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 supplicative 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 multiplication 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 lyzing 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 multinucleated 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.
In a similar experiment, cells infected with \textit{B. mallei} and \textit{B. thailandensis} form actin tails in J774.2 murine macrophage-like cells. Representative confocal micrographs of J774.2 cells infected with \textit{B. mallei} ATCC 23344(pBHR1-GFP) (a) or \textit{B. thailandensis} E30 (b). \textit{B. mallei} appears green in panel a owing to expression of GFP from a modified broad-host-range vector. \textit{B. thailandensis} E30 was stained red with rabbit anti-\textit{B. pseudomallei} 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). Scale bar = 4 \textmu m.

Having shown that \textit{B. mallei} and \textit{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 \textit{B. pseudomallei} factor (BimA) that is required for actin-based motility in J774.2 cells. BimA was identified by searching the translated \textit{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 \textit{Shigella flexneri} for actin-based motility (16).

BimA is a putative autosecreted protein with similarity at the carboxyl terminus to the \textit{Yersinia enterocolitica} YadA and \textit{Haemophilus influenzae} Hia autotransporters. We searched the available genome sequences of \textit{B. mallei} and \textit{B. thailandensis} at the nucleotide and amino acid levels and found proteins with carboxyl-terminal sequences nearly identical to that of the \textit{B. pseudomallei} BimA protein, which we have designated \textit{B. mallei} BimA (BimA\textsubscript{ma}) and \textit{B. thailandensis} BimA (BimA\textsubscript{th}). Both proteins also show considerable similarity to the carboxyl-termini of the \textit{Y. enterocolitica} YadA and \textit{H. influenzae} Hia proteins. The \textit{B. mallei} BimA homolog is derived from the gene locus BMAA0749 from the annotated \textit{B. mallei} strain ATCC 23344 (Uniprot/TrEMBL entry no. Q62CV6) (12). The \textit{B. thailandensis} homolog is derived from the partially sequenced \textit{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 BimA\textsubscript{ma} were 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 1, the 13-amino-acid NIPV-containing or PDAST repeats found in the BimA\textsubscript{ma} 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 \textit{Homo sapiens}, \textit{Caenorhabditis elegans}, \textit{Drosophila melanogaster}, and \textit{Saccharomyces cerevisiae} indicates that four residues within a predicted alpha-helical domain are highly conserved (14). These residues are conserved in the WH2 motifs 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 \textit{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 \textit{B. mallei}-infected cells, the \textit{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).

FIG. 1. \textit{Burkholderia mallei} and \textit{B. thailandensis} form actin tails in J774.2 murine macrophage-like cells. Representative confocal micrographs of J774.2 cells infected with \textit{B. mallei} ATCC 23344(pBHR1-GFP) (a) or \textit{B. thailandensis} E30 (b). \textit{B. mallei} appears green in panel a owing to expression of GFP from a modified broad-host-range vector. \textit{B. thailandensis} E30 was stained red with rabbit anti-\textit{B. pseudomallei} 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). Scale bar = 4 \textmu m.
in BimA<sub>ma</sub> and BimA<sub>th</sub> and are denoted by asterisks in Fig. 2b. BimA<sub>ps</sub> contains two WH2 domains in tandem while both the BimA<sub>ma</sub> and BimA<sub>th</sub> proteins each contain a single WH2 motif (Fig. 2a and 2b).

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

The predicted <i>B. thailandensis</i> protein is composed of 563 amino acids with a putative signal sequence of 47 residues (Fig. 2a). Similar to the BimA<sub>ma</sub> sequence, BimA<sub>th</sub> comprises a

FIG. 2. Putative domain organization of the BimA proteins from <i>Burkholderia pseudomallei</i>, <i>B. mallei</i>, and <i>B. thailandensis</i>. (a) The putative domains of the <i>B. pseudomallei</i>, <i>B. mallei</i>, and <i>B. thailandensis</i> BimA proteins are shown drawn to scale where: SP, predicted signal peptide; NIPV, repeat sequence in <i>B. pseudomallei</i> BimA with homology to human diaphanous 1 (hDia1); PRM, proline-rich motif 1 (IP<sub>7</sub>) with similarity to that found in hDia1, mouse formin and zyxin; WH2, WASP homology domain-2, actin monomer-binding motif; PDAST, repeat sequence in <i>B. pseudomallei</i> BimA, predicted casein kinase II sites for phosphorylation; TM, putative transmembrane anchor, containing regions of homology to <i>Yersinia enterocolitica</i> YadA and <i>Haemophilus influenzae</i> Hia type V autotransporters; Pro-rich, proline rich regions; CA, central and acidic domains that in combination with a WH2 domain (also known as V domain) are essential for actin and Arp2/3 binding in N-WASP and are involved in Arp2/3 induced actin polymerization in WAVE proteins. (b) Sequence alignment of the BimA WH2-like domains with WH2 motifs of human N-WASP and WIP (accession no.’s AAH12738 and Q8K117, respectively). * denotes residues essential for actin monomer binding by WASP family WH2 domains. (c) Sequence alignment of the CA-like domain of <i>B. thailandensis</i> BimA with the CA domains of human N-WASP, Scar1, <i>Listeria monocytogenes</i> ActA, and <i>Rickettsia rickettsii</i> RickA proteins (accession nos. AAH12738, NP_003922, S20887, and AJ293314, respectively). Alignments were generated using ClustalW. Residues conserved in WASP family members are shown in red.
single N-terminal WH2 domain and proline-rich domain. These domains are separated by a central and acidic (CA) region (Fig. 2c) (13) characterized in the Wasp family members, 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>ps</sub> (13). The CA domain is lacking in both the BimA<sub>ps</sub> and BimA<sub>ma</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 bimA<sup>-</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 endosome. We have previously shown that the BimA homologues 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 predict the possibility that accessory factors are required in addition to the BimA homologues for full actin-based motility in the respective organisms.

Homing BimA proteins are capable of restoring actin-based motility to the B. pseudomallei bimA<sup>-</sup> 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 S-transferase (GST) fusion (pGEX-BimA<sub>ps</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>-</sup>-CTCAGAATCTTCCAGGATGGGCCCTGATCGCCGACGACG-3<sup>′</sup>) and mallBimA<sup>-</sup>-CATCGAATTCACGCTCACCAGTCCGAGTCCGAATTC-3<sup>′</sup>) and encoded amino acids 47 to 384 of BimA<sub>ma</sub>. Expression constructs were electrotoporated into B. pseudomallei 10276 bimA<sup>-</sup>:pDM4 (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>-</sup>:pDM4 mutant, 10276 bimA<sup>-</sup>:pDM4(pME<sub>bimA</sub><sup>-</sup>), 10276 bimA<sup>-</sup>:pDM4(ME<sub>bimA</sub><sup>-</sup>), and 10276 bimA<sup>-</sup>:pDM4(pME<sub>bimA</sub><sup>-</sup>). 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>-</sup>:pDM4 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 predict the possibility that accessory factors are required in addition to the BimA homologues for full actin-based motility in the respective organisms.

For actin pull-down assays, GST fusion proteins were expressed in E. coli BL21 cells following induction with IPTG and glutathione Sepharose 4B beads (Amersham Pharmacia Biotech, St. Albans, United Kingdom) were coated with either GST, GST-BimA<sub>ps</sub>, GST-BimA<sub>ma</sub> or GST-BimA<sub>an</sub> protein following the manufacturer’s instructions. Beads were then mixed with murine splenic cell lysate (1 mg/ml protein concentration, homogenized in polymerization buffer: 10 mM Tris, pH 7.5, 50 mM KCl, 2 mM MgCl<sub>2</sub>, and cleared of debris by ultra-centrif-
Figure 4. B. mallei and B. thailandensis BimA proteins complement the actin-based motility defect of a B. pseudomallei bimA mutant in J774.2 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 \text{ps}) (c), 10276 bimA::pDM4 mutant trans-complemented with B. mallei bimA(pMEbimA \text{ma}) (d), or 10276 bimA::pDM4 mutant trans-complemented with B. thailandensis bimA(pMEbimA \text{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.

As we have previously reported, beads coated with GST-BimA \text{ps} but not GST specifically sequestered actin from the splenic lysate (16) (Fig. 4a). Furthermore, GST-BimA \text{ma} and GST-BimA \text{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\text{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).

The finding that BimA \text{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 \text{th} interacts with Arp2/3, we incubated Sepharose beads coated with GST, GST-BimA \text{ps}, GST-BimA \text{ma} and GST-BimA \text{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 \text{th} but not GST, GST-BimA \text{ps} or GST-BimA \text{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 interact directly with actin, since beads coated with GST-BimA \text{ps}, GST-BimA \text{ma} and GST-BimA \text{th} but not GST alone rapidly recruited rhodamine-labeled actin in the absence of any accessory proteins (Fig. 4b).
**FIG. 4.** 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.

dens is capable of generating actin tails within the infected host cell and have identified factors from *B. mallei* and *B. thailandensis* that can functionally substitute for the BimA protein of *B. pseudomallei*. Surprisingly, these factors are only similar to the *B. pseudomallei* BimA protein within the carboxy-terminal putative transmembrane anchor. Interestingly, the BimA<sub>th</sub> protein differs from both the BimA<sub>ps</sub> and BimA<sub>ma</sub> factors by the inclusion of a CA domain between the amino-terminal WH2 domain and the proline-rich region. Together with a WH2 domain, such domains are found within WASP family member proteins, where they are involved in the interaction and activation of the Arp2/3 complex. Indeed we have found that while all three BimA proteins interact directly with cellular actin, only BimA<sub>th</sub> associates with the Arp2/3 complex. Such findings may indicate a different mechanism of action for these actin-binding proteins in the formation of actin tails.

The identification of a family of actin-binding proteins capable of stimulating actin-based motility among *Burkholderia* species may lead to novel vaccines or treatments for the control of melioidosis and glanders, as well as provide valuable tools to dissect pathways of cellular actin assembly.

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