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# A *Burkholderia pseudomallei* Δ*purM* Mutant Is Avirulent in Immunocompetent and Immunodeficient Animals: Candidate Strain for Exclusion from Select-Agent Lists▽

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***Burkholderia pseudomallei*** **causes the disease melioidosis in humans and is classified as a category B select agent. Research utilizing this pathogen is highly regulated in the United States, and even basic studies must be conducted in biosafety level 3 (BSL-3) facilities. There is currently no attenuated *B. pseudomallei* strain available that is excluded from select-agent regulations and can be safely handled at BSL-2 facilities. To address this need, we created Bp82 and Bp190, which are Δ*purM* derivatives of *B. pseudomallei* strains 1026b and K96243 that are deficient in adenine and thiamine biosynthesis but replication competent *in vitro* in rich medium. A series of animal challenge studies was conducted to ensure that these strains were fully attenuated. Whereas the parental strains 1026b and K96243 and the complemented mutants Bp410 and Bp454 were virulent in BALB/c mice following intranasal inoculation, the Δ*purM* mutants Bp82 and Bp190 were avirulent even when they were administered at doses 4 logs higher than the doses used for the parental strains. Animals challenged with high doses of the Δ*purM* mutants rapidly cleared the bacterium from tissues (lung, liver, and spleen) and remained free of culturable bacteria for the duration of the experiments (up to 60 days postinfection). Moreover, highly susceptible 129/SvEv mice and immune incompetent mice (IFN-$\gamma^{-/-}$, SCID) were resistant to challenges with Δ*purM* mutant Bp82. This strain was also avirulent in the Syrian hamster challenge model. We concluded that Δ*purM* mutant Bp82 is fully attenuated and safe for use under BSL-2 laboratory conditions and thus is a candidate for exclusion from the select-agent list.**

Humans develop melioidosis following cutaneous or inhalational infection with the Gram-negative bacterium *Burkholderia pseudomallei*. Meliodosis in humans is associated with a diverse spectrum of diseases, including acute pneumonia, osteomyelitis, hepatic and splenic abscesses, and neurologic disease (7, 40). Septic shock is the most severe clinical manifestation of *B. pseudomallei* infection and is typically associated with bacterial dissemination to the lungs, liver, and spleen (40). Treatment of *B. pseudomallei* infections is complicated because this organism is refractory to antibiotic therapy (41). Moreover, recurrence of infection is common in patients, even following appropriate antimicrobial treatment (21).

Melioidosis occurs primarily in Southeast Asia and Northern Australia, but it has increasingly been found in other tropical and subtropical regions of the world (12), in visitors returning from regions where it is endemic (11, 20), or in tourists affected by natural disasters (2, 8, 10, 37). However, *B. pseudomallei* has received attention in the Western Hemisphere in recent years due to its potential for use as a biological weapon (13). Because of its biodefense implications, this pathogen is classified as a category B select agent by the U.S. Centers for Disease Control and Prevention. This select-agent listing means that research using *B. pseudomallei* is subject to strict federal guidelines that govern the acquisition, possession, and use of this organism (24, 33). Research with *B. pseudomallei* in the United States can be conducted only by cleared personnel in CDC-inspected biosafety level 3 (BSL-3) containment laboratories. For some bacteria on the select-agent list, including *Bacillus anthracis*, *Francisella tularensis*, and *Yersinia pestis*, bona fide attenuated strains (www.selectagents.gov/exclusions.htm) that are not included on select-agent lists are already available. These attenuated strains can be handled in BSL-2 laboratories, which has facilitated studies of these bacteria. However, in the case of *B. pseudomallei* progress has been slow because no approved attenuated strains are available. We believe that availability of such strains would greatly facilitate and accelerate sorely needed basic research on this emerging select agent and priority pathogen. Additionally, attenuated strains have the potential to be used as live vaccine strains since in many cases predosing of animals with attenuated strains has been shown to provide protection against challenges with wild-type bacteria (4, 5, 25, 35, 36). Therefore, the goal of our research was to obtain an attenuated *B. pseudomallei* strain that is avirulent in animal challenge studies and exempt from select-agent registration and thus can be widely distributed and used in BSL-2 laboratories.

Workers have identified diverse *B. pseudomallei* mutants that exhibit various degrees of attenuation in animal models, including mutants deficient in branched-chain amino acid biosynthesis (4) or aromatic compound synthesis (35), mutants

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with mutations affecting capsule biosynthesis (3, 27, 28), mutants lacking a type IV pilin (15), and mutants lacking components of the type III secretion system (36). Pilatz et al. conducted a transposon mutant screen aimed at identification of *B. pseudomallei* genes required for the intracellular life cycle and *in vivo* virulence (5, 26). Among the most highly attenuated mutants was a *purM* mutant. However, this mutant was created using a non-select-agent-compliant method (transposon mutagenesis with a tetracycline resistance marker), was not exhaustively studied using various animal models, and was generated using a strain that is not widely used by and available to the research community.

In the current study, we created Δ*purM* mutant derivatives of readily available and well-studied *B. pseudomallei* strains 1026b (14) and K96243 (18). These mutants were constructed by deleting the *purM* gene, which encodes phosphoribosyl formylglycinamide cycloligase. The product of the reaction catalyzed by this enzyme is aminoimidazole ribotide, a precursor of *de novo* adenine and thiamine biosynthesis. Here we report the results of studies conducted to evaluate the *in vivo* virulence of these Δ*purM* mutants of *B. pseudomallei*, with particular reference to virulence in immunodeficient and hypersusceptible animal models.

## MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** *B. pseudomallei* strain 1026b is a clinical isolate that was obtained from a case of human septicemic melioidosis in Thailand. This strain is lethal to mice and has been extensively studied in the laboratory (14). Bp82 is a Δ*purM* derivative of 1026b and is an adenine and thiamine auxotroph. K96243 is a clinical isolate that was obtained from a case of fatal human melioidosis in Thailand, and its sequence was the first sequence published for any *B. pseudomallei* strain (18). Strains Bp410 and Bp454 are Bp82 and Bp190 derivatives in which the Δ*purM* alleles have been replaced with wild-type *purM* sequences from 1026b. These strains are *purM*$^+$ and adenine and thiamine prototrophs. Strains were grown to saturation in Luria broth (LB) at 37°C with shaking and then stored at −80°C in 15% glycerol until they were used. For animal experiments, each strain was thawed just before use, and the bacteria were diluted to obtain the desired numbers of cells using sterile phosphate-buffered saline (PBS). To obtain growth curves, each strain was grown overnight at 37°C in LB broth. The overnight cultures were diluted 100-fold with either LB broth or M9 medium (22) with 10 mM glucose, and 200-μl aliquots of the diluted cultures were transferred to a sterile, 96-well, black assay plate with a clear bottom (catalog no. 3603; Corning Incorporated, Corning, NY). Growth was recorded using a Synergy HT multimode microplate reader (BioTek Instruments, Winooski, VT) using the following conditions: temperature, 37°C; and shaking at a low speed. The plate was read at 600 nm every 30 min for up to 48 h. Where indicated below, M9-glucose medium was supplemented with either 0.6 mM adenine or 0.0005% thiamine or with both 0.6 mM adenine and 0.0005% thiamine.

**Attenuated mutant strain construction.** A 1,545-bp fragment containing the *purM* gene and flanking DNA was PCR amplified from strain 1026b genomic DNA using *Taq* DNA polymerase (New England Biolabs, Ipswich, MA) and primers 1487 (5′-CACACGTAGAACGTGCGATC) and 1585 (5′-CTTTCGAG AAGCTTTCGACGG; underlining indicates a newly introduced HindIII site) purchased from Integrated DNA Technologies, Coralville, IA. The fragment was ligated into TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA), resulting in pPS2277. Next, a 114-bp NruI fragment was deleted from the *purM* coding sequence present in pPS2277 and replaced with a blunt-ended 776-bp SacI fragment containing a zeocin resistance marker from pFZE1 (9). This step resulted in pPS2336. A 2,253-bp HindIII fragment was purified from pPS2336 and used to transform 1026b utilizing a previously described procedure (9, 38). Zeocin-resistant transformants were obtained, and selected transformants were tested for purine auxotrophy by growing them on M9-glucose minimal medium plates (23) with and without 0.6 mM adenine. One representative colony, designated Bp80, which grew only in the presence of adenine, was retained and used for further study. A zeocin-susceptible derivative of Bp80 was derived by Flp recombinase-mediated excision of the zeocin resistance marker and curing of the Flp source plasmid pFLPe4 using previously described methods (9). The presence of the Δ*purM* mutation in the resulting strain, strain Bp82, was confirmed by PCR amplification of the fragment harboring Δ*purM* and the *FRT* scar using primers 1505 (5′-GATCTTCCATACCTGCTCGC) and 1508 (5′-GAATCCTC CGAAATCCGCTC) and sequencing of the resulting 975-bp PCR fragment. The K96243 Δ*purM* derivative Bp190 has been described previously (22). The Δ*purM* lesions in Bp82 and Bp190 were repaired by allele replacement using an EcoRI fragment containing the 1026b *purM* gene and a previously described pEXKm5-based sucrose counterselection method (22); the resulting adenine and thiamine prototrophs derived from Bp82 and Bp190 were designated Bp410 and Bp454, respectively.

**Animals.** Specific-pathogen-free female mice that were between 4 and 6 weeks old were purchased from Jackson Laboratories (Bar Harbor, ME). The mice used in these studies were BALB/c, 129/SvEv, IFN-γ$^{-/-}$ (with the BALB/c background), and severe combined immune deficiency (SCID) (with the BALB/c background) mice. Syrian hamsters that were 6 weeks old were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in microisolator cages under pathogen-free conditions. All animal studies were approved by the Institutional Animal Care and Use Committee at Colorado State University.

**Animal infections.** Every infection with *B. pseudomallei* was performed using intranasal (i.n.) inoculation. Animals were anesthetized with 100 mg/kg ketamine (Fort Dodge Animal Health, Overland Park, KS) plus 10 mg/kg xylazine (Ben Venue Labs, Bedford, OH). The desired challenge dose of *B. pseudomallei* was suspended in PBS, and 20 μl was delivered i.n. in alternating nostrils. Hamsters were infected in the same manner, but the inoculum was delivered in 60 μl (total volume). In all survival studies, animals were monitored for disease symptoms twice daily and were euthanized based on predetermined humane endpoints.

**Enumeration of viable *B. pseudomallei* in organs.** *B. pseudomallei* in lung, liver, and spleen tissues was quantified at 48 h postinfection in acute challenge studies and between days 30 and 60 postinfection in long-term survival studies. Lungs, spleens, and livers were removed aseptically and homogenized in sterile phosphate-buffered saline using a stomacher (Teledyne Tekmar, Mason, OH). Viable bacterial counts were determined for each organ by plating serial 10-fold dilutions of organ homogenates on LB agar. Plates were incubated at 37°C for 48 h, and then the numbers of colonies on the appropriate plates were scored visually and the bacterial burden was expressed as CFU/organ. Plates containing organ homogenates that were sterile after 48 h of incubation were incubated for an additional 2 to 3 days to ensure that they were sterile.

**Statistical analysis.** Statistical analysis was performed using Prism 5.0 software (GraphPad, La Jolla, CA). Survival times were compared using Kaplan-Meier survival curves and the log rank (Mantel-Cox) test. The bacterial burdens in organs for two groups of mice were compared using a one-sample *t* test. Data were considered to be statistically significantly different if the *P* value was $<0.05$.

## RESULTS

**Construction and properties of *B. pseudomallei* Δ*purM* mutants Bp82 and Bp190.** A previously described recombinant DNA fragment transformation procedure, coupled to Flp-mediated excision of the zeocin resistance gene employed to initially mark and select the *purM* deletion, was used to generate a markerless chromosomal Δ*purM* mutant, Bp82 (9). This mutant lacks codons 95 to 132 of *purM* and expresses a truncated 99-amino-acid PurM protein whose translation terminates at a stop codon in the *FRT* scar (Fig. 1A). As a result, Bp82 does not produce a functional PurM enzyme and is an adenine auxotroph (Fig. 1D and G). Bp190 has an in-frame *purM* deletion without the *FRT* scar, which results in a mutant PurM protein that lacks the same 38 amino acids as the protein remnant in Bp82 but terminates with the native stop codon (Fig. 1B). However, in contrast to the findings for Bp82, while the growth of the K96243 derivative Bp190 is significantly attenuated in M9-glucose minimal medium lacking adenine (Fig. 1F and H), this strain is not a strict adenine auxotroph. The growth rates of 1026b and Bp82 in LB medium are indistinguishable, as are those of K96243 and Bp190 (not shown). Repair of the PurM defects in Bp82 and Bp190 resulted in

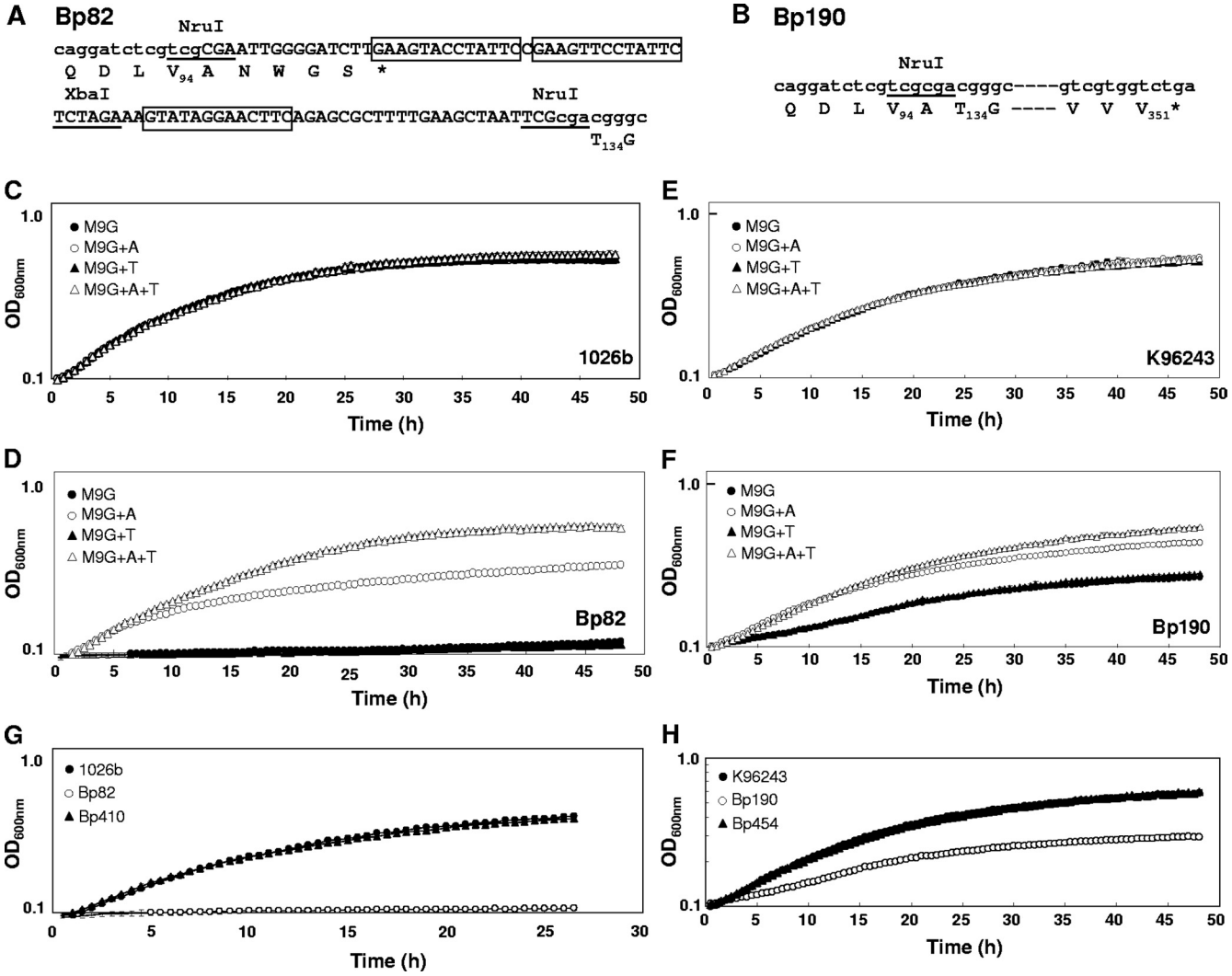


FIG. 1. *purM* mutant alleles and *in vitro* growth of *B. pseudomallei* Δ*purM* mutants Bp82 and Bp190 and their *purM*$^+$ derivatives Bp410 and Bp454. (A) Sequence of the *FRT* scar region in Bp82. Lowercase letters indicate *purM* sequences. Uppercase letters indicate the 86-bp *FRT* scar sequence with the XbaI site and the Flp recombination sequences, which are enclosed in boxes. The residual PurM amino acid sequence is shown below the nucleotide sequence. NruI sites indicate the original junction sequences of *purM* and a zeocin resistance-encoding cassette from pFZE1 and resulted from fusion of an NruI half-site with a T4 DNA polymerase-blunted SacI site. (B) Sequence of the deletion junction in Bp190. Deletion of an internal NruI fragment from *purM* resulted in deletion of 38 amino acids from PurM. The *purM* open reading frame terminates with the stop codon that naturally occurs after valine 351. (C to F) Growth of strains in minimal media. The following strains were tested: 1026b and its Δ*purM* derivative Bp82 and K96243 and its Δ*purM* derivative Bp190. The strains were inoculated into 200 μl of M9-glucose medium (M9G) and into this medium supplemented with 0.6 mM adenine (M9G+A), 0.0005% thiamine (M9G+T), or both adenine and thiamine (M9G+A+T), and growth at 37°C was monitored by determining the optical densities at 600 nm ($OD_{600nm}$) of the cultures at 30-min intervals. The symbols indicate the means of three independently monitored wells, and the error bars indicate standard deviations. (G and H) Growth of prototype, mutant, and complemented strains in minimal medium. The following strains were tested: 1026b and its Δ*purM* derivative Bp82 and K96243 and its Δ*purM* derivative Bp190. Bp410 and Bp454 are Bp82 and Bp190, respectively, with the Δ*purM* allele replaced by *purM*$^+$ from 1026b. The strains were grown in 200 μl of M9-glucose minimal medium without supplements, and growth at 37°C was monitored as described above.

prototrophs (Bp410 and Bp454) whose growth rates in M9-glucose minimal medium were indistinguishable from that of 1026b or K96243 (Fig. 1G and H).

Because the product of the PurM-catalyzed reaction, aminoimidazole ribotide (AIR), is a precursor of thiamine biosynthesis and *B. pseudomallei* possesses the genes for biosynthesis of thiamine from AIR, we assessed whether the Δ*purM* mutants were also thiamine auxotrophs. For both mutant strains, addition of thiamine alone did not affect growth in M9-glucose minimal medium (Fig. 1D and F). While the growth of both mutants in the same medium was significantly improved by addition of adenine alone, only addition of adenine and thiamine restored growth to levels that were indistinguishable from those observed for 1026b and K96243 (Fig. 1, compare panels D and F with panels C and E). The data confirm that both Δ*purM* mutants require both adenine and thiamine for normal growth in minimal medium.

**Deletion of *purM* renders *B. pseudomallei* nonvirulent in BALB/c mice.** *B. pseudomallei* 1026b is lethal to BALB/c mice following i.n. challenge, and the 50% lethal dose ($LD_{50}$) is

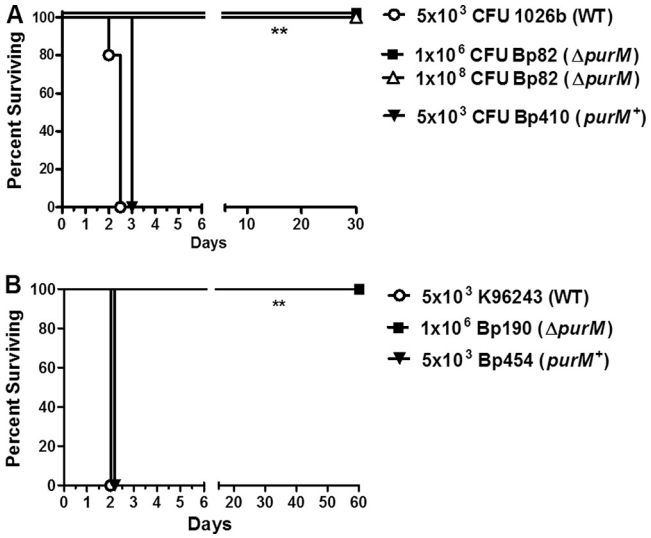


FIG. 2. *B. pseudomallei* Δ*purM* mutants Bp82 (A) and Bp190 (B) are attenuated in BALB/c mice. (A) Mice (5 animals per group) were challenged i.n. with either $5 \times 10^3$ CFU *B. pseudomallei* 1026b (wild-type strain), $1 \times 10^6$ CFU or $1 \times 10^8$ CFU Δ*purM* strain Bp82, or $5 \times 10^3$ CFU Bp410 (Bp82 Δ*purM* lesion repaired with *purM* gene sequence from 1026b). Animal survival was assessed as described in Materials and Methods. The statistical significance of differences in survival times was determined by using Kaplan-Meier curves, followed by a log rank test **, $P < 0.01$ for comparison of Bp82 and 1026b or for comparison of Bp82 and Bp410. (B) Mice (5 animals per group) were challenged i.n. with either $5 \times 10^3$ CFU *B. pseudomallei* K96243 (wild-type strain), $1 \times 10^6$ CFU Δ*purM* strain Bp190, or $5 \times 10^3$ CFU Bp454 (Bp190 Δ*purM* lesion repaired with *purM* gene sequence from 1026b). **, $P < 0.01$ for comparison of Bp190 and K92643 or for comparison of Bp190 and Bp454. WT, wild type.

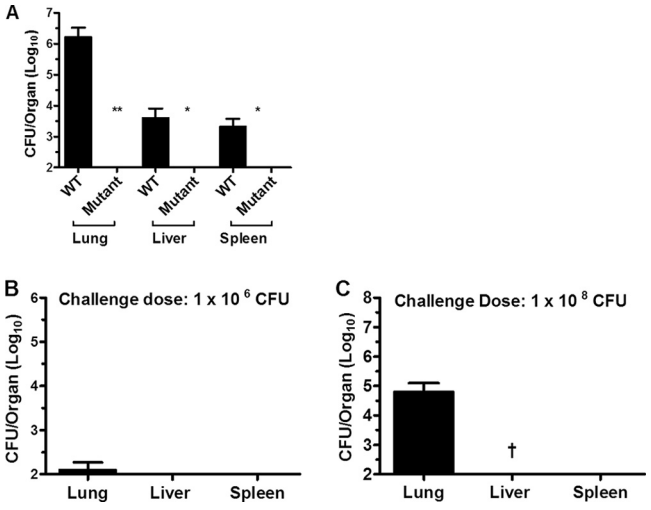


FIG. 3. Bacterial burdens in the lungs, liver, and spleen 48 h after i.n. challenge with wild-type *B. pseudomallei* 1026b or Δ*purM* mutant Bp82. (A) BALB/c mice (3 mice per group) were challenged with $6 \times 10^3$ CFU of *B. pseudomallei* 1026b or Bp82. The bacterial burden in each organ was determined 48 h after challenge. The statistical significance of differences was evaluated using a one-sample *t* test (**, $P < 0.01$; *, $P < 0.05$). WT, wild type. (B and C) BALB/c mice (3 mice per group) were challenged with $1 \times 10^6$ or $1 \times 10^8$ CFU Bp82 (challenge doses are indicated), and bacterial burdens were determined 48 h later. The dagger indicates that there was a single colony on the liver plate for one mouse.

approximately 900 CFU (17). We therefore first determined whether the *purM* deletion reduced the virulence of the resulting strain in BALB/c mice following i.n. challenge. BALB/c mice (5 mice per group) were challenged with 5 $LD_{50}$ (approximately 5,000 CFU) of wild-type *B. pseudomallei* 1026b, and survival was monitored. All mice reached an endpoint and were euthanized within 3 days postinfection (Fig. 2A). Next, BALB/c mice (5 mice per group) were challenged i.n. with high doses ($1 \times 10^6$ and $1 \times 10^8$ CFU) of Δ*purM* strain Bp82. None of the mice challenged with Bp82 died. The animals that received $1 \times 10^6$ CFU did not develop signs of infection, while the mice challenged with $1 \times 10^8$ CFU had ruffling and mild respiratory symptoms that lasted for 3 to 4 days following challenge. All mice were healthy at the time of sacrifice on day 30 postinfection, and the lung, liver, and spleen bacterial burdens for both groups challenged with the mutant strain were all below the limit of detection (100 CFU per organ) (data not shown). These data indicated that the *purM* deletion resulted in a significant ($P < 0.01$) reduction in virulence for wild-type BALB/c mice. The avirulent phenotype of Bp82 was attributable to deletion of the *purM* locus since repair of this locus with the 1026b *purM* gene by allelic exchange resulted in a strain (Bp410) which exhibited full virulence (Fig. 2A).

Although the K96243 derivative Bp190 was not a strict adenine and thiamine auxotroph, it was avirulent in BALB/c mice when very high doses were inoculated i.n. (Fig. 2B). As observed with Bp82, the avirulent phenotype of Bp190 was attributable to deletion of the *purM* locus since repair with the 1026b *purM* gene by allelic exchange resulted in a strain (Bp454) which exhibited full virulence (Fig. 2B). The mice challenged with $1 \times 10^6$ CFU Bp190 did not have clinical signs after challenge and appeared to be healthy during the course of infection. At the time of sacrifice on day 60 postinfection, the lung, liver, and spleen bacterial burdens for both groups challenged with the mutant strain were all below the limit of detection (50 CFU per organ) (data not shown). These data indicated that *purM* deletion from K96243 also resulted in a significant ($P < 0.01$) reduction in virulence for wild-type BALB/c mice.

**Genetic deletion of *purM* enables clearance of *B. pseudomallei* following i.n. challenge in immunocompetent mice.** The next experiments conducted were experiments to assess the degree to which Bp82 was able to replicate in BALB/c mice following i.n. challenge. BALB/c mice (3 mice per group) were infected i.n. with 6 $LD_{50}$ (approximately 6,000 CFU) of *B. pseudomallei* 1026b or Bp82. At 48 h after infection, the animals were sacrificed, and the bacterial burdens in the lungs, liver, and spleen were determined. Wild-type *B. pseudomallei* strain 1026b exhibited significant replication in the lungs of infected mice during the 48 h postchallenge, as shown by a >100-fold increase in the bacterial burden. Moreover, *B. pseudomallei* 1026b also disseminated to the liver and spleen following i.n. inoculation (Fig. 3A). In contrast, at 48 h following challenge, the level of Bp82 was below the limit of detection for the assay in the lungs, liver, and spleen.

To further assess replication and dissemination of Bp82, BALB/c mice were given higher i.n. challenge doses. Thus, one group of mice (3 mice per group) was challenged i.n. with 1,000

$LD_{50}$ ($1 \times 10^6$ CFU), and a second group of mice was challenged with 100,000 $LD_{50}$ ($1 \times 10^8$ CFU). Forty-eight hours after infection, the mice were euthanized, and the bacterial burdens in the lungs, liver, and spleen were determined. In a recent study, we reported that 40% of an i.n. *Burkholderia* inoculum reaches the lungs within 1 h after infection (16). In mice challenged i.n. with $1 \times 10^6$ CFU Bp82 (assuming that $4 \times 10^5$ CFU reached the lungs), there was a 3.5-log reduction in the bacterial burden in the lungs 48 h after challenge, and the bacterial counts in the liver and spleen were below the limit of detection for the assay (Fig. 3B). In mice that received $1 \times 10^8$ CFU Bp82 (assuming that $4 \times 10^7$ CFU reached the lungs), there was a >2.5-log reduction in the bacterial burden in the lungs in the 48 h following challenge. The bacterial burden in the spleen was below the limit of detection. A single colony grew on the liver plate for one mouse, whereas the burdens were below the limit of detection for the other mice (Fig. 3C). These results indicate that there is neither efficient pulmonary replication nor dissemination to the liver or spleen in wild-type mice following high-dose i.n. challenge with Bp82.

The replication and dissemination of Bp190 (Δ*purM* mutant derived from K92643) were also assessed. BALB/c mice (5 mice per group) were challenged i.n. with $1 \times 10^6$ CFU, and 48 h after infection the mice were euthanized and the bacterial burdens in the lungs, liver, and spleen were determined (data not shown). Assuming that 40% of the inoculum ($4 \times 10^5$ CFU) reached the lungs following challenge, there was 2-log reduction in the bacterial burden in the lungs at 48 h following challenge (mean concentration, 3.4 $\log_{10}$ CFU/lung). The bacterial burden in the spleen was below the limit of detection. For two of the five mice *B. pseudomallei* was detected in the liver at 48 h (mean concentration for all mice, 0.68 $\log_{10}$ CFU/liver) (data not shown). Compared to Bp82, strain Bp190 was not cleared as efficiently from the mice in the 48-h time period. This finding is consistent with *in vitro* growth data that indicated that Bp190 was not fully attenuated in M9 medium lacking adenine.

***B. pseudomallei* Δ*purM* strains Bp82 and Bp190 are avirulent in hypersusceptible mice.** We have previously observed that 129/SvEv mice are extremely susceptible to *Burkholderia* infection (A. Goodyear and S. Dow, unpublished observations). For example, the $LD_{100}$ of both *B. pseudomallei* and *Burkholderia mallei* following i.n. challenge in 129/SvEv mice was found to be less than 100 CFU (data not shown). Therefore, we assessed whether Bp82 and Bp190 were lethal when they were inoculated into these hypersusceptible mice. 129/SvEv mice (5 mice per group) were challenged i.n. with 100 CFU of wild-type *B. pseudomallei* 1026b or $1 \times 10^6$ CFU of Bp82. Mice (5 mice per group) were also challenged with 200 CFU *B. pseudomallei* K92643 or $1 \times 10^6$ CFU Bp190. The high challenge dose used for Bp82 and Bp190 was more than 10,000 $LD_{100}$ for wild-type *B. pseudomallei* strains 1026b and K92643 in 129Sv/Ev mice. In animals challenged i.n. with 100 CFU of *B. pseudomallei* strain 1026b, the euthanasia endpoint was reached by day 5 after infection. In contrast, challenge with $1 \times 10^6$ CFU Bp82 did not result in clinical signs or mortality in any of the animals (Fig. 4). Mice challenged with 200 CFU strain K92643 reached the endpoint on day 2 after infection, whereas challenge with $1 \times 10^6$ CFU Bp190 did not cause clinical symptoms or mortality in any of the mice (Fig. 4).

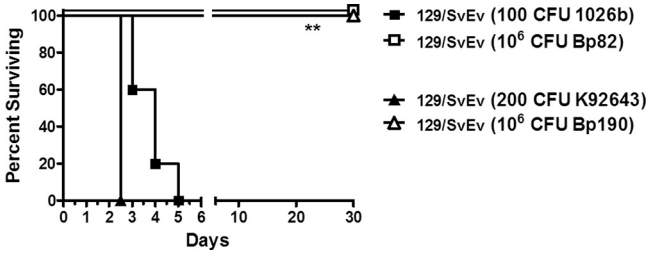


FIG. 4. *B. pseudomallei* Δ*purM* strains Bp82 and Bp190 are avirulent in 129/SvEv mice. Mice (5 animals per group) were challenged i.n. with either 100 CFU *B. pseudomallei* 1026b or $1 \times 10^6$ CFU Bp82. Mice (5 animals per group) were also challenged with either 200 CFU *B. pseudomallei* K92643 or $1 \times 10^6$ CFU Bp190. The statistical significance of differences in survival times was determined by using Kaplan-Meier curves, followed by a log rank test (**, $P < 0.01$ for comparison of Bp82 and 1026b or for comparison of Bp190 and K92643).

All mice infected with Bp82 appeared to be healthy at the time of sacrifice on day 30 postchallenge. The bacterial burdens in the lungs, liver, and spleen for all mice infected with Bp82 were below the limit of detection for the assay (data not shown). All mice infected with Bp190 were sacrificed on day 45 postchallenge, and the bacterial burdens in the lungs, liver, and spleen were below the limit of detection at this time (data not shown). These data indicate that the *purM* deletion in both 1026b and K92643 eliminated the virulence of *B. pseudomallei*, even in hypersusceptible mice.

Even though Bp190 was avirulent in both BALB/c and hypersusceptible 129/SvEv mice, this strain was less attenuated *in vitro* and not as dissemination deficient *in vivo* as Bp82. Thus, further animal testing was performed only with Bp82.

***B. pseudomallei* Δ*purM* strain Bp82 is avirulent in immunodeficient mice.** Previous studies clearly demonstrated that gamma interferon (IFN-γ) is vital for host defense against *B. pseudomallei* (30) and *B. mallei* (29) infection. To further investigate the degree to which Bp82 was attenuated, IFN-$\gamma^{-/-}$ mice (3 or 4 mice per group) were challenged with a low dose (approximately 500 CFU) of wild-type *B. pseudomallei* 1026b or a high dose ($1 \times 10^6$ CFU) of Bp82. While we did not experimentally determine the $LD_{50}$ of *B. pseudomallei* for IFN-γ knockout mice, we estimated that the $LD_{50}$ was in the range from 10 to 100 CFU. Therefore, the $10^6$-CFU challenge dose of Δ*purM B. pseudomallei* Bp82 used was approximately 100,000 times the estimated $LD_{50}$, and therefore the difference was similar to the difference in the doses given the BALB/c mice. We observed that all mice challenged with *B. pseudomallei* 1026b developed severe disease, and they were euthanized on day 2 following challenge (Fig. 5A). In contrast, none of the IFN-$\gamma^{-/-}$ mice challenged with a high dose of Bp82 developed clinical signs or succumbed to infection. The lung, liver, and spleen bacterial burdens for IFN-$\gamma^{-/-}$ mice infected with Bp82 were below the limit of detection at day 30 postinfection (data not shown).

The attenuation of *B. pseudomallei* Δ*purM* strain Bp82 was also evaluated using severe combined immune deficiency (SCID) mice. SCID mice are nearly devoid of B and T lymphocytes, which largely eliminates adaptive immune responses but leaves the innate immunity intact. SCID mice (5 mice per group) were challenged with $1 \times 10^4$ CFU of *B. pseudomallei*

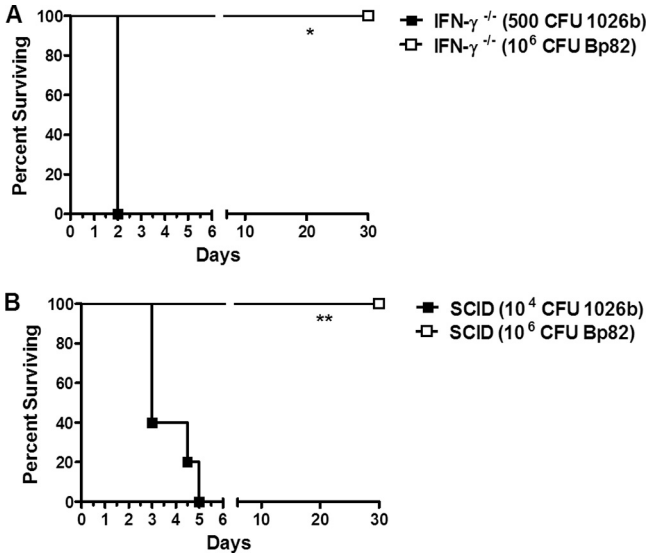


FIG. 5. *B. pseudomallei* Δ*purM* mutant Bp82 is avirulent in immunodeficient mice. (A) IFN-$\gamma^{-/-}$ mice (3 or 4 mice per group) were challenged i.n. with either 500 CFU of *B. pseudomallei* 1026b or $1 \times 10^6$ CFU of Δ*purM* strain Bp82. (B) SCID mice (5 mice per group) were challenged i.n. with either $1 \times 10^4$ CFU of *B. pseudomallei* 1026b or $1 \times 10^6$ CFU of Bp82. The statistical significance of differences in the survival times of Bp82 and 1026b was determined by using Kaplan-Meier curves, followed by a log rank test (**, $P < 0.01$; *, $P < 0.05$).

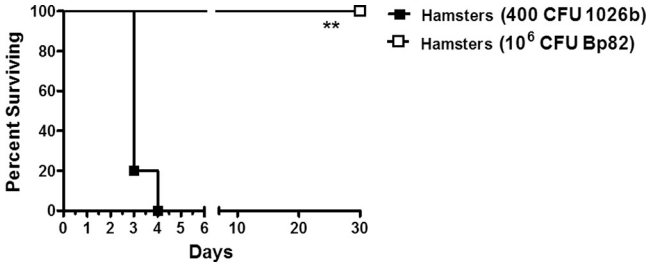


FIG. 6. *B. pseudomallei* Δ*purM* strain Bp82 is avirulent in Syrian hamsters. Hamsters (5 animals per group) were challenged i.n. with 400 CFU of *B. pseudomallei* 1026b or $1 \times 10^6$ CFU of Δ*purM* mutant Bp82. The statistical significance of differences in the survival times of Bp82 and 1026b was determined by using Kaplan-Meier curves, followed by a log rank test (**, $P < 0.01$).

1026b or with $1 \times 10^6$ CFU of Bp82. The SCID mice challenged with wild-type *B. pseudomallei* succumbed to infection within 5 days after challenge, which was a significantly longer time to death than that for the IFN-$\gamma^{-/-}$ mice ($P = 0.003$) or the wild-type BALB/c mice ($P = 0.004$) following challenge with 1026b. All of the SCID mice challenged with Bp82 remained healthy throughout infection and survived (Fig. 5B). The bacterial burdens at day 30 postinfection were below the limit of detection for mice challenged with Bp82 (data not shown).

***B. pseudomallei* Δ*purM* strain Bp82 is attenuated in Syrian hamsters.** Syrian hamsters have previously been shown to be exquisitely susceptible to *B. pseudomallei* infection (39). Therefore, we challenged Syrian hamsters i.n. with wild-type and mutant *B. pseudomallei* strains. Syrian hamsters (5 animals) were challenged i.n. with a low dose of wild-type *B. pseudomallei* 1026b (approximately 400 CFU) or with a high dose ($1 \times 10^6$ CFU) of Bp82 (Fig. 6). The animals challenged with *B. pseudomallei* 1026b developed acute illness, and all of them were euthanized by day 4 postchallenge. In contrast, there were no signs of disease in the animals challenged with Bp82. All hamsters infected with Bp82 remained healthy for 30 days postinfection, and the bacterial burdens in the lungs, liver, and spleen were all below the limit of detection at the time of sacrifice (data not shown).

## DISCUSSION

Despite the medical importance of melioidosis, studying *B. pseudomallei* is still cumbersome, especially in Western countries, where the awareness of melioidosis as a potential bioweapon has led to implementation of stringent security and containment requirements. Nowhere is this more evident than in the United States. The strict regulations governing the acquisition, possession, and use of *B. pseudomallei* in the United States hinder even basic studies of the physiology, genetics, antimicrobial resistance, etc., of this important yet understudied pathogen and make sharing of mutants difficult because of the permit requirements. Therefore, there is a growing need for safe, attenuated mutants of *B. pseudomallei* that can be utilized under BSL-2 containment conditions.

As mentioned above, several *B. pseudomallei* mutants that exhibit various degrees of attenuation in cell culture or animal models have been identified. These mutants were created mostly for live vaccine studies. Attenuated mutants fall into roughly two categories, mutants that do not express virulence factors (e.g., mutants that do not express capsule [3, 27, 28], mutants that lack a type IV pilin [15], and mutants that lack components of the type III secretion system [36]) and mutants that have metabolic defects (e.g., mutants that have defects in branched-chain amino acid biosynthesis [4], aromatic compound synthesis [35], and purine biosynthesis [5, 26]). Most of these mutants, however, either were not created using select-agent-compliant methods or were not well characterized at the molecular level or thoroughly evaluated in various animal models so that they met the criteria required for an organism to be considered for exclusion from the select-agent list.

In this study we created attenuated mutants, characterized them at the molecular level, and thoroughly evaluated them in various animal models with the goal of defining a strain that meets the criteria for exclusion from the select-agent list. We used select-agent-compliant methods to isolate and characterize defined Δ*purM* mutants. This target was chosen mainly for two reasons. First, transposon-induced *B. pseudomallei purM* mutants were shown to be severely replication deficient in cell culture and attenuated in a murine melioidosis model (5, 26). Second, the purine biosynthetic pathway is a validated target for attenuated mutant construction in bacteria. It has been successfully targeted in attempts to isolate attenuated mutants of *Francisella tularensis* (25), *Brucella abortus* (1), *Shigella flexneri* (6), *Actinobacillus pleuropneumoniae* (34), and *Mycobacterium tuberculosis* (19). In this study we constructed Bp82 and Bp190, which are Δ*purM* derivatives of strains 1026b and K96243, respectively. 1026b and K9623 were originally isolated from human melioidosis patients in Thailand. While 1026b is

amenable to genetic manipulation and has been widely used for research in North America, K96243 is the sequenced prototype strain.

*In vitro* growth experiments demonstrated that Bp82 was an obligate auxotroph and required both adenine and thiamine for normal growth in M9-glucose minimal medium. In contrast, the growth of Bp190 was only partially attenuated in the same medium, and normal growth required addition of both adenine and thiamine. At present, we do not understand why Bp190 is not an obligate adenine auxotroph. One possible explanation is that in contrast to the truncated 99-amino-acid PurM protein present in Bp82, Bp190 PurM has an internal in-frame 38-amino-acid deletion. The 313-amino-acid PurM protein may have enough enzymatic activity to sustain partial growth in minimal media in the absence of adenine and thiamine. We are currently constructing a K96243 Δ*purM* strain with the Bp82 allele to test this hypothesis. We did not observe any suppressors of adenine and thiamine auxotrophy *in vitro* for either Bp82 or Bp190.

Both Δ*purM* mutants constructed in this study have an internal deletion which is unlikely to be repaired by natural means. For increased safety and a decreased likelihood of suppression of the Δ*purM* mutation, we are currently constructing derivatives of Bp82 and Bp190 that have at least one additional auxotrophic mutation. The growth rates of 1026b and K96243 and their Δ*purM* derivatives Bp82 and Bp190 in rich medium were indistinguishable, indicating that the fitness of these strains under these conditions is very similar. These data indicate that Δ*purM* mutants are valid surrogates for many basic biological and applied studies (e.g., deciphering antibiotic resistance mechanisms or drug discovery research).

When i.n. inoculation was used, the Δ*purM* mutants were avirulent in acute BALB/c infection models even at high challenge doses (up to $10^6$ CFU).

For demonstration of complete safety and lack of virulence *in vivo*, it is often necessary to conduct challenge studies with strains of animals that are extremely susceptible to bacterial infection and with immunodeficient animals. The results of the present study show that *B. pseudomallei* Δ*purM* mutants Bp82 and Bp190 were fully attenuated in hypersusceptible 129/SvEv mice, and Bp82 was also avirulent in the Syrian hamster model. In addition, the mutant strains did not efficiently replicate *in vivo* or disseminate following i.n. challenge with high doses. It should also be noted that the animals used in this study were infected via the inhalational challenge route, which is the most lethal route of infection and the route by which healthy laboratory workers would most likely be infected (31). Moreover, *B. pseudomallei* Δ*purM* strain Bp82 did not cause mortality in immunodeficient mice, including IFN-$\gamma^{-/-}$ mice and SCID mice. Thus, based on very stringent animal challenge criteria, the *B. pseudomallei* Δ*purM* strains created here are fully attenuated. The attenuation was due solely to the *purM* defect since repair of the Bp82 and Bp190 Δ*purM* allele with wild-type sequences resulted in adenine and thiamine prototrophy and restored virulence.

In summary, extensive *in vitro* characterization and stringent animal challenge experiments showed that both *B. pseudomallei* Δ*purM* derivatives constructed and tested in this study are, in principle, viable candidates for exclusion from select-agent lists. However, given the overall evidence, we consider the *B. pseudomallei* 1026b Δ*purM* mutant Bp82 the superior attenuated strain candidate. This mutant is fully attenuated *in vitro* when it is grown in adenine- and thiamine-deficient growth medium. In addition, it is avirulent *in vivo*, even following high-dose challenge of extremely susceptible wild-type and immunodeficient animals. Moreover, this mutant does not replicate *in vivo* and also does not establish chronic infections. Thus, we concluded that to date, *B. pseudomallei* Δ*purM* mutant Bp82 is the most viable candidate strain for exclusion from select-agent lists and with good laboratory practice is safe for use under BSL-2 conditions. Federal regulations permit such exclusions from the list of select biological agents in cases when it has been established that an attenuated strain of a select biological agent does not pose a severe threat to public health and safety, animal health, or animal products. Unlike the situation for *B. mallei*, for which there are variants that are severely attenuated for virulence in their natural host and thus are likely candidates for exclusion from the select-agent list (32), clinically attenuated *B. pseudomallei* strains that grow normally in laboratory media have yet to be discovered. Until such strains are discovered, genetically engineered and well-characterized strains such as the strain described here are the only candidates for consideration for exclusion and provide useful tools for the extended research community.

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