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Actin-Binding Proteins from *Burkholderia mallei* and *Burkholderia thailandensis* Can Functionally Compensate for the Actin-Based Motility Defect of a *Burkholderia pseudomallei* bimA Mutant

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Recently we identified a bacterial factor (BimA) required for actin-based motility of *Burkholderia pseudomallei*. Here we report that *Burkholderia mallei* and *Burkholderia thailandensis* are capable of actin-based motility in J774.2 cells and that BimA homologs of these bacteria can restore the actin-based motility defect of a *B. pseudomallei* bimA mutant. While the BimA homologs differ in their amino-terminal sequence, they interact directly with actin in vitro and vary in their ability to bind Arp3.

The gram-negative bacteria *Burkholderia pseudomallei* and *Burkholderia mallei* are highly pathogenic to humans. They have been listed as biological risk category B agents, and due to their infectivity by the respiratory route are considered potential bioterror agents (15). *B. pseudomallei* is the causative agent of melioidosis in humans, which is endemic in Southeast Asia and northern Australia. This disease may present in a variety of ways from subacute and chronic suppurrative infections to rapidly fatal septicemia (18). *B. mallei* causes the zoonotic disease glanders, which mainly affects horses. *B. mallei* can also infect humans, an infection that is almost invariably fatal if untreated (19). The gram-negative soil saprophyte *B. thailandensis* is nonpathogenic in Syrian hamster models of infection (2) and was previously classified as an arabinose positive (Ara⁺) nonpathogenic variant of *B. pseudomallei* (3). Such Ara⁺ variants are rarely associated with human infections in areas where *B. pseudomallei* is endemic.

While these *Burkholderia* species differ in their virulence and host range they all subvert the host cell to promote their intracellular replication and survival. In this respect, *B. pseudomallei* is probably the best characterized. Upon uptake by either phagocytic or nonphagocytic host cells, *B. pseudomallei* escapes endocytic vacuoles by lysing the endosomal membrane (7) and utilizes the power of actin polymerization to propel itself within host cells (11, 16), as seen for several other intracellular pathogens (5). Intracellular actin-based motility is believed to underlie the ability of *B. pseudomallei* to spread from cell to cell and promote multineucleated giant cell formation, for the purpose of intercellular spread while evading host immune surveillance (11).

We recently identified a *B. pseudomallei* protein required for actin-based motility in J774.2 cells (16). Termed BimA (*Burkholderia intracellular motility A*), this protein was localized at the pole of the bacteria at which actin tails formed in cells. We demonstrated that this protein bound monomeric actin and stimulated actin polymerization independent of the cellular Arp2/3 complex in vitro. This observation fits well with other published data showing that while Arp2/3 associates with the actin tail of *B. pseudomallei* in HeLa cells, tail formation is not prevented by overexpression of the inhibitory fragment of Scar1 (1). Here we have extended our studies on *B. pseudomallei* intracellular actin tail formation to include the related species *B. mallei* and *B. thailandensis*.

It has previously been reported that *B. mallei* and *B. thailandensis* induce the formation of multinucleated giant cells in vitro through the fusion of adjoining cell membranes (6). However, it is not known whether intracellular motility is required for propulsion of the bacteria to the membranes at which cell fusion events occur or indeed, whether *B. mallei* and *B. thailandensis* are capable of actin-based intracellular motility. To address this we infected the murine macrophage-like J774.2 cell line with *B. mallei* ATCC 23344 or *B. thailandensis* E30.

To aid visualization of *B. mallei* by fluorescence microscopy, a plasmid for constitutive expression of green fluorescent protein (GFP) was introduced. This vector (pBHR1-GFP) was constructed by amplification of the GFP gene from pQBI T7-GFP (Qiagen Inc., Irvine, CA) and primers GARP-UP (5'-CTTTGTTAGCAGCCGGATCC-3') and GARP-DOWN (5'-CCCTCTAGAAATAATTTTG-3'). The 786-bp PCR product, which lacks the T7 promoter that drives transcription of the GFP gene, was cloned into pCR2.1-TOPO (Invitrogen, Paisley, United Kingdom), generating pCR2.1-GFP. The EcoRI insert from this plasmid was then subcloned into the corresponding site within the chloramphenicol resistance gene of the broad host range plasmid pBHR1 (MoBiTec GmbH, Goettingen, Germany). The GFP gene was cloned in the same orientation as the chloramphenicol resistance gene such that it is transcribed from the extraneous promoter. The resulting plasmid was conjugated into *B. mallei* ATCC 23344 from *Escherichia coli* S17-1pir with selection for kanamycin resistance.

J774.2 cells were infected with *B. mallei* ATCC 23344 (pBHR1-GFP) at a multiplicity of infection of 10:1. At 8 h postinfection, cells were fixed and permeabilized and filamentous actin was labeled with FITC-conjugated phalloidin. As seen in Figure 1A, adult cells infected with *B. mallei* show a clear intracellular localization of actin with the bacteria at the cell periphery. Pseudonuclei were positive for the presence of actin, suggesting that *B. mallei* is able to actin-based motility at the periphery of the host cell.
B. mallei-infected J774.2 cells (a) and B. thailandensis-infected J774.2 cells (b) were stained with various fluorescent dyes to visualize filamentous actin. Scale bar = 4 μm.

**FIG. 1.** *Burkholderia mallei* and *B. thailandensis* form actin tails in J774.2 murine macrophage-like cells. Representative confocal micrographs of J774.2 cells infected with *B. mallei* ATCC 23344(pBHR1-GFP) (a) or *B. thailandensis* E30 (b). *B. mallei* appears green in panel a owing to expression of GFP from a modified broad-host-range vector. *B. thailandensis* E30 was stained red with a rabbit antiserum against lipopolysaccharide and anti-rabbit immunoglobulin-Alexa 568 (b). Filamentous actin stained either red with tetramethylrhodamine isothiocyanate-phalloidin (a) or green with Alexa Fluor 488-phalloidin (b).

E30 at a multiplicity of infection of 100:1 were processed at 6 h postinfection for analysis by confocal laser scanning microscopy essentially as described (16). After fixation and permeabilization, bacteria were stained red following sequential incubation with rabbit antiserum against *B. pseudomallei* lipopolysaccharide (a kind gift from T. Pitt, Health Protection Agency, Colindale, United Kingdom) and anti-rabbit immunoglobulin-Alexa 568 (Molecular Probes, Cambridge, United Kingdom) and filamentous actin stained green following incubation with Alexa Fluor 488-phalloidin (Molecular Probes, Cambridge, United Kingdom). As was the case for *B. mallei*-infected cells, the *B. thailandensis* E30-infected cells exhibited numerous bacteria-tipped membrane protrusions which were accompanied by intense filamentous actin staining at a single pole of the bacteria (Fig. 1b).

Having shown that *B. mallei* and *B. thailandensis* induce the formation of actin tails in infected cells, we sought to identify the factors required for intracellular actin-based motility of these organisms. We have recently reported the identification of the *B. pseudomallei* factor (BimA) that is required for actin-based motility in J774.2 cells. BimA was identified by searching the translated *B. pseudomallei* genome for proline-rich autosecreted proteins based on the fact that it may be similar to the virulence-associated and autosecreted *IscA/VirG* protein required by *Shigella flexneri* for actin-based motility (16).

BimA is a putative autosecreted protein with similarity at the carboxyl terminus to the *Yersinia enterocolitica* YadA and *Haemophilus influenzae* Hia autotransporters. We searched the available genome sequences of *B. mallei* and *B. thailandensis* at the nucleotide and amino acid levels and found proteins with carboxyl-terminal sequences nearly identical to that of the *B. pseudomallei* BimA protein, which we have designated *B. mallei* BimA (BimAm) and *B. thailandensis* BimA (BimAt). Both proteins also show considerable similarity to the carboxyl-terminal portions of *Y. enterocolitica* YadA and *H. influenzae* Hia proteins. The *B. mallei* BimA homolog is derived from the gene locus BMAA0749 from the annotated *B. mallei* strain ATCC 23344 (Uniprot/TrEMBL entry no. Q62CV6) (12). The *B. thailandensis* homolog is derived from the partially sequenced *B. thailandensis* strain E264 (contig 493 of the unassembled genome at www.tigr.org).

However, it was surprising to find that while the carboxyl-terminal portions of the proteins corresponding to the putative membrane targeting and anchoring motifs in BimAm was conserved, the amino-terminal regions of the proteins that are exposed at the bacterial cell surface differed markedly (Fig. 2a). Neither protein contains the proline-rich motif I, the 13-amino-acid NIPV-containing or PDAST repeats found in the BimAm sequence, or shows any homology to Wiskott-Aldrich syndrome protein (WASP) family or bacterial factors mediating actin nucleation or polymerization. The BimA proteins also differ in their number of monomeric actin-binding WH2 (WASP homology domain 2) motifs, which are composed of approximately 35 amino acids and are conserved in cellular proteins that recruit actin monomers (14).

Alignment of the sequences of 50 WH2 motifs of proteins from *Homo sapiens*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Saccharomyces cerevisiae* indicates that four residues within a predicted alpha-helical domain are highly conserved (14).
in BimA\textsubscript{ma} and BimA\textsubscript{th} and are denoted by asterisks in Fig. 2b. BimA\textsubscript{ps} contains two WH2 domains in tandem while both the BimA\textsubscript{ma} and BimA\textsubscript{th} proteins each contain a single WH2 motif (Fig. 2a and 2b).

The predicted \textit{B. mallei} BimA protein is composed of 373 amino acids with a putative signal sequence comprising the first 54 residues (Fig. 2a). BimA\textsubscript{ma} contains a single N-terminal WH2 motif followed by a proline-rich region comprising a stretch of 18 prolines followed by five tandem SP\textsubscript{PPP} repeats. Proline-rich motifs are commonly found in proteins involved in the control of cellular actin dynamics (9).

The predicted \textit{B. thailandensis} protein is composed of 563 amino acids with a putative signal sequence of 47 residues (Fig. 2a). Similar to the BimA\textsubscript{ma} sequence, BimA\textsubscript{th} comprises a
single N-terminal WH2 domain and proline-rich domain. These domains are separated by a central and acidic (CA) region (Fig. 2c) (13) characteristically found in WASP family member proteins, *Listeria* ActA and *Rickettsia* RickA (4, 10). In combination with an upstream WH2 domain (also termed V domain), this may constitute a VCA domain which in WASP family members is involved in the concerted binding and activation of Arp2/3 (17). The acidic domains of WASP family members contain an invariant tryptophan residue that is required for binding of Arp2/3 and this residue is conserved in the acidic domain of BimA<sub>ma</sub> (13). The CA domain is lacking in both the BimA<sub>ma</sub> and BimA<sub>th</sub> proteins.

We next tested these putative BimA homologs for the ability to nucleate actin and mediate actin-based motility by determining whether these proteins could complement the actin-based motility defect of our *B. pseudomallei* 10276 bimA<sup>pDM4</sup> mutant in J774.2 cells (16). We previously shown that targeted disruption of the bimA gene renders *B. pseudomallei* unable to generate polar actin tails in J774.2 cells despite being able to escape the host cell endo-

To trans-complement the *B. pseudomallei* bimA<sup>pDM4</sup> mutant the *bimA* genes from *B. mallei* and *B. thailandensis* were cloned as follows. The BimA<sub>ma</sub> gene together with 235 bp upstream of the predicted translation initiation codon was amplified by PCR with Pfx DNA polymerase (Invitrogen, Paisley, United Kingdom) using Bm comp forward (5′-CATCGAGTCC ATGCGGTGAATAGCT-3′) and Bm comp reverse (5′-CTTC TCGAGTTACCCATGCGTGCAACAGTTGCT-3′) and cloned into BamHI- and EcoRI-digested pGEX-2T-1 via sites described elsewhere (16). A BglII/EcoRI DNA fragment encoding amino acids 47 to 222 of BimA<sub>ma</sub> was amplified by PCR from chromosomal *B. mallei* strain E30 DNA using the oligonucleotides mallBimA<sup>Bth</sup>-CATGAATTCCCATGCGTGCAACAGTTGCT-3′ and Bm comp reverse (5′-CTTC TCGAGTTACCCATGCGTGCAACAGTTGCT-3′) with *B. thailandensis* E30 chromosomal DNA as template. The resulting products were cloned under the control of the P<sub>act</sub> promoter in pME6032 via EcoRI and XhoI sites incorporated into the primers to give pGEX-BimA<sub>th</sub> and pME6032bimA<sub>ma</sub> Expression constructs were electroporated into *B. pseudomallei* 10276 bimA<sup>pDM4</sup> (16) using standard techniques with selection for tetracycline.

J774.2 cells were infected at a multiplicity of infection of 10 bacteria per cell with *B. pseudomallei* 10276, the 10276 bimA<sup>pDM4</sup> mutant, 10276 bimA<sup>pDM4</sup>(pME6bimA<sub>ma</sub>), 10276 bimA<sup>pDM4</sup>(MEbimA<sub>ma</sub>), and 10276 bimA<sup>pDM4</sup>(pMEbimA<sub>ma</sub>). Briefly, after 1 h of infection with bacteria diluted in RPMI media containing 10% (vol/vol) fetal calf serum at 37°C in a 5% CO<sub>2</sub> atmosphere, cells were washed several times and overlaid with media containing an inhibitory level of kanamycin (250 μg/ml) to kill extracellular bacteria. Incubation at 37°C was then continued for a further 5 h before washing in media containing kanamycin. Inducible expression of the BimA proteins during culture and cell infection studies was achieved by addition of 0.25 mM isopropyl-β-D-thiogalactoside (IPTG). At 6 h postinfection, cells were processed for microscopy. The infected cells were fixed in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline overnight at ambient temperature, permeabilized in 0.5% (vol/vol) Triton X-100 in phosphate-buffered saline for 15 min and then bacteria were stained red with rabbit anti-*B. pseudomallei* lipopolysaccharide followed by anti-rabbit immunoglobulin-Alexa 568 and filamentous actin stained green with Alexa Fluor 488-phalloidin.

Figure 3b shows that while the *B. pseudomallei* bimA<sup>pDM4</sup> mutant does not form membrane protrusions or nucleate actin at the pole of intracellular bacteria in J774.2 cells, expression of both the *B. mallei* and *B. thailandensis* BimA proteins in *trans* was able to restore tail formation (Fig. 3d and e). These observations indicate that the BimA homologs of *B. mallei* and *B. thailandensis* share the ability of *B. pseudomallei* BimA to stimulate actin assembly, despite a marked divergence in their amino-terminal sequences. Since defined mutants of *B. mallei* and *B. thailandensis* lacking the BimA homologues were not constructed, we cannot preclude the possibility that accessory factors are required in addition to the BimA homologues for full actin-based motility in the respective organisms.

Having shown that these proteins are capable of restoring actin-based motility to the *B. pseudomallei* bimA mutant, we next sought to determine whether these proteins interact with cellular actin. We have previously shown that the *B. pseudomallei* BimA protein directly interacts with actin without the need for any bridging molecules such as profilin (16).

Two expression constructs were generated for these experiments, each lacking the residues predicted to encode amino-terminal signal sequences. A vector for the expression of the *B. pseudomallei* BimA protein (residues 48 to 384) as a glutathione-one-S-transferase (GST) fusion (pGEX-BimA<sub>ma</sub>) has been described elsewhere (16). A BglII/EcoRI DNA fragment encoding amino acids 47 to 222 of BimA<sub>ma</sub> was amplified by PCR from chromosomal *B. mallei* strain ATCC 23344 DNA using the oligonucleotides mallBimA<sup>Bth</sup>-CATGAATTCCCATGCGTGCAACAGTTGCT-3′ and Bm comp reverse (5′-CTTC TCGAGTTACCCATGCGTGCAACAGTTGCT-3′). The digested PCR product was cloned into BamHI- and EcoRI-digested pGEX-2T-1 (Amersham Biosciences United Kingdom Ltd., Buckinghamshire, United Kingdom) to give pGEX-BimA<sub>ma</sub>. Similarly, a BamHI/EcoRI DNA fragment encoding amino acids 47 to 384 of BimA<sub>ma</sub> was amplified by PCR from chromosomal *B. thailandensis* strain E30 DNA using the oligonucleotides thailBimA<sup>Bth</sup>-CATGAATTCCCATGCGTGCAACAGTTGCT-3′ and thailBimA<sup>Bth</sup> (5′-CTCGAAT TCTCAAGTGTTTTGACCGGACGCATTTGCT-3′). The digested PCR product was cloned into BamHI- and EcoRI-digested pGEX-2T-1 (Amersham Biosciences United Kingdom Ltd., Buckinghamshire, United Kingdom) to give pGEX-BimA<sub>ma</sub>.
ugation prior to use) supplemented with 100 μM CaCl$_2$ and 500 μM ATP. After 15 min incubation at ambient temperature, beads were washed with ice-cold Tris-buffered saline and analyzed by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) and immunoblotting with anti-actin antibody as described (16).

As we have previously reported, beads coated with GST-BimA$_{ps}$ but not GST specifically sequestered actin from the splenic lysate (16) (Fig. 4a). Furthermore, GST-BimA$_{ma}$ and GST-BimA$_{th}$ proteins also precipitated actin from the splenic lysates indicating that these proteins could also interact with actin (Fig. 4a). To determine whether the BimA proteins of B. mallei and B. thailandensis interact directly with actin, GST and GST-BimA coated beads were mixed with polymerization buffer supplemented with 100 μM CaCl$_2$, 500 μM ATP and 1 μM rhodamine-labeled actin (Cytoskeleton, Denver, CO). After addition of the beads, 10 μl samples were mounted onto slides and visualized 5 min after mixing for the binding of rhodamine-labeled actin using a confocal laser scanning microscope. We found that similarly to BimA$_{ps}$, both the B. mallei and B. thailandensis BimA proteins could associate directly with actin, since beads coated with GST-BimA$_{ps}$, GST-BimA$_{ma}$ and GST-BimA$_{th}$ but not GST alone rapidly recruited rhodamine-labeled actin in the absence of any accessory proteins (Fig. 4b).

The finding that BimA$_{th}$ possesses a CA-like domain downstream of its WH2 domain raised the possibility that this protein may associate with the Arp2/3 complex of the host cell. To date we have failed to detect an interaction between the B. pseudomallei BimA protein and components of the Arp2/3 complex (16). To determine whether BimA$_{th}$ interacts with Arp2/3, we incubated Sepharose beads coated with GST, GST-BimA$_{ps}$, GST-BimA$_{ma}$, GST-BimA$_{th}$, and GST-BimA$_{th}$, with a murine splenic lysate, eluted any associated proteins and probed for the presence of the Arp2/3 complex component Arp3 by immunoblotting with specific anti-Arp3 antibody (Autogen Bioclear United Kingdom Ltd, Wiltshire, United Kingdom). As shown in Fig. 4a, beads coated with GST-BimA$_{th}$ but not GST, GST-BimA$_{ps}$ or GST-BimA$_{ma}$ specifically sequestered Arp3 from the splenic lysate, indicating that the B. thailandensis BimA homolog interacts with the Arp2/3 complex in host cells.

Here we have demonstrated that B. mallei and B. thailandensis BimA proteins complement the actin-based motility defect of a B. pseudomallei bimA mutant in J744.2 cells. Representative confocal micrographs of J774.2 cells infected with B. pseudomallei 10276 (a), the 10276 bimA::pDM4 mutant (b), 10276 bimA::pDM4 mutant trans-complemented with B. pseudomallei bimA(pMEbimA$_{ps}$) (c), 10276 bimA::pDM4 mutant trans-complemented with B. mallei bimA(pMEbimA$_{ma}$) (d), or 10276 bimA::pDM4 mutant trans-complemented with B. thailandensis bimA(pMEbimA$_{th}$) (e) under IPTG induction. Bacteria were stained red with rabbit anti-B. pseudomallei lipopolysaccharide followed by anti-rabbit immunoglobulin-Alexa 568 and filamentous actin stained green with Alexa Fluor 488-phalloidin. Scale bar = 4 μm.

FIG. 3. B. mallei and B. thailandensis BimA proteins complement the actin-based motility defect of a B. pseudomallei bimA mutant in J744.2 cells. Representative confocal micrographs of J774.2 cells infected with B. pseudomallei 10276 (a), the 10276 bimA::pDM4 mutant (b), 10276 bimA::pDM4 mutant trans-complemented with B. pseudomallei bimA(pMEbimA$_{ps}$) (c), 10276 bimA::pDM4 mutant trans-complemented with B. mallei bimA(pMEbimA$_{ma}$) (d), or 10276 bimA::pDM4 mutant trans-complemented with B. thailandensis bimA(pMEbimA$_{th}$) (e) under IPTG induction. Bacteria were stained red with rabbit anti-B. pseudomallei lipopolysaccharide followed by anti-rabbit immunoglobulin-Alexa 568 and filamentous actin stained green with Alexa Fluor 488-phalloidin. Scale bar = 4 μm.
The BimA proteins of *B. mallei* and *B. thailandensis* directly interact with actin. (a) Immunoblots showing association of cellular actin and Arp3 protein with GST-BimA fusion proteins following incubation of Sepharose beads coated with GST-BimA<sub>ps</sub>, GST-BimA<sub>ma</sub>, GST-BimA<sub>th</sub> or GST alone with murine splenic extract. (b) Representative confocal laser scanning micrographs of Sepharose beads coated with GST-BimA<sub>ps</sub>, GST-BimA<sub>ma</sub>, GST-BimA<sub>th</sub> or GST alone following incubation with rhodamine-labeled actin. Scale bar = 40 μm.

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