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Proteomic analysis of colony morphology variants of *Burkholderia pseudomallei* defines a role for the arginine deiminase system in bacterial survival

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ABSTRACT

Colony morphology variation of *Burkholderia pseudomallei* is a notable feature of a proportion of primary clinical cultures from patients with melioidosis. Here, we examined the hypothesis that colony morphology switching results in phenotypic changes associated with enhanced survival under adverse conditions. We generated isogenic colony morphology types II and III from *B. pseudomallei* strain 153 type I, and compared their protein expression profiles using 2D gel electrophoresis. Numerous proteins were differentially expressed, the most prominent of which were flagellin, arginine deiminase (AD) and carbamate kinase (CK), which were over-expressed in isogenic types II and III compared with parental type I. AD and CK (encoded by *arcA* and *arcC*) are components of the arginine deiminase system (ADS) which facilitates acid tolerance. Reverse transcriptase PCR of *arcA* and *arcC* mRNA expression confirmed the proteomic results. Transcripts of parental type I strain 153 *arcA* and *arcC* were increased in the presence of arginine, in a low oxygen concentration and in acid. Comparison of wild type with *arcA* and *arcC* defective mutants demonstrated that the *B. pseudomallei* ADS was associated with survival in acid, but did not appear to play a role in intracellular survival or replication within the mouse macrophage cell line J774A.1. These data provide novel insights into proteomic alterations that occur during the complex process of morphotype switching, and lend support to the idea that this is associated with a fitness advantage in vivo.

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1. Introduction

Burkholderia pseudomallei is an environmental Gram-negative bacillus and the cause of melioidosis, a life-threatening infection endemic to Southeast Asia and Northern Australia [1,2]. Melioidosis presents with a broad clinical spectrum ranging from mild localized infection to rapidly fatal septicemia. The more severe end of the spectrum is common, and infection is associated with a mortality rate of around 40% in northeast Thailand where the majority of cases are reported. Notable features relating to treatment include a slow response to antimicrobial drugs, the need for prolonged antimicrobial therapy, and relapse despite apparently adequate antibiotic treatment [1–3].

The colony morphology of *B. pseudomallei* on Ashdown agar (a selective medium used by laboratories that isolate this organism on a regular basis) is usually purple, wrinkled, and likened to a cornflower head. This appearance has been classified as type I, with the description of a further six less common types (types II to VII) during a study of primary plates from diagnostic cultures [4]. This observation can be explained by the process of colony morphology switching, a reversible event in which a given colony type can switch to an alternative type. Previously, we demonstrated that reversible alterations in *B. pseudomallei* morphotype were associated with reproducible and reversible changes in bacterial length, production of extracellular enzymes, biofilm formation, and flagella production [4]. We have proposed that such changes reflect a mechanism by which *B. pseudomallei* can survive unfavorable conditions. Although most likely to have evolved in response to survival in the environment, this may have important implications for host–pathogen interactions and persistence in the human host following infection.

Evidence for the role of *B. pseudomallei* colony morphology switching in disease comes from both animal and in vitro models. In an experimental mouse model, type II appeared to become adapted for persistence, and type III was associated with switching to type I or II [4]. Colony morphology switching has also been reported to occur after uptake of *B. pseudomallei* by macrophages in vitro, with a difference in the rate of bacterial replication between the different morphotypes after uptake [4,5].

The objective of this study was to expand on the number of proteins known to undergo differential expression during colony morphology switching, and to provide proof of concept that one or more of these proteins are involved in survival under adverse conditions. We generated isogenic types II and III from parental type I using starvation conditions, and compared protein expression by these three types using 2D gel electrophoresis. Numerous proteins were observed to be differentially expressed, the most prominent of which were flagellin, arginine deiminase (AD) and carbamate kinase (CK), which were over-expressed in isogenic types II and III compared with parental type I. AD and CK (encoded by *arcA* and *arcC*) are components of the arginine deiminase system (ADS) that catalyzes the metabolism of arginine to ornithine, ammonia, and CO_2 with the generation of ATP [6]. We hypothesized that the ADS facilitated adaptation and survival in adverse environments. We examined this question using transcript analysis of *arcA* and *arcC*, and by comparing survival of a parental strain versus mutants defective in each of these two genes after exposure to acid and following uptake by macrophages in vitro.

2. Materials and methods

2.1. *B. pseudomallei* strains and isolation of isogenic morphotypes

B. pseudomallei strain 153 type I was isolated from the blood of a patient admitted to Sappasithiprasong hospital, Ubon Ratchathani, Thailand in 2002. Two isogenic variants defined on the basis of colony morphology appearance as types II and III were obtained from the parental type I isolate using nutritional limitation, as described previously [4]. In brief, a single colony of type I was cultured in trypticase soy broth (TSB) at 37 °C under static conditions in air for 21 days. Serial dilutions were spread plated onto Ashdown agar, incubated at 37 °C in air for 4 days and the colony morphologies classified using a morphotyping algorithm described previously [4]. Isogenic types II and III were harvested and stored in TSB containing 20% glycerol at −80 °C, (a condition under which colony morphotype is stable) until use. The three isogenic types had identical banding patterns by pulsed-field gel electrophoresis (PFGE) using *SpeI* restriction enzyme digestion [4].

2.2. Protein extraction

Protein extraction was performed on two separate occasions for the three isogenic morphotypes. A single colony of *B. pseudomallei* picked from Ashdown agar was cultured in 100 ml of Luria-Bertani (LB) broth at 37 °C with shaking at 200 rpm for 18 h. Bacteria were centrifuged at 4500×*g* for 30 min at 4 °C, washed once with cold PBS and then resuspended in 1 ml of a cold lysis buffer containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF) [7]. The suspension was sonicated on ice at 22% amplitude at 1 second pulse intervals for 3 min, and the lysate centrifuged at 14,000×*g* for 3 min at 4 °C. The supernatant was collected and sterilized by passage through a 0.2 μm filter and stored at −80 °C until use.

2.3. Two-dimensional (2D) gel electrophoresis

Protein samples were cleaned using the 2D clean-up kit (GE Healthcare Bio-Sciences), and the concentration determined with the 2D quantification kit (GE Healthcare Bio-Sciences) using BSA as a standard. Seven hundred micrograms of protein was mixed with rehydration buffer (8 M Urea, 2% CHAPS, 60 mM DTT, 0.5% IPG buffer pH 4–7 and 0.002% bromophenol blue) to a total volume of 340 μl per immobiline dry strip. A 18-cm IPG strip pH 4–7 was rehydrated with this sample solution for 12 h at room temperature. Isoelectric focusing (IEF) was performed using an Ettan IPGphor 3 isoelectric focusing system (GE Healthcare Bio-sciences) at 20 °C. The running steps were set as follows: step 1: 500 V for 1 h, step 2: 1000 V for 1 h, step 3: 8000 V for 3 h and step 4: 8000 V for 1.30 h. On completion, the strip was equilibrated in 5 ml