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Characterization of Rationally Attenuated *Francisella tularensis* Vaccine Strains That Harbor Deletions in the *guaA* and *guaB* Genes

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Abstract

Francisella tularensis, the etiologic agent of tularemia, can cause severe and fatal infection after inhalation of as few as 10-100 CFU. *F. tularensis* is a potential bioterrorism agent and, therefore, a priority for countermeasure development. Vaccination with the live vaccine strain (LVS), developed from a Type B strain, confers partial protection against aerosal exposure to the more virulent Type A strains and provides proof of principle that a live attenuated vaccine strain may be efficacious. However LVS suffers from several notable drawbacks that have prevented its licensure and widespread use. To address the specific deficiencies that render LVS a sub-optimal tularemia vaccine, we engineered *F. tularensis* LVS strains with targeted deletions in the *guaA* or *guaB* genes that encode critical enzymes in the guanine nucleotide biosynthetic pathway. *F. tularensis* LVSΔ*guaA* and LVSΔ*guaB* mutants were guanine auxotrophs and were highly attenuated in a mouse model of infection. While the mutants failed to replicate in macrophages, a robust proinflammatory cytokine response, equivalent to that of the parental LVS, was elicited. Mice vaccinated with a single dose of the *F. tularensis* LVSΔ*guaA* or LVSΔ*guaB* mutant were fully protected against subsequent lethal challenge with the LVS parental strain. These findings suggest the specific deletion of these target genes could generate a safe and efficacious live attenuated vaccine.

1. Introduction

Francisella tularensis is a Gram-negative facultative intracellular bacterium and the causative agent of tularemia in humans. Depending of the route of exposure and biotype of the infecting strain, the disease may occur in ulceroglandular, oculoglandular, oropharyngeal, pneumonic, or septic forms. Two subspecies of *F. tularensis*, *F. tularensis* subsp. *tularensis* (Type A), and the less virulent *F. tularensis* subsp. *holarctica* (Type B), cause the majority of cases of

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tularemia in humans [44]. These two biotypes differ in geographic distribution as well as pathogenic manifestations. *F. tularensis* subsp. *holarctica* is found primarily in Europe, Asia, and to a lesser extent, in North America and causes a disease with reduced severity. *F. tularensis* subsp. *tularensis* is found almost exclusively in North America and aerosal exposure to as few as 10 CFU has the capacity to cause severe morbidity and mortality [25;37]. Accordingly, the CDC has designated *F. tularensis* as a Category A pathogen and a biodefense priority for vaccine development.

Currently, there is no licensed vaccine for *Francisella*. In the 1950s, a live attenuated strain of *F. tularensis* subsp. *holarctica* (LVS) was developed in the Soviet Union and transferred to the United States [10]. Despite the ability of LVS to decrease the incidence of laboratory acquired tularemia [5] as well as to confer partial protection against wild-type challenge, several factors prevented its licensure and widespread use. Specifically, these include an unknown and undefined molecular basis of attenuation [47], phenotypic inconsistencies [12; 23], and its ability to confer only partial protection against virulent Type A challenge [25;37].

While not an optimal vaccine, LVS serves as proof of principal that a live attenuated strain can be an effective tool to prevent human tularemia. A more valuable vaccine strain would contain precisely defined genetic mutations that render it safe for use in the general population and protective against virulent Type A challenge. Targeted mutations in genes composing metabolic pathways have been the basis of attenuating mutations in many bacterial pathogens. For example, the *guaBA* operon encodes two enzymes critical for guanine nucleotide biosynthesis. Deletions in the *Shigella flexneri guaBA* operon led to an attenuated auxotrophic strain that is both safe and immunogenic in volunteer studies [28;29]. As the synthesis of guanine nucleotides requires both IMP dehydrogenase, encoded by *guaB*, and GMP synthase, encoded by *guaA*, we hypothesized that deletion of either gene would render *Francisella* auxotrophic for guanine and attenuated for virulence. Interestingly, and unlike enteric organisms, the *Francisella guaB* and *guaA* genes are not contained within an operon, but rather, are present as independent loci separated by 312,613 bp.

Until recently, few tools were available for the genetic manipulation of *F. tularensis* [16]. In this report, we describe a system to generate precise deletions in chromosomal loci to construct *F. tularensis* LVS mutants that contained deletions in either the *guaB* or *guaA* genes. Here, we report characterization of two vaccine prototypes, LVSΔ*guaA* and LVSΔ*guaB*. Both are auxotrophic for guanine, attenuated for growth within macrophages, and exhibit reduced virulence in the mouse model. Most importantly, a single inoculation with either attenuated strain protected mice against a subsequent wild-type challenge.

2. Materials and Methods

2.1 Bacterial and growth conditions

Bacterial strains used in this study are listed in Table 1. *F. tularensis* LVS was kindly provided by Dr. Karen L. Elkins (CBER/FDA, Rockville, MD), and preserved in aliquots at −80°C. Mueller Hinton Broth (Becton Dickinson Microbiology Systems, Sparks, MD) supplemented with 1% IsoVitaleX (Becton Dickinson, Cockeysville, MD), 0.1% glucose and Ferric PPi (Sigma Chemical Co. St. Louis Mo.) was used for liquid cultures (MHB) and Mueller Hinton Agar containing 10% defibrinated sheep blood (MHA-B) was used for solid media when culturing *Francisella* strains. When needed, kanamycin (km) was added at a final concentration of 10 μg/ml and guanine added at a final concentration of 0.001%. The suicide plasmids used in this study were propagated in *E. coli* DH5α, grown in LB broth supplemented with ampicillin (Ap, 250 μg/ml) or Km (50 μg/ml). LVS vaccine stocks used for the animal studies were made from bacteria cultured in MHB and were stored in aliquots at −80°C. These were thawed for use, and viable bacteria were quantified by plating serial dilutions on MHA-B plates.

2.2 Construction of suicide vectors

The *guaB* and *guaA* genes were deleted by allelic exchange using the suicide plasmids, pFT724 and pFT695, respectively. Mutagenesis of both genes was accomplished by deletion of the locus and replacement with the *aphT* gene (encoding km resistance, Genbank accession #AY048743) amplified with primers AphTfor and AphTrev. Briefly, 500-bp flanking regions upstream and downstream of *guaA* (FTL_1071; location in the genome, 1023479 to 1025029) or *guaB* (FTL_1478; location in the genome, 1403186 to 1404646) were amplified from *F. tularensis* LVS using the primers listed in Table 2. Primers GuaA5for and GuaA5Arev amplified a 500 bp fragment upstream of *guaA*, and primers GuaA3for and GuaA3rev amplified a 500 bp fragment downstream of *guaA*. The fragments were cloned into pUC19 and the *aphT* gene was inserted in between the flanking fragments as a *Sma*I fragment to create pUCGA (*guaA5′-aphT-guaA3′* cassette). Similarly, 500 bp fragments upstream and downstream of *guaB* were amplified using primer pairs GuaB5for - GuaB5rev and GuaB3for - GuaB3rev, respectively. The fragments were cloned into pBlue-Script SK (Stratagene), with restriction sites *Bam*HI and *Hind*III and the *aph*T gene was inserted in between the 2 fragments via a *Sma*I site to create pBSGB (*guaB5′-aphT-guaB3′* cassette).

Subsequently, pFT695 (Figure 1A) was constructed by sub-cloning the *guaA5′-aphT-guaA3′* cassette into pSacB plasmid as a *Spe*I/*Sal*I fragment. pSacB is a pUC19 derivative plasmid that contains *sacB* (encoding levansucrase) to promote plasmid elimination during sucrose treatment. The efficiency of pFT695 in *Francisella* was improved by replacing the native *sacB* promoter (P_{sacB}) with the *Francisella* P_{groEL} promoter [14] (5′-TTT CTT GAA AAT TTT TTT TTT GAC TCA ATA TCT AGA CTT GCA AGA GCT TGG AAC TTT GAG ATT GTT CTA AGA TGC ATA CAA ATT CAA AAT GCT TAA ACA AAA ATA ATT TAA CAA AGG AGT AAG ATT CAT ATG-3′) amplified with primers GroELfor and GroELrev. Similarly pFT724 (Figure 1B) was obtained by sub-cloning the *guaB5′-aphT-guaB3′* cassette into pSacB plasmid as a *Spe*I/*Sal*I fragment. pFT695 and pFT724 were electroporated into LVS.

2.3 Deletion of *guaA* and *guaB loci*

Cells from one plate of confluent LVS growth were resuspended in 1.5 ml of fresh sucrose wash buffer (0.5 M sucrose) and centrifuged at 14,000 rpm. The pellet was re-suspended in wash buffer and centrifuged again for a total of four washes. The final pellets resulting from two bacterial plates were suspended in wash buffer to a total volume of 300 μl and mixed with the suicide plasmid. Electroporation conditions were 1.75 Kv, 25 μF and 600Ω for a total of 3 pulses. The electroporation mix was transferred to 1.5 ml of MHB. The bacteria were incubated with shaking for 3 h at 37°C, and plated on MHA-B with 10 μg/ml of km. Isolated colonies were analyzed by PCR to confirm the suicide plasmid integration in the *F. tularensis* genome. A positive cointegrant colony was selected and grown in MHB with 10% sucrose. When the optical density (OD) was ~0.4 at 600 nm, the bacteria were spread on MHA-B plates with guanine. Individual colonies were then replica plated onto MHA-B plates without guanine to identify guanine auxotrophs. Isolated guanine auxotrophic colonies were evaluated by PCR. Genomic DNA was isolated from colonies according to the Gnome®DNA KIT (Q-BIOgene, Carlsbad, CA) manufacturer's protocol and used as the PCR template. The primers used for genotype confirmation of the *guaA* mutation include OLIGA1, OLIGkm, and OLIGA2 (Table 2). The primers used for genotype confirmation of the *guaB* mutation include OLIGB1, OLIGkm, and OLIGB2 (Table 2).

2.4 Bacterial growth curves

The guanine auxotrophic phenotypes of *F. tularensis* LVSΔ*guaA* and LVSΔ*guaB* were evaluated *in vitro*. Isolated LVS derivatives were inoculated in 5 ml of MHB media supplemented with guanine and incubated overnight at 37° C. Aliquots containing 100 μl of

the overnight inocula were transferred to 50 ml of fresh media that either contained or lacked guanine supplementation (at 0.01 μg/ml final concentration). Both cultures were incubated with shaking at 37° C and the OD at 600 nm was evaluated every hour.

Reversion to guanine prototrophy was assessed by growing LVSΔ*guaA* and LVSΔ*guaB* in 10 ml broth cultures with serial passage every 24 hours for 2 days. At 6 hours and every 24 hour time point, bacteria were plated on MHB plates with or without guanine. The total number of bacteria in the culture was calculated from colony counts of serial dilutions plated on MHB plus guanine on day 3 post plating. The reversion rate was calculated by dividing the number of bacteria growing on MHB plates without guanine by the total number of bacteria in the culture. No colonies of LVSΔ*guaA* or LVSΔ*guaB* grew on MHB plates without guanine (allowed to incubate 3-5 days post plating). The reversion rate for each mutant was calculated to be $<10^{-10}$.

2.5 Intracellular Replication Assay

The intracellular replication of LVSΔ*guaA* and LVSΔ*guaB* mutants was evaluated in the murine macrophage cell line, J774 (American Type Culture Collection, Manassas, VA). Cells were cultivated in Dulbecco's modified essential medium (DMEM) (Cellgro® Herndon, VA), supplemented with 2 mM glutamine (Gibco, Grand Island, NY) and 10% heat-inactivated defibrinated fetal bovine serum (Gibco), and were maintained at 37° C in humidified air containing 5% CO_2 throughout the assay. Intracellular replication of the various strains was assessed in duplicate wells in 12-well plates (Costar, Corning, NY). Wells containing 3×10^5 cells per well were infected with bacteria at a multiplicity of infection (MOI) of 100 for 2 hours. Following this period, cells were washed 3 times with PBS, and incubated in DMEM medium containing 50 μg/ml of gentamicin (Gibco) for 1 hour. After 1 hour, the cells were washed and incubated in DMEM medium with 2 μg/ml gentamicin. *F. tularensis* replication in macrophages was evaluated at 0 - 48 hours post-gentamicin treatment by lysis of cells with a PBS-SDS (0.02%) solution and plating 10-fold serial dilutions on MHA-B plates. To determine if the intracellular growth defect of the LVSΔ*guaA* and LVSΔ*guaB* strains was specifically due to a failure to synthesize GMP *de novo* rather than an inability to use preformed guanine molecules, J774 cells were infected with LVS derivatives and incubated in DMEM medium with guanine (0.5μg/ml) or without guanine. Intracellular bacterial replication was evaluated at 0, 24, and 48 hours after gentamicin treatment.

2.6 Complementation of LVS mutants

pKK214*guaA* and pKK214*guaB* were constructed to complement the corresponding *gua* mutant in trans. Both pKK214*guaA* and pKK214*guaB* plasmids were derived from pKEK612 (kindly provided by Dr. Karl E. Klose, UTSA) [32]. First, pKEK612 was digested with *Xba*I to release the fragment araC-P_{BAD}-γβexo encoding λRed recombinase genes and re-ligated to obtain pKK214 [30]. The P_{guaB}-*guaB* fragment including the *guaB* gene and its promoter was amplified by PCR with primers CompB1 and CompB2 and cloned in *Xba*I-digested pKK214 to generate pKK214*guaB*. Since a *guaA* promoter has not been identified, the *guaB* promoter was used to drive *guaA* expression in pKK214. Previously, we engineered pFT906 to drive expression of genes in *Francisella*. pFT906 has the *Francisella guaB* promoter, P_{guaB}, (5′-TTT CTT GAA AAT TTT TTT TTT GAC TCA ATA TCT AGA CTT GCA AGA GCT TGG AAC TTT GAG ATT GTT CTA AGA TGC ATA CAA ATT CAA AAT GCT TAA ACA AAA ATA ATT TAA CAA AGG AGT AAG ATT CAT ATG-3′) followed by a multi-cloning site including *SpeI, XmaI, Sal* I, *NdeI* and *Eco*RV restriction sites.

The *guaA* gene was amplified by PCR using primers CompA1 and CompA2, digested with *Xma*I/*Sal*I and cloned into pFT906 to generate pFT916. The P_{guaB}*guaA* fragment was excised

from pFT916 and subcloned into pKK214 as an *Eco*RV fragment to generate pKK214*guaA*. pKK214*guaA* and pKK214*guaB* were electroporated into the corresponding LVS mutant.

2.7 Real-time PCR

Peritoneal macrophages were isolated from 6-8 week old female BALB/c mice 4 days after intraperitoneal (i.p.) injection of sterile 3% thioglycollate. Macrophages were plated in 12-well plates (2×10^6 cells/well) (Costar, Corning, NY USA). All experiments were performed in triplicate. After overnight incubation, cells were washed with PBS to remove non-adherent cells. Cells were cultured in RPMI 1640 containing 2% FBS and 2 mM L-glutamine during all experiments. Cells were infected with either the parental LVS or the LVS Δ*guaB* mutant at an MOI of 100. After 2 hours, the cells were washed twice in sterile PBS then incubated for 45 min in RPMI 1640 containing 50 μg/ml gentamicin, followed by replacement with antibiotic-free media. Time 0 was defined as the time at which the gentamicin-containing medium was replaced with antibiotic-free medium. Total RNA was isolated from macrophage cultures using TRIzol (Invitrogen., Carlsbad), at 0, 4, 8 and 24 hours. Real-time PCR was performed in a Sequence Detector System (ABI Prism 7900 Sequence Detection System and software; Applied Biosystems, Foster City, CA). Levels of mRNA for specific murine genes are reported as relative gene expression over background levels detected in uninfected control samples. The primer sets and reagents used in these studies were previously described [7;11]. All cellular treatments were performed in triplicate and data are representative of a single experiment.

2.8 *F. tularensis* growth in organs of naïve mice

Bacterial replication of LVS, LVSΔ*guaA*, and LVSΔ*guaB* was analyzed *in vivo*. Groups of 9 to 10 BALB/c mice (Charles River, N.Y.) were housed in microisolator cages in a barrier environment at the University of Maryland, School of Medicine Animal Facility. All experiments were performed according to protocols approved by the UMB Institutional Animal Care and Use Committee. The mice were injected i.p. with 100 CFU of LVSΔ*guaA*, 720 CFU of LVSΔ*guaB* or 860 CFU of LVS in 1 ml of gelatin 1%-PBS. Bacterial recovery from the lungs, spleens, livers, and blood of infected mice was evaluated at 24, 48 and 72 h post-infection. At each time point, 3-4 mice were euthanized and 100 μl of blood collected from the heart was plated on MHA-B to assess the systemic bacterial burden. The organs were dissected aseptically and homogenized. The homogenates were serially diluted in PBS and spread on MHA-B plates. The plates were incubated at 37° C for 2 to 5 days and the CFU in each organ was determined.

2.9 Immunogenicity of the LVS derivatives in mice

The virulence of the LVS derivatives was evaluated in three individual animal experiments. In a preliminary experiment, groups of 5 BALB/c mice were injected i.p. with varying amounts of LVS, LVSΔ*guaA*, or LVSΔ*guaB* suspended in 1 ml of 0.1% gelatin-PBS. A control group of mice was injected with 1 ml gelatin-PBS. The mice were monitored daily and survival ratio was determined at day 28.

The ability of the LVSΔ*guaA* and LVSΔ*guaB* strains to protect against subsequent LVS challenge was also evaluated. Twenty-eight days after immunization with varying doses of either LVSΔ*guaA* or LVSΔ*guaB* or 1 ml of gelatin-PBS, mice were challenged i.p with 2.5×10^3 CFU of the parental LVS strain. Challenged mice were observed daily and survival ratio was determined at day 28.

In a second experiment, groups of 10 BALB/c mice were immunized with a single dose of 2.2×10^7 CFU LVSΔ*guaA*, 3.6×10^7 CFU LVSΔ*guaB* or 1ml of gelatin-PBS and challenged at

day 28 with 2.8×10^5 CFU of LVS by the i.p. route. Survival ratios were determined at day 28.

In a third experiment, groups of 4 C57BL/6 mice were injected i.p. with approximately 10^4 CFU of LVS, or 10^7 or 10^8 CFU of LVSΔ*guaA*, or or 10^7 or 10^8 CFU of LVSΔ*guaB* suspended in 1 ml of 0.1% gelatin-PBS. The mice were monitored daily and the survival ratio was determined at day 11.

2.10 Statistical Methods

Linear regression modeling was used to assess associations of $\log_{10}$ counts with strains and experiments, separately for each time point (0, 24 hours, and 48 hours). In these models, LVS was taken as the reference strain, so that the p-value for each strain coefficient (LVSΔ*guaB* or LVSΔ*guaA)* indicated the statistical significance of that strain compared to LVS; $p < 0.05$ was considered statistically significant. The combined interaction terms (interactions of experiment with LVSΔ*guaB* and LVSΔ*guaA)* had $p > 0.05$ in all cases and $p > 0.10$ in all cases except one, so the final models had terms for strain and experiment, but not their interaction.

3.0 Results

3.1 Construction of *F. tularensis* LVSΔ*guaA* and LVSΔ*guaB* by allelic exchange

Because of their use as successful attenuating targets in enteric organisms, the *guaB* and *guaA* genes were selected for mutation in *F tularensis*. Using the sequences of the enteric *guaB* and *guaA* loci, homologues were identified in the genome sequence of *F tularensis* that were 62% and 60% identical to the respective enteric genes and separated in the chromosome by 312,613 bp. A deletion in each gene was generated independently using a two-step allelic exchange strategy. The LVS strain was used as the basis for genetic manipulation in order to allow refinement of genetic techniques without the need for high level containment.

The suicide system includes an *ori*E1 origin of replication that does not support plasmid replication in *Francisella*, the *aphT* gene encoding km resistance for selection of plasmid integration into the *Francisella* chromosome, and a *sacB* locus driven by the *Francisella gro*EL promoter to allow plasmid counter-selection following resolution. Suicide plasmids pFT695 (Figure 1A) and pFT724 (Figure 1B) were constructed to catalyze complete deletion of the *guaA* and *guaB* genes, respectively. The suicide plasmids contain DNA fragments that flank each target gene which was replaced with the *aphT* gene (km resistance marker) (Figure 1). Each plasmid was independently electroporated into *F. tularensis* LVS. Transformants were observed after 5-6 days of incubation at 37° C in 5% CO_2. Cointegrants were identified by PCR using a set of 3 primers in each reaction. For *guaA*, primers OLIGA1, OLIGA2, OLIGkm were used (Table 2). For *guaB*, primers OLIGB1, OLIGB2, OLIGkm were used (Table 2). Site-specific integration in the *Francisella* genome was confirmed in isolated *guaA* (LVS::pFT695) and *guaB* (LVS::pFT724) cointegrants. Using LVS::pFT695 genomic DNA as the template, amplification via PCR yielded two products corresponding to the native *guaA* gene (565 bp) and deleted *guaA* version (855 bp) (Figure 1C, lane 2). Similarly, PCR amplification of LVS::pFT724 genomic DNA resulted in two DNA fragments corresponding to the native *guaB* gene (550 bp) and the deleted *guaB* version (840 bp) (Figure 1D, lane 4).

Isolated, single cointegrant colonies were chosen for growth in the presence of sucrose to select for a second recombination event and excision of the plasmid from the chromosome. Colonies that were auxotrophic for guanine were evaluated by PCR using the 3 sets primers described above and in Table 2. A single DNA band, corresponding to the mutated allele, was amplified when LVSΔ*guaA* (Figure 1C, lane 3) and LVSΔ*guaB* (Figure 1D, lane 5) were analyzed via PCR. The integrity of *guaB* in LVSΔ*guaA* and the *guaA* gene in LVSΔ*guaB* was confirmed

by PCR (Figure 1D, lane 3 or Figure 1C, lane 5 respectively). These results demonstrated that LVSΔ*guaA* and LVSΔ*guaB* strains contain specific deletions in the target *gua* genes.

3.2 *F. tularensis* LVSΔ*guaA* and LVSΔ*guaB* are auxotrophic for guanine

Growth kinetics were measured for LVSΔ*guaA* and LVSΔ*guaB* in MHB in the presence or absence of guanine (Figure 2). While the parental LVS strain grew equally well in MHB with or without guanine supplementation, the LVSΔ*guaA* and LVSΔ*guaB* mutants were unable to grow in media without guanine. Addition of exogenous guanine fully restored the growth rate of LVSΔ*guaB* to parental LVS levels, but only partially restored the growth rate of LVSΔ*guaA* (Figure 2B). These results indicate that *guaA* and *guaB* encode essential enzymes that the bacteria use to synthesize guanine nucleotides *de novo*.

The ability of the mutants to revert to guanine prototrophy was assessed by growing LVSΔ*guaA* and LVSΔ*guaB* in broth cultures with serial passage for 2 days. At various time points bacteria were plated on media with or without guanine and the number of revertants calculated per total number of bacteria. No colonies of LVSΔ*guaA* or LVSΔ*guaB* grew on MHB plates without guanine. The reversion rate for each mutant was calculated to be $<10^{-10}$.

3.3 Mutations in *guaA* and *guaB* reduce bacterial growth in macrophages

A hallmark of the pathogenesis of *Francisella* infection is its ability to survive and replicate within macrophages. The ability of the *guaB* and *guaA* mutants to survive and replicate within J774 macrophages was compared to that of the parental strain, LVS. J774 murine macrophages were infected and intracellular growth was analyzed at 0, 24, and 48 hours post infection (Figure 3A). The initial uptake of bacteria was the same for all strains as evidenced by recovery of equal numbers of bacteria at time 0 post-infection. After 24 hours, the number of recovered *guaB* and *guaA* mutants significantly decreased ($<10^2$ CFU/well), while the parental LVS strain reached the highest levels of intracellular growth ($>10^6$ CFU/well). At the 48 hour time point, the number of mutant bacteria remained low and the number of LVS recovered was decreased due to the observed death of the macrophages at this late stage of infection. These findings demonstrate that the absence the metabolic genes *guaA* or *guaB* interferes with intracellular survival and replication within the J774 macrophage cell line.

Addition of guanine to the cell culture media restored the ability of LVSΔ*guaB* to survive and replicate (Figure 3B). In contrast, LVSΔ*guaA* reached higher numbers of intracellular growth at both 24 and 48 hours with the addition of guanine, but bacterial recovery was not restored to the level of parental LVS. This observation is consistent with the incomplete complementation of LVSΔ*guaA* growth *in vitro* even after the addition of guanine (Figure 2B).

Each mutant strain was transformed with a plasmid bearing the corresponding wild-type gene and tested for restoration of WT levels of intracellular replication. LVSΔ*guaB* (pKK214*guaB*) was able to grow to greater levels than LVSΔ*guaB* alone, but did not reach parental LVS levels at 24 or 48 hours post infection (Figure 3C). LVSΔ*guaA*(pKK214*guaA*) grew to higher levels than LVSΔ*guaA* at 24 hours, but did not reach parental LVS levels (Figure 3D). At 48 hours, LVSΔ*guaA*(pKK214*guaA*) was recovered at levels that were the same as the mutant alone.

3.3 *Francisella tularensis* LVSΔ*guaB* induces inflammatory cytokines by TLR2 activation

Despite the inability to replicate in macrophages, we previously demonstrated that LVSΔ*guaA* continued to signal through TLR2 equivalently to parental LVS [9]. Here we compared induction of a large panel of cytokine genes following macrophage infection with LVS or LVSΔ*guaB* using real-time PCR. Primary macrophages were infected with LVS or

LVSΔ*guaB* and mRNA levels for TNF-α, IL-1β, KC, IL-12p35, IL-12 p40, RANTES, and iNOS, were quantified at four time points. Expression of TLR2-dependent cytokines TNF-α, IL-1β, KC, and IL-12 p35, was increased at early time points following infection by both LVS and LVSΔ*guaB* (Figure 4A). The importance of IL-12 p40 in the clearance of *Francisella* infection in mice has been recognized, despite the fact that its biological mechanism of action is not completely understood [13]. In this study, we found that infection of macrophages with both LVS and LVSΔ*guaB* induced sustained mRNA expression of IL-12 p40 in the subsequent 24 hour post-infection period.

In previous studies, we showed that there is a group of cytokines including RANTES and iNOS that required both TLR2 and as well as activation of an unknown intracellular sensor to activate IRF-3 to be induced by infected macrophages [8]. In agreement with that finding, we observed that both RANTES and iNOS mRNA peaked at later time points (≥4 hours post-infection) (Figure 4B). Again, no major differences in cytokine expression levels were observed between macrophages infected with LVSΔ*guaB* or the parental LVS strain. While LVSΔ*guaB* is attenuated for virulence in mice and unable to replicate in macrophages, it is still able to induce a potent proinflammatory cytokine response.

3.4 *F. tularensis* LVSΔ*guaA* and LVSΔ*guaB* are attenuated in mice

While LVS is attenuated for humans, it is virulent in mice and causes death with a very low inoculum (*e.g.*, ~10 organisms i.p.). Accordingly, mice serve as a valuable model for studying the attenuating capacity of LVS mutations. Groups of 5 BALB/c mice were inoculated i.p. with 10^2 to 10^7 CFU of LVS or the mutant derivatives. Mice inoculated with the LVS parental strain became overtly sick (ruffled fur and hunched gait) between days 2 to 7 post-inoculation depending on the challenge dose, and a dose of 170 CFU or higher was lethal to 100% of the mice (Table 3). In contrast, mice inoculated with 10^2 to 10^7 CFU of LVSΔ*guaA* or LVSΔ*guaB* remained healthy through day 28 post-infection. The lethal dose for LVSΔ*guaA* delivered i.p. approached 9.0×10^7 as only 1/5 mice died after infection with this dose (Table 3).

In order to confirm the attenuation of the *guaB* and *guaA* mutations, virulence was assessed in a second mouse strain. One hundred percent (4/4) of C57BL/6 mice inoculated with 10^4 CFU of parental LVS succumbed to infection within 4-7 days post inoculation. In contrast, inoculation with LVSΔ*guaB* or LVSΔ*guaA* did not cause death in any mouse at doses of 10^7 or 10^8 CFU.

3.5 Replication of *F. tularensis* LVSΔ*guaA* and LVSΔ*guaB* mutants in mouse organs

To determine the extent of proliferation of the LVS attenuated mutants in the organs of infected mice, the numbers of bacteria in the spleen, liver, lung, and blood were enumerated at 1, 2 and 3 days post-infection. Groups of 9-10 mice were inoculated i.p. with parental LVS, LVSΔ*guaB*, or LVSΔ*guaA*. Three mice from each group were sacrificed at the specified time points post-infection and bacteria in the organs enumerated (Table 4). Both LVSΔ*guaA* and LVSΔ*guaB* mutants were completely cleared from the lungs, livers, and blood by day 1 post-infection. It should be noted that the inoculum size for LVSΔ*guaA* (100 CFU) was lower, than that of LVSΔ*guaB* or parental LVS (720 CFU and 860 CFU respectively) and could account for differences in organ counts. The LVSΔ*guaA* mutant was completely cleared from the spleen by day 1 post-infection; however, 2 out of 3 mice infected with LVSΔ*guaB* showed low levels of bacteria in the spleen on day 1. In contrast, the parental LVS strain was recovered from the blood, spleens, livers, and lungs of all infected animals at all time points examined. The recovery of LVS increased daily and correlated with the increase in disease severity.

3.6 A single inoculation with LVSΔ*guaA* and LVSΔ*guaB* mutants protects against parental LVS challenge

The mice that survived challenge with a single dose *F. tularensis* LVSΔ*guaA* or LVSΔ*guaB* in the virulence assay above were assessed for their ability to survive a subsequent LVS challenge. Twenty-eight days following the initial inoculation, mice were challenged with a dose of 2.5×10^3 CFU of parental LVS. All mice that received a single inoculation containing $10^4 - 10^7$ CFU of LVSΔ*guaA* or LVSΔ*guaB* were protected against LVS challenge (Table 5). The lower initial inoculation dose of 10^2 CFU of LVSΔ*guaA* or LVSΔ*guaB* protected 4/5 mice and 0/5 mice, respectively. Control, unimmunized mice succumbed to LVS challenge by day 5 following challenge. Similar findings were observed in a second high dose challenge experiment, where a single inoculation of either LVSΔ*guaA* (2.2×10^7 CFU) or LVSΔ*guaB* (3.6×10^7 CFU) conferred protection against a higher challenge dose of 2.8×10^5 CFU of LVS (Table 6). Unimmunized control mice did not survive LVS challenge. Taken together, these findings demonstrated that the vaccination of mice with a single dose of as few as 10^4 CFU LVSΔ*guaA* or LVSΔ*guaB* led to solid protection against systemic LVS challenge. All of the protected micesurvived without displaying any symptoms of disease.

4.0 Discussion

In the five years since its identification as a priority for biodefense countermeasure development in the United States, a surge of literature about *Francisella tularensis* has provided insights into mechanisms of pathogenesis and fostered efforts to develop vaccines against this pathogen [22;26]. Reports from studies performed in the 1960s on the protective efficacy of the LVS strain are proof of principal that a live attenuated strain can confer at least partial protection against tularemia in humans [25;37]. However LVS suffers from several shortcomings that prevent its licensure for broad use in the population including a lack of complete definition of the molecular basis of its attenuation despite the completion of genome sequences for Type A and B strains, phenotypic inconsistencies, and lack of complete protection against virulent Type A challenge [31;40;47]. Accordingly, an optimal live attenuated vaccine would possess precisely defined attenuating mutations, genetic stability, and the ability to confer robust protection against the most virulent strains of *Francisella*.

Towards the goal of constructing an improved vaccine, we created a set of genetic tools to allow precise genetic manipulation of chromosomal loci in *F. tularensis*. Earlier reports revealed a number of unique properties of *Francisella* genetics including the need for promoters native to *Francisella* to drive gene expression as well as the nonreplicative nature of *ori*E1 based plasmids [2;4;16;21;35;36]. Using this information we constructed a suicide plasmid system for allelic exchange in *F. tularensis.* Similar methods have recently been used by other investigators and are reported by Frank and Zahrt in a comprehensive review of genetic techniques used in *Francisella* [16]. We developed our system using the LVS strain as the template for mutagenesis so that molecular techniques could be refined without the need for high level containment. Using this suicide plasmid system, precise deletions in the *guaA* and *guaB* genes were introduced into the *F. tularensis* chromosome resulting in the guanine auxotrophic mutant derivatives LVSΔ*guaA* and LVSΔ*guaB.*

A key feature of *Francisella* pathogenesis is the ability to multiply to high levels within macrophages. While our LVS mutants were taken up into macrophages at levels equivalent to the parental LVS strain, the LVSΔ*guaA* and LVSΔ*guaB* mutant derivatives were unable to replicate intracellularly and decreased in numbers over time. By 24 hours post-infection, the parental LVS increased to levels that were 1-2 orders of magnitude greater than measured at time 0 h, whereas the LVSΔ*guaA* and LVSΔ*guaB* bacterial numbers were reduced 100- to 1,000-fold (Figure 3). Intracellular growth of LVSΔ*guaB* was restored by the addition of guanine to the media or by expression of the WT *guaB* gene *in trans*. In contrast, guanine or

trans expression of the WT *guaA* gene only partially complemented LVSΔ*guaA* growth *in vitro* and in macrophages. The lack of full complementation of LVSΔ*guaA* suggests that mutation of the *guaA* gene might be having unintended downstream polar effects. The next gene downstream from *guaA* is *dxs*. The *dxs* gene encodes 1-deoxy-D-xylulose-5-phosphate synthase, the loss of which could weaken the ability of LVS to grow *in vitro* and *in vivo*. As no promoter sequence is clearly defined for the *dxs* gene, it is possible that the *guaA* mutation disrupted an operon structure, thereby preventing full expression of the *dxs* gene. We subsequently constructed an unmarked nonpolar *guaA* deletion in a Type A strain. This new SCHU S4Δ*guaA* mutant was fully complemented with guanine supplementation as well as by introduction of the WT gene in *trans* (manuscript in preparation).

In concordance with the inability to replicate within macrophages, the mutant derivatives were also unable to replicate as effectively as LVS in mice following inoculation. Colony counts within livers, lungs, and blood of the infected mice confirmed a lack of replication of both mutant strains and only low level recovery of the LVSΔ*guaB* mutant in the spleen at the 24 hour time point. In contrast, *Ft* LVS was recovered from all organs and blood at every time point examined (Table 4). Both mutant strains were highly attenuated, at least 10,000-fold, compared to parental LVS. LVS killed 100% of mice inoculated with 170 CFU by the i.p. route, whereas, both mutants were avirulent in the mouse model with LD_{50} values greater than 10^7 CFU.

Infection of mice with LVS results in a pronounced inflammatory response [7;15;19;20;45]. We previously demonstrated that despite an inability to replicate in macrophages and mouse organs, LVSΔ*guaA* was able to activate NF-κB reporter activity in TLR2-transfected HEK293T cells comparably to parental LVS [9]. The proinflammatory cytokine response in infected macrophages has been shown to be TLR2-dependent [9;27]. We extended those studies by evaluating the ability of the replication-deficient LVSΔ*guaB* mutant to induce a cytokine response in macrophages. By real-time PCR, we demonstrated that LVS and LVSΔ*guaB* caused a rapid increase in the levels of transcription of TLR2-dependent cytokines including TNF-α, IL-1β, KC, and IL-12 p35 following infection of primary mouse macrophages. Expression of a second group of cytokines that are TLR2 and IRF-3-dependent was induced after 4 hours of infection, and included RANTES and iNOS. IL-12 p40 mRNA was induced later, but was expressed for a longer period of time than the other cytokine genes examined. All cytokines were induced by LVSΔ*guaB* to levels that were equivalent to the LVS parental strain. While we have only reported changes in mRNA expression, past experiments have demonstrated that overall there is a strong correlation between mRNA and protein levels for the cytokines measured [8;9].

Following uptake into macrophages *Francisella* resides within the phagosome before escaping into the cytoplasm where it can replicate and induce IFN-β transcription and IL-1β secretion [1;6;17;18]. The fact that IFN-β dependent genes iNOS and RANTES were induced following infection with LVS or LVSΔ*guaB* is an indirect indication that the bacteria escaped the phagosome and entered the cytosol [34]. Previously we demonstrated that LVSΔ*iglC*, a mutant incapable of phagosome escape, induced significantly lower levels of iNOS and RANTES transcription due to a lack of phagosomal escape [8]. Furthermore, LVSΔ*iglC* induced higher levels of a panel of TLR2-dependent cytokines as well as TLR2, TLR1, and TLR6, suggesting that retention within the phagosome allowed for enhanced and prolonged stimulation of TLR2 by the bacteria [8]. Taken together, these data suggest that while LVSΔ*guaB* does not replicate in macrophages or in mouse organs this strain is as capable as parental LVS at stimulating the pro-inflammatory cytokine cascade.

Inoculation with a single dose of LVSΔ*guaB* or LVSΔ*guaA* was able to protect mice from both low and high dose lethal challenges. A dose as low as 10^4 CFU of LVSΔ*guaB* or

LVSΔ*guaA* was able to protect against a lethal challenge dose of 2.5×10^3 CFU of LVS. Mice immunized with 10^7 CFU of either vaccine strain were fully protected against the more stringent high dose challenge of 2.8×10^5 CFU of LVS. This is in contrast to the LVSΔ*iglC* mutant which is unable to protect against a lethal LVS challenge [43]. The failure of the attenuated LVSΔ*iglC* mutant to provide protection against subsequent LVS challenge points to the importance of cytokine expression in the development of protective immunity. The LVSΔ*iglC* mutant is unable to escape from the phagosome and LVSΔ*iglC* infection of macrophages leads to little to no IFN-β and IFN-γ production and severely reduced activation of the inflammasome [8]. It has been postulated the activation of the inflammasome and type 1 IFN signaling pathways may be critical for priming protective responses [8;17;24;34;49]. Futhermore, cytokines have been shown previously to be important in the control of LVS infection; antibody neutralization of TNF-α or IFN-γ converts an otherwise non-lethal i.d. infection into one that is lethal and anti-cytokine treatment effectively abrogates the protective immunity provided by passive transfer of spleen cells from immune mice to naïve animals [33]. Therefore, it seems likely that any viable vaccine candidate cannot merely be attenuated. It must be able to induce an appropriate cytokine response. Accordingly, the generation of mutant strains that are avirulent, yet capable of inducing protective responses, provides models for further investigation of critical immune responses.

These two mutant strains have served as valuable tools for the elucidation of the immune response pathways of *Francisella* in the host. While these strains were protective against challenge with the homologous parental Type B LVS, we did not test their efficacy against Type A challenge. It has been hypothesized that an optimal live attenuated vaccine strain would be derived from a Type A strain in order to provide robust protection against the most virulent strains. This theory is supported by studies in rabbits, an arguably better model for *Francisella*, that established the superiority of Type A vaccination over LVS in protecting against a lethal Type A challenge [38]. While a few SchuS4 mutant strains have been reported to confer modest to no protective immunity against Type A challenge [39;41;46;48],a recent report by Qin and Mann described a *dsbA*-like mutant derivative of SchuS4 that was able to protect against i.n. challenge with SchuS4 in the stringent C57BL/6 mouse model [42]. These studies support the contention that an efficacious Type A derivative can be developed if the correct target genes are identified. Interestingly, Bakshi *et al.* demonstrated modest but significant short term protection against Type A challenge in C57BL/6 mice following immunization with a *sodB* mutant derivative of LVS [3]. This is remarkable in that parental LVS affords no protection in this model, and suggests potential for Type B-derived strains as well.

There may be multiple viable strategies for creating an attenuated *Francisella* vaccine that will protect against virulent Type A challenge. The *guaB* and *guaA* genes were chosen as targets for mutation based on the successful attenuating effects of their deletion in *Shigella*. In enteric organisms, the *guaBA* genes are contained within an operon and a single mutation deletes both genes. In *F. tularensis*, the *guaB* and *guaA* genes are widely separated in the chromosome. This arrangement proves beneficial for further development of *Francisella* vaccine candidates. Any live attenuated vaccine will require two independently attenuating mutations to ensure a high level of safety against the potential for reversion or repair to wild type. We observed similarly attenuated phenotypes and protective capacities for LVSΔ*guaB* and LV*S*Δ*guaA* and postulate that introduction of both mutations into a single strain will optimize safety by providing the requisite two independent mutations. It is expected that the combination of both *guaB* and *guaA* mutations would not result in additive attenuation since the encoded enzymes function consecutively in the terminal portion of the *de novo* pathway, both acting prior to the point of guanine entry into the salvage pathway. Finally, the genetic distance between the two loci makes the chance of repair of both genes minuscule. We have recently applied our genetic system to the SchuS4 prototype strain to create *guaB* and *guaA* mutant derivatives. Full

characterization of these candidates will determine the protective efficacy and potential for advancement of these live attenuated candidates.

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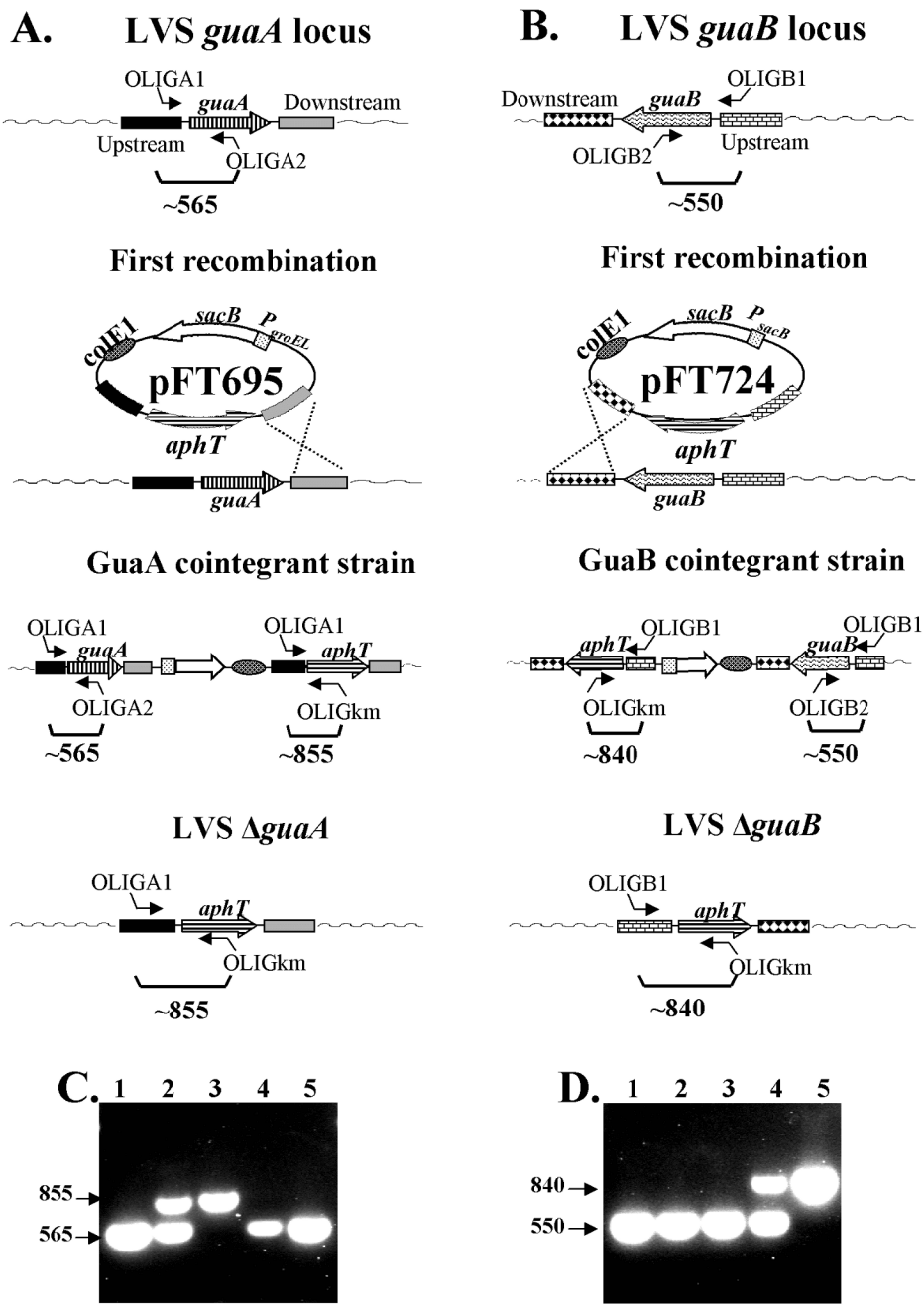

Figure 1.

Deletion of *guaA* (panel A) or *guaB* (panel B) in *F. tularensis* LVS was generated by allelic exchange. *F. tularensis* LVS::FTp695 and LVS::pFT724 cointegrants were generated by recombination of pFT695 or pFT724 into the LVS genome. Second recombination events for plasmid curing were promoted by growing the strains in sucrose. Colonies auxotrophic for guanine were isolated on MHA-B plates and evaluated by PCR. The genotypic PCR analysis utilized isolated genomic DNA from parental LVS, lane 1; LVS::pFT695 cointegrant, lane 2; LVSΔ*guaA* mutant, lane 3; LVS::pFT724 cointegrant, lane 4 and LVSΔ*guaB* mutant strain, lane 5. Primers were specific for *guaA* (OLIGA1, OLIGA2, OLIGkm) in panel C, or specific for *guaB* (OLIGB1, OLIGB2, OLIGkm) in panel D.

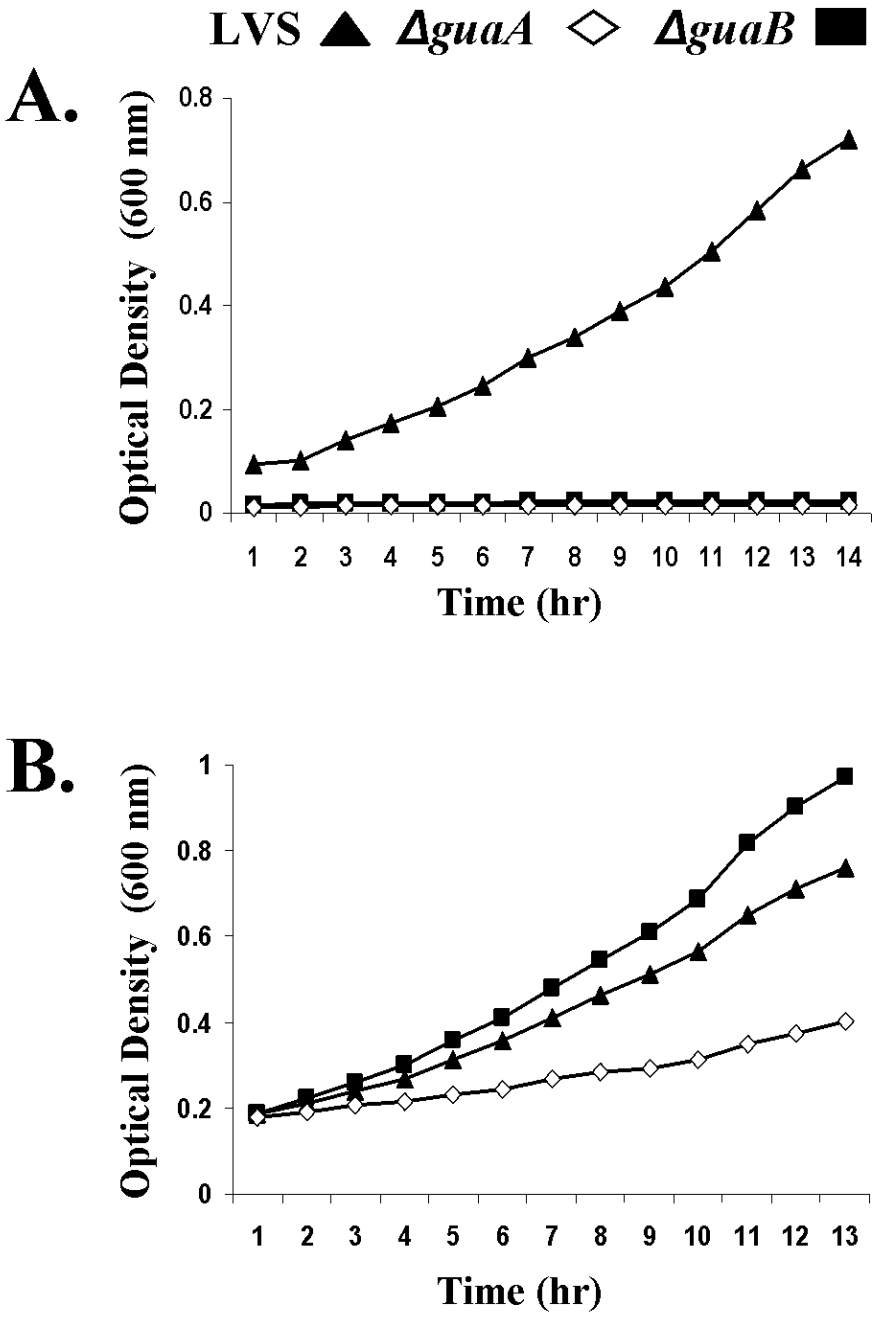

Figure 2.

Growth phenotypes of the LVS mutants were evaluated *in vitro*. *F tularensis* LVS (triangle), LVSΔ*guaA* (rhombus) and LVSΔ*guaB* (square) were grown in MHB (panel A) or MHB + guanine (panel B) and the optical density at 600 nm was recorded every hour.

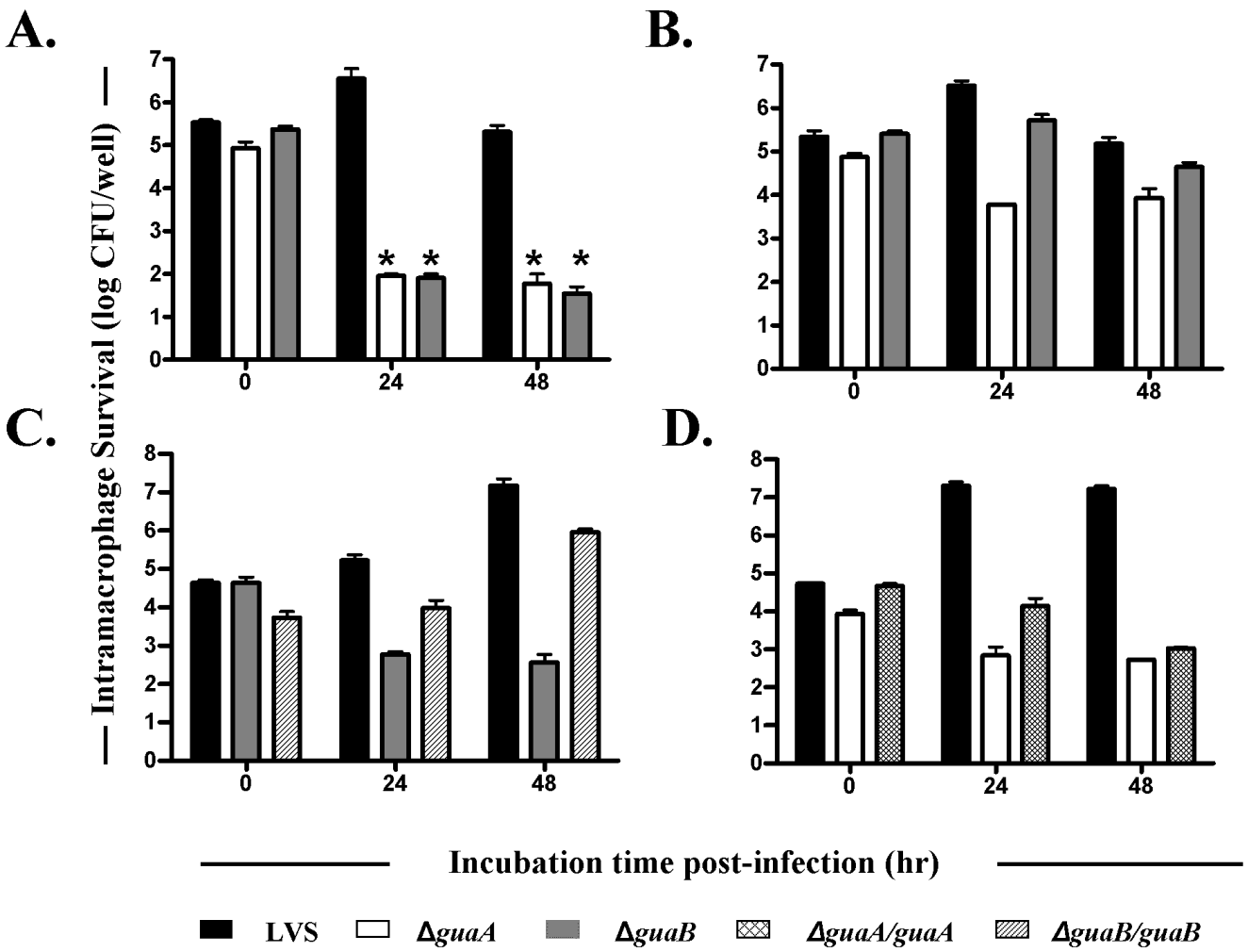

Figure 3.

Survival of *F. tularensis* strains within macrophages. In panel A, J774 cells (3×10^5) were infected with *F. tularensis* LVS (black bars), LVSΔ*guaA* (white bars), LVSΔ*guaB* (grey bars), LVSΔ*guaA*(pKK214*guaA*) (cross hatched bars), or LVSΔ*guaB*(pKK214*guaB*) (diagonal lined bars) at a multiplicity of infection of 100 in duplicate wells. In panel B, the media was supplemented with guanine (0.05 μg/ml). In panel C, complementation of LVSΔ*guaB* is evaluated. In panel D, complementation of LVSΔ*guaA* is evaluated. The number of bacteria in the cell monolayer was determined at 0, 24, and 48 h post-infection. Data represent the median ± standard deviations of two wells from one representative experiment of three. * $p<0.001$ compared to parental LVS.

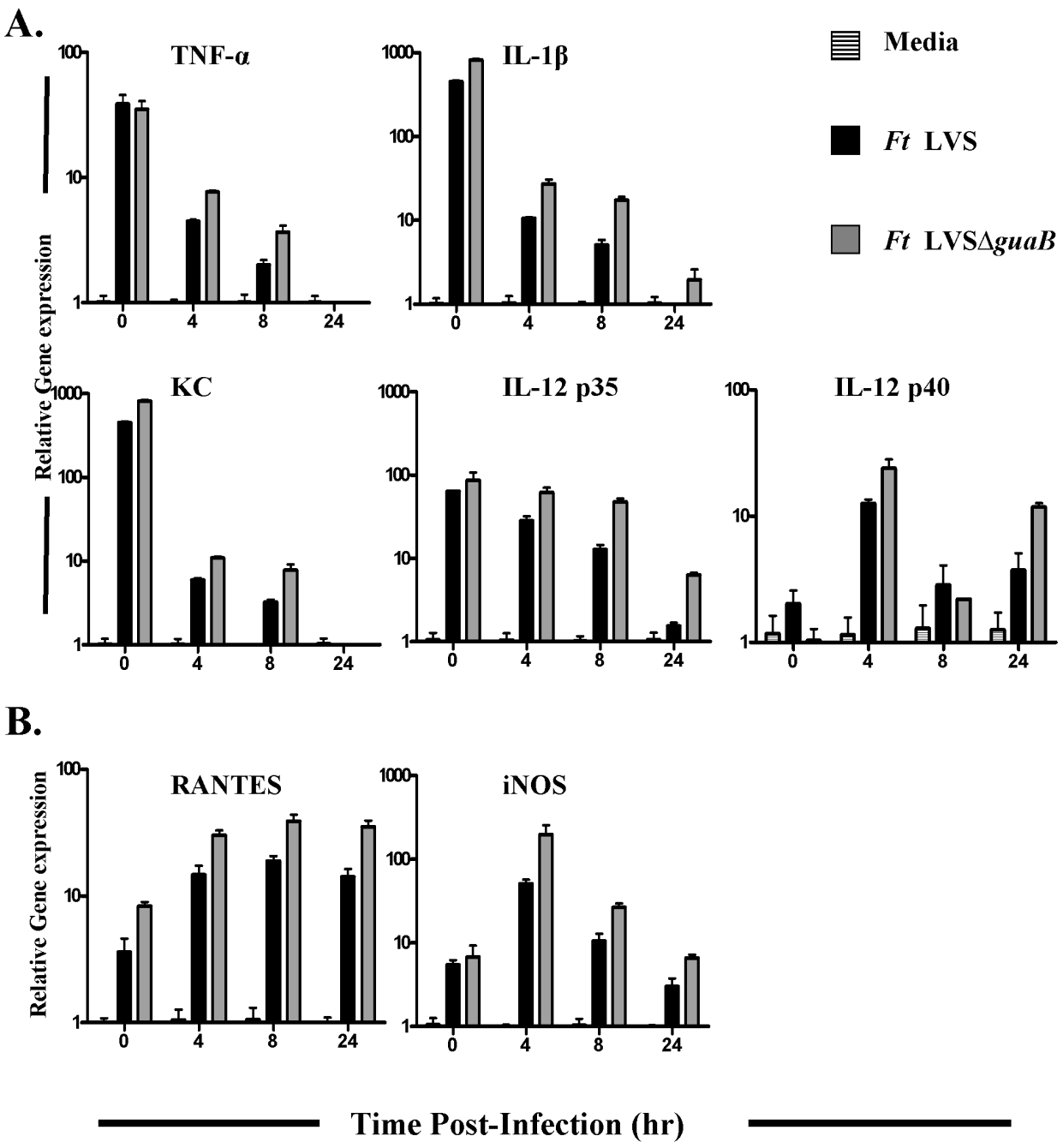

Figure 4.

Induction of proinflammatory cytokine gene expression in infected macrophages. Primary macrophages from BALB/c mice were exposed to media alone (lined bars), LVS (black bars) or LVSΔ*guaB* (gray bars) for 0 to 24 hours. At the indicated time points, total mRNA was extracted from the macrophages and analyzed by real-time PCR. Gene expression is reported as relative gene expression compared with macrophages exposed to media alone. Data are presented as mean ± SEM. Data are of a single representative experiment.

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
	Strains	
F. tularensis LVS	Attenuated *F. tularensis* vaccine strain	CBER/FDA
LVSΔ*guaA*	*F. tularensis* LVS mutant in *guaA* locus	This study
LVSΔ*guaB*	*F. tularensis* LVS mutant in *guaB* locus	This study
E coli DH5α	*deoR, endA1, gyrA96, recA1, relA1, supE44*	Laboratory collection
	Plasmids	
pUC19	Cloning vector (Amp^r)	Laboratory collection
pBlue-ScriptKS+	Cloning vector (Amp^r)	Laboratory collection
pUCGA	Containing *guaA5′-aphT-guaA3′* cassette	This study
pBSGB	Containing *guaB5′-aphT-guaB3′* cassette	This study
pSacB	Containing the *sacB* gene	This study
pFT695	*guaA* suicide plasmid	This study
pFT724	*guaB* suicide plasmid	This study
pFT906	Plasmid containing P_{guaB} promoter	This study
PFT916	Plasmid containing P_{guaB}-*guaA* fragment	This study
pKK214	Plasmid used to *trans* complement mutants	UTSA
pKK214*guaA*	pKK214 plasmid containing full-length *guaA*	This study
pKK214*guaB*	pKK214 plasmid containing full-length *guaA*	This study

Table 2
Oligonucleotides used in this study

Name	Oligonucleotide Sequences (5′ → 3′)	Combined primers and product size (bp)
	Upstream *guaA* region	
GuaA5for	CCC GGA TCC ATC TTT AGA GAT AAG TTT TCA CAT ATT GAG	GuaA5for-GuaA5rev (500)
GuaA5rev	GAT GAT ATC TTT TTA TAA AAA TAA ACA TTT TCT TAA AAG GTT ATT T	
	Downstream *guaA* region	
GuaA3for	GAT GAT ATC GCT ATG TCA AAA TAT ACT ATT TTA GAT AAA ATA A	GuaA3for-GuaA3rev (500)
GuaA3rev	CCC GTC GAC GCG GCC GCA GAT CTA TAC CGC CAG CAT GAT TTA AGG CTT CA	
	Upstream *guaB* region	
GuaB5for	CCC GGA TCC TAA ATA TAA GCC TAA GGC AGT GAT TGA TTT	GuaB5for-GuaB5rev (500)
GuaB5rev	GAT CCC CGA ATT CCT GCA GCC CGG GTT TTT GAT CTC CGT AAT TAA AAT CTA AAG AGT	
	Downstream *guaB* region	
GuaB3for	CCC GAA TTC CTG CAG AAC CTC TTA ATT ATG ATT TTA ATA AGT TAT AAT	GuaB3for-GuaB3rev (500)
GuaB3rev	CCC GTC GAC GCG GCC GCA ACA ACA CCG CTT ACA GCA AAC TTT TTA	
	Km resistance cassette (*aphT*)	
AphTfor	ATG CCC GGG GAA GTT CCT ATA CTT TCT AGA GAA TAG	AphTfor-AphTrev (1438)
AphTrev	ATG CCC GGG AGT TCC TAT TCC GAA GTT CCT ATT CT	
	GroEL promoter (P_{groEL})	
GroELfor	GAA TTC CGG ATC CTT TCT TGA AAA TTT TTT TTT TGA CTC AAT AT	GroELfor-GroELrev (132)
GroELrev	GAA TTC CCC CGG GCA TAT GAA TCT TAC TCC TTT GTT AAA TTA	
	Genotypic identification of LVS *guaA* mutant	
OLIGA1	GGA TAA AAT AAC CTT TTA AGA AAA TGT TT	OLIGA1-OLIGA2 (565)
OLIGA2	GTT TCT GGG TGA AAC TGC ACA CC	OLIGA1-OLIGkm (855)
	Genotypic identification of LVS *guaB* mutant	
OLIGB1	ACT CTT TAG ATT TTA ATT ACG GAG ATC	OLIGB1-OLIGB2 (550)
OLIGB2	TCT TTT TAA TCG CTC CTT GAG AAG C	OLIGB1-OLIGkm (840)
	***aphT* oligonucleotide**	
OLIGkm	ATG CAG CCG CCG CAT TGC ATC A	
	***Trans* complementation of *guaA* gene**	
CompA1	AGC TAG CTC CCG GGA TGA CAG ATA TAC ATA ATC ATA AGA TTT TGA TT	CompA1-CompA2 (1550)
CompA2	AGC TAG CTG ATA TCG TCG ACT TAT TCC CAT TCA ATT GTT CCA GGT GGT T	
	***Trans* complementation of *guaB* gene**	
CompB1	GGG ACT AGT CTA GAG CGA AAT AGT AAA TTC TAA TAC TTA TTT C	CompB1-ComB2 (1570)

Name	Oligonucleotide Sequences (5′ → 3′)	Combined primers and product size (bp)
CompB2	GGG ACT AGT CTA GAT TAA GAC TGG TAA TTA GG TGG TTC TTT AG	

Table 3
Virulence of *F. tularensis* derivatives in BALB/c mice

Experimental group[a]	Bacterial inoculum (CFU)	Survival ratio[b]	Time to death of individual mice (days)
LVS			
Group 1	1.7×10^1	3/5	5, 7, >28, >28, >28
Group 2	1.7×10^2	0/5	5, 5, 6, 6, 8
Group 3	1.7×10^3	0/5	4, 6, 6, 6, 7
Group 4	1.7×10^4	0/5	3, 3, 4, 5, 5
Group 5	1.7×10^5	0/5	3, 3, 3, 4, 6
LVSΔ*guaA*			
Group 6	9.0×10^2	5/5	>28, >28, >28, >28, >28
Group 7	9.0×10^3	5/5	>28, >28, >28, >28, >28
Group 8	9.0×10^4	5/5	>28, >28, >28, >28, >28
Group 9	9.0×10^5	5/5	>28, >28, >28, >28, >28
Group 10	9.0×10^6	5/5	>28, >28, >28, >28, >28
Group 11	9.0×10^7	4/5	3, >28, >28, >28, >28
LVSΔ*guaB*			
Group 12	1.05×10^2	5/5	>28, >28, >28, >28, >28
Group 13	1.05×10^3	5/5	>28, >28, >28, >28, >28
Group 14	1.05×10^4	5/5	>28, >28, >28, >28, >28
Group 15	1.05×10^5	5/5	>28, >28, >28, >28, >28
Group 16	1.05×10^6	5/5	>28, >28, >28, >28, >28
Group 17	1.05×10^7	5/5	>28, >28, >28, >28, >28
Gelatin 0.1%-PBS			
Group 18	None	5/5	>28, >28, >28, >28, >28

[a] Groups of 5 BALB/c mice were inoculated by intraperitoneal route with 10-fold dilutions of LVS (groups 1 to 5); LVSΔ*guaA* (groups 6 to 11) or LVSΔ*guaB* (groups 12 to 17) derivatives in 1.0 ml of gelatin 0.1%-PBS. As a control, group 18 was inoculated with 1.0 ml of gelatin 0.1%-PBS.

[b] Survival ratio, number of mice alive/total number of mice injected with the corresponding LVS derivative at day 28.

Table 4

Growth of *F. tularensis* in organs of naïve BALB/c mice

Group (No. mice)	*F. tularensis* strain[a]	Mean ± SD CFU of *Francisella* (log $_{10}$) / organ			
		Spleen	Lung	Liver	Blood (ml)
1 (n=3)	LVSΔ*guaA*	BDL[b]	BDL	BDL	BDL
2 (n=3)	LVSΔ*guaB*	1.03 ± 0.91 (2/3)[c]	BDL	BDL	BDL
3 (n=3)	LVS	4.31 ± 0.76	2.82 ± 1.18	4.48 ± 0.91	2.17 ± 0.96
4 (n=3)	LVSΔ*guaA*	BDL	BDL	BDL	BDL
5 (n=3)	LVSΔ*guaB*	BDL	BDL	BDL	BDL
6 (n=4)	LVS	5.91 ± 0.78	3.82 ± 1.69	5.87 ± 0.90	>2.00 [d]
7 (n=3)	LVSΔ*guaA*	BDL	BDL	BDL	BDL
8 (n=4)	LVSΔ*guaB*	BDL	BDL	BDL	BDL
9 (n=4)	LVS	6.77 ± 0.95	4.20 ± 0.95	6.92 ± 1.09	>2.00

[a] Groups 3 to 4 BALB/c mice were challenged with LVS derivatives in 1 ml of gelatin 1%-PBS. Groups 1, 4 and 7 were inoculated with 100 CFU of *F. tularensis* LVSΔ*guaA;* Groups 2, 5 and 8 received 720 CFU of LVSΔ*guaB* and groups 3, 6 and 9 were inoculated with 860 CFU of LVS by the intraperitoneal route. To determine bacterial burdens in lung, spleen and liver, inoculated mice were sacrificed on day 1 (groups: 1, 2, and 3), day 2 (groups: 4, 5 and 6) and day 3 of infection (groups: 7, 8 and 9). Tissue homogenates were serially diluted in PBS and plated on MHA-B plates, and the number of CFU per organ was calculated.

[b] BDL, below detection limit (~ 20 organisms/organ)

[c] Bacteria only detected in two out of three organs

[d] Bacteria detected in the organs is over 100 CFU/ml of blood

Table 5

Protective immunity in vaccinated BALB/c mice to re-infection with *F. tularensis* LVS

Vaccine Strain	Immunization dose	Survival ratio[a]	Median time to death (days)
LVS Δ*guaA*			
Group 6	9.0×10^2	4/5	>28
Group 8	9.0×10^4	5/5	>28
Group 10	9.0×10^6	5/5	>28
Group 11	9.0×10^7	4/4[b]	>28
LVS Δ*guaB*			
Group 12	1.05×10^2	0/5	5
Group 14	1.05×10^4	5/5	>28
Group 16	1.05×10^6	5/5	>28
Group 17	1.05×10^7	5/5	>28
Gelatin 0.1%-PBS			
Group 18	None	0/5	5

Groups of BALB/c mice pre-vaccinated (see table 3) with 10 fold dilutions of LVSΔ*guaA* (groups 6 to 11); LVSΔ*guaB* (groups 12 to 17) and gelatin 0.1% - PBS (group 18) were injected with 2.5×10^3 CFU of parental LVS strain by intraperitoneal route.

[a] Survival ratio, number of survival animal/ total number of animals immunized at day 28.

[b] One mouse dead after the immunization with LVSΔ*guaA* derivative strain

Table 6

Protective immunity in vaccinated BALB/c mice to challenge with *F. tularensis* LVS

Vaccine Strain	Immunization dose[a]	Survival ratio[b]	Median time to death (days)
LVSΔ*guaA*			
Group 1	2.2×10^7	10/10	>28
LVSΔ*guaB*			
Group 2	3.6×10^7	9/9[c]	>28
Gelatin 0.1%-PBS			
Group 3	None	0/10	3.5

[a] Groups of 10 BALB/c mice were immunized with a single dose of 2.2×10^7 LVSΔ*guaA* (group 1); 3.6×10^7 LVSΔ*guaB* (group 2) and gelatin 0.1%-PBS (group 3). The animals were challenged with 2.8×10^5 CFU of LVS by the i.p. route and survival was observed by 28 days.

[b] Survival ratio, number of survival animal/ total number of animals immunized at day 28.

[c] One mouse died after the immunization with the LVSΔ*guaB* derivative strain.