Paralogous Outer Membrane Proteins Mediate Uptake of Different Forms of Iron and Synergistically Govern Virulence in *Francisella tularensis tularensis* *

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*Running Title: Iron transport by Francisella outer membrane proteins*

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Background: FslE and FupA are *Francisella*-specific paralogous proteins involved in iron acquisition.

Results: *fslE* mutation disrupts siderophore-mediated ferric iron uptake, *fupA* mutation impairs high affinity ferrous iron uptake and both mutations impact virulence.

Conclusions: Optimal iron acquisition and virulence require both paralogs.

Significance: Iron acquisition mechanisms are potential targets for preventive or therapeutic intervention in *F. tularensis* infections.

SUMMARY

*Francisella tularensis* subsp. *tularensis* is a highly infectious bacterium causing acute disease in mammalian hosts. Mechanisms for the acquisition of iron within the iron-limiting host environment are likely to be critical for survival of this intracellular pathogen. FslE (FTT0025) and FupA (FTT0918) are paralogous proteins that are predicted to form beta-barrels in the outer membrane of virulent strain Schu S4 and are unique to *Francisella* species. Previous studies have implicated both FupA, initially identified as a virulence factor and FslE, encoded by the siderophore biosynthetic operon, in iron acquisition. Using single and double mutants, we demonstrated that these paralogs function in concert to promote growth under iron limitation. We used a ⁵⁵Fe transport assay to demonstrate that FslE is involved in siderophore-mediated ferric iron uptake while FupA facilitates high-affinity ferrous iron uptake. Optimal intracellular replication in J774A.1 macrophage-like cells required at least one of these uptake systems to be functional. In a mouse model of tularemia, the Δ*fupA* mutant was attenuated, but the Δ*fslE* Δ*fupA* mutant was significantly more attenuated, implying that the two systems of iron acquisition function synergistically to promote virulence. These studies highlight the importance of specific iron acquisition functions, and particularly that of ferrous iron, for virulence of *F. tularensis* in the mammalian host.

INTRODUCTION

*Francisella tularensis* is an aerobic Gram-negative proteobacterium and the etiological agent of the disease tularemia (1). Different subspecies of the organism are associated with varying severity of disease, with the *tularensis* subspecies responsible for the most severe Type A disease (2,3). Arthropod vectors are commonly involved in zoonotic transmission of infection, although infection can also be acquired by aerosol and oral routes. Many mammals are susceptible to infection, and the mouse is a commonly used model to study the disease (4,5). The bacteria are intracellular pathogens and can replicate within
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several types of host cells (6-9). Studies with infected macrophages and macrophage-like cell lines have revealed that after phagocytosis the bacteria first reside in phagosomes, but then escape and replicate in the cytoplasm (10,11).

As with any pathogen, \textit{F. tularensis} derives several nutrients from its host. Iron is an essential nutrient, but being largely sequestered within the host, is particularly challenging to obtain. Microbial pathogens typically possess several redundant mechanisms to acquire iron in this limiting environment, and these can be critical for optimal virulence of the pathogen (12,13). Secretion of siderophores to chelate ferric iron from the environment for subsequent uptake is a common iron acquisition strategy for many microorganisms. We and others have demonstrated that strains of \textit{F. tularensis}, and the closely related \textit{F. novicida}, produce a polycarboxylate siderophore under iron limitation (14,15). Resembling rhizoferrin in structure, the siderophore appears to be identical in the \textit{F. tularensis} \textit{holarctica} Live Vaccine Strain (LVS), the \textit{F. tularensis} \textit{tularensis} strain Schu S4, and the \textit{F. novicida} strain U112 and are interchangeable in growth assays (16), (data not shown). Siderophore production is governed by genes encoded by the \textit{fsl} operon and, as in many bacteria, is under control of the Ferric Uptake regulator (Fur) (14-17).

The product of the \textit{fslE} gene (FTT0025) was identified as the likely siderophore receptor in the Type A strain Schu S4 as well as in \textit{F. novicida} U112 based on cross-feeding assays on iron limiting agar (16,18). Gram-negative bacteria rely on a largely ubiquitous inner membrane complex of proteins: the TonB-ExbB-ExbD complex to facilitate receptor-mediated transport of siderophore-iron complexes across the outer membrane (19). \textit{F. tularensis} is among a small group of bacteria with genomes that do not encode an obvious TonB complex and the first for which a receptor (FslE) was identified (16). Characterization of siderophore uptake facilitated by FslE therefore is of particular interest as it represents a new paradigm.

Studies to date suggest that the siderophore-mediated iron uptake impacts virulence to different extents depending on the \textit{Francisella} strain and/or infection model used.

The siderophore biosynthetic gene \textit{fslA} gene was found to be important for virulence of LVS in a mouse model of respiratory tularemia, but not for Schu S4 virulence in an intradermal model of infection (20,21). Genes in the \textit{fsl} operon of \textit{F. novicida} are required for virulence in mice and arthropod vectors as well as for intracellular replication (22-25).

FslE is one of a family of five sequence-related proteins encoded in the Schu S4 genome (26). The FslE paralogs are unique to \textit{Francisella} species although they show some variation among the different strains. The paralog with the greatest similarity, FupA was initially characterized as a virulence factor in Schu S4 based on attenuation of a \textit{fupA} mutant in a mouse model of tularemia (27). \textit{fupA} was found to be important for intracellular replication of Schu S4 as well as \textit{F. novicida} (23,27), and the related \textit{fupA/B} hybrid protein in LVS has also been linked to virulence in mice (28).

Growth assays and transcriptional analysis of a \textit{fupA} mutant linked this FslE paralog with iron metabolism and iron acquisition in Schu S4 although its exact function was not established (21). Here we have sought to define the primary roles played by FslE and FupA in iron acquisition in Schu S4 and to determine their relative importance for virulence. We used single and double deletion mutants of \textit{fslE} and \textit{fupA} to show that both paralogs contribute to optimal growth in vitro. We used $^{55}$Fe transport assays to show that FslE functions in the siderophore-mediated uptake process and that FupA mediates high affinity ferrous iron uptake. Our results indicate that while FupA functions as the principal virulence determinant, both are required for full virulence in a mouse model of tularemia.

**EXPERIMENTAL PROCEDURES**

Bacterial strains and culture- \textit{F. tularensis} \textit{tularensis} strain Schu S4 (obtained from the CDC, Fort Collins, CO) and derivatives were maintained on Mueller-Hinton agar supplemented with serum, cysteine and iron salts (MHA). Chamberlain's defined medium (CDM) (29) was used for routine liquid culture. Iron limiting agar plates were prepared using CDM without addition of FeSO$_4$ (CDM-Fe). Chelex-100 (BioRad) treated CDM (che-CDM) was supplemented with 0.55 mM...
MgSO₄ and 5 µM CaCl₂ and defined levels of ferric pyrophosphate (FePPi) were added as noted. C-CDM was prepared similarly, but was additionally supplemented with 1.5 µM ZnCl₂, 0.2 µM CuSO₄ and 1 µM MnCl₂ (14, 21). *Escherichia coli* strain MC1061.1 (*araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL (Strr) hsdR2 (rK- mK+)) mcrA mcrB1 recA) was used for routine cloning and was grown in Luria broth. Strain S17-1 (RP4-2-TC::Mu-Km: ΔTn7, Pro, res-, mod+) was used in conjugation experiments and BL21(DE3) was used for expression of recombinant protein. Ampicillin for selection of *E. coli* transformants was used at a concentration of 50 µg/ml (for liquid culture) or at 100 µg/ml (agar plates). Kanamycin was used to select for plasmid integrants in *F. tularensis* at 15 µg/ml concentration. Polymyxin B was used at 100 µg/mL to select against *E. coli* in conjugation experiments.

**Construction of ΔfupA and ΔfupB mutants and complementing plasmids** - Deletion mutants of *fupA* and *fupB* were generated using a sacB plasmid as previously detailed (16). A 1.977 kb region upstream of and including the first 10 codons was amplified with primers 243 and 244 (Table S1) using FastStart High Fidelity DNA polymerase (Roche). The 3' flanking sequences included the last 13 codons and 1.958 additional kb downstream and were amplified with primers 245 and 246. For the *fupB* deletion construct, the 1.713 kb of DNA starting 14 bp upstream of the start codon was amplified with primers 256 and 257 to generate the 5' flanking sequence. An insertion element IS7TU1 is located 65 bp downstream of the *fupB* stop codon. As in our previous work, we avoided use of the transposon sequences in the 3' flanking sequences (28), and instead, amplified the 1.532 kb sequence located downstream of the insertion element using primers 360 and 361. The 5' and 3' flanking sequences were cloned in suicide vector pGIR463 to generate plasmids pGIR476 (for *fupA* deletion) and pBAS4 (for *fupB* deletion). The 5' flanking sequences used in pGIR476 were combined with the 3' flanking sequences from pBAS4 to generate the Δ*fupA* Δ*fupB* deletion construct pBAS5. pGIR476 was introduced into Schu S4 and the Schu ΔfslE strain GR211 by electroporation, while pBAS4 and pBAS5 were electroporated into Schu S4. PCR analysis of genomic DNA was used to identify deletion mutants isolated from sucrose plates. PCR products from deletion mutants GR218 (Schu Δ*fupA*), GR219 (Schu ΔfslE Δ*fupA*), GR242 (Schu Δ*fupA* Δ*fupB*) and GR243 (Schu Δ*fupB*) were sequenced to verify the deletions. The mutation in *fupA* resulted in an in-frame deletion of the central 524 amino-acid coding region of *fupA*. The *fupB* mutant had a complete deletion of the gene including 14 nucleotides upstream.

Complementation in *cis* was achieved by integration of a suicide plasmid targeted to the 3' region of *feoB* as previously detailed (28). The integrative plasmids had the promoter of *fslA* driving expression of the different genes. Plasmid pGIR474 expressing *fslA* has been described previously (28). For construction of the *fupA* complementing plasmid pGIR477, the *fupA* sequences were amplified with primers 256 and 257 using FastStart High Fidelity polymerase (Roche). The plasmids were introduced into the different bacterial strains by electroporation or by conjugation from *E. coli* S17-1. Integrants were confirmed by PCR analysis of genomic DNA.

**Growth in iron-replete and iron-limiting media** - Bacteria were grown in che-CDM containing 2.5 µg/mL (3.36 µM, iron-replete) or 0.125 µg/mL (0.168 µM, iron-limiting) FePPi for analysis of growth under iron limitation as previously described (28). In specific experiments as noted, FeSO₄ was added at 2 µg/mL or 0.2 µg/mL to generate iron-replete and iron-limiting media, respectively. Bacteria from overnight cultures in CDM were washed and inoculated into iron-replete or iron-limiting media to an optical density (A₅₉₅) of 0.01 and grown at 37°C with shaking. Growth was monitored as increase in optical density. For depletion of intracellular iron pools, the CDM-grown bacteria were washed and allowed to grow for seven hours in che-CDM or for eleven hours in C-CDM containing no iron. For testing growth on iron-limiting agar, washed bacteria from overnight cultures in CDM were normalized to an optical density of 1.0, diluted ten-fold serially in che-CDM and 5 µL of the different dilutions were spotted on the CDM-Fe agar plates. Specific plates contained the standard supplement FeSO₄ (7.19 µM) or FePPi (6.8 µM). The plates were incubated at 37°C, and growth...
recorded over a period of 4 days. Growth assays were repeated at least twice.

**Generation of FupA antibody** - The DNA sequences corresponding to amino acid 25 through 176 of FupA were amplified from Schu S4 genomic DNA using primers 332 and 333 (Table S1) by PCR with FastStart High Fidelity polymerase (Roche) and cloned in the expression vector pGST-parallel (30). Expression of protein in BL21(DE3) cells was induced overnight at room temperature with 5mM IPTG and the GST-FupA’ protein was purified by affinity purification on glutathione-agarose (Thermo-Fisher). Recombinant protein was used to raise antibody in guinea-pigs (Covance, Denver, PA).

**Western blotting** - Cultures of *F. tularensis* in iron-replete or iron-limiting media were normalized to cell density and lysates were separated by SDS PAGE on 10% gels. Proteins were transferred to PVDF and incubated with different primary and HRP-conjugated secondary antibodies for detection by chemiluminescence. The primary antibodies were rabbit antiserum raised to a FslE peptide (28) used at a 1:2500 dilution, rat antiserum raised to FupB (FTT0919, a kind gift from Michael Norgard) used at 1:1000 dilution, guinea-pig antibody to FupA used at 1:100,000 dilution and rabbit antibody to GroEL (Sigma) used at 1:10,000 dilution.

**55Fe uptake assays** - 

$^{55}$FeCl$_3$ (Perkin-Elmer, 25.04 mCi/mg, 36.88 mCi/mL) was diluted as required for use in transport assays carried out in 96-well filter plates (Millipore). Bacteria were cultured for 18-24 hours in iron-limiting medium and cell pellets were washed once in che-CDM to remove any iron and siderophore remaining from the culture supernatants. Bacteria were then resuspended in cold uptake medium to an optical density ($A_{595}$) of 0.2 and stored on ice for no longer than one hour. The wells of the filter plate were rinsed once with milli-Q water and 100 µL bacteria were aliquoted into wells along with 90 µL uptake medium. The plate was incubated on a heat block at 37°C for ten minutes to pre-warm the cells. Uptake reactions were initiated by addition of 10 µL of the $^{55}$Fe labeling mix at different times, and the bacteria collected and washed by vacuum filtration at 12” Hg pressure. All experiments used samples in triplicate or quadruplicate. Ten µL of 0.1% SDS was added into each well and the plates warmed to 56°C for one hour to kill bacteria and to dry the plates. Individual wells were punched out into scintillation vials and counted after 3-4 hours at room temperature in 4 mL of scintillation fluid (Ecoscint A, National Diagnostics). A media control with no bacteria was used to obtain a value for background correction in some experiments. Bacteria in wells were used in a BCA assay (Pierce) after extraction of proteins with 0.1%SDS in order to normalize results to protein in place of cell density for some of the experiments.

Siderophore mediated uptake was carried out in che-CDM containing 10 mM sodium citrate. Siderophore was purified from LVS culture supernatants by anion-exchange chromatography on AG1X-8 columns as previously described (14). Siderophore concentrations were assessed by a Cu-CAS assay (31) in relation to deferroxamine standards. A labeling mix was prepared by incubating 30 µM $^{55}$Fe with an excess of siderophore (100 µM) in uptake medium at room temperature for 30-60 minutes before use in the assay. 10 µL of mix were added to 190 µL bacteria in wells so as to have a final concentration of 1.5 µM $^{55}$Fe and 5 µM siderophore in the uptake reaction.

For ferrous iron uptake, $^{55}$FeCl$_3$ was diluted in 0.1 M ascorbate in order to keep the iron in the reduced form. 10 µL of $^{55}$Fe in ascorbate at varying concentrations was added to 190 µL bacteria in che-CDM to attain the desired final concentration of iron with 5 mM ascorbate in the uptake reaction.

For assessment of uptake rates, incorporation of label by bacteria at time points 4 or 5 minutes apart early in the uptake process was determined. The rate was calculated by normalizing the incorporation to time and to optical density ($A_{595}$) or to protein content. Kinetic parameters of transport were determined by non-linear regression analysis and were plotted using Prism4.0 software (GraphPad Software, Inc., San Diego, CA).

**Intracellular invasion and replication** - The ability of bacteria to replicate intracellularly was assessed as previously detailed (28). Mouse macrophage-like J774.1 cells (ATCC TIB-67) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco/Invitrogen) at 37°C with 5%
CO₂. The cells were seeded in 24-well plates at a density of 2X10^5 cells/well one day prior to infection. F. tularensis bacteria grown to mid-exponential phase in CDM were used to infect the J774A.1 cells at a multiplicity of infection (MOI) of 10. The numbers of bacteria present in the inocula were verified by plating on MHA. After one hour at 37°C, the medium was replaced with fresh medium containing gentamycin (100 µg/mL) and the cells were incubated for one hour more. The wells were washed with DMEM lacking FBS and replenished with fresh DMEM+FBS. Cells in sets of 4 wells were lysed with 1% saponin at 2, 9.5 and 23 hours post-infection and the lysates were serially diluted and plated on MHA plates to determine the titer in colony forming units (CFU). The results were analyzed using the t-test function of Excel 2003 (Microsoft). Experiments were performed three separate times with similar results.

Analysis of virulence in mice- Six to eight week old C57BL/6 mice (Jackson Labs, Bar Harbor, ME) were infected with Schu S4 and mutants using aliquots of frozen stocks grown in CDM and titered prior to use in infection. Bacteria were diluted in phosphate-buffered saline (PBS) and delivered by subcutaneous injection behind the neck in a volume of 100 µl. The actual numbers of bacteria delivered were verified by plating on MHA. Animals were monitored daily and those demonstrating signs of irreversible morbidity were humanely euthanized. Mice surviving the initial challenge were re-challenged with Schu S4 at doses indicated. All mouse infection experiments were conducted with the approval of the Animal Care and Use Committee of the University of Virginia.

RESULTS

Generation of Schu ∆fupA and Schu ∆fslE ∆fupA mutants- Our studies using a Schu ∆fslE mutant in cross-feeding assays on iron-limiting CDM agar suggested that FslE functions as a receptor in siderophore-mediated iron uptake (16). Using a similar growth assay on agar, Lindgren et al. concluded that FupA is also involved in siderophore-dependent growth in Schu S4, but phenotypic differences with a siderophore-deficient mutant suggested an additional siderophore-independent role for FupA in iron acquisition. (21). To help identify the primary contributions of the two paralogs to iron uptake processes in Schu S4, we generated an in-frame deletion mutant in fupA and a strain with both fslE and fupA deleted.

Whole cell lysates from these strains grown in iron-limiting or iron-replete media were used in Western blotting to test for expression of the proteins. The predicted 56 kDa band corresponding to FupA was present in Schu S4 lysates, while the ∆fupA mutant was missing this band (Fig. 1A, 1B). FupA expression was not regulated in Schu S4 by iron levels in the growth medium, consistent with transcriptional data reported previously (21). Deletion of fslE or of the fur gene had no effect on FupA levels (Fig. 1A).

Lindgren et al reported that the fupA mutant had increased siderophore production correlating with transcriptional deregulation of the fsl operon under iron-replete conditions (21). Our ∆fupA and ∆fslE ∆fupA mutant strains also had significant siderophore production following growth in iron-replete liquid medium (data not shown). We examined FslE levels in cell lysates by western blotting (Fig. 1 B). As expected, a 50 kDa band corresponding to FslE was present in lysates of the parent Schu S4 strain grown under iron limitation but not under iron-replete conditions. The ∆fupA mutant had elevated levels of FslE, with detectable levels even after growth in iron-replete medium. The chaperone GroEL was used as a loading control for the lysates. The protein levels of FslE correlate with the reported transcriptional deregulation of the fsl operon. Our results confirmed that unlike FslE, protein levels of FupA are not influenced by iron availability. Deletion of FupA resulted in increased expression of FslE even under iron-replete growth conditions. This suggested that FupA function was constitutively required and that in its absence, FslE over-expression was a compensatory mechanism.

FslE and FupA independently contribute to growth under iron limitation- Lindgren et al reported that deletion of fupA affects growth in defined liquid medium with limiting ferrous iron, particularly after iron-starvation (21). We therefore tested our ∆fupA strain for a growth deficiency in che-CDM similarly supplemented with FeSO₄. The cells were first cultured in iron-rich CDM and then allowed to grow in che-CDM
with no added iron in order to deplete internal iron pools. When these starved cells were inoculated into media with FeSO₄ supplementation, growth in iron-replete and in iron-limiting medium was significantly reduced relative to Schu S4 (Fig. 2A). We employ ferric salts such as FePPi to study siderophore-mediated iron acquisition since the siderophore is specific for ferric iron. We therefore tested growth of iron-starved strains in che-CDM supplemented with limiting or sufficient levels of FePPi and found that the ΔfupA strain was not deficient in these media, but even consistently exceeded growth of Schu S4 under iron limitation (Fig. 2A). The results presented in Fig 2A suggested that the fupA mutant had a defect associated specifically with ferrous iron utilization.

In the Lindgren et al study, deletion of fupA was found to affect growth even in iron-replete medium (2 μg/mL FeSO₄) if the bacteria had previously been iron-starved (21). The C-CDM medium used in the above study contained additional micronutrient supplements of zinc, copper and manganese. Although we used these supplements when we first started work on this organism (14), we subsequently determined that they are not required for growth by Schu S4 and therefore no longer routinely include them in our media. We tested the fupA mutant in C-CDM and consistent with the published results, found that growth was affected in ferrous iron supplemented medium regardless of iron levels (Fig. 2B). However, as shown in Fig. 2B, we found that growth in ferric iron was also affected in this medium. Thus the ΔfupA strain appeared to lose viability when iron-starved in medium containing these additional metal ions.

Schu S4 and the single mutants showed similar growth kinetics when inoculated without prior iron-starvation into che-CDM made iron-replete with FePPi, (Fig. 2C). Under ferric iron limiting conditions, the ΔfslE mutant showed a significant growth defect as expected, but the ΔfupA mutant grew as well or better than the parent Schu S4 strain. The double mutant had a slower growth rate and grew to a lower density than the single mutants even in iron-replete media. When iron levels were limiting, the attenuation in growth of the double mutant was greater than that of the ΔfslE mutant. These results indicated that FslE played a primary role in iron acquisition under ferric iron limitation, and that FupA influenced the process in some manner.

We next assessed growth of the mutants on agar. Serial dilutions of washed bacteria were spotted onto iron-limiting CDM (CDM-Fe) agar and compared to growth on the rich medium MHA. The parent Schu S4 strain grew out to several dilutions on the CDM-Fe plate (Fig. 2D). The ΔfslE mutant showed barely any growth, as previously reported (16); we observed growth on the outline of the spot at the highest density, and a faint shadowy growth with increasing dilution that was qualitatively very different from the dense growth seen with Schu S4. The ΔfupA mutant had a different attenuated phenotype than the ΔfslE mutant, with heavy growth similar to Schu S4 at the highest density spots, and no growth at all with increasing dilution. The ΔfslE ΔfupA mutant showed the most severe growth phenotype with no hint of growth at even the highest density spot. The different growth phenotypes of the ΔfslE and ΔfupA mutants suggested that the paralogs function in different capacities in iron acquisition.

Complementation of the fslE and fupA mutants - We sought to better clarify the roles of the two paralogs by reintroducing the fslE gene or the fupA gene individually back into the chromosome of the different mutants. In these strains, the introduced gene was placed under control of the fslA promoter on a suicide plasmid, rendering expression iron responsive. The expression of the paralogs in the singly complemented ΔfslE ΔfupA mutant is shown in Fig. 1C. Although iron regulated, these constructs expressed detectable levels of protein even in iron-replete medium, and the proteins accumulated to significant levels after prolonged growth.

We found that each of the single mutants could be fully rescued for growth on iron limiting agar by the corresponding wild type gene (Table 1), indicating that the defective growth phenotype was a result of the specific mutation. Furthermore, we found that fslE expression in cis partially rescued growth of the ΔfupA as well as the ΔfupA ΔfslE mutants, but restoration of fupA expression in cis could not rescue growth of the ΔfslE or the ΔfslE ΔfupA double mutant on these plates. These results were consistent with a role for both fslE
and fupA in growth on iron-limiting agar, with FslE appearing to be more important.

Siderophore-mediated $^{55}$Fe uptake—We developed a filter plate assay that could permit us to follow bacterial siderophore-mediated uptake of ferric $^{55}$Fe using siderophore purified from LVS. Schu S4 bacteria grown either in iron-limiting or iron-replete CDM were washed in che-CDM to remove secreted siderophore. They were then incubated for varying periods of time with $^{55}$Fe in complex with purified siderophore in the presence of citrate and the $^{55}$Fe taken up by cells was quantified by scintillation counting. As shown in Fig 3A, a steady increase in accumulation of $^{55}$Fe by Schu S4 occurred in bacteria grown under iron limitation whereas cells grown in iron-replete media showed little uptake. This is consistent with the derepression of iron uptake genes in response to iron limitation. The accumulation of $^{55}$Fe was dependent on the added siderophore; in the absence of purified siderophore, a far lower level of $^{55}$Fe was taken up by cells (Fig. 3B). This basal uptake was likely mediated by residual endogenous siderophore produced by the bacteria as a siderophore-deficient ΔfslA mutant showed even lower siderophore-independent uptake of $^{55}$Fe in this assay (data not shown). Siderophore-mediated $^{55}$Fe uptake was inhibited by 10 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), demonstrating that this was an active process dependent on the proton motive force (PMF). Reactions incubated at 4°C also showed poor $^{55}$Fe transport. These assays demonstrated that even though F. tularensis lacks a TonB homolog, the siderophore uptake process in the organism is energy-dependent as in other Gram-negative bacteria (32).

We tested the fslE and fupA mutants for siderophore-mediated $^{55}$Fe uptake. The kinetics of uptake are shown in Fig. 3C and the deduced rates of uptake are presented in Fig. 3D. The ΔfslE mutant showed no appreciable accumulation of $^{55}$Fe, consistent with a key role for FslE as receptor mediating siderophore-mediated iron uptake (Fig. 3C). Unexpectedly, the ΔfupA strain was extremely efficient at siderophore-mediated uptake of $^{55}$Fe, with a significantly higher rate of uptake than the parent strain Schu S4 (Fig. 3C). In the ΔfupA ΔfslE mutant however, the siderophore uptake was negligible, similar to the single fslE mutant. These results indicated that FslE is essential for siderophore-mediated iron uptake while FupA is dispensable for this process. The higher level of uptake observed in the ΔfupA mutant might be ascribed to the high expression of fsl genes in this strain.

We tested the singly complemented ΔfslE ΔfupA strains in the siderophore-mediated $^{55}$Fe uptake assay. Figure 3E shows the rates of $^{55}$Fe uptake for the different strains. The ΔfslE ΔfupA strain with control vector integrated in the chromosome had a negligible rate of uptake. This was significantly enhanced when the fslE gene was reintroduced into the strain. The fupA complement had very low levels of uptake, similar to the parental strain. These experiments definitively demonstrated that i) fslE can function independently of fupA as siderophore receptor and ii) that FupA is not itself a receptor for the siderophore.

Transport of ferrous iron in Schu S4 and mutants—Siderophores are specific for ferric iron, and siderophore uptake mechanisms typically are distinct from mechanisms for acquisition of the reduced (ferrous) form of iron. We sought to characterize ferrous iron uptake in Schu S4 and determine if the FslE and FupA proteins play a role in this process.

We grew Schu S4 in iron-limiting medium and followed transport of $^{55}$Fe by these cells in the presence of ascorbate to keep the iron in the reduced form. A plot of the uptake with 100 nM Fe over a period of 20 minutes is shown in Fig. 4A. The uptake was initially linear and accumulation reached a plateau at later times (data not shown). Uptake at the early time points could be used to plot the transport rate. The transport rates for iron concentrations ranging from 12.5 nM to 6.2 μM plotted as a function of iron concentration followed a curve consistent with Michaelis-Menten kinetics, as shown in Fig. 4B. Maximal uptake ($V_{max}$) of 21.94 pmol min$^{-1}$ mg$^{-1}$ was reached at around 3 μM iron while half-maximal uptake (corresponding to $K_M$) was seen at 357 nM iron. Concentrations of 100 nM and 3 μM, taken to represent high and low affinity uptake, respectively, were used in subsequent experiments to characterize ferrous iron uptake further.
We compared the kinetics of $^{55}$Fe (ferrous) uptake of Schu S4 grown under iron-limitation and in iron-replete media. Bacteria grown under iron-limitation had a seventeen-fold higher rate of uptake at 100 nM ferrous iron and a four-fold higher rate of uptake at 3 $\mu$M iron (Fig. 5A). All subsequent experiments were carried out with bacteria grown under iron limitation.

The protonophore CCCP had a drastic effect on ferrous iron uptake both at 100nM and 3 $\mu$M concentrations (Fig. 4C). Incubation at 4°C did not support transport at either concentration. This indicated that ferrous iron uptake is also dependent on proton motive force and on temperature.

We assessed the ferrous uptake capability of different mutants (Fig. 5). The $\Delta$fupA mutant was significantly defective for uptake at 100 nM iron concentration. The difference in uptake was much less pronounced, but remained significant at 3 $\mu$M iron concentration (Fig. 5A, 5C). To get a better understanding of the uptake deficiency of the $\Delta$fupA mutant, we compared transport rates of Schu S4 and the $\Delta$fupA mutant with $^{55}$Fe concentrations ranging from 100nM to 3.2 $\mu$M. The relative rates changed with concentration with the very large difference in rates at low concentrations tapering with increasing iron concentration (Fig. 5B). These results clearly demonstrate that the $\Delta$fupA mutant is defective in ferrous iron uptake, and specifically high affinity ferrous iron uptake.

In contrast to the $\Delta$fupA mutant, the $\Delta$fslE mutant had rates of ferrous uptake that were comparable to Schu S4 both at 100 nM and at 3 $\mu$M (Fig. 5C). The $\Delta$fslE $\Delta$fupA mutant was however even more affected than $\Delta$fupA alone, displaying a much lower transport rate than the other strains even at the higher iron concentration. Thus the double mutant is defective even for the low affinity uptake process. We examined the singly complemented double mutant strain to see if restoring FupA expression would abrogate the uptake defect. As shown in Fig. 5D, reintroduction of $fupA$ on a plasmid restored both high affinity and low affinity ferrous uptake to the mutant demonstrating that the ferrous iron transport defect was entirely due to loss of $fupA$.

We also examined the transport rate for the siderophore-deficient $fsI$A mutant GR206 at 100nM $^{55}$Fe$^{2+}$ and found that it was not statistically different from the Schu S4 rate (107± 3%). This ruled out the formal possibility that the siderophore was in some way involved in the high affinity ferrous iron uptake process.

These experiments clearly demonstrated that FupA was necessary for optimal ferrous iron uptake in Schu S4. At higher iron concentrations, other transporters could partially compensate for FupA function, but the high affinity uptake important for iron acquisition at low iron concentrations was lacking in the mutant. Our results also demonstrated that FslE played no significant role in high affinity ferrous iron uptake.

**Rescue of growth on CDM agar by different forms of iron**- Iron in an aerobic environment is largely in the ferric form. Since the $\Delta$fupA mutant is capable of siderophore-mediated ferric iron uptake, it was not clear as to why it had a growth defect on CDM-Fe agar which presumably contains trace amounts of iron in the ferric form. We therefore investigated the ability of the two different forms of iron to rescue growth of the mutants on CDM-Fe agar. Supplementation of the agar with 7 $\mu$M FeSO$_4$ (the normal iron supplement in CDM) completely rescued growth of the $\Delta$fslE mutant, while growth of the $\Delta$fupA mutants was only partially rescued by the iron supplement after 4 days of growth (Fig. 2B). FePPi at a similar concentration (6.8 $\mu$M) in the CDM agar also rescued the $\Delta$fslE mutant completely. While it did not completely rescue growth of the $\Delta$fupA mutant, the bacteria grew to a higher dilution than with ferrous sulfate. Increasing the ferric supplement concentration ten-fold to 68 $\mu$M allowed a more complete, albeit slow recovery of growth in contrast to supplementation with an equivalent concentration of FeSO$_4$ (data not shown).

The difference in growth on ferric and ferrous iron supplemented CDM agar was observed only when freshly poured plates were used for the assay; with plates that were more than a few days old, the distinction between ferrous and ferric supplemented plates was hard to discern. One explanation is that there is some level of inter-conversion of the iron between ferrous and ferric forms in the agar that is influenced by a complex set of factors including oxidation following exposure to air on the surface and reduction promoted by cysteine in the medium.
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(CDM contains 0.02% cysteine as supplement since the bacteria are defective in sulfate assimilation and require cysteine for growth (26). Cysteine is known to reduce ferric salts in bacterial media (33). We found that increasing cysteine levels in the CDM agar reduced the extent of growth promotion of the ∆fupA mutant by ferric iron, presumably because more of the iron was converted to the ferrous form (data not shown). We propose that the iron in agar plates used for growth of F. tularensis is in a dynamic equilibrium between ferrous and ferric forms, and that optimal growth requires functional acquisition systems for both. This may also explain the different growth phenotypes of the ∆fslE and ∆fupA mutants on agar. Furthermore, in a shaking iron-limiting liquid culture, the oxidized form is probably more prevalent; this could explain why the fslE mutant had a strong growth phenotype in medium containing FePPi, but the growth defect of the fupA mutant in medium supplemented with FeSO₄ was more modest.

The fslE paralog fupB does not play a key role in iron acquisition- FslE and FupA are the only two of five paralogs encoded in the Schu S4 genome for which any function is known. A third paralog, fupB (FTT0919) is encoded adjacent to fupA, and likely forms part of the same transcription unit. We considered the possibility that it may also in some way play a part in iron acquisition. We therefore created deletion mutants Schu ∆fupB (GR243), and Schu ∆fupA ∆fupB (GR242). On western blots with rat antiserum, the Schu S4 and ∆fupA strains showed a reactive ~45 kDa band corresponding to FupB; this band was missing in lysates of the ∆fupB mutant (Fig. 1B). The fupB mutant expressed FslE and FupA at levels similar to Schu S4.

The ∆fupB mutant grew similarly to Schu S4 on iron-limiting agar, while the ∆fupA ∆fupB mutant behaved similarly to the ∆fupA mutant (data not shown). Siderophore-mediated ⁵⁵Fe uptake by the ∆fupB mutant was not significantly different from Schu S4 (Fig. 3D), while the rate of high affinity ferrous iron uptake with 100 nM ⁵⁵Fe was 78% of Schu S4 (Fig. 5A). Overall, the fupB deletion did not have large effects on the iron acquisition capability of the bacteria in our assays. We conclude that not all fslE paralogs play a major role in these iron acquisition processes.

Optimal intracellular growth requires at least one paralog functional in iron acquisition- We tested the ability of the fslE and fupA mutants to replicate intracellularly in the mouse macrophage-like cell line J774A.1. We evaluated the ability of the bacterial strains to invade and replicate by assessing bacterial numbers at different times following infection of the monolayers. As shown in Fig. 6, all the strains had similar intracellular numbers at 2 hours, indicating a similar level of invasion. The intracellular numbers of the parental Schu S4 strain titer increased by ~2.5 logs over a period of ~24 hours. The ∆fslE mutant did not show differences relative to Schu S4 at 9.5 and 23 hours. The ∆fupA mutant showed a relative defect at 9.5 hours, but at 23 hours had caught up to Schu S4. The double mutant, however, was significantly reduced in titer at every time point post invasion. This may reflect the general slow growth of the strain that we observed in vitro, likely due to the poor ability to assimilate iron.

The mammalian intracellular milieu is considered to be an iron-limiting environment. We found that that there was a growth defect in the fupA mutant in J774A.1 cells at 9.5 hours, but this did not persist to later times. A fupA mutant was previously found to be defective for intracellular replication in mouse peritoneal macrophages at 15 hours post infection, although intracellular bacterial titers at later time points in this assay were not reported (27). Furthermore, biological differences between J774 tissue culture cells and peritoneal murine macrophages may contribute to differences in experimental outcome. Our results indicated that while neither of the iron uptake mechanisms mediated by the two paralogs was essential, at least one needed to be functional for intracellular replication in the J774A.1 cell line. FslE and FupA govern alternate pathways for iron acquisition in this system.

Both paralogs synergistically contribute to virulence- Schu S4 is highly virulent in mice, requiring less than 10 CFU for a lethal infection. We evaluated the ability of the different mutants to kill C57/BL6J mice following infection by the subcutaneous route (Fig. 7). We initially challenged mice with ~25 CFU (Fig. 7A); at this dose, Schu S4-infected mice all succumbed within 6 days of infection. The fslE mutant was equally virulent in this model. When infected with the
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ΔfupA mutant, 2/5 mice succumbed within 6 days while the remaining 3 survived. Mice infected with the ΔfslE ΔfupA mutant all survived. The survivors from both groups were immune to subsequent challenge with 12 CFU Schu S4.

In a second experiment, we tested higher doses of the ΔfupA mutants (Fig. 7B). At doses of 528 and 2112 CFU of the ΔfupA strain, 3/4 mice succumbed, while all mice receiving the highest dose succumbed, albeit with some delay in time to death. Mice survived infection with the ΔfslE ΔfupA mutant at all doses tested. Surviving mice from both groups were largely resistant to subsequent challenge with 500 CFU Schu S4, with just 1/4 mice in two of the groups succumbing to the challenge.

We also tested a ΔfupB mutant and found it to be as virulent as wild type (data not shown). Upon challenge with a ΔfupA ΔfupB mutant, three of four infected mice survived and were immune to further Schu S4 challenge. These data suggest that fupB does not significantly contribute to virulence, in agreement with earlier studies (27).

Previous reports with Schu fupA mutants found the strains to be highly attenuated following intradermal infection of Balb/c mice (21,27). Our ΔfupA mutant was far less attenuated in our model. This may reflect differences in the route of infection and additionally, the mouse genotype is known to influence outcome (4,34).

In our experiments the ΔfslE ΔfupA mutant demonstrated extreme attenuation relative to the single fupA mutant. Additionally, the survivors demonstrated significant resistance to subsequent Schu S4 challenge. This indicates potential for the combined mutations to be included in development of a live vaccine strain. Our results indicate that the two paralogs involved in iron acquisition synergistically function to govern virulence in F. tularensis.

DISCUSSION

Several lines of evidence suggest that mechanisms for acquisition of iron in the limiting host environment are critical for establishment of infection by pathogenic Francisella strains. Early studies with LVS showed that intracellular replication is dependent on availability of iron (35). In addition, the intracellular pathogen was shown to modulate host iron metabolism to increase the labile iron pool (36). Interfering with iron uptake by use of the competitor gallium inhibited bacterial replication in vitro and in vivo (37). Genetic screens and studies with defined mutants have linked the fsl and fupA genes to virulence in different strains (20-25,27,38).

FslE and FupA are paralogous proteins that share 63% identity and 79% similarity and localize to the outer membrane in Schu S4 (39). The PRED-TMBB program (40,41) predicts that these proteins form beta-barrels with large periplasmic plugs of 152 residues and 201 residues for FslE and FupA, respectively. Our studies with 55Fe transport in conjunction with the predicted structure of the proteins have confirmed the role of FslE as the siderophore receptor and identified FupA as a high affinity ferrous iron transporter in Schu S4. While each protein is able to function independently of the other, the two paralogs function in concert to promote growth under iron limitation.

We have demonstrated here that uptake of ferric 55Fe by FslE is dependent on the siderophore. Siderophore-dependent iron transport by TonB-dependent outer membrane transporters has been characterized in a number of Gram-negative bacteria (19). How the transport is facilitated by FslE across the outer membrane in F. tularensis in the absence of a TonB complex remains to be determined. An analogous TonB-independent siderophore uptake system has been recently identified in Legionella pneumophila (42), suggesting that this paradigm may extend to other bacteria lacking TonB.

We have established that the PMF is important in facilitating the siderophore uptake process in F. tularensis. The fsl operon in F. tularensis encodes two members of the Major Facilitator Superfamily (MFS) of transporters, fslB and fslD. In a recent study with siderophore uptake in L. pneumophila, the authors noted that the L. pneumophila inner membrane transporter LbtC is a close homolog of FslD (43). Based on this similarity, FslD may be the inner membrane transporter involved in the siderophore uptake process in F. tularensis. In the TonB-dependent siderophore uptake systems, transport across the outer membrane relies on the PMF (32). Whether the PMF is important for transport across only one
or across both membranes in *F. tularensis* is not yet clear.

The involvement of FupA in ferrous iron uptake was suggested by growth assays where the nature of the iron supplement influenced the growth phenotype, but the function was most clearly defined on the basis of $^{55}$Fe transport. Growth assays can be confounding because of the inherent tendency of ferrous iron to oxidize in an aerobic environment. The presence of cysteine in the medium may also contribute to alterations in the relative levels of ferrous and ferric iron. As such, the forms of iron may be in flux over the period of growth. Although siderophore-mediated uptake plays a prominent role, our results suggest that both ferrous and ferric uptake contribute to optimal growth on iron-limited agar. *fupA* expression is not regulated by iron levels and ferrous iron transport by FupA may well be the default iron uptake mechanism in Schu S4. Under ferric iron-replete conditions, there may be sufficient ferrous iron for uptake by FupA due to the presence of cysteine in the medium. In a *fupA* mutant, siderophore-mediated ferric iron uptake or low affinity ferrous iron uptake by FslE may compensate for loss of the ferrous uptake pathway. This could explain the reported lower intracellular free iron pool and the deregulation of *fsl* gene expression in the ∆*fupA* mutant (21).

To our knowledge, this is the first report of an outer membrane ferrous iron transport protein in Gram-negative bacteria and our studies suggest that specificity for ferrous iron is also a feature of outer membrane transport. A manganese-specific outer membrane channel protein has been recently characterized in *Bradyrhizobium japonicum* (44). Selective channels, rather than non-specific porins may be more widely involved in transport of different divalent cations across the outer membrane.

We have shown that siderophore-mediated iron uptake capability is enhanced following growth under iron limitation, consistent with Fur-regulation of *fsl* genes in response to iron levels. That FupA mediates high affinity ferrous iron uptake but is not regulated by iron or Fur is surprising; FupA maintains a relatively steady rate of ferrous iron uptake over a wide range of iron levels and may play a housekeeping role in transport of iron across the outer membrane. Subsequent transport of ferrous iron across the inner membrane is likely mediated by the ortholog of the ferrous iron transporter FeoB (45). FeoB in *F. tularensis* is Fur-regulated (GR, unpublished data) and would provide the selective step in ferrous iron uptake. Since *fupA* mutation leads to attenuation of virulence of Schu S4, ferrous iron is probably the predominant or preferred source for the pathogen within the host. Siderophore-mediated iron uptake plays a less significant role and thus the *fslE* mutant shows no loss of virulence. The *fupA* mutant retains some level of virulence possibly because compensatory FslE over-expression permits salvage of ferric iron. However, loss of both uptake pathways is debilitating to the pathogen.

A recent study in *F. novicida* identified a role for FupA (referred to as a lipoprotein, FopC) in down-regulation of the interferon-gamma signaling pathway as a mechanism for enhancing virulence (46). The authors also proposed that FupA helps to maintain outer membrane integrity based on their findings of cellular proteins in the culture supernatant of a *fupA* mutant. Our studies identified a ferrous uptake role for FupA, but additional functions related to stabilization of outer membrane proteins, including FslE, are also possible.

FupA was initially identified as a virulence factor based on the fact that *fupA* mutation results in attenuation in a mouse model of tularemia (27). In a subsequent study, a siderophore-deficient *fslA* mutant was reported to be virulent while the *fupA* mutant was not (21). In our experiments, we found that the ∆*fslE* mutant was also as virulent as wild-type, as was a ∆*fslA* mutant (data not shown). Our ∆*fupA* mutant showed some attenuation in virulence, but not as much as that observed in published studies (21), and this may be related to the model of infection employed. The ∆*fslE* ∆*fupA* mutant had defective growth both in culture and in intracellular infection and was extremely attenuated in virulence in our mouse model. Our data suggest that the two iron acquisition systems work synergistically to support pathogen survival in the host.

In most strains of *Francisella, fupA* and *fupB* are independent and adjacent genes on the chromosome. These genes have undergone a recombination to form the hybrid *fupAB* gene in LVS (27), which nevertheless contributes both to
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Iron acquisition and virulence to some extent (47). Full virulence could be restored by returning the full length *fupA* to the genome (48). We have shown here that *fupB* does not significantly contribute to iron acquisition. The *fupB* mutation also did not alter virulence, as has been previously reported (27).

*F. tularensis* Schu S4 has a relatively small genome of 1.89 Mb and 2097 CDS as most recently catalogued by the Pathosystems Resource Integration center (PATRIC) database (49). Sixteen *F. tularensis* and 8 *F. novicida* genomes have been analyzed at PATRIC (http://www.patricbrc.org). Although the pathogenic *tularensis* strains have accumulated a number of gene inactivation events that are considered to be unimportant to the evolutionary niche that they occupy, they have retained an intact set of iron acquisition related genes found in all naturally occurring Francisella strains this far (50,51). These include the *fur* repressor and *fsl* operon, the *fupA* locus and the *feoB* gene encoding the ferrous iron transporter. Conservation of these genes may reflect the importance of iron acquisition mechanisms to the pathogenic lifestyle of *F. tularensis*.

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FOOTNOTES
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Abbreviations- LVS, Live Vaccine Strain; FePPi, ferric pyrophosphate; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; PMF, proton motive force; CFU, colony forming units.

FIGURE LEGENDS

FIGURE 1. Expression of paralogous proteins in bacteria. Lysates of bacteria grown in iron-replete (H) or iron-limiting (L) medium were analyzed by SDS-PAGE and western blotting. A, expression of FupA in Schu S4 and mutants. B, expression of FupA, FupB, FslE and GroEL in Schu S4 and mutants. C, Expression of FslE and FupA in Schu ΔfupA ΔfupB complemented in cis with fslE or fupA.

FIGURE 2. Growth of ΔfslE and ΔfupA strains under iron limitation. A and B, Maximal growth with ferrous or ferric iron source in liquid. Schu S4 and the ΔfupA mutant were first iron-starved to deplete internal iron pools and then grown in media made replete or limiting with ferrous (FeSO4) or ferric (FePPi) iron. Che-CDM was used for experiments in A and C-CDM (containing additional metal ions) in B. The maximal density reached was recorded and means ± standard deviation are shown.*
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C, Kinetics of growth in liquid medium with ferric iron. Bacteria were grown in medium made iron-replete or iron-limiting with FePPI and growth was followed by change in optical density (OD595). The readings are expressed as means ± standard error. D, Growth on agar. Bacteria were serially diluted in che-CDM and spotted onto iron-replete MHA, iron-limiting (CDM-Fe) agar plates and CDM plates supplemented with 7 μM ferrous sulfate (CDM+Fe²⁺) or with 6.8 μM ferric pyrophosphate (CDM+Fe³⁺).

FIGURE 3. FslE enables siderophore-mediated ⁵⁵Fe transport. A, Growth under iron-limitation promotes siderophore-mediated uptake ability. Schu S4 bacteria grown in iron-replete or iron-limiting medium were incubated with ⁵⁵Fe-siderophore mix and the incorporation of ⁵⁵Fe over time was determined by scintillation counting. B, Siderophore-mediated ferric iron uptake is energy dependent. Schu S4 bacteria grown in iron limiting media were incubated with ⁵⁵Fe in the presence or absence of siderophore and incorporation monitored over time. Parallel reactions with siderophore were carried out at 4°C or following pretreatment of bacteria with 10 μM CCCP. C, FslE is required for siderophore-mediated ⁵⁵Fe uptake. The incorporation of ⁵⁵Fe-siderophore was followed in Schu S4 and mutant strains after growth under iron limitation. D and E, Rates of siderophore-mediated ⁵⁵Fe transport in strains. (D) Schu S4 and mutant derivatives (E) Schu ΔfslE ΔfupA mutant complemented in cis with control vector sequences (-) or with fslE or with fupA. All values are expressed as means ± standard error. * p<0.001, **p<0.005

FIGURE 4. Characterization of ferrous iron transport. A, Kinetics of ⁵⁵Fe²⁺ transport at 0.1 μM concentration by Schu S4 and by ΔfupA mutant. B, Rate of ferrous iron uptake by Schu S4 plotted as a function of ferrous iron concentration. The apparent Kᵢ is 357 nM. C, Rates of high-affinity (0.1 μM iron) and low-affinity (3 μM iron) ferrous iron transport and the effect of CCCP pretreatment or incubation at 4°C. All values are expressed as means ± standard error.

FIGURE 5. FupA mediates high-affinity ferrous iron uptake. Rates of high-affinity (0.1 μM iron) and low-affinity (3 μM iron) ferrous iron uptake by various strains. Rates were normalized to optical density (A and B) or to protein (C and D). A, Schu S4 grown in iron-replete (hi) or iron limiting medium (lo) and ΔfupA and ΔfupB mutants grown in iron limiting medium (lo). B, Schu S4, ΔfslE and ΔfupA mutants grown under iron limitation. C, Comparison of ferrous iron transport rates in Schu S4 and ΔfupA mutant over a range of iron concentrations. D, Rates of ferrous iron transport in Schu ΔfslE ΔfupA mutant complemented in cis with control vector sequences or with fslE or with fupA. All values are expressed as means ± standard error. Significance was calculated relative to Schu S4 grown under iron limitation. * p<0.001, # p<0.002, ** p<0.05

FIGURE 6. Intracellular replication is dependent on FslE and FupA. Schu S4 and mutant derivatives were used to infect J774A.1 cells in 24-well plates and bacterial load was assessed at different times post-infection. The Y-axis represents CFU yield per well. All values are expressed as means ± standard error. * p<0.001, ** p=0.02

FIGURE 7. Virulence of strains in mice. Groups of C57BL/6 mice were subcutaneously infected with the doses of bacterial strains as noted and survival of the mice was plotted over time. A, low dose infection of mice. Survivors were challenged on day 35 with 12 CFU of Schu S4 and all survived for greater than 21 days. B, dose response to ΔfupA mutants. Survivors were challenged with 500 CFU Schu S4 and all survived for greater than 16 days except for one mouse in the lowest and highest dose groups of ΔfslE ΔfupA.
TABLE 1. Complementation for growth on iron-limiting agar\(^a\).

| Complement | \(\Delta fslE\) | \(\Delta fupA\) | \(\Delta fslE \Delta fupA\) |
|------------|----------------|----------------|-----------------------------|
| none\(^b\) | (-)\(^c\)      | + (2)          | -                           |
| \(fslE^+\) | + (6)          | + (4)          | + (2)                       |
| \(fupA^+\) | (-)\(^c\)      | + (6)          | (-)\(^c\)                   |

\(^a\) Ten-fold serial dilutions of the in \textit{cis}-complemented bacteria were spotted on iron-limiting CDM agar and results are presented after 4 days incubation at 37\(^o\)C. - represents absence of growth, + represents growth. Numbers in parentheses indicate the number of dilutions for which growth was observed on the plate.

\(^b\) control vector integrated in chromosome

\(^c\) represents growth only on the outline of the first spot
FIGURE 1

A

| Schu | ΔfsIE | ΔfupA | Δfur |
|------|-------|-------|------|
| L    | L     | L     | L    |
| H    | H     | H     | H    |

FupA  

- 75 kDa
- 50 kDa
- 37 kDa
- 25 kDa

B

| Schu | ΔfupA | ΔfupB |
|------|-------|-------|
| H    | L     | H     |
| L    | L     | L     |

FupA

- 50 kDa

FupB

- 50 kDa
- 37 kDa

FslE

- 50 kDa

GroEL

- 75 kDa
- 50 kDa

C

| - | fslE+ | fupA+ |
|---|-------|-------|
| L | H     | L     |
| L | H     | L     |

FslE

- 50 kDa

FupA

- 50 kDa
FIGURE 3
FIGURE 4

A

B

C

| Treatment | 0.1 mM Fe$^{2+}$ | 3 mM Fe$^{2+}$ |
|-----------|------------------|----------------|
|           | Rate $(\text{pmol min}^{-1} \text{mg}^{-1})$ | % control | Rate $(\text{pmol min}^{-1} \text{mg}^{-1})$ | % control |
| None      | 28.99±1.86       | 100          | 59.11±12.57      | 100        |
| CCCP      | 0.95±0.24        | 3.3          | 10.28±5.43       | 17.4       |
| 4°C       | 0.04±0.28        | 0.15         | 0.19±0.32        | 0.32       |
FIGURE 5

A

B

C

D

Iron transport by Francisella outer membrane proteins
Paralogous outer membrane proteins mediate uptake of different forms of iron and synergistically govern virulence in *Francisella tularensis tularensis*

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