The FupA/B protein uniquely facilitates transport of ferrous iron and siderophore-associated ferric iron across the outer membrane of *Francisella tularensis* live vaccine strain

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*Francisella tularensis* is a highly infectious Gram-negative pathogen that replicates intracellularly within the mammalian host. One of the factors associated with virulence of *F. tularensis* is the protein FupA that mediates high-affinity transport of ferrous iron across the outer membrane. Together with its parologue FslE, a siderophore–ferric iron transporter, FupA supports survival of the pathogen in the host by providing access to the essential nutrient iron. The FupA orthologue in the attenuated live vaccine strain (LVS) is encoded by the hybrid gene *fupA/B*, the product of an intergenic recombination event that significantly contributes to attenuation of the strain. We used $^{55}$Fe transport assays with mutant strains complemented with the different paralogues to show that the FupA/B protein of LVS retains the capacity for high-affinity transport of ferrous iron, albeit less efficiently than FupA of virulent strain Schu S4. $^{55}$Fe transport assays using purified siderophore and siderophore-dependent growth assays on iron-limiting agar confirmed previous findings that FupA/B also contributes to siderophore-mediated ferric iron uptake. These assays further demonstrated that the LVS FslE protein is a weaker siderophore–ferric iron transporter than the orthologue from Schu S4, and may be a result of the sequence variation between the two proteins. Our results indicate that iron-uptake mechanisms in LVS differ from those in Schu S4 and that functional differences in the outer membrane iron transporters have distinct effects on growth under iron limitation.

INTRODUCTION

The Gram-negative bacterium *Francisella tularensis* is the causative agent of the zoonotic disease tularemia (Sjöstedt, 2007). Strains of *F. tularensis* subsp. *tularensis* are restricted to North America and induce a more severe form of disease than strains of the more widespread *F. tularensis* subsp. *holarctica*. The highly infectious pathogen can access the mammalian host by multiple routes: by aerosol, entry through cuts or wounds or by the gastrointestinal route. The live vaccine strain (LVS) is an attenuated *F. tularensis* subsp. *holarctica* derivative that can provide limited protection against virulent type A strains (Burke, 1977). Although highly attenuated by the intradermal or subcutaneous route of infection in mice, LVS is able to cause a fatal infection in mice at low dose when introduced by the intraperitoneal route (Fortier et al., 1991), and can invade and replicate within isolated mammalian cells.

Genome sequencing and molecular analyses have identified several differences between LVS and virulent strains that may contribute to virulence differences (Rohmer et al., 2006; Salomonsson et al., 2009; Svensson et al., 2005). One such difference rests with neighbouring paralogous genes *fupA* and *fupB* of the virulent *Francisella* species, which in LVS have undergone a recombination event leading to formation of the unique *fupA/B* hybrid gene (Rohmer et al., 2006). Introduction of the *fupA* gene from a virulent strain into LVS was shown to significantly enhance virulence following subcutaneous infection of mice (Salomonsson et al., 2009). Spontaneous generation of a similar fusion protein also led to attenuation in a strain of *F. tularensis* subsp. *tularensis*, as did targeted deletion of *fupA* (Twine et al., 2005). Based on these findings, the FupA/B fusion protein is predicted to have altered function compared with the intact FupA.

FupA is an outer membrane protein that belongs to a protein family unique to *Francisella* species (Larsson et al., 2005) and is associated with acquisition of the essential nutrient iron in *F. tularensis* subsp. *tularensis* strain Schu S4 (Lindgren et al., 2009; Ramakrishnan et al., 2012).
Genetic analysis and $^{55}$Fe transport assays in Schu S4 demonstrated that FupA mediates high-affinity transport of ferrous iron (Ramakrishnan et al., 2008, 2012). FupB, encoded by the gene adjacent to fupA, is not important for ferrous iron transport (Ramakrishnan et al., 2012). The FupA/B protein of LVS has the amino-terminal 297 aa residues of FupA fused to the carboxy-terminal 241 residues of FupB at a central conserved stretch of 12 aa. This fusion protein might therefore be expected to be defective in ferrous iron transport.

FslE, a parologue of FupA, was shown to mediate siderophore-dependent iron acquisition in Schu S4 using growth assays on iron-limiting agar, and $^{55}$Fe uptake assays subsequently confirmed its role as siderophore transporter (Ramakrishnan et al., 2008, 2012). FslE is encoded within the fsl siderophore operon, which is conserved and is similarly expressed in different Francisella strains including LVS and Schu S4 (Sullivan et al., 2006; Deng et al., 2006; Milne et al., 2007). Despite similarities in siderophore production, siderophore utilization assays indicated that LVS differs in siderophore utilization; FslE_LVS appeared to be unimportant for siderophore-dependent growth promotion on agar and, rather paradoxically, FupA/B seemed to be more critical in these assays (Sen et al., 2010). The current study aimed at characterizing iron acquisition in LVS and determining how the involvement of FupA/B and FslE in these processes differs from that of their orthologues in virulent Schu S4. Our data indicate that FupA/B functions both in siderophore-iron and in ferrous iron acquisition, thereby playing a dominant role in iron acquisition by LVS.

**Methods**

**Bacterial strains and culture.** *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* strains used in this study are listed in Table 1. Strains were maintained on modified Mueller–Hinton agar supplemented with serum, cysteine and iron salts (MHA). Chamberlain’s defined medium (CDM) (Chamberlain, 1965) was used for routine liquid culture of strains. Iron-limiting agar plates used CDM without addition of iron salts (CDM-Fe) (Sullivan et al., 2006). Chelex-treated CDM (che-CDM) (Ramakrishnan et al., 2012) was supplemented with MgSO$_4$ and CaCl$_2$ and defined levels of ferric pyrophosphate (FePPI) (2.5 μg ml$^{-1}$ for iron-replete and 0.125 μg ml$^{-1}$ for iron-limiting medium) (Ramakrishnan et al., 2012). *Escherichia coli* strain MC1061.1 (araD139 araE139 strain MC1061.1 (araD139 araE139) was grown in CDM-Fe to late-exponential growth and utilized the siderophore secreted by the donor strain (seeded on the plate) to grow on the iron-limiting plate utilizing the siderophore secreted by the donor strain (seeded on the plate). Experiments were repeated on at least two different occasions with similar results.

**$^{55}$Fe uptake assays.** $^{55}$FeCl$_3$ (25.04 and 36.88 mCi ml$^{-1}$; Perkin-Elmer) was used in transport assays carried out in 0.2 μm 96-well filter plates (Millipore) as previously described (Ramakrishnan et al., 2012). Bacteria were cultured for 20–24 h in iron-limiting che-CDM; for assessing transport rates of LVS over a range of ferrous iron concentrations, the bacteria were more completely iron-starved by further dilution and growth in iron-limiting medium for an additional 16 h. Bacteria were washed to remove any iron and secreted siderophore and resuspended in che-CDM without iron to an OD$_{600}$ of 0.2. Bacteria were incubated in wells of the filter plate on a heat block at 37 °C and uptake reactions were initiated by addition of $^{55}$Fe labelling mix. Bacteria were collected and washed by vacuum filtration. All experiments used samples in triplicate or quadruplicate. Individual wells were punched out and counted in scintillation fluid (Ecocint A; National Diagnostics). Filter wells with unlabelled bacteria were used in a bichinoninic acid assay (Pierce) to normalize results to protein. Ferrous iron-uptake reactions contained 5 mM ascorbate to keep the iron in the reduced form. Siderophore-mediated ferrous iron uptake was carried out in che-CDM containing 10 mM sodium citrate. Siderophore purified from LVS culture supernatants (Sullivan et al., 2006) was incubated with $^{55}$FeCl$_3$ in a 3:1 molar ratio to form complexes prior to the uptake assay and used at a concentration of 1.5 μM $^{55}$Fe and 5 μM siderophore in the uptake reaction. Incorporation of label by bacteria at two time points 5 min apart early in the uptake process was determined. The change in incorporation was normalized to time and to OD$_{600}$ or to protein content to determine the rate of transport. Kinetic parameters of transport were determined by non-linear regression analysis and graphs were plotted using Prism 4.0 software (GraphPad Software). Results comparing different strains were analysed for statistical significance by Student’s t-test using Microsoft Excel.
RESULTS

Ferrous iron uptake in LVS and role of FupA/B

We first assessed ferrous iron uptake in LVS after growth in iron-limiting media to fully derepress iron-acquisition mechanisms, as done previously for the virulent Schu S4 (Ramakrishnan et al., 2012). We followed transport of $^{55}$Fe in the presence of ascorbate to keep the iron in the reduced form and found that $^{55}$Fe incorporation increased with time at 37 °C (Fig. 1a). This accumulation was inhibited at 4 °C and by the protonophore carbonyl cyanide m-chlorophenyl hydrazone, indicating a dependence on temperature and the proton motive force (Fig. 1a). We assessed rates of uptake over a range of ferrous iron concentrations from 6.2 nM to 6.4 µM (Fig. 1b). Based on our understanding of bacterial growth, transport at the low end of the concentration range would depend on mechanisms active under conditions of iron limitation (high-affinity uptake systems) (Sullivan et al., 2006). The transport rates were plotted as a function of iron concentration and fitted a curve representative of Michaelis–Menten kinetics, with a maximal uptake value ($V_{\text{max}}$) of 232.5 pmol min$^{-1}$ OD$_{600}^{-1}$ and substrate concentration for half-maximal transport ($K_{c}$) of 815 nM (Fig. 1b). While generally resembling transport in Schu S4, the high $V_{\text{max}}$ and $K_{c}$ for LVS compared with the corresponding values for Schu S4 (21.94 pmol min$^{-1}$ OD$_{600}^{-1}$ and 357 nM, respectively) (Ramakrishnan et al., 2012) suggested that LVS possessed an intrinsically higher capacity for ferrous iron transport when iron was abundant. This was confirmed in a direct comparison of transport rates of the two strains with 3 µM ferrous iron, with LVS showing an ~4.5-fold higher rate of transport than Schu S4 (Fig. 1c). This difference between the two strains could either result from a conserved mechanism that was more active in LVS or be due to occurrence of different mechanisms for ferrous iron acquisition.

Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
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<tr>
<td><em>F. tularensis</em></td>
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<tr>
<td>LVS</td>
<td><em>F. tularensis</em> subsp. holarctica LVS</td>
<td>K. Elkins</td>
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<tr>
<td>GR7</td>
<td>LVS ΔfisA</td>
<td>Sullivan et al. (2006)</td>
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<td>LVS ΔfisE</td>
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<td>LVS ΔfupA/B</td>
<td>Sen et al. (2010)</td>
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<td>GR20</td>
<td>GR16 (pGIR459); vector integrant</td>
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<td>GR17 (pGIR459); vector integrant</td>
<td>Sen et al. (2010)</td>
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<td>GR17 (pGIR474); fisELvs plasmid integrant</td>
<td>Sen et al. (2010)</td>
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<td>GR16 (pGIR477); fupA plasmid integrant</td>
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<tr>
<td>GR228</td>
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We compared rates of $^{55}\text{Fe}^{2+}$ transport in LVS and ΔfslE, ΔfupA/B and the ΔfslE ΔfupA/B mutant strains at 0.1 μM Fe$^{2+}$, reflecting high-affinity transport, and at 3 μM Fe$^{2+}$, corresponding to a low-affinity process (Fig. 1d). The ΔfslE strain showed transport similar to LVS at both the limiting and the high iron concentrations but the ΔfupA/B and the double mutant were defective in uptake at both concentrations. We compared LVS and the ΔfupA/B strain at ferrous iron concentrations ranging from 50 nM to 1 μM (Fig. 1e). The fupA/B mutant was more defective at lower concentrations; at 50 nM there was a 20-fold difference in transport rates, while at 1 μM the difference was only 2.5-fold. These results indicated that FupA/B functioned in high-affinity ferrous iron transport, similar to FupA in Schu S4.

To understand how FupA/B may differ in function from FupA, we compared ferrous iron transport rates of the LVS ΔfslE ΔfupA/B mutant complemented in cis with either fupA/B or with fupA derived from the Schu S4 genome. As seen in Fig. 2(a), the low level of transport at 0.1 μM Fe$^{2+}$ by the mutant transformed with vector alone was increased sixfold when fupA/B was reintroduced into the strain. Introduction of fupA in place of fupA/B resulted in a further ∼150% increase in transport rate. At 3 μM Fe$^{2+}$, fupA/B and fupA appeared to have equivalent activity. Introduction of fupB along with fupA did not significantly alter iron transport in the LVS background. This is consistent with our previous findings that fupB is not critically important for iron transport in Schu S4 (Ramakrishnan et al., 2012).

To test if transport by FupA and FupA/B might be influenced by the strain background, we performed the complementary experiment, introducing fupA/B and fupA individually into the Schu S4 ΔfslE ΔfupA strain. We found that fupA/B was able to promote ferrous iron transport in the Schu S4 background, but as seen with the LVS strain, the transport rate at 0.1 μM Fe$^{2+}$ was lower than that conferred by fupA (Fig. 2b). At 3 μM iron, there was no difference in the rates of transport. Thus, fupA/B appeared to function less efficiently than fupA in high-affinity ferrous iron transport regardless of the genetic background of the host strain.
To confirm that the transport differences with the *fupA/B* and *fupA* complements were not caused by differences in expression of these genes, we tested lysates from the different strains in Western blotting with antibody raised to the amino-terminal domain of FupA that is also present in FupA/B. In this complementation system, expression of the gene of interest is under control of the Fur-regulated *fslA* promoter and is enhanced relative to the wild-type strain, although it is still influenced by iron levels (Ramakrishnan et al., 2012). We determined that the complementing gene products were expressed at comparable levels in the outer membranes of the LVS and Schu S4 backgrounds (~300 versus ~60 pmol min$^{-1}$ mg$^{-1}$). This indicated that the capacity for ferrous iron uptake under iron-replete conditions was not influenced by FupA or FupA/B, but was instead set by some additional mechanism that differs between the LVS and Schu S4 backgrounds.

**FupA/B is more effective than FupA at supporting growth on iron-limiting agar**

A Δ*fupA/B* mutant of LVS shows reduced growth on iron-limiting agar while deletion of both *fupA/B* and *fslE* results in a more severe growth defect (Sen et al., 2010). We compared the ability of *fupA* and *fupA/B* introduced in cis to support growth of LVS Δ*fupA/B* and LVS Δ*fslE ΔfupA/B* under iron limitation. Serial dilutions of washed bacteria were spotted on CDM-Fe agar and growth was compared with that on iron-replete MHA (Fig. 4). While the LVS vector control grew out to four dilutions on the iron-limiting agar, the Δ*fupA/B* vector control grew only to the second dilution and the double mutant control showed even poorer growth. As seen previously (Sen et al., 2010), complementation with *fupA/B* restored growth similarly in both strains, indicating that overexpression of FupA/B can compensate in some way for loss of FslE function. Interestingly, the *fupA* and the *fupA* + *fupB* complements showed differences in growth recovery in the two mutant backgrounds. The single mutant complemented with *fupA* S4 background (~300 versus ~60 pmol min$^{-1}$ mg$^{-1}$). This indicated that the capacity for ferrous iron uptake under iron-replete conditions was not influenced by FupA or FupA/B, but was instead set by some additional mechanism that differs between the LVS and Schu S4 backgrounds.
or with fupA+fupB grew similarly to the fupA/B complement; in contrast, the fupA and fupA+fupB complemented double mutant displayed poorer growth that was only marginally better than the vector control. These results demonstrated that fupA could compensate for loss of fupA/B only in conjunction with fslE. Additional loss of fslE gene function could be compensated for by restoration of fupA/B expression, but not of fupA.

To determine if the growth promotion by fupA/B is specific to the LVS background, we similarly tested iron-limited growth of the fupA/B complemented Schu ΔfslE ΔfupA mutant (Fig. 5). As previously shown, restoration of the Schu S4-derived fslE (fslEs) in cis to this mutant can partially rescue growth, whereas restoration of fupA cannot (Ramakrishnan et al., 2012). Interestingly, the introduction of fupA/B resulted in growth that was better than the fslE complement and almost comparable to the parent Schu S4 vector control. This indicated that FupA/B but not FupA possessed a strain-independent ability to promote growth under iron limitation and could compensate for loss of fupA and of fslE.

**FupA/B but not FupA promotes siderophore utilization**

Siderophore-promoted growth assays on iron-limiting agar using different mutant strains showed that FslE was essential for siderophore utilization in Schu S4 and in the related *Francisella novicida* strain U112 (Kiss et al., 2008; Ramakrishnan et al., 2008). In LVS, however, fupA/B and not fslE was primarily required for growth in this cross-feeding assay (Sen et al., 2010).

To better understand the basis for differences in growth promotion by FupA, FupA/B and FslE, we tested the different complemented strains for the ability to utilize siderophore for growth (Fig. 6a). The test bacteria were spread on iron-limiting agar and cells of siderophore-producing LVS were spotted in the centre. The siderophore secreted from the bacteria in the centre could promote growth of the surrounding LVS control bacteria. The single and double mutants bearing the vector control were unable to produce growth haloes around the siderophore-producing strain, as shown previously (Sen et al., 2010). We found that introduction of fupA or fupA+fupB in the single mutant promoted robust growth, similar to restoration of fupA/B expression. The ΔfslE ΔfupA/B mutant, however, was only able to grow upon restoration of fupA/B expression, but not by introduction of fupA or fupA+fupB. That the growth halo is siderophore-dependent may be confirmed by the inability of a siderophore-deficient ΔfslA strain to promote this growth; thus, LVS seeded on the iron-limiting agar can form a halo around an LVS spot but not around a spot of ΔfslA (Sullivan et al., 2006) (Fig. 6b). Likewise, the LVS ΔfslE ΔfupA/B mutant complemented with fupA/B was only able to grow around the LVS spot but not the ΔfslA cells (Fig. 6c). Our results thus indicated that fupA/B could mediate siderophore utilization whereas fupA was unable to facilitate siderophore-mediated iron acquisition on its own, although it could do so in conjunction with fslELVS. We also found that fupA/B was similarly functional in siderophore utilization in the Schu S4 background (data not shown).

**FslE<sub>LVS</sub> is less effective than FslE<sub>Schu</sub> at mediating siderophore utilization for growth under iron limitation**

The importance of FupA/B for siderophore-dependent growth and the apparent minor role for FslE<sub>LVS</sub> is in contrast to the situation in Schu S4, where FslE is primarily responsible for the process (Sen et al., 2010; Ramakrishnan et al., 2012). The FslE sequence in LVS (FslELVS) differs at five amino-acid residues from that of Schu S4 (FslESchu) and we considered the possibility that the mutations may either destabilize the FslELVS protein, leading to reduced levels in the outer membrane, or might reduce the functionality of the protein. To test the first possibility, we used Western blotting to examine levels of the proteins in lysates and in the outer membrane of LVS and the single mutant strains (Fig. 7a). FipB was used as loading control. FupA/B was present at similar levels in lysates and in membrane fractions of LVS and the ΔfslE mutant, while no band corresponding to FupA/B was detected in the ΔfupA/B mutant. FslE was detectable in the lysate, FslE<sub>LVS</sub> and also in the outer membrane fraction of LVS. The fsl operon is
deregulated in the ΔfupA/B mutant (Sen et al., 2010); as expected, levels of FsIE were much greater in the ΔfupA/B mutant lysate and this deregulation was observed also in the outer membrane protein fraction (Fig. 7a). The results demonstrated that FsIE\textsubscript{LV5} does localize to the outer membrane and that its assembly is not dependent on FupA/B.

We introduced the fsIE orthologues independently into the LVS ΔfsIE ΔfupA/B strain and tested the levels of these proteins in lysates and in the outer membrane by Western blotting, with FipB as loading control (Fig. 7b). FsIE\textsubscript{LV5} in lysates and in the membrane fraction was detected at levels not lower but higher than FsIE\textsubscript{Schu}. These results suggested that the sequence alterations in FsIE\textsubscript{LV5} do not destabilize the protein. To test if the sequence differences might lead to reduced functionality of FsIE\textsubscript{LV5}, we compared growth of the complements on iron-limiting CDM agar. As shown in Fig. 4, there was an obvious difference in the restoration of growth by the two orthologues. The fsIE\textsubscript{Schu} complement showed more robust growth than the fsIE\textsubscript{LV5} complement, suggesting that FsIE\textsubscript{LV5} is indeed less effective than FsIE\textsubscript{Schu} at promoting growth under iron limitation.

We tested the LVS ΔfsIE ΔfupA/B strain complemented with each of the fsIE orthologues in the siderophore utilization assay on iron-limiting CDM agar. As shown in Fig. 6(d, e), both complements formed growth haloes around siderophore-producing LVS but not around the siderophore-deficient ΔfsA mutant spots. Of note, the ability to form growth haloes was dependent on the number of c.f.u. seeded on the iron-limiting plate; while ~3 × 10^6 c.f.u. of LVS seeded on the plate were sufficient for growth halo formation, the fsIE\textsubscript{Schu} Complement of LVS ΔfsIE ΔfupA/B required ~3 × 10^7 c.f.u. and the fsIE\textsubscript{LV5} complement required ~3 × 10^7 c.f.u. These results are consistent with differences in growth of serial dilutions of the strains (Fig. 4).

**FupA/B and FsIE\textsubscript{LV5} jointy mediate siderophore–\textsuperscript{55}Fe transport**

Although growth of Schu S4 on iron-limiting CDM agar primarily requires FsIE function, optimal growth relies on both FsIE and FupA, indicating that the trace iron in the medium is acquired both in the ferrous form and through siderophore–ferric iron complexes (Ramakrishnan et al., 2012). The most direct read-out for siderophore-mediated iron acquisition is bacterial incorporation of \textsuperscript{55}Fe complexes to siderophore in real-time and we have previously used this assay to establish the role of FsIE as siderophore transporter in Schu S4 (Ramakrishnan et al., 2012). We characterized siderophore-dependent ferric iron uptake in LVS so that we could more definitively understand the roles of fupA/B and fsIE\textsubscript{LV5} in this process. LVS bacteria grown for an extended period under iron limitation were incubated with \textsuperscript{55}Fe complexed to purified siderophore in the presence of citrate and accumulation of \textsuperscript{55}Fe was followed over time. The bacteria showed a siderophore-dependent increase in internalized \textsuperscript{55}Fe with time (Fig. 8a).
A slow increase in the absence of added siderophore was probably due to endogenous siderophore produced by the bacteria.

We compared rates of siderophore-mediated $^{55}$Fe transport in LVS and the $fsIE$ and $fupA/B$ mutants (Fig. 8b). Somewhat surprisingly, both of the single mutants showed marked reduction in transport rates, suggesting that both FslE and FupA/B individually contribute to siderophore-mediated acquisition of iron in LVS. The double mutant showed no appreciable transport of $^{55}$Fe, suggesting that these two proteins are the sole mediators of siderophore–iron transport in LVS.

To further analyse the roles of $fupA/B$ and $fsIE$ in siderophore–iron transport, we examined the rates of siderophore-mediated $^{55}$Fe transport in $\Delta fupA/B$ mutants complemented with the different paralogues (Fig. 8c). The $\Delta fupA/B$ mutant with control vector had a low rate of transport compared with LVS. The rate was restored to wild-type levels when $fupA/B$ was provided in cis. Introduction of $fupA$ or $fupA + fupB$ resulted in a twofold
enhancement of uptake over the vector control, but not to wild-type levels. We examined transport in the double mutant complemented with either fupA/B or fslELVS in cis. Restitution of fupA/B and fslELVS individually partially restored transport, but neither alone restored activity to wild-type levels (Fig. 8d), demonstrating that each of the paralogues contributes to siderophore-mediated iron acquisition. Transport in the fslEschu complemented double mutant was notably increased over the fslELVS complement, demonstrating that FslELVS is less effective at siderophore–iron acquisition than FslEschu. Neither the fupA nor the fupA+fupB complement showed increased siderophore–iron transport over the vector control (Fig. 8e). We also examined siderophore-mediated iron transport in the Schu ΔfslE ΔfupA mutant complemented with the different paralogues. The fupA/B but not the fupA complement showed an increase in siderophore-mediated ferric iron transport, as in the LVS background (Fig. 8f).

Overall, these experiments demonstrated that both FupA/B and FslELVS independently contribute to siderophore-mediated iron acquisition. Comparison of transport by the single mutants with LVS in Fig. 8(b) suggests, however, that the paralogues may function not additively but cooperatively in transport. Although FupA did not have siderophore-transport activity, its ability to increase transport activity in the ΔfupA/B mutant (Fig. 8c) suggested that FupA could support FslELVS function in this process in some manner.

**DISCUSSION**

Specialized mechanisms for acquiring iron are critical for survival under iron limitation and bacterial pathogens typically possess multiple redundant systems for obtaining this nutrient within the host milieu (Ratledge & Dover, 2000). The small 1.892 Mb genome of Schu S4 (Larsson et al., 2005) encodes only two transporters for moving iron across the outer membrane under limiting conditions: FslE is a siderophore–ferric iron transporter, while the paralogous protein FupA mediates high-affinity ferrous iron transport (Ramakrishnan et al., 2012). The LVS genome shares 99.3% identity with that of Schu S4 and one of the earliest noted genomic differences between the strains (RD18) was the deletion in the fupA–fupB region leading to formation of the fupA/B hybrid gene in LVS (Broekhuijsen et al., 2003; Rohmer et al., 2006; Svensson et al., 2005). The switch from FupA to FupA/B in LVS profoundly influences virulence (Salomonsson et al., 2009), suggesting that this altered protein is altered in function as well. Here we have shown that the FupA/B protein of LVS retains high-affinity ferrous iron transport ability, albeit at a somewhat diminished level compared with FupA of Schu S4. We found additionally that FupA/B is capable of siderophore–ferric iron transport in conjunction with FslELVS while FslELVS itself has reduced functionality in siderophore–iron transport as compared with FslEschu. These differences may render LVS less efficient at extracting nutrient iron within the limiting host environment.

Strains of *F. tularensis* subsp. *tularensis* and subsp. *holarctica* show differences in iron metabolism, as evidenced by the larger internal iron stores of subsp. *holarctica* strains following growth under standard iron-replete conditions (Hubálek et al., 2004; Lindgren et al., 2011). A greater susceptibility to oxidative stress accompanies the increased iron stores in the *F. tularensis* subsp. *holarctica* strains and was suggested as contributing to the reduced virulence of these strains (Lindgren et al., 2011). An inherent difference in capacity for ferrous iron uptake may contribute to the altered iron stores; we have shown here that under iron-replete conditions, LVS has a four- to fivefold greater rate of ferrous iron uptake than Schu S4. This increased rate of transport is not determined by the presence of FupA/B in the outer membrane, but by some other aspect of metabolism.

Iron-acquisition mechanisms are mechanistically conserved in Gram-negative bacteria. In the overwhelming majority of Gram-negative bacteria, transport of the siderophore–iron complex across the outer membrane is an energy-requiring process and is enabled by the TonB–ExB–ExBD complex, which transduces energy from the
proton motive force (Faraldo-Gómez & Sansom, 2003). The Francisella genome does not encode proteins of the TonB complex and iron–siderophore transport by FslE represents a novel paradigm (Ramakrishnan et al., 2008). A TonB-independent siderophore transporter that may be mechanistically similar to FslE has also been identified in Legionella species (Chatfield et al., 2011). FslE and the FupA and FupA/B paralogues are predicted by the PRED-TMBB program (Bagos et al., 2004a, b) to assemble in the outer membrane as beta-barrels with extracellular loops and amino-terminal periplasmic extensions, resembling the plug and barrel structure of classic TonB-dependent transporters (Noinaj et al., 2010). While the proton motive force is important for siderophore–iron transport in F. tularensis (Ramakrishnan et al., 2012, and data not shown), it remains to be determined whether an energy-transduction mechanism analogous to TonB is associated with transport across the outer membrane.

Fig. 8. Siderophore-mediated $^{55}$Fe transport. (a) Siderophore-dependent accumulation of $^{55}$Fe by LVS. LVS bacteria were grown for 16 h under iron limitation, and diluted and further grown for 24 h in iron-limiting media. The bacteria were washed and incubated with $^{55}$Fe in the presence (+sid) or absence (-sid) of siderophore and incorporation of $^{55}$Fe over time was determined by scintillation counting. (b) Siderophore-mediated $^{55}$Fe transport rates in LVS and ΔfsIE, ΔfupA/B or ΔfslE ΔfupA/B mutants grown in iron-limiting media for 20 h. (c) Rates of siderophore-mediated $^{55}$Fe transport in LVS ΔfupA/B complemented with different genes as indicated. (d) and (e) Rates of siderophore-mediated $^{55}$Fe transport in LVS ΔfsIE ΔfupA/B complemented by fupA/B and by fsIE orthologues from the LVS and Schu S4 backgrounds. (f) Rates of siderophore-mediated $^{55}$Fe transport in Schu ΔfsIE ΔfupA/B complemented with different genes as indicated. $^{55}$Fe accumulation was normalized to protein content. Values are plotted as means with se. Significance was calculated relative to LVS values in (b) and (c) and relative to vector control in (d), (e) and (f): #P<0.05, *P<0.01, **P<0.002, ***P<0.001; n.s., not significant.
We have demonstrated here that although siderophore-\(^{55}\!)Fe transport in LVS displays similarity to the process in Schu S4, FslE\(_{LVS}\) is functionally less efficient than FslE\(_{Schu}\). FslE\(_{LVS}\) and FslE\(_{Schu}\) display differences in five amino-acid residues across their length of 509 aa. Two of the sequence alterations in FslE\(_{LVS}\) compared with FslE\(_{Schu}\) are conservative substitutions (V154I and M169I) mapping to the predicted plug domain, while the third other changes (T216A, K439E, S472A) map to the predicted barrel domain. Substrate specificity in the TonB-dependent transporters is conferred by sequences of the barrel domain, extracellular loops and the periplasmic plug (Noinaj \textit{et al.}, 2010). The sequence changes in FslE\(_{LVS}\) could conceivably impact siderophore-iron transport function of the protein by altering affinity for substrate. Interestingly, these sequence changes are conserved in the different sequenced strains of \textit{F. tularensis} subsp. \textit{holarctica} in the GenBank database and may represent an evolutionary characteristic of this subspecies.

Previous studies indicated that while FslE functions in siderophore-iron transport in Schu S4, the contribution of FslE to iron acquisition is secondary to FupA/B in LVS (Ramakrishnan \textit{et al.}, 2008, 2012; Sen \textit{et al.}, 2010). Optimal growth on iron-limiting CDM agar requires both ferrous and ferric iron-uptake capability, and the dual involvement of FupA/B in ferrous iron uptake and in siderophore-iron transport coupled with the weaker transport activity of FslE\(_{LVS}\) could account for the dominant role played by FupA/B in growth-based assays with LVS. The \(^{55}\!)Fe transport assays have clarified roles for the two paralogues in LVS.

FupA (557 aa) shares 63\% identity and 79\% similarity overall with FslE and has high specificity for ferrous iron but no appreciable siderophore-iron transport capability. FupB (481 aa), a parologue with 50\% identity and 66\% similarity to FupA and with lower sequence similarity to FslE (42\% identity, 59\% similarity) is also not associated with transport of either form of iron. FupA/B largely retains the ferrous iron transport capability of FupA while having acquired some level of siderophore-iron transport capability. While both FslE\(_{LVS}\) and FupA/B have siderophore-ferric iron transport capability, our \(^{55}\!)Fe transport studies suggest that these paralogues may function co-operatively in transport. How FupA/B co-operates with FslE\(_{LVS}\) to carry out siderophore-mediated iron uptake remains to be discerned in future studies. Neither FupA nor FupB in combination with FupB is capable of promoting siderophore transport, but we found that they could enhance FslE\(_{LVS}\) transport function in some manner that may be related to a co-operative interaction. Identification of the residues in the predicted plug and barrel domains that contribute to altered substrate specificity could provide insight into the transport mechanism. Ferrous iron transport in Schu S4 and LVS is also dependent on the proton motive force, and given the homology between FupA and FupA/B with FslE, a potential involvement of an energy-transduction mechanism in the transport of ferrous iron across the outer membrane is also a question to investigate.

The \textit{fupA/B} recombination event in LVS is associated with attenuation in virulence by the intradermal or subcutaneous route, although the strain remains highly virulent if inoculated intraperitonitely in mice (Fortier \textit{et al.}, 1991; Salomonsson \textit{et al.}, 2009). However, deletion of the \textit{fupA/B} gene results in significant attenuation also by the intraperitoneal route of infection and deletion of the \textit{fslE} locus in addition leads to further attenuation (Sen \textit{et al.}, 2010). FupA/B and FslE proteins appear to be the only significant outer membrane transporters in LVS that operate under iron limitation, consistent with the roles of FupA and FslE in Schu S4. The accumulated evidence suggests that the iron-acquisition functions of these paralogues are critical for survival of \textit{F. tularensis} as pathogens in the mammalian host.

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