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Vaccines against tularemia

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Abstract

Francisella tularensis is a Category A select agent for which vaccine and countermeasure development are a priority. In the past eight years, renewed interest in this pathogen has led to the generation of an enormous amount of new data on both the pathogen itself and its interaction with host cells. This information has fostered the development of various vaccine candidates including acellular subunit, killed whole cell and live attenuated. This review summarizes the progress and promise of these various candidates.

Keywords

public health; Francisella; tularemia; vaccine; bacteria; infection; biodefense

Introduction

Francisella tularensis, a non-spore forming, encapsulated Gram-negative coccobacillus, is the etiologic agent of the potentially fatal zoonotic disease tularemia. Following the anthrax bioterror attacks in the US in 2001, *F. tularensis* was placed on the Category A select agent list as one of six pathogens that were the highest priority for the development of preventative countermeasures. Since then significant progress has been made in both the understanding of the *F. tularensis* pathogenic process as well as the host immune response. This, in turn, has spurred development of new and exciting tularemia vaccine candidates.

F. tularensis was first identified as the cause of tularemia in 1911 during an outburst of a plague-like disease among squirrels inhabiting Tulare Lake in California. It has since been shown that *F. tularensis* can infect a wide range of animals including mammals, birds, amphibians, fish and invertebrates.[73] This diversity helps to explain the various colloquial names associated with tularemia including rabbit fever, hare fever, deerfly fever and lemming fever.[73]*F. tularensis* is capable of invading and replicating within macrophages as well as non-phagocytic cells (including hepatocytes and alveolar epithelial cells).[22,31,42]*F. tularensis* invades cells by both a novel asymmetric pseudopod loops mechanism[19] and by a receptor-dependent mechanism that has been shown to involve class A scavenger receptors,[79] the complement factor C3 receptor (CR3 and CR4),[5,7,90] IgG receptor (FcγR), surfactant protein A and the mannose receptor.[90] Once internalized, *F. tularensis* is able to escape the degradative environment of the phagolysosome[13,15,17,59] into the cytoplasm where it replicates. The high virulence of *F. tularensis* results from many factors including its ability to proliferate to high numbers in host tissues and organs as well as its ability to elicit a pronounced inflammatory response.[8,23,28,39] In humans, the disease syndrome varies with both the route of inoculation and the virulence of the infecting strain. Infection by the dermal, oral or pulmonary routes results in ulceroglandular, oropharyngeal or pneumonic

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(formerly called typhoidal) tularemia, respectively and the highest mortality rates are associated with the pneumonic form of the disease.[94] Two subspecies, *F. tularensis* subspecies holarctica (also called Type B) and *F. tularensis* subspecies tularensis (Type A) are responsible for the vast majority of human tularemia cases worldwide. The less virulent Type B strains are found in North America, Europe and Asia and the more virulent Type A strains are found primarily in North America.[54] A third subspecies, *F. tularensis* subspecies novicida, while rarely a human pathogen, is widely studied as a model for tularemia. While the *F. tularensis* organism is widespread in the United States, incidence of tularemia is not, as approximately 100 cases of human tularemia are reported each year. These cases result mainly from direct contact with infected animals or bites from arthropod vectors (e.g., ticks), although pneumonic disease from inhalation of aerosols generated by mowing lawns or brush in tick-infected areas has also been described.[27,45,67]

The attractiveness of *F. tularensis* as a potential bioweapon stems from its ability to be disseminated via the aerosol route, its extremely low infectious dose, and its potential to cause severe morbidity and mortality.[23] Additionally, *F. tularensis* has a history of weaponization first documented by the Japanese for purposes of warfare between 1932–1945,[43] and later by both the former Soviet Union and the United States.[18,23] This history has elevated concerns that *F. tularensis* could be used as a bioweapon in the future.[74,75] The current standard of care for tularemia is treatment with antibiotics as this therapy is highly effective if implemented early in infection.[98] However, the nonspecific symptoms of tularemia, which include swollen lymph nodes, fever and lethargy, might lead to misidentification of the pathogen that could delay appropriate therapy. Therapeutic options could be further limited by the development of natural antibiotic resistance or the engineering of resistant strains. Therefore a safe and effective vaccine able be used both in a prophylactic manner in targeted populations such as the military or health care providers as well as in the general population in a crisis situation would be a very valuable public health tool.

Two key pieces of evidence support the feasibility of developing a Francisella vaccine. First, immunospecific protection against reinfection has been demonstrated following natural infection.[11,97] Second, immunization with the live vaccine strain (LVS), has demonstrated efficacy against wild type challenge in humans. LVS originated from an attenuated Type B strain that was developed and used for mass vaccination in the Soviet Union in 1946.[101] LVS was transferred from the Gamaleia Institute in Moscow to the US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD in 1956. It was shown that vaccination of at risk laboratory personnel with LVS reduced the incidence of laboratory-acquired respiratory tularemia.[12] LVS, while safe in humans, can be lethal in mice and has therefore been a valuable tool for use in the murine model of tularemia infection. Although LVS demonstrated proof of principal that a protective response may be elicited by a vaccine it remains unlicensed for use in the general population. In response to the desire to develop a safe and effective tularemia vaccine, researchers have focused their efforts towards the rational design of tularemia vaccines using three main methods, acellular subunit, killed whole cell and live attenuated vaccines.

Acellular Subunit Vaccines

Acellular subunit vaccines are cell-free vaccines which are prepared from synthesized or purified antigenic components of a microorganism. The main advantage of acellular subunit vaccines is that they are not infectious. Antigens recognized by either the T cells or immune sera represent possible acelluar subunit vaccine candidates.

Within two weeks of a tularemia infection or immunization, humans develop a robust antibody response that is primarily directed against LPS.[2,50,56,57,97] Accordingly, LPS has been investigated as a potential *F. tularensis* vaccine candidate. *F. tularensis* LPS is tetraacylated and therefore only weakly activates TLR4.[20,25,41] It fails to induce production of inflammatory cytokines in vivo and in vitro[20] yet pretreatment with *F. tularensis* LPS is able to protect mice against subsequent LVS challenge.[20,21,24,35,36,86] This protection has been shown to be primarily humoral as passive infusion of sera from *F. tularensis* LPS immunized mice protects naïve mice against subsequent LVS challenge.[36] However this passive protection is not truly passive, T cells are required as the transfer of serum does not protect mice that had been depleted of $CD4^+$ or $CD8^+$ T cells.[36] IFNγ is also required as neither passive transfer of immune serum nor direct immunization with LPS provided protection to $IFN\gamma^{-/-}$ mice.[24,55]

Studies using immunization with whole bacteria have suggested that antibodies against the O-antigen of *F. tularensis* LPS are responsible for LPS mediated protection. Passive administration of antibodies elicited to whole cell LVS protect against an otherwise lethal LVS challenge, while antibodies elicited by immunization with the O-antigen-deficient strain, *F. tularensis* LVS *wbtA*, do not.[91,92] Furthermore, passively administered rabbit anti-*F. tularensis* LVS antisera, but not antisera depleted of anti-O antibodies, protects mice against lethal challenge.[91] However other studies have suggested that the protective antibodies are not limited to the LPS O-antigen. Serum taken from mice immunized with a heat killed O-antigen LVS mutant (a *wbtC* mutant that is completely deficient of O antigen expression) was able to protect 80% of naïve mice against subsequent i.p. challenge with LVS.[60]

That protection against *F. tularensis* could be mediated through humoral immunity has been a controversial matter because *F. tularensis* is an intracellular pathogen. The prevailing methodology in vaccine development suggests that humoral immunity plays a critical role in protection against extracellular pathogens, while cell-mediated immunity is far more important for protection against intracellular pathogens. However recent studies have demonstrated that the majority of *F. tularensis* recovered from the blood of infected mice was located in plasma rather than within leukocytes.[29] This distribution pattern was observed irrespective of inoculation route or size, time after inoculation or virulence of the infecting strain.[29]

One significant drawback to the use of LPS as a vaccine is its inability to protect against the most virulent strains. Immunization with LPS purified from LVS only fully protected mice against challenge with LVS and some virulent Type B strains. LVS LPS vaccination increased the mean time to death but did not protect against challenge with the Type A strain Schu S4.[36] It is possible that these differences in survival relates to the inherent differences in LPS from Type A and B strains. However, studies have shown that the structure of the O-antigens are identical in Type A and B strain[41,80,100] and immunization with LPS purified from Schu S4 was unable to protect mice against Schu S4 challenge and only increased the mean time to death.[80] Therefore the disparate outcomes of LVS and Type A challenge following LPS immunization most likely relates to differences in virulence between the strains and their distinct requirements for protection.

One possible means to increase the protective ability of LPS might be to couple LPS immunization to induction of Francisella specific cell mediated immune response. This idea has shown some promise as LPS-immunized mice boosted with live LVS were protected against Schu S4 challenge.[36] Additionally, immunization of mice with LPS in combination with *Neisseria meningitidis* PorB, a TLR2/1 ligand that has been shown to enhance the T-cell costimulatory activity of antigen-presenting cells both in vitro and in vivo,[65,66,93]

greatly improved survival after intranasal LVS challenge when compared to immunization with *F. tularensis* LPS alone.[16]

As an alternative subunit vaccine formulation, Huntley et al. investigated the potential utility of *F. tularensis* outer membrane proteins (OMP) as an acellular subunit vaccine. Immunization with 3 doses of native OMPs with adjuvant provided protection in 50% of the mice against intranasal challenge with Schu S4.[49]

Acellular subunit vaccines can also utilize antigens that activate T-cells. A screen of T lymphocyte antigens identified a pool of candidate epitopes from Schu S4 antigens for inclusion in a rationally designed tularemia vaccine.[69] HLA transgenic mice immunized with a subset of these epitopes incorporated into a string-of-beads DNA prime, peptide boost vaccine regimen were protected against an otherwise lethal intratracheal *F. tularensis* LVS challenge.[69]

Another *F. tularensis* specific T cell epitope is comprised of amino acids 86–99 from the 17-kDa lipoprotein Tul4 (also known as LpnA). These amino acids function as an immunodominant $CD4^+$ T cell epitope in B6 mice and T cells specific for this epitope can account for as much as 20% of the responding $CD4^+$ T cells in an acute Francisella infection.[95,104] However immunization with *Salmonella typhimurium* expressing Tul4,[96] as well as immunization with Tul4 incorporated into immunostimulating complexes[38] only provided partial protection against LVS challenge.

Killed Whole Cell Vaccines

Successful killed whole cell vaccines are biologically complex, non infectious, preparations of infectious agents that are able to induce a protective immune response. In the 1940's Lee Foshay developed killed whole cell tularemia vaccine formulations by phenolization or acetone extraction.[32,33,51] Immunization of non human primates with the Foshay vaccine prevented death after challenge with 740 CFU of Schu S4. However, immunization caused adverse reactions in the animals including local necrotic lesions and regional lymphadenopathy.[51] Administration of the Foshay vaccine to volunteers led to the development of milder reactions but was unable to prevent development of lesions after intracutaneous challenge with 10 CFU of Schu S4.[88] Furthermore, administration of the Foshay vaccine neither prevented nor modified the development of overt tularemia in individuals who inhaled of 50 CFU of Schu S4.[87] Though there has been minimal attention devoted to the development of a killed whole *F. tularensis* vaccine in recent years,[40] in 2007, Lavine et al. reported that immunization with heat-killed *F. tularensis* LVS alone or in combination with an IL-12-expressing vesicular stomatitis virus-based vector protected mice against subsequent i.p. challenge with LVS. This protection appeared to be antibody mediated as sera from mice immunized with heat killed LVS was able to protect naïve animals against subsequent i.p. challenge with LVS.[60] However, Baron et al. found that i.n. inoculation with inactivated LVS only protected against subsequent i.n. challenge with live LVS when the inactivated bacteria were given in conjuction with recombinant IL-12.[6]

Live Attenuated Vaccines

Live attenuated vaccines are broadly defined as vaccines prepared from live organisms that, while attenuated for virulence, are still immunogenic. The most extensively tested tularemia live vaccine is LVS. Multiple challenge studies in non human primates as well as in humans demonstrated the efficacy of LVS vaccination in conferring at least partial protection against challenge with the Schu S4; although the degree of protection varied with the route and dose of both vaccine and challenge administration.[47,48,68,89] Yet, while LVS demonstrated proof of principal that a live attenuated strain could protect against challenge, it suffers from

several drawbacks that make it a sub-optimal vaccine. LVS is based on a Type B strain and confers only partial protection against virulent Type A challenge, the molecular mechanism of its attenuation is not defined and LVS demonstrates an unstable colony phenotype.[26,44,78,102] Accordingly, researchers have attempted to replicate and improve upon the protective ability of LVS by generating fully defined, stable, attenuated mutants. Modern molecular techniques have allowed the engineering of precise genetic mutations resulting in the generation of completely defined mutant strains.[34]

Genes that have been targeted for mutation can be broadly classified into three groups: metabolic enzymes, virulence factors and regulatory proteins (Table 1). Most of the targeted mutations were first constructed and tested in LVS or *F. novicida* because of the ease of manipulation of these strains and ability to work under BSL-2 conditions. This has allowed researchers to identify promising target genes prior to their mutation in Type A strains and the requirement for higher level containment.

Mutants in metabolic enzymes

Targeted mutations in genes encoding critical enzymes in metabolic pathways have been the basis of attenuating mutations in many bacterial pathogens.[61] Analysis of Francisella genomes revealed the presence of enzymes that are involved in the aromatic amino acid biosynthetic pathways.[53,58] While *F. novicida*, *purA*, *purCD* or *purM* mutants were attenuated in mice, they did not protect against wild type homologous challenge. In contrast, i.p. injection of the *F. novicida purF* mutant induced an immune response in mice that provided protection against challenge with the parental strain, but not against challenge with Schu S4.[83,99] Deletions in *purMCD*, *guaA* or *guaB* highly attenuated Francisella LVS.[76,77,86] These three mutant strains did not disseminate in the organs of infected mice nor were they able to replicate intracellularly in macrophages.[76,86] Mice vaccinated with LVS *purMCD*, *guaA* or *guaB* mutants were protected against lethal challenge with the parental LVS strain. However, a single i.n. immunization with LVS *purMCD* did not protect mice against i.n. and i.d. challenge with low doses of Type A Schu S4.[77] These findings are in contrast to the results seen after immunization with parental LVS, as one i.n. dose of LVS protected mice against subsequent low dose i.d. and i.n. challenge with Schu S4.[77] The Schu S4 *guaA* and *guaB* mutants and the Schu S4 *purMCD* mutant were attenuated in mice.[77,85] However, immunization with either the Schu S4 *guaA* or *guaB* mutant was unable to protect against homologous challenge.[85] Intranasal immunization with a single dose of the Schu S4 *purMCD* mutant provided only partial protection against i.n. challenge with Schu S4 and provoked tissue damage in the lungs.[77]

γ-glutamyl transpeptidase (GGT) is an essential enzyme that catalyzes the first step in the degradation of the tripeptide glutathione (GSH). In *F. tularensis*, GGT allows the utilization of γ-glutamyl as a source of cysteine during intracellular replication. Mutation of *ggt* in LVS resulted in a significant growth defect in J774 macrophages and reduced virulence in mice; the LD_{50} of the mutant was three orders of magnitude lower then the LD_{50} for LVS when mice were challenged by the i.p route.[1]

Mutants in virulence factors

Virulence factors offer another rational target for mutation. The LPS of Francisella, like other Gram negative bacteria, is composed of the lipid A, a core oligosaccharide and an O-antigen polysaccharide (O-PS).[41] Unlike other many other Gram negative pathogenic bacteria, the LPS of *F. tularensis* is tetraacylated and does not provoke an overt proinflammatory cytokine response.[20,25,41] However mutations affecting *F. tularensis* LPS attenuate bacterial virulence. Deletions in *wbtA*-encoded epimerase/dehydratase of the Francisella O-PS locus resulted in a Δ*wbtA* LVS strain that was attenuated for virulence in

mice.[84,91] Mutations in the sugar transamine/perosamine synthetase gene, *wbtI*, resulted in the complete loss of O-antigen expression. The *wbtI* mutant was highly susceptible to the bactericidal action of serum however, it was still able to multiply to wild type levels in J774 macrophages which may explain why this strain was only moderately attenuated in mice.[62] Mutants in three enzymes required for *F. novicida* lipid A carbohydrate modifications (*flmF1*, *flmF2* and *flmK*) were generated and evaluated in mice. The *flmF1* mutant was not attenuated in mice but the *flmF2* and *flmK* mutants were attenuated after challenge by both aerosolized and subcutaneous routes of infection.[52]

In terms of their protective ability, immunization with either the LVS *wbtA* or *wbtI* mutants protected mice against low level LVS challenge (25 LD_{50}s).[62,91] However immunization with LVS Δ*wbtA* was not able to induce protection against challenge with Schu S4.[91] Mice immunized with *flmF2* or *flmK* mutants by the pulmonary route were protected against a lethal *F. novicida* challenge, but only the *flmK* mutant induced protective immunity when the mice where immunized by subcutaneous injection.[52]

Other virulence factors that have been targeted for deletion include the superoxide dismutase (*sodB*).[3,4] A *F. tularensis sodB* mutant strain was significantly attenuated for virulence in mice. BALB/c mice vaccinated with the LVSΔ*sodB* mutant strain were partially protected against low dose intranasal Schu S4 challenge and the protection levels were improved in boosted mice.[3] While only modest and short term protection was induced following immunization with the LVSΔ*sodB* mutant, it is notable that immunization with this LVS mutant induced better protection against Schu S4 challenge than the parental LVS strain.

Acid phosphatases hydrolyze a wide variety of substrates including proteins with phosphorylated tyrosines. In Francisella five acid phosphatases have been described (AcpA, AcpB, AcpC, Hap and Hap homolog). The acid phosphatase A (AcpA) is required for intramacrophage survival and efficient escape from the phago-some.[70] A *F. novicida* derivative mutated in four of these genes, *acpA*, *acpB*, *acpC* and *hap*, was defective for growth and survival in macrophages, unable to escape from the phagosome, and was highly attenuated in mice. Mice vaccinated with this quadruple mutant survived a stringent wild type *F. novicida* challenge.[72]

The *dsbB*- and the *dsbA*-encoded enzymes are required to catalyze the formation of disulfide bonds in Gram negative bacteria. Both DsbA and DsbB proteins participate in the assembly of several virulence factors in bacteria.[46] Mann and colleagues introduced mutations into FTT0107c and FTT1103, which encode DsbB- and DsbA-like proteins respectively, in Schu S4. Both mutants were unable to replicate intracellularly, and the FTT1103 mutant also showed impaired ability to escape the phagosome. Both mutant strains were highly attenuated in mice, however only the FTT1103 mutant induced protection against wild type Schu S4 challenge.[81,82] It is significant to note that the Schu S4 FTT1103 mutant is the only live attenuated strain that has shown a high level of protection against wild type Type A challenge in the stringent C57BL/6 mouse model.

FTT918 encodes a hypothetical protein of 58 kDa that is a virulence factor of unknown function. Deletion of this gene in Schu S4 led to a reduction in the intracellular growth rate in peritoneal mouse macrophages. Mice vaccinated with the FTT918 deletion mutant were protected against low challenge doses (~10 CFU) of the virulent Type A strain FSC033.[103]

Type IV pili are considered virulence factors in a wide spectrum of bacteria and the genes encoding Type IV pili have been identified in the Francisella genomes.[58] In *F. tularensis* subspecies holarctica, deletion of pilin genes resulted in attenuation of virulence in mice and impaired ability to spread from the initial site of infection to the spleen.[30] Studies in LVS showed that deletions in *pilF*, encoding the assembly ATPase, and *pilT*, encoding the

disassembly ATPase, caused a complete loss of pili. While both *pilF* and *pilT* LVS mutants were able to multiply intracellularly in cells, both mutants were defective for adherence to macrophages, epithelial cells and hepatocytes. The mutants were attenuated in mice when introduced via the intradermal route.[14]

Catalase is encoded by *katG* and is used by bacteria to detoxify bactericidal compounds such as H_2O_2 and $ONOO^-$. Schu S4 and LVS mutants in *katG* demonstrated enhanced susceptibility to H_2O_2 in vitro, but were not affected in their ability to replicate intracellularly in murine peritoneal macrophages. The LVS *katG* mutant was attenuated in mice while the Schu S4 *katG* mutant retained its virulence.[63]

Mutants in regulatory proteins

Mutations in regulatory proteins can also attenuate virulence. Four transcriptional regulators, *mglA, sspA, fevR* and *pmrA*, have been shown to regulate genes that are contained in the Francisella Pathogenecity Island.[10,59,71] A *F. novicida pmrA* mutant was defective in survival and intracellular growth within human and murine macrophages.[71] The mutant was highly attenuated in mice, and a single immunization protected against a high dose challenge with the homologous wild type strain but failed to induce protection against Schu S4 challenge. A *F. novicida mglA* mutant was attenuated in mice and did not replicate as effectively as the parental strain in infected organs. However immunization with this strain was unable confer protection against a subsequent challenge with wild type *F. novicida*.[59,106] Studies in mice suggested that FevR is required for bacterial replication in macrophages. In mice, a *fevR* mutant is unable to replicate in spleen and skin.[9]

Taken together, these studies underscore the differences between Type A and B strains and suggest different requirements for protective vaccines against each. Furthermore these studies illustrate that attenuation and protective ability are not synonymous; multiple engineered strains are attenuated, but few have demonstrated the ability to protect against subsequent challenge with a Type A strain (Table 1).

Summary

The requirements for a successful tularemia vaccine are clear; an effective tularemia vaccine will safely provoke long lasting protective immunity in the general population in a relatively short period of time. The search for this elusive product has led to the development of multiple new vaccine candidates and, succeed or fail, these attempts all provide valuable information about the requirements for the generation of a protective immune response. While the data is complicated by the use of different Francisella strains as well as different animal and cell models, a clearer picture is emerging of both the pathogenic pathways of *F. tularensis* and host response. The fact that *F. tularensis* is an intracellular pathogen has led to the conclusion that a cell-mediated response will be required for protection. While this assumption has been born out in many studies, a role for antibody has also been clearly established. This suggests that any successful vaccine will need to induce both a humoral and cell mediated response.

Concurrent advances in the broader vaccine fields of adjuvants and co-stimulatory molecules, administrative routes, as well as vaccine formulation have provided an abundance of options for the development of a tularemia vaccine. Accordingly, the search for a tularemia vaccine has included the investigation of new vaccine regimens including heterologous prime boost, new administration options e.g., nasal injection and new possible adjuvants such as IL-12.[6] Novel strategies such as these may be required to induce an efficacious response against tularemia. Additionally, a viable vaccine for use against a potential biothreat must also take into account several practical considerations. This vaccine

must be safe for use in the general population and effective in individuals of varying ages and levels of immunocompetence. Since it is highly unlikely that a vaccine against a potential biothreat would be routinely administered to the general populous, routes of administration must allow for speed and ease of deployment and this vaccine must be able to be manufactured quickly, or stored in a formulation conferring long term stability.

Animal models are crucially important in the study of human pathogens; however there are limitations that must be recognized. The majority of *F. tularensis* research has been, and continues to be carried out in mice. While this work is very valuable, findings in mice and humans are not necessarily equivalent. For example, mice can be lethally infected with strains that are not pathogenic in humans, i.e., LVS. Therefore, advancement of any vaccine candidate will require the use of additional animal models to confirm safety, immunogenicity and protection. Models under investigation include the rabbit, rat and nonhuman primates.[64,107]

Historically, vaccines have served as one of the most effective public health tools. While a great deal of effort has been applied towards the development of a tularemia vaccine, much work remains. Our greater understanding of the protective immune response to *F. tularensis* will help to direct research towards the most effective vaccine candidates or regimes.

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Table 1

Live attenuated vaccine candidates

Gene	Principal function	Findings in mice	Reference
		***F. tularensis subsp. holarctica* LVS derivatives**	
purMCD	Purines biosynthesis	Attenuated and protective against LVS challenge	(76)
tolC, ftlC	TolC and TolC homologous	*tolC* is attenuated and *ftlC* is not attenuated in C3H/HeN mice	(37)
sodB	Superoxide Dismutase B	Moderately attenuated in mice, modest protection against Schu S4 challenge	(3, 4)
wbtA	O-antigen biosynthesis	Attenuated and protective against Type B strains LVS and FSC108 challenge, but not protective against Schu S4 challenge	(84, 91)
wbtl	Transamine/perosamine synthetase	Moderately attenuated and protective against low dose challenge with LVS	(62)
katG	Catalase	Attenuated in mice	(63)
pitF, pitT	Type IV Pili assembly	Moderately attenuated in C3H/HeN mice	(14)
ggt	Gamma-glutamyl transpeptidase	Moderately attenuated in BALB/c mice	(1)
guaB, guaA	Synthesis of GMP	Attenuated in mice and protective against LVS challenge in BALB/c mice	(86)
		***F. tularensis subsp. Tularensis* Schu S4 derivatives**	
FTT0918	58 kDa protein	Attenuated in mice, induces modest protection against Type A strain FSC033 challenge (10 CFU/aerosol)	(103)
FTT0107c *dsbB*	Disulfide bond formation	Attenuated in C57BL/6 mice, not protective against Schu S4 challenge	(81)
FTT1103 *dsbA*-like	Lipoprotein	Attenuated in mice and protective against Schu S4 challenge in C57BL/6 mice (100–1,000 CFU/i.n route)	(82)
purMCD	Purines biosynthesis	Attenuated in mice, modest protection against Schu S4 challenge	(77)
guaA/guaB	Synthesis of GMP	Attenuated in mice, not protective against Schu S4 challenge	(85)
katG	Catalase	Not attenuated in C57BL/6 mice	(63)
		***F. novicida* U112 derivatives**	
purA/purF	Purines biosynthesis	*purA* is attenuated but not protective against U112 challenge in mice. *purF* is attenuated and induces protection against U112 challenge but not against Schu S4	(83)
lpxF	4'-Phosphatase	Attenuated in mice	(105)
acpA, acpB, acpC, hap	Acid phosphatases	Δ*acp*ABCH attenuated in BALB/c mice and protective against U112 challenge	(70, 72)
flmF1, ImF2, flmK	Lipid A biosynthesis	*flmF1* mutant is not attenuated while *flmF2* and *flmK* mutants are modestly attenuated in mice	(52)
mglA	Transcriptional factor	Attenuated, not protective against U112 challenge	(106)
pmrA	Response regulator protein	Attenuated, induces protection against U112 challenge but not against Schu S4	(71)
fevR	Regulator protein	*fevR* mutant is unable to multiply in spleen and skin	(9)