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Iron transport in *Francisella* in the absence of a recognizable TonB protein still requires energy generated by the proton motive force

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Abstract The mechanism of iron transport in *Francisella* is still a puzzle since none of the sequenced *Francisella* strains appears to encode a TonB protein, the energy transducer of the proton motive force necessary to act on the bacterial outer membrane siderophore receptor to allow the internalization of iron. In this work we demonstrate using kinetic experiments of radioactive Fe^{3+} utilization, that iron uptake in *Francisella novicida*, although with no recognizable TonB protein, is indeed dependent on energy generated by the proton motive force. Moreover, mutants of a predicted outer membrane receptor still transport iron and are sensitive to the iron dependent antimicrobial compound streptonigrin. Our studies suggest that alternative pathways to internalize iron might exist in *Francisella*.

Keywords *Francisella* · Iron transport · Proton motive force

Introduction

Francisella tularensis, the causative agent of tularemia, is a small Gram-negative coccobacillus and facultative intracellular pathogen. *Francisella* can infect a variety of animals including rodents and lagomorphs (rabbits) with or without overt symptoms. Insects are important vectors for transmission to humans, although any contact with an infected animal or laboratory specimens can transmit the infection. In the antibiotic era few people die from tularemia. The incidence of the disease has steadily declined since the 1920s in direct correlation with the decline in the rural population. However, due to the fact that this bacterium is highly infectious as an aerosol and causes a severe pneumonia that cannot be easily distinguished from influenza it is considered a potential biowarfare agent.

The pathogenic mechanisms employed by *Francisella* are only now being uncovered. In the past 5 years the DNA sequences of several *Francisella* species and serovars have been reported, accelerating many research efforts. We have studied *Francisella novicida* strain Utah 112 (U112), as this strain is more than 95% identical to the highly infectious serovar *tularensis*; it retains its infectivity for mice, but is not infectious for immunocompetent humans. This allows culture and manipulation under BSL2 safety conditions, thus speeding discovery.

Like many other bacteria, *Francisella* employs iron as a cofactor for many enzymes, and iron utilization is required for virulence in many bacterial pathogens (Crosa et al. 2004). An infected host limits the amount of iron as part of the acute phase response and thus the pathogens have evolved complex mechanisms to steal

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iron from the host. In this vein, most bacteria synthesize and secrete siderophores, small molecules that chelate iron, but then release it following reuptake of the ferric siderophore by the bacteria. A siderophore was first identified in *Francisella* (Halmann et al. 1967) as an endogenously produced substance essential for growth initiation of *Pasteurella tularensis* (now *Francisella tularensis*). They noticed that it was similar to a substance produced by the fungus *Rhizopus microsporus* var. *rhizopodiformis*, later shown to be rhizoferrin, a polyhydroxycarboxylate siderophore that complexes Fe^{+3} (Drechsel et al. 1991). Most recently it was demonstrated that indeed *Francisella* synthesizes rhizoferrin (Sullivan et al. 2006). This siderophore exists as two enantiomers, R, R-rhizoferrin, produced by *Rhizopus*, and S,S-rhizoferrin identified as a siderophore secreted by the opportunistic human pathogen *Ralstonia pickettii* (Münzinger et al. 1999). Other bacteria, like *Morganella morganii* (Kuhn et al. 1996) and *Mycobacterium smegmatis* (Matzanke et al. 1999) are able to utilize R,R-rhizoferrin.

Several groups have examined *Francisella* mutations to identify the biosynthetic pathway for rhizoferrin synthesis as well as its receptor (Milne et al. 2007; Kiss et al. 2008; Ramakrishnan et al. 2008). Genetic mutations in genes associated with iron uptake, such as the siderophore receptor, are starved for iron and therefore should produce increased amounts of the siderophore. Titball et al. noted that mutations located within a specific open reading frame of *Francisella novicida*, FTN1686, produced a larger halo on CAS agar plates, indicating increased siderophore production (Milne et al. 2007). The polypeptide encoded by FTN1686 (ORF FTT0125c in *F. tularensis* SchuS4, designated *fslE* or *srfA* by other authors' (Kiss et al. 2008)), was localized in the outer membrane of two strains of *F. tularensis*, SchuS4 and LVS (Huntley et al. 2007). An in-frame deletion mutant of FTT0125c, identical to FTN1686 in *F. novicida* U112, was able to secret siderophore but was defective in its utilization (Ramakrishnan et al. 2008). In iron limited media a mutation in FTN1686, also resulted in a slower growth as compared to the wild type strain (Kiss et al. 2008).

In most bacteria that secrete siderophores, when the ferric-siderophore binds to its receptor, this undergoes an energy dependent conformation change allowing iron internalization. The energy necessary for this process is usually transduced via TonB, a protein that links the inner membrane to proteins present in the outer membrane. TonB acts as a vehicle to channel the energy provided by the proton motive force generated across the inner membrane to the outer membrane receptor. TonB has not been found in any of the sequenced *Francisella* strains or in several other bacteria such as *Legionella*, *Chlamidia*, *Halobacte*r and other ones (Chu et al. 2007).

In this study we have screened a comprehensive transposon library of *Francisella novicida* U112 for mutations that are altered in iron metabolism focusing on a subset of underproducers or overproducers of siderophore as measured by a whitish halo diameter on CAS agar plates. Among mutations that are expected from this screen are those located in the previously described so-called rhizoferrin receptor. We analyzed the kinetics of the uptake of radioactive Fe^{3+} for a subset of these mutations as well as the sensitivity to the iron dependent antimicrobial compound streptonigrin. We conclude that iron uptake is energy dependent and that there are multiple genes whose mutations increase production of the siderophore, some of which have not been previously reported. In addition, we present preliminary evidence for the existence of two iron uptake pathways in *Fancisella novicida*.

Materials and methods

Bacteria and mutants

Francisella novicida U112 was provided by Dr. Francis Nano (University British Columbia). A library of transposition mutants was obtained from the University of Washington (Gallagher et al. 2007). Bacteria and mutants are listed in Table 1. All bacteria were cultured at 37°C with aeration; in solid media we used Cysteine Heart Agar, CHA (DIFCO). For liquid cultures we used Trypticase Soy Media (DIFCO) with 2% cysteine added (TSBC). When iron limitation was needed, 50–200 μM 2,2′-dipyridyl (Sigma) was added. For specific assays, *Francisella novicida* was cultured in Chamberlain's defined media (Chamberlain 1965) without ferric pyrophosphate or with it.

Table 1 Bacterial strains

Name	Strain	Source
U112	Wild type *Francisella novicida*	UBC
M*fur*	FTN1681 transposon mutant-*tnfn1* pw060420p01q146	UW
M1682	FTN1682 (*frgA*) transposon mutant-*tnfn1* pw060328p05q150	UW
M1683	FTN1683 transposon mutant-*tnfn1* pw060418p02q193	UW
M1684	FTN1684 transposon mutant-*tnfn1* pw060328p03q166	UW
M1685	FTN1685 transposon mutant-*tnfn1* pw060323p06q117	UW
M1686	FTN1686 transposon mutant-*tnfn1* pw060510p01q185	UW
M1687	FTN1687 transposon mutant-*tnfn1* pw060323p08q140	UW
M1272	FTN1272 transposon mutant-*tnfn1* pw060420p01q122	UW
M*aroG*	FTN0842 transposon mutant-*tnfn1* pw060510p04q131	UW

CAS assay and siderophores

Siderophore from *Francisella novicida* was prepared by growing bacteria overnight in Chamberlain media without added iron. The supernatant was filtered sterilized and partially purified by a Sephadex LH-20 column.

Ralstonia pickettii siderophore was obtained by growing the bacteria in complete M9 minimal media, without iron added. Supernatant was filtered through a 0.22 μm filter before using it in bioassays. Rhizoferrin from *Rhizopus* was purchased at EMC micro collections, GmbHSindelfinger Str. 3D-72070 Tuebingen.

Bioassays

Screening of siderophore production in wild type strains and mutants was done in chrome azurol S (CAS) plates (Schwyn and Neilands 1987). More specific bioassays were performed using a lawn of complete minimum media (Chamberlain's media without iron) containing 0.7% agarose, 50 μM 2,2-dipyridyl and 70 μl overnight cultures of a recipient strain (U112, *Ralstonia picketti*, or M*aroG* or M1682) washed in PBS and resuspended in the same volume of Chamberlain's media.

Measurement of $^{55}Fe^{3+}$ uptake

Bacterial strains were grown overnight in TSBC and used to inoculate Chamberlain's media (1:50) without iron up to OD600 = 0.3–0.4. Cells were washed in PBS and resuspended to a density of 0.4×10^8 cells/ml in similar medium lacking aminoacids and containing 100 μM nitriloacetate. Carrier free $^{55}FeCl_3$ (1 μM/ml; Perking Elmer) was added to shaking cells suspensions, and 1 ml samples were removed at intervals and filtered through membrane filters (0.45 μm pore size; Millipore Corp.) and washed with 10 ml of 100 mM sodium citrate. Potassium cyanide or 2,4-dinitrophenol (Sigma) were added prior to the incorporation of the radioactive iron when inhibition of the iron uptake was tested. Filters were washed with sodium citrate, dried and counted in a Beckman Coulter LS6500 liquid scintillation counter using 10 ml of biodegradable scintillation fluid (Ecolite, MP).

Streptonigrin sensitivity

Overnight cultures of the strains to be tested for streptonigrin resistance were washed with PBS and resuspended in PBS at OD600 = 0.5. Fifty microliters of each preparation were plated on CHA agar plates containing 50 μM dipyridyl. Filter paper disks with 10 μl of 1 μg/ml streptonigrin (Sigma) were laid on the plates and incubated overnight at 37°C aerobically or in CO_2 5%. The diameter of inhibition halos is measured.

Results

Rhizoferrin production

A recent report showed that *Francisella novicida* U112 secretes the siderophore rhizoferrin (Sullivan et al. 2006). Since the plant pathogen *Ralstonia picketti* also secretes rhizoferrin (Münzinger et al. 1999), we wondered whether this siderophore could

be taken up by *F. novicida* and conversely whether *Ralstonia picketti* could recognize and transport the *F. novicida* siderophore. To answer this question we used a plate bioassay with either one of the two strains seeded in an iron limiting lawn (see Materials and methods) in which we spotted filtered supernatants from cultures of the two species grown under iron limiting conditions. As shown in Table 2 we find that the *F. novicida* and *Ralstonia pickettii* siderophores are interchangeable. In contrast, in our hands, the purified fungal optical antipode does not seem to substitute for the native rhizoferrin.

We next screened a bank of mutants of *F. novicida* U112 (see Materials and methods) to identify siderophore overproducers, underproducers and non-producers, as measured by the diameter of the halo on CAS agar plates. There were a large number of under producer mutants, but because the assay is very sensitive to differences in growth rate they have not been completely characterized. However, two non-producers were used in subsequent assays including a mutation in the aromatic amino acid biosynthesis pathway *(aroG)*, essential for the biosynthesis of siderophore and another in *fslA* (FTN1682), another biosynthetic gene for rhizoferrin (Sullivan et al. 2006). These two mutant strains were used to verify the results shown in Table 2 that *F. novicida* could take up the *Ralstonia* siderophore by spotting it onto a lawn seeded with either of the two mutants, confirming the previous results.

Iron cluster

Several of the genes involved in iron metabolism in *Francisella* are located together including the gene for the general iron regulator *fur* (see Fig. 1). A six-gene cluster is located downstream of *fur* (Ramakrishnan et al. 2008; Kiss et al. 2008). The first gene of the cluster in *F. novicida* U112, FTN1682 (*frgA*) is

Table 2 Sensitivity to 1 μg/ml streptonigrin

Strain	Size of inhibition[a] (cm)
U112	0.4 + 0.2
M*frg*A	0.0 + 0.1
M1686	2.0 + 0.3
M1272	0.1 + 0.2

[a] Diameter of clear halo around filter minus filter diameter

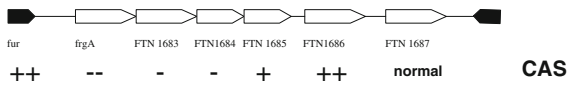

Fig. 1 Iron cluster in *Francisella novicida* U112 preceded by *fur*. Indicated under the diagram is the production of siderophore as measured by the diameter of a halo around a transposon mutant in each gene shown above plated on a CAS plate. **–,** indicates no halo; -, halo smaller than wild type; +, larger than wild type; ++, much larger halo than wild type; normal: same halo as wild type strain

essential for rhizoferrin biosynthesis (Sullivan et al. 2006) but the function of the other genes in the cluster is not clear. As shown in Fig. 1, we determined the phenotype of mutations in each of these genes on CAS agar plates and our results generally agree with others (Kiss et al. 2008). Mutations in the first three genes resulted in a decreased zone of clearing on CAS agar suggesting a decrease or absence of rhizoferrin production. Mutations in the two downstream genes FTN1685 and 1686 showed an increased zone of clearing suggesting that the bacterium is being starved for iron and consequently they have an increase in rhizoferrin production. FTN1686 has been proposed to be the rhizoferrin receptor, consistent with the siderophore overproduction.

Radioactive iron $^{55}Fe^{3+}$ uptake by cells of *F. novicida* U112 and mutants

To study iron uptake by *F. novicida*, exponentially growing cells of *F. novicida* were cultured in limiting iron conditions to an OD600 between 0.30 and 0.40, centrifuged, resuspended in the same media lacking all the essential aminoacids (to prevent growth) and then exposed to ^{55}Fe as described in "Materials and methods". To determine whether Fe^{3+} uptake is an energy dependent process, we inhibited energy production using either potassium cyanide (electron transport inhibitor) or 2,4-dinitrophenol (DNP, uncoupling agent) before adding the radioactive iron. As shown in Fig. 2a, these two respiration inhibitors block iron uptake in *F. novicida* U112. Similar results were found with *Ralstonia pickettii* (Fig. 2b).

In addition, we would expect that mutants that have decreased rhizoferrin production would show lower levels of iron uptake and that mutants that are unable to take up rhizoferrin would also show poor iron uptake. As seen in Fig. 3 the mutants in each of the genes of the iron cluster showed indeed a

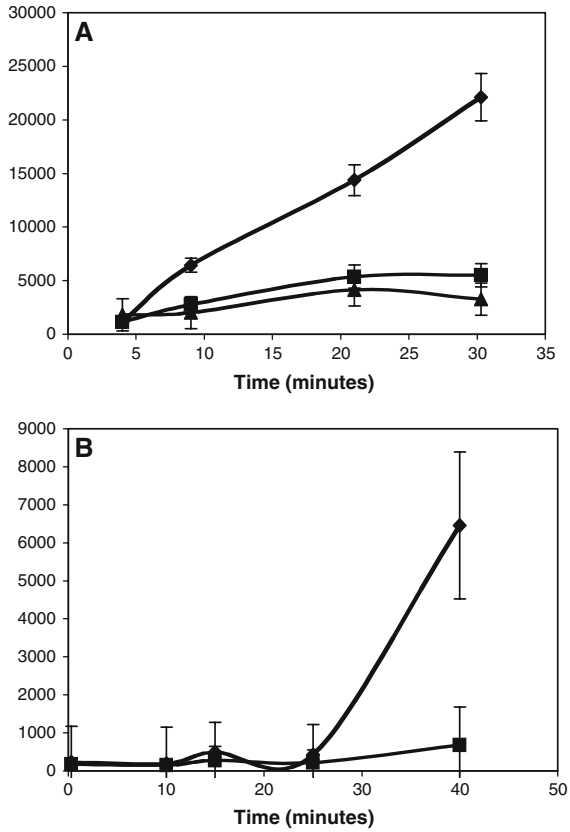

Fig. 2 Effect of respiration inhibitors in the uptake of radioactive iron-starved. **a** *Francisella novicida* U112 ◆; U112 with: potassium cyanide added ■, or DNP added ▲. **b** *Ralstonia picketti* ◆; *Ralstonia picketti* with DNP added ■

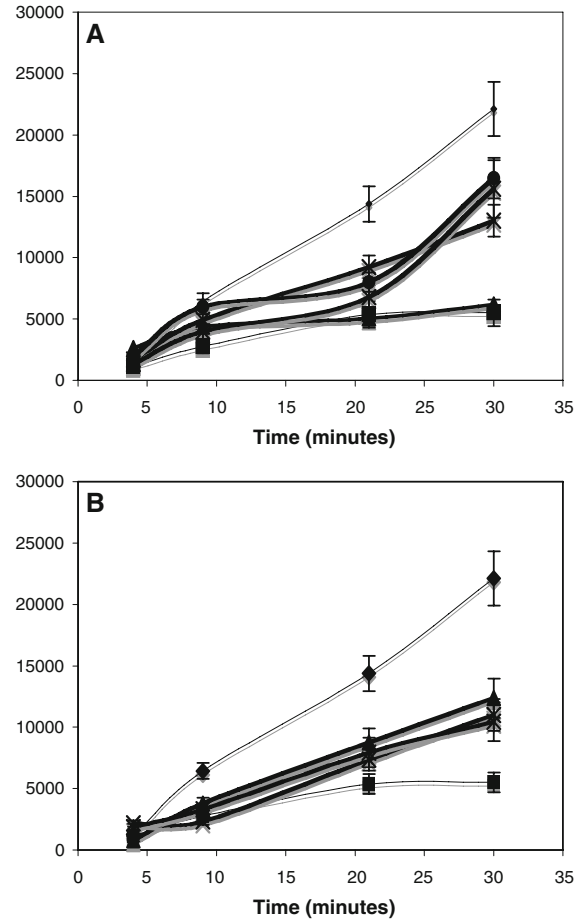

Fig. 3 $^{55}Fe^{3+}$ uptake of mutants in the genes of the iron cluster of *Francisella novicida*. **a** U112 ◆; U112, with potassium cyanide added ■; M1682 ▲; M1683 **X**; M1684 ●; and M1687 ○. **b** *Francisella novicida* U112 ◆; U112 with potassium cyanide added ■; M1685 ○; M1686 **X**; and M1272 ▲

decrease in iron uptake. It is remarkable that M1685 and M1686 still transport iron significantly, which was not expected for siderophore uptake mutants. One caveat is that insertion mutations might be polar on expression of downstream genes.

Streptonigrin sensitivity as a probe for free intracellular iron

The antibiotic streptonigrin is bactericidal in the presence of iron (Fe^{3+}). Streptonigrin passes through the cell membrane and, in the presence of iron, promotes the formation of hydroxyl radicals and hydrogen peroxide that are highly toxic to the cell (White and Yeowell 1982; Yeowell and White 1982). The wild type U112, and mutants *frg*A (M1682) and M1686 were tested for streptonigrin resistance (see Materials and methods and Table 2). As expected, M1682 is resistant to streptonigrin because it does not make rhizoferrin; but, to our surprise, M1686 is highly sensitive and thus it can still internalize iron, suggesting the presence of an alternative iron uptake system.

Evidence for a second uptake mechanism

Although we observed a decrease in iron uptake in mutants of all the cluster genes, we noticed that the putative rhizoferrin receptor M1686 still shows significant iron uptake (Fig. 3). Likewise we observed that M1686 is highly sensitive to streptonigrin suggesting that iron can be taken up by an alternative mechanism. Thus, to investigate possible candidates for this iron uptake function we carried out a

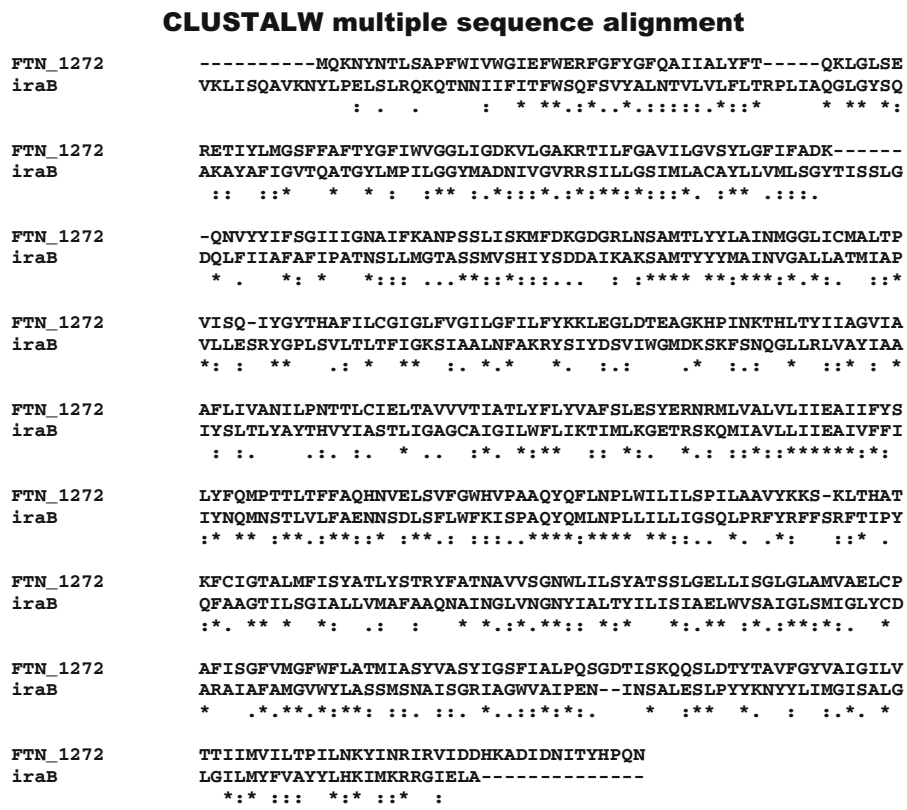

Fig. 4 CLUSTAL W comparison between *iraB* of *Legionella pneumophila* and FTN1272 of *Francisella novicida* U112

homology search to identify other potential iron acquisition genes. We compared the *F. novicida* genome sequence with other bacterial genomes without an annotated TonB as well as with the genomes of bacteria that produce or use rhizoferrin (Chu et al. 2007). Our reasoning was that bacteria missing *tonB*, which is normally required for iron assimilation, may encode an alternative mechanism of energy transduction. Based on our observations of radioactive iron uptake, such an uptake mechanism would still require the proton motive force (Fig. 2). Since no homolog's to traditional siderophore-Fe^{3+} outer membrane receptors have been found in *Francisella* sequenced genomes, it is possible that there is a novel system of iron uptake and energy transduction and that the protein(s) involved is quite different because it does not contain a TonB interacting domain. We were struck by the finding that a gene in *Legionella pneumophila*, *iraB*, involved in a possible novel iron uptake system (Viswanathan et al. 2000) is closely related to the open reading frame FTN1272, which is annotated in *F. novicida* as a proton-dependent oligopeptide transporter. Figure 4 shows a CLUSTAL alignment of FTN1272 and *iraB*.

It is of interest that other genes with high homology to FTN1272 exist in *Francisella* and in *Legionella*. Because FTN1272 shows some homology to a rhizoferrin receptor, *rumB*, identified in the opportunistic enterobacteria *Morganella morganii* (Kuhn et al. 1996), we decided to first analyze the function of this ORF in more detail. Our results in this work demonstrate that a mutation in FTN1272 transports radioactive iron poorly as shown in Fig. 3b, comparable to that of a mutation in the putative rhizoferrin receptor FTN1686. Furthermore the FTN1272 mutation shows an increased streptonigrin resistance as compared to the wild type (Table 2) as well as a small increase in siderophore production as assessed with the CAS agar plates.

Discussion

The vast majority of Gram-negative bacteria need iron for survival and growth (*Lactobacillus*, *Borrelia burgdorferi* being exceptions) (Jakubovics and Jenkinson 2001). During host infection, pathogens that require iron may use a siderophore to take up iron

or alternatively directly capture iron bound to hemin, transferrin, or lactoferrin. *Francisella* produces the siderophore rhizoferrin to complex the necessary iron for survival. We find that *Francisella novicida* U112 can also utilize the *S*-*S*′ rhizoferrin secreted by the plant pathogen *Ralstonia pickettii;* similarly this bacterium recognizes the *Francisella* rhizoferrin. Conversely, in our hands the enantiomer R,R-rhizoferrin from the fungus *Rhizopus* is not utilized by *F. novicida* U112, although it was previously reported that culture filtrates of a *Rhizopus* culture were found to initiate *Francisella tularensis* growth (Halmann and Mager 1967).

In most bacterial iron uptake systems there is an outer membrane receptor specific for the Fe^{3+}-siderophore compound, which is dependent on the energy-transducing protein TonB to internalize the iron in the cytoplasm. These outer membrane receptors show homology to each other and structural similarities as well (Crosa et al. 2004). In the case of *Francisella*, some obligate intracellular bacteria and but a few others, a TonB homologue is absent. This may explain why *Francisella* does not encode an outer membrane receptor homologous to the prototype of iron receptors in other protobacteria. Nevertheless, we show that *Francisella novicida* requires energy to internalize radioactive $^{55}Fe^{3+}$ suggesting that the proton motive force is transduced to the rhizoferrin receptor by a different mechanism. FTN1686 is thought to be the rhizoferrin receptor (Ramakrishnan et al. 2008); however, another iron uptake system is suggested by our results with the FTN1686 mutant.

Based on the fact that FTN1686 is still sensitive to iron toxicity when exposed to streptonigin and continues to take up radioactive iron we searched for an alternative uptake mechanism by computer aided analysis of other intracellular pathogens and bacteria that are missing *tonB*. We identified one open reading frame that encodes a peptide transporter homologous to *iraB*, that mediates iron uptake in *Legionella* (Viswanathan et al. 2000). It is believed that this protein is an antiporter that delivers a novel peptide-iron complex to the cell, possibly using the proton motive force for energy. The structure of the peptide-iron complex is not yet known. In *Legionella pnumophila*, it was suggested (Viswanathan et al. 2000) that *iraA* is critical for virulence of this bacterium while *iraB* is involved in a novel method of iron acquisition, which may utilize the iron-loaded peptide. There are no *iraA* homologues in *F. novicida*.

Many other *Francisella* genes are also induced in response to iron starvation (Deng et al. 2006) and our laboratories (unpublished results). The function of these genes as well as the homologues of FTN1272 requires further investigation.

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