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Live Attenuated Tularemia Vaccines: Recent Developments and Future Goals

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Abstract

In the aftermath of the 2001 anthrax attacks in the U.S., numerous efforts were made to increase the level of preparedness against a biological attack both in the US and worldwide. As a result, there has been an increase in research interest in the development of vaccines and other countermeasures against a number of agents with the potential to be used as biological weapons. One such agent, *Francisella tularensis*, has been the subject of a surge in the level of research being performed, leading to a substantial increase in knowledge of the pathogenic mechanisms of the organism and the induced immune responses. This information has facilitated the development of multiple new *Francisella* vaccine candidates. Herein we review the latest live attenuated *F. tularensis* vaccine efforts. Historically, live attenuated vaccines have demonstrated the greatest degree of success in protection against tularemia and the greatest promise in recent efforts to develop of a fully protective vaccine. This review summarizes recent live attenuated *Francisella* vaccine candidates and the lessons learned from those studies, with the goal of collating known characteristics associated with successful attenuation, immunogenicity, and protection.

Keywords

Francisella tularensis; live attenuated vaccines; LVS; Schu S4

1. Introduction

Francisella tularensis is a non-motile, non-spore-forming Gram-negative coccobacillus and the causative agent of tularemia. Of the four recognized subspecies, two cause the majority of disease in humans. These include the highly virulent subspecies *tularensis* (type A) and the less virulent subspecies *holarctica* (type B). Exposure to as few as ten organisms of subspecies *tularensis* by the aerosol route can cause disease in humans, which can lead to a 30–50% mortality rate if left untreated [1, 2]. Due to its high capacity for infection by the aerosol route, its low infectious dose, and its extreme virulence, *F. tularensis* was developed for use as a biological weapon during World War II and the Cold War era. Countries including Japan, the Soviet Union, and the United States had programs devoted to the development of *F. tularensis* as an offensive weapon [2, 3]. Because of its highly infectious, aerosolizable nature, ability to cause severe morbidity and mortality, and history of weaponization, *F. tularensis* is considered a potential biological weapon and has been

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designated a Category A select agent by the CDC. The development of an effective vaccine that is protective against a type A challenge is therefore a priority in the biodefense community.

Attempts to develop a vaccine against tularemia have employed a number of different strategies, and though some candidates have shown promise in human volunteers [4, 5] and in animal models (Tables 3 and 4), there is currently no licensed vaccine against tularemia. Along with the weaponization efforts during WWII and the Cold War, there were efforts toward vaccine development. The history of 20th century *Francisella* vaccine development has been extensively reviewed elsewhere [6, 7]. The earliest vaccines developed against tularemia were killed whole cell vaccines, which became known as Foshay vaccines [6, 8]. These vaccines were administered to laboratory personnel, but were not effective against exposure to fully virulent strains [6]. A number of live attenuated vaccine candidates were made from *F. tularensis* subsp. *holarctica* strains by the Soviets in the 1940s and 1950s [6]. One of these live attenuated derivatives was transferred to the US, and after serial passage to achieve further attenuation, became known as *F. tularensis* Live Vaccine Strain (LVS). Although LVS provided partial protection against a type A challenge depending on the dose and route of both vaccination and challenge in humans [1, 4, 5, 9], it is not licensed in the USA due to the uncertainty of its history, instability of colony type, and incompletely understood basis of attenuation. Recently, attempts have been made to improve this vaccine. A new lot of LVS manufactured under cGMP conditions was produced and evaluated for safety and immunogenicity in rabbits and humans [10, 11]. This new LVS lot was well tolerated at all doses in both rabbits and humans, and high IgG, IgM, and IgA antibody titers were elicited [10, 11], though protection has not yet been demonstrated. In addition to these efforts to reassess the LVS vaccine, multiple new live vaccine candidates have been developed.

In the aftermath of 9/11 and the 2001 anthrax attacks in the US, there has been a renewed focus on vaccine development against pathogens with potential for use in a bioterror attack. In the past 10 years there has been a surge of research interest in *Francisella* and a number of new vaccine candidates have been developed using modern technologies. Subunit based vaccine technologies have yielded several candidates, but none have conferred protection against homologous challenge in animal models [12, 13]. Due to the proof of principle supplied by the LVS vaccine that a live attenuated vaccine could provide at least partial protection against virulent challenge, numerous live attenuated candidates have been engineered with defined deletions of genes that encode virulence factors or enzymes responsible for metabolic, housekeeping, or other critical functions. These live attenuated vaccine candidates are based on different strain backgrounds and have been tested in various *in vitro* and *in vivo* models. This review will focus on these candidates and summarize their attenuation and protective capacity.

2. LVS As a Model Organism

In addition to demonstrating the potential protective capacity of a live attenuated vaccine candidate, the LVS strain has also proved to be valuable as a model organism in mouse studies of tularemia. While type A strains are potentially lethal to humans at low doses and therefore require BSL-3 containment, LVS can be manipulated in BSL-2 containment, and causes a lethal infection in mice that closely resembles the disease course of type A tularemia in humans. Early studies established the LD₅₀ of LVS by several routes of infection in several different mouse strains typically used in infection and challenge studies [14, 15]. These studies defined the LD₅₀ by the intranasal (i.n.), intraperitoneal (i.p.), and intradermal (i.d.) routes to be 1,000 CFU, 100 CFU, and 10⁷ CFU, respectively. These standards have been used as a barometer to establish the attenuation level of numerous live

attenuated mutant strains engineered by other researchers. LVS shares 99% genetic identity with the most widely used model type A strain, Schu S4. This level of similarity allows for the deletion of candidate genes first in the LVS background to establish attenuation and initial characterization prior to moving into the more dangerous Schu S4 background, an approach that has been taken by a number of researchers [16–21].

3. Summary of Recent Live Attenuated LVS Mutants

Numerous mutant strains engineered in the LVS background have demonstrated impressive levels of attenuation in mouse models of infection, and even more importantly, protective capacity against a subsequent challenge with lethal doses of LVS. A summary of mutants can be found in Table 3.

One of several recent examples of LVS mutants reported to be attenuated is a mutant of the *capB* gene, which encodes a protein which functions in capsule biosynthesis [20, 22]. In this study, the authors demonstrated that the *capB* mutant was outcompeted by LVS in human macrophage THP-1 cells. Since *F. tularensis* depends on the ability to survive in macrophages and a number of other cell types as part of its pathogenic process, a first step to measure potential attenuation of a mutant is often to assay its ability to survive and replicate intracellularly (Tables 1 and 2). The *capB* mutant was attenuated when administered by both the intradermal (i.d.) and intranasal (i.n.) routes in BALB/c mice. In i.d. studies, the authors demonstrated that a dose of 10^8 CFU of LVS killed 100% of infected mice, whereas 100% of infected mice survived a similar dose of $\text{LVS}\Delta\text{capB}$. In i.n. studies, LVS was 100% lethal to mice at a dose of 10^3 CFU, whereas 100% of infected mice survived a dose of 10^7 CFU of $\text{LVS}\Delta\text{capB}$. The $\text{LVS}\Delta\text{capB}$ mutant was also shown to be protective, as mice immunized either i.d. or i.n. with 10^5 CFU of the mutant survived an i.n. challenge with 4,000 CFU of LVS.

In another report, the attenuation of a mutant of the *wzy* gene, which is involved in O-polysaccharide synthesis, was characterized [23]. The *wzy* mutant was unable to replicate in RAW264.7 and MH-S murine macrophage cell lines. The mutant was attenuated for virulence in BALB/c mice by the intraperitoneal (i.p.), i.d., and i.n. routes. Mice infected with the *wzy* mutant survived at a rate of 100% at doses up to 10^8 CFU by the i.d. route and up to 10^7 CFU by the both the i.n. and i.p. routes. The *wzy* mutant was also shown to be protective against LVS challenge, as mice immunized with two doses of $\text{LVS}\Delta\text{wzy}$ (10^7 CFU) by the i.n. route were fully protected against an i.n. challenge of 10^5 CFU of LVS. More importantly, the $\text{LVS}\Delta\text{wzy}$ mutant was shown to provide protection against a type A Schu S4 challenge. Mice immunized three times by the i.n. route had an 84% survival rate against an i.n. challenge with 8 CFU of Schu S4 (8 times the LD_{50}), which exceeds the previously documented survival rate for BALB/c mice challenged i.n. by a type A strain following i.n. immunization with LVS [24].

Another attenuated LVS mutant that was protective against homologous challenge was a mutant of the *galU* gene, which encodes a UDP-glucose phosphorylase [25]. While the $\text{LVS}\Delta\text{galU}$ mutant was reported to replicate to similar levels as the parental LVS strain in both RAW264.7 and J774.1 cells, it was shown to be attenuated for virulence in C57BL/6 mice by the i.n. route. Mice infected with $\text{LVS}\Delta\text{galU}$ at doses up to 10^7 CFU survived at a rate of 100%. Additionally, these mice were protected against an i.n. challenge with 10^4 CFU of LVS.

One of the more recent reports demonstrated the attenuation of three LVS mutants of a family of genes predicted to encode transporter proteins of the MFS family [26]. $\text{LVS}\Delta\text{fptB}$, $\text{LVS}\Delta\text{fptE}$, and $\text{LVS}\Delta\text{fptG}$ were all found to display altered intracellular replication kinetics in either J774.1 cells and/or HepG2 cells. All three mutant strains were attenuated in BALB/

c mice when delivered by the i.p. route; LVS is most virulent when delivered via this route. The *fpdG* mutant was the most attenuated of the three, as 100% of mice infected with this mutant survived doses up to 10^6 CFU. Importantly, a single vaccination with only 10^3 CFU of any of these three mutants conferred complete protection against an LVS challenge of 10^5 CFU, and this protection was maintained in mice that were challenged up to 90 days after vaccination. These findings are significant, as there is only one other LVS mutant reported to provide a similar level protection against LVS challenge after a single vaccination using a dose as low as 10^3 CFU. That was a mutant of the FTL_0439 gene, which is involved in iron uptake [27].

Two of the most recent reports of attenuated mutants engineered in the LVS background characterized mutations in the FTL_0883 gene, which encodes a protein involved in spermine response [28], and the FTL_0724 gene, which encodes a protein involved in folate metabolism [29]. Both mutants failed to replicate in murine bone marrow-derived macrophages (BMDMs) and were found to be attenuated for virulence in C57BL/6 mice. Mice infected with 10^4 CFU of the FTL_0883 mutant (intratracheally) or the FTL_0724 mutant (i.p.) survived at a rate of 100% [28, 29]. Neither of these mutants was tested for protection against homologous challenge.

4. Summary of Recent Live Attenuated Schu S4 Mutants

While a number of the mutants discussed above demonstrated significant protection against a lethal LVS challenge by various routes, only two were tested for protective capacity against an aerosol-like challenge with a type A strain (Schu S4) [20, 23], which is the ultimate goal of a successful *Francisella* vaccine. In fact, multiple examples in the literature demonstrate that vaccine-induced protection against LVS challenge does not predict protection against type A challenge [16, 30]. This suggests that an effective vaccine will need to be constructed in the Schu S4 background in order to provide full protection against type A challenge. The LVS studies provide valuable data pointing towards candidate genes in Schu S4. A number of live attenuated Schu S4 mutants have been generated in the last 10 years, many of which have provided some degree of protection against a type A challenge (Table 4).

A $\Delta capB$ mutant strain was engineered in the Schu S4 background [21], which survived and replicated similarly to the parental strain in J774.1 cells. This mutant was attenuated in BALB/c mice up to 10^6 CFU by the s.c. route (80% survival). In contrast, all mice infected with only 120 CFU of Schu S4 by this route succumbed to the infection. Vaccination with 10^4 CFU of the Schu S4 $\Delta capB$ mutant conferred 60% protection against s.c. challenge with 10^3 CFU of Schu S4.

Another Schu S4 mutant strain that has been assayed for attenuation and protection recently was a mutant of the *clpB* gene, which encodes a heat shock protein. No studies to determine whether this mutant exhibited any defects in intracellular survival were reported, but it was attenuated for virulence in mice infected i.d. with 10^5 CFU (100% survival) [22]. Interestingly, only BALB/c, but not C57BL/6 mice, were protected from challenge (an increased mean time-to-death (MTD) versus naïve mice) against i.n. challenge with 100 CFU of Schu S4 following i.d. immunization [31]. This underscores previous findings that C57BL/6 mice represent a more stringent model for protection against type A challenge. In a later studies it was reported that an i.d. immunization of BALB/c mice with 10^5 CFU of $\Delta clpB$ protected 80% of mice from an i.n. challenge with 100 CFU of Schu S4 [32] and that full protection of BALB/c mice against a 75 CFU i.n. Schu S4 challenge was conferred following an i.d. vaccination of 10^5 CFU [33]. These results are important, since $\Delta clpB$ is one of few mutants to demonstrate significant protection against a type A respiratory

challenge, and protection against a systemic type A challenge has not been shown to predict protection against a respiratory challenge.

In addition to the $\Delta capB$ mutant, there are other examples of studying a mutant in the Schu S4 background that had previously demonstrated attenuation in the LVS background. A mutant of the *ggt* gene in Schu S4, which encodes a gamma-glutamyl transferase, failed to replicate in J774.1 cells and was found to be attenuated for virulence in BALB/c mice [34] following infection by the s.c. route at doses up to 8.75×10^5 CFU. Protection conferred against an s.c. Schu S4 challenge was dose dependent; vaccination with 8.75 , 87.5 , and 875×10^2 CFU of the mutant conferred partial protection against a 100 CFU Schu S4 challenge. However, vaccination with 8.75×10^3 CFU was sufficient to provide full protection against that same challenge.

The FTT_0615c gene is the Schu S4 homolog of the LVS FTL_0883 gene encoding spermine response. Unlike its LVS counterpart, the Schu S4 FTT_0615c mutant did not exhibit any intracellular growth defects in either murine BMDMs or human MDMs. However, the mutant was shown to be somewhat attenuated for virulence in C57BL/6 mice [28]. Mice infected i.t. with 100 CFU of Schu S4 all succumbed with an MTD of 6 days, but 33% of mice infected with the same dose of the mutant survived with an MTD of 15 days. As with its LVS counterpart, the FTT_0615c mutant was not tested for protection against Schu S4 challenge.

The most recent study of an attenuated mutant in the Schu S4 background reported the results of deleting two different genes, FTT_0369c or FTT_1676, as well as a double mutant with both genes deleted [35]. All three mutant strains failed to replicate in mBMDMs and exhibited a high degree of attenuation in BALB/c mice following infection by both the i.d. (50 CFU) and i.n. (10 CFU) routes. Some degree of protection was conferred against a Schu S4 challenge when mice were vaccinated by either route with any of the three mutant strains. The best results occurred when the mice were vaccinated i.d. and then challenged i.n. The FTT_1676 mutant was the most protective, with 100% of vaccinated mice surviving challenge. The FTT_0369c mutant was the next most protective, with a vaccinee survival rate of 90%. The double mutant was the least protective strain, with a vaccinee survival rate of 60%. This study also reported that mice infected i.d. with up to 5×10^4 CFU of FTT_0369c survived at a rate of 100%, and that these mice were fully protected against an i.n. Schu S4 challenge of 50 CFU, though protection was lost at a challenge dose of 200 CFU. These findings are important, as i.d. vaccination may be more practical than i.n., and this represents only the second mutant strain to provide this level of protection against a high dose Schu S4 challenge [36].

5. Summary and Conclusions

The data summarized here and previously reviewed demonstrate that it is possible to create genetically defined deletion mutants in the LVS background that are significantly attenuated for virulence and protective against high level LVS challenges. However, there are few examples of mutant strains in the LVS background that confer meaningful protection against a Schu S4 or other type A challenge. While LVS based mutants such as the *wzy* mutant and the *sodB* mutant (previously reviewed) [30] have demonstrated some protection against a Schu S4 challenge, most mutants in the LVS background have not.

Since the ultimate goal of a *Francisella* vaccine is to confer full protection against a high dose type A aerosol exposure, the kind that might be seen in the event of a biological attack, the evidence gathered in recent years would suggest that an effective vaccine will likely need to be engineered in the Schu S4 (or other type A strain) background. While it is difficult to directly compare the protective capacity of attenuated strains developed in

different laboratories due to differences in study design, several strains have shown promise towards protection against a type A challenge (Table 4). To date, four Schu S4 mutant strains have stood out as the most effective vaccine candidates against a high dose type A challenge. The $\Delta fipB$ mutant [36, 37] is attenuated in C57BL/6 mice infected i.n. up to 10^{10} CFU. An i.n. immunization with 10^7 CFU of the $\Delta fipB$ mutant conferred full protection against a Schu S4 challenge of 68 CFU in C57BL/6 mice, and i.n. immunization with 10^8 CFU conferred full protection against a Schu S4 challenge of 95 CFU in BALB/c mice [36, 37]. These results are significant not only because of the high level of attenuation and protection demonstrated by the $\Delta fipB$ mutant, but also because protection was achieved after a single vaccination. Many of the strains reported to be protective against a lethal challenge required multiple vaccinations. Multi-dose vaccine regimens are common for typical vaccines and could be implemented for the application of a *Francisella* vaccine to high risk populations including health care workers, first responders, or military personnel. However, a single dose regimen would be most useful in an emergency situation such as a biological attack. The other three candidates are among the most recently reported, the FTT_0369c, FTT_1676, and $\Delta clpB$ mutants discussed above.

In addition to the identification and characterization of an increasing number of live attenuated vaccine candidates, research in the pursuit of a *Francisella* vaccine has produced an expanded base of knowledge about the organism and its pathogenic process. We have learned that phagosomal escape is required for intramacrophage replication and is critical to a mutant's ability to confer protection [38, 39]. While we would expect that an inability to survive or replicate intracellularly would correlate with *in vivo* attenuation (and many reports support this hypothesis (see tables)), some reports have also shown that mutant strains can demonstrate significant *in vivo* attenuation even without demonstrated defects in intracellular replication. The LVS $\Delta galU$ and Schu S4 $\Delta capB$ mutants are examples of such mutant strains [21, 25]. Additionally, the immune response to *Francisella* infection has been well characterized both *in vitro* [38, 40–42] and *in vivo* [41, 43, 44]. The *in vitro* and *in vivo* immune response to infection with many of the mutant strains reviewed here have been compared to their wild-type parent, allowing researchers to begin to understand the mechanisms behind the attenuation of their strains. For example, the murine immune response to vaccination with the Schu S4 $\Delta clpB$ mutant and subsequent Schu S4 challenge was extensively studied [31].

While it is encouraging to see a growing number of mutant strains that are attenuated and confer protection against a type A challenge, it is also important to note that the level of protection conferred has often been dependent on the dose and route of the vaccination. Since the most likely need for a *Francisella* vaccination would be to protect the public from a biological attack, protection from an aerosol challenge is the goal of an effective vaccine. The i.n. route is commonly used to mimic an aerosol exposure in the mouse model, and the majority of vaccine candidates that demonstrated protection against an i.n. challenge have been delivered with an i.n. vaccination (Tables 3 and 4). Recent advances in mucosal vaccine delivery support the viability of this route. The licensed FluMist® live attenuated influenza vaccine, delivered intranasally with the accuSpray device, has proven to be well accepted and highly efficacious [45]. Additionally, in attempts to optimize mass campaigns for measles vaccination, aerosol immunization was utilized and demonstrated to be safe and efficacious [46–48]. Three Schu S4 mutant strains (the FTT_1676, FTT_0369c, and $\Delta clpB$ mutants) were shown to confer significant protection against an i.n. Schu S4 challenge following vaccination by the i.d. route. This is very encouraging, because having multiple vaccine delivery options would be beneficial should the need for widespread vaccination arise.

Live attenuated vaccines offer a number of advantages that would be useful in conferring protection. A successful live attenuated tularemia vaccine would be able to infect cells and present a complete array of antigens to the innate immune system but also persist long enough for the host to develop a strong adaptive immune response and develop immunological memory. These advantages come with a set of concerns that are particular to live attenuated vaccines and must be addressed for successful implementation.

One hypothetical concern is reversion to virulence through re-acquisition of a virulence trait in a deletion mutant. Though the probability of such an event occurring is exceedingly low, this concern is addressed by constructing attenuated strains which feature multiple independent mutations, creating an even lower probability of a return to virulence. However, secondary mutations must be carefully selected. The balance between attenuation and immunogenicity is tenuous, and combining multiple mutations can result in over-attenuation and loss of protective capacity (such as what was seen when the FTT_0369c and FTT_1676c mutants were combined [35]). Another consideration is the safety of the vaccine in the broad population, including individuals who may be immunocompromised. This issue is somewhat allayed for a live tularemia vaccine (and could potentially be addressed in specialized animal models or clinical trials [49]), since the vaccine would likely only be used in specific populations such as military personnel or first responders, and widespread use would be unlikely except in the event of a bioterror attack. Person to person transmission of a live vaccine must be taken into account; however, tularemia is not transmitted in this fashion.

Looking forward to advancement of this vaccine includes optimization of manufacturing and storage. A recent report demonstrated the success of foam drying for large scale production of *F. tularensis* using LVS as a model [50]. This suggests an economical strategy for a tularemia vaccine would be formulation as a dried powder and storage as bulk product until a need for large scale vaccination arises.

It is also important to note that certain hurdles would need to be cleared for any of the candidates summarized above to become a licensed vaccine. The current regulatory environment requires testing in two relevant animal models if human testing is not an option. Nearly all the candidates discussed in this and previous reviews have been tested in only one animal model. An ideal animal model for simulating human tularemia has not yet been developed. While mice have largely served as the primary animal model for attenuation and protection studies, they are far more sensitive to type B strains than humans, and thus are not an ideal model for tularemia. Recent advances have been made in modeling human tularemia infection in rabbits, rats, and monkeys [11, 51–56]. One recent non-human primate study demonstrated that Schu S4 mutants of the *dsbB* and *pdpB* genes were shown to be attenuated in rhesus macaque monkeys and partially protective against an i.n. challenge with Schu S4 following an s.c. vaccination. Monkeys immunized by the s.c. route with these strains had lower bacterial burdens, measured at necropsy, following i.n. challenge with Schu S4 than naïve animals [57].

Establishing a reliable correlate of protection against tularemia would aid the development of a live tularemia vaccine. Historically, the literature suggests that a high antibody titer is a reliable marker for prior exposure/vaccination and that cell-mediated immunity is likely required for long-term protection. Immunity conferred following natural infection might be expected to be different from immunity elicited by vaccination, especially depending on the vaccine type. However, and establishment of a protective correlate would contribute to efforts in targeting vaccine responses. The literature suggests that immunity to *Francisella* is complex and includes cell-mediated responses that are likely required for long-term protection [6, 58]. Recent data suggests that IL-6 is required for murine survival of a

primary LVS infection and that T and B cells along with IL-12 and IFN- γ are required for murine survival of a primary Schu S4 infection [59, 60]. It should also be noted that most mutant strains that have been identified as protective against a type A challenge have only demonstrated protection against the Schu S4 strain. While this strain represents the most widely used type A strain in *F. tularensis* research, a vast array of type A strains across multiple clades exist in the environment [2, 61, 62], and an ideal vaccine would protect against challenge with a majority of those strains. While no studies have been performed that tested cross-protection against multiple type A strains by a single live attenuated strain (though studies have performed challenges with type A strains other than Schu S4 [63]), given the extremely high genetic similarity (>99%) among sequenced type A strains and the fact that LVS confers partial protection against Schu S4, it is reasonable to expect that a vaccine that is protective against a single type A strain would be protective against a variety these strains.

In summary, the last 10 years have been a very productive time in the development of a potential vaccine against *Francisella* infection. The development of numerous vaccine candidates, further characterization of the immune mechanisms induced against *Francisella*, and the continued development of animal models have propelled the field forward. These advances represent remarkable progress, especially for a pathogen with little prior study, which is expected to continue into the future as technologies and models continue to expand and support the development of a safe and efficacious tularemia vaccine.

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Highlights

- Interest in vaccine development against bioterror agents has increased since 2001.
- One such agent of interest is *Francisella tularensis*.
- Live attenuated strains have shown promise as vaccine candidates in mouse models.
- Survival and protection data from recent examples is reviewed in this article.

Table 1

Intracellular growth characteristics of live attenuated LVS mutant strains.

Gene	Function	Intracellular Phenotype	Cell Types Tested	Reference
<i>purMCD</i>	Purine biosynthesis	Escapes phagosome, fails to replicate, induce apoptosis	J774A.1	[16]
<i>tolC</i>	TolC	Replicates normally	murine BMDM	[64]
<i>sodB</i>	Superoxide Dismutase B	Not determined	N/A	[65]
<i>wbtA</i>	O-antigen biosynthesis	Fails to replicate	J774A.1	[66, 67]
<i>wbtI</i>	Transamine/perosamine synthesis	Reduced growth during first 48 hours, rapid growth thereafter	J774A.1	[68]
<i>katG</i>	Catalase	Similar growth to wild type	murine PEC	[18]
<i>pilF, pilT</i>	Type IV pili assembly	Reduced intracellular growth	murine BMDM, A549, FL83B	[69]
<i>ggt</i>	Gamma-glutamyl transpeptidase	Reduced intracellular growth	J774A.1, RAW264.7, murine BMDM, THP-1	[70]
<i>guaB, guaA</i>	GMP synthesis	Fail to replicate	J774A.1	[19]
<i>capB</i>	Capsule biosynthesis	Does not replicate to wild type levels	THP-1, MH-S, U937	[20]
<i>wzy</i>	O-polysaccharide biosynthesis	Fails to replicate	RAW 264.7, MH-S	[23]
<i>galU</i>	UDP-glucose phosphorylase	Replicates similarly to wild type	J774A.1, RAW264.7	[25]
FTL_0883	Spermine responsiveness	Fails to replicate	murine BMDM, human MDM	[28]
FTL_0439	Iron uptake	Fails to replicate to wild type levels	J774A.1	[27]
<i>fptB, fptE, fptG</i>	Phagosomal transport	Altered replication kinetics	J774A.1, HepG2	[26]
FTL_0724	Folate metabolism	Does not replicate to wild type levels	murine BMDM	[29]

Table 2

Intracellular growth characteristics of live attenuated Schu S4 mutants.

Gene	Function	Intracellular Phenotype	Cell Types Tested	Reference
FTT_0918	58 kDa protein	Fails to replicate to wild type levels in murine macrophages	murine PEC	[63]
FTT_0107c/ <i>dsbB</i>	Disulfide bond formation	Fails to replicate	J774A.1, HepG2	[71]
<i>lipB</i>	Lipoprotein	Fails to replicate in hepatic cells, does not escape phagosome in macrophages	HepG2, J774A.1	[36]
<i>purMCD</i>	Purine biosynthesis	Fails to replicate	human MDM, A549	[17]
<i>guaA</i> , <i>guaB</i>	GMP synthesis	Fails to replicate	J774A.1	[72]
<i>katG</i>	Catalase	Similar growth to wild type	murine PEC	[18]
<i>capB</i>	Capsule biosynthesis	Similar growth to wild type	J774A.1	[21]
<i>clpB</i>	Heat shock protein	Not determined	N/A	[31]
<i>ggt</i>	Gamma-glutamyl transpeptidase	Fails to replicate	J774A.1	[34]
FTT_0615c	Spermine responsiveness	Similar growth to wild type	murine BMDM, human MDM	[28]
FTT_0369c, FTT_1676	Unknown	Fail to replicate	murine BMDM	[35]

Table 3

In vivo attenuation and protection profile of LVS-derived mutant strains.

Gene	<i>In vivo</i> Phenotype	Survival Percentage and Dose in CFU (Route)	Challenge Results	Challenge Dose in CFU; Route	Reference
<i>purMCD</i>	Attenuated in BALB/c mice	100%, 5×10^6 (IP)	Full protection against LVS challenge	5×10^3 ; IP	[16]
<i>tolC</i>	Attenuated in C3H/HeN mice	100%, 10^6 (ID)	N/A	N/A	[64]
<i>sodB</i>	Attenuated in BALB/c, C57BL/6 mice	60%, 1×10^4 (IN), 80%, 1×10^4 (IN)	40% of C57BL/6 mice survived Schu S4 challenge	14; IN	[65]
<i>whiA</i>	Attenuated in BALB/c mice	100%, 10^8 (ID), 10^6 (IN), 10^7 (IP)	Full protection against LVS	25x LD50; ID, IN, IP	[66, 67]
<i>whiI</i>	Attenuated in BALB/c mice	100%, 2.8×10^7 (IP)	Full protection against LVS (ID), 60% survival against LVS (IP)	25x LD ₅₀ (ID), 75x LD ₅₀ (IP)	[68]
<i>katG</i>	Attenuated in C57BL/6 mice	100%, 10^8 (ID)	Not tested	Not tested	[18]
<i>pilF</i>	Moderately attenuated in C3H/HeN mice	90%, 10^6 (ID)	Not tested	Not tested	[69]
<i>pilT</i>	Moderately attenuated in C3H/HeN mice	70%, 10^6 (ID)	Not tested	Not tested	[69]
<i>ggT</i>	Moderately attenuated in BALB/c mice	100%, 10^3 (IP)	Not tested	Not tested	[70]
<i>guaB</i>	Attenuated in BALB/c mice	100%, 1.05×10^7 (IP)	Full protection against LVS	2.8×10^5 ; IP	[19]
<i>guaA</i>	Attenuated in BALB/c mice	100%, 9×10^6 (IP)	Full protection against LVS	2.8×10^5 ; IP	[19]
<i>capB</i>	Attenuated in BALB/c mice	100%, 10^7 (ID and IN)	Full protection against LVS, Schu S4	4×10^3 ; IN (LVS) 10; IN (Schu S4)	[20]
<i>wzy</i>	Attenuated in BALB/c mice	100%, 10^8 (ID) 100%, 10^7 (IN) 100%, 10^7 (IP)	Full protection against LVS, 84% survival against Schu S4	1×10^5 ; IN (LVS) 8; IN (Schu S4)	[23]
<i>galU</i>	Attenuated in C57BL/6 mice	100%, 1×10^7 (IN)	Full protection against LVS	5×10^4 ; IN	[25]
FTL_0883	Attenuated in C57BL/6 mice	100%, 1×10^4 (IT)	Not tested	Not tested	[28]
FTL_0439	Attenuated in C57BL/6 mice	100%, 2.3×10^3 (IP)	Full protection against LVS	1.2×10^5 ; IP	[27]
<i>fpiB</i>	Attenuated in BALB/c mice	75%, 3×10^3 (IP)	Full protection against LVS	1.2×10^5 ; IP	[26]
<i>fpiE</i>	Attenuated in BALB/c mice	100%, 3×10^3 (IP)	Full protection against LVS	1.2×10^5 ; IP	[26]
<i>fpiG</i>	Attenuated in BALB/c mice	100%, 3×10^6 (IP)	Full protection against LVS	2×10^6 ; IP	[26]
FTL_0724	Attenuated in C57BL/6 mice	100%, 3×10^4 (IP)	Not tested	Not tested	[29]

Table 4

In vivo attenuation and protection profile of Schu S4-derived mutant strains.

Gene	<i>In vivo</i> Phenotype	Survival Percentage and Dose in CFU (Route)	Challenge Results	Challenge Dose in CFU; Route	Reference
FTT0918	Attenuated in BALB/c mice	100%, 10 ³ (ID)	Full protection against ID FSC033 challenge, delayed MTD against IN FSC033 challenge	500; ID 10; IN	[63]
FTT0107c <i>dsbB</i>	Attenuated in C57BL/6 mice	100%, 2 × 10 ³ (IN)	Not protective against Schu S4 challenge	13; IN	[71]
<i>fipB</i>	Attenuated in C57BL/6 mice	100%, 1.8 × 10 ¹⁰ (IN)	Full protection against Schu S4 challenge	37; IN	[36]
<i>purMCD</i>	Attenuated in BALB/c mice	100%, 10 ⁶ (IN, ID)	40% survival against Schu S4 challenge	500; IN	[17]
<i>guaA</i>	Attenuated in mice	100%, 10 ⁹ (IN)	Not protective against Schu S4 challenge	1,000; IN	[72]
<i>guaB</i>	Attenuated in mice	100%, 7 × 10 ⁵ (IN)	Not protective against Schu S4 challenge	1,000; IN	
<i>katG</i>	Not attenuated in C57BL/6 mice	0%, 10 (ID)	Not tested	Not tested	[18]
<i>capB</i>	Attenuated in BALB/c mice	100%, 1.6 × 10 ⁴ (SC)	60% survival against Schu S4 challenge	10 ³ ; SC	[21]
<i>clpB</i>	Attenuated in BALB/c mice	100%, 10 ⁷ (ID)	80% survival against Schu S4 challenge	100; IN	[31]
<i>ggf</i>	Attenuated in BALB/c mice	100%, 8.75 × 10 ⁵ (SC)	Full protection against Schu S4 challenge	100; SC	[34]
FTT0615c	Attenuated in C57BL/6 mice	33%, 1 × 10 ⁴ (IT)	Not tested	Not tested	[28]
FTT0369c	Attenuated in BALB/c mice	95%, 50 (ID) 95%, 10 (IN)	90% survival against Schu S4 challenge	50; IN	[35]
FTT1676	Attenuated in BALB/c mice	80%, 50 (ID) 90%, 10 (IN)	Full protection against Schu S4 challenge	50; IN	[35]