



Iron and Virulence in *Francisella tularensis*

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Francisella tularensis, the causative agent of tularemia, is a Gram-negative bacterium that infects a variety of cell types including macrophages, and propagates with great efficiency in the cytoplasm. Iron, essential for key enzymatic and redox reactions, is among the nutrients required to support this pathogenic lifestyle and the bacterium relies on specialized mechanisms to acquire iron within the host environment. Two distinct pathways for iron acquisition are encoded by the *F. tularensis* genome- a siderophore-dependent ferric iron uptake system and a ferrous iron transport system. Genes of the Fur-regulated *fsIABCDEF* operon direct the production and transport of the siderophore rhizoferrin. Siderophore biosynthesis involves enzymes FslA and FslC, while export across the inner membrane is mediated by FslB. Uptake of the rhizoferrin- ferric iron complex is effected by the siderophore receptor FslE in the outer membrane in a TonB-independent process, and FslD is responsible for uptake across the inner membrane. Ferrous iron uptake relies largely on high affinity transport by FupA in the outer membrane, while the Fur-regulated FeoB protein mediates transport across the inner membrane. FslE and FupA are paralogous proteins, sharing sequence similarity and possibly sharing structural features as well. This review summarizes current knowledge of iron acquisition in this organism and the critical role of these uptake systems in bacterial pathogenicity.

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INTRODUCTION

Francisella tularensis, the etiological agent of the zoonosis tularemia, is a Gram-negative gamma-proteobacterium with a small genome of 1.89 Mb (Sjöstedt, 2007). The species is further differentiated into three subspecies, of which *tularensis* causes a more severe disease than *holarctica*, while *mediasiatica* is less well-studied. The closely related species *F. novicida*, considered to be of a more ancestral lineage (Svensson et al., 2005) and with a genome sequence identity of ~98% (Larsson et al., 2009) is an opportunistic human pathogen, but can cause a virulent tularemia-like disease in mice.

In the laboratory, studies have largely focused on virulent strain Schu S4 of the *tularensis* subspecies, the attenuated live vaccine strain (LVS) derived from a *holarctica* isolate and the strain U112 of *F. novicida*. These three isolates share many biological attributes although their genetic and functional differences significantly impact virulence (Jones et al., 2012; Celli and Zahrt, 2013; Kingry et al., 2014). For this reason, all three strains are referred to in this review as *F. tularensis* unless specifically identified in order to highlight particular differences.

F. tularensis is a facultative intracellular pathogen infecting a wide variety of cells, including mammalian and arthropod cells (Ozanic et al., 2015). Following uptake into the macrophage, the bacteria at first reside within a phagosome, but then rapidly escape into the cytoplasm. Phagosomal

escape is dependent on the *igl* operon and associated genes in the *Francisella* Pathogenicity Island (FPI) that encode components of a putative Type VI secretion system (Barker et al., 2009; de Bruin et al., 2011). The bacteria replicate to high numbers in the cytoplasm resulting finally in apoptotic death of the host cell. Adaptation to the specialized intracellular lifestyle is associated with evolutionary loss of genes for many metabolic pathways (Rohmer et al., 2007; Larsson et al., 2009), but *F. tularensis* has retained or evolved mechanisms to efficiently acquire essential nutrients within the intracellular niche of the different cell types that it infects (Meibom and Charbit, 2010).

Mice have been extensively used to model animal infection (Lyons and Wu, 2007). Phagocytic cells are thought to be the first infected (Hall et al., 2008); subsequently infection is disseminated to other tissues in the body. *F. tularensis* exercises several strategies to evade immune responses and is able to replicate to high levels in the liver, spleen and lungs before the immune system is provoked to respond with a destructive cytokine storm (Sharma et al., 2011; Jones et al., 2012).

Iron and *Francisella*

Francisella requires iron for essential cellular functions. Early studies reported that infection with *F. tularensis* induces an iron-withholding response typical of the innate nutritional immunity defense mechanism (Pekarek et al., 1969). However, the intracellular pathogen manipulates host cell iron metabolism to support growth; LVS induces infected macrophages to increase iron flow through the cell by enhanced expression of the transferrin receptor TfR1 for uptake of iron and in parallel, increased expression of Dmt1 that moves endosomal iron into the cytoplasm and a slight increase in ferroportin that promotes outflow of iron from the cell (Pan et al., 2010). A functional Nramp1 protein that also transports endosomal iron into the cytoplasm restricts growth of endosome-resident bacteria but enhances *Francisella* growth (Kovářová et al., 2000, 2002), highlighting the importance of cytoplasmic iron availability for pathogenesis. However, the nature of the host iron sources accessed by the organism remains to be characterized. Iron-limitation restricts growth of bacteria in culture (Deng et al., 2006; Sullivan et al., 2006) as well as within the macrophage (Fortier et al., 1995).

As might be predicted, growth of *F. tularensis* is inhibited by gallium, which competes with ferric iron for uptake and also interferes with iron-dependent biological processes (Olananmi et al., 2010; Lindgren and Sjöstedt, 2016). Inhibition of the iron-associated enzymes catalase and superoxide dismutase leads to increased susceptibility to oxidative stress (Bakshi et al., 2006; Lindgren et al., 2007; Olananmi et al., 2010; Binesse et al., 2015).

Iron metabolism appears to differ among *F. tularensis* isolates. LVS expresses higher levels of bacterioferritin as compared to Schu S4 (Hubálek et al., 2003, 2004). Consistent with these findings, isolates of the *holarctica* subspecies have greater iron stores than *tularensis* isolates, and since iron is closely associated with generation of reactive oxygen species, *holarctica* strains are more susceptible to oxidative stress (Lindgren et al., 2011).

Fur and Iron Regulation of Genes

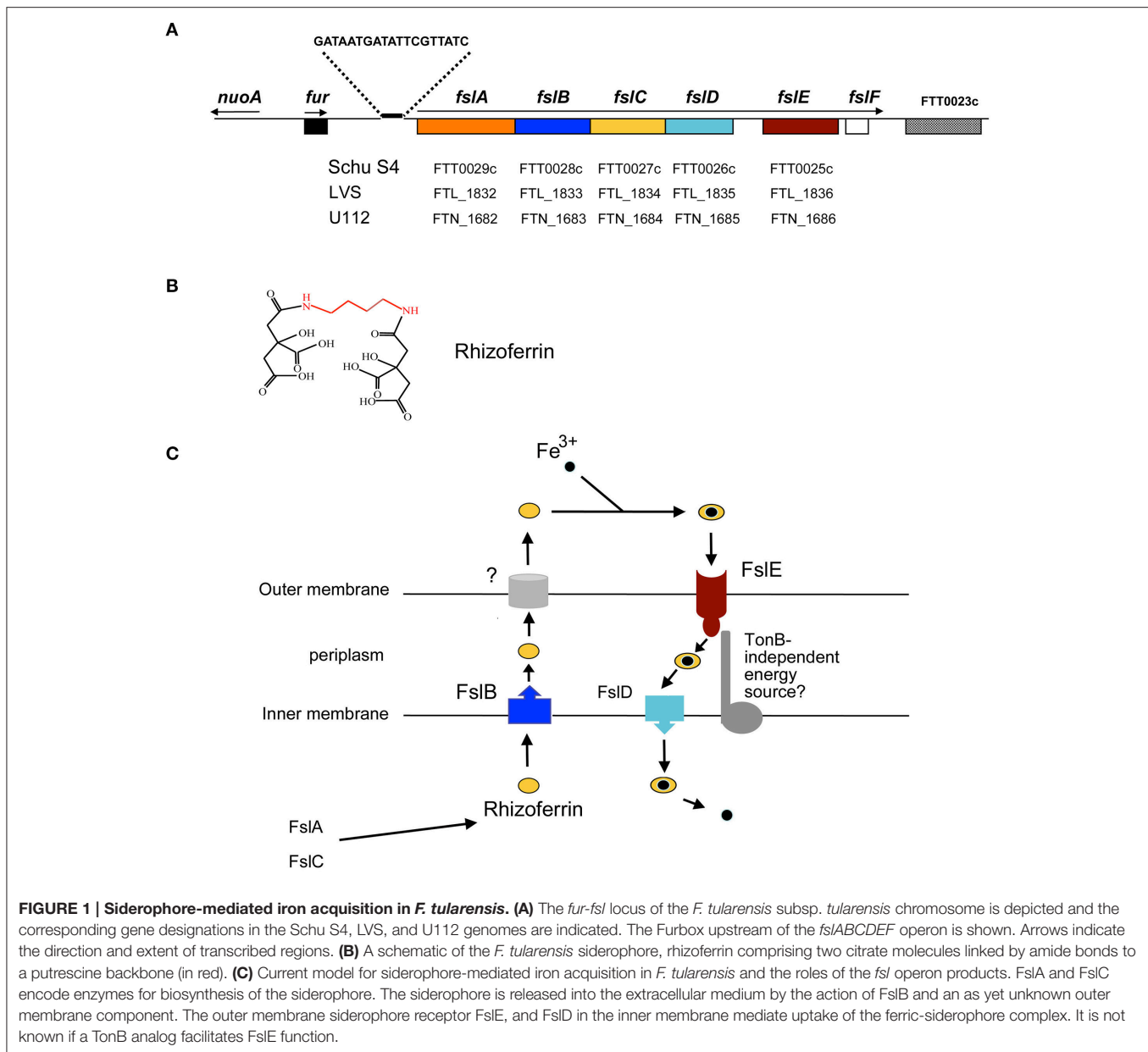
A *fur* ortholog is encoded in the *F. tularensis* genome and the predicted Fur protein contains elements known to be important for Fur function (Pérard et al., 2016). The *fur* gene is adjacent to the *fsl* operon encoding components of a siderophore-mediated iron uptake pathway, and a canonical Furbox is located upstream of the first gene of the operon, *fslA* (Figure 1A). Expression of the *fsl* operon is induced in iron-limiting media (Deng et al., 2006; Sullivan et al., 2006; Buchan et al., 2008). Loss of the *fur* gene results in deregulated transcription of the *fsl* operon and increased siderophore production (Buchan et al., 2008; Ramakrishnan et al., 2008). Expression of the inner membrane ferrous iron transporter *feoB* is also upregulated in a *fur* mutant (Pérez and Ramakrishnan, 2014).

Exposure to iron limitation was also shown to increase virulence of an *F. tularensis* isolate, suggesting that pathogenicity is influenced by iron levels (Bhatnagar et al., 1995). Microarray analysis of RNA indicated that besides the *fsl* operon, transcription of the *igl* operon was increased under iron limitation (Deng et al., 2006). Proteomic analysis also confirmed that iron limitation results in increased levels of the IglC protein (Lenco et al., 2007). However, although an *fslB-lacZ* reporter could be repressed by overexpression of Fur, an *iglB-lacZ* reporter was not similarly repressible (Buchan et al., 2008), suggesting that a mechanism besides Fur regulates the *igl* genes in response to iron levels in *F. tularensis*.

Siderophore-Mediated Iron Acquisition

A “growth inducing substance (GIS)” that promoted growth of *F. tularensis* bacteria from small inocula was reported in the 1960s (Halmann and Mager, 1967; Halmann et al., 1967); in all likelihood this substance was the siderophore now identified as the polycarboxylate rhizoferrin (Drechsel et al., 1991; Thieken and Winkelmann, 1992; Sullivan et al., 2006). Rhizoferrin is structurally simple, comprising 2 citrate moieties linked through amide bonds to a putrescine backbone (Figure 1B). Originally identified as a fungal siderophore (Drechsel et al., 1991; Thieken and Winkelmann, 1992), rhizoferrin was subsequently also isolated from a strain of the bacterium *Ralstonia pickettii* (Münzinger et al., 1999). The identification of the siderophore made by *F. tularensis* as rhizoferrin (Sullivan et al., 2006), and the subsequent identification of the *Legionella pneumophila* siderophore legiobactin as also rhizoferrin (Burnside et al., 2015) suggests that this siderophore may be more widely prevalent in bacteria than suspected. Structurally related siderophores made by bacteria include staphyloferrin A (Konetschny-Rapp et al., 1990) and corynebactin (Zajdowicz et al., 2012), where D-ornithine and lysine, respectively, constitute the siderophore backbone in place of the putrescine present in rhizoferrin. The *R. pickettii* rhizoferrin was shown by CD spectroscopy to be an S-S enantiomer in contrast to the R-R fungal molecule (Münzinger et al., 1999). Whether all bacterial rhizoferrins adopt the S-S conformation is not clear, but bacteria making rhizoferrin are capable of utilizing the fungal form for iron uptake (Münzinger et al., 1999; Kiss et al., 2008).

Genes for synthesis and transport of *Francisella* rhizoferrin are located on the siderophore operon *fslABCDEF* (also designated



figABCDEF; (Deng et al., 2006; Sullivan et al., 2006; Milne et al., 2007; Buchan et al., 2008; Ramakrishnan et al., 2008); **Figures 1A,C**). Analysis of individual mutants as well as complementation of a strain carrying a complete deletion of the *fslA-F* genes helped to determine the roles played by the different genes in siderophore-mediated iron acquisition, as detailed below.

fslA and *fslC* and share homology with genes found in siderophore biosynthetic loci of other bacteria. *FslA* is similar to the aerobactin synthetases *IucA/IucC* and a member of the non-ribosomal peptide synthetase-independent siderophore (NIS) synthetases, enzymes that assemble non-peptide siderophores using dicarboxylic acids and diamines or amino-alcohols

(Challis, 2005). *FslC* is predicted to be a member of the pyridoxal phosphate-dependent decarboxylases. Mutant analysis demonstrated that both *fslA* and *fslC* are required for *Francisella* rhizoferrin production (Deng et al., 2006; Sullivan et al., 2006; Lindgren et al., 2009; Thomas-Charles et al., 2013). Rhizoferrin biosynthesis in *F. tularensis*, involving just two dedicated enzymes, may be the simplest siderophore biosynthetic pathway identified thus far.

fslB encodes a transporter of the Major Facilitator superfamily (MFS) and deletion of this gene in *F. novicida* results in reduced levels of siderophore activity in the culture medium (Kiss et al., 2008). Additionally, detection of siderophore activity in culture supernatants of an LVS $\Delta fslA-F$ mutant

required complementation with the *fslB* gene in addition to the biosynthetic genes *fslA* and *fslC* (Pérez et al., 2016). These observations support a role for FslB in export of the siderophore across the cytoplasmic membrane. How the siderophore is channeled through the outer membrane into the extracellular space is currently not known.

fslD encodes an inner membrane MFS protein and deletion of this gene was found to have little effect on siderophore production (Kiss et al., 2008). The role of this protein in siderophore-mediated iron uptake across the cytoplasmic membrane was deduced on the basis of complementation studies: a Schu S4 $\Delta fslA$ -*F* mutant is able to transport $^{55}\text{Fe}^{3+}$ complexed to siderophore only when complemented with the *fslD* gene in addition to the *fslE* receptor gene (see below; Pérez et al., 2016).

fslE, the fifth gene in the operon, encodes an outer membrane protein unique to the *Francisella* genus Larsson et al., 2005; Huntley et al., 2007). $\Delta fslE$ mutants are impaired for growth in iron-limiting media and are unable to utilize exogenous siderophore for growth (Kiss et al., 2008; Ramakrishnan et al., 2008). In transport assays, *fslE* mutants proved incapable of siderophore-mediated $^{55}\text{Fe}^{3+}$ uptake, establishing a role for FslE as receptor for the siderophore (Ramakrishnan et al., 2012). FslE can also transport the iron mimic gallium in complex with rhizoferrin as shown by the resistance of *fslA* and *fslE* mutants to gallium (Pérez et al., 2016).

The last gene of the *fsl* operon, *fslF*, varies structurally among the *tularensis* and the *novicida* species, being truncated in the *tularensis* isolates. Studies with a $\Delta fslF$ mutant in Schu S4 indicate that the gene does not influence iron transport in *F. tularensis* (Pérez et al., 2016).

Siderophore-mediated iron transport by outer membrane receptors in Gram-negative bacteria is typically dependent on the proton motive force transduced by the TonB-ExbB-ExbD complex (Noinaj et al., 2010). The *Francisella* genome, however, does not encode orthologs of *tonB*, *exbB*, and *exbD*, implying that alternative mechanisms must facilitate siderophore-iron uptake.

Ferrous Iron Uptake

The *F. tularensis* genome encodes an inner membrane ferrous iron transport system comprising unlinked genes *feoA* and *feoB*. $\Delta feoB$ mutants of LVS and Schu S4 are deficient for growth on iron-limiting media (Thomas-Charles et al., 2013; Pérez and Ramakrishnan, 2014; Pérez et al., 2016). ^{55}Fe uptake assays demonstrated that the *F. tularensis* $\Delta feoB$ mutants are completely deficient in ferrous iron uptake (Pérez and Ramakrishnan, 2014; Pérez et al., 2016), implying that the Feo system is the sole ferrous iron transporter across the inner membrane (Figure 2). It is likely that FeoA supports FeoB function as seen in *Salmonella* Typhimurium (Kim et al., 2012) and *Vibrio cholerae* (Weaver et al., 2013; Stevenson et al., 2016).

Given the soluble nature of ferrous iron, the general assumption has been that it diffuses into the periplasmic space through non-specific porin proteins in the outer membrane. However, growth and ^{55}Fe transport assays indicate that *F. tularensis* is capable of high-affinity uptake of ferrous iron mediated by the specific outer membrane protein FupA (Ramakrishnan et al., 2012). FupA was initially characterized as

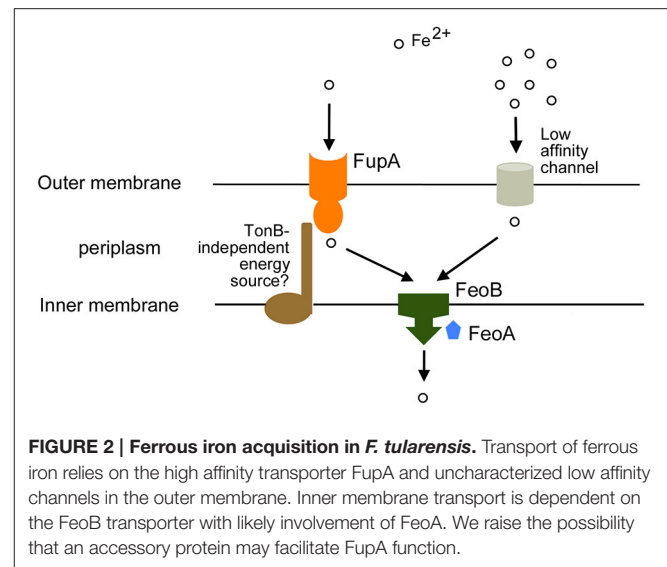


FIGURE 2 | Ferrous iron acquisition in *F. tularensis*. Transport of ferrous iron relies on the high affinity transporter FupA and uncharacterized low affinity channels in the outer membrane. Inner membrane transport is dependent on the FeoB transporter with likely involvement of FeoA. We raise the possibility that an accessory protein may facilitate FupA function.

a virulence factor in Schu S4 (Twine et al., 2005) and found to influence bacterial intracellular replication (Twine et al., 2005; Asare et al., 2010). An involvement in iron acquisition was established by the finding that Schu S4 $\Delta fupA$ mutant grew poorly under iron limitation, had lowered internal iron levels and was deregulated for siderophore production (Lindgren et al., 2009). A link to ferrous iron uptake was indicated by the finding that ferrous and ferric iron supplements supported growth of a *fupA* mutant to different extents on agar plates (Ramakrishnan et al., 2012). ^{55}Fe transport assays clearly demonstrated that the *fupA* mutant was unable to transport ferrous iron at limiting concentrations ($\sim 0.1 \mu\text{M}$) although low affinity ferrous iron transport at high concentrations ($\sim 3 \mu\text{M}$) could still be observed, and siderophore-iron uptake was not perturbed (Ramakrishnan et al., 2012). These findings suggest that FupA serves as a *F. tularensis* adaptation to efficiently acquire ferrous iron even in low abundance settings when general diffusion-based transport across the outer membrane might prove inadequate (Figure 2).

FslE and FupA: Related High-Affinity Iron Transport Proteins

FupA and FslE are paralogs belonging to a family of proteins unique to *Francisella* (Larsson et al., 2005). Both proteins have been localized to the outer membrane of *F. tularensis* (Huntley et al., 2007; Ramakrishnan and Sen, 2014) and share a global 54% identity and 69% similarity in amino acid sequence. FupA with 557 amino acid residues is larger than FslE (509 residues). The Hidden Markov Model-based PRED-TMBB program (Bagos et al., 2004a,b) predicts that both FupA and FslE fold as β -barrels in the outer membrane with amino-terminal periplasmic domains. FupA is predicted to form a 16-stranded barrel with a periplasmic domain of 201 residues while FslE could form a 14-stranded barrel with a 152 residue periplasmic domain. This structure is reminiscent of typical TonB-dependent transporters (Noinaj et al., 2010). The greatest similarity between FslE and FupA is in the predicted β -barrel domains.

The *fupA* gene is located adjacent to a paralog *fupB* on the chromosome. Infrequent recombinational deletion events have been observed leading to formation of *fupA/B* hybrid genes (Twine et al., 2005; Rohmer et al., 2006); such recombination accounts for a significant reduction in virulence as seen in LVS, which can be reversed by restoration of the full length *fupA* gene (Salomonsson et al., 2009). The FupA/B hybrid protein encoded by LVS is less efficient at high-affinity ferrous iron uptake than FupA, but gains siderophore-iron uptake capability (Sen et al., 2010; Ramakrishnan and Sen, 2014). The structural and functional overlap in protein function raises the intriguing possibility that a common mechanism may underlie transport by FslE and FupA.

fupA expression is independent of iron and *fur* regulation, suggesting that FupA may have functions in addition to iron transport. *fupA* mutants have increased resistance to copper, and transport assays indicated that copper competes with ferrous iron for transport (Pérez et al., 2016). A role for FupA in maintenance of outer membrane integrity has also been proposed (Nallaparaju et al., 2011). Although, the *fsl* operon is regulated in response to iron levels, it was reported recently that calcium and magnesium limitation also result in increased *fslE* transcription (Wu et al., 2016). These observations are consistent with the idea that the high affinity iron transport proteins in the outer membrane of *F. tularensis* may assume roles in transport of additional substrates under stress.

Interestingly, FslE appears structurally different from rhizoferrin receptors of other bacteria. The LbtU siderophore receptor of *L. pneumophila* is not predicted to have a distinct periplasmic domain (Chatfield et al., 2011). RumA, the rhizoferrin receptor in *Morganella morganii* is a TonB-dependent transporter (Kühn et al., 1996). Mechanisms for rhizoferrin transport thus appear to have evolved independently in different bacteria.

Iron Uptake and Pathogenesis

Analysis of the transcriptome of Schu S4-infected macrophages demonstrated that the *fsl* genes are among the most highly induced genes in the intracellular niche (Wehrly et al., 2009). Similar results were obtained using LVS infected hepatocytes (Thomas-Charles et al., 2013), suggesting that the siderophore uptake system contributes to survival within different tissue types. *fsl* mutants were identified in a negative selection screen of a U112 transposon mutant library in mice (Weiss et al., 2007) and a signature-tagged mutant screen identified *fslA* and *feoB* to be important for pulmonary infection by LVS (Su et al., 2007). Nevertheless, individual mutants in the *fsl*, *fupA*, and *feoB* genes

are capable of intracellular growth although the reduced growth of the *feoB* mutants in hepatocytes suggests that ferrous iron is likely the major iron source within these cells (Lindgren et al., 2009; Ramakrishnan et al., 2012; Thomas-Charles et al., 2013; Pérez and Ramakrishnan, 2014; Pérez et al., 2016). Both iron uptake pathways appear to contribute to utilization of iron from heme for growth (Lindgren et al., 2015), suggesting that iron needs to be released from the heme for use by the bacteria. Screens with U112 mutants have implicated additional genes in iron acquisition, but they have not been definitively characterized (Crosa et al., 2009).

A Schu $\Delta fslE \Delta fupA$ mutant deficient for both siderophore and high affinity ferrous iron uptake grows slowly, is attenuated for growth in macrophages and completely avirulent in mice (Ramakrishnan et al., 2012). $\Delta fslA \Delta feoB$ mutants of LVS and Schu S4 deficient for siderophore biosynthesis and for all ferrous iron uptake have an even more severe defect, with dependence on extraneous siderophore for growth, loss of all capacity for intracellular growth and complete loss of virulence (Pérez and Ramakrishnan, 2014; Pérez et al., 2016). These findings indicate that both subspecies of *F. tularensis* have a similar repertoire of iron uptake mechanisms, limited to just the *fsl* and *feo*-mediated mechanisms. Interestingly, although the $\Delta fslE \Delta fupA$ and the $\Delta fslA \Delta feoB$ mutants are avirulent in mice, exposure to these strains protects from subsequent challenge with the virulent strain (Ramakrishnan et al., 2012; Pérez et al., 2016), making them good candidates for further exploration as live vaccines.

CONCLUSIONS

With a reduced genome, *F. tularensis* has evolved to efficiently support its lifestyle as an intracellular pathogen with a minimal set of two iron acquisition pathways. Host iron sources utilized and mechanisms regulating the transport proteins FslE and FupA are interesting questions for future investigations.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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