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A disruption of *ctpA* encoding carboxy-terminal protease attenuates *Burkholderia mallei* and induces partial protection in CD1 mice

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ABSTRACT

Burkholderia mallei is the etiologic agent of glanders in solipeds (horses, mules and donkeys), and incidentally in carnivores and humans. Little is known about the molecular mechanisms of *B. mallei* pathogenesis. The putative carboxy-terminal processing protease (CtpA) of *B. mallei* is a member of a novel family of endoproteases involved in the maturation of proteins destined for the cell envelope. All species and isolates of *Burkholderia* carry a highly conserved copy of *ctpA*. We studied the involvement of CtpA on growth, cell morphology, persistence, and pathogenicity of *B. mallei*. A sucrose-resistant strain of *B. mallei* was constructed by deleting a major portion of the *sacB* gene of the wild type strain ATCC 23344 by gene replacement, and designated as strain 23344Δ*sacB*. A portion of the *ctpA* gene (encoding CtpA) of strain 23344Δ*sacB* was deleted by gene replacement to generate strain 23344Δ*sacB*Δ*ctpA*. In contrast to the wild type ATCC 23344 or the *sacB* mutant 23344Δ*sacB*, the *ctpA* mutant 23344Δ*sacB*Δ*ctpA* displayed altered cell morphologies with partially or fully disintegrated cell envelopes. Furthermore, relative to the wild type, the *ctpA* mutant displayed slower growth *in vitro* and less ability to survive in J774.2 murine macrophages. The expression of mRNA of *adtA*, the gene downstream of *ctpA* was similar among the three strains suggesting that disruption of *ctpA* did not induce any polar effects. As with the wild type or the *sacB* mutant, the *ctpA* mutant exhibited a dose-dependent lethality when inoculated intraperitoneally into CD1 mice. The CD1 mice inoculated with a non-lethal dose of the *ctpA* mutant produced specific serum immunoglobulins IgG1 and IgG2a and were partially protected against challenge with wild type *B. mallei* ATCC 23344. These findings suggest that CtpA regulates *in vitro* growth, cell morphology and intracellular survival of *B. mallei*, and a *ctpA* mutant protects CD1 mice against glanders.
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1. Introduction

Burkholderia mallei, the causative agent of glanders, is a Gram-negative, aerobic bacillus. Primarily a disease of solipeds, glanders is generally confined to equines (horses, mules and donkeys) [1,2]. Glanders in solipeds presents as a chronic or as an acute disease. The chronic form occurs either as a pulmonary disease, an upper respiratory disease, or a cutaneous disease. The symptoms of the acute form of the disease include high temperature, depression, shortness of breath, diarrhea, and rapid weight loss with mortality [1,2]. Death may occur after a few weeks from an acute infection, whereas the chronic form of glanders may persist for years and may end in death. Nearly 90% *B. mallei* infections in horses are latent [37], and chronically infected horses serve as reservoirs for transmitting the disease to uninfected animals [3]. *B. mallei* is an obligate animal pathogen that also can infect mice, guinea pigs, rabbits, monkeys, hamsters, and carnivores including lions and dogs that consume contaminated meat [2,4].

In humans, glanders is primarily an occupational disease that affects individuals who have close contact with infected animals [1,2,5]. Infection results primarily from exposure to affected animals via wounds, abrasions or mucous membranes. Glanders in humans is almost always fatal unless treated with antibiotics [33,36,37]. *B. mallei* is a category-B biological agent because it is highly infectious by aerosol [34,35], resulting in a debilitating acute disease in humans [6].

Relatively little is known about the mechanisms of *B. mallei* pathogenesis. Previous reports indicate that a capsular polysaccharide [7], a type III secretion system [8], a quorum sensing

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network [9], and a type VI secretion system [10] are important for the virulence of this bacterium. Molecular characterization of the *in vivo* survival process (or pathogenicity) of *B. mallei* is important because it would provide guidance for the development of measures for the prevention and control of glanders. Information on the effective treatment of infections due to this organism with antibiotic therapy is sparse. Currently, there is no effective animal or human vaccine that provides protection against infection with *B. mallei*.

Inactivation of individual genes by allelic exchange or transposon mutagenesis has been the most precise way of elucidating the specific roles of genes in the pathogenicity of a bacterium. *B. mallei* strain SR1 was previously used as the platform in making genetically-defined deletion mutants of this bacterium [10,11]. Strain SR1 was resistant to sucrose, a feature that was used as a non-antibiotic selection marker in these genetic manipulations. Strain SR1 harbors an IS*407*A-mediated deletion of a 78-kb region of chromosome II encompassing the *sacB* gene [12]. In this communication, we report the successful construction of an isogenic Δ*sacB* strain of *B. mallei* that can also be used as a platform to delete targeted genes. This new strain is sucrose-resistant due to a deletion that specifically removes only the *sacB* gene, leaving the adjacent genes intact.

The proteins destined for extracytoplasmic locations are initially synthesized as precursor forms and processed into mature forms by proteolytic cleavage to remove short peptide sequences, near either the amino terminus or the carboxyl terminus. The endoproteases responsible for cleaving of amino-terminal peptides are called signal peptidases or amino-terminal processing proteases and have been well characterized [13]. A relatively new class of endoproteases with carboxyl-terminal processing activities has been identified from higher plants, algae, and bacteria [14–18]. These carboxyl-terminal proteases (Ctp) are serine proteases that utilize a Ser/Lys catalytic dyad instead of the well-known Ser/His/Asp catalytic triad [19].

The Ctp of *Escherichia coli* is responsible for cleavage of C-terminal 11 amino acid residues of the precursor penicillin-binding protein-3 (PBP-3) [20,21]. A Ctp mutant *E. coli* strain that was defective in PBP-3 processing exhibited filamentous cell morphology, in contrast to the cocco-bacillus morphology of the wild type *E. coli*. These observations confirmed the role of Ctp on PBP-3 processing and thereby on cell morphology of *E. coli* [20,21]. The Ctp protein is also believed to be involved in protection of the bacterium from thermal and osmotic stresses [20,21] and degradation of certain aberrant cellular proteins [22]. In *Borrelia burgdorferi*, Ctp is involved in up- or down-regulation of protein expression [14,16,21]. The putative Ctp protein of *Brucella suis* influences cell morphology, salt-sensitive growth, and *in vitro* and *in vivo* persistence [23]. The genome of *B. mallei* strain ATCC 23344 carries a gene, BMA3209, which shares up to 66% identity at the amino acid level with the Ctps of other bacteria. This putative protein is designated as CtpA. Using isogenic mutant strains, we report the influence of CtpA on growth, cell morphology, *in vitro* persistence, and *in vivo* pathogenicity of *B. mallei*. In addition, we report the successful use of a CD1 mouse model to study the pathogenicity of *B. mallei*.

2. Results

2.1. Nucleotide and protein sequence of ctpA

The ctpA gene (BMA3209) is 1574-bp long and located on chromosome I of *B. mallei* strain ATCC 23344 (GenBank accession NC_006348). Just upstream of *ctpA* is *gpmA* (BMA3208) encoding an enzyme catalyzing the interconversion of 2-phosphoglycerate to 3-phosphoglycerate. Downstream of *ctpA* is a gene (BMA3210) encoding an ATP-dependent adenylate transferase (*adtA*), involved in transfer of adenyl moiety to the MoeD subunit of molybdopterin synthase. The DNA sequence analyses predicted that CtpA has a 29 amino acid long N-terminal signal sequence (signal peptide probability: 1.00; maximum cleavage site probability: 0.424 between amino acid positions 29 and 30). The predicted subcellular localization of the putative CtpA protein is the periplasmic space (Reliability Index: RI = 4; Expected Accuracy = 92%). At the amino acid level, *B. mallei* CtpA shared 100% identity with *B. pseudomallei* putative CtpA, 98% identity with *B. thailandensis* CtpA, and up to 92% identity with the putative CtpA of other *Burkholderia* species, including *B. vietnamiensis*, *B. cepacia*, *B. cenocepacia*, and *B. dolosa*. Furthermore, *B. mallei* CtpA also shared considerable identity with the putative CtpAs of other bacterial species including *Brucella*, *Ralstonia*, *Polaromonas*, *Methylibium*, *Bordetella*, *Neisseria*, *Pseudomonas*, and *Legionella*.

2.2. Genomic characterization of sacB and ctpA mutants of B. mallei

A Δ*sacB* mutant strain of *B. mallei* was constructed by disrupting the *sacB* gene of wild type strain ATCC 23344, and designated as 23344Δ*sacB*. A PCR assay with the primer pair Sac5/Sac8 (see Section 5) produced a predicted 3.3-kb amplicon from the wild type *B. mallei* strain ATCC 23344, and an approximately 2.0-kb product from the *sacB* mutant strain 23344Δ*sacB* (data not shown), indicating that due to a homologous recombination event, a 1243-bp region was deleted from the *sacB* gene.

A Δ*ctpA* mutant strain of *B. mallei* was constructed by disrupting the *ctpA* gene of the mutant 23344Δ*sacB*, and designated as 23344Δ*sacB*Δ*ctpA*. A PCR assay with the primer pair CtpA-1/CtpA-4 (see Section 5) produced a predicted 1.3-kb amplicon from wild type *B. mallei*, and an approximately 1.0-kb amplicon from the *ctpA* mutant strain 23344Δ*sacB*Δ*ctpA* (data not shown), indicating that due to homologous recombination event, a 299-bp region was deleted from the *ctpA* gene. Sequencing data suggest that due to the recombination event, the *ctpA* mutant lost 299-bp region in the middle of the *ctpA* gene. In addition, this recombination event produced a frame-shift at the 3′ end of the deletion site, but no stop codons were introduced as a result of this event. While the wild type, the *sacB* mutant, and the *ctpA* mutant were resistant to polymyxin and sensitive to gentamicin, both mutants were resistant to sucrose.

Reverse-transcription (RT)-PCR results suggest that both the wild type and the *sacB* mutant expressed a full-length *ctpA* mRNA (lanes 1 and 2 of Fig. 1A). As a result of the homologous recombination event in the *ctpA* gene, the *ctpA* mutant expressed a *ctpA* mRNA that was nearly 0.3-kb shorter than the full-length mRNA (lane 3 of Fig. 1A). In order to characterize any polar effect

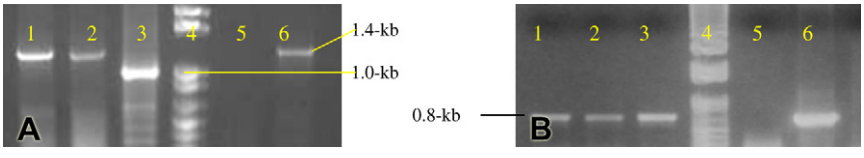

Fig. 1. Expression of *ctpA* (A) and *adtA* (B) mRNA by *B. mallei* strains as determined by reverse-transcription PCR. For PCR assays, cDNA from the wild type (lane-1), *sacB* mutant (lane-2), or *ctpA* mutant (lane-3) was used. Genomic DNA from the wild type (lane-6), and DNase-digested genomic DNA from the wild type (lane-5) were used as controls in PCR. Primers AdtA-Forward and AdtA-reverse were used in PCR with *adtA*, whereas, CtpA-1 and CtpA-4 were used with *ctpA*. Lane-M represents 1-kb molecular size marker.