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Construction of a reporter system to study *Burkholderia mallei* type III secretion and identification of the BopA effector protein function in intracellular survival

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Summary *Burkholderia mallei*, the aetiological agent of glanders disease, is a Gram-negative facultative intracellular bacterium. Despite numerous studies, the detailed mechanism of its pathogenesis is almost unknown. The presence of a type III secretion system (TTSS) is one of the known mechanisms associated with virulence. An intact TTSS indicates that *B. mallei* is able to secrete proteins in response to different environmental conditions, which could play an important role in pathogenesis. Therefore, characterization of the TTSS and identification of the secreted proteins associated with bacterial pathogenesis could provide crucial information for the development of a candidate vaccine. In the current study, we used an enzymatic reporter system to establish some of the conditions enabling TTS. Construction of the TTSS *bopA* mutant revealed that BopA is important for *B. mallei* invasion and intracellular survival. Overall, our study elucidates how BopA can aid in the optimization of TTS and defines the function of TTS effectors in bacterial intracellular survival and invasion.

1. Introduction

Burkholderia mallei, the aetiological agent of glanders disease, is a Gram-negative, capsulated, non-motile, facultative intracellular bacterium. Glanders commonly infects horses, which are considered to be the natural reservoir for infection, although mules and donkeys are also susceptible.[1] Direct contact with the skin can lead to a localized cutaneous infection. Inhalation of aerosol or dust containing *B. mallei* can lead to septicaemic, pulmonary, or chronic infections of the muscle, liver and spleen. The use of *B. mallei* as a biological warfare weapon has been reported and is presently on the Centers for Disease Control and Prevention select agent list.[2,3] Despite the history and highly infective nature of *B. mallei*, as well as the potential for use as a biological warfare weapon, research into *B. mallei* remains limited. A comparative analysis of *B. mallei* with virulence-associated genes found in pathogenic bacteria has revealed the possession of multiple secretion systems.[4] The type III secretion system (TTSS) is one of at least five different types of protein secretion employed by Gram-negative bacteria to transport proteins from the cytoplasm to either the host cytosol or external milieu. While the TTS apparatus itself is substantially conserved, each bacterial species has effector molecules and subsequent biochemical activities unique to that species. For example, intracellular pathogens such as *Salmonella* and *Shigella* use a TTSS for invasion of, and/or multiplication within, host cells.[5–8] *Yersinia* species employ a TTSS to resist the uptake of bacteria by phagocytic cells.[9] In enteropathogenic *Escherichia coli* (EPEC), a TTSS

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Table 1 Bacterial strains and plasmids used in this study

Strain	Relevant characteristics[a]	Reference/source
Escherichia coli		
E2348/69	Prototype enteropathogenic *E. coli*	Levine et al.[21]
CVD 452	*escN* mutant of E2348/69	Jarvis et al.[22]
86-24	Prototype enterohaemorrhagic *E. coli*	Tarr et al.[23]
SM10 (λ*pir*)	*thi thr leuB tonA lacY supE recA*::RP4-2-Tc::Mu-Km, Km^r	Simon et al.[24]
Burkholderia mallei		
ATCC 23344	1944 human isolate, Pxb^r	BEI Resources/American Type Culture Collection
GCW006	ATCC 23344:pGCW03, Gm^r	This study
GCW005	ATCC 23344:pGCW02; *bopA*$^-$, Gm^r	This study
Plasmids		
pGY100	Full length *yplA* in pTM100 vector, Tc^r	Young and Young[25]
pGY661	C-terminal *yplA* in pTM100 vector, Tc^r	Young and Young[25]
pGEM-T	Easy PCR cloning vector, Ap^r	Promega
pGSV3	Mobilizable Gm^r suicide vector	DeShazer et al.[13]
pGCW01	pGY661 with *BglII* 653 bp *bopA* N-terminal, Tc^r	This study
pGCW02	ATCC 23344 *bopA* internal 300 bp amplimer in pGSV3, Gm^r	This study
pGCW03	*EcoRI* digested *bopA-yplA* from pGCW01 in pGSV3, Gm^r	This study

[a] Ap^r: ampicillin; Tc^r: tetracycline; Km^r: kanamycin; Pxb^r: polymyxin B; Gm^r: gentamicin.

remodels the cytoskeleton of enterocytes, leading to the formation of a pedestal underneath the bacterium.[10]

In silico analysis has shown the presence of a TTSS in *B. mallei*, having a genetic similarity to the *B. pseudomallei* animal pathogen-like TTSS locus, as well as both *Salmonella inv/spa/prg* and *Shigella ipa/mxi/spa* systems.[11,12] *Burkholderia mallei* TTSS genes (two regions; animal-like and plant-like TTSS) are clustered in a distinct region of chromosome 2 sharing homology with TTSS genes from other organisms encoding proteins associated with the assembly of the secretion apparatus and its regulation, as well as effector proteins and their chaperones (as reviewed by Whitlock et al.[4]). Currently, findings from studies of the identification of the virulence mechanisms utilized by *B. mallei* in an animal model of infection (BALB/c mice) have been limited to an extracellular capsule and a TTSS.[13,14] Recent studies have shown the ability of *B. mallei* to invade murine J774.2 macrophages, escape into the host cell cytoplasm, replicate intracellularly, and induce actin polymerization.[15,16] Polar mutations in the *B. mallei* ATCC 23344 animal pathogen-like TTSS have demonstrated its requirement for intracellular survival in murine J774.2 macrophages, as disruption of this TTSS reduces intracellular replication, prevents phagosomal escape into the host cell cytoplasm and inhibits membrane protrusion, although the identity of the putative effector molecules involved in these phenotypes remains unknown.[15]

The identification and function of TTSS proteins belonging to *B. mallei* remain largely unknown, with the exception of genetic homology correlates. One of the effectors secreted via TTSS by intracellular *Shigella*, namely IcsB, has recently been shown to play a role in intracellular replication.[17,18] IcsB shares some sequence similarity with *B. mallei* BopA, though the role of BopA in *Burkholderia* infection is unknown.[19] *Burkholderia pseudomallei* mutants lacking the putative effectors BopA and BopB show a significant delay in time-to-death when challenged by the intraperitoneal route, demonstrating the potential for TTSS effectors to serve as vaccine candidates.[20] The aim of the current study was to construct a reporter system to examine the secretion properties of *B. mallei* and to identify the function of the BopA protein during its intracellular survival and replication.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* was grown on Luria–Bertani (LB) agar or in LB broth at 37ºC and *B. mallei* strains were grown at 37ºC on LB agar or LB broth containing 4% glycerol (LBG). *Burkholderia mallei* strain ATCC 23344 was obtained from BEI Resources (Manassas, VA, USA). When appropriate, antibiotics were added at the following concentrations: 15 μg/ml of polymyxin B (PxB) and 5 μg/ml of gentamicin (Gm) for *B. mallei* and 100 μg/ml of ampicillin (Ap) and 25 μg/ml of tetracycline (Tc) for *E. coli*. Experiments were conducted in the UTMB Keiller BSL-3 facility.

2.2. DNA manipulations

Plasmid DNA was isolated using QIAGEN QIAprep (Valencia, CA, USA) plasmid preparation kit from 3 ml of overnight bacterial culture. Reagents were used according to the manufacturers' protocols. Plasmids were introduced into clinical isolates of *E. coli* by electroporation as described by Dower et al.[26] Restriction endonuclease analyses, ligation and transformation of plasmid DNA were performed following standard methods.[27] *Burkholderia mallei* DNA transfer was accomplished using conjugation methods as previously described.[28]

2.3. Construction of bopA-'yplA fusion plasmid and the Burkholderia mallei bopA-'yplA reporter strain

We used a novel reporter system approach to define the secretion properties of *B. mallei*. The reporter system was constructed as part of the transposon mTn-*'yplA*, which carries a reporter of protein secretion (S.M. Warren et al., unpublished data) consisting of the phospholipase activity domain of YplA from *Y. enterocolitica*.[29] Because YplA