Introduction

Iron is fundamental to basic metabolic processes and homeostasis of every domain of life; nevertheless, aerobic organisms need to tightly regulate levels of free iron in order to limit the formation of damaging reactive oxygen species (Freinbichler et al. 2011). Microbial pathogens, therefore, face iron limitation within the mammalian host where the majority of iron is sequestered; additional iron restriction results from mechanisms that are activated in response to perceived infection including an increase in levels of lipocalin to bind up bacterial siderophores, transferrin to scavenge free iron, and the hormone hepcidin, which influences systemic iron metabolic pathways (Nairz et al., 2010).

Iron in living systems transitions between two forms that have intrinsically different properties: the reduced ferrous form (Fe^{2+}) is highly soluble and is thought to be the primary constituent of the cytoplasmic labile iron pool, while the relatively insoluble oxidized ferric form (Fe^{3+}) is believed to mainly be contained within protein complexes (Diouhy and C. E 2013; Hider and Kong 2013). Bacterial pathogens, therefore, need different mechanisms to access these two forms of iron.

Francisella tularensis, the etiological agent of tularemia, is a Gram-negative bacterium with a very low infectious dose (<10 CFU) (Jones et al. 2012; Celli and Zahrt 2013). It is commonly transmitted by arthropod vectors in the wild and can infect a wide range of mammalian hosts. The bacterium is internalized by and replicates within the cytoplasm of a variety of host cells, most notably macrophages and hepatocytes. The high virulence and rapid intracellular growth of this pathogen implies that it possesses mechanisms to efficiently acquire...
essential nutrients from the host (Abu Kwaik and Bumann 2013).

The importance of iron for intracellular growth of *F. tularensis* was first demonstrated using a macrophage infection model where bacterial replication was inhibited by deferoxamine treatment and iron acquired by the macrophage through transferrin or nontransferrin-dependent mechanisms was able to support bacterial growth (Fortier et al. 1995). Several studies have demonstrated that *F. tularensis* encodes separate mechanisms for ferrous and ferric iron acquisition to support its pathogenic lifestyle (Deng et al. 2006; Sullivan et al. 2006; Lindgren et al. 2009; Ramakrishnan et al. 2012; Thomas-Charles et al. 2013).

The three subspecies of *F. tularensis: tularensis*, *holarctica* and *mediasiatica* display differences in virulence, and there is great interest in discerning the mechanisms that contribute to these differences. Variations in iron metabolism appear to influence the physiology of the subspecies; the *holarctica* strains have higher levels of bacterioferritin and increased internal iron stores relative to *tularensis* leading to differences in susceptibility to hydrogen peroxide–induced killing (Hubálek et al., 2004; Lindgren et al., 2011). Differences in mechanisms of iron acquisition could potentially contribute to these variations.

Many bacteria produce siderophore molecules that chelate ferric iron in the environment for subsequent uptake by dedicated transport systems (Miethke and Marahiel 2007). Strains of *F. tularensis* and the related *Francisella novicida* species secrete a polycarboxylate siderophore similar to rhizoferrin, and siderophore production is governed by the conserved *Francisella* siderophore locus (*fsl*) (frg) operon under control of the Fur repressor (Deng et al. 2006; Sullivan et al. 2006; Buchan et al. 2008; Ramakrishnan et al. 2008) (Fig. 1A represents the chromosomal *fsl* locus in the *tularensis* subspecies strain Schu S4). The *fslA* and *fslC* genes encode a siderophore synthetase and a decarboxylase, respectively, required for *Francisella* rhizoferrin production (Deng et al. 2006; Sullivan et al. 2006; Lindgren et al. 2009; Thomas-Charles et al. 2013). *fslB* and *fslD* encode putative inner-membrane proteins belonging to the Major Facilitator Superfamily (MFS). Mutation in *fslB* in the *F. novicida* strain U112 led to diminished production of siderophore (Kiss et al. 2008). *fslD* function in *Francisella* has not been explored.

The *Francisella* rhizoferrin receptor FslE, which bears no significant sequence similarity to known siderophore receptors, is also encoded by the *fsl* operon (Milne et al. 2007; Kiss et al. 2008; Ramakrishnan et al. 2008). Transport of the siderophore–iron complex across the outer membrane of Gram-negative bacteria is typically powered by the TonB–ExbB–ExbD complex in the inner membrane (Miethke and Marahiel 2007; Chu et al. 2010). The *F. tularensis* genome lacks the genes for TonB, ExbB, and ExbD, and therefore, siderophore-mediated iron uptake in this Gram-negative organism is atypical. The last gene of the operon, *fslF*, shows sequence differences between the *F. tularensis* and *F. novicida* strains; *fslF*<sub>Schu S4</sub> encodes a 114-amino acid protein predicted to be in the inner membrane, while *fslF*<sub>U112</sub> is a larger protein that includes sequences of an independent ORF downstream in *F. tularensis* (FTT0023c in Fig. 1A). Whether FslF retains a siderophore-associated function in the different strains is therefore of question.

Dedicated outer and inner-membrane proteins for transport of ferrous iron have been identified in *Francisella* species. The outer-membrane protein FupA, a paralog of the siderophore receptor FslE, is also associated with iron metabolism (Lindgren et al. 2009) and mediates high-affinity transport of ferrous iron (Ramakrishnan et al. 2012). The inner-membrane ferrous iron transporter FeoB is encoded by all sequenced *Francisella* genomes and has been functionally characterized in the live vaccine strain (LVS) of the *F. tularensis* holarctica subspecies (Thomas-Charles et al. 2013; Pérez and Ramakrishnan 2014).

Transcriptional profiling of Schu S4–infected mouse bone marrow derived macrophages showed that genes within the *fsl* were upregulated during the course of infection (Wehrly et al. 2009). Screens of transposon mutant libraries and testing of individual mutants indicated that the *fsl* genes and the ferrous iron transporter gene *feoB* are required for optimal virulence of *F. novicida* and of LVS (Su et al. 2007; Weiss et al. 2007; Thomas-Charles et al. 2013; Pérez and Ramakrishnan 2014). Deletion of *fupA* in Schu S4 led to partial attenuation in mice (Twine et al. 2005; Ramakrishnan et al. 2012), while a mutant lacking both FslE and FupA remained avirulent for mice even with 1000 LD<sub>50</sub> delivered by the subcutaneous route (Ramakrishnan et al. 2012). Screens with mutants of U112 and Schu S4 have suggested that additional genes could be involved in iron acquisition, but their roles have not been definitively characterized (Crosa et al. 2009; Lindgren et al. 2015).

We previously showed using in vitro and intracellular growth assays and a mouse infection model that the *Francisella* rhizoferrin and *feoB*-dependent transport mechanisms were the only operational iron acquisition systems in LVS (Pérez and Ramakrishnan 2014). An important cause of attenuation in LVS is the genomic recombination event that generated a hybrid gene between *fupA* and the downstream gene *fupB* (Salomonsson et al. 2009). The FupA paralog of LVS FupA/B is less efficient at ferrous iron uptake; in addition, FslE<sub>LVS</sub> is less
efficient than FslE_{Schu S4} and FupA/B contributes to siderophore-mediated iron uptake in LVS (Sen et al. 2010; Ramakrishnan and Sen 2014). These findings highlight differences in iron uptake capability of LVS and Schu S4.

To gain a comprehensive understanding of iron acquisition in the virulent *tularensis* subspecies, we have queried gene function and defined attributes of iron uptake in *Francisella* rhizoferrin-mediated ferric iron acquisition and FupA–FeoB-dependent ferrous iron uptake are parallel and independent, and that in conjunction, these two systems satisfy the iron requirements for growth and full virulence of the organism.
Results

The fsl operon encodes requisite functions for siderophore biosynthesis, transport, and siderophore-dependent iron acquisition

We generated mutants lacking the entire fsl operon (ΔfslA-ΔfslF) (Fig. 1A) in both Schu S4 (Δfsl) and LVS (Δfsl). As might be predicted, deletion of the entire fsl locus resulted in loss of siderophore production as determined by the Chrome Azurol S (CAS) assay (Fig. 1B). We then introduced combinations of fsl genes back into the Δfsl mutants and tested for restoration of siderophore-associated function. Specifically, we reintroduced biosynthetic gene fslA in combination either with fslB, fslC or fslB + fslC back into the Δfsl mutant and assessed siderophore production. As shown in Figure 1C, fslA, fslB, and fslC were all required and were sufficient to restore siderophore production. This is consistent with previous findings that mutants in the individual genes are deficient in siderophore production (Deng et al. 2006; Sullivan et al. 2006; Kiss et al. 2008; Thomas-Charles et al. 2013). We conclude that siderophore synthesized by action of the FslA and FslC enzymes is transported out of the cytoplasm by the MFS transporter FslB.

The Δfsl mutants are predicted to be deficient at siderophore utilization and the ΔΔfsl mutant showed loss of siderophore-mediated 55Fe uptake capability (Fig. 1D). Expression of the siderophore receptor gene fslE alone was not sufficient to restore siderophore uptake capability to the mutant, but introduction of fslD along with fslE did restore this function. These results indicated that the outer-membrane receptor FslE and the inner-membrane MFS transporter FslD are involved in siderophore-iron uptake.

Introduction of fslF with fslD and fslE did not further augment siderophore-mediated 55Fe uptake. This complementation assay could potentially mask a subtle involvement of fslF in iron uptake, and we, therefore, generated a Schu ΔfslF strain lacking only the last gene of the operon. The ΔΔfslF mutant, however, showed no differences relative to the parent strain in assays for siderophore production and for siderophore-mediated uptake (data not shown), suggesting that FslF does not contribute to siderophore utilization in F. tularensis.

Although deficient in siderophore-mediated 55Fe uptake, the ΔΔfsl mutant was capable of ferrous iron (55Fe2+) acquisition (Fig. 1E); in fact, the mutant strain was more proficient than the parent strain in ferrous iron uptake, with the difference being statistically significant at the lower iron concentrations (100 nmol/L). This suggests that ferrous iron uptake is deregulated in the mutant to compensate for loss of siderophore function.

In summary, complementation studies with the Δfsl mutants definitively established that the fslA, fslB, and fslC genes were responsible for siderophore production while fslD and fslE were essential for siderophore-mediated iron uptake. Additionally, these studies demonstrated clearly that ferrous iron uptake functions independently of the fsl siderophore operon.

FeoB is necessary for optimal growth under iron limitation

Previous studies indicated that inner-membrane ferrous iron transport in LVS is primarily mediated by FeoB (Pérez and Ramakrishnan 2014). To determine the importance of FeoB function in Schu S4, we generated a deletion mutant predicted to express a truncated FeoB protein lacking the carboxy-terminal half (ΔfeoB′).

Growth of the Schu ΔfeoB′ mutant was compared to the parent Schu S4 and to ΔfupA and ΔfslA mutants known to be important for ferrous iron acquisition and siderophore biosynthesis, respectively (Sullivan et al. 2006; Lindgren et al. 2009; Ramakrishnan et al. 2012). Tenfold serial dilutions of the bacteria were spotted on iron-replete Mueller–Hinton agar (MHA)+ or on iron-limiting MHA−. Growth of the Schu ΔfeoB′ mutant was compared to the parent Schu S4 and to ΔfupA and ΔfslA mutants known to be important for ferrous iron acquisition and siderophore biosynthesis, respectively (Sullivan et al. 2006; Lindgren et al. 2009; Ramakrishnan et al. 2012). Tenfold serial dilutions of the bacteria were spotted on iron-replete Mueller–Hinton agar (MHA)+ or on iron-limiting MHA−. While the different strains grew similarly on MHA+, there was a dramatic difference on MHA− (Fig. 2A). Schu S4 was able to grow to 10−6 dilution on MHA−, while the mutants showed different levels of reduction in growth. The ΔfslA mutant grew to 10−4 dilution and the ΔfeoB′ and the ΔfupA mutant grew less well. The ΔfupA mutant had a more severe growth defect than the ΔfeoB′ mutant, suggesting that FupA may have additional functions besides that of ferrous iron acquisition. The ΔfeoB′ mutant complemented with a wild-type copy of feoB in cis was able to grow like the wild-type Schu S4 on MHA− plates (Fig. 2B). Taken together, these results indicated that the ferric-siderophore system and FeoB-mediated ferrous iron acquisition are both required for normal growth on iron-limiting MHA plates.

FeoB is essential for transport of 55Fe2+ by Schu S4

We previously showed that the outer-membrane protein FupA is associated with high-affinity uptake of ferrous iron (Fe2+), important at limiting iron concentrations (Ramakrishnan et al. 2012). We compared 55Fe2+ uptake in the ΔfeoB′ mutant, the ΔfupA mutant, and parent Schu S4. As expected, the ΔfupA mutant was deficient at iron transport particularly at the lower ferrous iron concentration (0.1 μmol/L), but the ΔfeoB′ mutant was defective for uptake of ferrous iron both at the low and at the high 3.0 μmol/L iron concentration (Fig. 3). This uptake
defect was abrogated upon complementation with a wild-type copy of feoB. These results indicate that whereas the outer membrane has low-affinity channels in addition to the high-affinity FupA transporter, FeoB is the only functional inner-membrane transporter for ferrous iron in Schu S4.

**Schu ΔfeoB’ mutant shows increased expression of the fsI operon**

A ΔfsI A mutant is unable to produce siderophore while a ΔfupA mutant secretes siderophore in both iron-replete and iron-limiting conditions (Sullivan et al. 2006; Lindgren et al. 2009). Using the CAS assay with cultures grown in liquid Chamberlin’s defined medium (CDM), we found that siderophore production is also deregulated in the Schu S4 ΔfeoB’ mutant under iron-replete (high iron) conditions, similar to the ΔfupA mutant (Fig. 4A).

Siderophores can bind gallium in a manner similar to ferric iron and gallium-siderophore complexes are recognized and taken up by microbial siderophore-transport systems (Emery and Hoffer 1980). Gallium is toxic to bacteria, and siderophore-mediated uptake of gallium leads to bacterial growth inhibition (Carrano et al., 1996). We evaluated siderophore activity in the *F. tularensis* strains by testing for growth sensitivity in the presence of gallium.

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**Figure 2.** Growth of Schu S4, iron acquisition mutants and complemented strains on iron-replete or iron-limiting agar. Except for the ΔfeoB’ ΔfsI A mutant, all strains were grown overnight in iron-replete che-CDM, washed and resuspended to an OD₅₉₅ of 1.0. Ten-fold serially diluted suspensions were spotted on iron-replete MHA+ (A,D,E) or iron-limiting MHA- (A,B) and grown for 3 days at 37°C before recording the images. (C-E) The ΔfupA ΔfsI A mutant was grown on MHA+ supplemented with siderophore and bacteria were scraped into che-CDM and washed before serial dilutions were spotted on the agar plates as indicated. In C, two sets of dilutions of the ΔfupA ΔfsI A were spotted on the iron-limiting CDM- plate, and Schu S4 bacteria were streaked in the vicinity of one set of dilutions.

**Figure 3.** Uptake of ⁵⁵Fe²⁺ by Schu S4 and iron acquisition mutants. Iron-starved cultures of Schu S4, the ΔfupA and ΔfeoB’ mutants and the complemented ΔfeoB’ mutant were assayed for the ability to take up ⁵⁵Fe²⁺ when present at 0.1 μmol/L (A) or 3 μmol/L (B) concentration. Values are expressed as means ± standard error of the mean (SEM) of quadruplicate assays. Values were compared to those of Schu S4. *P < 0.05, **P ≤ 0.0001.
We first seeded the different strains on iron-rich MHA+ or iron-limiting MHA− plates and spotted gallium nitrate on 6 mm paper discs, and then examined subsequent growth of the lawn. The siderophore is normally only expressed under iron limitation, and as expected, Schu S4 showed a zone of growth inhibition around the gallium discs only on MHA− and not on MHA+ plates (Fig. 4B). The ΔfslA and ΔfslE mutants showed no zone of inhibition on either plate confirming that an intact fsl siderophore uptake system is required for gallium toxicity. The fupA and the feoB mutants, in contrast, showed zones of growth inhibition around the paper discs on MHA+ and even larger zones on MHA− plates compared to Schu S4. These results confirmed that siderophore-mediated iron uptake is deregulated in the fupA and feoB mutants and suggested that loss of ferrous iron uptake capability shifts the reliance of the bacterial growth to the siderophore system.

A Schu ΔfeoB′ ΔfslA mutant relies on exogenous siderophore for growth

We generated a ΔfeoB′ ΔfslA mutant to test if Schu S4 possesses iron uptake mechanisms separate from fsl siderophore and feoB encoded systems. As shown in Figure 2C, serial dilutions of the double mutant could grow in the vicinity of, but not distant from Schu S4 on iron-limiting CDM agar (CDM−). However, even in iron-replete conditions (MHA+), the double mutant could only grow adjacent to a siderophore producer (Schu S4 or Schu ΔfeoB′), but not the siderophore-deficient ΔfslA mutant (Fig. 2D, 2E). Complementation with feoB restored its ability to grow as well as the parent strain on MHA+ (Fig. 2E). These data made it clear that virulent Schu S4, like LVS, has only the two iron uptake mechanisms of fsl and feoB for growth in vitro. Since supplementation with purified Francisella rhizobium ferrin was required for routine maintenance of this strain, all further experiments with this strain were conducted with bacteria grown on siderophore-containing MHA+ plates.

feoB and fslA functions support intracellular growth of Schu S4

The Schu S4 iron acquisition mutants were tested for their ability to enter and replicate within cell lines of different lineage, J774A.1 and human liver carcinoma cell line (HepG2) (Fig. 5). In the murine macrophage J774A.1 cell line, the single deletion mutants of Schu S4, ΔfslA, and ΔfeoB′ were able to replicate to numbers comparable to wild type 24 h post infection (Fig. 5A). This implies that both ferrous and ferric iron sources are available to the intracellular pathogen in this cell type. The fslA mutant was able to grow to levels comparable to wild-type Schu S4 also in the human hepatocyte cell line, HepG2 (Fig. 5B). The feoB′ mutant, however, showed a reduced ability to replicate within this cell line suggesting that levels of ferric iron are limiting and that ferrous iron is the more available iron source in this cell line. The Schu S4 ΔfeoB′ ΔfslA mutant was unable to grow in either the J774A.1 or HepG2 strains. This defect for intracellular growth indicates that fsl and feoB code the only iron acquisition systems for growth in the intracellular environment of these cell types.

The Schu ΔfeoB′ ΔfslA mutant is avirulent in mice

A small dose of <10 CFU Schu S4 is sufficient to kill C57BL/6 mice by any route of infection. We infected
mice by the subcutaneous route with approximately 25 CFU of wild-type Schu S4 or the different iron acquisition mutants to evaluate the importance of these systems for virulence. Mice infected with Schu S4 all died between 5 and 7 days postinfection. The single mutants in siderophore acquisition genes (ΔfslA, ΔfslE) showed no reduction in time to death of the infected mice (Fig. 6A). Mice infected with the ΔfeoB′ mutant also died within the same time frame (Fig. 6B). The Schu ΔfeoB′ ΔfslA strain, however, lived beyond 21 days post infection. These surviving mice were subsequently challenged with 25 CFU Schu S4 and monitored for 14 days more. Four of the five mice previously infected with the ΔfeoB′ ΔfslA mutant survived lethal Schu S4 challenge. In an additional experiment, we attempted to calculate the LD₅₀ of the Schu S4 ΔfeoB′ ΔfslA mutant with increasing doses and found that even 25,000 CFU did not kill a mouse. Results with the Schu ΔfeoB′ ΔfslA mutant resemble those seen with the ΔfupA ΔfslE mutant which is also highly attenuated for virulence, and is similarly able to protect mice from subsequent challenge with Schu S4 (Ramakrishnan et al. 2012). Overall, these experiments confirm that no cryptic pathways for iron acquisition are induced and that the fsl and feo pathways satisfy the iron requirements to support full virulence of Schu S4 in mice.

**FupA promotes transport of copper in addition to ferrous iron**

Although 55Fe uptake experiments indicated that fupA and feoB are both involved in ferrous iron uptake, mutants in these genes demonstrated different growth phenotypes; the ΔfupA mutant that is affected in high-affinity ferrous iron uptake had a more severe growth defect on

**Figure 5.** Intracellular growth of Schu S4 and mutants. Tissue culture cells seeded in 24 well plates were infected with bacteria at multiplicity of infection (MOI) as stated below. Bacterial entry at 2 h and replication over a 24 h period were determined by lysis and plating on Mueller–Hinton agar (MHA+) agar to determine bacterial load. Purified *F. tularensis* siderophore was topically applied to MHA+ plates for growth of the ΔfeoB′ ΔfslA mutant. Values are expressed as the CFU means from quadruplicate wells ± standard error of the mean (SEM). Significance calculations were made relative to Schu S4. **P < 0.01, ***P < 0.0005. (A) J774A.1 infected at a MOI 15–25 and (B) Human liver carcinoma cell line (HepG2) infected at a MOI of 80–140.

**Figure 6.** Virulence of Schu S4 and iron acquisition mutants in mice. 6–8 week old C57/BL6 mice were subcutaneously infected with ~25 CFU of the different strains. Actual CFUs delivered were determined by plating on Mueller–Hinton agar (MHA+), with additional siderophore supplementation for growth of the ΔfeoB′ ΔfslA mutant. Groups of 4 mice were used in (A) while 5 mice were used in (B) Survival curves were not significantly different among the single mutants. All mice survived infection with the ΔfeoB′ ΔfslA mutant. Survivors did not succumb when subsequently challenged with 25 CFU Schu S4.
iron-limiting agar than the ΔfeoB′ mutant that has lost all ability to transport ferrous iron (Fig. 2A). Although an insertion mutation in fupA was found to cause membrane destabilization in F. novicida (Nallaparaju et al. 2011), we have not observed such effects in the Schu ΔfupA mutant. We considered the possibility that FupA mediates transport of substrates besides iron and tested the ability of other metals to compete with high-affinity transport of $^{55}\text{Fe}^{2+}$ in Schu S4 (data not shown). The presence of ascorbate in the transport assay buffer ensures that the metals ions stay in the reduced form. In these preliminary experiments, we determined that transport of 0.1 μmol/L $^{55}\text{Fe}^{2+}$ by Schu S4 could be inhibited in the presence of an excess of nonradioactive Fe or Cu salts. To discern the specific role of FupA without potential influence of FslE, we compared transport in Schu S4 and a ΔfupA ΔfslE mutant as well as a mutant complemented with fupA. As expected, the ability to transport $^{55}\text{Fe}$ at 0.1 μmol/L, lost in the double deletion mutant, was regained upon restoration of fupA to the strain (Fig. 7A).

We then compared the ability of a 10-fold excess of metal ions to compete with 0.1 μmol/L $^{55}\text{Fe}^{2+}$ in the fupA complement. $^{55}\text{Fe}^{2+}$ uptake in both Schu S4 and the fupA complement was effectively outcompeted by added Fe and Cu, but not by Zn salts (Fig. 7B). This suggested that FupA might specifically facilitate transport of copper in addition to iron.

Copper at low levels is a micronutrient for bacteria, but an excess leads to destruction of iron sulfur clusters of dehydratases in the cell, and is therefore toxic (Macomber and Imlay 2009). We assessed the sensitivity of the different strains to copper using disc diffusion assays. Consistent with a role for FupA in copper transport, the ΔfupA mutant specifically showed greater resistance to CuCl$_2$ (Fig. 8). Additionally, Schu S4 and the other mutant strains showed a gray coloration at the perimeter of growth suggesting an accumulation of copper sulfide in the cells at the margin of growth. In notable contrast, the ΔfupA mutant had a more diffuse growth boundary and showed no gray coloring. Complementation with the fupA gene restored the sensitivity to Cu and the gray margin phenotype.

These studies suggested that besides ferrous iron transport, FupA in the outer membrane has additional functions such as transport of copper that may be a micronutrient for F. tularensis. FeoB, on the other hand, appears specific for ferrous iron transport.

**Discussion**

In the iron-limiting host environment, high-affinity acquisition systems for iron are an essential feature of pathogenic organisms. While bacteria in general possess multiple redundant mechanisms for iron acquisition, a limited number of highly specialized strategies may suffice to support the lifestyle adapted to specific niches; *Shigella flexneri* for instance was shown to require only three iron uptake systems to support intracellular growth (Runyen-Janecky et al. 2003). Reduction in genome size might be expected to limit the number of encoded uptake systems; our studies suggest that the highly virulent *F. tularensis* with a small genome of 1.89 Mb has adapted to support its lifestyle with just two primary mechanisms – the *fsl* system that transports ferric iron complexed to *Francisella* rhizoferrin and the FupA–FeoB pathway for ferrous iron uptake (Fig. 9).

The *fsl* operon encodes five proteins that are conserved across all the closely related strains of *F. tularensis*, and siderophore preparations from *tularensis* and *novicida* strains are identical in structure based on MS-MS analysis ((Sullivan et al. 2006), data not shown). Our results indicate that the inner-membrane MFS transporter FslB exports the rhizoferrin synthesized by the enzymes FslA and FslC. The outer-membrane protein that facilitates siderophore export has not been identified, but our studies suggest that the gene for the protein must lie outside of the *fsl* locus. Our studies further demonstrated that the pathway for uptake of rhizoferrin-conjugated iron involves the outer-membrane receptor FslE and the MFS protein FslD that functions as an inner-membrane channel. These findings are consistent with the observation that FslD shares sequence similarity with the LbtC siderophore transporter in *Legionella pneumophila* (Chatfield et al.,

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**Figure 7.** High-affinity uptake of $^{55}\text{Fe}^{2+}$ in the presence of competing metal ions. High-affinity ferrous iron transport using 0.1 μmol/L $^{55}\text{Fe}^{2+}$ was studied in Schu S4 and a fupA complemented ΔfslE ΔfupA mutant as described in the legend to Figure 1E. (A) High-affinity transport of ferrous iron by Schu S4 is dependent on FupA. Uptake of 0.1 μmol/L $^{55}\text{Fe}^{2+}$ is only restored to a ΔfslE ΔfupA mutant when it is complemented by a wild-type copy of fupA and not by vector (v) alone. (B) $^{55}\text{Fe}^{2+}$ uptake in the presence of competing metal ions. Reactions contained a 10-fold molar excess of nonradioactive metal ions added as FeCl$_3$, CuCl$_2$, or ZnCl$_2$. Rates of uptake are represented as mean ± standard error of the mean (SEM) of assays done in triplicate. Experiments were repeated at least once more with similar results.
Figure 8. Copper sensitivity of Schu S4 and iron acquisition mutants. Bacteria resuspended in Chamberlin’s defined medium (CDM) to an OD$_{595}$ of 1 were spread on Mueller–Hinton agar (MHA)+. 10 μL of 10 mmol/L copper chloride was spotted on paper discs placed on the seeded plates and the effect on growth was evaluated after 48 h. The diameter of growth inhibition was measured and tabulated as mean ± standard error of the mean (SEM). The images document the phenotype and the gray coloration of the growth margins that was absent in the ΔfupA mutant.

| Strain       | Schu S4 | ΔfslE | ΔfupA | ΔfslB$^\Delta$ | ΔfupA (v) | ΔfupA (ΔfupA) |
|--------------|---------|-------|-------|----------------|------------|---------------|
| Diameter of growth inhibition zone (mm) | 26.87 ± 0.58 | 26.83 ± 0.58 | 20.33 ± 0.29 | 26.83 ± 0.29 | 20.0 ± 0.58 | 20.7 ± 0.29 |

Figure 9. Model for the iron acquisition mechanisms operational in F. tularensis. The fsl operon encodes cytoplasmic siderophore biosynthetic enzymes FslA and FslC, inner-membrane proteins FslB and FslD, and outer-membrane receptor FslE that together effect siderophore-dependent transport of ferric iron. This system is under Fur control and is induced under iron limitation. The ferrous iron uptake system comprises a high-affinity transporter FupA and uncharacterized low-affinity channels in the outer-membrane and the inner-membrane transporter FeoB, whose function may be modulated by FeoA. These two systems work as parallel, independent, and mutually compensatory mechanisms to support growth and virulence of the organism.
Ferric and Ferrous Iron Acquisition in Virulent Francisella Tularensis

We have shown here that the fsl mutant showed limited intracellular growth in a mammalian cell line. The Francisella Tularensis genome additionally encodes a FeoA ortholog that may also contribute to FeoB function (Kim et al. 2012; Weaver et al. 2013). Several lines of evidence demonstrate the limited redundancy of iron acquisition pathways in F. tularensis: (1) fsl genes for ferric-siderophore transport and fupA and feoB for ferrous iron uptake are highly conserved in all F. tularensis strains, but neither pathway is essential. Previous studies as well as the current report show that single mutants in the fupA, feoB, and fsl genes are all viable and virulent, suggesting the existence of compensatory systems to support growth and virulence; the Δfsl mutant demonstrates an increased capacity for ferrous iron uptake and conversely, the siderophore-mediated uptake pathway is deregulated in ΔfupA and ΔfeoB mutants. (2) We previously demonstrated that a mutant lacking the high-affinity outer-membrane transporters FslE and FupA, specific for ferric-siderophore and ferrous iron, respectively, grew slowly even in iron-replete conditions (Ramakrishnan et al. 2012). This mutant showed limited intracellular growth in a macrophage cell line. We have shown here that the ΔfeoB ΔfslA mutant that can neither acquire ferrous iron nor synthesize siderophore has an even more severe defect, being completely dependent for growth on exogenously supplied rhizoferrin. This mutant also was unable to grow within mammalian cells. (3) The previously characterized ΔfupA ΔfslE mutant and the ΔfeoB ΔfslA mutant reported here are both avirulent even when mice were infected with >10,000 × LD₅₀ CFUs. These results suggest that no cryptic iron acquisition system is induced upon entry of F. tularensis into the host environment.

While levels of ferric and ferrous forms of iron may vary among different tissues within the host, the retention of virulence by the fsl and the feoB single mutants demonstrates that both ferric and ferrous sources are utilized by the invading pathogen. Ferric iron is the form of iron stored in ferritin or bound to transferrin and lactoferrin while ferrous iron is thought to be the form that is rapidly mobilized and translocated within the cell (Hider and Kong 2013). The Francisella siderophore structure appears identical to that of rhizoferrin (Sullivan et al. 2006); with a relatively low affinity for ferric iron (pFe = 19.7) (Carrano et al. 1996), it is not predicted to have the capacity to directly chelate away iron that is more tightly bound to host sources such as transferrin (pFe = 23.6) (Turcot et al. 2000). Rhizoferrin is also produced by isolates of diverse bacteria, both environmental and pathogenic (Münzinger et al. 1999; Burnside et al. 2015). This might suggest that the siderophore is not specialized for use in the mammalian environment but targets iron sources commonly available in multiple niches. The ability of F. tularensis to grow on transferrin or heme (Olakanmi et al. 2010; Lindgren et al. 2015) is likely dependent on release of iron from the source by other mechanisms prior to internalization by the fsl and/or the feoB systems. These uptake systems may also be active within tick vectors, where iron availability after a blood meal is predicted to be abundant.

Siderophore uptake across the outer membrane in Gram-negative bacteria is typically energized by the proton motive force and is dependent on the interaction of the TonB–ExbB−ExbD complex with the siderophore receptor. However, the F. tularensis genome does not encode orthologs of the TonB complex. The FslE and FupA transporters share a high level of sequence similarity, but have different substrate specificity; FslE for the Francisella rhizoferrin–iron complex and FupA for ferrous iron and potentially for copper as well. The proteins are also predicted by the PRED-TMBB program (Bagos et al. 2004a,b) to both fold as beta-barrels in the outer membrane with periplasmic plug domains similar to TonB-dependent receptors. The similarity suggests that a common mechanism underlies functioning of the two proteins.

Soluble metal ions like ferrous iron and copper are generally considered to diffuse in through porins in the outer membrane of Gram-negative bacteria. As of now, functional porin proteins have not been identified in F. tularensis. The ability of FupA to function at low concentrations of substrate would suggest that it is involved in an active transport process rather than in general diffusion like the MspA porin of Mycobacterium smegmatis which acts as a low-affinity channel for both iron and copper (Jones and Niederweis 2010; Speer et al. 2013). A TonB-dependent transporter from the cyanobacterium Anabaena with specificity for both iron and copper has been previously reported (Nicolaïsen et al. 2010); this protein was thought to transport iron as citrate, but the mechanism was not definitively characterized. Despite the lack of an identifiable TonB complex in F. tularensis, it is intriguing to consider that these disparate systems may nevertheless share common mechanistic features.

Like iron, copper at high levels is toxic and one strategy of macrophages for controlling internalized pathogens is to mobilize copper into the phagosome (Hodgkinson and Petris 2012). Following entry into the macrophage, F. tularensis passes through the phagosome fairly quickly and is able to suppress innate immune responses, and so it is unlikely to encounter toxic copper levels within the macrophage. In fact, FupA may provide a necessary high-affinity transport function for copper as it does for iron, nutrients that are normally at limiting levels in the host environment.

The Francisella genus is diverse and encompasses human and fish pathogens, tick endosymbionts and...
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Methods

Bacterial strains and media

*Francisella tularensis* subspecies *tularensis*, Schu S4 (from the Centers for Disease Control and Prevention, Fort Collins, CO), and mutant derivatives were maintained on modified MHA supplemented with horse serum, cysteine, and defined amounts of iron salts including ferrous sulfate (FeSO$_4$) and ferric pyrophosphate (FePPi). *F. tularensis* strains were grown in liquid CDM (Chamberlain 1965) or in tryptic soy broth supplemented with 0.1% cysteine (TSB/c) at 37°C with shaking. Bacterial culture optical densities were determined at 595 nm (OD$_{595}$) using a microplate reader (iMark, Bio-Rad Laboratories, Hercules, CA, USA). For growth determinations in defined media, bacterial cultures were grown initially in iron-replete CDM overnight. Cultures were then washed in chelate treated CDM three times and inoculated into che-CDM supplemented with known quantities of FePPi (2.5 and 0.125 mg/L) and FeSO$_4$ (2 mg/L and 0.2 mg/L). Bacteria in the exponential growth stage were inoculated to an OD$_{595}$ of 0.01 in the respective growth media and grown at 37°C with shaking for 24 h. The Schu ΔfeoB ΔfslA mutant was maintained on MHA agar supplemented with siderophore purified from LVS (as described in Pérez and Ramakrishnan 2014).

Generation of Schu S4 iron acquisition mutants and complementation

Marker less deletion mutants were generated by a two-step process using suicide plasmids with 5’ and 3’ flanking sequences as previously described (Sullivan et al. 2006; Ramakrishnan et al. 2008). ΔfslA and ΔfeoB' mutants in the Schu S4 background were obtained as described previously for LVS (Sullivan et al. 2006; Pérez and Ramakrishnan 2014). Generation of the ΔfslF mutant and deletion of the entire fslABCDEF locus (Δfsl) in Schu S4 and LVS backgrounds was similarly accomplished using primers listed in (Table S1). The suicide plasmids were introduced into Schu S4 or LVS by electroporation and integrants were selected on MHA+ containing 15 μg/mL kanamycin. Mutants were obtained by selection on sucrose containing MHA+, screened for loss of the kanamycin resistance marker and confirmed by PCR. The Schu ΔfslA ΔfeoB' strain required supplementation with purified *F. tularensis* siderophore for growth, as previously seen with the LVS ΔfslA ΔfeoB' mutant (Pérez and Ramakrishnan 2014).

Complementation

Plasmids for complementation in cis or in trans were introduced by electroporation and selected on media containing kanamycin

*pfEOB*

Both the ΔfeoB' and double deletion mutants were complemented in cis with a wild-type copy of *feoB* present on an integrating suicide plasmid, p463_feoB previously described for the complementation of the LVS *feoB* mutants (Pérez and Ramakrishnan 2014). Complemented mutants were selected with kanamycin and confirmed by PCR from DNA purified from the bacteria.

*pfsIA, pfsIAc, and pfsIABC*

The LVS Δfsl mutant was complemented in trans to assess *fsl* genes required for siderophore production.
Complementation plasmids were derived from the plasmid pGIR458 (Sullivan et al. 2006) that contains a wild-type copy of Schu S4 fslA with its native promoter. To generate pfslAC, the fslC gene was amplified from Schu S4 genomic DNA with primers 5′-ctactggagctcTTAAATCTACATTTTTTTTATTAG 3′ and 5′-ctactggagctcTTATGATGTGGCTAACTC 3′ and then cloned downstream of fslA in pGIR458 at SacI and BamHI restriction enzyme sites. The plasmid pfslAB was generated by the amplification of fslB containing sequences with primers 5′-ctactggagctcTTAAATCTACATTTTTTTTATTAG 3′ and 5′-ctactggagctcTTATGATGTGGCTAACTC 3′ and cloning into pGIR458 at the NotI and SacI restriction enzyme sites. The plasmid pfslABC was generated with the amplification of fslABC with primers 5′-ctactggagctcTTAAATCTACATTTTTTTTATTAG 3′ and 5′-ctactggagctcTTATGATGTGGCTAACTC 3′ and ligation into pGIR458 at NotI and BamHI restriction enzyme sites. The parent plasmid pFNLTPE (V) (Maier et al. 2004) was used as a negative control in our studies.

**psfIE, psfIDE, and psfIDEF**

The Schu Δfsl mutant was complemented in cis to identify fsl genes involved in siderophore-mediated iron uptake. The psfIE plasmid pGIR474 that has the fslE gene under control of its native fslA promoter in the integrative plasmid pGIR458 has been previously described (Sen et al. 2010). The forward primer 5′-ctactgtccggagctcTTATGATGTGGCTAACTC 3′ with reverse primers 5′-ctactggagctcTTATGATGTGGCTAACTC 3′ and 5′-ctactggagctcTTATGATGTGGCTAACTC 3′ were used to amplify fslDE and fslDEF sequences, respectively, from Schu S4 chromosomal DNA. The sequences were cloned under control of the fslA promoter in pGIR459 to generate pfslDE and pfslDEF, respectively. The parent plasmid pGIR459 was used as vector control for complementation studies.

**F. tularensis siderophore detection with the Chrome Azurol S (CAS) assay**

As previously described in Pérez and Ramakrishnan (2014), an adaptation of the liquid CAS assay was used to determine the presence of the *F. tularensis* siderophore under iron limitation. Bacterial cultures were grown in iron replete or iron-limiting FePi (2.5 and 0.125 μg/mL) supplemented che-CDM overnight at 37°C with shaking. Cultures were centrifuged at 9,000g for 5 min and supernatants were collected and added to equal parts CAS solution (100 μL) and 2 μL of the shuttle solution in a 96-well plate. The CAS activity was normalized to cell density

**Growth on agar plates**

To assess the growth of Schu S4 and Schu S4 iron acquisition mutants on agar, overnight cultures were washed in che-CDM and 5 μL of 10-fold serially diluted suspensions were spotted on modified Muller Hinton agar plates supplemented with FePi or FeSO4 or on CDM plates without iron.

**Disc diffusion test**

Overnight cultures grown to logarithmic phase in TSB/c were resuspended to OD 1 in CDM. The cells were uniformly plated on MHA using 100 μL of each of the OD 1 cultures. The plates were dried at room temperature for 15 min. Sterile 6-mm paper discs were placed on the plates and 5 μL of the test solutions were spotted. All experiments were carried out in triplicate. After drying, the plates were incubated at 37°C for 2 days. The effect on growth was evaluated by measuring the diameter of the inhibition zone.

**55Fe uptake assays**

55Fe uptake with 55FeCl3 (PerkinElmer Life Sciences; 21.95 mCi/mg, 38.59 mCi/mL) was studied using previously described protocols (Ramakrishnan et al. 2012; Ramakrishnan and Sen 2014). Prior to 55Fe uptake assays, bacterial strains (except for the ΔfeoB ΔfslA mutant) were grown overnight in low iron liquid che-CDM at 37°C with shaking. For experiments involving the ΔfeoB ΔfslA mutant, the double mutant bacteria were scraped off after growth on MHA+ plates supplemented with siderophore on the day of the experiment and incubated in che-CDM without iron for 3 h at 37°C with shaking. Control cultures grown in low iron che-CDM were washed with fresh che-CDM and similarly incubated in che-CDM. Bacterial cells were brought to an OD595 of 0.2 in che-CDM, and 0.1 mL of each bacterial suspension was added to wells of 96-well filter plates (Millipore, Billerica, MA, USA) containing 90 μL of che-CDM. After a 10-min incubation at 37°C, uptake assays were initiated by the addition of 10 μL of 55Fe labeling mix to the bacteria in the filter wells. 55Fe3+ siderophore uptake reactions were carried out in the presence of 10 mmol/L citrate. Siderophore complexes were prepared by incubating 30 μmol/L 55FeCl3 with 100 μmol/L siderophore for 30–60 min at room temperature. The uptake reaction contained 1.5 μmol/L 55Fe in complex with siderophore. For ferrous iron uptake studies, the final transport assay contained 55FeCl3 at concentrations of 0.1 μmol/L (high-affinity transport) or 3 μmol/L (low-affinity uptake) in the presence of 5 mmol/L ascorbate to keep the iron reduced.
55Fe accumulation was assessed at 5 and 10 min by scintillation counting of filtered cells. Bacterial protein concentration was analyzed by the Pierce BCA assay (Thermo-Fisher Scientific, Middletown, VA, USA). All strains were further tested in either triplicate or quadruplicate and rates of transport were normalized to protein concentration (pmol/min/mg).

**Intracellular replication**

Intracellular replication of Schu S4 and mutants was assessed in murine macrophage-like cells J774A.1 (ATCC TIB-67) as previously described (Sen et al. 2010; Pérez and Ramakrishnan 2014). J774A.1 cells were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS at 37°C with 5% CO2 and split 1:10 per passage. Cells were counted on an automated cell counter (TC10, Bio-Rad Corporation, Hercules, CA, USA) and seeded at a concentration of 2 x 10^5 cells per well in 24-well plates the day before the assay. Bacteria were added at a multiplicity of infection (MOI) of 10 into four wells per group. HepG2 cells also used to assess replication were maintained in DMEM supplemented with 10% FBS, 5% glutamate, and grown at 37°C with 5% CO2 and split 1:10 per passage. Cells were seeded at a concentration of 2 x 10^5 per well in a 24-well plate the day before the assay. Bacteria were added at an MOI of 100 into four wells per group.

**Mouse infection studies**

All animal protocols were approved by the Animal Care and Use Committee (ACUC) of the University of Virginia, and the vivarium is accredited by the Association for Assessment Accreditation of Laboratory Animal Care International. Mice were anesthetized with a cocktail of ketamine-HCl-xylazine. Previously titered frozen bacterial cultures were thawed to room temperature and diluted in 0.9% sterile saline solution to 250 CFU/mL and 10 μL (25 CFU) aliquots were subcutaneously (S.C) injected into 4–6 week old C57BL/6 male mice (five mice per group) (Jackson laboratories, Bar Harbor, ME) (Ramakrishnan et al. 2012). CFUs were determined after plating bacterial dilutions on MHA plates. MHA topically supplemented with F. tularensis siderophore was used to determine CFUs of the double deletion mutant. Clinical scores were determined for mice over the course of infection and mice were euthanized at a humane endpoint if symptoms of irreversible morbidity were observed. Survivors were subsequently challenged by S.C. delivery of 25 CFU of wild-type Schu S4 and monitored over 21 days.

LD<sub>50</sub> studies: Three groups of male C57BL/6 (4–6 weeks old) mice were S.C. injected with 0.1-mL saline solution containing either 14,700 CFU, 3000 CFU, or 173 CFU of the ΔfeoBΔfpsA mutant. CFUs were determined by plating bacterial dilutions on MHA plates supplemented with F. tularensis siderophore. Mice were monitored for 21 days and then challenged with 33 CFU of wild-type Schu S4. CFUs were determined by plating on MHA plates.

**Statistical analysis**

Prism 4.0 (GraphPad Software, Inc., San Diego, CA) was used for analysis of data. Statistical comparison of values was accomplished using t test function and the log-rank test function was used to evaluate mouse survival curves.

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**Conflict of Interest**

None declared.

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Supporting Information
Additional supporting information may be found in the online version of this article:
Table S1. Primers used in the study.