been identified and the complete repertoire of proteins secreted by the system has not yet been fully characterized. We constructed a mutant lacking *bsaP*, a homolog of the T3SS “gatekeeper” family of proteins that exert control over the timing and magnitude of effector protein secretion. Mutants lacking *BsaP*, or the BsaT3SS translocon protein BipD, were observed to hypersecrete the known Bsa effector protein BopE, providing evidence of their role in post-translational control of the Bsa T3SS and representing key reagents for the identification of its secreted substrates. Isobaric Tags for Relative and Absolute Quantification (iTRAQ), a gel-free quantitative proteomics technique, was used to compare the secreted protein profiles of the Bsa T3SS hypersecreting mutants of *B. pseudomallei* with the isogenic parent strain and a *bsaZ* mutant incapable of effector protein secretion. Our study provides one of the most comprehensive core secretomes of *B. pseudomallei* described to date and identified 26 putative Bsa-dependent secreted proteins that may be considered candidate effectors. Two of these proteins, BprD and BapA, were validated as novel effector proteins secreted by the Bsa T3SS of *B. pseudomallei*. 

Author contributions: C.W.V., K.J.C., M.P.S., and J.M.S. designed research; C.W.V. performed research; C.W.V., K.J.C., and J.M.S. analyzed data; C.W.V. and J.M.S. wrote the paper.

*Burkholderia pseudomallei* is a Gram-negative environmental saprophyte found in free-standing water and soil (1). The causative agent of melioidosis, *B. pseudomallei* causes severe disease in humans and animals with a range of clinical manifestations including severe pneumonia and septic shock, acute or chronic suppurative infections, genitourinary disease, and asymptomatic carriage (2). An emerging infectious disease, melioidosis is found primarily in South East Asia and Northern Australia, yet there is a growing body of evidence of a more widespread distribution in subtropical regions of Asia, the Americas, and Africa (3). Melioidosis is an important cause of community-acquired septicaemia in North-East Thailand, where it can cause mortality rates of up to 40% (4). Because of the lack of an effective vaccine, intrinsic antibiotic resistance, and the high mortality rates, there is a need to better understand the molecular mechanisms of pathogenesis to inform the design of treatments and vaccines (5).

*B. pseudomallei* is a facultative intracellular pathogen capable of invading both phagocytic and nonphagocytic cells (6). Once inside the cell, the bacteria can rapidly escape the endosome and polymerize host cell actin at one pole causing actin-based motility, a process dependent on the bacterial BimA protein (7). *B. pseudomallei* also causes host cell fusion creating multinucleated giant cells (MNGCs) that facilitate cell to cell spread while evading immune detection (8). The formation of MNGCs by *B. pseudomallei* is dependent on secretion of the protein VgrG5 by the virulence-associated type VI secretion system-5 (9–12). One important virulence factor of *B. pseudomallei* that has also been shown to be important for infection in many other Gram-negative bacteria including pathogenic *Escherichia coli*, *Salmonella*, *Shigella*, and *Yersinia*, is the Type III Secretion System (T3SS) (13–16). T3SSs act as a molecular syringe.
projecting outwards from the surface of the bacteria allowing for the transport of bacterial proteins into target eukaryotic cells (17). These bacterial proteins, termed effectors, subvert host cell processes to the advantage of the bacteria and rely on a second class of T3SS-secreted proteins (translocators) for delivery (18). B. pseudomallei contains three T3SSs, of which T3SS-1 and T3SS-2 share homology with T3SSs found in plant pathogens (19, 20), also known as the Burkholderia Secretion Apparatus (Bsa) T3SS, is similar to the Inv-Spa (Salmonella pathogenicity island (SPI)-1) and Mxi-Spa T3SSs of the animal pathogens Salmonella spp. and Shigella flexneri respectively (21). The Bsa T3SS is important in invasion, endosome escape and net intracellular replication in cultured cells and in virulence in murine and Syrian hamster models of melioidosis (21–24).

As the Bsa system influences pathogenesis, one may infer that its effectors also influence the outcome of infection. However, only three effectors, BopE, BopC, and CHBP, have so far been proven to be secreted by B. pseudomallei in a Bsa T3SS-dependent manner (25–27). BopE is a guanine nucleotide exchange factor that is involved in invasion of nonphagocytic cells (26). BopC has been shown to be important for invasion of A549 epithelial cells and endosome escape, and intracellular survival in J774A.1 macrophage-like cells (25, 28). CHBP is a homolog of E. coli Cif, a cyclomodulin that disrupts the eukaryotic cell cycle (29), for which evidence of Bsa-dependent translocation into host cells was recently presented (27). A fourth candidate effector, BopA, prevents LC3-associated host autophagy of intracellular bacteria and has been shown to be secreted in a Bsa T3SS-dependent manner in a surrogate enteropathogenic E. coli (EPEC) host (30, 31). Compared with the large number of effectors secreted by T3SSs in other Gram-negative pathogens, it is likely that there are other effectors of the Bsa T3SS yet to be characterized (32). Bsa-secreted proteins are known to be potent B- and T-cell antigens in humans (33–36) and subunit vaccines based on needle components or effectors are protective against other pathogens that deploy T3SSs (e.g. Shigella IpaB and IpaD, Yersinia V antigen, Salmonella Ssel) (37–41).

One of the problems facing T3SS effector protein discovery is the tight regulation of the T3SS under laboratory conditions with different T3SSs responding to very different environmental cues (42–47). Although some genes involved in transcriptional control of the Bsa T3SS were recently identified (48), little is known about the post-translational regulation of the system and what environmental signals may activate it. Indeed, a recent proteomics study of B. pseudomallei failed to identify any of the known Bsa T3SS effectors in bacterial culture supernatants (49). To override the tight control of the T3SS, one solution employed in the study of other Gram-negative bacteria (for example EPEC and Citrobacter rodentium) was to generate mutations of T3SS components that dysregulate the system, thereby creating a hypersecretion phenotype. The bacterial supernatants were then analyzed using gel-free quantitative proteomics techniques to identify a range of novel effector proteins (50, 51).

In Shigella, deletion of IpaD, the needle tip protein, creates a “leaky” phenotype, in which higher levels of both translocators and effectors are seen in the supernatant (46, 52). The homolog of IpaD in B. pseudomallei is BipD (53) (supplemental Fig. S1). In a previous study, we have shown a 10276 bipD::pDM4 mutant secreted higher levels of the effector protein BopE into the bacterial culture supernatant by Western blot analysis, but it is unknown what effect bipD disruption may have on the secretion of other effectors and translocators (26).

Another group of T3SS proteins that have been shown in many systems to be involved in the control of effector and translocator secretion are the so-called “gatekeeper” family of proteins, InvE/MxiC/Sepl/YopN-TyeA of Salmonella/Shigella E. coli/Yersinia, respectively (17). Investigation of these proteins has given insight into the temporal regulation of their respective T3SSs. Although deletion of all of these proteins causes an increase in levels of secreted effectors there are also differences between them. Deletion of InvE and Sepl cause a reduction in secreted levels of translocators, deletion of MxiC has no effect on their levels, and deletion of YopN increases levels of secreted translocators (54–60). The closest homolog to this family of proteins in B. pseudomallei is the uncharacterized protein BsaP (BPSS1544) (supplemental Fig. S1).

Here, a 10276 Δbasp deletion mutant was generated and its effect on secreted levels of the known effector BopE was assessed. To define the total and Bsa-dependent repertoire of secreted proteins, Isobaric Tags for Relative and Absolute Quantification (iTRAQ), a gel-free quantitative proteomics technique, was used to quantify protein levels in the bacterial culture supernatants of B. pseudomallei mutant strains 10276 bipD::pDM4 and 10276 Δbasp. In order to identify candidate Bsa effector proteins, the secreted protein profiles from these hypersecreting mutants were then compared with the isogenic parent strain (10276 WT) and a T3SS structural-null mutant (10276 bsaZ::pDM4), which has been shown to be unable to secrete BipD and BopE (21). BsaZ is a homolog of YscU in Yersinia, which forms a key component of the inner membrane ring and is required for a functional T3SS (61) (supplemental Fig. S1). From this analysis we provide the first evidence of the involvement of both BipD and BsaP in control of known effector/translocator secretion in the Bsa T3SS. We also present a comprehensive general secretome for B. pseudomallei. Furthermore, we have identified 26 candidate effector proteins, including all known Bsa-secreted effectors, of which we show two proteins are novel substrates for secretion by the Bsa T3SS.

**EXPERIMENTAL PROCEDURES**

Strains, Media, Plasmids, and Oligonucleotides—Bacterial strains and plasmids are listed in supplementary Table S1. The insertion
mutants 10276 bipD::pDM4 and 10276 bsaZ::pDM4 were produced by integration of the pDM4 suicide vector into the coding sequence of the target gene (21). Primers, purchased from Sigma Aldrich (Gillingham, UK), are listed in supplemental Table S2. Bacteria were routinely cultured at 37 °C on LB agar (Miller) or LB broth (Lennox). Antibiotic selection was performed at the following concentrations unless otherwise stated: ampicillin (Amp) 100 μg/ml, kanamycin (Kan) 50 μg/ml, chloramphenicol (Cm) 34 μg/ml, and tetracycline (Tet) 15 μg/ml. IPTG was used at a final concentration of 0.5 mM where appropriate. Unless otherwise stated all chemicals were purchased from Sigma Aldrich (Gillingham, UK).

**B. pseudomallei Mutagenesis** — The 10276 ΔbsaP in-frame deletion mutant was made using allelic exchange and the sacB containing positive-selection suicide vector pDM4 essentially as previously described (62). DNA fragments upstream and downstream of bsap (BPS51544) were amplified by PCR from *B. pseudomallei* 10276 genomic DNA using primer pair's bsap-del-1/bsap-del-2 and bsap-del-3/bsap-del-4. The resulting DNA fragments were combined using PCR-ligation-PCR (63). A-tailing was performed to add an adenine to the blunt-ended PCR product, which was then ligated into pGEM-T resulting in the plasmid pGEM-T-ΔbsaP. The insert was sequenced and a SpeI and BglII fragment containing the 10276 bsaP/H9004 was cloned into pGEM-T by blunt-end ligation. The resulting plasmid was transformed into TOP10 PIR1 cells (Life Technologies, Paisley, UK) before transfer into *E. coli* (S17-1) with conjugation with *B. pseudomallei* K96243 to create strain 10276 ΔbsaP. The plasmid was transformed into TOP10 PIR1 cells (Life Technologies, Paisley, UK) before transfer into *E. coli* (S17-1) with conjugation with *B. pseudomallei* K96243 to create strain 10276 ΔbsaP. The resulting strain designated 10276 ΔbsaP was used as a recipient for allelic exchange and the (Gillingham, UK). otherwise stated all chemicals were purchased from Sigma Aldrich (Gillingham, UK).

Protein Precipitation — Proteins were precipitated using a Pyrogallol red-molybdate methanol (PRMM) solution (0.5 mM pyrogallol red, 0.5 mM sodium molybdate, 0.5 mM sodium oxalate, 50 mM succinic acid, 20% MeOH, pH 2.0) as previously described (65). In brief, equal volumes of bacterial culture supernatant and PRMM solution were thoroughly mixed and the pH was adjusted to 2.8 ± 0.1 with HCl. The samples were then precipitated overnight at 4 °C. The resulting precipitate was centrifuged at 3220 RCF for 10 min at 4 °C. The culture supernatant was collected and centrifuged a second time to ensure no intact bacteria remained. The supernatant was then passed through a 0.2 μm low protein binding cellulose acetate membrane filter (Millipore, Watford, UK) and sterility tested.

**SDS-PAGE and Immunoblotting** — Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Equal volumes of protein samples and Laemmli buffer were mixed and heated to 90 °C for 5 min. Samples were separated on any kDa™ Mini-PROTEAN® TGX gels (Bio-Rad, Hemel Hempstead, UK) at 150V for 45 min in Tris-Glycine running buffer. Proteins were visualized by Coomassie staining. Western blots were used to identify particular proteins of interest. Proteins were transferred onto a nitrocellulose membrane using the Trans-Blot Turbo system (Bio-Rad, Hemel Hempstead, UK) at 2.5 A for 3 min. α-Blo (26), α-BipD (22), or α-cMyc (Insight Biotechnology, Wembley, UK) rabbit polyclonal antibodies were used at 1 μg/ml. For detection by infrared fluorescence, goat-α-rabbit IgG (H + L) (DyLight™ 800 Conjugate) was used at a 1:10,000 dilution (Cell Signaling Technology, Hitchin, UK). Blots were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences, Cambridge, UK) according to the manufacturer’s instructions. Images were captured and analyzed using Image Studio Lite (LI-COR Biosciences, Cambridge, UK).

**iTRAQ Experimental Design** — Bacterial culture supernatants of *B. pseudomallei* 10276 WT, 10276 bsazP::pDM4, 10276 bipD::pDM4, and 10276 ΔbsaP were generated in three independent experiments. Equal amounts of protein from each replicate were combined creating a single pooled sample for each strain. These samples were enzymatically digested, peptides were labeled using iTRAQ reagents, and then pooled and submitted to LC MS/MS in three technical replicates as follows:

1. **Protein Digestion and Peptide Fractionation** — Protein samples were prepared for MS analysis using a protocol adapted from (66). Following protein quantification, samples were reduced in 10 mM DTT and alkylated in 50 mM iodoacetamide prior to heating in Laemmli buffer, and then separated by one-dimensional SDS-PAGE (4–12% Bis-Tris Novex mini-gel, Life Technologies, Paisley, UK) and visualized by colloidal Coomassie staining (Life Technologies). The entire protein gel lanes were excised and cut into 10 slices each. Every gel slice was subjected to in-gel digestion with trypsin overnight at 37 °C. The resulting tryptic peptides were extracted by formic acid (1%) and acetonitrile, lyophilized in a speedvac, and resuspended in 1% formic acid.
(2) Peptide Concentration—To determine the concentration of the peptides, the dried samples were resuspended in 100 mM TEAB 1 M ACN. To determine the volume of sample needed for an even protein load across all samples, 1 µl was injected onto an LTQ Velos Mass Spectrometer (Thermo Fisher Scientific, MA) and the TIC trace was used.

(3) iTRAQ Labeling—Samples were labeled according to the manufacturer’s instructions using an iTRAQ Reagents Multiplex kit – fourplex (AB Sciex UK Ltd., Warrington, UK). To each vial of labeling reagent (labels- 114, 115, 116, and 117), 70 µl ethanol was added, after which they were vortexed and centrifuged. A single vial of label was added to a single vial of sample and mixed. The samples used with each label were: 10276 bsaZ::pDM4–114, 10276 WT-115, 10276 bipP::pDM4–116, and 10276 ΔbsaP-117. Samples were incubated with shaking at room temperature for 1 h, after which 100 µl of water was added and incubated for a further hour. The four vials of labels/samples were pooled into one vial and then dried under vacuum. Samples were desalted using a standard method.

(4) SCX Fractionation – Salt Cuts—To a Millipore (Watford, UK) C18 ziptip, 10 µl Poros HS resin (Life Technologies, Paisley, UK) was added and washed with 3 × 25 µl Buffer A (35% acetonitrile (ACN), 0.1% formic acid (FA)). Samples were resuspended in Buffer A and loaded onto the resin, washed with 3 × 25 µl Buffer A, and eluted with 2 × 25 µl of each of 11 salt cuts ranging from 5 mM to 800 mM NaCl. A final elution was performed using 2 × 25 µl 50% isopropanol, 0.4% ammonium hydroxide. Samples were centrifuged and speed vacuum until dry. Samples were resuspended in 1% FA and pooled as six samples containing the salt cuts as follows: JS-1 (5 mM, 10 mM, 20 mM), JS-2 (50 mM), JS-3 (100 mM), JS-4 (150 mM), JS-5 (200 mM), and JS-6 (250 mM, 300 mM, 400 mM, 800 mM).

Mass Spectrometry—Peptides were separated using an Ultimate 3000 RSLC nanoflow LC system (Thermo Fisher Scientific, MA). With a constant flow of 5 µl/min, 15 µl of sample was loaded onto an Acclaim PepMap100 nanoViper C18 trap column (100 µm inner diameter, 2 cm) (Thermo Fisher Scientific, MA). After trap enrichment, peptides were eluted onto an Acclaim PepMap RSLC nanoViper, C18 column (75 µm, 15 cm) (Thermo Fisher Scientific, MA) with a linear gradient of 2–45% solvent B (80% ACN with 0.08% FA) over 125 min with a constant flow of 300 nl/min. The HPLC system was coupled to a linear ion trap Orbitrap hybrid mass spectrometer (LTQ-Orbitrap Velos) (Thermo Fisher Scientific, MA) via a nanoelectrospray ion source (Thermo Fisher Scientific, MA). The spray voltage was set to 1.2kV and the temperature of the heated capillary was set to 250 °C. Full-scan MS survey spectra (m/z 335–1800) in profile mode were acquired in the Orbitrap with a resolution of 60,000 after accumulation of 1,000,000 ions. The ten most intense peptide ions from the preview scan in the Orbitrap were fragmented sequentially by higher-energy collisional dissociation (HCD) (two-step (6%)) normalized collision energy (NCE), 40%; and activation time, 0.1 ms) prior to analysis in the Orbitrap after accumulation of 50,000 ions. Maximal filling times were 500 ms for the full scans and 200 ms for the MS/MS scans. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged species were rejected. The lock mass option was enabled for survey scans to improve mass accuracy (Δ7). Data were acquired using Xcalibur software (Thermo Fisher Scientific, MA).

Quantification and Bioinformatic Analysis—The raw mass spectral data files obtained for each experiment were collated into a single data set using Proteome Discoverer version 2.0 (Thermo Fisher Scientific, MA) and the Mascot search engine version 2.4 (www.matrixscience.com) using a translated B. pseudomallei K96243 genome as a reference for protein identification. Enzyme specificity was set to that of trypsin, allowing for cleavage N-terminal to proline residues and between aspartic acid and proline residues. Other parameters used were: 1) variable modifications, methionine oxidation, protein N-acetylation, gln → pyro-glu; 2) fixed modifications, cysteine carbamidomethylation; 3) database: B. pseudomallei K96243, 20120601 (compiled 01/06/2012, containing 5728 proteins); 4) iTRAQ labels: standard iTRAQ 4-plex quant method with labels 114–117; 5) MS/MS tolerance: FTMS- 10ppm, FTMS/MS- 0.06Da; 6) minimum peptide length, 6; 7) maximum missed cleavages, 2; and 8) false discovery rate, 1%. Peptide ratios were calculated for samples using the 10276 bsaZ::pDM4–114 label as the denominator. Data are available via ProteomeXchange with identifier PXD001656.

Protein ratios were normalized using the overall median ratio for all quantified peptides in each sample based on the assumption that the abundance of the majority of secreted proteins was identical in each condition. Proteins were selected as potential T3SS effector protein candidates if they had at least two unique peptides used in quantification with a Mascot peptide score ≥21 (which corresponds to the threshold value for a 95% confidence level, a 1 in 20 chance that the match is random), and a "high" iTRAQ ratio in at least one strain, which we defined as a value greater than 1.5 × IQR. IQR is defined as $Q_3 = \frac{(3(n+1))}{4}$ term − $Q_1 = \frac{(n+1)}{4}$ term; where $n$ complete set of ratios over 10276 bsaZ::pDM4 for all quantified proteins in a given sample.

RESULTS

Construction and Characterization of Hypersecreting bsa Mutants—We hypothesized that BsaP may control the timing and magnitude of effector secretion based on homology to “gatekeeper” proteins from the T3SSs of other Gram-negative bacterial pathogens. BLAST analysis of available genomes indicated that BsaP shares 33% identity over 92% coverage (E-value 1e-41) with the Shigella MxiC “gatekeeper” protein and 34% identity over 88% coverage (E-value 4e-32) with the Salmonella InvE “gatekeeper” protein (supplemental Fig. S2). A nonpolar bsaP deletion mutant was therefore constructed in B. pseudomallei strain 10276 by double homologous recombination using a positive-selection suicide vector containing short regions of DNA flanking the bps1544 gene (62). The chromosomal deletion was confirmed by sequencing across the bsaP adjoining regions (data not shown). Because bsaP is predicted to reside in the middle of an operon, with seven genes upstream and two downstream, the deletion of bsaP was complemented using a constitutive expression vector encoding bsaP to confirm any phenotypes were not caused by polar effects. Alongside the bsaP mutant we also analyzed 10276 bipP::pDM4 lacking the BipD needle tip protein that has been previously reported to secrete elevated levels of the effector protein BopE (26).

Bacteria-free culture supernatants were collected from B. pseudomallei 10276 WT, 10276 bsaZ::pDM4, 10276 bipP::pDM4, 10276 ΔbsaP, and 10276 ΔbsaP (pbsaP) and analyzed by SDS-PAGE followed by Coomassie staining. The secreted protein profile of B. pseudomallei LB-culture supernatant is highly complex with few obvious differences between the strains (supplemental Fig. S3). The bacterial supernatants and whole cell lysates were also analyzed by Western blot using antibodies to detect the BopE effector protein and the BipD translocon protein (Fig. 1). The absence of both...
BopE and BipD in the supernatants of the 10276-bsaZ::pDM4 insertion mutant indicates that there was no detectable cell lysis at the time when the secretome was sampled. When compared with the 10276 WT strain, both 10276 bipD::pDM4 and 10276 ΔbsaP strains demonstrated increased secretion of BopE. Interestingly, the levels of BipD secreted into the supernatant were reduced in 10276 ΔbsaP compared with 10276 WT. This pattern of increased levels of the effector BopE and reduced secretion of the translocator BipD provides the first evidence of a role for BsaP as a "gatekeeper" protein, controlling a switch between the secretion of translocators and effectors. In the case of both BopE and BipD, the phenotype of 10276 ΔbsaP was partially restored by plasmid-mediated complementation, indicating the effects are unlikely to be caused by polar or secondary defects. Differences in the levels of BopE and BipD across the different strains sampled are not caused by differences in the number of bacteria at six hours subculture, because the growth rates of all strains were shown to be identical by viable count assessment (data not shown).

Characterization of the total B. pseudomallei Secretome under Standard Laboratory Conditions—Having identified two bsa mutant strains with dysregulated BopE secretion profiles in bacterial culture supernatants, we then sought to identify the total secreted protein profile from these two strains in comparison to the isogenic parent strain (10276) and a bsa null mutant strain. In three independent experiments, culture supernatants were prepared from the four test strains at 6 h post subculture. The strains used were B. pseudomallei 10276 WT, 10276 bsaZ::pDM4, 10276 bipD::pDM4, and 10276 ΔbsaP (supplemental Fig. S1). The samples were all assessed for a lack of cell lysis by immunoblotting for BopE. All 10276 bsaZ::pDM4 samples lacked BopE, indicating the samples were suitable for analysis (supplemental Fig. S4). After mixing equimolar amounts of each of the three biological replicates, the samples were subjected to iTRAQ labeling and MS/MS analysis as described under "Experimental Procedures." iTRAQ allowed the simultaneous identification and relative quantification of peptide fragments in all four separate samples, resulting in the positive identification of a total of 475 proteins in all samples (listed in supplemental Table S3). The data was expressed as ratios of relative protein abundance either in the 10276 WT, 10276 bipD::pDM4, or 10276 bsaP samples over the 10276 bsaZ::pDM4 sample as a denominator (i.e. 10276 WT/10276 bsaZ::pDM4, 10276 bipD::pDM4/10276 bsaZ::pDM4, and 10276 ΔbsaP/10276 bsaZ::pDM4). The ratios were normalized to the median for each strain and a density plot showed that the majority of protein ratios clustered around the median of one, indicating that the mutations made did not radically change the secretion profile of the bulk of proteins (supplemental Fig. S5). From this analysis we identified a core set of 426 proteins, out of the predicted total proteome of B. pseudomallei K96243 of ~5900 proteins (68), that were present in all samples. These are listed in supplemental Table S4. Strikingly, the majority of these proteins (~80%) are encoded by Chromosome 1.

Of the proteins identified in the core secretome, 54 are annotated with reference to the K92643 genome as hypothetical (uncharacterized) proteins, several more are annotated with metabolic functions (supplemental Table S4). The core secretome also consisted of several elongation factors (Ts, G, P, Tu) and heat shock/chaperone proteins (GroES, GroEL), many of which have previously been reported to be present on the surface of B. pseudomallei or part of the outer membrane proteome and recognized by the sera of recovering melioidosis patients (35, 69–71). In addition to the presence of a collagenase, chitobiase, bacterioferritin, and Sec secretory pathway constituents, the secretome also harbored several proteases and lipoproteins. Several phase components, Type I fimbriae, and flagellar subunits were also present, although there was a notable lack of any of the predicted T6SS structural or effector proteins, Type V family of autotransporters or the Type IV pilus protein PilA.

Prediction of the Bsa Effector Repertoire—Putative T3SS effector proteins were identified in the data sets primarily on the calculated iTRAQ protein abundance ratios. The protein abundances obtained in each of the WT or mutant strain samples were each expressed as a ratio of the 10276 bsaZ::pDM4 sample and proteins with an iTRAQ ratio higher than the 1.5 × interquartile range (IQR), together with at least two unique peptides with a Mascot peptide score ≥21, were taken to be T3SS effector protein candidates. The 1.5 × IQR values were calculated and the cut-offs set at 1.56 for the 10276 WT/10276 bsaZ::pDM4 data set, 1.79 for the 10276 bipD::pDM4/10276 bsaZ::pDM4 data set, and 1.46 for the 10276 ΔbsaP/10276 bsaZ::pDM4 data set, based on the calculation described under “Experimental Procedures.”
the data was plotted as a boxplot, the data showed a trend toward more proteins with larger ratios in the 10276 bipD::pDM4 (bipD) or 10276 ΔbsaP (bsaP) samples compared with the 10276 bsaZ::pDM4 (bsaZ) sample. The “cut-off” values (calculated using R software) are shown by the dotted lines. Points shown above the "cut-off" value are candidate T3SS effectors. A, Boxplot generated by R software (www.r-project.org) showing the distribution and median values of the ratios of the protein abundances in the 10276 WT (WT), 10276 bipD::pDM4 (bipD), or 10276 ΔbsaP (bsaP) samples compared with the 10276 bsaZ::pDM4 (bsaZ) sample. The "cut-off" values (calculated using R software) are shown by the dotted lines. Points shown above the "cut-off" value are candidate T3SS effectors. B, A Venn diagram (constructed with Venny; www.bioinfogp.cnb.csic.es/tools/venny) depicting the number of strain-specific and shared candidate T3SS effector proteins identified in each of the strains 10276 WT (WT), 10276 bipD::pDM4 (bipD), and 10276 ΔbsaP (bsaP). C, Distribution of the candidate T3SS effector proteins plotted according to B. pseudomallei gene number across both Chromosomes and their ratios. Gene numbers refer to the B. pseudomallei K96243 reference strain. The bsa T3SS locus is indicated by the red box.

Fig. 2. Proteins with high iTRAQ protein abundance ratios are enriched in hypersecreting B. pseudomallei strains and encoded within the bsa T3SS locus. A, Boxplot generated by R software (www.r-project.org) showing the distribution and median values of the ratios of the protein abundances in the 10276 WT (WT), 10276 bipD::pDM4 (bipD), or 10276 ΔbsaP (bsaP) samples compared with the 10276 bsaZ::pDM4 (bsaZ) sample. The “cut-off” values (calculated using R software) are shown by the dotted lines. Points shown above the "cut-off" value are candidate T3SS effectors. B, A Venn diagram (constructed with Venny; www.bioinfogp.cnb.csic.es/tools/venny) depicting the number of strain-specific and shared candidate T3SS effector proteins identified in each of the strains 10276 WT (WT), 10276 bipD::pDM4 (bipD), and 10276 ΔbsaP (bsaP). C, Distribution of the candidate T3SS effector proteins plotted according to B. pseudomallei gene number across both Chromosomes and their ratios. Gene numbers refer to the B. pseudomallei K96243 reference strain. The bsa T3SS locus is indicated by the red box.

The data was plotted as a boxplot, the data showed a trend toward more proteins with larger ratios in the 10276 bipD::pDM4/10276 bsaZ::pDM4 and 10276 ΔbsaP/10276 bsaZ::pDM4 data sets when compared with the 10276 WT/10276 bsaZ::pDM4 data set (Fig. 2A). A total of 26 proteins met the selection criteria and can be considered putative effector proteins (Table I). Of the 26 proteins there were strain-specific subsets, with eight proteins with high ratios in two of the data sets and three proteins with high ratios in all three (Fig. 2B). The three proteins that displayed high ratios in all three data sets, BopC, BopA, and BopE, are all known B. pseudomallei Type III secreted (T3S) effector proteins or predicted to be T3S proteins based on their T3SS-dependent secretion in a surrogate host system (25, 26, 31) (Fig. 2B). Of the 26 proteins, 20 were only found in the hypersecreting mutant strain samples and would not have been identified had our study focused solely on the study of the secreted proteins of the 10276 WT strain (Fig. 2B). Plotting the iTRAQ ratios of the 26 putative effector proteins against their B. pseudomallei K96243 genetic locus number, it is clear that there is a concentration of eight proteins with especially high ratios encoded within the annotated bsa T3SS locus on Chromosome 2 compared with that of the rest of the genome (Table I, Fig. 2C).
As expected we observed Bsa-dependent secretion of BopE in the 10276 WT strain that increased in 10726 bipD::pDM4 and 10276 ∆bsaP culture supernatants that displayed protein abundance ratios (compared to the 10276 bsaZ::pDM4 mutant) higher than the calculated cut-off values for each comparison. The cut-off values were set at 1.56 for the 10276 WT/10276 bsaZ::pDM4 dataset, 1.79 for the 10276 bipD::pDM4/10276 bsaZ::pDM4 dataset, and 1.46 for the 10276 ∆bsaP/10276 bsaZ::pDM4 data set, as described in “Experimental Procedures.” Additional criteria for protein identification included the presence of at least two unique peptides with a MASCOT peptide score of ≥21 corresponding to a 95% confidence level. The proteins are listed in order of gene number with genes from Chromosome one listed first. Those proteins fulfilling the criteria for selection as candidate T3SS effector proteins are highlighted in the gray shaded boxes. Protein score refers to the adjusted sum of scores of the individual peptide scores as calculated by MASCOT. Number of unique peptides refers to the number of significant peptides specific to a protein used in quantification.

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</tbody>
</table>

As expected we observed Bsa-dependent secretion of BopE in the 10276 WT strain that increased in 10726 bipD::pDM4 and 10276 ∆bsaP strains, in full agreement with Western blot data (Fig. 1). BopC and BopA, the latter of which has only been proven to be secreted in a T3SS-dependent manner in EPEC, followed a similar pattern of secretion to BopE. Although both of the two putative translocator proteins, BipB and BipC, were secreted at higher levels in 10276 WT than 10276 bsaZ::pDM4, only BipB was secreted at levels above the cut-off ratio of 1.56. Similar to the effect of Shigella ipaD mutation, the mutation of bipD in B. pseudomallei 10276 caused an increase in secretion of both BipB and BipC into the supernatant compared with 10276 WT. Where 10276 bipD::pDM4 had increased ratios of the translocators, 10276 ∆bsaP showed a decrease in ratios below that of 10276 WT in agreement with Western blot data (Fig. 1). This is further evidence that BsaP plays a similar role in B. pseudomallei to its homolog InvE in Salmonella as a “gatekeeper” protein. The only proven effector missing from this screen was CHBP, however this is not surprising as we have recently reported that the gene encoding CHBP, bpss1385, is absent from the genome of the B. pseudomallei 10276 strain used here (27).

Besides the needle tip protein BipD, both putative translocators BipB and BipC, BopA, BopC, and BopE, there are also two hypothetical proteins encoded within the bsa T3SS locus identified by the screen that have high iTRAQ ratios. The first, BapA (BPSS1528), is a predicted 880 amino acid protein with no conserved domains and homologs only in the closely related species B. mallei and B. thailandensis. Unlike the
known effector proteins in the iTRAQ screen, although secretion of BapA appears to be Bsa-dependent in 10276 WT and the ratio increases in the 10276 ΔbsaP data set, it does not appear to be secreted abundantly by the 10276 bipD::pDM4 mutant. Interestingly, under the conditions used we did not detect BapB or BapC, which are encoded downstream of BapA in the same operon and predicted to be substrates of the T3SS (21).

The other candidate effector protein encoded in the bsa T3SS locus is BprD (BPSS1521), which is annotated as a hypothetical regulator (48). Predicted to be much smaller than BapA at 147aa, BprD is another protein with no known conserved domains and homologs that exist only within closely related Burkholderia species. The iTRAQ ratios suggest that it is not secreted at detectable levels by 10276 WT B. pseudomallei, but secreted by both 10276 bipD::pDM4 and 10276ΔbsaP, with the ratio of 13.4 in the 10276 ΔbsaP/10276 bsaZ::pDM4 data set being the highest of any protein described in this iTRAQ study.

Outside of the Bsa T3SS locus the remaining 18 proteins with high ratios are distributed across both B. pseudomallei chromosomes. One of the proteins encoded by a gene closely flanking the bsa locus is the virulence factor TssM (BPSS1512), which is known to be coregulated with the Bsa T3SS as well as secreted in a Type II-dependent manner (72).

Validation of iTRAQ Data by Analysis of the Secretion of Epitope-Tagged Candidate Effector Proteins—To confirm a number of targets are secreted in a Bsa-dependent manner, a number of candidates were selected and cloned into an inducible expression vector as in-frame C-terminal c-Myc tagged fusion proteins. The targets chosen were BprD, BapA, BPSS0860, BPSS1512, BopE, and BopA, as well as BPSS1916, a protein with a very high iTRAQ ratio in all strains that did not meet the initial selection criteria because of being quantified with only a single unique peptide. Targets were chosen to represent proteins with both high and low protein scores, high and low numbers of peptides, and to be encoded inside and outside of the bsa T3SS locus. The plasmids were transformed into strains 10276 WT, 10276 bsaZ::pDM4, 10276 bipD::pDM4 and 10276 ΔbsaP, and bacterial supernatants were collected and assessed by Western blot for the presence of the tagged proteins using a c-Myc tag-specific polyclonal antibody. Initial studies in which BopE was cloned as a c-Myc fusion in the widely used shuttle vector pBHR1 under a constitutive chloramphenicol resistance gene promoter indicated that BopE-c-Myc was secreted in a BsaZ-independent manner, possibly owing to the high level of expression (data not shown). We therefore elected to clone effector-c-Myc fusions under an IPTG-inducible expression vector pME6032 in B. pseudomallei strains 10276 WT, 10276 bsaZ::pDM4, 10276 bipD::pDM4 or 10276 ΔbsaP. Supernatant and whole cell fractions were collected after 6 h of incubation at 37 °C in the presence of 0.5 mM IPTG. Equal quantities of protein were separated by reducing SDS-PAGE, blotted onto nitrocellulose membranes and probed using polyclonal rabbit α-c-Myc antibody.

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of the Bsa T3SS, not just the locus of enterocyte effacement-encoded T3SS in EPEC (31). Furthermore, both of the putative effector proteins located within the bsa locus, BprD-c-Myc and BapA-c-Myc, were secreted in a BsaZ-dependent manner (Fig. 3), confirming that these are novel effectors of the Bsa apparatus.

Interestingly, we were unable to validate Bsa-dependent secretion of proteins encoded outside of the Bsa T3SS locus. BPSS0860 and BPSS1512 appeared in the supernatants of all strains, including 10276 bsaZ::pDM4, indicating that although they are secreted, the Bsa system is not required for their secretion (Fig. 3). Secretion of the protein BPSS1916 could not be detected in any of the strains tested (Fig. 3). These examples highlight the need for independent validation of such data sets.

**DISCUSSION**

*B. pseudomallei* is an important cause of invasive clinical disease in South-East Asia and Northern Australia and its
potential as a bioterrorism agent is cause for concern. One of the known virulence factors that is important for invasion, intracellular net replication and virulence in mice is the Bsa T3SS (21–24), but the repertoire of effector proteins secreted by the system has not yet been fully characterized. To establish a better understanding of the Bsa T3SS, we made mutations in key structural components shown to dysregulate other T3SSs. We show these mutations lead to hyperssecretion of known effector proteins. Using these mutants we have performed a quantitative proteomic analysis of the total secretome of the Bsa T3SS system. This iTRAQ analysis identified all known effector proteins and translocators present in B. pseudomallei 10276, as well as identifying a number of novel putative effector proteins, two of which were independently validated. Also, examining the changes in the abundance of effectors and translocators secreted by the different mutants provides insight into the role the proteins likely play in the temporal regulation of the Bsa T3SS compared with other T3SSs of other Gram-negative bacteria.

The inability of previous proteomics studies to identify known Bsa T3SS effectors highlights the need for the use of more sensitive techniques such as iTRAQ in combination with genetic dysregulation. The B. pseudomallei secretome is very complex (supplemental Table S4, supplemental Fig. S3). This is not surprising when taking into account the large 7.2 Mb genome (68), which encodes ~5900 proteins, of which over 55% are expressed in LB (73). This study produced one of the most comprehensive secretomes of B. pseudomallei to date, identifying a core secretome of 426 proteins secreted under standard laboratory conditions.

Although known effectors were absent from the secreted proteome of the bsaZ mutant by Western blotting (Fig. 1) and largely undetectable by iTRAQ (Table I) implying a lack of cell lysis, we detected a number of ribosomal proteins that are enriched in the supernatant when compared with the supernatant harvested from the 10276 bsaZ::pDM4 strain. This observation has been made in several other proteomic screens of T3SS effector repertoires (50, 74–76) and may be because of a combination of the high abundance of ribosomal proteins in the bacterial cell coupled with the sensitivity of iTRAQ.

Concurrent to this study, a proteome analysis investigating Type II secreted proteins of B. pseudomallei strain MSHR688 was published (72). From our screen, 27 of the 38 proteins identified as being substrates of Type II secretion in LB by Burtnick et al. (72) were also identified in our B. pseudomallei 10276 culture supernatants. Whether all 38 Type II secreted proteins identified in B. pseudomallei MSHR688 are encoded by B. pseudomallei 10276 is unknown because the genome sequence for B. pseudomallei 10276 is not yet available. Interestingly, three of the Type II secreted proteins were enriched in the supernatants of the hypersecreting mutants (BPSL0707, BPSS1512, BPSS1555) and two of the proteins are encoded by regions flanking the bsa T3SS locus, BPSS1512 and BPSS1555. The deubiquitinase BPSS1512 (TssM) has been shown to be secreted inside host cells where it suppresses the host innate immune response (77, 78). Although TssM is secreted independently of the Bsa T3SS, its expression appears to be coregulated with the Bsa T3SS and T6SS-5 (78, 79). This coregulation may explain the increased levels of TssM in the supernatants of the hypersecreting mutants in this study and again highlights the importance of validating candidate effector proteins.

The profile of secreted proteins encoded within the bsa T3SS locus provides a comprehensive picture of the secreted levels of the three known effector proteins. The profiles of both BopE and BopC in the iTRAQ screen correspond with previous studies in which they were shown to be secreted in a Bsa-dependent manner (25, 26). Although BopA has been shown to be Type 3 secreted in a surrogate host (31), we have confirmed its secretion to be dependent on the Bsa T3SS in B. pseudomallei and not one of the other T3SSs.

Two of the putative Bsa T3SS substrates encoded by the bsa T3SS locus (BapA and BprD) were validated as Bsa-dependent by Western blotting for a C-terminal c-Myc tag. Although the function of BapA is currently unknown, a bapA insertion mutation was not attenuated in a Syrian hamster model of acute melioidosis (23). BprD was previously annotated as a transcriptional regulator (48). In a previous study, when bprD was deleted along with bprB and bprC, there was no change in levels of transcription of other Bsa T3SS genes leaving the role of BprD as a regulator in doubt (48). More recently, a bprD mutant of B. pseudomallei K96243 was shown to have increased expression of bprC (80), a downstream gene in the same operon shown to be involved in regulation of the virulence-associated T6SS (81). The bprD mutant also showed decreased time to death in intraperitoneally inoculated BALB/c mice demonstrating its importance in vivo and indicating its possible role as a negative regulator of virulence (80). It is not without precedent that a regulator of the T3SS is also a substrate for secretion (e.g. LcrQ of Yersinia) (82).

Another candidate protein identified in this study, BPSS0860, appears to be a fliD homolog. There is a second copy of fliD (BPSS3320) located within the flagellar locus on Chromosome 1, which had protein abundance ratios that were similar between all strains. Interestingly, the expression of BPSS3320 was up-regulated, whereas the expression of BPSS0860 was down-regulated in the organs of hamsters infected by B. pseudomallei when compared with bacteria grown in vitro (83). Using our c-Myc expression system we were unable to establish Bsa-dependent secretion of BPSS0860; however, it is possible it may be coregulated with the Bsa T3SS in a similar manner to BPSS1512. One recent study has shown an effector protein may be secreted by more than one secretion system, thereby masking its secretion by the T3SS (84). This could also be the case for BPSS0860.
At the time of writing, little is known about the post-translational control of the Bsa T3SS. Generally it is accepted that T3SSs are under tight temporal regulation to ensure substrates are secreted in the correct order (17). This starts with assembly of the membrane-associated structural components, assembly of the needle with needle tip, secretion of translocators to form a pore in the host cell membrane, followed by secretion of effector proteins. Changes in the patterns of secreted translocators and effector proteins in different mutant strains can inform us of the possible role these proteins play in the hierarchy of secretion.

Inactivation of the needle tip protein of B. pseudomallei, BipD, shows a large increase of levels of both translocators and effectors in the culture supernatant, in agreement with data published for the homologous Shigella protein IpaD (46, 52). IpaD is the needle cap protein, acting to block secretion of effector proteins until host cell contact has taken place (85). Because of its high similarity in both sequence and structure (S3) it is perhaps unsurprising that BipD would have a similar effect on the levels of substrates secreted by the Bsa T3SS. Interestingly there was a high ratio of BipB, but not BipC in the WT B. pseudomallei 10276 strain culture supernatant compared with 10276 bsaZ::pDM4 (Table I). In Shigella, IpaB is present in the needle tip in complex with IpaD, whereas IpaC is secreted later, only upon host cell contact or activation (86). With this in mind it is possible that growth in LB lacks the stimuli required for BipC secretion in WT B. pseudomallei 10276.

Here we present evidence that BsaP functions as a “gatekeeper” protein for effectors in a manner similar to the homologous T3SS proteins InvE/Sepl/MxiC/YopP-TyeA (54–60). Members of this family of proteins function differently from each other, but are all thought to be important in allowing effectors to be secreted by the mature T3SS. Here we show that deletion of bsaP creates a phenotype in which effector proteins are hypersecreted and levels of translocators decrease. To fully understand BsaP in the context of the other members of the “gatekeeper” family of proteins, more work would be required to determine the molecular interactions of BsaP with other components of the Bsa T3SS.

This study is another example of the power of a combination of classic targeted mutagenesis and a gel-free quantitative proteomics approach, such as iTRAQ, to study a complex secretion system, providing insights into the involvement of certain proteins on the regulation of the T3SS itself. Two new substrates of the Bsa T3SS were revealed by the study, confirming the effector repertoire is more complex than reported so far.

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This article contains supplemental Figs. S1 to S5 and Tables S1 to S4.

17. Böttner, D. (2012) Protein export according to schedule: architecture, assembly, and regulation of type III secretion systems from plant- and


