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NOTE

Comparative Assessment of the Intracellular Survival of the *Burkholderia pseudomallei bopC* Mutant

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***Burkholderia pseudomallei*, the causative agent of melioidosis, is a Gram-negative saprophytic bacterium capable of surviving within phagocytic cells. To assess the role of BopC (a type III secreted effector protein) in the pathogenesis of *B. pseudomallei*, a *B. pseudomallei bopC* mutant was used to infect J774A.1 macrophage-like cells. The *bopC* mutant showed significantly reduced intracellular survival in infected macrophages compared to wild-type *B. pseudomallei*. In addition, the *bopC* mutant displayed delayed escape from endocytic vesicles compared with the wild-type strain. This indicates that BopC is important, and at least in part, needed for intracellular survival of *B. pseudomallei*.**

***Keywords*:** *Burkholderia pseudomallei*, BopC, type III secretion effector, intracellular survival, phagocyte

Burkholderia pseudomallei, a Gram-negative, saprophytic bacterium, is the etiologic agent of melioidosis, which is a clinically diverse and frequently fatal cause of infectious disease. This organism is present in soil and water across much of Southeast Asia and in Northern Australia (Currie, 2008). The disease is acquired typically through inhalation, skin penetration or possibly through ingestion (Cheng and Currie, 2005). The incidence of melioidosis in northeast Thailand, where melioidosis is endemic, has increased from 8.0 cases per 100,000 people per year in 2000 to 21.3 per 100,000 in 2006 (Limmathurotsakul *et al*., 2010). In 2006, melioidosis was the third most common cause of death from infectious diseases in northeast Thailand (Limmathurotsakul *et al*., 2010). Currently, no vaccine or other approved prophylactics for melioidosis exist.

B. pseudomallei can invade and replicate within host cells including both epithelial and phagocytic cells; the latter are a key component of innate immunity (Jones *et al*., 1996; Harley *et al*., 1998; Kespichayawattana *et al*., 2000). After internalization, *B. pseudomallei* can escape from endocytic vacuoles into the host cell cytoplasm where the bacteria induce actin polymerization at one bacterial pole. These motile bacteria can then move into neighbouring cells via protrusions from the infected cell (Stevens *et al*., 2002, 2005), leading to the formation of multinucleated giant cells (MNGC) (Kespichayawattana *et al*., 2000).

The *Burkholderia* secretion apparatus (*Bsa*) type III secretion system (T3SS) cluster, one of three T3SSs found in *B. pseudomallei*, is homologous to the SPI-1 pathogenicity island of *Salmonella enterica* serovar Typhimurium (Attree and Attree, 2001). Mutants affecting the *B. pseudomallei Bsa* T3SS and/or its effector proteins have been shown to be attenuated in various animal models and displayed intracellular phenotypes, including reduced endocytic vesicle escape, reduced replication, and delayed MNGC formation (Stevens *et al*., 2004; Suparak *et al*., 2005; Warawa and Woods, 2005; Burtnick *et al*., 2008; Muangsombut *et al*., 2008). However, at present, only two *B. pseudomallei* T3SS effector proteins, BopE and BopA, have been functionally characterized. BopE has been demonstrated to play a minor role in bacterial invasion, with *bopE* mutants reduced in their ability to invade epithelial-like cell lines but shown to display a wild-type level of invasion in macrophage-like cell lines (Stevens *et al*., 2002, 2003). BopA has been shown to play a role in evasion of LC3-associated phagocytosis by *B. pseudomallei*, including autophagic vesicles (Cullinane *et al*., 2008; Gong *et al*., 2011).

Recently, we identified a third effector of the Bsa T3SS, BopC, encoded by *B. pseudomallei bpss1516* (Muangman *et al*., 2011). A recombinant *B. pseudomallei* BopC (56 kDa) fusion protein was found to interact with its upstream chaperone, BPSS1517. Additionally, the first 20 amino acids of BopC were found to be sufficient to mediate T3SS-dependent translocation in a heterologous *Escherichia coli* host (Muangman *et al*., 2011). Furthermore, a *bopC* mutant was found to be less invasive than wild-type in epithelial-like cell lines (Muangman *et al*., 2011).

In this study, we continued investigations into the contribution of BopC protein in the intracellular lifestyle of *B. pseudomallei*. Different *B. pseudomallei* strains were used to infect J774A.1 murine macrophage-like cells and several

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intracellular characteristics were investigated including intracellular replication, the ability to escape from phagosomes, and the formation of actin tails and MNGC. The intracellular survival of the *bopC* mutant strain was compared to both the wild-type strain and the *bipB* mutant. The *B. pseudomallei bipB* mutant, which is disrupted in one of genes encoding the BSa T3SS translocator proteins, was previously reported to have delayed escape from endocytic vesicles and a dramatically reduced MNGC formation in macrophage-like J774.1 cells (Suparak *et al*., 2005).

The bacterial strains used in this study were *B. pseudomallei* K96243 wild-type and two previously described T3SS mutant strains including *bopC* and *bipB* (Suparak *et al*., 2005; Muangman *et al*., 2011). These mutants were constructed by inactivation of the *B. pseudomallei bopC* and *bipB* genes using the pKNOCK-Km and pKNOCK-Tet plasmid vectors, respectively (Alexeyev, 1999). The mutants were initially isolated, and subsequently maintained, on agar plates containing 400 μg/ml kanamycin and 60 μg/ml tetracycline (Sigma-Aldrich, USA), respectively.

Complementation of *B. pseudomallei bopC* mutant was carried out by insertion of the effector-encoding gene (*bpss 1516*) along with its upstream chaperone (*bpss1517*) under the control of the P*tac* promoter in pME6032. Essentially, *B. pseudomallei* K96243 genomic DNA was used as a template in a PCR with primers 1516cfor (5′-CTGGTACCCG CCCGTATGTAAACCGACC-3′) and 1517crev (5′-GAG AGCTCGCTATGCGGCCTGATTCAAG-3′) to generate a 2,139-bp DNA fragment encoding a full length copy of *bopC* (*bpss1516*) and its upstream chaperone *bpss1517*. The PCR-generated DNA fragment was digested with *Kpn*I and *Sac*I and cloned into the plasmid pME6032 vector (Heeb *et al*., 2002) to yield pBopC-BPSS1517, which was introduced into the *B. pseudomallei bopC* mutant by electroporation. To confirm that the resulting *B. pseudomallei* trans-complemented strain contained the pBopC-BPSS1517, plasmid DNA was extracted and sequenced.

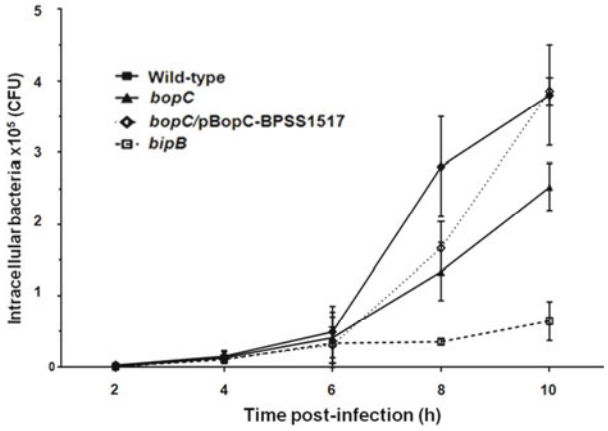

Fig. 1. Intracellular growth of *B. pseudomallei* in J774A.1 macrophage-like cells. *B. pseudomallei* K96243 wild-type (filled square), *bopC* mutant (filled triangle), *bopC*/pBopC-BPSS1517 (*bopC* mutant harbouring pBopC-BPSS1517; open rhombus) and *bipB* mutant (open square) were used to infect J774A.1 cells. At 4, 6, 8, 10 h post-infection, the infected cells were lysed and released bacteria were plated onto agar plates for enumeration of bacterial loads. Error bars represent standard error of the means for triplicate experiments. Where shown, asterisks indicate statistically significant differences ($P<0.05$, *t*-test) relative to the wild-type strain at each time point.

To assess the role of the BopC effector on the survival and multiplication of *B. pseudomallei* in host cells, bacterial replication within murine macrophage-like cells (J774A.1) was assessed as described previously (Muangsombut *et al*., 2008). J774A.1 cells were obtained from the American Type Culture Collection and cultured in DMEM (Gibco, USA), supplemented with 10% v/v fetal bovine serum (FBS, HyClone, USA) at 37°C in 5% CO_2. The J774A.1 macrophages were infected with the *B. pseudomallei* K96243 wild-type or the mutant strains, including the *bopC* mutant, the trans-complemented *bopC* mutant and the *bipB* mutant, at a multiplicity of infection (MOI) of 2 for 2 h. Infected J774A.1 cell monolayers were overlaid with DMEM medium containing 128 μg/ml gentamicin (Sigma-Aldrich) and 256 μg/ml spectinomycin (Sigma-Aldrich) to kill extracellular bacteria. During infection, the expression of genes encoded by the plasmid pBopC-BPSS1517 was induced by the addition of 0.5 mM Isopropyl-β-D-thiogalactoside (IPTG, Sigma-Aldrich). The infected cell monolayers were subsequently lysed at 2, 4, 6, 8, and 10 h post-infection (p.i.) with 0.1% v/v Triton X-100 (Sigma-Aldrich). The number of intracellular bacteria were quantitated by dilution and plating on tryptic soy agar (Difco Laboratories, USA). Bacterial colony forming units (CFU) were counted after 36–48 h of incubation at 37°C.

A similar number of viable intracellular bacteria was seen at 4 and 6 h p.i. for all *B. pseudomallei* strains, indicating similar levels of survival within J774A.1 macrophage cells at these time points (Fig. 1). In contrast, at 8 and 10 h p.i. the number of viable bacteria for the *bopC* mutant was significantly less than that of the wild-type strain ($P<0.05$, *t*-test). It was found that the *B. pseudomallei bipB* mutant displayed a comparatively lower number of viable bacteria than the *bopC* mutant suggesting that BopC is not the only T3SS effector required for intracellular survival. The impaired survival of the *B. pseudomallei bopC* mutant at 8 and 10 h p.i. in macrophage cells could be restored by the presence of the plasmid pBopC-BPSS1517 encoding the BopC effector (BPSS1516) and its chaperone (BPSS1517). This result indicates a direct role for the *B. pseudomallei* BopC effector in the intracellular survival of *B. pseudomallei*. Growth rates for the *B. pseudomallei bopC* and *bipB* mutants were not significantly different to the wild-type in LB medium (data not shown) suggesting the impaired intracellular survival does not result from an *in vitro* growth defect. As the *B. pseudomallei bopC* mutant was complemented with a multicopy plasmid, the effect of high expression of the gene insert on the observed phenotype of the complemented strain could not be ruled out.

We proposed that the impaired intracellular survival of *B. pseudomallei bopC* mutant may result from the delayed bacterial escape from endocytic vesicles within the macrophages. The ability of bacteria to escape from endocytic vacuoles before fusion with lysosomes can be monitored by co-staining infected host cells for bacteria and the lysosomal marker LAMP-1. Therefore, J774A.1 macrophage cells were infected with *B. pseudomallei* wild-type or the mutant strains at an MOI of 10. At 2 h p.i., the bacteria were stained red with

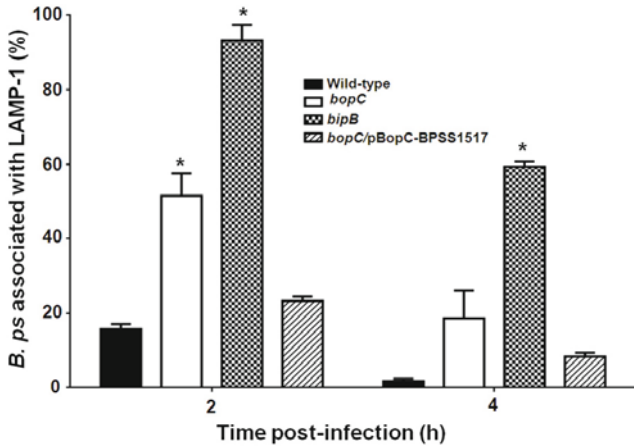

Fig. 2. Quantitative analysis of *B. pseudomallei* co-localization with LAMP-1 in J774A.1 infected cells. *B. pseudomallei* K96243 wild-type (solid bars), *bopC* mutant (open bars), *bopC*/pBopC-BPSS1517 (*bopC* mutant harbouring pBopC-BPSS1517; striped bars) and *bipB* mutant (checkered bars) were used to infect J774A.1 cells. At 2 and 4 h post-infection, the infected macrophage and bacterial cells were stained with anti-LAMP-1 and anti-*B. pseudomallei* antibodies. The percentage of *B. pseudomallei* co-localization with LAMP-1 was calculated by the number of bacteria associated with LAMP-1/total number of intracellular bacteria. Error bars represent standard error of the mean for triplicate experiments. Where shown, asterisks indicate statistically significant differences ($P<0.05$, *t*-est) relative to the wild-type strain at each time point.

rabbit anti-*B. pseudomallei* polyclonal antibody (kindly provided by Prof. R.W. Titball, Exeter University) and anti-rabbit IgG antibody, Cy3 conjugate (α *B. pseudomallei*). LAMP-1 was stained green with rat monoclonal antibody (1D4B) and Alexa Fluor 488 goat anti-rat IgG antibody (α LAMP-1), and nuclei were stained blue with 4′,6-diamidino-2-phenylindole (DAPI). Bacteria were considered LAMP-1 associated if any region of the red fluorescent stained bacterial cells co-localized with the green fluorescent stained LAMP-1 vesicle membrane proteins to merge into a yellow stained region. The percentage of co-localization was calculated by the number of bacterial co-localizations with LAMP-1/total number of intracellular bacteria. By 2 h p.i., most of the *B. pseudomallei* wild-type cells had escaped from the endocytic vesicles; therefore, were infrequently co-localized with LAMP-1 (16.15% co-localization) (Fig. 2). In contrast, the *B. pseudomallei bopC* and *bipB* mutants predominately co-localized with LAMP-1-positive phagosomes. These mutants had 53% and 94% co-localization respectively, demonstrating that they were significantly impaired for escape from endocytic vesicles at this time point ($P<0.05$). The impaired phenotype of the *bopC* mutant could be largely restored to the wild-type level by complementation with plasmid pBopC-BPSS1517 (22.99% co-localization with LAMP-1 at 2 h p.i.). However, at a later time point (4 h p.i.), the number of the *bopC* mutant bacteria that had escaped from endocytic vesicles increased (as judged by a decrease in bacteria associated with the LAMP-1 marker from 53% to 16%) indicating that the mutation of *bopC* did not abolish, but merely delayed, escape of *B. pseudomallei* from endocytic vesicles. The degree of delayed vesicle escape of the *B. pseudomallei bopC* mutant was comparatively less than the *bipB* mutant strain but still demonstrated an impaired intracellular survival. Similar results have also been described for the phenotypes associated with the *B. pseudomallei bopA* effector mutant (Gong *et al.*, 2011) suggesting that each effector has an additive effect to the intracellular survival of *B. pseudomallei*.

B. pseudomallei employs the BimA protein to recruit host actin polymerization within the cytoplasm of infected cells (Stevens *et al.*, 2005). To determine whether the BopC effector is involved in *B. pseudomallei*-induced host actin tail formation, J774A.1 macrophage cells were infected with the *B. pseudomallei* wild-type and mutant strains and evaluated by immunofluorescence staining (Sitthidet *et al.*, 2011). Bacteria were stained green with rabbit anti-*B. pseudomallei* polyclonal antibody and anti-rabbit IgG Alexa Fluor 488, host actin was stained red with Alexa Fluor 568 phalloidin, and nuclei were stained blue with DAPI. Analysis of the stained cells by confocal microscopy revealed that at 8 h p.i., the *B. pseudomallei bopC* mutant exhibited many intracellular bacteria with intense filamentous actin staining proximal to one bacterial pole similar to the wild-type strain (Figs. 3A and 3B). In contrast, at the same time point (8 h p.i.), no such membrane protrusions or actin rearrange-

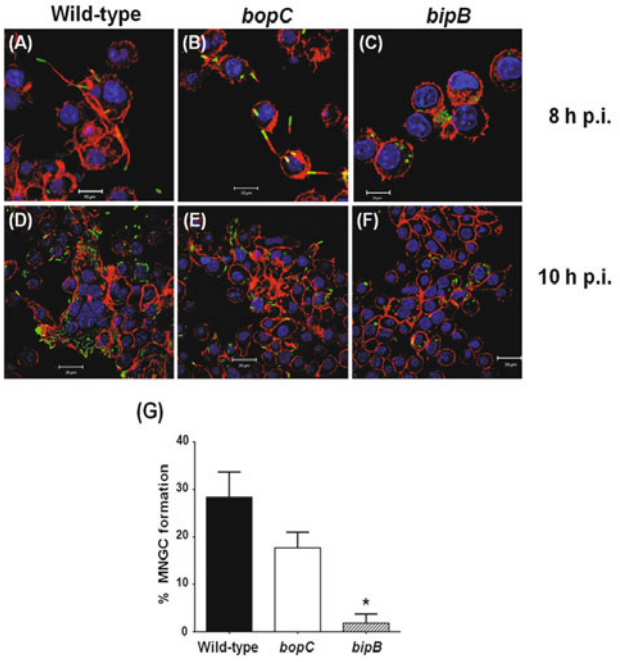

Fig. 3. Actin tail formation and MNGC formation in macrophage cells induced by *B. pseudomallei*. (A-G) Confocal micrographs of J774A.1 macrophage cells infected with *B. pseudomallei* (wild-type), *bopC* mutant (*bopC*), or *bipB* (*bipB*) mutant at an MOI of 10. After 8 h (A to C) and 10 h (D to F) post-infection, the infected macrophages and bacteria were stained and examined by confocal microscopy. Bacteria were stained green with rabbit anti-*B. pseudomallei* polyclonal antibody and anti-rabbit IgG Alexa Fluor 488, host actin was stained red with Alexa Fluor 568 phalloidin, and nuclei were stained blue with DAPI. (G) Quantitation of the percentage of MNGC formation induced by *B. pseudomallei* wild-type (solid bar), *bopC* mutant (open bar), or *bipB* mutant (striped bar). MNGCs were defined as a cell with three or more nuclei per cell. Percentage of MNGC formation was calculated by the number of nuclei within MNGC/total number of nuclei counted. Error bars represent standard error of the means for triplicate experiments. Asterisks indicate statistically significant differences ($P<0.05$, *t*-test) relative to the wild-type strain.

ments were detected in any of the cells infected with the *B. pseudomallei bipB* mutant (Fig. 3C). The exact reason for this *bipB* phenotype is unknown but it is likely to be due to the more pronounced delay in escape of the *bipB* mutant from the phagosome. Taken together, these results suggest that BopC is not involved in *B. pseudomallei*-induced host actin polymerization.

In addition to the ability to induce host actin polymerization, *B. pseudomalle* is able to induce fusion of infected host cells, leading to MNGC formation (Kespichayawattana *et al*., 2000). Thus, we assessed whether the impaired intracellular survival and delayed escape from endocytic vesicles of the *B. pseudomallei bopC* mutant had an effect on MNGC formation. J774A.1 macrophage cells were infected with *B. pseudomallei*, and subjected to immuno-fluorescence staining. At 8 h p.i., MNGC formation was rarely observed in macrophage cell monolayers infected with the *B. pseudomallei bopC* or *bipB* mutants (Figs. 3B and 3C). However, at 10 h p.i., MNGCs containing numerous bacilli could be readily observed in the *B. pseudomallei* wild-type and the *bopC* mutant (Figs. 3D and 3E), but no MNGCs were observed in macrophages infected with the *bipB* mutant (Fig. 3F). To further investigate the difference in MNGC formation, we enumerated the percentage of MNGC formation according to previously described methods (Kespichayawattana *et al*., 2000). We found that at 10 h p.i., the percentage of MNGC formation in the *B. pseudomalle bopC* mutant was slightly lower (18%) than the wild-type strain (28%), but this was not statistically significant (Fig. 3G). These data suggest that BopC is not directly involved in MNGC formation; however, the delayed phagocytic escape of the *bopC* mutant may still affect the kinetics of the MNGC formation. Such a phenotype and its significance for virulence would be challenging to assess. In comparison, the percentage of MNGC formation of cells infected with the *bipB* mutant (1.5%) was significantly lower than both the *bopC* mutant and the wild-type strains (Fig. 3G). These data are consistent with the observation that the *bopC* mutant is less affected in its ability to escape from phagocytic vesicles than the *bipB* mutant. Thus, in this work we demonstrated that, in addition to its earlier described role in epithelial cell invasion, the BopC effector of *B. pseudomallei* contributes to the rapid Bsa-T3SS-dependent escape of bacteria from phagocytic vesicles. This is consistent with the notion that multiple effector proteins (BopA, BopC, BopE and possibly several others), delivered through the Bsa secretion pathway, additively contribute to different aspects of the intracellular lifestyle of *B. pseudomallei*. Defects associated with each of the individual effector mutations are less pronounced than those observed for the T3SS apparatus *bsa* or *bip* mutants; however, concerted actions of effectors sustain each of the Bsa-associated phenotypes. It is important to identify the complete repertoire of Bsa secretion effectors and to assess the molecular mechanisms underlying their activities.

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