A *Francisella tularensis* SCHU S4 mutant deficient in γ -glutamyltransferase activity induces protective immunity: characterization of an attenuated vaccine candidate

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Francisella tularensis is an intracellular pathogen which causes tularaemia. There is no licensed vaccine currently available for prophylaxis. The γ -glutamyl transpeptidase (GGT) encoded by the *ggt* gene has been shown to be important for the intracellular survival of *F. tularensis*. In this study we have constructed a *ggt* deletion mutant in the highly virulent *F. tularensis* strain SCHU S4. Characterization of the mutant strain confirmed the function of *ggt*, and confirmed the role of GGT in cysteine acquisition. The mutant strain was highly attenuated both *in vitro* and *in vivo* using murine models of infection. Moreover, we have demonstrated that the attenuated mutant is able to induce protective immunity against an *F. tularensis* SCHU S4 challenge, and thus may be a candidate for the development of an attenuated vaccine.

INTRODUCTION

Francisella tularensis, the aetiological agent of tularaemia, is a highly infectious, facultative intracellular bacterium (Dorofe'ev, 1947; McCoy & Chapin, 1912; Sjöstedt, 2006). This highly virulent member of the class γ -proteobacteria of Gram-negative bacteria is capable of infecting a wide range of mammalian species, including man (Mörner, 1992; Sjöstedt, 2006). There are currently three accepted subspecies of *F. tularensis* with differing geographical distributions: the highly virulent subspecies *tularensis* (also known as type A) is found almost exclusively in North America (Olsufiev *et al.*, 1959; Olsufjev & Meshcheryakova, 1982); the less virulent subspecies *holarctica* (also known as type B) is found in both North America and Eurasia (Olsufiev *et al.*, 1959; Olsufjev & Meshcheryakova, 1982); and subspecies *mediasiatica* is of similar virulence to subspecies *holarctica* and is only found in the central Asian republics of the former Soviet Union (Olsufiev *et al.*, 1959; Olsufjev & Meshcheryakova, 1982). A fourth subspecies, *novicida*, has been proposed, but at the time of writing *Francisella novicida* remains a separate species (Euzéby, 1997; Forsman *et al.*, 1994; Hollis *et al.*, 1989).

There are several forms of human tularaemia, dependent upon the route of infection (Dennis *et al.*, 2001; Hubálek *et al.*, 2004). Ulceroglandular tularaemia, acquired by

dermal routes of infection, is the most common natural presentation, but pneumonic tularaemia following inhalation is the most acute form, with the highest associated mortality (Gill & Cunha, 1997). Upon entry into the host, *F. tularensis* is able to evade the immune system and replicate in the cytosol of infected macrophages (Checroun *et al.*, 2006; Clemens *et al.*, 2004). From there, *F. tularensis* spreads from the site of infection via the regional lymph nodes and disseminates systemically to organs, including the liver, spleen and lungs (Checroun *et al.*, 2006; Hubálek *et al.*, 2004).

The enzyme γ -glutamyl transpeptidase (GGT) catalyses the hydrolysis of γ -glutamyl compounds, mediating the transfer of γ -glutamyl moieties to amino acids and peptides (Tate & Meister, 1981). It is present in both eukaryotes and prokaryotes; the bacterial enzyme has been shown to be associated with either the inner membrane or the periplasm, whereas eukaryotic GGT is anchored at the N terminus to the plasma membrane (Ikeda *et al.*, 1995; Tate & Meister, 1981; Xu & Strauch, 1996). GGT is reported to have a variety of functions, with one of the major roles being participation in the γ -glutamyl cycle, where it catalyses the degradation of the antioxidant molecule reduced glutathione (GSH) to L- γ -glutamyl and L-cysteinylglycine in the first step of a reaction resulting in the acquisition of cysteine (Gregus *et al.*, 1987; Tate & Meister, 1981). Interestingly, in light of the cysteine auxotrophy displayed by *F. tularensis*, GGT is reported to play a role in the supply of cysteine from environmental peptides to *Neisseria meningitidis* during periods of cysteine shortage (Takahashi *et al.*, 2004). In a previous study to elucidate

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Abbreviations: GGT, γ -glutamyl transpeptidase; MLD, median lethal dose; sc, subcutaneous(ly).

virulence mechanisms employed by *Francisella*, transcriptional analysis was undertaken on *F. novicida* cultured *in vitro* under iron-starvation conditions (LeButt, 2008; Milne *et al.*, 2007). A number of genes were observed to be upregulated under these conditions, one of which was homologous to *ggt* enzymes, indicating an important role for GGT *in vivo*.

A recent investigation using the empirically derived, attenuated strain *F. tularensis* subsp. *holarctica* LVS demonstrated that GGT plays a role in cysteine synthesis, and that deletion of *ggt* attenuates the residual virulence that is displayed by the vaccine strain in mice (Alkhuder *et al.*, 2009). An *F. tularensis* SCHU S4 *ggt* mutant, identified by transposon mutation, is attenuated in hepatic carcinoma cell line HepG2 cells (Qin & Mann, 2006), and a defined SCHU S4 *ggt* mutant is attenuated in a murine model (Kadzhaev *et al.*, 2009). However, these mutants were not characterized any further, for example, gene function was not restored through complementation.

F. tularensis LVS was originally derived through *in vitro* subculture (Tigertt, 1962), and as such contains multiple genetic defects (Conlan & Oyston, 2007). LVS can induce protective immune responses in man, but as the genetic basis of attenuation remains to be elucidated, LVS is yet to receive full licensure (Conlan & Oyston, 2007). There are an increasing number of targeted mutants created in *F. tularensis* SCHU S4 that are attenuated in the murine model of infection (Kadzhaev *et al.*, 2009), but although some have been shown to offer protection, such as strains lacking *clpB*, *fupA* or *capB* (Shen *et al.*, 2010), many when used to vaccinate mice rarely offer sufficient protection against homologous challenge (Kadzhaev *et al.*, 2009). We

wished to evaluate a mutant of *F. tularensis* SCHU S4 Δ ggt as a live attenuated vaccine strain. The challenge of a live vaccine is balancing attenuation, required for safety, with immunogenicity. If a strain is overly attenuated the correct immune response will not be induced, and mere persistence has been shown not to be sufficient for immunogenicity (Twine *et al.*, 2005).

In this study an isogenic in-frame deletion mutant for *ggt* was generated in the *F. tularensis* strain SCHU S4. In order to confirm the function of *ggt* in a fully virulent isolate, as reported for the attenuated LVS strain (Alkhuder *et al.*, 2009), the effect of this mutation on GGT activity and the ability of the subspecies *tularensis* SCHU S4 *ggt* mutant to obtain and use cysteine from GSH was investigated. To confirm that this mutation was attenuating in a highly virulent strain, the ability of bacteria to infect macrophages and mice was also investigated. The protective efficacy of this mutant against a fully virulent SCHU S4 challenge was also evaluated for the first time.

METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. Strains of *F. tularensis* were grown routinely on blood cysteine glucose agar supplemented with 4 % cysteine, 4 % histidine, 5 % glucose and 10 % fresh filtered horse blood (BCGA), solidified Chamberlain's defined medium (CDM) (Chamberlain, 1965) or Thayer–Martin agar (Atlas, 2004). Where required, media were supplemented with 100 μ g polymyxin ml⁻¹, 12.5 μ g kanamycin ml⁻¹ and 13.5 μ g tetracycline ml⁻¹. Strains of *Escherichia coli* were grown in Luria–Bertani (LB) medium (Atlas, 2004) or on LB agar at 37 °C, supplemented as required with 25 μ g kanamycin ml⁻¹. All work undertaken with *Francisella* strains was

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description*	Source or reference
Strains		
<i>E. coli</i>		
JM109	Host cloning strain	Promega
S17- λ pir	Host strain used for transfer of suicide plasmid into <i>F. tularensis</i> SCHU S4 by conjugation	Simon <i>et al.</i> (1983)
<i>F. tularensis</i>		
SCHU S4	Wild-type <i>F. tularensis</i> subsp. <i>tularensis</i> strain	Hesselbrock & Foshay (1945); human ulcer, 1941, OH, USA
SCHU S4 Δ ggt	<i>F. tularensis</i> SCHU S4 with unmarked, in-frame deletion of <i>ggt</i>	This study
Plasmids		
pGEM-T Easy Vector System I	Cloning vector, Amp ^R	Promega
pSMP22	Suicide vector containing <i>sacB</i> selection	Thomas <i>et al.</i> (2007)
pSMP75	Modified pSMP22 suicide vector; Kan ^R introduced under the control of FTT1441 promoter using <i>mull</i> and <i>psII</i> restriction sites	Thomas <i>et al.</i> (2011)
pSMP75 Δ ggt	pSMP75 containing 1787 bp Δ ggt left and right flanking regions inserted at <i>MluI</i> site	This study
pSMP6oriT	Complementation plasmid, Tet ^R	Quarry <i>et al.</i> (2007)

*Amp^R, ampicillin resistant; Chl^R, chloramphenicol resistant; Kan^R, kanamycin resistant; Tet^R, tetracycline resistant.

performed under appropriate laboratory containment conditions in accordance with relevant legislative requirements. The use of tetracycline as a marker was approved for this work and was reviewed locally by a genetic manipulation safety committee and also approved nationally by our health and safety authorities.

Plasmids and manipulation of DNA. Generally, the manipulation of DNA was carried out as outlined elsewhere (Owens & Hartman, 1986). Genomic DNA was isolated using the Gentra Puregene DNA Isolation kit (Qiagen). Plasmid DNA was isolated using Qiagen Plasmid Mini and Maxi kits. Plasmids used in this study are shown in Table 1. Southern blotting was performed under high-stringency conditions as detailed by Sambrook & Russell (2001). Standard PCR was performed with 25 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s.

Generation of an *F. tularensis* Δ ggt mutant. Primers were designed from the genome sequence of *F. tularensis* SCHU S4, such that a 1787 bp deletion was generated in the *ggt* (FTT1181c) ORF. Primers were used to amplify DNA regions flanking the deletion (left flank forward, ggtF1: 5'-ACGCGTTAGGTTACCTTGGGCTTGAG-3'; reverse, ggtR1: 5'-AGATCTGGTTATTAACACTTTAATAG-3'; right flank forward, ggtF2: 5'-AGATCTACGACGATTAAGAAAC-3'; reverse, ggtR2: 5'-ACGCGTTGTGGGATTAAGTGGGAAACC-3'). These amplicons were ligated with pGEM-T Easy (Promega) according to the manufacturer's protocol to generate pGEMggt1 and pGEMggt2 containing left and right flanks, respectively. The two plasmids were digested with *Bgl*II to linearize them, and they were then ligated. The ligated plasmids were used as template in a PCR to join the two flanks, using primers ggtF1 and ggtR2. The product was ligated with pGEM-T Easy as above and used to transform *E. coli* JM109. Plasmids were isolated and the correct sequence was confirmed by PCR. One correct clone was digested with *Mlu*I, and the insert was isolated and ligated with similarly digested pSMP75 to produce pSM75 Δ ggt. Plasmid pSM75 Δ ggt was introduced into *F. tularensis* SCHU S4 by conjugation using the method of Golovliov *et al.* (2003). Briefly, *E. coli* S17- λ pir pSM75 Δ ggt was grown overnight in LB-kanamycin, a 1 ml aliquot was removed, and the bacteria were sedimented by centrifugation and resuspended in 50 μ l fresh LB broth. The *Francisella* were grown on BCGA overnight at 37 °C in a confluent lawn. Bacteria were removed using a sterile loop and resuspended in the *E. coli* suspension, and the mixture spotted onto BCGA. After incubation overnight at 25 °C, the bacteria were removed with a sterile loop and suspended in 500 μ l PBS before being inoculated onto Thayer–Martin agar supplemented with 100 μ g polymyxin ml⁻¹ (to inhibit growth of the *E. coli*) and 12.5 μ g kanamycin ml⁻¹. Colonies were subcultured onto Thayer–Martin agar supplemented with 5% sucrose. Isolated colonies that grew in the presence of sucrose were analysed by PCR for the deletion of the *ggt* ORF and loss of the vector. This was subsequently confirmed by Southern blotting. The strain was designated *F. tularensis* SCHU S4 Δ ggt.

Complementation of *F. tularensis* SCHU S4 Δ ggt. The *F. tularensis* SCHU S4 Δ ggt strain was complemented using the native promoter by cloning the region 1052 bp upstream to 942 bp downstream of the *ggt* gene using the primers 5'-GGTACCAATTATTGCTAAGCTTATTTATACACCACAG-3' forward and 5'-GGTACCTAGCTTAGATTAATTATTCAAATTACTAAG-3' reverse. The amplicon was ligated with pSMP6oriT that had been digested using *Kpn*I and contained a tetracycline-resistance cassette. The plasmid was introduced into *F. tularensis* SCHU S4 Δ ggt by conjugation, again using the method of Golovliov *et al.* (2003). The strain was designated *F. tularensis* SCHU S4 Δ ggt_C.

Assay for GGT activity. The GGT activity of both mutant and wild-type *F. tularensis* was determined by measuring optically the release of *p*-nitroaniline from L- γ -glutamyl-*p*-nitroanilide as described

elsewhere, with minor modifications (Huseby & Strömme, 1974). Briefly, reaction buffer, consisting of 1 mM L- γ -glutamyl-*p*-nitroanilide, 20 mM Gly-Gly, and 60 mM Tris-HCl (pH 8.0) was prepared, and 180 μ l was applied to each well of a 96-well microtitre plate. Bacteria were suspended in PBS to an OD₅₉₀ of 0.6, and 20 μ l was also applied to each well. Assays were performed at 37 °C for 23 h, at which point the A₄₀₅ of each well was measured.

Growth in the absence of exogenous cysteine. Overnight cultures of bacteria on BCGA were suspended in PBS and used to inoculate media to a starting OD₅₉₀ of 0.14. Cultures were incubated at 37 °C with shaking at 150 r.p.m. for 19 h, following which the OD₅₉₀ was measured as an indication of bacterial growth. Growth was measured in the following media: CDM, CDM prepared without cysteine (CDM -cys), and CDM -cys supplemented with 0.6 mM GSH.

Survival in J774.A1 macrophages. Bacterial survival in J774.A1 murine macrophages was studied with the method described by Golovliov *et al.* (1997), using an m.o.i. of 10. Briefly, J774.A1 cells were cultured in 24-well tissue culture plates at 37 °C with 5% (v/v) CO₂ until confluent monolayers of approximately 1 $\times$ 10⁶ cells per well were established. The culture medium was removed from the cells and replaced with 0.1 ml bacterial suspension at 1 $\times$ 10⁸ c.f.u. ml⁻¹. Infected monolayers were incubated as before for 30 min to allow bacterial invasion of the cells. The inoculum was aspirated and the monolayer washed with PBS three times. Cells were then overlaid with culture medium containing 10 μ g gentamicin ml⁻¹ and incubated as before for 30 min to kill any remaining extracellular bacteria. The medium was aspirated and the monolayer again washed with PBS three times. Finally, the cells were overlaid with maintenance medium containing 2 μ g gentamicin ml⁻¹ and incubated as before. This was denoted time point 0 h post-infection. At time points 1, 24 and 48 h, bacteria were recovered from duplicate monolayers by aspiration of the media, washing in PBS, and lysis using sterile water. Cell lysates were decimally diluted in PBS, and the number of viable, internalized bacteria was determined by counting the resultant colonies on BCGA. This experiment was repeated in triplicate.

Virulence and protection of BALB/c mice. Groups of six 6–8 week-old, female BALB/c mice (Charles RiverUK) were dosed by the subcutaneous (sc) route with serial dilutions of bacteria in PBS. The concentration of bacteria in the inoculum was determined by retrospective viable count. Mice surviving the initial challenge were subsequently challenged (sc) 49 days later with wild-type bacteria to determine whether a protective immune response had been induced. Humane end points were strictly observed, and animals deemed incapable of survival were humanely killed by cervical dislocation. The median lethal dose (MLD) to induce morbidity or death is approximately 1 c.f.u. for *F. tularensis* SCHU S4 given by the sc route (Green *et al.*, 2005).

Statistical analyses. Statistical differences in recovered c.f.u. from infected J774.A1 and in GGT activity were determined using a repeated measures analysis of variance (ANOVA) with Bonferroni's multiple comparisons test and Student's *t* test, respectively.

RESULTS

To determine the function of GGT in *F. tularensis*, a mutant was constructed in strain SCHU S4. PCR and Southern blot analysis confirmed the double crossover and successful allelic replacement of the gene (data not shown). The GGT activity of *F. tularensis* SCHU S4 and *F. tularensis*

Fig. 1. GGT activity of *F. tularensis* SCHU S4, *F. tularensis* SCHU S4 Δ ggt and *F. tularensis* SCHU S4 Δ ggt_C as determined by incubation of bacterial cells with L- γ -glutamyl-*p*-nitroanilide and Gly-Gly; the increase in A₄₀₅ caused by the release of *p*-nitroaniline is directly proportional to GGT activity. Data are presented as means and SEM of three experiments.

SCHU S4 Δ ggt was determined by assaying the release of *p*-nitroaniline from L- γ -glutamyl-*p*-nitroanilide at 37 °C for 23 h. The mean GGT activity was observed to be approximately 11-fold higher in *F. tularensis* SCHU S4 than in *F. tularensis* SCHU S4 Δ ggt ($P < 0.001$) (Fig. 1). Complementation of the *ggt* mutation with a plasmid containing the gene resulted in full restoration to the wild-type phenotype.

To test whether GGT in *F. tularensis* SCHU S4 plays a role in the acquisition of cysteine from GSH, the growth of *F. tularensis* SCHU S4 and *F. tularensis* SCHU S4 Δ ggt was compared *in vitro* in cysteine-depleted media that was supplemented with GSH. The results of triplicate assays are shown in Fig. 2. The growth of both the wild-type and

mutant was reduced by approximately fivefold in CDM –cys compared with CDM. The addition of 0.6 mM GSH to the CDM –cys media restored the growth of *F. tularensis* SCHU S4 to a similar level to that observed in CDM, whereas the growth of *F. tularensis* SCHU S4 Δ ggt was only restored following complementation with a *ggt*-expressing plasmid.

The potential role of GGT in *F. tularensis* virulence was tested by comparing the mutant and wild-type strains in a well-established murine macrophage model of infection. At 24 h post-infection, with an m.o.i. of 10, the number of viable *F. tularensis* SCHU S4 recovered from J774A.1 murine macrophage-like cells was approximately two times higher than the number of viable *F. tularensis* SCHU S4 Δ ggt recovered ($P < 0.001$), a trend which was continued at 48 h post-infection (Fig. 3). Complementation of the *ggt* mutation resulted in partial restoration of wild-type virulence, such that the c.f.u. recovered were intermediate between those of SCHU S4 and Δ ggt and significantly different from both ($P < 0.001$). The potential attenuation of the *F. tularensis* SCHU S4 mutant strain was also investigated using a murine model of infection. BALB/c mice received doses of *F. tularensis* SCHU S4 Δ ggt ranging from 8.75 c.f.u. to 8.75×10^6 c.f.u. in one-log increments via the sc route. Control mice that received 1.3×10^3 c.f.u. *F. tularensis* SCHU S4 had all reached the humane end point by 4 days post-infection. Mice that received *F. tularensis* SCHU S4 Δ ggt survived in a dose-dependent manner (Table 2). The MLD of *F. tularensis* SCHU S4 Δ ggt was calculated as approximately 2×10^6 c.f.u., compared with a wild-type *F. tularensis* MLD of approximately 1 c.f.u.

The protective efficacy of *F. tularensis* SCHU S4 Δ ggt against a wild-type challenge was also investigated using the murine model of infection. Mice that survived vaccination with *F. tularensis* SCHU S4 Δ ggt were

Fig. 2. Growth of *F. tularensis* SCHU S4, *F. tularensis* SCHU S4 Δ ggt and *F. tularensis* SCHU S4 Δ ggt_C in CDM with and without free cysteine, and without free cysteine but supplemented with GSH (0.6 mM). Growth was monitored at 5 h (white bars), 24 h (grey bars) and 48 h (black bars) post-inoculation. Data points represent means and SEM of three experiments.

Fig. 3. Recovery of viable counts for *F. tularensis* SCHU S4 (white bars), *F. tularensis* SCHU S4 Δggt (grey bars) and *F. tularensis* SCHU S4 Δggt_C (black bars) from J774A.1 cells inoculated with an m.o.i. of 10 bacteria per macrophage. Each time point represents the means and SEM of triplicate wells performed on three separate occasions (** $P < 0.001$).

subsequently challenged with 100 MLD *F. tularensis* SCHU S4. Mice vaccinated with 8.75×10^2 c.f.u. *F. tularensis* SCHU S4 Δggt or less did not show total protection, whereas mice that received 8.75×10^3 c.f.u. *F. tularensis* SCHU S4 Δggt or more were fully protected (Fig. 4).

DISCUSSION

There is a pressing need for a licensable *F. tularensis* vaccine candidate. Much effort has been expended attempting to license the LVS strain (Conlan & Oyston, 2007), but as yet this has not been achieved. Similarly, attempts to identify protective subunit formulations have been unsuccessful to date (Oyston & Griffiths, 2009). As such, a novel, defined, live attenuated vaccine candidate may offer a safe and

Table 2. Virulence of *F. tularensis* SCHU S4 Δggt in BALB/c mice

Mice challenged with 1300 c.f.u. of wild-type *F. tularensis* SCHU S4 all reached the humane end point at 4 days post-challenge. NA, Not applicable.

Challenge Δggt dose (c.f.u.)	Survivors/total number	Time to humane end point (days post-inoculation)
8.75	6/6	NA
87.5	6/6	NA
8.75×10^2	5/6	11
8.75×10^3	5/6	10
8.75×10^4	4/6	8, 9
8.75×10^5	6/6	NA
8.75×10^6	2/6	4, 7, 7, 7

Fig. 4. Protection of BALB/c mice against SCHU S4 following sc vaccination with SCHU S4 Δggt [8.75 c.f.u. (○), 87.5 c.f.u. (+), 8.75×10^2 c.f.u. (▲), 8.75×10^3 c.f.u. (▼), 8.75×10^4 c.f.u. (◆), 8.75×10^5 c.f.u. (●) or 8.75×10^6 c.f.u. (■)] and naïve mice (x) subsequently challenged with 100 MLD of *F. tularensis* SCHU S4.

effective alternative. There has thus been a drive to identify genes which, when inactivated, produce safe immunogenic mutants (Kadzhaev *et al.*, 2009; Michell *et al.*, 2010; Pechous *et al.*, 2008; Qin *et al.*, 2008; Shen *et al.*, 2010; Thomas *et al.*, 2007; Twine *et al.*, 2005). Preliminary results indicate that *ggt* may be a suitable target for inactivation, as it is attenuating in LVS and SCHU S4 (Alkhuder *et al.*, 2009; Kadzhaev *et al.*, 2009; Qin & Mann, 2006). However, the function of the encoded protein in the highly virulent subspecies has not been confirmed, nor has the induced immune response been characterized for protective efficacy. Extrapolating observations between *Francisella* species and subspecies is not always appropriate. For example, *F. tularensis* subsp. *tularensis* O antigen has been identified as important for intracellular survival, but in *F. novicida* this gene does not confer such an advantage, being instead important for viability before entering a host cell (Thomas *et al.*, 2007). In addition, the deletion of pilin homologues in *F. tularensis* LVS produces significant attenuation, in contrast to SCHU S4, in which attenuation is not observed, suggesting a different role in the fully virulent strain (Ark & Mann, 2011). Similar discrepancies between mutants created in LVS and SCHU S4 have been observed in an RND-like efflux pump (Qin & Mann, 2006) and catalase (*katG*) (Bina *et al.*, 2008), emphasizing the need fully to characterize phenotypes in virulent strains. We therefore created an in-frame deletion mutant to undertake these studies and confirm the role of GGT in SCHU S4.

The results of the *p*-nitroanilide assay for GGT activity carried out using wild-type, mutant and complemented strains demonstrated that, as in *F. tularensis* LVS, the *ggt* of *F. tularensis* SCHU S4 does encode GGT (Alkhuder *et al.*, 2009). Growth of the mutant and wild-type strains *in vitro* was comparable, suggesting that GGT is not essential for *in*

vitro growth of *F. tularensis* (Fig. 2). This is in agreement with previously published *Aggt* strains constructed in *Campylobacter jejuni* and *N. meningitidis* (Barnes *et al.*, 2007; Takahashi *et al.*, 2004). However, the expression of active GGT by the wild-type strain cultured *in vitro* seems to indicate that, although not essential, GGT may still contribute to the ability of *F. tularensis* to overcome growth-limiting factors.

The restoration of growth of *F. tularensis* strains encoding intact *ggt* upon addition of GSH (in the absence of free cysteine) demonstrates that strain SCHU S4 does utilize GGT to acquire cysteine from GSH. This supports the findings of Alkhuder *et al.* (2009) in that GSH was used to restore the growth of *F. tularensis* LVS, but not *F. tularensis* LVS *Aggt*, cultured in the absence of cysteine. However, restoration of the growth of the attenuated LVS strain was only partial upon addition of GSH, compared with the full restoration of *F. tularensis* SCHU S4 growth. *F. tularensis* LVS was originally derived through *in vitro* subculture (Thomas *et al.*, 2007), and despite the completion of the genome sequence (P. Chain and others, unpublished results), the physiological nature of *F. tularensis* LVS attenuation remains to be elucidated. It is likely that *F. tularensis* LVS contains a number of disrupted functions, and this, taken together with a lack of full restoration of growth by GSH, could indicate that in the vaccine strain GGT serves some additional purpose, the absence of which could not be overcome by the addition of GSH.

It is often difficult through complementation *in trans* to completely restore observed phenotypes in targeted deletion mutants (Ark & Mann, 2011; Brotcke *et al.*, 2006; Lai *et al.*, 2004). Partial restoration (Fig. 3) of the GGT mutant in macrophages may have been a consequence of expression above wild-type levels due to copy number. Additional breakdown of the potentially beneficial GSH by *Aggt_C* may account for the absence of full restoration, as GSH is a well-documented antioxidant molecule, and has been demonstrated to provide protection to all cells against reactive oxygen and nitrogen species through electron donation (Masip *et al.*, 2006; Wills, 1966). It has also been shown to protect bacteria against acid stress, chlorine compounds and osmotic stress (Masip *et al.*, 2006; Owens & Hartman, 1986; Riccillo *et al.*, 2000). Therefore, while these data do provide evidence of a role for GGT in *F. tularensis* cysteine acquisition *in vitro*, they may also indicate that the addition of GSH provides some other, GGT-independent, benefit to *F. tularensis* SCHU S4 cultured under these conditions.

In addition to the attenuation for virulence demonstrated by *F. tularensis* SCHU S4 *Aggt*, and by the LVS mutant in the same gene (Alkhuder *et al.*, 2009; Kadzhaev *et al.*, 2009), there are several prior examples of a role for GGT in virulence. In the intracellular fungal pathogen *Histoplasma capsulatum*, which resides in macrophages, GGT has been shown to play a role in both the γ -glutamyl cycle and also in iron acquisition: the thiol group of the dipeptide released upon GGT-mediated degradation of GSH is able to

efficiently reduce ferric iron to the more usable ferrous form (Zarnowski *et al.*, 2008). In *C. jejuni*, GGT has been shown to be required for persistent colonization of the avian gut (Barnes *et al.*, 2007). GGT has also been shown to play a role in the colonization of murine gastric mucosa by *Helicobacter pylori*, potentially enabling survival through participation in the *de novo* synthesis of essential amino acids (Chevalier *et al.*, 1999; Gong & Ho, 2004; McGovern *et al.*, 2001). In addition, an *F. tularensis* SCHU S4 *ggt* transposon mutant is defective for intracellular replication in the hepatic carcinoma cell line HepG2 (Qin & Mann, 2006).

The level of attenuation for virulence (in BALB/c mice) of *F. tularensis* SCHU S4 *Aggt* described here is in agreement with an *F. tularensis* SCHU S4 *ggt* mutant identified elsewhere (Kadzhaev *et al.*, 2009). Moreover, this one millionfold level of attenuation of *F. tularensis* SCHU S4 *Aggt* in mice is reflective of, but far exceeds, the thousandfold level of attenuation reported for *F. tularensis* LVS *Aggt* in a murine model of infection (Alkhuder *et al.*, 2009). The reduced level of attenuation observed in *F. tularensis* LVS *Aggt* (compared with *F. tularensis* SCHU S4 *Aggt*), both *in vivo* and *in vitro*, may be due to the high level of attenuation already displayed by *F. tularensis* LVS masking the effect of the *ggt* mutation. This, together with its undefined nature, makes *F. tularensis* LVS a suboptimal strain for the evaluation and characterization of mutants. Conversely, fully virulent strains, such as *F. tularensis* SCHU S4, display the full effects of mutations in a more clinically relevant framework.

Attenuated mutant strains need to balance attenuation, for safety, with the ability to induce a protective immune response. Additionally, it is hard to predict whether an attenuated strain will induce protective immune responses against challenge with a fully virulent strain. For example, Δ *purA* mutants are overly attenuated, as they fail to induce a protective immune response (Quarry *et al.*, 2007; Tempel *et al.*, 2006), whereas mutants defective in genes further down the purine biosynthetic pathway provide protective immunity (Pechous *et al.*, 2009). In this study, we have demonstrated that the *ggt* mutant is able to induce protective responses in mice, and that it may be a candidate for inactivation in a live attenuated vaccine, although for safety reasons more than one attenuating mutation will need to be included in a defined vaccine strain, and the effects of combining mutations is hard to predict.

In conclusion, the data presented here demonstrate that GGT is required for virulence in *F. tularensis* SCHU S4. Deletion of *ggt* is attenuating, and the mutant elicits a protective immune response against a fully virulent wild-type challenge. We have confirmed a function of GGT in fully virulent *F. tularensis* subsp. *tularensis* SCHU S4, and that starvation for cysteine is likely to be the cause of attenuation. The undefined nature of *F. tularensis* LVS, together with the fact that it can cause a tularemia-like disease in some individuals, means that there is a requirement for an improved vaccine against *F. tularensis*. The attenuated and protective nature of *F. tularensis* SCHU

S4 *ΔggT* makes this gene an excellent candidate for further study towards the production of a defined live vaccine.

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