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

1 Defence Science and Technology Laboratory, Porton Down, Salisbury SP4 0JQ, UK
2 Swedish Defence Research Agency, SE-901 82 Umeå, Sweden
3 Department of Infection, Immunity and Inflammation, Maurice Shock Building, University of Leicester, PO Box 138, Leicester LE1 9HN, UK
4 Department 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 ΔFTT0025 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 ΔFTT0025 mutant was attenuated in a BALB/c mouse model of infection relative to wild-type *F. tularensis* subsp. *novicida* U112.

**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 & Schriever, 2005). Of the four described *F. tularensis* subspecies, *F. tularensis* subsp. *tularensis* is...
considered the most infectious for humans with an LD\textsubscript{50} 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 \textit{F. tularensis} subsp. novicida (Whipp \textit{et al.}, 2003), although analysis of rRNA sequences has suggested a high degree of similarity at the genetic level amongst the different \textit{F. tularensis} subspecies (Forsman \textit{et al.}, 1994). The genetic relatedness of the subspecies enables less-virulent subspecies to serve as models for investigation of \textit{Francisella} with less risk to laboratory personnel. Two recent independent \textit{Francisella} studies highlighted a four-gene operon that was responsible for siderophore biosynthesis, positioned directly downstream of the \textit{fur} global iron-regulator gene (Deng \textit{et al.}, 2006; Sullivan \textit{et al.}, 2006). The aims of this study were to confirm that \textit{FTT0025}, an additional gene adjacent to this novel \textit{Francisella} operon, is also involved in siderophore functionality and to examine the effects of deleting this gene on the virulence of \textit{F. tularensis} subsp. novicida U112. (\textit{FTT0025} has been used as the designation of the gene reported in this study in accordance with the \textit{F. tularensis} subsp. \textit{tularensis} SchuS4 annotation, although this gene has been denoted \textit{FTN\_1686} in the recently released \textit{F. tularensis} subsp. \textit{novicida} U112 annotation.)

**METHODS**

**Bacterial strains and culture media.** All bacterial strains and plasmids used are described in Table 1. \textit{F. tularensis} subsp. \textit{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 limitations experiments, CDM was prepared without FeSO\textsubscript{4}·7H\textsubscript{2}O. 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 \textit{F. tularensis} subsp. \textit{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) IsoVitalex, 100 μg polymixin B ml\textsuperscript{−1} and 20 μg chloramphenicol ml\textsuperscript{−1}] were incubated at 37°C for 24–72 h for the selection of \textit{F. tularensis} subsp. \textit{novicida} U112 integrants. Mutants were counter-selected by streaking colonies onto modified chloramphenicol-free Thayer–Martin agar supplemented with 5% (w/v) sucrose. All \textit{Escherichia coli} strains were grown in Luria–Bertani medium at 37°C.

**Iron-replete and iron-deficient growth of \textit{F. tularensis} subsp. \textit{novicida} U112.** An overnight \textit{F. tularensis} subsp. \textit{novicida} U112 culture was prepared by inoculating 20 ml CDM with approximately 3 × 10\textsuperscript{8} cells and cultured to an OD\textsubscript{600} of 1.9. Bacteria were then subcultured into a 30 ml total volume of pre-warmed CDM to a concentration of 1.6 × 10\textsuperscript{8} cells ml\textsuperscript{−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 FeSO\textsubscript{4}·7H\textsubscript{2}O to a density of 4.5 × 10\textsuperscript{7} cells ml\textsuperscript{−1}. At T\textsubscript{0} and every subsequent hour, 1 ml of culture was removed and the OD\textsubscript{600} 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\textsuperscript{8} 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 ml\textsuperscript{−1}.

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

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

---

**Table 1.** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Escherichia coli}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>Host cloning strain</td>
<td>Promega</td>
</tr>
<tr>
<td>S17-\textit{pir}</td>
<td>Host strain used for transfer of suicide plasmid into \textit{F. tularensis} subsp. \textit{novicida} U112 by conjugation</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{F. novicida}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U112</td>
<td>Wild-type \textit{F. tularensis} subsp. \textit{novicida} U112</td>
<td>This study</td>
</tr>
<tr>
<td>U112 \textit{ΔFTT0025}</td>
<td>\textit{F. tularensis} subsp. \textit{novicida} U112 with unmarked in-frame deletion of \textit{FTT0025}</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T Easy Vector System 1</td>
<td>Cloning vector; Amp\textsuperscript{R}</td>
<td>Promega</td>
</tr>
<tr>
<td>pTM6a</td>
<td>pGEM-T containing 1274 bp \textit{FTT0025} left flanking region</td>
<td>This study</td>
</tr>
<tr>
<td>pTM7a</td>
<td>pGEM-T containing 1074 bp \textit{FTT0025} right flanking region</td>
<td>This study</td>
</tr>
<tr>
<td>pTM8</td>
<td>pGEM-T containing PCR product of 2337 bp \textit{FTT0025} left and right flanking regions</td>
<td>This study</td>
</tr>
<tr>
<td>pSMP62</td>
<td>\textit{Francisella} suicide vector containing \textit{sucB} selection gene; Chlor\textsuperscript{R}, Amp\textsuperscript{R}</td>
<td>This study</td>
</tr>
<tr>
<td>pTM9Δ\textit{FTT0025}</td>
<td>pSMP62 containing 2337 bp \textit{ΔFTT0025} left and right flanking regions inserted at \textit{Spd} site</td>
<td>This study</td>
</tr>
</tbody>
</table>
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, significance deficient dataset. To test the expression levels statistically, a each gene, with those showing upregulation of discovery rate was applied. Fold change levels were calculated for and the Benjamini–Hochberg procedure for controlling false deficient growth considered biologically relevant.

Microarray hybridization. Test and control Cy3/Cy5-labelled Oligonucleotide primers (25-mers) were designed for Microarray hybridization 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 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 (BlueGene) and saved as Excel 97 format. For Southern blot hybridization, For Southern blot hybridization, Conjugation transfer of plasmids. Alleric 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 plated on Thayer–Martin agar supplemented with 5% (w/v) sucrose (Pelhic 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'-CAGGCGCAGTTGCGGTAGTAC-3'; P6: 5'-TACCGGTGCTATTACGAGGACACCACTACCC-3').

Southern blot hybridization. For Southern blot hybridization, EcoRV digests of 2-3 μg genomic DNA from F. tularensis subsp. novicida U112 wild-type and AFTT0025 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 AFTT0025 mutant in CDM. Volumes known to contain approximately 1.57 × 10^11 cells of F. tularensis subsp. novicida U112 wild-type and its isogenic AFTT0025 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 T0, and each subsequent hour,
1 ml culture was removed and the OD_{600} 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 ΔFTT0025 mutant containing approximately 5 × 10^6 c.f.u. ml^{-1} were prepared as described previously. From these, 50 μl 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).

**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. 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.
Expression of \textit{FTT0025} is upregulated during iron deficiency

To confirm the upregulation of \textit{FTT0025} expression, we employed RT-PCR as described in Methods. PCR primers were designed to amplify a 1301 bp product of the \textit{FTT0025} gene. As an internal housekeeping control, primers were designed to amplify a 584 bp PCR product of the \textit{fopA} gene, an ORF whose expression is known to be unaffected by the amount of iron in the culture medium (Deng \textit{et al.}, 2006). The PCR results confirmed that \textit{FTT0025} was indeed upregulated by \textit{F. tularensis} subsp. \textit{novicida} U112 during iron-deficient growth (Fig. 3; compare lanes 4 and 5). The equal amounts of \textit{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 H$_2$O negative controls confirmed that there was no DNA contamination in the PCR master mixes.

\textit{FTT0025} is co-transcribed with the siderophore operon

To determine whether \textit{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 \textit{FTT0025} and \textit{FTT0026}. The data presented in Fig. 4 revealed that transcription of mRNA encoding both \textit{FTT0025} and \textit{FTT0026} was upregulated by \textit{F. tularensis} subsp. \textit{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 \textit{FTT0025} is co-transcribed as part of this operon.
Production of the *F. tularensis* subsp. *novicida* U112ΔFTT0025 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* U112ΔFTT0025 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 ΔFTT0025 mutant. In liquid medium, the ΔFTT0025 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 ΔFTT0025 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. 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.

**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* U112ΔFTT0025. Expected wild-type band, 2405 bp; expected mutant band, 2572 bp.
may serve this essential function. It is important to note that F. tularensis subsp. novicida U112ΔFTT0025 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 U112ΔFTT0025 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 U112ΔFTT0025 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 U112ΔFTT0025 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 U112ΔFTT0025, this result was not statistically significant \((P=0.0996)\).

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.

<table>
<thead>
<tr>
<th>Challenge strain</th>
<th>Dose (c.f.u.)</th>
<th>No. of survivors</th>
<th>Time to death (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. tularensis subsp. novicida U112ΔFTT0025</td>
<td>4.05 × 10⁴</td>
<td>2/6</td>
<td>3, 4, 4, 4</td>
</tr>
<tr>
<td></td>
<td>4.05 × 10³</td>
<td>4/6</td>
<td>3, 5</td>
</tr>
<tr>
<td>F. tularensis subsp. novicida U112</td>
<td>4.2 × 10⁴</td>
<td>0/6</td>
<td>3, 3, 3, 4, 4</td>
</tr>
<tr>
<td></td>
<td>4.2 × 10³</td>
<td>0/6</td>
<td>3, 4, 4, 4, 4</td>
</tr>
</tbody>
</table>

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. Results confirming its suitability as a rationally attenuated protective mutant will lead to the generation of the ΔFTT0025 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


