A 55 kDa hypothetical membrane protein is an iron-regulated virulence factor of *Francisella tularensis* subsp. *novicida* U112

Timothy S. Milne,¹ Stephen L. Michell,^{1†} Helen Diaper,¹ Per Wikström,² Kerstin Svensson,² Petra C. F. Oyston^{1,3} and Richard W. Titball^{1,4†}

Correspondence

Timothy S. Milne
TSMILNE@dstl.gov.uk

¹Defence Science and Technology Laboratory, Porton Down, Salisbury SP4 0JQ, UK

²Swedish Defence Research Agency, SE-901 82 Umeå, Sweden

³Department of Infection, Immunity and Inflammation, Maurice Shock Building, University of Leicester, PO Box 138, Leicester LE1 9HN, UK

⁴Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK

Iron is an important nutritional requirement for bacteria due to its conserved role in many essential metabolic processes. As a consequence of the lack of freely available iron in the mammalian host, bacteria upregulate a range of virulence factors during infection. Transcriptional analysis of *Francisella tularensis* subsp. *novicida* U112 grown in iron-deficient medium identified 21 genes upregulated in response to this condition, four of which were attributed to a siderophore operon. In addition, a novel iron-regulated gene, *FTT0025*, was identified which is part of this operon and encodes a 55 kDa hypothetical membrane protein. When grown on chrome azurol S agar, the *F. tularensis* subsp. *novicida* U112 Δ *FTT0025* mutant produced an increased reaction zone compared with the wild-type, suggesting that siderophore production was unaffected but that the bacteria may have a deficiency in their ability to re-sequester this iron-binding molecule. Furthermore, the Δ *FTT0025* mutant was attenuated in a BALB/c mouse model of infection relative to wild-type *F. tularensis* subsp. *novicida* U112.

Received 26 January 2007

Accepted 13 June 2007

INTRODUCTION

The onset of infection by pathogenic bacteria is usually achieved by invading and colonizing a particular host niche. Initially, a primary goal for invading cells is obtaining essential metabolites to promote successful bacterial replication. As iron is an essential nutrient, due in part to its conserved role in many vital microbial cellular processes (Wooldridge & Williams, 1993), iron acquisition is viewed as an important virulence trait for many bacterial pathogens. However, several paradoxical issues relate to the general acquisition and utilization of iron by biological systems. Firstly, although iron is the most abundant transition metal, its availability is restricted by the formation of insoluble ferric hydroxide under aerobic physiological conditions (Andrews, 1998). Furthermore, oxidative metabolism of free iron can form toxic hydroxyl radicals, which pose a threat to core cellular molecules (Halliwell & Gutteridge, 1984). Therefore, iron atoms are commonly solubilized and

maintained *in vivo* by incorporation into specific iron-binding proteins (Clarke *et al.*, 2001), thus restricting the availability of host iron to potential invading organisms. To counteract the iron-restricted host status, microbes can quickly adapt to this environmental cue by triggering the increased expression of specific virulence-associated mechanisms such as iron acquisition, via tightly regulated feedback (Litwin & Calderwood, 1993). One commonly deployed bacterial iron-acquisition mechanism is the production of a siderophore, a high-affinity iron-chelating molecule that can acquire iron from host iron-binding proteins including transferrin and lactoferrin. Following secretion, siderophores bind host iron and are transported back into the bacteria via cell-surface receptor-mediated uptake (Ratledge & Dover, 2000). Siderophores have been identified in many human pathogenic species, including *Pseudomonas*, *Burkholderia*, mycobacteria and *Salmonella* (Crosa, 1997; De Voss *et al.*, 1999; Ratledge & Dover, 2000).

The Gram-negative coccobacillus *Francisella tularensis* is an endemic zoonotic pathogen and the causative agent of the debilitating disease tularemia (Johansson *et al.*, 2000; Petersen & Schriefer, 2005). Of the four described *F. tularensis* subspecies, *F. tularensis* subsp. *tularensis* is

[†]Present address: School of Biosciences, Geoffrey Pope Building, University of Exeter, Exeter EX4 4QD, UK.

Abbreviations: CAS, chrome azurol S; LVS, live vaccine strain; MLD, median lethal dose; s.c., subcutaneous.

considered the most infectious for humans with an LD₅₀ of <10 c.f.u. delivered via the subcutaneous (s.c.) route (Gurycova, 1998). This subspecies is recognized as a cause for concern by the World Health Organization as a potential biological weapon (Casadevall & Pirofski, 2004). The least-virulent subspecies for humans is the rarely isolated *F. tularensis* subsp. *novicida* (Whipp *et al.*, 2003), although analysis of rRNA sequences has suggested a high degree of similarity at the genetic level amongst the different *F. tularensis* subspecies (Forsman *et al.*, 1994). The genetic relatedness of the subspecies enables less-virulent subspecies to serve as models for investigation of *Francisella* with less risk to laboratory personnel. Two recent independent *Francisella* studies highlighted a four-gene operon that was responsible for siderophore biosynthesis, positioned directly downstream of the *fur* global iron-regulator gene (Deng *et al.*, 2006; Sullivan *et al.*, 2006). The aims of this study were to confirm that *FTT0025*, an additional gene adjacent to this novel *Francisella* operon, is also involved in siderophore functionality and to examine the effects of deleting this gene on the virulence of *F. tularensis* subsp. *novicida* U112. (*FTT0025* has been used as the designation of the gene reported in this study in accordance with the *F. tularensis* subsp. *tularensis* SchuS4 annotation, although this gene has been denoted *FTN_1686* in the recently released *F. tularensis* subsp. *novicida* U112 annotation.)

METHODS

Bacterial strains and culture media. All bacterial strains and plasmids used are described in Table 1. *F. tularensis* subsp. *novicida* U112 was routinely cultured in Chamberlain's defined medium (CDM) (Chamberlain, 1965) at 37 °C with aeration. CDM was produced in sterile plasticware to prevent contamination with trace levels of iron, and was filter-sterilized (Nalgene 500 ml capacity, pore size 0.2 µm) prior to storage at 2–8 °C for 1 month. For iron-limitation experiments, CDM was prepared without FeSO₄·7H₂O. To

ensure further elimination of trace iron, filter-sterilized deferoxamine was added to pre-warmed media prior to use, giving a final concentration of 10 µM. Blood cysteine glucose agar (BCGA) plates containing 4 % (w/v) cysteine, 4 % (w/v) histidine, 5 % (w/v) glucose and 10 % (v/v) horse blood were used for plating out serial dilutions when determining *F. tularensis* subsp. *novicida* U112 culture cell counts and purity. Modified Thayer–Martin agar plates [100 ml GC agar base, 100 ml defibrinated horse blood, 1 % (w/v) IsoVitalax, 100 µg polymyxin B ml⁻¹ and 20 µg chloramphenicol ml⁻¹] were incubated at 37 °C for 24–72 h for the selection of *F. tularensis* subsp. *novicida* U112 integrants. Mutants were counter-selected by streaking colonies onto modified chloramphenicol-free Thayer–Martin agar supplemented with 5 % (w/v) sucrose. All *Escherichia coli* strains were grown in Luria–Bertani medium at 37 °C.

Iron-replete and iron-deficient growth of *F. tularensis* subsp. *novicida* U112.

An overnight *F. tularensis* subsp. *novicida* U112 culture was prepared by inoculating 20 ml CDM with approximately 3×10^9 cells and cultured to an OD₆₀₀ of 1.9. Bacteria were then subcultured into a 30 ml total volume of pre-warmed CDM to a concentration of 1.6×10^9 cells ml⁻¹ and incubated for a further 2.5 h. Cells were washed three times with PBS and resuspended in 30 ml pre-warmed CDM or 10 µM deferoxamine-supplemented CDM without FeSO₄·7H₂O to a density of 4.5×10^7 cells ml⁻¹. At *T*₀, and every subsequent hour, 1 ml of culture was removed and the OD₆₀₀ was measured. This experiment was repeated three separate times for the construction of accurate growth curves.

Nucleic acid isolation. RNA extraction was carried out using an RNeasy midi preparation kit (Qiagen) following treatment of approximately 4×10^9 c.f.u. with 2 vols RNAProtect bacterial reagent (Qiagen). RNA quantification was performed by spectrophotometric analysis using a NanoDrop ND-1000 (NanoDrop Technologies) and each RNA sample was adjusted to give a final concentration of 1 µg µl⁻¹.

All genomic DNA preparations were isolated using a Puregene DNA Isolation kit (Gentra Systems) and plasmid DNA was isolated using a PerfectPrep kit (Eppendorf), both according to the manufacturer's instructions.

Production of a *Francisella*-specific DNA microarray. A microarray containing 1937 *F. tularensis* subsp. *tularensis* SchuS4

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Description	Source
Strains		
<i>Escherichia coli</i>		
JM109	Host cloning strain	Promega
S17-λpir	Host strain used for transfer of suicide plasmid into <i>F. tularensis</i> subsp. <i>novicida</i> U112 by conjugation	This study
<i>F. novicida</i>		
U112	Wild-type <i>F. tularensis</i> subsp. <i>novicida</i> U112	This study
U112 Δ <i>FTT0025</i>	<i>F. tularensis</i> subsp. <i>novicida</i> U112 with unmarked in-frame deletion of <i>FTT0025</i>	This study
Plasmids		
pGEM-T Easy Vector System I	Cloning vector; Amp ^R	Promega
pTM6a	pGEM-T containing 1274 bp <i>FTT0025</i> left flanking region	This study
pTM7a	pGEM-T containing 1074 bp <i>FTT0025</i> right flanking region	This study
pTM8	pGEM-T containing PCR product of 2337 bp Δ <i>FTT0025</i> left and right flanking regions	This study
pSMP62	<i>Francisella</i> suicide vector containing <i>sacB</i> selection gene; Chlor ^R , Amp ^R	This study
pTM9Δ <i>FTT0025</i>	pSMP62 containing 2337 bp Δ <i>FTT0025</i> left and right flanking regions inserted at <i>SphI</i> site	This study

ORFs with a minimum size of 120 bp was constructed. This was based on ORF predictions from a concatenated version of the pre-annotated genome, obtained using GLIMMER and GLIMMER2 software. Each ORF was represented by one 50-mer oligonucleotide, designed and optimized by MWG Biotech. The array also contained control spots comprising 20 ORFs from 10 unrelated organisms. Arrays were printed on Nexterion A glass slides (SCHOTT Glass), coated with aminosilane, using a Biorobotics TAS Microgrid II Arrayer (Genomics Solutions). Each oligonucleotide was printed twice on each array, and the entire array was printed twice on each slide to give a total of four replicate spots per oligonucleotide. Oligonucleotides were fixed to the slides by baking in a dry oven at 180 °C for 2 h.

Microarray labelling reactions. Reverse transcription and incorporation of Cy3-dCTP or Cy5-dCTP fluorescent dye during cDNA synthesis from *F. tularensis* subsp. *novicida* U112 RNA was performed as described previously (Stewart *et al.*, 2002), with the exception that CyScript reverse transcriptase (GE Healthcare) was used in place of Superscript II. To enable a reference design experiment, *F. tularensis* subsp. *tularensis* SchuS4 genomic DNA was used as a background control for each slide, enabling global scaling during data analysis. For every RNA sample, a dye swap experiment was performed.

Microarray hybridization. Test and control Cy3/Cy5-labelled samples were mixed, co-purified using a MinElute DNA purification kit (Qiagen) and eluted into 10 µl dH₂O, to which 10 µl 1 × DIG Easy Hyb buffer (Roche) was added, followed by heating at 95 °C for 2 min. A 22 × 22 mm LifterSlip (Erie Scientific) was placed over each slide, under which 20 µl co-purified, labelled DNA was pipetted. Hybridization was performed by incubating the slides in sealed chambers at 42 °C overnight. After removing the coverslip, slides were then immersed in pre-warmed FT1 washing buffer (50 ml 20 × SSC, 25 ml 10 % SDS, 425 ml dH₂O) for two separate 15 min washes at 42 °C with gentle rotation. This process was repeated with FT2 washing buffer (6.25 ml 20 × SSC, 25 ml 10 % SDS, 468.75 ml dH₂O). The microarray slides were dried by centrifugation at 257 g for 7 min and stored in the dark until scanning.

Microarray analysis. Each microarray slide was scanned in the Cy3 channel (510–550 nm) and Cy5 channel (630–660 nm) using a GenePix 4000B scanner and GENEPix PRO software (Molecular Devices Corporation). The resultant TIFF digital image files were quantified using BLUEFUSE v3.1 software (BlueGnome) and saved as Excel 97 output files. To determine the most appropriate form of normalization, MA plots were constructed for each corresponding iron-replete/iron-deficient dataset for a given time point and replicate. From the data scatter pattern observed from these plots, linear normalization was then applied individually to each separate dataset. For this, quality control was performed by excluding positive- and negative-control gene spots from each dataset, as well as those with confidence values <0.1 (denoted in BLUEFUSE by confidence flag E). A normalizing constant, defined as the median channel 1/channel 2 log₂ ratio across all probes for a given array, was then calculated and subtracted from each probe log₂ ratio. After global scaling, the relative gene expression for each time point was determined by calculating the mean log₂ ratio difference across each paired iron-replete and iron-deficient dataset. To test the expression levels statistically, a significance *P* value was determined using a paired two-tailed *t*-test, and the Benjamini–Hochberg procedure for controlling false discovery rate was applied. Fold change levels were calculated for each gene, with those showing upregulation of ≥1.5-fold under iron-deficient growth considered biologically relevant.

RT-PCR. Oligonucleotide primers (25-mers) were designed for amplification of the *FTT0025* gene (forward: 5'-AGCAAGGTATA-ACCAACTACAATC-3'; reverse: 5'-TGCGCGTTGTGAGGAACT-ATAATC-3') and a region spanning *FTT0025* and *FTT0026* (forward:

5'-CACCTACTTGGAGCCGCCATATGTG-3'; reverse: 5'-CAAACC-AAAGCTGCGCATCAGCTTC-3') using CLONE MANAGER software (Scientific and Educational Software). RNA was pre-treated with a Turbo DNA-free kit (Ambion) according to the manufacturer's instructions and reversed transcribed using an Enhanced Avian RT First Strand Synthesis kit (Sigma). PCR was performed in 20 µl reactions containing 125 mM dNTPs, 1 µM oligonucleotides, 50 ng cDNA, 2 µl PCR buffer containing magnesium (Roche) and 0.1 µl *Taq* polymerase. PCRs were performed under the following conditions: denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final elongation at 72 °C for 7 min. The *F. tularensis* *fopA* gene was used as an internal control (forward: 5'-GGCCAGTGGTACTTAGGTGTAGATG-3'; reverse: 5'-GTATAGCACGCGACACCATCTTCAG-3'). Genomic DNA was included as a positive control, and dH₂O and RNA controls were performed to exclude the possibility of DNA contamination. Band sizes were compared under UV light after samples had been electrophoresed through a 1 % agarose gel.

Construction of a Δ FTT0025 mutagenesis plasmid. DNA manipulations were performed as described by Sambrook *et al.* (1989). The suicide plasmid for deletion of *FTT0025* was constructed as follows. Regions of DNA flanking the *FTT0025* gene were PCR-amplified from *F. tularensis* subsp. *novicida* U112 genomic DNA using the primer pairs P1/P2 and P3/P4 (P1: 5'-ATGCATGCCATTCTTGCTGCCATTACTACCTTTAC-3'; P2: 5'-GACGTACGAAAGGGGGTAATTGATAATACTAAAAAG-3'; P3: 5'-TACGTACGACAATAGATATGGCTGTATATCTTTAAG-3'; P4: 5'-CAGAGCTCGCATGCTAAGCCCAAATTAGGTAATCCA-3'; engineered restriction sites are underlined). The PCR products were cloned into the plasmid pGEM-T Easy (Promega) to give plasmids pTM6 and pTM7. These plasmids were digested with *Bsi*WI and ligated. This ligation mixture was used as template in a PCR using the primer pair P1/P4. The product of 2337 bp from this reaction was digested with *Sph*I and cloned into pSMP62 [a gift from Dr S. Michell, Defence Science and Technology Laboratory, Porton Down, UK, derived from pSMP22 (Thomas *et al.*, 2007), containing a *Bst*EII chloramphenicol-resistance cassette], to generate pTM9. This resultant plasmid was electroporated into *E. coli* S17- λ pir.

Conjugal transfer of plasmids. Allelic replacement by conjugation was performed based on the methods described by Golovliov *et al.* (2003). Transconjugants were selected on chloramphenicol-supplemented Thayer–Martin agar plates and merodiploids arising from chromosomal integration of the suicide plasmid were resolved by plating on Thayer–Martin agar supplemented with 5 % (w/v) sucrose (Pelicic *et al.*, 1996). Sucrose-resistant colonies were screened for the presence of a wild-type or mutant copy of *FTT0025* by PCR using the primer pair P5 and P6 (P5: 5'-CAGCGGCAGTTGGCGTAGTATC-ACC-3'; P6: 5'-TACCGGTGCTATTACCAGGAGCAAC-3').

Southern blot hybridization. For Southern blot hybridization, *Eco*RV digests of 2–3 µg genomic DNA from *F. tularensis* subsp. *novicida* U112 wild-type and Δ FTT0025 mutant were separated by electrophoresis on a 1 % agarose gel and transferred to a nylon membrane. The pTM6 insert was labelled with DIG-11-dUTP during PCR amplification with the primer pair P1/P2 and used as a probe to hybridize to the membrane. DNA fragments to which the probe hybridized were detected in a chemiluminescent assay (CSPD substrate, 30 min exposure to X-ray film).

Growth of *F. tularensis* subsp. *novicida* U112 wild-type and Δ FTT0025 mutant in CDM. Volumes known to contain approximately 1.57×10^{11} cells of *F. tularensis* subsp. *novicida* U112 wild-type and its isogenic Δ FTT0025 mutant were subcultured into 30 ml fresh pre-warmed CDM. Incubation was carried out aerobically at 37 °C for 8 h, with shaking at 180 r.p.m. At *T*₀, and each subsequent hour,

1 ml culture was removed and the OD₆₀₀ was measured. This experiment was repeated three times for the construction of accurate growth curves.

Chrome azurol S (CAS) assay. CAS-CDM agar plates were prepared as described previously (Deng *et al.*, 2006). Standardized CDM suspensions of *F. tularensis* subsp. *novicida* U112 wild-type and its isogenic Δ FTT0025 mutant containing approximately 5×10^8 c.f.u. ml⁻¹ were prepared as described previously. From these, 50 μ l was inoculated onto the centre of separate CAS-CDM plates. This procedure was performed in triplicate and plates were incubated at 37 °C for 3 days. At 24 h intervals, electronic callipers were used to measure the observable zone sizes (Machuca & Milagres, 2003).

Attenuation study of *F. tularensis* subsp. *novicida* U112 Δ FTT0025 mutant. Challenge doses of *F. tularensis* subsp. *novicida* U112 wild-type and its isogenic Δ FTT0025 mutant were prepared by serially diluting cultures of approximately 10^9 c.f.u. ml⁻¹ (as determined by OD₆₀₀). All challenges were prepared in PBS, and bacterial enumeration was confirmed by plating out onto BCGA plates. To investigate the level of attenuation, groups of six age-matched (8–10-week-old) adult female BALB/c mice (Charles River) were challenged via the s.c. route. Mice showing obvious signs of distress were culled humanely. Significance of attenuation was determined by the Mantel–Haenszel log rank method using GRAPHPAD PRISM v4.0. The median lethal dose (MLD) of the *F. tularensis* subsp. *novicida* U112 Δ FTT0025 mutant was calculated using the method of Reed & Muench (1938). Virulence studies were carried out in accordance with legislative and welfare requirements and after prior authorization by an on-site ethics committee.

RESULTS AND DISCUSSION

Growth restriction of *F. tularensis* subsp. *novicida* U112 occurs in the absence of free iron

The growth of bacteria in iron-deficient medium was used as a strategy to mimic the *in vivo* iron-restricted status of the host. As shown in Fig. 1, growth of wild-type *F.*

Fig. 1. Dependence of growth of *F. tularensis* subsp. *novicida* U112 on iron. Bacteria were cultured in iron-replete (◆) or iron-deficient (■) CDM for 4 h as described in Methods. The data represent the mean of three separate experiments, with error bars indicating 95 % confidence limits.

tularensis subsp. *novicida* U112 was similar in iron-replete and iron-deficient media for up to approximately 2 h. Subsequently, bacteria in iron-replete medium continued to grow exponentially, whilst bacteria in iron-deficient medium appeared to enter stationary phase. These results suggest that cells grown without a source of exogenous iron deplete their internal stores of iron within about 4 h, after which growth ceases.

Identification of a previously characterized *F. novicida* subsp. *novicida* U112 iron-regulated operon

As iron becomes limiting, cells are likely to express genes required to adapt to and survive this environment. To identify these genes in *F. tularensis* subsp. *novicida* U112, we examined gene expression at three separate time points during growth in iron-replete or iron-deficient medium. As described in Methods, a *Francisella*-specific DNA microarray was used to analyse RNAs expressed at 1, 2 and 3.5 h of growth in the presence or absence of iron. Using a false discovery rate of 0.05 and focusing on changes in expression of >1.5-fold, 21 genes were identified as upregulated under iron-deficient conditions (data not shown). Four upregulated genes from this list (*FTT0029*, *FTT0028*, *lysA* and *FTT0026*) are positioned directly downstream of *fur*, the global iron regulator, indicating the presence of an iron-regulated operon (see Fig. 2). This observation is in good agreement with results reported in two recent publications that defined an iron-regulated operon present in both *F. tularensis* subsp. *tularensis* and the less-virulent *F. tularensis* subsp. *holarctica* and indicated that this operon is involved in siderophore biosynthesis (Deng *et al.*, 2006; Sullivan *et al.*, 2006). Sullivan and co-workers detected the presence of a siderophore in the culture filtrate of *F. tularensis* subsp. *holarctica* live vaccine strain (LVS) and *F. tularensis* subsp. *tularensis* SchuS4, and demonstrated that this operon was essential for siderophore biosynthesis, whilst Deng and colleagues employed microarray technology to study gene expression in LVS cultured under iron-replete or iron-deficient conditions, identifying the same four-gene operon as the most highly upregulated genes in LVS when iron was limiting.

Within our microarray data was the novel observation that a fifth gene, *FTT0025*, a hypothetical membrane protein, located directly adjacent to *FTT0026*, was upregulated coordinately with the four genes of this operon. Expression of *FTT0025* was upregulated 4.54-fold ($P=0.08$) at the 3.5 h time point. The data also suggested that *FTT0025* expression was upregulated at 1 h (1.51-fold, $P=0.68$) and 2 h (3.10-fold, $P=0.11$); however, these results did not achieve statistical significance. Neither Deng *et al.* (2006) nor Sullivan *et al.* (2006) identified *FTT0025* as a member of the siderophore biosynthetic operon, and thus we undertook efforts to confirm the association of this gene with the operon.

Fig. 2. Organization of the chromosome region containing an iron-regulated operon responsible for siderophore production in *Francisella* species. ORFs on the complementary DNA strand are indicated with a 'c'.

Expression of *FTT0025* is upregulated during iron deficiency

To confirm the upregulation of *FTT0025* expression, we employed RT-PCR as described in Methods. PCR primers were designed to amplify a 1301 bp product of the *FTT0025* gene. As an internal housekeeping control, primers were designed to amplify a 584 bp PCR product of the *fopA* gene, an ORF whose expression is known to be unaffected by the amount of iron in the culture medium (Deng *et al.*, 2006). The PCR results confirmed that *FTT0025* was indeed upregulated by *F. tularensis* subsp. *novicida* U112 during iron-deficient growth (Fig. 3; compare lanes 4 and 5). The equal amounts of *fopA* product (Fig. 3; lanes 11 and 12) confirmed that equal masses of RNA were used in these reactions. The presence of RNA-negative controls ensured that no DNA contamination was present in the initial RNA samples after Turbo DNA-free treatment, whilst H₂O negative controls

confirmed that there was no DNA contamination in the PCR master mixes.

FTT0025 is co-transcribed with the siderophore operon

To determine whether *FTT0025* is co-transcribed as part of the siderophore biosynthesis operon, the same RT-PCR procedure was followed, this time using primers designed to amplify a 1075 bp region bridging *FTT0025* and *FTT0026*. The data presented in Fig. 4 revealed that transcription of mRNA encoding both *FTT0025* and *FTT0026* was upregulated by *F. tularensis* subsp. *novicida* U112 under conditions of iron deficiency. As in Fig. 3, controls for RNA normalization and absence of DNA contamination supported this conclusion. This result confirmed that expression of the siderophore biosynthesis operon is upregulated under iron-deficient conditions and, moreover, that *FTT0025* is co-transcribed as part of this operon.

Fig. 3. RT-PCR examination of the expression of *FTT0025* by *F. tularensis* subsp. *novicida* U112 in the presence and absence of iron. Lanes: 1, 8 and 15, molecular mass markers; 2–7, amplification of *FTT0025*; 9–14, amplification of *fopA*. Lanes: 2 and 9, genomic DNA positive control; 3 and 10, no-DNA controls; 4 and 11, cDNA from iron-replete conditions; 5 and 12, cDNA from iron-deficient conditions; 6 and 13, RNA from iron-replete conditions; 7 and 14, RNA from iron-deficient conditions.

Fig. 4. RT-PCR examination of the co-transcription of *FTT0025* and *FTT0026* in *F. tularensis* subsp. *novicida* U112 in the presence or absence of iron. Lanes: 1, 8 and 15, molecular mass markers; 2–7, amplification of fragment bridging *FTT0025* and *FTT0026*; 9–14, amplification of *fopA*. See Fig. 3 legend for details of lanes.

Production of the *F. tularensis* subsp. *novicida* U112Δ*FTT0025* mutant

Having shown that *FTT0025* was a member of the siderophore operon, we wanted to determine the role of this gene in siderophore production and/or function. The approach we adopted was to generate a targeted deletion of the ORF of *FTT0025* in *F. tularensis* subsp. *novicida* U112. An in-frame unmarked *F. tularensis* subsp. *novicida* U112Δ*FTT0025* mutant was generated by allelic replacement (see Methods). Of 20 sucrose-resistant colonies, 15 had a mutant phenotype. This deletion phenotype was confirmed by Southern blot hybridization (Fig. 5). Due to the presence of an *EcoRV* site in the *FTT0025* gene, it was determined that a DIG-labelled left flank region probe would hybridize to a 2405 bp *EcoRV* fragment of wild-type genomic DNA, whilst this probe would recognize a 2572 bp *EcoRV* fragment from a genome from which the *FTT0025* ORF has been deleted. As shown in Fig. 5, we indeed observed only the 2572 bp fragment in genomic DNA isolated from the presumptive mutant (Fig. 5; lane 3), thus confirming the deletion of this ORF in this strain.

Deletion of *FTT0025* affects siderophore activity

The function of *FTT0025* is unknown due to the lack of homologues or orthologues of this gene in other bacterial genomes. However, certain sequence elements led to the annotation of *FTT0025* as a hypothetical membrane protein. For example, PSORT predicts *FTT0025* to be an inner-membrane protein (Huntley *et al.*, 2007). To elucidate further the function of *FTT0025*, a series of simple characterization experiments were performed in which we compared the phenotype of *F. tularensis* subsp. *novicida* U112 wild-type and the Δ*FTT0025* mutant. In liquid medium, the Δ*FTT0025* mutant appeared to enter exponential-phase growth more slowly than wild-type, after which its growth in exponential phase paralleled that of wild-type (Fig. 6). In addition, both strains appeared to achieve a similar cell density upon reaching stationary phase. To determine whether siderophore secretion was affected by the deletion of *FTT0025*, cells were cultured on CAS agar as described previously (Schwyn & Neilands, 1987). A larger zone of siderophore secretion was produced

by the Δ*FTT0025* mutant compared with the wild-type (Fig. 7a). This disparity was observed over the course of 3 days of growth on CAS agar (Fig. 7b), suggesting that deletion of *FTT0025* resulted in increased siderophore secretion or an inability to retrieve siderophore back into the cell. These findings, coupled with the predictions of PSORT, suggest that *FTT0025* plays a role in siderophore uptake. To date, genes thought of as essential for promoting uptake of iron by bacteria, such as *tonB*, are not found in the *Francisella* genome. Therefore, *FTT0025*

Fig. 5. Confirmation of the targeted deletion of *FTT0025* from *F. tularensis* subsp. *novicida* U112. DNA isolation and Southern blot hybridization were performed as described in Methods. Lanes: 1 and 4, molecular mass markers; 2, *EcoRV* digest of wild-type *F. tularensis* subsp. *novicida* U112; 3, *EcoRV* digest of *F. tularensis* subsp. *novicida* U112Δ*FTT0025*. Expected wild-type band, 2405 bp; expected mutant band, 2572 bp.

Fig. 6. Dependence of growth of *F. tularensis* subsp. *novicida* on expression of *FTT0025*. Growth curves were performed as described in Methods for *F. tularensis* subsp. *novicida* U112 wild-type (◆) and *F. tularensis* subsp. *novicida* U112Δ*FTT0025* (■). The data represent the mean of three separate experiments, with error bars indicating 95 % confidence limits.

may serve this essential function. It is important to note that *F. tularensis* subsp. *novicida* U112Δ*FTT0025* did reach a cell density similar to that of wild-type, suggesting that other iron-uptake mechanisms are available to the bacteria. As the CAS assay demonstrated, siderophore production and secretion was by no means hindered, confirming that the in-frame deletion of *FTT0025* had no upstream effects on the other siderophore genes contained within the biosynthesis operon. It is also possible that the increased siderophore levels we observed related to further upregulation in an attempt to compensate for the lack of uptake.

The *F. tularensis* subsp. *novicida* U112Δ*FTT0025* mutant is attenuated

Given the limited availability of iron within the normal hosts of *F. tularensis* subsp. *novicida* U112, it was expected that a reduced ability to acquire iron would result in reduced virulence. To test this hypothesis directly, we performed an attenuation study in BALB/c mice as described in Methods. The results of this study are summarized in Table 2. All mice challenged via the s.c. route with 10^3 or 10^4 c.f.u. of wild-type *F. tularensis* subsp. *novicida* U112 died by day 4. However, four of the six mice challenged with 10^3 c.f.u. of *F. tularensis* subsp. *novicida* U112Δ*FTT0025* survived; this represents a significant level of attenuation ($P=0.0197$). The MLD for wild-type *F. tularensis* subsp. *novicida* U112 has been calculated previously to be 3.4×10^2 c.f.u. via the s.c. route in BALB/c mice (P. C. F. Oyston, personal communication). However, the MLD of *F. tularensis* subsp. *novicida* U112Δ*FTT0025* was calculated as 5.3×10^3 c.f.u. via the s.c. route, corresponding to a greater than 10-fold increase for this strain. Although two of six mice survived challenge with 10^4 c.f.u. of *F. tularensis* subsp. *novicida* U112Δ*FTT0025*, this result was not statistically significant ($P=0.0996$).

Fig. 7. (a) Siderophore production by *F. tularensis* subsp. *novicida* U112 wild-type and the Δ*FTT0025* mutant. Colorimetric detection on CAS agar was performed as described in Methods for *F. tularensis* subsp. *novicida* U112 wild-type (A) and *F. tularensis* subsp. *novicida* U112Δ*FTT0025* mutant (B). (b) Dependence of secretion of siderophore over time on *FTT0025*. CAS-based determination of siderophore production was performed as described in Methods for *F. tularensis* subsp. *novicida* U112 wild-type (◆) and *F. tularensis* subsp. *novicida* U112Δ*FTT0025* mutant (■). The data represent the mean of three separate experiments, with error bars indicating 95 % confidence limits.

Targeting siderophore receptors has attenuated other bacterial pathogens. In *Bacillus anthracis*, deletion of the siderophore biosynthesis gene *asbA* attenuated virulence in BALB/c mice (Cendrowski *et al.*, 2004). Similarly, alcaligin biosynthesis by *Bordetella bronchiseptica* was shown to be essential for maximal virulence in a swine infection model (Register *et al.*, 2001). Generating a *fepA iron cir* triple mutant in *Salmonella enterica*, each gene encoding a different siderophore receptor, resulted in attenuation, and the mutant was capable of inducing a protective immune response in mice (Rabsch *et al.*, 2003; Williams *et al.*, 2006).

Table 2. Dependence of virulence on expression of *FTT0025*

In vivo assessment of virulence was performed as described in Methods.

Challenge strain	Dose (c.f.u.)	No. of survivors	Time to death (days)
<i>F. tularensis</i> subsp. <i>novicida</i> U112Δ <i>FTT0025</i>	4.05×10^4	2/6	3, 4, 4, 4
	4.05×10^3	4/6	3, 5
<i>F. tularensis</i> subsp. <i>novicida</i> U112	4.2×10^4	0/6	3, 3, 3, 4, 4, 4
	4.2×10^3	0/6	3, 4, 4, 4, 4, 4

Although *F. tularensis* subsp. *holarctica* LVS is effective at preventing human cases of tularemia, the lack of a definition for the mechanism of attenuation for this strain has delayed the license of LVS as a vaccine. However, the ability of this attenuated *Francisella* strain to induce a protective immune response in humans lends promise to the generation of a well-characterized attenuated vaccine. The results from this study show that the previously described *Francisella* siderophore biosynthesis operon also includes the 55 kDa hypothetical membrane protein *FTT0025*, which appears to play an important role in siderophore uptake. As observed for a number of bacterial pathogens, deleting this putative siderophore receptor results in a significant loss of bacterial virulence, once again confirming the association between bacterial iron acquisition from the host and establishment of infection. Further work is currently under way to assess the protective efficacy of the *F. tularensis* subsp. *novicida* U112Δ*FTT0025* mutant. Results confirming its suitability as a rationally attenuated protective mutant will lead to the generation of the Δ*FTT0025* mutant in the most virulent subspecies, *F. tularensis* subsp. *tularensis* SchuS4.

ACKNOWLEDGEMENTS

We acknowledge Dr K. Svennson at FOI, Sweden, for the assignation of putative microarray ORFs using GLIMMER. We thank Dr N. Silman and J. Oshota at the Health Protection Agency, Porton Down, UK, and Dr P. Butcher and the rest of the Bacterial Microarray Group at St George's Hospital, London, UK, for printing the *Francisella* arrays. We express gratitude to R. Dean and Dr M. Nelson for their technical assistance. We thank A. Hunter and Dr T. Laws at Dstl for their kind help with statistical analysis of the data. We also thank Dr M. Bolanowski for his critical reading of the manuscript. This contract has been funded in whole or in part with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, USA, under contract no. NO1-AI-50041.

REFERENCES

- Andrews, S. C. (1998). Iron storage in bacteria. *Adv Microb Physiol* **40**, 281–351.
- Casadevall, A. & Pirofski, L.-A. (2004). The weapon potential of a microbe. *Trends Microbiol* **12**, 259–263.
- Cendrowski, S., MacArthur, W. & Hanna, P. (2004). *Bacillus anthracis* requires siderophore biosynthesis for growth in macrophages and mouse virulence. *Mol Microbiol* **51**, 407–417.
- Chamberlain, R. E. (1965). Evaluation of live tularemia vaccine prepared in a chemically defined medium. *Appl Microbiol* **13**, 232–235.
- Clarke, T. E., Tari, L. W. & Vogel, H. J. (2001). Structural biology of bacterial iron uptake systems. *Curr Top Med Chem* **1**, 7–30.
- Crosa, J. H. (1997). Signal transduction and transcriptional and posttranscriptional control of iron-regulated genes in bacteria. *Microbiol Mol Biol Rev* **61**, 319–336.
- Deng, K., Blick, R. J., Liu, W. & Hansen, E. J. (2006). Identification of *Francisella tularensis* genes affected by iron limitation. *Infect Immun* **74**, 4224–4236.
- De Voss, J. J., Rutter, K., Schroeder, B. G. & Barry, C. E., III (1999). Iron acquisition and metabolism by mycobacteria. *J Bacteriol* **181**, 4443–4451.
- Forsman, M., Sandstrom, G. & Sjostedt, A. (1994). Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. *Int J Syst Bacteriol* **44**, 38–46.
- Golovliov, I., Sjostedt, A., Mokrievich, A. & Pavlov, V. (2003). A method for allelic replacement in *Francisella tularensis*. *FEMS Microbiol Lett* **222**, 273–280.
- Gurycova, D. (1998). First isolation of *Francisella tularensis* subsp. *tularensis* in Europe. *Eur J Epidemiol* **14**, 797–802.
- Halliwell, B. & Gutteridge, J. M. C. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* **219**, 1–14.
- Huntley, J. F., Conley, P. G., Hagman, K. E. & Norgard, M. V. (2007). Characterization of *Francisella tularensis* outer membrane proteins. *J Bacteriol* **189**, 561–574.
- Johansson, A., Ibrahim, A., Goransson, I., Eriksson, U., Gurycova, D., Clarridge, J. E. & Sjostedt, A. (2000). Evaluation of PCR-based methods for discrimination of *Francisella* species and subspecies and development of a specific PCR that distinguishes the two major subspecies of *Francisella tularensis*. *J Clin Microbiol* **38**, 4180–4185.
- Litwin, C. M. & Calderwood, S. B. (1993). Role of iron in regulation of virulence genes. *Clin Microbiol Rev* **6**, 137–149.
- Machuca, A. & Milagres, A. M. F. (2003). Use of CAS-agar plate modified to study the effect of different variables on the siderophore production by *Aspergillus*. *Lett Appl Microbiol* **36**, 177–181.
- Pelacic, V., Reyat, J.-M. & Gicquel, B. (1996). Expression of the *Bacillus subtilis* *sacB* gene confers sucrose sensitivity on mycobacteria. *J Bacteriol* **178**, 1197–1199.
- Petersen, J. M. & Schrieffer, M. E. (2005). Tularemia: emergence/re-emergence. *Vet Res* **36**, 455–467.
- Rabsch, W., Methner, U., Voight, W., Tschape, H., Reissbrodt, R. & Williams, P. H. (2003). Role of receptor proteins for enterobactin and 2,3-dihydroxybenzoylserine in virulence of *Salmonella enterica*. *Infect Immun* **71**, 6953–6961.
- Ratledge, C. & Dover, L. G. (2000). Iron metabolism in pathogenic bacteria. *Annu Rev Microbiol* **54**, 881–941.
- Reed, L. J. & Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *Am J Epidemiol* **27**, 493–497.
- Register, K. B., Ducey, T. F., Brockmeier, S. L. & Dyer, D. W. (2001). Reduced virulence of a *Bordetella bronchiseptica* siderophore mutant in neonatal swine. *Infect Immun* **69**, 2137–2143.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

- Schwyn, B. & Neilands, J. B. (1987).** Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**, 47–56.
- Stewart, G. R., Wernisch, L., Stabler, R., Mangan, J. A., Hinds, J., Laing, K. G., Young, D. B. & Butcher, P. D. (2002).** Dissection of the heat-shock response in *Mycobacterium tuberculosis* using mutants and microarrays. *Microbiology* **148**, 3129–3138.
- Sullivan, J. T., Jeffery, E. F., Shannon, J. D. & Ramakrishnan, G. (2006).** Characterization of the siderophore of *Francisella tularensis* and role of *fslA* in siderophore production. *J Bacteriol* **188**, 3785–3795.
- Thomas, R. M., Titball, R. W., Oyston, P. C., Griffin, K., Waters, E., Hitchen, P. G., Michell, S. L., Grice, I. D., Wilson, J. C. & other authors (2007).** The immunologically distinct O antigens from *Francisella tularensis* subspecies *tularensis* and *Francisella novicida* are both virulence determinants and protective antigens. *Infect Immun* **75**, 371–378.
- Whipp, M. J., Davis, J. M., Lum, G., de Boer, J., Zhou, Y., Bearden, S. W., Petersen, J. M., Chu, M. C. & Hogg, G. (2003).** Characterization of a *novicida*-like subspecies of *Francisella tularensis* isolated in Australia. *J Med Microbiol* **52**, 839–842.
- Williams, P. H., Rabsch, W., Methner, U., Voigt, W., Tschape, H. & Reissbrodt, R. (2006).** Catecholate receptor proteins in *Salmonella enterica*: role in virulence and implications for vaccine development. *Vaccine* **24**, 3840–3844.
- Wooldridge, K. G. & Williams, P. H. (1993).** Iron uptake mechanisms of pathogenic bacteria. *FEMS Microbiol Rev* **12**, 325–348.