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# A Mutant of *Burkholderia pseudomallei*, Auxotrophic in the Branched Chain Amino Acid Biosynthetic Pathway, Is Attenuated and Protective in a Murine Model of Melioidosis

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**Using a transposon mutagenesis approach, we have identified a mutant of *Burkholderia pseudomallei* that is auxotrophic for branched chain amino acids. The transposon was shown to have interrupted the *ilvI* gene encoding the large subunit of the acetolactate synthase enzyme. Compared to the wild type, this mutant was significantly attenuated in a murine model of disease. Mice inoculated intraperitoneally with the auxotrophic mutant, 35 days prior to challenge, were protected against a challenge dose of 6,000 median lethal doses of wild-type *B. pseudomallei*.**

*Burkholderia pseudomallei* is the causative agent of melioidosis, a severe disease of humans and animals (27). Melioidosis can present in a number of forms described as acute septicemic, acute pulmonary, subacute, and chronic disease (5, 8, 20, 27). In some cases a persistent subclinical infection is established with the subsequent ability to become septicemic. The factors influencing the outcome of disease are not known, although it has been suggested that differences in the virulence of different strains (13, 20) might contribute to the clinical outcome of disease. In addition, melioidosis is most frequently seen in diabetics, those with impaired cellular immunity, or those with a history of drug or alcohol abuse, suggesting that differences in the immunological status of the host might also influence the outcome of the disease (8, 32).

Presently no vaccine exists to protect against melioidosis. *B. pseudomallei* is a heterogenous species with different strains displaying various surface antigens. For example, naturally occurring variants lacking the capsule have been reported and have recently been reclassified as a new species, *Burkholderia thailandensis* (7). Similarly, different strains have previously been shown to produce two types of lipopolysaccharide (LPS, termed OPSI and OPSII [3, 31]) and different forms of flagellin have also been reported (39). LPS (8) or conjugates of LPS and flagellin have been evaluated as vaccines against melioidosis (9). 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.

Auxotrophic bacterial mutants have been used to induce protective immunity against wild-type challenge with a number of bacterial pathogens, including *Salmonella enterica* serovar Typhi (15, 18, 30), *Neisseria gonorrhoeae* (10), and *Mycobacterium tuberculosis* (38). The level of attenuation demonstrated in auxotrophic mutants in vivo often dictates the level of protection achieved when they are used as vaccines. Mutations in genes in the shikimate pathway, which is normally required for the generation of aromatic amino acids and other compounds, or mutations in the purine biosynthesis pathway have previously been shown to be attenuating in a range of pathogens (1, 6, 18, 22, 34, 11, 21, 23, 30). An attenuated *B. pseudomallei* purine auxotroph has previously been identified following the exposure of bacteria to UV radiation (23). Mice that had previously been dosed with this mutant were protected against challenge with wild-type *B. pseudomallei* (28). However, the use of radiation as a mutagen introduces the possibility of multiple mutations, and it is therefore difficult to conclusively show that the auxotrophic nature of the mutant was responsible for the attenuation described. Indeed, unlike the case for a purine mutant of *S. enterica* serovar Typhimurium, the virulence of the *B. pseudomallei* mutant was not restored by hypoxanthine, supporting the possibility that additional mutations were present.

In contrast to the aromatic amino acid and purine biosynthetic pathways, the pathway responsible for the synthesis of branched chain amino acids has rarely been targeted for attenuation. However, several studies have reported the attenuation and vaccine efficacy of mutants of *M. tuberculosis* that are unable to synthesize the amino acid leucine (4, 19, 29). It is thought that these mutants cannot survive within mononuclear phagocytes (4) and hence are attenuated in virulence.

Using a transposon mutagenesis approach, we have set out to identify an auxotrophic mutant of *B. pseudomallei* that is attenuated in a murine model of melioidosis. This would then allow us to determine whether a defined genetic mutant can be used to protect mice against challenge with wild-type *B. pseudomallei*.

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**Identification of an auxotrophic mutant.** Throughout this work chemicals and enzymes were obtained from the Sigma-Aldrich Chemical Co. (Poole, Dorset, United Kingdom) unless otherwise stated. *B. pseudomallei* strain 576 was isolated initially from a clinical case of fatal melioidosis in Thailand and was provided by Ty Pitt, Central Public Health Laboratory, Colindale, United Kingdom This strain and others described below were cultured at 37°C on Luria-Bertani (LB) broth media supplemented as required with antibiotics (36). The median lethal dose (MLD) of *B. pseudomallei* strain 576 by the intraperitoneal route in BALB/c mice, calculated by the method of Reed and Muench (33), was 80 CFU. It was calculated on a total of 30 mice, six groups of five, at 5 weeks postinfection.

To generate a bank of transposon mutants, *B. pseudomallei* 576 was transformed by electroporation (2.5 kV, 200 Ω, and 25 μF capacitance) using a Bio-Rad Gene Pulser II (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, United Kingdom) with plasmid pUTminiTn5Km2 (17). Transformed cells were recovered for 3 h at 37°C with shaking (220 rpm) and were then selected on LB agar containing 700 μg of kanamycin/ml. To ensure plasmid DNA loss, randomly selected bacterial mutants were screened by PCR for the loss of the nucleotide sequence encoding the transposase enzyme. No mutants were identified that had the transposase gene sequence indicating loss of the suicide plasmid.

Pools of transposon mutants were assembled into groups of 96, and approximately 500 mutants were screened for their ability to grow on LB agar and inability to grow on minimal M9 media. Mutant 2D2 was selected for further analysis. Six groups of five BALB/c mice were challenged with increasing doses of this mutant ($10^7$ to $10^1$ CFU) and monitored for up to 5 weeks. From this experiment the MLD was calculated as described above to be greater than $10^7$ CFU.

*B. pseudomallei* mutant 2D2 had properties similar in vivo to those of the previously described *B. pseudomallei* purine auxotroph (28). In this reported study the 50% lethal dose of the parent *B. pseudomallei* strain at 6 weeks postinoculation was 80 organisms and that of the purine auxotroph was approximately $10^8$ organisms.

**Identification of the gene interrupted by the transposon.** To determine the nucleotide sequence of the DNA flanking the transposon, DNA was first prepared from *B. pseudomallei* mutant 2D2 (36). The PCR was used (25) to amplify DNA fragments adjacent to the transposon. The nucleotide sequences of the PCR products were determined using a *Taq* Dyedeoxy kit supplied by Applied Biosystems (Warrington, United Kingdom). This sequence was used to search the GenBank database with BLASTX (2). The gene interrupted showed significant sequence identity with the gene encoding the large subunit of the acetolactate synthase enzyme (*ilvI*) from *Neisseria meningitidis*. The nucleotide sequences adjacent to the putative *ilvI* gene in *B. pseudomallei* strain K96243 were identified from the partially completed genome sequence of this strain (http://www.sanger.ac.uk/projects/B_pseudomallei). A map of this region of the *B. pseudomallei* genome was deduced using the annotation tool ARTEMIS (http://www.sanger.ac.uk/software/Artemis), and the flanking genes were identified on the basis of the sequence similarity of the encoded proteins with proteins in GenBank.

The transposon in *B. pseudomallei* 2D2 was inserted in the *ilvI* gene, which encodes the large subunit of the acetolactate synthase enzyme. This enzyme is made up of a large catalytic subunit associated with a small, regulatory subunit and is necessary for the biosynthesis of the branched chain amino acids along with pantothenate and coenzyme A. The genes encoding the large and small acetolactate synthase subunits are often located adjacently on the bacterial chromosome, and this was the case for *B. pseudomallei* strain K96243. The *B. pseudomallei* *ilvI* gene was located in a operon, with the gene encoding the small acetolactate synthase subunit (*ilvH*) downstream, followed by the *ilvC* gene, which potentially encodes ketol acid reductoisomerase, an enzyme also involved in the biosynthesis of the branched chain amino acids along with pantothenate and coenzymeA. Assuming that the *ilv* operon is organized similarly in *B. pseudomallei* K96243 and 576, then the presence of the transposon in this operon, with a putative promoter region and possible Rho-independent termination site downstream of *ilvC*, means that any potential polar effects due to the transposon insertion are likely to be limited to this operon.

**Correlation of the deduced genotype with an auxotrophic phenotype.** *B. pseudomallei* 2D2 or 576 was inoculated into 10 ml of M9 minimal media with or without added leucine, isoleucine, or valine. *B. pseudomallei* 576 was able to grow in minimal media lacking all of these amino acids, indicating a functional branched chain amino acid biosynthetic pathway. In contrast, *B. pseudomallei* 2D2 was unable to grow in unsupplemented M9 minimal media (Fig. 1). M9 media supplemented with all branched chain amino acids were capable of supporting the growth of *B. pseudomallei* 2D2, but supplementation with the individual branched chain amino acids did not allow the growth of *B. pseudomallei* 2D2.

Some bacteria, such as *Escherichia coli*, have several acetolactate synthase isozymes, which differ in their expression patterns and substrate specificity (16). The fact that interruption of the *ilvI* gene has rendered the *B. pseudomallei* mutant 2D2 auxotrophic for all three branched chain amino acids suggests that it possesses only one acetolactate synthase. A search of the *B. pseudomallei* K96243 genome supported this suggestion, as the only genes likely to encode acetolactate synthase large and small subunits are the *ilvIH* genes reported here. Located at the start of the branched chain amino acid pathway, acetolactate synthase is common to the branches leading to synthesis of isoleucine, valine, and leucine. Mutating an enzyme required at this early stage of the biosynthetic pathway has resulted in auxotrophy for all three branched chain amino acids in *B. pseudomallei* 2D2.

***B. pseudomallei* mutant 2D2 protects against challenge with wild-type bacteria.** Two groups of 10 female BALB/c mice were intraperitoneally dosed with $10^6$ CFU of *B. pseudomallei* 2D2. After 5 weeks the mice were challenged with $10^6$ CFU of *B. pseudomallei* 576.

Eighty percent of mice that had received the auxotrophic mutant prior to challenge survived until the termination of the experiment. On the other hand, all 10 of the naive control group mice died. At lower challenge doses ($10^3$ and $10^4$ CFU), all the mice survived challenge with wild-type *B. pseudomallei*.

To our knowledge the only other pathogens in which the branched chain amino acid biosynthetic pathway has been disrupted, resulting in a protective attenuated mutant, are *Myco-*

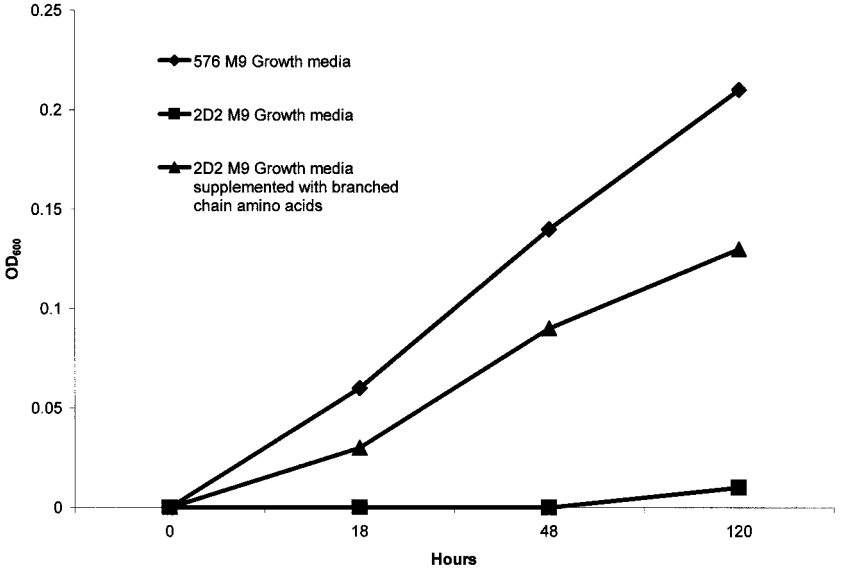


FIG. 1. Growth of *B. pseudomallei* mutant 2D2 and strain 576 in defined growth media. M9 growth medium was prepared as previously described (36) and was supplemented with leucine, isoleucine, and valine (40 μg/ml) where appropriate. The results represent the mean of three experiments. $OD_{600}$, optical density at 600 nm.

*bacterium bovis* and *M. tuberculosis*. However, in these cases, the attenuating mutations were in the *leuD* gene, which encodes a protein in the branch of the pathway specific to leucine biosynthesis (19, 29). These mycobacteria are able to survive and multiply within mononuclear phagocytes. In this environment essential nutrients required for bacterial survival are likely to be limited. It is thought that the ability of the pathogenic mycobacteria to synthesize amino acids (e.g., leucine) is essential for survival and growth in host phagocytes. The exact mechanism of stimulation of the specific immune response by the *leuD* mycobacterial mutant, protective against challenge with wild-type mycobacteria, has not yet been determined.

We also challenged mice with *B. pseudomallei* strain BRI, which had an MLD by the intraperitoneal route in BALB/c mice of 3 CFU. A group of 10 BALB/c mice was dosed with *B. pseudomallei* mutant 2D2, and 5 weeks later these mice were challenged with $10^6$ CFU of *B. pseudomallei* strain BRI. All 10 mice that had received *B. pseudomallei* 2D2 prior to challenge with *B. pseudomallei* BRI survived, compared to a naive control group in which 8 of the 10 mice died over the 32 days of the experiment.

For the challenges with strains 576 and BRI, we used age-matched, approximately 6-week-old female mice. The animals were grouped together in cages of five with free access to food and water and were subjected to a 12-h light-dark cycle.

The strains that we have used for challenge (576 and BRI) were both isolated from human cases of melioidosis but produce immunologically distinct forms of LPS. Although LPS has been suggested as one component of a subunit vaccine against melioidosis, our findings suggest that immune responses to antigens other than LPS are able to provide protection against *B. pseudomallei*.

***B. pseudomallei* mutant 2D2 does not persist in vivo.** In order to study the kinetics of infection of *B. pseudomallei* strain 2D2 or 576, BALB/c mice were challenged, intraperitoneally with $10^4$ CFU of either strain and tissues were sampled at various times postchallenge. Initially, *B. pseudomallei* 2D2 colonized spleen, liver, kidney, and lung tissues and culturable bacteria persisted in these tissues for up to 30 days postchallenge. After this time no culturable bacteria were recovered from any of the tissue samples. In contrast, the numbers of wild-type bacteria present in the tissues sampled increased over the course of the experiment (Fig. 2).

**Protection demonstrated using *B. pseudomallei* 2D2 is specific for *B. pseudomallei*.** It is known that under some conditions *B. pseudomallei* is able to establish a persistent infection (12, 37) in which viable bacteria may not be recovered from animal tissues. We therefore considered that the protection that we have seen may not be specific for *B. pseudomallei* but might be due to persistent organisms stimulating an innate immune response. In order to discount this possibility, we dosed mice with *B. pseudomallei* 2D2 and then challenged them with *Francisella tularensis* strain LVS. We chose to use *F. tularensis* strain LVS in this experiment for several reasons. Firstly, like *B. pseudomallei*, *F. tularensis* is a facultative intracellular human pathogen. In addition, dosing of mice with DNA containing CpG motifs has previously been shown to induce protection against challenge with *F. tularensis* (14, 26) as a consequence of the induction of cytokine expression by the host. This mechanism of protection has also been demonstrated for the bacterial pathogen *Listeria monocytogenes*, which is also often used to probe for the induction of nonspecific immunity. *F. tularensis* strain LVS was grown overnight on blood glucose cysteine agar as previously reported (24). *F. tularensis* strain LVS has been shown previously to have an MLD of less than 1 CFU in the murine model (35).

Groups of 10 BALB/c mice were used in this study, which was carried out over a period of 5 weeks postchallenge. When BALB/c mice were challenged with $10^3$ or $10^5$ CFU of *F. tularensis* LVS or $10^4$ CFU of *B. pseudomallei* 576, all of the

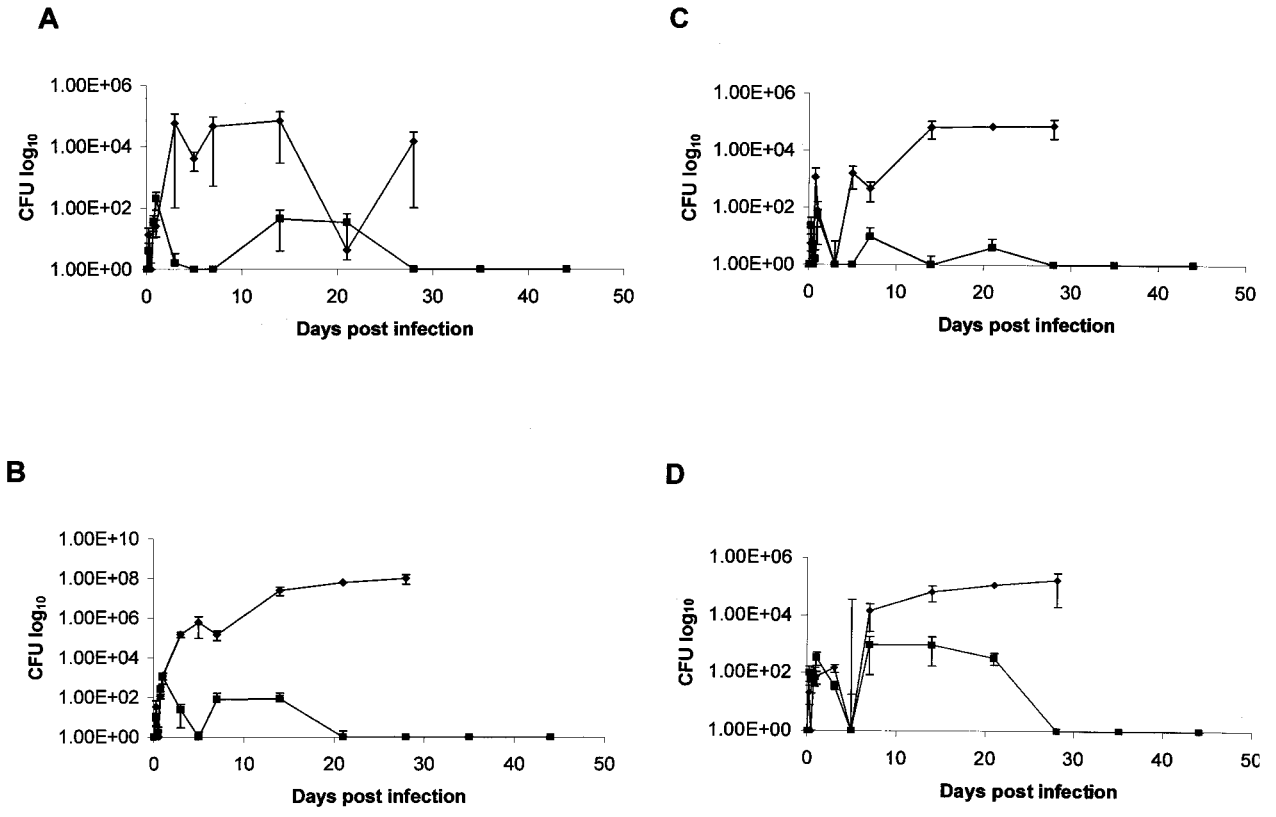


FIG. 2. Clearance of *B. pseudomallei* mutant 2D2 (■) or strain 576 (♦) from animal tissue. Groups of five female BALB/c mice were inoculated with $10^4$ CFU by the intraperitoneal route of infection. At time points throughout the duration of the experiment mice were killed and liver (A), spleen (B), kidney (C), and lung (D) were harvested. The number of *B. pseudomallei* organisms present in each organ was calculated (error bars represent mean bacterial counts from three individual animals).

animals died over the course of the experiment. Groups of BALB/c mice that had been dosed with $10^4$ CFU of *B. pseudomallei* 2D2 5 weeks previously were protected against a challenge with $10^4$ CFU of *B. pseudomallei* 576. However, mice that had been dosed with *B. pseudomallei* 2D2 prior to challenge with $10^3$ or $10^5$ CFU of *F. tularensis* were not protected.

Live attenuated vaccines have been used successfully to prevent a number of human infectious diseases, including polio and tuberculosis. The use of a live attenuated vaccine allows the generation of an immune response against a number of antigens, and both the antibody-mediated and cellular arms of the immune response are stimulated. Therefore, we believe that live attenuated vaccines may provide protection against a wide range of *B. pseudomallei* strains.

Further work is now required to determine the precise nature of the protective response induced by *B. pseudomallei* 2D2. Genes encoding enzymes necessary for a functional branched chain amino acid biosynthetic pathway are also found in a wide range of other pathogenic bacteria. This observation, along with the finding that mutations in this pathway attenuate *M. bovis* and *M. tuberculosis*, suggests that this pathway might be targeted to devise rationally attenuated mutants as vaccines against other diseases.

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