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Development of Signature-Tagged Mutagenesis in *Burkholderia pseudomallei* To Identify Genes Important in Survival and Pathogenesis▽

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***Burkholderia pseudomallei*, the causative agent of melioidosis, is an important human pathogen in Southeast Asia and northern Australia for which a vaccine is unavailable. A panel of 892 double signature-tagged mutants was screened for virulence using an intranasal BALB/c mouse model of infection. A novel DNA tag microarray identified 33 mutants as being attenuated in spleens, while 6 were attenuated in both lungs and spleens. The transposon insertion sites in spleen-attenuated mutants revealed genes involved in several stages of capsular polysaccharide biosynthesis and DNA replication and repair, a putative oxidoreductase, ABC transporters, and a lipoprotein that may be important in intercellular spreading. The six mutants identified as missing in both lungs and spleens were found to have insertions in *recA* involved in the SOS response and DNA repair; putative auxotrophs of leucine, threonine, *p*-aminobenzoic acid, and a mutant with an insertion in *aroB* causing auxotrophy for aromatic compounds were also found. Murine challenge studies revealed partial protection in BALB/c mice vaccinated with the *aroB* mutant. The refined signature-tagged mutagenesis approach developed in this study was used to efficiently identify attenuating mutants from this highly pathogenic species and could be applied to other organisms.**

Burkholderia pseudomallei is a gram-negative, facultative, intracellular pathogen of both animals and humans. The bacterium is a soil saprophyte and the causative agent of melioidosis, a disease endemic to Southeast Asia and northern Australia. In Thailand, the bacterium is an important cause of community-acquired septicemia and accounts for 39% of deaths due to community-acquired septicemia in the Ubon Ratchatani province (7). Human infection is thought to initiate by the inhalation or inoculation of cuts and abrasions with contaminated soil particles (43). Symptoms of infection vary greatly but can generally be described as being acute, subacute, or chronic (5). The bacterium is reportedly able to persist in a host for decades prior to reactivation (26). The mechanism by which the organism is able to persist in the host is unknown. Due to the severity of acute infection, *B. pseudomallei* is classified as an Advisory Committee on Dangerous Pathogens category 3 pathogen and category B bioterrorism agent. Currently, no vaccine against the organism exists, and antibiotic treatment is complicated by the fact that *B. pseudomallei* is intrinsically resistant to many antibiotics including aminoglycosides and the early β-lactams (4). A number of virulence determinants including flagella (10), a capsular polysaccharide-encoding region (33), and an Inv/Mxi-Spa-like type III secretion system involved in the intracellular behavior of the pathogen (38, 39) have been identified in this organism. However, further studies are required to understand this complex infection.

Signature-tagged mutagenesis (STM) is a technique that allows the simultaneous screening of up to 96 mutants using an in vivo selection model. Typically, a single 40-bp sequence, the signature, is inserted within the transposon used to generate the random mutants. Comparison of the relative abundance of tag sequences detectable before infection to that after infection reveals which mutants within the pool are attenuated. The technique was first used in *Salmonella enterica* serovar Typhimurium and resulted in the identification of a new pathogenicity island, SPI2 (18). This locus was later found to code for a previously unknown type III secretion system critical for virulence and bacterial proliferation within macrophages (19, 36). STM studies of other intracellular pathogens such as *Mycobacterium tuberculosis* (6), *Listeria monocytogenes* (3), *Yersinia pestis* (14), and *Brucella suis* (15) successfully identified a number of genes not previously linked to virulence, so we aimed to use a modified version of the technique on the genome-sequenced *B. pseudomallei* reference strain K96243 (21).

Previously, we screened a limited panel of 96 *B. pseudomallei* strain 576 transposon mutants in the intraperitoneal Porton outbred mouse model. The study identified the acapsular mutant, 1E0, with a transposon insertion site in a mannosyltransferase-encoding gene within the capsular polysaccharide-encoding region. A loss of capsule and increased time to death in Porton outbred mice vaccinated with this mutant were demonstrated (2).

We developed a refined STM approach with two DNA tags to identify mutants attenuated in the intranasal (i.n.) BALB/c murine model of melioidosis. This route of infection mimics natural inoculation via inhalation, which is also the most likely route of infection should *B. pseudomallei* be used as a bioterrorism agent. Two target organs were studied simultaneously

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to attempt to identify tissue-specific tropisms. Of the 892 mutants tested, 39 were confirmed to be attenuated in the spleens of mice, and 6 of these mutants were identified as being attenuated in both lungs and spleens. An *aroB* mutant was tested for vaccine potential.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. *B. pseudomallei* K96243 was a gift from Tyrone Pitt (Health Protection Agency, London, United Kingdom) and was maintained in Luria Bertani (LB) broth or LB agar. *Escherichia coli* 19851 (*pir*$^+$), used for direct mating, was maintained in LB agar and LB broth supplemented with kanamycin (50 μg/ml) and ampicillin (100 μg/ml). This host strain enabled the maintenance of the modified *pir*-dependent plasmid pUTminiTn*5*Km2 (12, 23). Medium used for the selection of *B. pseudomallei* transposon mutants consisted of LB agar supplemented with kanamycin (400 μg/ml) to counterselect against wild-type *B. pseudomallei* and zeocin (100 μg/ml; Invitrogen) to counterselect against the *E. coli* donor. Colonies produced on this medium were subcultured in the same selective medium to confirm mutant generation. Input pools were assembled in 96-well plates and stored at −70°C.

Infection studies. All experiments were carried out using 7- to 10-week-old female BALB/c mice (Charles Rivers Laboratories). Mice were maintained with access to food and water ad libitum under animal biohazard containment level 3 conditions. Experiments were performed in accordance with the Animals (Scientific Procedures) Act of 1986 and were approved by the local ethical review committee. For i.n. infection, mice were anesthetized intraperitoneally with 50 ng/kg ketamine (Ketaset; Fort Dodge Animal Health) and 10 mg/kg xylazine (Rompum; Bayer, Germany) diluted in saline. Bacterial inocula were delivered in 50-μl volumes.

Inocula were prepared by inoculating 10 ml of LB broth with a single colony from a culture of pure mutant or wild-type bacteria growing on LB agar. The culture was then incubated for 24 h before being centrifuged at 3,000 × *g* for 10 min. The supernatant was removed, and the pellet was washed once in 10 ml phosphate-buffered saline (PBS) before being centrifuged again. Finally, the supernatant was removed, and the pellet was resuspended in 3 ml PBS–30% glycerol. Aliquots were stored at −80°C, and postfreeze counts were carried out to enumerate the number of bacteria prior to infection. To determine the median lethal dose, groups of six mice were infected i.n. with increasing inocula: 10, 10^2, 10^3, and 10^4 CFU per 50-μl inoculum. Three mice were infected with PBS only as a control. Infections were allowed to continue for 65 days, and animal survival was observed. To screen the transposon mutant library, groups of two mice were infected i.n. with an inoculum dose of 5×10^4 CFU. Approximately 48 h postinoculation, lungs and spleens were extracted and homogenized. Dilutions of this homogenate were plated onto LB agar and incubated for 48 h before genomic DNA was extracted to identify mutants lost following infection.

Selected mutants were tested as pure cultures to confirm attenuation and test organ load kinetics. For both assays, mice were infected i.n. with 1×10^3 CFU of mutant bacteria and compared to a control group of mice infected i.n. with *B. pseudomallei* K96243; the detection limit for both assays was 100 CFU. For survival assays, groups of six mice were infected, and animal survival was observed daily. Upon experiment termination, lungs and spleens were extracted from surviving mice and plated onto nonselective medium to check for the presence of bacteria. For organ load kinetic assays, groups of five mice per time point were infected, and at 24, 48, and 72 h postinoculation, a group of mice was killed, and organ loads in lungs and spleens were enumerated by plating onto nonselective medium. Survival data were analyzed using the log rank test (GraphPad Prism version 4 for Windows software) (www.graphpad.com). Organ load data were visualized using Prism software and analyzed for statistical significance using the Student's *t* test.

Genomic DNA extraction. Genomic DNA was extracted from bacteria colonizing the organs of infected animals as previously described (21), with some modifications. Mice were killed by cervical dislocation, and lungs and spleens were extracted into 10 ml PBS. Organs were then homogenized by passing through a 100-μm cell strainer (Becton Dickinson Biosciences, Oxford, United Kingdom); for each organ, dilutions of this homogenate were plated onto five agar plates and incubated at 37°C for 48 h. Bacteria from plates containing approximately 1×10^4 colonies were isolated by scraping, and the resulting pellet was resuspended in 10 ml lysis solution (100 μg/ml proteinase K, 10 mM NaCl, 20 mM Tris HCl [pH 8.0], 1 mM EDTA, and 0.5% sodium dodecyl sulfate [SDS]) and incubated at 55°C overnight. Three milliliters of 5 M sodium perchlorate was added and incubated for 1 h at room temperature. DNA was purified using phenol-chloroform-isoamyl alcohol (25:24:1 ratio), precipitated with ethanol, and dissolved in water. Once the DNA was confirmed to be sterile, it was subjected to two further phenol-chloroform-isoamyl alcohol washes followed by one wash in chloroform-isoamyl alcohol (24:1 ratio) to remove traces of phenol and finally precipitated in ethanol before being spooled into water.

Signature tag amplification and labeling. Tag sequences were amplified from input and output pool genomic DNA in two separate reactions. For primary tag amplification, primers Cy5_Tag1_dir (5′-AGAGACCTCGTGGACATC) and Cy5_Tag1_rev (5′-GTGGCAAAGCAAAAGTTCAA) were used.

Secondary tag amplification was achieved using primers Cy5_Tag2_dir (5′-GTCGACCTGCAGCGTACG) and Cy5_Tag2_rev (5′-GATGTCCACGAGGTCTCT). DNA tag microarrays were produced by the Bacterial Microarray Group at St. George's Hospital. Microarray slides were prehybridized by incubating them in a solution containing 3.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS, and 10 mg/ml bovine serum albumin at 55°C for 20 min. After prehybridization, the slides were washed with agitation in distilled water for 1 min, followed by isopropanol for 1 min. Slides were dried by centrifugation (1,500 × *g* for 5 min). Cy5-labeled tags from primary and secondary PCRs were mixed and added to the hybridization solution (19.5 μl of each PCR product in a solution containing 4× SSC and 0.3% SDS), denatured by heating for 5 min at 95°C, and applied to the prehybridized microarray slide underneath a 22- by 25-mm Lifterslip (Erie Scientific Company). Each slide was placed into a waterproof cassette and incubated overnight at 55°C. After hybridization, slides were washed once in wash buffer A (1× SSC, 0.05% SDS) preheated to 55°C for 2 min, followed by two 2-min washes at room temperature in wash buffer B (0.06× SSC). Finally, slides were dried by centrifugation. Slides were scanned using a GMS 418 scanner (Genetic Microsystems). Images produced were visualized and analyzed using Imagene 5.5 software (Biodiscovery).

Identification of transposon insertion sites. Attenuated mutants were grown as a pure culture, and genomic DNA was extracted as previously described (21). The nucleotide sequence surrounding the transposon insertion site was determined using either single-primer PCR (24) or arbitrary PCR (29). Briefly, arbitrary primers ARB1 (5′-GGCCACGCGTCGACTAGTACNNNNNNNNNG ATAT-3′), ARB3 (5′-GGCCACGCGTCGACTAGTACNNNNNNNNNNTGA CG-3′), ARB4 (5′-GGCCACGCGTCGACTAGTACNNNNNNNNNNACGC C-3′), and ARB5 (5′-GGCCACGCGTCGACTAGTACNNNNNNNNNNTAC NG-3′) and transposon-specific primer P7M1 (5′-GTCATTAAACGCGTATTC AGGCTGAC-3′) were used in a first round of PCR under the following conditions: 5 min at 95°C and 6 cycles of 30 s at 95°C, 30 s at 30°C, and 1.5 min at 72°C, followed by 30 cycles of 30 s at 95°C, 30 s at 45°C, and 2 min at 72°C. The PCR product was purified using the QIAquick PCR purification kit (QIAGEN) and eluted in water. Subsequently, 5 μl of the purified product was used as the template in a second round of PCR with primers ARB2 (5′-GGCCACGCGTC GACTAGTAC-3′) and P7M (5′-GCCGAACTTGTGTATAAGAGTC-3′) under the following conditions: 30 cycles of 30 s at 95°C, 30 s at 45°C, and 2 min at 72°C. PCR products were purified as described above. Sequencing of the transposon insertion site was carried out using primer P7U (CTGCAGGCATGCA AGCTTCG) (ABI Prism Ready Reaction Mix; Amersham Biosciences) and a PTC-225 Peltier Thermal Cycler. Sequencing data were generated by use of an ABI 3730 DNA analyzer and examined with BLASTn, BLASTx (www.ncbi.nlm.nih.gov/BLAST/), and VGE BLAST (www.vge.ac.uk/BLAST/) software.

Capsular polysaccharide detection. To obtain information about the effects of transposon insertions in the capsular polysaccharide-encoding region, *B. pseudomallei* K96243 and three transposon derivatives were tested by immunofluorescence staining. Ten milliliters of LB broth was inoculated with a single colony of mutant or wild-type bacteria; this culture was incubated overnight at 37°C. Ten microliters of this culture was then applied onto a coverslip. The immunofluorescence staining continued as previously described (2), except that the secondary antibody used was a goat anti-mouse Alexa Fluor 488 conjugate (Invitrogen, Paisley, United Kingdom). The samples were examined using confocal microscopy (Zeiss AX10 Vert 200 M confocal microscope; Carl Zeiss Ltd., Hertfordshire, England) and Zeiss LSM 510 Image Browser software. Each sample was tested in triplicate, and images are representative of at least 10 fields examined.

Characterization of mutant 13B11. The arbitrary PCR result obtained from mutant 13B11 was confirmed by amplifying across the transposon insertion junction using *aroB*-specific primer aroB2rev (5′-GGTATTTCGCTCACCGTG TC-3′) and transposon-specific primer P6M3 (5′-ACTTGACGGGACGGCGG T-3′). Primer P6M3 was then used to sequence the PCR product. An insertion in the *aroB* gene of *B. pseudomallei* K96243 was predicted to render the mutant unable to grow on M9 minimal medium (40). To confirm this predicted phenotype, a single colony of mutant 13B11 or *B. pseudomallei* K96243 was used to inoculate 10 ml LB broth and incubated for 24 h statically at 37°C. The cultures

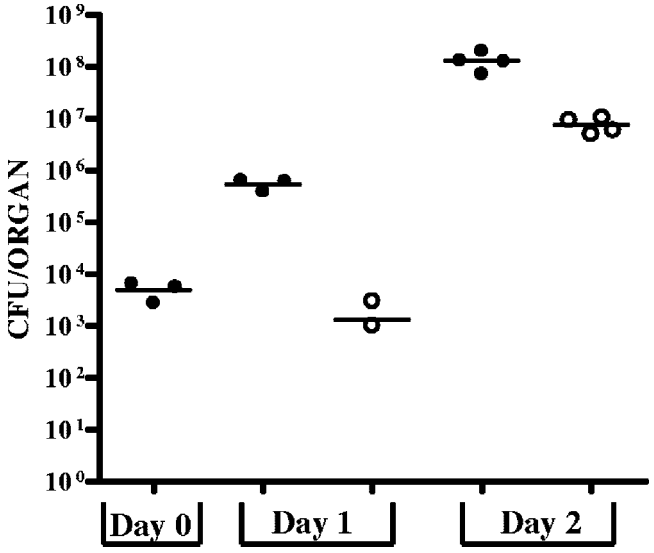

FIG. 1. Organ load in BALB/c mice infected i.n. with 1×10^4 CFU of *B. pseudomallei* K96243. ●, lungs; ○, spleens. Symbols indicate counts per animal, and bars indicate means per group.

were centrifuged at 3,000 × *g* for 10 min to pellet the bacterial cells and washed twice in M9 minimal medium before being resuspended in 10 ml M9 minimal medium. This was used to inoculate M9 minimal medium alone or M9 minimal medium supplemented with tyrosine, tryptophan, and phenylalanine at a concentration of 40 μg/ml and *p*-aminobenzoic acid (PABA) and 2,3-dihydroxybenzoate at 10 μg/ml. Optical density readings at 600 nm were taken at various time points. The reading were used to generate a graph using Microsoft Excel software (Microsoft Limited, United Kingdom).

RESULTS

Construction of a *B. pseudomallei* mutant library. A total of 96 plasmids were modified to carry two individual tag sequences during a previous STM study of *Yersinia pseudotuberculosis* (23). Direct mating of *E. coli* 19851 *pir*$^+$ carrying pUTminiTn*5*Km*2* with *B. pseudomallei* K96243 was used to construct a library of 892 mutants. Single and random transposon insertions were confirmed by Southern blotting using genomic DNA from 10 randomly chosen mutants generated in a single conjugation reaction. DNA was digested with a restriction enzyme and probed using the kanamycin cassette amplified from pUTminiTn*5*Km*2* (data not shown).

Median lethal dose determination. The median lethal dose of *B. pseudomallei* K96243 when BALB/c mice were infected i.n. was found to be less than 10 CFU (data not shown). Due to the susceptibility of the mice, a 100-fold representation of each mutant in an input pool of 96 individually tagged mutants was sufficient to prevent variable hybridization patterns noted in previous studies when lower doses were used while also preventing the host immune system from becoming overwhelmed (9, 28). Thus, an inoculum of 1×10^4 CFU was shown to kill all mice by day 3 postinfection. An organ load kinetic experiment was carried out with a dose of 1×10^4 CFU in order to ascertain if replication could take place in mouse lungs and spleens within 48 h of inoculation. Results indicated an increase of over 1,000-fold in lungs and spleens of mice infected (Fig. 1).

We established that a "bottleneck" effect (8) was not occurring in this STM model by assembling an input pool of 63 separately tagged mutants and infecting a group of five mice

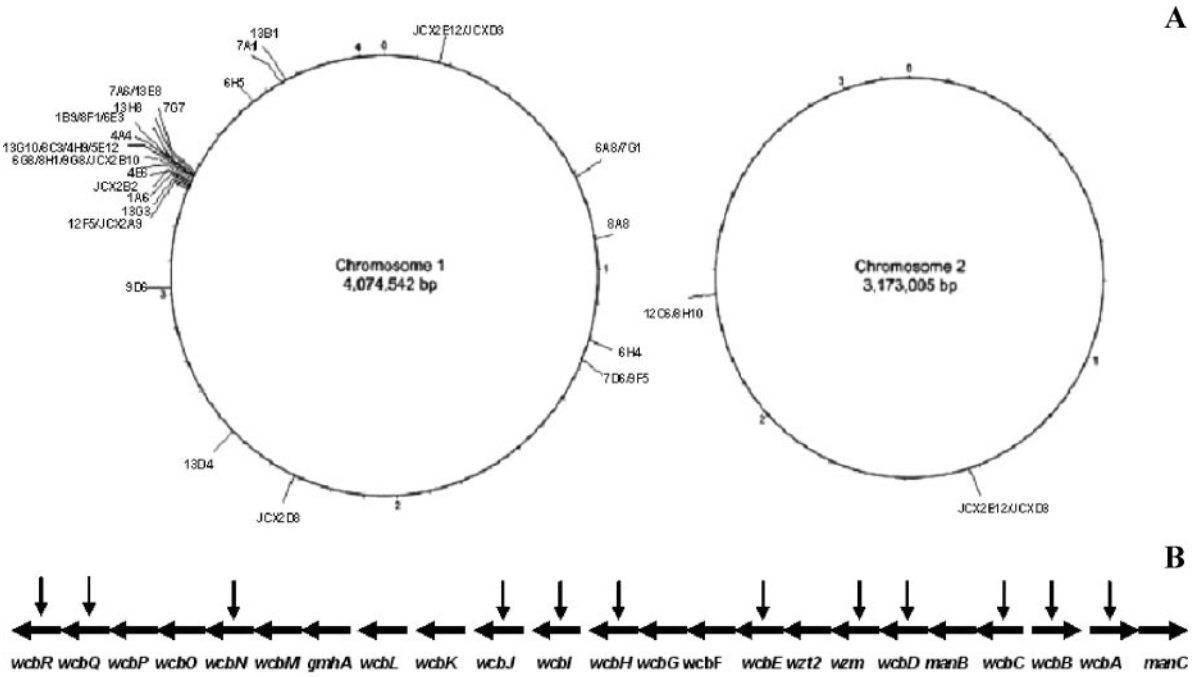

FIG. 2. (A) Attenuating transposon insertion sites in *B. pseudomallei* K96243. (B) Transposon insertion sites within the capsular polysaccharide biosynthesis region of *B. pseudomallei* K96243. Transposon insertion sites are indicated by vertical arrows. Genes and regions are not drawn to scale.

TABLE 1. Transposon insertion sites identified in this study[a]

Mutant	Disrupted gene	Product	Putative function	Insertion distance from start codon (bp)[b]
7A6	*wcbA*	Putative capsular polysaccharide export protein	Capsular polysaccharide biosynthesis	1,301
13E8	*wcbA*	Putative capsular polysaccharide export protein	Capsular polysaccharide biosynthesis	713
13H8	*wcbB*	Putative capsular polysaccharide glycosyltransferase biosynthesis protein	Capsular polysaccharide biosynthesis	46
1B9	*wcbC*	Putative capsular polysaccharide export protein	Capsular polysaccharide biosynthesis	1,035
8F1	*wcbC*	Putative capsular polysaccharide export protein	Capsular polysaccharide biosynthesis	1,018
6E3	*wcbC*	Putative capsular polysaccharide export protein	Capsular polysaccharide biosynthesis	518
4A4	*wcbD*	Putative ABC transporter transmembrane protein	Capsular polysaccharide biosynthesis	961
JCX2C5	*wzm*	Putative capsule polysaccharide export ABC transporter transmembrane protein	Capsular polysaccharide biosynthesis	466
8C3	*wcbE*	Putative glycosyltransferase	Capsular polysaccharide biosynthesis	196
13G10	*wcbE*	Putative glycosyltransferase	Capsular polysaccharide biosynthesis	945
4H9	*wcbE*	Putative glycosyltransferase	Capsular polysaccharide biosynthesis	1,464
5E12	*wcbE*	Putative glycosyltransferase	Capsular polysaccharide biosynthesis	1,346
6G8	*wcbH*	Putative glycosyltransferase	Capsular polysaccharide biosynthesis	625
8H1	*wcbH*	Putative glycosyltransferase	Capsular polysaccharide biosynthesis	731
9G8	*wcbH*	Putative glycosyltransferase	Capsular polysaccharide biosynthesis	474
JCX2B10	*wcbH*	Putative glycosyltransferase	Capsular polysaccharide biosynthesis	75
4E6	*wcbI*	Putative capsular polysaccharide biosynthesis protein	Capsular polysaccharide biosynthesis	459
JCX2B2	*wcbJ*	Putative capsular polysaccharide biosynthesis protein	Capsular polysaccharide biosynthesis	516
1A6	*wcbN*	Putative D-*glycero*-D-*manno*-heptose 1,7-bisphosphate phosphatase	Capsular polysaccharide biosynthesis	258
13G3	*wcbQ*	Putative capsular polysaccharide transmembrane export protein	Capsular polysaccharide biosynthesis	711
12F5	*wcbR*	Putative type I polyketide synthase	Capsular polysaccharide biosynthesis	761
JCX2A9	*wcbR*	Putative type I polyketide synthase	Capsular polysaccharide biosynthesis	451
8A8	BPSL0776	RecA	Homologous recombination, SOS response	207
7G7	BPSL2825	Putative PabB	Biosynthesis of *para*-aminobenzoate	1,352
9D6	*serC*	Phosphoserine aminotransferase	Serine biosynthesis	360
6H5	*trpG*	Anthranilate synthase component II	Tryptophan biosynthesis	447
12C6	*leuB*	Isopropylmalate dehydrogenase	Leucine biosynthesis	769
8H10	*leuB*	Isopropylmalate dehydrogenase	Leucine biosynthesis	899
13B11	*aroB*	Dehydroquinate synthase	Chorismate biosynthesis	532
7A1	BPSL3147	Putative lipoprotein	39.16% amino acid identity to *Shigella flexneri* VacJ lipoprotein	618
6H4	BPSL1039	Putative ABC transporter	Putative transmembrane ABC transporter	1,293
7G1	BPSL0634	Putative oxidoreductase	Energy production and conversion	2,306
6A8	BPSL0634	Putative oxidoreductase	Energy production and conversion	3,191
13D4	*ppsA*	Putative phosphoenol pyruvate synthase	Carbohydrate transport and metabolism	704
JCX2D8	Upstream of *ihfA*	Putative integration host factor alpha subunit IhfA	DNA replication, recombination, and repair	NA
9F5	*nth*	Putative endonuclease III	DNA replication, recombination, and repair	150
7D6	*nth*	Putative endonuclease III	DNA replication, recombination, and repair	134
JCX2E12	BPSL0175/BPSL1060	Putative hypothetical bacteriophage protein	Unknown	149
JCXD3	BPSL0175/BPSL1060	Putative hypothetical bacteriophage protein	Unknown	123

[a] Insertion sites within the capsule region are indicated in Fig. 4b.
[b] NA, not applicable.

i.n. with 1×10^4 CFU of *B. pseudomallei*. Hybridization patterns from all five animal lungs and spleens were compared and found to have minimal variability (data not shown).

Characterization of attenuated mutants. Eight hundred ninety-two mutants were divided into 10 input pools and tested in the i.n. BALB/c mouse model. Two mice were infected with each input pool, and the presence of mutants in lungs and spleens before and after infection was identified using a DNA tag microarray. We selected putatively attenuated mutants by looking for a complete loss of signal in either lungs or spleens of mice tested. In total, 45 mutants were identified as being attenuated in this first screen. To validate the results of this screening, these mutants were incorporated into two new pools, which also contained previously untested mutants. The pools were retested in mice, and the attenuation was evidenced by the lack of a mutant-specific signal from the lungs or the spleens or both in at least four of five of the challenged mice. Thirty-two mutants were confirmed to be attenuated, and seven new attenuated transposon derivatives were also identified. Due to time constraints, these seven new mutants were not put into animals again in order to confirm attenuation. Transposon insertion sites were mapped to the *B. pseudomallei* chromosomes (Fig. 2A). Overall, 4.4% of mutants in this study were identified as being attenuated. This percentage of attenuating mutants is similar to that of most other in vivo STM studies with bacterial pathogens (27). A summary of transposon insertion sites is shown in Table 1 and Fig. 2.

Mutants involved in capsular polysaccharide biosynthesis. Twenty-two insertions were identified in the capsule polysaccharide synthesis locus. A loss or maintenance of capsular polysaccharide was investigated in selected transposon derivatives by immunofluorescence (Fig. 3). This confirmed previous data indicating that insertions in *wcbB* resulted in a loss of capsular polysaccharide (2, 33). A summary of transposon insertions identified within the capsule polysaccharide biosynthesis region is shown (Fig. 2B). Mutants with insertions in *wcbN*, and to a lesser extent *wcbC*, were observed to have maintained the epitope reacting to 4V1H12. In order to confirm the attenuation indicated by array data, survival assays of mutants 1A6 and 1B9 (insertions in *wcbN* and *wcbC*, respectively) using pure cultures were carried out (Fig. 4a and b). The organ load kinetics of mice infected with the *wcbC* mutant were also

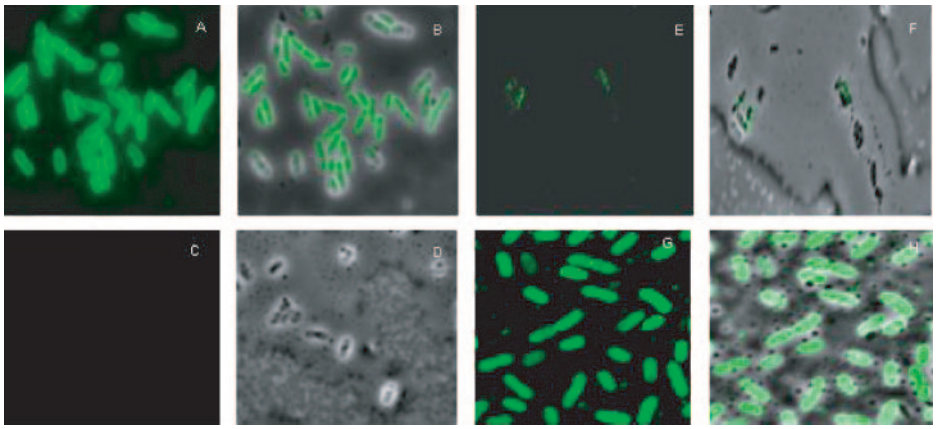

FIG. 3. Binding of monoclonal antibody 4V1H12 to the capsular polysaccharide of *B. pseudomallei* K96243 and selected transposon derivatives with insertions in the capsular polysaccharide coding region. (A) *B. pseudomallei* K96243 fluorescence view; (B) fluorescence view superimposed on phase-contrast image; (C and D) mutant 13H8 (*wcbB*); (E and F) mutant 1B9 (*wcbC*); (G and H) mutant 1A6 (*wcbN*).

investigated (Fig. 5a and b). Lung loads comparable to those of wild-type-infected mice at 24 h postchallenge were observed; however, by 48 h, the counts had dropped to a level comparable to that of the inoculum, while wild-type-infected mice had considerably higher bacterial loads. No *wcbC* mutants were detected in the spleens of mice at any time point investigated, and wild-type-infected mice registered some counts at 24 h and had an average of 1×10^5 CFU by 48 h. A Student's *t* test was used to identify statistically significant data.

Mutants putatively involved in the SOS response, DNA replication, and repair. Mutant 8A8 was confirmed to have a transposon insertion in BPSL0776. The encoded amino acid sequence shared 96.54% identity to the *Burkholderia cepacia* recombinase A protein RecA. This protein is involved in the induction of the SOS response, DNA recombination, single- and double-stranded DNA repair, and recombination-dependent replication (22). Mutants 9F5 and 7D6 were found to have a insertions in *nth*, and the JCX2D8 mutant had an insertion in an intergenic region upstream of *ihfA*. Both of these genes play roles in DNA replication, recombination, and repair.

Other insertions resulting in attenuation. Mutant 7A1 was identified as having a transposon insertion in BPSL3147; the coded amino acid sequence shared 39.16% identity to a *Shigella flexneri* VacJ lipoprotein. Mutants 6A8 and 7G1 were found to have insertions in BPSL0634, coding for a putative oxidoreductase; the genes found to have insertions in 4A4, 6H4, and JCX2C5 are related to ABC transporters, a class of proteins involved in the export-import of a range of molecules. Mutants JCXD3 and JCX2E12 were found to have insertions within conserved hypothetical phage proteins of genomic island 2 or 15. Mutant 9D6 had an insertion in *serC*, encoding a putative phosphoserine aminotransferase. Finally, mutant 13D4 was found to have an insertion in *ppsA*, putatively coding for phosphoenol pyruvate synthase, an enzyme involved in carbohydrate metabolism.

Lung- and spleen-attenuated mutants. Six transposon derivatives were identified as being missing in the lungs and spleens of all mice tested: five were putative auxotrophs, while the remaining mutant had an insertion in BPSL0776. Mutants 12C6 and 8H10 were the only mutants identified in this study as having insertions in chromosome 2. Both transposon insertion sites were in the *leuB* gene, encoding a putative isopropylmalate dehydrogenase. Mutant 6H5 was found to have a transposon insertion in *trpG*, encoding the putative anthranilate synthase component II. Mutant 7G7 had an insertion in BPSL2825; the protein encoded shared 41.3% identity to the *Neisseria meningitidis* putative *para*-aminobenzoate synthetase component I, an enzyme involved in the biosynthesis of *p*-aminobenzoate. Finally, mutant 13B11 was found to have a transposon insertion in *aroB*, a gene encoding 3-dehydroquinate synthase, an enzyme known to play a role in chorismate biosynthesis.

AroB mutant growth in minimal medium. The transposon insertion in mutant 13B11 was confirmed and found to have caused a severe growth defect when cultured in M9 minimal medium (Fig. 6). The aromatic compounds tyrosine, tryptophan, phenylalanine, PABA, and 2,3-dihydroxybenzoate were required to restore the growth of the mutant in minimal medium. We also assayed bacterial growth in M9 minimal medium supplemented with individual aromatic compounds and found that growth could not be restored by any single compound (data not shown).

AroB mutant protection studies. To ascertain the potential of an *aroB* vaccine candidate, mice were vaccinated i.n. with mutant 13B11 at doses of 1×10^5 CFU or 1×10^6 CFU. Animal survival was monitored for 35 days, after which five mice from each vaccination group were killed and lungs and spleens were homogenized and plated to verify the presence of latent surviving bacteria. All organs were found to be clear of bacteria. Vaccinated mice or naive controls were challenged i.n. with 1×10^3 CFU of *B. pseudomallei* K96243, and survival was monitored. The median survival time increased from 4.5 days in unvaccinated mice to 6 days in mice vaccinated with 1×10^5 CFU of the *aroB* mutant and 7 days in mice vaccinated with 1×10^6 CFU of the *aroB* mutant (Fig. 7) ($P < 0.05$ with both doses). The clearance kinetics of mice challenged with a dose of 7.5×10^5 CFU of the *aroB* mutant were also investigated (Fig. 8).

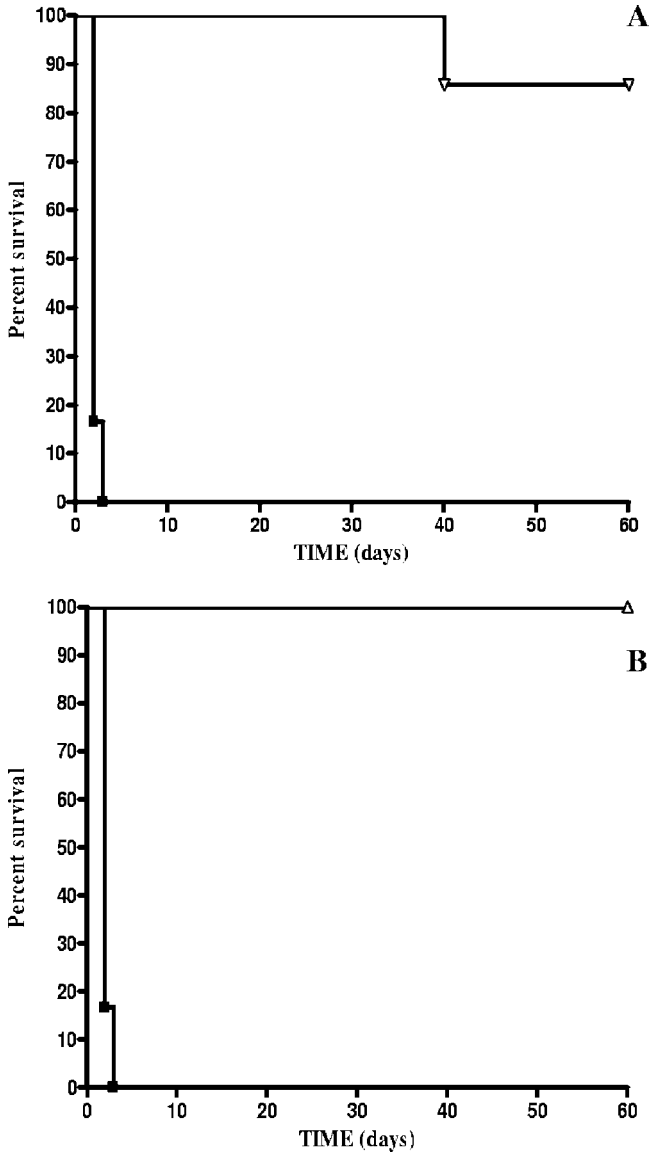

FIG. 4. (A) Survival assay of mice infected with 1×10^3 CFU of *B. pseudomallei* K96243 (■) or the *wcbN* mutant (▽). (B) Survival assay of mice infected with 1×10^3 of CFU *B. pseudomallei* K96243 (■) or the *wcbC* mutant (△).

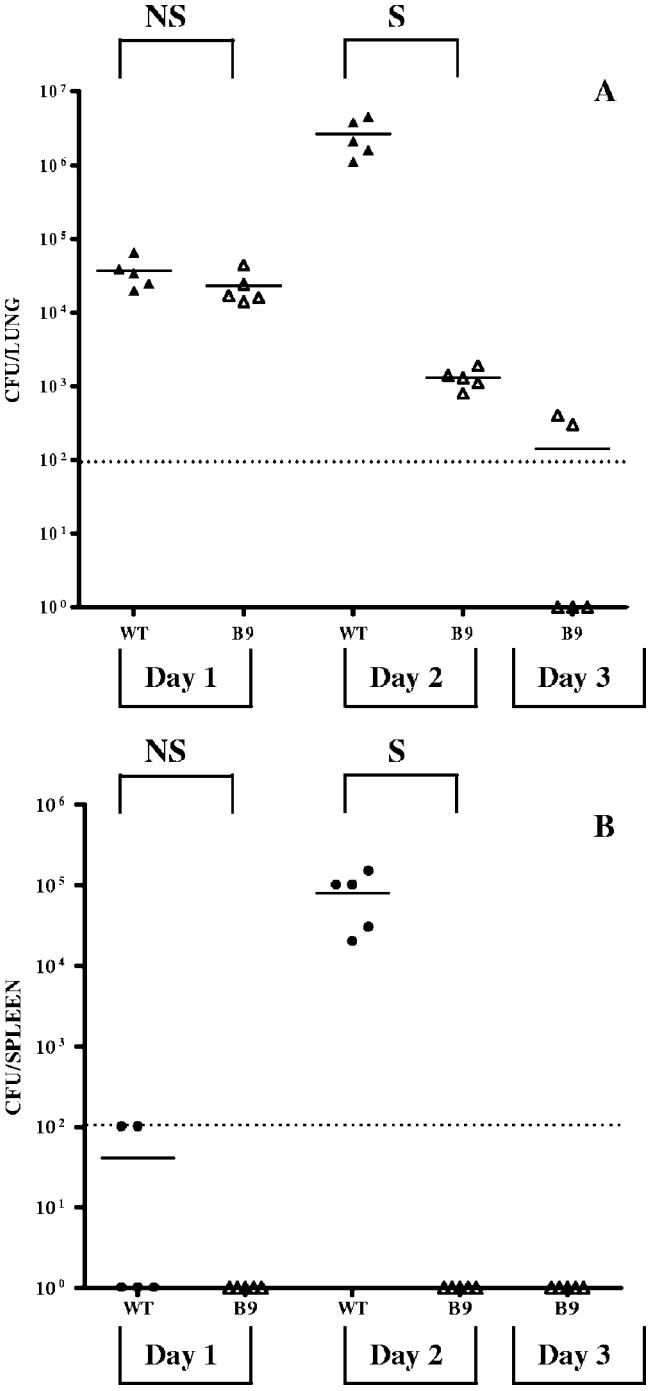

FIG. 5. (A) Organ loads obtained from lungs of BALB/c mice infected i.n. with 1×10^3 CFU of wild-type (WT) *B. pseudomallei* K96243 (▲) or *wcbC* mutant strain 1B9 (B9) (△). (B) Organ loads obtained from spleens of BALB/c mice infected i.n. with 1×10^3 CFU of *B. pseudomallei* K96243 (●) or *wcbC* mutant strain 1B9 (△). Each symbol indicates counts from an individual mouse. Solid lines indicate mean values per group, and the broken lines show the limit of detection for this assay. NS, not statistically significant; S, significant ($P < 0.05$).

DISCUSSION

STM was originally developed by Hensel and coworkers to test up to 96 mutants simultaneously for survival using an in vivo selection model (18). To minimize the number of false-positive identifications of attenuated mutants and to maximize the information return, we have developed a refined STM methodology that further reduced the number of animals required and the labor-intensive studies required on containment level 3 pathogens such as *B. pseudomallei*. Eight hundred ninety-two mutants were screened in the i.n. BALB/c murine model of melioidosis, 39 of which were shown to have a significant reduction in numbers following passage in animals. A novel DNA tag microarray to measure the relative abundance of mutants from input and output pools was developed. The DNA tag regions were selected during a previous STM study of *Y. pseudotuberculosis* to have minimal cross-hybridization potential and similar melting temperatures (23). A recent study of the anaerobic sediment survival of *Desulfovibrio desulfuri-*

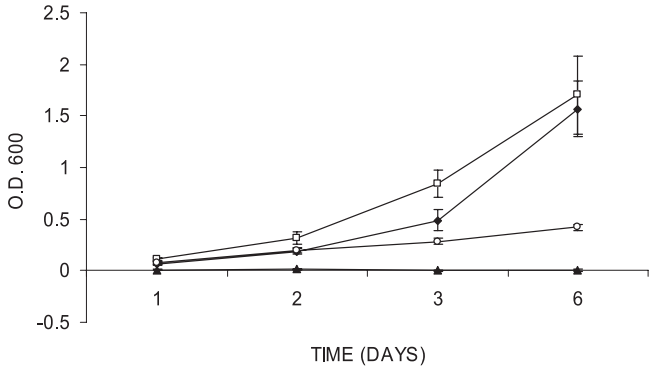

FIG. 6. Growth of *B. pseudomallei* K96243 and mutant strain 13B11 in M9 minimal medium with and without aromatic compound supplementation. ◆, *B. pseudomallei* K96243 in M9 minimal medium; ▲, 13B11 in M9 minimal medium; □, *B. pseudomallei* K96243 in M9 minimal medium with aromatic compounds; ○, 13B11 in M9 minimal medium with aromatic compounds. The results indicate the means of three experiments each carried out in triplicate. Bars indicate standard deviations. O.D. 600, optical density at 600 nm.

cans and *Shewanella oneidensis* also used microarray technology to identify mutants (16), independently confirming the viability of this technology. The use of a DNA tag microarray to screen mutants allows samples to be processed efficiently and rapidly. The method does not require the removal of the invariable arms from the PCR product prior to hybridization, as the tag sequences alone are printed on the array. By attaching Cy dyes to the 5′ end of the tag amplification primers, amplification and labeling are achieved simultaneously. The array is inexpensive compared to photolithography-based technologies (23) and can be considered portable, as any group with access to microarray scanners could make use of it. Finally, the DNA tag array is semiquantitative compared to traditional STM using radioactivity, and because each of the mutants has two individual tag sequences, the likelihood of false results is minimized. This efficient STM method reduces the number of animals necessary and minimizes the number experiments required to generate reliable data under containment level 3 conditions. During our investigation, two independent organs were analyzed simultaneously, a feature rarely reported in other STM studies.

A large number of transposon insertions were found in

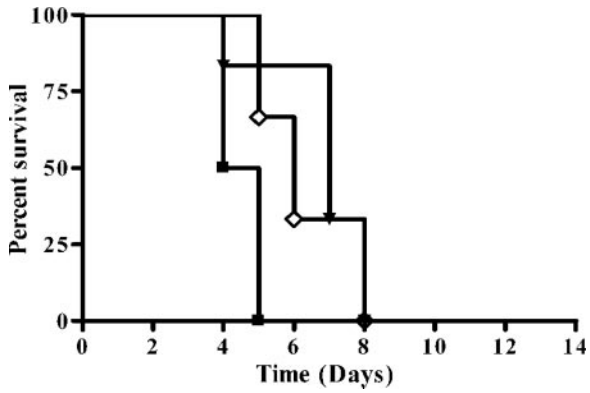

FIG. 7. Protection assay of BALB/c mice vaccinated with *B. pseudomallei* mutant strain 13B11. ■, naive mice; ◇, mice vaccinated with 1×10^5 CFU of mutant strain 13B11; ▼, mice vaccinated with 1×10^6 CFU of mutant strain 13B11. Mice were challenged with 1×10^3 CFU of *B. pseudomallei* K96243 at day 35 postvaccination.

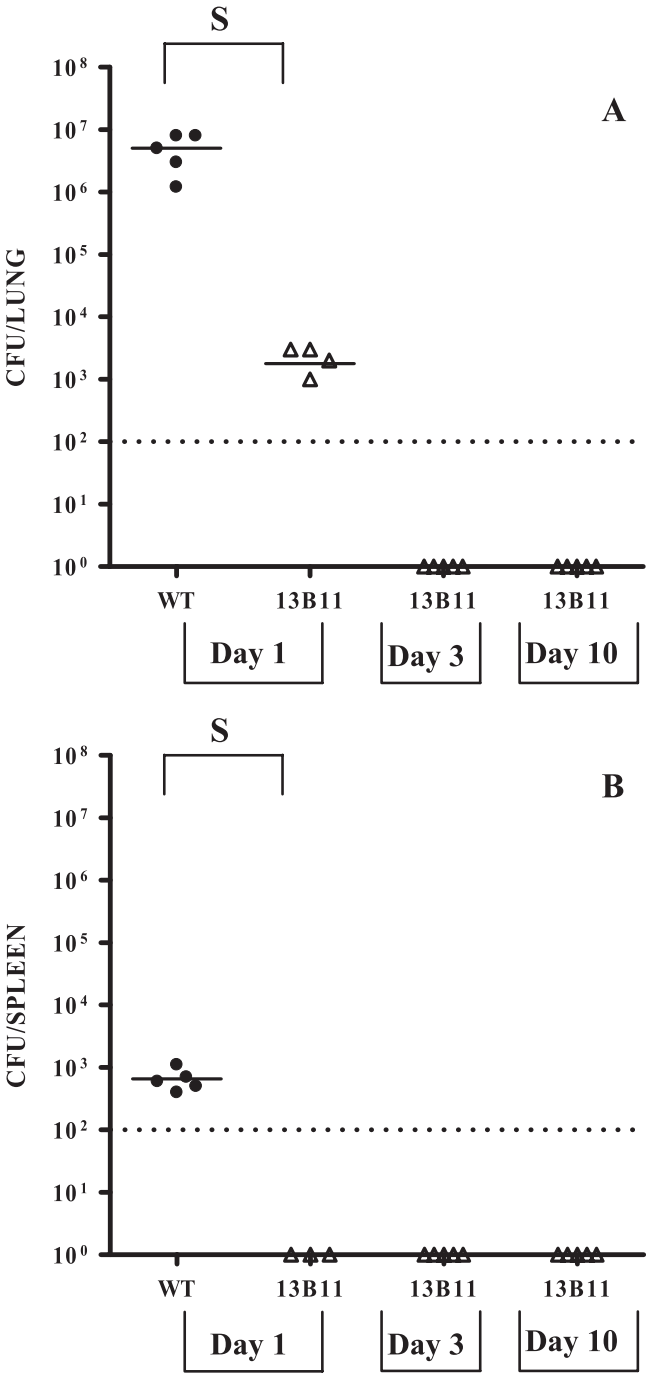

FIG. 8. (A) Organ loads obtained from lungs of BALB/c mice infected i.n. with 6×10^5 CFU of wild-type (WT) *B. pseudomallei* K96243 (●) or 7.5×10^5 CFU of *aroB* mutant strain 13B11 (△). (B) Organ loads obtained from spleens of BALB/c mice infected i.n. with 6×10^5 CFU of wild-type *B. pseudomallei* K96243 (●) or 7.5×10^5 CFU of *aroB* mutant strain 13B11 (△). Solid lines indicate mean values per group, and broken lines show the limit of detection for this assay. S, significant ($P < 0.05$).

the capsular polysaccharide cluster first described by Reckseidler et al. in 2000 (33). The capsular polysaccharide of *B. pseudomallei* has been shown to be critical for virulence in a number of previous studies (2, 33, 34). In order to better understand the reason behind the apparent attenuation, the capsular polysaccharide of *B. pseudomallei* K96243 and a selected panel of transposon derivatives were visualized by immunofluorescence (Fig. 3). The monoclonal antibody 4V1H12, which binds to capsular polysaccharide, was used to detect capsule (the exact epitope reacting to this antibody is not yet known). Mutant 13H8, with an insertion in *wcbB*, putatively coding for a glycosyltransferase, was tested and found to have lost the epitope reacting to 4V1H12 (Fig. 3C and D). The attenuation and loss of polysaccharide observed in this mutant seem to correlate with previously published studies (2, 33, 34).

Vaccination with a live mutant lacking capsular polysaccharide failed to provide complete protection against a subsequent infection (2), indicating that the polysaccharide was an important protective antigen. Three independent insertion mutations in mutants 1B9, 8F1, and 6E3 were found to have occurred in the *wcbC* gene, coding for a putative capsular polysaccharide export protein. A previous study indicated that knocking out this gene function did not result in a loss of capsular polysaccharide and also indicated that the mutant was still fully virulent in the Syrian hamster model of acute melioidosis (33). Mutant 1B9 was tested by immunofluorescence in order to establish if this polysaccharide could still be detected (Fig. 3E and F). Our investigations indicated that this structure was still present in this mutant but that the amount of visible capsular polysaccharide was reduced. Therefore, we postulate that the WcbC protein does play a role in capsular polysaccharide export, a role that is enhanced by the presence of other export proteins within the region.

To confirm array data indicating the presence of bacteria in lungs but absence in spleens at 48 h postinfection, the organ load kinetics of mice challenged with 1×10^3 CFU of *wcbC* mutant strain 1B9 were monitored. The results showed lung loads comparable to those of wild-type-infected mice at 24 h postchallenge; however, by 48 h, the counts had dropped to a level comparable to that of the inoculum, while wild-type-infected mice had considerably higher bacterial loads. At no point were we able to detect the *wcbC* mutant in the spleens of mice, while wild-type-infected mice registered counts at 24 h and 48 h (Fig. 5a and b). The difference in attenuation between Syrian hamsters and BALB/c mice infected with a *wcbC* knockout mutant may be due to the high susceptibility of the hamster model to infection by *B. pseudomallei*. This was also demonstrated when an aflagellate mutant was identified as being fully virulent in hamsters, while a subsequent study of BALB/c mice found the same mutant to be attenuated (10, 13). Mutant 1A6, with a transposon insertion in *wcbN*, coding for a putative D-*glycero*-D-*manno*-heptose 1,7-bisphosphate phosphatase, was also examined by fluorescence microscopy (Fig. 3G and H). The gene coded for an enzyme that putatively catalyzed the removal of a phosphate group from D-*glycero*-β-D-*manno*-heptose 1,7-PP and resulted in the formation of D-*glycero*-β-D-*manno*-heptose 1-P, an intermediate step in the biosynthesis of nucleotide-activated *glycero-manno*-heptose (42). The image obtained revealed that the epitope reacting to the monoclonal antibody 4V1H12 was still intact in this mutant. We postulate that the capsular polysaccharide structure may have been altered but that the epitope reacting with antibody 4V1H12 was still intact.

B. pseudomallei is an environmental saprophyte, so it is likely that the capsule plays a role not only as a defense to the host immune system but also in preventing desiccation when in the soil. The rate at which mutants with transposon insertions in the capsule polysaccharide-coding region are cleared from the lungs and spleens of infected mice indicates that the capsule is an important barrier to the host's innate immune system. To the best of our knowledge, this is the first demonstration of capsule mutant attenuation after i.n. infection. Recent studies of Syrian hamsters have shown that this capsular polysaccharide decreases the effectiveness of opsonization and phagocytosis, leading to a decreased clearance of the organism from the blood (34). The number of mutants identified in the capsule locus suggests that it is an insertion hot spot. Twenty-five genes in the genome were identified as coding for capsular polysaccharide biosynthesis; the genome comprises 5,855 predicted coding sequences. One would estimate that if transposon integration were random, four genes would be identified within this region when 892 mutants were tested. This study identified 22, 4 within *wcbE* and *wcbH* and 3 within *wcbC*. The genes in the capsule polysaccharide gene cluster located between BPSL2787 and BPSL2810 have an average G+C content of 58%, 10% lower than the rest of the genome, suggesting that they may have been acquired as a result of horizontal gene transfer and may be more prone to insertion by Tn*5*. Interestingly, a number of other potential polysaccharide export clusters have been found in the genome of *B. pseudomallei* (21), but these were not identified during this STM study. The reason for this may be that they do not play a role in this particular infection model. Furthermore, analysis of the G+C content of the other potential surface polysaccharide-encoding regions (BPSS1825 to BPSS1834 and BPSS0417 to BPSS0429) found them to match the average G+C content of the *B. pseudomallei* genome.

We identified a novel putative *B. pseudomallei* lipoprotein; a tblastx (1) comparison of the BPSL3147 gene sequence with data in the National Center for Biotechnology Information database revealed 39.16% amino acid identity to a VacJ lipoprotein in *S. flexneri*. This protein was experimentally determined to play a critical role in the intercellular spread of the organism; *S. flexneri vacJ* mutants were capable of spreading within the cytoplasm of infected epithelial cells but were unable to move into the cytoplasm of adjacent cells (41). *B. pseudomallei* is also a facultative intracellular pathogen; intercellular spread plays an important role during infection, as the bacterium has been demonstrated to be able to invade and spread through actin-associated membrane protrusions in both phagocytic and nonphagocytic cell lines (25). We postulate that the BPSL3147 mutant may be attenuated due to a reduced ability to spread intercellularly.

The simultaneous investigation of two target organs resulted in the identification of six mutants that differed from other transposon derivatives by being unable to colonize mouse lungs within 48 h of infection. We postulate that these mutants had insertions in biosynthetic pathways that affected their growth. Five mutants were identified as being putative auxotrophs of leucine, threonine, *p*-aminobenzoic acid, and aro-

matic compounds, while the sixth mutant had an insertion in a putative *recA* gene.

Auxotrophic mutants have been proven to be effective as live attenuated vaccine candidates in many bacterial species including *M. tuberculosis* (37), *Brucella melitensis* (11), *Pseudomonas aeruginosa* (32), and *S. enterica* serovar Typhimurium (20). Live vaccines have advantages over subunit vaccines because they have a wider repertoire of antigens presented to the host immune system, which may lead to stronger immune responses against virulence challenge. Auxotrophs generated by mutations in the shikimate pathway cannot produce chorismate, the precursor for the biosynthesis of aromatic compounds such as PABA, dehydroxybenzoic acid, tryptophan, tyrosine, and phenylalanine. Mutants in the shikimate pathway are attenuated and protective as vaccines in a number of pathogens including *S. enterica* serovar Typhimurium (17), *L. monocytogenes* (40), and *P. aeruginosa* (32). By contrast, in *Y. pestis* strain GB, an *aroA* mutant was found to be virulent in BALB/c mice, suggesting that a functional shikimate pathway does not diminish virulence in this pathogen. This suggests that the organism may be able to bypass the mutation through an alternative mechanism (30).

Given that *aroA* and *aroB* mutants are attenuated in other bacteria (17, 20, 32, 40), we chose *B. pseudomallei* mutant strain 13B11, with a transposon insertion in *aroB*, as a possible vaccine. *aroB* mutant strain 13B11 was unable to grow in minimal medium. Growth could be restored with the addition of tryptophan, tyrosine, phenylalanine, PABA, and 2,3-dihydroxybenzoate. The addition of aromatic compounds could not completely restore the growth rate of the mutant to that of wild-type bacteria. This may have been due to nutrient depletion in the minimal medium. The *aroB* mutant had a growth rate comparable to that of wild-type bacteria up to day 2. We postulate that at this point, the mutant had exhausted the exogenous supply of aromatic compounds and that the growth rate slowed accordingly. When tested individually, no individual aromatic compound was capable of restoring growth (data not shown). This indicates that *B. pseudomallei* does not have an alternative biosynthetic pathway capable of bypassing the mutation and suggests that compounds targeting 3-dehydroquinate synthase may be of use in the treatment of melioidosis. Given that the shikimate pathway is missing in mammals but present in bacteria, fungi, algae, and plants and also in apicomplexan parasites such as *Toxoplasma gondii* and *Plasmodium falciparum* (35), AroB in *B. pseudomallei* may be an interesting drug target.

Our studies indicated a small but significant statistical increase in time to death in mice vaccinated with the *aroB* mutant and subsequently challenged with a 100-fold minimal lethal dose of *B. pseudomallei* K96243 compared to naive mice. However, all mice eventually succumbed to infection and died. An organ load kinetic study of mice challenged with 6.5×10^5 CFU of the *aroB* mutant was carried out to understand the reason behind this low level of protection. The investigation demonstrated that the mutant was cleared rapidly upon infection, resulting in complete clearance from the host by day 3 (Fig. 8).

Mutant 7G7 was found to have a transposon insertion in BPSL2825; the predicted protein shares 50.88% amino acid identity to *Ralstonia solanacearum* putative *p*-aminobenzoate synthetase component I protein PabB. This putative *p*-aminobenzoate auxotroph was found to be unable to grow in minimal medium (data not shown). A recent study by Pilatz et al. demonstrated that a mutant with an insertion in BPSL2825 was severely attenuated in the i.n. BALB/c mouse model; this mutant was also shown to have a defect in iron acquisition and a reduced ability to grow intracellularly (31). In total, putative auxotrophs of five different compounds were identified during this study.

Complementation was not attempted during this study due to the time constraints and the inherent difficulties of carrying out this procedure in *B. pseudomallei*. Using STM, we were able to detect a number of attenuating mutations by more than one route of infection; these data not only confirmed data from independent investigations but also revealed a number of novel attenuating mutations, which may lead to a better understanding of *B. pseudomallei* pathogenesis. The refined mutagenesis strategy used to investigate this highly pathogenic bacterium may be widely applicable to other pathogens.

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