Aerogenic vaccination with a *Burkholderia mallei* auxotroph protects against aerosol-initiated glanders in mice

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Abstract

Burkholderia mallei is an obligate mammalian pathogen that causes the zoonotic disease glanders. Two live attenuated *B. mallei* strains, a capsule mutant and a branched-chain amino acid auxotroph, were evaluated for use as vaccines against aerosol-initiated glanders in mice. Animals were aerogenically vaccinated and serum samples were obtained before aerosol challenge with a high-dose (>300 times the LD_{50}) of *B. mallei* ATCC 23344. Mice vaccinated with the capsule mutant developed a Th2-like Ig subclass antibody response and none survived beyond 5 days. In comparison, the auxotrophic mutant elicited a Th1-like Ig subclass antibody response and 25% of the animals survived for 1 month postchallenge. After a low-dose (5 times the LD_{50}) aerosol challenge, the survival rates of auxotroph-vaccinated and unvaccinated animals were 50 and 0%, respectively. Thus, live attenuated strains that promote a Th1-like Ig response may serve as promising vaccine candidates against aerosol infection with *B. mallei*.
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Keywords: Glanders; Vaccine; Allelic exchange

1. Introduction

Burkholderia mallei, the causative agent of glanders, is a gram-negative bacillus. It is a highly adapted parasite of equines and cannot persist in nature outside of its horse host [1]. Glanders was eradicated in many countries through the use of the mallein test, but sporadic cases still occur in Asia, Africa, the Middle East, and south America. Approximately 90% of *B. mallei* infections in horses are latent or non-clinical [2]. Chronically infected horses serve as efficient reservoirs for transmitting the disease to uninfected animals, especially under conditions of crowding [3]. Clinical cases of glanders in horses are characterized by nasal discharge from one or both nostrils with or without visible ulceration of the nasal septum, enlargement and induration along the lymphatics and lymph nodes, and the presence of nodules, pustules, and ulcers on the skin, lungs, and other organs.

Humans are incidental hosts for *B. mallei* and glanders in humans is almost always fatal without antibiotic intervention [2,4,5]. Human infections typically occur in people who have occupations that put them in contact with glanderous animals, such as veterinarians, stablemen, slaughter-house employees, and farmers. The most likely route of transmission involves the direct contact of infectious exudates with cuts and abrasions or with mucosal membranes. *B. mallei* is also highly infectious by aerosol [6,7] and it is widely believed that it has the potential for use as a weapon of biological terrorism [8–10]. The incubation period in human glanders is 1–14 days. The course of the disease in humans is highly variable and both chronic and acute infections occur [2,4,5].

No vaccines are currently available for preventing glanders in horses or humans. *B. mallei* is a facultative intracellular pathogen and a live attenuated vaccine may be the

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Report Documentation Page

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
14 MAR 2005	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Aerogenic vaccination with a Burkholderia mallei auxotroph protects against aerosol-initiated glanders in mice. Vaccine 23:1986 - 1992	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Ulrich, RL Amemiya, K Waag, DM Roy, CJ DeShazer, D	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD	**RPP-04-357**

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Burkholderia mallei is an obligate mammalian pathogen that causes the zoonotic disease glanders. Two live attenuated B. mallei strains, a capsule mutant and a branched-chain amino acid auxotroph, were evaluated for use as vaccines against aerosol-initiated glanders in mice. Animals were aerogenically vaccinated and serum samples were obtained before aerosol challenge with a high-dose (>300 times the LD50) of B. mallei ATCC 23344. Mice vaccinated with the capsule mutant developed a Th2-like Ig subclass antibody response and none survived beyond 5 days. In comparison, the auxotrophic mutant elicited a Th1-like Ig subclass antibody response and 25% of the animals survived for 1 month postchallenge. After a low-dose (5 times the LD50) aerosol challenge, the survival rates of auxotroph-vaccinated and unvaccinated animals were 50 and 0%, respectively. Thus, live attenuated strains that promote a Th1-like Ig response may serve as promising vaccine candidates against aerosol infection with B. mallei.

15. SUBJECT TERMS
Burkholderia mallei, glanders, vaccine, auxotroph, aerosol exposure, laboratory animals, mice

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE	**SAR**	**10**	
unclassified	**unclassified**	**unclassified**			

best strategy to induce both cellular and humoral immune responses to this organism. Live attenuated bacterial vaccines harbor genetically-defined mutations in virulence factors and/or biosynthetic pathways that hinder replication in vivo [11,12]. Such vaccines often establish limited infections that mimic natural infections and generate systemic and mucosal immune responses. In addition, they can often induce the production of cytokines that recruit elements of the immune system that ordinarily do not respond to subunit and inactivated vaccines. Live attenuated vaccines are relatively inexpensive to produce and are immunogenic and protective against a variety of facultative intracellular pathogens, including *Mycobacterium tuberculosis*, *Shigella* spp., *Salmonella* spp., *Yersinia* spp., *Listeria monocytogenes*, *Francisella tularensis*, *Brucella melitensis*, and *Burkholderia pseudomallei* [11–16]. In this report, we describe the construction of a *B. mallei* branched-chain amino acid auxotroph, and its use as a live attenuated vaccine against an aerosol challenge in mice.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli TOP10 (Invitrogen, Carlsbad, CA) was used for general cloning and blue/white screening, and *E. coli* S17-1 [17] was used as a mobilizing strain for constructing mutants. The *B. mallei* mutant strains used in this study were derivatives of ATCC 23344, a highly pathogenic clinical isolate and type strain of the species [18–20]. *B. mallei* SR1, Δ(BMAA0437-BMAA0497), is a sucrose-resistant mutant that harbors an IS*407*A-mediated deletion of a 78-kb region of chromosome 2 encompassing the *sacB* gene [19]. SR1 exhibits full virulence in BALB/c mice and Syrian hamsters. *B. mallei* ILV1, Δ(*ilvI*) Δ(BMAA0437-BMAA0497), is a branched-chain amino acid auxotroph derived from SR1 (see below). *B. mallei* DD3008, *wcbB*::pGSV3, is a capsule mutant that is avirulent in animals [18,21]. Bacteria were grown at 37 °C on LB agar (Lennox L agar, Sigma, St. Louis, MO) or in LB broth (Lennox L broth). The medium was supplemented with 4% glycerol for the growth of *B. mallei*. Five percent sucrose was added to LB agar for experiments utilizing the gene replacement vector pGRV2. When appropriate, antibiotics were added at the following concentrations: 15 µg of polymyxin B (Pm) and 5 µg of gentamicin (Gm) per ml for *B. mallei* and 100 µg of ampicillin (Ap), 25 µg of kanamycin (Km), and 15 µg of Gm per ml for *E. coli*. *B. mallei* ILV1 was grown on Difco™ M9 Minimal Salts (Becton, Dickinson, and Company, Sparks, MD) agar containing 0.4% glucose (M9 glucose) supplemented with isoleucine, leucine, and valine, each at 40 µg/ml.

2.2. DNA manipulation

Restriction enzymes, T4 DNA ligase, Klenow, and T4 DNA polymerase were purchased from Roche Diagnostics Corporation (Indianapolis, IN) and were used according to the manufacturer's instructions. DNA fragments used in cloning procedures were excised from agarose gels and purified with a GeneClean III Kit (Q●BIOgene, Carlsbad, CA). Bacterial genomic DNA was prepared using a previously described protocol [22]. Plasmids were purified from overnight cultures using the Wizard *Plus* SV Minipreps DNA Purification System (Promega, Madison, WI). The allelic exchange vector pGRV2 was constructed by replacing the Ap resistance (Ap^r) gene of pEX100T [23] with the Gm resistance (Gm^r) gene from pKNOCK-Gm [24]. The 800 bp Gm^r fragment was released with *Mlu*I, blunted with Klenow and T4 DNA polymerase, and cloned into the unique *Sca*I site within the Ap^r gene. Because, the resulting vector contained few useful cloning sites, the 319 bp polylinker from pMOLUC [25] was excised with *Nco*I and *Spe*I, blunted with Klenow and T4 DNA polymerase, and cloned into the unique *Sma*I site. The resulting gene replacement vector, pGRV2, is shown schematically in Fig. 1A.

2.3. PCR amplifications

PCR products were sized and isolated using agarose gel electrophoresis, cloned using the pCR2.1 TOPO TA Cloning Kit (Invitrogen), and transformed into chemically competent *E. coli* TOP10. PCR amplifications were performed in a final reaction volume of 100 µl containing 1X Taq PCR Master Mix (Qiagen, Valencia, CA), 1 µM oligodeoxyribonucleotide primers, and approximately 200 ng of genomic DNA. PCR cycling was performed using a PTC-150 MiniCycler with a Hot Bonnet accessory (MJ Research, Inc., Reno, NV) and heated to 97 °C for 5 min. This was followed by 30 cycles of a three-temperature cycling protocol (97 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s) and one cycle at 72 °C for 10 min.

2.4. Construction of B. mallei ILV1

The *B. mallei ilvI* gene and flanking regions were identified by performing a blastn search of the *B. mallei* ATCC 23344 genomic sequence (http://www.tigr.org/) with the *B. pseudomallei ilvI* gene [13]. PCR amplicons flanking the 5′ and 3′ ends of *ilvI* were obtained using ATCC 23344 genomic DNA and the primer pairs ILV1/ILV2 (971-bp) and ILV3/ILV4 (1039 bp), respectively. The nucleotide sequences of the primers (5′ to 3′) were as follows: ILV1, TTGAGCGCCAACGCCAATGC; ILV2, ATTCCGACGTGGAGAATTCC; ILV3, CGCTGCCCGATTTCGTGAAG; and ILV4, AGACCGACCGTCACGTTCAC. The PCR products were directionally cloned into pGRV2 and the resulting plasmid was designated pDD120. This plasmid was mobilized into *B. mallei* SR1 via biparental mating with *E. coli* S17-1 (pDD120), as previously described [21]. Pm was used to counterselect *E. coli* and Gm was used to select for transconjugants containing pDD120 integrated into the chromosome via homologous recombination. Numerous Gm^r
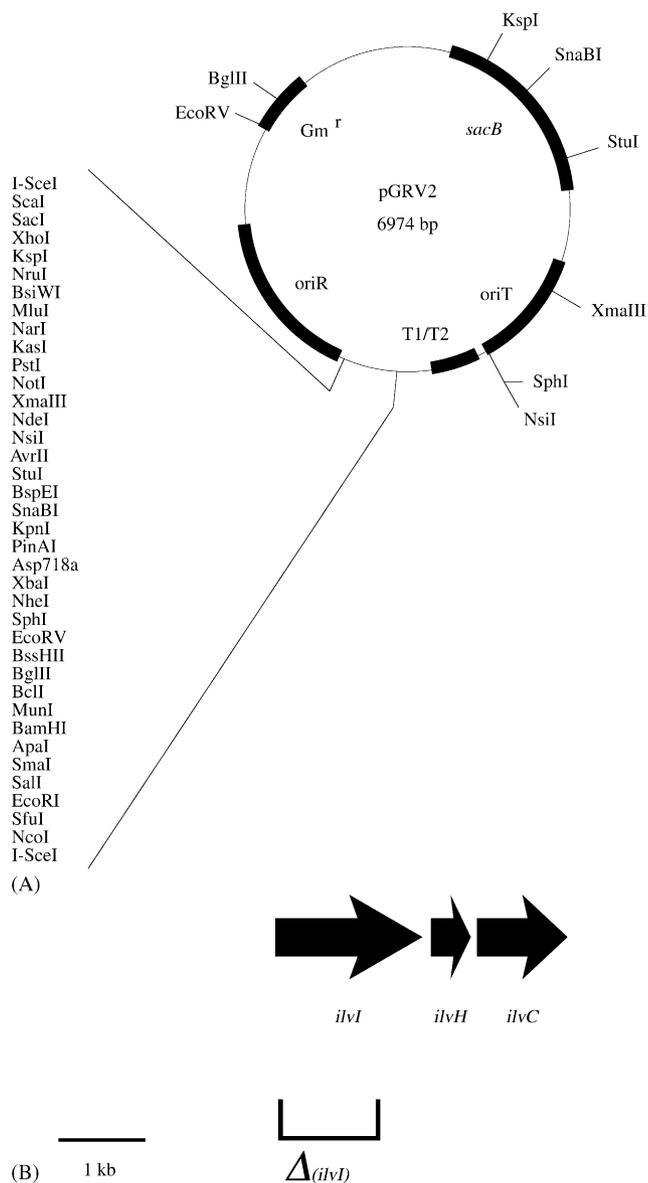
(A)

(B)   1 kb $\Delta_{(ilvI)}$

Fig. 1. Schematic view of pGRV2 and the *B. mallei* ATCC 23344 *ilvIHC* locus. (A) Gene replacement vector pGRV2. Gentamicin resistance gene, Gm^r; *sacB*, *Bacillus subtilis* gene encoding levansucrase; oriR, pMB1-based origin of replication; oriT, origin of transfer; T1/T2, double transcriptional terminators. (B) Construction of *B. mallei* ILV1. The *ilvIHC* locus on *B. mallei* chromosome 1 is depicted schematically. The extent of the Δ(*ilvI*) mutation in *B. mallei* ILV1 is shown. *ilvI*, gene encoding acetolactate synthase (large subunit); *ilvH*, gene encoding acetolactate synthase (small subunit); *ilvC*, gene encoding ketol-acid reductoisomerase. The genes *ilvI*, *ilvH*, and *ilvC* are also known as BMA1848, BMA1847, and BMA1846, respectively. A scale bar, representing 1 kb, is shown.

colonies (SR1::pDD120) were obtained and one was used to inoculate LBG broth without antibiotic selection. Ten-fold dilutions of the overnight culture were spread onto LBG plates with and without 5% sucrose. Sucrose-resistant colonies were recovered at a frequency of 6.5×10^{-8}. To select for bacteria that underwent a second homologous recombination resulting in a deletion of *ilvI*, 100 sucrose-resistant colonies were transferred to petri plates containing M9 glucose and M9

glucose supplemented with isoleucine, leucine, and valine. Eight percent of the sucrose-resistant colonies were unable to grow on M9 glucose without branched-chain amino acid supplementation, suggesting that they harbored the 1510 bp internal deletion of *ilvI* (Fig. 1B). DNA was isolated from the auxotrophs and PCR was performed with ILV1 and ILV4 to confirm the Δ(*ilvI*) mutation. One of the isoleucine, leucine, and valine auxotrophs was arbitrarily chosen and designated as *B. mallei* ILV1.

2.5. Animal studies

Female BALB/c mice were obtained from Charles River Laboratories (National Cancer Institute, Frederick, MD) and were 6- to 8-week-old at the time of use. Animals were provided with rodent feed and water ad libitum and maintained on a 12-h light cycle. Whole-body exposures to aerosols of *B. mallei* were performed as described by Roy et al. [26]. When necessary, mice were euthanized (CO_2 chamber) and spleens, livers, and lungs were removed, homogenized in sterile phosphate-buffered saline (PBS), serially diluted, and plated on LBG. Three weeks after primary vaccination and boost, ILV1- and DD3008-vaccinated mice (three animals per group) were exsanguinated by intracardial puncture. Serum samples were pooled and stored at $-20\,^\circ\text{C}$ until further use. Bronchoalveolar lavage (BAL) fluid was also collected immediately after exsanguination. BAL fluid samples were pooled and stored at $-20\,^\circ\text{C}$ until further use. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals* (http://www.nap.edu/readingroom/books/labrats/chaps.html). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.6. Antibody assays

Antibody titers were determined as described previously [27]. Briefly, two-fold dilutions of antisera were made in triplicate in 96-well, Immulon 2HB plates (Thermo Labsystems, Franklin, MA), previously containing 50 μl of nonviable, irradiated *B. mallei* ATCC 23344 (10 μg/ml in 0.1 M carbonate buffer, pH 9.5). After incubating the plates for 1 h at 37 °C, the plates were washed, and 50 μl of 1/5000-diluted anti-immunoglobulin (Ig)-horseradish peroxidase conjugate (anti-Ig class or subclass conjugate) obtained from Southern Biotechnology Associates (Birmingham, AL) was added to each well, and plates incubated for 1 h at 37 °C. After the plates were washed, the color was developed by adding 50 μl of a buffered hydrogen peroxide and 3,3′, 5,5′-tetramethylbenzidine solution (BD Pharmingen, San Diego, CA) was added to each well. Plates were incubated for 15 min at room temperature. The reaction was stopped with 25 μl of

2N sulfuric acid, and the amount of antibody bound was determined by reading the optical density at 450 nm with a reference filter at 570 nm on a Dynex MRX instrument (Dynex Technologies, Inc., Chantilly, VA). The results are reported as the reciprocal of the highest dilution giving a mean OD of at least 0.1, which is at least twice the background, ±1 S.D.

3. Results

3.1. Construction and use of a B. mallei gene replacement vector

There are few plasmids or methods available for constructing unmarked mutations by allelic exchange in *B. mallei* [28]. Allelic exchange involves replacing a gene with an in vitro-modified allele and requires the use of a suicide vector containing a counterselectable marker such as *rpsL* or *sacB* [29]. We constructed a gene replacement vector based on *sacB* for use in *B. mallei* (Fig. 1A). The pGRV2 vector was used to construct *B. mallei* ILV1, a branched-chain amino acid auxotroph. ILV1 contained a 1510 bp internal deletion of *ilvI*, the gene encoding the large subunit of acetolactate synthase (Fig. 1B). This enzyme is required for the biosynthesis of branched-chain amino acids, and ILV1 did not grow on M9 glucose medium without isoleucine, leucine, and valine supplementation.

3.2. B. mallei ILV1 is attenuated in mice and generates a Th1-like Ig subclass antibody response

The focus of this investigation was to develop a vaccine that prevents glanders delivered by aerosol. We aerogenically vaccinated mice with two genetically defined *B. mallei* mutants, ILV1 and DD3008 [21]. ILV1-vaccinated animals received a predicted inhaled dose of 7.3×10^4 bacteria and DD3008-vaccinated animals received a predicted inhaled dose of 1.2×10^3 bacteria. Three mice from each group were euthanized 1 week postexposure and their spleens, livers, and lungs were homogenized, diluted in PBS, and spread onto LBG plates. No bacteria were present in tissue homogenates, suggesting both strains were cleared from the animals within

7 days of aerosol exposure. In fact, no animals died from vaccination with ILV1 or DD3008 during this study. Pooled sera and BAL fluid samples from three animals in each group were examined for Ig class and Ig subclass antibody responses 3 weeks after vaccination. The BAL fluid IgG and IgA titers of ILV1- and DD3008-vaccinated animals were not significantly different than naive control mice (data not shown). Serum IgG and IgM titers of mice exposed to ILV1 were 20- and 60-fold higher than naive controls, respectively (Table 1). The predominate IgG subclass elicited by ILV1 was IgG2a and the ratio of IgG2a to IgG1 was 16.0. In comparison, the IgG and IgM sera titers of animals exposed to DD3008 were 10- and 60-fold higher than unvaccinated controls, but the IgG2a subclass titer was only 50 (Table 1). On day 22, the animals were revaccinated (boosted) with whole-body aerosols of ILV1 and DD3008. The predicted inhaled doses for ILV1 and DD3008 were 4.7×10^4 and 1.3×10^5 bacteria, respectively. Serum and BAL fluid samples were obtained from three animals in each group 3 weeks postboost (Table 1). The BAL fluid IgG and IgA titers from ILV1- and DD3008-boosted mice were still not measurable (titer < 50), but there was a substantial increase in serum IgG titers in both groups (Table 1). The ratio of IgG2a to IgG1 was 4.7 for ILV1-boosted mice and 0.3 for DD3008-boosted mice. We conclude from these results that (1) ILV1 was attenuated in mice by the aerosol route of infection, (2) ILV1 elicited a Th1-like Ig subclass antibody response, (3) DD3008 induced a Th2-like Ig subclass antibody response, and (4) neither strain generated a significant mucosal immune response.

3.3. Vaccination with ILV1, but not DD3008, protected against a high-dose aerosol challenge with ATCC 23344

BALB/c mice are susceptible to infection with *B. mallei* ATCC 23344 given by aerosol and the LD_{50} is approximately 10^3 bacteria [30,31]. Animals vaccinated with ILV1 or DD3008 (see above) were exposed to a high-dose aerosol challenge with ATCC 23344 3 weeks postboost. Seventeen DD3008-vaccinated animals and 10 control mice were challenged with an inhaled dose of 3.4×10^5 bacteria (340 times the LD_{50}). Fig. 2A shows that on day 3 there was 94% survival in the DD3008-vaccinated group, but only 20% survival in

Table 1
Serum antibody class response of BALB/c mice vaccinated via the aerosol route with live attenuated *B. mallei* strains

Vaccine[a] (P or B)	Serum titer[b]					Serum ratio IgG2a/IgG1
	IgG	IgM	IgA	IgG1	IgG2a	
Control	40 ± 0	213 ± 74	0	20 ± 17	0	ND[c]
DD3008 (P)	400 ± 0	12,800 ± 0	0	50 ± 0	50 ± 0	ND
DD3008 (B)	32,000 ± 0	3200 ± 0	128 ± 0	42,667 ± 8475	12,800 ± 0	0.3
ILV1 (P)	800 ± 0	12,800 ± 0	0	100 ± 0	1600 ± 0	16.0
ILV1 (B)	53,333 ± 8475	3200 ± 0	0	32,000 ± 0	149,333 ± 97,762	4.7

[a] Antibody titers were determined 3 weeks post primary vaccination (P) and 3 weeks post boost (B). Control animals were not vaccinated.

[b] Pooled sera from three mice/group were used to determine the titer in triplicate using irradiation-killed *B. mallei* ATCC 23344 whole cells as the antigen in an ELISA. The titers were reported as the calculated average ±S.D.

[c] Not determined, because, titers less than or equal to 50 were considered to be negative.
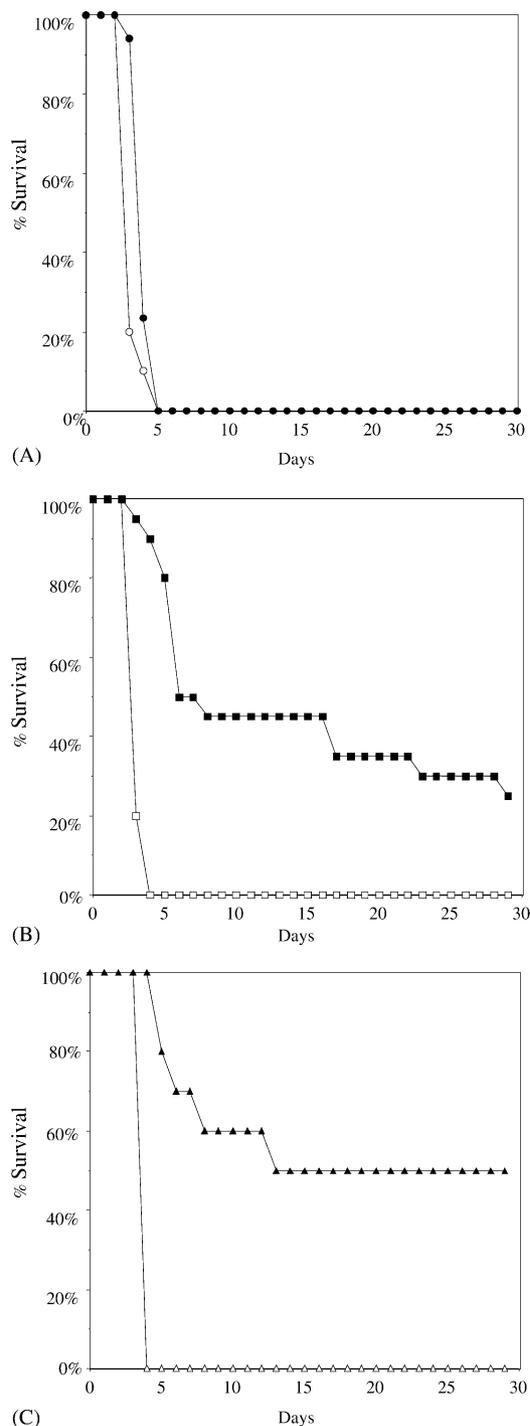
(A)

(B)

(C)

Fig. 2. ATCC 23344 aerosol challenge of mice vaccinated with live attenuated strains of *B. mallei*. (A) High-dose aerosol challenge (340 times the LD_{50}) of unvaccinated mice (open circles, $n = 10$) and mice vaccinated with DD3008 (closed circles, $n = 17$). (B) High-dose aerosol challenge (440 times the LD_{50}) of unvaccinated mice (open squares, $n = 10$) and mice vaccinated with ILV1 (closed squares, $n = 20$). (C) Low-dose aerosol challenge (five times the LD_{50}) of unvaccinated mice (open triangles, $n = 10$) and mice vaccinated with ILV1 (closed triangles, $n = 10$). Animals were aerogenically vaccinated twice and challenged with ATCC 23344. The percentage survival of vaccinated animals is shown for up to 30 days postchallenge.

the unvaccinated group. However, all animals in both groups were dead by day 5, indicating that DD3008 failed to protect against a high-dose ATCC 23344 aerosol challenge (Fig. 2A). Twenty ILV1-vaccinated and 10 control mice were challenged with an inhaled dose of 4.4×10^5 bacteria (440 times the LD_{50}). There were no surviving animals in the control group 4 days postchallenge, but 90% of the ILV1-vaccinated mice remained alive during this period (Fig. 2B). In fact, there was 45% survival 2 weeks postchallenge and 25% survival 1 month postchallenge (Fig. 2B). These results demonstrate that ILV1 can significantly protect against a high-dose ATCC 23344 aerosol challenge, and establishes a correlation between a Th1-like Ig subclass antibody response and protection of mice against aerosol-initiated glanders.

3.4. ILV1-vaccinated animals that survive a low dose aerosol challenge with ATCC 23344 are chronically infected

It is not surprising that ILV1 did not protect all of the animals against an overwhelming aerosol challenge (>400 times the LD_{50}) of virulent *B. mallei*. In an attempt to better characterize the protective efficacy of ILV1, we conducted an experiment in which vaccinated animals were challenged with a low-dose aerosol of ATCC 23344. Ten mice were treated with ILV1 and challenged according to the same schedule as previously described (see above). The ILV1-vaccinated mice and 10 unvaccinated mice were exposed to an inhaled dose of 5.0×10^3 bacteria (five times the LD_{50}). Fig. 2C shows that unvaccinated control mice all died 4 days postchallenge, whereas all of the ILV1-vaccinated animals survived during this period. Two vaccinated mice died on day 5, and one died on day 6, day 8, and day 13 (Fig. 2C). However, 50% of the ILV1-vaccinated group were still alive 1 month postchallenge (Fig. 2C). The five surviving mice were euthanized on day 29, necropsies were performed, and the bacterial loads within the lungs, liver, and spleen were assayed. Abscesses and pyogranulomas were present on the livers and spleens of all ILV1-vaccinated mice. The surviving animals experienced splenomegaly and bacterial counts within the spleen were $>1 \times 10^5$ cfu/g (Table 2). In addition, *B. mallei* was present

Table 2

Post-mortem characteristics of five ILV1-immunized mice that survived an aerosol challenge with 5×10^3 cfu of *B. mallei* ATCC 23344

Mouse[a]	Bacterial burden[b]			Splenomegaly
	Lungs	Liver	Spleen	
1	1.2×10^4	197	$>2.4 \times 10^5$	+
2	375	94	$>5.4 \times 10^5$	+
3	$>3.8 \times 10^6$	1.1×10^3	$>5.6 \times 10^5$	+
4		2.5×10^3	$>4.1 \times 10^5$	+
5		9.7×10^4	$>3.3 \times 10^5$	+

[a] Twenty-nine days postchallenge with *B. mallei* ATCC 23344 the surviving ILV1-immunized mice (Fig. 2C) were euthanized, and their lungs, livers, and spleens were examined for evidence of infection.

[b] cfu/g of tissue.

in the liver of all animals and in the lungs of three animals (Table 2). Taken together, the results suggest that the level of protection provided by ILV1 was dependent on the aerosol challenge dose and that surviving animals were chronically infected.

4. Discussion

This investigation describes the construction of a new allelic exchange vector (pGRV2) and its use to genetically engineer an unmarked mutation in *B. mallei*. The resulting strain, *B. mallei* ILV1, has a defined *ilvI* deletion mutation that makes it auxotrophic in the branched-chain amino acid pathway and attenuated in the mouse model of glanders infection. Rationally designed live attenuated vaccine strains often harbor deletion mutations, because, they are unlikely to revert to wild-type. We did not detect reversion of ILV1 in vitro or in vivo, suggesting that the auxotrophic phenotype of this strain is stable. The pGRV2 allelic exchange vector (Fig. 1A) should be useful for future gene replacement studies in *B. mallei* and other gram-negative bacteria.

The aerosol LD_{50} of *B. mallei* ATCC 23344 in mice is $\sim 10^3$ bacteria [30,31] and the intraperitoneal (ip) LD_{50} is $\sim 10^6$ bacteria [32]. Given the increased virulence of aerosolized *B. mallei*, the goal of this study was to protect mice against an aerosol glanders challenge. The live attenuated *B. mallei* vaccine candidates, ILV1 and DD3008, were delivered aerogenically because, parenteral vaccination does not always protect against aerosol challenge in mice. Live attenuated tularemia and melioidosis vaccines delivered intradermally or by ip protected against challenge by these routes, but not against an aerosol challenge [33,34]. In addition, DD3008 delivered ip did not protect against a low-dose aerosol challenge with ATCC 23344 [21]. Finally, Atkins et al. [13] demonstrated that a live attenuated *B. pseudomallei ilvI* transposon mutant protected mice against a lethal ip melioidosis challenge, but it did not protect against an ATCC 23344 aerosol challenge with 1.9×10^4 bacteria (19 times the LD_{50}). Surprisingly, aerogenic exposure with ILV1 and DD3008 did not stimulate a significant mucosal IgA or IgG antibody response, which may be the reason they did not provide 100% protection against aerosol challenge. In future studies, it may be necessary to include a mucosal adjuvant with live attenuated *B. mallei* strains to generate productive mucosal IgA and IgG antibody responses.

Vaccination with ILV1, but not DD3008, protected against a high-dose aerosol challenge (Fig. 2A and Fig. 2B). Twenty-five percent of the ILV1-vaccinated animals survived for 1 month after a challenge with 440 times the LD_{50}, while all of the DD3008-vaccinated mice died within 5 days after a challenge with 340 times the LD_{50}. This represents the best protection that we have achieved against a high-dose *B. mallei* aerosol challenge. Other vaccine candidates that have been tested include irradiation-killed *B. mallei* whole cells and live attenuated type three secretion (TTS) mutants. Par-

enteral vaccination with irradiation-killed ATCC 23344 did not protect against an aerosol challenge with 76 times the LD_{50}, but there was an increased time to death of 2 days over that of unvaccinated controls. Similar to the results obtained with DD3008 (Fig. 2A), mice vaccinated by the aerosol route with live attenuated *B. mallei* TTS mutants were not protected against an aerosol challenge with 170 times the LD_{50} [35]. It is interesting that *B. mallei* strains with attenuating mutations in virulence determinants (TTS and capsule) failed to protect against challenge, but an attenuating mutation in a biosynthetic pathway does. ILV1 produces all of the known virulence determinants and we hypothesize that live attenuated *B. mallei* vaccine strains need to synthesize all virulence factors to generate a protective immune response against a lethal aerosol challenge. It should be emphasized that vaccination with ILV1 did not fully protect against high- or low-dose aerosol challenge and the surviving animals were chronically infected. ILV1 was cleared from vaccinated animals within 7 days, which may not be long enough to generate sterilizing immunity. It may be necessary to construct mutations in different biosynthetic pathways to make live attenuated strains that can persist longer in vivo.

B. mallei is a facultative intracellular pathogen and it is likely that a Th1-like cellular and humoral immune response will be necessary to protect against a glanders infection. Animals boosted with ILV1 and DD3008 developed IgG titers of 53,000 and 32,000, respectively (Table 1). Interestingly, there was a dramatic difference in the IgG subclass antibody response elicited by these strains. The IgG2a to IgG1 ratio generated by ILV1 was 4.7, which indicates a Th1-like Ig subclass antibody response [36]. On the other hand, DD3008 generated a IgG2a to IgG1 ratio of 0.3, which represents a Th2-like Ig subclass antibody response. Similarly, Amemiya et al. [27] showed that parenteral vaccination of mice with nonviable *B. mallei* elicited a Th2-like Ig subclass antibody response and did not protect against a high-dose ip challenge with virulent *B. mallei*. The results presented here suggest that an IgG2a/IgG1 ratio of > 1 is a good serological correlate of protection against inhalational glanders in mice.

Taken together, the results of this study suggest that live attenuated *B. mallei* strains containing biosynthetic mutations, but not virulence factor mutations, may be potential vaccine candidates against inhalational glanders in mice. Further experiments are required to identify and characterize a live attenuated *B. mallei* vaccine strain that would be safe and efficacious in horses and/or nonhuman primates. It is currently unknown if live attenuated *B. mallei* strains are capable of eliciting a good immune response in horses, because few glanders vaccination studies have been conducted. One of the strains used in this study (DD3008) was recently shown to be attenuated in miniature horses [18], suggesting that there is a correlation between attenuation in mice and horses. Given the results of our study, it seems likely that a genetically defined auxotroph would be a good vaccine candidate for use in equines. Future equine vaccination studies should explore this possibility.

Acknowledgements

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