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 A2Δ*aroC*, 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, A2Δ*aroC* was highly attenuated for virulence following intraperitoneal introduction into BALB/c and C57BL/6 mice. BALB/c mice immunized intraperitoneally with A2Δ*aroC* 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 A2Δ*aroC* 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

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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- dihydroxybenzoate. 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 37ºC 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 *Sau*3AI. A genomic DNA library consisting of a 3-5 kb *Sau*3AI fragments were ligated into pUC18 previously digested with *Bam*HI. 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 37ºC. 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 *Xho*I fragment present in the cloned *aroC* gene (Fig 1) was deleted. The resultant plasmid, pPHE149, was digested with *Sma*I and *Hin*dIII. The 1.8 kb *Sma*I/*Hin*dIII fragment containing the Δ*aro*C 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 for 12 hours. Aliquots were then spread onto 2YT agar containing 10% sucrose. After incubation for 48 hours at 37°C, sucrose-resistant, nonmucoid colonies were tested for the tetracycline sensitivity phenotype. The tetracycline sensitive colonies were then tested for auxotrophy for aromatic compounds. The aromatic compound-dependent strain, designated A2Δ*aroC*, was confirmed by PCR analysis with specific primers *aroC*-F and *aroC*-R and by Southern hybridization using the 2.0 kb *Kpn*I fragment containing the whole *aroC* gene as probe. To complement the *aroC* mutant, the fragment containing the cloned *aroC* gene was cloned into pMR4, a broad host range plasmid (provided by Prof Christian Mohr, University of Minnesota, USA) (Mohr *et al*, 2001), and the resultant construct was transformed into the A2Δ*aroC* mutant.

Virulence and vaccination studies in mice

Female BALB/c and C57BL/6 mice (6-8 weeks old) purchased from National Laboratory Animal Center, Mahidol University, Thailand, were used. All animal experiments complied with Khon Kaen University guidelines regarding the use of animals in research. Bacteria were grown to mid-log phase 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 x 10^4, 2 x 10^5, 2 x 10^6 and 2 x 10^7 cfu and four groups of five C57BL/6 mice with 5 x 10^5, 5 x 10^6, 5 x 10^7 and 5 x 10^8 cfu of the constructed A2Δ*aroC* 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 A2Δ*aroC* 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 x 10^7 cfu and of 5 x 10^8 cfu respectively, of the A2Δ*aroC* 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

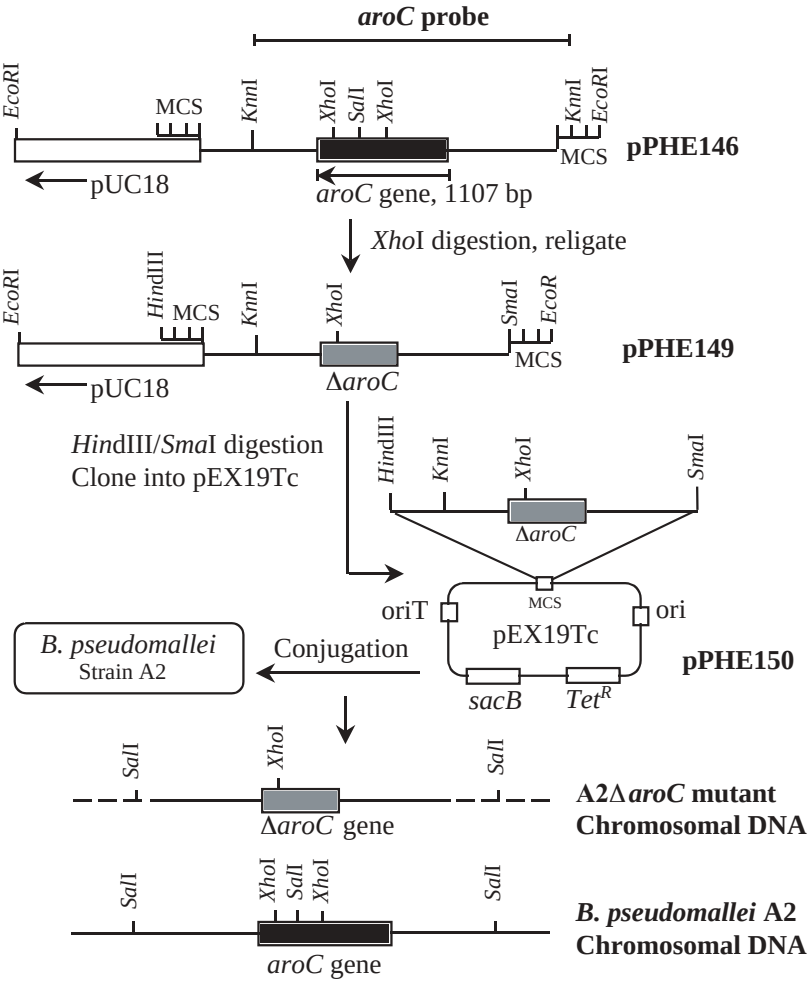

Fig 1-Construction of an unmarked *aroC* deletion mutant of *B. pseudomallei* strain A2. The black box represents the cloned *B. pseudomallei aroC* gene and its orientation with respect to the *lac* promoter in pUC18. The grey box represents the partially deleted *aroC* gene. A brief description of cloning 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.

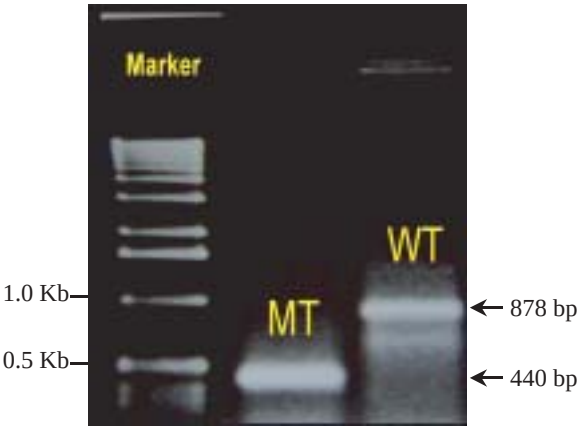

Fig 2-PCR analysis of wild type *B. pseudomallei* strain A2 and constructed *aroC* mutant. Specific primers were used to amplify the *aroC* gene in wild type (WT) and Δ*aroC* gene in the mutant (MT) strain. DNA marker is shown on the left.

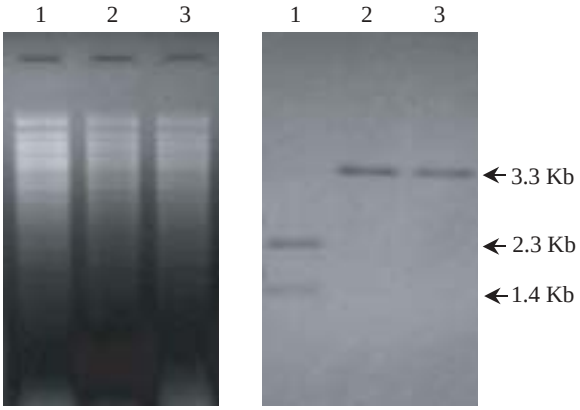

Fig 3-Southern hybridization of genomic DNA from wild type *B. pseudomallei* strain A2 and two independent "aromix" dependent clones. Total cell DNA was digested with *Sal*I and, following electrophoresis (A) and blotting onto a membrane, was hybridized with *Kpn*I fragment (*aroC* probe) from pPHE146 (B). Lane 1, *B. pseudomallei* strain A2; Lane 2, "aromix"-dependent clone no.1; Lane 3, "aromix"-dependent clone no. 2. The positions of *Kpn*I fragments are shown on the right.

E. coli AB2849, indicating that the insert contained functional *B. pseudomallei* A2 *aroC* gene.

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 Δ*aroC* 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 Δ*aroC* mutant was 440 bp in length (Fig 2). For Southern analysis of the parental strain A2 and the two Δ*aroC* mutants, chromosomal DNA was digested with *Sal*I (the *Sal*I site in the native *aroC* gene was removed by the 438-bp internal *Xho*I 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 to only one band at 3.3 kb for the two Δ*aroC* mutants were obtained as predicted (Fig 3). One Δ*aroC* mutant, designated A2Δ*aroC*, was selected for further study.

Attenuation in virulence of A2Δ*aroC* 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.

Group	First immunizing dose (cfu)[a]	Second immunizing dose (cfu)	Wild type challenge dose (cfu)	Percent survival
BALB/c mice				
1	3.5×10^7	3.0×10^7	5×10^4 (2,500 LD50)	0
2	3.5×10^7	3.0×10^7	5×10^3 (250 LD50)	0
3	3.5×10^7	3.0×10^7	5×10^2 (25 LD50)	0
4	PBS control	PBS control	5×10^2 (25 LD50)	0
C57Bl/6 mice				
1	5.0×10^8	5.0×10^8	6×10^5 (2,000 LD50)	20
2	5.0×10^8	5.0×10^8	6×10^4 (200 LD50)	60
3	5.0×10^8	5.0×10^8	6×10^3 (200 LD50)	80
4	PBS control	PBS control	6×10^3 (200 LD50)	0

[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 A2Δ*aroC* was attenuated at least 10^6-fold. Complementation of A2Δ*aroC* 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 A2Δ*aroC* were dead within 4 days (data not shown).

Protection against experimental challenge

BALB/c mice immunized with two doses of A2Δ*aroC* 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 A2Δ*aroC* 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 A2Δ*aroC* mutant was unable to grow in the absence of aromatic amino acid supplementation. The A2Δ*aroC* was highly attenuated in both BALB/c and C57BL/6 mice. Doses up to 10^8 cfu of A2Δ*aroC* could be given i.p. without any apparent adverse reaction.

A2Δ*aroC* 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. A2Δ*aroC* 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 A2Δ*aroC* 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 A2Δ*aroC* can protect BALB/c mice against a lower challenge dose.

On the other hand, the A2Δ*aroC* 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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