An intracellularly inducible gene involved in virulence and polyphosphate production in *Francisella*

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Francisella tularensis is an intracellular pathogen capable of multiplying to high levels in macrophages. By protein analysis, only a few proteins have been shown previously to be expressed at high levels in macrophages relative to bacteria grown in culture media. To identify additional genes that show increased expression during intracellular growth, we developed a plasmid for use in *Francisella* based on the induction of expression of green fluorescent protein. Clones of *F. tularensis* subsp. *novicida* were identified that were fluorescent only intracellularly and not when grown *in vitro*. Sequencing identified a range of genes comprising some such as *dnaK* that are already known to be expressed intracellularly and some novel targets. One of these newly identified regulated genes, FTN1472/FTT1564, was selected for further study. Isogenic mutants were generated in *F. tularensis* subsp. *novicida* and subsp. *tularensis* by allelic replacement. Inactivation of the gene resulted in abolition of polyphosphate production by *F. novicida*, strongly supporting the bioinformatic analysis, which had suggested that the gene may encode a polyphosphate kinase. The mutants exhibited defects for intracellular growth in macrophages and were attenuated in mice, indicating a key role for the putative polyphosphate kinase in the virulence of *Francisella*.

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INTRODUCTION

Francisella tularensis is the causative agent of the zoonotic disease tularemia. There are four subspecies of *F. tularensis*, differing in their virulence for man: *F. tularensis* subsp. *tularensis* is the most virulent for man, subsp. *holarctica* and *mediasiatica* are moderately virulent, and subsp. *novicida* is relatively avirulent (Oyston *et al.*, 2004). The presentation of disease depends on the route of infection (Ellis *et al.*, 2002). Typhoidal and respiratory tularemia are the most serious forms of disease and have high associated mortality rates. Although infection can be treated with appropriate antibiotics, there is no vaccine available for prophylaxis. An undefined attenuated Live Vaccine Strain (LVS) was identified during the last century, but as yet this strain has failed to achieve regulatory approval.

F. tularensis is fastidious, requiring a rich medium supplemented with cysteine for growth in the laboratory, but it is primarily an intracellular pathogen, able to grow to high numbers within a wide range of cell types. However, invasion and proliferation in most cell types does not occur to the same level as is observed in macrophages and

amoebae. Although intracellular multiplication is essential for pathogenicity of the bacterium, very little is known about the intracellular lifestyle of this organism, hindered in part by the lack of genetic tools available for working with *Francisella*.

Proteomic studies have revealed upregulation of very few proteins during intracellular growth (Golovliov *et al.*, 1997). Although the molecular mechanisms of pathogenesis are not well understood, replication inside macrophages is essential for the organism to cause disease (Fortier *et al.*, 1994). Until recently, only a limited number of genes had been shown to be required for intracellular growth, most notably the *Francisella* pathogenicity island carrying the *igl* genes (Golovliov *et al.*, 2003; Lauriano *et al.*, 2004; Lindgren *et al.*, 2004; Twine *et al.*, 2005). More recently, as genetic tools have been developed for use with *F. tularensis* (LoVullo *et al.*, 2006), and aided by the publication of the genome sequence (Larsson *et al.*, 2005), other genes involved in intracellular growth have been identified (Maier *et al.*, 2007; Pechous *et al.*, 2006; Tempel *et al.*, 2006). In this study, we undertook promoter trapping to identify *Francisella* genes induced intracellularly. This allowed the identification of a gene encoding polyphosphate kinase, which we showed to be required for intracellular growth and virulence.

Abbreviations: GFP, green fluorescent protein; i.p., intraperitoneal; LD₅₀, median lethal dose to induce morbidity or death; s.c., subcutaneous.

METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *F. tularensis* strains were grown routinely on blood cysteine glucose agar (BCGA) containing 4% cysteine, 4% histidine, 5% glucose and 10% fresh filtered horse blood, solidified Chamberlain's defined medium (CDM) (Chamberlain, 1965) or Thayer–Martin agar (Thomas *et al.*, 2007). Where required, the medium was supplemented with 100 µg polymyxin ml⁻¹ and 10 µg chloramphenicol ml⁻¹. *Escherichia coli* strains were grown in Luria–Bertani (LB) medium or on LB agar at 37 °C, supplemented as required with 30 µg chloramphenicol ml⁻¹. All work undertaken with *Francisella* strains was performed under appropriate laboratory containment conditions in accordance with relevant legislative requirements.

Plasmids and manipulation of DNA. Generally, the manipulation of DNA was carried out as outlined previously (Sambrook *et al.*, 1989). Genomic DNA was isolated using a Puregene DNA Isolation kit (Gentra Systems). Plasmid DNA was isolated using Qiagen Mini and Maxi Plasmid kits. The plasmids used in this study are shown in Table 1. Southern blotting was performed under high-stringency conditions as described by Sambrook *et al.* (1989). The oligonucleotide primers used for PCR in this study are given in Table 1. A standard PCR was performed with 25 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s.

Construction of the promoter trap vector. The plasmid pKK202 is stably maintained in *F. novicida* (Norqvist *et al.*, 1996). To facilitate

conjugation, the *oriT* locus was subcloned into pKK202 from pPV2 (Golovliov *et al.*, 2003) to create pKK202oriT. To release the *oriT* locus, pPV2 was digested with *Bam*HI and the 1.7 kb fragment was isolated following agarose gel electrophoresis. The fragment was ligated into pKK202 that had been linearized with *Bcl*I. The gene encoding green fluorescent protein (GFP) was amplified from pGFPuv (Clontech) by PCR using primers GFPFor/GFPRev (Table 1). The PCR product was ligated into pGEM-T Easy according to the manufacturer's instructions. The oligonucleotide primers were designed to include *Apa*LI restriction sites, allowing the insert to be isolated from the pGEM-T Easy vector and cloned into pKK202oriT linearized with *Apa*LI. This plasmid was designated pKKoriTGFP. One of the oligonucleotide primers used to amplify the gene from pGFPuv was designed to incorporate an *Fba*I site directly upstream. Digestion with *Fba*I produces overhangs compatible with those produced by *Sau*3AI. As *Fba*I is methylation-sensitive, the plasmid was transformed into *E. coli* SCS110 to produce an unmethylated plasmid preparation.

Production of a genomic library. Genomic DNA was isolated from *F. novicida* strain U112 and partially digested with *Sau*3AI (Roche). The DNA fragments were separated on a 0.7% agarose gel and a region covering <2 kb was selected. The DNA was purified from the gel and ligated into pKKoriTGFP, which had been linearized with the restriction enzyme *Fba*I. This process was repeated several times to generate different sublibraries. The plasmids were introduced into *E. coli* S17λpir by electroporation for conjugation into *F. novicida* U112. Clones were selected on Thayer–Martin agar supplemented with chloramphenicol (to identify clones containing the plasmid) and

Table 1. Bacterial strains, primers and plasmids used in this study

Strain/primer/plasmid	Details	Source/reference
Strains		
<i>Escherichia coli</i>		
JM109	<i>endA1 recA1 gyrA96 thi hsdR17</i> (r _k ⁻ , m _k ⁺) <i>relA1 supE44 Δ(lac-proAB)</i> [F' <i>traD36 proAB lacI</i> ^q ΔM15	Promega
SCS110	<i>rpsL</i> (<i>Strr</i>) <i>thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44 Δ(lac-proAB)</i> [F' <i>traD36 proAB lacI</i> ^q ΔM15	Stratagene
S17λpir	<i>recA thi pro hsdR-M</i> ⁺ <RP4:2-Tc:Mu:Kn:Tn7>Tp ^R Sm ^R	Simon <i>et al.</i> (1983)
<i>F. tularensis</i>		
U112	<i>F. tularensis</i> subsp. <i>novicida</i>	
SCHU S4	<i>F. tularensis</i> subsp. <i>tularensis</i>	
SCHUS4ΔFTT1564::CAM	<i>FTT1564</i> ⁻ , Cam ^R	This study
U112ΔFTN1472::CAM	<i>FTN1472</i> ⁻ , Cam ^R	This study
Primers (5'→3')*		
1564RFF	ATCTCGAGAGATCTCAAAACAAAAATAGTCCTTGAC	
1564RFR	TAACGCGTAGAACTTATTCCATATATAAGG	
1564LFF	CTCGAGACGCGTACATCACTACTATATTAAGCTTTC	
1564LFR	AGATCTGCTTAACCTATTTAATATATTATC	
GFPFor	GTGCACTAAGTAAGTAATGATCAGGAGGAAGAAAAAATGAGTAAAGGAGA	
GFPRev	GTGCACTTATTTGTAGAGCTCATCCATGCCATGTG	
Plasmids		
pGEM-T Easy	Ap ^R , PCR cloning vector, supplied with 3' T overhang	Promega
pGFPuv	Ap ^R , P _{lac} driving expression of 'cycle 3' variant of GFP	Clontech
pKK202	Tet ^R Cam ^R , <i>E. coli</i> – <i>Francisella</i> shuttle vector	Norqvist <i>et al.</i> (1996)
pPV2	<i>sacB</i> , Ap ^R Cam ^R , <i>oriT</i>	Golovliov <i>et al.</i> (2003)
pKK202oriT	A <i>Bam</i> HI fragment of pPV2 containing the <i>oriT</i> , cloned into the <i>Bcl</i> I site of pKK202	This study
pKKoriTGFP	The <i>gfp</i> gene from pGFPuv cloned into the <i>Apa</i> LI site of pKK202oriT	This study
pSMP22	Suicide vector for mutagenesis of <i>F. tularensis</i>	Thomas <i>et al.</i> (2007)

*The *Bgl*II site is shown in *italic*, the *Xho*I or *Apa*LI site in **bold** and the *Mlu*I site is underlined.

polymixin (to inhibit growth of the *E. coli* donor). Clones were picked onto BCGA medium supplemented with chloramphenicol and in duplicate onto LB+chloramphenicol agar. Colonies growing on LB+chloramphenicol agar were examined under UV illumination and fluorescent colonies were removed from the library.

Macrophage assays. The J774 mouse macrophage cell line was maintained in Dulbecco's modified Eagle's medium (Gibco) and incubated at 37 °C in 5% CO₂. The macrophages were passaged a maximum of 25 times. Bacterial cells were resuspended in 3 ml L-15 medium containing GlutaMAX and 10% fetal calf serum (Life Technologies). The OD₆₀₀ of this suspension was measured and the suspension was suitably diluted to give an approximate concentration of 1×10^9 c.f.u. ml⁻¹. The L-15 medium was aspirated from each well and 100 µl bacterial suspension was overlaid onto the macrophages to give an m.o.i. of 10 bacteria per macrophage. Control wells were overlaid with L-15 medium alone. The cells were incubated for 30 min at 37 °C, after which time the medium was replaced with L-15 containing 10 µg gentamicin ml⁻¹. The cells were incubated at 37 °C for 30 min. The medium was then removed and replaced with L-15 containing 2 µg gentamicin ml⁻¹. To identify intracellularly expressed promoters, macrophages were examined at intervals up to 24 h for fluorescence using a confocal microscope (Olympus Instruments). Fluorescent pools were broken down into single clones to identify the fluorescent clones.

To follow bacterial survival, at selected intervals two wells of infected macrophages per strain were washed twice with 1 ml PBS, and 1 ml dH₂O was added to each well. The macrophages were disrupted by aspiration 30 times. The cell lysate was serially diluted in PBS and dilutions were plated onto BCGA, which were incubated at 37 °C for 2 days. At the final sampling time, the uninfected controls were similarly processed and plated to detect possible contamination. A two-way analysis of variance was performed with Bonferroni's post-test using GraphPad Prism v4.0.

Generation of Δ1564::CAM mutants in subsp. *novicida* and *tularensis*. Primers were designed from the genome sequence of *F. tularensis* subsp. *tularensis* strain SCHU S4 to generate an 831 bp deletion in the FTT1564 ORF as follows. Primers 1564LFF/1564LFR and 1564RFF/1564RFR were used to amplify DNA regions flanking the deletion. These were ligated into pGEM-T Easy according to the manufacturer's protocol to generate p1564L and pGEM1564R containing the left and right flanks, respectively. The plasmids were digested with *Bgl*II and *Xho*I to linearize p1564L and to isolate the insert from p1564R. The p1564R insert was then ligated into p1564L to generate pGEM-T Easy containing both left and right flanks, designated p1564LR. The internal *Bgl*II restriction site between the two flanks allowed the insertion of a chloramphenicol resistance cassette (Quarry *et al.*, 2007). The plasmid was digested with *Mlu*I and the insert was isolated and ligated into similarly digested pSMP22 to produce pMR1564::CAM. Plasmid pMR1564::CAM was introduced into *F. tularensis* subsp. *novicida* U112 or subsp. *tularensis* SCHU S4 by conjugation using the method of Golovliov *et al.* (2003). Briefly, *E. coli* S17λpir pMR1564::CAM was grown overnight in LB+chloramphenicol; a 1 ml aliquot was removed and the bacteria were pelleted by centrifugation and resuspended in 50 µl fresh LB broth. *Francisella* was grown on BCGA overnight at 37 °C in a confluent lawn. Bacteria were removed using a sterile loop, resuspended in the *E. coli* suspension and the mixture was spotted onto BCGA. After incubation overnight at 25 °C, the bacteria were removed with a sterile loop and resuspended in 500 µl PBS before being inoculated onto Thayer–Martin agar supplemented with 100 µg polymixin ml⁻¹ (to inhibit growth of the *E. coli*) and 10 µg chloramphenicol ml⁻¹. Colonies were subcultured onto Thayer–Martin agar supplemented with 5% sucrose and 10 µg chloramphenicol ml⁻¹. Isolated colonies that grew in the presence of sucrose were analysed by PCR for the deletion of the FTTN1472/FTT1564 ORF and loss of the vector (mutants

U112ΔFTN1472::CAM and SCHUS4ΔFTT1564::CAM, respectively). This was subsequently confirmed by Southern blotting.

Animal studies. Groups of six 6–8-week-old female BALB/c mice (Charles River Laboratories) were dosed by the intraperitoneal (i.p.) or subcutaneous (s.c.) route with serial dilutions of bacteria in PBS. The concentration of bacteria in the inoculum was determined by viable count. Mice surviving the initial challenge were subsequently challenged 56 days later with wild-type strains to determine whether a protective immune response had been induced. Humane end points were strictly observed and animals deemed incapable of survival were killed humanely by cervical dislocation. The median lethal dose to induce morbidity or death (LD₅₀) has been shown to be approximately 1 c.f.u. for SCHU S4 given by the s.c. route and for U112 by the i.p. route (Quarry *et al.*, 2007), and 3×10^2 c.f.u. for U112 by the s.c. route (P. Oyston, personal communication).

Polyphosphate analysis. Wild-type and mutant strains of *F. novicida* were grown overnight in CDM. The inorganic phosphate concentration in CDM is 5 mM. Bacteria were then subcultured into 28 ml pre-warmed CDM to a concentration of approximately 1.6×10^9 c.f.u. ml⁻¹ (Milne *et al.*, 2007) and incubated for a further 24 h, resulting in cultures with a density of $2\text{--}2.57 \times 10^{10}$ c.f.u. ml⁻¹. Polyphosphate extraction was performed using a modification of the acidic extraction method (Ghorbel *et al.*, 2006). One millilitre aliquots of each bacterial culture were removed and the bacterial cells were pelleted by centrifugation. The pellet was resuspended in 1 ml 0.5 M HClO₄ and incubated on ice for 30 min with occasional inversion. The bacterial cells were pelleted by centrifugation and the supernatant was retained for analysis. Nucleoside phosphates were removed by adsorption on acid-washed activated charcoal (Sigma). The free phosphates in the acid-soluble extract were determined before and after hydrolysis at 100 °C for 10 min. An increase in detectable free phosphate following hydrolysis is a measure of the amount of polyphosphate present in the bacteria. The concentration of free phosphate was determined using a spectrophotometric phosphorus assay kit based on phosphate complex formation with malachite green and molybdate (BioAssay Systems). The mean results from eight replicates were analysed by a two-way analysis of variance with Bonferroni's post-tests using GraphPad Prism v4.0.

RESULTS AND DISCUSSION

Identification of a putative polyphosphate kinase expressed by *Francisella* within macrophages

F. tularensis has been described as a 'stealth pathogen' (Sjostedt, 2006) in that very little is known about how the organism causes disease. However, as an intracellular pathogen it has long been assumed that an inability to multiply within cells would cause attenuation, and that genes key for intracellular survival would help elucidate the basis of pathogenicity. The ability to identify such genes has been hampered firstly by the lack of genetic tools for use with *Francisella* and secondly by the difficulties encountered in transformation. The development of vectors capable of mobilization from *E. coli* into *Francisella* allows the efficient transfer of these shuttle plasmids. Due the paucity of genetic tools for use with *Francisella*, it was necessary to construct a suitable vector to facilitate this study. To this end, the plasmid pKKoriTGFP was developed (Fig. 1). The backbone of the vector was derived from pKK202, which is stably maintained in both

Fig. 1. Plasmid pKKoriTGFP: a mobilizable plasmid suitable for use in *Francisella*, carrying a chloramphenicol-resistance cassette for plasmid stabilization and a promoterless gene encoding GFP. An *FbaI* restriction site immediately preceding the gene encoding GFP allowed insertion of *Sau3AI* genomic digest fragments.

E. coli and *Francisella*. As electroporation is poorly efficient in *Francisella* compared with conjugation, the *oriT* locus of pPV2 was inserted into pKK202 to produce a mobilizable vector. A promoter trapping vector based on induction of chloramphenicol acetyltransferase has been shown to be able to identify intracellularly induced genes (Kuoppa *et al.*, 2001). The drawback of the use of antibiotic resistance as an inducible marker is that it requires the introduction of multiple resistance genes into a human pathogen. To reduce the dependence on multiple antibiotic resistance markers, we developed a promoter trapping vector, pKKoitTGFP, carrying a promoterless gene encoding the cycle 3 variant of GFP (Cramer *et al.*, 1996). GFP has been used to identify genes induced in *Erwinia chrysanthemi* during plant infection (Yang *et al.*, 2004), and GFP expression was used to identify genes expressed by *Salmonella enterica* serovar Typhimurium, *Mycobacterium tuberculosis* and *Listeria monocytogenes* during growth inside macrophages (Barker *et al.*, 1998; Valdivia & Falkow, 1997; Wilson *et al.*, 2001). However, because the detection of induced fluorescence was by visual inspection of macrophages by confocal microscopy, the pools in this study could not be as complex as an antibiotic selection approach would allow.

F. novicida is frequently used as a model for the more pathogenic subspecies, as the subspecies are genetically closely related and the mechanisms of intracellular survival are likely to be conserved in general (Brotcke *et al.*, 2006). Not only is *F. novicida* safer to work with than the highly virulent strains, but it grows more quickly and is more amenable to genetic manipulation. However, extrapolating

from *F. novicida* to the highly virulent subspecies must be undertaken with caution. It is apparent that the immune response required for protection against challenge with subsp. *tularensis* strains is different to that required for protection against *F. novicida* (Fulop *et al.*, 2001; Shen *et al.*, 2004). Despite this, studies in *F. novicida* are useful in identifying attenuation targets for further study in subsp. *tularensis*. Genomic DNA was isolated from *F. tularensis* subsp. *novicida* U112 and partially digested with *Sau3AI*. Fragments of <2 kb in size were ligated into the *FbaI* site of pKKoriTGFP. The pool of plasmids was transferred to *F. tularensis* subsp. *novicida* U112 by conjugation from *E. coli* S17 λ pir and chloramphenicol-resistant colonies were isolated. Over 550 colonies were picked into gridded libraries. Of these, approximately 1% fluoresced on agar when examined under UV light and were removed from the library. This is similar to the proportion of *in vitro*-expressed promoters identified by Kuoppa *et al.* (2001) when screening for *cat* activity. The non-fluorescent clones were assembled into pools of eight for screening in macrophages. It was found that increasing the complexity of the pools reduced the ability to detect fluorescent bacteria (data not shown). The infected macrophages were examined at 24 h for bacteria expressing GFP (Fig. 2). In total, 77 pools of mutants were screened in macrophages. Eighteen positive pools were then examined in pure culture to identify the intracellularly fluorescent clones and 23 fluorescent clones were identified. Inserts were sequenced and the DNA sequence was compared with the genome sequences for strain SCHU S4, the only published *Francisella* genome at the time. Subsequently, the *F. tularensis* subsp. *novicida* genome became available and the insert sequences were then compared with this as confirmation. This allowed the identification of intergenic

Fig. 2. Macrophages infected with an *F. novicida* pKKoriTGFP pool containing a clone (subsequently identified as *dnaK*) with an intracellularly inducible promoter, at 24 h post-infection.

regions that may encode intracellularly induced promoters (Table 2).

Acid phosphatase (AcpA) and DnaK were identified in this study, and are both known to be expressed intracellularly. AcpA is proposed to be involved in inhibition of the respiratory burst (Reilly *et al.*, 1996), but recently phospholipase activity has also been proposed for AcpA, with a role in phagosomal trafficking of the bacterium (Felts *et al.*, 2006; Mohapatra *et al.*, 2007). However, there are conflicting reports on whether AcpA is essential for virulence and intracellular replication (Baron *et al.*, 1999; Mohapatra *et al.*, 2007), which are yet to be resolved. DnaK was first demonstrated to be upregulated in macrophages using a proteomic approach (Golovliov *et al.*, 1997) and was identified as being essential for intracellular growth and virulence of subsp. *novicida* (Tempel *et al.*, 2006). Identification of these promoters already known to be induced intracellularly provided confirmation that this GFP-based approach could identify intracellularly inducible promoters.

Of the remaining genes identified as being induced intracellularly, none had been identified previously as being involved in the intracellular growth of *Francisella*. Two-component regulatory systems have been extensively studied for their contribution to intracellular growth. One promoter identified in this study was upstream of the *kdpD* gene. KdpD is the sensor of a two-component regulatory system, which in *E. coli* monitors K⁺ concentration and turgor pressure (Sugiura *et al.*, 1994). An associated response regulator has not been reported in *Francisella*, but the *kdpE* homologue, encoding the cognate response regulator in *E. coli*, is a pseudogene in strains SCHU S4, LVS and OSU18.

One of the genes identified in this study was *yfdH*, predicted by sequence homology to encode a glycosyl-transferase group 2 family protein. The same gene was identified during a genome comparison study as a potential cause of attenuation of the LVS strain (Rohmer *et al.*,

Table 2. Identification of *F. novicida* inserts driving GFP expression in macrophages

Clone	Gene	FTN locus*	FTT locus†	Proposed function
pFPOSV8	<i>acpA</i>	FTN0090	FTT0221	Acid phosphatase
pVIII82-097 pGFPOP1 pGFPOP3 pGFPOP6 pGFPOP7 pGFPOP9	<i>yfdH</i>	FTN0545	FTT0454	Glycosyl transferase
pFPOSX4		FTN0994	FTT0689	Unknown
pFPOSAJ2		FTN1472	FTT1564	Polyphosphate kinase
pGFNOP2 pGFNOP5	<i>pepA</i>	FTN0660	FTT1318c	Cytosol aminopeptidase
pFPOSV4	<i>kdpD</i>	FTN1715	FTT1736c	Kinase – two-component system
pFPOSS3 pFPOSM5 pFPOSL2 pFPOSE2 pFPOSA3	<i>rpoA2</i>	FTN1412	FTT1442c	DNA-directed RNA polymerase subunit α
pKK3.4 pKK3.5 pKK3.6 pKK3.7	<i>dnaK</i>	FTN1284	FTT1269c	Chaperone – heat-shock protein
pKKGFN02		FTN0041	No hit in BLASTN	Unknown (internal region of coding sequence)
pKKGFN05		FTN1388	FTT1422	Oxidoreductase (internal region of coding sequence)

*FTN, *F. tularensis* subsp. *novicida*.

†FTT, *F. tularensis* subsp. *tularensis*.

SCHU S4

U112

Fig. 3. Organization of the genomes of *F. tularensis* strain SCHU S4 and *F. novicida* strain U112 in the region of FTN1472/FTT1564.

2006), as it is a pseudogene in LVS but was intact in 15 further strains examined. The locus appeared to be under strong purifying selection, indicating that its product plays an important role in bacterial survival or fitness (Rohmer *et al.*, 2006). The putative glycosyltransferase is predicted to be involved in cell wall biogenesis or LPS biosynthesis. The LPS of strain LVS lacks galactosamine 1-phosphate compared with the LPS of another subsp. *holarctica* strain, and the inactivation of *yfdH* may be the cause of this difference.

Two genes identified as having intracellularly inducible promoters, FTN0994/FTT0689 and FTN1472/FTT1564, were annotated as hypothetical proteins (Table 2). At the time they were identified, the proteins encoded by these genes had not been identified previously as having a role that may be important *in vivo*, either by microarray analysis (Brotcke *et al.*, 2006; Deng *et al.*, 2006) or by proteomics-based approaches (Twine *et al.*, 2006). However, recently the FTN1472/FTT1564 homologue in the attenuated subsp. *holarctica* strain LVS (FTL0544) was identified as playing a role in intracellular survival and virulence (Maier *et al.*, 2007). However, the suggested identity of the genes as encoding a polyphosphate kinase was not confirmed. Therefore, we wished to confirm whether the gene we had identified did in fact encode a polyphosphate kinase and determine what role this played in the virulence of *F. tularensis* subsp. *tularensis* and subsp. *novicida*.

Fig. 4. Quantification of inorganic phosphates from *F. novicida* U112 and *F. novicida* U112ΔFTN1472::CAM. ■, Unhydrolysed; □, hydrolysed.

FTN1472/FTT1564 is required for intracellular growth and virulence of *F. tularensis*

Polyphosphate has been shown to be key in determining how bacteria respond to stress, possibly by contributing to the production of ppGpp (Manganelli, 2007), and has been shown to be required for virulence for a range of pathogens including *Pseudomonas aeruginosa* (Rashid *et al.*, 2000),

Fig. 5. Survival of *F. tularensis* SCHUS4ΔFTT1564::CAM and *F. novicida* U112ΔFTN1472::CAM in macrophages. J774.1 macrophages were infected with strain SCHU S4 or the SCHUS4ΔFTT1564::CAM mutant (a) or with strain U112 or the U112ΔFTN1472::CAM mutant (b) at an m.o.i. of 10. At selected times post-infection, macrophages were lysed and bacteria were enumerated by culture on agar. The results are shown as means ± SEM, and the statistically significant difference ($P < 0.001$) is indicated by *.

Campylobacter jejuni (Candon *et al.*, 2007), *Salmonella enterica* and *Shigella flexneri* (Kim *et al.*, 2002). We therefore wished to determine what effect inactivation of this putative polyphosphate kinase had on the virulence of *Francisella*.

Mutants of *F. tularensis* subsp. *tularensis* and subsp. *novicida* were generated containing a deletion encompassing the majority of the FTN1472/FTT1564 ORF, replacing it with a chloramphenicol-resistance cassette. Examination of the genome sequence data for strains SCHU S4 (Larsson *et al.*, 2005) and U112 (Rohmer *et al.*, 2007) indicated that the FTT1564 gene was not part of an operon (Fig. 3) and that the observed phenotypes would therefore be unlikely to be due to polar effects on downstream genes. The plasmid was introduced into *Francisella* by conjugation, and mutants were selected by chloramphenicol resistance and sucrose tolerance. Replacement of the gene with the antibiotic-resistance cassette was confirmed by PCR and Southern blotting. The colony morphology of the mutants was identical to that of the wild-type strains and they grew to a similar density in liquid culture (data not shown). Bioinformatic analysis has suggested FTN1472/FTT1564 to be a polyphosphate kinase (Maier *et al.*, 2007), but this function has not been proven. Therefore, we quantified the polyphosphate content of *F. novicida* U112 and the

U112ΔFTN1472::CAM mutant (Fig. 4). Polyphosphate is a linear polymer of hundreds of phosphate residues, found in bacterial, fungal, plant and animal cells (Kulaev & Kulakovskaya, 2000). The hydrolysis of polyphosphates to free phosphate resulted in a significant increase in phosphate titre for the wild-type ($P < 0.05$), indicating that the organism produced detectable polyphosphate. There was no significant difference in phosphate titre pre- and post-hydrolysis for the samples isolated from cultures of the mutant, indicating that polyphosphate production had been abolished by inactivation of FTN1472.

Having been identified in our promoter trapping screen as a gene apparently induced intracellularly, the effect of inactivation of FTT1564 on intracellular growth of *Francisella* was evaluated in the J774.1 macrophage cell line. Initially, the mutants appeared to be taken up by macrophages similarly to wild-type; however, a growth defect was apparent by 24 h post-infection and significant ($P < 0.001$) by 48 h compared with the wild-type (Fig. 5). The experiment was terminated at 48 h, as *Francisella* induces destruction of the monolayer after this time. The growth defect was not as marked as that observed following mutation of *iglC* or its regulator MglA (Baron & Nano, 1998; Golovliov *et al.*, 2003). Thus, similarly to the function of the homologue in the attenuated subsp.

Fig. 6. Attenuation of *F. novicida* U112ΔFTN-1472 and *F. tularensis* SCHUS4ΔFTT1564 in the mouse model. (a) Groups of six BALB/c mice were dosed s.c. with dilutions of the mutant U112ΔFTN1472 or wild-type U112 (WT) and survival was monitored. (b) Groups of six BALB/c mice were dosed s.c. with dilutions of the mutant SCHUS4ΔFTT1564 or wild-type SCHU S4 (WT) and survival was monitored.

Table 3. Protection of mice induced by SCHUS4ΔFTT1564::CAM against homologous challenge

Primary challenge	Secondary challenge	Survivors*	Day of death
None	U112	0/6	3, 3, 3, 3, 3, 4
U112ΔFTN1472::CAM (6.9×10^4 c.f.u.)	U112	6/6	–
U112ΔFTN1472::CAM (6.9×10^6 c.f.u.)	U112	4/4	–
None	SCHU S4	0/6	6, 6, 6, 6, 6, 7
SCHUS4ΔFTT1564::CAM (4.5×10^2 c.f.u.)	SCHU S4	0/6	6, 6, 6, 7, 7, 7
SCHUS4ΔFTT1564::CAM (4.5×10^4 c.f.u.)	SCHU S4	2/2	–

*No. surviving secondary challenge/no. surviving primary challenge. Mice dosed with the mutant strains were challenged 56 days later with 100 LD₅₀ wild-type strain.

holarctica strain LVS (Maier *et al.*, 2007), polyphosphate kinase was shown to be essential for intracellular survival and growth for both subsp. *novicida* and the highly virulent subsp. *tularensis*. Inactivation of polyphosphate production has been shown to have a detrimental effect on intracellular survival of other intracellular pathogens such as *M. tuberculosis* (Sureka *et al.*, 2007) and *Salmonella enterica* (Kim *et al.*, 2002).

Most mutants of *Francisella* defective for intracellular survival, such as the *iglC* mutant, are attenuated, even though they may persist for significant periods (Twine *et al.*, 2005). We wished to determine whether the observed intracellular growth defect correlated with attenuation in the mouse model of disease, as has been indicated by results obtained with a transposon insertion mutant of strain LVS (Maier *et al.*, 2007). BALB/c mice were inoculated by the s.c. route with U112 or U112ΔFTN1472::CAM. Retrospective counts determined that mice received 6.9×10^4 or 6.9×10^6 c.f.u. of the mutant and 7.25×10^4 or 7.25×10^6 c.f.u. of wild-type. Mice were also inoculated by the s.c. route with either SCHU S4 or SCHUS4ΔFTT1564::CAM. Retrospective counts determined that mice received 4.5×10^2 or 4.5×10^4 c.f.u. of the mutant and 2×10^2 or 2×10^4 c.f.u. of wild-type. Both mutants were attenuated such that all mice survived the lower dose challenge, but there were some deaths at the higher challenge levels (Fig. 6a, b). Thus, as was observed following i.p. challenge with the transposon mutant of the attenuated subsp. *holarctica* strain LVS (Maier *et al.*, 2007), polyphosphate kinase was shown to be essential for virulence for both subsp. *novicida* and the highly virulent subsp. *tularensis*.

FTN1472/FTT1564 mutants induce a protective immune response against homologous challenge with subsp. *novicida* but not subsp. *tularensis*

To determine whether infection with the attenuated mutants was sufficient to induce a protective immune response, the survivors from the attenuation study were challenged with 100 LD₅₀ of each wild-type strain. The U112ΔFTN1472::CAM mutant was able to induce a robust protective immune response against homologous

challenge at both dose levels (Table 3). However, mice receiving the lower dose of SCHUS4ΔFTT1564::CAM were not protected against wild-type SCHU S4 and there was no delayed time to death. Naïve mice challenged in parallel all died. This may in part reflect the differences in the basis of protection against high- and low-virulence strains, with antibody being sufficient to protect against subsp. *novicida*, whilst cell-mediated immunity is key to a protective immune response against subsp. *tularensis* infection (Fulop *et al.*, 2001). The lack of protection at lower immunizing doses of SCHUS4ΔFTT1564::CAM may indicate that the mutant is unable to replicate sufficiently to induce a protective immune response. Previously, overattenuation due to a growth defect was reported for a *F. tularensis* subsp. *novicida* Δ*purA* mutant (Quarry *et al.*, 2007). Alternatively, polyphosphate may regulate expression of an antigen that contributes to the protective immune response against tularemia: loss of polyphosphate kinase has profound effects on cellular composition and morphology (Fraley *et al.*, 2007). Mere persistence by *Francisella* *in vivo* has been shown not to correlate with the ability of mutants to induce a protective immune response (Twine *et al.*, 2005) and the correct interaction of the attenuated mutant with the host is key to developing a protective immune response. Further work will be required to elucidate the molecular mechanisms by which FTT1472/FTT1564 contributes to the virulence of *Francisella* and its intracellular lifestyle.

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