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A dissertation partially satisfying the requirements for the degree

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in Microbiology, Immunology and Molecular Genetics

by

Christopher Todd French

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ABSTRACT OF THE DISSERTATION

Protein secretion systems of *Bordetella* and *Burkholderia* species and their roles in virulence

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University of California, Los Angeles, 2012

Professor Jeffery F. Miller, Chair

Bordetella and *Burkholderia* species are significant human and animal pathogens. *Bordetella pertussis*, *B. parapertussis* and *B. bronchiseptica* colonize respiratory epithelium to cause human pertussis and pertussis-like respiratory illnesses, while *Burkholderia pseudomallei* and its less-virulent relative *Burkholderia thailandensis* are soil organisms that can behave as opportunistic pathogens and survive as facultative intracellular parasites. *Bordetella* and *Burkholderia* are well equipped with virulence determinants, including multiple protein secretion systems. In the first section of this dissertation I describe an analysis of the *Bordetella* type III secretion (T3SS) effector protein BteA, which is necessary and sufficient for the induction of

rapid toxicity in a variety of mammalian cell lines. We identify a N-terminal domain of BteA that mediates its localization to lipid raft domains of mammalian cells following ectopic expression and T3SS-mediated translocation by *B. bronchiseptica*. The rest of the dissertation is focused on the secretion systems and virulence mechanisms in *B. pseudomallei* and *B. thailandensis*. Unlike *Bordetella*, which contains a single injection-type T3SS and one known effector protein BteA, *Burkholderia pseudomallei* contains three injection T3SSs that function in mediating its interactions with other organisms in the rhizosphere and in mammalian pathogenesis. In the second section of the dissertation, the roles of T3SS and other virulence mechanisms are closely examined in the intracellular lifecycle of *Burkholderia*. Bacteria were delivered directly into the cytoplasm of mammalian cells using a photothermal nanoblade device, bypassing invasion and the need to escape from endosomes. Activity of the *Burkholderia* secretion apparatus T3SS (T3SS$_{Bsa}$) was critical for endosome escape following infection, but was not required for subsequent actin polymerization, intercellular spread and the fusion of host cell membranes to form multinucleate giant cells (MNGCs) following cytosolic nanoblade delivery of bacteria. Motility, mediated by either the Fla2 flagellar system or actin polymerization, was required for cell-cell spread and MNGC formation by *B. thailandensis* and *B. pseudomallei*, as was the activity of a type VI secretion (T6SS) system. We conclude that the primary means for intercellular spread involves cell fusion, as opposed to pseudopod engulfment and bacterial escape from double-membrane vacuoles. The third section of the dissertation describes further analysis of the Fla2 flagellar T3SS and *B. pseudomallei* injection T3SS effector proteins. Using confocal immunofluorescence microscopy, we discovered that *fla2* encodes multiple, lateral flagellar filaments and is activated following infection of mammalian cells. Surprisingly, constitutive expression of a Fla2 response regulator protein, Frr, strongly reduced

plaquing efficiency in *B. pseudomallei* and *B. thailandensis*. I also describe the identification and analysis of novel <u>B</u>. *pseudomallei* <u>e</u>ffector <u>p</u>rotein (Bep) candidates. Specific phenotypes for six Bep proteins were observed following transfections of HeLa cells; BepA exhibited perinuclear localization, BepB resulted in cytotoxicity and alterations in cell morphology, BepC bundled actin, BepD decorated microtubules, and BepE and BepF rearranged actin and altered cell morphology. However, strains carrying deletion mutations in single Bep effector candidate loci did not exhibit defects following infection of mammalian cells. A single strain containing deletions in BopA, BopC and BopE, the three previously known T3SS$_{Bsa}$ effectors, exhibited reduced plaque formation in RAW264.7 macrophages. Of the three effectors, only BopA was seen to play a major role in *B. pseudomallei* intracellular pathogenesis.

The dissertation of Christopher Todd French is approved.

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…FOR DAD.

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Chapter 1. Introduction: secretion systems and virulence mechanisms in *Bordetella* and

Burkholderia species

A living cell continuously exchanges information with its extracellular space by importing nutrients and secreting waste products, sensing the environment and communicating with its neighbors. Both eukaryotic and prokaryotic cells may move about to seek nutrients or flee from predators. Bacterial pathogens have an additional requirement of communicating with the host and its various defense mechanisms. These tasks are accomplished by one of the most basic functions of biological systems: the transport of biomolecules across the cell envelope. In Gram-negative bacteria, the second outer membrane poses an additional obstacle for the transport of proteins, while facilitating the colonization of surfaces and acting as a barrier against antimicrobial compounds. As such, Gram-negative species have evolved a remarkable array of mechanisms for the secretion of proteins and biomolecules that function in biogenesis, nutrient acquisition, motility and virulence (Gerlach and Hensel, 2007). The secretion systems are designated as types 1-VI due to the different mechanisms that have evolved to accommodate the transport needs of various classes of compounds. Although this dissertation will focus mainly on type III and type VI secretion systems as virulence determinants, a brief overview of the different Gram-negative secretion systems is presented below. A schematic is shown in Fig. 1.

Secretion systems in Gram-negative species

Protein transport across the outer membrane of Gram-negative bacteria can be subdivided into Sec-independent and Sec-dependent pathways. The three main pathways allowing further transport of Sec-transported periplasmic intermediates to the cell surface or the extracellular space are: the chaperone-usher pathway for the synthesis of fimbrial adhesins, the autotransporter (T5SS) pathway and the complex type II secretion system (T2SS). No stable periplasmic intermediates are found in type I, type III, and type IV secretion systems (T1SS, T3SS, T4SS), where the secretion mechanism for Type VI secretion is still poorly resolved. Each

of the three export mechanisms features a protein-conducting channel able to span the two bacterial membranes and, in the case of T3SS, T4SS and T6SS, one additional membrane of the target cell.

The general secretory pathway Components for the general secretory pathway (i.e. the Sec-dependent pathway) are found in bacteria, archaea and in eukaryotic organelles (chloroplasts and the endoplasmic reticulum). Proteins targeted for the Sec pathway possess an N-terminal signal peptide, and are transported in an unfolded state into the periplasm by a multi-subunit translocon composed of SecA (Rusch and Kendall, 2007) and two heterotrimeric complexes, SecYEG and SecDFYajC (Yahr and Wickner, 2000). The Sec translocon is inserted into the inner membrane (IM), and upon translocation of the protein, the signal peptide is cleaved by specific periplasmic signal peptidases, and the mature protein is released in the periplasmic space. If additional signals are present, these might be recognized by components of the terminal branch pathways for subsequent outer membrane (OM) insertion or translocation into the extracellular milieu (i.e. type II secretion; see below). Numerous bacterial proteins utilize this pathway; analysis of proteobacteria genomes indicate that up to 17% of the coding capacity of some organisms may be specific for proteins exported by the Sec-mediated pathway (Gerlach and Hensel, 2007).

Type I secretion systems Type one secretion systems (T1SS) are generally heterotrimeric complexes consisting of an inner membrane ATP-binding cassette (ABC) exporter protein, a membrane fusion protein (MFP) and a pore-forming, outer membrane protein (OMP). T1SS allows the single-step secretion of a wide range of protein and non-protein substrates from the cytoplasm to the extracellular space (Binet *et al.*, 1997). The adenylate cyclase toxin of *Bordetella* (Shrivastava and Miller, 2009) and the antibiotic efflux pumps and secreted

hemolysin of *B. pseudomallei* are relevant examples of export by T1SS (Andersen, 2003; Harland *et al.*, 2007).

Type II secretion systems The T2SS pathway is a main terminal branch of the general secretory pathway and depends on the Sec machinery. It is used by Gram-negative bacteria to secrete enzymes and toxins across the OM. The T2SS consists of a multi-subunit complex inserted into the IM, with some of the components containing extensive cytoplasmic domains (Gerlach and Hensel, 2007). Cholera toxin of *Vibrio cholerae* is a notable example of a substrate exported by T2SS (Stathopoulos *et al.*, 2000).

Type III secretion systems The type III secretion system (T3SS) is a complex structure spanning the bacterial inner membrane, periplasm and outer membrane. T3SS have been identified in several Gram-negative species, many of which are important pathogens including *Bordetella, Burkholderia, E. coli, Pseudomonas, Salmonella, Shigella* and *Yersinia*. The T3SS is composed of more than 20 different subunits enabling the translocation of protein "effectors" directly into the cytoplasm of the host cell where they manipulate numerous cellular processes to benefit the pathogen (Ghosh, 2004).The T3SS is also referred to as an 'injectisome' or 'molecular syringe' (Cornelis, 2006). Injection-type T3SSs are closely related to **t**he flagellum. (Woestyn *et al.*, 1994; Van Gijsegem *et al.*, 1995). Both systems consist of a secretion apparatus for the sequential export of structural components and secretion substrates (Macnab, 2003). The injectisome is as ancient as the flagellum and both share a common ancestor (Kubori *et al.*, 1998). Therefore, T3SS is a protein export pathway used by two different nanomachines, and both the injection-type and flagellar systems are known as "type III secretion".

The *Yersinia* Ysc apparatus is one of the best characterized T3SSs, and a general attempt has been made in the literature to adopt the genetic nomenclature laid out by Cornelis, although it has not by any means been universally followed (Cornelis, 2006). Structurally, two oligomeric rings form the basal body of the T3SS. The ring complex inserted into the IM consists of multiple proteins. The complex is composed of YscR, S,T, U and V. A central periplasmic cylinder, the 'inner rod', is built of YscD subunits and protrudes from an IM ring socket structure (Marlovits *et al.*, 2004; Diepold *et al.*, 2010). The OM ring "secretin" is composed of a 12-14mer of YscC . To the cytoplasmic face of the basal body the YscN ATPase is bound, presumably as a double-hexameric ring, that energizes the translocation (Muller *et al.*, 2006). A characteristic of T3SS is the utilization of effector chaperones – small acidic proteins with a characteristic fold (Panina *et al.*, 2005; Cornelis, 2006). T3SS chaperones stabilize and prevent terminal folding of effector proteins. The energy of ATP hydrolysis by theT3SS YscN ATPase is conducted by release of the chaperone from an effector chaperone complex and subsequent loading of the unfolded effector into the T3SS apparatus (Akeda and Galan, 2005). Another function of the effector-specific chaperones could be the masking of domains needed for membrane targeting in the host cell, or prevention of multimerization and aggregation of effector proteins in the bacterial cytoplasm (Letzelter *et al.*, 2006). The rigid extracellular needle forms a protein-conducting channel extending the inner rod. The hollow cylinder is made of several hundred copies of proteins of the YscF family (Cornelis, 2006), and shows an inner diameter of 2-3 nm, which only supports translocation of at least partially unfolded substrates. The heteromultimeric translocon complex is present at the tip of the needle and is thought to mediate the formation of a pore in the host cell membrane. In *Yersinia* the translocon has been shown to consist of YopB and YopD (Viboud and Bliska, 2005). Reconstitution of pore complexes has been performed

using purified translocon proteins from *Pseudomonas aeruginosa* (Schoehn *et al.*, 2003). These studies revealed asymmetrical pores with an inner diameter ranging between 30 and 50 Å.

Type IV secretion systems (T4SS) T4SS are characterized by the ability to translocate protein complexes with single stranded DNA (type IVA) or protein effectors (type IVB) (Gerlach and Hensel, 2007). Based on sequence similarities, the system is believed to have evolved from bacterial conjugation machineries (Cascales and Christie, 2003). Recent work demonstrated the roles of T4SS in several important human bacterial pathogens. Secretion of pertussis toxin by *B. pertussis* requires a functional Ptl T4SS, while CagA of *Helicobacter pylori* is a translocated T4SS effector that induces inflammatory responses and cytoskeletal alterations in the host cell. Virulence-associated T4SSs have also been identified in *Legionella, Brucella* and *Bartonella,* and the translocated effectors have central functions in the intracellular lifestyle of these pathogens (Gerlach and Hensel, 2007).

Type V secretion systems (T5SS) Type V secretion system (T5SS) proteins, also known as autotransporter proteins, include the two-partner system (TPS) and the oligomeric coiled-coil adhesin system. Autotransporters are targeted by an N-terminal signal sequence to the IM Sec machinery. Following cleavage of the signal sequence, the protein is released into the periplasmic space, where it inserts into the OM. A *β*-barrel secondary structure forms which allows translocation across the outer membrane. The "effector" domain may be cleaved off after passing the OM and released into the extracellular environment. A group of non-fimbrial adhesins belonging to the family of surface-attached autotransporters form oligomeric complexes. Their C-terminal translocation units interact to form a *β*-barrel pore which functions as a membrane anchor (Koretke *et al.*, 2006). The most prominent member of this family of membrane proteins is YadA from *Yersinia* spp. (Roggenkamp *et al.*, 2003). *Bordetella* pertactin

is a T5SS transported surface adhesin (Inatsuka *et al.*, 2010). *Burkholderia* BimA, which functions to bind and polymerize host cell actin, is also a member of this group (Lazar Adler *et al.*, 2011).

Type VI secretion systems (T6SS) Genome-wide screens for homologs of the type IVB secretion system component IcmF revealed a group of pathogenicity islands in Gram-negative bacteria that have been named the IcmF-associated homologous protein locus (Das and Chaudhuri, 2003). The identification of these elements in *V. cholerae* resulted in the designation of an additional group of secretion systems, termed type VI. T6SSs manage the export of substrates without the requirement of hydrophobic N-terminal signal sequences. Using the model host *Dictyostelium discoideum*, a virulence function for T6SS-secreted substrates could be shown in *V. cholerae*, and suggested translocation of effectors into the cytoplasm (Pukatzki *et al.*, 2006). Another T6SS was identified in *P. aeruginosa* (Mougous *et al.*, 2006). In this study an ATPase, ClpV, was identified, that presumably energizes secretion. Furthermore, the T6SS structural component Hcp was secreted in a T6SS-dependent manner and was identified in cystic fibrosis patients with *P. aeruginosa* infection. T6SS's have since been identified in numerous species, mostly in the context of virulence, including pathogenic *Burkholderia* species (Burtnick *et al.*, 2010; Burtnick *et al.*, 2011) which is discussed in more detail below and in Chapters 3 and 5.

A hallmark of all T6SSs is the presence of Hcp (hemolysin co-regulated protein) and VgrG (valine-glycine repeat protein G) proteins in culture supernatants (Pukatzki *et al.*, 2009). The structures of Hcp and VgrG suggest a relationship between T6SSs and the puncturing machinery of bacteriophages. (Pukatzki *et al.*, 2007; Leiman *et al.*, 2009). Hcp forms hexameric rings that can polymerize *in vitro* into tubules (Mougous *et al.*, 2006). VgrG proteins show

structural similarities to the spike complex of the T4 bacteriophage. VgrGs form needle-like structures that could potentially puncture host cell membranes. Consistent with this, direct cell-to-cell contacts are required for T6SS-dependent virulence (Pukatzki *et al.*, 2006). Although the actual secretion mechanism of T6SSs is complex and poorly understood, two T6SS proteins, VipA and VipB, form tubular structures that can be depolymerized by ClpV (Bonemann *et al.*, 2009). VipA/VipB tubules visually resemble the contracted T4 tail sheath and suggests that a sheath-like structure might power translocation by a contraction mechanism. In agreement with this, Basler and colleagues recently demonstrated that that the type VI secretion system in *V. cholerae* cycles between assembly, quick contraction, disassembly and re-assembly, supporting a mechanism where the contraction of the type VI secretion system sheath provides the energy needed to translocate proteins into target cells (Basler *et al.*, 2012).

In summary, Gram-negative bacteria possess a variety of systems for secretion of proteins across the outer membrane, from relatively simple T1SS and T5SS apparatuses to the complex T3SS, T4SS and T6SSs "injectisomes" that facilitate the direct communication of a bacterium with a eukaryotic host cell. Chromosomal loci encoding the virulence-related secretion systems can be found near other chromosomal elements encoding functions for different phases of pathogenesis. Some organisms such as *Burkholderia, Salmonella* and *Yersinia* may possess multiple copies of T3SSs, indicating the repetitive acquisition or divergence of these gene clusters for new or multiple functions. Much remains to be learned regarding the temporal and spatial control of the expression and function of various protein secretion systems in bacterial pathogens.

Bordetella

Bordetella species are Gram-negative Betaproteobacteria in the order Burkholderiales. *Bordetella* sp. are well-endowed with virulence determinants and are important pathogens of humans and animals. *B. pertussis* is the cause of whooping cough in humans, which manifests as a severe respiratory infection and paroxysmal coughing (Mattoo and Cherry, 2005; de Gouw *et al.*, 2011). Pertussis was first described in the Middle Ages, when it was called "the kink," which in the Scottish vernacular of the time was synonymous with "paroxysm", and "kindhoest", denoting a child's cough. Guillaume de Baillou (1536-1616), described the first pertussis epidemic in 1578 (Cherry, 1996). Pertussis illness may have fatal complications in infants. Its close relative, *B. parapertussis* is associated with milder, pertussis-like disease. Despite extensive vaccination strategies, pertussis remains a leading cause of vaccine preventable deaths (He and Mertsola, 2008). *B. bronchiseptica*, the evolutionary progenitor of *B. pertussis* and *B. parapertussis*, usually causes asymptomatic infections in humans, although acute and chronic disease in domesticated animals is often reported (Cotter and Miller, 1994). *B. pertussis*, however, is exclusively a human pathogen. Genomic data suggest that host restriction and the ability to cause acute disease is a recently evolved characteristic of *B. pertussis* (Preston *et al.*, 2004).

Bordetella is equipped with protein export pathways that function in pathogenesis, many of which are adhesins that facilitate bacterial attachment and colonization of the respiratory epithelium (Shrivastava and Miller, 2009). Otherwise, only three substrates secreted by canonical systems are known to enter target cells. Two of these, adenylate cyclase toxin and pertussis toxin have been thoroughly studied, whereas the third, BteA, is a recently discovered T3SS substrate (Panina *et al.*, 2005). A description and analysis of BteA is presented in Chapter 2.

***Bordetella* type III secretion** The Bsc T3SS locus is highly conserved in *B. bronchiseptica*, *B. pertussis* and *B. parapertussis*. Due to the lack of suitable animal models involving the use of *B. pertussis*, most studies of the *Bordetella* T3SS *in vivo* have utilized *B. bronchiseptica* (Yuk *et al.*, 1998; Yuk *et al.*, 2000). The Bsc apparatus and its associated chaperones and regulatory factors are encoded by twenty-two contiguous genes. Most have close homologs in the prototypical *Yersinia* Ysc T3SS with the notable exception of *bsp22*. Bsp22 self-polymerizes into a filamentous, flexible conduit connecting the T3SS needle to the pore-forming translocation apparatus (Medhekar *et al.*, 2009). Bsp22 antibodies protect epithelial cells from T3SS-dependent killing *in vitro*, and immunization with Bsp22 provides protection in mouse models of pathogenesis, presumably by blocking the connection between the T3SS needle and translocation pore (Medhekar *et al.*, 2009).

The Bsc T3SS causes rapid cytotoxicity in a variety of cell types *in vitro*. The mechanism is complex and neither fully apoptotic nor necrotic, and is dependent on the function of *bteA* (Stockbauer *et al.*, 2003; Panina *et al.*, 2005). Strains containing *bteA* null mutations exhibit dramatically reduced cytotoxicity following infection of cells in culture. In fact, *bteA* null deletions recapitulate BscN ATPase mutant phenotypes that eliminate T3SS function altogether (Panina *et al.*, 2005). BteA will kill virtually any cell type tested, yet paradoxically, most *B. bronchiseptica* infections are asymptomatic and do not result in tissue destruction. Efforts to understand cytotoxicity have been hampered since BteA is the only known T3SS effector so far discovered for *Bordetella*. Although the hunt for additional effectors continues, the possibility exists that the Bsc apparatus is dedicated to secreting BteA as its only translocated substrate.

BscN, bsp22 or *bteA* null mutants display a reduced ability to establish a persistent lower respiratory tract infection in mice following intranasal inoculation (Yuk *et al.*, 1998; Yuk *et al.*,

2000) (Ahuja U, Richards DM, Miller JF, unpublished). Evidence is mounting that the *Bordetella* T3SS possesses an immunomodulatory function. Infection of mice with wild type *B. bronchiseptica* results in decreased IFN-γ production and increased IL-10 production in splenocytes compared to animals infected with Δ *BscN* mutant strains. Furthermore, IFN-γ−/− mice do not clear *B. bronchiseptica* from the lower respiratory tract as quickly as wild type animals, whereas IL-10−/− mice display more rapid clearance (Skinner *et al.*, 2005; Pilione and Harvill, 2006). These results suggest that the T3SS alters the equilibrium between immunostimulatory and immunosuppressive cytokine production. Interestingly, *B. bronchiseptica* Δ *BscN* Δ*cyaA* mutants are highly attenuated in animals yet capable of conferring effective immunity against infection with wild type strains (Mattoo *et al.*, 2004; Mann *et al.*, 2007).

Perhaps the most relevant issue regarding the Bsc system is its role during *B. pertussis* infections in humans. Earlier studies indicated that T3SS-associated phenotypes were not observed with laboratory strains of *B. pertussis*. This was surprising since the T3SS genes are intact, highly conserved, transcribed, and regulated in *B. pertussis* (Mattoo *et al.*, 2004). Next, the vast majority of nucleotide substitutions between the *B. bronchiseptica* and *B. pertussis* Bsc loci are silent or result in conservative amino acid changes, implying positive selection. Finally, the *bteA* locus is conserved and functionally interchangeable between *B. pertussis* and *B. bronchiseptica* (Panina *et al.*, 2005). Recent studies have begun to explain these findings (Fennelly *et al.*, 2008). T3SS activity was not detected in common *B. pertussis* laboratory strains, but was observed in low-passage strains and clinical *B. pertussis* isolates. Although BteA effector secretion was not examined and there was no indication of T3SS-mediated cytotoxicity *in vitro*, infection with Δ *BscN* mutants did result in elevated proinflammatory cytokine levels

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and accelerated clearance from the lungs of aerosol-infected mice. A description and analysis of

BteA is presented in Chapter 2.

Burkholderia

　　Burkholderia species (family Burkholderiaceae, order Burkholderiales), are ubiquitous Gram-negative rod-shaped bacteria that occupy diverse ecological niches. Previously classified as *Pseudomonas,* the *Burkholderia* genus encompasses over forty species (Estrada-De Los Santos *et al.*, 2001; Coenye and Vandamme, 2003; Konstantinidis *et al.*, 2006), whose metabolic diversity is thought to facilitate adaptation to a variety of environmental conditions including nutrient limitation, temperature extremes, pH variations and the presence of antibiotics and toxic compounds. Many Burkholderiaceae are plant symbionts and may fix nitrogen from the environment (Vandamme *et al.*, 2002; Bontemps *et al.*, 2010; Gyaneshwar *et al.*, 2011). Some, such as *B. caryophylli* (Hang *et al.*, 2002), *B. plantari* (Mitchell and Teh, 2005), and *B. glumae* (Cottyn *et al.*, 2001) are recognized phytopathogens, while others, including the *B. cepacia* complex, can infect plants and also cause opportunistic infections in humans (Taylor *et al.*, 1992; Ellis and Cooper, 2010). *Burkholderia pseudomallei*, one of the subjects of this dissertation, is the agent of melioidosis; a serious and often lethal infection in humans and animals (Wiersinga *et al.*, 2006). *Burkholderia mallei*, the causative agent of glanders in equids, is a host-restricted descendent of *B. pseudomallei* (Galyov *et al.*, 2010). Both organisms are considered to be category B select agent pathogens and potential bioterrorism threats due to their ease of aerosol transmission, low infectious doses, lack of available vaccines and historical use in biowarfare (Galyov *et al.*, 2010). Together with the related but less virulent *Burkholderia thailandensis* (Brett *et al.*, 1998), these organisms may be collectively referred to as the "pseudomallei group" of pathogenic *Burkholderia*.

Melioidosis Nearly all human melioidosis infections are acquired from the environment, and agricultural workers are especially at risk (Peacock, 2006; Limmathurotsakul and Peacock, 2011;

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Rolim *et al.*, 2011). As such, human and animal infections with *B. pseudomallei* are likely accidental albeit life-threatening due to the organisms adaptability and high degree of hardiness (Nandi *et al.*, 2010). Although melioidosis is most commonly recognized in Thailand and Northern Australia, the disease is endemic in many other countries, including Malaysia, Singapore, Vietnam, and Burma (Wiersinga *et al.*, 2006). Other possible endemic areas include Brunei, Southern China, Hong Kong, Cambodia, Laos, Southern India, and Taiwan. Additional cases have been reported in Africa, Brazil, the Caribbean, the Middle East, and the Pacific (Wiersinga *et al.*, 2006), and have been known to occur following travel to endemic areas, including instances involving American military personnel deployed to tropical regions (Howe *et al.*, 1971; Currie *et al.*, 2000). Seroprevalence of *B. pseudomallei* may reach over 24% in the most endemic areas and is established at an early age (Kanaphun *et al.*, 1993). Melioidosis has been termed "the great mimicker", with protean clinical manifestations resembling those caused by other infectious agents. Pulmonary infections with associated dissemination and septicemia are the most serious forms of the infection (Dance, 1991; Dance, 2002; Cheng and Currie, 2005). The mortality rate in Thailand and Australia among treated individuals averages 50% and 20%, respectively. If untreated, the infection is almost always fatal (Stone, 2007). *B. pseudomallei* is known for its ability establish latency in human and animal hosts, resulting in asymptomatic infection, and can reactivate up to several decades later (Yap *et al.*, 1991; Leelarasamee, 1998).

Ecology *B. pseudomallei* poses an emerging global infectious disease problem as well as a bioterrorism threat (Dance, 2000). Despite its ability to cause serious illness in humans and animals, infections are most often acquired from the environment, while person to person transmission is rare (Holden *et al.*, 2004; Inglis and Sousa, 2009). No single host organism has been identified. Accordingly, mammalian disease is likely to represent more of an "accident"

than the product of direct evolutionary selection, and the virulence mechanisms that operate in mammals would thus be a reflection of those that are important for environmental interactions. There are good reasons to consider pathogenesis in its ecological context. Virulence and colonization factors that are important for environmental persistence are subject to coordinate regulation and the linkages between these systems can be used to identify common virulence and regulatory networks that are relevant for mammalian infection. Finally, viewing *B. pseudomallei* virulence in terms of its ecology affords a highly relevant perspective from which to learn about this fascinating organism and an outstanding opportunity to study how pathogens evolve.

Areas of *B. pseudomallei* endemicity are mostly tropical regions between 20° latitude north and south of the equator (Currie *et al.*, 2008), and the organism can often be isolated from the soil and water in these locations (Inglis and Sousa, 2009; Corkeron *et al.*, 2010; Baker *et al.*, 2011). Since it is commonly found in soil and water, *B. pseudomallei* is thought to prefer to inhabit the rhizosphere as opposed to a lifestyle as a professional pathogen. The rhizosphere is home to intense biological activity, competition and predation (Fig. 2). The arsenal of *B. pseudomallei* virulence factors have likely evolved to facilitate competition for nutrients and as defensive measures against other soil organisms. *B. pseudomallei* is capable of interactions with *Acanthamoeba* (Inglis *et al.*, 2000; Inglis *et al.*, 2003), nematodes (Gan *et al.*, 2002; Lee *et al.*, 2011), and insects (Warawa, 2010; Wand *et al.*, 2011). A recent study by Schell and colleagues identified the Bsa T3SS as a central virulence determinant in survival against predation by *Dictyostelium discoideum* (Hasselbring et al., 2011). The natural host or hosts of *B. pseudomallei* remain to be positively identified, however the organism has been recently found in roots and tissues of exotic Australian grasses (Kaestli *et al.*, 2011), indicating that plants may be at least one possible environmental niche of *B. pseudomallei*.

Like other intracellular bacterial pathogens such as *Legionella pneumophila* and *Listeria monocytogenes* (Rowbotham, 1980; Ly and Muller, 1990), one largely unexplored reservoir for *B. pseudomallei* may be within unicellular environmental organisms, such as protists, fungi and algae. Similar to *Legionella*, which is also an environmental saprophyte capable of intracellular survival, *B. pseudomallei* infections rarely spread from person to person. Intracellular growth within protozoa and subsequent acquisition by human hosts has been proposed to account for some aspects of *Legionella* virulence (O'Brien and Bhopal, 1993). Initial adhesion of *B. pseudomallei* to the protozoan *Acanthamoeba astronyxis* involves polar attachment via flagella (Inglis *et al.*, 2003). Following engulfment by pseudopodia, the viable bacteria are observed both within vacuoles and free in the cytoplasm (Inglis *et al.*, 2000). It is predicted that mechanisms similar to those used for invasion and survival within protists are also used during infection of human phagocytes.

Most meliodosis cases are acquired from environmental sources. However, there is some evidence that infected humans and other animals might play an inadvertent role in the spread of the organism to new environments. *B. pseudomallei* is found in the sputum, pus, urine and waste of infected animals and humans, which is destined to contaminate their surrounding environment (Whitmore, 1913; Kaestli *et al.*, 2007) . Indeed, there is a higher probability of isolating *B. pseudomallei* from the soil of pens and paddocks where animals are kept, however it has not been established whether this is due to spread from infection or from increased agitation of the soil in these areas (Kaestli *et al.*, 2009). It is known that *B. pseudomallei* is highly virulent in most domesticated animals (Warawa, 2010), and outbreaks have occurred following the importation of animals from endemic areas (Fournier, 1965). Although the overall picture of *B. pseudomallei* etiology remains somewhat enigmatic, these observations taken together suggest

that *B. pseudomallei* is capable of opportunistic interactions with denizens of the rhizosphere and numerous animal species, in addition to its ability to obtain nutrients from decaying organic matter.

Diversity and evolution *B. pseudomallei* is an exceptionally diverse species with a highly dynamic genome subject to frequent mutation, rearrangement and lateral gene transfer (Holden *et al.*, 2004). Genomic regions common to all strains that are involved in essential functions are referred to as the core genome and the variable portion is known as the accessory genome. Genes on genomic islands – regions of DNA acquired by lateral gene transfer, may play an important role in adaptation and pathogenesis (Kim *et al.*, 2005; Sim *et al.*, 2008; Tuanyok *et al.*, 2008; Tumapa *et al.*, 2008; Ronning *et al.*, 2010). Indeed, intraspecies diversity is likely to be largely driven by lateral gene transfer in *B .pseudomallei*. Recently, Pearson *et al.* claimed that the Thai and Australian populations of *B. pseudomallei* conform to biogeographic patterns found in many plant and animal species that are separated by Wallace's Line. *B. pseudomallei* likely originated on the Australian subcontinent, and subsequently spread up the Malay Archipelago into Southeast Asia in a single introduction event sometime between 16 thousand to 225 thousand years ago during recent periods of glaciation and low sea levels. Further, *B. pseudomallei* and *B. thailandensis* appear to have diverged between 307 thousand to 4.27 million years ago on the Australian subcontinent, although this assertion is based on very few *B. thailandensis* and *B. thailandensis*-like isolates. Finally, the highly homogeneous *B. mallei* clade diverged from *B. pseudomallei* in Australia before the current Southeast Asian population was established (Pearson *et al.*, 2009).

Physical properties and genomics *B. pseudomallei* is a bipolar-staining rod that measures between 2-5 μm in length and 0.4 - 0.8 μm in diameter. Although non-sporulating, the organism

is exceptionally robust and exhibits resistance to extremes in temperature and pH, nutrient deprivation and desiccation. Optimal growth temperature is reported to be around 40° C, but it can survive for extended periods at temperatures up to 45° C. It grows optimally in pH-neutral environments (pH 6.5–7.5) but can survive in pH ranges from 4.0–8.0 (Chen *et al.*, 2003). *B. pseudomallei* generally exhibits a high degree of resistance to aminoglycoside antibiotics (e.g. streptomycin and gentamicin) as well as to macrolides (azithromycin, erythromycin) and fluoroquinolones (Levoquin, Moxifloxacin). It is susceptible to tetracyclines and fourth-generation cephalosporins, such as ceftazidime and carbapenems (imipenem and meropenem). Ceftazidime or trimethoprim/sulfamethoxazole are recommended as first choices in the treatment of infections (Schweizer, 2003; Trunck *et al.*, 2009; Estes *et al.*, 2010). The majority of strains are capable of fermentation of sugars without gas formation (predominantly glucose and galactose). Colonies have a characteristic dry and wrinkled appearance and may exhibit multiple morphologies; even from a single isolate. The organism is most reliably identified by its growth on Ashdown's selective agar and the use of the API20NE identification system (Lagatolla *et al.*, 2002; Deepak *et al.*, 2008). Its distinctive pattern of antibiotic resistance and inability to assimilate arabinose helps differentiate *B. pseudomallei* from *P. aeruginosa* and the closely related *B. thailandensis* (Chaiyaroj *et al.*, 1999; Wuthiekanun *et al.*, 2002).

The large genome of *B. pseudomallei* consists of two chromosomes. In the prototypical *B. pseudomallei* strain K96243, a clinical isolate from Thailand (Table 1), chromosome 1 is 4.07 Mb and chromosome 2 is 3.17 Mb, encoding 3,460 and 2,395 ORFs, respectively (Holden *et al.*, 2004). Chromosome 1 encodes many genes involved in core functions, such as macromolecule biosynthesis, amino acid metabolism, nucleotide and protein biosynthesis, chemotaxis and flagellar motility. In contrast, chromosome 2 contains the greater portion of genes encoding

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accessory functions including adaptation to growth conditions, iron acquisition, secondary metabolism, regulation, virulence and horizontal gene transfer. Characterized or partially characterized virulence determinants are found throughout the genome, but a higher proportion of them, including the gene clusters encoding the three T3SSs and the virulence-associated T6SS-1, are located on chromosome 2 (Holden *et al.*, 2004).

Virulence determinants and intracellular survival

The ability of pseudomallei-group *Burkholderia* to survive intracellularly is thought to be relevant to pathogenesis, since initial infection presumably occurs at abraded skin or mucosal surfaces, and bacteria may invade epithelial cells at these locations (Galyov *et al.*, 2010). Alternatively, the extracellular space at the sites of inoculation may be the initial site of establishment of infection, requiring the bacterium be able to survive following uptake by resident phagocytes. Following attachment to cell surfaces, pseudomallei-type *Burkholderia* invade cells and are taken up into an endocytic vesicle. Invasion and uptake into epithelial and phagocytic cells is sensitive to cytochalasin-D, demonstrating a dependence on host cell actin dynamics. The bacterium subsequently escapes the endocytic vesicle into the cytoplasm, where it replicates, polymerizes host cell actin and spreads to neighboring cells (French *et al.*, 2011). Actin polymerization requires the polarly-localized BimA protein and the cellular Arp2/3 actin nucleating complex (Gouin *et al.*, 2005; Sitthidet *et al.*, 2010). *B. pseudomallei*, *B. mallei* and *B. thailandensis* are unique in their ability to induce multinucleate giant cell formation in a variety of cell types following intercellular spread (Harley *et al.*, 1998; Kespichayawattana *et al.*, 2000; Burtnick *et al.*, 2010). The relevance of intracellular survival to establishing and maintaining infection in mammals is poorly understood, although bacteria residing inside spleen cells have been observed by electron microscopy following infection of mice (Gauthier *et al.*, 2001).

B. pseudomallei contains an extensive repertoire of known and putative virulence factors that facilitate intracellular survival and cell-cell spread. Prominent examples that are relevant to these studies and their functions are described below.

Bsa T3SS The genome of *B. pseudomallei* encodes three injection-type T3SSs and at least one flagellar T3SS, depending on the strain origin. The third injection-type system, known as T3SS-3 or the *Burkholderia* secretion apparatus (T3SS$_{Bsa}$), is similar to those in *Salmonella* and *Shigella* (Stevens *et al.*, 2002; Sun and Gan, 2010), and is conserved in *B. mallei* and *B. thailandensis*. T3SS$_{Bsa}$ mediates escape from primary endocytic vesicles (Burtnick *et al.*, 2008; French *et al.*, 2011) and other roles in virulence, such as suppression of cellular immunity and evasion of autophagy cannot be ruled out (Whitlock *et al.*, 2008; Gong *et al.*, 2011). T3SS$_{Bsa}$ is required for full virulence of *B. pseudomallei*, *B. mallei* and *B. thailandensis* in animal models (Warawa and Woods, 2005; Haraga *et al.*, 2008). The Bsa and other T3SSs of *Burkholderia* are further discussed in Chapters 3 and 4.

Type VI secretion system (T6SS) *B. pseudomallei* contains six T6SS loci, and homologs of four of these are found in the genomes of *B. mallei* and *B. thailandensis* (Schell *et al.*, 2007; Schwarz *et al.*, 2010a; Schwarz *et al.*, 2010b; Burtnick *et al.*, 2011). While T6SS clusters 1–3 appear to comprise a single transcriptional unit, clusters 4–6 are divided into two or more units. T6SS-1– T6SS-3 are present in all three species and are highly conserved (> 80%), while T6SS-4 is unique to pathogenic *B. mallei* and *B. pseudomallei*. T6SS-5 exists in both *B. pseudomallei* and *B. thailandensis*, but not in *B. mallei*. T6SS-6, the only one on chromosome 1, appears to be widely conserved among many soil-dwelling species, including *Burkholderia* and *Ralstonia*. Incidentally, this cluster in *B. mallei* contains a deletion that spans the last seven genes. Using the nomenclature of Schell, *et al.*, (Schell *et al.*, 2007), the expression of T6SS-1, also known as

T6SS-5 (Schwarz *et al.*, 2010b) in *B. pseudomallei* and *B. mallei* is induced inside murine macrophages, suggesting that it plays a role in the intracellular lifecycle (Shanks *et al.*, 2009; Burtnick *et al.*, 2010). *B. mallei* T6SS-1 mutants are avirulent in hamsters (Schell *et al.*, 2007) and a *B. pseudomallei* T6SS-1 mutant was highly attenuated in mice (Pilatz *et al.*, 2006). Null mutations affect the cell-cell spreading ability of all three species (Burtnick *et al.*, 2010; Burtnick *et al.*, 2011; French *et al.*, 2011). T6SS-6, which is the most deteriorated of the T6SSs remaining in *B. mallei*, is reported to mediate bacterial-bacterial interactions (Schwarz *et al.*, 2010b).

Flagella The Southeast Asian *B. pseudomallei* isolates produce polar flagella from a single flagellar T3SS gene cluster located on chromosome 1 (*fla1*) (Tuanyok *et al.*, 2007; French *et al.*, 2011). *fla1* flagellin (FliC1) is required for motility *in vitro* (Brett and Woods, 1996; DeShazer *et al.*, 1997; French *et al.*, 2011). *B. pseudomallei fliC1* mutants are attenuated in mice when delivered intranasally but not following intraperitoneal inoculation (Chua *et al.*, 2003; Chuaygud *et al.*, 2008). Similarly, *fliC1* mutants are not attenuated in hamsters or infant diabetic rats when inoculated intraperitoneally (DeShazer *et al.*, 1997). Passive exposure studies demonstrated that *fliC1*-specific antiserum was capable of protecting infant diabetic rats from challenge with *B. pseudomallei* (Brett *et al.*, 1994).

B. thailandensis and Australian *B. pseudomallei* isolates encode a second flagellar T3SS system on chromosome 2 (*fla2*) (Tuanyok *et al.*, 2007; French *et al.*, 2011). We recently demonstrated that Fla2-mediated motility contributes to cell-cell spread and MNGC formation following infection of HEK293 cells with *B. thailandensis*. Little is known about the environmental signals required for expression and activity of *fla2* in *B. pseudomallei* and *B. thailandensis*, but Fla2 activity is not required for swarming in soft agar (French *et al.*, 2011). Questions regarding the structural distribution of flagellin filaments on the surface of the

bacterium, as well as the regulatory networks controlling expression of the flagellar loci are being addressed and are presented in-part in Chapter 4.

Intracellular life cycle

The coordinated activity of T3SSBsa and T6SS-1, presumably in concert with other currently unrecognized factors, facilitates the ability of pseudomallei-group *Burkholderia* to opportunistically parasitize eukaryotic cells and to survive and escape predation . The intracellular life cycle proceeds through the phases described below.

Adherence Despite its high pathogenic potential, *Burkholderia* is only weakly adherent to cells in culture, compared to other pathogens such as *Salmonella enterica* (Jones *et al.*, 1996). In studies with either HeP-2 (HeLa) or A549 epithelial cells, Balder *et al.* identified two YadA-type autotransporters in *B. pseudomallei* (BPSS0796 and BPSL1705) and their orthologs in *B. mallei* that appear to function as adhesins (Balder *et al.*, 2010). Flagella, capsule polysaccharide and type IV pili have also been implicated in attachment (Ahmed *et al.*, 1999; Essex-Lopresti *et al.*, 2005). Inhibition studies indicate that *B. pseudomallei* binds to the asialoganglioside of the αGM1–aGM2 receptor complex on HeLa cells, although the bacterial factors mediating this interaction have not been elucidated (Gori *et al.*, 1999).

Invasion and endosome escape To date, no virulence determinants have been identified that definitively contribute to *Burkholderia* invasion of non-phagocytic cells. Studies by Stevens *et al.* suggested that null mutations in *bopE* (BPSS1525), a T3SS$_{Bsa}$ secreted effector and guanine nucleotide exchange factor (Stevens *et al.*, 2003) reduced the number of intracellular *B. pseudomallei* 10276 by 50% at 6 hr. after infection of HeLa cells (Stevens *et al.*, 2003). Deletions in *bopC* (BPSS1516), a T3SS$_{Bsa}$ substrate of unknown function are reported to slightly

reduce invasion efficiency of *B. pseudomallei* K96243 in A549 cells (Muangman *et al.*, 2011).

However, similar studies in our laboratory using Δ*bopE* and Δ*bopC* derivatives of *B. pseudomallei* 1026b to infect HeLa cells yield wild-type invasion efficiencies, and the same is seen with *bsaS* (*sctN*) T3SS$_{Bsa}$ ATPase mutants, which cannot secrete apparatus components or effectors (French *et al.*, 2011). Therefore, it does not appear that T3SS$_{Bsa}$ significantly contributes to invasion.

Following invasion, mediated by unrecognized factors, *Burkholderia* escapes the primary endosome into the cytoplasm. Rapid escape is facilitated by the T3SS$_{Bsa}$ (Burtnick *et al.*, 2008; Haraga *et al.*, 2008) although the specific T3SS structural components or effectors that meditate this process are unclear. Results by Devenish *et al.* and others have implicated the T3SS effector BopA in endosome escape (Cullinane *et al.*, 2008; Whitlock *et al.*, 2009; Gong *et al.*, 2011). Intriguingly, many *B. pseudomallei* strains are capable of delayed endosomal escape even in the absence T3SS$_{Bsa}$ function. How the bacterium accomplishes late escape from endosomes is unknown, but the other *B. pseudomallei* T3SSs (T3SS-1 and -2) are not required (Burtnick *et al.*, 2008). It is clear that T3SS$_{Bsa}$ plays an important role in pathogenesis, since loss of function mutants are attenuated in the murine and hamster models of melioidosis, as is a *bipD* translocon mutant (Stevens *et al.*, 2002; Stevens *et al.*, 2004; Warawa and Woods, 2005), although mutations in individual effector and T3SS accessory loci (*bopA, bopE, bapA, bapC*) are curiously not attenuated (Stevens *et al.*, 2004).

Intracellular replication Following escape from endosomes, pathogenic *Burkholderia* multiply within the cytoplasm of phagocytic and non-phagocytic cells. In macrophages, monocytes and neutrophils, the ability of *B. pseudomallei* to modulate the bactericidal response is an important mechanism of pathogenesis. The bacterium is reported to suppress inducible nitric oxide

synthase (iNOS) suppression of cytokine signaling 3 (SOCS3) and cytokine-inducible src homology 2-containing protein (CIS) through an uncharacterized process (Ekchariyawat *et al.*, 2005; Ekchariyawat *et al.*, 2007). Intracellular survival has been shown by our laboratory and others to require the activity of T6SS-1 (Burtnick *et al.*, 2011; Chen *et al.*, 2011; French *et al.*, 2011). Aside from the requirement for the T6SS-1 components ClpV1, Hcp1 and VgrG1, which are necessary for T6SS-1 function, little is understood regarding the precise mechanism of T6SS-1-mediated intracellular survival. Deshazer *et al.*, report the identification and partial characterization of a predicted T6SS-1 substrate that possesses ubiquitin hydrolase activity (BPSS1512 TssM). TssM was found to be co-regulated with T6SS-1 through the VirAG two component system and expressed following infection of RAW264.7 monocytes. It was subsequently found that TssM was responsible for suppression of cellular immunity through downregulation of NF-κB and the type I IFN pathway (Shanks *et al.*, 2009; Tan *et al.*, 2010). Otherwise, the T3SS$_{Bsa}$ effector BopA has been shown by our laboratory and other investigators to be a key mediator of intracellular survival. Several studies have shown the importance of BopA in the ability of *B. pseudomallei* to avoid LC3-associated autophagocytosis (Cullinane *et al.*, 2008; Gong *et al.*, 2011). In concert with other unidentified factors, it is conceivable that T3SS$_{Bsa}$ and T6SS-1 perform non-overlapping but complementary roles in promoting the intracellular survival of *B. pseudomallei*.

Actin motility and intercellular spread Existing models of the *B. pseudomallei* intracellular lifestyle and mechanisms of cell-cell spread has been based largely on extrapolation from studies of *Listeria* and *Shigella* (Ray *et al.*, 2009). In the canonical intracellular lifecycle, bacteria replicate in the cytoplasm and polymerize host cell actin. In *Burkholderia* this process is mediated by the bacterial BimA protein, a polarly-localized autotransporter that binds and

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polymerizes actin in a fashion analogously to ActA of *Listeria* and IcsA of *Shigella* (Stevens *et al.*, 2005a; Stevens *et al.*, 2005b; Sitthidet *et al.*, 2008; Sitthidet *et al.*, 2011). Actin-based motility produces growing membrane protrusions that extend away from the cell with bacteria at their tips. In *Listeria* and *Shigella*, actin motility provides the force that drives bacteria into neighboring cells, where they subsequently escape from double membrane vesicles and initiate additional rounds of cellular infection (Ray *et al.*, 2009). Bacteria-mediated cell fusion, a phenomenon which *Burkholderia* are uniquely suited to accomplish, makes escape from double membrane vesicles unnecessary (French *et al.*, 2011). The implications of this are described below and in Chapter 3.

Multinucleate giant cell (MNGC) formation Infection of cells in culture with *B. mallei*, *B. pseudomallei* and *B. thailandensis* results in cell fusion and the formation of multinucleate giant cells (MNGCs) (Harley *et al.*, 1998; Kespichayawattana *et al.*, 2000). Although MNGC formation can be induced by infection with viruses (HIV and Nipah are notable examples) (Diederich and Maisner, 2007; Helming and Gordon, 2007; Helming and Gordon, 2009; Huerta *et al.*, 2009), and is exhibited by alveolar macrophages as a result of *M. tuberculosis* infection (Puissegur *et al.*, 2007), the pseudomallei group of pathogenic *Burkholderia* are unique in that they readily and efficiently induce MNGC formation in a wide variety of cell types (Kespichayawattana *et al.*, 2000). MNGCs have been observed in melioidosis patients (Sirikulchayanonta V, 1994; Wong *et al.*, 1995), in post-mortem samples from human melioidosis victims (Wong *et al.*, 1995) and from biopsies from *B. pseudomallei*-infected bone (Sirikulchayanonta V, 1994), indicating that cell entry, intracellular survival and proliferation and MNGC development does occur *in vivo*. The precise relevance of MNGCs in *B. pseudomallei* pathogenesis is not well understood, although it could conceivably be a mechanism

of escaping detection by the host immune system (Boddey *et al.*, 2007). Almost nothing is known about how cell fusion occurs and what bacterial and host cell molecules are involved; only the global regulatory factor RpoS and the T3SS$_{Bsa}$ translocon component BipB have been previously implicated in MNGC formation by *B. pseudomallei* (Suparak *et al.*, 2005; Utaisincharoen *et al.*, 2006). In light of current understanding, the latter observation regarding the requirement of BipB is most likely due to endosomal entrapment of mutants in the initially infected cell, and not a failure of cell-cell spread itself. The implications of this are further discussed in Chapter 3.

Other virulence factors

Though they are not direct subjects of this dissertation, other factors play central roles in *Burkholderia* virulence. *B. pseudomallei* capsular polysaccharide is required for optimal survival in macrophages (Wikraiphat *et al.*, 2009) and may reduce complement-mediated opsonization and phagocytosis (Reckseidler-Zenteno *et al.*, 2005). Capsule mutants are highly attenuated in animal models (Sarkar-Tyson *et al.*, 2007), and the same is true for *B. mallei* (DeShazer *et al.*, 2001). The LPS O-antigen of *B. pseudomallei* confers resistance to bactericidal macrophage killing (Perry *et al.*, 1995; Arjcharoen *et al.*, 2007) and promotes resistance to serum complement (DeShazer *et al.*, 1998). A phospholipase BPSS0067 (*plc-3*) was transcriptionally upregulated in the hamster model of infection and the LD50 of mutants was more than 100 fold higher than that of the parental strain (Tuanyok *et al.*, 2006; Korbsrisate *et al.*, 2007). The roles of *plc-3* in *B. mallei* and its homolog in *B. thailandensis* have not been evaluated. Finally, the quorum sensing systems of *B. pseudomallei* (Ulrich *et al.*, 2004) may contribute to virulence (Valade *et al.*, 2004), but the virulence-associated genes regulated by these complex quorum-sensing systems are still under investigation.

Regulation of T3SS$_{Bsa}$ and T6SS-1 loci Only recently has insight been gained into the regulation of specific *B. pseudomallei* virulence determinants. The regulatory network controlling the Bsa T3SS, actin motility and T6SS-1 has been deciphered by Sun and colleagues and is the best understood example of virulence-associated regulatory networks in *B. pseudomallei* (Sun *et al.*, 2010). Sitting atop the hierarchy, a TetR family regulator, BPSL1105 BspR, is responsible for the control of T3SS$_{Bsa}$ gene expression. BspR acts through BPSS1553 BprP, a novel transmembrane regulator located adjacent to T3SS$_{Bsa}$. BprP in turn regulates the expression of structural and secretion components of T3SS$_{Bsa}$ and the AraC family regulator BsaN. Likely forming a complex with BsaN, the regulatory chaperone BicA regulate the expression of T3SS$_{Bsa}$ effectors and other regulators which in turn affect the expression of T6SS-1 (Chen *et al.*, 2011). BsaN/BicA affect expression of the T3SS$_{Bsa}$ effector loci and regulators for T6SS-1 such as the VirAG two component system and BprC. Whereas T6SS-1 gene expression was completely dependent on BprC when bacteria were grown in medium, the expression of T6SS-1 inside host cells was dependent on VirAG. Further, VirAG and BprC initiate different transcriptional start sites within T6SS-1. Furthermore, *bspR, virAG* and *bprC* mutants demonstrated reduced intracellular survival and were avirulent in mice (Sun and Gan, 2010). VirAG also activates expression of the *bim* actin motility locus (Schell *et al.*, 2007). The precise signals or environmental conditions that activate these pathways are still under investigation.

Preface

My dissertation will focus on the secretion systems and other virulence mechanisms of *Bordetella* and *Burkholderia* species, and the majority is dedicated to *Burkholderia pseudomallei* and the closely related *B. thailandensis*. The rationale is as follows; at the initiation of my graduate research, my objectives were to investigate and characterize secreted proteins and

toxins from bacterial pathogens. Following the publication of the study on the *Bordetella* T3SS effector BteA in *Cellular Microbiology*, which appears in modified form as Chapter 2, I serendipitously made several new and exciting discoveries with *Burkholderia*, prompting a shift in my research priorities. As new opportunities became available, my research objectives changed to take advantage of them. My new and ongoing research goals are thus the analysis of *Burkholderia* virulence determinants and their roles in pathogenesis *in vitro* and *in vivo*. Chapter 2 describes an analysis of a functionally important N-terminal domain of the *Bordetella* T3SS effector BteA, and how this domain possesses lipid raft targeting activity in mammalian cells. In Chapter 3, the focus switches to *Burkholderia*, and I present a genetic dissection of its intracellular lifecycle. The study involves the use of a novel device – the photothermal nanoblade, to deliver bacteria into the cytosol of mammalian cells to bypass invasion and escape from endocytic vesicles. Chapter 4 is a compendium of current and ongoing studies involving type III secretion systems of *Burkholderia*. A description of the discovery and partial characterization of *B. pseudomallei* T3SS effectors, their mutational analysis and infection phenotypes is presented. Additional studies on the Fla2 flagellar system and its roles in the intracellular lifecycle is included. Since flagellar and injection-type T3SSs descend from a common ancestor, the two topics are presented in a single chapter. Finally, Chapter 5 describes current and future research involving the molecular pathogenesis of T3SS, T6SS and flagellar systems in *Burkholderia* and perspectives on melioidosis vaccine development and therapeutic solutions.

Table Legend

Table 1. *Burkholderia pseudomallei* and *Burkholderia thailandensis* strains used in this dissertation.

Figure Legends

Fig. 1. Secretion systems in Gram-negative bacteria. Systems can be divided into Sec-independent (T1SS, T4SS, T3SS) and Sec–dependent (T2SS, T5SS)pathways. A membrane-spanning conduit and extension is present in the case of T4SS and T3SS, facilitating direct communication with eukaryotic cells. (Gerlach and Hensel, 2007). Used with permission.

Fig. 2. The denizens of the rhizosphere. *B. pseudomallei* and related species are soil saprophytes and opportunistic pathogens of humans and animals. In this regard, the analysis of *Burkholderia* virulence factors should be viewed as having evolved for survival and self defense in the environment.

Table 1

Strain	origin	remarks
K96243	Thailand	*B. pseudomallei*, fatal melioidosis (year)
1026b	Thailand	*B. pseudomallei* recrudescent, fatal melioidosis (year)
Bp340	Thailand	*B. pseudomallei* 1026b ΔamrAB-oprA efflux mutant
MSHR668	Australia	*B. pseudomallei* fatal meliodosis
NAU14B6	Australia	*B. pseudomallei* environmental isolate. Virulent in mice.
E264	Thailand	*B. thailandensis* environmental isolate.

Fig. 1
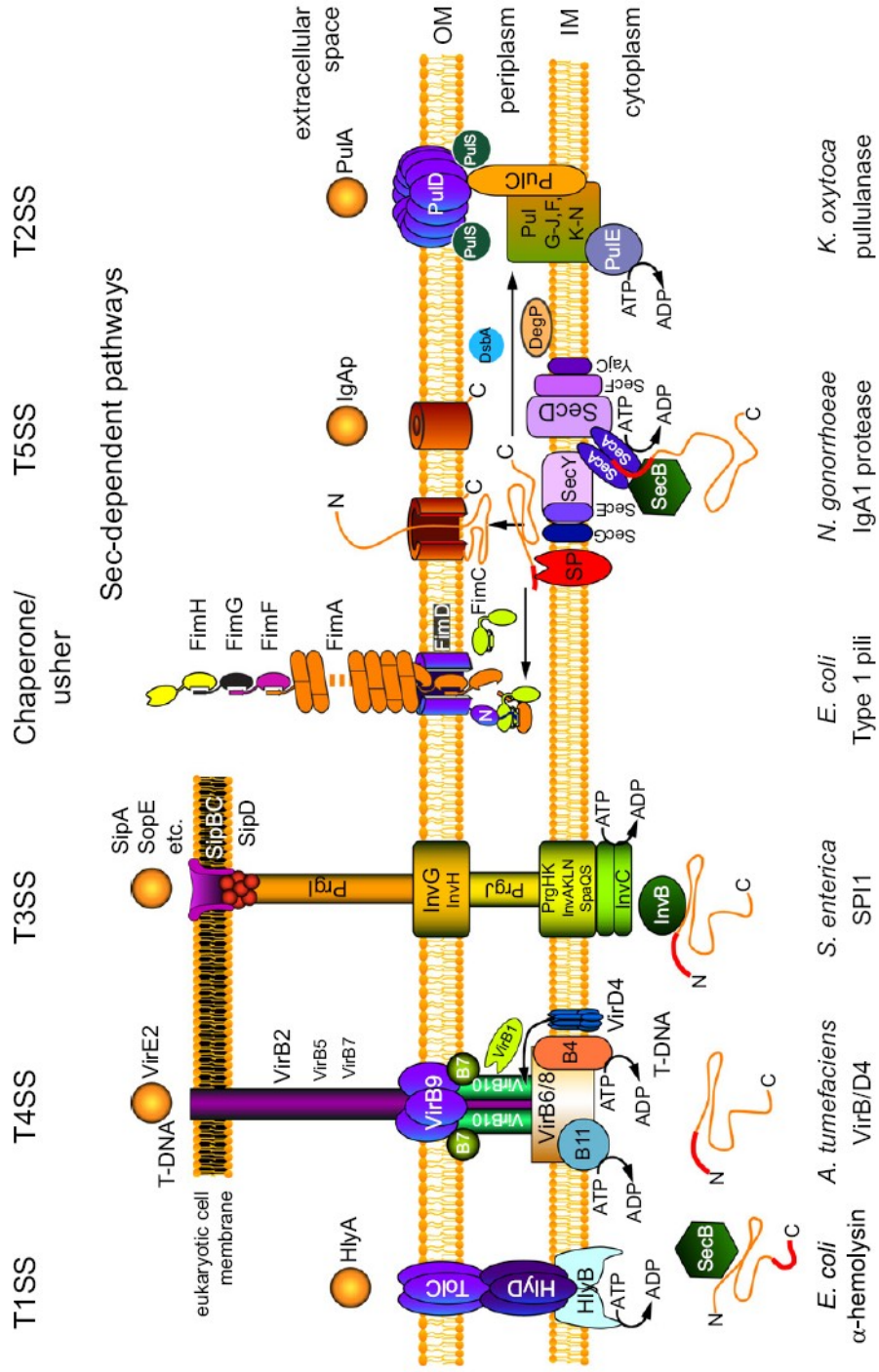
Fig. 2

Rhizosphere

- *B. pseudomallei*
- *B. thailandensis*

Oryza sativa

amoebae
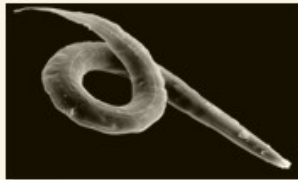
nematodes

bacteria

insects
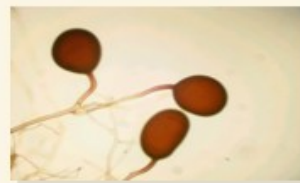
fungi

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Chapter 2. The *Bordetella* T3SS effector BteA contains a conserved N-terminal motif that

guides bacterial virulence factors to lipid rafts

Fig. S1. Journal cover image. 2009, *Cellular Microbiology 11 (12)*: 1735-1749.

Fig. S1. (Journal Cover) BteA colocalizes to membrane domains rich in cortical actin and lipid rafts. In this cover image for the December 2009 issue of *Cellular Microbiology*, HeLa cells were transfected with BteA$_{644}$-EGFP (blue) and stained for actin (green) and GM1 ganglioside (red). Near perfect colocalization is seen with BteA, actin and GM1, which appears white in the transfected cell overlay image (lower left) (French *et al.*, 2009).

Abstract

The *Bordetella* effector protein BteA is necessary and sufficient for rapid cytotoxicity in a wide range of mammalian cells. We show that BteA is highly conserved and functionally interchangeable between *B. bronchiseptica, B. pertussis,* and *B. parapertussis*. The identification of BteA sequences required for cytotoxicity allowed the construction of non-cytotoxic mutants for localization studies. BteA derivatives were targeted to lipid rafts following transfection and showed clear co-localization with cortical actin, ezrin, and the lipid raft marker GM1. We hypothesized that BteA associates with the cytoplasmic face of lipid rafts to locally modulate host cell responses to *Bordetella* attachment. *B. bronchiseptica* adhered to host cells almost exclusively to GM1-enriched lipid raft microdomains and BteA co-localized to these same sites following T3SS-mediated translocation. Disruption of lipid rafts with methyl-β-cyclodextrin protected cells from T3SS-induced cytotoxicity. Localization to lipid rafts was mediated by a 130 amino acid lipid raft targeting (LRT) domain at the N-terminus of BteA, and homologous domains were identified in known and putative virulence factors from other bacterial species. LRT sequences from a T3SS effector (Plu4750) and an RTX-type toxin (Plu3217) from *Photorhabdus luminescens* directed fusion proteins to lipid rafts in a manner identical to the N-terminus of BteA.

Introduction

Lipid membrane rafts (rafts), which are prominent targets for bacterial, viral and protozoan pathogens (Manes *et al.*, 2003; Lafont *et al.*, 2004; Riff *et al.*, 2005; Mejia *et al.*, 2008), are specialized membrane microdomains enriched in cholesterol and sphingolipids that produce ordered structures distinct from surrounding areas of disordered, predominantly unsaturated lipid species (Simons and Ikonen, 1997; Laude and Prior, 2004). Unique sets of proteins preferentially localize to rafts, including GPI-anchored exofacial proteins (Paulick and Bertozzi, 2008), acylated cytofacial signaling proteins such as Src family kinases (Song *et al.*, 1997), G proteins (Song *et al.*, 1996; Moffett *et al.*, 2000), growth factor receptors (Liu *et al.*, 1996; Waugh *et al.*, 1999), integrins (Baron *et al.*, 2003), and cholesterol-binding proteins such as caveolin (Sargiacomo *et al.*, 1993) and Sonic Hedgehog (Rietveld *et al.*, 1999). Lipidomic analysis has revealed that rafts are enriched in signaling molecules such as phosphatidylserine, phosphatodylinositol 4,5-biphosphate and arachidonic acid (Pike and Casey, 1996; Fridriksson *et al.*, 1999; Pike *et al.*, 2002). The clustering of signaling proteins and lipid cofactors within rafts suggests they function as platforms where close proximity increases efficiency and specificity of signaling cascades (Brown and London, 1998; Simons and Toomre, 2000). Although rafts are presumed to play important roles in key biological events such as cytoadhesion, transport, and signal transduction, considerable controversy exists regarding their size, function, and precise physical nature (Edidin, 2003; Munro, 2003; Laude and Prior, 2004; Skwarek, 2004). Despite uncertainty regarding the most appropriate experimental criteria for defining lipid rafts, considerable evidence supports their importance for attachment of bacterial pathogens (e.g. *Mycoplasma, Shigella*, enteropathogenic *E. coli*, *Porphyromonas, Yersinia*) (Lafont *et al.*, 2004; Riff *et al.*, 2005; Allen-Vercoe *et al.*, 2006; Hawkes and Mak, 2006; Mejia *et al.*, 2008; Hayward

et al., 2009), bacterial invasion (*Shigella, Salmonella, Listeria, Mycobacterium, Chlamydia*) (Manes *et al.*, 2003; Lafont *et al.*, 2004; Lafont and van der Goot, 2005a), oligomerization of pore-forming toxins (aerolysin, streptolysin O, listeriolysin O) (Gekara *et al.*, 2005), and cell-surface binding by A-B toxins (cholera toxin, shiga toxin) (Takenouchi *et al.*, 2004; Lencer and Saslowsky, 2005). Recent evidence suggests that association of *Salmonella* and *Shigella* T3SS translocon components with target cell membranes is cholesterol-dependent (Lafont and van der Goot, 2005b). Although interactions between bacterial components and the exofacial surface of lipid rafts have been extensively studied, virulence factor targeting events occurring on the cytoplasmic side of rafts are less well understood.

In this study we report an analysis of the activity and intracellular localization of BteA, a 658 amino-acid effector protein secreted by the *Bordetella Bsc* T3SS (Panina *et al.*, 2005; Kuwae *et al.*, 2006). The *Bsc* T3SS is most extensively characterized in *B. bronchiseptica*, the broad host-range evolutionary progenitor of *B. pertussis* and *B. parapertussis* which cause acute respiratory diseases in humans (Yuk *et al.*, 1998; Yuk *et al.*, 2000). Although nearly identical T3SS loci are encoded in the genomes of all three subspecies, the ability to readily detect T3SS activity *in vitro*, and the availability of natural-host animal models to study respiratory infection have made *B. bronchiseptica* the subspecies of choice for studying type III secretion by *Bordetella*. The loci encoding *bteA* and the *Bsc* T3SS apparatus are co-regulated by the alternative sigma factor BtrS, which in turn is dependent on the BvgAS phosphorelay for expression (Mattoo *et al.*, 2004). In addition to transcriptional control, the partner-switching proteins BtrU, BtrV and BtrW regulate secretion through a complex series of protein-protein interactions, serine-phosphorylation, and serine-dephosphorylation events (Mattoo *et al.*, 2004; Kozak *et al.*, 2005). BteA is an unusually potent cytotoxin capable of inducing rapid, non-

apoptotic death in a diverse array of cell types, and precise, multi-level control of expression and secretion may be essential for its role in promoting persistent colonization of the respiratory epithelium (Stockbauer *et al.*, 2003; Panina *et al.*, 2005; Shrivastava and Miller, 2009). BteA is the only *Bordetella* effector that has been identified and its central importance is illustrated by the observation that null mutations in *bteA* recapitulate the *in vitro* and *in vivo* phenotypes associated with mutations that eliminate type III secretion altogether (Panina et al., 2005). Despite its central role in pathogenesis, little is known regarding the events that occur following BteA injection into target cells. In the course of analyzing the intracellular localization of BteA, we have identified a lipid raft targeting motif found in virulence determinants expressed by diverse bacterial pathogens.

Results

BteA is functionally conserved between *Bordetella* subspecies The *Bsc* T3SS loci encoded by

B. pertussis, *B. parapertussis* and *B. bronchiseptica* are highly conserved, with apparatus genes

typically displaying 93% to 100% amino acid sequence identity. *bteA* and *btcA*, which encode

the BteA effector and its chaperone, respectively, are present in the genomes of all three

subspecies and are predicted to encode nearly identical proteins (Fig. 1A). As shown in Fig. 1B,

bteA alleles from *B. pertussis* and *B. parapertussis* were able to complement a Δ*bteA* derivative

of *B. bronchiseptica* strain RB50. Cytotoxicity against rat lung epithelial (L2), human epithelial

(HeLa) and mouse lung epithelial (MLE12) cells was restored to levels equal to or greater than

those observed with the cognate RB50 *bteA* allele. Moreover, the morphological characteristics

of dying cells were identical (data not shown). These results demonstrate that the BteA effector

proteins encoded by *Bordetella* subspecies that cause respiratory infections in mammals are

functionally interchangeable *in vitro*.

Identification of non-cytotoxic BteA derivatives A characteristic of the wild-type BteA protein

is the induction of rapid, non-apoptotic cytotoxicity in a wide range of target cells (Stockbauer *et

al.*, 2003; Panina *et al.*, 2005). In an attempt to identify a time interval where BteA might be

detected and localized prior to the onset of cell death, we performed the time-course experiment

shown in Fig. 2A. *bteA* was inserted downstream from the CMV promoter in plasmid pEGFP-

N1, fusing EGFP to the C-terminus of BteA. Expression of the resulting fusion construct,

BteA[658]-EGFP, was analyzed by fluorescence microscopy at time intervals beginning 2 hr. after

transfection. With the vector control, GFP-positive cells could be identified as early as 4 hours

(hr.), while no GFP-positive cells were found at any time point following transfection with the

construct encoding BteA[658]-EGFP (Fig. 2B), and the fusion protein was not detectable by

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Western blot analysis (data not shown). LDH release assays showed that expression of BteA with the C-terminal EGFP tag resulted in a level of cytotoxicity comparable to the native untagged protein (Fig. 2B). These data show that even trace amounts of BteA[658]-EGFP expression are sufficient to induce cytotoxicity and prevent detection of the fusion protein by fluorescence microscopy or Western blot analysis.

We reasoned that BteA derivatives with reduced cytotoxicity would be required for localization studies to proceed. A series of *bteA* fragments truncated at their 3' termini were cloned into pEGFP-N1 to create C-terminal fusions with EGFP (Fig. 2B). Following transfection into HeLa cells, expression of BteA-EGFP derivatives was monitored by fluorescence microscopy and cytotoxicity was measured by LDH release assays (Fig. 2B). Although BteA[652]-EGFP retained full cytotoxicity, further truncations eliminated activity. The markedly reduced LDH release following transfection with BteA[644]-EGFP indicates that the last 14 aa of BteA are critical for full cytotoxicity. To determine if the BteA[644] mutant retains structural features of the wild-type protein, we assessed its ability to self-associate; a characteristic of native BteA (Panina et al., 2005). Full-length BteA resolves as high molecular weight multimers on SDS PAGE (Fig. 2C), a property that correlates with the formation of fibers in buffered aqueous solution that can be visualized by electron microscopy (data not shown). BteA[644] retains this ability to form SDS-insoluble high molecular weight species, suggesting that it maintains its core structural scaffold despite a significant loss of cytotoxicity.

BteA[644] localizes to ezrin- and GM1-rich membrane sites We next examined the localization of BteA[644]-EGFP in HeLa and L2 cells by scanning confocal microscopy. As shown in Fig. 3A, BteA[644]-EGFP clearly colocalized with cortical actin. BteA was observed to associate with regions of the cell surface (Fig. 3A, arrowheads), but not actin stress fibers, suggesting that it

targets proteins and/or lipids positioned in the vicinity of actin-associated membrane sites. Cortical actin participates in the formation of structural platforms for membrane-bound signaling proteins, including transmembrane receptors as well as downstream signaling components, which may self-associate or be found in complexes with receptor cytoplasmic domains (Xavier *et al.*, 2004; Gajate and Mollinedo, 2005; Halayko and Stelmack, 2005; Chichili and Rodgers, 2009). These complexes are often anchored to the actin cytoskeleton via adaptor proteins of the ERM (ezrin-radixin-moesin) family. ERM proteins contain C-terminal domains that bind actin and N-terminal domains that associate with a variety of cellular receptors such as EGFR, CD44, ICAM-1, ICAM-3, β2AR, PDGF-R, and NHE-1 (Bretscher *et al.*, 2002; Hoeflich and Ikura, 2004). In addition to structural roles, ERM proteins participate in signal transduction cascades, including protein kinase C signaling and the activation of Rho-family GTPases (Takahashi *et al.*, 1997; Bretscher *et al.*, 2002; Lajoie *et al.*, 2009). ERM proteins often localize to receptor-rich membrane rafts involved in signaling, and we were interested to determine whether BteA co-localizes with ezrin, an ERM protein prominently expressed by HeLa cells. Cells transfected with constructs expressing BteA[644]-EGFP or EGFP alone were stained using antibodies against ezrin. Confocal microscopy revealed a high degree of colocalization between BteA[644]-EGFP and ezrin, but not EGFP and ezrin, suggesting that BteA is specifically targeted to ezrin-enriched membrane sites (Fig. 3B-C).

In many cells examined, BteA[644]-EGFP was particularly prominent in filopodia. Filopodia are rod-like projections on the cell surface that are supported by cortical actin and function in sampling and exploration of the environment (Fig. 3B) (Bryant, 1999; Small *et al.*, 2002; Faix *et al.*, 2009). BteA[644] expression appeared to increase both the number and length of these structures in HeLa cells (Fig. 3B). In contrast, EGFP alone (Fig. 3C) did not localize to

filopodia and did not result in abnormal filopodia formation. Due to their sensory and exploratory function, filopodia possess a large number of transmembrane receptors and raft-associated signaling complexes, which vary according to cell type and growth conditions (Lidke *et al.*, 2005; Ladwein and Rottner, 2008). Since filopodia usually contain high concentrations of lipid rafts, we next examined the colocalization of BteA with GM1 ganglioside, which serves as a marker for lipid rafts. Cells expressing BteA[644]-EGFP were stained using fluorescently labeled cholera toxin subunit B (CtxB), which binds with high avidity and specificity to ganglioside GM1 (Schon and Freire, 1989). Nearly complete colocalization was observed between BteA[644]-EGFP and GM1 (Fig. 3D). High magnification XZ scans of the region indicated by the arrowhead in Fig. 3D further demonstrate that transfected BteA localizes precisely with GM1 in cell membranes (Fig. 3E).

BteA contains a conserved, multifunctional N-terminal domain The BteA proteins expressed by *Bordetella* subspecies are highly homologous, especially within an N-terminal 130 aa segment (Fig.1A). Using iterative PSI-BLAST, six homologs of this domain were found in *Photorhabdus luminescens* and two others in *Vibrio splendidus* and *Photorhabdus asymbiotica* (Fig. 4A,B). We had previously predicted that two of the *P. luminescens* proteins (Plu0822 and Plu4750) function as T3SS effectors on the basis of their close proximity to class I chaperones (Panina et al., 2005). The remaining four *P. luminescens* proteins (Plu1341, Plu1344, Plu3217, Plu3324) and the proteins from *V. splendidus* and *P. asymbiotica* are homologous to RtxA, an atypical RTX toxin with actin cross-linking activity (Lin *et al.*, 1999; Fullner and Mekalanos, 2000).

Given the presence of homologous sequences in known and putative virulence determinants from multiple species, we hypothesized that the N-terminal 130 aa segment of

BteA may perform a conserved cellular function, possibly by directing localization. This was tested by fusing the first 130 aa of BteA to EGFP (BteA[130]-EGFP) and examining transfected cells by confocal microscopy. BteA[130]-EGFP localized to GM1-positive lipid rafts in a manner indistinguishable from BteA[644]-EGFP (Fig. 3D and 5B respectively). Similarly, the localization patterns of all C-terminal truncated derivatives of BteA (Fig. 3B) were indistinguishable from BteA[644]-EGFP, while the first 30 aa of BteA was unable to direct EGFP to membrane sites (data not shown). When a construct lacking the N-terminal 130 aa region of BteA (BteA[131-644]-EGFP) was tested, it too failed to localize to GM1-stained raft microdomains (Fig. 5C). These observations demonstrate that a lipid raft targeting (LRT) signal of BteA is contained between aa 1 and 130. Although the BteA LRT motif is essential for localization, it is not required for cytotoxicity following transfection (Fig. 5D). Expression of BteA[131-658]-EGFP conferred a high level of cytotoxicity, even though there was no detectable localization to the cell membrane. Although overexpression of toxic domains may compensate for the lack of localization following transfection, membrane association is likely to be important during T3SS-mediated translocation (see Discussion).

N-terminal sequences of T3SS effectors contain secretion signals and often mediate chaperone-effector interactions (Harrington *et al.*, 2003). *bteA* is directly adjacent to a divergently transcribed locus, *btcA*, which is predicted to encode a class I chaperone. As shown in Fig. 5E, full length BteA associates with BtcA in a bacterial two-hybrid system (Dove and Hochschild, 2004) and the 130 aa LRT domain is both necessary and sufficient for this interaction. Taken together, our results show that the N-terminus of BteA is multi-functional, mediating chaperone interactions and directing the localization of BteA to lipid raft microdomains in eukaryotic cells.

BteA fractionates with detergent resistant membrane domains The relative insolubility of cholesterol in Triton X-100 (TX100) facilitates the separation of cholesterol-enriched lipid rafts during cell fractionation (Ignoul *et al.*, 2007). Detergent-resistant membrane (DRM) fractions were prepared from HeLa cells transfected with BteA-EGFP derivatives to further investigate the association between BteA and lipid rafts. HeLa cells expressing EGFP, BteA^{644}-EGFP, BteA^{130}-EGFP or BteA$^{130-644}$-EGFP were fractionated by TX100 solubilization followed by isopycnic sucrose gradient centrifugation. Cellular fractions were separated by SDS-PAGE and Western blots were probed with antibody to EGFP. As expected, fusion constructs containing the BteA LRT domain (BteA^{644}- and BteA^{130}-EGFP) were abundant in the low density TX100-insoluble DRM fractions, suggesting that BteA associates with rafts, raft proteins, or associated protein complexes (Fig. 6). Notably, the distribution pattern of BteA in DRMs was nearly identical to that of ezrin. The lipid raft marker caveolin-1 also preferentially fractionated with DRMs. In contrast, BteA$^{130-644}$-EGFP appeared in the dense, non-membrane associated TX100-soluble fractions along with the EGFP negative control. The LRT domain-dependent fractionation of BteA within DRMs coincides with results from colocalization studies.

BteA is translocated to the cytoplasmic face of membrane raft locations that function as sites for bacterial attachment The observation that BteA targets discrete locations on host cell membranes prompted us to examine whether a correlation exists with sites of *Bordetella* attachment. HeLa cells were infected with wild-type *B. bronchiseptica* strain RB50 for 15 minutes (min.), a time point before cytotoxicity is observed. Cells were washed to remove unbound bacteria, fixed, and stained with CtxB. Remarkably, almost every cell-associated bacterium was found to colocalize with GM1-positive rafts (Fig. 7A). We reasoned that *B. bronchiseptica* may specifically adhere to lipid rafts and that BteA functions locally following

injection, or that BteA and/or other T3SS-associated factors actively recruit GM1-rich structures to sites of *Bordetella* attachment. To distinguish these possibilities we analyzed the attachment pattern of a T3SS ATPase mutant, RB50 Δ *BscN* (Yuk et al., 1998), which adhered to GM1-enriched sites in a manner indistinguishable from wild-type RB50 (Fig. 7B). Association with lipid rafts, therefore, is independent of T3SS activity and is likely mediated by adhesin specificity.

We next determined if BteA localizes to lipid rafts following T3SS-mediated translocation by adherent *Bordetella*. HeLa cells were infected with RB50 derivatives expressing hemagglutinin (HA) tagged BteA, (BteA^{644}-HA), permeabilized with TX100, and stained with DAPI, CtxB, and an □-HA monoclonal antibody. BteA^{644}-HA closely associated with adherent bacteria (Fig. 8A) and colocalized with GM1-positive lipid rafts (Fig. 8B). XZ confocal scans of the region marked in Fig. 8B (arrow) show that BteA is translocated into lipid rafts that coincide with sites of bacterial attachment (arrowheads, Fig. 8C). T3SS-translocated BteA^{644}-HA could not be visualized when the TX100 membrane permeabilization step was omitted, indicating that BteA was not localized to the outer surface of the plasma membrane (data not shown). Deletions in loci encoding the BscN ATPase (RB50 Δ *BscN*), or the BopB translocon component (RB50 Δ*bopB*), eliminated the ability to detect raft-associated BteA^{644}-HA, demonstrating a requirement for T3SS-mediated translocation. These data demonstrate that translocated BteA associates with the cytoplasmic face of lipid rafts that serve as attachment sites for *Bordetella*, and suggest that BteA acts locally following bacterial attachment. It is interesting to note that only a subset of adherent bacteria are associated with translocated BteA at any given time (Fig. 8A). The mechanistic basis for this lack of synchrony is currently unknown.

Disruption of lipid rafts decreases T3SS-induced cytotoxicity Since both *B. bronchiseptica* and BteA preferentially localize to lipid rafts, we were curious to determine if the integrity of cholesterol-enriched membrane domains is important for attachment and/or T3SS-mediated cytotoxicity. Methyl-β-cyclodextrin (MBCD), a cyclic glucose oligomer that forms soluble inclusion complexes with cholesterol, was used to disrupt lipid rafts (Shaw, 2006). HeLa cells were pretreated with 2.5 m*M* MBCD or media alone for 30 min., washed, and briefly infected with *B. bronchiseptica* RB50. Mild treatment conditions were employed to minimize the MBCD-dependent cytotoxicity observed with higher concentrations or longer exposure. As shown in Fig. 9A, a modest decrease in bacterial attachment was observed at 20 min., a time before T3SS-induced cytotoxicity could be detected. In the experiment in Fig. 9B, HeLa cells were first infected with *B. bronchiseptica* RB50 for 15 min., followed by a 30 min. treatment with MBCD or media alone. In this case, no significant difference was observed between MBCD-treated cells and untreated controls, indicating that treatment with MBCD has little effect on previously-attached *Bordetella*.

Having identified a MBCD treatment protocol that was non-toxic and did not disrupt bacterial attachment, we next determined the effects of cholesterol depletion on T3SS-induced cytotoxicity. HeLa cells were infected with *B. bronchiseptica* RB50 for 15 min., treated with MBCD or media alone for 30 min., and washed extensively. The infection was then allowed to proceed for several hr. to a point where high levels of cytotoxicity are normally observed. As shown in Fig. 9C, cytotoxicity was reduced to background levels in cells subjected to mild treatment with MBCD. These results suggest that BteA-mediated cytotoxicity following *Bordetella* infection is dependent on the integrity of lipid rafts.

Functional conservation of LRT domains in T3SS effectors and RTX toxins The relative

positions of LRT domains differ according to the known or predicted nature of the protein; T3SS

effectors bear them near their N-termini while RTX-type toxins encode them near the middle of

their 3500-4900 aa-long sequences (Fig. 4A). Having demonstrated that the BteA LRT domain is

necessary and sufficient for membrane localization, we determined whether putative LRT

sequences from two *Photorhabdus luminescens* ORFs; one predicted to encode a T3SS effector

(Plu4750) and the other an RTX toxin (Plu3217), were capable of directing EGFP fusion

constructs to lipid rafts. Sequences encoding the *P. luminescens* LRT domains were fused to

EGFP at their C-termini and transfected into HeLa cells. As predicted, both LRT sequences

localized the EGFP fusion constructs to ezrin-rich sites, despite their presence in otherwise-

unrelated proteins (Fig. 10A-C). Moreover, the same localization pattern was observed when

full-length Plu4750 was fused to EGFP, confirming that the LRT motif functions as predicted in

the context of the entire protein (Fig. 10C). On the basis of these observations we conclude that

the N-terminal LRT motif of BteA defines a newly-identified family of localization domains that

target diverse bacterial proteins to membrane rafts in eukaryotic cells.

Discussion

BteA is the only *Bordetella* T3SS effector protein described to date (Panina et al., 2005). This stands in marked contrast to other T3SS-expressing species where numerous effectors have been identified by proteomic, bioinformatic and translocation screens (Fouts *et al.*, 2002; Chang *et al.*, 2005). Although BteA has no full-length homologs among proteins of previously characterized function, it is highly conserved and functionally interchangeable between *Bordetella* subspecies. BteA is both necessary and sufficient for the induction of rapid cell death in a broad range of cell types *in vitro*, and mutations in *bteA* have *in vivo* phenotypes that are indistinguishable from mutations that completely eliminate type III secretion (Panina et al., 2005). Despite its central role in virulence, the molecular function and intracellular targets of BteA have remained elusive.

The remarkably potent cytotoxicity of BteA precludes the ability to detect even trace amounts of full length protein in mammalian cells. A deletion of 14 amino acids from the C-terminus was sufficient to eliminate cytotoxicity. This derivative, BteA[644], retained the ability to form high molecular weight, detergent-insoluble complexes that were similar to full length BteA, suggesting that the structural scaffold of the protein remains intact. Most importantly, the reduction of cytotoxicity permitted the efficient detection of intracellular protein. Following transfection or T3SS-mediated translocation, BteA[644] clearly localized to ezrin- and GM1-rich membrane microdomains in HeLa cells as observed by confocal microscopy. Localization was dependent on the 130 aa LRT motifs present at the N-terminus of BteA. Identical patterns of localization were observed with BteA derivatives that retain the LRT sequence, and its removal disrupted normal targeting. As predicted, BteA associated with low density, detergent-insoluble membrane fractions from transfected cells, indicating direct or indirect association with lipid

rafts. Importantly, BteA displayed a pattern of localization following T3SS-mediated translocation that was identical to that observed following transfection. When sites of *Bordetella* attachment were examined, nearly every cell-associated bacterium was found to co-localize with GM-1, and there was a striking correlation between the position of extracellular bacteria and the location of translocated BteA. These observations tempt the hypothesis that BteA modulates localized cellular responses to *Bordetella* infection, and we speculate that this may be especially relevant to interactions with phagocytic antigen presenting cells. This is consistent with previous results showing T3SS-dependent modulation of T cell and B cell-mediated responses during *B. bronchiseptica* infections (Skinner *et al.*, 2005; Pilione and Harvill, 2006; Siciliano *et al.*, 2006).

Although lipid raft localization is likely to be essential under physiologically relevant conditions, a BteA[130-658] construct lacking LRT sequences displayed cytotoxic activity following transfection. This contrasts with observations that transfected and translocated BteA behave in an otherwise identical manner, and that depletion of membrane cholesterol with MBCD abrogates BteA-dependent cell death following T3SS-mediated delivery. A likely explanation is that the LRT domain plays an essential role in facilitating interactions between BteA and host cell targets during T3SS-mediated translocation, and overexpression of BteA following transfection eliminates the need for LRT-dependent localization. In addition to its role in membrane association, the BteA LRT domain also contains sequences that mediate secretion and chaperone binding. The identification of mutations that disrupt targeting without affecting secretion or translocation will facilitate further studies on the importance of lipid raft targeting for BteA function. Experiments aimed at identifying BteA binding partners responsible for localization and/or cytotoxicity are also underway. On the basis of results presented here, we expect that BteA targets will be associated with the host cell membrane and cholesterol-rich domains. Likely

candidates include components of membrane-associated signaling complexes that are found in lipid rafts and are known to be associated with the cortical actin cytoskeleton.

The BteA LRT domain represents a newly identified motif capable of directing proteins to lipid rafts. Although the identification of similar sequences at the N-termini of other T3SS effectors was not unexpected, we were intrigued to find homologous motifs in RTX-type toxins. A model has been recently proposed for *V. cholerae* RtxA which postulates that both N-terminal and C-terminal sequences associate with the plasma membrane and mediate translocation of the central portion of the protein into the cytosol. A cysteine protease domain then cleaves the toxin between L3428 and A3429 (Fig. 4A) and additional cleavage events liberate the actin crosslinking and Rho-inactivation domains from their membrane anchors (Sheahan *et al.*, 2007; Prochazkova and Satchell, 2008). Since homologous cysteine protease domains are found in all of the RTX-type toxins shown in Fig. 4A., proteolytic processing following cytosolic delivery is likely to be a common feature (Sheahan et al., 2007). For the *P. luminescens* and *V. splendidus* RTX-type toxins, we propose that adjacent LRT motifs target activity domains released by proteolysis to GM1-rich lipid rafts. The relative positions of cleavage sites would determine exactly which domains co-localize with the LRT. Although LRT-containing proteins represent distinct classes of virulence determinants with completely different translocation mechanisms, the ability of the LRT domains from a *Photorhabdus* effector and an RTX-type toxin to guide fusion proteins to lipid rafts clearly demonstrates conserved function.

P. luminescens is a bacterial symbiont of entomopathogenic nematodes. It is transported to the hemocoel of an insect prey, which is then killed by an arsenal of toxins and T3SS effectors (Derzelle *et al.*, 2004). *Vibrio splendidus* is a dominant *Vibrio* in coastal sediments and seawater, which is pathogenic for bivalves and fish and shares an ecological niche with *V. cholerae*

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(Lacoste *et al.*, 2001; Gay *et al.*, 2004). *B. bronchiseptica* infects the respiratory epithelia of a wide range of mammals, including humans, and appears to have an environmental reservoir as well (Akerley *et al.*, 1995). The evolutionary journey the LRT domain has taken between pathogens of insects, fish, and mammals provides a poignant example of the conservation of virulence mechanisms in nature and exemplifies the ancient origins of bacterial-host interactions.

Materials and Methods

Bacterial Strains and Cell Culture HeLa cells were maintained in DMEM (Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Hyclone) and penicillin-streptomycin. L2 cells were maintained in DMEM or F12K media (Invitrogen) supplemented with 10% FBS and pen-strep. Both cell lines were grown at 37C in a 5% CO_2 atmosphere. *Bordetella bronchiseptica* strains RB50, RB50 Δ *BscN*, Δ*bteA and* Δ*bopB* have been previously described (Cotter and Miller, 1994; Yuk *et al.*, 1998; Panina *et al.*, 2005). Full length or truncated *bteA* loci from *B. bronchiseptica*, *B. pertussis* Tohama I or *B. parapertussis* strain 12822 (Parkhill *et al.*, 2003) were cloned into pBBR1MCS-5 for expression in *Bordetella*. All *B. bronchiseptica*-derived strains were cultured in Stainer-Scholte liquid medium or on Bordet-Gengou agar (Becton Dickinson Microbiology Systems, San Jose, CA) containing 7.5% defibrinated sheep blood (Mission Laboratories, Rosemead, CA) and appropriate antibiotics for plasmid selection.

Construction of BteA–EGFP and LRT-EGFP fusions Genes encoding full-length BteA (BteA^{658}) and BteA derivatives with C-terminal deletions (BteA^{30}, BteA^{300}, BteA^{400}, BteA^{500}, BteA^{540}, BteA^{580}, BteA^{620}, BteA^{628}, BteA^{644}, and BteA^{652}) were cloned into the Nhe I and Hind III restriction sites in pEGFP-N1 (Clontech), in-frame with the C-terminal EGFP tag. *bteA* fragments were PCR amplified from *B. bronchiseptica* RB50 using Herculase (Stratagene). All fragments include the first codon of BteA and terminate at the codon indicated by their superscript designation (e.g. BteA^{300} includes codons 1 to 300). EGFP-tagged mutants containing N-terminal BteA deletions (BteA$^{131-644}$, BteA$^{131-658}$) were constructed by cloning BteA fragments into the same sites on pEGFP-N1 and adding ATG start codons for translation initiation. LRT domains from Plu4750 (codons 1 to 132), Plu3217 (codons 1808 to 1907), and the full-length Plu4750 protein (codons 1 to 316) were cloned from *P. luminescens* sp. *luminescens* (ATCC)

into pEGFP-N1 to construct EGFP fusions. An N-terminal ATG codon was added immediately upstream of LRT sequences from Plu3217.

Transfection, LDH release assays, and MBCD treatments HeLa or L2 cells grown in 12-well plates were transfected with 1.6 μg of plasmid DNA using Lipofectamine LTX (Invitrogen). For LDH release assays, transfected cell cultures were briefly centrifuged to remove cell debris, and 50 μl of media was removed and assayed for LDH activity using the Promega CytoTox 96 assay kit according to the manufacturer's protocol. For MBCD protection or attachment assays, HeLa cells were washed with PBS and incubated in DMEM + 2.5 m*M* MBCD (Sigma Aldrich) for 30 min. MBCD-containing medium was removed, cells were washed with PBS and cultured in reduced serum DMEM (1.0% FBS) lacking MBCD. *B. bronchiseptica* RB50 or RB50 Δ *BscN* grown to mid-log phase were added at an MOI of 50:1 and centrifuged onto cultured cells at 640 x *g* for 5 min. 15 min. after centrifugation, colony forming units (CFU) of attached bacteria were assayed by spreading serial dilutions of the cell lysate on BG agar following washing and solubilization of the cell monolayer in 0.2% Triton X-100. *Bordetella*-induced cytotoxicity was measured as described above at time intervals up to 3 hr. post infection.

Confocal microscopy Cells were grown in multiwell plates on glass coverslips and transfected as described above. 18 hr. post transfection, cells were washed and fixed with 4% paraformaldehyde in 1X PBS for 30 min. at room temp. To visualize EGFP fusion proteins, glass slides were mounted using Vectashield mounting media (Vector Laboratories) and sealed with clear nail polish. For actin and ezrin staining, cells were permeabilized with 0.1% Triton X-100 for 10 min., and stained with either Alexa Fluor -conjugated phalloidin (Invitrogen) or anti-ezrin primary antibody (BD Biosciences) followed by fluorescently-labeled secondary antibody (Invitrogen). GM1 staining using Alexa-fluor 555-labeled CtxB was performed as described

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(Chen *et al.*, 2007). HeLa cells infected with *B. bronchiseptica* were washed twice with PBS, fixed as described above, and *B. bronchiseptica* was visualized using Vectashield mounting media with DAPI (Vector Laboratories). All images were taken with a Leica SP2-AOBS laser scanning confocal microscope setup. Images are representative of multiple independent experiments and were produced with Leica's LCS confocal software.

Western blot analysis *B. bronchiseptica* strains were grown overnight in Stainer-Scholte medium and cell cultures were separated into pellet and supernatant fractions. Whole cell extracts were prepared by dissolving pellets in Laemmli buffer (Laemmli, 1970). Proteins from supernatant fractions were precipitated with 10% trichloroacetic acid for 6 hr. at 4°C. OD equivalents of *B. bronchiseptica* whole-cell lysates and supernatants were separated on 10% SDS-PAGE gels and transferred to PVDF membranes (Millipore). Membranes were incubated with mouse anti-FLAG antibody (Sigma Aldrich) and antigen-antibody complexes were detected with horseradish peroxidase-conjugated anti-mouse immunoglobulin (Amersham Biosciences) and visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Bacterial two hybrid analysis *B. bronchiseptica* sequences were cloned into the BacterioMatch (Stratagene) two-hybrid "bait" vector pBT, creating fusions with the C terminus of the lambda cI protein, and/or into the cognate "target" vector pTRG, creating fusions with the amino-terminal domain of the alpha subunit of RNA polymerase. Clones were confirmed by sequence analysis and co-transformed into the BacterioMatch I reporter strain (XL1Blue MRF' *laqI*^{q} *bla lacZ* Kan^r). To assay ß-galactosidase activity, overnight cultures were subcultured to an optical density (OD$_{600}$) of 0.07 in LB medium containing tetracycline (15 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}). Cultures were incubated at 30°C until the OD$_{600}$ reached 0.4, at which point isopropyl ß-D-

thiogalactoside (IPTG) was added to a final concentration of 10 µM. After 2 hr. of IPTG induction, cells were harvested and permeabilized with sodium dodecyl sulfate (SDS) and CHCl$_3$, and ß-galactosidase activity was measured using the method described by Miller (Sommer *et al.*, 1978).

Detergent resistant membrane (DRM) fractionation Detergent resistant membrane fractions were prepared as described (Ignoul et al., 2007). Approximately 18 hr. after transfection, 1-2x10^7 cells were washed with PBS, scraped into 1 mL of lysis buffer containing 1.0% Triton X 100 and protease inhibitor cocktail (Roche), and adjusted to a final sucrose concentration of 45%. The cell lysate was overlaid with a 30%-5% sucrose step gradient, and separated for 16 hr. at 38,000 RPM by isopycnic flotation in a Beckman SW-41 rotor. 1mL fractions were collected, and sedimented in a Ti70.1 rotor at 40,000 RPM for 1 hr. The pellets were resuspended in 1X Laemmli buffer (Laemmli, 1970) and electrophoresed on 4-16% gradient SDS-PAGE gels (BioRad). Proteins were transferred to PVDF-Plus membranes (Amersham) and probed with rabbit anti-EGFP (Abcam), mouse anti-ezrin (BD-Biosciences), or mouse anti-caveolin-1 (Santa Cruz). Chemiluminescent detection was performed using Amersham's ECL reagents and Kodak BioMax MS film.

Bacterial attachment assays HeLa cells preincubated with MBCD or media alone as described above were infected with *B. bronchiseptica* RB50 at an MOI of 50:1 for 25 min. Infected cultures were briefly centrifuged at 640 x g for 5 min. followed by a thorough wash with PBS. Cells were solubilized in 0.2% Triton X-100, and aliquots assayed for CFU on BG plates. In parallel, attached *B. bronchiseptica* were stained, visualized by light microscopy, and manually counted.

Figure Legends

Fig. 1. BteA loci of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* are functionally interchangeable. (A) Schematic representation of *bteA* loci from three *Bordetella* subspecies. Percent identities between *B. bronchiseptica* proteins and their homologs in human-adapted *B. pertussis* and *B. parapertussis* are indicated. Positions of residues that differ between *B. bronchiseptica* and other subspecies are indicated by vertical bars. (B) BteA expressed by human-adapted bordetellae is functionally interchangeable with BteA from *B. bronchiseptica*. HeLa, L2 and MLE-12 epithelial cell lines were infected with wild type *B. bronchiseptica* RB50 wild-type (WT) or a Δ*bteA* derivative. The Δ*bteA* allele was complimented with vector alone (pvec) or the *bteA* locus from *B. bronchiseptica* (p*bteA*$_{Bb}$), *B. pertussis* (p*bteA*$_{Bp}$), or *B. parapertussis* (p*bteA*$_{Bpp}$).

Fig. 2. BteA is a potent cytotoxin for mammalian cells. (A) Full-length BteA is not detected in eukaryotic cells by fluorescence microscopy. HeLa cells were transfected with BteA^{658}-EGFP or EGFP alone for 2, 4, 6 or 21 hr. and visualized via fluorescence microscopy. Typical images of the same field showing green fluorescence and DAPI nuclear staining are presented. (B.) Full-length BteA (aa 1 to 658) and C-terminally truncated mutants were fused to EGFP (green bars) on the plasmid pEGFP-N1. The EGFP tag is not drawn to scale. The N-terminal BteA domain (aa 1-130) is shown in red and residues at EGFP fusion junctions are indicated. Constructs were transfected into HeLa cells and cytotoxicity was measured 4 hr. later by LDH release assays. (C) Western Blot analysis of complex formation by full-length BteA^{658}-FLAG (658 aa) and the noncytotoxic derivative BteA^{644}-FLAG (644 aa) using anti-FLAG monoclonal antibody. Both proteins were expressed in a Δ*bteA* derivative of *B. bronchiseptica* strain RB50. RB50 expressing untagged full-length BteA was used as a negative control. High MW complexes

(arrowheads) were observed for both BteA[658]-FLAG and BteA[644]-FLAG in whole cell extracts and culture supernatants.

Fig. 3. BteA localization. BteA[644]-EGFP was transfected into HeLa cells. 18 hr. later cells were fixed, permeabilized, and visualized by laser scanning confocal microscopy. Images represent average composite Z-stacks processed by Leica's LCS software. BteA644-EGFP colocalization was assessed using Alexa-Fluor 568-conjugated phalloidin to stain actin (A), Alexa Fluor 633 anti-ezrin (B, C), and Alexa-Fluor 555 labeled CtxB to stain GM1 (D). (E), XZ scans of the region indicated by the arrow in (D). Colocalization appears as yellow, for two-color overlays, or white in three color overlay (4D).

Fig. 4. Distribution of LRT domains in putative bacterial virulence factors. (A) Schematic representation of known and predicted proteins with LRT domains (red bars) and their closest homologs lacking LRT sequences. The schematic is not drawn to scale. Homologous domains are colored as indicated. The cysteine protease domain in RtxA and related toxins, and a cleavage site identified in RtxA, are indicated by hatched boxes and an arrow, respectively (Sheahan et al., 2007), (see Discussion). Species origin is indicated in parentheses by a two-letter code: *Bb, B. bronchiseptica; Bp, B. pertussis; Bpp, B. parapertussis; Pl, P. luminescens; Pa, Photorhabdus asymbiotica, Vs, V. splendidus.* (B) Alignment of LRT domains in known and predicted virulence factors. The central region of the BteA LRT domain (aa 34-112) is shown, with identical amino acids present in all 9 sequences in red and conserved residues in blue.

Fig. 5. The N-terminal LRT domain of BteA mediates chaperone binding and host cell localization. (A) Constructs used in this experiment. Notations are the same as in Fig. 3A. HeLa cells were transfected with BteA[130]-EGFP (B) or BteA[130-644]-EGFP (C). EGFP fusion proteins

and GM1 bound to Alexa-Fluor 555 labeled CtxB were visualized by fluorescence confocal microscopy. Images are single Z-sections. Co-localization between transfected proteins and GM1 appears as yellow on the overlay images. (D) Cytotoxicity assays. Full length (BteA[658]) or truncated BteA-EGFP fusion constructs were transfected into HeLa cells and LDH release was measured 25 hr. post-transfection. E. Bacterial two-hybrid analysis of interactions between BtcA, BteA and BteA domains. The pTRG target vector alone (vec), or pTRG expressing full length BtcA fused to the amino-terminal domain of the alpha subunit of RNA polymerase was transformed with the pBT bait vector (vec), or pBT expressing full length BteA (BteA[658]), or fragments truncated at their N- and/or C-termini fused to the C-terminus of the lambda repressor.

Fig. 6. BteA is present in detergent resistant membrane (DRM) fractions from transfected cells. HeLa cells were transfected with the constructs indicated (left sidebar), and fractionated as described in Materials and Methods. Western blots were probed with anti-EGFP or antibodies to ezrin and caveolin as indicated in the left sidebar. Transfected (T) or untransfected (U) cell controls are shown in the right sidebar. Lanes within the red outline are low-density DRM fractions.

Fig. 7. *B. bronchiseptica* RB50 adheres to GM1-containing lipid rafts. Images are representative of single Z-sections. HeLa cell monolayers were infected with *B. bronchiseptica* strain RB50 (A) or the T3SS-defective strain RB50 Δ *BscN* (B) for 25 min., washed, fixed, and stained. DAPI staining (green) reveals bacteria adherent to HeLa cells. GM1 appears red, and overlap is yellow in the merged images.

Fig. 8. BteA is translocated by the *Bsc* T3SS into lipid rafts that coincide with sites of bacterial attachment. A single HeLa cell is shown, infected with RB50 expressing an HA-tagged BteA^{644} (BteA^{644}-HA) and stained using DAPI (bacteria) and Alexa Fluor 488-conjugated secondary antibody to HA. (A) BteA644-HA (red) colocalizes with adherent bacteria (green). (B) 100X magnification, XY confocal image of BteA^{644}-HA stained with DAPI for bacteria (blue), anti-HA (red), and GM1 ganglioside (green). (C) 100X magnification section XZ scan and merged images of the same portion of the cell as in (B). The arrows indicate the position of bacterial cells. Colocalization appears yellow in two color overlays, and white in triple color overlays. Image scans are single Z sections.

Fig. 9. MBCD treatment interferes with bacterial attachment and protects cells from T3SS-mediated cytotoxicity. (A) HeLa cells incubated with media alone (black bars) or 2.5 m*M* MBCD (grey bars) for 30 min. were washed and infected with WT *B. bronchiseptica* RB50 for 15 min. at an MOI of 50:1. Following infection, cells were extensively washed, solubilized, and adherent bacteria measured as colony forming units (CFUs) per well. All statistical analyses were performed using the Student's t-test. Asterisk indicates a p value < 0.001. (B) HeLa cells were first infected with WT *B. bronchiseptica* RB50 for 15 min, washed, and incubated with media alone (black bars) or 2.5 m*M* MBCD (grey bars) for 30 min. Cells were extensively washed, solubilized, and adherent bacteria measured as colony forming units (CFUs) per well. There was no statistically significant difference between untreated and MCDB-treated cells. (C) HeLa cells preinfected with *B. bronchiseptica* RB50 for 15 min, or uninfected, were incubated with media alone (black bars) or 2.5 m*M* MBCD (striped bars) for 30 min as described in (B). Cells were then washed and incubated in fresh media for 2 h 15 min. Bars indicate LDH release

as a percentage of maximum cell lysis. Wells designated as "Max lysis" were treated with

MBCD as described above and chemically lysed to generate maximum values for LDH release.

Fig. 10. Functional conservation of LRT domains from T3SS effectors and RTX-type toxins. Co-localization of ezrin with the isolated LRT domains from Plu3217 (A) and Plu4750 (B) fused to EGFP, and with the full-length Plu4750 protein fused to EGFP (C). HeLa cells were transfected with expression plasmids and EGFP fusion proteins, or GM1 were visualized by fluorescence microscopy. Colocalization between transfected proteins and GM1 appears as yellow on the merged images.

Fig. 1

A.

B.

82

Fig. 2

A.

B.

% wt cytotoxicity

C.

83

Fig. 3

A. BteA644-EGFP   Actin   Merge

B. BteA644-EGFP   Ezrin   Merge

C. EGFP   Ezrin   Merge

D. BteA644-EGFP   GM1   Merge

E. BteA644-EGFP   GM1   Merge   Ezrin   Merge + Ezrin

84

Fig. 4

85

A.

T3SS effectors
- BteA (*Bb,Bp,Bpp*) 658
- Plu4750 (*Pl*) 316
- Plu0882 (*Pl*) 408

LRT

RTX-type toxins
- Plu1341 (*Pl*) 3672
- Plu1344 (*Pl*) 4070
- Plu3217 (*Pl*) 3528
- Plu3324 (*Pl*) 3531
- Rtx (*Pa,Vs*) 4872

Legend:
- LRT domain
- BteA cytotoxicity
- polyproline linker
- actin ADP-ribosyltransferase
- N-terminal "novel repeats"
- ACT domain
- C-terminal GD repeats
- Cys protease
- cleavage site
- class I chaperones
- conserved domains
- unknown function

B.

```
Plu1341   LLKEGSKLFINPDERTLGQKRP--LSQAMTAVRDIFYKTMSHFDE--EHVLQFEQVIADWQQHSPKEFALRANQVNLIRFRMG
Plu1344   LLKEGNKLFINPNVRTLGRKRK--LSTALATVRDTFYKTMSHFDE--EHILLERAIADWQQHSPKEFALRTNQVNLVRFKMG
Plu3217   LLSQGDKRFIASTKRYLGQINTDRPSKALVAVREAFNNAAEQPDE--QHVLQLEQALAHWQQHDPNEFAKRGRLVKSLRFEMG
Plu3324   LLKKDNKRFIDSTKRRLGSINTDLPSKALVAVREAFNRTVEQPDE--QHVLQLEQTLAHWQQHDPKEFTQRSKLVKSLRFEMG
Plu0882   LLEQGNRLFEQSVRR--GPLHFQ--SSSLKHLCAELRQLQNAPSS--MQARRVQDAIQHWENHHPKEVMARSTRLAELKQAL--
Plu4750   LLEQGNRLFEQSVRR--GPLHFQ--SSDLKHLITELRQLQSAPNS--AQAQRVQDAIQHWESHHPKEVAARSTRLAELKQALA
Rtx (Pa)  LLNKGDKRFIDSTKRRLGAINTDRPSKALVAVREVFNSTVKQPDE--QHVLQLEQALAHWQQHDPKEFAQRSKLVKSLRFEMG
Rtx (Vs)  LLGPDDVRFVDSTKRHLGKLSTDLPSKELKAVRSHIQSIQRIPSE--SNLAVLELAVEQWQQNNPKEFAQRGEMVKTLQFEIV
BteA      LLEPNNDEFVRSVAS--PRLHHS--SEALREVKHDVRQFQASGDRSLQQLRDLEVALNHWEASQPREFAKRGGMVAELRTAID
```

Fig. 5

A.

B. BteA¹³⁰-EGFP   GM1   Merge

C. BteA¹³¹⁻⁶⁴⁴-EGFP   GM1   Merge

D. LDH release

E. Bacterial two hybrid analysis

86

Fig. 6

Rafts (DRMs)

Density

EGFP

BteA[133-644] GFP

BteA[1-133] GFP

BteA[1-644] GFP

caveolin

ezrin

T   U

α-EGFP

Fig. 7

A.

RB50 wt.   GM1   Merge

B.

Δ*bscN*   GM1   Merge

Fig. 8

A.

B. bronchiseptica   BteA⁶⁴⁴-HA   Merge

B.

XY

B. bronchiseptica   BteA⁶⁴⁴-HA   GM1   Merge   + *B. bronchiseptica*

C.

XZ

88

Fig. 9

A.

B.

C.

Fig. 10

A. Plu3217 LRT   GM1   Merge

B. Plu4750 LRT   GM1   Merge

C. Plu4750 full length   GM1   Merge

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Chapter 3. Dissection of the *Burkholderia* intracellular lifecycle using a photothermal

nanoblade

Abstract

Burkholderia pseudomallei and *B. thailandensis* are related pathogens that invade a variety of cell types, replicate in the cytoplasm and spread to nearby cells. We have investigated temporal and spatial requirements for virulence determinants in the intracellular lifecycle using genetic dissection and photothermal nanoblade delivery, which allows efficient placement of bacterium-sized cargo into the cytoplasm of mammalian cells. The conserved Bsa type III secretion system (T3SS$_{Bsa}$) is dispensable for invasion, but is essential for escape from primary endosomes. By nanoblade delivery of *B. thailandensis* we demonstrate that all subsequent events in intercellular spread occur independently of T3SS$_{Bsa}$ activity. Although intracellular movement was essential for cell-cell spread by *B. pseudomallei* and *B. thailandensis*, neither BimA-mediated actin polymerization nor the formation of membrane protrusions containing bacteria were required for *B. thailandensis*. Surprisingly, the cryptic (*fla2*) flagellar system encoded on chromosome 2 of *B. thailandensis* supported rapid intracellular motility and efficient cell-cell spread. Plaque formation by both pathogens was dependent on the activity of a type VI secretion system (T6SS-1) that functions downstream from T3SS$_{Bsa}$-mediated endosome escape. A remarkable feature of *Burkholderia* is their ability to induce the formation of multinucleate giant cells (MNGCs) in multiple cell types. By infection and nanoblade delivery, we observed complete correspondence between mutant phenotypes in assays for cell fusion and plaque formation, and time course studies showed that plaque formation represents MNGC death. Our data suggest that the primary means for intercellular spread involves cell fusion, as opposed to pseudopod engulfment and bacterial escape from double-membrane vacuoles.

Introduction

Burkholderia pseudomallei (*Bp*) is a robust Gram-negative bacillus endemic to warm, fecund soils of tropical regions (Wiersinga *et al.*, 2006; Galyov *et al.*, 2010). Infections acquired from the environment can lead to melioidosis, a serious and sometimes fatal human disease. Accumulating evidence supports the notion that adaptations selected in the rhizosphere are responsible for "accidental virulence" in mammals (Nandi *et al.*, 2010). *Bp* has a large (7.2 Mb) genome that has been shaped by extensive horizontal exchange (Holden *et al.*, 2004). *B. mallei* (*Bm*) is a clonal descendent of *Bp* that has undergone genome decay and has lost the capacity for environmental survival. *Bm* is the agent of equine glanders and it can also cause fatal human infections (Galyov et al., 2010). Resistance to antibiotics and their low infectious dose have led to the classification of *Bp* and *Bm* as biowarfare threats.

The geographic distribution of *Bp* overlaps with that of *B. thailandensis* (*Bt*) and their genomes are highly similar and syntenic (Kim *et al.*, 2005). Although *Bt* is rarely associated with human disease and is considered relatively non-pathogenic, this assessment is not absolute. Following aerosol challenge of mice, *Bt* causes fulminant, lethal infections that are dependent on virulence determinants that are shared with *Bp* and *Bm* (West *et al.*, 2008; Galyov *et al.*, 2010). *Bp*, *Bt* and *Bm* invade and replicate in a wide range of cell types and exhibit nearly identical intracellular lifecycles (Wiersinga *et al.*, 2006; Galyov *et al.*, 2010). Following invasion and escape from endosomes, replication in the cytoplasm is accompanied by actin-based motility and cell-cell spread, analogous to *Shigella flexneri* and *Listeria monocytogenes* (Tilney and Portnoy, 1989; Stevens *et al.*, 2006; Ray *et al.*, 2009). Actin motility is mediated by BimA, a polarly localized surface protein that binds actin and promotes polymerization (Stevens et al., 2006). An unusual feature of infection is the induction of cell fusion and the formation of multinucleate

giant cells (MNGCs) (Kespichayawattana *et al.*, 2000). For *Bp* and *Bm* this requires BimA and has been observed with multiple cell types *in vitro* and in tissues from patients with melioidosis. (Galyov et al., 2010).

Bp possesses a generous endowment of specialized export systems including three "injection" type III secretion systems (T3SS), two of which are similar to those in phytopathogenic bacteria. The third, T3SS$_{Bsa}$, is homologous to the *Shigella* Mxi-Spa and *Salmonella* SPI-1 T3SSs and is highly conserved in *Bp*, *Bt* and *Bm* (Wiersinga *et al.*, 2006; Galyov *et al.*, 2010). T3SS$_{Bsa}$ is required for virulence in hamster and murine models of pathogenesis (Galyov et al., 2010) and has been implicated in invasion of epithelial cells, escape from endosomes, intracellular survival, and evasion of autophagy (Gong *et al.*, 2011). In addition, *Bp* encodes six type VI secretion systems (T6SSs) (Pukatzki *et al.*, 2006). Using the nomenclature of Schell et al. (Schell *et al.*, 2007), T6SS-1 (also referred to as T6SS-5) (Schwarz *et al.*, 2010b) is critical for virulence in the *Bt* murine model of acute melioidosis and contributes to the lethality of *Bm* in hamsters (Schell et al., 2007). Recently, T6SS-1 mutants in *Bp* were shown to be capable of endosome escape in RAW264 cells but were defective in MNGC formation (Burtnick *et al.*, 2011).

For intracellular pathogens, understanding the roles of virulence determinants is complicated by their involvement in temporally and spatially staged events. T3SS$_{Bsa}$ has been proposed to be required for late events associated with cell-cell spread, but direct investigation of this has been difficult since mutants are defective in earlier steps in the intracellular lifecycle. To address this conundrum, we have used a photothermal nanoblade to deliver live bacteria directly into the cytoplasm of mammalian cells (Wu *et al.*, 2011). The photothermal nanoblade device uses a laser pulse to excite a thin titanium coating on the tip of a glass capillary pipette. Rapid

thermal excitation of the metallic nanostructure produces an explosive nanoscale vapor bubble that creates a small incision in the cell membrane at the point of pipette contact. This incision provides a transient delivery portal through which variably sized cargo - from molecules to bacteria - can be efficiently delivered with high cell viability. We have combined the use of this technology with traditional genetic ablation techniques and infection analysis to probe virulence mechanisms participating in the intracellular lifecycle of *Burkholderia*.

Results

T3SS$_{Bsa}$ is required for plaque formation and endosome escape but is dispensable for invasion HEK293 cells are efficiently invaded by *Bp* and *Bt* and are highly amenable to photothermal nanoblade-mediated cytosolic delivery. In the experiment in Fig. 1*A*, HEK293 monolayers were infected with *B. thailandensis* E264 (Brett *et al.*, 1998), *B. pseudomallei* Bp340 (Mima and Schweizer, 2010) or derivatives containing in-frame deletions in *sctN*, which encodes the ATPase required for activity of the Bsa T3SS. Plaque formation, a hallmark of cell-cell spread, was eliminated in Δ*sctN* mutants and restored by complementation *in trans*. These observations are consistent with previous reports that plaque formation by *Bp* is dependent on T3SS$_{Bsa}$ (Pilatz *et al.*, 2006).

It has been suggested that T3SS$_{Bsa}$ is required for invasion of nonphagocytic cells, a prerequisite for plaque formation (Stevens *et al.*, 2003). For Bp340 and BtE264, invasion was inhibited by cytochalasin D as expected, but it was unaffected by Δ*sctN* alleles (Fig. 1*B*). These results indicate that while invasion requires actin polymerization, it occurs independently of T3SS$_{Bsa}$ activity. Analogous results were obtained using HeLa cells (Fig. S1*A*). To determine if T3SS$_{Bsa}$ facilitates endosome escape in HEK293 cells, monolayers were stained 8 hr. after infection for F-actin. As shown in Fig. S1*B*, BtE264 and Bp340 formed actin tails, indicating successful escape, while Δ*sctN* mutants and Δ*bimA* control strains did not. Moreover, Δ*sctN* mutants colocalized with the late endosomal marker LAMP1 (Fig. S1*C*). At later timepoints, only scattered bacterial debris was detected for Δ*sctN* mutants, suggesting they were killed and degraded due to endosomal entrapment (Fig. S1*D*). Although our results do not support a role for T3SS$_{Bsa}$ in invasion, they are consistent with previous reports of its essential contribution to endosome escape (Haraga *et al.*, 2008; Galyov *et al.*, 2010).

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Intercellular spread following cytoplasmic delivery by a photothermal nanoblade The inability of Δ*sctN* mutants to escape from primary endosomes confounds efforts to understand the involvement of T3SS$_{Bsa}$ in subsequent steps required for cell-cell spread. Solutions to this dilemma require the ability to bypass early events that are otherwise essential during infection. To accomplish this, we exploited the capabilities of our recently developed photothermal nanoblade to place bacteria directly into the cytoplasm of host cells (Fig. 2*A*) (Wu et al., 2011). Since a nanoblade has not yet been customized and approved for use with *Bp* in a select agent BSL-3 facility, we focused our analysis on *Bt* which can be safely manipulated under BSL-2 conditions.

In Fig. 2, wild-type (WT) BtE264 or mutant derivatives were introduced into HEK293 cells by infection or by photothermal nanoblade delivery. Plaque formation following infection was absolutely dependent on T3SS$_{Bsa}$ activity (Fig. 2*B*). When Δ*sctN* mutants were delivered into the cytosol using the nanoblade, they divided and polymerized actin as well as wild type bacteria (Fig. 2*C*), demonstrating that actin motility functions independently of T3SS$_{Bsa}$ and does not require passage through the endosomal environment. We were surprised, however, to find that Δ*sctN* mutants were capable of forming plaques following nanoblade delivery that were indistinguishable in size and morphology from those formed by WT *Bt* following infection *or* nanoblade delivery (Fig. 2*D*-2*F*). The ability to bypass early events in the intracellular lifecycle allows us to conclude that the *only* requirement for T3SS$_{Bsa}$ in cell-cell spread and plaque formation is for escape from primary endosomes following invasion. It had been assumed that for cell-cell spread to occur, T3SS$_{Bsa}$ would be required to lyse double membrane secondary vacuoles formed during penetration of adjacent cells (Wiersinga et al., 2006). Our observations

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indicate that either some other factor(s) perform this function, or that intercellular spread occurs by an entirely different mechanism.

Two distinct motility systems facilitate plaque formation A deletion mutant in *bimA* was included as a control for plaque formation in photothermal delivery experiments. As shown in Fig. S1*B* and Fig. 2*C*, the Δ*bimA* allele eliminates actin polymerization as previously described (Sitthidet *et al.*, 2010), and it also eliminates the formation of membrane protrusions containing *Burkholderia* at their tips. Although we assumed that actin motility would be required as a driving force for cell-cell spread, this was clearly incorrect. Following infection or nanoblade delivery, Δ*bimA* mutants formed plaques that were similar in size and morphology to those of their WT parent (Fig. 2*B*, 2*D* and 2*E*). BimA-mediated actin motility and the formation of membrane protrusions are therefore dispensable for cell-cell spread by *B. thailandensis*.

Intrigued by these results, we examined live infected cells by microscopy and discovered that *Bt* exhibits remarkably rapid intracellular motility. Bacteria move at speeds of >20 μm/sec and often reverse course and change direction (Fig.S2*A*). Since this was independent of BimA (Fig. 3*A*), we explored the possibility that flagellar motility was involved. Deletion of *motA1*, a motor component locus in the chromosome 1 flagellar biosynthesis gene cluster (*fla1*), eliminated swarming in soft agar but had no effect on intracellular motility or plaque formation (Fig. S2*;*, Fig. 3*A*, 3*B*; Table S1). Previous studies identified potential chemotaxis and flagellar loci on chromosome 2 in *Bt* (Tuanyok *et al.*, 2007), and on closer inspection we found a full complement of flagellar structural and regulatory genes that could encode a second, functional motility system (*fla2*; Fig. S3). Deletion of *motA2* from the *fla2* flagellar cluster had no effect on swarming in agar or actin polymerization following invasion (Fig. S2*B*, 3*C*), but it eliminated rapid intracellular motility (Fig.3*A*), as did deletion of *fliC2* which is predicted to encode

flagellin (Table S1). Although significant differences were not observed in plaquing efficiency (Fig. 3*B*), Δ*motA2* mutants formed plaques that were smaller than those produced by WT or Δ*bimA* strains (Fig. 3*D;* Table S1). When *motA2* was deleted from a Δ*bimA* background, plaque formation was almost completely abolished, and the defect was reversed by complementation with *motA2* (Fig. 3*B*).

These observations demonstrate that MotA2-dependent flagellar motility can drive intercellular spread independently of BimA-mediated actin polymerization, and at least one of the two motility systems must be active for plaque formation. Although flagellar motility does not affect invasion or endosome escape (Fig. 3*E;* Table S1), an interesting phenotype is observed in intracellular growth assays. As shown in Fig. 3*F*, WT, Δ*bimA* and Δ*motA2* strains multiply, plateau at 12 hr., and decrease in numbers due to cell disruption and exposure to extracellular antibiotics. In contrast, Δ*bimA*Δ*motA2* mutants continue to multiply and reach significantly higher levels, suggesting a relationship between intracellular movement and cell death (see below).

T6SS-1 facilitates plaque formation Of the multiple T6SSs encoded by *Burkholderia* species, T6SS-1 has been repeatedly linked to host-pathogen interactions (Pilatz *et al.*, 2006; Pukatzki *et al.*, 2006; Schell *et al.*, 2007; Schwarz *et al.*, 2010b; Burtnick *et al.*, 2011). These correlations with virulence led us to investigate the role of T6SS-1 following infection and nanoblade delivery. T6SS-1 was inactivated by deletion of the *clpV1* ATPase (Shalom *et al.*, 2007). Δ*clpV1* mutants were fully invasive, escaped from endosomes, replicated in the cytoplasm, polymerized actin and displayed rapid intracellular motility similar to WT (Fig. 4*A*-4*C;* Table S1). In contrast, plaque formation following infection (Fig. 4*D*) or nanoblade delivery (Table S1) was reduced to

near background levels, demonstrating that *clpV1*-dependent T6SS activity is crucial for intercellular spread.

Motility and T6SS-1 mutants exhibited similar phenotypes. In both cases major defects in plaque formation are observed, and as shown in Fig. 4*C*, Δ*clpV1* mutants replicate to higher numbers in intracellular growth assays, similar to the Δ*bimA*Δ*motA2* double mutant (Fig. 3*F*). As shown in Fig. 4*E*, the kinetics of cell death are significantly delayed following infection by Δ*clpV1* mutants, suggesting that increased bacterial numbers reflects prolonged cell survival. In considering how these and earlier observations might be related, a closer look at the process of plaque formation itself is revealing.

Intercellular spread and plaque formation occur through cell fusion *Bp, Bm and Bt* induce cell fusion and multinucleate giant cell (MNGC) formation with remarkable efficiency in a variety of cell types (Kespichayawattana *et al.*, 2000; Boddey *et al.*, 2007; Galyov *et al.*, 2010). In observing cell monolayers following nanoblade delivery, we often noticed the appearance of MNGCs in areas that developed to become plaques. To examine the process more closely, we constructed HEK293 cell lines that constitutively express green fluorescent protein (GFP) or monomeric strawberry RFP by stable transduction with recombinant lentivirus. In Fig. 5*A*, GFP and RFP-expressing cells were seeded at a 1:1 ratio and *Bt* or mutant derivatives were introduced by infection or nanoblade delivery. The progression of events leading to plaque formation is readily apparent; individual cells (red or green) initially fuse to form one or more MNGCs (yellow), which later lyse to form a clear zone in the monolayer surrounded by a ring of fused cells (i.e. a plaque). A MNGC containing numerous DAPI-stained nuclei prior to lysis is shown in Fig. 5*B*.

Fig. 5*C* shows early (12 hr., upper panel) and late (24 hr., lower panel) timepoints in cell fusion-plaque assays with BtE264 or mutant strains. Although Δ*sctN* mutants failed to form MNGCs or plaques following infection due to endosomal entrapment, both events occurred normally after nanoblade delivery, demonstrating that T3SS$_{Bsa}$ is not required for MNGC formation. In BtE264, deletion of *bimA* or *motA2* individually had little effect, whereas a Δ*bimA*Δ*motA2* double mutant was greatly delayed in MNGC formation. A similar phenotype was observed with the Δ*clpV1* strain. A Δ*sctN*Δ*clpV1* mutant was incapable of MNGC formation following infection, but resembled the Δ*clpV1* single mutant after nanoblade delivery, showing that T6SS-1 affects cell-cell spread downstream of T3SS$_{Bsa}$-mediated endosomal escape (Fig. 5*C;* Table S1). Fig. 5*D* shows that plaque formation by *B. pseudomallei* also occurs through a process that involves MNGC formation and lysis, and is dependent on *clpV1*. Since Bp340 lacks *fla2* (Fig. S3 and Discussion), this process required *bimA*. Based on these and other results, we propose that cell fusion is the central mechanism for cell-cell spread by *B. thailandensis* and *B. pseudomallei*, and that plaque formation occurs through a process that is dependent on the formation of MNGCs. This differs fundamentally from intercellular spread by *Listeria monocytogenes* which requires actin-based motility, engulfment of bacterial protrusions by adjacent cells and escape from double membrane vacuoles (Tilney and Portnoy, 1989). Indeed, as shown in Fig. 5*E*, plaque formation by *Listeria* occurs in the absence of detectable cell fusion.

Discussion

Our results are incorporated into a model for the *Burkholderia* intracellular lifecycle shown in Fig. S4. This is based on observations with HEK293 cells and may not take into account factors that are specifically required for survival and replication in professional phagocytes.

Invasion of HEK293 by *Bp* or *Bt* requires host-cell actin polymerization but not the activity of T3SS$_{Bsa}$. This contrasts with a clear requirement for the Mxi-Spa and SPI-1 T3SSs in invasion by *Shigella* (Ray et al., 2009) and *Salmonella* (Patel and Galan, 2005) respectively, and with expectations for *Burkholderia* based on analyses of BopE, a T3SS$_{Bsa}$ substrate homologous to *Salmonella* SopE and SopE2 (Stevens et al., 2003). In an experiment often cited as supporting a role for T3SS$_{Bsa}$ in invasion (Stevens et al., 2003), insertion mutations in *bipD* (a T3SS$_{Bsa}$ translocon gene) or *bopE* conferred modest decreases in invasion (35-40%) 6 hr. after infection of HeLa cells. The discrepancy between these data and ours is likely due to the use of a late time point where endosomally trapped mutants loose viability (6 hr. in ref. (Stevens et al., 2003) *vs.* 2 hr. in Fig. 1), giving the appearance of an invasion defect. A similar conclusion was previously reported by Haraga *et al.* using BtE264 and HeLa cells (Haraga et al., 2008). Their study and ours support the conclusion that for *Bp* and *Bt*, invasion of nonphagocytic cells can occur by mechanisms that are independent of T3SS$_{Bsa}$. Virulence determinants that mediate invasion await discovery.

The Bsa T3SS T3SS$_{Bsa}$ is required for escape from endosomes following invasion of HEK293 cells by *Bp* or *Bt*, consistent with numerous prior studies with a variety of cell types (Wiersinga *et al.*, 2006; Galyov *et al.*, 2010). Cytosolic delivery of *Bt* using a photothermal nanoblade

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allowed us to bypass endosome escape and directly examine the role of T3SS$_{Bsa}$ in downstream events: actin polymerization, cell-cell spread and MNGC formation. In all cases the results were remarkably clear; plaque formation following infection was absolutely dependent on T3SS$_{Bsa}$, whereas plaque formation following nanoblade delivery was independent of its activity. The same was true for MNGC formation. We also show that replication and actin polymerization occur normally when Δ*sctN* mutants are placed directly into the cytosol.

These observations have several implications; first, they allow us to conclude that in our system, the *only* role for T3SS$_{Bsa}$ is to facilitate escape from primary endosomes of initially infected cells. Next, our results help explain a perplexing lack of phenotypes associated with known or putative effectors. In contrast to obvious requirements for the Bsa T3SS *in vitro* and *in vivo* (Galyov et al., 2010), to our knowledge no effectors have been *definitively* shown to be required for invasion, replication in nonphagocytic cells, cell-cell spread, MNGC formation or virulence in animals. BopA, a suspected T3SS$_{Bsa}$ substrate, is reported to facilitate survival and evasion of autophagy in phagocytic cells (Gong et al., 2011), but *bopA* mutants are not significantly attenuated in mice (Galyov et al., 2010). The precise mechanism of T3SS-mediated endosome escape is unknown for any intracellular pathogen, however it could conceivably be a function of translocon insertion in the endosomal membrane and occur in the absence of additional effectors. Finally, there is an instructive contrast between the roles of the *Burkholderia* Bsa and *Shigella* Mxi-Spa T3SSs. The *Shigella* system is essential for both endosome escape and for escape from double membrane vacuoles formed during the process of cell-cell spread (Ray et al., 2009). For *Burkholderia*, our results support a model for intracellular spread that obviates the need for membrane lysis after the primary endosome has been breached.

Intracellular motility Polarized, unidirectional actin polymerization is a hallmark of cell-cell spread and plays an essential role for *Shigella*, *Listeria*, and other intracellular pathogens (Stevens et al., 2006). The discovery that *Bt* remains fully capable of plaque formation in the absence of BimA was quite unexpected. Even more surprising was the observation that a predicted flagellar system on chromosome 2 (*fla2*) can compensate for the lack of actin motility and drive intercellular spread and MNGC formation.

The *Bt* and *Bp fla1* flagellar gene clusters on chromosome 1 are highly conserved. *fla1* encodes polar flagella, which in *Bp* have been implicated in invasion of epithelial cells and virulence in animal models (Galyov et al., 2010). In *Bt*, mutation of *motA1* (*fla1*) or *motA2* (*fla2*) individually or in combination had no effect on invasion. Although this is the first characterization of dual flagellar motility systems in an intracellular pathogen, their occurrence and functions have been described in *Vibrio* and *Aeromonas* spp. where they facilitate motility in response to different environmental signals (McCarter, 2004). Not surprisingly, *fla1* and *fla2* in *Bt* were observed to function under different conditions; deletion of *motA1* eliminated swarming in soft agar but had no effect on motility following infection. Conversely, deletion of *motA2* had no effect in soft agar but eliminated rapid intracellular motility.

Our sequence analysis suggests that *fla2* encodes lateral flagella (Fig. S3*B*), but this prediction awaits direct conformation. It is also unknown how the system is regulated, or if intracellular bacteria are simultaneously capable of BimA-mediated actin polymerization and Fla2 dependent motility. Since flagellin monomers are known to activate assembly of the NLRC4 inflammasome, resulting in cytokine production and inflammatory cell death (Miao *et al.*, 2010), the use of flagella for intra- and intercellular motility is surprising. It is presently unknown whether Fla2 flagellin induces inflammasome-dependent cytoplasmic responses or if

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mechanisms to overcome them exist. Perhaps the most important question involves the relevance of our observations with *Bt* to pathogenesis in *Bp* and *Bm*. A recent analysis of the global population structure of *Burkholderia* species pathogenic for mammals predicts an Australian origin for *Bp*, with a single introduction event leading to the expansion of Southeast Asian isolates (SEA *Bp*) and *Bm* (Pearson *et al.*, 2009). Interestingly, the *fla2* gene cluster is absent in SEA *Bp* isolates such as Bp340, which is dependent on BimA for plaque formation (Fig. 5*D*), but it is highly conserved in sequenced genomes from Australian strains (Fig. S3) (Tuanyok et al., 2007). The potential role of the *fla2* locus in pathogenesis by Australian *Bp* remains to be investigated.

T6SS-1 T6SSs are widely distributed among pathogenic and non-pathogenic Gram-negative species; they have broad roles in survival and fitness and have been linked to virulence in numerous pathogens, including *Bp*, *Bm*, and *Bt* (Galyov *et al.*, 2010; Schwarz *et al.*, 2010b). In our analysis, T6SS-1 was found to facilitate intercellular spread following infection or photothermal delivery. Consistent with recent reports (Pilatz *et al.*, 2006; Schwarz *et al.*, 2010b; Burtnick *et al.*, 2011), deletion of *clpV1* resulted in a defect in plaque formation in HEK293 cells and a delay in the formation of MNGCs. Δ*clpV1* mutants exhibited robust actin-polymerization and flagellar-mediated motility inside cells, and genetic dissection using the photothermal nanoblade established that T6SS-1 functions downstream of invasion and T3SS$_{Bsa}$-mediated endosome escape. Concomitant with decreased efficiency of MNGC formation, we observed an increase in cell survival following infection with Δ*clpV1* mutants and an accumulation of intracellular bacteria. These data are consistent with the hypothesis that T6SS-1 participates in events that can alternatively facilitate intercellular spread by fusing cell membranes, or kill cells by compromising their integrity.

Cell fusion and intercellular spread *Burkholderia* efficiently induce MNGC formation in both phagocytic and non-phagocytic cells (Kespichayawattana et al., 2000). We propose that cell fusion represents the primary path for intercellular spread and plaque formation by *Bt* and *Bp* (Fig. S4), and the same is likely to hold true for *Bm*. This is consistent with timecourse observations showing that the formation of MNGCs and their eventual lysis gives rise to the open cores of plaques, and the lack of a requirement for T3SS$_{Bsa}$ in cell-cell spread following cytosolic delivery of bacteria using our photothermal nanoblade. While *Bt* T3SS$_{Bsa}$ mutants are incapable of endosome escape following infection, *Bp* T3SS$_{Bsa}$ mutants are reported to exhibit delayed escape that eventually leads to MNGC formation (Burtnick *et al.*, 2008). This is consistent with our proposal that cell-cell spread occurs independently of T3SS$_{Bsa}$ activity for both *Bt* and *Bp*. Furthermore, mutations that eliminate intracellular motility or inactivate T6SS-1 have analogous effects on MNGC formation and cell-cell spread following photothermal delivery or infection. Our model also explains the lack of published reports demonstrating double-membrane vacuoles following engulfment of protrusions with *Burkholderia* at their tips, as observed with *L. monocytogenes*, *S. flexneri* and other pathogens with similar lifestyles (Tilney and Portnoy, 1989; Ray *et al.*, 2009). Although membrane protrusions are readily formed by wild type *Bt*, they are not observed with Δ*bimA* mutants. The ability of Δ*bimA* strains that retain Fla2 motility to efficiently form plaques shows that membrane protrusions are not required for intercellular spread.

Our results, along with a recent report from Stevens *et al.* (Sitthidet et al., 2010), demonstrate a clear link between motility and the efficiency of cell fusion by intracellular *Burkholderia*. We hypothesize that flagellar and/or actin-mediated motility increases the frequency of contact between bacteria and host cell membranes, and that contact is prerequisite

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for membrane fusion through a process facilitated by T6SS-1. It is tempting to speculate that a bacterially-encoded fusogenic factor is involved in this novel mechanism of cell-cell spread.

Materials and Methods

Bacterial strains, growth and mutant construction BtE264 (Brett et al., 1998) and Bp340 (SEA *Bp* 1026b Δ*amrAB-oprA*) (Mima and Schweizer, 2010) were routinely grown in L-medium with or without NaCl (LB, LB-NS). In-frame mutations were constructed using allelic exchange with the *pheS** negative selection marker on M9 agar containing 0.1% cholorophenylalanine (cPhe) (Barrett *et al.*, 2008). Gene loci that were targeted for mutational analysis: in BtE264; *sctN* (Bth_II0832), *bimA* (Bth_II0875), *clpV1* (Bth_II0864), *motA1* (Bth_I3185), *motA2* (Bth_II0153), and *fliC2* (Bth_II0151). In Bp340; *sctN* (BPSS1541), *bimA* (BPSS1492), and *clpV1* (BPSS1502). Complementation of mutants was performed using derivatives of the pBBR1-MCS2 broad host-range plasmid (Kovach *et al.*, 1995) containing the *nptII* kanamycin resistance gene. For infection studies and cytosolic delivery with the photothermal nanoblade, 2 mL of LB-NS was inoculated with a small amount of bacteria swabbed from a fresh L-NS agar plate and grown with shaking to an OD600 of approx. 0.5.

Cell culture, infection and cytotoxicity assays HEK293 (ATCC CRL-1573) and HeLa (ATCC CCL-2) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% bovine growth serum (BGS, Hyclone) and 5% CO_2. Prior to experiments, plate wells were incubated at room temp for 30 min with a 1:40 dilution of Matrigel liquid (BD) in serum free medium to enhance retention of cells during washing and manipulation. For infection studies, cells were seeded at $1.8-2.0 \times 10^6$ cells per well in 6-well plates. Following the addition of bacteria, plates were gently centrifuged to bring bacteria into contact with cells (200 x *g* for 5 min).

Cells were infected using a multiplicity (MOI) of 1 for cytotoxicity and intracellular replication experiments, an MOI of 10 for invasion, and an MOI of 3×10^{-4} for plaque assays. One hour after infection, cells were washed thoroughly with Hank's balanced salts and extracellular

bacteria were eliminated by the addition of 1000 µg/mL Km. In invasion and replication experiments, infected cells were washed with Hank's, harvested by trypsinization and lysed with 0.2% Triton X-100 + 20 mM MgSO$_4$ and 50 µg/mL DNase I (to reduce lysate viscosity). Assays for intracellular colony forming units (CFU) were performed on serial dilutions of the lysate. Intracellular growth and replication timecourse experiments were done similarly, except cells were harvested at indicated timepoints. For plaque assays, cells were infected as described above and overlaid with DMEM containing 0.2% NaHCO$_3$, 50mM HEPES (pH 7.4), 10% BGS and antibiotics (see below) in 1.2% low melting temp. agarose (BioRad). Plaques were counterstained for photography by adding 200 µl DMEM containing 0.1% neutral red onto the agarose 24 hr. later. For immunofluorescence microscopy, agarose and neutral red staining were not used. Antibiotics to suppress growth of extracellular bacteria were used as follows: 125 µg/mL kanamycin (BtE264 and Bp340), 150 µg/ml gentamicin (pBBR-MCS Km-complemented *Bp* strains) or 10 µg/mL pipericillin/tazobactam (Zosyn) (complemented *Bt* and *Bp* strains).

Cell fusion assays HEK293 cells were transduced with 3rd generation recombinant lentivirus (Dull *et al.*, 1998) encoding enhanced green fluorescent protein (GFP) or mStrawberry red fluorescent protein (RFP). Stable cell lines were isolated by limiting dilution and clonal expansion in DMEM + BGS containing 3 µg/ml puromycin. RFP and GFP cells were seeded for experiments as described above. Starting at 12 hr. after infection or nanoblade delivery, live cells were examined by fluorescence microscopy for the appearance of MNGCs or plaques, or were fixed and stained as described below and in refs. (French *et al.*, 2009; Wu *et al.*, 2011).

Cytosolic delivery of *B. thailandensis* using a photothermal nanoblade Photothermal delivery was performed essentially as described (Wu et al., 2011). The photothermal nanoblade uses a laser pulse to induce ultrafast bubble cavitation at the tip of titanium (Ti)-coated micropipette for efficient cell membrane opening and large cargo delivery. HEK293 cells were cultured in multiwell plates or in LabTek glass bottom dishes. Approximately $5x10^8$ *Bt* cells were washed three times in Hank's Balanced Salts and resuspended at 10^8-10^9 CFU/ml. Ti-coated pulled glass capillary pipets were fabricated by pulling (P-97, Sutter Instrument) a 1 mm diameter borosilicate glass capillary tube, followed by Ti thin film deposition onto the tapered ends. Pipets were loaded with 1-2 µl of $5x10^8$ CFU/ml bacterial suspension. The laser pulse system was a Q-switched, frequency-doubled Nd:YAG laser (Minilite I, Continuum) operated at 532 nm wavelength and 6 ns pulse width. The laser beam was sent into the fluorescence port of an inverted microscope (AxioObserver, Zeiss) and then through the objective lens (40X, 0.6 NA), to generate a 260 µm-wide laser spot on the sample plane. The optimized laser fluence used for cargo delivery was 180 mJ/cm^2. The excitation laser pulse was synchronized with a liquid delivery system (FemtoJet, Eppendorf) using a switch. A pressure of 100-350 hPa was used to deliver 1-2 pl of a bacterial suspension per pulse. Approximately one bacterium was successfully delivered into a cell every two pulses. Following delivery, cells were washed and treated with antibiotics as described above.

Microscopy HEK293 cells were grown as described above on glass coverslips or in glass-bottom dishes treated with dilute Matrigel liquid. Following infection or nanoblade delivery of bacteria, cells were washed with Hank's and fixed with ambient temp. 4% paraformaldehyde in PBS containing 3 mM MgCl$_2$ and 10 mM EGTA for 15 min. Cells were permeabilized using PBS

containing 0.2% Triton X-100. Nonspecific binding sites were blocked in PBS containing 1%

bovine serum albumin (BSA), 10% calf serum and 2% goat serum for 1 hr. at room temp.

Antibody incubations were carried out for 1 hr. at room temp in blocking buffer using the

following dilutions: rabbit *Bt* antiserum (Burtnick et al., 2008); 1:1000 (for *Bt*), 1:50 (*Bp*); mouse

α-LAMP-1 monoclonal antibody (Santa Cruz), 1:40; Alexa-Fluor 488 or 555- labeled phalloidin

and secondary antibodies, 1:150 (Molecular Probes). Permanent mounts of specimens were

created using ProLong Gold (Invitrogen). Glass-bottom dishes and slides were analyzed using a

Leica SP5-II AOBS confocal microscope setup or an Olympus BX51 upright fluorescent

microscope. Live cell brightfield and fluorescence imaging and video recording was performed

using a Zeiss Axiovert 40CFL inverted fluorescence microscope with a Canon digital camera.

Image processing was performed with Leica's LAS-AF software suite or Adobe Photoshop CS2.

Image and Data Analysis Figures, tables and graphs were prepared using Microsoft PowerPoint

and Excel. Statistical analysis was performed with the Student's *t*-test using Excel and

GraphPad.

Figure Legends

Fig. 1. T3SS$_{Bsa}$ is required for endosome escape but not invasion. (*A*) Plaque forming units (PFU) per bacteria 18 hr. after infection in HEK293 cells. Plaque assays were performed with BtE264, Bp340, Δ*sctN* mutants or complemented derivatives (p*sctN*). Dashed line; limit of detection. (*B*) CFU recovered per bacteria in 2 hr. invasion assays. HEK293 cells were untreated (black bars) or treated with 2 µg/ml cytochalasin D (grey bars) prior to infection. All assays were performed in triplicate and error bars represent +/- standard error of the mean (SEM). * = *P* < 0.005.

Fig. 2. Intercellular spread following photothermal nanoblade-mediated delivery. (*A*) Top; invasion by BtE264 is followed by T3SS$_{Bsa}$-mediated endosome escape and BimA-mediated actin polymerization in the cytoplasm. Bottom; photothermal excitation of Ti-coated microcapillary pipets coupled with a 6 ns, 549 nm laser pulse facilitates pressurized delivery of bacteria directly into the cytosol. (*B*) Plaque formation on HEK293 monolayers following infection with BtE264 (top), Δ*sctN* (middle) or Δ*bimA* mutants (bottom). Scale = 1 cm. (*C*) *Bt* and mutants (red) were delivered into HEK293 cells using a photothermal nanoblade and stained for actin (green) 12 hr. later. Scale = 20 µm. (*D*) Plaque formation on HEK293 monolayers following nanoblade delivery. Scale = 1 cm. (*E*) Plaques in (*D*) were stained for bacteria (red) and actin (green). Scale = 500 µm. (*F*) Magnified edges of plaques in (*E*) stained for bacteria (red) and actin (green). Scale = 20 µm.

Fig. 3. Fla2-mediated flagellar motility facilitates plaque formation. (*A*) Fraction of infected HEK293 cells containing rapidly motile bacteria 8 hr. after infection with BtE264 and derivatives. Approx. 300 cells were monitored per strain. (*B*) Plaque forming efficiency on

HEK293 cell monolayers 18 hr. after infection. (*C*) Representative infected cells stained for bacteria (red) and actin (green). (*D*) Plaque diameters 24 hr. after infection of HEK293 cell monolayers. (*E*) HEK293 cell invasion efficiencies by BtE264 or mutant strains 2 hr. post infection. (*F*) Timecourse of intracellular replication in HEK293 cells. All assays were performed in triplicate and error bars represent +/- SEM. * = $P < 0.005$, Un = undetectable.

Fig. 4. T6SS-1 is critical for efficient intercellular spread. (*A*) HEK293 cell invasion efficiencies by BtE264 or Δ*clpV1* derivatives 2 hr. post infection. (*B*) HEK293 cells were infected and stained for bacteria (red) and actin (green) 8 hr. post infection. Scale = 20 µm. (*C*) Timecourse of intracellular replication in HEK293 cells. (*D*) Plaque forming efficiency on HEK293 cell monolayers 18 hr. after infection. (*E*) Cytotoxicity assays in HEK293 cells. All assays in (*A*), (*C*)-(*E*) were performed in triplicate and error bars represent +/- SEM. ** = $P < 0.05$, * = $P < 0.005$. Un = undetectable.

Fig. 5. Intercellular spread and plaque formation occur through cell fusion. (*A*) Progression of events leading to plaque formation. HEK293-RFP (red) and -GFP (green) cells immediately after infection (left). 12 hr. later, a MNGC is formed (yellow, middle), which undergoes lysis and forms a plaque at 24 hr. (right). Scale = 500 µm. (*B*) A MNGC (left) was stained with DAPI (blue, center); triple-color merged image (right). (*C*) MNGC and plaque formation by BtE264 or mutant strains on HEK293 RFP+GFP cell monolayers 12 hr. (upper panels) or 24 hr. (lower panels) following infection or photothermal nanoblade delivery ("n"; Δ*sctN*, Δ*sctN*Δ*clpV1*). (*D*) MNGC and plaque formation 12 hr. (upper) and 24 hr. (lower) after infection with Bp340 and mutants. (*E*) Plaque formation 56 hr. following infection with *L. monocytogenes* 10403S does not involve MNGC formation. The slight appearance of yellow at the edge of plaques is due to

physical overlap of red and green cells and does not indicate cell fusion. Images are

representative of multiple independent experiments.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

A indiv. cells   cell fusion   plaque

B MNGC   DAPI   merge

C BtE264   Δ*sctN* (n)   Δ*bimA*   Δ*motA2*   Δ*bimA*Δ*motA2*   Δ*clpV1*   Δ*sctN*Δ*clpV1* (n)

D Bp340   Δ*bimA*   Δ*clpV1*

E *L.m.*
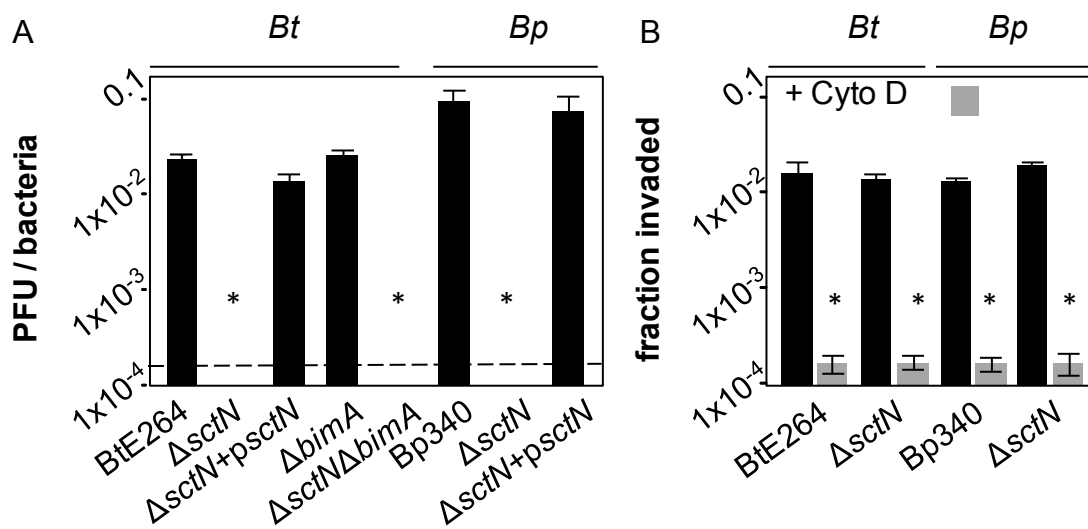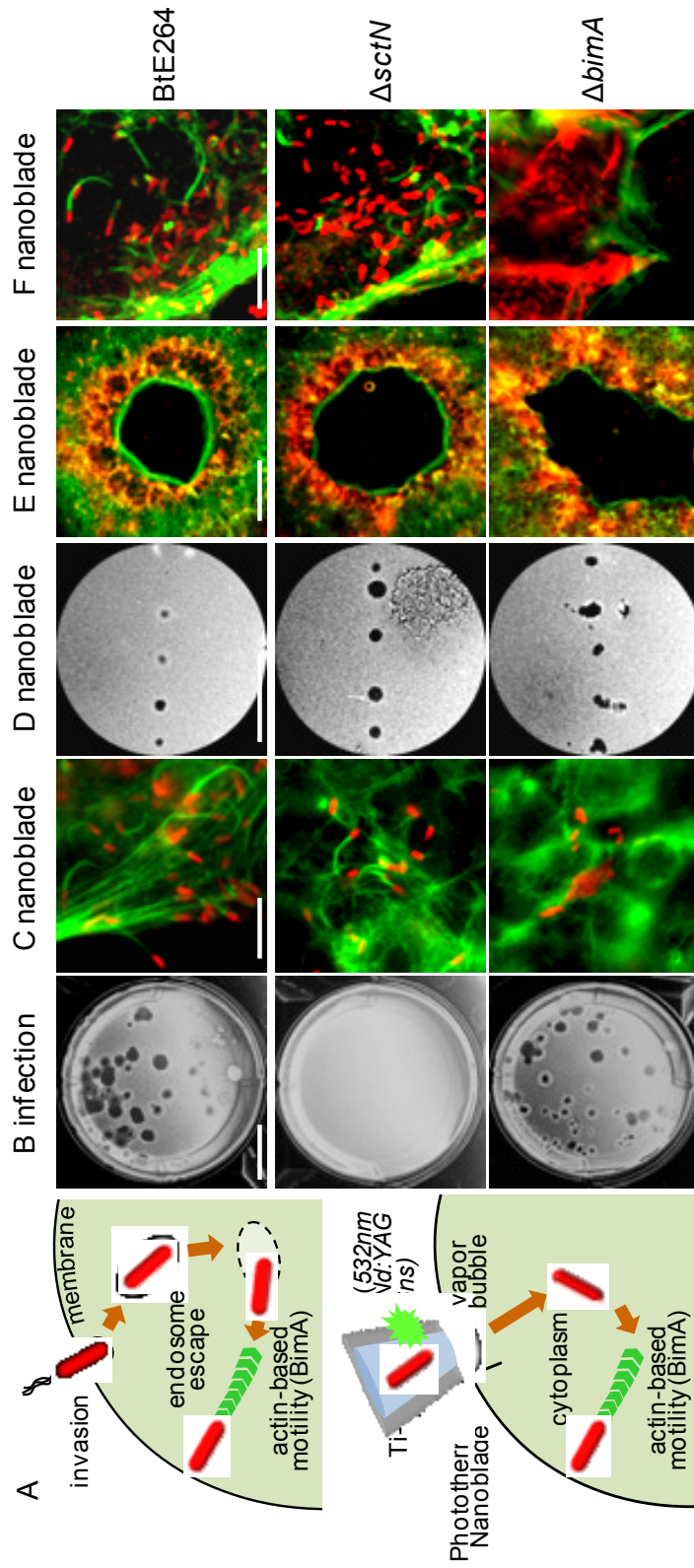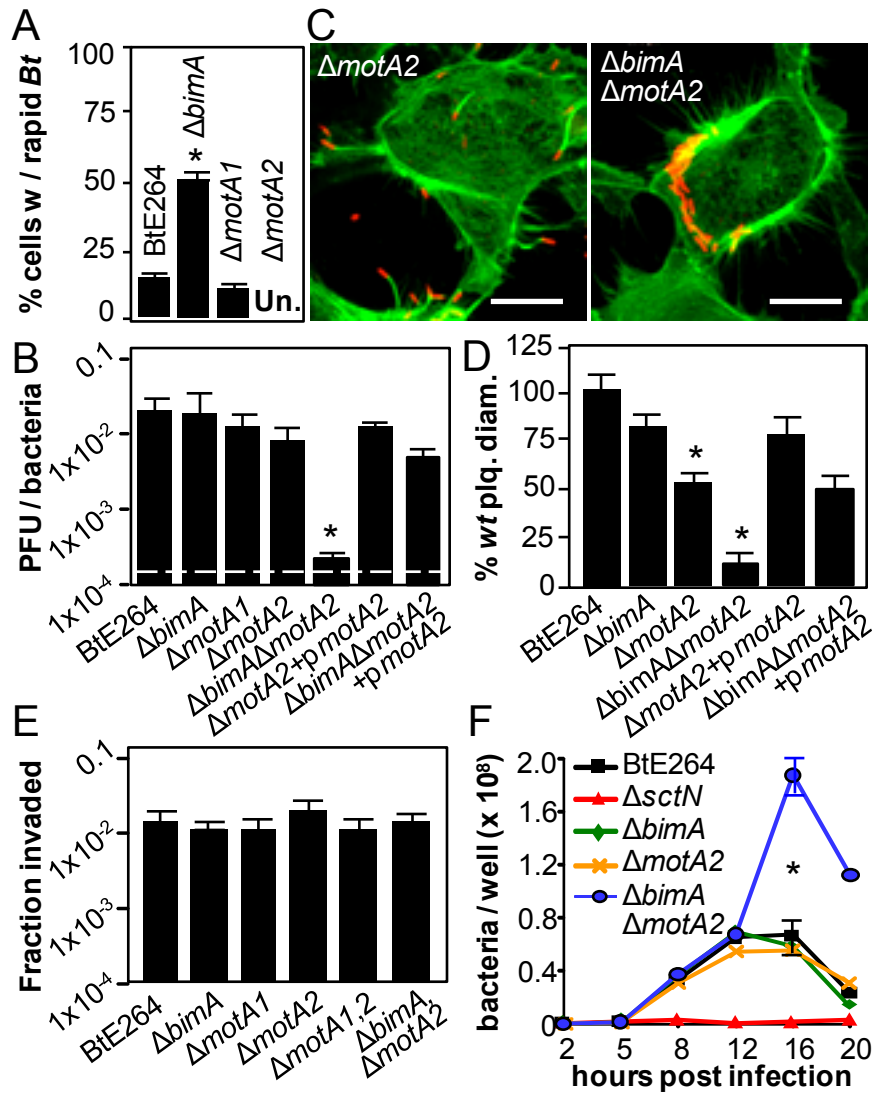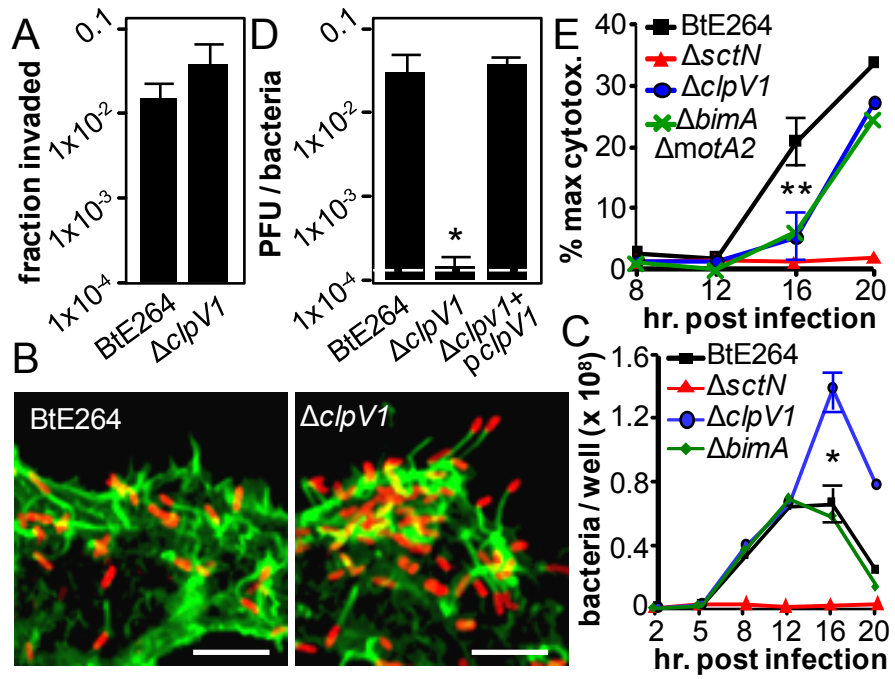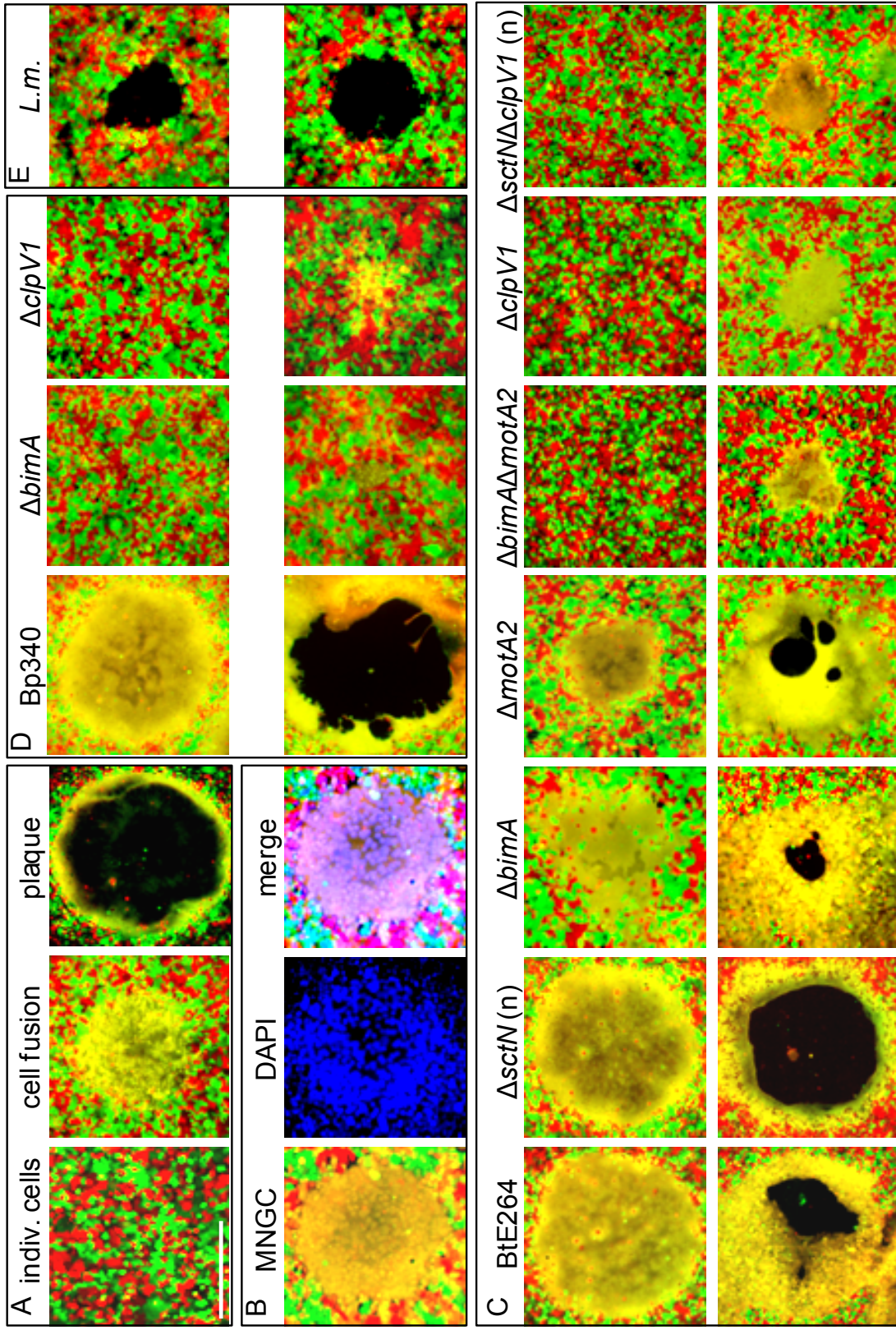
Supplementary Material

Table Legend

Table S1. Summary of intracellular lifecycle dissection following infection. (*A-E*) or photothermal nanoblade delivery (*F-I*) of BtE264 WT and indicated mutants in HEK293 cells, phenotypes were scored for endosome escape, actin motility, flagellar motility, multinucleate giant cell (MNGC) formation and plaque formation. Observed (+); near limit of detection (+/-); not observed (-); not done (ND). (*A*) Endosome escape (infection only) was assessed by fluorescence microscopy of cells fixed and stained for LAMP1 endosome marker and bacteria 8 hr. after infection. (+) means at least 50% of intracellular bacteria were observed free in the cytoplasm or not colocalized with LAMP1. (*B, F*) Actin motility; (+) denotes that at least 10% of bacteria in cells were associated with actin tails 8 hr. after infection or nanoblade delivery, respectively. (*C,G*) Rapid flagellar motility of intracellular bacteria; (+) denotes rapid intracellular motility exhibited by bacteria 8 hr. after infection or nanoblade delivery in a significant portion (>10%) of infected cells. (*D, H*) Multinucleate giant cell (MNGC) formation; (+) indicates that MNGCs were observed in the red-green mixed cell assay for cell fusion 12 hr. after infection or bacterial delivery with the photothermal nanoblade. (*E, I*) Plaque formation 24 hr. following infection or nanoblade delivery; (+) indicates average plaque size greater than 250 μm in diameter; (+/-) indicates plaques smaller than 250 μm, and (-) indicates that plaques were not observed. Data is representative of at least three independent experiments.

Figure Legends

Fig. S1. T3SS$_{Bsa}$ does not facilitate invasion in HeLa epithelial cells. (*A*) Invasion efficiency of *B. thailandensis* E264 and *B. pseudomallei* Bp340 and Δ*sctN* mutants 2 hr. after infection of HeLa cells. Cells were untreated (black bars) or treated in parallel with 2 µg/mL cytochalasin D, which blocks rearrangement of cortical actin, 30 min before infection with bacteria using an MOI of 10. Values shown are averages of three independent experiments performed in triplicate. Error bars are +/- SEM. * = *P* < 0.001; ** = *P* < 0.05. (*B*) Fluorescence confocal microscopy of HEK293 cells 8 hr. post infection with non-mutant BtE264 (upper) or Bp340 (lower) and Δ*sctN* and Δ*bimA* derivatives. Actin (green), bacteria (red). Scale bar = 20 µm. (*C*) HEK293 cells infected with BtE264 (upper) or Bp340 (lower) and indicated mutants were stained for LAMP1 (green) and bacteria (red) 8 hr. after infection. Arrowheads (middle) indicate LAMP1 positive endosomal vesicles containing bacteria. Scale = 20 µm. (*D*) Bt and Bp *sctN* mutants eventually lose viability and are degraded as a result of endosomal entrapment. HEK293 cells were infected with BtE264 or Bp340 Δ*sctN* mutants and stained for actin (green) and bacteria (red). Bacterial debris remains 16 hr. after infection with Δ*sctN* mutants. Images are single 0.3 µm Z-sections and are representative of four experiments. Scale = 10 µm.

Fig. S2. The *fla2* locus of *B. thailandensis* is required for rapid intracellular motility but does not facilitate swarming in soft agar. (*A*) HEK293 cells were infected with BtE264 Δ*bimA*Δ*motA1* (MOI=1) and examined 8 hr. later by live phase contrast microscopy. Shown is a 200 ms timelapse image series of two, motile bacterial cells joined end-to-end (approx. 6 µm in total length) inside a HEK293 cell. The red pointer indicates the lagging pole of the rear-most bacterium, and green pointers denote the distance traveled. From this information, the movement rate of the bacteria is estimated to be 25 µm/sec. (*B*) Soft agar swarming assays (Gardel and

Mekalanos, 1996). BtE264 WT, Δ*fliC1*, Δ*motA1* (*fla1*) mutants or a Δ*motA2* (*fla2*) mutant were inoculated into 0.4% L-agar and incubated at 37°C for 18 hr. Swarming motility is indicated by bacterial growth (dark grey) radiating from the central site of inoculation. Images are representative of multiple independent experiments. Scale = 1 cm.

Fig.S3. The *fla2* loci of *B. thailandensis* and *B. pseudomallei* are highly conserved and are predicted to encode lateral flagella. (*A*) Map of the BtE264 and *B. pseudomallei* MSHR668 *fla2* chromosome 2 flagellar and chemotaxis loci. As described by Tuanyok et al. (Tuanyok *et al.*, 2007; Tuanyok *et al.*, 2008), *fla2* loci are highly conserved and syntenic between *B. thailandensis* and Australian *B. pseudomallei* species (red shading) but are not present in southeast Asian isolates of *B. pseudomallei* or in *B. mallei*. Color coding: putative flagellar-accessory/modification genes (blue); *che* chemotaxis (orange); *lfi* basal body/export/assembly (green); *lfg* flagellar hook/rod/ring components (brown); *mot/laf* motor components (black), *fliC/lafA* flagellin (grey); putative regulatory loci (red); hypothetical protein/unknown function (white). Position of Δ*motA2* and Δ*fliC2* mutations used in this study is indicated by the position of the yellow arrow. Map is to scale. Scale bar = 3.5 kb. The predicted *fla2* gene products from *Bt* E264 and *Bp* MSHR668 genomic sequence data were analyzed by protein BLAST (BLAST-P). Numbers below the locus map in (*A*) indicate a representative subset of gene loci for which the analysis is shown in (*B*). Listed are the *Bt* and *Bp* locus tag designations, predicted gene products and highly similar homologs in other species that are known or indicated to possess lateral flagella.

Fig. S4. Model of the intracellular lifecycle. T3SS$_{Bsa}$-independent invasins, which have yet to be identified, facilitate actin-dependent internalization of *B. thailandensis* and *B. pseudomallei* into single membrane primary endosomes. The activity of T3SS$_{Bsa}$ is required for escape from

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the primary endosome and entry into the cytoplasm. Once the primary endosome has been breached, T3SS$_{Bsa}$ is dispensable for all subsequent events in the intracellular lifecycle, including intracellular replication, motility, cell-cell spread and MNGC formation. Either one of two independent motility systems; flagellar (Fla2) or actin-based (BimA), are required for efficient cell-cell spread. T6SS-1 facilitates intercellular spread and multinucleate giant cell (MNGC) formation, possibly through the fusion of host cell membranes. Membrane interaction facilitated by motility and T6SS-1 can also lead to cell death. The lifecycle model for *B. thailandensis* and *B. pseudomallei* contrasts with that of *Listeria* and *Shigella* since it does not require engulfment of membrane protrusions with bacteria at their tips, obviating the need for lysis of double-membrane vesicles for cell-cell spread.

Table S1

	endosome escape	actin motility	flagellar motility	MNGC formation	plaque formation		actin motility	flagellar motility	MNGC formation	plaque formation
	A	*B*	*C*	*D*	*E*		*F*	*G*	*H*	*I*
			Infection					Nanoblade delivery		
*Bt*E264 (WT)	+	+	+	+	+		+	+	+	+
Δ*sctN*	-	-	-	-	-		+	+	+	+
Δ*bimA*	+	-	+	+	+		-	+	+	+
Δ*sctN*Δ*bimA*	-	-	-	-	-		-	+	+	+
Δ*clpV1*	+	+	+	-	+/-		+	+	-	+/-
Δ*sctN*Δ*clpV1*	-	-	-	-	-		+	ND	-	+/-
Δ*bimA*Δ*clpV1*	+	-	+	-	+/-		-	ND	-	+/-
Δ*sctN*Δ*bimA*Δ*clpV1*	-	-	-	-	-		-	ND	-	+/-
Δ*motA1*	+	+	+	+	+		ND	ND	ND	ND
Δ*motA2*	+	+	-	+	+		ND	ND	ND	ND
Δ*bimA*Δ*motA2*	+	-	-	-	+/-		ND	ND	ND	ND
Δ*fliC2*	+	+	-	+	+		ND	ND	ND	ND

Fig. S1

A

BtE264   Bp340

= 2µg/mL cytochalasin D

Fraction invaded

5.0×10^{-4}

5.0×10^{-5}

5.0×10^{-6}

5.0×10^{-7}

*

**   **

*

wt   ΔsctN   wt   ΔsctN

B

BtE264   ΔsctN   ΔbimA

Bp340   ΔsctN   ΔbimA

C

BtE264   ΔsctN   ΔbimA

Bp340   ΔsctN   ΔbimA

D

BtE264 ΔsctN   Bp340 ΔsctN

Fig. S2

A

0.00"   **0.10"**   **0.20"**

B

BtE264 (*wt*)   Δ*fliC1*

Δ*motA2*   Δ*motA1*

Fig. S3

B. thalandensis E264 fla2 Bth_II0146-0197

B. pseudomallei MSHR668 fla2 BURPS668_A0194-0251

100% locus synteny
93% nucleotide identity

3.5 kb

B

	Bt, Bp locus tags	**product name**	**homolog**	**species**	**E**
1	BTH_II0151, BURPS668_A0199	flagellin FliC2	LafA	*Citrobacter rodentium, Vibrio parahaemolyticus*	9e-58, 4e-55
2	BTH_II0153, BURPS668_A0201	flagellar motor protein MotA2	LafU	*E. coli, Aeromonas samonicida*	2e-58, 5e-79
3	BTH_II0154, BURPS668_A0202	flagellar motor protein MotB2	LafT	*E. coli, V. parahaemolyticus*	2e-44, 8e-43
4	BTH_II0163, BURPS668_A0211	flagellar biosynthesis protein FlhA	LfhA	*E. coli, A. salmonicida*	0.0, 0.0
5	BTH_II0165, BURPS668_A0214	flagellar biosynthetic protein FliR	LfiR	*E. coli, A. salmonicida*	6e-40, 2e-35
6	BTH_II0170, BURPS668_A0222	flagellar hook-basal body component FliE	LfiE	*C. rodentium, A. salmonicida*	3e-15, 2e-10
7	BTH_II0180, BURPS668_A0233	RNA pol. σ factor 28 for flagellar operon FliA	LfiA	*Chromobacterium violaceum, C. rodentium*	4e-58, 7e-53
8	BTH_II0184, BURPS668_A0237	flagellar basal body protein FlgB	LfgB	*E. coli, A. salmonicida*	2e-27, 2e-26
9	BTH_II0194, BURPS668_A0247	flagellar hook associated protein FlgL	LfgL	*E. coli, C. rodentium*	2e-24, 3e-22

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Fig. S4

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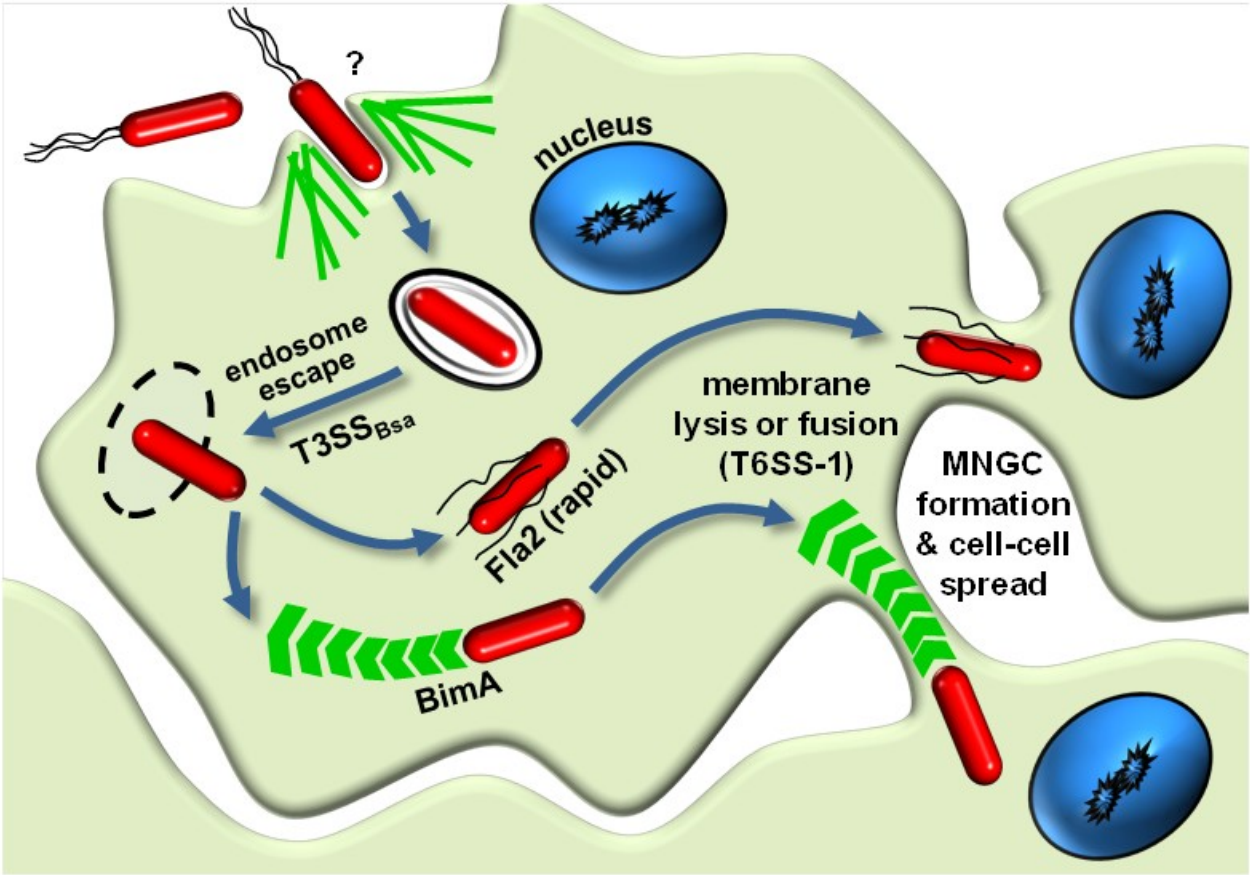
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Chapter 4. *Burkholderia* **flagellar and injection type III secretion systems**

Abstract

Pathogenic *Burkholderia* species contain up to five type III secretion systems (T3SSs); three injection-T3SSs and two flagellar T3SSs in strains of Australian origin, and three injection-type and a single flagellar system in southeast Asian isolates. Two of the injection T3SSs and both of the flagellar loci (*fla1* and *fla2*) are conserved in *B. thailandensis,* a closely-related BSL-2 surrogate organism. Fla1 facilitates swimming in liquid medium and swarming motility in laboratory soft agar medium. Fla2 mediates intracellular motility of *B. thailandensis,* and is capable of driving cell-cell spread in the absence of BimA-mediated actin polymerization and the formation of membrane protrusions. By immunofluorescence microscopy of a *B. thailandensis* strain producing epitope tagged Fla2 flagellin (*fliC2::HA*), we confirm that the *fla2* T3SS encodes lateral flagella that are expressed following infection of HEK293 cells. Constitutive expression of a putative response regulator protein, Frr induces Fla2-mediated swarming motility in a *B. thailandensis* Δ*fla1* mutant. Expression of Frr dramatically reduced the plaque forming ability of Australian and southeast Asian *B. pseudomallei* strains and of *B. thailandensis*, suggesting that Frr interferes with other conserved virulence pathways. Little is known regarding the temporal, spatial and environmental conditions that govern *fla2* expression. Using a tandem green- and red fluorescent protein reporter system, we show that the *bim* actin motility and *fla2* loci are only expressed following infection of cells. In addition to the Fla2 flagellar T3SS, the conserved Bsa injection T3SS (T3SS$_{Bsa}$) is expressed following invasion of cells and facilitates the escape of *B. mallei, B. pseudomallei* and *B. thailandensis* from endosomes. Despite the plethora of T3SSs in *B. pseudomallei*, there is a dearth of known effector proteins that influence its interactions with eukaryotes. We implemented a series of bioinformatic screens designed to identify novel T3SS effector candidate loci from *B. pseudomallei* genome sequence data. The

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results of these screens yielded 16 _Burkholderia_ effector protein (Bep) candidates. Eight of these were considered high-confidence T3SS effector candidates, being located in the genome near the T3SS-1, -2, or-3 gene clusters, and were chosen for further analysis: _bepB_, _bepC_, _bepD_, _bepE_, _bepF_, _bopA_, _bopC_ and _bopE_. BopA and BopE have been previously identified as likely T3SS$_{Bsa}$-exported effectors. Ectopic expression of five candidates in mammalian cells yielded interesting phenotypes: BepB caused alterations in cell morphology, BepC was observed to bundle actin, BepD was observed to decorate microtubules, and BepE and BepF induced changes in the actin cytoskeleton and altered cell adhesion properties. Deletion mutations in the candidate effector loci were constructed in _B. pseudomallei_ Bp340 and analyzed for infection-related phenotypes in cell-based assays. Following invasion analysis, Δ_bopC_ and Δ_bopE_ strains invaded cells as efficiently as wild-type _B. pseudomallei_, contradicting earlier reports. We confirm that export of BopE and BopC requires activity of the Bsa T3SS (also known as T3SS-3 in _B. pseudomallei_), although ultimately only BopA was seen to play a significant role in survival and spread in RAW264.7 mouse macrophages.

Introduction

As described in Chapter 3, *B. thailandensis* and Australian *B. pseudomallei* strains contain two, highly conserved flagellar biosynthesis gene clusters, *fla1* and *fla2*, that encode distinct systems that function under different conditions. Southeast Asian *B. pseudomallei* isolates lack the *fla2* gene cluster, having acquired a *Yersinia*-like fimbrial adhesin in its place by horizontal transfer (Tuanyok *et al.*, 2007). In *B. thailandensis*, fla2 – and not fla1 – is capable of driving cell-cell spread in the absence of BimA-mediated actin polymerization and the formation of membrane protrusions. Our hypothesis is that *fla2* encodes lateral flagella due to the similarity of *fla2* to the systems in *Aeromonas* and *Vibrio*, which are known to utilize lateral flagella. Almost nothing is known concerning the environmental signals leading to the activation of *fla2* or how the system is regulated.

The flagellum is closely related to injection-type T3SSs (Woestyn *et al.*, 1994; Van Gijsegem *et al.*, 1995). Both systems consist of a secretion apparatus for the sequential export of needle components or translocon subunits and effector proteins, in the case of the T3SS injectisome, and of the flagellar hook and filament (FliC) components in the flagellar system (Macnab, 2003). In some instances, the flagellar apparatus also exports non-flagellar proteins (Young *et al.*, 1999). Although, it is often assumed that the T3SS injectisome derives from the flagellum, some phylogenetic studies do not support this view, indicating instead that the injectisome is as ancient as the flagellum and that both share a common ancestor (Kubori *et al.*, 1998). Therefore, T3SS is a protein export pathway used by two different nanomachines, and both the injection-type and flagellar systems are known as "type III secretion".

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T3SSs represent one of the most complex mechanisms of protein translocation in biology and provide a striking example of the ability of bacteria to manipulate eukaryotes (Mueller *et al.*, 2008). The effects of a particular T3SS on target cells is governed by the constellation of effectors it injects. The *B. pseudomallei* genome contains three injection-type T3SS gene clusters (Rainbow *et al.*, 2002) and up to two flagellar systems (Tuanyok *et al.*, 2007; French *et al.*, 2011). Of the injection-type T3SSs, T3SS-1and T3SS-2 are most similar to the systems in the phytopathogens *Xanthomonas campestris* and *Ralstonia solanacearum*. T3SS-2 appears to represent a partial duplication of T3SS-1 followed by a considerable amount of divergence. T3SS-3, also known as the Bsa system, or $T3SS_{Bsa}$ is required for virulence *in vitro* and *in vivo* (Stevens *et al.*, 2002; Ulrich and DeShazer, 2004). T3SS-3 is most similar to the SPI-1 T3SS in *Salmonella enterica* and the *ipa/mxi/spa* system in *Shigella*, and is highly conserved in *B. pseudomallei, B. thailandensis and B. mallei*. Pathogenic *Burkholderia* can invade and survive within a variety of epithelial and macrophage-like cell lines *in vitro* (Kespichayawattana *et al.*, 2004). Once inside, *B. pseudomallei* escapes the phagocytic vesicle, polymerizes host actin, and moves within and between neighboring cells via actin-based motility. *B. pseudomallei* strains containing mutations in T3SS-3 genes are unable to escape the phagocytic vesicle, polymerize actin or spread from cell to cell and show decreased virulence in mouse and hamster models of melioidosis, while mutations in T3SS-1 and T3SS-2 had no significant effect (Stevens *et al.*, 2002; Ulrich and DeShazer, 2004). The T3SS-3-secreted effector, BopE, is reported to function as a GTP exchange factor and to be important for inducing actin cytoskeletal rearrangements and invasion of epithelial cells (Stevens *et al.*, 2003). Otherwise, there are few identified T3SS effectors in *B. pseudomallei*. Here I report the identification and partial characterization of new,

putative T3SS effector proteins in *B. pseudomallei* and undertake an analysis of the structure and

regulation of the Fla2 flagellar T3SS.

Results

The *Burkholderia* Fla2 flagellar T3SS

Surface arrangement of Fla2 filaments *B. thailandensis* was used for many of these experiments since it is a tractable BSL-2 surrogate model organism for *B. pseudomallei* host pathogen interactions. Although the *B. thailandensis* and Australian *B. pseudomallei fla2* loci are similar to those in other species that encode lateral flagella, the arrangement of flagellar filaments on the bacterial surface has not been examined. To address this, an HA epitope tag (YPYDVPDYA) was inserted in the central, variable region of *B. thailandensis* FliC2; the flagellin protein encoded by the *fla2* gene cluster, by allelic exchange. The FliC2-HA construct was integrated in a Δ*fliC1*Δ*bimA* genetic background to ensure that all motility functions were mediated by Fla2. This yielded the *B. thailandensis* E264 derivative Δ*fliC1*Δ*bimA fliC2::HA*. Since rapid Fla2-dependent motility has only been observed for intracellular bacteria, this strain was used to infect HEK293 cells which were then stained with fluorescent *B. thailandensis* antiserum and antibody to HA. As shown in Fig. 1 A-C, multiple, peritrichous flagella extend from the sides of the bacterium, confirming our hypothesis that *fla2* encodes lateral flagella. The configurations of the flagellar filaments are seen to vary from one bacterium to another. Since the images were obtained in infected, paraformaldehyde-fixed HEK293 cells, they are merely representative snapshots of the polymorphic transitions inherent in flagellar dynamics,. In Fig. 1A (left & middle), flagella are observed to be spreading away from the bacterium, which is indicative of counter-rotation as bacteria attempt to change direction (Bray and Duke, 2004). Flagella were also observed to extend in coils beyond a single pole of the bacterium, which signifies directional swimming mode (Fig. 1A, right). Lateral Fla2 flagella are only expressed when bacteria are inside cells, as shown in Fig. 1 B and C. Polar flagella, produced by the Fla1

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system, were visible following growth in laboratory medium but not following infection (Fig. 1B), suggesting that *fla2* and *fla1* are differentially regulated depending on external conditions. *B. pseudomallei* NAU14B6 of Australian origin has also been observed to exhibit rapid intracellular flagellar motility, and we anticipate that a similar arrangement of flagellar filaments will be observed, since the *fla2* locus in Australian *B. pseudomallei* is almost 100% identical to *fla2* in *B. thailandensis*. Flagellar motility has not been observed in cells infected with southeast Asian *B. pseudomallei* 1026b or its derivatives, which is consistent with the fact that these strains lack the *fla2* locus.

***fla2* regulation** Swarming in soft agar is mediated by Fla1 (Fig. 2A) (French *et al.*, 2011). In contrast, Fla2 is not involved in swarming motility *in vitro* and its activity only observed following intracellular localization of bacteria. Since nothing more is known about the signals or conditions that lead to Fla2 activation, we examined the *B. thailandensis* genome for potential regulatory loci. Two canonical response regulators and their cognate sensor kinases flanking the *fla2* gene cluster were indicated by BLAST-P analysis (Bth_II0141 – 0144) (Fig. 2E). The sequences encoding the response regulators (Bth_II0141 and Bth_II0143) were cloned for overexpression in *B. thailandensis*. No swarming phenotype was observed following expression of Bth_II0141 (French, C.T and Miller, JF unpublished). Expression of Bth_II0143 (<u>F</u>la2 <u>r</u>esponse <u>r</u>egulator–Frr) (Fig. 2E, green arrowhead) resulted in a partial rescue of swarming motility (Fig. 2A). The Δ*fliC1* parent shown in this figure cannot utilize Fla1 for motility and is unable to swarm in soft agar. Therefore, the restoration of swarming motility is dependent on constitutive expression of Frr and indicates Fla2-mediated swarming.

To examine potential cellular infection phenotypes resulting from constitutive activation of Fla2, plaque formation assays were carried out on HEK293 cells. Surprisingly, increased

levels of Frr expression resulted in marked decrease in plaque formation in *B. thailandensis* as well as *B. pseudomallei* strains of Australian (NAU14B6) and Southeast Asian (1026b) origin (Fig. 2B, 2C). We determined this was not due to the reduced ability of p*frr* strains to invade cells (Fig. 2D), as there was no significant difference in intracellular bacterial loads 2 hr. following infection of HEK293 with *B. thailandensis* p*frr* compared to WT.

***fla2* and *bimA* expression** Where the rate of bacterial movement from the polymerization of cellular actin is on the order of 0.3 µm / min (Theriot *et al.*, 1992), Fla2 is capable of propelling *B. thailandensis* up to 500 fold more rapidly – around 25 µm / sec – in the intracellular environment (French *et al.*, 2011). It is difficult to envision a survival strategy where actin polymerization and flagella would be utilized simultaneously, since activity of the two motility systems seems redundant and energetically costly. As described above, the Fla1 and Fla2 motility systems are differentially activated in response to changes in environmental conditions, and we wished to investigate whether a similar relationship exists between the utilization of Fla2 and BimA actin-based motility. We thus examined temporal and spatial gene expression patterns for the *bim* and *fla2* gene clusters by transcriptionally fusing the predicted promoter elements for the *bim* actin motility locus (P-*bimB*) and *fla2* (P-*fliE2*) to the coding sequences for EGFP and mCherry RFP (P*bimB-EGFP* and P*fliE2-mcRFP*) respectively. Green fluorescence (EGFP) would indicate bacteria dedicated to expression of the *bim* actin motility system, while red (mCherry) fluorescence would signal expression of *fliE2*, a critical Fla2 inner membrane component. The plasmid construct containing the promoters and fluorescent reporter elements (Fig. 3A), was transformed into *B. thailandensis* E264. In the experiment shown in Fig. 3B, no fluorescent bacteria were observed when bacteria were grown in L-broth at 37°C or immediately (1 hr.) after infection of HEK293 cells (Fig. 3C). This result demonstrates that neither the *bim*

nor the *fla2* motility operons are actively expressed under these conditions. This concurs with our earlier observation that only Fla1 is active under these conditions. Beginning at 2 hr. following infection of HEK293 cells, however, EGFP and RFP expression began to appear in intracellular bacteria (Fig. 3C). At 5 hr. after infection, the majority of intracellular bacteria exhibited green fluorescence, indicating preferential expression of the *bim* operon, while red and yellow bacteria were relatively rare. This is consistent with the high proportion of bacteria that are seen to exhibit actin tails following infection of mammalian cells (Fig. 3D) compared to the portion that exhibit rapid flagellar motility. Indeed, as shown in Fig. 3D, all green-fluorescent bacteria were associated with actin tails (Fig.3D, right). At the same timepoint, a smaller set of intracellular bacteria exhibited mCherry red fluorescence indicative of *fla2* expression (Fig. 3D, left). A few red bacteria were seen to polymerize actin (Fig. 3D, left), indicating the intriguing possibility that the Fla2 and Bim motility systems may be simultaneously expressed. Similar results were seen at 7 hr. post infection (Fig. 3C). Overall, it appears that expression of the *bim* operon (P*bimB-EGFP*) was more prominent at later timepoints, while the proportion of bacteria exhibiting red (P*fliE2-mcRFP*) or dual (P*bimB-EGFP* + P*fliE2-mcRFP*) fluorescence remained relatively constant throughout. The implications are discussed below.

Burkholderia injection type T3SSs

Identification of new putative T3SS effector loci In addition to the Fla2 flagellar T3SSs and the Bim actin motility system, the conserved Bsa injection T3SS (T3SS$_{Bsa}$) is expressed inside cells and facilitates the escape of *B. mallei, B. pseudomallei* and *B. thailandensis* from endocytic vesicles. Despite the plethora of T3SSs in *B. pseudomallei*, there is a dearth of known effector proteins that influence its interactions with eukaryotes. The exported substrates of flagellar and injection-type T3SSs do not contain cleavable signal sequences or commonly-recognized

sequence motifs. In known cases involving the flagellar hook and filament proteins as well as translocated effector molecules, T3SS substrates contain a segment of 15–30 amino acids in the N-terminal region which directs export (Figs.4-7) (Vegh *et al.*, 2006). Since no universally conserved T3SS "secretion signals" are known, identifying T3SS effector substrates based on genome sequence data is quite challenging. To partially address this issue, a computational algorithm was developed to identify T3SS class I chaperones in bacterial genomes (Panina *et al.*, 2005). This screen is based on the tendency of genes encoding class I chaperones to localize in the genome near their cognate effectors and on shared biophysical characteristics of T3SS chaperones, such as low molecular weight, acidic isoelectric point and conserved tertiary structure. When combined with additional screening methods based on the presence of conserved eukaryotic functional domains and similarity to known T3SS effectors in other bacteria, the sensitivity of the screen increases significantly. Using this combined approach we have identified 16 predicted T3SS effector loci, all of which are novel and conserved in the genomes of *B. pseudomallei* strains K96243 (Table 3) (Holden *et al.*, 2004) and 1026b (Warawa and Woods, 2005). Of these, six <u>B</u>urkholderia <u>e</u>ffector <u>p</u>rotein (Bep) candidates; BepB (BPSS1411), BepC (BPSS1528), BopC (BPSS1516), BepD (BPSS1609), BepE (BPSS1385), BepF (BPSS1386), have been partially characterized.

Ectopic expression of putative T3SS effectors We have examined transfection-associated phenotypes associated with six Bep candidates following expression in HeLa cells. BepB (BPSS1411), which flanks T3SS-1, was identified by genomic colocalization with its cognate chaperone, BecB (BPSS1410). BepB is similar to the Tcd family of insecticidal toxins and ectopic expression results in cytotoxicity and the formation of tubular structures which remain attached to the substratum (Fig. 4A). BepE (BPSS1386), which flanks T3SS-1, was identified

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through a screen for eukaryotic functional domains. BepE contains a predicted P-loop ATP-GTP hydrolysis and nucleotide binding domain fused to a putative α-actinin-type actin binding motif. Expression leads to the disruption of stress fibers and actin reorganization to the cell periphery (Fig. 4B). We hypothesize this factor is a novel actin-binding protein that controls cytoskeletal dynamics. BepF (BPSS1385), also flanking T3SS-1, contains partial homology to Cif, an EPEC effector that functions as a cyclomodulin to arrest cell cycle progression at the G2-M transition (Marches *et al.*, 2003). BepF also has a predicted P-loop ATP-GTP hydrolysis domain and is flanked by transposases, suggesting recent horizontal acquisition. BepF expression results in membrane ruffling, alterations in actin distribution and an increase in the size of focal adhesions (Fig. 4C). These phenotypes are similar to those associated with Cif and represent downstream effects of alterations in cell cycle control (Yao *et al.*, 2009). BepD (BPSS1609), which flanks T3SS-2, was identified by colocalization with the BecD chaperone (BPSS1610) and does not display sequence similarity to other known effectors. As shown in Fig. 5, BepD displays a striking pattern of colocalization with cytoplasmic microtubules. Tubulin was identified in immunoprecipitation complexes with BepD-EGFP by tandem mass spectroscopy (MS-MS), along with the heavy chain component of the dynein minus-end directed microtubule motor complex. These and other observations support the hypothesis that BepD is a unique T3SS effector protein that associates with microtubules. Other proteins identified by MS-MS, such as γ-spectrin, clathrin heavy chain are associated with the cell membrane and may be contaminants. The presence of the Hsp70 and Hsp90 heat shock proteins in the IP complex likely indicates a cellular response to overexpressed and misfolded BepD-EGFP.

bepC (BPSS1528) and *bopC* (BPSS1516) are putative effector loci flanking T3SS-3. BepC and BopC exhibit no similarity to other known proteins. BepC-EGFP bundles actin

filaments following expression in HeLa cells by transfection (Fig. 6A). Immunoprecipitation and MS-MS analysis confirms the presence of α-actin and actin-associated proteins – notably myosin I, α-actinin and α-spectrin (Fig. 6B). BopC was initially designated as BepA, and is secreted by *B. pseudomallei* T3SS-3 (Muangman *et al.*, 2011). Our results confirm this as described below (Fig.9A). Using our screening methodologies, BopC/BepA was identified by two criteria; colocalization with a class I chaperone (BicC/BecA – BPSS1517), and probably horizontal acquisition due to the presence of flanking transposase genes. In our analysis, BopC-EGFP exhibits perinuclear localization following transfection (Fig. 7A). Fam38A-Mib, an endoplasmic reticulum membrane protein of unknown function, and α-actin were identified by MS-MS in immunoprecipitation experiments with BopC-EGFP (Fig. 7).

Infection analysis of *B. pseudomallei* effector mutants We did not observe invasion or plaque formation defects in *B. pseudomallei* strains containing deletions in the *sctNBp1* or *sctNBp2* ATPases of T3SS-1 and T3SS-2, respectively (Fig.8A). On the other hand, the Bsa T3SS (T3SS-3) was absolutely required for plaque formation, and this agrees with previous reports (Fig. 8A) (Stevens *et al.*, 2002; Stevens *et al.*, 2004; Warawa and Woods, 2005; Pilatz *et al.*, 2006; Burtnick *et al.*, 2008; Haraga *et al.*, 2008; Sun and Gan, 2010; French *et al.*, 2011). In light of these results, we did not further pursue the search for infection phenotypes for individual effectors linked to T3SS-1 and -2, and instead refocused our analysis on T3SS-3 and its associated effectors, BopA, BopE and BopC/BepA. BopE, a secreted T3SS-3 substrate with guanine nucleotidyl GTPase-enhancing (GEF) activity has been reported to affect invasion of epithelial cells (Stevens *et al.*, 2003; Muangman *et al.*, 2011). As shown in Fig. 8B, we were unable to confirm this result for a *B. pseudomallei* Bp340 Δ*bopE* mutant. There was no significant difference in the invasion efficiency of Δ*bopE* compared to WT in non-phagocytic

HeLa epithelial cells (P > 0.05). Moreover, the Δ*bsaS* T3SS-3 null mutant strain invaded HeLa

cells comparably to WT, and the same was found for a Δ*bopA* single mutant and a Δ*bopACE*

triple deletion strain. Bacterial uptake by RAW264.7 phagocytes was similarly not influenced by

the function of T3SS-3 (Fig. 8C). Taken together, these results demonstrate that T3SS-3 and its

effector proteins do not significantly affect invasion or uptake of *B. pseudomallei*. Plaque

formation efficiency by the Δ*bopA* strain, however, was significantly reduced in RAW264.7 cells

(P < 0.05) (Fig. 8D). The Δ*bsaN* regulatory mutant, which cannot secrete T3SS-3 effectors (Sun

et al., 2010; Chen *et al.*, 2011) and the Δ*bopACE* triple effector mutant were similarly

attenuated, and plaquing ability was completely eliminated in the Δ*bsaS* T3SS-3 ATPase

deletion strain. In the Δ*bopA* single mutant and the Δ*bopACE* triple mutant, plaquing defects

were rescued by *in trans* complementation of *bopA* (p*bopA*). Altogether, these results suggest

that only BopA plays a major role in the lifecycle of *B. pseudomallei* inside mammalian cells.

T3SS dependent secretion of effector proteins Considering that *B. pseudomallei* contains three

injection-type T3SSs, it is curious that the only two effector proteins ever previously shown to be

secreted are BopE and BopC (Sun *et al.*, 2010; Muangman *et al.*, 2011). Our results

independently confirm T3SS-dependent secretion of HA-tagged BopE and BopC (Fig. 9A, B) by

Western blot. Protein bands corresponding to BopE-HA and BopC-HA were detected in

supernatants from non mutant (WT) bacteria, but not from control strains containing Δ*bsaS* null

mutations (T3SS-3). For HA-tagged BepB, BepC, and BepE, effectors were found in whole cell

lysates and supernatants of WT *and* control strains carrying mutations in all three T3SS loci

(T3SS-1,2,3) (Fig. 9C,D and F). T3SS-dependent secretion of BepB, BepC, and BepE effectors

therefore does not occur under the conditions used for these experiments. BepF, an

enteropathogenic *E. coli* Cif homolog, was observed in the bacterial pellet as shown in Fig. 9G,

but no secreted protein was detected in the supernatant. Secretion of BopA was not observed (Fig. 9B), which is surprising in light of its clear role in promoting plaque formation discussed in the previous section (Fig. 8D). Further, not even trace amounts of BopA were visible in the bacterial pellet (Fig. 9B), and a similar situation was seen for BepD (Fig. 9E). This phenomenon has also been observed by other investigators and could be due to inherent instability or high turnover of these proteins when constitutively expressed (Dr. Yunn H. Gann, National Univ. Singapore, personal communication). Another likely possibility is that BopE and BopC are the only T3SS effectors exported in the assay conditions. The success of efforts to analyze T3SS-1 and -2 effectors in *B. pseudomallei* is predicated upon the expression and activation of T3SS-1 and T3SS-2, and the conditions required for the activity of these systems are currently unknown.

Discussion

Role of Fla2 in virulence Flagellar motility is important for survival and colonization of specific niches. In most organisms with dual flagellar systems, polar flagella provide maximal swimming efficiency whereas numerous lateral flagella support biofilm formation and motility over surfaces (McCarter, 2004). Whether Fla2 contributes to biofilm formation and virulence in animal models requires additional study. We anticipate that Fla2 activation occurs following bacterial uptake in response to an uncharacterized cytoplasmic signal. Another possibility is that Fla2 is activated inside cells in response to a small molecule produced by bacteria following cytoplasmic localization. Mutational analysis of the quorum sensing systems of pathogenic *Burkholderia* is underway to examine the potential effects on Fla2 expression following infection of cells. The potential roles of Fla2 in mammalian pathogenesis will also be examined in an aerosol model of murine melioidosis.

The Frr response regulator Another important question concerns the role of Frr on the regulation of virulence determinants. Frr expression significantly decreased plaque formation in *B. thailandensis* E264*, B. pseudomallei* NAU14B6 (Australian origin) and 1026b (from northeast Thailand). This result points to the possibility that Frr directly or indirectly affects expression of one or multiple conserved virulence pathways that function in the intracellular lifecycle. Quantitative PCR analysis is underway to identify the pathways affected by Frr. Transcript levels of T3SS, T6SS, flagellar and motility-associated loci in *B. pseudomallei* and *B. thailandensis* strains overexpressing Frr will be compared to empty vector controls. Global transcriptome analysis to analyze the effects of Frr expression will be carried out using RNAseq.

Regulation of motility systems following infection As shown in Fig. 3A-D, the proportion of bacteria exhibiting green fluorescence; indicative of *bim* operon expression, and those exhibiting red fluorescence, denoting *fliE2* expression, were approximately equal at 2 hr. following infection. At later timepoints, a progressively greater proportion of green fluorescent bacteria were present, indicating their utilization of the *bim* operon, and a positive correlation was seen with their association with actin tails. 1 hr. after infection, most bacteria were attached to the cell surface (Fig. 3C, arrows). Induction of the *bim* and *fliE2* operons, as indicated by the appearance of green or red fluorescent marker proteins respectively, began to occur 2 hr. following infection. Since escape from the endosome must occur prior to cytoplasmic localization of bacteria, it is conceivable that the signal(s) for induction of the *bim* or *fla2* motility systems occur in the endocytic vesicle, before escape into the cytosol. The maturation times and half-lives of EGFP and mCherry fluorescence proteins pose an additional consideration for temporal-spatial analysis of gene expression. Maturation times for EGFP and mcRFP are on the order of 15 minutes to over 1 hr. (Macdonald *et al.*, 2012), and the half lives of both proteins exceeds 4 hr. (Tsien, 1998; Laxman *et al.*, 2010). Taking these factors into consideration, dual red and green fluorescence of individual bacteria could indicate simultaneous *bim* and *fliE2* expression, or that cells are in the process of switching from one system to another. However, it is clear that some bacteria do exhibit Fla2 activity following endosome escape, as discussed in Chapter 3. The temporal and spatial regulation of these motility systems, as well as the nature of the signals leading to their activation remain to be elucidated.

T3SS Effectors The Fla2 flagellar T3SSs provides intracellular motility in the cell cytosol, while the Bsa injection T3SS (T3SS$_{Bsa}$) facilitates the prior escape from endosomes. Roles for the phytopathogen-like T3SS-1 and -2 in the infection of mammalian cells have not been reported,

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however these systems may function to mediate interactions with plants or other organisms in the environment (Lee *et al.*, 2010). Although BepB, C, D, E and F show interesting phenotypes when expressed ectopically in mammalian cells, T3SS-dependent secretion of these effector candidates could not be verified. Even though each of these effector candidates are associated with one of the three T3SSs, we have not yet determined which T3SS normally secretes these proteins. Because of their genomic locations, we anticipate that secretion of BepB, E and F will be dependent on the activity of T3SS-1, while BepD will require activation of T3SS-2. Similarly, BepC clustered with actin filaments in HeLa cells, and was expected to be an important T3SS-3 effector, but specific secretion could not be shown. Furthermore, there was no detectable invasion, survival or cell-cell spread phenotype observed for a Δ*bepC* mutant (French, C.T. unpublished), raising the possibility that BepC is not a secreted effector after all. T3SS-3 has been shown to be moderately active in L broth *in vitro* (Sun *et al.*, 2010), while the conditions required for induction of T3SS-1 and -2 are unknown. It is similarly mysterious whether their natural targets are mammalian, protozoan, nematode or plant cells. The most relevent question may be whether the *B. pseudomallei* T3SSs play a role in environmental persistence and, if so, what their role might be.

T3SS-3 and the BopE and BopC effectors have been previously implicated in invasion of non-phagocytic cells (Stevens *et al.*, 2003; Muangman *et al.*, 2011). Stevens *et al.* claim a modest (30-50%) decrease in intracellular bacterial load following infection of *bipB* translocon and *bopE* mutants. We have found that such measurements of subtle differences in intracellular bacterial loads are difficult to consistently reproduce. Numerous experimental variations (pipetting, dilutions, growth rates, CFU normalization counts, bacterial aggregation, cell density, etc.), affect the apparent invasion efficiency of bacteria. Our results indicate that mutants

containing deletions in single and multiple T3SS-3 effectors invaded cells as efficiently as WT, and the same was true for the null-function T3SS-3 Δ*bsaS* ATPase mutant. As such, our data do not support a significant role for T3SS-3 or its effectors in invasion or internalization of pathogenic *Burkholderia* regardless of the host cell type used. Of the putative *B. pseudomallei* effectors identified in our and others' studies, only BopA appears to play a role in intracellular survival and cell-cell spread. BopA is similar to the *Shigella* IcsB effector, which is known to be involved in promoting intracellular survival through evasion of the autophagic pathway. In *Shigella*, it is thought that this is accomplished via binding of IcsB to IcsA; the *Shigella* BimA actin-polymerizing analog, masking IcsA from recognition by the autophagy pathway (Ogawa *et al.*, 2005; Levine *et al.*, 2011). Although BopA has been recently implicated in avoidance of autophagy by *B. pseudomallei* (Cullinane *et al.*, 2008; Gong *et al.*, 2011), there is no evidence to support a similar interaction of BopA with *Burkholderia* BimA. Regardless of the mechanism, it is clear that BopA is important for the intracellular livelihood of *B. pseudomallei*. The mechanism and intracellular targets of BopA will be the subject of future investigations.

With a large (7 MB) and complex genome, it is curious that only the T3SS-3 and T6SS-1 secretion systems have been linked to *Burkholderia* virulence and intracellular pathogenesis (Burtnick *et al.*, 2008; Burtnick *et al.*, 2010; Schwarz *et al.*, 2010b; Sun and Gan, 2010; Burtnick *et al.*, 2011; Chen *et al.*, 2011; French *et al.*, 2011). The Fla1 flagellum has been previously linked to attachment and internalization of *B. pseudomallei* by phagocytic mammalian cells (Chuaygud *et al.*, 2008) and *Acanthamoeba* (Inglis *et al.*, 2003). Our results (French *et al.*, 2011) and those of other studies (Wikraiphat *et al.*, 2009) do not support such a for Fla1 in attachment. It is perhaps illuminating to consider that humans and other mammals are likely to be "accidental" hosts for *B. pseudomallei*. In this light, many and perhaps all of the virulence

mechanisms that are important for human disease have evolved to mediate interactions and self defense from protist, bacterial and fungal denizens of the rhizosphere. While many soil-dwelling plant symbionts fix nitrogen from the environment, there are no nitrogen fixation loci in *B. pseudomallei* genomes. In contrast, T3SS-1 and -2 bear striking similarities to T3SS loci of phytopathogenic bacteria, raising the possibility that *B. pseudomallei* has evolved pathogenic relationships with plants, potentially to facilitate nutrient acquisition or environmental persistence. This notion is supported by the recent observation that *B. pseudomallei* was found to colonize native and exotic grasses in northern Australia (Kaestli *et al.*, 2011). Although T3SS-3 clearly plays a role in mammalian pathogenesis, it is similarly possible that its evolved natural function is to fend off protozoan and nematode predators, thereby facilitating environmental persistence and further acquisition of nutrients. An additional role for T3SS-3 in the colonization of plant roots or the nutrient and moisture-rich leaves seems unlikely but cannot be excluded. A better understanding of the *B. pseudomallei* T3SSs will be essential for understanding its ability to persist and thrive in the environment.

Materials and Methods

Bacterial strains, growth and mutant construction *B. thailandensis* E264 (Brett *et al.*, 1998)*, B. pseudomallei* Bp340 (SEA *Bp* 1026b Δ*amrAB-oprA*) (Mima and Schweizer, 2010) and Australian *B. pseudomallei* NAU14B6 were routinely grown in L-medium with or without NaCl (LB, LB-NS). In-frame mutations were constructed using allelic exchange with the *pheS** negative selection marker on M9 agar containing 0.1% cholorophenylalanine (cPhe) (Barrett *et al.*, 2008). Complementation of mutants was performed using derivatives of the pBBR1-MCS2 broad host-range plasmid (Kovach *et al.*, 1995) containing the *nptII* kanamycin resistance gene. For flagellar swarming motility assays, bacterial colonies were lifted with a flame-sterilized needle and inoculated into 0.3% L-agar. Bacteria were allowed to swarm through the semisolid medium for 18-24 hr. before examination.

Cell culture, infection and cytotoxicity assays HEK293 (ATCC CRL-1573), HeLa (ATCC CCL-2) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% bovine growth serum (BGS, Hyclone) and 5% CO_2. Prior to experiments, plate wells were incubated at room temp for 30 min with a 1:40 dilution of Matrigel (BD) in serum free medium to enhance retention of cells during washing and manipulation. For transfections, HeLa cells grown to 80% confluence in 6-well plates were transfected with the derivatives of the pEGFP-N1 plasmid containing *B. pseudomallei* effector genes using 3 μg of plasmid DNA and 8 μl of FuGENE HD (Roche), according to manufacturer's protocol. Cell were transfected for 8-24 hr. prior to functional assays. For co-immunoprecipitation experiments, cells were transfected as described above. Lysis and capture of the effector-EGFP fusions and co-precipitating proteins was performed using camelid EGFP nanobody-conjugated magnetic beads according to the manufacturer's directions (Allele Biotech).

For infection studies, cells were seeded at 1.8-2.0x10^6 cells per well in 6-well plates. Following the addition of bacteria, plates were gently centrifuged to bring bacteria into contact with cells (200 x g for 5 min). Cells were infected using a multiplicity (MOI) of 1 for cytotoxicity and intracellular replication experiments, an MOI of 10 for invasion, and an MOI of 3x10^{-4} for plaque assays. One hour after infection, cells were washed thoroughly with Hank's balanced salts and extracellular bacteria were eliminated by the addition of 1000 µg/mL Km. In invasion and replication experiments, infected cells were washed with Hank's, harvested by trypsinization and lysed with 0.2% Triton X-100 + 20 mM MgSO$_4$ and 50 µg/mL DNase I (to reduce lysate viscosity). Assays for intracellular colony forming units (CFU) were performed on serial dilutions of the lysate. Intracellular growth and replication timecourse experiments were done similarly, except cells were harvested at indicated timepoints. For plaque assays, cells were infected as described above and overlaid with DMEM containing 0.2% NaHCO$_3$, 50mM HEPES (pH 7.4), 10% BGS and antibiotics (see below) in 1.2% low melting temp. agarose (BioRad). Plaques were counterstained for photography by adding 200 µl DMEM containing 0.1% neutral red onto the agarose 24 hr. later. For immunofluorescence microscopy, agarose and neutral red staining were not used. Antibiotics to suppress growth of extracellular bacteria were used as follows: 125 µg/mL kanamycin (BtE264 and Bp340), 150 µg/ml gentamicin (pBBR-MCS Km-complemented Bp strains) or 10 µg/mL pipericillin/tazobactam (Zosyn) (complemented Bt and Bp strains).

Microscopy HEK293 or RAW264.7 cells were grown as described above on glass coverslips or in glass-bottom dishes treated with dilute Matrigel liquid. Following infection cells were washed with Hank's and fixed with ambient temp. 4% paraformaldehyde in PBS containing 3 mM MgCl$_2$ and 10 mM EGTA for 15 min. Cells were permeabilized using PBS containing 0.2%

Triton X-100. Nonspecific binding sites were blocked in PBS containing 1% bovine serum albumin (BSA), 10% calf serum and 2% goat serum for 1 hr. at room temp. Antibody incubations were carried out for 1 hr. at room temp in blocking buffer using the following dilutions: rabbit *Bt* antiserum (Burtnick *et al.*, 2008); 1:1000 (for *Bt*), 1:50 (*Bp*); mouse α-LAMP-1 monoclonal antibody (Santa Cruz), 1:40; Alexa-Fluor 488 or 555- labeled phalloidin and secondary antibodies, 1:150 (Molecular Probes). Permanent mounts of specimens were created using ProLong Gold (Invitrogen). Glass-bottom dishes and slides were analyzed using a Leica SP5-II AOBS confocal microscope setup or an Olympus BX51 upright fluorescent microscope. Live cell brightfield and fluorescence imaging and video recording was performed using a Zeiss Axiovert 40CFL inverted fluorescence microscope with a Canon digital camera. Image processing was performed with Leica's LAS-AF software suite or Adobe Photoshop CS2.

Image and Data Analysis Figures, tables and graphs were prepared using Microsoft PowerPoint and Excel. Statistical analysis was performed with the Student's *t*-test using Excel and GraphPad.

Screen for class 1 T3SS effector chaperones Candidate T3SS chaperone genes were identified via a series of six consecutive filters applied to all open reading frames (ORFs) from the *B. pseudomallei* K96243 genome (Fig. 2.1A). First, proteins of estimated molecular weight between 12 and 20 kDa were selected. Second, theoretical isoelectric points (pI) of the selected proteins were calculated to identify those with a pI between 3.5 and 6.5, followed by elimination of proteins with assigned enzymatic activity indicative of non-chaperone related functions. Phylogenetic footprinting analysis21 was then applied to the remaining ORFs and proteins with homologs found in genomes lacking a T3SS were eliminated. Next, three dimensional folds of the remaining proteins were estimated via a threading method and proteins that matched the

canonical class I chaperone structure (E<0.05) were selected. finally, the threading procedure was repeated for all homologs of selected proteins, and only those candidates whose homologs also adopted chaperone-like structures were hypothesized to function as T3SS class I chaperones.

Effector secretion assays *B. pseudomallei* Bp340 parental and the Δ*bsaS* mutant expressing either the C-terminally hemagglutinin (HA) epitope-tagged BopC and BopE control construct were grown in acidic (pH 5.2) medium A (MA) (Sambrook *et al.*, 1989) for 4 hours. Following centrifugation, total protein from the bacterial pellet and culture supernatant fractions was harvested and precipitated using 15% trichloroacetic acid (TCA). Protein concentration was normalized with respect to the optical density (OD600) of the bacterial cultures. Proteins were separated on 4-16% gradient polyacrylamide gels, transferred to PVDF membranes and probed with a mouse monoclonal antibody to HA (HA.11, Covance).

Table Legend

Table 1. **Results of bioinformatic screens for *B. pseudomallei* T3SS effector candidates.**

Summary of putative effector loci by applying screening techniques to the B. *pseudomallei* K96243 genome sequence (Holden *et al.*, 2004). The screen for class I chaperones was applied, yielding three chaperone / *Burkholderia* effector putative (Bep) pairs (Panina *et al.*, 2001). Clues involving genomic context, homology to other known effectors, and the presence of eukaryotic functional domains yielded another 5 effector candidates. The entire repertoire of T3SS effectors from known plant and animal pathogens was applied to the K96243 genome and the results were manually inspected for the highest confidence hits, yielding 8 possible effectors. The currently known effectors in *B. pseudomallei* include BopA (BPSS1524) and BopE (BPSS1525), for a total of 18 T3SS effector candidates.

Figure Legends

Fig. 1. ***B. thailandensis fla2* encodes lateral flagella that are expressed following infection of mammalian cells.** A. HEK293 cells were infected with *B. thailandensis* Δ*motA1*Δ*bimA fliC2::H*A for 6 hr., then stained for microscopy using α-*B. thailandensis* antiserum or α-HA (red). Lateral, HA-tagged flagellin filaments are red, while the bacterium is green. B. (left) Flagellar expression was examined for *B. thailandensis* E264 WT in LB and following infection of HEK293 cells. In WT, polar flagella, from expression of *fla1*, are visible only in broth culture (top), and the majority of cells lack Fla1 flagella but instead possess actin tails following infection (bottom). The Δ*motA1*Δ*bimA fliC2::HA* strain does not possess polar flagella when grown in broth (top) and cannot polymerize actin, thus it expresses numerous lateral flagella (bottom).

Fig. 2. **Expression of the Frr response regulator leads to constitutive *F* activity and dramatically reduces plaque formation following infection**. A. Swarming assay of *B. thailandensis* E264 wild-type (WT), ΔfliC1 pfrr, and ΔfliC1 in 0.3% L agar. Strains were inoculated and allowed to swarm for 20 hr. at 37°C before examining. Partial restoration of fla2-mediated swarming is indicated by the arrow. B. Frr expression inhibits plaque formation in all *B. thailandensis* genetic backgrounds and similar results were obtained with Australian and Southeast Asian B. *pseudomallei* strains. C. defective plaque formation in p*frr* strains is not due to reduced invasion efficiency, as p*frr* strains invaded cells comparably to WT. E. Comparison of *fla2* loci in *B. thailandensis* and Australian B. *pseudomallei* NAU14B6. Color coding: putative flagellar-accessory/modification genes (blue); *che* chemotaxis (orange); *lfi* basal body/export/assembly (green); *lfg* flagellar hook/rod/ring components (brown); *mot/laf* motor

components (black), *fliC/lafA* flagellin (grey); putative regulatory loci (red); hypothetical

protein/unknown function (white). Map is to scale. Scale bar = 3.5 kb.

Fig. 3. **Fluorescent expression reporter system to monitor *fla2* and *bim* operon expression**.

A. The coding sequences for EGFP or mCherry RFP were placed under control of the predicted

promoter elements for the *B. thailandensis bim* actin motility operon (P*bimB*) and the *fliE2* gene

(P*fliE2*). B. Neither promoter was active when bacteria were grown in laboratory medium . C.

Timecourse of fluorescent protein expression. Most bacteria remained attached to the

extracellular surface of HEK293 cells 1 hr. after infection (arrows) and did not express EGFP or

RFP from the *bim* or *fliE2* promoters, suggesting that the activity of these systems is dependent

on intracellular localization of bacteria. At 2 hr. following infection, red and green fluorescent

bacteria began to appear. The fraction of the total intracellular bacteria exhibiting red (PbimB),

green (PfliE2) or red + green) (PbimB + P fliE2) are indicated by the red, green or yellow bars,

respectively. The graph (3C, upper) corresponds to the image examples (3C, lower). D.

Occasional PfliE2-mcRFP expressing bacteria were seen to polymerize actin (left and inset),

while all PbimB-EGFP expressing bacteria were associated with actin tails (right and inset).

Fig. 4. Phenotypes from ectopic expression of Bep effector candidates in mammalian cells.

Effectors were expressed by transient transfection of HeLa cells. Bep effector candidates were

cloned as N-terminal translational fusions to EGFP under control of the strong, constitutive

cytomegalovirus (CMV) immediate early promoter. Cells were stained for common cytoskeletal

markers (tubulin, actin) and compared to non-transfected controls to gauge whether changes in

cytoskeletal structure or cell morphology were evident following transfection. A. BepB-EGFP

(BPSS1411). B. BepE-EGFP (BPSS1386). C. BepF-EGFP (BPSS1385). Upper row. Effector-

EGFP fusions (green). Middle row. Tubulin/actin staining (red). Bottom row. Merged images. D.

Diagrams of effector candidates indicating the presence of putative conserved functional domains and, if present, homology to effectors in other species. Diagrams are approximately to scale. Color codes: yellow, presumed non-conserved T3SS secretion signal; orange, putative chaperone interaction domain; red, functional domain of protein; dark blue, chaperone; light blue, putative conserved eukaryotic functional domain.

Fig. 5. BepD-EGFP decorates microtubules and is found in immunoprecipitation complexes with tubulin and microtubule associated proteins. A. Transfection analysis of BepD-EGFP in HeLa cells (green, top); tubulin staining (red, middle); merged image (bottom). B. Immunoprecipitation of BepD-EGFP from transfected HeLa cells using α-EGFP antibody-coupled magnetic beads. Proteins were separated on 4-16% gradient polyacrylamide gels, and potential interacting partner proteins were excised from the gel and identified by tandem mass spectrometry (MS-MS). Left, Coomassie stain of protein molecular weight marker (M). Middle, BepD-EGFP. Right, MS results. C. Western blot of immunoprecipitated protein from cell lysate of BepD-EGFP transfected cells. Left; tubulin antibody. Right; EGFP antibody.

Fig. 6. BepC-EGFP bundles actin and is found in immunoprecipitation complexes with actin and actin-associated proteins. A. Transfection analysis of BepC-EGFP in HeLa cells (green, top); actin staining (red, middle); merged image (bottom). B. Immunoprecipitation of BepC-EGFP from transfected HeLa cells using α-EGFP antibody-coupled magnetic beads. Proteins were separated on 4-16% gradient polyacrylamide gels, and potential interacting partner proteins were excised from the gel and identified by tandem mass spectrometry (MS-MS). Left, Coomassie stain of protein molecular weight marker (M). Middle, BepC-EGFP. Right, MS results. C. Western blot of immunoprecipitated protein from cell lysate of BepC-EGFP transfected cells. Left; EGFP antibody. Right; actin antibody.

Fig. 7. **BopC-EGFP exhibits perinuclear localization following transfection.** A. Transfection analysis of BopC-EGFP in HeLa cells (green, top); tubulin staining (red, middle); merged image (bottom). B. Immunoprecipitation of BopC-EGFP from transfected HeLa cells using α-EGFP antibody-coupled magnetic beads. Proteins were separated on 4-16% gradient polyacrylamide gels, and potential interacting partner proteins were excised from the gel and identified by tandem mass spectrometry (MS-MS). Left, Coomassie stain of protein molecular weight marker (M). Middle, BopC-EGFP. Right, MS results.

Fig. 8. Analysis of *B. pseudomallei* T3SSs and effector proteins in cellular pathogenesis. A. Plaque formation assay following infection of HEK293 cell monolayers with Bp340 T3SS-1 (Δ*sctNBp1*), T3SS-2 (Δ*sctNBp2*) and T3SS-3 (Δ*sctNBp3* / Δ*bsaS*) mutants. A MOI of 1 x 10^{-4} was used. B. Invasion of HeLa cells with Bp340 and mutant derivatives. B. Uptake by phagocytic RAW264.7 mouse monocytes. C. Plaque formation 40 hr. following infection of RAW264.7 cells using an MOI of 1 x 10^{-5} CFU/cell. * = P < 0.05. Error bars are +/- SEM of three separate experiments.

Fig. 9. Secretion assays of T3SS effector candidates. Western blots of bacterial cell lysates (P) or TCA-precipitated supernatants (S) using Bp340 wild-type (WT) or T3SS-1, 2- or -3 Δ*sctNBp* ATPase mutants containing plasmid constructs encoding HA-tagged effectors. Proteins were separated on 4-16% gradient polyacrylamide gels, transferred to membranes and probed with mouse monoclonal to HA. A. The secretion BopE and BopC is dependent on T3SS-3. Neither expression nor secretion of BopA (C.) nor BepD (E.) was detected. C. Expression and secretion of BepB was observed, although it was independent of T3SS activity. The same was observed for BepC (D.), BepE (F.) and BepF (G.).

Fig. 1

α-Bt E264 + α-HA

A

WT   ΔfliC1ΔbimAfliC2::HA   ΔfliC1ΔbimAfliC2::HA

B   C

BROTH

HEK 293

α-Bt E264 + α-HA   HEK 293

Fig. 2

A

Bt E264   Δ*motA1*

Bt E264 p*frr*   Δ*motA1* p*frr*

B

pfu/cfu

1.00E-01

1.00E-02

1.00E-03

BtE264   *Bt E264 pfrr*   Δ*bimA*Δ*motA2*

C

Pfu/cfu

1.00E-01

1.00E-02

1.00E-03

Bp NAU14B6   *Bp NAU14B6 pfrr*   *Bp 1026b*   *Bp 1026b pfrr*

D

Invasion Efficiency

1.00E-01

1.00E-02

Bt E264   *Bt E264 pfrr*   Δ*motA2*Δ*bimA*

E

B. thailandensis E264

RRSKRRSK lafV(lafA) *fliCd* *motA2B2 (lafTU)* *cheYAW* *mcp* *RDBB* *lfhAB* *lfiRQPNMEFGHIJDS* *lfiKLA*$_{\sigma 28}$ *lfgM* *lfgNJ*$_1$*ABCDEFGHIJ*$_2$*KL* *tetR*

100% synteny
93% nucleotide identity

B. pseudomallei NAU14B6 (Australia)

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Fig. 3

A

B Broth 1 hr

Broth 7 hr

C % of fluorescent bacteria over time

PfliE2 PbimB PbimB+
              PfliE2

1 hr   2 hr   5 hr   7 hr

PfliE2   PbimB   PbimB+
                    PfliE2

HEK293

D PfliE2-mRFP/Actin   PbimB-EGFP   Actin

HEK293 7 hr

Table 1

Chaperone Screen	Cognate effector(s)	Homology	Putative conserved domains / other comments
BPSS1410	BPSS1411	*Photorhabdus* Tcd, *Yersinia* Yit/Yip	BepB, insecticidal toxin
BPSS1517	BPSS1516	none	BopC, partial internal IS element, eff.&chap. flanked by transposases
BPSS1611	BPSS1609	none	BepD, proline-rich
Homology Screens, Conserved Domains, Contextual Clues			
	BPSS1385	EPEC Cif	BepF, ATP/GTP binding
	BPSS1386	none	BepE, actin binding, ATP/GTP binding
	BPSS1387	none	acetylase
	BPSS1389	none	contextual
	BPSS1608	none	acetylase
Automated BLASTX of effector database			
	BPSL0006	*Burkholderia* BapC	
	BPSS2102	*Chlamydia spp.*	T3SS effector, Ser/Thr kinase
	BPSL2506	*Ralstonia*	Put. exp. glycosylase
	BPSL0892a	*Erwinia, Ralstonia* PopA, HrpW	
	BPSS1416	*P. syringae* HopAN1	
	BPSL1705	*Ralstonia*	LRR-GALA T3SS protein (w/in genomic island)
	BPSL1317	*Ralstonia* AvrBS3	
	BPSS1439	*Chlamydia* Tarp	Possibly Autotransporter
Previously Identified			
	BPSS1524	*Shigella* IcsB	BopA
	BPSS1525	*Salmonella* SopE	BopE, GEF

Fig. 4

A   B   C

BepB-GFP   BepE-GFP   BepF-GFP

tubulin   actin   actin

merge   merge   merge

D

T3SS-1 // [blue box]   1   20   100   371   BepB (BPSS1411)

BecB Chap. (BPSS1410)   *Photorhabdus* Tcd Insecticidal Toxin Domain

ATP/GTPase domain   α-actinin-type actin- binding signature

BepE (BPSS1386)   1   20   100   1200   // T3SS-1

Novel actin-binding effector

ATP/GTPase domain

BepF (BPSS1385)   1   20   100   300   // T3SS-1

EPEC / EHEC Cif T3SS effector

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Fig. 5

BepD BPSS1609

A

BepD-EGFP

α-tubulin

Merge

B

Coomassie

M   BepD-EGFP   MS-MS results

250
150
100
75
50
37
25
20

Dynein HC
γ-spectrin
Clathrin HC
Hsp90
Hsp70
β tubulin
BepD-EGFP

C

EGFP   BepD-EGFP   EGFP   BepD-EGFP

50   50
25   25

WB   β Tubulin   EGFP

BepD (BPSS1609)

1 20 100   215
BecD Chap. (BPSS1611)   T3SS-2

Fig. 6

BepC BPSS1528

A BepC-EGFP

Actin

Merge

B

Coomassie

M BepC-EGFP MS-MS results

250 — ◀ γ-spectrin
150 — ◀ α -actinin
100 — ◀ BepC –GFP &
75 — Myosin IB
50 —
◀ Actin
37 —
25 —
20 —

C

EGFP EGFP BepC-EGFP

Actin monomer (44kD) ◀

WB EGFP actin

BepC (BPSS1528) 1 20 100 880 // T3SS-3

Fig. 7

BopC BPSS1516

A
BopC-EGFP

α-tubulin

Merge

B

Coomassie

M   BopC -GFP

250 ◄ Fam38A-Mib (ER
Membr. Prot.)
150
100
75 ◄ BopC -EGFP
50
◄ Actin
37

25

20

1   20   100   469

Tn'ase   BopC/BepA (BPSS1516)   BicC/BecA Chap.   Tn'ase   // T3SS-3
(BPSS1517)

Fig. 8

A   HEK293

B   HeLa

C   Raw264.7

D   Raw264.7

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Fig. 9

A

	BopE		BopC		BopE		BopC	
	wt	T3SS3	wt	T3SS3	wt	T3SS3	wt	T3SS3

75 —
50 —
37 —
25 —

PELLET   SUPERNATANT

B

BopA-HA (57 KDa)

BopE-HA (34 KDa)

BopE-HA   BopA-HA   BopE-HA   BopA-HA

P   S

C

BepB-HA

(45 KDa)

P   S

D

BepC-HA

(103 KDa)

P   S

E

BepD-HA

(22 KDa)

P   S

F

146 KDa

30 KDa

P   S

BepE-HA

(146 KDa)

G

BepF-HA

(41 KDa)

P   S

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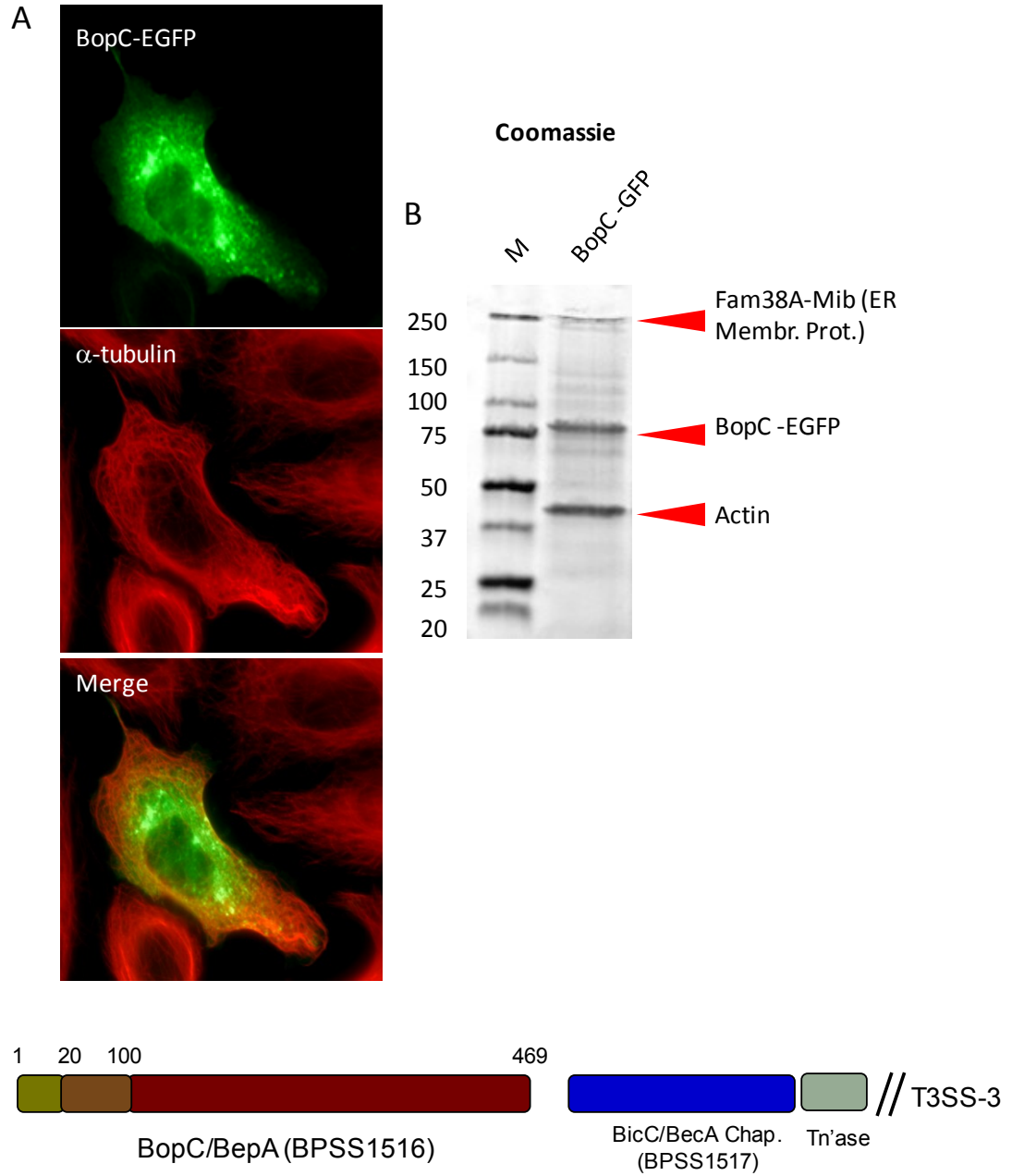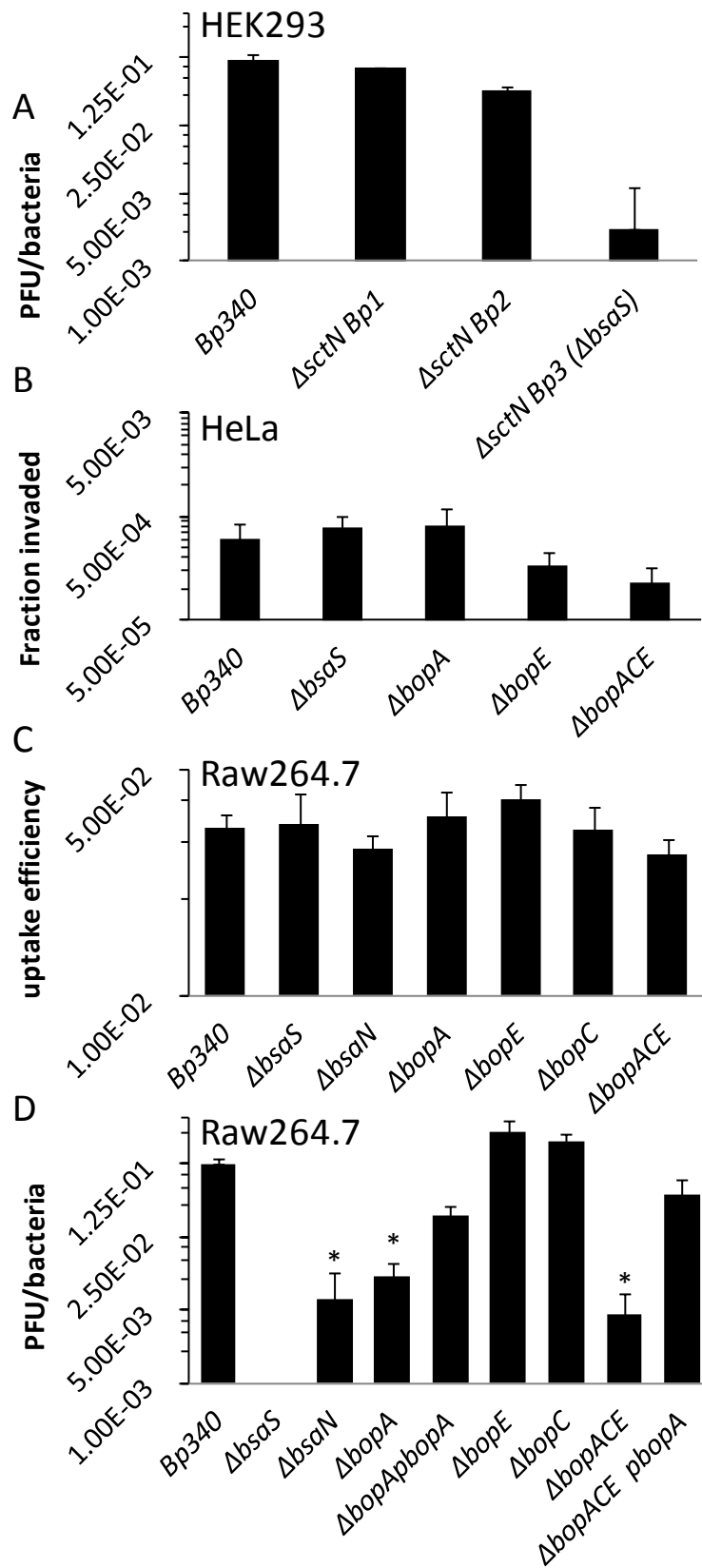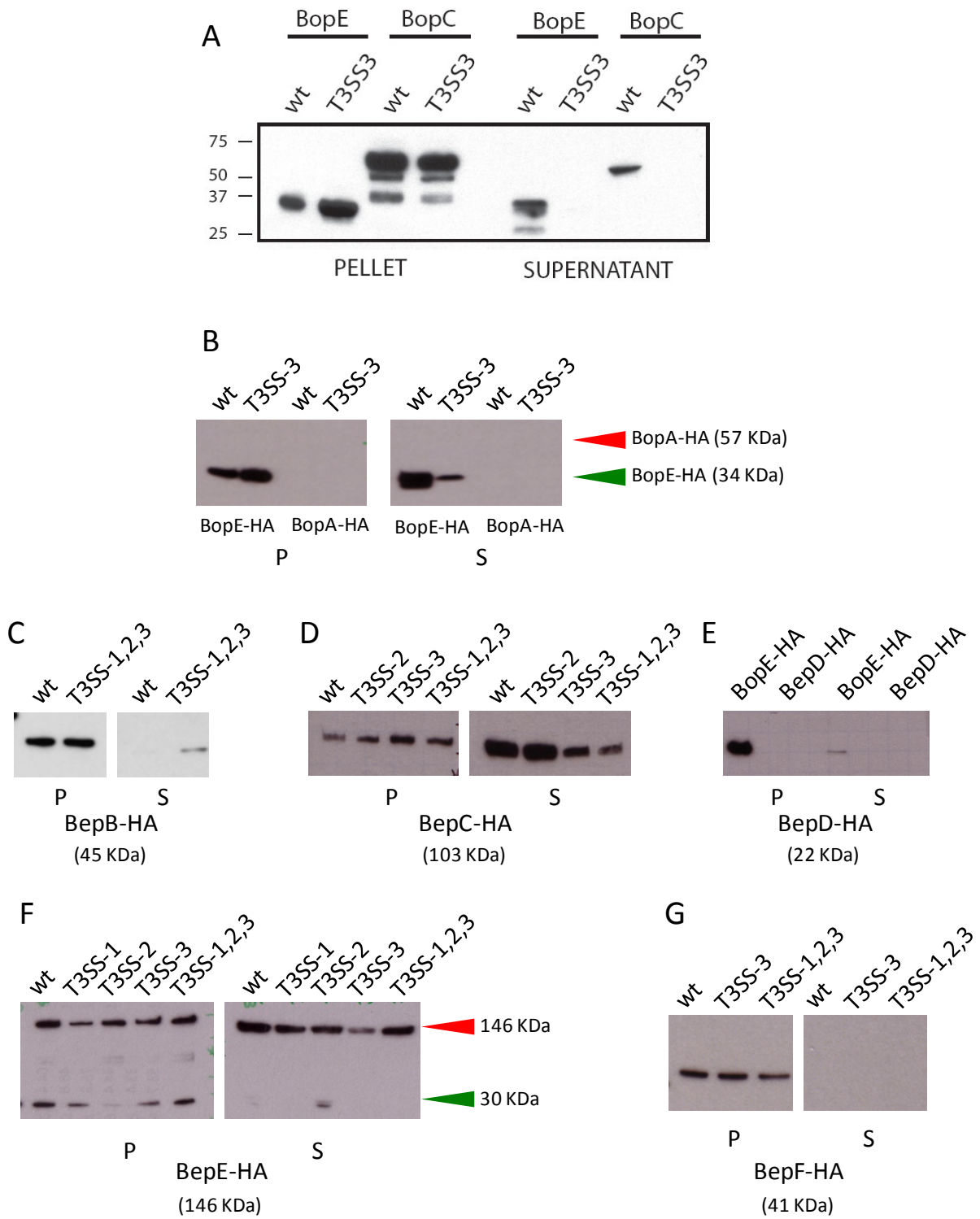
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Chapter 5. Future research and perspectives

Following Alfred Whitmore's description of melioidosis in Rangoon, Burma in 1913 (Whitmore, 1913), few laboratories have historically focused on *Burkholderia pseudomallei* until its designation as a Centers for Disease Control and Prevention select agent pathogen. Although melioidosis research has recently experienced significant growth, efforts to identify and characterize virulence factors have been complicated by the organism's enormous degree of ecologic and genetic diversity. Our knowledge of *B. pseudomallei* virulence mechanisms and ecology remains fragmentary despite recent progress. As I have described in the preceding sections, many putative *B. pseudomallei* virulence determinants do not appear to contribute to infection in mammals or mammalian cells, and thus have probably evolved to facilitate survival in the environment. This perplexing aspect of *B. pseudomallei* pathogenesis presents a unique opportunity to study virulence mechanisms in a fascinating, diverse bacterial species and to understand how pathogens evolve. This understanding should pave the way for the development of new vaccines and therapeutics for the prevention and treatment of melioidosis.

Ongoing and future research objectives

Genetic dissection T3SS$_{Bsa}$-mediated endosome escape The Bsa T3SS and T6SS-1 are required for pathogenesis in animals, and are highly expressed upon infection of cells *in vitro* and in animal models (Tuanyok *et al.*, 2006; Sun *et al.*, 2010). The roles of T3SS$_{Bsa}$ and of T6SS-1 in promoting virulence are well described and definitive (Galyov *et al.*, 2010). On the other hand, little is known mechanistically regarding how specific components this system operate to promote survival and virulence inside mammalian cells and in alternative host models. Since *B. thailandensis sctN/bsaS* mutants spread and fuse mammalian cells into MNGCs indistinguishably from wild-type following cytosolic nanoblade delivery, T3SS$_{Bsa}$ is not required for cell-cell spread once the primary endosome of the initially infected cell has been breached.

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These results and those of others suggest that the main, and perhaps only role for the T3SS$_{Bsa}$ may be lysis of the primary endosome (Burtnick *et al.*, 2008; French *et al.*, 2011). Additional roles for T3SS$_{Bsa}$, such as suppression of the host immune response cannot be excluded. Since bacteria only exhibit robust growth following escape from the endocytic vesicle into the cell cytosol, intracellular replication timecourse experiments are useful albeit indirect methods of assessing the kinetics of endosome escape (Chapter 3) (Fig. 1A) (French *et al.*, 2011). Moreover, fluorescence microscopy to localize fluorescent intracellular bacteria with endosomal markers, such as Rab 5 for early endosomes (Bucci *et al.*, 1992) or LAMP1 (Gong *et al.*, 2011) for late endosomes, while widely utilized, is frequently unreliable and results can be difficult to interpret. To address this, we are developing methods to directly analyze the kinetics of *B. pseudomallei* endosome escape. This will be based on differential permeabilization of the plasma and endosomal membranes using the detergent digitonin. A similar approach has been used to examine endosome escape by other intracellular pathogens such as *Francisella tularensis* (Checroun *et al.*, 2006). This procedure will be modified to work with red or green fluorescent *Burkholderia* and is summarized in Fig. 1B. Results will be validated using electron microscopy of infected cells.

What are the T3SS factors that participate in *Burkholderia* endosome escape? Is the BipBCD translocon complex necessary and sufficient, or does vesicle lysis depend on the action of one or more effector proteins? Insights should be provided by mutational analysis of *B. pseudomallei* T3SS structural, regulatory and effector components followed by cell-based assays. As I have already shown (Chapters 3 and 4), activity of the *sctN/bsaS* ATPase, which energizes secretion of needle, translocon and effector components, is required for plaque formation following infection. Gong *et al.* has suggested that the BopA effector may be involved in

endosome escape (Gong *et al.*, 2011), although no direct evidence was shown. I have demonstrated that elimination of BopA *reduces* plaque formation in RAW264.7 macrophages, but does not *eliminate* it as observed for Δ*sctN/bsaS* ATPase mutants (Chapter 4, Fig. 8D). The plaque phenotype for a Δ*bopA* single mutant is comparable to a Δ*bopACE* triple effector mutant and a Δ*bsaN* T3SS$_{Bsa}$ regulatory mutant, which synthesizes the injectisome but neither expresses nor secretes effectors (Sun *et al.*, 2010). Together, these observations suggest that of the three known effectors, BopA plays the major role in the intracellular life cycle. Further, unlike Δ*sctN/bsaS* ATPase mutants, the Δ*bsaN*, Δ*bopA* single mutant and the Δ*bopACE* triple effector mutants *do* form plaques, which suggests some degree of successful endosome escape. One possibility is that Δ*bsaN* and Δ*bopACE* mutants do not survive well once in the cytosol, possibly due to attack from cellular autophagy processes.

Although the endosome escape mechanism is not understood for any intracellular pathogen, it could be mediated by insertion of the pore-forming T3SS needle translocon tip components into the endosomal membrane. Assuming there are no additional, undiscovered T3SS$_{Bsa}$ effectors at work, the BipBCD T3SS translocon complex is the logical culprit for endosomal lysis. To test this possibility, plaque formation and intracellular replication timecourse assays will be performed using Bp340 WT, Δ*sctN/bsaS,* Δ*bipB*, Δ*bopA* and Δ*bopACE* strains. A Δ*bipB* translocon mutant cannot translocate effectors (Diepold *et al.*, 2010; Montagner *et al.*, 2011), and if our hypothesis is correct, we do not expect it to escape from the endosome, multiply in the cytoplasm, or spread and form plaques. Thus, Δ*bipB* should recapitulate the no-plaque phenotype of the Δ*sctN/bsaS* ATPase strain and intracellular bacterial loads are expected to resemble Δ*sctN/bsaS,* as measured with intracellular replication assays. Regarding the role of BopA, higher numbers of intracellular bacteria seen for Δ*bopA*, Δ*bsaN* and

Δ*bopACE* compared to Δ*sctN/bsaS* will indicate an ability of these strains to escape from endosomes and multiplication in the cytosol. Conversely, numbers of intracellular bacteria are expected to remain low if BopA is important for intracellular survival.

Experiments to analyze endosome escape kinetics will be conducted in parallel with the phagosome integrity assay described above (Fig. 1B). To accomplish this, *B. pseudomallei* strains will be labeled with plasmid-encoded RFP. Cells will be fixed with paraformaldehyde at 1, 2, 4 and 8 hr. after infection and the plasma membranes will be selectively permeabilized by a brief incubation with digitonin. Cells will then be incubated with green-fluorescent *Burkholderia* antiserum. Bacteria remaining inside endocytic vesicles will exhibit only red fluorescence, where those that have escaped into the cytosol will exhibit dual red-green fluorescence and appear yellow. To establish the proper conditions, the escape rate of Bp340 WT will be compared to that of the Δ*sctN/bsaS* mutant. The Δ*sctN/bsaS* mutant is expected to remain trapped within vesicles and thus should only exhibit RFP fluorescence. Translocon and effector mutants will then be analyzed and their escape efficiency will be quantitated by the number of red and yellow intracellular bacteria over time using confocal microscopy. Finally, infected cells will be fixed with glutaraldehyde, and analyzed by electron microscopy of ultrathin sections. Overall, this plan is expected to provide a thorough assessment of the genetic requirements for T3SS$_{Bsa}$-mediated endosomal escape.

T6SS as a potential fusogenic factor in cell-cell spread *B. pseudomallei* Δ*sctN/bsaS* mutants, which carry a null mutation in the ATPase required for T3SS$_{Bsa}$ activity, fail to form MNGCs or plaques following infection due to endosomal entrapment. However, nanoblade delivery of mutants results in normal MNGC and plaque formation (French *et al.*, 2011), demonstrating that T3SS$_{Bsa}$ is not required for MNGC formation. In contrast, deletion of the *clpV1* encoded ATPase,

which energizes T6SS-1, abrogates MNGC formation and cell-cell spread following bacterial delivery by either infection or nanoblade delivery, illustrating the crucial role of T6SS-1 (French *et al.*, 2011).

T6SSs, which are evolutionarily related to the injection devices of tailed bacteriophages, are able to translocate cargo proteins across cell surfaces of Gram-negative bacteria and through the plasma membranes of infected eukaryotic cells. They are unique secretion machines that operate through a mechanism that is complex and poorly resolved (Veesler and Cambillau, 2011) Fig. 2A). Hcp and VgrG proteins are highly conserved components of the translocation machinery that are mutually dependant for their own secretion, and some VgrG proteins possess "evolved" carboxy-terminal domain (CTD) extensions which perform effector functions following injection across target cell membranes (Ma *et al.*, 2009). *B. pseudomallei* contains six T6SS gene clusters which are important for its interactions with mammalian cells and other bacterial species (Schwarz *et al.*, 2010b). *B. pseudomallei* also contains twelve *vgrG* loci, some of which appear to encode "orphan" VgrGs in that they are not localized within or adjacent to one of the T6SS clusters. Other T6SS loci are associated with multiple VgrG proteins, which are likely the products of duplication and divergence (Fig.2B). Comparison of the *B. pseudomallei* VgrG1 protein, which is encoded in the virulence-associated T6SS-1 gene cluster, to VgrGs in other bacterial pathogens reveals a conserved N-terminal domain homologous to the trimeric phage T4 tail spike proteins gp27 and gp5 (Fig. 2B.). VgrG1 also contains an evolved CTD that does not contain strong matches to known motifs or functional domains, but is highly conserved between *B. pseudomallei*, *B. mallei* and *B. thailandensis*. Null mutations in the *B. pseudomallei* Bp340 *vgrG1* locus eliminate MNGC and plaque formation, similar to what is observed in Δ*clpV1* mutants. In light of these and other observations, we propose that the CTD of VgrG1

possesses a unique activity that triggers membrane fusion in a contact-dependent manner to facilitate cell-cell spread. Specifically, we hypothesize that in *Burkholderia*-infected cells, motility facilitates contact with the cytoplasmic face of the plasma membrane and triggers T6SS-dependent translocation of the VgrG1 CTD across the cell surface. Simultaneous or subsequent contact with a neighboring (uninfected) cell would then result in membrane fusion (Fig. 3A). This hypothesis evokes a process that is analogous in many ways to infection by enveloped viruses. If true, it will represent an entirely novel mechanism in bacterial pathogenesis and a potential new target for small-molecule inhibitors and vaccines.

In the experiment summarized in Fig. 3B-C, full length or truncated *vgrG1* alleles were expressed on a replicating plasmid in Bp340 Δ*vgrG1*, which is defective in cell-cell spread as indicated by plaque formation and MNGC assays (French *et al.*, 2011). Full-length *vgrG1* restored plaque and MNGC formation in Bp340 Δ*vgrG1*, although a version containing a C-terminal HA epitope tag did not (Fig. 3B). This observation suggests that modification of the VgrG1 CTD is detrimental to its function. To more closely examine requirements for cell fusion, the Δ*vgrG1* mutant was complemented with expression constructs containing *vgrG1* constructs with C-terminal truncations. MNGC and plaque formation assays showed that none of the truncated mutants were capable of restoring function, including a construct that lacked only the last 10 C-terminal amino acids. We propose that the N-terminal region of VgrG1 is required for secretion by the T6SS-1 apparatus, and that secretion activity is separable from the unique role of the CTD in cell fusion. To directly test this, experiments are in progress to measure T6SS-1 activity in strains carrying truncations of the VgrG1 CTD. Since T6SS-1 is inactive under standard laboratory growth conditions, secretion of Hcp1 is being measured in Bp340 Δ*vgrG1* derivatives engineered to overexpress the VirAG two component system, which activates

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expression of T6SS-1 loci leading to secretion activity *in vitro*. If our hypothesis is correct, mutations in sequences encoding the VgrG1 CTD that retain an intact N-terminal region will be competent for secretion.

To further test our hypothesis that the VgrG1 CTD is responsible for cell fusion, three additional approaches are being pursued. First, we will determine if the CTD is *sufficient* to promote cell fusion following transfection of mammalian cells. Sequences encoding the first 182 amino acids of the Nipah virus Glycoprotein (NiV-G) have been fused to VgrG1 CTD coding sequences on a CMV-based expression vector. NiV-G is a type II transmembrane protein and is predicted to orient the VgrG1 CTD on the external face of the plasma membrane following transfection. The ability of this construct to mediate cell fusion will be measured using our GFP/RFP MNGC assay described in Chapter 3. Second, our model predicts that the VgrG1 CTD will be displayed on the surface of *B. pseudomallei*-infected cells in a manner dependent on intracellular motility and the ClpV1 (T6SS-1) ATPase. Antibody-based detection assays are under development to test this prediction. Finally, we will determine if polyclonal antisera against the CTD can inhibit cell fusion and intercellular spread. If successful, these experiments will support the development of VgrG1 as a potential component of a melioidosis vaccine.

Regulation of virulence loci Even as headway is being made in deciphering the regulatory networks that control expression of *Burkholderia* pathogenicity loci, relatively little is known compared to better-studied pathogens. The cascade that controls expression of the Bsa T3SS and T6SS-1 is one of the better characterized pathways in *B. pseudomallei* is the one (Fig. 4) (Sun *et al.*, 2010; Sun and Gan, 2010; Chen *et al.*, 2011). At the top of the regulatory ladder, a TetR-type regulator BspR (BPSL1105) exerts indirect control over T3SS-3 apparatus genes through a hierarchy involving the ToxR/S-like two component system BprP/Q. BprP activates the AraC-

type regulator BsaN and the regulatory class I chaperone BicA, which are encoded within the T3SS$_{Bsa}$ cluster. The combination of BsaN/BicA goes on to activate expression of T3SS effector genes and additional regulators important for T6SS-1 gene expression. $\Delta bsaN$ and $\Delta bspR$ strains are avirulent in mice (Sun *et al.*, 2010; Chen *et al.*, 2011). Although this establishes the existence of a regulatory link between T3SS$_{Bsa}$ and T6SS-1, it is less clear whether alternate control pathways exist, how this network ties into others, or the precise environmental signals needed to trigger expression of T6SS-1 and T3SS$_{Bsa}$.

Our discovery of a Fla2 response regulator (Frr) and its cognate sensor histidine kinase (Fsk) has important and exciting implications for the study of *Burkholderia* virulence regulation. In *B. thailandensis*, constitutive expression of Frr on a plasmid vector enabled Fla2-mediated swarming motility *in vitro*. To our surprise, increased levels of *frr* expression greatly reduced plaque formation in *B. thailandensis* and *B. pseudomallei* isolates (described in Chapter 4). It is not known whether Frr overexpression interferes with the ability to escape the primary endosome, or whether the lack of plaque formation stems from reduced intracellular survival or cell-cell spread. Frr overexpression could inhibit the deployment of the Bsa T3SS, implicating defects in endosome escape. Alternatively, Frr expression could result in dysregulation of T6SS-1 or motility functions, leading to reduced intracellular survival or spread. Current and future analysis will focus on identifying the specific steps in the intracellular life cycle and plaque formation that are affected by mutation or overexpression of Frr/Fsk, and identifying global alterations in gene expression resulting from manipulation of the Frr/Fsk system using RNASeq and quantitative RT-PCR.

Perspectives on future research

Alternate host models The picture is emerging that many of the presumed *B. pseudomallei* virulence determinants do not contribute to its interaction with mammalian cells. After all, *B. pseudomallei* is an environmental organism and an "accidental pathogen" of animals and humans. The significant diversity of *B. pseudomallei* has evolved from the horizontal acquisition of DNA during its interactions with the multitude of other organisms in the rhizosphere. Most, if not all the virulence mechanisms of *B. pseudomallei* have thus evolved to facilitate the acquisition of nutrients, survival and self defense in the environment. The use of alternate host models may be helpful in identification of factors that facilitate environmental persistence and opportunistic interactions of *B. pseudomallei* in the rhizosphere. Ironically, these "alternate" models are almost certainly the most relevant ones for investigating *Burkholderia* pathogenic mechanisms, as I described in Chapter 1. Such studies may well yield new information on virulence pathways that operate in mammalian infections.

B. pseudomallei and B. thailandensis are capable of infecting both *Dictyostelium* and *Acanthamoeba* species, and both protists are easily cultivated in the laboratory (Inglis *et al.*, 2000; Inglis *et al.*, 2003) (French, C.T., unpublished observations). Utilized in combination with a *B. pseudomallei* transposon mutagenesis library, protist models could serve as a high-throughput platform to screen for factors facilitating *B. pseudomallei* resistance to environmental predation. Protist host models are also suitable for investigating possible environmental roles for known virulence factors that facilitate mammalian infections. *Dictyostelium* strains containing mutations in vesicle trafficking and cell signaling pathways are available (Dictybase.org), and could be utilized to analyze the roles of T3SS, T6SS and motility functions in the lifecycle inside amoeba, as has been modeled for intracellular *Mycobacterium* and *Dictyostelium* (Hagedorn *et*

al., 2009). *B. pseudomallei* would be expected to interact with nematodes in the soil, making *Caenorhabditis elegans* potentially useful for host-pathogen interaction studies (Gan *et al.*, 2002; Lee *et al.*, 2011). *C. elegans* has been widely used to characterize virulence pathways in other pathogenic bacteria (Darby, 2005; Dorer and Isberg, 2006).

Plant models may also be applicable to the study of *Burkholderia* host interactions. Recently, *B. pseudomallei* was found to colonize the xylem of Tully Grass, Paspalum and Mission Grass at field sites near Darwin, Australia that were highly positive for *B. pseudomallei*. The bacterium was found around the stomata, at the surface of these openings as well as within the stomatal guard cells. Curiously, *B. pseudomallei* was observed to cause a dose-dependent pathogenic effect on Mission Grass, but not on wild rice (*Oryza rufipogon*) (Kaestli *et al.*, 2011). A model system using these species would be useful for testing the prediction that the *B. pseudomallei* T6SSs and the phytopathogen-like T3SS-1 and/or T3SS-2 play roles in mediating its interactions with plants.

Treatments and vaccines

Even though the ecology and environmental interactions of *B. pseudomallei* provide an interesting context in which to study the evolution of virulence mechanisms, there is little doubt that the organism is still a significant cause of fatal infections in highly endemic areas. Melioidosis is the third most common cause of death from infectious disease in northeast Thailand. It is concerning that the incidence of melioidosis appears to be increasing and is expected to soon surpass tuberculosis, placing second only behind HIV/AIDS (Limmathurotsakul *et al.*, 2010). The cause for this increase is not well understood, but it could be due in part to an aging population that is more susceptible to infection. Efforts have increased

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to promote greater awareness of the risks in endemic areas. There is also greater interest in the development and implementation of improved diagnosis and treatment strategies for melioidosis. Currently, the standard panel of broad-spectrum antibiotics; β-lactams, fluoroquinolones, aminoglycosides and macrolides, that have been effective against bacterial infections for years, are also useful for treating infections caused by many "priority pathogens", such as anthrax (*Bacillus anthracis*), tularemia (*Francisella tularensis*) and plague (*Yersinia pestis*) (http://www.cdc.gov). Organisms such as *B. pseudomallei* and *Mycobacterium tuberculosis*, however, present a special challenge for the development of effective therapeutic countermeasures; only a handful of antibiotics are effective due to extensive antibiotic resistance and the difficulty of eradicating the organisms following infection. Owing to the additional tendency for clinical misdiagnosis, victims of *B. pseudomallei* infection often succumb to their illness after treatment with ineffective antibiotics. Proper antibiotic therapy can be successful, however the options for treating *B. pseudomallei* infections are limited and current treatment regimens are intensive and lengthy. The current treatment strategy is biphasic; an initial "intensive phase" with intravenous ceftazidime plus trimethoprim/sulfamethoxazole (TMP/SMX), or meropenem, for 2-4weeks, followed by an oral "eradication phase" of TMP/SMX, doxycycline or chloramphenicol for 12-20 weeks (Inglis *et al.*, 2006). The side effects from such treatment are significant. Moreover, ceftazidime resistance in *B. pseudomallei* is emerging in endemic areas (Behera *et al.*, 2012; Sarovich *et al.*, 2012). A new monosulfactam has recently been tested *in vitro* against a collection of clinical and environmental *B. pseudomallei* strains from Thailand, and possesses a median inhibitory concentration (MIC) of 0.01 μg/mL, which compares favorably to the MICs of 1.5 μg/mL for ceftazidime, 0.5 μg/mL for imipenem and 1 μg/mL for meropenem (Mima *et al.*, 2011). This is an example of a new

antibiotic that could potentially replace or supplement current treatments in the near future. Such antimicrobials are sorely needed for the management of melioidosis, but unfortunately there are few in current development.

Compared to melioidosis, which has seen an upswing in research interest only recently, the etiology of *Bordetella pertussis* is much better understood, and pertussis illness has been acknowledged as a significant health concern for centuries (Cherry, 1996). Current trends with regard to pertussis are more aimed at mitigation by "herd immunity" through improved vaccine formulations and strategies (Coudeville *et al.*, 2009). Adopting a similar approach in endemic areas for the prevention of melioidosis, however, is considerably less straightforward, and is attributed at least in part to the relatively weak immunogenicity of *B. pseudomallei*. It is currently unclear whether traditional vaccine formulation strategies will even be efficacious at providing adequate protection against *B. pseudomallei* in humans. A range of candidate vaccines that have demonstrated partial protection in mice suggests at least the feasibility of a human vaccine (Peacock *et al.*, 2012). All of the *B. pseudomallei* vaccine candidates have been evaluated in mouse models, but strains, doses and routes of challenge have been variable, and sterilizing immunity has rarely been observed. For biodefense purposes, protection against inhalation is believed to be most valuable. This might be difficult to achieve since protection is greatest following intraperitoneal inoculation rather than intranasal or inhalational. Subunit vaccines from bacterial polysaccharide and proteins have achieved limited success (Allwood *et al.*, 2008), although immunization with *B. pseudomallei* outer membrane vesicles provided protection against an aerosol challenge (Nieves *et al.*, 2011). The most promising vaccine candidates consist of either live-attenuated *B. pseudomallei* or heat-killed *B. pseudomallei* preparations (Stevens *et al.*, 2004; Peacock *et al.*, 2012). Fears over reversion to a virulent

phenotype or establishment of latent infection makes a live-attenuated vaccine less desirable. Additional research is needed to determine whether the best vaccine candidates protect against multiple routes of infection, and whether they are effective in immunocompromised individuals without undue side effects.

Conclusion

The field of bacterial pathogenesis has made significant leaps in recent decades through the advent of DNA cloning and other technological improvements. Advances in the detection and identification of pathogenic species have translated to improved diagnostics and more rapid treatment of bacterial infections. New and continuously refined experimental methods have facilitated the development of reproducible *in vitro* and *in vivo* disease models and safe, effective recombinant vaccines. Basic research, however, continues to lead the way in characterizing the evolutionary origins and functions of bacterial virulence mechanisms. Studies such as the ones described in this dissertation are steadily yielding clues that will have important implications for understanding the infection process and treating infections caused by *Bordetella*, *Burkholderia* and other pathogens. For example, studies on the *Bordetella* T3SS in our laboratory recently found that the T3SS component Bsp22 was immunoprotective in mice. Bsp22 is thus an excellent candidate for incorporation into the next generation of *Bordetella* subunit vaccines (Fennelly *et al.*, 2008; Medhekar *et al.*, 2009). Progress also continues on understanding the roles of the *Bordetella* T3SS and the BteA effector protein in pathogenesis and modulation of immunity. It is similarly crucial from a public health and national security perspective to further our knowledge of pathogenic mechanisms in *Burkholderia*. Investigating *Burkholderia* virulence factors in the context of how they promote infection and survival within the host and in the environment will reveal new information on potential targets for vaccines and small molecule

inhibitors. Such studies are bound to enhance our understanding of the natural habitat and the

evolution of virulence determinants in bacterial pathogens and how these relate to human

infection.

Figure legends

Fig. 1. Kinetics of *Burkholderia* endosome escape. A. Intracellular replication timecourse of *B. pseudomallei* Bp340 wild-type (WT) and T3SS$_{Bsa}$ mutant ($\Delta sctN/\Delta bsaS$) following infection of mammalian cells. WT bacteria escape from endosomes and replicate explosively at around 5 hr., while T3SS$_{Bsa}$ mutants are killed as a result of entrapment. A hypothetical growth curve for BopA is shown (green) if it is needed for survival in the cytosol but not if it is critical for escape from the endosome. B. Schematic of the phagosome protection assay (Checroun *et al.*, 2006). In this example, cells are infected with red-fluorescent protein-labeled bacteria, which escape from endosomes. Cells are permeabilized at different timepoints by a brief treatment with digitonin, which is selective for the outer plasma membrane. Cells are then incubated with green-fluorescent *Burkholderia* antiserum. Bacteria that have escaped into the cytoplasm will exhibit dual (red and green) fluorescence, while those still enclosed within endosomes retain only the red fluorescence. Control samples are permeabilized with saponin, which destroys plasma and endosomal membranes.

Fig. 2. The type VI secretion system and VgrG proteins of *B. pseudomallei*. A. Schematic of the type VI secretion system components. B. VgrG proteins in *B. pseudomallei*. Color legend: light blue, conserved N-terminus of VgrG proteins. Black – possible membrane-spanning regions of VgrG1. Green, red, dark blue, purple and orange; predicted C-terminal domains of "evolved" VgrGs, which are likely to confer independent functions for the interactions of *Burkholderia* with mammalian cells, as is the case with VgrG1. (Cascales, 2008), used with permission.

Fig. 3. Analysis of type VI secretion system 1 (T6SS-1) for fusogenic activity in mammalian cells. A. proposed model of T6SS-1 VgrG1-mediated cell fusion. Following bacterial contact

with the inner face of the cell membrane, T6SS-1 activity is triggered resulting in translocation of VgrG1 (red) through the cell surface and possibly into neighboring cells. By an unknown process, VgrG1 is predicted to catalyze membrane fusion leading to MNGC formation. B. Summary of *B. pseudomallei* VgrG1 truncation analysis. Bp340 ΔvgrG1 was complemented in trans with the indicated VgrG1 constructs (left). The results of the complementation analysis were assessed by MNGC forming ability (right). VgrG1 from Australian *B. pseudomallei* MSHR668 and *B. thailandensis* E264 were included (bottom). C. MNGC assay using mixed RFP- and EGFP-labeled HEK293 cells. Infections were carried out with the indicated strains and analyzed for MNGC formation 12 hr. later.

Fig. 4. The regulatory hierarchy of the Bsa T3SS and T6SS-1 converge at BsaN/BicA. The master upstream regulator BspR activates BprP, which in turn required for activation of BsaN. The interaction of BsaN and the regulatory chaperone BicA is required for activation of T3SS effector loci and other regulators such as *virAG*, which controls expression of the *bim* actin motility locus and T6SS-1(Sun and Gan, 2010). Colors: blue, T3SS apparatus genes; red, regulatory loci; green, putative effector loci; purple, translocon components; grey, unknown function.
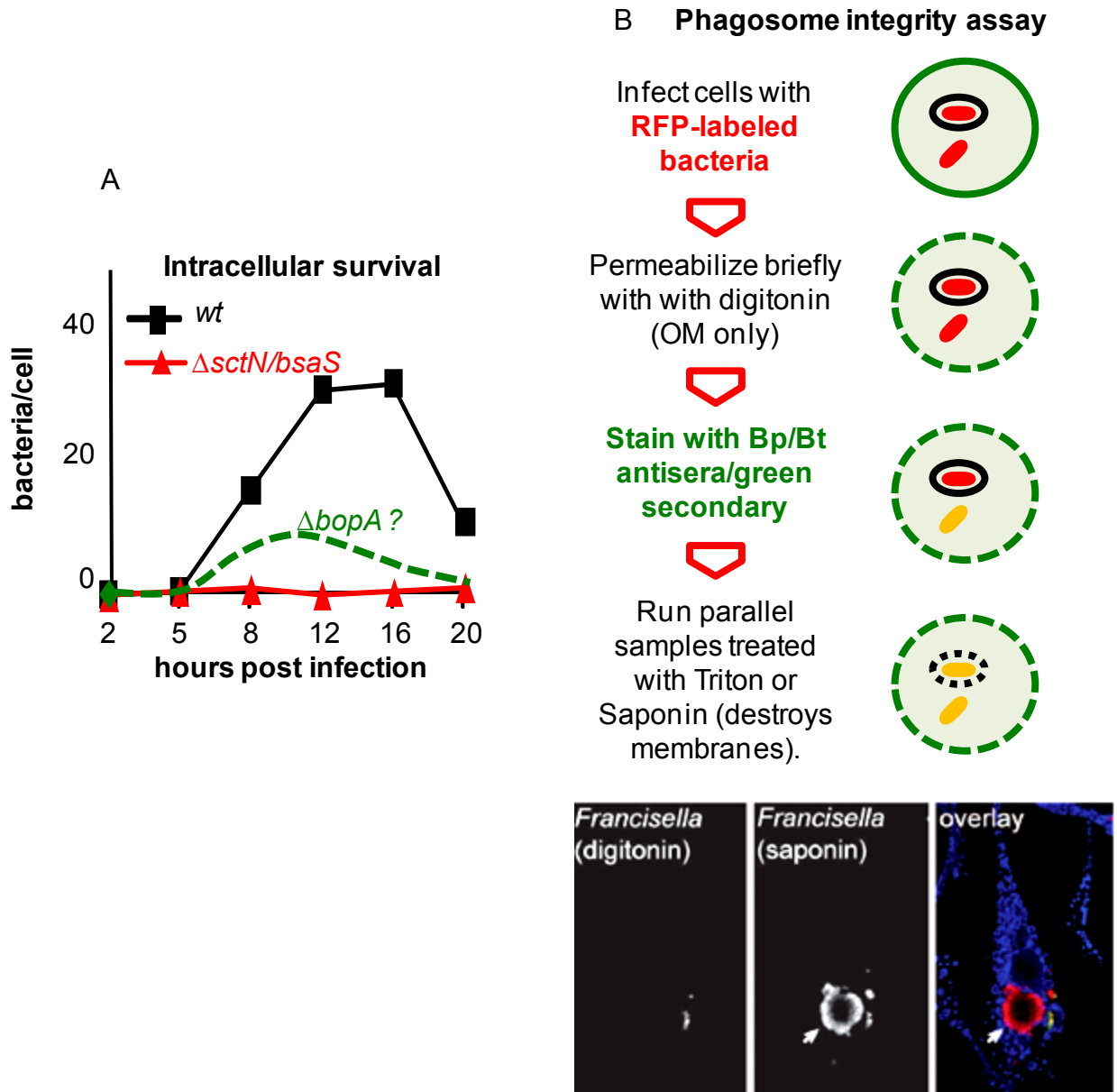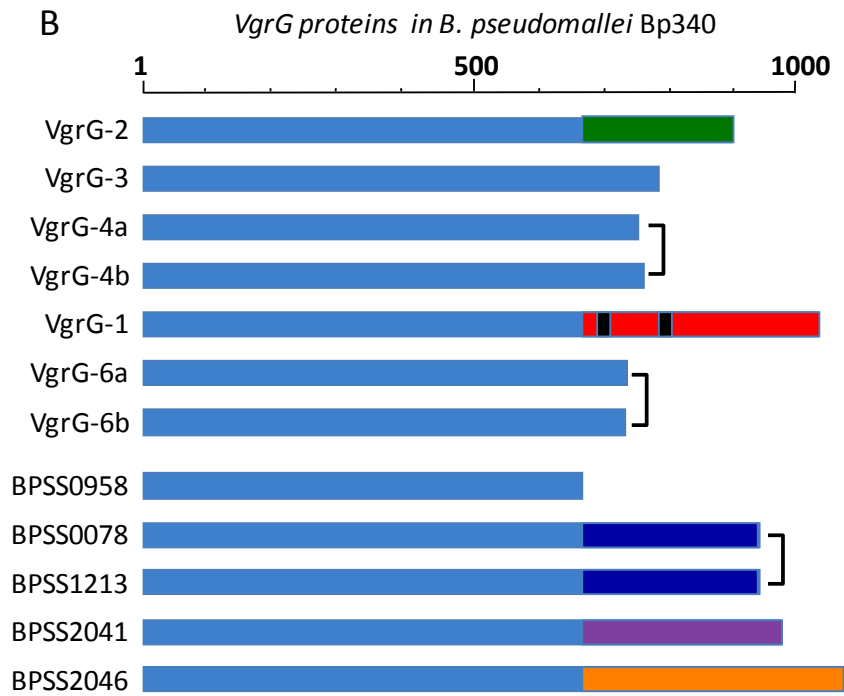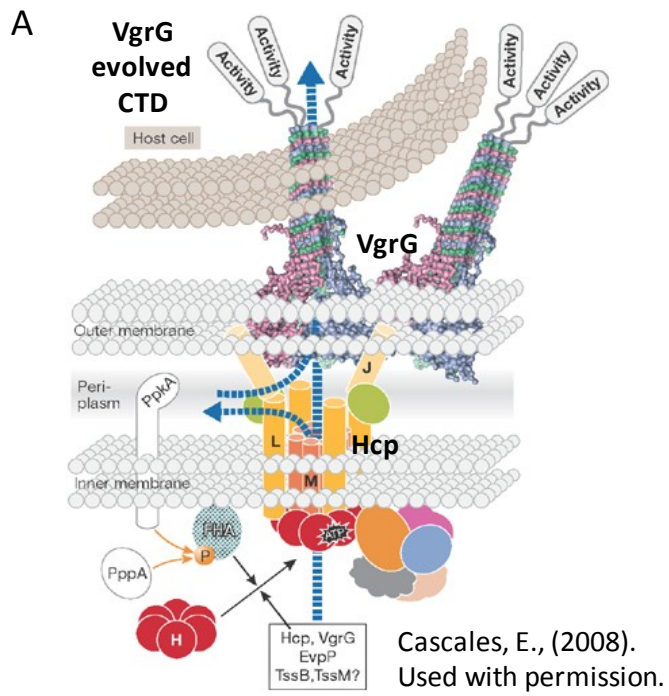
Fig. 1

A

Intracellular survival

B   **Phagosome integrity assay**

Infect cells with
**RFP-labeled
bacteria**

Permeabilize briefly
with with digitonin
(OM only)

**Stain with Bp/Bt
antisera/green
secondary**

Run parallel
samples treated
with Triton or
Saponin (destroys
membranes).

Francisella
(digitonin)

Francisella
(saponin)

overlay

Fig. 2

A

VgrG evolved CTD

VgrG

Cascales, E., (2008). Used with permission.

B

VgrG proteins in B. pseudomallei Bp340
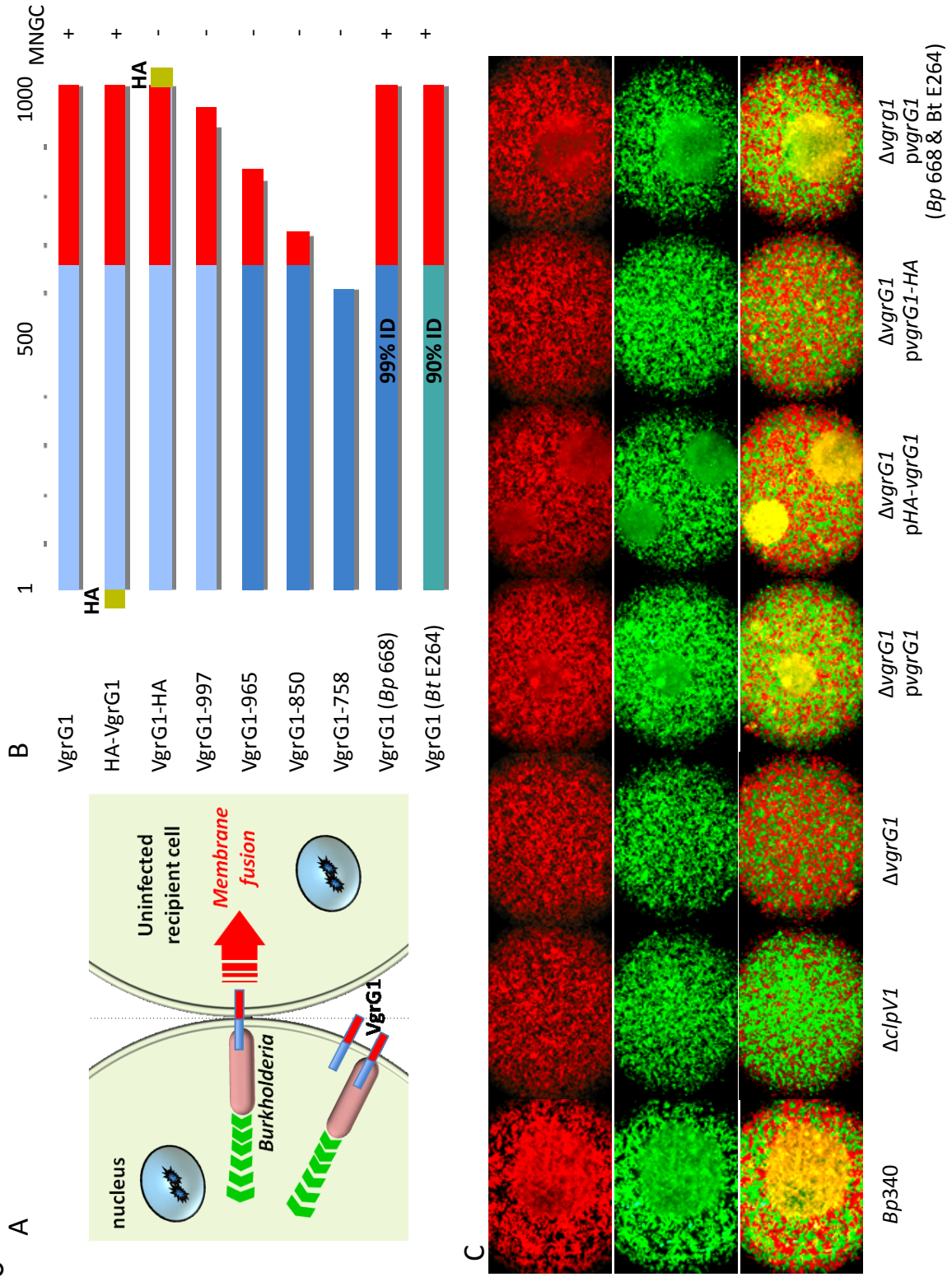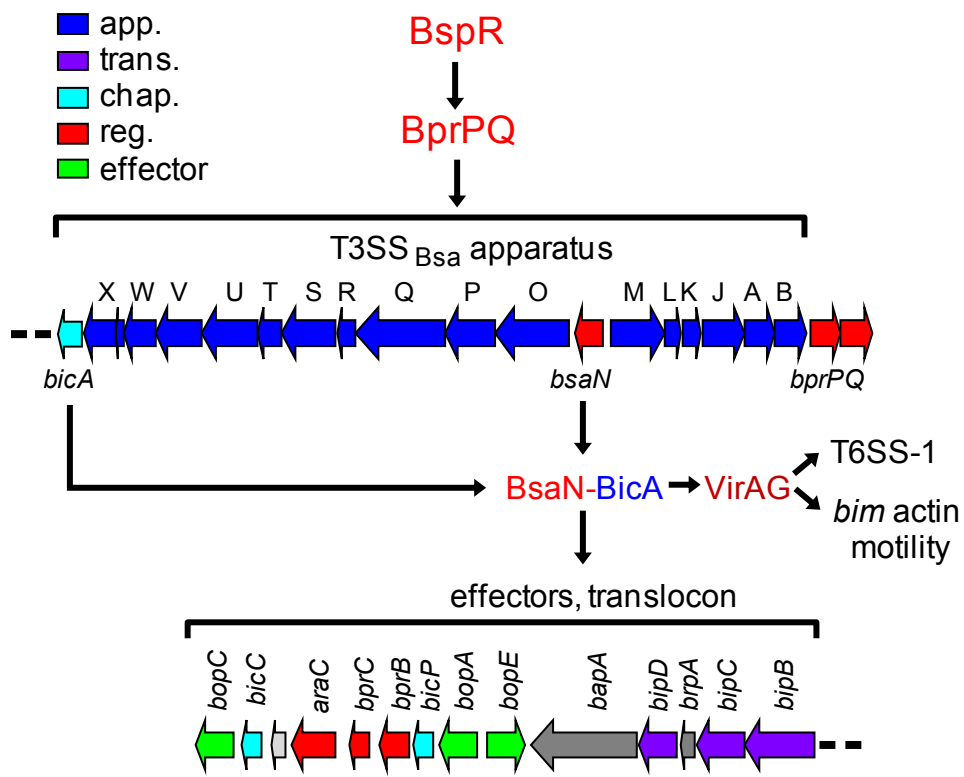
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Fig. 3

A

B

C

Fig. 4

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