CONSTRUCTION AND CHARACTERIZATION OF AN UNMARKED aroC DELETION MUTANT OF BURKHOLDERIA PSEUDOMALLEI STRAIN A2

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Abstract. Using a sucrose counter-selection strategy, we have constructed an unmarked aroC deletion mutant of B. pseudomallei strain A2 in order to investigate the possibility of an effective live-attenuated vaccine. The aroC deletion in the resultant mutant, designated A2ΔaroC, was confirmed by PCR, Southern hybridization and failure of the mutant to grow in a defined medium without aromatic compounds. Compared to the parental wild type strain, A2ΔaroC was highly attenuated for virulence following intraperitoneal introduction into BALB/c and C57BL/6 mice. BALB/c mice immunized intraperitoneally with A2ΔaroC were not protected against a challenge dose of 500 cfu (25 x LD50) of the parental strain A2, whereas C57BL/6 mice similarly immunized intraperitoneally with A2ΔaroC were significantly protected against a challenge dose of 6,000 cfu (20 x LD50).

INTRODUCTION

Burkholderia pseudomallei, a facultative intracellular bacterium, is the causative agent of melioidosis, a life-threatening disease affecting both human and animals (Wiersinga et al., 2006). This bacterium is widely distributed in the soil and surface water of endemic regions such as Southeast Asia and northern Australia. However, active cases have also been reported from many areas where the disease is not endemic including southern China, Taiwan, and the south of India (Dance, 2000; White, 2003). The organism is also currently considered to have potential in germ warfare and is regarded as a potential bioterrorist weapon (Jeddeloh et al, 2003) making it a disease of worldwide concern.

At present, no effective vaccine exists to protect against B. pseudomallei infection. Current approaches under investigation include conjugative, DNA, attenuated and heterologous vaccines (Warawa and Woods, 2002). An attenuated mutant of B. pseudomallei that is auxotrophic for branched-chain amino acids induced protective immunity in a murine model of melioidosis (Atkins et al., 2002). Lipopolysaccharide (LPS) or conjugates of LPS and flagellin have been evaluated as vaccine against melioidosis (Brett and Woods, 2000). However, the degree of
antigenic variation that occurs between different strains of *B. pseudomallei* suggests that a vaccine based on a single form of surface antigen may not induce protection against all strains. In addition, mice immunized with a *B. pseudomallei* bipD mutant were partially protected against subsequent challenge with wild type *B. pseudomallei* (Stevens *et al.*, 2004).

Rational attenuation of bacteria pathogens for vaccine applications is to mutate a gene or genes encoding key enzymes in a biosynthetic pathway that is essential for *in vivo* growth and survival of the organism. The *aro* genes are required in the biosynthesis of aromatic amino acids and various key metabolic compounds. Bacterial *aro* mutants are auxotrophic for certain aromatic compounds, such as tryptophan, tyrosine and phenylalanine, as well as for ρ-aminobenzoic acid and 2,3-dihydroxybenzoic acid. This *aro* pathway is not present in mammalian cells, meaning that some of these compounds are not available at sufficient levels to complement the requirement of an auxotroph. Mutation in *aroC* gene, encoding chorismate synthase that catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate to chorismate, the final branch-point intermediate of the “common” *aro* pathway, has been shown to be attenuating in *Brucella suis* and *Salmonella typhi* (Lowe *et al.*, 1999; Foulongne *et al.*, 2001).

In this report, we describe the cloning of *B. pseudomallei* *aroC* gene and the construction and characterization of an unmarked *aroC* mutant of *B. pseudomallei* strain A2 in two mouse models for the intended purpose of developing a melioidosis vaccine.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, media and growth conditions**

Wild-type *B. pseudomallei* strain A2 was isolated from a melioidosis patient at Srinakarind Hospital, Khon Kaen, Thailand. *Escherichia coli* and *Burkholderia* strains were cultured at 37°C in 2YT broth or on 2YT agar. To test the auxotrophy for aromatic amino acids, *B. pseudomallei* *aroC* mutant was grown in M9 minimal medium. When added to medium, the “aromix” supplementation consisted of phenylalanine, tryptophan and tyrosine at a final concentration of 40 µg ml\(^{-1}\) and ρ-aminobenzoic acid and 2,3-dihydroxybenzoic acid at 10 µg ml\(^{-1}\). When required, antibiotics were added at the following concentrations: ampicillin 100 µg ml\(^{-1}\); tetracycline 12 µg ml\(^{-1}\); streptomycin 200 µg ml\(^{-1}\). Sucrose at final concentration of 10% was used when required.

**DNA manipulations**

Unless otherwise stated, DNA manipulations were carried out as described by Sambrook and Russell (2001). Restriction enzymes, *Taq* DNA polymerase, dNTPs, T4 DNA ligase, calf intestine alkaline phosphatase and Klenow enzyme were obtained from either Promega (WI, USA) or New England Biolabs (MA, USA) and the manufacturer’s instructions were followed. Chromosomal DNA of *B. pseudomallei* was isolated by the method of Ausubel *et al.* (1999); plasmid DNA was purified by using kit from Qiagen (Crawley, United Kingdom); and DNA fragments were isolated from agarose gels using the QIAquick gel extraction kit. Agarose gel-electrophoresis was employed to estimate DNA concentration. One kb DNA ladders (500 ng) were routinely included as DNA molecular weight markers. Stained DNA fragments in gels were visualized using a UV transluminator and recorded by Gel Doc 1000 (BioRad Labs, USA).

**Cloning of *B. pseudomallei* *aroC* gene**

*B. pseudomallei* *aroC* gene was cloned by functional complementation of an *E. coli* *aroC* mutant (AB2849) (Pittard and Wallace, 1966). In brief, chromosomal DNA of *B. pseudomallei*
strain A2 was partially digested with Sau3AI. A genomic DNA library consisting of a 3-5 kb Sau3AI fragments were ligated into pUC18 previously digested with BamHI. The ligated products were used to transform E. coli aroC mutant by electroporation using a Gene Pulser® II apparatus (Bio Rad Labs, Richmond, CA) set at 1.8 kV, 25 µF and 200 Ω. Bacteria transformants were incubated for 1 hour with moderate shaking at 37°C. The desired complementing clones were selected on M9 minimal medium agar containing ampicillin.

**Construction of unmarked aroC deletion mutant of B. pseudomallei**

A sucrose counter-selection strategy was used to construct B. pseudomallei aroC mutant without antibiotic resistance marker. In brief, the internal 438 bp XhoI fragment present in the cloned aroC gene (Fig 1) was deleted. The resultant plasmid, pPHE149, was digested with SmaI and HindIII. The 1.8 kb SmaI/HindIII fragment containing the ΔaroC gene was cloned into pEX19Tc replacement vector (generous gift from Prof Herbert Schweizer, Colorado State University, USA), which contains the Bacillus subtilis sacB gene as a counterselectable marker. The resultant plasmid, pPHE150, was transferred into B. pseudomallei A2 by conjugation. Tetracycline- and streptomycin-resistant transconjugants were cultured in 2YT broth, washed and resuspended in phosphate-buffered saline pH 7.4 (PBS). For virulence tests, four groups of five BALB/c mice were injected intraperitoneally (i.p.) with $2 \times 10^4$, $2 \times 10^5$, $2 \times 10^6$ and $2 \times 10^7$ cfu and four groups of five C57BL/6 mice with $5 \times 10^5$, $5 \times 10^6$, $5 \times 10^7$ and $5 \times 10^8$ cfu of the constructed A2ΔaroC mutant in PBS and monitored for up to 30 days. As control, a group of five mice of each strain was injected with sterile PBS. The lethal 50% dose (LD50) value was calculated by the method of Reed and Muench (1938). To assess vaccine efficacy of the A2ΔaroC mutant, three groups of five BALB/c mice and three groups of five C57BL/6 mice were immunized i.p. with two doses of $3 \times 10^7$ cfu and of $5 \times 10^8$ cfu respectively, of the A2ΔaroC mutant at an interval of a week. As control, a group of five mice of each strain was injected with sterile PBS. Two weeks after the second dose, the immunized mice were challenged i.p. with increasing doses of wild type B. pseudomallei A2 (Table 1). The control mice were challenged with the lowest dose of the wild type. Infected mice, which were moribund and considered incapable of survival, were euthanized.
RESULTS

Cloning of *B. pseudomallei* aroC gene

Following electroporation and transformation of *E. coli* AB2849 with the *Sau*3AI DNA library constructed in pUC18, a number of complementing clones were selected. Recombinant plasmids were prepared from five independent colonies, digested with a variety of restriction enzymes and the restriction profiles were analyzed. One recombinant plasmid, designated pPHE146, was chosen for construction of a full restriction map using the published genome sequences of *B. pseudomallei* K96243 as reference (Fig 1). pPHE146 was then used to retransform *E. coli* AB2849 and was found capable of complementing aroC mutation in

![Diagram](image-url)

Fig 1-Construction of an unmarked aroC deletion mutant of *B. pseudomallei* strain A2. The black box represents the cloned *B. pseudomallei* aroC gene and its orientation with respect to the lac promoter in pUC18. The grey box represents the partially deleted aroC gene. A brief description of cloning steps is shown next to the vertical arrows. The portion of pHE146 used as an aroC probe in Southern blotting is indicated. MCS, multiple cloning site.
Construction of unmarked aroC deletion mutant of B. pseudomallei strain A2

Preliminary experiments showed that expression of sacB gene in B. pseudomallei strain A2 produced a mucoid and liquefied colony phenotype on 2YT agar containing 10% sucrose after 48 hours of incubation. Therefore, lack of expression of sacB and the resulting absence of the mucoid phenotype in the presence of sucrose was used as a convenient indicator for allelic exchange and loss of vector sequences containing the sacB gene. Two nonmucoid colonies were isolated from 3,200 colonies screened. Both colonies were sensitive to tetracycline and were auxotrophic for aromix supplement. These colonies should be clones in which chromosomal aroC gene had been replaced by the plasmid-borne ΔaroC gene. The two aromix-dependent colonies were subjected to PCR and Southern analysis to confirm allelic exchange at the aroC locus. As predicted, the PCR amplicon of the parental strain A2 was 878 bp in length, while that of the ΔaroC mutant was 440 bp in length (Fig 2). For Southern analysis of the parental strain A2 and the two ΔaroC mutants, chromosomal DNA was digested with SalI (the SalI site in the native aroC gene was removed by the 438-bp internal XhoI deletion) and following electrophoresis and blotting the membrane was hybridized with aroC probe. Two bands at 1.4 kb and 2.3 kb for the parental strain A2, but only one band at 3.3 kb for the two ΔaroC mutants were obtained as predicted (Fig 3). One ΔaroC mutant, designated A2ΔaroC, was selected for further study.

Attenuation in virulence of A2ΔaroC in mice

All mice survived all the infective doses (data not shown). LD50 values of >2 x 10^7.
**Table 1**

Assessment of vaccine potential of aroC mutant of *B. pseudomallei* strain A2 in BALB/c mice and C57BL/6 mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>First immunizing dose (cfu)</th>
<th>Second immunizing dose (cfu)</th>
<th>Wild type challenge dose (cfu)</th>
<th>Percent survival</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BALB/c mice</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>$3.5 \times 10^7$</td>
<td>$3.0 \times 10^7$</td>
<td>$5 \times 10^4 \ (2,500 \ LD50)$</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>$3.5 \times 10^7$</td>
<td>$3.0 \times 10^7$</td>
<td>$5 \times 10^3 \ (250 \ LD50)$</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>$3.5 \times 10^7$</td>
<td>$3.0 \times 10^7$</td>
<td>$5 \times 10^2 \ (25 \ LD50)$</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>PBS control</td>
<td>PBS control</td>
<td>$5 \times 10^2 \ (25 \ LD50)$</td>
<td>0</td>
</tr>
<tr>
<td><strong>C57Bl/6 mice</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>$5.0 \times 10^8$</td>
<td>$5.0 \times 10^8$</td>
<td>$6 \times 10^5 \ (2,000 \ LD50)$</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>$5.0 \times 10^8$</td>
<td>$5.0 \times 10^8$</td>
<td>$6 \times 10^4 \ (200 \ LD50)$</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>$5.0 \times 10^8$</td>
<td>$5.0 \times 10^8$</td>
<td>$6 \times 10^3 \ (200 \ LD50)$</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>PBS control</td>
<td>PBS control</td>
<td>$6 \times 10^3 \ (200 \ LD50)$</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* cfu, colony forming unit.

cfu per BALB/c mouse and $>5 \times 10^8$ cfu per C57BL/6 mouse for the constructed aroC mutant were obtained compared with LD50 value of 20 cfu and 300 cfu for BALB/c and C57BL/6 respectively, for the parental strain A2. Thus A2ΔaroC was attenuated at least $10^6$-fold. Complementation of A2ΔaroC with pMR4 containing the cloned aroC gene resulted in restoration of virulence as all 5 BALB/c mice injected i.p. with 200 cfu of the complemented A2ΔaroC were dead within 4 days (data not shown).

**Protection against experimental challenge**

BALB/c mice immunized with two doses of A2ΔaroC were not protected against a challenge with the lowest dose of 500 cfu (25 x LD50) of the parental strain A2, whereas C57BL/6 mice immunized with two doses of A2ΔaroC were significantly protected against a challenge of 6,000 cfu (ca. 20 x LD50) (Table 1). All surviving C57Bl/6 mice did not show any signs of disease for up to 5 months post-challenge, indicating a sterile immunity had developed. No control unimmunized mice survived the challenge.

**DISCUSSION**

This study resulted in the cloning of aroC gene, and construction of an unmarked aroC deletion mutant of *B. pseudomallei* strain A2, with vaccine potential in BALB/c and C57Bl/6 mouse models of acute and chronic human melioidosis. The deletion construct was fully defined at the molecular level. The A2ΔaroC mutant was unable to grow in the absence of aromatic amino acid supplementation. The A2ΔaroC was highly attenuated in both BALB/c and C57BL/6 mice. Doses up to $10^8$ cfu of A2ΔaroC could be given i.p. without any apparent adverse reaction.

A2ΔaroC was unable to induce significant protection in BALB/c mice against a challenge dose of 500 cfu of the virulent parental strain. The failure to induce protective immunity could be due to the inability of this mutant to persist *in vivo* long enough to induce protective immunity. A2ΔaroC could not be recovered from a spleen of infected BALB/c mouse at 24 hours post-infection (data not shown). This mouse strain is known to develop preferentially a T helper
cell type 2 immune response against challenge infection with *B. pseudomallei* (Hoppe et al, 1999). However, a single dose of a mutant of *B. pseudomallei* that is auxotrophic for branched-chain amino acids (strain 2D2) induces a complete protection in BALB/c mice against a lower challenge dose of the wild type (Atkins et al, 2002). This 2D2 mutant was found to persist in various mouse tissues for up to 30 days post-challenge. Our constructed A2ΔaroC mutant might be too attenuated or the immunization protocols used in this study were not suitable to induce protective immunity in BABL/c mice. Experiments are under way to determine if nasal inoculation with more than two immunizing doses of A2ΔaroC can protect BALB/c mice against a lower challenge dose.

On the other hand, the A2ΔaroC mutant was able to induce significant protection in C57Bl/6 mice. This mouse strain, when infected by an intracellular pathogen such as *Leishmania major*, shows a protective T helper cell type 1 (Th1) immune response and resistance to the disease (Reiner and Locksley, 1995). Previous study has shown that a Th1-type immune response is associated with resistance to infection with *B. pseudomallei* (Chen et al, 2006).

In summary, the results of this study demonstrated that the constructed unmarked aroC mutant of *B. pseudomallei* is attenuated in both BALB/c and C57Bl/6 mice and is able to confer significant protection in C57Bl/6 mice. Future work is required to determine if this live vaccine candidate can induce protection against heterologous strain challenge.

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**REFERENCES**


