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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 paralogue 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.

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INTRODUCTION

The Gram-negative bacterium *Francisella tularensis* is the causative agent of the zoonotic disease tularaemia (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).

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Abbreviation: LVS, live vaccine strain.

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 paralogue 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) 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* Δ*(ara-leu)7696 galE15 galK16* Δ*(lac)X74 rpsL* (Str^r) *hsdR2* ($r_K^-m_K^+$) *mcrA mcrB1 recA*) was grown in Luria broth and used for routine cloning. Ampicillin for *E. coli* transformants was used at 50 μg ml^{-1} (for liquid culture) or at 100 μg ml^{-1} (agar plates). Kanamycin at 15 μg ml^{-1} was used to select for plasmid integrants in *F. tularensis*.

Work with LVS strains was conducted in a Biosafety level 2 laboratory while work with Schu S4 derivatives and with LVS strains complemented with Schu S4-derived genes was conducted in accordance with Select Agent Regulations of the US Centers for Disease Control in an approved Biosafety level 3 facility.

Complementation of Δ*fupA/B* and Δ*fslE* Δ*fupA/B* strains. Use of a suicide plasmid targeted to the 3′ region of *feoB* for complementation with *fupA*, *fupA/B* and $fslE_{Schu}$ *in cis* has been previously described (Sen *et al.*, 2010; Ramakrishnan *et al.*, 2012). The *fslE* gene from the LVS genome ($fslE_{LVS}$) was amplified and cloned in plasmid pSC1 as detailed previously for the *fslE* from Schu S4 ($fslE_{Schu}$) (Sen *et al.*, 2010). For construction of the *fupA*+*fupB* complementing plasmid pBAS3, the *fupA*+*fupB* sequences from Schu S4 genomic DNA were amplified with primers 5′-*CTACTGTCCGGA*-TTTGGTTTGCCAATTTTTTATTAG and 5′-*CTACTGGCGGCCGC*-CTAAATATGTCTAGATATAAACTG (letters in italics indicate sequences not present in the *F. tularensis* genome) using FastStart High Fidelity polymerase (Roche) and cloned as an *Nhe*I–*Not*I fragment in the suicide vector. Expression of the complementing genes on the integrative plasmids was under control of the *fslA* promoter. Integrants were confirmed by PCR analysis of genomic DNA from kanamycin-resistant transformants.

Growth and siderophore utilization. Growth of *F. tularensis* on iron-limiting agar was essentially as previously described (Sen *et al.*, 2010). Bacteria grown in CDM were washed and resuspended in che-CDM to an OD_{600} of 1.0. The cells were tenfold serially diluted in che-CDM and 5 μl of the dilutions was spotted on a CDM-Fe plate and, in parallel, on an iron-rich MHA plate. Growth at 37 °C was monitored over a period of 4 days. Cells were tested for siderophore utilization as described previously (Sullivan *et al.*, 2006). Cells grown in CDM were washed and ~3×10^5 c.f.u. (10^{-3} dilution of cells at an OD_{600} of 1.0) were spread on the CDM-Fe plates and allowed to dry. Then, 2.5 μl of the siderophore-producing control strain at an OD_{600} of 1.0 was spotted at the centre of the seeded plates and the plates were incubated at 37 °C for 2–4 days. A growth halo of colonies around a spot demonstrated the ability of the indicator strain (seeded on the plate) to grow on the iron-limiting plate utilizing the siderophore secreted by the donor strain (spotted in the centre of 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 additional16 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 (Ecoscint A; National Diagnostics). Filter wells with unlabelled bacteria were used in a bicinchoninic 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 ferric 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.

Preparation of outer membranes. Outer membranes from LVS and the Δ*fslE* and Δ*fupA/B* mutants were prepared following osmotic lysis using a modification of the protocol of Huntley *et al.* (2007). Briefly, bacteria were grown in iron-limiting che-CDM to late-exponential

Table 1. Strains used in this study

Strain	Description	Source or reference
F. tularensis		
LVS	*F. tularensis* subsp. *holarctica* LVS	K. Elkins
GR7	LVS Δ*fslA*	Sullivan *et al.* (2006)
GR13	LVS Δ*fslE*	Sen *et al.* (2010)
GR16	LVS Δ*fupA/B*	Sen *et al.* (2010)
GR17	LVS Δ*fslE* Δ*fupA/B*	Sen *et al.* (2010)
GR20	GR16 (pGIR459); vector integrant	Sen *et al.* (2010)
GR21	GR17 (pGIR459); vector integrant	Sen *et al.* (2010)
GR23	GR17 (pGIR474); $fslE_{Schu}$ plasmid integrant	Sen *et al.* (2010)
GR24	GR16 (pGIR479); *fupA/B* plasmid integrant	Sen *et al.* (2010)
GR25	GR17 (pGIR479); *fupA/B* plasmid integrant	Sen *et al.* (2010)
GR34	GR16 (pGIR477); *fupA* plasmid integrant	This study
GR36	GR17 (pGIR477); *fupA* plasmid integrant	This study
GR38	GR17 (pBAS3); *fupA* + *fupB* plasmid integrant	This study
GR39	GR17 (pBAS3); *fupA* + *fupB* plasmid integrant	This study
GR68	GR17 (pSC1); $fslE_{LVS}$ plasmid integrant	This study
GR215	Schu S4 (pGIR459); vector integrant	Ramakrishnan *et al.* (2012)
GR219	Schu S4 Δ*fslE* Δ*fupA*	Ramakrishnan *et al.* (2012)
GR224	GR219 (pGIR459); vector integrant	Ramakrishnan *et al.* (2012)
GR225	GR219 (pGIR479); *fupA/B* plasmid integrant	Ramakrishnan *et al.* (2012)
GR227	GR219 (pGIR474); $fslE_{Schu}$ plasmid integrant	Ramakrishnan *et al.* (2012)
GR228	GR219 (pGIR477); *fupA* plasmid integrant	Ramakrishnan *et al.* (2012)

phase. The pelleted cells were first resuspended in 0.75 M sucrose in 10 mM Tris, pH 7.8, and subsequently adjusted to 10 mM EDTA. Lysozyme was added at 300 μg ml^{-1} and after 2 h of incubation on ice, the spheroplast solution was poured very slowly over four volumes of MilliQ water. After further incubation at room temperature for 30 min to effect complete cell lysis, unlysed cells were removed by centrifugation at 10 000 ***g*** for 30 min. The supernatant was centrifuged at 200 000 ***g*** for 2 h at 4 °C in an Optimax Max-XP ultracentrifuge (Beckman-Coulter) to obtain total membrane pellets. The pellet with membrane was washed once with 10 mM HEPES, pH 6.8, and then incubated in 10 mM HEPES and 0.5 % Sarkosyl at room temperature for 2 h to solubilize the inner membrane. The outer membrane fraction was obtained by centrifugation at 200 000 ***g*** for 2 h at 4 °C. The outer membrane fraction was resuspended in 100 μl of 0.1 % SDS and the concentration of proteins present was assessed using a bicinchoninic acid assay kit (Pierce).

Western blotting. Bacteria grown in iron-replete or iron-limiting media were normalized to cell density and lysates corresponding to ~3×10^8 c.f.u. were separated by SDS-PAGE on 10 % gels. Outer membrane proteins in amounts as specified were also analysed on gels. Following transfer to PVDF membranes and incubation with primary and horseradish-conjugated secondary antibodies, proteins of interest were detected by chemi-luminescence. Rabbit antiserum raised to an FslE peptide (Sen *et al.*, 2010) was used at a 1 : 2500 dilution and guinea pig antibody to the FupA amino-terminal domain (Ramakrishnan *et al.*, 2012) was used at 1 : 5000 dilution. Rabbit antibody to FipB (Qin *et al.*, 2011), a kind gift from Dr Barbara Mann (University of Virginia), was used at a dilution of 1 : 25 000.

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_s) of 232.5 pmol min^{-1} $OD_{600}{}^{-1}$ and substrate concentration for half-maximal transport (K_s) of 815 nM (Fig. 1b). While generally resembling transport in Schu S4, the high V_{max} and K_s 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.

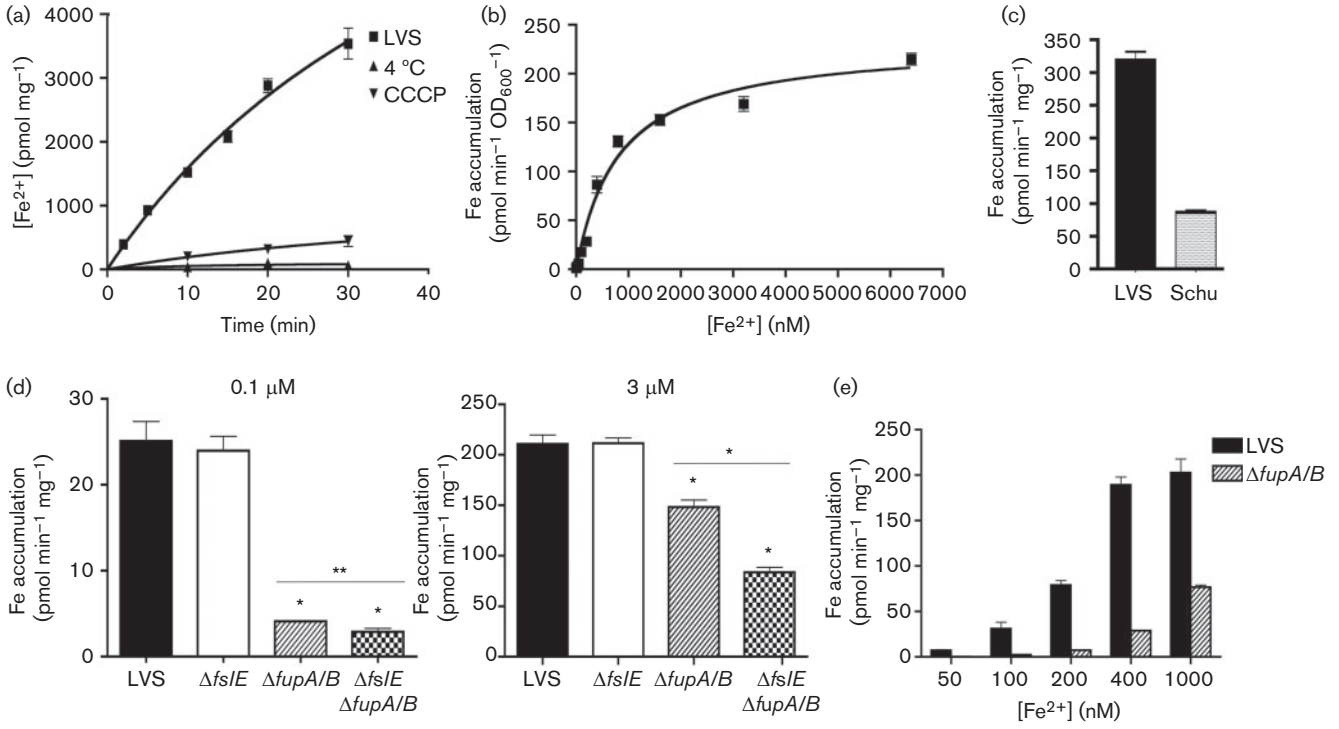

Fig. 1. Ferrous iron uptake in LVS and mutants. (a) Kinetics of $^{55}Fe^{2+}$ transport by LVS. LVS bacteria were grown in iron-limiting che-CDM for 16 h and then diluted and further grown under iron limitation for 22 h. The bacteria were washed and incubated with 7.4 μM $^{55}Fe^{2+}$ at 37 °C and incorporation of $^{55}Fe^{2+}$ over time was determined by scintillation counting. Transport reactions were carried out in parallel at 4 °C and with bacteria that had been pretreated with carbonyl cyanide *m*-chlorophenyl hydrazone. (b) Rate of $^{55}Fe^{2+}$ transport by LVS plotted as a function of ferrous iron concentration in the uptake reaction. (c) Rate of $^{55}Fe^{2+}$ transport by LVS and Schu S4 at 3 micromol ferrous iron. (d) Rates of high-affinity (0.1 μM) and low-affinity (3 μM) ferrous iron transport in LVS and the Δ*fslE* and Δ*fupA/B* mutants after growth in iron-limiting media for 20 h. (e) Comparison of ferrous iron transport rates of LVS and Δ*fupA/B* mutant over a range of iron concentrations. ^{55}Fe accumulation was normalized to protein content or to cell density (OD_{600}). Values are plotted as means with SE. Significance was calculated relative to LVS values: **P*<0.001, ***P*<0.02.

We compared rates of $^{55}Fe^{2+}$ transport in LVS and Δ*fslE*, Δ*fupA/B* and the Δ*fslE* Δ*fupA/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 Δ*fslE* strain showed transport similar to LVS at both the limiting and the high iron concentrations but the Δ*fupA/B* and the double mutant were defective in uptake at both concentrations. We compared LVS and the Δ*fupA/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 Δ*fslE* Δ*fupA/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 Δ*fslE* Δ*fupA* strain. Wd 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.

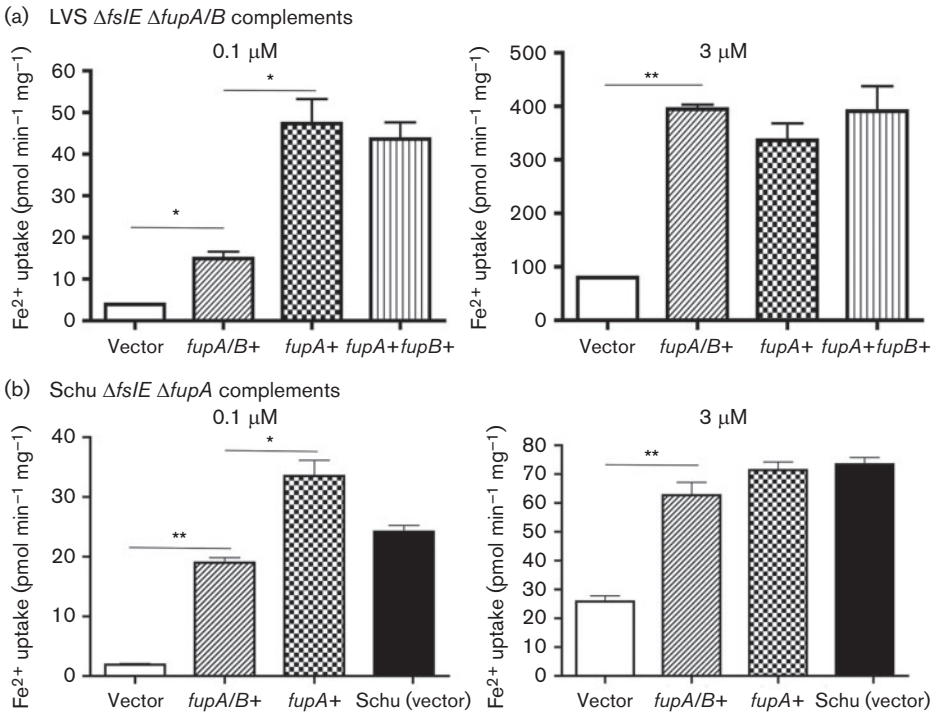

Fig. 2. Ferrous iron uptake in *fupA/B* and *fupA* complemented strains. Rates of high-affinity (0.1 μM) and low-affinity (3 μM) ferrous iron transport in (a) LVS Δ*fslE* Δ*fupA/B* and (b) Schu Δ*fslE* Δ*fupA* complemented *in cis* with *fupA/B*, *fupA* and *fupB* as indicated. Vector indicates control strains in which the vector plasmid alone was integrated in the chromosome. Values are plotted as means with SE; **P*<0.01; ***P*<0.001.

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 different transformants of LVS Δ*fslE* Δ*fupA/B* (Fig. 3a) and Schu S4 Δ*fslE* Δ*fupA* (Fig. 3b). We also assessed levels of the FupA and FupA/B proteins in the outer membranes of the Schu S4 Δ*fslE* Δ*fupA* complements. As a loading control, we used antibody to FipB (FTL_1096 in LVS, orthologue of FTT1103 in Schu S4) (Qin *et al.*, 2011), a lipoprotein that fractionates with the outer membrane (Huntley *et al.*, 2007). Both FupA and FupA/B were present at comparable levels in the outer membrane, implying that the FupA/B fusion protein is intrinsically less efficient at ferrous iron transport than FupA.

Low-affinity ferrous iron transport (at 3 μM Fe^{2+}, Fig. 2) remained distinctly different between the LVS- and Schu S4-derived strains, with the rate in all the LVS-based complements being about fivefold higher than in the Schu 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.

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* Δ*fupA/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*

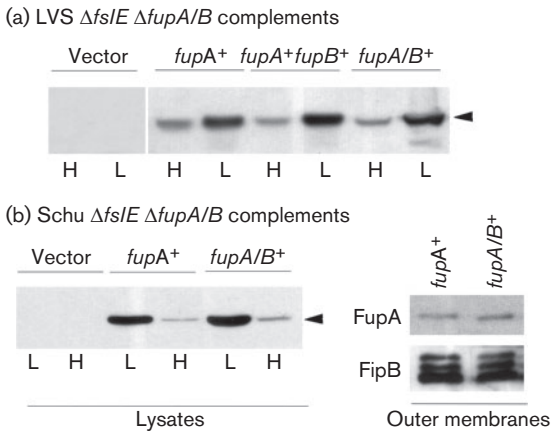

Fig. 3. Expression of *fupA* and *fupA/B* in complemented strains. Lysates of bacteria grown in iron-replete (H) or iron-limiting (L) media were analysed by SDS-PAGE and Western blotting with antibodies to FupA (FupA/B) and control FipB as indicated. The arrowhead indicates the FupA band. Vector indicates strains carrying the control vector plasmid. (a) Whole-cell lysates of LVS Δ*fslE* Δ*fupA/B* complemented with different genes as indicated were tested for expression of FupA (FupA/B). (b) Whole-cell lysates of Schu Δ*fslE* Δ*fupA* complemented with *fupA* and *fupA/B* mutants were tested for levels of FupA (FupA/B). Corresponding outer membrane proteins (1 μg) were analysed by Western blotting for levels of FupA (FupA/B) and for control protein FipB.

or with *fupA*+*fupB* grew similarly to the *fupA/B* complement; in contrast, the *fupA* and *fupA*+*fupB* complemented double mutant displayed poor 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 Δ*fslE* Δ*fupA* mutant (Fig. 5). As previously shown, restoration of the Schu S4-derived *fslE* (*fslE*$_S$) *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 Δ*fslE* Δ*fupA/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 Δ*fslA* 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 Δ*fslA* (Sullivan *et al.*, 2006) (Fig. 6b). Likewise, the LVS Δ*fslE* Δ*fupA/B* mutant complemented with *fupA/B* was only able to grow around the LVS spot but not the Δ*fslA* 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 *fslE*$_{LVS}$. We also found that *fupA/B* was similarly functional in siderophore utilization in the Schu S4 background (data not shown).

FslE$_{LVS}$ is less effective than FslE$_{Schu}$ 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$_{LVS}$ 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 (FslE$_{LVS}$) differs at five amino-acid residues from that of Schu S4 (FslE$_{Schu}$) and we considered the possibility that the mutations may either destabilize the FslE$_{LVS}$ 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 Δ*fslE* mutant, while no band corresponding to FupA/B was detected in the Δ*fupA/B* mutant. FslE was detectable in the lysate and also in the outer membrane fraction of LVS. The *fsl* operon is

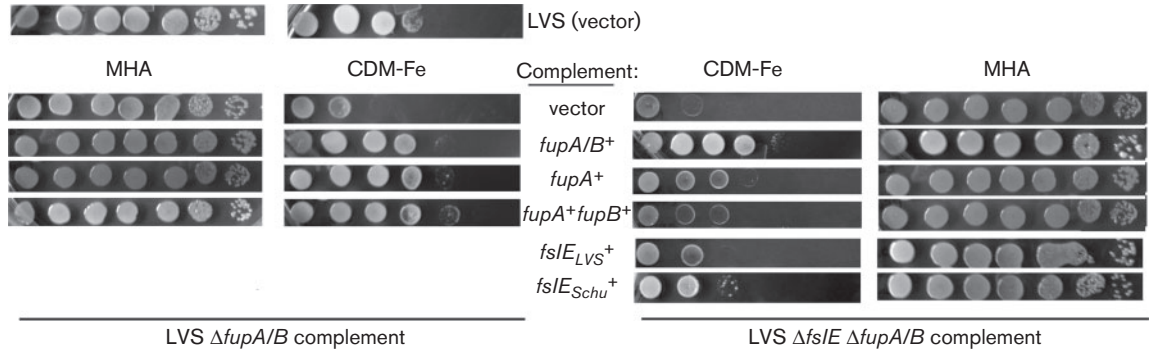

Fig. 4. Growth of LVS Δ*fupA/B* and LVS Δ*fslE* Δ*fupA/B* complements on iron-limiting agar. Tenfold serial dilutions of LVS Δ*fupA/B* or LVS Δ*fslE* Δ*fupA/B* complemented with different genes as indicated were spotted on iron-replete (MHA) or iron-limiting (CDM-Fe) plates. LVS carrying the vector alone was used as control. Growth was recorded after 2 days (MHA) or 4 days (CDM-Fe).

deregulated in the Δ*fupA/B* mutant (Sen *et al.*, 2010); as expected, levels of FslE were much greater in the Δ*fupA/B* mutant lysate and this deregulation was observed also in the outer membrane protein fraction (Fig. 7a). The results demonstrated that $FslE_{LVS}$ does localize to the outer membrane and that its assembly is not dependent on FupA/B.

We introduced the *fslE* orthologues independently into the LVS Δ*fslE* Δ*fupA/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). $FslE_{LVS}$ in lysates and in the membrane fraction was detected at levels not lower but higher than $FslE_{Schu}$. These results suggested that the sequence alterations in $FslE_{LVS}$ do not destabilize the protein. To test if the sequence differences might lead to reduced functionality of $FslE_{LVS}$, 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 $fslE_{Schu}$ complement showed more robust growth than the $fslE_{LVS}$ complement, suggesting that $FslE_{LVS}$ is indeed less effective than $FslE_{Schu}$ at promoting growth under iron limitation.

We tested the LVS Δ*fslE* Δ*fupA/B* strain complemented with each of the *fslE* orthologues in the siderophore utilization assay on iron-limiting CDM agar. As shown in Fig. 6(d, e), both complements formed growth haloes around sideropore-producing LVS but not around the siderophore-deficient Δ*fslA* 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 $\sim 3 \times 10^5$ c.f.u. of LVS seeded on the plate were sufficient for growth halo formation, the $fslE_{Schu}$ complement of LVS Δ*fslE* Δ*fupA/B* required $\sim 3 \times 10^6$ c.f.u. and the $fslE_{LVS}$ complement required $\sim 3 \times 10^7$ c.f.u. These results are consistent with differences in growth of serial dilutions of the strains (Fig. 4).

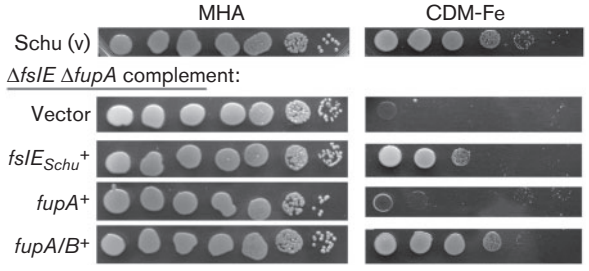

Fig. 5. Growth of Schu Δ*fslE* Δ*fupA* complements on iron-limiting agar. Serial dilutions of Schu Δ*fslE* Δ*fupA* complemented with endogenous *fslE* ($fslE_{Schu}$), *fupA* or *fupA/B* as indicated were tested for growth on iron-replete (MHA) or on iron-limiting (CDM-Fe) agar as in Fig. 4. Growth of the control Schu S4 bearing the vector alone is shown.

FupA/B and $FslE_{LVS}$ jointly mediate siderophore–^{55}Fe transport

Although growth of Schu S4 on iron-limiting CDM agar primarily requires FslE function, optimal growth relies on both FslE 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 ^{55}Fe complexed to siderophore in real-time and we have previously used this assay to establish the role of FslE 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 $fslE_{LVS}$ in this process. LVS bacteria grown for an extended period under iron limitation were incubated with ^{55}Fe complexed to purified siderophore in the presence of citrate and accumulation of ^{55}Fe was followed over time. The bacteria showed a siderophore-dependent increase in internalized ^{55}Fe with time (Fig. 8a).

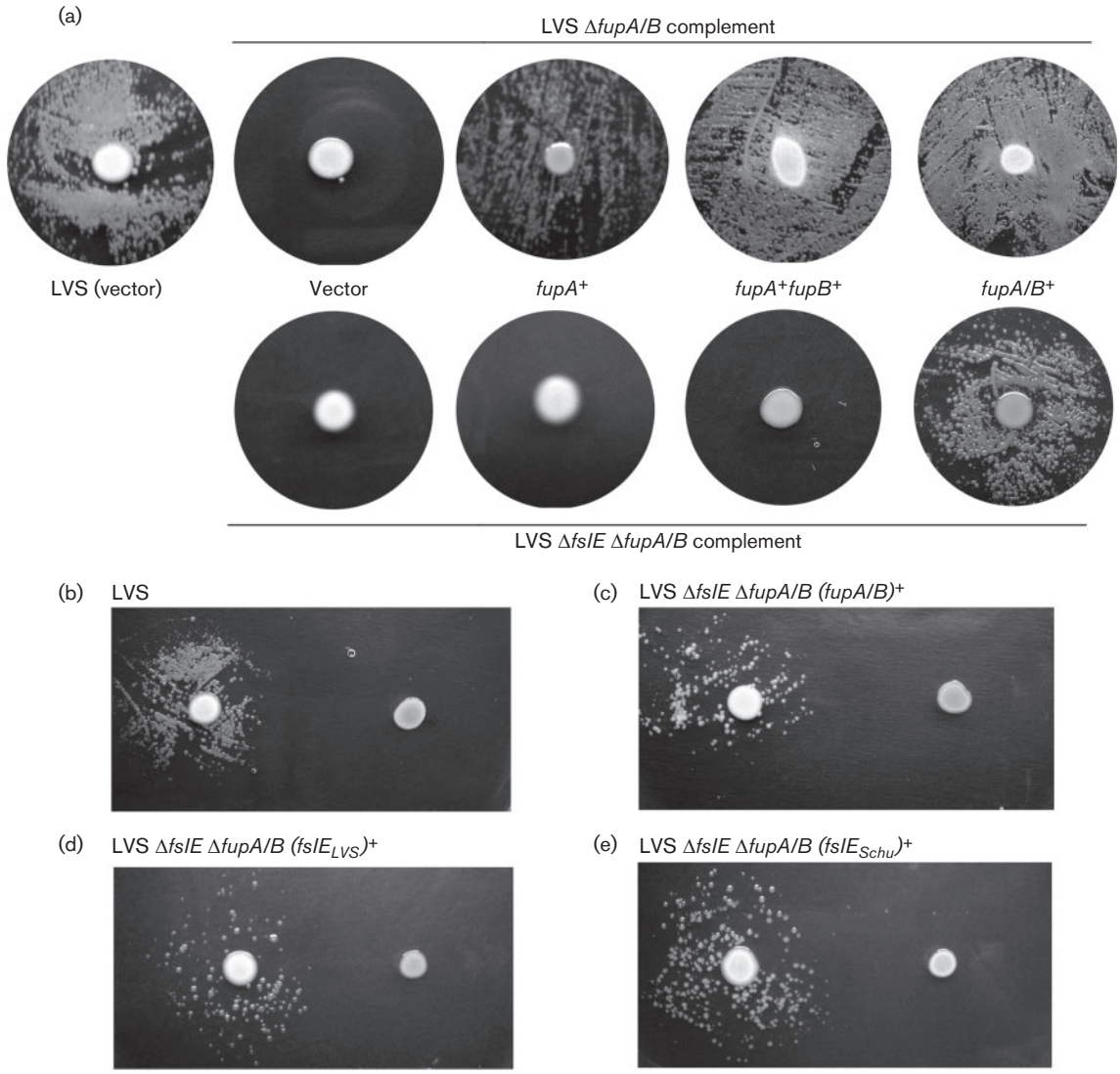

Fig. 6. Growth promotion by cross-feeding of siderophore. (a) Growth promotion of complements by LVS: 3×10^5 c.f.u. of bacterial strains as indicated were seeded on iron-limiting CDM agar and siderophore-producing LVS bacteria carrying the vector control were spotted in the centre. Growth halo formation around the spot was recorded after 3 days at 37 °C. (b–e) Siderophore dependence of growth halo formation. Bacteria as indicated were seeded on iron-limiting agar and tested for the ability to form growth haloes around spots of siderophore- producing LVS (left) and siderophore-deficient Δ*fslA* bacteria (right) individually spotted on the same plates. The strains were seeded at titres as follows: (b) 3×10^5 c.f.u. LVS; (c) 3×10^6 c.f.u. LVS Δ*fslE* Δ*fupA/B* complemented with *fupA/B*; (d) 3×10^7 c.f.u. LVS Δ*fslE* Δ*fupA/B* complemented with *fslE*$_{LVS}$; (e) 3×10^6 c.f.u. LVS Δ*fslE* Δ*fupA/B* complemented with *fslE*$_{Schu}$.

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 *fslE* 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 *fslE* in siderophore–iron transport, we examined the rates of siderophore-mediated ^{55}Fe transport in Δ*fupA/B* mutants complemented with the different paralogues (Fig. 8c). The Δ*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

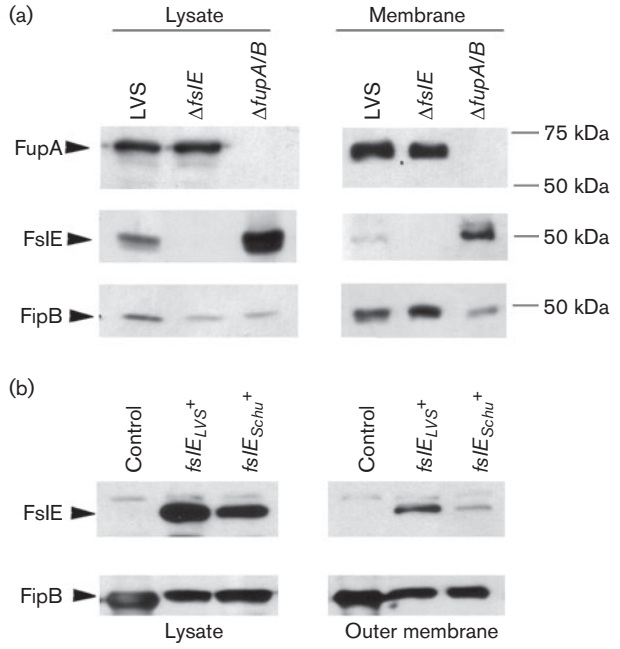

Fig. 7. Expression of *fslE* in bacterial strains. Whole-cell lysates and outer membrane preparations from bacteria as indicated were analysed by Western blotting for expression of FslE and FupA/B. FipB was used as loading control. (a) Lysates of LVS and Δ*fslE* and Δ*fupA/B* mutants and corresponding outer membrane proteins (5 μg) were analysed. (b) Lysates of LVS Δ*fslE* Δ*fupA/B* complemented with *fslE* derived from LVS ($fslE_{LVS}$) or from Schu S4 ($fslE_{Schu}$) and corresponding outer membrane proteins (2 μg) were analysed.

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 $fslE_{LVS}$ *in cis*. Restitution of *fupA/B* and $fslE_{LVS}$ 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 $fslE_{schu}$ complemented double mutant was notably increased over the $fslE_{LVS}$ complement, demonstrating that $FslE_{LVS}$ is less effective at siderophore–iron acquisition than $FslE_{Schu}$. 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 Δ*fslE* Δ*fupA* 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 $FslE_{LVS}$ 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 co-operatively in transport. Although FupA did not have siderophore-transport activity, its ability to increase transport activity in the Δ*fupA/B* mutant (Fig. 8c) suggested that FupA could support $FslE_{LVS}$ 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 $FslE_{LVS}$ while $FslE_{LVS}$ itself has reduced functionality in siderophore–iron transport as compared with $FslE_{Schu}$. 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–ExbB–ExbD complex, which transduces energy from the

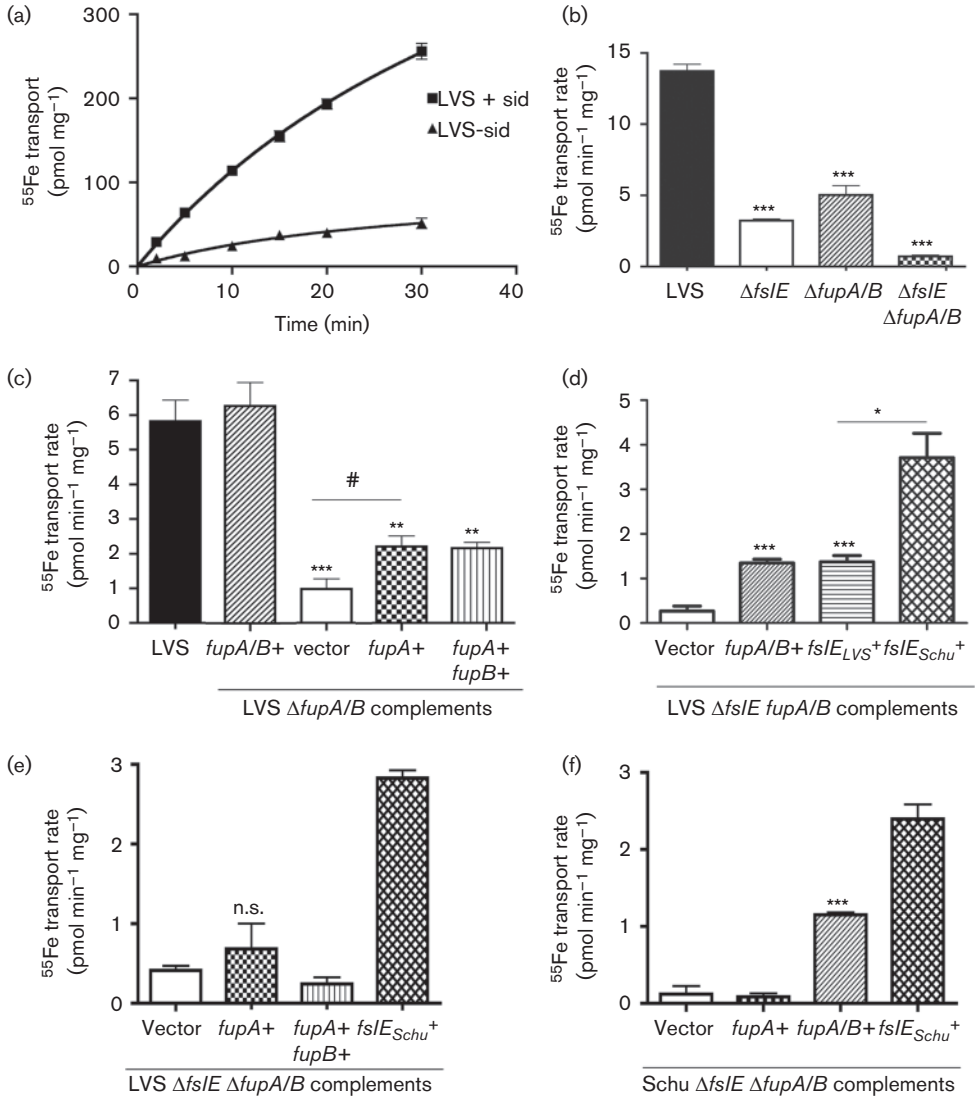

Fig. 8. Siderophore-mediated ^{55}Fe transport. (a) Siderophore-dependent accumulation of ^{55}Fe by LVS. LVS bacteria were grown for 16 h under iron limitation, and diluted and further grown for 24 h in iron-limiting media. The bacteria were washed and incubated with ^{55}Fe in the presence (+sid) or 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 Δ*fslE*, Δ*fupA/B* or Δ*fslE* Δ*fupA/B* mutants grown in iron-limiting media for 20 h. (c) Rates of siderophore-mediated ^{55}Fe transport in LVS Δ*fupA/B* complemented with different genes as indicated. (d) and (e) Rates of siderophore-mediated ^{55}Fe transport in LVS Δ*fslE* Δ*fupA/B* complemented by *fupA/B* and by *fslE* orthologues from the LVS and Schu S4 backgrounds. (f) Rates of siderophore-mediated ^{55}Fe transport in Schu Δ*fslE* Δ*fupA/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.

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.

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 three 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 *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 *F. tularensis* subsp. *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 *et al.*, 2008, 2012; Sen *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 paralogue 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 FupA 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 *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 intraperitoneally in mice (Fortier *et al.*, 1991; Salomonsson *et al.*, 2009). However, deletion of the *fupA/B* gene results in significant attenuation also by the intraperitoneal route of infection and deletion of the *fslE* locus in addition leads to further attenuation (Sen *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 *F. tularensis* as pathogens in the mammalian host.

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