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Short Communication

SHORT COMMUNICATION

Characterization of *Francisella tularensis* Schu S4 defined mutants as live-attenuated vaccine candidates

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One sentence summary: Mutations in guanine biosynthesis genes, but not in four other hypothetical virulence factors in highly virulent *Francisella tularensis* strain Schu S4 resulted in attenuation in macrophage replication and mouse virulence.
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ABSTRACT

Francisella tularensis (Ft), the etiological agent of tularemia and a Tier 1 select agent, has been previously weaponized and remains a high priority for vaccine development. *Ft tularensis* (type A) and *Ft holarctica* (type B) cause most human disease. We selected six attenuating genes from the live vaccine strain (LVS; type B), *F. novicida* and other intracellular bacteria: FTT0507, FTT0584, FTT0742, FTT1019c (*guaA*), FTT1043 (*mip*) and FTT1317c (*guaB*) and created unmarked deletion mutants of each in the highly human virulent *Ft* strain Schu S4 (Type A) background. FTT0507, FTT0584, FTT0742 and FTT1043 Schu S4 mutants were not attenuated for virulence *in vitro* or *in vivo*. In contrast, Schu S4 *gua* mutants were unable to replicate in murine macrophages and were attenuated *in vivo,* with an i.n. $LD_{50} > 10^5$ CFU in C57BL/6 mice. However, the *gua* mutants failed to protect mice against lethal challenge with WT Schu S4, despite demonstrating partial protection in rabbits in a previous study. These results contrast with the highly protective capacity of LVS *gua* mutants against a lethal LVS challenge in mice, and underscore differences between these strains and the animal models in which they are evaluated, and therefore have important implications for vaccine development.

Keywords: *Francisella tularensis*; vaccines; live attenuated; Schu S4; mutants; tularemia

Francisella tularensis (*Ft*) is the etiological agent of tularemia and includes two subspecies that cause most disease in humans. *Ft* subsp. *tularensis* (type A) causes the most severe form of disease following an aerosol infectious dose as low as 10 CFU. *Ft* subsp. *holarctica* (type B) causes disease with reduced severity. Considering the low infectious dose, high mortality rate and the previous weaponization of this bacterium, *Ft* is classified as a Tier 1 select agent, and there is an immediate need for a vaccine against this pathogen. One vaccine studied in depth was the live vaccine strain (LVS), a subsp. *holartica* derivative which

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was passaged *in vitro* until attenuated. LVS was tested in humans in the 1960s (Saslaw *et al.* 1961; Hornick and Eigelsbach 1966) and demonstrated partial protection against pulmonary challenge with a highly human virulent subsp. *tularensis* strain, demonstrating proof of principle that a live-attenuated vaccine can protect against tularemia. We hypothesized that improved efficacy in humans against a highly virulent type A challenge would be achieved by developing a live-attenuated vaccine derived from a type A strain, Schu S4.

Using previously described techniques (Santiago *et al.* 2009), we generated 7 Schu S4 mutant strains containing unmarked, targeted deletions in the following *Ft* genes: FTT0507, FTT0584, FTT0742, FTT1019c (*guaA*), FTT1043, FTT1317c (*guaB*) and a double knockout of FTT1019c and FTT1317c (*guaAguaB*), as homologs of these genes have been previously demonstrated to be attenuating in *Francisella* spp. or other intracellular bacteria. In *F. novicida*, FTT0584 was required for suppression of the host ASC/caspase-1 pathway, which is important for innate immune defense (Henry and Monack 2007; Henry *et al.* 2007; Weiss *et al.* 2007; Monack 2008), and FTT0742 encodes a hypothetical lipoprotein that is predicted to form part of the *F. novicida* cell wall (Tempel *et al.* 2006); both were attenuating mutations in *F. novicida*. FTT0507 was identified in *Ft* subsp. *tularensis* as a third member of the thioredoxin (TRX) family of proteins; TRX proteins play a major role in maintaining the redox environment of the cell (Inaba 2008, 2009; Inaba and Ito 2008; Ito and Inaba 2008; Qin *et al.* 2008, 2009; Heras *et al.* 2009). The presence of multiple TRX members in a single bacterium suggests that these proteins play a crucial role in the correct folding of many secreted or exposed virulence determinants (Inaba and Ito 2008; Ito and Inaba 2008; Heras *et al.* 2009). FTT1043 was identified as encoding a protein with similarity to macrophage infectivity potentiator (mip). Mip proteins have been well characterized in several human pathogens, including *Legionella* (Bangsborg, Cianciotto and Hindersson 1991; Cianciotto and Fields 1992; Wagner *et al.* 2007), *Neisseria* (Starnino *et al.* 2010; Hung *et al.* 2011), *Coxiella* (Mo, Cianciotto and Mallavia 1995; Seshu, McIvor and Mallavia 1997), *Burkholderia* (Norville *et al.* 2011) and *Chlamydia* (Lundemose *et al.* 1991; Rockey *et al.* 1996; Neff *et al.* 2007; Bas *et al.* 2008; Lu *et al.* 2013), and are required by *Legionella pneumophila* for invasion and proper intracellular establishment of infection in macrophages and protozoa (Cianciotto and Fields 1992). Finally, deletions in genes encoding metabolic enzymes including *guaA* and *guaB* have been demonstrated to attenuate *Salmonella* and *Shigella* spp. (Chatfield *et al.* 1994; Cersini, Salvia and Bernardini 1998; Kotloff *et al.* 2000, 2007). The *gua*A and *gua*B genes encode essential enzymes in guanine nucleotide biosynthesis and deletion of either gene is highly attenuating in LVS; additionally, LVS *gua* mutants were protective against subsequent lethal LVS challenge (Santiago *et al.* 2009).

The successful deletion of each gene(s) in Schu S4 was confirmed by PCR, and growth kinetics were evaluated in both broth and J774 macrophages, a preferential host cell type for *Ft*. Schu S4 mutants in FTT0507, FTT0584, FTT0742 and FTT1043 exhibited no defects and replicated in broth and macrophages with no significant differences in kinetics compared to WT. As expected, the Schu S4Δ*gua* single and double mutants were unable to grow in broth without exogenously added guanine, and growth of the mutants was restored by either addition of guanine to the media or *trans*-complementation of the gene (data not shown). Additionally, the Schu S4 Δ*guaA*, Δ*guaB* and Δ*guaA*Δ*guaB* mutants failed to replicate in macrophages, exhibiting decreased bacterial counts over the time course ($P < 0.01$ for all three mutants compared to WT at 24 h, Fig. 1). Each single *gua* mutant

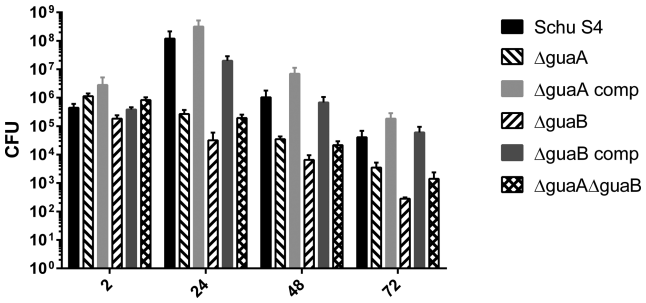

Figure 1. Growth of Schu S4 mutants in macrophages. J774.1 murine macrophages (3×10^5 cells/well) were infected with an MOI = 100 of each designated strain for 2 h, then washed and treated with gentamicin for 1 h. Cells were lysed with 0.02% SDS-PBS, and serial dilution plating was used to enumerate intracellular bacteria at defined time points post-infection. Data are representative of two independent experiments. Two-way ANOVAs with multiple comparisons were carried out to compare growth of individual strains over the time course and growth of all strains at each time point. WT Schu S4 showed significant growth from 2 to 24 h ($P < 0.001$), followed by a significant decrease from the 24 to 48 h time point ($P < 0.001$). The Δ*guaA*, Δ*guaB* and Δ*guaA*Δ*guaB* strains were significantly attenuated compared to WT at 24 h ($P < 0.01$ for all three strains). No significant differences were seen between *trans*-complemented strains and WT across the time course.

derivative was effectively complemented in *trans*, following a growth pattern that was comparable to that of the WT Schu S4 strain. The double Δ*guaA*Δ*guaB* strain could not be complemented since the two genes could not be cloned and effectively expressed in a single plasmid. Results similar to those seen in J774.1 cells (Fig. 1) were also seen in primary murine peritoneal macrophages (data not shown).

The mutants were then assessed for attenuation *in vivo* using the C57BL/6 mouse model and compared to WT Schu S4 (intranasal LD_{50} of Schu S4 is <10 CFU; Chen *et al.* 2003). Our studies revealed that Schu S4ΔFTT0507 and Schu S4ΔFTT0584 retained WT levels of virulence; no animals survived intranasal challenge. Schu S4 ΔFTT0742 and Schu S4ΔFTT1043 were minimally attenuated, with 2/5 and 1/5 mice respectively surviving intranasal inoculation (Table 1). As these four strains did not show growth defects *in vitro*, these results were not unexpected. Interestingly, we determined that protein sequence variations may contribute to differing functions and levels of attenuation between mutants in *Ft* and *F. novicida*. Alignment of the *F. novicida* and *Ft* protein sequences for FTT0584 showed 88% identity over the first 1015 amino acids but the *F. novicida* homolog FTN0757 contains 506 C-terminal amino acids that are lacking in the *Ft* version. Similarly, FTT0742 is 79% identical to its *F. novicida* homolog FTN0714 but FTT0742 is truncated by 1190 residues. Truncation of these two *F. novicida* genes in *Ft* suggests the possibility that these may be pseudogenes in *Ft*, a common occurrence with *Francisella*, and would explain why these deletions show no phenotype in *Ft*. Likewise, FTT0507 and FTT1043, predicted to be important virulence factors in *Ft*, did not affect macrophage replication or significantly affect virulence in the mouse model. The lack of attenuation in the FTT1043 mutant is especially interesting given its homology to other *mip* genes in intracellular bacteria that have been demonstrated to be critical virulence factors (Lundemose *et al.* 1991; Cianciotto and Fields 1992; Mo, Cianciotto and Mallavia 1995; Rockey *et al.* 1996; Seshu, McIvor and Mallavia 1997; Neff *et al.* 2007; Wagner *et al.* 2007; Bas *et al.* 2008; Starnino *et al.* 2010; Hung *et al.* 2011; Norville *et al.* 2011; Qin *et al.* 2011; Qin, Scott and Mann 2013).

Only mutations in genes encoding enzymes in metabolic pathways including *guaA* and *guaB* significantly attenuated Schu

Table 1. *In vivo* attenuation and protective efficacy of Schu S4 mutants.

		Attenuation in mice			Protection against Schu S4 challenge				
Gene/ strain	Function	Dose (i.n.)	Survival	Average time to death*	Priming dose (i.n.)	Booster dose (i.n.)	Challenge dose (i.n.)	Survival	Average time to death*
WT Schu S4		2×10^3	0/4	5					
ΔFTT0507	Thioredoxin-like oxoreductase	2×10^3	0/5	5.4					
ΔFTT0584	Innate immune response in *Fn*	3×10^3	0/5	6					
ΔFTT0742	Putative lipoprotein	1×10^3	2/5	14.2					
ΔFTT1043	Macrophage infectivity potentiator (mip)-like protein	9×10^2	1/5	9.8					
PBS					–	–	100	0/2	4
WT Schu S4		4×10^2	0/4	5					
ΔFTT1019c	GMP synthetase (guaA)	7×10^5	3/3	28	7×10^5	–	100	0/3	4
		7×10^6	1/4	12.75					
		7×10^7	0/4	7.25					
ΔFTT1317c	IMP dehydrogenase (guaB)	1×10^4	4/4	28	1×10^9	–	95	0/4	6
		1×10^5	3/4	22.5	6×10^7	6×10^7	100	0/4	4
		1×10^6	4/4	28					
		1×10^7	4/4	28					
		6×10^7	4/4	28					
ΔFTT1019c ΔFTT1317c	*guaAguaB* double mutant	1×10^8	4/4	28	1×10^8	1×10^8	100	0/4	4

*Average time to death, in days, of mice that were euthanized; mice surviving for 28 days were not included in this calculation.

S4 in the mouse model (Table 1). All mice receiving 7×10^5 CFU of Schu S4Δ*guaA* survived with no adverse clinical signs (Table 1, $P < 0.05$ compared to WT Schu S4). Schu S4Δ*guaB* was more highly attenuated and 100% of mice survived i.n. inoculation with 1×10^9 CFU ($P < 0.01$ compared to WT). As expected, the double Δ*guaA*Δ*guaB* strain was also highly attenuated and 100% of mice survived a dose of 1×10^8 CFU ($P < 0.01$ compared to WT).

The high level of attenuation of the *gua* derivatives made them potential vaccine candidates. Their ability to induce protective responses was assessed in mice following either a single immunizing dose or a prime/boost regimen. Twenty-eight days following the last dose, mice were challenged via the i.n. route with a lethal dose of WT Schu S4. In contrast to the protective capacity, we documented with LVSΔ*guaA* or LVSΔ*guaB* mutants against a lethal LVS challenge in mice (Santiago *et al.* 2009), the Schu S4 Δ*gua* derivatives did not confer protection against Schu S4 challenge (Table 1). None of the mice immunized with the highest safe dose of Schu S4Δ*guaA*, Schu S4Δ*guaB* (1 or 2 doses) or Schu S4Δ*guaA*Δ*guaB* (2 doses) survived a challenge dose of 95–100 CFU Schu S4 (>10 LD_{50}). In addition, time to death was not significantly increased following vaccination.

We also assessed cytokine expression in murine peritoneal macrophages infected with each of the *gua* mutants and found no significant differences in cytokine production between Schu S4 and either the Schu S4 Δ*guaA*, Δ*guaB* or Δ*guaA*Δ*guaB* strains (Fig. S1, Supporting Information). Interestingly, all three *gua* mutant strains elicited cytokine profiles similar to that of WT Schu S4 within 24 h of macrophage infection, with a rapid induction of TLR2 and TLR2-dependent transcription, including the cytokines TNF-α, IL-1β and neutrophil-attracting chemokine KC following infection of primary mouse macrophages, that decreased, but remained above uninfected levels at the 4 and 8-h time points. Expression of a second group of cytokines that are both TLR2- and IRF-3-dependent was induced after 4 h of infection when the TLR2 gene transcription wanes, and included IL-12 (p35 and p40), RANTES and iNOS. Transcription of these genes remained high until 8 h and decreased by 24 h post-infection. These observations were not entirely surprising, since analysis of *Ft* Schu S4 genome reveals several potential lipoproteins that may be involved in the activation of TLR2. Moreover, two lipoproteins (TUL4 and FTT1103) that can engage the TLR2 signaling pathway were not compromised during the *gua* mutagenesis process (Thakran *et al.* 2008). It is also possible that the

initial internalization of Ft, whether mutant or WT, triggers this cascade of responses which is not dependent on the ability of the bacteria to replicate within the macrophage. Alternatively, the mutant strains may be scavenging guanine from the host allowing for completion of the intracellular Ft life cycle, albeit at a slower pace. While critical for immune activation, this profile of cytokine induction is not sufficient to predict protective immunity since the mutant and WT strains induced similar levels of activation yet none were protective against a subsequent challenge.

Targeting critical biosynthetic pathway components has been a successful attenuating strategy for the Gram-negative enteric organisms *Salmonella* and *Shigella* (Hoiseth and Stocker 1981; Hone *et al*. 1991; Chatfield *et al*. 1994; Cersini, Salvia and Bernardini 1998; Kotloff *et al*. 2000, 2007) as well Ft LVS (Santiago *et al*. 2009). Furthermore, the Ft LVS Δ*guaA* and Δ*guaB* mutant strains elicited robust protection against a lethal LVS challenge in the mouse model (Santiago *et al*. 2009). It was therefore surprising that the Schu S4Δ*guaA* and Schu S4Δ*guaB* vaccine strains did not induce protection against a lethal Schu S4 challenge (Table 1), as these two gene sequences in LVS and Schu S4 are 99% identical. Pechous and colleagues reported similar findings where *purMCD* mutations in LVS were both highly attenuating and protective against virulent LVS challenge, but that the same mutations in Schu S4 provided limited protection against low dose Schu S4 challenge (Pechous *et al*. 2006, 2008). Other reports have documented differences in efficacy conferred by vaccines created in different background strains containing the same deletion when alternative animal models were employed (Cong *et al*. 2009; Signarovitz *et al*. 2012; Chu *et al*. 2014). Our data, although negative, underscore the importance of the background strain that is used in vaccine construction and emphasize the importance of demonstrating protection against the target type A strain. The Schu S4Δ*guaA*Δ*guaB* strain, which was shown here to not be protective against challenge in mice, has recently been demonstrated to be partially protective against aerosol Schu S4 challenge in New Zealand white rabbits (Reed *et al*. 2014), providing evidence of the value of using more than one animal model to assess Ft vaccines. While the mouse model can provide critical information regarding the contributions of host genetics and immune responses to protective immunity, viable live-attenuated vaccine candidates may be eliminated because they are still too virulent for vaccination/challenge studies in the highly sensitive murine model. Alternative small animal models (including rabbits and rats), which are more resistant to Ft, may more accurately reflect the levels of reactivity and protection that would be seen in humans. As such, these findings highlight the importance of the differences between subspecies of *F. tularensis* and the use of appropriate models in tularemia vaccine studies.

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SUPPLEMENTARY DATA

Supplementary data are available at FEMSPD online.

Conflict of interest. None declared.

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