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Working toward the Future: Insights into *Francisella tularensis* Pathogenesis and Vaccine Development

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INTRODUCTION

Francisella tularensis is a gram-negative coccobacillus that has an exceedingly low infectious dose. It is a category A select agent and is one of the most infectious bacteria known. Following the terrorist attacks of 2001 and subsequent anthrax mailings in the fall of that year, there has been a renewed interest in the study of this organism. Advancements in the field of *F. tularensis* genetics have lead to a dramatic expansion in the generation of mutant strains of various *F. tularensis* subspecies. Collectively, this has led to an improved understanding of *F. tularensis* biology, host responses to infection, and virulence factors required for infection and/or disease elicitation. Many investigators in this field have focused on the development of a vaccine capable of protecting against the most virulent biovars of *F. tularensis*. Of particular interest are those providing substantive protection against type A strains delivered by the respiratory route. Here, we review the major characteristics of *F. tularensis* and provide an update regarding genes required for pathogenesis and determinants being targeted for vaccine development.

FRANCISELLA TULARENSIS AND TULAREMIA

Classification

Francisella tularensis is one of the most infectious and pathogenic bacteria known. It is the etiological agent of the debilitating febrile illness tularemia. The bacterium is a gram-negative, capsulated, facultative intracellular pathogen and is one of the members of the genus *Francisella* of the *Gammaproteobacteria* class. *Francisella* has no close pathogenic relatives but exists in a sister clade with the arthropod endosymbiont *Wolbachia persica*. It is also distantly related to human pathogens *Coxiella burnetii* and *Legionella pneumophila* (109). *F. tularensis* is commonly classified into three subspecies, *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, and *F. tularensis* subsp. *mediasiatica*, based on genetic makeup, virulence, ability to produce acid from glycerol, and citrulline ureidase activity (49) (Table 1). *Francisella novicida* is also often considered a subspecies of *F. tularensis*; however, recent whole-genome single-nucleotide polymorphism analysis indicates that it is likely an independent species (104). *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* are the primary biovars associated with disease in humans. *F. tularensis* subsp. *tularensis*, also known as type A *Francisella*, is found primarily in North America and is highly virulent in humans. This subspecies is responsible for roughly 70% of *Francisella* disease cases in North America (186). Type A strains have an infectious dose of <10 CFU in humans (174, 175) and can lead to life-threatening illness, particularly when infection occurs via the respiratory route. Molecular subtyping techniques indicate that *F. tularensis* subsp. *tularensis* can be further divided into two genetically distinct clades (A.I and A.II) that differ with respect to disease outcome, transmission, and geographic location (59, 98, 104, 187, 194). *F. tularensis* subsp. *holarctica*, or type B strains, is found throughout much of the Northern Hemisphere and is the primary cause of tularemia in Europe (141). These organisms have an infectious dose of $<10^3$ CFU and cause a milder form of tularemia in humans. The live vaccine strain (LVS) that was developed in the former Soviet Union and gifted to the United States in the 1950s is a human-attenuated type B derivative. *F. tularensis* subsp. *mediasiatica* and *F. novicida* are focally distributed and are rarely associated with disease in humans. *F. tularensis* subsp. *mediasiatica* is primarily isolated to central Asian regions of the former USSR, while *F. novicida* is found in North America and Australia (57, 141, 147). *F. novicida* has been extensively studied as a model organism in the laboratory setting due to its enhanced genetic tractability relative to other subspecies and its relative avirulence in humans. All *F. tularensis* subspecies are highly pathogenic in animal models, particularly in rabbits and mice. *F. novicida* is also highly pathogenic in mice, but its virulence remains less characterized outside this model system.

Comparative Genomics

There are currently eight completely sequenced *Francisella* genomes (Table 1) and an additional nine genomes for which shotgun sequencing is currently under way. Sequence analysis of *F. tularensis* subsp. *tularensis* (FSC198 [NC 008245], Schu S4 [NC 006570], and WY96-3418 [NC 009257]), *F. tularensis* subsp. *holarctica* (FTNF002-00 [NC 009749], OSU18 [NC 008369], and LVS [NC 067880]), *F. tularensis* subsp. *mediasiatica* (FSC147 [NC 010677]), and *F. novicida* (U112 [NC 008601]) indicates that these strains are highly similar at the genetic level. The genome of each strain is roughly 1.8 Mb, with *F. novicida* U112 having the largest genome at 1.91 Mb. All genomes have a G+C content of approximately 32%, with between 1,800 and 2,000 putative coding sequences depending on the subspecies and strain. Between 70 and 90% of open reading frames within these isolates are predicted to code for functional proteins. Interestingly, the more virulent subspecies, *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*, contain roughly 200 to 300 pseudogenes, depending on the strain, while the less pathogenic *F. novicida* U112 contains only 14 pseudogenes (109, 203). Additionally, nearly 30% of annotated genes within an *F. tularensis* isolate are characterized as hypothetical proteins with unknown function, suggesting that *Francisella* is likely to encode novel virulence determinants. A 30-kb region with low G+C content (27.5%) that is unique to *Francisella* among the 17 gammaproteobacterial genomes exists in duplicate in type A and B strains of *F. tularensis* but is present in single copy in *F. novicida*. This locus has been identified as a pathogenicity island and is required for aspects of *F. tularensis* survival within host cells.

Comparative genomic studies have indicated that there is a high level of nucleotide identity between and within *F. tularensis* subspecies, ranging from roughly 97% to 99%. Despite this, there are numerous DNA rearrangements present between subspecies, particularly between type A and type B strains, and

TABLE 1. Characteristics of *Francisella* species[a]

Species or subspecies	LD_{50} (CFU) in:		Strain[d]	Accession no.	Length (bp)	No. of:	
	Mice[b]	Humans[c]				Genes	Pseudogenes[e]
F. tularensis subsp. *tularensis*	<10	<10	Schu S4	NC 006570	1,892,819	1,852	201
			FSC198	NC 008245	1,892,616	1,852	199
			WY96-3418	NC 009257	1,898,476	1,872	0
F. tularensis subsp. *holarctica*	<10	$<10^3$	LVS	NC 007880	1,895,994	2,020	213
			OSU18	NC 008369	1,895,727	1,932	328
			FTNF002-00	NC 009749	1,890,909	1,887	2
F. tularensis subsp. *mediasiatica*	NR[f]	NR	FSC147	NC 010677	1,893,886	1,750	297
F. novicida	<10	$>10^3$	U112	NC 008601	1,910,031	1,781	14

[a] Adapted from Table 1 in reference 140 with kind permission from Springer Science + Business Media (© Springer 2005) and from reference 203 with kind permission of Wiley-Blackwell.
[b] Doses delivered subcutaneously.
[c] Estimated LD based on virulence in animal models and case studies.
[d] Several additional *Francisella* isolates are currently being sequenced, including *F. tularensis* subsp. *tularensis* (strains MA00-2987 [NZ ABRI00000000] and FSC033 [NZ AAYE00000000]), *F. tularensis* subsp. *holarctica* (strains FSC200 [NZ AASP00000000], FSC022 [NZ AAYD00000000], and 257 [NZ AAUD00000000], and *F. novicida* (strains FTG [NZ ABXZ00000000], FTE [NZABSS00000000], GA99-3548 [NZ ABAH00000000], and GA99-3549 [NZ AAYF00000000]).
[e] Refers to sequences resembling functional genes but thought to have lost protein-coding capability.
[f] NR, doses have not been reported.

among different type A strains (148). These rearranged sequences are flanked by repeated DNA insertion sequence elements, indicating that they likely evolved from homologous recombination events. In contrast, little genomic reorganization is observed in type B strains. Though the precise impact of these rearrangements remains unclear, it is of note that they exist primarily in the more virulent type A strains.

Comparisons of deletion events, repeat sequences, and single-nucleotide polymorphisms between sequenced *Francisella* genomes have provided important insights into the evolution of these organisms. The presence of conserved genomic deletion events and single-nucleotide variations in *F. tularensis* and *F. novicida* isolates suggest that these species have evolved vertically, with *F. novicida* being the most ancestral. Additionally, the highly virulent type A strains appeared before the less virulent type B strains (59). The reduced genomic heterogeneity of type B strains compared with type A strains and the recovery of type B strains from around the world indicate that *F. tularensis* subsp. *holarctica* has evolved recently and spread rapidly (59). The evolution of *F. tularensis* from a common ancestor appears to have resulted from both a loss and a gain of genetic information over time, as type A strains have undergone a reduction in their genomic content relative to *F. novicida*, but type B strains contain additional genomic content that is otherwise absent from their type A counterparts. These observations indicate either that rearrangements occurred in type A *Francisella* after type A and B strains diverged evolutionarily or that type B strains were derived from one type A strain that lost the ability to undergo such rearrangements (148).

Epidemiology of *F. tularensis*

Though its primary environmental niche remains unknown, *F. tularensis* has a broad and complex host distribution, infecting a number of wildlife species, including lagomorphs, rodents, insectivores, carnivores, ungulates, marsupials, birds, amphibians, fish, and invertebrates (14, 18, 136, 196). *F. tularensis* is most frequently found in rodents, hares, and rabbits; however, these are unlikely reservoirs for *F. tularensis*, considering that infection often leads to acute disease in these animals. It has also been suggested that protozoa may play a role as hosts in aquatic cycles, which is supported by the demonstration of *F. tularensis* in amoebal cysts (2, 111, 141, 202). Finally, although most arthropod vectors serve only as transient hosts, *F. tularensis* may be transmitted by ticks throughout their life cycle, raising the possibility that a single tick may infect multiple hosts (90, 149).

A primary route of *F. tularensis* transmission to humans and other animals is through arthropod vectors such as ticks, biting flies, and possibly mosquitoes. Infection by *F. tularensis* can also occur through direct contact with contaminated water, food supplies, or infected animals (49). *F. tularensis* is occasionally acquired by inhalation of organisms that have been aerosolized through disruption of contaminated materials. For these reasons, high-risk groups include hunters or trappers, who might come into contact with infected animals, and landscapers, who may encounter aerosolized organisms through mechanical disruption of contaminated soil or animal carcasses. Though *F. tularensis* organisms are readily aerosolized, transmission via human-to-human contact has yet to be reported.

F. tularensis-mediated disease was first recognized as a plague-like illness in rodents during an outbreak in Tulare County, CA, in 1911, resulting in the first isolation of the bacterium (70). Three years later, human disease caused by *F. tularensis* in two patients in Ohio who had recent contact with wild rabbits was described (215). In 1919, Edward Francis established that a number of clinical symptoms were specifically caused by "*Bacterium tularense*," named for the county in which the disease was found to be endemic, and the name "tularemia" was subsequently used to describe them (68, 70). Tularemia has been referred to as "rabbit fever," "market men's disease," and "meat-cutter's disease," all named for the

frequent incidence of disease associated with dressing rabbits for meat. The terms "deer-fly fever" and "glandular type of tick fever" have also been used to describe tularemia in the context of symptoms arising from a tick or fly bite resulting in a noted enlargement of lymph nodes. Identification of symptoms and potential sources led to the subsequent accumulation of tularemia reports in the United States, with roughly 14,000 cases reported by 1945 (96) and a peak incidence of 2,291 cases in 1939 (60, 183). Concurrently, reports of a similar disease were emerging from Japan and Russia. Large waterborne outbreaks in the 1930s and 1940s further solidified the epidemic potential of this organism and prompted further investigation into the characteristics of *F. tularensis*. The largest recorded tularemia outbreak occurred via airborne transmission of the European biovar *F. tularensis* subsp. *holarctica* and involved more than 600 individuals between 1966 and 1967 in a farming area of Sweden (45). In this case, most individuals acquired tularemia while doing farm work that created aerosols, such as sorting hay.

Today, the worldwide occurrence of human tularemia is likely underestimated and underreported due to the generic nature of the disease symptoms. It is well established that natural tularemia outbreaks are typically highly localized, with areas of endemicity often encompassing only a few hundred square kilometers. Outbreaks of tularemia often occur in parallel with outbreaks in rodents, hares, rabbits, and other small mammals (196). Tularemia is known to be dispersed throughout much of the Northern Hemisphere, particularly in parts of North America, Europe, and northern Asia (57, 91). There have been reports of human tularemia in every state in the United States except Hawaii, with a localization of most cases to south-central and western states (18, 24a, 48). Overall, reported cases of tularemia have dropped from several thousand per year prior to 1950 to fewer than 200 in the 1990s (18, 24a, 48). Cases are typically sporadic or occur in small clusters during June through September, correlating with the incidence of arthropod-borne transmission (18, 48, 58). A summary of a number of tularemia reports during the 1980s in the United States revealed that 63% of infected individuals reported an attached tick, and 23% reported contact with wild rabbits (197). The most recent major incident involving *F. tularensis* in the United States occurred on Martha's Vineyard in 2000 and involved 15 patients with one fatality; 11 of the patients had acquired pneumonic tularemia (61). Many of those infected were landscapers, and it is speculated that lawn mowing or brush cutting was a major risk factor (61). Though less numerous, reports of tularemia continue to arise from Martha's Vineyard annually, with landscapers representing a majority of the infected. Most tularemia reports in Europe are from the northern and central countries, particularly Scandinavian countries (196). Disease in many of these countries occurs in an uneven geographical distribution, with high percentages of reports coming from localized rural regions. Furthermore, a strain similar to *Francisella novicida* was recently isolated from a patient in Australia, indicating that the geographic distribution of *Francisella* is likely more widespread than previously reported (216).

Tularemia

Tularemia is an acute febrile illness, the type and severity of which depend on the route of infection and the infecting biovar. *F. tularensis* can infect humans through the skin, mucous membranes, gastrointestinal tract, and respiratory tract. Major target organs include the lymph nodes, lungs, spleen, liver, and kidneys (58, 69, 116, 154, 190). Infection acquired through the skin or mucous membranes results in ulceroglandular tularemia, which comprises up to 90% of all cases (195). Ulceroglandular tularemia results from direct contact of the organism with the skin, often while handling infected animals or animal tissues or as a result of vector-borne transmission. A primary ulcer develops at the infection site, followed by painful swelling of the nearby lymph nodes. After an incubation period that can last up to 21 days, there is a rapid onset of high fever accompanied by flu-like symptoms. *F. tularensis* may further disseminate to and replicate in other organs in the body, particularly the lungs, liver, and spleen. Ulceroglandular tularemia has a mortality rate of less than 5% (58), though dissemination and replication within the lung may lead to a more serious respiratory disease. Inhalation of live organisms or accumulation of organisms in the lung following dissemination from other infection routes often leads to respiratory tularemia, the most severe form of the disease. Outbreaks resulting from respiratory transmission are rare but can involve a large number of cases, depending on the mechanism of dispersion. Symptoms for respiratory tularemia can be somewhat variable. While inhalation of *F. tularensis* subsp. *holarctica* results in a mild and generally non-life-threatening respiratory infection, inhalation of *F. tularensis* subsp. *tularensis* results in an acute, serious infection that presents with a high fever, chills, malaise, and cough. Organisms deposited into the lung readily spread to the draining lymph nodes and further disseminate to the liver and spleen, where severe inflammation and tissue damage can occur. Tularemia resulting from respiratory infection of type A *Francisella* has mortality rates approaching 30% to 60% if untreated (50, 174, 175). However, the fatality rate is reduced to less than 2% when antibiotics are administered in a timely fashion (58). Other, less common forms of the disease include oculoglandular tularemia, which results from direct contact of organisms with the eye, accounting for 1% to 4% of all cases (141). Ingestion of food or water contaminated with *F. tularensis* may also cause oropharyngeal and/or gastrointestinal tularemia, which is the least common form of the disease. Typhoidal tularemia is a term used to describe infection with severe systemic symptoms without regional ulcerations or swollen lymph nodes indicative of a site of inoculation (49). Though less common, these additional disease forms highlight the ability of *Francisella* to infect humans via multiple routes.

Potential as a Biological Weapon

F. tularensis has long been considered a potential biological weapon based on its ability to cause severe disease upon inhalation of doses as low as 10 CFU (174). The biological weapons programs in several countries, including Japan, the former Soviet Union, and the United States, developed weapons containing *F. tularensis* (49, 87). In the 1960s, *F. tularensis* was one of a number of agents stockpiled by the United States military

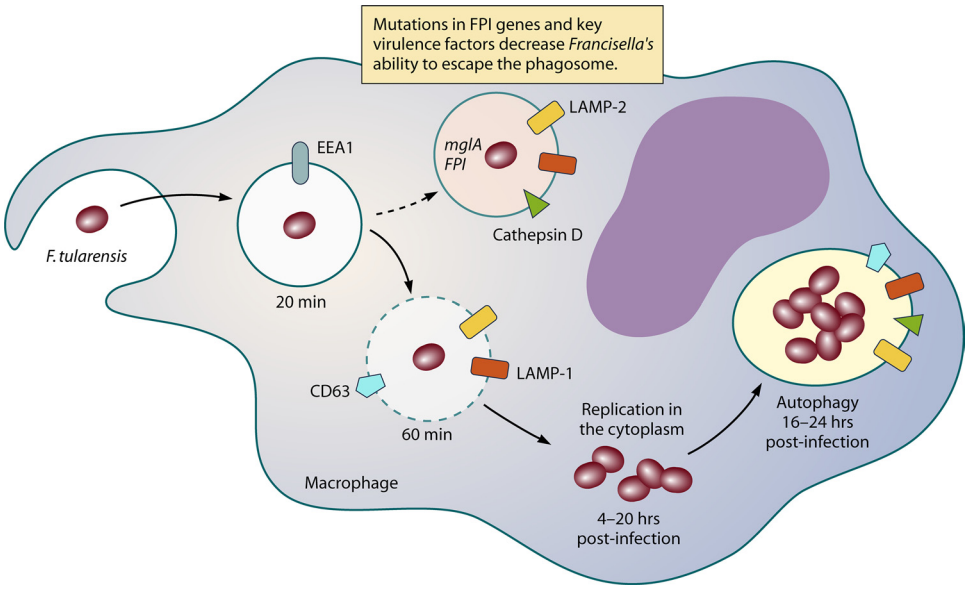

FIG. 1. Illustration of *Francisella* survival inside macrophages. *Francisella* is taken up by macrophages through looping phagocytosis (33) into an endosomal compartment that transiently acquires late endosome-associated markers (29, 32). *Francisella* then exits the phagosomal compartment and replicates to high numbers in the cellular cytosol. Prior to lysis of the cell, *Francisella* has been shown to reside in an autophagy-like compartment (27).

as part of a biological weapons development program that was eventually terminated by executive order in 1970 (30). Despite efforts to disengage biological weapons programs around the world, former Soviet Union biological weapons senior scientist Ken Alibeck reported that weaponization efforts occurred in the Soviet Union well into the 1990s (3). In light of recent world events, the extreme infectivity and the ability to potentially disseminate aerosolized organisms over an urban area continue to drive concerns regarding *Francisella* weaponization and/or intentional release. In 1969, a report from a World Health Organization committee assessed the bioweapon threat of *F. tularensis*. It estimated that an aerosol release of 50 kg of *F. tularensis* over an urban area with a population of nearly 5 million individuals would result in 250,000 incapacitating casualties and 19,000 deaths (220a). More recently, the Centers for Disease Control and Prevention (CDC) estimated that the total base costs to society resulting from such an attack would approach $5.4 billion for every 100,000 persons exposed (102). In the event of an intentional release of *F. tularensis*, it is likely that prompt treatment of at-risk individuals would dramatically reduce the impact of the event.

F. TULARENSIS PATHOGENESIS

The success of *Francisella* as a pathogen is intimately associated with its ability to survive and replicate within a wide variety of host cell types. Upon entering a mammalian host, *Francisella* is known to target macrophages. However, it has become increasingly clear that these organisms can infect and survive in a number of additional cell types, including dendritic cells, neutrophils, hepatocytes, and lung epithelial cells. While the importance of these cell types to infection is not completely understood, it is well documented that *Francisella* replicates within mononuclear phagocytes in vivo and exhibits a disease cycle within these cells that appears to differ little between strains or subspecies.

Intracellular Life Cycle of *F. tularensis* in Phagocytic Cells

Francisella enters cells through the process of phagocytosis. It has been reported that *F. tularensis* may utilize an unusual mechanism involving the formation of spacious asymmetric pseudopod loops. This process, termed "looping phagocytosis" (33), involves actin rearrangement through phosphatidylinositol 3-kinase signaling and is strongly dependent on the presence of complement factor C3 and complement receptor CR3 (32, 33). *Francisella* may also enter cells via the mannose receptor, type I and II class A scavenge receptors, and the Fcγ receptor (11, 151, 178). Following internalization into host cells, *F. tularensis* is able to alter normal bactericidal processes. It prevents induction of the respiratory burst (66), limiting its exposure to superoxide or other reactive oxygen by-products. It alters phagosome maturation and as a result only transiently interacts with components of the endocytic trafficking network (Fig. 1). The organism initially resides in a membrane-bound compartment that acquires limited amounts of early endosomal and late endosomal-lysosomal markers, including EEA1, CD63, LAMP1, and LAMP2 (35). The *F. tularensis*-containing vacuole (FCV) fails to acquire the acid hydrolase cathepsin D and does not fuse with lysosomes (35). In addition, *F. tularensis* alters host cell trafficking by escaping from the phagosome and entering the host cell cytosol, where it undergoes extensive replication (27, 35, 82, 173). While the relative timing of these events appears to differ between the various *Francisella* species and the host cell types infected (27, 35, 82, 173), mutants that fail to prevent fusion with the lysosome and/or are unable to escape from the phagosome are highly attenuated in virulence in vitro and in vivo (19, 117, 133, 135).

There are conflicting reports regarding the extent to which the FCV acidifies as it transiently interacts with components of the endocytic pathway. It also remains controversial whether exposure to acidic pH is necessary and/or sufficient for *F. tularensis* egress from the phagosome. Studies conducted by Clemens et al. using THP1 cells (a human macrophage-like cell line) or primary macrophages derived from peripheral blood monocytes have demonstrated that FCVs harboring LVS or type A *F. tularensis* become only minimally acidified (pH of 6.7) and acquire limited amounts of the proton vacuolar ATPase (34, 35). Additionally, use of the proton pump inhibitor bafilomycin A prior to infection of these macrophage types with *F. tularensis* strains does not alter the efficiency of *F. tularensis* phagosomal escape (34). In contrast, studies published by Santic et al. and Chong et al. have reported significant levels of FCV acidification and vacuolar ATPase acquisition in primary human and murine macrophages infected with *F. tularensis* Schu S4, LVS, and *F. novicida* (29, 173). Treatment of these macrophages with bafilomycin A prior to infection significantly reduced the efficiency with which these *F. tularensis* derivatives were able to escape from the phagosome (29, 173).

Phagosomal escape requires viable *F. tularensis* and occurs via an unknown mechanism that involves degradation of the surrounding lipid bilayers (27, 34, 35, 78, 173). At roughly 12 h postinfection, *Francisella* begins to replicate to high numbers within the host cell cytosol, eventually leading to cell death, egress of *Francisella*, and presumably infection of nearby cells. Escape of *F. tularensis* from the phagosome and replication within the host cell cytosol is dependent on genes present in the *Francisella* pathogenicity island (FPI) (29, 78, 83, 110, 117, 138, 171, 173, 176). *Francisella* has also been shown to reside in vesicles similar to autolysosomes at $\geq$24 h postinfection, prior to host cell death, indicating that *Francisella* may reenter the endocytic pathway through host cell autophagy (27). The significance of this process for either *Francisella* infection or the immune response to infection remains unclear. *Francisella* may also exhibit an extracellular phase, as both LVS and Schu S4 have been found in the plasma following infection of mice via various inoculation routes (63). Whether this observation correlates to humans or plays a significant role in the ability of the organism to cause disease awaits further investigation.

IMMUNITY AND HOST RESPONSE TO INFECTION

Successful development of a *Francisella* vaccine will ultimately rely on a comprehensive understanding of the host immune response to infection. Many of the details regarding the host response to *F. tularensis* infection have come from studies using the less virulent *F. novicida* or the *F. tularensis* subsp. *holarctica* LVS, both of which are thought to differ from the more virulent type A strains in certain aspects of infection. Studies carried out with various murine infection models have shown that low doses of the attenuated LVS strain can be cleared by innate host defense mechanisms, while the fully virulent type A and B strains are able to rapidly kill mice prior to generation of a cell-mediated immune response. The precise mechanisms by which virulent strains avoid and overcome murine immune responses remain unknown. Differences in the host response to these subspecies and the route of infection highlight the complexities of this issue and suggest that the correlates of immunity need to be evaluated for each potential infection scenario.

Innate Immunity

The innate immune responses to *F. tularensis* infection share much in common with the responses seen with other intracellular pathogens. *Francisella* infection results in an early pronounced inflammatory response, with initial induction of proinflammatory and Th1-type cytokines, including interleukin-12, tumor necrosis factor alpha, and gamma interferon (IFN-γ) (38, 81, 189, 217). Tumor necrosis factor alpha and IFN-γ are essential for control of infection, as depletion of either converts typically sublethal infections into lethal ones (55, 56, 112). Macrophages, dendritic cells, and natural killer (NK) cells are likely responsible for the cytokine induction seen almost immediately postinfection (17, 118). Activation of proinflammatory cytokines in murine macrophages occurs in a Toll-like receptor 2 (TLR2)-dependent manner, indicating that TLR2-mediated signaling may be crucial for early pathogen recognition (37). IFN-γ activation of macrophages and other professional phagocytes is also particularly important for initial containment of *Francisella*, as these cells are a primary target of the organism for infection and replication. In addition to macrophages, neutrophils have been shown to be important in the initial control of infection, but their importance may differ with respect to the different tissues infected. In mouse infection models, depletion of neutrophils increases sensitivity to systemic infection but has little effect on respiratory infection with *Francisella* (41, 56, 184).

There is evidence that *Francisella* evades and modulates the host immune response beyond its ability to inhibit maturation of the host phagosome and escape lysosomal degradation. *Francisella* diminishes the capacity of macrophages to respond to engagement of TLRs with secondary stimuli such as lipopolysaccharide (LPS) (198). Similar effects have also been demonstrated in dendritic cells (81). A recent report by Bosio et al. has indicated that when delivered via the respiratory route, the type A strain Schu S4 actively suppresses early inflammatory responses in the lung (16). In particular, Schu S4 fails to activate pulmonary macrophages and dendritic cells and actively interferes with induction of proinflammatory cytokines, in part through the induction of transforming growth factor β (16). In addition, Woolard et al. have recently demonstrated that *Francisella* infection of bone marrow-derived macrophages results in secretion of prostaglandin E_2, which inhibits interleukin-2 production and promotes a Th2-type response, a T-cell response that is ineffective against the clearing of intracellular organisms (220). This increase in prostaglandin E_2 has also been confirmed in the lung in vivo (219). Finally, *Francisella* has been shown to infect and replicate within neutrophils and inhibit the respiratory burst, thus evading neutrophil killing mechanisms (127, 179). Though the precise contributions of these findings to infection remain unclear, it is likely that immune evasion and/or suppression is essential to the highly virulent nature of *Francisella* and differences between subspecies.

Adaptive Immunity

Exposure to sublethal concentrations of *Francisella* induces strong protective immunity against secondary exposure in humans and in experimental animal models (51, 188). Though specific antibodies are readily detectable in sera upon *F. tularensis* infection, their importance to immunity remains unclear. Passive antibody transfer studies carried out in animals suggest that antibodies may play a role in combating infection with lower-virulence strains while playing a lesser role against the more virulent subspecies (51, 54, 188). Although *Francisella* antibodies may prove beneficial in some situations, they are likely not essential. Rather, they must be coupled with an effective cellular immune response to fully control infection. Adaptive immunity to *F. tularensis* infection is largely dependent on T-cell-mediated immunity, particularly that mediated by $CD4^+$ and $CD8^+$ T cells (56). In mice, either $CD4^+$ or $CD8^+$ cells are able to control infection by *F. novicida* or LVS, while both cell types seem to be required for successful defense against the highly virulent type A strains (43, 74, 222). Similar to the case for mice, $CD4^+$ and $CD8^+$ T-cell responses are prominent in humans vaccinated with LVS (107, 192). The T-cell effector functions are likely very closely linked to the ability to activate macrophage intracellular killing mechanisms. Despite the known requirement of $CD4^+$ and $CD8^+$ T cells for resolving *F. tularensis* infection, little is known regarding the T-cell receptors, coreceptors, memory profiles, or major histocompatibility complex restriction of T-cell responses to infection.

Immunity to Infection by Different Routes

An effective vaccine against *F. tularensis* will require generation of an immune response that is protective against pulmonary infection. However, much of the work examining and characterizing the immune responses to *Francisella* infection have involved infection by the systemic route. Though there are many general consistencies, correlates of immunity to *F. tularensis* infection differ in certain aspects depending on the route of infection. A number of recent reports highlight potentially key differences in the host immune response to respiratory versus systemic infection (41). In addition to the diminished role of neutrophils and reactive nitrogen species, there exists disparity in the timing of initial host inflammation when comparing respiratory versus systemic infection. During murine infection initiated via the intradermal or subcutaneous route, there is an immediate onset of inflammation within the first 2 days postinfection that includes the rapid induction of IFN-γ (36, 54). During respiratory infection this response is delayed, not occurring until 3 to 5 days postinfection. By this time, significant bacterial burdens have begun to accumulate in the livers and spleens of infected mice, and it has been speculated that systemic disease contributes to the morbidity observed in these animals (40). The delay in inflammation onset is consistent with what has been seen in human disease (6). The precise reasons for this delay remain unclear, but it may play a contributing factor in the general difficulty in vaccinating against respiratory forms of the disease. Other recent reports also highlight potential differences in T-cell responses between respiratory and systemic infection. Woolard et al. have demonstrated that intranasal infection of mice produces much lower levels of IFN-γ-secreting T cells than systemic infection (219). Furthermore, intranasal inoculation results in a delayed accumulation of T cells in the spleen and lung, along with a significant increase in the amounts of prostaglandin E_2. Collectively, these observations suggest that virulent *F. tularensis* subspecies alter T-cell responses to the detriment of the host (220).

Though our understanding of *Francisella* host/pathogen interactions is advancing, there is still a great deal that remains unclear. Of particular interest, the host immune response to infection by highly virulent type A strains has only now been investigated in any great detail. Further evaluation of the host immune response to infection, as well as identification of key *Francisella* virulence mediators, will be necessary to gain a more complete understanding of the interplay between *Francisella* and the host immune system, particularly for the development of novel prophylactic treatments.

FRANCISELLA GENETICS AND VIRULENCE FACTORS

The ability to effectively colonize or parasitize a diverse array of hosts suggests that *F. tularensis* is capable of adapting to a wide variety of growth environments. Despite its extreme virulence and fairly well-characterized intracellular life cycle, very little is known about the mechanisms of *F. tularensis* pathogenesis or the virulence factors encoded by this organism. Initial assessments of the completed genomic sequences from different *F. tularensis* subspecies have indicated that *F. tularensis* does not encode any toxins or secretion systems that are commonly present in other intracellular pathogens. In addition, *F. tularensis* does not encode homologs of genes that mediate phagosomal escape in other organisms, such as *Listeria* and *Shigella*. Due in large part to an increase in the development and efficiency of genetic tools, recent studies have begun to shed light on the specific virulence genes necessary for successful infection by *F. tularensis*.

Genetic Tools

Shuttle and integration vectors. The field of *Francisella* genetics has undergone an extensive expansion over the past 10 years (71). Until recently, few vectors or selectable markers were amenable for use in *Francisella*, and the available methods for introducing DNA were generally inefficient. In 1994, identification of a 3,990-bp cryptic plasmid (pFNL10) from an *F. novicida*-like strain designated F6168 helped to usher in the first generation of useful genetic vectors for this organism (144). pFNL10 could be introduced by standard procedures and maintained in various subspecies of *F. tularensis*, although it was not capable of replicating in *Escherichia coli* and lacked any antibiotic resistance markers (144). Further modifications to pFNL10 led to the construction of second-generation vectors that carried replication origins for *E. coli* and selectable antibiotic resistance markers. pFNL100 included sequences from both pFNL10 and cloning vector pBR328 (143). pFNL200 was a deletion derivative of pFNL100 and expressed tetracycline and chloramphenicol resistance; however, it suffered from stability issues (143). pKK202 was a more stable derivative of pFNL200 that carried the p15A origin of replication from *E. coli* (139). Finally, the generation of pKK214

and its variants expanded the utility of pKK202 by incorporating a promoterless chloramphenicol acetyltransferase or green fluorescent protein reporter gene in place of the chloramphenicol acetyltransferase gene (2, 108).

More recently, Maier et al. have constructed a series of *E. coli-Francisella* shuttle vectors, termed pFNLTP, that are hybrids between pFNL10 and cloning vector pCR2.1-TOPO (125). These vectors can be efficiently transformed into *F. tularensis* subspecies by electroporation, are stably maintained even in the absence of antibiotic selection, and do not alter virulence characteristics of *F. tularensis* in vitro or in vivo (124, 125, 145, 146). A variety of pFNLTP1 variants have been generated, and these include derivatives that carry antibiotic resistance elements amenable for use in type A strains of *F. tularensis*, multiple cloning sites, reporter genes and counterselectable markers, and temperature-sensitive origins of replication (93, 125). In addition to their use as complementation and reporter gene platforms, pFNLTP1-based vectors (or vectors that have been derived from them) have been used as delivery vehicles to carry out transposon mutagenesis, targeted allelic exchange, and promoter-trap library construction (22, 125, 128).

LoVullo et al. have recently developed a series of shuttle vectors, pMP, that are based on the minimal regions of pFNL10 required for replication and regions from *E. coli*-mycobacterial shuttle vector pMV261 carrying the *aphA1* antibiotic resistance determinant and ColE1 replication origin (121). While the original plasmid, pMP393, could be efficiently introduced by electroporation and was stable in various *F. tularensis* subspecies, it was frequently lost in the absence of selection (121). Second-generation variants of pMP393 have corrected maintenance issues and expanded the choice and utility of antibiotic resistance determinants for selection within *F. tularensis* (120). Third-generation pMP-based vectors have also been developed, in which stability has been further enhanced, useful multiple cloning sites introduced, and heterologous promoters added for gene expression studies (120). Finally, a single-copy chromosomal integration system for *Francisella* has been developed by that group (119). Vectors designed for this system include plasmids allowing integration at the attachment site for the Tn*7* transposon (located downstream of the *glmS* gene) or within the *blaB* gene, encoding resistance to the antibiotic ampicillin (119). Development of an integration system for *F. tularensis* represents a major advancement for the field, as it alleviates some of the previous issues inherent with use of multicopy shuttle vectors, including lack of stability, use of heterologous antibiotic resistance determinants, and multicopy expression artifacts.

Gene disruption vectors. Much of the lack of understanding of *Francisella* virulence can be directly attributed to the difficulty in generating defined genetic lesions within this family. While genetic tools and methodologies have been available for some time to disrupt genes in *F. novicida*, construction of mutant derivatives in the type A or B genetic background was not reported until 2004 (82). Gene disruptions in *F. novicida* have been generated using a variety of approaches, including allelic exchange of linear substrates (Table 2) (110). Initial efforts to disrupt genes in type A or type B strains were based largely on utilization of pUC19-derived suicide vectors (82). Optimization of these vectors, along with the development of additional vectors, has allowed the list of *Francisella* mutants to expand significantly (Tables 3 and 4). This list includes mutants that are defective for putative virulence factors as well as metabolic genes that may be utilized for the construction of live attenuated vaccine candidates. More recently, the TargeTron group II intron mutagenesis system has been adapted for use with various *F. tularensis* subspecies (162, 163). This system has proven efficient, and it is advantageous as it allows simultaneous disruption of genes that are present in more than one copy (162, 163).

The development of *Francisella* genetic tools has also allowed for the implementation of global mutagenesis strategies. These include transposon mutagenesis of *F. novicida*, the LVS, and type A strain Schu S4. In 2004, Kawula et al. described the use of Tn*5* transposon-transposase complexes to create random, stable insertion mutations in the LVS chromosome (103). Another strategy, employed by Maier et al., involved the utilization of either a temperature-sensitive version of pFNLTP1 or a derivative of pFNLTP1 in which the origin of replication for *Francisella* had been removed as a delivery vehicle for *Himar* transposon mutagenesis (124, 126). This library has since been used to identify mutants of LVS defective for intracellular growth within macrophages (124). More recently, a number of negative selection strategies including transposon site hybridization and signature-tagged mutagenesis have been optimized for use with *Francisella* and employed to identify genes required for growth of *F. novicida* or LVS in mice (106, 191, 213). In 2007, Gallagher et al. completed a full-scale genomic mutagenesis of the *F. novicida* U112 genome in which each putative nonessential open reading frame had been interrupted, thereby contributing a comprehensive mutagenesis library available for analyses (76). In addition, a Tn*5*-based transposon mutagenesis system has also been developed (23). This system expands upon the utility of current transposon tools by allowing unmarking of the antibiotic resistance determinant following transposition into the genome and inclusion of promoterless *lux* and *lacZ* alleles for analyzing gene expression using reporter gene technology (23). Mutagenesis using this transposon system has allowed identification of genes from *F. tularensis* LVS that are required for inhibition of the respiratory burst in neutrophils and for intracellular growth or survival within this cell type (179). Additionally, this system was used to identify FevR, a novel regulator of *iglB* (22). Finally, useful genetic surrogates of *F. novicida* which lack all of the resident restriction-modification systems that otherwise act as a barrier to gene transfer have now been developed (75). Clearly, the ongoing genetic work with *Francisella* is rapidly expanding our understanding of *Francisella* pathogenesis, and it will no doubt lead to identification of key virulence mediators that can be exploited for the development of potential vaccines and therapeutics.

Virulence Factors

Developments in the field of *F. tularensis* genetics have played an important role in identifying the repertoire of virulence genes utilized by this organism during infection within the host. While the characterization of these determinants is still in its infancy, the large numbers of mutants that exhibit defects in virulence indicate that the pathogenic lifestyle of *F.*

TABLE 2. *F. novicida* genes involved in pathogenesis

Locus tag	Name	Function	Method(s)[a]	Cells/animals in which mutant strain is attenuated[b]	Reference(s)
FTN_0008	FTN_0008	Ten-transmembrane-spanning drug/metabolite exporter protein	Tp	C57BL/6	106
FTN_0012	FTN_0012	Hypothetical protein	Tp	C57BL/6	106
FTN_0019	*pyrB*	Aspartate carbamoyltransferase	Tp	C57BL/6	213
FTN_0020	*carB*	Carbamoylphosphate synthase large chain	Tp	J774A.1, RAW, BMDM, BALB/c	199, 213
FTN_0021	*carA*	Carbamoylphosphate synthase small chain	Tp	C57BL/6	213
FTN_0023	*tmpT*	Thiopurine *S*-methyltransferase	Tp	C57BL/6	106
FTN_0028	FTN_0028	Conserved hypothetical membrane protein	Tp	C57BL/6	106
FTN_0031	FTN_0031	Transcriptional regulator, LysR family	Tp	C57BL/6	106
FTN_0035	*pyrF*	Orotidine 5′-phosphate decarboxylase	Tp	C57BL/6	213
FTN_0036	*pyrD*	Diyroorotate dehydrogenase	Tp	C57BL/6	213
FTN_0045	FTN_0045	Protein of unknown function	Tp	C57BL/6	106
FTN_0055	*tyrA*	Prephenate dehydrogenase	Tp	C57BL/6	106
FTN_0090, FTN_1556, FTN_1061, FTN_0954	*acpABC*, *hap*	Acid phosphatases[c]	Ar	J774A.1, activated THP-1, BALB/c	13, 133, 135
FTN_0096	FTN_0096	Conserved hypothetical membrane protein	Tp	C57BL/6	213
FTN_0097	FTN_0097	Aromatic amino acid transporter of the hydroxy/aromatic amino acid permease family	Tp	C57BL/6	213
FTN_0098	*gidB*	Methyltransferase, glucose-inhibited cell division protein	Tp	C57BL/6	213
FTN_0111	*ribH*	Riboflavin synthase beta-chain	Tp	C57BL/6	106
FTN_0112	*ribA*	RibA/GTP-cyclohydrolase II	Tp	C57BL/6	213
FTN_0113	*ribB*	Riboflavin synthase a-subunit 3,4-digydroxy-2-butanone 4-phosphate synthetase	Tp	C57BL/6	213
FTN_0132	FTN_0132	Protein of unknown function	Tp	C57BL/6	106, 213
FTN_0133	FTN_0133	RNase II family protein	Tp	C57BL/6	106
FTN_0143	FTN_0143	Monovalent cation:proton antiporter	Tp	C57BL/6	106
FTN_0169	FTN_0169	Conserved hypothetical membrane protein	Tp	C57BL/6	106
FTN_0177	*purH*	AICAR transformylase/IMP cyclohydrolase	Tp	J774A.1, RAW, BMDM, BALB/c	199, 213
FTN_0178	*purA*	Adenylosuccinate synthetase	Ar/In	J774A.1, BALB/c	158
FTN_0196	*cyoB*	Cytochrome *o* ubiquinol oxidase subunit I	Tp	C57BL/6	213
FTN_0197	*cyoC*	Cytochrome *o* ubiquinol oxidase subunit III	Tp	C57BL/6	213
FTN_0198	*cyoD*	Cytochrome *o* ubiquinol oxidase subunit IV	Tp	C57BL/6	213
FTN_0199	*cyoE*	Heme O synthase	Tp	C57BL/6	106
FTN_0202	*pdxY*	Pyridoxal/pyridoxine/pyridoxamine kinase	Tp	C57BL/6	213
FTN_0205	FTN_0205	Conserved hypothetical protein	Tp	C57BL/6	106
FTN_0208	FTN_0208	Hypothetical membrane protein	Tp	C57BL/6	106
FTN_0210	FTN_0210	Conserved hypothetical protein	Tp	C57BL/6	213
FTN_0211	*pcp*	Pyrrolidone-carboxylate peptidase	Tp	C57BL/6	213
FTN_0217	FTN_0217	L-Lactate dehydrogenase	Tp	C57BL/6	106
FTN_0229	*pyrH*	Uridylate kinase	Tp	C57BL/6	213
FTN_0240	*rplD*	50S ribosomal protein L4	Tp	C57BL/6	213
FTN_0241	*rplW*	50S ribosomal protein L23	Tp	C57BL/6	213
FTN_0248	*rpsQ*	30S ribosomal protein S17	Tp	RAW	199
FTN_0265	*rplQ*	50S ribosomal protein L17	Tp	C57BL/6	213
FTN_0266	*htpG*	Chaperone Hsp90, heat shock protein HtpG	Tp	J774A.1, RAW, BMDM, BALB/c	199, 213
FTN_0280	FTN_0280	Hypothetical protein	Tp	C57BL/6	106
FTN_0281	FTN_0281	Protein of unknown function	Tp	C57BL/6	106
FTN_0289	FTN_0289	ProP osmoprotectant transporter, fragment	Tp	C57BL/6	213
FTN_0292	FTN_0292	Protein of unknown function	Tp	C57BL/6	106
FTN_0295	*lpxF*	Phosphatidic acid phosphatase, PAP2 superfamily	Ar	C57BL/6	209
FTN_0296	FTN_0296	Amino acid transporter	Tp	C57BL/6	213
FTN_0297	FTN_0297	Conserved protein of unknown function	Tp	C57BL/6	106, 213
FTN_0298	*gplX*	Fructose 1,6-bisphosphatase II	Tp	C57BL/6	106, 213
FTN_0299	*putP*	Proline:Na^+ symporter	Tp	C57BL/6	106
FTN_0300	FTT1629c	Glycosyl transferase, group 2	Tp	RAW	199
FTN_0302	FTN_0302	Hypothetical protein	Tp	C57BL/6	106
FTN_0326	FTN_0326	Conserved hypothetical protein	Tp	C57BL/6	106
FTN_0330c	*minD*	Septum formation inhibitor-activating ATPase	In	PM, BMDM, C57BL/6NCrIBR	8
FTN_0337	*fumA*	Fumarate hydratase, class I	Tp	J774A.1, RAW, BMDM, BALB/c	199
FTN_0340	FTN_0340	Protein of unknown function	Tp	C57BL/6	106
FTN_0344	FTN_0344	Aspartate:alanine exchanger family	Tp	C57BL/6	106
FTN_0347	*fkpB*	FK506 binding protein-type peptidyl-prolyl, *cis-trans*	Tp	C57BL/6	106
FTN_0351	FTN_0351	Hypothetical protein, novel liver	Tp	C57BL/6	106
FTN_0365	FTN_0365	Conserved hypothetical membrane protein	Tp	C57BL/6	106
FTN_0393	FTN_0393	Conserved protein of unknown function	Tp	C57BL/6	106
FTN_0395	FTN_0395	Transcriptional regulator, ArsR family	Tp	C57BL/6	106
FTN_0397	*gpsA*	Glycerol-3-phosphate-dehydrogenase	Tp	C57BL/6	106
FTN_0400	FTN_0400	Protein of unknown function	Tp	C57BL/6	106
FTN_0402	*aroC*	Chorismate synthase	Tp	C57BL/6	106
FTN_0416	FTN_0416	Conserved hypothetical membrane protein	Tp	C57BL/6	213
FTN_0417	*folD*	Methylene tetrahydrofolate	Tp	C57BL/6	106, 213
FTN_0419	*purM*	Phosphoribosylformylglycinamide cyclo-ligase	Tp	J774A.1, RAW, BMDM, BALB/c	199, 213

Continued on following page

TABLE 2—*Continued*

Locus tag	Name	Function	Method(s)[a]	Cells/animals in which mutant strain is attenuated[b]	Reference(s)
FTN_0420	*purCD*	SAICAR synthetase/phosphoribosylamine-glycine ligase	Tp	J774A.1, RAW, BMDM, BALB/c	199, 213
FTN_0422	*purE*	Phosphoribosylaminoimidazole carboxylase, catalytic subunit	Tp	C57BL/6	213
FTN_0426	FTN_0426	Conserved protein of unknown function	Tp	C57BL/6	106
FTN_0427	*tul4*	Lipoprotein of unknown function	Ar/In	J774A.1[c]	110
FTN_0429	FTN_0429	Hypothetical protein	Tp	C57BL/6	213
FTN_0430	*lpnB*	Protein of unknown function	Tp	C57BL/6	106, 213
FTN_0431	FTN0431	Type IV pilus glycosylation protein	Tp	C57BL/6	213
FTN_0436	FTN_0436	Hypothetical protein	Tp	C57BL/6	213
FTN_0437	FTN_0437	Hydrolase, HD superfamily	Tp	C57BL/6	106
FTN_0438	*rrmJ*	23S rRNA methylase	Tp	C57BL/6	106
FTN_0443	*maeA*	NAD-dependent malic enzyme	Tp	RAW	199
FTN_0444	FTT0918	Membrane protein of unknown function	Tp	C57BL/6	181
FTN_0480	*fevR*	Protein of unknown function	Ar	BMDM, C57BL/6	20
FTN_0488	*cspC*	Cold shock protein,	Tp	C57BL/6	106
FTN_0493	*mtn*	Adenosylhomocysteine nucleosidase	Tp	C57BL/6	213
FTN_0494	FTN_0494	Hypothetical membrane protein	Tp	C57BL/6	213
FTN_0494	FTN_0494	BNR/Asp box repeat protein	Tp	C57BL/6	213
FTN_0503	*lolD*	Lipoprotein releasing system, subunit D, ABC transporter, ATP-binding protein	Tp	C57BL/6	213
FTN_0504	*cadA*	Lysine decarboxylase, inducible	Tp	C57BL/6	213
FTN_0505	*gcvT*	Glycine cleavage complex T protein (aminomethyltransferase)	Tp	C57BL/6	213
FTN_0506	*gcvH*	Glycine cleavage system H protein	Tp	C57BL/6	213
FTN_0507	*gcvP1*	Glycine cleavage system P protein, subunit 1	Tp	C57BL/6	213
FTN_0514	*pgm*	Phosphoglucomutase	Tp	C57BL/6	213
FTN_0536	*yjjK*	ABC transporter, ATP binding protein	Tp	C57BL/6	213
FTN_0537	FTN_0537	Proton-dependent oligopeptide	Tp	C57BL/6	106
FTN_0544	FTN_0544	Conserved hypothetical protein	Tp	C57BL/6	213
FTN_0545	*flmF2*	Glycosyl transferase, group 2	Tp	BALB/c, C57BL/6	101, 213
FTN_0546	*flmK*	Dolichyl-phosphate-mannose-protein, mannosyltransferase family protein	Tp	BALB/c, C57BL/6	101, 213
FTN_0554	FTN_0554	RNA methyltransferase,	Tp	C57BL/6	106
FTN_0559	*surA*	Peptidyl-prolyl *cis*-trans isomerase	Tp	C57BL/6	213
FTN_0560	*ksgA*	Dimethyladenosine transferase, kasugamycin resistance	Tp	C57BL/6	213
FTN_0571	*xasA*	Amino acid-polyamine-organocation	Tp	C57BL/6	106, 213
FTN_0578	FTN_0578	Major facilitator superfamily transport	Tp	C57BL/6	106
FTN_0582	*gph*	Phosphoglycolate phosphatase	Tp	C57BL/6	106
FTN_0594	*sucC*	Succinyl-coenzyme A synthetase, beta chain	Tp	RAW	199
FTN_0599	FTN_0599	Conserved hypothetical protein	Tp	C57BL/6	213
FTN_0609	*pnp*	Polyribonucleotide nucleotidyltransferase	Tp	C57BL/6	106
FTN_0624	*sdaC1*	Serine transporter	Tp	C57BL/6	213
FTN_0643	FTN_0643	Hypothetical protein	Tp	C57BL/6	213
FTN_0646	*cscK*	ROK family protein	Tp	C57BL/6	106
FTN_0651	*cdd*	Cytidine deaminase	Tp	C57BL/6	213
FTN_0669	*deoD*	Purine nucleoside phosphorylase	Tp	C57BL/6	213
FTN_0678	FTN_0678	Drug:H^+ antiporter-1 (DHA1) family	Tp	C57BL/6	106
FTN_0689	*ppiC*	Parvulin-like peptidyl-prolyl isomerase	Tp	C57BL/6	106
FTN_0690	*deaD*	DEAD box subfamily, ATP-dependent	Tp	C57BL/6	106
FTN_0710	FTN_0710	Type I restriction-modification system	Tp	C57BL/6	106
FTN_0713	*ostA2*	Organic solvent tolerance protein OstA	Tp	C57BL/6	106
FTN_0714	FTT0742	Protein of unknown function	Tp	J774A.1, RAW, BMDM, BALB/c	199
FTN_0720	FTN_0720	Transcriptional regulator, LclR family	Tp	C57BL/6	106, 213
FTN_0728	FTN_0728	Cation efflux family protein	Tp	C57BL/6	213
FTN_0737	*potI*	ATP binding cassette putrescine uptake	Tp	C57BL/6	106
FTN_0740	FTN_0740	Protein of unknown function	Tp	C57BL/6	106
FTN_0756	*fopA*	OmpA family protein	Tp	RAW	199
FTN_0757	FTN_0757	Hypothetical protein	Tp	C57BL/6	213
FTN_0768	*tspO*	Tryptophan-rich sensory protein	Tp	C57BL/6	106
FTN_0787	*rep*	UvrD/REP superfamily I	Tp	C57BL/6	106
FTN_0806	FTN_0806	β-Glucosidase-related glycosidase	Tp	C57BL/6	213
FTN_0810	FTN_0810	ROK family protein	Tp	C57BL/6	106
FTN_0814	*bioF*	8-Amino-7-oxononanoate synthase	Tp	C57BL/6	213
FTN_0815	*bioB*	Biotin synthase	Tp	C57BL/6	213
FTN_0816	*bioA*	Oxononanoate aminotransferase	Tp	C57BL/6	213
FTN_0817	FTN_0817	Conserved hypothetical protein	Tp	C57BL/6	213
FTN_0818	FTN_0818	Lipase/esterase	Tp	C57BL/6	213
FTN_0821	FTN_0821	AMP binding family protein	Tp	C57BL/6	213
FTN_0822	FTN_0822	Chorismate binding family protein	Tp	C57BL/6	213
FTN_0823	*trpG*	Anthranilate synthase component II	Tp	C57BL/6	213
FTN_0824	FTN_0824	Major facilitator superfamily	Tp	C57BL/6	106
FTN_0825	FTN_0825	Aldo/keto reductase family protein	Tp	C57BL/6	106
FTN_0840	*mdaB*	NADPH-quinone reductase	Tp	C57BL/6	106
FTN_0848	FTN_0848	Amino acid antiporter	Tp	C57BL/6	213
FTN_0853	*sufD*	SufS activator complex, SufD subunit	Tp	C57BL/6	106
FTN_0857	FTN_0857	Conserved hypothetical protein	Tp	C57BL/6	106
FTN_0869	FTT0989	Conserved protein of unknown function	Ar/In	BMDM, C57BL/6	21
FTN_0873	*dcd*	dCTP deaminase	Tp	C57BL/6	106

Continued on following page

TABLE 2—*Continued*

Locus tag	Name	Function	Method(s)[a]	Cells/animals in which mutant strain is attenuated[b]	Reference(s)
FTN_0877	*cls*	Cardiolipin synthetase	Tp	C57BL/6	106
FTN_0881	FTN_0881	Fe2/Zn2 uptake regulator protein	Tp	C57BL/6	106
FTN_0891	*ruvB*	Holliday junction DNA helicase, subunit B	Tp	C57BL/6	213
FTN_0918	FTN_0918	Conserved protein of unknown function	Tp	C57BL/6	106
FTN_0925	FTN_0925	Hypothetical protein	Tp	C57BL/6	213
FTN_0933	FTN_0933	Protein of unknown function	Tp	C57BL/6	106
FTN_0938	FTN_0938	Hypothetical protein	Tp	C57BL/6	106
FTN_0998	FTN_0998	Potassium channel protein	Tp	C57BL/6	213
FTN_1006	FTN_1006	Transporter-associated protein	Tp	C57BL/6	106
FTN_1015	FTN_1015	Isochorismatase family protein	Tp	C57BL/6	106
FTN_1016	FTN_1016	Conserved protein of unknown function	Tp	C57BL/6	106, 213
FTN_1029	FTN_1029	Conserved protein of unknown function	Tp	C57BL/6	106
FTN_1030	*lipA*	Lipoic acid synthetase	Tp	C57BL/6	106
FTN_1038	FTN_1038	Conserved hypothetical membrane protein	Tp	C57BL/6	213
FTN_1055	*lon*	DNA binding, ATP dependent	Tp	C57BL/6	106
FTN_1063	FTN_1063	tRNA-methylthiotransferase MiaB protein	Tp	C57BL/6	106
FTN_1064	*phoH*	PhoH-like protein	Tp	C57BL/6	213
FTN_1066	FTN_1066	Metal ion transporter protein	Tp	C57BL/6	213
FTN_1090	FTN_1090	Hypothetical protein	Tp	C57BL/6	213
FTN_1098	FTN_1098	Conserved hypothetical membrane protein	Tp	C57BL/6	106
FTN_1107	*metIQ*	Methionine uptake transporter family	Tp	C57BL/6	106
FTN_1111	FTN_1111	Conserved hypothetical protein	Tp	C57BL/6	213
FTN_1112	*cphA*	Cyanophycin synthetase	Tp	C57BL/6	213
FTN_1131	*putA*	Multifunctional protein, transcriptional repressor of proline utilization, proline dehydrogenase, pyrroline-5-carboxylate dehydrogenase	Tp	C57BL/6	213
FTN_1132	FTN_1132	Conserved hypothetical protein	Tp	C57BL/6	213
FTN_1133	FTN_1133	Protein of unknown function, novel lung, spleen	Tp	C57BL/6	106
FTN_1146	*aspC2*	Aspartate aminotransferase	Tp	RAW	199
FTN_1148	FTN_1148	Glycoprotease family protein	Tp	C57BL/6	106
FTN_1157	FTN_1157	GTP binding translational elongation	Tp	C57BL/6	106
FTN_1165	FTN_1165	Predicted ATPase of the PP loop	Tp	C57BL/6	106
FTN_1177	*sbcB*	Exodeoxyribonuclease I	Tp	C57BL/6	106
FTN_1186	*pepO*	M13 family metallopeptidase	Ar/In	C57BL/6	21
FTN_1196	FTN_1196	Conserved protein of unknown function	Tp	C57BL/6	106
FTN_1199	FTN_1199	Conserved protein of unknown function	Tp	C57BL/6	106
FTN_1200	*capC*	Capsule biosynthesis protein CapC	Tp	C57BL/6	106
FTN_1201	*capB*	Capsule biosynthesis protein CapB	Tp	C57BL/6	213
FTN_1209	*cphB*	Cyanophycinase	Tp	C57BL/6	106, 213
FTN_1211	FTN_1211	Haloacid dehalogenase-like hydrolase family protein	Tp	C57BL/6	213
FTN_1212	FTN_1212	Glycosyltransferase group 1 family protein	Tp	C57BL/6	213
FTN_1213	FTN_1213	Glycosyltransferase family protein	Tp	C57BL/6	213
FTN_1214	FTN_1214	Glycosyltransferase family protein	Tp	C57BL/6	213
FTN_1217	FTN_1217	ABC transporter, ATP binding and membrane protein	Tp	C57BL/6	213
FTN_1218	FTN_1218	Glycosyltransferase, group 1	Tp	C57BL/6	106, 213
FTN_1219	*gale*	UDP-glucose 4-epimerase	Tp	C57BL/6	213
FTN_1220	FTN_1220	Bacterial sugar transferase family protein	Tp	C57BL/6	213
FTN_1232	FTN_1232	Conserved hypothetical membrane protein	Tp	C57BL/6	106
FTN_1241	*dedA2*	DedA family protein	Tp	RAW	199
FTN_1242	*dedA1*	DedA family protein	Tp	C57BL/6	213
FTN_1243	*recO*	RecFOR complex, RecO component	Tp	C57BL/6	106
FTN_1254	FTN_1254	Hypothetical protein	Tp	C57BL/6	213
FTN_1255	FTN_1255	Glycosyl transferase family 8 protein	Tp	C57BL/6	213
FTN_1256	FTN_1256	Hypothetical membrane protein	Tp	C57BL/6	213
FTN_1257	FTN_1257	Hypothetical membrane protein	Tp	C57BL/6	213
FTN_1259	*glyA*	Serine hydroxymethyltransferase	Tp	RAW	199
FTN_1272	FTN_1272	Proton-dependent oligopeptide	Tp	C57BL/6	106
FTN_1273	*fadD1*	Acyl coenzyme A synthetase	Tp	C57BL/6	213
FTN_1276	*emrA1*	HlyD family secretion protein	Tp	C57BL/6	213
FTN_1277	FTN_1277	Outer membrane efflux protein	Tp	C57BL/6	213
FTN_1284	*dnaK*	Chaperone, heat shock protein, HSP 70 family	Tp	J774A.1, RAW, BMDM, BALB/c	199
FTN_1290	*mglA*	Macrophage growth locus, protein A	Ar/In	J774A.1, *Acanthamoeba castellanii*, BALB/cAnNHsd, BALB/c	110, 111, 214
FTN_1292	FTN_1292	Solute:sodium symporter	Tp	C57BL/6	106
FTN_1298	FTN_1298	GTPase of unknown function	Tp	C57BL/6	106
FTN_1309	*pdpA*	Protein of unknown function	Ar	CE, BALB/cByJ	176, 177, 213
FTN_1310	*pdpB*	Protein of unknown function	Ar/In, Tp	BMDM, C57BL/6, J774A.1, RAW, BALB/c	21, 199, 213
FTN_1311	FTN_0311	Hypothetical protein	Tp	C57BL/6	213
FTN_1312	FTN_1312	Conserved hypothetical protein	Tp	C57BL/6	106, 213
FTN_1313	FTN_1313	Conserved hypothetical protein	Tp	C57BL/6	213
FTN_1314	FTN_1314	Conserved hypothetical protein	Tp	C57BL/6	213
FTN_1315	FTN_1315	Conserved hypothetical protein	Tp	C57BL/6	213
FTN_1316	FTN_1316	Conserved hypothetical protein	Tp	C57BL/6	213

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TABLE 2—*Continued*

Locus tag	Name	Function	Method(s)[a]	Cells/animals in which mutant strain is attenuated[b]	Reference(s)
FTN_1317	FTN_1317	Conserved hypothetical protein	Tp	C57BL/6	213
FTN_1318	*cds2*	Hypothetical protein	Ar/In, Tp	BMDM, C57BL/6	21, 213
FTN_1319	FTN_1319	Conserved hypothetical protein	Tp	C57BL/6	213
FTN_1320	FTN_1320	Conserved hypothetical protein	Tp	C57BL/6	213
FTN_1321	*iglD*	Intracellular growth locus protein D	Ar/In, Tp	BALB/c, hMDMs, C57BL/6	106, 172, 213
FTN_1322	*iglC*	Intracellular growth locus, protein C	Ar/In	J774A.1, *Acanthamoeba castellanii*, BALB/cAnNHsd, C57BL/6	110, 111, 142, 213
FTN_1323	*iglB*	Intracellular growth locus, protein B	In	J774, BALB/c	39, 213
FTN_1324	*iglA*	Intracellular growth locus protein A	Ar	J774A.1, CE	46, 213
FTN_1325c	*pdpD*	Protein of unknown function	Ar/In	BMDM, BALB/cByJ	138, 213
FTN_1326	FTN_1326	Conserved hypothetical protein	Tp	C57BL/6	213
FTN_1333	*tktA*	Transketolase I	Tp	J774A.1, RAW, BMDM, BALB/c	199
FTN_1349	FTN_1349	Hypothetical protein	Tp	C57BL/6	106
FTN_1351	FTN_1351	Conserved hypothetical protein	Tp	C57BL/6	106
FTN_1355	FTN_1355	Regulatory factor, Bvg	Tp	C57BL/6	106
FTN_1357	*recB*	Exodeoxyribonuclease V b-chain	Tp	C57BL/6	213
FTN_1362	FTN_1362	Hypothetical protein	Tp	C57BL/6	106
FTN_1400	FTN_1400	*S*-Adenosylmethionine dependent	Tp	C57BL/6	106
FTN_1403	*flmF1*	Glycosyltransferase	Tp	BALB/c, C57BL/6	101
FTN_1415	FTN_1415	Thioredoxin	Tp	C57BL/6	106
FTN_1417	*manB*	Phosphomannomutase	Tp	C57BL/6	213
FTN_1421	*wbtH*	Asparagine synthase	Tp	C57BL/6	213
FTN_1423	*wbtG*	Glycosyl transferase	Tp	C57BL/6	213
FTN_1425c-FTN_1427c	*wbtDEF*	LPS O-antigen biosynthetic gene cluster	Ar/In	BALB/c	200, 213
FTN_1432	*wbtA*	dTDP-glucose 4,6-dehydratase	Tp	C57BL/6	213
FTN_1439	*fadA*	Acetyl coenzyme A acetyltransferase	Tp	C57BL/6	106
FTN_1440	FTN_1440	Conserved hypothetical protein	Tp	C57BL/6	106
FTN_1444	*ocd*	Ornithine cyclodeaminase, mu-crystallin homolog	Tp	RAW	199
FTN_1455	FTN_1455	Conserved hypothetical protein	Tp	C57BL/6	106
FTN_1459	FTN_1459	Short-chain dehydrogenase	Tp	C57BL/6	106
FTN_1464	*lepB*	Signal peptidase I	Tp	C57BL/6	106
FTN_1465	*pmrA*	Two-component response regulator, orphan	Ar	J774A.1, activated THP-1, HeLa, BALB/c	134
FTN_1467	*proC*	Pyrroline-5-carboxylate reductase	Tp	C57BL/6	106
FTN_1471	*pcs*	(CDP-alcohol) phosphatidyltransferase	Tp	C57BL/6	106
FTN_1500	FTN_1500	Hypothetical protein	Tp	C57BL/6	213
FTN_1501	FTN_1501	Na^+/H^+ antiporter	Tp	C57BL/6	213
FTN_1507	FTN_1507	Pilus assembly protein	Tp	C57BL/6	106
FTN_1513	*xerC*	Integrase/recombinase XerC	Tp	C57BL/6	213
FTN_1518	*relA*	GTP pyrophosphokinase	Tp	C57BL/6	213
FTN_1433	FTN_1433	Hypothetical membrane protein	Tp	C57BL/6	213
FTN_1438	*fadB-acbP*	Fusion product of 3-hydroxacyl-coenzyme A dehydrogenase and acyl coenzyme A binding protein	Tp	C57BL/6	213
FTN_1470	*ispA*	Geranyltransferase	Tp	C57BL/6	213
FTN_1471	*pcs*	Phosphatidylcholine synthase	Tp	C57BL/6	213
FTN_1534	FTN_1534	Conserved protein of unknown function	Tp	C57BL/6	106
FTN_1536	FTN_1536	Amino acid-polyamine-organocation	Tp	C57BL/6	106
FTN_1538	*groL*	Chaperone protein, GroEL	Tp	C57BL/6	213
FTN_1548	FTN_1548	Conserved hypothetical lipoprotein	Tp	C57BL/6	213
FTN_1551	*ampD*	*N*-Acetylmuramoyl-L-alanine amidase	Tp	C57BL/6	213
FTN_1558	*xerD*	Integrase/recombinase	Tp	C57BL/6	213
FTN_1567	*rpoC*	DNA-directed RNA polymerase, beta′ subunit/160-kDa subunit	Tp	RAW	199
FTN_1582	FTN_1582	Hypothetical membrane protein	Tp	C57BL/6	213
FTN_1587	FTN_1587	Protein of unknown function	Tp	C57BL/6	106
FTN_1588	FTN_1588	Major facilitator superfamily transport	Tp	C57BL/6	106
FTN_1592	*oppB*	Peptide/opine/nickel uptake transporter (PepT) family protein	Ar/In	C57BL/6	21
FTN_1597	*prfC*	Peptide chain release factor 3	Tp	C57BL/6	213
FTN_1602	*deoB*	Phosphopentomutase	Tp	C57BL/6	213
FTN_1607	*cca*	tRNA nucleotidyltransferase	Tp	C57BL/6	213
FTN_1608	*dsbB*	Disulfide bond formation protein	Tp	J774A.1, RAW, BMDM, BALB/c, C57BL/6	199, 213
FTN_1611	FTN_1611	Major facilitator superfamily transport	Tp	C57BL/6	106
FTN_1617	*qseC*	Sensor histidine kinase	Tp	C57BL/6	213
FTN_1633	*apt*	Adenine phosphoribosyltransferase	Tp	C57BL/6	213
FTN_1634	*sucB*	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex	Tp	C57BL/6	213
FTN_1639	*sdhC*	Succinate dehydrogenase, cytochrome b556	Tp	C57BL/6	213
FTN_1645	FTN_1645	AtpC ATP synthase, F1 sector, subunit epsilon	Tp	C57BL/6	106
FTN_1653	FTN_1653	Hypothetical membrane protein	Tp	C57BL/6	213
FTN_1654	FTN_1654	Transport protein	Tp	C57BL/6	213
FTN_1655	*rluC*	Ribosomal large subunit pseudouridine synthase C	Tp	C57BL/6	213
FTN_1656	FTN_1656	ATPase	Tp	C57BL/6	213
FTN_1657	FTN_1657	Major facilitator superfamily transport protein	Tp	C57BL/6	213

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TABLE 2—*Continued*

Locus tag	Name	Function	Method(s)[a]	Cells/animals in which mutant strain is attenuated[b]	Reference(s)
FTN_1658	*hisS*	Histidyl-tRNA synthetase	Tp	C57BL/6	213
FTN_1659	*rbfA*	Ribosome binding factor A	Tp	C57BL/6	213
FTN_1665	FTN_1665	Magnesium chelatase	Tp	C57BL/6	106
FTN_1673	*nuoH*	NADPH dehydrogenase I, H subunit	Tp	C57BL/6	106
FTN_1682	*figA (fslA)*	Siderophore biosynthesis protein	Ar/In	J774A.1	47, 213
FTN_1683	FTN_1683	Conserved membrane protein	Tp	C57BL/6	213
FTN_1684	*lysA*	Diaminopimelate decarboxylase	Tp	C57BL/6	213
FTN_1686	*figE*	Hypothetical membrane protein involved in siderophore uptake	Ar	BALB/c	132
FTN_1699	*purL*	Phosphoribosylformylglycinamide synthase	Tp	J774A.1, RAW, BMDM, BALB/c	199, 213
FTN_1700	*purF*	Amidophosphoribosyltransferase	Ar/In	J774A.1, BALB/c	158, 213
FTN_1715	*kdpD*	Two-component sensor protein KdpD	Tp	C57BL/6	213
FTN_1726	FTN_1726	Pyridoxal-dependent decarboxylase	Tp	C57BL/6	106
FTN_1731	*pip*	Proline iminopeptidase	Tp	C57BL/6	106
FTN_1743	*clpB*	ClpB protein	Tp	RAW	199, 213
FTN_1744	FTN_1744	Chitinase	Tp	C57BL/6	213
FTN_1745	*purT*	Phosphoribosylglycinamide formyltransferase 2	Tp	C57BL/6	213
FTN_1753	FTN_1753	Rieske (2Fe-2S) domain protein	Tp	C57BL/6	106
FTN_1756	*bcp*	Bacterioferritin comigratory protein	Tp	C57BL/6	106
FTN_1760	FTN_1760	Zinc binding alcohol dehydrogenase	Tp	C57BL/6	106
FTN_1778	*trpE*	Anthranilate synthase component I	Tp	C57BL/6	106

[a] Ar, allelic replacement; In, insertion; Tp, transposon insertion.
[b] BMDM, bone marrow-derived macrophages; PM, peritoneal macrophages; CE, chicken embryos; hMDMs, human monocyte-derived macrophages; RAW, RAW264.7 murine macrophages.
[c] Attenuated phenotype more pronounced with accumulated mutations.

tularensis is both complex and multifaceted. Further characterization of these genes will be necessary to fully understand *F. tularensis* infection and the type of immunity required to control infection.

Surface structures. (i) Capsule. *F. tularensis* is thought to encode a carbohydrate-rich capsule that may assist the bacterium in mediating resistance to various environmental and host-generated stresses. Capsule-negative mutants were first reported for *F. tularensis* LVS following mutagenesis with acridine orange (166). Mutants exhibited a rough rather than smooth colony phenotype on agar medium and failed to produce an electron-dense surface layer, which was readily observable on the wild-type parent by electron microscopy (166). Rough mutants of LVS were found to be more sensitive to killing by serum complement, and they exhibited a diminished capacity to cause disease in mice (166). Consistent with the potential importance of capsule production in virulence, removal of capsule-like material by exposure to hypertonic solutions of sodium chloride was also found to attenuate virulence of *F. tularensis* following infection of guinea pigs (89). A locus in *Francisella* (*capBCA*) containing genes with similarity to determinants encoding the poly-γ-D-glutamic capsule in *Bacillus anthracis* has recently been described (191). This locus has been shown to be essential for virulence of *F. tularensis* strains LVS and U112 in a murine model of tularemia using two independent negative-selection screens (191, 213). However, definitive demonstration that these determinants encode components of the capsule has not yet been realized.

(ii) LPS. LPS is a major structural component of most gram-negative bacteria and is a predominant eptiope recognized by components of the innate immune system. LPS is comprised of a hydrophobic lipid A portion that anchors the structure to the outer membrane, an oligosaccharide core, and an O-polysaccharide chain that specifies antigenicity. Compared to other gram-negative bacteria such as *E. coli*, the LPS produced by *F. tularensis*, particularly by the more virulent subspecies including *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*, elicits relatively low immunobiological activity (5, 167). While the exact reasons for this are unclear, the lipid A moiety of *F. tularensis* differs from that present in most other organisms (131, 150, 206). In particular, lipid A from *Francisella* lacks the 4′-phosphate moiety commonly found on the nonreducing glucosamine dimer in *E. coli* LPS and is hypoacylated compared to other LPS species. Wang et al. have shown that the 4′-phosphate is removed postproduction by the lipid A 4′-phosphatase LpxF (208). Deletion of *lpxF* leads to decreased virulence following intradermal inoculation in the footpads of mice (209). Interestingly, the deletion of *lpxF* also leads to retention of the 3′-*O*-acyl chain on the core glucosamine, possibly further contributing to increased immunogenicity (209). Several studies have established the importance of LPS in the virulence of *F. tularensis*. "Rough" LPS phase derivatives of type A strain Schu S4 (i.e., the blue-to-gray color variants observed under obliquely transmitted light and first described by Eigelsbach in 1951) are less virulent and less immunogenic in mice than their "smooth" wild-type counterparts (52). A similar pattern of reduced virulence and immunogenicity in mice and guinea pigs is also observed with gray variants of *F. tularensis* LVS (53). More recent studies have revisited the blue-to-gray phase variation in *F. tularensis* and the importance of LPS in resistance to bactericidal factors made by host cells (7, 31, 44, 56, 88). Finally, other studies have described LPS biosynthetic mutants and shown them to be attenuated for virulence in vitro and/or in vivo (114, 124, 155, 191, 213).

(iii) Type IV pili. Pili are complex filamentous bacterial surface structures that have been shown to play an important role in numerous physiological processes in bacteria, including association of organisms with host cell surfaces and protein secretion (153). Pili are comprised of a main structural pilus shaft that contains a terminal adhesin that promotes interactions with specific surface-exposed host cell determinants.

TABLE 3. *F. tularensis* subsp. *holarctica* genes involved in pathogenesis

Locus tag	Name	Function	Method(s)[a]	Cells/animals in which mutant strain is attenuated[b]	Reference(s)
FTL_0009	FTT1747	Outer membrane protein	STM	BALB/c	191
FTL_0010	*glpE*	Thiosulfate sulfurtransferase	STM	BALB/c	191
FTL_0012	*recA*	Recombinase A protein	STM	BALB/c	191
FTL_0028-FTL_0030	*carA*, *carB*, *pyrB*	Uracil biosynthesis	Tp	MDM	179
FTL_0050	FTT1645	Hypothetical protein	Tp	J774A.1, BALB/c	124
FTL_0058	FTT1688	Aromatic amino acid transporter of the hydroxy/aromatic amino acid permease family	Tp	J774A.1	124
FTL_0073	FTT1676	Membrane protein	STM	BALB/c	191
FTL_0094	*clpB*	Caseinolytic protease	Ar, Tp, STM	BMM, J774A.1[c], BALB/c	124, 129, 191
FTL_0111	*iglA*	Intracellular growth	STM	BALB/c	191
FTL_0112	*iglB*	Intracellular growth	STM	BALB/c	191
FTL_0113	*iglC*	Intracellular growth	STM	BALB/c	191
FTL_0113, FTL_1159	*iglC1*, *iglC2*	Intracellular growth locus C	Ar	J774A.1, PM, PEC, BALB/c	82, 117
FTL_0133	*feoB*	Ferrous iron transport protein	STM	BALB/c	191
FTL_0193	*cyoC*	Cytochrome *o*-ubiquinol oxidase subunit III	STM	BALB/c	191
FTL_0257	*rpmJ*	50S ribosomal protein L36	STM	BALB/c	191
FTL_0304	FTT1490	Na^+/H^+ antiporter	Tp	J774A.1	124
FTL_0337	FTT0843	Unknown	STM	BALB/c	191
FTL_0382	*rocE*	Amino acid permease	STM	BALB/c	191
FTL_0387	*aspC1*	Aspartate aminotransferase	STM	BALB/c	191
FTL_0395-FTL_0396	*purMCD*	Purine biosynthesis	Ar/In, Ar	J774A.1, THP-1, PM, A549, BALB/c	145, 146
FTL_0421	*tul4*	Lipoprotein, T-cell-stimulating antigen	STM	BALB/c	191
FTL_0428	*parB*	Chromosome partition protein B	STM	BALB/c	191
FTL_0430	FTT0910	Unknown	STM	BALB/c	191
FTL_0439	FTT0919	Hypothetical outer membrane protein	Tp	J774A.1, BALB/c	124
FTL_0439	FTT0918	Unknown	STM	BALB/c	191
FTL_0440	FTT0920	Transposase	STM	BALB/c	191
FTL_0456	*rpsU1*	30S ribosomal protein S21	STM	BALB/c	191
FTL_0483	*glgB*	Glycogen branching enzyme, GlgB; polysaccharide metabolism	Tp, STM	J774A.1, BALB/c	124, 191
FTL_0485	*glgC*	Glucose-1-phosphate adenylyltransferase	STM	BALB/c	191
FTL_0514	FTT1611	Unknown	STM	BALB/c	191
FTL_0519	*minD*	Division inhibitor ATPase	STM	BALB/c	191
FTL_0520	*minC*	Septum site-determining protein	STM	BALB/c	191
FTL_0525	*fumA*	Fumarate hydratase	STM	BALB/c	191
FTL_0540	*lpxB*	Lipid A-disaccharide synthase	STM	BALB/c	191
FTL_0544	FTT1564	Hypothetical protein	Tp	J774A.1, BALB/c	191
FTL_0552	*pmrA*	Two-component response regulator, orphan	Ar/In	J774A.1, PM, MH-S, BALB/c, C57BL/6	165
FTL_0584	*fadB*	Acyl coenzyme A binding protein	STM	BALB/c	191
FTL_0589	FTT1525c	Hypothetical membrane protein	STM	BALB/c	191
FTL_0592	*wbtA*	dTDP-glucose 4,6-dehydratase	Ar, Tp	J774A.1, BALB/c, BALB/cByJ	124, 160, 180, 191
FTL_0594	*wbtC*	UDP-glucose-4-epimerase	Tp	J774A.1	124
FTL_0597	*wbtF*	NAD-dependent epimerase, LPS modification	STM	BALB/c	191
FTL_0601	*wbtI*	Sugar transaminase/perosamine synthetase	Pm	BALB/c	115
FTL_0606	*wbtM*	dTDP-glucose 4,6-dehydratase	Tp	J774A.1	124
FTL_0616	*rpoA2*	DNA-directed RNA polymerase, subunit	STM	BALB/c	191
FTL_0617	*bfr*	Bacterioferritin	STM	BALB/c	191
FTL_0645	FTT1416c	Lipoprotein	STM	BALB/c	191
FTL_0663	FTT1400c	Unknown	STM	BALB/c	191
FTL_0706	FTT1238c	Hypothetical membrane protein	Tp	J774A.1, BALB/c	124
FTL_0723	FTT1221	Unknown	STM	BALB/c	191
FTL_0766	*ggt*	Gamma-glutamyltranspeptidase	Tp	J774A.1, RAW, THP-1, BMM, BALB/c	4, 124
FTL_0768	*bipA*	GTP-binding translational elongation factor Tu and G family protein	STM	BALB/c	191

Continued on following page

TABLE 3—*Continued*

Locus tag	Name	Function	Method(s)[a]	Cells/animals in which mutant strain is attenuated[b]	Reference(s)
FTL_0789	*aspC2*	Aspartate aminotransferase, amino acid biosynthesis	Tp, STM	J774A.1, BALB/c	124, 191
FTL_0803	FTT1152	Unknown	STM	BALB/c	191
FTL_0837	*metIQ*	D-Methionine transport protein (ABC transporter)	Tp, STM	J774A.1, BALB/c	124, 191
FTL_0838	*metN*	D-Methionine transport protein (ABC transporter)	Tp	J774A.1	124
FTL_0846	FTT1117c	Isochorismatase hydrolase family protein	STM	BALB/c	191
FTL_0878	FTT0610	DNA/RNA endonuclease family	Tp	J774A.1	124
FTL_0886	FTT0618c	Conserved hypothetical protein YleA	Tp	J774A.1, BALB/c	124
FTL_0891	*tig*	Molecular chaperone	STM	BALB/c	191
FTL_0892	*clpP*	ATP-dependent Clp protease subunit P	STM	BALB/c	191
FTL_0893	*clpX*	ATP-dependent Clp protease subunit X	STM	BALB/c	191
FTL_0894	*lon*	ATP-dependent protease Lon	STM	BALB/c	191
FTL_0899	*hflX*	Protease, GTP binding subunit	STM	BALB/c	191
FTL_0903	*hflK*	Protease modulator	STM	BALB/c	191
FTL_0928	*elbB*	DJ-1/PfpI family protein	STM	BALB/c	191
FTL_0950	*rplY*	50S ribosomal protein L25	STM	BALB/c	191
FTL_0960	*sthA*	Soluble pyridine nucleotide transhydrogenase	STM	BALB/c	191
FTL_1029	*pilF*	Type IV pilus lipoprotein	Ar	C3H/HeN	25
FTL_1030	*rluB*	Ribosomal large subunit pseudouridine synthase B	STM	BALB/c	191
FTL_1071, FTL_1478	*guaA*, *guaB*	Guanine biosynthesis	Ar/In	J774, BALB/c	170
FTL_1075	FTT1015	Unknown	STM	BALB/c	191
FTL_1096	FTT1103	Hypothetical lipoprotein	Tp	J774A.1, BALB/c	124, 191
FTL_1096	FTT1103	Lipoprotein	STM	BALB/c	191
FTL_1097	FTT1102	Macrophage infectivity potentiator	STM	BALB/c	191
FTL_1134	NA	Membrane protein	STM	BALB/c	191
FTL_1158, FTL_0112	*iglB*	Intracellular growth locus B	Ar	J774A.1	19
FTL_1225	FTT0975	Unknown	STM	BALB/c	191
FTL_1233	FTT0968c	Amino acid antiporter	STM	BALB/c	191
FTL_1240	*aroG*	Phospho-2-dehydro-3-deoxyheptonate aldolase	STM	BALB/c	191
FTL_1262	FTT0945	Chorismate family binding protein, aromatic amino acid and folate biosynthesis	Tp	J774A.1	124
FTL_1266	*lipP*	Lipase/esterase	STM	BALB/c	191
FTL_1273	*bioF*	8-Amino-7-oxononanoate synthase	STM	BALB/c	191
FTL_1274	*bioC*	Biotin synthesis	STM	BALB/c	191
FTL_1275	*bioD*	Dethiobiotin synthetase	STM	BALB/c	191
FTL_1328	*fopA*	Outer membrane-associated protein	STM	BALB/c	191
FTL_1354	FTT0759	Membrane protein	STM	BALB/c	191
FTL_1392	*deaD*	Cold shock DEAD box protein A	STM	BALB/c	191
FTL_1393	*ppiC*	Peptidyl-prolyl *cis-trans* isomerase or parvulin	STM	BALB/c	191
FTL_1404	*rplT*	50S ribosomal protein L20	STM	BALB/c	191
FTL_1414	*capA*	Transmembrane HSP60 family protein	STM	BALB/c	191
FTL_1414-FTL_1416	*capACB*	Capsule biosynthesis	Ar, Tp	J774A.1, BALB/c	124, 191
FTL_1415	*capC*	Capsular polyglutamate biosynthesis protein CapC	STM	BALB/c	191
FTL_1416	*capB*	Capsular polyglutamate biosynthesis protein CapB	STM	BALB/c	191
FTL_1419	*cphB*	Cyanophycinase	STM	BALB/c	191
FTL_1452	*rpmA*	50S ribosomal protein L27	STM	BALB/c	191
FTL_1458	*secA*	Preprotein translocase, subunit A	STM	BALB/c	191
FTL_1461	*deoD*	Purine nucleoside phosphorylase	STM	BALB/c	191
FTL_1473	*uvrA*	DNA excision repair enzyme, subunit A	STM	BALB/c	191
FTL_1474	*greA*	Transcription elongation factor	STM	BALB/c	191
FTL_1475	FTT1314c	Type IV pilus fiber building block protein	STM	BALB/c	191

Continued on following page

TABLE 3—*Continued*

Locus tag	Name	Function	Method(s)[a]	Cells/animals in which mutant strain is attenuated[b]	Reference(s)
FTL_1504	*katG*	Catalase	STM	BALB/c	191
FTL_1528	FTT0708	Major facilitator superfamily transport protein	STM	BALB/c	191
FTL_1542	*migR*	Transcriptional regulator	Ar	MDM	22
FTL_1553	*sucC*	Succinyl coenzyme A synthetase beta chain	STM	BALB/c	191
FTL_1554	*sucD*	Succinyl coenzyme A synthetase alpha chain	STM	BALB/c	191
FTL_1581	*tivA*	Hypothetical lipoprotein	Ar/In	CE, MDM	93
FTL_1583	*xasA*	Glutamate-aminobutyric acid antiporter, XasA; amino acid transport	Tp	J774A.1	124
FTL_1601	*yibK*	tRNA/rRNA methyltransferase	STM	BALB/c	191
FTL_1622	FTT0444	Multidrug transporter	STM	BALB/c	191
FTL_1623	FTT0443	Unknown	STM	BALB/c	191
FTL_1664	*deoB*	Phosphopentomutase	Ar/In	CE, MDM, DC, HEK-293	93
FTL_1670	*dsbB*	Disulfide bond formation protein, DsbB	Tp	J774A.1	124
FTL_1672	*acrB*	RND efflux pump	In, STM	BALB/c	15, 191
FTL_1678	FTT0101	Membrane protein	STM	BALB/c	191
FTL_1701	*gplX*	Fructose-1,6-bisphosphatase	Tp, STM	J774A.1, BALB/c	124, 191
FTL_1750	*secE*	Preprotein translocase, subunit E	STM	BALB/c	191
FTL_1771	*pilT*	Twitching motility protein PilT	Tp	C3H/HeN	25
FTL_1793	*sodB*	Fe-superoxide dismutase	Ar	BALB/c, C57BL/6, MH-S	9
FTL_1806	FTT0053	Major facilitator superfamily transporter	Tp	J774A.1	124
FTL_1832	FTT0029c	Unknown	STM	BALB/c	191
FTL_1865	*tolC*	Glutamate decarboxylase	Ar	C3H/HeN	77
FTL_1867	*yegQ*	Protease	STM	BALB/c	191
FTL_1912	*rpsA*	30S ribosomal protein S1	STM	BALB/c	191
FTL_1914	*ripA*	Hypothetical protein	Ar	J774A.1, TC-1, C57BL/6	72
FTL_1936	FTT0209c	Periplasmic solute binding family protein	STM	BALB/c	191
FTL_1947	*yjjk*	ABC transporter ATP binding protein	STM	BALB/c	191
FTL_R0003	16S rRNA	16S rRNA	STM	BALB/c	191
FTL_R0004	tRNA-Ile	tRNA-isoleucine	STM	BALB/c	191
FTT0890	*pilA*	Type IV pilus fiber building block protein	Recomb.	C57BL/6	65

[a] Ar, allelic replacement; In, insertion; Tp, transposon insertion; STM, signature-tagged mutagenesis; Recomb., direct repeat-mediated deletion.
[b] MDM, monocyte-derived macrophages; PM, peritoneal macrophages; PEC, peritoneal exudate cells; CE, chicken embryos; DC, dendritic cells.
[c] Intermediate attenuation phenotype.

Analysis of genomic sequences from various *Francisella* strains indicates that these organisms contain clusters of genes that share homology with type IV pilus determinants in other species, including *Neisseria meningitidis* and *Pseudomonas aeruginosa* (64, 77, 109, 223). Pili have been observed on the surface of several *Francisella* species following growth in laboratory medium (77, 223). Importantly, disruption of genes predicted to encode pilus biosynthetic factors abolishes expression of these structures on the bacterial surface of *Francisella* (25, 65). Interestingly, a subset of pilus-like genes does not appear to be directly involved in pilus production but rather is required for protein secretion (86, 223). Expression of pilus biosynthetic genes from *Francisella* has also been shown to functionally complement pilus-negative strains of *Neisseria gonorrhoeae* with deletions in the analogous determinant (164). Several lines of evidence indicate that production of pili or expression of pilus-like genes is important for virulence by *Francisella*. Pilus-like genes are regulated by MglA (223), a well-established virulence determinant of *F. tularensis*. Disruption of specific pilus-like genes also attenuates the ability of *Francisella* to adhere to various host cell types (25) or grow intracellularly (223). Finally, mutations in pilus-like genes alter virulence of *Francisella* in a mouse model of tularemia (25, 65, 86, 223).

The FPI. An FPI of approximately 30 kb is present in duplicate in virulent *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* and in single copy in *F. novicida*. The FPI is comprised of roughly 17 open reading frames (137), a majority of which have been shown to be required for aspects of *F. tularensis* pathogenesis. It is currently thought that several FPI genes encode components of a secretion system, similar to the type VI system recently described in other organisms (46, 122). Genes present in the FPI are arranged into two major transcriptional units (138) and are induced during intracellular growth of *F. tularensis* (29, 111, 212). FPI genes have also been shown to be regulated by other environmental conditions, including iron (22, 23, 47, 105, 113, 177) and hydrogen peroxide (80), and by several regulatory factors, including MglAB (21, 46, 85, 111, 177), SspA (26), PmrA (134, 165), FevR (20), and

TABLE 4. *F. tularensis* subsp. *tularensis* genes involved in pathogenesis

Locus tag	Name	Function	Method(s)[a]	Cells/animals in which mutant strain is attenuated[b]	Reference(s)
FTT0026c	*fslE*	Siderophore uptake	Tp, Ar	BALB/c	100, 159
FTT0056c	FTT0056c	Major facilitator superfamily transport protein	Tp	Hep G2	155
FTT0069c	FTT0069	Unannotated	Tp	BALB/c	100
FTT0107c	*dsbB*	Transposon mutant	Tp, Ar	C57BL/6	157
FTT0107c	*dsbB*	Disulfide bond formation protein	Tp	Hep G2, J774A.1	155
FTT0118	*prfC*	Peptide chain release factor 3	Tp	BALB/c	100
FTT0129	FTT0129	Major facilitator superfamily transport protein	Tp	Hep G2	155
FTT0132	*glpA*	Glycerol-3-phosphate dehydrogenase	Tp	BALB/c	100
FTT0141	*rplA*	50S ribosomal protein L1	Tp	BALB/c	100
FTT0156	FTT0156	Acid phosphatase	Tp	BALB/c	100
FTT0203c	*purH*	Bifunctional purine biosynthesis protein	Tp	Hep G2	155
FTT0204	*purA*	Adenylosuccinate synthetase	Tp	Hep G2	155
FTT0245	*usp*	Universal stress protein	Tp	BALB/c	100
FTT0250	*ppdK*	Phosphoenolpyruvate synthase/pyruvate phosphate dikinase	Tp	BALB/c	100
FTT0290	*moxR*	MoxR-like ATPase	Tp	BALB/c	100
FTT0425	*asd*	Aspartate semialdehyde dehydrogenase	Tp	BALB/c	100
FTT0435	*ctv*	Carbon-nitrogen hydrolase	Ar	BMDM, MH-S, BALB/c	123
FTT0444	*tet*	Drug:H^+ antiporter-1 (DNA1) family protein	Tp	BALB/c	100
FTT0486	*mutL*	DNA mismatch repair enzyme with ATPase activity	Tp	BALB/c	100
FTT0588	*aroA*	3-Phosphoshikimate I-carboxyvinyl transferase	Tp	Hep G2	155
FTT0609	FTT0609	Unannotated	Tp	BALB/c	100
FTT0623	*tig*	Trigger factor protein (peptidyl-prolyl *cis*/*trans* isomerase), chaperone	Tp	BALB/c	100
FTT0626	*lon*	DNA binding, ATP-dependent protease LA	Tp	BALB/c	100
FTT0654	*elbB*	DJ-1/PfpI family protein	Tp	BALB/c	100
FTT0687c	*hslU*	ATP-dependent protease HslVU, ATPase subunit	Tp	BALB/c	100
FTT0862c	*htpX*	Zn-dependent protease with chaperone function	Tp	BALB/c	100
FTT0876c	*aroC*	Chorismate synthase	Tp	BALB/c	100
FTT0891	FTT0891	Conserved hypothetical membrane protein	Tp	BALB/c	100
FTT0893-FTT0894	*purMCD*	Purine biosynthesis	Ar	MDM, J774A.1, A549, BALB/c	146
FTT0918	FTT0918	Hypothetical protein	Ar	BALB/c	204
FTT1087c	*rep*	UvrD/REP superfamily I DNA and RNA helicases	Tp	BALB/c	100
FTT1103	FTT1103	Conserved hypothetical lipoprotein	Tp/Ar	Hep G2, BALB/c, C57BL/6	155, 157
FTT1120c	*tgt*	Queuine tRNA-ribosyltransferase	Tp	BALB/c	100
FTT1130c	*cphA*	Cyanophycin synthetase	Tp	BALB/c	100
FTT1158c	*pilO*	Type IV pilus glycosylation protein	Tp	BALB/c	100
FTT1179	*bipA*	GTP binding translational elongation, factor Tu and G family protein	Tp	BALB/c	100
FTT11802c	*trpE*	Anthranilate synthase component I	Tp	BALB/c	100
FTT1181c	*ggt*	Gamma-glutamyltranspeptidase	Ar, Tp	BALB/c, HepG2	100, 155
FTT1234	FTT1234	Choloylglycine hydrolase family protein	Tp	Hep G2	155
FTT1236	FTT1236	Hypothetical protein	Tp	Hep G2, J774A.1	155
FTT1244c	*yfiO*	Conserved hypothetical lipoprotein	Tp	Hep G2	155
FTT1278c	*rnhB*	RNase HII	Tp	BALB/c	100
FTT1310c	*hflB*	ATP-dependent metalloprotease	Tp	Hep G2	155
FTT1312c	*uvrA*	DNA excision repair enzyme, subunit A	Tp	BALB/c	100
FTT1356, FTT1711	*idlD*	Intracellular growth locus D	Ar	BALB/c	100
FTT1357c, FTT1712c	*iglC2*, *iglC1*	Intracellular growth locus C	Ar	BALB/c	204
FTT1358, FTT1713	*iglB*	Intracellular growth locus B	Ar	BALB/c	100
FTT1455	*wbtI*	Sugar transamine/perosamine synthetase	Ar	BALB/c	100
FTT1455	*wbtI*	LPS O-antigen synthesis	Tp	BALB/c	100
FTT1459c-FTN1461c	*wbtDEF*	LPS O-antigen biosynthetic cluster	Ar/In	J774A.1, BALB/c	200
FTT1490	FTT1490	Na^+/H^+ antiporter	Tp	Hep G2	155
FTT1542c	*omp26*	Protein of unknown function	Tp	BALB/c	100
FTT1561	*kdtA*	3-Deoxy-D-*manno*-octulosonic-acid transferase	Ar	BALB/c	100
FTT1631	*glpX*	Fructose 1,6-bisphosphatase II	Ar	BALB/c	100
FTT1640c	FTT1640	Activator of osmoprotectant transporter ProP, fragment	Tp	BALB/c	100
FTT1663	*carA*	Carbamoylphosphate synthase small chain	Tp	Hep G2	155
FTT1664	*carB*	Carbamoylphosphate synthase large chain	Tp	Hep G2	155
FTT1665	*pyrB*	Aspartate carbamoyltransferase	Tp	Hep G2	155
FTT1720c	*purL*	Phosphoribosylformylglycinamide synthase	Tp	BALB/c, Hep G2	100, 155
FTT1721	*purF*	Amidophosphoribosyltransferase	Tp	BALB/c, Hep G2	100, 155
FTT1724c	*tolC*	Outer membrane efflux protein	Tp	BALB/c	100
FTT1744c	*ipdC*	Indolepyruvate decarboxylase	Tp	BALB/c	100
FTT1762c	FTT1762c	Acetyltransferase protein	Tp	Hep G2	155
FTT1773c	*trpB*	Tryptophan synthase beta chain	Tp	BALB/c	100
FTT1782c	*yjjK*	ABC transporter ATP binding protein	Tp	BALB/c	100

[a] Ar, allelic replacement; In, insertion; Tp, transposon insertion.
[b] BMDM, bone marrow-derived macrophages; MDM, moncyte-derived macrophages; HepG2, human hepatocellularcarcinoma cells; MH-S, murine alveolar macrophages.

MigR (22). One major transcriptional unit encodes genes of the *i*ntracellular *g*rowth *l*ocus (i.e., *iglABCD*). Mutation within any of these genes attenuates growth of *Francisella* in macrophages, in insect cells, and/or in mice (78, 83, 100, 110, 117, 161, 173, 207). IglA and IglB are interacting cytoplasmic proteins and have been predicted to be involved in effector protein secretion (46). *iglC* encodes a 23-kDa hypothetical protein. Although the exact function of this protein has not yet been defined, *iglC* mutants generated in *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, and *F. novicida* are highly attenuated for growth in vitro and in vivo (12, 78, 83, 110, 111, 117, 161, 173, 191, 198, 204, 213). In particular, IglC is required

for the inhibition of phagosomal maturation and/or escape from the host phagosome by *F. novicida* (173). Finally, *iglD* has been shown to be essential for intracellular replication of *Francisella* in human monocyte-derived macrophages as well as for intracellular replication in mice (29, 100, 171).

pdpA and *pdpB* represent the first two genes in the second major transcriptional unit located within the FPI. *pdpA* and *pdpB* encode hypothetical proteins that are required for virulence of *F. novicida* in insect cells, in chicken embryos, and/or in mice (21, 138, 161, 176, 199, 213). While little is known regarding the role of PdpB, mutants with deletions in *pdpA* localize with markers of lysosomes following infection into host cells (176). This phenotype is reminiscent of that observed with *iglC* mutants, suggesting that PdpA may be required for phagosomal maturation modification and/or escape of *F. tularensis* from the phagosome. *Francisella* PdpA contains a motif in its N-terminal region that is characteristic of proteins that interact with components of the eukaryotic ubiquitin-proteosome pathway (176). However, this protein does not contain an identifiable secretion signal, it localizes to the cytoplasm in *F. novicida*, and it has not been detected inside host cells following infection by the bacterium (176). Thus, the roles of PdpA and PdpB and their localization during an infection currently remain undefined.

Transcriptional regulators. (i) MglAB. MglAB (*m*acrophage *g*rowth *l*ocus) was first identified in a screen for suppressors of acid phosphatase expression in *F. novicida* (12). *mglA* and *mglB* are organized in an operon and encode proteins exhibiting homology to SspA and SspB of *E. coli* and *Haemophilus* spp. (12). SspA is an RNA polymerase-associated protein in *E. coli*, and it has been shown to regulate expression of numerous genes in response to stress (95, 218). Mutations in either *mglA* or *mglB* attenuate growth of *F. tularensis* in macrophages (12), in mosquito cells (161), in amoebas (12, 111), in fruit flies (207), and in mice (12, 111). Microarray comparisons between wild-type strains and *mglA* mutants indicate that MglA functions as a global transcription factor in *F. tularensis*, regulating expression of more than 100 genes, including those within and outside of the FPI (21). Of note, disruption of *mglA* attenuates the ability of *Francisella* to inhibit phagosome maturation and escape into the host cell cytosol, likely due to regulation of genes, including *iglABCD*.

(ii) SspA. SspA is a transcription factor in the *Francisella* genome that exhibits high homology to MglA. In *F. tularensis* LVS, both SspA and MglA associate with RNA polymerase (26). Furthermore, SspA and MglA directly interact, and association of MglA with RNA polymerase requires SspA (26). Targeted gene and global gene expression studies carried out using DNA microarrays indicate that SspA and MglA coregulate the same gene set in *F. tularensis*, providing further evidence that these proteins likely work together in combination with RNA polymerase to regulate gene expression (26).

(iii) PmrA. *pmrA* encodes an orphaned response regulator that shares homology with the *Salmonella enterica* serovar Typhimurium response regulator PmrA (134). Response regulators (and their cognate histidine sensor kinases) function to regulate gene expression through phosphorylation-based signal transduction cascades initiated following exposure of the histidine kinase to extracytoplasmic signals. In general, *F. tularensis* encodes a paucity of two-component signal transduction systems and other transcription factors relative to other organisms of similar genome size. The consequences of *pmrA* disruption have been examined in both *F. novicida* and in *F. tularensis* LVS (134, 165). Mutants generated in either strain background are attenuated for virulence in macrophages and in mice. In addition, *pmrA* mutants of *F. novicida* are more sensitive to antimicrobial peptides than their wild-type counterparts (134). DNA microarray studies carried out with these mutants indicate that PmrA regulates a large group of genes, including those contained within the FPI (134, 165). However, PmrA does not appear to regulate MglA or vice versa (134).

(iv) FevR. *fevR* (*Francisella* effector of *v*irulence *r*egulation) encodes a hypothetical protein and was identified in a screen of genes requiring MglA/SspA for their expression (20). FevR is required for replication of *F. novicida* and *F. tularensis* LVS in murine and human macrophages (20, 22), in the human epithelial cell lines HEp-2 and A549 (22), and in mice (20). While the mechanism(s) of FevR's activity remains unclear, *fevR* mutants of *F. tularensis* LVS remain trapped within a phagosome that retains late endosomal marker LAMP-1 but not lysosomal marker cathepsin D (22). *fevR* mutants are also compromised in their ability to block NADPH oxidase activity following infection into neutrophils (22). Based on these and other observations not discussed here, FevR has been postulated to act in parallel with MglA/SspA in a feed-forward loop to positively regulate virulence factors essential for *Francisella* pathogenesis, including genes within the FPI (20). *fevR* expression is also positively regulated by response regulator PmrA (134). Thus, expression of this regulatory determinant is complex and is mediated by multiple input signals.

(v) MigR. *migR* (*m*acrophage *i*ntracellular *g*rowth *r*egulator) is a newly identified regulator of virulence gene expression in *F. tularensis* LVS (22). Isolated in a transposon mutagenesis screen for transcriptional activators of *iglB*, *migR* encodes a hypothetical protein containing a predicted AMP binding domain (22). *F. tularensis* LVS *migR* mutants are attenuated for growth in human monocyte-derived macrophages relative to their wild-type counterparts; however, they exhibit no growth differences following infection into human epithelial cell lines, including HEp-2 and A549 pneumocytes (22). In addition to its ability to regulate the *igl* locus in LVS, MigR is also a positive regulator of transcription factor *fevR* (22). However, it remains unclear whether this regulation is direct or indirect or whether the observed regulation on *igl* expression occurs indirectly through FevR.

Additional virulence factors. Apart from the genetic determinants described above, a number of other virulence factors involved in *Francisella* pathogenesis have also been identified (Tables 2, 3, and 4). While a comprehensive discussion of these genes is beyond the scope of this review, it is possible to make several general observations regarding genes contained within this list and phenotypes of selected mutants carrying disruptions within these genes. First, advancements in genetic tools and procedures for doing allelic replacement and transposon mutagenesis have greatly expanded the number of mutants that have been generated from type A strains of *F. tularensis*. While the first report of a nonspontaneous mutant of a type A strain did not occur until 2005 (204), nearly 70 Schu S4 derivatives carrying targeted or randomly generated transposon insertions have been reported to date. Second, a large percentage of the genes identified in screens for virulence factors in the various *F. tularensis* subspecies encode hypothetical proteins with unknown function. This suggests that

TABLE 5. *Francisella* subunit vaccine candidates

Antigen	Adjuvant	Dose, μg (route)[a]	Challenge strain	Boost	Challenge route (dose, CFU)[b]	Host mouse strain	Protection	Reference
Ethanol-inactivated LVS	Freund's adjuvant	20 (i.p.)	Schu S4	No	i.n. (40)	C3H/HeN	Yes	94
LPS	Freund's adjuvant	20 (i.p.)	Schu S4	No	i.n. (40)	C3H/HeN	No	94
Outer membrane proteins	Freund's adjuvant	20 (i.p.)	Schu S4	No	i.n. (40)	C3H/HeN	Yes	94
LPS	Freund's adjuvant	50 (s.c.)	LVS	Yes	i.p. (2.5×10^6)	BALB/c	Yes	73
OMP	Freund's adjuvant	100	LVS	Yes	i.p. (2.5×10^6)	BALB/c	Yes	73
LPS	Bovine serum albumin	20 (s.c.)	Type B no. 108	No	i.d. (80)	BALB/c	Yes	42
					Aerosol (30)	BALB/c	No	42
			Type A no. 33	No	i.d. (60)	BALB/c	No	42
					Aerosol (50)	BALB/c	No	42

[a] i.p., intraperitoneal; s.c., subcutaneous.
[b] i.d., intradermal; i.n., intranasal.

many of the mechanisms utilized by this organism to infect, be maintained, and/or cause disease within its host are likely to be novel. Third, there is a high correlation between the requirement of a given gene for intracellular growth and/or survival and its requirement for *F. tularensis* to cause disease in vivo. While there have been exceptions to this rule, the ability of *F. tularensis* to infect cells, alter phagosome trafficking, egress into the host cell cytosol, and undergo extensive multiplication is an essential part of the overall life cycle of this organism. Finally, phenotypes observed following the disruption of a given gene in one *Francisella* species or subspecies do not necessarily correlate with the phenotype observed in another strain. This characteristic will be particularly important as more and more mutant strains are generated and target genes characterized for their potential utilization as vaccine targets.

DEVELOPING A *FRANCISELLA* VACCINE

Early Studies

Foshay vaccine. As the potential for weaponization of *F. tularensis* has been realized for some time, efforts to develop a *Francisella* vaccine have been ongoing since the 1940s. Early efforts involved preparations of killed whole-cell *F. tularensis*. However, these materials offered limited efficacy in both human and animal studies (67). Administration of such preparations resulted in poor or incomplete protection in most cases and in adverse side effects that ultimately limited utility (205). A vaccine developed by Foshay et al. using acid extraction and preservation in phenol was less reactogenic and was used to vaccinate several thousand volunteers in Ohio between 1933 and 1941 (67). However, due to the limited sample size and inadequate reporting of infection in the general population, the effectiveness of this vaccine could not be directly determined (67). The Foshay preparation was able to protect nonhuman primates from death after challenge with 740 CFU of Schu S4, but in many cases it was unable to prevent disease symptoms (84). Further studies with mice indicated that the Foshay vaccine offered only low levels of protection against highly virulent strains (67, 99).

LVS. The isolation of an LVS in Russia and its subsequent transfer to the United States in the 1950s constituted a significant breakthrough in vaccination against *Francisella*. LVS was developed by multiple passages in vitro and in vivo of a virulent *F. tularensis* subsp. *holarctica* strain (201). This strain was gifted to the United States in 1956 and was approved as an investigational new drug by the Federal Drug Administration (FDA) in the early 1960s (39a). Early evaluation of LVS as a live vaccine was performed in human volunteers by the U.S. Army in the late 1950s (174, 175). LVS was given by scarification for evaluation against subsequent subcutaneous or aerosol challenges with *F. tularensis* type A strain Schu S4. Vaccination with LVS protected against high-dose (1,000 CFU) subcutaneous and low-dose (10 to 100 CFU) aerosol challenge with Schu S4 but did not confer significant protection against aerosol challenge with doses approaching 1,000 CFU (174, 175). Improved efficacy of LVS was observed following vaccination of volunteers via the respiratory route; however, LVS was capable of inducing human tularemia in a subset of volunteers when given at the dose necessary to confer immunity (92). Regardless, LVS was used to vaccinate laboratory workers at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRID), after which the incidence of laboratory-acquired tularemia decreased significantly. It remains unclear whether a part of this decrease may also have resulted from improved laboratory practices (24). Despite its successes, the unknown nature of its attenuation and residual virulence following vaccination by the aerosol route has limited the overall utility of LVS and resulted in its removal from the Investigational New Drug list by the FDA. Consequently, it remains unlicensed for use in the United States. Even so, LVS remains the benchmark to which other *Francisella* vaccine candidates are compared.

Subunit Vaccines

Efforts toward developing subunit vaccines against *F. tularensis* have met with limited success (Table 5). Early attempts to identify protective antigens were based on identification of immunoreactive surface-associated proteins. While these proteins were found to stimulate in vitro responses using T cells from LVS-vaccinated animals or individuals (79, 168, 185, 193), the level of in vivo protective immunity conferred by some of these immunoreactive complexes in mice was below that observed with LVS vaccination (79). Other surface-exposed antigens, including carbohydrates and LPS, have also been investigated as to their potential to stimulate an immunoreactive

and/or immunoprotective response (42, 73, 74, 169, 200). While immunization with LPS has been shown to generate a protective antibody response to challenge with less virulent *Francisella* species or subspecies of *F. tularensis*, it provides only limited protection against high-dose type A challenge (42, 73, 74, 200). A number of specific immunoreactive protein and lipoprotein antigens have also been investigated, including GroEL, KatG, and Tul4. Despite their immunogenicity, evaluations of these and other protein antigens have yet to demonstrate significant protection as subunit vaccine candidates (39a, 73, 79). These results, along with the failure of the Foshay preparation years before, indicate that generation of protective immunity against *F. tularensis* likely requires recognition of multiple antigenic determinants capable of stimulating both humoral and cell-mediated immune responses. Consistent with this observation, administration of an LVS boost following primary vaccination with LPS provides almost complete protection in mice against 1,000 50% lethal doses (LD_{50}s) of type A *F. tularensis*, a level of protection better than that seen with LVS alone (74). More recently, Huntley et al. have demonstrated that vaccination with a mixture of outer membrane preparations from LVS provides partial protection against 40 CFU of type A Schu S4 delivered via the respiratory route (94). Given these recent advances and the reduced risks associated with subunit preparations relative to other vaccination types, development of protective subunit vaccines for *F. tularensis* remains a plausible goal.

Live Vaccines

The utility of live attenuated strains as vaccines has been demonstrated for a number of bacterial pathogens and has resulted in the development of commercially available vaccines for *Salmonella enterica* serovar Typhi (Ty21a), *Vibrio cholerae* (CVD 103-HgR), and *Mycobacterium tuberculosis* (*Mycobacterium bovis* BCG). A live vaccine typically contains one or more defined genetic lesions in pathways that are essential for in vivo growth of the bacterium. Vaccination with these strains is advantageous, as the organism is generally unable to survive for extended periods or at high levels within host cells but remains capable of expressing its repertoire of antigenic determinants. Conversely, an inherent caveat of working with live vaccines is that they must strike an intricate balance between attenuation and stimulation of immunity. Bacterial derivatives that are overattenuated may not prime substantive immunity. On the other hand, derivatives that are able to prime substantive immunity may retain partial virulence, undermining their use as a safe vaccination tool. Despite its limitations, the successes of LVS as a vaccine suggest that developing a rationally attenuated *Francisella* strain is both feasible and warranted.

***F. novicida*.** Many of the studies examining the initial feasibility of defined *F. tularensis* mutants as live attenuated vaccine candidates have been conducted using *F. novicida* as a surrogate organism (Table 6). Mutant derivatives examined have included those carrying gene disruptions in the FPI (*iglC* and *pdpB*), in metabolic pathways (*purA*, *purF*, *carB*, and *fumA*), in transcription factors (*mglA*, and *pmrA*), in acid phosphatases (*acpABCH*), in LPS biosynthesis (*fmlK*), in disulfide bond formation (*dsbB*), and in other, as-yet-uncharacterized processes. While results from these studies indicate that many of the generated mutants are able to prime robust immune responses in mice following vaccination, host responses generated to these mutants, or even to sublethal concentrations of the wild-type *F. novicida* parent, have led to protection only against challenge with the homologous species (Table 6). To date, no *F. novicida* mutants have been shown to confer adequate protective immunity against challenge with virulent type A or type B *F. tularensis* isolates (Table 6) (134, 158, 182). This suggests that despite the high level of genetic similarity between the *Francisella* species, *F. novicida* exhibits significant differences either in immunodominant antigens or in the profile of immunological responses that it elicits relative to the more virulent subspecies. Alternatively, the in vivo lifestyle of *F. novicida* may differ in some as-yet-unapparent aspect from that of its more virulent type A or type B counterparts.

Infection-vaccination studies with specific *F. novicida* mutants have also provided important insights into characteristics that will be necessary for successful *F. tularensis* vaccine development. Mutants unable to escape from the phagosome (i.e., *iglC* and *mglA* mutants) do not function well as live vaccine candidates even though they are extremely attenuated and can be administered at high doses in vivo (Table 6) (142, 214). This suggests that replication within the host cell cytosol is essential for priming a protective immune response against *Francisella*. The variable effectiveness of metabolic mutants as vaccine candidates also indicates that limited intracellular (or extracellular) replication is an important criterion for generating a protective immune response. For example, *F. novicida* mutants disrupted in *purA*, a gene involved in the early steps of purine biosynthesis, are unable to protect against homologous challenge (158). In contrast, protective immunity against homologous challenge is observed following vaccination of mice with mutants defective in a later step of the purine biosynthetic pathway (i.e., *purF*) (158). It is likely that differences in the ability of these mutants to persist in host tissues contribute to this phenotype.

***F. tularensis* subsp. *holarctica*.** While LVS remains problematic as a host for vaccine development for several reasons, its previous success as a live vaccine against type A *F. tularensis* challenge indicates that it may be a more suitable surrogate for evaluating the efficacies of specific genes as vaccine targets. In this regard, a variety of mutants have been generated in the LVS background and examined for their ability to confer protective immunity against challenge with type A or type B strains (Table 6) (10, 62, 129, 145, 146, 165, 170, 180). These have included LVS derivatives mutated in the *clpB* heat shock protein gene, purine biosynthetic genes *purMCD* and *guaAB*, response regulator gene *pmrA*, predicted lipoprotein gene *lpnA*, superoxide dismutase gene *sodB*, and LPS biosynthetic gene *wbtA* (Table 6). In general, results from these studies indicate that vaccination with sublethal doses of wild-type LVS or LVS-derived mutants confer high levels of protective immunity against challenge with wild-type LVS (10, 62, 129, 145, 170). Vaccination with these derivatives also provides variable protection against virulent type A or type B strains when administered by the parenteral route (146, 180) and against relatively low doses of Schu S4 delivered via the respiratory route (10, 146, 165). Not surprisingly, mice vaccinated with LVS mutants are not well protected against respiratory infection with higher doses of type A or type B *F. tularensis* (10, 146), and this level of protection is similar to that observed following sublethal vaccination with wild-type LVS (146).

TABLE 6. *Francisella* live vaccine candidates

Species or subspecies	Locus tag for attenuating mutation	Annotation	Vaccination route[a] (dose, CFU)	Challenge strain	Boost	Challenge route (dose, CFU)	Host mouse strain(s)	Protection	Reference
F. novicida	None (wild type)		i.d. (100)	FSC033	No	Aerosol (10)	BALB/c	No	182
	FTN_1645	*atpC*	i.n. (149)	U112	No	i.n. (25)	C57BL/6	Yes	106
	FTN_0178	*purA*	i.p. (3.3×10^6)	U112	No	i.p. (170)	BALB/c	No	158
	FTN_1700	*purF*	i.p. (6.95×10^3)	U112	No	i.p. (170)	BALB/c	Yes	158
				Schu S4	No	i.p. (100)	BALB/c	No	158
	FTN_1465	*pmrA*	i.n. (10^6)	Schu S4	No	i.n. (100)	BALB/c	No	134
				U112	No	i.n. (10^6)	BALB/c	Yes	134
	FTN_1608	*dsbB*	i.n. (6×10^5)	U112	No	i.n. (6×10^7)	BALB/c	Yes	199
	FTN_0714	FTT0742	i.n. (6×10^7)	U112	No	i.n. (6×10^7)	BALB/c	Yes	199
	FTN_1310	*pdpB*	i.n. (6×10^7)	U112	No	i.n. (6×10^7)	BALB/c	Yes	199
	FTN_0337	*fumA*	i.n. (6×10^5)	U112	No	i.n. (6×10^7)	BALB/c	Yes	199
	FTN_0020	*carB*	i.n. (6×10^3)	U112	No	i.n. (8×10^5)	BALB/c	Yes	199
	FTN_1322	*iglC*	i.n. (10^6)	U112	No	i.n. (10^3)	BALB/c	Yes	142
						i.n. (10^4)	BALB/c	Yes	142
						i.n. (10^5)	BALB/c	No	142
	FTN_1322	*iglB*	i.n. (10^6)	U112	No	i.n. (10^3)	BALB/c	Yes	39
				LVS	No	i.n. (3×10^4)	BALB/c	Yes	39
			Oral (10^3)	Schu S4	Yes	i.n. (52)	BALB/c	No	39
	FTN_1290	*mglA*	Aerosol (10^5)	U112	No	Aerosol (35–8×10^4)	BALB/c, C57BL/6	No	214
	FTN_0050, FTN_1556, FTN_1061, FTN_0954	*acpABCH*	i.n. (10^3)	U112	No	i.n. (10^6)	BALB/c	Yes	135
			i.n. (10^6)	U112	No	i.n. (10^6)	BALB/c	Yes	135
	FTN_0090, FTN_1061, FTN_0954	*acpCH* or *acpACH*	i.n. (10^3)	U112	No	i.n. (10^3)	BALB/c	No	135
	FTN_0546	*flmK*	s.c. (400–500)	U112	No	s.c. (660)	BALB/c, C57BL/6	Yes	101
			Aerosol (100)	U112	No	i.n. (50)	BALB/c	Yes	101
F. tularensis subsp. *holarctica*	None (wild type)		i.n. (200)	Schu S4	No	i.n. (100)	BALB/c	Yes	146
						i.n. (2,000)	BALB/c	No	146
					Yes	i.d. (100, 2,000)	BALB/c	Yes	146
	FTL_0094	*clpB*	i.p. (13)	LVS	No	i.p. (1×10^4)	BALB/c	Yes	129
			i.p. (1,300)	LVS	No	i.p. (1×10^4)	BALB/c	Yes	129
	FTL0395-FTL0396	*purMCD*	i.n. (10^6)	Schu S4	No	i.n. (100, 2,000)	BALB/c	No	146
						i.d. (100, 2,000)	BALB/c	No	146
					Yes	i.n. (100)	BALB/c	Yes	146
						i.n. (2,000)	BALB/c	No	146
						i.d. (100, 2,000)	BALB/c	Yes	146
	FTL0552	*pmrA*	i.n. (10^5)	Schu S4	No	i.n. (10^2)	C57BL/6, BALB/c	Yes	165
	FTL0421	*lpnA*	i.d. (10^5)	LVS	No	i.d. (10^7)	C3H/HeN	Yes	62
	FTL_1071	*guaA*	(2.2×10^7)	LVS	No	2.8×10^5	BALB/c	Yes	170
	FTL_1478	*guaB*	(3.6×10^7)	LVS	No	2.8×10^5	BALB/c		170
	FTL_1793	*sodB*	i.n. (5,000)	Schu S4	No	i.n. (14×10^3)	C57BL/6	Yes	9
			i.n. (500)	LVS	Yes	i.n. (1.3×10^6)	C57BL/6	Yes	9
				Schu S4	Yes	i.n. (104)	C57BL/6	No	9
	FTL_0592	*wbtA*	i.d. (1.5×10^8)	FSC 108	Yes	i.d. (17)	BALB/c	Yes	180
				Schu S4	Yes	i.d. (10)	BALB/c	Yes	180
F. tularensis subsp. *tularensis*	FTT0107c	*dsbB*	i.n. (6.8×10^3)	Schu S4	No	i.n. (13–13,000)	C57BL/6	No	156
	FTT0893-FTT0894	*purMCD*	i.n. (10^4)	Schu S4	No	i.n. (100, 2,000)	BALB/c	No	146
						i.d. (100, 2,000)	BALB/c	Yes	146
					Yes	i.n. (100)	BALB/c	Yes	146
						i.n. (2,000)	BALB/c	No	146
						i.d. (100, 2,000)	BALB/c	Yes	146
	FTT0918	FTT0918	i.d. (10^5)	FSC033	No	i.d. (500)	BALB/c	Yes	204
						Aerosol (10)	BALB/c	Yes	204
	FTT1357c, FTT1712c	*iglC*, *iglC1*	i.d. (10^6–10^7)	FSC033	No	i.d. (500), aerosol (10)	BALB/c	No	204
	FTT1459c-FTT1461c	*wbtDEF*	s.c. (10^5)	Schu S4	No	s.c. (100)	BALB/c	No	200
	FTT1103	FTT1103	i.n. (1.6×10^8)	Schu S4	No	i.n. (1,000)	C57BL/6, BALB/c	Yes	157

[a] i.d., intradermal; i.n., intranasal; i.p., intraperitoneal; s.c., subcutaneous.

***F. tularensis* subsp. *tularensis*.** It has been speculated that the limited protection offered by LVS or its derivatives against aerosol challenge with type A strains of *F. tularensis* might be related to differences in protective antigens and could be countered by vaccination with an attenuated type A strain (39a, 40, 84, 204, 221). Alternatively, the types of immune responses elicited or cell types encountered during type A pulmonary infection may be different than those generated with other subspecies. Consistent with this idea, Wu et al. have observed that boosting with sublethal doses of the type A strain NMFTA after LVS vaccination affords mice superior protection against NMFTA challenge compared to simply boosting with LVS (221). While only a limited number of *F. tularensis* subsp. *tularensis* mutants have been evaluated for their potential as live attenuated vaccine candidates (Table 3), results from these studies have indicated that it is possible to generate highly attenuated derivatives in this genetic background, including some that are able to provide low to

modest levels of protection against virulent type A or type B isolates delivered by the respiratory route (146, 157, 204). However, given the highly infectious nature of type A strains of *F. tularensis* and the broad host range that these strains exhibit in nature, development of vaccine candidates that are safe, immunogenic, and highly protective will undoubtedly be a formidable task.

Heterologous hosts. The inherent problems with the use of live attenuated *Francisella* vaccines, as well as the limitations of subunit/component vaccines, have led some groups to explore the use of heterologous bacteria to express *Francisella* antigens (73, 97, 186) (Table 7). Of the various outer membrane protein, lipoprotein, and virulence factor genes studied to date, only *iglC* expressed in a *Listeria monocytogenes* Δ*actA* mutant confers protection against challenge with type A or B *F. tularensis* (97).

Respiratory versus parenteral vaccination. A growing body of literature indicates that pulmonary vaccination may provide optimal protection against respiratory pathogens, rather than vaccination via the typical parenteral routes (39b, 92, 221). Aerosol delivery of a tularemia vaccine may be important for the stimulation of lung-associated lymph nodes and key immune mediators present in the lung. While inoculation of vaccines via the intradermal or intramuscular route typically induces systemic immune responses, vaccination via these routes often fails to induce substantial responses in mucosal tissues such as the lungs (130). This may in part explain the fact that while offering considerable protection against subsequent parenteral Schu S4 challenge, LVS delivered by scarification confers poor protection against respiratory challenge with Schu S4. An advantage of vaccination via the respiratory route is the generation of immune responses at local as well as at distal mucosal sites, thus generating the necessary response in lymphoid organs (130). Chen et al. demonstrated that intranasal vaccination of mice with LVS confers superior protection against subsequent aerosol challenge with 10 CFU of type A *F. tularensis* relative to that observed following vaccination with LVS via the intradermal route (28). Importantly, immunization via this route also maintains protective efficacy against intradermal challenge (28). These results are also consistent with findings by Wu et al. demonstrating that intranasal vaccination with LVS confers superior protection against subsequent intranasal challenge with type A strain NMFTA (221). These findings and others indicate that a live attenuated *Francisella* strain delivered via the respiratory route may provide optimal protection against subsequent aerosol challenge.

CONCLUDING REMARKS

F. tularensis is a highly virulent pathogen with the potential to cause severe disease in humans. Very few infectious bacteria have as broad a host range and the capacity to replicate to such high numbers in a variety of host tissues. Humans can be infected via a number of routes, resulting in a variety of disease manifestations. Inhalation of as few as 10 live organisms can result in potentially lethal disease. The biological weapons programs of several countries included *F. tularensis* as a bioagent due to its potential to cause debilitating disease. However, since the conclusion of these weapons programs, little work has been done to understand the genetic determinants

TABLE 7. Expression of *Francisella* antigens in heterologous hosts

F. tularensis antigen(s)	Host	Attenuating mutation(s)	Vaccination route[a] (dose, CFU)	Challenge strain	Boost	Challenge route[b] (dose, CFU)	Host mouse strain	Protection	Reference
IglC	*L. monocytogenes*	Δ*actA*	i.d. (10^6–10^7)	LVS	Yes	i.n. (4,400)	BALB/c	Yes	97
				Schu S4	Yes	Aerosol (1 [10 LD_{50}])	BALB/c	Yes	97
KatG	*L. monocytogenes*	Δ*actA*	i.d. (10^6–10^7)	LVS	Yes	i.n. (4,400)	BALB/c	No	97
				Schu S4	Yes	Aerosol (1 [10 LD_{50}])	BALB/c	No	97
AcpA, Bfr, DnaK, GroEL, Pld Tul4	*L. monocytogenes*	Δ*actA*	i.d. (10^6–10^7)	LVS	Yes	i.n. (4,400)	BALB/c	No	97
	S. enterica serovar Typhimurium	Δ*asd*, Δ*cya*, Δ*crp*	i.p. (5×10^3)	LVS	No	i.v. (160–230)	BALB/cJ	No	186
FopA	*S. enterica* serovar Typhimurium	Δ*aroA*	i.v. (1×10^7)	LVS	Yes	i.p. (10^1–2.5×10^6)	BALB/c	No	73

[a] i.d., intradermal; i.p., intraperitoneal; i.v., intravenous.
[b] i.n., intranasal.

required for *Francisella* disease and pathogenesis. The recent influx of investigators and funding initiatives in the *Francisella* field has lead to a number of advancements in the study of genetics, pathogenesis, and vaccine development for this organism. Continued support of initiatives in these areas will undoubtedly continue to uncover important new aspects of the *F. tularensis* lifestyle and/or the mechanisms by which this organism interacts with its host. Undoubtedly, these insights will prove crucial as studies into the development of a safe and protective *Francisella* vaccine continue forward.

As increasing amounts of literature examining vaccination strategies against virulent *F. tularensis* subspecies have become available, a picture of the characteristics necessary for protective immunity is emerging. It is likely that a successful vaccine candidate will need to induce aspects of both cell-mediated and humoral immunity. Both $CD4^+$ and $CD8^+$ T-cell responses are necessary for clearance of highly virulent strains, and induction of such responses is essential to generate *F. tularensis* immunity. Despite efforts to identify key antigenic mediators, it is evident that live attenuated vaccines may offer the best protection, as has been demonstrated in mouse infection models. Vaccination with attenuated derivatives of type A *F. tularensis* strains appears thus far to offer no significant advantage over vaccination with LVS, indicating that work toward developing a live attenuated vaccine candidate based on utilization of a type B background may be more suitable. Regardless of the subspecies utilized, it may be necessary to disrupt bacterial determinants involved in initial suppression of the host immune response. Particularly during respiratory infection, *F. tularensis* uses a number of strategies to limit the host response, allowing for significant bacterial proliferation and dissemination early after infection. It is likely no coincidence that respiratory tularemia is the most difficult disease manifestation to prevent with vaccination. Identification and elimination of *F. tularensis* immunosuppressive mediators in already-attenuated strains may also be a plausible strategy that leads to a safer and less reactogenic vaccine. Finally, the route of vaccination needs to be considered when developing vaccine candidates, as evidence indicates that respiratory vaccination may provide better protection against aerosol challenge. Despite difficulties in vaccinating against highly virulent *F. tularensis* strains, the multitude of vaccine candidates emerging as a result of recent advancements in *F. tularensis* genetics continues to yield important information regarding the elements necessary to elicit immunity against this highly virulent pathogen.

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ADDENDUM IN PROOF

Presently, the genome for *Francisella tularensis* subsp. *holarctica* strain URFT1 (NZABAZ00000000) is undergoing shotgun sequencing.

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