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Melioidosis: Molecular Aspects of Pathogenesis

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SUMMARY

Burkholderia pseudomallei is a Gram-negative bacterium that causes melioidosis, a multifaceted disease that is highly endemic in Southeast Asia and northern Australia. This facultative intracellular pathogen possesses a large genome that encodes a wide array of virulence factors that promote survival in vivo by manipulating host cell processes and disarming elements of the host immune system. Antigens and systems that play key roles in *B. pseudomallei* virulence include capsular polysaccharide, lipopolysaccharide, adhesins, specialized secretion systems, actin-based motility and various secreted factors. This review provides an overview of the current and steadily expanding knowledge regarding the molecular mechanisms used by this organism to survive within a host and their contribution to the pathogenesis of melioidosis.

Keywords

Burkholderia pseudomallei; melioidosis; pathogenesis; virulence factors; facultative intracellular pathogen; capsule; lipopolysaccharide; secretion systems

INTRODUCTION

Burkholderia pseudomallei, the etiologic agent of melioidosis, is an environmental saprophyte found in moist soils and surface waters in tropical and subtropical regions. This Gram-negative bacterium can be acquired by humans via inhalation, inoculation, or ingestion, with the highest number of infections occurring during the rainy season [1]. Melioidosis is highly endemic in Southeast Asia and northern Australia although scattered cases have been described globally [2]. Based on increasing numbers of case reports from various areas of the world including South America, Africa, and India, melioidosis is considered to be an emerging infectious disease. Human cases that have occurred in temperate regions have, for the most part, been attributed to recent travel to endemic areas.

The clinical presentations of melioidosis are varied with manifestations ranging from localized abscess formation to acute pneumonia and overwhelming septicemia [1,3]. Risk factors, most commonly diabetes mellitus, alcoholism, and chronic pulmonary disease, are

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present in up to 80% of patients. Acute cases typically present within 1-21 days after infection while chronic cases are characterized by less severe symptoms that persist for weeks or months [1,4]. *B. pseudomallei* is resistant to numerous antibiotics so treatment is prolonged, requiring antibiotic regimens of up to six months for clearance of infections [5,6]. In addition, recrudescence is common, occurring in up to ~9% of patients [4]. Mortality can be high, ranging from 14% in northern Australia to 43% in northeast Thailand, and case fatality rates are as high as 61.5% in Cambodia [4,7,8]. Diagnosis of melioidosis can be challenging and no vaccine is currently available [1]. Further complicating matters, *B. pseudomallei* is categorized as a U.S. Centers for Disease Control and Prevention Tier 1 select agent, restricting work to select agent compliant biosafety level 3 containment facilities [9].

The genome of *B. pseudomallei* is large at 7.2 Mb and encodes for numerous virulence factors [10]. Included amongst these are surface polysaccharides such as capsular polysaccharide (CPS) and lipopolysaccharide (LPS) which are involved in inhibiting opsonophagocytosis and conferring resistance to killing by host complement [11], and specialized secretion systems, in particular the cluster 3 type III secretion system (T3SS-3) and cluster 1 type VI secretion system (T6SS-1), that facilitate optimal survival and growth of the organism within host cells [12]. Additional virulence factors including adhesins, flagella, various secreted proteins (e.g. phospholipases and proteases) and secondary metabolites have also been described. Many of the virulence associated systems expressed by *B. pseudomallei* appear to be regulated by two component systems (TCSs) or quorum sensing (QS) suggesting that environmental cues and bacterial cross-talk may mediate activation of some of these factors.

In this review, we discuss the molecular mechanisms used by *B. pseudomallei* to evade constitutive immune defenses and to then survive and replicate inside phagocytic and nonphagocytic cells. As a facultative intracellular pathogen, the life cycle of *B. pseudomallei* involves adherence and entry into host cells, escape from the phagosome, replication within the cytosol and spread to neighboring cells (**Figure 1**). Based on the current literature, it appears that various adhesins mediate attachment to non-phagocytic cells and that cell contact triggers T3SS-3 expression. Once inside a eukaryotic cell, the iron limiting environment of the phagosome appears to lead to activation of the VirAG TCS and T6SS-1 transcription. T3SS-3 effectors enable escape from phagosomes and once free in the cytosol, *B. pseudomallei* can polymerize host actin and propel itself throughout the cell. Upon contact with host cell membranes T6SS-1 mediates membrane fusion with adjacent cells, resulting in multinucleated giant cell (MNGC) formation. Lysis of MNGCs results in release of the intracellular bacteria and can lead to plaque formation *in vitro*.

SURFACE POLYSACCHARIDES

B. pseudomallei strains express a number of cell surface exposed polysaccharides that play important roles in the pathogenesis of melioidosis. The organism is capable of expressing at least five different CPS antigens as well as the O-polysaccharide (OPS) portion of LPS [13-15]. Four CPS biosynthetic gene clusters (encoding for CPS I, II, II and IV) and one OPS biosynthetic gene cluster have been identified in the *B. pseudomallei* genome [10,16].

Of the CPS antigens described to date, only CPS I (encoded by the *wcb* gene cluster, BPSL2786-BPSL2810; commonly referred to as CPS) has been shown to play a major role in virulence. This antigen is an unbranched homopolymer consisting of monosaccharide repeats having the structure →3)-2-O-acetyl-6-deoxy-β-D-*manno*-heptopyranose-(1→ and is expressed by both *B. pseudomallei* and *Burkholderia mallei*, the causative agent of glanders, and some strains of *Burkholderia thailandensis*, a closely related non-pathogenic environmental saprophyte [15,17]. Expression of CPS I is up-regulated in response to normal human serum (NHS) and this antigen has been shown to reduce phagocytosis by host cells by preventing complement factor C3b deposition on the surface of the bacterium [11,18]. *B. pseudomallei* CPS I mutants have been shown to exhibit significantly attenuated virulence, with a >10,000-fold increase in the 50% lethal dose (LD_{50}), in comparison to wild type strains in Syrian hamsters and mice [18,19]. Recent studies have demonstrated that CPS I is both a protective antigen and promising vaccine candidate [20,21]. Investigations focused on examining the function of the individual ORFs within the *wcb* operon may provide a better understanding of the mechanism of CPS I biosynthesis and export in *B. pseudomallei* and further insights into the role of this important antigen in pathogenesis [22].

CPS III (BPSS1825-BPSS1835) is composed of galactose, glucose, mannose, rhamnose, and xylose residues and has been shown to be down-regulated in the presence of NHS and in animal tissues, but up-regulated in water suggesting a role in environmental survival [15]. A CPS III mutant retained wild type virulence in hamsters, but was attenuated in BALB/c mice, an observation that could be reflective of differences in animal models [15,23]. Little work has examined the roles of CPS II (BPSS0417-BPSS0429) and CPS IV (BPSL2769-BPSL2785) in the pathogenesis of melioidosis. Growth of *B. pseudomallei* in numerous different laboratory conditions did not induce CPS IV expression, however, CPS II was up-regulated in response to divalent cation supplementation of TSB-DC growth media [24]. Virulence testing of CPS II mutants in BALB/c mice demonstrated an increased mean time to death compared to the wild type strain [23]. In addition to these CPS antigens, a 1,3-linked mannan CPS as well as a tetrameric exopolysaccharide composed of three repeating galactose and a Kdo residue are also produced by *B. pseudomallei*, but their genetic loci and functions are unknown [13,25,26].

Three *B. pseudomallei* OPS types have been described: the typical and predominant type A as well as the atypical and serologically related types B and B2 [27]. Rough strains lacking OPS have also been reported. Type A OPS is best described as an unbranched polymer consisting of disaccharide repeats having the structure →3)-β-D-glucopyranose-(1→3)-6-deoxy-α-L-talopyranose-(1→ in which the 6-deoxy-α-L-talopyranose residues are variably substituted with *O*-acetyl and *O*-methyl modifications [14,28]. The presence of this moiety confers serum resistance and enables *B. pseudomallei* to grow in 10-30% NHS while rough type strains are rapidly killed [16]. Consistent with this function, mutant strains lacking type A OPS exhibited LD_{50}s ~10-fold higher in hamsters and guinea pigs and ~100-fold higher in rats relative to the wild-type strains [16]. Passive protection studies in animals using anti-OPS monoclonal antibodies indicate that this carbohydrate moiety is a protective antigen and potential vaccine candidate [20]. The structures and roles of the type B and B2 OPS are unknown but would be predicted to function similarly to the type A OPS as strains

expressing type B are able to grow in 30% NHS while type B2 strains are susceptible to killing [27]. Collectively, these studies indicate that surface polysaccharides play important roles in resisting host innate immune killing mechanisms and likely help to facilitate survival of *B. pseudomallei* during the early stages of infection. In addition, both CPS and LPS represent attractive targets for the development of future countermeasures.

ADHERENCE TO HOST CELLS

Bacteria commonly use type IV pili (TFP) or fimbriae to adhere to eukaryotic cells. Eight TFP gene clusters have been identified in the *B. pseudomallei* genome and three of these (TFP1, 4 and 7) have been preliminarily characterized. Deletion of TFP1 (*pilA*, BPSL0782) reduced adherence to human epithelial cell lines RPMI-2650, A549, and BEAS2-B, with adherence nearly abolished to the latter two [29]. A subsequent study using ME-180 human cervical cancer cells found that PilA mediated formation of microcolonies of *B. pseudomallei* which in turn adhered to the eukaryotic cells [30]. Two genes in the TFP4 pili cluster, *pilW* and *pilV* (BPSL2754-2755), were required for full virulence of *B. pseudomallei* in a BALB/c mouse model, however, their role in mediating adherence to host cells was not determined [31]. The TFP7 protein PilV (BPSS1593) was found to be highly immunogenic in mice suggesting it is surface-exposed during infection [32]. Interestingly, however, PilV failed to induce protective immunity in mice. A recent study demonstrated that the TFP7 gene cluster (BPSS1593-BPSS1602) is orthologous to the R64 plasmid thin pilus and to a *Salmonella enterica* TFP, which aids in adhesion to intestinal epithelial cells and to other bacteria via biofilm formation [33,34]. It is likely that TFP7 may act in a similar manner although this remains to be experimentally determined.

Autotransporters are a large family of Type V secreted proteins that are bound in the outer membrane and contain an effector domain, and often acts as adhesins. These proteins are subdivided into classical or trimeric autotransporter adhesins (TAAs). The *B. pseudomallei* genome contains one confirmed classical autotransporter (BcaA), one predicted classical autotransporter (BPSL2237) and nine predicted TAAs, six of which display homology to the adhesin YadA of *Yersinia enterocolitica* [35-39]. Two important TAA adhesins expressed by *B. pseudomallei* are BoaA and BoaB [35]. When cloned and expressed in *E. coli*, both BoaA and BoaB increased adhesion to A549, HEp2, and NHBE epithelial cells. Accordingly, *B. pseudomallei boaA* and *boaB* mutants exhibited decreased adherence to these cell lines and no difference was found in the rate of phagocytosis. A double *boaA/boaB* mutant adhered to host cells at levels comparable to those for each single mutant, and was also phagocytosed equivalently. Curiously, this double mutant had a 60% decrease in survival within J774.1 murine macrophages [35]. Similar experiments conducted with BpaC demonstrated that expression of this TAA in *E. coli* increased adherence to A549, HEp2, and NHBE cells 5-7 fold [40]. A *B. pseudomallei bpaC* mutant had no change in adherence to A549 or HEp2 cells, but exhibited a 61% reduction in adherence to NHBE cells. There was no change in invasion of epithelial cells, phagocytosis by macrophages, or intracellular replication suggesting that BpaC may be specific for attachment to ciliated mucosal epithelial cells such as NHBE [40]. These findings are in contrast to those of Campos et al who found that a *bpaC* mutant displayed reduced adherence (~10-fold) to A549 cells [38]. It is possible that these differences could be attributable to culture methods, length of infection

time, or the nature of the *bpaC* mutation since both studies used derivatives of *B. pseudomallei* 1026b [40]. Another TAA, BbfA (BPSS1439 or BpaF), was shown to play a role in microcolony formation, biofilm production, and adherence when grown on glass coverslips. While no change in motility or intracellular replication within A549 and J774.2 cells was observed, the BbfA mutant was attenuated for virulence in BALB/c mice [36]. Taken together, these data suggest that adherence of *B. pseudomallei* to host cells is multi-factorial and that further investigations will be necessary to more fully understand the specific role(s) of each of these proteins during interactions with host cells.

TYPE III SECRETION SYSTEM

Type III secretion systems (T3SSs) are syringe-like mechanisms used by Gram-negative pathogens to translocate effector proteins directly into target cells and are often associated with virulence [41]. The *B. pseudomallei* genome encodes three T3SS gene clusters, two of which are homologous to systems found in the plant pathogen *Ralstonia solanacearum*, and a third (T3SS-3, *bsa* locus, BPSS1520-BPSS1554) which is homologous to the Inv/Mxi-Spa T3SS of *Salmonella typhimurium* (SPI-1 T3SS) and *Shigella flexneri* [42]. Several studies have shown that T3SS-3 expression is triggered following contact with host cells and has been associated with invasion of non-phagocytic cells, rapid escape from endocytic vacuoles, and virulence [43-46].

T3SS-3 structural proteins BsaQ (BPSS1543) and BsaZ (BPSS1534) are predicted to be components of the integral membrane export apparatus necessary for secretion of effectors. Disruption of *bsaQ* prevented secretion of BopE and BipD, resulted in reduced invasion and plaque formation in A549 cells as well as decreased phagosomal escape and MNGC formation in J774A.1 macrophages [47]. In contrast, no differences were observed in invasion, replication, or vacuolar escape in human HEK293T cells [48]. Stevens et al showed that a *bsaZ* mutant was unable to escape phagosomes and thus could not replicate intracellularly or form actin filaments in J774.2 macrophages [43]. This mutant was later found to be capable of delayed vacuolar escape, limited replication and MNGC formation in RAW 264.7 macrophages [49]. These data are consistent with experiments demonstrating that *bsaZ* mutants exhibit attenuated virulence in mice and hamsters [45,46].

To date, at least five putative T3SS-3 effector proteins have been described in *B. pseudomallei* including BopE (BPSS1525), BopA (BPSS1524), BopB (BPSS1514), BopC (BPSS1516) and CHBP (BPSS1385). BopE, the first effector to be characterized, is a guanine nucleotide exchange factor that targets Cdc42 and Rac1 and induces actin rearrangements that aid in bacterial invasion of non-phagocytic cells. Ectopic expression of BopE in HeLa cells resulted in actin polymerization and ruffling of host cell membranes [44]. A *B. pseudomallei bopE* mutant exhibited reduced invasion of HeLa cells compared to the wild type strain, however, no change was observed in its ability to escape from phagosomes, replicate, or form actin tails in J774.2 cells or murine bone-derived macrophages, and full virulence was retained in mice and hamsters [43,44,46,50]. These findings suggest that although BopE may promote invasion of non-phagocytic cells, this protein plays only a minor role in virulence.

Investigations focused on BopA have revealed that this effector protein is involved in avoidance of host autophagy since a *B. pseudomallei bopA* mutant showed increased co-localization with autophagosomal microtubule-associated protein light chain 3 (LC3) compared to the wild type strain following infection of RAW 264.7 macrophages [51,52]. The *bopA* mutant also exhibited delayed phagosomal escape and reduced intracellular survival, but was fully virulent in hamsters [46,51,52]. Deletion of *bopB,* a predicted phosphatase, had no effect on intracellular growth or actin tail formation in J774.2 macrophages and did not attenuate virulence in BALB/c mice [45]. A *bopC* mutant displayed decreased invasion of A549 cells, and reduced phagosomal escape and intracellular survival in J774A.1 macrophages, however, no change was noted in actin tail or MNGC formation [53].

The CHBP effector protein is encoded for outside of the T3SS-3 gene cluster and is a cycle inhibiting factor (Cif) homolog that is predicted to be a cysteine protease. CHBP secretion was shown to occur following infection of human U937 macrophages with *B. pseudomallei,* require a functional T3SS-3, and was postulated to be host cell contact dependent [54]. A *chbP* mutant survived at wild type levels in U937 cells, retained the ability to form actin tails, and induced MNGC formation in J774A.1 macrophages [54]. Interestingly, following infection of HeLa cells the plaque forming efficiency of the *chbP* mutant was significantly decreased, cytotoxicity was reduced, and smaller plaques were consistently observed in comparison to the wild type [54]. When ectopically expressed in HEK293T and HeLa cells, CHBP induced actin stress fiber formation and arrested the cell cycle at the G2/M boundary by deamidation of Gln^{40} in ubiquitin and ubiquitin-like proteins [55-57].

The T3SS-3 translocator proteins BipB, BipC and BipD facilitate the delivery of effectors proteins into host cells. A *B. pseudomallei bipD* mutant exhibited significantly reduced invasion of HeLa cells, inefficient endosomal escape, and decreased intracellular survival and actin tail formation in J774.2 and RAW 264.7 macrophages [43-45]. A *bipB* mutant was unable to invade A549 cells, did not spread between HeLa cells, and failed induce MNGC formation or apoptosis in J774A.1 macrophages. Consistent with these phenotypes, both *bipD* and *bipB* mutants were highly attenuated for virulence in an intranasal BALB/c mouse model [45,58]. Collectively, these data suggest that while T3SS-3 promotes invasion of non-phagocytic cells, it appears that a key a function of this system is to facilitate rapid escape of *B. pseudomallei* from endocytic vacuoles allowing entry into host cell cytosol.

TYPE VI SECRETION SYSTEMS

The T6SS is a relatively recently described secretion system resembling an inverted bacteriophage-like structure that functions to deliver effector proteins directly into eukaryotic and prokaryotic target cells [59]. T6SS gene clusters typically encode 15-20 proteins involved in the assembly, structure and function of the system. Two conserved components of the T6SS apparatus, hemolysin co-regulated protein (Hcp) and valine glycine repeat protein (VgrG), are predicted to form a spear-like structure that is propelled into target cells to facilitate effector delivery [60]. In many instances VgrG proteins also function as effector molecules.

The *B. pseudomallei* genome harbors six type VI secretion system (T6SS) gene clusters. At present, only T6SS-1 (BPSS1494-BPSS1511) has been shown to play a role in virulence in animals [12,61,62]. While T6SS-1 is not typically expressed during the routine culture of *B. pseudomallei* in rich media, it is expressed in minimal media and following uptake by host cells, and is required for virulence in both mice and hamsters [61-65]. Several studies have shown that disruption of this system significantly influences the intracellular behavior of the organism. Notably, *hcp1* (BPSS1498) deletion mutants exhibited attenuated intracellular growth in RAW 264.7 cells, defects in actin-based motility, an inability to stimulate MNGC formation and a corresponding decrease in macrophage cytotoxicity [61]. Similar phenotypes were observed with *tssF* (BPSS1504) and *tssK* (BPSS1509) mutants which displayed impaired MNGC formation in macrophages, reduced plaque formation in Ptk2 cells, and attenuated virulence in BALB/c mice [65,66].

Recent studies focused on *B. pseudomallei* VgrG1 (BPSS1503, also known as VgrG5) indicate that the C-terminal extended region of this protein is required for MNGC formation and virulence in mice [61,67]. Additional studies with *B. thailandensis* a VgrG1-GFP fusion protein have revealed that T6SS-1 localizes to the bacterial poles, either with or opposite to actin tail formation [68]. Consistent with these observations, Toesca et al showed that *B. pseudomallei vgrG1* mutants escaped from endosomes in HEK 293 cells and could polymerize actin [67]. Complementation of a *B. pseudomallei vgrG1* mutant with orthologous *B. mallei*, *B. thailandensis*, or *Burkholderia oklahomensis vgrG1* genes restored the ability to induce MNGC formation [67]. Based on this evidence, it has been postulated that the C-terminal domain (CTD) of VgrG1 facilitates host cell fusion, however, the specific mechanism and factors involved remain to be determined. Identification of additional T6SS-1 associated effectors may provide insight into how T6SS-1 is modulating host cell processes to enable bacterial proliferation.

REGULATION OF T3SS-3 AND T6SS-1

In general, the expression of T3SSs and T6SSs is precisely regulated so that these systems are only expressed when appropriate. In keeping with this notion, a complex regulatory cascade involving multiple proteins governing the coordinated expression of T3SS-3 and T6SS-1 in *B. pseudomallei* has been identified and partially characterized [64,69]. A TetR family regulator designated BspR (BPSL1105) is encoded by neither gene cluster but is believed to act on the T3SS-3 regulator BprP (BPSS1553). BprP then activates transcription of T3SS-3 structural components and the regulator BsaN which, along with the chaperone BicA, activates the expression of secreted effectors such as BopE and the AraC family transcriptional regulator BprC [69]. BsaN/BicA also activate BprABD, predicted transcriptional regulators within the T3SS-3 gene cluster whose targets are unknown [42]. Both BprC and the VirAG two-component system positively regulate expression of T6SS-1 [61,64,69].

Activation of this signal cascade is dependent upon contact between the bacterium and eukaryotic cells [64]. T6SS-1 transcription occurs after the bacteria has entered the host cell and is dependent on VirAG, with BprC activating transcription of *tssA* (BPSS1496) and *tssB* (BPSS1497), encoding predicted structural components required for Hcp1 secretion [64]. It

has been hypothesized that VirA may recognize nutrient deprived conditions found in the phagosome, activating VirG and the transcription of T6SS-1 genes. This is supported by the finding that *virAG* is expressed during growth of *B. pseudomallei* in minimal media [63]. Furthermore, the observation that T6SS-1 expression is negatively regulated by iron and zinc suggests that divalent cation depletion may serve as a signal for expression of this system [63]. This observation is consistent with the finding that *B. mallei* T6SS-1 expression is activated within the iron-limiting environment of the phagosome [70].

OTHER SECRETED PROTEINS

B. pseudomallei secretes a multitude of proteins with numerous different activities. The importance of some of these secreted products has been examined in the context of host cells or in animal models of infection. One recently identified protein that has gained considerable attention is TssM (BPSS1512), a potent deubiquitinase that is secreted inside host cells. The *tssM* gene is physically linked to both the T3SS-3 and T6SS-1 gene clusters and is co-regulated with T6SS-1, but neither system is involved in TssM export [71]. It has recently been shown that secretion of TssM is instead dependent on a functional type II secretion system (T2SS) [72]. When secreted inside macrophages, TssM interferes with the toll-like receptor (TLR) mediated NF-κB signaling pathways associated with innate immune responses [71]. In *B. mallei*, which has an identical copy of *tssM*, expression was found to occur exclusively within macrophages; however, deletion of this gene had no effect on intracellular survival and replication [73]. Consistent with these findings, *B. pseudomallei tssM* mutants were hyper-inflammatory in mice and resulted in a decreased time to death indicating that this protein may manipulate host immune responses to the benefit of the bacteria [71].

Several proteases and phospholipases have been described which are secreted by the T2SS [72,74]. MprA (BPSS1993) is a serine metalloprotease that is responsible for most of the extracellular proteolytic activity of *B. pseudomallei* [72,75]. Three phospholipase C paralogs are present in the *B. pseudomallei* genome, *plcN1* (BPSL2403), *plcN2* (BPSL0338), and *plcN3* (BPSS0067). PlcN1 and PlcN2 are acidic proteins which contribute to plaque formation in HeLa cells [76]. A *plcN2* mutant also demonstrated reduced cytotoxicity in RAW 264.7 macrophages, however, no change was observed in MNGC formation or cell lysis [76]. PlcN3 is a basic protein and deletion of *plcN3* resulted in attenuation in a hamster model [77]. BPSL2198 is a phospholipase A2-like protein that when ectopically expressed in HeLa cells localized to the nuclear periphery and caused cell death [78]. Additionally, BPSL1549 or *Burkholderia* Lethal Factor 1 (BLF1), is a glutamine deamidase which destroys the RNA helicase ability of elongation factor 4A at the start of translation [79]. While the mechanism of its secretion is uncertain, BLF1 is highly cytotoxic toward host cells and lethal when administered to BALB/c mice.

SECONDARY METABOLITES

The *B. pseudomallei* genome contains a large number of biosynthetic gene clusters encoding polyketide- and nonribosomal peptide-based small molecules [80,81]. Many of these gene clusters are not expressed *in vitro* and their biological functions have not been identified.

Recently, Biggins *et al.* have demonstrated that several of these small molecules are critical for *Burkholderia* virulence [80,81]. They devised methods to artificially express these large gene clusters *in vitro*, structurally characterize the encoded natural products, and test the virulence of mutant derivatives in animal models of infection. The *B. pseudomallei* 1026b *mpnA-E* genes (BP1026B_II1742-1746) encode malleipeptin A and malleipeptin B, 12 amino acid lipopeptides with a novel peptide sequences and a rarely seen 13-membered terminal lactone [80]. The malleipeptins function as biosurfactants and are critical for virulence in BALB/c mice. The *B. pseudomallei* 1026b *syrA-I* genes (BP1026B_II1345-1353), encode glidobactin C and deoxyglidobactin C. Syrbactins are 20S proteasome inhibitors that have been found in plant pathogenic bacteria, but glidobactin C and deoxyglidobactin C are the first syrbactins described in a mammalian bacterial pathogen [80]. A mutant strain that could not synthesize the syrbactins was severely attenuated in BALB/c mice, suggesting that these molecules play an important role in *B. pseudomallei* virulence. Finally, the *malA-M* gene cluster (BP1026B_II0328-II0340) encodes a polyketide synthase-derived cytotoxic siderophore termed malleilactone [81]. *B. thailandensis* strains harboring mutations in the *mal* gene cluster are less virulent in the *Caenorhabditis elegans* nematode and the *Dictyostelium discoideum* co-culture models of infection, suggesting that malleilactone is a *Burkholderia* virulence determinant.

INTRACELLULAR MOTILITY

Once *B. pseudomallei* has escaped from phagosomes/endosomes and entered into the cytosol, it is able to utilize actin-based motility to move about the host cell. It is known that binding and polymerization of host actin by *B. pseudomallei* involves the polarly localized protein BimA, but the mechanism(s) that facilitate this process appear to be distinct from those described for other intracellular pathogens [82,83]. Mutagenesis of *bimA* resulted in abolishment of actin tail formation in J774.2 cells and plaque formation in HEK293 and A549 cells, but had no effect on vacuolar escape [84-86]. When BimA was expressed in HeLa cells, monolayer detachment was observed and cellular shape was spherically distorted. Actin was found to polymerize with BimA and corresponded to a decrease in actin stress fiber formation [86].

Additional gene products have been identified which may contribute to actin-based motility in *B. pseudomallei* including BPSL1057F1, BPSL1528, and BPSS1727 [31]. Revised genome annotation of *B. pseudomallei* K96243 identified a novel gene, *BPSL1057F1*, which contains a putative signal sequence for extracellular secretion. A marked increase in actin stress fiber formation, very similar in effect to BopE, was observed when *BPSL1057F1* was expressed in HeLa cells [31]. A mutant of the hypothetical protein BPSL1528 had severely reduced actin tail formation in HeLa cells while expression of the hemagglutanin FhaB (BPSS1727) in J774.2 macrophages yielded actin projections stretching towards adjacent cells [65,87]. The BPSL1528 mutant also displayed reduced swimming motility and intracellular growth in phagocytic and non-phagocytic cells, suggesting BPSL1528 may act pleiotropically in a regulatory manner [65].

While BimA facilitates actin polymerization and intracellular motility in Southeast Asian isolates of *B. pseudomallei*, a flagellar gene cluster designated BTFC (*fla2*) that is present in

B. thailandensis and most *B. pseudomallei* isolates of Australian origin may serve a similar function [88]. French et al demonstrated that *fla2* encodes for lateral flagella which enable rapid intracellular motility by *B. thailandensis* that can compensate for the lack of BimA-mediated actin-based motility [84]. Inactivation of *fla2* resulted in reduced plaque diameter in infected HEK293 cells while a *fla2*/*bimA* double mutant was unable to induce plaque formation [84]. Although *B. thailandensis* carries a different *bimA* allele ($bimA_{Bt}$) than *B. pseudomallei* ($bimA_{Bp}$), $bimA_{Bt}$ can complement *B. pseudomallei* actin polymerization when $bimA_{Bp}$ is deleted [89]. *B. mallei* contains a different *bimA* allele ($bimA_{Bm}$) which is also found in 12% of Australian *B. pseudomallei* isolates (54% ID to $bimA_{Bp}$) [90]. The implications of these findings are unclear, although Australian *B. pseudomallei* strains with the $bimA_{Bp}$ allele are more likely to cause pneumonia and strains with the $bimA_{Bm}$ allele are more likely to cause encephalomyelitis [91].

MNGC FORMATION

MNGC formation has been observed in both phagocytic and non-phagocytic cell lines infected with *B. pseudomallei* as well as in tissues isolated from melioidosis patients. The role of MNGCs in pathogenesis is unknown, but it has been speculated that these structures facilitate bacterial survival and replication, yielding a direct path to neighboring cells while avoiding extracellular immune responses [3,82,83]. This phenomenon is associated with actin-based motility and results from host cell fusion events. In support of this, French et al demonstrated that a *B. pseudomallei* Δ*bimA* mutant failed to stimulate MNGC and plaque formation in HEK293 cells [84]. As discussed above, a functional T6SS-1 is required for *Burkholderia*-induced MNGC formation, and current evidence suggests that the CTD of VgrG1 plays a critical role in this process. While it has been postulated that the effector function associated with VgrG1 may be as a “fusogenic factor”, the exact mechanism(s) of T6SS-1 mediated cell fusion remains to be elucidated.

Several additional genes have been linked to *B. pseudomallei*-induced MNGC formation although their specific roles in the process are not well understood. The RNA polymerase sigma subunit *rpoS* (BPSL1505) has been implicated in this process as a *B. pseudomallei rpoS* mutant stimulated reduced MNGC formation (3%) in comparison to the wild-type strain (17%) in RAW 264.7 cells. It is possible that RpoS may play a role in regulating the transcription of genes whose products are involved in promoting macrophage fusion [84,92]. Ecotopic expression of BPSL0590 and BPSL0591, putative homologs of *Photorhabdus luminescens* insecticidal toxins, in J774.2 macrophages induced MNGC formation and nuclear apoptosis [87]. BPSS0945, a putative exported peptidase, localized to the nucleus when expressed in HeLa cells and resulted in increased MNGC formation. This protein also contains a human microtubule-associated serine/threonine kinase-like (MASTL) domain; MASTL proteins aid in facilitation of mitosis, anaphase, and cytokinesis, which could implicate host cell growth and expansion as ideal conditions for MNGC formation [78]. The senescence marker LfpA (BPSS2074) was able to modify macrophages to resemble osteoclasts; these osteoclastic markers are also detected on MNGCs [93]. A lipoate-protein ligase *lipB* mutant (BPSL0413) did not affect intracellular growth, but had a delayed time to plaque formation and was unable to grow in iron deplete media conditions. This protein is proposed to modify proteins with lipoyl groups, possibly in a regulatory manner [65].

INTRACELLULAR SURVIVAL

Host cells, particularly macrophages, are capable of killing intracellular pathogens through a variety of mechanisms including the production of reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), phagolysosome fusion and autophagy. While T3SS-3 plays an important role in facilitating phagosomal escape and avoidance of autophagy, *B. pseudomallei* possesses additional mechanisms to contend with exposure to potent ROS such as superoxide (O^{2-}) and RNI such as nitric oxide (NO). These include strategies ranging from the production of specific proteins to combat oxidative stress to suppression of pathways that normally lead to the generation of RNI.

Bacterial resistance to killing via superoxide involves a family of enzymes designated superoxide dismutases (SOD) which convert O_2^- to hydrogen peroxide (H_2O_2), a less harmful compound that can be detoxified by other enzymes (e.g. KatG or AhpC). In *B. pseudomallei* SodC (BPSL1001), a Cu^{2+}- and Zn^{2+}-dependent SOD, confers resistance against exogenous O_2^-, contributing to survival within J774A.1 macrophages and virulence in BALB/c mice [94]. A SodB homolog (BPSL0880) has also been identified in *B. pseudomallei*, but remains to be investigated. Enzymes that degrade H_2O_2 such as KatG (a catalase/peroxidase), AhpC (an alkyl hydroperoxide reductase), and DpsA (a DNA-binding protein) have been preliminarily characterized and shown to mediate resistance of *B. pseudomallei* to oxidative stress [95-97]. In addition to providing protection against oxidative stress, AhpC also helps *B. pseudomallei* to evade killing by RNI [96]. Expression of these genes (*katG*, *dpsA* and *ahpC*) was shown to be dependent on the global regulator OxyR, and consistent with this, *B. pseudomallei oxyR* mutants were hypersensitive to H_2O_2 [95-98].

In activated macrophages, inducible nitric oxide synthase (iNOS) is responsible for generating high levels of nitric oxide (NO) which is rapidly oxidized to nitrogen dioxide, dinitrogen trioxide, and when superoxide is present, peroxynitrite is also generated. All of these RNI play important roles in the clearance of intracellular bacteria. Previous studies have demonstrated that following uptake by murine macrophage cell lines, *B. pseudomallei* fails to activate expression of interferon-beta (IFN-β), iNOS, and NO production [99,100]. By interfering with RNI generating responses the organism essentially promotes its own survival within host cells. Recent studies aimed at understanding how *B. pseudomallei* subverts these defense mechanisms have revealed that this phenomenon is complex and involves modulation of the expression of several host proteins including the sterile-α and armadillo motif (SARM)-containing protein, a known negative regulator of the MyD88-independent (TRIF dependent) pathway, and signal regulatory protein alpha (SIRPα), a negative regulator of Toll-like receptor signaling [99,101,102]. By up-regulating SARM and preventing down-regulation of SIRPα, *B. pseudomallei* effectively inhibited MyD88-independent signaling and ultimately lead to the suppression IFN-β and iNOS expression. Elucidation of the mechanisms used by *B. pseudomallei* to interfere with RNI production will be important for understanding how the organism manipulates host signaling pathways in order to escape killing by macrophages.

Additional factors that have been identified in *B. pseudomallei* that may help to facilitate survival of the organism in host cells include an ecotin homolog (*eco*), a macrophage infectivity potentiator (Mip)-like protein (BPSS1823) with peptidyl-prolyl isomerase (PPIase) activity belonging to the FK-506-binding protein (FKBP) family, a disulfide oxidoreductase (*dsbA*), and a second FKBP homolog (BPSL0918) lacking PPIase activity. In *E. coli* ecotin is a periplasmic protein which inhibits host serine proteases and is involved in resisting the degradative enzymes found in lysosomes. A *B. pseudomallei eco* homolog (BPSL1054) was required for replication in J774A.1 macrophages and full virulence in an intraperitoneal BALB/c mouse model [103]. A *B. pseudomallei* mutant in the Mip-like protein BPSS1823, proposed to play a role in resisting phagolysosomal degradation, demonstrated reduced growth in J774A.1 macrophages and A549 cells, and was attenuated in BALB/c mice. In addition, the mutant displayed increased sensitivity to low-pH conditions and decreased protease secretion and flagella production [104]. Likewise, *B. pseudomallei dsbA* (BPSL0381) and BPSL0918 mutants were attenuated in both macrophages and in BALB/c mice [105,106]. DsbA is responsible for introducing disulfide bonds into unfolded or partially folded proteins while BPSL0918 thought to play a role in protein folding and chaperoning.

QUORUM SENSING AND TWO COMPONENT SYSTEMS

QS is a cell density-dependent form of communication between bacteria that uses extracellular signaling molecules known as *N*-acyl homoserine lactones (AHLs). Most QS systems consist of two proteins LuxI, an AHL synthase, and LuxR, a transcription regulator that binds AHLs and then activates expression of target genes. This process leads to coordinated gene expression in entire populations. The *B. pseudomallei* genome contains three *luxI* homologs and five *luxR* homologs [107]. Six of the eight genes were inactivated by mutagenesis resulting in a delayed time to death in Syrian hamsters and reduced colonization in BALB/c mice [107]. This was corroborated by a *luxI1* (BPSS0885) mutant in a Swiss mouse model [75]; however a mutant with all three *luxI* genes deleted showed no difference in bacterial clearance in an intranasal BALB/c mouse model [108]. Interestingly, the triple *luxI* mutant also displayed a markedly increased formation rate of MNGCs resulting from the fusion of RAW 264.7 murine macrophages [108]. LuxI1 and LuxR1 (BPSS0887) have also been linked to regulation of siderophore synthesis, secretion of phospholipase C, and the MprA protease [75,109].

TCSs sense and respond to environmental stimuli and are commonly involved in the regulation of bacterial virulence gene expression. At present, three *B. pseudomallei* TCSs (IrlRS, VirAG and BPSL0127-0128) have been shown to influence interactions with host cells or to be required for virulence in animals. Jones et al identified the IrlRS (BPSS1039-1040) TCS via transposon mutagenesis and demonstrated that this locus was required for efficient invasion of A549, CHO, and HeLa epithelial cells [110]. Interestingly, an *irlRS* mutant retained wild type virulence in both Syrian hamsters and infant diabetic rats. No differences were observed in uptake by macrophages suggesting that these genes may specifically modulate invasion of nonphagocytic cells. The VirAG TCS was initially identified in *B. mallei* as a major virulence factor and shown to be a positive regulator of T6SS-1, *bimBCADE* and *tssM* [12,111]. Consistent with these findings, Chen et al showed

that *B. pseudomallei virAG* mutants exhibited attenuated survival in RAW 264.7 cells and were avirulent in mice [64]. The BPSL0127-0128 TCS was identified using the phagocytic amoeba *Dictyostelium discoideum* as a model system. Mutation of the sensor kinase (BPSL0127) in *B. pseudomallei* resulted in decreased resistance to *D. discoideum* predation, reduced survival in RAW 264.7 cells, and was avirulent in BALB/c mice [112]. The specific genes controlled by BPSL0127-0128 remain to be determined.

ANTIBIOTIC RESISTANCE

In addition to having a complex intracellular lifestyle and producing numerous secreted products that influence virulence, *B. pseudomallei* is resistant to the killing action of cationic antimicrobial peptides (CAPs) including human neutrophil peptides (defensins), protamine sulfate, poly-L-lysine, magainins, and polymyxins [113,114]. Investigations aimed at determining the molecular basis for this resistance have revealed that the expression of smooth LPS and maintenance of outer membrane permeability are critical for conferring high level resistance of *B. pseudomallei* to CAPs. Mutations that disrupted LPS core biosynthesis and lipid A modifications, or compromised outer membrane integrity resulted in increased susceptibility of *B. pseudomallei* to CAPs and other antibiotics [113]. More recent studies, however, indicate that *B. pseudomallei* is highly susceptible to human cathelicidin peptide LL-37 and lactoferrin antimicrobial peptide derivatives, and that these may be useful in the development of novel therapeutic agents for treatment of melioidosis [115-117].

B. pseudomallei is also intrinsically resistant to numerous commonly used antibiotics, including aminoglycosides, penicillins, rifamycins, and third-generation cephalosporins [6]. Thus, the treatment options for melioidosis are limited with intravenous ceftazidime, imipenem or meropenem followed by oral trimethorpim-sulfamethoxazole or amoxicillin-clavulanate reported as effective treatments for acute disease [1,5]. While primary resistance to ceftazidime in clinical isolates is relatively rare, it has been reported. In patients with multiple isolates collected over hospitalization stays, mutation of *penA* (BPSS0946), a Class A β-lactamase, is common and associated with increased resistance to ceftazidime and clavulanic acid in as little as 17 days [118-121]. Notably, a point mutation leading to a C69Y transition in *penA* can increase ceftazidime resistance >170 fold [118-120]. Additionally, *B. pseudomallei* has been reported to undergo genome reduction during chronic infection leading to the deletion of drug targets such as penicillin-binding proteins [122,123]. Understanding the molecular basis for antimicrobial resistance mechanisms in *B. pseudomallei* should allow the rapid detection of resistant strains and help to facilitate the use of appropriate melioidosis therapies.

EXPERT COMMENTARY & FIVE YEAR VIEW

Melioidosis is an emerging infectious disease in many parts of the world and is a significant public health concern in endemic regions. In addition, *B. pseudomallei* is recognized as a biothreat agent with the potential for malicious use. Because of this, research into the biology and pathogenesis of this organism has steadily increased over the past decade. It is anticipated that this will continue over the next 5 years and that the knowledge gained from

present and ongoing studies can be exploited to identify novel therapeutic targets and antigens for use in future diagnostics and vaccines.

Although *B. pseudomallei* virulence is multifactorial, it has become apparent that surface polysaccharides (CPS and LPS) and specialized secretion systems (T3SS-3 and T6SS-1) are key factors that contribute to ability of this pathogen to cause disease. Not only do CPS and LPS enable the organism to subvert innate host immune defenses following initial infection, both have been shown to be protective antigens and promising diagnostic candidates. T3SS-3 and T6SS-1 are attractive as anti-bacterial targets and preliminary studies indicate that components of these systems (e.g. BopA and Hcp1) demonstrate promise as future melioidosis vaccine candidates. Additional antigens including adhesins and autotransporter proteins may also be useful in this regard.

B. pseudomallei is a versatile pathogen with a complex intracellular lifestyle that is only beginning to be appreciated at the molecular level. Mechanistic studies focused on understanding the functions of *B. pseudomallei* virulence determinants will not only shed light on their roles in the pathogenesis of melioidosis, but may also provide insights for the development of novel treatment strategies and countermeasures. Many interesting questions relating to multiple aspects of *B. pseudomallei* pathogenesis remain to be addressed.

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**__Of considerable interest:__

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KEY ISSUES

- Melioidosis is an emerging infectious disease that can be difficult to treat due to the intrinsic resistance of *B. pseudomallei* to many commonly used antibiotics.
- Diagnosis of melioidosis can be challenging and no vaccines are currently available for prevention of *B. pseudomallei* infections.
- The complex intracellular lifestyle of *B. pseudomallei* involves numerous factors and is only beginning to be understood at a molecular level.
- *B. pseudomallei* expresses several major virulence determinants that are required for survival of the organism in animal models of melioidosis.
- Surface polysaccharides (CPS and LPS) are important for evasion of host innate immune defenses, are protective antigens, and represent promising vaccine candidates.
- Adherence of *B. pseudomallei* to host cells is multi-factorial and the contribution of various autotransporter proteins in this process is only beginning to be explored.
- Virulence associated Type III and Type VI secretion systems play major roles in the intracellular behavior of *B. pseudomallei*. Research aimed at determining how these systems function at a molecular level and how they are regulated will provide important insights into the pathogenesis of melioidosis.
- The mechanistic details regarding intracellular actin-based motility and *B. pseudomallei*-induced MNGC formation are poorly understood; the role of MNGCs in disease is unclear.
- A better understanding of the molecular mechanisms underlying *B. pseudomallei* pathogenesis may lead to the identification of new therapeutic targets, diagnostic antigens, and vaccine candidates.

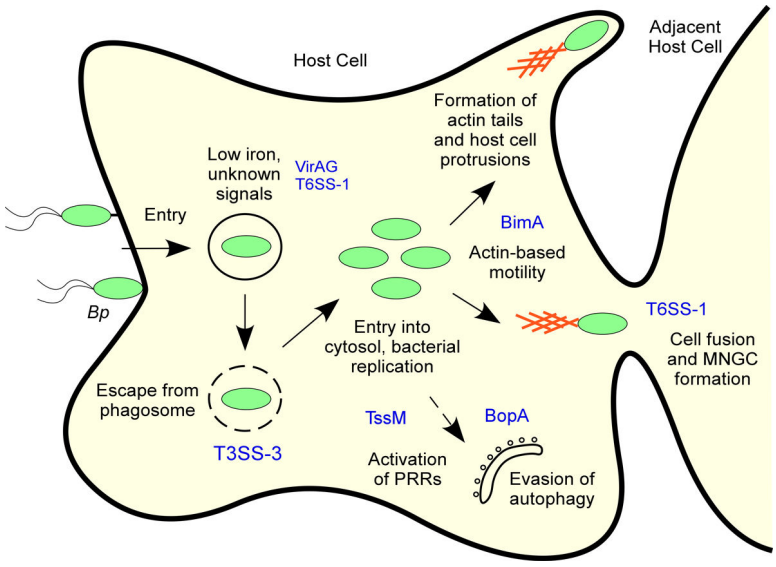

Figure 1. Proposed model of the intracellular lifestyle of *B. pseudomallei* in phagocytic cells

Following entry in host cells, *B. pseudomallei* (*Bp*) rapidly escapes from the phagosome, enters into the cytosol where it can replicate, polymerize host cell actin (red), spread cell to cell, and induce host cell fusion resulting in the formation of multinucleated giant cells (MNGC). Within the cytosol *B. pseudomallei* can activate pattern recognition receptors (PRRs) and evade host cell autophagy. Gene products and systems that are important at various points are indicated in blue text. VirAG senses a signal within the phagosome that activates T6SS-1 gene expression; T3SS-3 is required for escape from the phagosome; BimA facilitates actin-based motility and actin tail formation; BopA is important for avoidance of autophagy; TssM interferes with host cell signaling and activation of PRRs; T6SS-1 is critical for MNGC formation.