The Ability to Acquire Iron Is Inversely Related to Virulence and the Protective Efficacy of Francisella tularensis Live Vaccine Strain

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Francisella tularensis is a highly infectious bacterial pathogen that causes the potentially fatal disease tularemia. The Live Vaccine Strain (LVS) of F. tularensis subsp. holarctica, while no longer licensed as a vaccine, is used as a model organism for identifying correlates of immunity and bacterial factors that mediate a productive immune response against F. tularensis. Recently, it was reported that two biovars of LVS differed in their virulence and vaccine efficacy. Genetic analysis showed that they differ in ferrous iron homeostasis; lower Fe^{2+} levels contributed to increased resistance to hydrogen peroxide in the vaccine efficacious LVS biovar. This also correlated with resistance to the bactericidal activity of interferon γ-stimulated murine bone marrow-derived macrophages. We have extended these findings further by showing that a mutant lacking bacterioferritin stimulates poor protection against Schu S4 challenge in a mouse model of tularemia. Together these results suggest that the efficacious biovar of LVS stimulates productive immunity by a mechanism that is dependent on its ability to limit the toxic effects of oxidative stress by maintaining optimally low levels of intracellular Fe^{2+}.

Keywords: Francisella tularensis, iron acquisition mechanism, microbial pathogenesis, inflammatory mechanisms, virulence mechanisms, intracellular pathogen

INTRODUCTION

Francisella tularensis is a highly infectious bacterial pathogen that parasitizes the cytosol of host cells and causes the lethal disease tularemia in humans. The low infectious dose and high morbidity and mortality associated with tularemia have led the United States Centers for Disease Control and Prevention to designate F. tularensis a Tier 1 Select Agent. Fear of the intentional misuse of F. tularensis has spurred research into understanding the pathogenesis of the organism and the development of a vaccine. No licensed vaccine for F. tularensis infection is currently available for widespread use, although, the F. tularensis Live Vaccine Strain (LVS) has, in recent years, been used to vaccinate laboratory staff in the US and in other countries and large numbers of individuals have been vaccinated in the past (Eigelsbach and Downs, 1961). LVS is an attenuated derivative of F. tularensis subsp. holarctica that was generated in the Soviet Union in the mid-twentieth century but an incomplete understanding of the genetic changes, and therefore the underlying mechanisms, leading to the attenuation of the strain have led to its discontinuation as a licensed
vaccine. Although not available as a human vaccine, LVS is routinely used as a model to understand the genetic requirements of a *F. tularensis* strain to stimulate host immunity and as a tool to discover and characterize correlates of immunity in the host. Each of these areas of understanding are critical in the rational design of a future vaccine against virulent *F. tularensis* species.

Despite significant differences in virulence, members of the *Francisella* genus are very similar at the genetic level; many of the observed differences are genomic rearrangements and single nucleotide polymorphisms (SNPs) (Rohmer et al., 2006, 2007). The genome similarities of the *Francisella* strains have made identification of specific factors that mediate the high level of virulence displayed by Type A and Type B strains difficult, although genome comparisons between *F. tularensis* subsp. *tularensis* Schu S4 and less virulent LVS have identified some of the attenuating mutations. These mutations include fusion of the *fupA/B* genes and deletion of *pilA* (Lindgren et al., 2009). Another noted difference between the highly virulent Type A strains and the intermediate virulent type B strains (including LVS) is the levels of intracellular iron, with virulence being inversely correlated with iron levels (lower intracellular iron correlates with higher virulence and higher intracellular iron correlates with lower virulence) (Lindgren et al., 2011). Iron is an essential micronutrient for bacteria, and numerous studies have shown that it is highly sought after by bacterial pathogens, as host sequestration of iron (a part of nutritional immunity) can restrict the growth of numerous pathogens (Cassat and Skaar, 2013; Parrow et al., 2013). Apparently, the virulence strategy of *F. tularensis* represents an exception to this paradigm as Lindgren et al., found that higher virulence subspecies had lower levels of intracellular iron (Lindgren et al., 2011).

*Francisella* species import iron via siderophore-bound ferric iron via the Fsl and Fup systems, and ferrous iron via FeoB and FupA/B; the latter system is unique to LVS (Ramakrishnan et al., 2008; Thomas-Charles and et al., 2013; Perez and Ramakrishnan, 2014; Ramakrishnan and Sen, 2014). The *feo* operon typically consists of *feoAB*, and less frequently *feoABC*; the *Francisella* chromosome encodes *feoA* and *feoB* separately, and lacks *feoC* (Cartron et al., 2006; Perez and Ramakrishnan, 2014). FeoB is a large transmembrane protein that has an N-terminal G-protein domain and a multi-pass transmembrane C-terminal domain (Marlovits et al., 2002; Andrews et al., 2003; Hantke, 2003). In other organisms, the Fe$^{2+}$ import activity of FeoB requires interaction with the small protein FeoA, though the details of how FeoA stimulates FeoB activity are unknown (Su et al., 2010; Kim et al., 2012; Lau et al., 2013; Weaver et al., 2013). The general importance of iron in bacterial pathogenesis is reflected by the numerous ways that bacterial pathogens have evolved to obtain iron in a host, and several research groups have demonstrated that *feoB* contributes to or is required for full pathogenesis of *Salmonella*, *Campylobacter*, *Helicobacter*, *Legionella*, and others (Velayudhan et al., 2000; Robey and Cianciotto, 2002; Naikare et al., 2006; Aranda et al., 2009; Cassat and Skaar, 2013; Nagy et al., 2014). Multiple studies have characterized the Fe$^{2+}$ uptake function of the *F. tularensis* FeoB and have linked this to its pathogenesis (Thomas-Charles et al., 2013; Perez and Ramakrishnan, 2014; Lindgren et al., 2015). However, it is not clear from these studies if the modulation of growth among *feoB* mutants is a direct result of differential ability to use and take up iron or if there are indirect effects of excess iron that impact bacterial replication. For example, while iron is necessary, excess Fe$^{2+}$ can contribute to toxicity for an organism as ferrous iron can participate in the Fenton reaction with H$_2$O$_2$, poisoning iron-sulfur cluster enzymes in essential metabolic pathways (Imlay, 2013).

*Francisella tularensis* virulence is connected to the ability of the organism to avoid the detrimental effects of reactive oxygen species (ROS), such as H$_2$O$_2$ (Bou-Abdallah et al., 2002; Lindgren et al., 2007; McCaffrey et al., 2010; Lindemann et al., 2011; Crane et al., 2014; Ma et al., 2014). The bacterium couples glutamate metabolic pathways to H$_2$O$_2$ neutralization, and also maintains optimally low levels of intracellular Fe$^{2+}$ such that Fenton reaction-mediated damage appears to be minimized (Lindgren et al., 2011; Ramond et al., 2014). When this optimal iron threshold is surpassed, numerous antioxidant enzymes are employed to protect the organism from toxic H$_2$O$_2$; mutants lacking enzymes like the Dyp peroxidase, superoxide dismutase and catalase are more sensitive to H$_2$O$_2$ damage, fail to inhibit host inflammatory signaling, and are often attenuated in murine infection models (Bakshi et al., 2006; Melillo et al., 2009, 2010; Llewellyn et al., 2011; Binesse et al., 2015; Rabadi et al., 2015; Shakerley et al., 2015). A growing body of literature has shown that *F. tularensis* utilizes numerous strategies to avoid activation of ROS-dependent host signaling pathways and killing by host-generated reactive oxygen and reactive nitrogen species (Buchan et al., 2009; Melillo et al., 2009, 2010; McKenna et al., 2010; Langmead and Salzberg, 2012; Binesse et al., 2015; Griffin et al., 2015; Rabadi et al., 2015; Shakerley et al., 2015). Furthermore, at least one phenotypic difference between the virulent *F. tularensis* Schu S4 strain and the non-pathogenic *F. novicida* strain is that the latter is considerably more sensitive to H$_2$O$_2$; this sensitivity is associated with activation of the AIM2 inflammasome (Zubay et al., 1972).

In this work we describe how regulation of iron uptake by *F. tularensis* LVS has a critical impact on the mouse virulence of the organism and on the resulting ability of the strain to induce effective protection against challenge with virulent *F. tularensis*. We first demonstrate how genetic variability in FeoB-mediated Fe$^{2+}$ uptake observed in low vs. highly efficacious strains of LVS contributes to H$_2$O$_2$ sensitivity of *F. tularensis* LVS. We show that a LVS biovar that exhibits greater virulence to H$_2$O$_2$ is not exclusive to *feoB*, but can be extended to other iron homeostasis systems in the bacterium. We previously identified a *F. tularensis* gene involved in iron homeostasis from a mutagenesis screen to identify genes important for growth in human monocyte derived macrophages (MDMs).
(Salomonsson et al., 2009). This early work identified the bacterioferritin (bfr) gene as important for \textit{F. tularensis} Schu S4 growth in MDMs infected with pools of transposon mutants (Salomonsson et al., 2009). Specifically, we demonstrate that deletion of bacterioferritin (bfr) increases sensitivity of the bacterium to \text{H}_2\text{O}_2, decreases virulence \textit{in vivo}, and renders LVS poorly protective against challenge with virulent \textit{F. tularensis}. Together, our findings connect the nutrient acquisition of LVS with its ability to provoke strong vaccine induced immunity.

**MATERIALS AND METHODS**

**Mutant Construction**

Deletion of \textit{feoB} (FTL\textsubscript{0133}) and \textit{bfr} (FTL\textsubscript{0617}) genes was achieved by homologous recombination using derivatives of the non-replicating plasmid pJ\textsubscript{C84}. Primer sequences are shown in Table 1. Briefly, upstream and downstream flanking DNA was amplified via PCR, and amplicons were cloned into the multiple cloning site of pCR\textsubscript{2.1} (Life Technologies). A spectinomycin resistance cassette was cloned into the AvrII site in the 3’ end of each upstream PCR fragment. The upstream-spectinomycin resistance fragment was removed by digestion with Ascl and cloned into the Ascl site in the 5’ region of the downstream PCR plasmid. The entire upstream-spectinomycin resistance-downstream fragment was cloned into the BamH1 site of the suicide plasmid pJ\textsubscript{C84}. Finally, the spectinomycin resistance cassette was removed by digestion with AvrII and the plasmid was re-ligated. Plasmids were electroporated into LVS (2.5 kV, 25 \mu F, and 600 \Omega), and the bacteria were plated on MMH agar and supplemented with 50 \mu g/mL of kanamycin or spectinomycin, as needed. Mueller-Hinton agar was also prepared without ferric pyrophosphate supplementation to assay for growth in lower iron conditions. For some experiments, bacteria were cultured in Chamberlain’s defined medium with either 35 \mu M FeSO\textsubscript{4} or 350 nM FeSO\textsubscript{4}, supplemented with antibiotics as needed. Agar plates were incubated at 37\degree C with humidity and 5% CO\textsubscript{2} while broth cultures were grown at 37\degree C, shaken at 200 rpm.

**Murine Infection**

Eight to Ten week old female C57BL/6 mice were maintained on corncob bedding for 1 week prior to infection. Mice were intranasally infected with 25 \mu L of various doses of \textit{F. tularensis} LVS strains and mutant derivatives that had been resuspended in PBS. Inocula were calculated by measuring the OD\textsubscript{500} value of mid- to late-log phase grown organisms, and were confirmed by serial dilution onto modified Mueller-Hinton agar and were plated onto MMH agar with 8% sucrose for \textit{SacB}-mediated counter-selection. Kanamycin sensitive colonies were screened by colony PCR to detect deletion of the \textit{feoB} or \textit{bfr} gene. Primers were designed so that they flanked the coding sequence of each gene such that an amplicon would be produced regardless of genotype.

| TABLE 1 | Primers used in this study. |
| --- | --- |
| 5’feoB.up.asc1-bamh1–gggccggcgcgtaccAGCATATCTAAGACACAAAGAGAAAG AAGATTAG | 3’feoB.up.avr2–ctctgACATACGAGCTGTTAATGTAAGTATG |
| 5’feoB.down.asc1.avr2–gggccggccctggATCTCTGCCAGGACCTGTTAATGTAAGTATG | 3’feoB.down.bgl2–agatctTTAGCATTTCACTAAGATTTGC |
| 5’feoB.mut.check–AGATGTAGCTCAGATAAAGACCACTTGGC | 3’feoB.mut.check–CATTAAAGATAGATCTCATCTCATTAAAATACCTC |
| 5’feoB.SNP–AGACTCTGCGATATTTCTCAGATTGC | 3’feoB.SNP–TTAGCGGCATCCAGCTGCTTG |
| 5’feoB.SNP–TTTAGCCGATGCATTTTGCTCAGAACCTGG | 3’feoB.Bc.kpn1–gtgaccATGAAATATGCTCTAGTTGGCAATCC |
| 5’feoB.Bc.sal1–gtcgacATATTTAAAGCTGTATATTCAAATTAG | 3’feoB.SNP–TTTACCGGCATATTCAAGTGCTGTGG |
| 5’feoB.SNP–ATAGCTGCGATATTTCTCAGATTGC | 3’feoB.SNP–TTTACGGCCATCTTCAGGATGAGTTTTTCTTC |
| 5’feoB.Bc.kpn1–gtgaccATGAAATATGCTCTAGTTGGCAATCC | 3’feoB.SNP–TTTACCGGCATATTCAAGTGCTGTGG |
| 5’feoB.Bc.sal1–gtcgacATATTTAAAGCTGTATATTCAAATTAG | 3’feoB.mut.check–ATAAATACTTTAAGTCACTAAATATCTCG |
| 5’bfr.mut.check–ATAATCATTATTTAAGATATCGATTG | 3’bfr.mut.check–ATATAACATTTAAGTCACTAATATCG |

| TABLE 2 | Plasmids. |
| --- | --- |
| Plasmids | Description | Source |
| pCR2.1TOPO | Invtrogen | Promega |
| pGEMT Easy | Francisella-E. coli shuttle vector, specR | Buchan et al., 2009 |
| pBB103 | Francisella-E. coli shuttle vector containing the \textit{P}_{\text{PsaA-}}\text{-lacZ} reporter | Wehrly et al., 2009 |
| pBB133 | Francisella shuttle vector | Wehrly et al., 2009 |
| pJC84 | Primer | This study |
| pJC84 | Primer | This study |
| pJC84 | Primer | This study |
| pJC84 | Primer | This study |
| pJC84 | Primer | This study |
| pJC84 | Primer | This study |
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| pJC84 | Primer | This study |
| pJC84 | Primer | This study |
enrollment after 2–3 days growth at 37°C with humidity, 5% CO₂. Moribund animals (defined as having lost 25% of the initial body weight) were sacrificed in accordance with the protocol approved by the University of Iowa Institutional Animal Care and Use Committee.

### Genome Sequencing

Genomic DNA from RML LVS and ATCC LVS was prepared from overnight cultures using DNeasy tissue kit (Qiagen) according to the instructions of the manufacturer. DNA was sequenced using an Illumina HiSeq2500 platform by the Institute for Genomic Medicine Genomic Center (University of California, San Diego, San Diego, CA), resulting in ~7 million reads per sample. Reads were trimmed for adapter sequence and trimmed and filtered for low quality bases using the FASTX-Toolkit (Hannon Lab, CSHL). Resulting reads were mapped to the LVS genome (NC_007880) using Bowtie2 (Ashkenazy et al., 2010). Analysis of genetic modifications, e.g., SNPs and gene deletions was performed using GATK comparing both RML LVS and ATCC LVS genomes to each other and the annotated LVS genome (Landau et al., 2005).

### Iron-Regulated β-Galactosidase Reporter Activity

To assess iron uptake-regulated gene expression, Miller assays were performed with Iowa LVS, RML LVS, and ATCC LVS carrying the fslA promoter-lacZ fusion on the pBB103 plasmid (Troxell and Hassan, 2013). Bacteria were grown in Chamberlains’s defined medium (supplemented with 50 μg/mL spectinomycin) overnight, and β-galactosidase activity was assayed using the standard Miller assay (Sullivan et al., 2006).

### FeoB Function in a Heterologous Reporter System

The coding sequence of feoB was PCR-amplified from both Iowa and RML LVS using the high fidelity Phusion polymerase (New England Biolabs). Sanger sequencing was performed to confirm that the SNP observed in the RML LVS background was present. The feoA coding sequence was cloned into the KpnI/Sall sites of a derivative of pTrc99a, immediately downstream of the groE promoter. The entire P\text{groE-feoA} fragment was removed by digestion with BamHI and Sall, and ligated into the same sites in pBB103. Both this plasmid and the pWKS30 containing feoB were transformed into the E. coli H1771 strain (MC4100 arbo feoB7 fhuF::plac Mu). Control strains were also generated that carried only one of the plasmids. Miller assays were performed to measure FeoB Fe²⁺ import activity via the readout of Fur repression of fhuF::placZ. All strains were grown in LB supplemented with 50 μg/mL of spectinomycin and 100 μg/mL of ampicillin, as necessary.

### Hydrogen Peroxide Sensitivity

To measure resistance to H₂O₂-mediated killing, bacteria were grown in modified Mueller-Hinton broth and mid- to late-log phase organisms were pelleted at 13,200 RPM for 5 min, washed in PBS, and ~10⁵ CFU were resuspended in 200 μL PBS with or without 100 μM hydrogen peroxide (H₂O₂) in a 96-well dish. The samples were incubated at 37°C with humidity and 5% CO₂ for 1 h. The culture from each well was then serially diluted in sterile PBS, plated onto modified Mueller-Hinton agar (with antibiotic when necessary) and the number of surviving organisms for each strain was enumerated after 2–3 days.

### In Vitro Infections

LVS infections of bone marrow derived macrophages (BMM) were performed as previously described (Su et al., 2007). Briefly, BMM were differentiated from femurs of C57Bl/6 mice over the course of 7 days in complete DMEM (cDMEM, DMEM supplemented with 10% heat-inactivated fetal calf serum [FCS], 0.2 mM L-glutamine, 1 mM HEPES, and 0.1 mM non-essential amino acids [NEAA], all from Life Technologies) supplemented with M-CSF (Peprotech). Immediately prior to infection medium was removed and reserved for addition after infection. The indicated bacterial strains were added at a MOI of 50 and co-incubated with BMM for 90 min. Bacteria were then removed and fresh medium containing 10 U/ml of IFN-γ (Peprotech) was then added to the indicated groups at a final concentration of 10 U/ml. At the indicated time points, medium was collected and assessed for cytokines as described below. BMM were washed 3 times with D-PBS followed by lysis with water. Lysates were serially diluted and plated on to MMH agar for enumeration of viable organisms.
Statistics
All statistics were calculated with Graphpad Prism software (San Diego, CA). To compare β-galactosidase activity across multiple strains we used one-way ANOVA with Dunnett’s multiple comparisons test. For comparisons of multiple strains across multiple conditions, we utilized two-way ANOVA with either Sidak’s or Tukey’s multiple comparisons tests. When applicable, an unpaired two-tailed t-test was used. The LD₅₀ values were calculated using the method of Reed and Muench. To compare survival after F. tularensis SchuS4 challenge, a log-rank sum test was utilized. The error bars in the presented data represent the standard error of the mean. A $p < 0.0001$ is represented by $****p < 0.01$ is represented by **$p < 0.05$ is represented by *, and "ns" indicates “not significant.”

RESULTS
Genome Sequencing of F. tularensis LVS Strain
It was recently reported that there are differences in virulence and vaccine efficacy between LVS isolates (Deng et al., 2006; Su et al., 2007). Specifically, a low passage strain, termed RML LVS, had increased virulence in C57Bl/6 mice but also engendered increased complete protection against challenge with virulent F. tularensis subsp tularensis. In contrast, LVS strain ATCC29684 (ATCC LVS) had lower virulence in C57Bl/6 mice and failed to protect animals from lethality following F. tularensis challenge. The specific genetic changes that could account for the dramatic in vivo differences between these two strains were not identified. Therefore, in this work we have sequenced the genomes of these two LVS biovars to identify and characterize these genetic differences. Surprisingly, we detected very few differences in the sequences between RML LVS and ATCC LVS. We detected a 93 base pair deletion in the gene encoding a Dyp-type peroxidase that has previously been characterized (Binesse et al., 2015). Additionally, we detected a single nucleotide polymorphism in the feoB gene encoded by RML LVS. The C to A substitution leads to an aspartate to tyrosine mutation in the coding sequence of feoB. The D471Y mutation maps to a cytoplasmic loop of the FeoB protein, adjacent to a highly conserved glycine residue that is predicted to be functionally important by the Consurf program (Kammler et al., 1993; Buchan et al., 2008). Although the genome of the lab stock of Iowa LVS from the Jones lab has not been completely sequenced, the strain was also included in the experiments described here. Sanger sequencing confirmed that the Iowa LVS feoB encodes an aspartate at residue 471, similar to the published LVS genome. FeoB is an inner membrane protein that imports ferrous iron into the bacterial cytoplasm and has previously been linked to virulence as one of two major iron uptake pathways in LVS (Schulert et al., 2009; Thomas-Charles et al., 2013; Perez and Ramakrishnan, 2014). The substitution of a large, hydrophobic tyrosine for an aspartate at residue 471 led us to hypothesize that the FeoB protein encoded by the RML LVS allele imports Fe²⁺ poorly, or not at all. We determined if differences in iron acquisition could account for the varied virulence and vaccine efficacy of RML LVS vs. ATCC LVS. Since iron plays an integral role in host-induced oxidative stress, it is likely that controlling intracellular iron levels is a significant component of the virulence strategy of F. tularensis. Furthermore, sensitivity to oxidative stress may contribute to the vaccine efficacy of LVS given that the ATCC biovar of LVS has an aspartate at residue 471 in FeoB and the 93 base pair dyp deletion, and is both relatively attenuated in murine infections and stimulates a weak immune response against a challenge with virulent F. tularensis Schu S4 (Griffin et al., 2015).

RML LVS Displays Phenotype Consistent With Lower Intracellular Fe²⁺ Than the ATCC LVS or Iowa LVS
Our first set of experiments was designed to test the hypothesis that RML LVS has less intracellular iron than either the Iowa LVS or the ATCC LVS. Strains were grown overnight in standard modified Mueller-Hinton (MMH) broth, serially diluted in PBS, and ten-fold dilutions of liquid bacterial cultures were spotted onto MMH agar with varying concentrations of iron (Figure 1A). Growth was identical among each strain when spotted onto the control agar containing the typical concentration of iron (0.0025% ferric pyrophosphate) routinely used for propagation of F. tularensis. When the iron concentration was reduced by 50%, both the Iowa LVS and ATCC LVS had growth patterns similar to that observed on the control plate, however, RML LVS exhibited growth restriction at this concentration of iron. Fewer isolated colonies were observed and lawn growth was less luxurious when compared to the Iowa and ATCC LVS. On MMH agar lacking added iron, all three strains exhibited growth restriction, however the reduced growth phenotype of RML LVS was exacerbated, with extremely poor growth even at the lowest dilution plated. Iowa and ATCC LVS both grew as lawns at these dilutions, indicating that they were able to scavenge sufficient iron from the agar plate environment, while RML LVS could not. The decrease in RML LVS growth was approximately two orders of magnitude greater than that observed for either the Iowa or ATCC LVS, consistent with the hypothesis that RML LVS has less intracellular iron under conditions of iron limitation. We interpreted these results to mean that RML LVS had inherently lower levels of intracellular iron and that when growing the strain on agar with less iron, the amount that RML LVS could acquire for growth was limiting, thus the strain grew poorly relative to the Iowa LVS or ATCC LVS.

To confirm that the mutation carried in feoB by RML LVS had a direct consequence on iron levels, we performed experiments designed to give a semi-quantitative measure of intracellular iron levels. To avoid toxicity associated with the Fenton reaction that include lipid peroxidation, DNA damage, and poisoning of the iron-sulfur cluster enzymes of essential metabolic pathways, bacteria have evolved regulatory mechanisms to maintain iron homeostasis. One such example is the Fur system (Troxell and Hassan, 2013). The Fur transcriptional repressor is an allosteric regulator that uses Fe²⁺ as a co-repressor; when iron levels are sufficient, the Fur-Fe²⁺ complex binds to Fur Box sequence motifs in the promoters of iron uptake genes to mediate their repression. When iron is limiting,
less Fe$^{2+}$ is bound to Fur, decreasing its ability to bind
DNA. This leads to de-repression of iron uptake genes. Since
several studies have demonstrated that *Francisella* has a Fur
regulatory system that functions similarly to other organisms
(Deng et al., 2006; Sullivan et al., 2006; Buchan et al., 2008;
Ramakrishnan et al., 2008), we were able to design experiments
to indirectly measure *F. tularensis* LVS intracellular iron levels.
A plasmid carrying a Fur-regulated P$_{fslA}$-lacZ construct was
introduced into the RML LVS, Iowa LVS, and ATCC LVS.
Iron limitation is known to relieve Fur repression at the fslA
promoter, so increased P$_{fslA}$-lacZ reporter activity correlates with
decreasing concentrations of intracellular Fe$^{2+}$ (Buchan et al.,
2009). Each LVS strain was grown in Chamberlain's defined
medium (CDM) with 35 µM (high iron) or 350 nM (low iron)
FeSO$_4$, and β-galactosidase activity was measured after overnight
growth (Figure 1B). As predicted, iron starvation induced high
expression of P$_{fslA}$-lacZ in all three strains; however, the lacZ
reporter expression was approximately 2-fold higher in RML
LVS than that observed for Iowa LVS or ATCC LVS. The
higher activity of the P$_{fslA}$-lacZ construct in the RML LVS
is consistent with the hypothesis that this strain has lower
levels of intracellular Fe$^{2+}$ when iron is limiting in the growth
medium.

To assess intracellular iron levels in RML LVS by another
method, we reduced the ability of each strain to import iron
by overexpression of Fur (which downregulates genes encoding
iron uptake systems). The *F. tularensis* fur gene was placed
under the control of the groE promoter, and the construct was
introduced into each strain. Bacteria were grown in MMH broth
with standard iron concentrations overnight (no differences
in growth rate were observed between the two strains in iron
replete conditions), serially diluted in PBS and plated onto
standard MMH agar (Figure 1C). Iowa LVS and ATCC LVS,
when overexpressing Fur, exhibited no significant difference
in colony growth compared to vector controls, indicating that
intracellular pools of iron were still sufficiently high to support
normal growth, even with Fur overexpression. In contrast, RML
LVS had much smaller colony size and poor lawn formation.
We interpret these results to indicate that Fur overexpression
in RML LVS had a greater impact on growth because Fur
repression of iron uptake genes decreased the intracellular Fe$^{2+}$
concentration to levels such that the strain could not grow
normally, even though sufficient iron was present in the agar
media. These results are consistent with the hypothesis that
intracellular Fe$^{2+}$ pools of RML LVS are lower than in Iowa
LVS or ATCC LVS and can be manipulated to become limiting

![Figure 1](image_url)
for growth in conditions that are not limiting for the other two strains.

**FeoB D471Y Does Not Complement E. coli ΔfeoB fhuF::lacZ**

The data demonstrating that RML LVS has lower intracellular pools of iron than either Iowa LVS or ATCC LVS suggest that the single nucleotide change in the feoB gene of the RML strain encodes a protein with significantly reduced Fe\(^{2+}\) import activity. To assess the ability of the FeoB D471Y protein to transport iron, we made use of a well-characterized E. coli iron reporter strain that lacks *feoB* (Kammler et al., 1993; Weaver et al., 2013). This strain, *E. coli* H1771, has a chromosomal transcriptional lacZ reporter in the Fur-regulated gene *fhuF* which can be used to report the relative intracellular iron concentrations within the strain. When Fe\(^{2+}\) levels are high, expression of the *fhuF::lacZ* reporter is low and when Fe\(^{2+}\) levels are low, high β-galactosidase activity is observed. The *feoB* alleles from Iowa LVS and RML LVS (D471Y) were introduced into *E. coli* H1771 on the low copy number plasmid pWKS30, and Miller assays were performed (Figure 2). Initial experiments supplying only *feoB* (either allele) showed no repression of *fhuF::lacZ*, indicating that FeoB alone is not sufficient for Fe\(^{2+}\) import in this reporter system (data not shown). In most bacterial species encoding a *feo* system, the small *feoA* gene is encoded either in an operon with *feoB* or elsewhere on the chromosome and the encoded protein is required to stimulate FeoB Fe\(^{2+}\) import activity (Cartron et al., 2006; Lau et al., 2013; Weaver et al., 2013). When the *Francisella feoA* was supplied on pBB103 in concert with *feoB* (Iowa/ATCC allele) on pWKS30, significant repression of the *fhuF::lacZ* reporter was observed (∼80% reduction in expression relative to the empty vector control), indicating that Fe\(^{2+}\) import was significantly increased. In contrast, complementation of H1771 with *feoA* and *feoB* D471Y (RML allele) failed to repress *fhuF::lacZ*. This result is consistent with the hypothesis that the FeoB D471Y protein from the RML LVS is significantly impaired in its ability to import Fe\(^{2+}\).

**The feoB d471Y Allele Is Associated With Resistance to H\(_2\)O\(_2\)**

Since we have provided several lines of evidence consistent with the hypothesis that RML LVS has less intracellular Fe\(^{2+}\) than either the Iowa or ATCC LVS strains and Lindgren *et al.* demonstrated that strains with lower intracellular iron concentrations are more resistant to H\(_2\)O\(_2\), we hypothesized that RML LVS would be more resistant to killing by H\(_2\)O\(_2\) than Iowa LVS or ATCC LVS. Bacteria were exposed to PBS alone or PBS with 100 μM H\(_2\)O\(_2\) in a 96-well dish for 1 h at 37°C with 5% CO\(_2\) and humidity. Following incubation, the bacteria were serially diluted in PBS and plated onto MMH agar (Figure 3A). The RML LVS was typically ten-fold more resistant to H\(_2\)O\(_2\) than the Iowa strain, and approximately 100-fold to 1,000-fold more resistant than ATCC strain. The genome of ATCC encodes a Dyp peroxidase with a 93-base pair deletion, and it has been reported that this deletion can contribute to increased sensitivity to H\(_2\)O\(_2\); an Iowa LVS Δdyp mutant matches the H\(_2\)O\(_2\) sensitivity of ATCC LVS (data not shown) and is in agreement with the data from Binesse *et al.* (2015).

Given the increased sensitivity of Iowa LVS to H\(_2\)O\(_2\), and the functional complementation of an *E. coli feoB* mutant by the Iowa *feoB* allele, we next tested if the RML LVS could be sensitized to H\(_2\)O\(_2\) by increasing the intracellular Fe\(^{2+}\) pool via overexpression of a functional *feoB*. This was achieved by transforming the strain with a plasmid carrying constructs P\(_{gro}\)-feoB (Iowa/ATCC allele–high Fe\(^{2+}\) transport) and P\(_{gro}\)-feoB (D471Y; RML allele–low Fe\(^{2+}\) transport) which both overexpress *feoB*. H\(_2\)O\(_2\) sensitivity assays were performed as described previously, except that a 30 min H\(_2\)O\(_2\) exposure time point was also included in case overexpression of the *feoB* genes led to substantially faster killing by hydrogen peroxide (Figure 3B). Nearly one log of killing was observed at 30 min for the RML + P\(_{gro}\)-feoB (Iowa/ATCC), while RML + P\(_{gro}\)-feoB (D471Y) only had a two-fold reduction in viability. The effect was significantly more pronounced at the 1 h time point, with no viable bacteria recovered at the level of detection (10\(^5\) CFU) from RML + P\(_{gro}\)-feoB (Iowa/ATCC). In contrast, over 10\(^4\) CFU were recovered at 1 h from the RML + P\(_{gro}\)-feoB (D471Y). These data demonstrate that the single nucleotide change in the *feoB* gene (D471Y0 encoded in the RML LVS genome results in significantly higher resistance to H\(_2\)O\(_2\) compared to the *feoB* allele in either Iowa LVS or ATCC LVS.
To confirm that the H$_2$O$_2$ resistance phenotype conferred by $\text{feoB}$ D471Y is not unique to the RML genetic background, we constructed a $\Delta\text{feoB}$ mutant in the Iowa LVS background and complemented it with the P$_{\text{gro}^{-}}\text{feoB}$ constructs. The Iowa LVS strain background was chosen over the ATCC LVS background, as the latter strain was approximately 100-fold more sensitive to H$_2$O$_2$ than the RML LVS due to the 93 base pair deletion in the dpy peroxidase gene, while RML LVS and Iowa LVS both encode a full length $\text{dyp}$. H$_2$O$_2$ sensitivity assays were performed as described previously and, consistent with results in the RML strain background, overexpression of $\text{feoB}$ D471Y mediated significant resistance to H$_2$O$_2$ in the Iowa LVS $\Delta\text{feoB}$ mutant, relative to that when the Iowa/ATCC $\text{feoB}$ was overexpressed (Figure 3C).

**Bacterioferritin Protects Against Hydrogen Peroxide**

To assess the role of the $\text{bfr}$ gene in *F. tularensis* iron homeostasis and oxidative stress resistance, a $\Delta\text{bfr}$ strain was constructed in the RML LVS and P$_{\text{bla}^{-}}\text{lacZ}$ assays, as well as H$_2$O$_2$ sensitivity assays were performed as described above. No growth differences were observed in the $\Delta\text{bfr}$ mutant compared to the wild type strain. The RML LVS $\Delta\text{bfr}$ strain exhibited a modest decrease (~20%) in P$_{\text{bla}^{-}}\text{lacZ}$ reporter activity relative to the parent strain (Figure 4A), while in the H$_2$O$_2$ sensitivity assay, as before, the RML LVS exhibited relatively high level resistance to H$_2$O$_2$.

The $\Delta\text{bfr}$ mutant had ~10-fold fewer viable organisms, similar to Iowa LVS (Figure 4B). A possible explanation for these results is that in the absence of the Bfr iron storage protein, intracellular ferrous iron levels are elevated relative to the parent RML LVS, which may lead to increased ferrous iron available for the Fenton reaction in the presence of H$_2$O$_2$ and the increased toxicity observed in the assay. Consistent with this, P$_{\text{bla}^{-}}\text{lacZ}$ activity was modestly reduced (~20%) in the RML LVS $\Delta\text{bfr}$ mutant (Figure 4B). As a result, we conclude that bacterioferritin contributes to protection against oxidative stress, consistent with bacterioferritin function in *Neisseria gonorrhoea* and *Mycobacterium tuberculosis*.

**Expression of the Iowa/ATCC LVS feoB Allele Is Deleterious in IFN-γ-Stimulated Macrophages**

One mechanisms by which IFN-γ can control intracellular bacteria is to provoke production of reactive nitrogen and reactive oxygen species. It has been reported that IFN-γ mediated control of *Francisella* replication can be independent of RNS and ROS (Edwards et al., 2010). However, this may be due to its ability to limit toxicity of ROS by restricting iron. Therefore, we hypothesized that strains of LVS that were unable to control iron acquisition similarly to wild type RML LVS would be more sensitive to host cell IFN-γ mediated killing. We first compared RML LVS, ATCC LVS, RML LVS $\Delta\text{feoB}$ and RML...
LVS overexpressing RML _feoB_ or Iowa/ATCC _feoB_. In agreement with our hypothesis RML LVS was the least sensitive to IFN-γ mediated killing among the strains tested (Figures 5A, B). Deletion of the _feoB_ gene rendered the bacteria significantly more sensitive to IFN-γ mediated killing (Figures 5A, B). However, resistance to IFN-γ was partially restored in bacteria complemented with RML _feoB_. Complementation with ATCC _feoB_ did not significantly change RML LVS sensitivity to IFN-γ mediated killing. We next tested the ability of RML LVSΔ_bfr_ to resist IFN-γ mediated killing. Similar to RML LVSΔ_feoB_, RML LVSΔ_bfr_ was significantly more sensitive to IFN-γ mediated killing compared to wild type controls (Figures 5C, D). Together these data suggest that alterations in ferrous iron homeostasis confer sensitivity to IFN-γ mediated control of intracellular replication of LVS.

**RML LVS Δ_bfr_ Is Attenuated in Murine Infection and Fails to Protect Against _F. tularensis_ SchuS4**

As strains with increased sensitivity to H₂O₂ are associated with IFNγ-mediated killing in BMMs, we sought to determine if strains with increased sensitivity to H₂O₂ _in vitro_ might also be attenuated in a mouse model of respiratory tularemia. Disappointingly, several efforts to assess virulence of Iowa LVS, its isogenic Δ_feoB_ mutant and complemented derivatives gave inconclusive results, likely due to plasmid instability in the LVS strains in the absence of antibiotic selection _in vivo_. Thus, we pursued an alternative approach to assay for the role of altered ferrous iron homeostasis _in vivo_ and the ability of RML LVS to provide protection against a challenge with _F. tularensis_ SchuS4. To this end, we used the H₂O₂ sensitive RML LVS Δ_bfr_ mutant in murine intranasal infections. RML LVS was previously reported to have a LD₅₀ of 174 CFU in C57BL6 mice (Griffin et al., 2015). We confirmed this value by performing a similar infection experiment and calculated a LD₅₀ value for RML LVS in C57BL/6 mice of 195 CFU (Figure 6A). To test if bacterioferritin contributed to the virulence of the _F. tularensis_ RML LVS, three groups of mice (_n_ = 5 per group) were intranasally infected with 215 CFU, 2.15 × 10⁶, or 2.15 × 10⁸ CFU of the RML LVS Δ_bfr_ mutant, and their weight and health status was followed daily over the course of the experiment. All mice that received 215 CFU survived (average weight loss of ~12 percent) while forty percent of the mice survived infection with 2 × 10³ CFU (average weight loss of ~22%); none survived 2 × 10⁴ CFU. Importantly, it took >2,000 CFU of the RML LVS Δ_bfr_ strain to yield survival curves and weight losses in mice that approximated that observed for mice infected with only 133 CFU of RML LVS, a >15-fold difference in inoculum (Figures 6B, C). The estimated LD₅₀ of the RML LVS Δ_bfr_ mutant was 1,464 CFU. These results demonstrate that the bacterioferritin protein contributes to the virulence of RML LVS in mice; however, future work is needed to determine if this is a consequence of the H₂O₂ sensitivity of this mutant.

Wild type RML LVS uniformly protects C57Bl/6 mice against challenge with virulent _Francisella_ whereas ATCC LVS does not (Griffin et al., 2015). We have established that an important difference between RML LVS and ATCC LVS is their acquisition of iron and subsequent sensitivity to ROS that is inversely correlated with protective efficacy. Since mutation of _bfr_ in RML LVS led to a decrease in P₉₀₆₄-lacZ reporter activity, increased sensitivity to H₂O₂, and decreased virulence we hypothesized that vaccination with RML LVS Δ_bfr_ would provide poor protection against challenge with virulent _F. tularensis_ SchuS4. Accordingly, 6 weeks after infection with the RML LVS Δ_bfr_ mutant, we challenged all animals that survived with _F. tularensis_ SchuS4 (Figure 7). As a control, mice that had been intranasally infected with 121 CFU of RML LVS (_n_ = 3) were also challenged with 25 CFU of _F. tularensis_ SchuS4. As expected all mice that had previously been infected with RML LVS survived SchuS4 challenge. In contrast, none of the mice previously infected with RML LVS Δ_bfr_ strain were protected from the SchuS4 infection and all mice died within 8 days of challenge. Thus, while prior infection with RML LVS Δ_bfr_ did extend the mean time to death, it provoked incomplete protection against subsequent infection with fully virulent SchuS4. Together this suggests that _Francisella_ iron homeostasis and its interplay with host elements that control bacterial replication are important factors that contribute to both survival of primary infection and induction of adaptive immune responses _in vivo_.

![Figure 4](image-url)
FIGURE 5 | Mutations in iron acquisition enhance sensitivity of LVS to IFN-γ mediated killing. BMM were infected with the indicated strains of LVS and were treated with IFN-γ (10 U/ml) immediately after infection. Three and 24 h after infection intracellular bacteria were enumerated. (A,B) RML LVS is more resistant to IFN-γ mediated killing compared to all other strains tested. Deletion of the feoB gene rendered RML LVS more sensitive to IFN-γ mediated killing and complementation with homologous feoB, but not ATCC feoB partially restored resistance to IFN-γ. (C,D) Deletion of the bacterioferritin (bfr) also increased RML LVS sensitivity to IFN-γ mediated killing. Error bars represent SD. Significance was tested via two-way ANOVA followed by Tukey’s multiple comparison of means. Significance was set at $p < 0.05$. *, significantly greater than all other samples. **, significantly greater than RML ΔfeoB. ***, significantly less than RML LVS. Data is representative of three experiments of similar design.

DISCUSSION

*Francisella tularensis* is a highly pathogenic bacterium for which no effective licensed vaccine exists. Due to concerns that the extreme pathogenicity of the organism might lead to its intentional release there is increased interest in developing an effective vaccine for *F. tularensis* infections. A complication with the LVS is that the genetic mutations that attenuated LVS are undefined, leaving open the possibility that the strain could revert to full virulence. While LVS is unlikely to be reapproved for human vaccination, it is used extensively as a standard by which other candidate *Francisella* vaccines can be measured in animal models, as well as a tool to discover correlates of immunity to *F. tularensis*. Indeed, in a previous report it was found that the RML LVS biovar stimulated protective immunity in a mouse model of vaccine against virulent *F. tularensis* Schu S4 by inducing higher numbers of effector T cells (Griffin et al., 2015). Building on this observation, we have sought to identify the genetic differences between biovars of LVS and explore how they may affect the outcomes of virulence and immunity in mouse models of infection. In this report, we have demonstrated that there is a significant difference between the RML LVS and the Iowa LVS or ATCC LVS biovars in ferrous iron homeostasis.

Iron is one of the most abundant metals on the planet but is usually found in the environment in an insoluble oxidized state, which has required bacteria to develop mechanisms to scavenge iron from their local environment (Crichton and Pierre, 2001). The need for bacterial pathogens to acquire iron within a host environment presents additional challenges to the microorganism as they must also contend with host responses, which include mounting an immune response and sequestering iron, both of which can severely limit the ability of the pathogen to grow within the host (Cassat and Skaar, 2013). As expected, *F. tularensis* requires iron for productive infection of host cells, as mutants in ferrous and ferric iron uptake systems in *F. tularensis* LVS and *F. tularensis* Schu S4 were incapable of intracellular growth in vitro and were attenuated for virulence in mice (Perez and Ramakrishnan, 2014; Perez et al., 2016). Other work has shown that *F. tularensis* significantly increases expression of host iron-related genes, including transferrin receptor, presumably to increase the labile iron pools in the cytosol of infected host cells (Pan et al., 2010). Although *F. tularensis* requires iron for growth in a host, it appears to be
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FIGURE 6 | Bacterioferritin is required for optimal RML LVS virulence and. (A) Groups of mice (n = 5) were intranasally infected with 25 µl inocula containing 133 or 1,330 CFU of RML LVS in PBS. Mice were weighed daily and humanely sacrificed after weight loss ≥ 25% of their initial weight. LD₅₀ values were calculated via the method of Reed and Muench (1938). (B) Groups of mice (n = 5) were intranasally infected with 25 ul inoculant containing 215, 2,150 or 21,500 CFU of RML LVS Δbfr in PBS. Mice were weighed daily and humanely sacrificed after weight loss ≥ 25% of their initial weight. LD₅₀ values were calculated via the method of Reed and Muench (1938). (C) The average weight loss of mice infected with 10² or 10³ of the RML LVS strain or 10² or 10³ of the RML LVS Δbfr is represented by the bars in the graph. The significance of the weight loss of the mice infected with the RML LVS strain was compared to the weight loss of the mice infected with the RML LVS Δbfr strain. Mice infected with RML LVS were closer to or at clinical endpoint at all doses, while mice infected with RML LVS Δbfr lost significantly less weight at the lowest infectious dose. Significance was set at p < 0.05. ****Indicates a significant difference between the two samples bracketed. Significance was tested via log-rank sum test.

FIGURE 7 | RML requires bacterioferritin to elicit protection against an intranasal challenge of 25 CFU F. tularensis Schu S4. Mice were infected with either 121 CFU of RML LVS or 215 CFU of the bacterioferritin mutant and allowed to recover for fix weeks before challenge. The challenge inoculum was suspended in 25 µl sterile PBS and administered intranasally. Health was monitored for the duration of infection and mice were humanely sacrificed if ≥25% of initial weight was lost. Data shown is from one replicate. Significance was tested via log-rank sum test.

unusual among bacterial pathogens in that its virulence may depend, in part, on maintaining optimally low levels of iron within its cytoplasm, as the highly virulent subsp. *tularensis* strains had approximately 4- to 5-fold less intracellular iron than the moderately virulent subsp. *holarctica* strains (Lindgren et al., 2011).
Consistent with this model of *Francisella* virulence, we have shown that the more virulent RML LVS biovar encodes a non-

non-synonymous mutation in the *feoB* gene that confers phenotypes consistent with lower ferrous iron levels. The strain grows similarly to the Iowa and ATCC LVS biovars in standard propagation media, yet is restricted for growth when the iron concentrations are significantly reduced. This indicates that when extracellular iron levels become limiting, the RML LVS may not be as proficient at scavenging iron (via the *feoB* system) as Iowa LVS and ATCC LVS. Numerous labs have reported that expression of the *fsl* iron uptake operon genes are tightly regulated (repressed in high iron, induced in low iron) by iron concentrations in the media, so we used a *P$_{fslA}$-lacZ* reporter as an indirect measure of relative ferrous iron levels between the various LVS biovars (Deng et al., 2006; Ramakrishnan et al., 2008; Lindgren et al., 2009). As with growth phenotypes, there were no detectable differences in *P$_{fslA}$-lacZ* expression in growth media with normal iron concentrations; however, the RML LVS had increased reporter activity (consistent with lower intracellular iron levels) relative to the Iowa LVS and ATCC LVS after growth in iron limiting media. We probed *feoB* function of the various LVS strains further by performing functional complementation experiments. Using an *E. coli* Δ*feoB* mutant with a Fur-repressed *fhuF-lacZ* reporter as the complementation strain, we observed that the RML LVS *feoB* allele did not mediate repression of the *fhuF-lacZ* reporter (low intracellular iron), while both the Iowa LVS and ATCC LVS alleles did (high intracellular iron concentrations). Collectively, these experimental results provide compelling genetic evidence that the RML LVS likely has lower basal levels of intracellular ferrous iron than the Iowa LVS and ATCC LVS biovars. To our knowledge this is the first direct genetic evidence that explains differences in iron content between *F. tularensis* strains, though it is likely unique to LVS biovars. A significant contribution of this work is that it helps to provide a rationale for why virulent *F. tularensis* strains may carefully control their levels of intracellular iron as it appears that they balance the requirement of iron for growth against the effects of the Fenton reaction, which may induce oxidant damage to the bacteria that could expose the organisms to the host immune response (Binesse et al., 2015).

The low ferrous iron phenotypes exhibited by subsp. *tularensis* have been reported to correlate with resistance to the lethal effects of *H$_2$O$_2*, so we sought to characterize the relative sensitivity of each LVS biovar *in vitro*. Indeed, the RML LVS exhibited nearly ten-fold more resistance to hydrogen peroxide than the Iowa LVS, which encodes a functional FeoB, and 100-fold or more resistance than the ATCC LVS. The latter strain is significantly more sensitive to *H$_2$O$_2* as it is competent for Fe$^{2+}$ uptake through its functional FeoB, and because it encodes a Dyp peroxidase lacking 31 amino acids near the C-terminal portion of the protein. Mutation of the *dyp* gene has been shown previously to increase *H$_2$O$_2* sensitivity (Binesse et al., 2015), and a clean deletion of *dyp* in the Iowa LVS recapitulates this phenotype (unpublished data). We found that the RML LVS became significantly more sensitive to *H$_2$O$_2* by introducing a functional *feoB* allele under constitutive expression on a plasmid; the *H$_2$O$_2* sensitivity was significantly lower when the nonfunctional RML allele was introduced into the strain. We further demonstrated that the increased *H$_2$O$_2* lethality associated with overexpression of a functional *feoB* was not unique to the RML LVS genetic background, as an Iowa LVS Δ*feoB* mutant complemented with the mutant allele was more resistant to *H$_2$O$_2* than that complemented with the functional allele. These experiments are significant, as the strains only differed by a single nucleotide in *feoB*, yet exhibited nearly ten-fold differences in their sensitivities to *H$_2$O$_2*, irrespective of genetic background. In sum, these experiments strongly suggest that RML LVS is better at avoiding Fe$^{2+}$-associated toxicity in the context of the host environment virtue due to its lower functioning FeoB system.

In addition to characterizing the *feoB* D471Y mutation in RML LVS, we also characterized a mutant lacking the putative iron storage protein bacterioferritin. Bacterioferritin is known to oxidize ferrous iron and store the ferric oxide mineral in the hollow cavity formed by a 24-mer sphere in other organisms (Honarmand Ebrahimi et al., 2015). We reasoned that a *F. tularensis* RML LVS mutant lacking bacterioferritin may be unable to sequester free Fe$^{2+}$ as effectively as the wild type strain, and that this would be reflected in iron-related gene expression. The *P$_{fslA}$-lacZ* reporter indeed showed a modest decrease in expression in the Δ*bfr* mutant, indicating that the strain may have more Fe$^{2+}$ available to interact with Fur and repress expression of the reporter. This result is consistent with the hypothesis that *F. tularensis* LVS uses bacterioferritin to alter the pools of intracellular Fe$^{2+}$ levels by sequestration. The *P$_{fslA}$-lacZ* reporter expression decreased by only ~20% suggesting that the bacteria may utilize other iron storage mechanisms as well. As the iron-regulated reporter indicated that the Δ*bfr* mutant may have increased levels of intracellular ferrous iron, we hypothesized that the strain would be more sensitive to *H$_2$O$_2*. We found that the Δ*bfr* mutant had approximately ~90% reduction in viability relative to the parental strain, indicating that bacterioferritin has a role in resistance to oxidative stress in *F. tularensis* LVS.

As the plasmid-based complementation strategy for the Iowa LVS Δ*feoB* was unsuitable for *in vivo* studies, we utilized the RML LVS Δ*bfr* mutant as an alternative for proof of principle, given the lower *P$_{fslA}$-lacZ* reporter activity and the increased sensitivity to *H$_2$O$_2* of this mutant. Intranasal infections of mice confirmed that bacterioferritin was required for full virulence of the strain, with the LD$_{50}$ of the mutant estimated to be ~1,500 CFU, nearly an eight-fold increase relative to the parental strain (~200 CFU). Additionally, the mice exhibited differences in the severity of disease, as mice infected with 133 CFU of RML LVS lost considerable weight during the infection, approximately twenty-three percent on average. In contrast, the mice infected with 215 CFU of the Δ*bfr* mutant lost significantly less body weight during infection (~12%), even though the dose was almost two times higher. Only at a ten-fold higher dose of the Δ*bfr* mutant did the mice lose similar amounts of weight as those infected with the lowest dose of the parent strain.

As the RML LVS has been shown to be more effective at stimulating a protective response to challenge with *F. tularensis* Schu S4 than the more *H$_2$O$_2* sensitive biovar of LVS (ATCC), we hypothesized that loss of *H$_2$O$_2*$ resistance would render it a poor vaccine strain. Consistent with our hypothesis, the RML
LVS Δbfr did not stimulate robust protection in mice infected with this strain, as all succumbed from Schu S4 infection by day eight. These data suggest that H₂O₂ resistance may contribute to the efficacy of the RML LVS as a vaccine against tularemia in mice; however, it is possible that other interpretations of the data are valid. For example, the bacterioferritin protein is known to stimulate antibody production and T cell proliferation in LVS immunized mice, however it isn’t clear that antibody responses are effective at controlling infection with virulent \( F. \text{tularensis} \) Schu S4 (Bakshi et al., 2008). In \textit{in vitro} studies, \( F. \text{tularensis} \) Schu S4 is able to bind the active form of the host protease plasmin, which can cleave \textit{Francisella} specific antibodies to avoid the ensuing bactericidal and inflammatory consequences of phagocytosis via antibody mediated opsonization (Crane et al., 2009). Furthermore, introduction of monoclonal antibodies against \textit{Francisella} bacterioferritin into mice did not provide protection against lethal LVS challenge, even though other antibodies can be highly effective at protecting against this strain (Cole et al., 2009; Savitt et al., 2009). Taken together, these observations suggest that the lack of a bacterioferritin-specific antibody response is not likely to explain the poor efficacy of the RML LVS Δbfr at protecting against Schu S4.

This work is significant for several reasons. The observation that the RML LVS is better able to induce host immunity than other LVS strains and has phenotypes consistent with relatively lower levels of free ferrous iron indicates that there is an important inverse correlation between the two. This finding is of particular importance for research groups using \( F. \text{tularensis} \) LVS as a model organism for studying tularemia. Care should be taken as lab workers plate LVS cultures during routine work to not overpassage LVS and/or inadvertently select for isolates that carry mutations that lead to higher acquisition of iron. This may have the unintended consequence of working with LVS isolates that have reduced immunogenicity and/or virulence. In addition, our findings highlight further the virulence strategy of \( F. \text{tularensis} \) which appears to be optimizing its ability to grow while avoiding, as much as possible, its visibility to the host’s immune surveillance systems. Presumably, this contributes significantly to the extreme virulence of this bacterial pathogen. Finally, we believe that this work provides new information that should help to guide efforts to design a rational vaccine for tularemia.

**ETHICS STATEMENT**

All experiments using recombinant DNA techniques or reagents with \( F. \text{tularensis} \) LVS were approved by the Institutional Biosafety Committee. All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the University of Iowa Animal Care and Use committee (ACURF #1305086). Guidelines provided by the NIH were followed in all experimentation. The University of Iowa is PHS assured.

**AUTHOR CONTRIBUTIONS**

JF designed and performed a majority of experiments and was responsible for writing the manuscript. DC and TW designed and performed experiments in which LVS strains were used to infect macrophages and stimulate cytokine production. They also participated in editing the manuscript. CM performed bioinformatics analysis of the genomic sequences of strains and was responsible for editing the manuscript. CB oversaw experiments in her lab, she is responsible for the first observation of virulence differences in LVS strains and she edited and helped to write the manuscript. BJ oversaw research work performed by JF in his lab, is responsible for the final state of the manuscript and is the corresponding author.

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