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Construction and Characterization of an Attenuated Purine Auxotroph in a *Francisella tularensis* Live Vaccine Strain

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***Francisella tularensis* is a facultative intracellular pathogen and is the etiological agent of tularemia. It is capable of escaping from the phagosome, replicating to high numbers in the cytosol, and inducing apoptosis in macrophages of a variety of hosts. *F. tularensis* has received significant attention recently due to its potential use as a bioweapon. Currently, there is no licensed vaccine against *F. tularensis*, although a partially protective live vaccine strain (LVS) that is attenuated in humans but remains fully virulent for mice was previously developed. An *F. tularensis* LVS mutant deleted in the *purMCD* purine biosynthetic locus was constructed and partially characterized by using an allelic exchange strategy. The *F. tularensis* LVS Δ*purMCD* mutant was auxotrophic for purines when grown in defined medium and exhibited significant attenuation in virulence when assayed in murine macrophages in vitro or in BALB/c mice. Growth and virulence defects were complemented by the addition of the purine precursor hypoxanthine or by introduction of *purMCDN* in *trans*. The *F. tularensis* LVS Δ*purMCD* mutant escaped from the phagosome but failed to replicate in the cytosol or induce apoptotic and cytopathic responses in infected cells. Importantly, mice vaccinated with a low dose of the *F. tularensis* LVS Δ*purMCD* mutant were fully protected against subsequent lethal challenge with the LVS parental strain. Collectively, these results suggest that *F. tularensis* mutants deleted in the *purMCD* biosynthetic locus exhibit characteristics that may warrant further investigation of their use as potential live vaccine candidates.**

Francisella tularensis is a facultative intracellular pathogen responsible for the zoonotic disease tularemia. Several forms of tularemia are recognized, the type and severity of which depend on the route of exposure and biotype of the infecting strain. The most infectious strains for humans include the highly virulent strain *F. tularensis* subsp. *tularensis* (biotype A) and the less virulent strain *F. tularensis* subsp. *holarctica* (biotype B). Infection with type A *F. tularensis* is associated with mortality rates approaching 30% in untreated individuals (7). In contrast, infection with type B *F. tularensis* is rarely fatal (33). The Centers for Disease Control (CDC) has recently classified *F. tularensis* as a select agent due to its low infectious dose, multiple routes of infection, and ease of dissemination. Currently, there is no licensed vaccine against *F. tularensis* infection.

A live vaccine strain (LVS) derived from the less virulent strain *F. tularensis* subsp. *holarctica* was previously developed in Russia in the 1950s and is the only current means of vaccination against *F. tularensis* infection (5). Several limitations associated with the use of this vaccine have prevented its licensure and use in the United States (12, 28). The genetic basis of *F. tularensis* LVS attenuation remains unknown, and it has been reported that LVS offers poor or incomplete protection against certain forms of the disease (11, 31). Though limited in its vaccine efficacy, LVS remains a good model organism for elucidating *Francisella* pathogenesis or generating attenuated strains, as it is attenuated in humans and retains full virulence in mice (2, 6).

Work leading to the development of new live vaccine candidates in *Francisella* has been hindered by the lack of useful genetic tools and paucity of information regarding the genetic factors required for pathogenesis of this organism. The recent sequencing of several *Francisella* species, including *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*, has indicated that these organisms encode all of the genes necessary for de novo purine biosynthesis (14, 20). Introduction of mutations in the purine biosynthetic pathways of several intracellular bacterial pathogens, including *Salmonella* spp. (1, 21, 25, 32, 34), *Mycobacterium tuberculosis* (13), and *Brucella melitensis* (4), renders these organisms less able to replicate intracellularly and results in an attenuation in their virulence in vitro and in vivo. When administered as vaccines, several of these auxotrophic mutants also confer protective immunity, indicating that the generation of purine biosynthetic mutants is a rational approach for generating immunoreactive live vaccine candidates (13, 25, 34). Here, we describe the construction by allelic exchange of an *F. tularensis* LVS mutant that is deleted in the *purMCD* purine biosynthetic locus and report its initial characterization using in vitro and in vivo model systems.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *F. tularensis* LVS derivatives were grown aerobically at 37°C in modified Mueller-Hinton (MH) broth or agar (Difco) as described previously (22) or in Chamberlain's defined medium (CDM) (3). When required, medium was supplemented with proteose peptone (1.0%; Difco), fetal bovine serum (2.5%; Invitrogen), hypoxanthine (50 μg/ml;

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TABLE 1. Bacterial strains, plasmids, and primers

Strain, plasmid, or primer	Description[a]	Source or reference
Strains		
F. tularensis		
LVS	*F. tularensis* subsp. *holarctica* live vaccine strain	K. L. Elkins
TCZ1013	LVS Δ*purMCD*::*groE-aph*	This work
TCZ1062	LVS Δ*purMCD*::*groE-aph* with pTZ753	This work
E. coli		
DH5α	F$^-$ Φ80d*lacZ*Δ*M15 endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169*	Invitrogen
Plasmids		
pCR2.1-TOPO	3.9-kb plasmid for cloning PCR products; Ap^r Km^r	Invitrogen
pTZ699	pUC19 with *groE-sacB* cloned into the BamHI site; Ap^r	This work
pTZ717	pTZ699 with *purMCD*upstream and downstream 1.0-kb regions cloned into the SmaI-NdeI sites; Ap^r	This work
PTZ732	pCR2.1-TOPO containing *groE-aph* with SpeI ends; Ap^r Km^r	This work
pTZ736	pTZ717 with *groE-aph* cloned into SpeI site; Ap^r Km^r	This work
pTZ750	pCR2.1-TOPO containing *purMCDN*; Ap^r Km^r	This work
pTZ752	pFNLTP6 with *purMCDN* cloned into EcoRI site; Ap^r Km^r	This work; 22
pTZ753	pTZ752 with *groE-hyg* amplified from pTZ744 and cloned into NotI site; Ap^r Km^r Hyg^r	This work
Primers		
PurCDF5	CCCGGGGATTTTAATCGATGGTAAGTCTCTCTCAA; forward primer for *purMCD* upstream region incorporating a SmaI restriction site	
PurCDR5	ACTAGTAATATTTGTCATTCCGGACTTGATCCAG; reverse primer for *purMCD* upstream region incorporating a SpeI restriction site	
PurCDF6	ACTAGTGTGGTGATAAATATCAGGAGCTTAAATAAAT; forward primer for *purMCD* downstream region incorporating a SpeI restriction site	
PurCDR6	CATATGCTTGATTTAACTGGTACACCTAATACTGGAT; reverse primer for *purMCD* downstream region and generation of *purMCDN* complementing fragment incorporating an NdeI restriction site.	
GroEF	ACTAGTTTGTATGGATTAGTCGAGC; forward primer for amplifying *groE-aph* incorporating a SpeI restriction site	
KanRSpeI	TTACTAGTGCGACACGGAAATGTTGAAT; reverse primer for amplifying *groE-aph* incorporating a SpeI restriction site	
purCDFlankF1	ATATTTTTCAGTTATACAGATAAGCTATC; forward primer used for cloning *purMCDN* locus and confirmation of mutant allele	
purCDFlankR1	GGAATACCCGCTGGCATCTG; reverse primer used for confirmation of mutant allele	
groEFNotI	AAGCGGCCGCTTGTATGGATTAGTGGAGC; forward primer used for amplifying *groE-hyg* incorporating a NotI restriction site	
HygRNotI	AAGCGGCCGCCCCAGAGGAACTGCGCCA; reverse primer used for amplifying *groE-hyg* incorporating a NotI restriction site	

[a] Restriction sites are underlined in primer sequences.

Sigma), kanamycin (10 μg/ml; Fisher Scientific), or hygromycin B (100 μg/ml; A. G. Scientific). *Escherichia coli* DH5α was grown at 37°C in Luria-Bertani (LB) medium (Difco) supplemented with kanamycin (50 μg/ml), ampicillin (100 μg/ml; Sigma), or hygromycin B (150 μg/ml) when required. In vitro growth kinetics of *F. tularensis* LVS derivatives were measured with an Ultraspec 3100 Pro spectrophotometer (Amersham Biosciences) at 550 nm.

DNA manipulations. Restriction enzyme digests, cloning, subcloning, and DNA electrophoresis were done according to standard techniques (30). All oligonucleotide primers were synthesized by Operon and are described in Table 1. PCR was performed using high-fidelity Platinum PCR Supermix or *Taq* polymerase (Invitrogen). Ligations were performed using a quick ligation kit (New England Biolabs). Plasmid and genomic DNA were prepared using a QIAprep Spin miniprep kit or a QIAGEN genomic-tip kit (QIAGEN) as recommended by the manufacturer. DNA fragments were purified using either a QIAquick gel extraction kit or a QIAquick PCR purification kit (QIAGEN). DNA sequencing was performed using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit and an automated long capillary method (ABI PRISM 3100 genetic analyzer; Applied Biosystems). Electroporation or transformation of plasmid DNA into *F. tularensis* or *E. coli* was conducted as previously described (22). Southern blotting was carried out as described previously (35), using probes specific to the *purMCD* upstream region (primers purCDF5 and purCDR5) or the *aph* gene (primers groEF and KanRSpeI) that were generated by PCR and random prime labeled (Invitrogen) with [α-^{32}P]dCTP (6,000 Ci mmol^{-1}; MP Biomedical).

Construction *of purMCD* mutagenesis and complementation vectors. Mutagenesis of *purMCD* was accomplished by deletion of the locus and replacement with the *groE-aph* kanamycin resistance cassette. Briefly, 1.0-kb upstream and downstream regions flanking *purMCD* were amplified from *F. tularensis* LVS by use of primer sets purCDF5-purCDR5 and purCDF6-purCDR6, respectively. Resulting fragments were individually cloned into pCR2.1-TOPO (Invitrogen), digested with SmaI and SpeI (for release of upstream region) or SpeI and NdeI (for release of downstream region), gel purified, and directionally subcloned into corresponding sites present in *Francisella* suicide plasmid pTZ699. This is a derivative of pUC19 containing the *groE-sacB* counterselectable marker (22). The resulting construct, pTZ717, was then digested with SpeI and ligated with SpeI-digested *groE-aph* from pTZ732 to generate pTZ736. For genetic complementation, the wild-type *purMCDN* locus, including ~1,000 bp of DNA upstream of *purM*, was amplified from *F. tularensis* LVS by use of primers pur CDFlankF1 and purCDR6. This fragment was cloned into pCR2.1-TOPO, digested with EcoRI, gel purified, and subcloned into pFNLTP6 (22), resulting in pTZ752. Finally, the hygromycin resistance determinant *groE-hyg* was amplified from pTZ744 by using groEFNotI and HygRNotI, digested with NotI, and cloned into the NotI site present in pTZ752. The resulting construct, pTZ753,

expresses the *purMCDN* locus from the native promoter and is resistant to hygromycin.

Allelic replacement. For mutagenesis of *purMCD* via allelic replacement, pTZ736 was electroporated into wild-type *F. tularensis* LVS and transformants were selected on MH medium containing kanamycin. Integration of plasmid into the homologous region was confirmed by PCR using primer sets purCDFlankF1-KanRSpeI and groEF-purCDFlankR1. Merodiploids were then grown on MH-kanamycin medium containing 10% sucrose to enrich for *F. tularensis* LVS Δ*purMCD*::*groE-aph* recombinants that had resolved the plasmid vector sequence. Δ*purMCD*::*groE-aph* mutants were confirmed by PCR using primers purCDFlankF1 and purCDFlankR1 or by Southern blot analyses.

Murine macrophages. The murine macrophage line J774A.1 (ATCC TIB-67) or macrophages derived from the peritoneal cavities or bone marrow of 6- to 8-week-old female BALB/c mice were used in infection assays with *F. tularensis* LVS derivatives. J774A.1 cells and peritoneal macrophages were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine in humidified air containing 5% CO_2. Peritoneal macrophages were obtained by injecting mice intraperitoneally (i.p.) with 2 ml of 2% thioglycolate (Sigma) and collecting peritoneal cells by lavage with sterile phosphate-buffered saline (PBS) after 3 days (16). To generate bone marrow-derived macrophages (BMMs), bone marrow cells were collected from dissected femurs of mice, and macrophages were derived in 150-mm non-tissue culture-treated dishes in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 10% L929 fibroblast-conditioned medium (complete medium) in humidified air containing 5% CO_2. After 5 days, loosely adherent BMMs were washed with PBS, harvested by incubation in chilled, cation-free PBS on ice for 10 min, and suspended in complete medium for seeding.

***F. tularensis* LVS intracellular growth assays.** *F. tularensis* derivatives were grown to mid-exponential phase (optical density at 550 nm of 0.3 to 0.7), diluted in prewarmed DMEM, and added to macrophages at various multiplicities of infection (MOIs). Macrophages were allowed to internalize *F. tularensis* for 2 h before extracellular bacteria were removed by two washes with PBS. For some experiments, gentamicin (5 μg/ml; Sigma) was added to DMEM after the 2-h incubation period to kill extracellular bacteria that were not internalized or that were released into the medium following infection (18). At specific times after infection, macrophage monolayers were washed twice with PBS and lysed with sterile water, and the number of CFU was determined by plating serial 10-fold dilutions on MH agar medium. On average, the number of *F. tularensis* bacteria internalized by macrophages was between 1% and 5% of the initial inoculum (data not shown).

Macrophage cytopathic assays. J774A.1 macrophages (2×10^5) were seeded into wells of 12-well tissue culture plates and left uninfected or infected with *F. tularensis* LVS derivatives at an MOI of 10 bacteria per macrophage. Macrophages were incubated for 2 h before extracellular bacteria were removed by two washes with PBS. At specific times after infection, macrophage monolayers were washed once with PBS and incubated at room temperature with 1 ml of a saturated crystal violet solution. After 5 min, crystal violet was removed and macrophages were washed once briefly with 1 ml water. For quantification of crystal violet staining, stained macrophages were suspended in 1 ml 20% acetone-80% ethanol and diluted fivefold, and A_{570} was measured (26).

Annexin V staining. Apoptosis of *F. tularensis*-infected J774A.1 macrophages was determined using a Vybrant apoptosis assay kit no. 2 (Invitrogen). Briefly, round, 2-cm-diameter, sterile glass coverslips were placed in the wells of 12-well tissue culture plates before seeding with 1×10^5 macrophages per well. Macrophages were left uninfected or infected with *F. tularensis* LVS derivatives at an MOI of 10 bacteria per macrophage for 2 h before extracellular bacteria were removed by two washes with PBS. At 2 h, 1 day, or 2 days after infection, coverslips were removed and transferred into wells containing 1 ml ice-cold PBS. PBS was aspirated, and macrophages were incubated with annexin V (5.0 μl) and propidium iodide (200 ng) in a final volume of 0.1 ml 1× annexin binding buffer for 15 min at room temperature with constant agitation. Macrophages were then washed with 1.0 ml 1× annexin binding buffer (10 mM HEPES, 140 mM NaCl, 25 mM $CaCl_2$ [pH 7.4]) and mounted with Prolong Gold antifade reagent (Invitrogen) on glass slides for fluorescence microscopy. Epifluorescent images were captured using a Photometrics Coolsnap ES digital camera connected to a Nikon Eclipse TE2000-U inverted microscope. Fluorescence of Alexa Fluor 488 (annexin V) was captured using a shutter speed of 5,000 ms, while red fluorescent propidium iodide was captured using a shutter speed of 4,000 ms.

Transmission electron microscopy. BMMs (1×10^5) were seeded on 12-mm Aclar coverslips in 24-well plates and infected with *F. tularensis* LVS derivatives at an MOI of 50 bacteria per macrophage. Twenty minutes postinfection, macrophages were washed five times in DMEM and incubated for an additional 40 min in complete medium to allow phagosome maturation. Infected macrophages were then incubated with complete medium containing gentamicin (100 μg/ml) for 1 h to kill extracellular organisms and suspended in complete medium for the duration of the experiment. At 2 h and 8 h postinfection, infected macrophages were fixed at 4°C for 24 h in 0.1 M cacodylate buffer, pH 7.0, containing 2.5% glutaraldehyde, 4.0% paraformaldehyde, and 50 mM sucrose. Samples were washed with 0.1 M cacodylate buffer alone and then in water and postfixed in 1% OsO_4 in water for 30 min at room temperature. After three washes in water, samples were treated with 0.1% tannic acid for 15 min at room temperature, washed again in water, and then stained en bloc in 0.1% aqueous uranyl acetate, pH 3.9, overnight at 4°C. Samples were dehydrated through graded ethanol series and embedded in Spurr's low-viscosity resin (Ted Pella, Inc.). Following poststaining with uranyl acetate and lead citrate, thin sections were viewed at 80 kV with a Hitachi H7500 transmission electron microscope fitted for image capture with a Hamamatsu C4742-95 charge-coupled-device camera and Advantage HR/HR-B digital image software (AMT, Danvers, MA).

LAMP-1 immunofluorescence microscopy. BMMs (1×10^5) were seeded on 12-mm glass coverslips in 24-well plates and were infected with *F. tularensis* LVS derivatives at an MOI of 25 bacteria per macrophage. Twenty minutes postinfection, macrophages were washed five times in DMEM and incubated for an additional 40 min in complete medium to allow phagosome maturation. Infected macrophages were then incubated with complete medium containing gentamicin (100 μg/ml) for 1 h and suspended in complete medium for the duration of the experiment. At 2 h or 8 h postinfection, infected macrophages were washed three times with PBS and fixed at 37°C for 10 min with 3% paraformaldehyde, pH 7.4. Fixed cells were washed three times with PBS and incubated for an additional 10 min in PBS containing 50 mM NH_4Cl to quench free aldehyde groups. Samples were blocked and then incubated in permeabilization buffer (PBS containing 10% horse serum and 0.1% saponin) for 30 min at room temperature. Cells were labeled by incubating inverted coverslips for 45 min at room temperature in permeabilization buffer containing mouse anti-*F. tularensis* lipopolysaccharide (1:5,000; U.S. Biological) and rat anti-mouse LAMP-1 (1D4B, 1:400; developed by J. T. August and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242) antibodies. Bound antibodies were detected by incubation with 1:500 dilutions of Alexa Fluor 488 donkey anti-mouse and Alexa Fluor 568 donkey anti-rat antibodies for 45 min at room temperature. Cells were washed twice with 0.1% saponin in PBS, once in PBS, and once in water and then mounted in Mowiol 4-88 mounting medium (Calbiochem). Samples were observed with a Nikon Eclipse E800 epifluorescence microscope equipped with a Plan Apo 60×/1.4 objective for quantitative analysis. A total of 100 bacteria were scored for LAMP-1 colocalization at each time point. Values are expressed as the means ± standard deviations (SD) from experiments performed in triplicate.

Animal infections. Six- to 8-week-old female BALB/c mice (Harlan Sprague) were infected i.p. in a final volume of 0.2 ml with *F. tularensis* LVS derivatives. For in vivo growth experiments, mice were infected with wild-type LVS, the Δ*purMCD* mutant, or the complemented mutant strain at a dose of ~1 log above the 50% lethal dose (LD_{50}) reported for *F. tularensis* LVS (6, 10). At specific times after infection, groups of three mice were sacrificed by cervical dislocation, and the spleens and livers were removed aseptically. Infected tissues were homogenized and diluted in PBS, and total CFU were determined by plating on MH agar medium. For time-to-death studies, groups of 10 mice were infected i.p. with *F. tularensis* LVS derivatives and the mean time for animals to become moribund was determined. LD_{50} experiments were conducted essentially as described previously (29). Briefly, groups of 10 mice were infected i.p. with serial 10-fold dilutions of the *F. tularensis* LVS Δ*purMCD* mutant. Infected animals were closely monitored for 21 days, and the number of moribund animals during this time was determined. Surviving mice were then challenged with 10, 100, or 1,000 LD_{50}s of wild-type *F. tularensis* LVS. The absence of the *F. tularensis* LVS Δ*purMCD* mutant in vaccinated animals prior to challenge was confirmed by plating spleen and liver homogenates from an individual animal from each vaccination group. The actual number of bacteria delivered in all experiments was determined by performing plate counts of the initial inoculum on MH agar medium. All animal infection experiments were approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of the Medical College of Wisconsin.

Statistical analyses. All statistical analyses (analysis of variance [ANOVA] and Fisher's protected least significant difference) were performed with ANOVA (version 1.11; Abacus Software, Abacus Concepts, Berkeley, CA).

RESULTS

Construction of Δ*purMCD* mutants of *F. tularensis* LVS. *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* are predicted to encode the complete complement of genes required for de novo purine biosynthesis (14, 20). To determine whether disruption of genes in this biosynthetic pathway would attenuate growth of *Francisella* in vitro or in vivo, a *purMCD* deletion derivative was constructed in *F. tularensis* LVS and characterized. *purMCD* deletion mutants were generated with a pUC19-based suicide vector by use of a two-step allelic exchange strategy (9). This vector (pTZ736) expressed the counterselectable marker *sacB* under control of the *F. tularensis* LVS *groE* promoter and included 1.0-kb regions of DNA flanking *purMCD* that were interrupted with *groE-aph*. More than 50 Km^r Suc^r *F. tularensis* LVS mutants were isolated following electroporation and resolution of this vector, and ~35% were found to be auxotrophic when screened on CDM (data not shown). PCR and Southern blot analyses of four such auxotrophs confirmed that these mutants carried the Δ*purMCD*::*groE-aph* allele (Fig. 1). These mutants have been designated *F. tularensis* LVS Δ*purMCD* mutants.

Growth kinetics of *F. tularensis* LVS Δ*purMCD* mutants. To examine the nutritional requirements of constructed mutants in more detail, growth kinetics of wild-type *F. tularensis* LVS, the Δ*purMCD* mutant, and the Δ*purMCD* mutant complemented in *trans* with wild-type *purMCDN* were compared over time in defined (CDM) and complex (MH) broth media. The *F. tularensis* LVS Δ*purMCD* mutant was unable to grow in CDM broth (Fig. 2A). This growth defect was specifically due to an inability to synthesize purines de novo, as introduction of pTZ753 expressing wild-type *purMCDN* in *trans* (Fig. 2A) or addition of the purine precursor hypoxanthine to the growth medium (Fig. 2B) fully restored growth kinetics to wild-type levels in this strain. The *F. tularensis* LVS Δ*purMCD* mutant was also impaired for growth in MH broth (Fig. 2C), suggesting that this medium may contain limiting concentrations of purines. Supplementation of MH broth with proteose peptone and fetal bovine serum, enrichments normally included in MH agar medium, reversed this growth defect (Fig. 2D). Taken together, these results indicate that the *F. tularensis* LVS Δ*purMCD* mutant is a bona fide purine mutant that is unable to grow in medium containing limiting concentrations of purines.

Growth characteristics of the *F. tularensis* LVS Δ*purMCD* mutant in murine macrophages. It is generally thought that the cytosol of eukaryotic cells contains limiting concentrations of certain nutrients, including purines. To determine whether the deletion of *purMCD* attenuated intracellular growth of *F. tularensis* LVS, J774A.1 or peritoneal macrophages were infected with *F. tularensis* LVS derivatives and bacterial growth was monitored every 16 h for 2 days. A defect in intracellular growth of the *F. tularensis* LVS Δ*purMCD* mutant in J774A.1 macrophages was observed (Fig. 3A), and this growth defect was restored to wild-type levels upon the addition of 50 μg/ml hypoxanthine to DMEM culture medium or complementation in *trans* with wild-type *purMCDN* (Fig. 3B). The restoration in growth was specific to intracellular bacteria, as all strains remained unable to grow in hypoxanthine-supplemented DMEM in the absence of macrophages (data not shown). When infection assays were repeated in the presence of gentamicin to kill

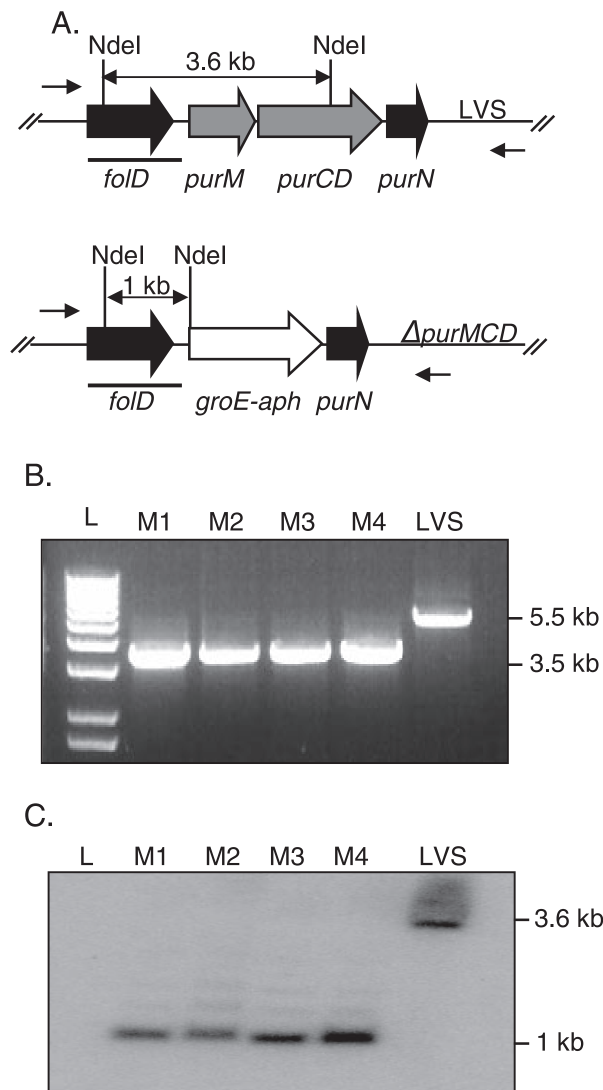


FIG. 1. PCR and Southern blot analyses of four constructed *F. tularensis* LVS Δ*purMCD* mutants. (A) Genomic organization of the *purMCD* region in *F. tularensis* LVS. NdeI restriction enzyme sites are present within the coding sequences for *folD* and *purCD* and in the beginning of the *groE-aph* kanamycin resistance gene. Primers used for PCR confirmation of mutants lie outside the cloned region and are indicated with arrows. The thick solid line indicates DNA from the *purMCD* upstream region used as the probe for Southern blotting. (B) PCR products resulting from amplification of *F. tularensis* LVS or *F. tularensis* LVS Δ*purMCD*::*groE-aph* genomic DNA with primers purCDFlankF1 and purCDFlankR1. DNA (2.2 kb) between *folD* and *purN* was removed following deletion of *purMCD* and replacement with *groE-aph*. (C) Southern blot of total genomic DNA from wild-type LVS or Δ*purMCD*::*groE-aph* mutants digested with NdeI and hybridized to radiolabeled probe from the *purMCD* upstream region. Replacement of *purMCD* with *groE-aph* reduces the size of the hybridized band from 3.6 kb to 1.0 kb due to the presence of an NdeI site within *groE-aph*. L, ladder; M1 to M4, mutants 1 to 4.

extracellular organisms, the *F. tularensis* LVS Δ*purMCD* mutant remained unable to grow and was killed upon increased incubation within J774A.1 macrophages (Fig. 3C). Increasing the MOI of the *F. tularensis* LVS Δ*purMCD* mutant failed to reverse this phenotype (Fig. 3C). Killing of the *F. tularensis*

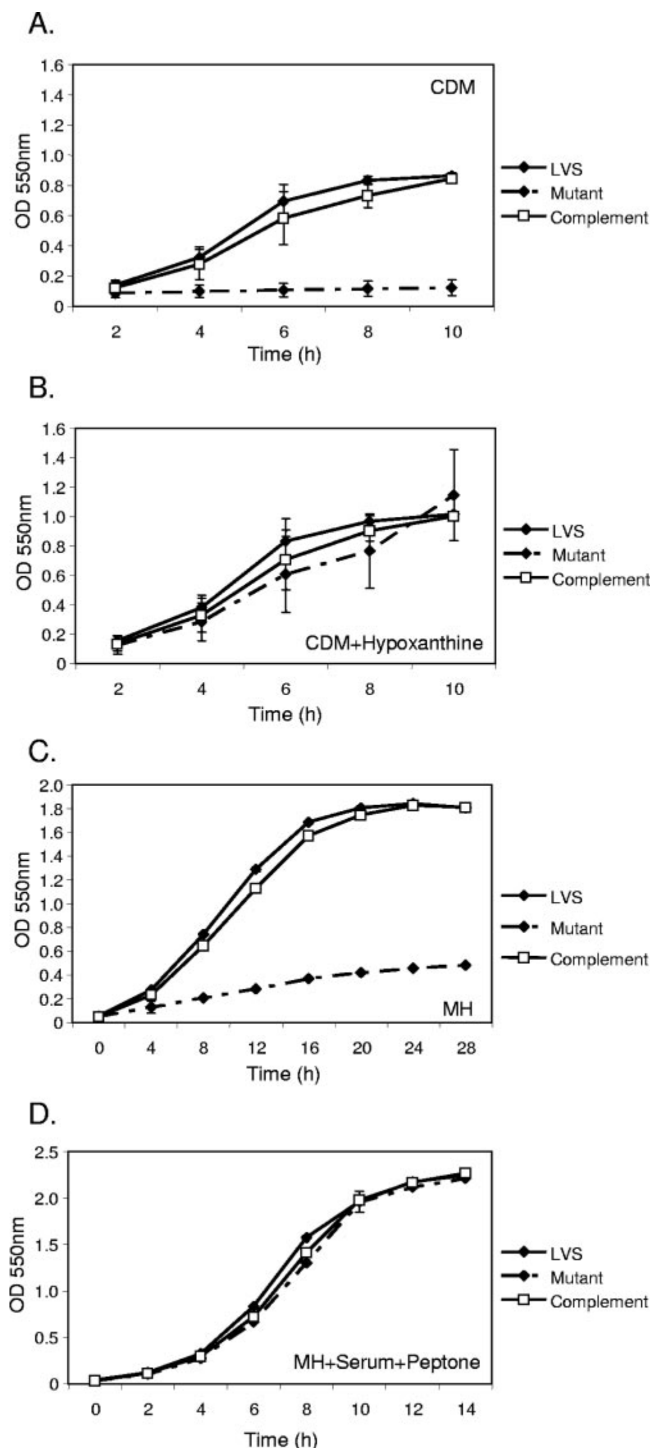


FIG. 2. Growth kinetics of *F. tularensis* LVS derivatives in defined and rich media. Wild-type *F. tularensis* LVS, the Δ*purMCD* mutant, and the complemented Δ*purMCD* mutant were grown in CDM in the absence (A) or presence (B) of 50 μg/ml purine precursor hypoxanthine or in modified MH broth medium lacking (C) or containing (D) 2.5% fetal bovine serum and 1.0% proteose peptone. Growth was monitored by measuring the optical density at 550 nm (OD 550nm). The means and standard errors of experiments performed in triplicate are shown.

LVS Δ*purMCD* mutant but not wild-type LVS or the complemented mutant was also observed during infection of primary peritoneal macrophages (Fig. 3D). Thus, the *F. tularensis* LVS Δ*purMCD* mutant is defective for intracellular growth and is killed upon continued incubation in macrophage-like cells or primary murine macrophages.

Lack of cytopathic and apoptotic responses in *F. tularensis* LVS Δ*purMCD* mutant-infected macrophages. The cytotoxicity of *F. tularensis* LVS in macrophages has been linked to the ability of the bacterium to escape from the phagosome, replicate in the cytosol, and initiate apoptosis (17, 18). As intracellular growth of the *F. tularensis* LVS Δ*purMCD* mutant was attenuated in macrophages, we reasoned that the mutant may be unable to induce a cytopathic response in these cells. We used a crystal violet staining assay (27) to measure the retention of macrophages to plastic dishes after infection. Macrophages infected with wild-type *F. tularensis* LVS or the complemented mutant demonstrated a significant reduction in dye binding, suggesting a loss of adherence and cell death (Fig. 4A). In contrast, the number of adherent macrophages observed following infection with the *F. tularensis* LVS Δ*purMCD* mutant remained indistinguishable from that seen in the uninfected macrophage control over the time course examined. To determine whether the observed differences in cytotoxicity were a consequence of apoptosis, infected macrophages were also stained with fluorescently labeled annexin V and propidium iodide. While no annexin V staining was observed in macrophages infected with wild-type LVS, the Δ*purMCD* mutant, or the complemented mutant at 2 h or 24 h after infection (data not shown), significant differences in annexin V staining patterns were observed by 48 h after infection. Annexin V staining was readily observed in macrophages infected with wild-type LVS and the complemented mutant at this time point (Fig. 4B). In contrast, only background levels of annexin V staining were observed in uninfected macrophages or macrophages infected with the *F. tularensis* Δ*purMCD* mutant (Fig. 4B). In addition, few if any infected macrophages that were stained with annexin V were also stained with propidium iodide (Fig. 4B), indicating that the cells infected with wild-type LVS or the complemented mutant were undergoing apoptosis. Taken together, these results indicate that the *F. tularensis* LVS Δ*purMCD* mutant fails to initiate a cytopathic response in infected macrophages due to an inability to induce apoptosis.

The *F. tularensis* LVS Δ*purMCD* mutant escapes from the phagosome but fails to replicate in the cytosol. To determine whether the failure of the *F. tularensis* LVS Δ*purMCD* mutant to induce apoptosis and cytotoxicity in macrophages was due to an inability to escape from the phagosome, murine BMMs were infected with *F. tularensis* derivatives and analyzed by transmission electron microscopy (TEM) and immunofluorescence microscopy. Wild-type *F. tularensis* LVS, the Δ*purMCD* mutant, and the complemented mutant were all observed free in the cytosol by 2 h postinfection (Fig. 5A). However, unlike wild-type LVS or the genetic complement, the *F. tularensis* LVS Δ*purMCD* mutant failed to replicate in the cytosol of infected BMMs as measured 8 h after infection (Fig. 5A). Consistent with these observations, only ~25 to 30% of the *F. tularensis* LVS Δ*purMCD* mutant bacteria were found in

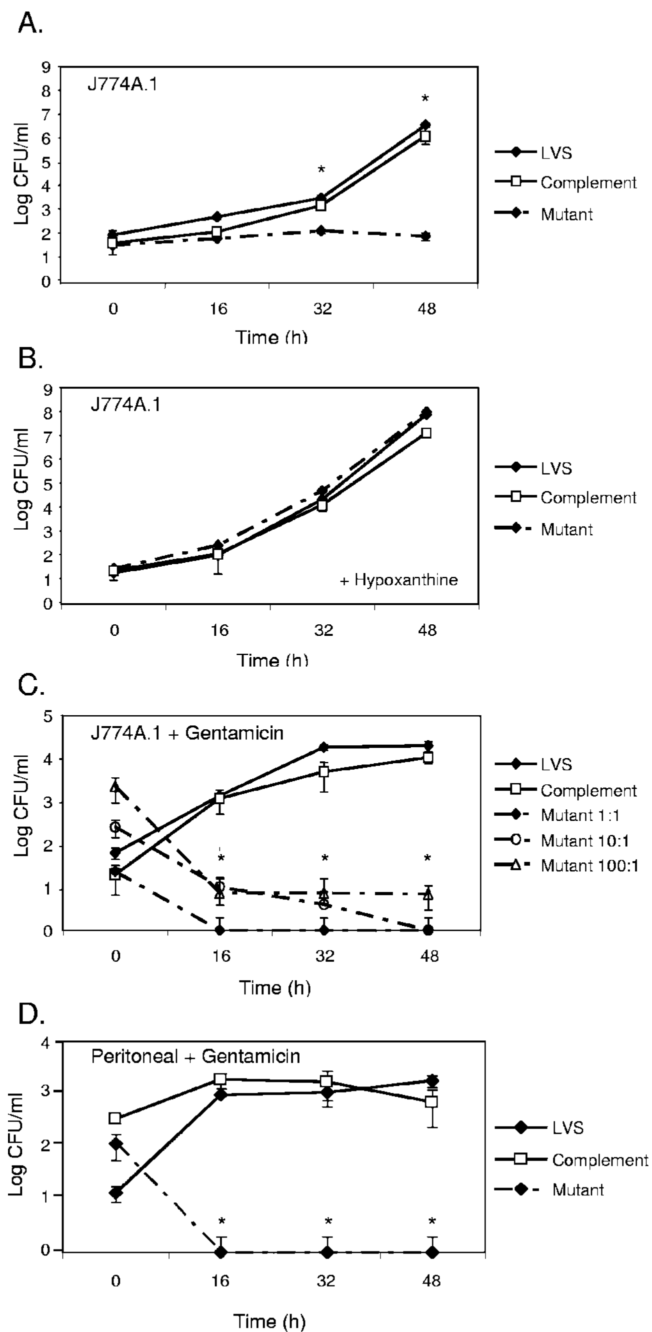


FIG. 3. Intracellular growth of *F. tularensis* LVS derivatives in macrophages. J774A.1 macrophage-like cells (A to C) or peritoneal macrophages (D) were infected with wild-type *F. tularensis* LVS, the Δ*purMCD* mutant, and the complemented Δ*purMCD* mutant, and intracellular growth was monitored by lysing macrophages at the indicated time points and determining CFU. All infections were conducted at an MOI of 1 unless noted otherwise. Gentamicin (5 μg/ml) was added to culture medium after infection (C and D) to kill extracellular organisms that were not ingested or that were released from infected macrophages. Asterisks indicate time points where growth of the *F. tularensis* LVS Δ*purMCD* mutant was significantly different ($P < 0.05$; ANOVA) from growth of LVS or the genetic complement. The means and standard errors of experiments performed in triplicate are shown.

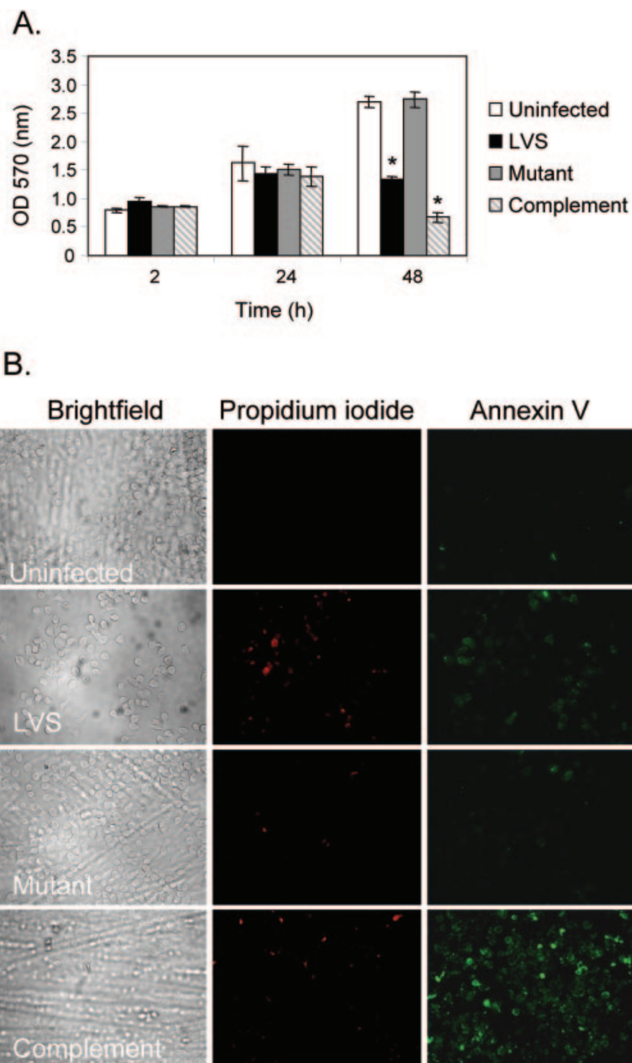
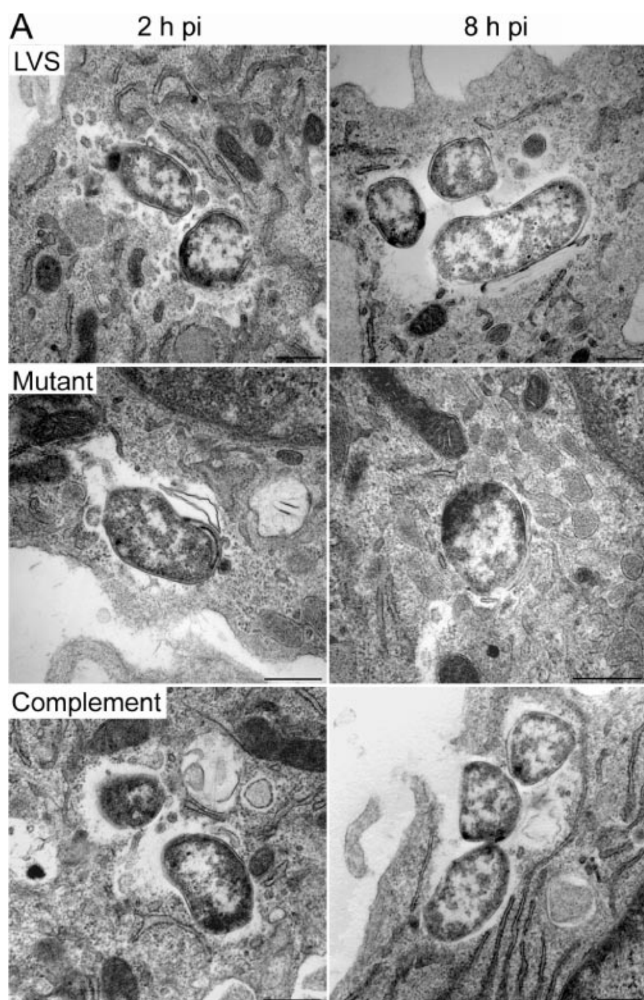


FIG. 4. Effect of *F. tularensis* LVS growth on macrophage adherence. (A) Retention of crystal violet stain was used as an indicator of adherence of J774A.1 macrophages infected with *F. tularensis* LVS derivatives. A significant drop (asterisks, $P < 0.05$; ANOVA) in adherence was observed 48 h after infection in macrophages infected (MOI of 10) with wild-type LVS or the complemented mutant. No differences in staining were observed between the *F. tularensis* Δ*purMCD* mutant and the uninfected controls. OD 570 (nm), optical density at 570 nm. (B) Apoptosis in *F. tularensis*-infected J774A.1 macrophages was examined using a Vibrant apoptosis assay kit no. 2 to stain annexin V and propidium iodide to measure permeability. Macrophages were seeded onto coverslips in tissue culture dishes and were left uninfected or infected with wild-type *F. tularensis* LVS, the Δ*purMCD* mutant, or the complemented mutant for 48 h. Infected cells were washed, stained with annexin V conjugated to Alexa Fluor 488 and red fluorescent propidium iodide, and mounted onto glass slides for analysis by epifluorescence microscopy. Note the absence of annexin V staining in macrophages infected with the *F. tularensis* Δ*purMCD* mutant. Representative images from experiments performed in triplicate are shown.

LAMP-1-positive phagosomes at 2 or 8 h postinfection (Fig. 5B). While slightly higher than those observed for wild-type *F. tularensis* LVS or the complemented mutant (Fig. 5B), these results indicate that the majority of the *F. tularensis* LVS Δ*purMCD* mutant bacteria escape from the phagosome but are unable to replicate in the cytosol of infected macrophages.



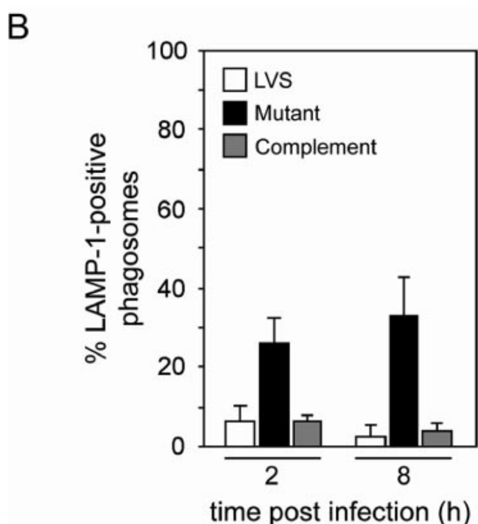


FIG. 5. Intracellular localization of *F. tularensis* LVS derivatives inside macrophages. Murine BMMs were infected with *F. tularensis* LVS derivatives and analyzed by TEM (A) or epifluorescence microscopy following *Francisella* and LAMP-1 immunostaining (B). BMMs were infected with *F. tularensis* derivatives at an MOI of 50 (for TEM) or 25 (for epifluorescence microscopy) and processed at 2 h and 8 h postinfection (pi). The *F. tularensis* LVS Δ*purMCD* mutant escapes from the phagosome but is unable to replicate in the cytosol of infected macrophages. The *F. tularensis* LVS Δ*purMCD* mutant bacteria are also found at low frequency in LAMP-1-positive phagosomes in infected macrophages. LAMP-1 colocalization was determined for each strain by counting 100 bacteria at each time point. Values are expressed as the means ± SD from experiments preformed in triplicate. Bar, 0.5 μm.

Growth and virulence of the *F. tularensis* LVS Δ*purMCD* mutant in mice. To determine whether the *F. tularensis* LVS Δ*purMCD* mutant was attenuated for virulence in vivo, BALB/c mice were infected with *F. tularensis* LVS strains and the numbers of viable organisms in the spleens and livers were determined at various times after infection. Mice were injected i.p. with between 25 and 50 total CFU, a dose of ~1 log above the reported LD_{50} (6, 10). Whereas increasing numbers of both wild-type LVS CFU and complemented mutant CFU were recovered from the spleens and livers of infected animals during the course of infection, the *F. tularensis* LVS Δ*purMCD* mutant was not recovered from these tissues at any time point examined (Fig. 6A and B). Consistent with this observation, between 80 and 90% of mice infected with wild-type LVS or the complemented mutant at this dose became moribund within 7 days after infection, while 100% of the mice infected with the *F. tularensis* LVS Δ*purMCD* mutant at this dose survived (Fig. 6C). To determine the extent of attenuation mediated by the *purMCD* deletion, the LD_{50} for this strain was determined by injecting groups of mice ($n = 10$) i.p. with serial 10-fold dilutions containing between 5×10^1 and 5×10^6 total CFU of the *F. tularensis* LVS Δ*purMCD* mutant. Of the mice infected, all but one survived up to the maximum dose of 5×10^6 CFU administered, indicating that the LD_{50} for this strain is greater than 5×10^6 CFU (Table 2). In addition, mice infected with the *F. tularensis* LVS Δ*purMCD* mutant were protected in a dose-dependent manner against secondary challenge with up to 1,000 LD_{50}s of wild-type *F. tularensis* LVS (Table 2). Taken together, these results suggest that the *F. tularensis* LVS Δ*purMCD* mutant is severely attenuated in vivo but remains able to confer protective immunity against challenge with wild-type *F. tularensis* LVS.

DISCUSSION

Infection by *F. tularensis* can result in a debilitating or fatal disease if left untreated. The low infectious dose of this bacterium and its ability to be disseminated by aerosolization have prompted a renewed interest in generating an efficacious vaccine capable of eliciting protective immunity in the event of an intentional biological attack. Though *F. tularensis* LVS offers some protection, retrospective studies of its vaccine efficacy have demonstrated that it provides limited protection against typhoidal, ulceroglandular, and pneumonic tularemia (2, 12, 28). In the present study, recently developed genetic tools (22) were utilized to construct and complement an *F. tularensis* LVS derivative deleted in the *purMCD* purine biosynthetic locus. Initial evaluation of this auxotrophic mutant indicates that it is severely attenuated for growth and virulence in macrophage and animal model systems and is capable of conferring protec-

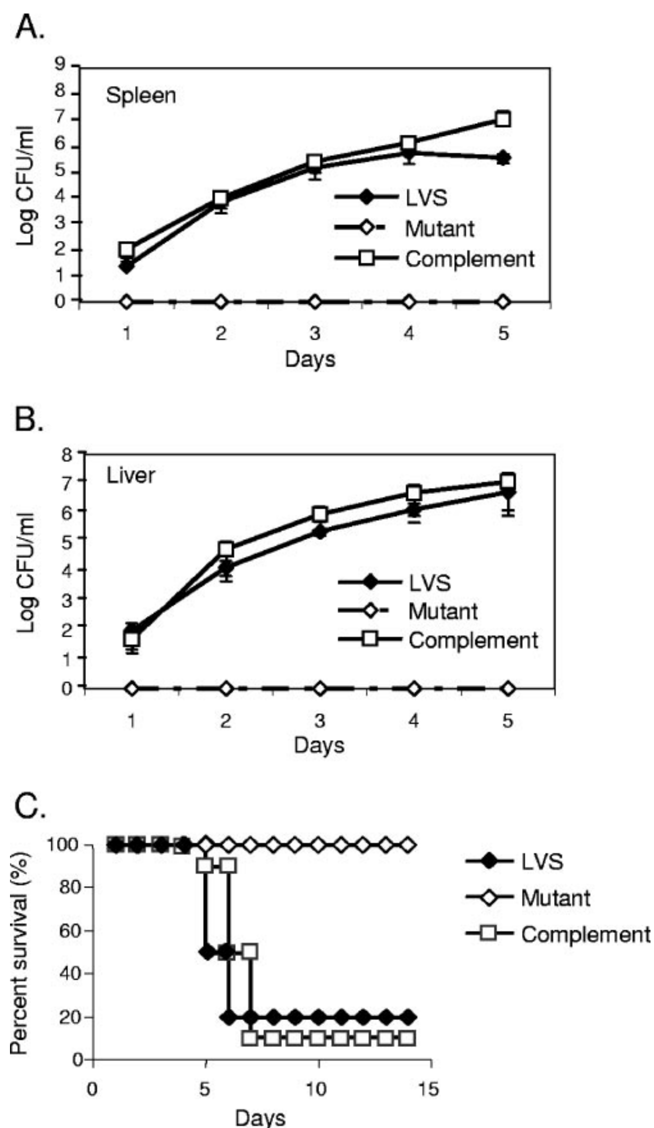


FIG. 6. Growth characteristics of *F. tularensis* LVS derivatives in BALB/c mice. Groups of mice were infected intraperitoneally with wild-type LVS (50 CFU), the Δ*purMCD* mutant (48 CFU), or the complemented mutant (26 CFU). At specific times after infection, subsets of mice were sacrificed and the numbers of bacteria present in the spleens (A) or livers (B) were determined by plating organ homogenates on MH agar medium. Values depicted at each time point represent the mean log CFU per ml ± standard errors of the means from groups of three animals. The *F. tularensis* LVS Δ*purMCD* mutant was not recovered from spleen or liver tissues at any time examined. The remaining subset of infected animals was used to define the mean time required by each bacterial strain to induce morbidity (C). Percent survival was determined from groups of 10 mice per infecting bacterial strain.

tive immunity against an otherwise lethal challenge with the wild-type parent.

Targeted disruption of purine biosynthetic genes has long been a viable strategy for generating live attenuated vaccine candidates in a variety of intracellular pathogens (4, 13, 25, 32). The utility of this approach stems from the fact that these mutants have a limited ability to undergo replication in the purine-limiting environment of the host cell cytosol but maintain their ability to stimulate cell-mediated immunity. The extent of attenuation and level of protective immunity conferred by purine auxotrophy are largely dependent on the organism studied and the enzymatic step disrupted. For example, *Brucella* and *Mycobacterium* strains with genes required for initial steps in purine biosynthesis (i.e., IMP generation) disrupted are severely attenuated in virulence (4, 13) and are capable of conferring protective immunity (13), while *Salmonella* strains harboring similar genetic lesions are ineffective vaccines due to their incomplete attenuation in virulence (24, 25). The *purMCD* locus was targeted for disruption in *F. tularensis* LVS, as these genes are found in an apparent single transcriptional unit and encode enzymes involved in multiple steps of the purine biosynthetic pathway (14, 20). *purM* is predicted to encode 5′-phosphoribosyl-*N*-formylglycinamidine (FGAM), while *purCD* is a single open reading frame predicted to encode a bifunctional enzyme having both 5′-phosphoribosyl-5-aminoimidazole carboxylic acid (CAIR) and 5′-phosphoribosylamine (PRA) activities (14). The *purN* gene encoding 5′-phosphoribosyl-1-glycinamide (GAR) is also present in this apparent operon but was not targeted for deletion. As expected, replacement of *purMCD* in *F. tularensis* LVS with the

TABLE 2. *F. tularensis* Δ*purMCD* vaccination and LVS challenge

Immunizing dose (CFU)[a]	No. of mice that survived immunizing dose/total no. of mice[b]	Challenge dose (CFU)[c]	No. of mice that survived against WT challenge/total no. of mice[d]	Time to death (days)[e]
5×10^1	10/10	5×10^1	3/3	>21, >21, >21
		5×10^2	2/3	7, >21, >21
		5×10^3	0/3	4, 4, 5
5×10^2	10/10	5×10^1	3/3	>21, >21, >21
		5×10^2	3/3	>21, >21, >21
		5×10^3	1/3	4, 5, >21
5×10^3	9/10	5×10^1	3/3	>21, >21, >21
		5×10^2	3/3	>21, >21, >21
		5×10^3	2/2	>21, >21
5×10^4	10/10	5×10^1	3/3	>21, >21, >21
		5×10^2	3/3	>21, >21, >21
		5×10^3	3/3	>21, >21, >21
5×10^5	10/10	5×10^1	3/3	>21, >21, >21
		5×10^2	3/3	>21, >21, >21
		5×10^3	3/3	>21, >21, >21
5×10^6	10/10	5×10^1	3/3	>21, >21, >21
		5×10^2	3/3	>21, >21, >21
		5×10^3	3/3	>21, >21, >21
None		5×10^1	2/5	5, 5, 5, >21, >21

[a] Mice were vaccinated i.p. (0.2 ml) with serial 10-fold dilutions of the *F. tularensis* LVS Δ*purMCD* mutant. The actual number of organisms injected was determined from CFU counts on MH agar medium.

[b] Survival was determined 21 days after infection.

[c] Mice were challenged i.p. (0.2 ml) with 10, 100, or 1,000 LD_{50}s of wild-type *F. tularensis* LVS 21 days after vaccination with the *F. tularensis* Δ*purMCD* mutant.

[d] Survival against wild-type (WT) challenge was determined 21 days after infection.

[e] Time to death was determined for each mouse in challenge studies carried out for 21 days.

groE-aph kanamycin resistance determinant resulted in an auxotrophic phenotype when the bacterium was cultured on defined medium. Interestingly, impaired growth was also observed when the *F. tularensis* LVS Δ*purMCD* mutant was cultured in MH broth, indicating that this otherwise rich medium may contain insufficient concentrations of purines and possibly other nutrients. This deficiency might explain, in part, the low frequency of auxotrophs observed following screening of random *F. tularensis* LVS transposon mutant libraries (23). Importantly, the *F. tularensis* LVS purine auxotroph could be complemented by addition of the purine precursor hypoxanthine or by introduction of a complementing plasmid encoding wild-type *purMCDN* in *trans*, indicating that the observed phenotype is not due to polar effects.

Since growth of the *F. tularensis* LVS Δ*purMCD* mutant was attenuated in purine-limiting medium, we were encouraged to compare its growth to that of the wild-type LVS parent and the complemented mutant in more relevant systems, including tissue culture and animal models. In both J774A.1 macrophage-like cells and peritoneal-derived murine macrophages, *F. tularensis* LVS and the genetically complemented mutant replicated in macrophages over the time course examined, consistent with their escape from the phagosome and rapid growth in the cytosol. In contrast, the *F. tularensis* LVS Δ*purMCD* mutant was unable to grow or was killed in these macrophages even when administered at higher MOIs. The inability to observe killing of the *F. tularensis* LVS Δ*purMCD* mutant in macrophages incubated in the absence of gentamicin is likely due to the presence of residual extracellular organisms not removed by PBS washings. While transmission electron microscopy and LAMP-1 immunofluorescence studies confirmed that the *F. tularensis* LVS Δ*purMCD* mutant escaped from the phagosome, albeit at frequencies slightly lower than those of wild-type LVS and the complemented mutant, the lack of cytosolic replication prevented subsequent induction of apoptotic and cytopathic host responses that were observed with these other strains. These results are consistent with those observed by other groups (8, 15, 17–19) and point to an essential role for phagosomal escape and cytosolic multiplication in the *Francisella* intracellular life cycle.

When assayed in in vivo growth experiments, time-to-death studies, and LD_{50} assays with BALB/c mice, the *F. tularensis* LVS Δ*purMCD* mutant was also highly attenuated. Eighty to 90% of mice infected i.p. with either wild-type LVS (50 CFU) or the complemented mutant (26 CFU) became moribund 5 to 7 days after infection, while 99% of mice infected with up to 5×10^6 CFU of the *F. tularensis* LVS Δ*purMCD* mutant were able to survive and clear the infection. Although one mouse from the group vaccinated with 5,000 CFU of the *F. tularensis* LVS Δ*purMCD* mutant died after 4 days, it is unlikely that this animal died from *F. tularensis* infection, as it did not exhibit any overt symptoms characteristic of this disease. Regardless, this indicates that the *F. tularensis* LVS Δ*purMCD* mutant is attenuated by at least 6 orders of magnitude relative to wild-type LVS or the complemented mutant. Importantly, the attenuation in in vivo growth did not impair the ability of the mutant to confer protective immunity against an otherwise lethal challenge with wild-type LVS. Vaccination of mice with a single dose of as few as 5,000 CFU of the *F. tularensis* LVS Δ*purMCD* mutant i.p. was sufficient to provide complete protection against challenge with up to 1,000 LD_{50}s of the wild-type *F. tularensis* LVS parent given by the same route 21 days later, indicating that the *F. tularensis* LVS Δ*purMCD* mutant displays characteristics consistent with its potential utility as a vaccine candidate. It should be noted that these studies were conducted only with a single challenge using an isogenic strain that was administered shortly after vaccination. Additional experiments are under way to investigate the ability of the *F. tularensis* LVS Δ*purMCD* mutant to confer protective immunity against lethal challenge with *F. tularensis* strains administered by more relevant routes of infection and at various times after vaccination, as are analogous mutagenesis and characterization studies in the background of the more virulent clinical type A strains of *F. tularensis* subsp. *tularensis*.

In summary, the present study describes the generation of a *purMCD* deletion mutant of *F. tularensis* LVS and its initial characterization in macrophage and animal infection models. The *F. tularensis* LVS Δ*purMCD* mutant is auxotrophic for growth in vitro and is highly attenuated for virulence in vivo. Although able to escape from the phagosome, the *F. tularensis* LVS Δ*purMCD* mutant fails to replicate in the host cytosol and is unable to induce host apoptotic or cytopathic response. Importantly, a single vaccination with this mutant at a low dose confers protective immunity against an otherwise lethal challenge with the wild-type parent. Collectively, these attributes indicate that targeted disruption of purine biosynthetic genes may represent a legitimate strategy for generating a safe and immunogenic live vaccine strain active against *F. tularensis* infection.

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