SITE-DIRECTED MUTAGENESIS IN *FRANCISELLA TULARENSIS* BY ALLELIC REPLACEMENT

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ABSTRACT

Francisella tularensis is a Gram-negative, facultative intracellular coccobacillus and the etiologic agent of tularemia for a wide variety of vertebrate and invertebrate animal species. Several species and subspecies of *Francisella* are currently recognized. However, the majority of infections are caused by *F. tularensis* subspecies *tularensis* (type A) and subspecies *holarctica* (type B). Given the low infectious dose, multiple transmission routes, severity of illness, and lack of licensed vaccines, *F. tularensis* has long been considered a potential biological weapon and is now classified as a category A select agent by the National Institutes of Health and the Centers for Disease Control and Prevention.

The investigation of the mechanisms of pathogenesis by *F. tularensis* type A and B strains is hindered by the difficulty and lack of methods to mutate the putative genes that encode for virulence factors. New genetic tools have been developed that have enabled mutagenesis of *F. tularensis* type A and type B stains. However, site-specific mutations remain difficult to execute or these methods generate random mutations. In this study a novel method was developed to create site-directed mutations in a putative capsule biosynthesis locus to knock out encapsulation of the attenuated *F. tularensis* live vaccine strain. Two suicide vectors for mutagenesis of *F. tularensis* were constructed based on the commercial PCR cloning vector pSC-A. These vectors were created by inserting into the cloning site a kanamycin resistance

gene boarded upstream by 1.3 kb of N-terminal DNA and downstream by 1.3 kb of C-terminal DNA that flanks the target gene. Cryotransformation was used to introduce the vectors into *F. tularensis*. Open reading frame (ORF) FTT0793, which may encode for an ABC transporter involved in capsule export, was initially selected for mutagenesis in order to generate a mutant that was nonencapsulated, but could still synthesize capsule and induce a host immune response. Mutagenesis of this gene was successful. However, phenotypic assays could not confirm that the mutant was nonencapsulated compared to the parent. Therefore, adjacent ORFs FTT0798 and FTT0799, which may encode for a galactosyl transferase and mannosyl transferase, respectively, were also deleted to completely knock out capsule synthesis. The resulting mutant appeared to be nonencapsulated as determined by negative staining transmission electron microscopy.

In this study, a plasmid and method for generating allelic exchange mutants is reported, which should be useful for generating additional mutants of *F. tularensis* for use in clarifying the roles of specific genes. This vector is currently being used to make a nonencapsulated mutant of a virulent type A strain to determine the role of capsule in virulence.

DEDICATION

I would like to dedicate this work to my parents, Fan Wang and Dongxia Xue. I cannot imagine a life without your constant love and support!

ACKNOWLEDGEMENTS

ATTRIBUTION

Dr. Thomas J. Inzana is my supervisor for the research presented in this thesis. He provided the original ideas and suggestions through out the research. My experiments were performed under his directions.

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CHAPTER 1

Review of Literature and Introduction

1.1. Review of Literature

1.1.1. Description and history

Francisella tularensis is a Gram-negative, facultative intracellular coccobacillus and the etiologic agent of tularemia for a wide variety of vertebrate and invertebrate animal species; tularemia is a zoonotic disease for humans (51, 74, 90, 101, 104). It has been recognized as a human pathogen since the beginning of the 20th century. The original isolation of *F. tularensis* occurred during a plague-like disease of ground squirrels in Tulare County, California in 1911 (67). Although first isolated in the United States, isolation of *F. tularensis* has been reported from many countries of the Northern Hemisphere (91). The first verified case of tularemia occurred in Ohio 1914 (108). Human cases have occurred in every state of the United States except Hawaii (2). To date, outbreaks have been reported globally, the largest recorded was in 1966 in a farming region of Sweden (21). Outbreaks are often associated with inhalation of *F. tularensis*. As one of the most infectious bacteria known, inoculation or inhalation of as few as 10 organisms can cause severe disease (87). Given the low infectious dose, multiple transmission routes and severity of illness, *F. tularensis* has long been considered a potential biological weapon.

1.1.2. Species, subspecies and geographic distribution

Thus far, several species and subspecies of *Francisella* have been recognized. There are four subspecies of *F. tularensis* (**Table 1.1**): (a) subspecies *tularensis* (type A) is found in North America, (b) subspecies *holarctica* (type B) is found in Europe, Asia, and throughout North America, (c) subspecies *mediasiatica* is found in central Asia and parts of the former Soviet Union, and (d) subspecies *novicida* is found in North America and Australia (45, 92, 102). However, the international Systematics Committee recognizes *F. novicida* as a separate species (http://www.bacterio.cict.fr/aldl.html)(62). There is a high degree of genetic conservation

among the subspecies despite differences in virulence (8).

F. tularensis LVS (live vaccine strain) is derived from subspecies *holarctica*. It has been a widely used model strain for studies on *F. tularensis*. It was also a human live vaccine strain that provides good protection against both natural and laboratory-acquired tularemia (9), but is no longer approved for use as a vaccine due to its instability and lack of genetic characterization.

Table 1.1. Subspecies of *Francisella* and their distribution

Biotype	Previous names	Geographical locations
Francisella tularensis subsp. *tularensis*	*Francisella tularensis* type A *Francisella tularensis* subsp. *nearctica*	Mainly North America
Francisella tularensis subsp. *holarctica*	*Francisella tularensis* type B *Francisella tularensis* subsp. *palaearctica*	Mainly Europe, former Soviet Union, Far east, Japan, and North America
Francisella tularensis subsp. *mediasiatica*	*Francisella tularensis*	Mainly central Asia and parts of the former Soviet Union.
Francisella tularensis subsp. *novicida*	*Francisella novicida*	North America and Australia.

1.1.3. A facultative intracellular bacterium

F. tularensis is a facultative intracellular pathogen that is capable of infecting a large number of cell types, such as type II alveolar epithelial cells (47) and neutrophils (66), but it is clear that mononuclear phagocytes and hepatocytes are important host cells for *F. tularensis* (15). During normal phagosome maturation, the bacteria containing phagosome fuses with lysosomes and acidifies to degrade the bacteria. Normally, the phagosomes of human macrophages containing killed bacteria acidify to pH 5.5. However, recent studies have shown that phagosomes containing live *F. tularensis* only acidify to pH 6.7 (16). Therefore, *F. tularensis* is thought to neutralize the phagosomal environment through disruption of the membrane. It can persist in both macrophages of human and mouse origin, and within 2-4 hours, bacteria begin to escape the phagosomes, and after 4-8 hours a large majority of bacteria appear to be free in the cytoplasm (16, 40, 84), where they proceed to replicate. However, the mechanism by which the bacteria escape from the phagosome is not yet known (72).

Although little is known about the genes responsible for virulence of *F. tularensis*, some genes required for intracellular growth have been identified. The *iglC* (igl stands for intracellular growth locus) gene in the *igl* locus is essential for the intracellular proliferation of *F. tularensis* subsp. *holarctica* and subsp. *novicida* in both macrophage and amoebae (44, 98). It was reported that *F. tularensis* LVS-infected cells of the mouse macrophage cell line J774 are killed by apoptosis with 24-48 hours (57, 58). In contrast, the *iglC* mutant of *F. tularensis* LVS induces no apoptosis. An Δ*iglC* mutant of *F. tularensis* LVS is also avirulent in mice (42). Taken together *F. tularensis* uses a unique mechanism to survive intracellularly.

1.1.4. Epidemiology and clinical manifestations

The first description of tularemia was published in the paper "A Plague-like Disease of Rodents" in 1911 written by George McCoy (67). Tularemia occurs globally, and in the United

States human cases have been reported from every state except Hawaii (2). Both terrestrial and aquatic animals can maintain *F. tularensis*. Mice, rabbits, voles, water rats, squirrels, and Atlantic cod act as the natural reservoirs of the bacteria (23, 71, 72). Humans becomes infected with *F. tularensis* by various modes, including bites by arthropods, handling infectious animal tissue or fluids, direct contact with or ingestion of contaminated water, food or soil, and inhalation of infective aerosols (21, 28). Most cases of *F. tularensis* causing infection are transmitted via arthropods, mainly *Ixodid* ticks (27). Humans, regardless of age and sex, are equally susceptible to tularemia (23). Laboratory personnel are especially vulnerable to infection by accidental inoculation, or inhalation of contaminated aerosols. To date, person to person transmission of *F. tularensis* has not been reported (23).

The onset of tularemia normally presents after an inoculation period of approximately 3-5 days. Symptoms include fever, malaise, chills, and headache (13, 26). Other clinical manifestations depend on the route of infection and the subspecies of *F. tularensis*. Oropharyngeal tularemia is acquired by drinking contaminated water or ingesting contaminated food. Infected persons may develop exudative pharyngitis or tonsillitis (23). Swellen lymph nodes (lymphadenopathy) may be observed. Ulceroglandular lesions may develop if the infection is transmitted through skin or mucous membranes (72); this form of tularemia is rarely fatal. Respiratory tularemia results from inhalation of *F. tularensis*, which by far is the most dangerous form of tularemia and has a case fatality rate of up to 30% if untreated (24). However, in the United States the fatality rate has been reduced to less than 2% with antibiotic treatment (72). Type B infections are rarely fatal.

1.1.5. Virulence

As a facultative intracellular pathogen, *F. tularensis* can rapidly grow to large numbers in the cytosol of the infected cells without disrupting their physical integrity (18). To date, the virulence factors of *F. tularensis* are not well known. The bacteria do not produce any obvious

exotoxins, and its lipopolysaccharide (LPS) is not toxic. Thus far, several *Francisella* pathogenicity islands (FPI) have been identified. These islands include the *mglAB, iglABCD, minD*, and *pdpAD* genes. Studies have shown that *iglA* and *iglC* are essential for intra-macrophage multiplication in *F. tularensis* subsp. *holarctica* and subsp. *novicida* (60, 85). Failure to express IglC leads to complete loss of virulence. The *mglAB* (mgl stands for macrophage growth locus) operon is essential for the intracellular survival of *F. tularensis* subsp. *novicida* and is also found in *F. tularensis* subsp. *tularensis* (72). MglA has been described as a transcriptional regulator, coordinately regulating *iglC* in *F. tularensis* subsp. *novicida* (85). *pdpAD, iglA* and *iglD* also appear to be regulated by MglA and MglB (61, 70, 85). The majority of these genes represent novel bacterial factors that have not been previously associated with *F. tularensis* virulence (95). In addition to the genes described above, there are several other virulence factors associated with this island (103).

1.1.6. Virulence factors

Lipopolysaccharide (LPS). As an important virulence factor, the LPS of *F. tularensis* has attracted significant interest because of its unusual biological and structural properties. The LPS of *F. tularensis* types A and B is unusual in that the O-antigen consists entirely of dideoxy glycoses, the core oligosaccharide contains mannose in the place of heptose (106, 107), and the lipid A of LVS is tetraacylated and lacks phosphate (106). It shows an unusual low toxicity *in vitro* and *in vivo* (4, 83). Probably as a result of the atypical structure of the lipid A, the LPS does not signal through Toll Like Receptor 4 (TLR4), and therefore does not induce an inflammatory response (11, 17, 46). The O-antigen deficient strains are able to survive in infected host cells without mutiplification for at least three days (77). However, the O-antigen appears to be critical for serum resistance in *F. tularensis* (41, 62, 77, 88).

"Capsule". In addition to LPS, capsular polysaccharide is another important surface

polysaccharide in pathogenic microorganisms such as *Bacillus anthracis* and *Neisseria meningitides*, etc. Capsule helps protect bacteria against phagocytosis as well as desiccation. Moreover, it is considered one of the key virulence determinants that cover the surface of the organism and confers resistance to host-mediated defense mechanisms, such as opsonization and complement-mediated lysis (25). Several reports have described a capsular structure on *F. tularensis* (12, 36, 50, 82, 94). Cherwonogrodzky *et al.* (12) described that when LVS was subcultured on Chamberlain's synthetic medium (10), the strain became extensively encapsulated and the virulence for mice increased about 1000-fold. It is possible that the synthetic medium mimics the intracellular growth environment for *F. tularensis* and the bacteria respond by a derepression of virulence factors. The capsule seems to be a necessary component for expression of full virulence (92).

It was also found that capsule may limit the frequency of DNA transformation. The efficiency of DNA transformation could by increased by approximately 10-fold after the treatment of the N-hexadecyl-N,N-dimethyl-3-ammonio-1-propane-sulphonate (HDAP), a mild detergent that removes the capsule from encapsulated strains (5). Similarly, utilization of a Cap⁻ mutant strain of LVS increases the efficiency of DNA introduction relative to the wild-type parent (5). While the mechanism responsible for this increased DNA transformation efficiency in *F. tularensis* is unclear, the capsule likely acts as a physical barrier that limits the entry of DNA into the bacterium (109). However, the extent of inhibition mediated by the capsule is minimal, and efficient introduction of DNA still proceeds even in capsule-expressing strains.

Although the makeup of the poorly characterized capsule encircling *F. tularensis* is not known, a gene cluster (FTT0789−FTT0801) in *F. tularensis* type A and type B strains has been identified that could encode a polysaccharide addition to the LPS O-antigen. Homologues of the genes *capB* (FTT0805) and *capC* (FTT0806), which are required for capsule biosynthesis in *B. anthracis* have also beeb identified (59). Capsule of *F. tularensis* has been considered as a

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potential virulence factor. However, its role as a vaccine candidate has not been studied (92).

Type IV pili. Type IV pili are surface polysaccharides and iron-acquisition systems (59)**.** They are common virulence factors for a variety of pathogenic bacteria (19). The presence of pili on the surface of *F. tularensis* has been reported (38), and all the currently known genes necessary for Type IV pili biosynthesis have been found in the genome. However, the exact role of Type IV pili in *Francisella* is not yet known. It was reported Type IV pili are critical for the virulence of subsp. *holarctica* in which they appear to be involved in dissemination of the pathogen from the initial infection site (29).

Based on current findings, the virulence of *F. tularensis* appears to stem from its ability to escape from the phagosomes and proliferate in large numbers within various host cells, thereby disrupting their normal functions (19).

1.1.7. Genetic characterization

Francisella is the only genus of the family *Francisellaceae*, which belongs to the γ subclass of proteobacteria. The genomes of at least five strains of *F. tularensis* types A and B and *F. tularesis* subsp. *novicida* have recently been sequenced, and the genome of type A strain SchuS4 has been annotated (59). The genome of *F. tularensis* SchuS4 consists of a 1.89 Mbp circular chromosome with a G+C content of 32.9% and 1,804 predicted coding sequences (59). Through homology searches no new virulence genes of *F. tularensis* have been identified and the gene products of the previously reported *iglA, iglC* and *pdpD* do not show sufficient homology with any other genes in GenBank (59). Thus, little is known regarding the virulence mechanisms of *F. tularensis*, and more virulence genes are expected to be identified.

1.1.8. Vaccines

A multitude of strategies have been employed to develop experimental and clinical vaccines against a wide variety of bacterial pathogens (75). Several different kinds of vaccines have been developed against *F. tularensis*. They include:

1.1.8.1. Killed whole cell vaccines

Killed whole cell vaccines were the earliest vaccines developed to prevent human tularemia. They were devised by Lee Foshay, and are referred to in the literature as "Foshay" vaccines in honor of their inventor (19, 30). The "Foshay" vaccines involved acid extraction followed by phenol preservation and were used to immunize thousands of individuals (31). But efficacy of these kinds of whole-cell vaccines appears to be questionable, because many recipients of the "Foshay" vaccine still developed severe infection requiring therapeutic intervention following exposure to highly virulent strains (30). The failure of the killed whole-cell vaccines may be due to the destruction of antigens caused by the severe conditions of the treatments (43); or due to the inability to elicit a robust cellular immune response, which is considered necessary for protection against type A strains (97).

1.1.8.2. Subunit vaccines

Rather than exposing an inactivated or attenuated bacterium to the immune system, a fragment of it can also induce an immune response. To date the LPS induces the greatest humoral immune response in the host. Immunization with LPS provides good protection against systemic challenge with attenuated and virulent type B stains, but not against challenge with type A stains, or aerosol challenge with type A or B strains (20, 32, 33, 100). In addition, immunization with purified LPS from *F. tularensis* subsp. *novicida* protected mice against

homologous challenge, but not against challenge from *F. tularensis* subsp. *holarctica* (100), and *vice versa*. The reason is because the O-antigens are identical in type A and type B stains, but altered in subsp. *novicida.*

In addition to LPS, several protein antigens have also been evaluated for their ability to elicit protection. However, to date little success has been made in this approach (19). With the recent completion of the *F. tularensis* genome sequence and the development of proteomics, more efforts are focusing on identifying putative protein vaccine candidates.

1.1.8.3. Live attenuated vaccines

Live attenuated strains were developed prior to World War II in the former Soviet Union. Attenuated strains were developed by either repeatedly sub-culturing a virulent strain of *F. tularensis* subsp. *holarctica* on media containing antiserum or by drying the organisms (56). Although, *F. tularensis* subsp. *novicida* is relatively attenuated in humans, it is not suitable as a vaccine against tularemia because it fails to induce a protective immune response effective against the more virulent subspecies (89). The live vaccine strain (LVS) was widely used in the former Soviet Union where as many as 60 million people were reportedly immunized with it with great success (93).

In the US, LVS vaccine has been used for at-risk personnel working with type A strains in the laboratory. Compared to the "Foshay" vaccine, LVS significantly reduced laboratory-acquired tularemia from 5.7 to 0.27 cases per 1000 at-risk employee years (9). However, the LVS vaccine still reminds unlicensed in the US today due to variety of reasons. First, the genetic basis of attenuation of the LVS strain is as yet unknown. Secondly, the LVS stain retains virulence for mice. Finally, the LVS vaccine does not confer protection to every vaccinated individual (68, 86), and is also unstable. Further work is required in order to develop a

licensable vaccine that is highly safe and effective. Currently, the LVS strain is widely used as a model strain for tularemia research.

1.1.9. Biological weapon

Many cases of tularemia have been recorded during wars. According to Ken Alibeck, a former Soviet Union scientist, the tularemia outbreaks affecting large number of German and Soviet soldiers on the eastern European front during World War II may have been the result of intentional use of *Francisella* (3). Outbreaks of tularemia were also reported during the Continuation War between Finland and the Soviet Union in Karelia between 1941 and 1944 (37) and from the civil wars in Bosnia and Kosovo in the 1990's and early 2000's, where tularemia had not been described before (78).

Why is *F. tularensis* considered a threat as a bioweapon in the U.S.? Whereas about 10,000 *B anthracis* (anthrax) spores are required to cause disease, the infectious dose of *F. tularensis* can be as low as 10 organisms (23). In addition, pneumonic tularemia has a high mortality rate of up to 30 % without treatment (25). The low infectious does, various transmission routes, and high mortality rate are among the reasons this bacterium has been considered a potential agent for weaponization. Between 1932 and 1945 Japanese germ-warfare units examined the feasibility of deliberately causing tularemia in humans (48). In the 1950s and 1960s, the US military developed weapons that would disseminate *F. tularensis* aerosols (14), and according to Alibeck, the development of *F. tularensis* as a biological weapon continued until the 1990s and resulted in weapons production of engineered strains that were resistant to antibiotics and vaccines (3). In 1969, a World Health Organization expert committee estimated that an aerosol dispersal of 50 kg virulent *F. tularensis* over a metropolitan area with 5 million inhabitants would result in 250,000 incapacitating casualties, including 19,000 deaths (1). Based of these facts, *F. tularensis* has been classified as a category A select agent by the National Institutes of Health

and the Centers for Disease Control (22, 23).

1.1.10. Genetic manipulation

1.1.10.1. Methods for introducing DNA

In order to genetically study *Francisella*, there must be a way to introduce and manipulate the DNA. Thus far a variety of efficient methods for delivery of DNA into *Francisella* have been described. Cryotransformation, electroporation and conjugation are the most widely used methods.

A. Cryotransformation

Cryotransfroamtion is a method involves flash freezing the DNA, bacteria, and transformation buffer mixture with liquid nitrogen followed by thawing at room temperature. This method was first reported to work in *Francisella* by Pavlov *et al.* 1996. The *Francisella* culture is washed and concentrated in 0.2 M KCl buffer. Cells are then mixed with transformation buffer (0.2 M MgSO$_4$, 0.01 M Tris-acetate pH 7.5) and DNA at room temperature for 10 min, and then flash frozen in liquid nitrogen for 5 min. The mixture is thawed at room temperature for about 5 min and the bacteria are recovered on non-selective medium for several hours. The bacteria are then collected in PBS and spread onto selective medium for mutant isolation (73). When testing with pFNLTP6, which is a shuttle vector that can replicate in *Francisella*, the efficiency can be more than 10^3 transformants per ng of plasmid DNA. This method has been tested to work in type A, type B, and *F. tularensis* subsp. *novicida* strains. However, the requirement for liquid nitrogen is a limitation of this technique and may be problematic for those studies involving the use of virulent clinical strains in biosafety level three laboratories (109).

B. Electroporation

Electroporation is a significant increase in the electrical conductivity and permeability of the cell plasma membrane caused by a controlled electrical field. It is another effective way to introduce DNA into *Francisella.* To transform *Francisella* by electroporation, the bacterial cells are first made electrocompetent by washing 3-4 times with 0.5 M sucrose. The cells are then mixed with a certain amount of DNA and electroporated. The standard settings for *Francisella* are a voltage of 12.5-15 kv/cm, capacity of 25 μF, and a resistance of 200-400 Ω (7, 63). Following electroporation, the cells are recovered on non-selective agar or liquid medium for several hours. Transformants are then isolated on selective medium. High efficiency is an advantage to electroporation. When using pFNLTP6，the efficiency of the plasmid is about 10^4 transformants per ng of plasmid DNA, which is more than 10-fold higher than by cryotransformation. The procedures for inducing competent cells, preparation, and cell recovery are also easy to follow. However, the electroporation parameters need to be optimized for each experiment. The success of elecroporation depends greatly on the purity of the plasmid solution, especially on its salt content. Impure solutions might cause electrical arcing of the solution, which can kill many of the bacteria, requiring repetition of the procedure.

C. Conjugation

Bacterial conjugation is the transfer of genetic material between bacteria through direct cell-to-cell contact. The genetic information transferred can be beneficial to the recipient, such as in conferring antibiotic resistance, or an enzyme that provides broader adaptation to its environment (49). To work with *Francisella*, cultures of donor and recipient bacteria are prepared separately. They are then concentrated, mixed, and plated on non-selective Luria agar or McLeod agar plates (41, 42). After incubation, the cells are recovered and plated on selective medium. *Escherichia coli* S17-1 strain is often used as the donor strain. The plasmid DNA to be transferred is marked with a mobilization sequence (origin of transfer, *oriT*), so that it can be

recognized by a protein complex and transferred through the mating apparatus. In *Francisella*, approximately 1% of cells can be transformed under optimal conditions when using *E. coli* donors to conjugate DNA (42). Furthermore, successful conjugation has been reported for several different *Francisella* subspecies including *novicida* (69, 100), *holarctica* (6, 29, 39, 42), and *tularensis* (100, 105). The shortcomings of this method are that the efficiency is relatively low compared with other methods, and the amount of DNA that is transferred depends on the how long the bacteria remain in contact. Larger DNA may also reduce the transformation efficiency.

1.1.10.2. Mutagenesis methods

Mutagenesis is an important way to identify the genetic determinants required for virulence and immunogenicity. In *Francisella*, several different mutagenesis strategies have been used.

A. Chemical mutagenesis

Chemical mutagenesis is the interaction of certain chemical compounds and cell metabolism intermediates that may result in genetic changes in DNA structure, affecting one or more genes. These chemical compounds include base analogues, substitutes and intercalating agents. In *Francisella*, chemical mutagenesis was used in early studies to induce random mutations. The first capsule mutants in wild-type type B strains and LVS were generated following exposure of these organisms to acridine orange (82), which is an intercalating agent. The generation of chemically induced mutants is easy and quicker than screening for spontaneous mutations, especially when there was no other efficient mutagenesis strategy. However, the generated mutants are unmarked making it difficult to elucidate their locations in the genome (109). In addition, many mutations have no observable phenotype *in vitro*. Furthermore, random mutations cannot be controlled.

B. Allelic exchange

Allelic exchange is a widely used method to generate insertion or deletion mutants. In *Francisella*, different allelic replacement procedures have been recently developed.

a. Linear PCR substrates

Creation of antibiotic-marked mutants by allelic exchange using PCR products has been reported in *F. tularensis* subsp. *novicida* by Laurano *et al*. (60) (**Fig. 1.1A**). In this method, the upstream and downstream flanking regions of the gene of interest are amplified and ligated into a plasmid. An antibiotic resistance cassette is then inserted between the ligated flanking regions. The resulting mutated DNA fragment is then PCR-amplified and introduced into the recipient strain. Recombinants are isolated on selective medium.

The requirement for two concomitant double-crossover events greatly reduces the frequency of generating an allelic exchange mutant. The generation of long DNA substrate is often difficult due to problems associated with amplification efficiency and fidelity (109). Thus far there has been no successful report of allelic exchange in type A and type B strain mutants using this method.

b. Suicide vectors

A suicide vector is a plasmid that cannot replicate in a particular host or cannot replicate under certain conditions. In *Francisella*, use of a suicide vector has been the most common strategy for generating marked or unmarked gene alleles by allelic exchange(109) (**Fig 1.1B & 1.1C**). To date, site-directed mutations have been made in several *Francisella* subspecies including *novicida, holarctica,* and *tularensis*. The creation of mutagenesis plasmids is similar to the PCR-based methods, but instead of introducing a PCR product, a non-replicating plasmid is

introduced into the recipient strain. The mutagenesis plasmid should be able to replicate in *E. coli* in order to be genetically manipulated. If a selectable marker is located between the homologous regions, a marked gene deletion can be generated. On the other hand, if the selectable marker is outside the homologous region, an unmarked gene deletion can be obtained. For unmarked allelic exchange, the *sacB* gene is often used as a counterselectable marker. The *sacB* gene encodes the secreted *Bacillus subtilis* enzyme levansucrase, the expression of which is toxic for Gram-negative bacteria in the presence of sucrose (35, 79). For *Francisella*, inclusion of 5-10% sucrose into the agar medium is adequate to promote counterselection. However, resistance to sucrose through the generation of spontaneous *sacB* mutations is common and has been reported to occur at frequencies ranging from 0 to 30% (63).
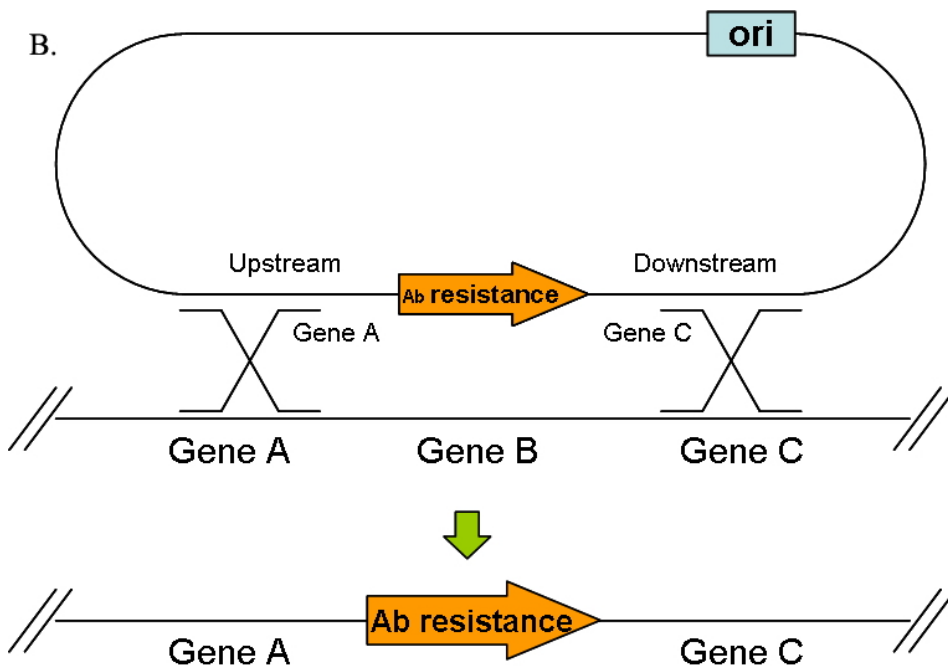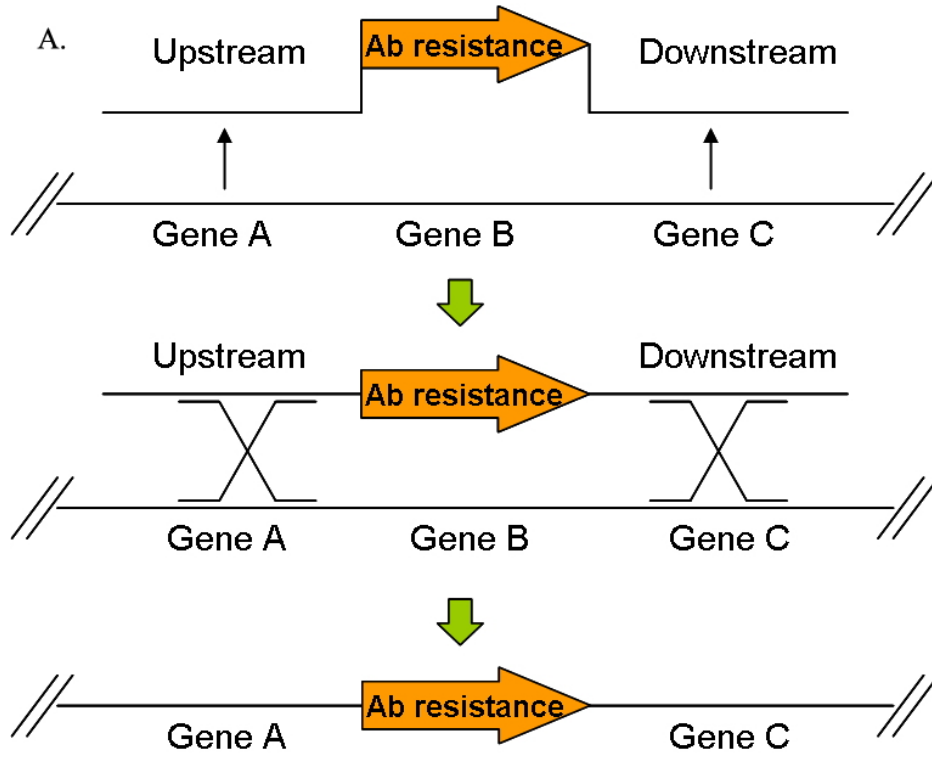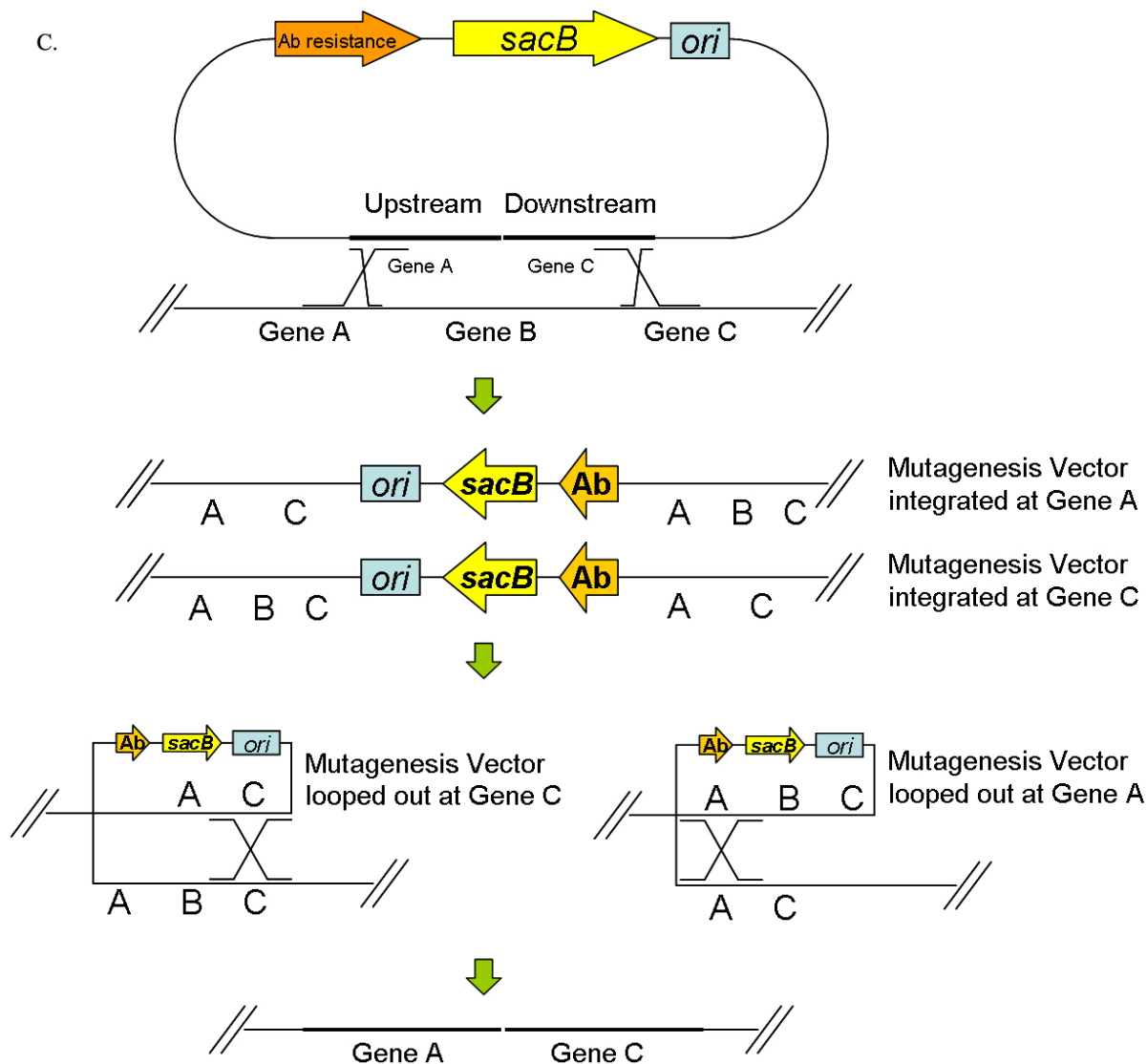
Figure 1.1. Allelic exchange strategies for *Francisella*. A. PCR substrate-based allelic exchange strategy. B. Suicide vector-based one-step allelic exchange strategy. This method generates mutants with antibiotic resistance genes marked. C. Suicide vector based two-step allelic exchange strategy. This method generates unmarked mutants. The *sacB* gene is used as a counterselectable marker in the second selection step. Diagram modified from Zahrt, T. C. *et al.* 2007.

C. Transposon random mutagenesis

A transposon is a mobile genetic element containing additional genes unrelated to transposition functions, which is the ability of the foreign DNA to move to different positions within the genome of the cell. During this process they can cause mutations and change the amount of DNA in the genome. Thus, transposons are useful tools for genetic studies of *Francisella*. To date, transposon mutagenesis libraries have been successfully created in subspecies *novicida* (34) and *tularensis* Schu S4 (76). A recent study has demonstrated the ability of generating a LVS mutagenesis library using a mariner family *himar1* transposon (65).

In early studies, insertions of some transposons used in *Francisella*, i.e. Tn10, Tn1721, were unstable, and no mutagenesis library could be generated. It was reported that bacterial gene-encoded transposase-complementing activity may function to promote the movement of these transposons (60). In 2004, Kawula *et al.* (55) described the use of a transoposome complex (transposon-transposeas complex) to create insertion mutations in LVS, which are stable. In this method a Tn5 derived EZ::TN transposon system$^{®}$ <kan-2> (Epicentre, Madison, WI) was used (**Fig 1.2A**). This method does not require the expression of transposase in the host cell. Instead a purified transposase is supplied along with the transposon. The transposase is stably associated with the transposon, but is inactive in the absence of Mg^{2+}. Magnesium ions present inside the bacterium activate the transposase following transformation (55). The strategy was modified and used to create insertional mutation libraries of *novicida* (34, 99) and Schu S4 (type A) (76) (**Fig. 1.2A**). In 2006, Maier *et al.* reported work that used a similar strategy to develop a Himar1-based random mutagenesis system for *F. tularensis* (*HimarFT*) (65) (**Fig 1.2B**). Their results showed the insertion was stable and the orientation of integration was unbiased. Himar-1 was chosen because the insertion sequence requires a TA dinucleotide (80); the *F. tularensis* genome is composed of approximately 70% TA nucleotides (72).

A.

EZ-Tn5™ <R6Kγ *ori* / KAN-2> Transposon
(2001 bp.)

B.

HimarFT

pFNLTP16H3 (9,681 bp)
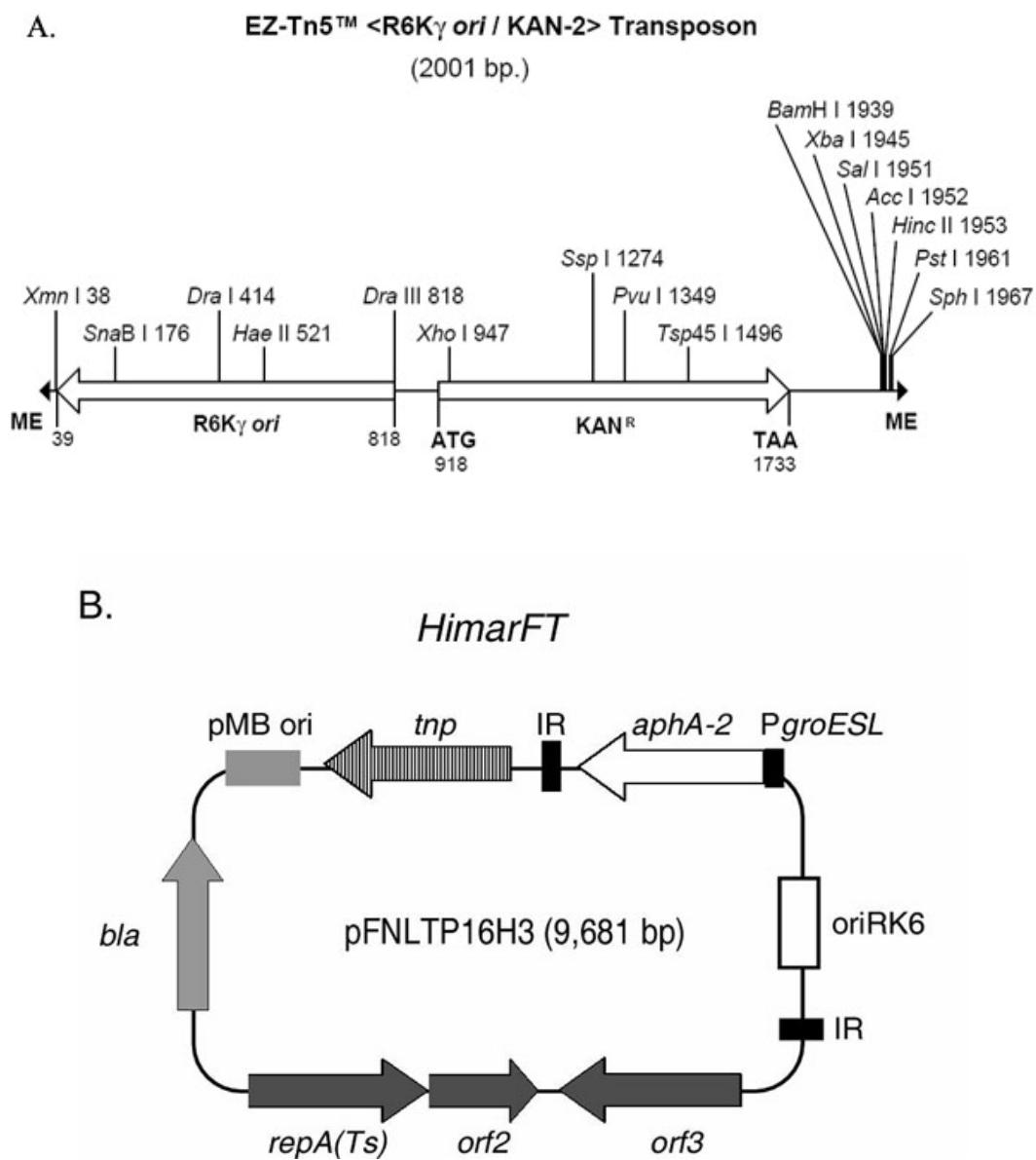
Figure 1.2. Transposition systems for *Francisella*. A. EZ-TN5™ based *in vitro* transposition system, which contains an R6Kγ conditional origin and the Tn903 kanamycin resistance gene. (Epicentre EZ-TN5™ Transposon User Manual). B. *Francisella* transposition vector that encodes the *HimarFT* transposon and the associated kanamycin resistance cassette. (Zahrt, T. C. *et al.* 2007).

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1.2. Introduction

Lack of suitable genetic tools have hampered the study of biology and molecular pathogenesis of *F. tularensis* subspecies *holarctica* and *tularensis*. With the development of molecular biology technology, some genetic tools have been proven to work in *Francisella*. Prior to this study, several research groups have published mutagenesis methods for *Francisella*. Nano *et al.* (60) reported site-directed mutagenesis method in *F. tularensis* subsp. *novicida* using a linear PCR substrate (60). Sjostedt *et al.* (42) developed the suicide conjugative *sacB* plamid pPV. This plasmid encodes chloramphenicol resistance and was shown to work in both LVS (42) and Schu S4 strains (105). However, due to the spontaneous *sacB* mutants, the pPV plasmid is not very convenient to use. The primary objective of our study aims to develop an alternate site-directed mutagenesis system that is easier to use in *F. tularensis* type A and type B strains.

Although the makeup of the poorly characterized capsule encircling *F. tularensis* is not known, a gene cluster (FTT0789−FTT0801) (**Fig 1.3**) in *F. tularensis* type A and type B strains has been identified that could encode a polysaccharide distinct from the lipopolysaccharide O-antigen (59). The gene functions and similarity are listed in **Table 1.2**. In this study, three genes in this locus were deleted by allelic replacement. One gene is open reading frame (ORF) FTT0793 that may encode for an ABC transporter in this locus, and could be involved in exopolysaccharide or capsule export. Mutagenesis of this putative gene may result in a mutant that is attenuated due to the lack of export of polysaccharide, but still make the antigen in the cell and hence induce an immune response in the host. ORFs FTT0798 and FTT0799 may encode for a galactosyl transferase and a mannosyl transferase, respectively. They were selected for deletion in case the transporter is compensated by the many other *Francisella* ATPases. By studying the phenotypic effects of the mutants, we aimed to investigate the role of this putative exopolysaccharide locus in *F. tularensis* capsule biosynthesis.
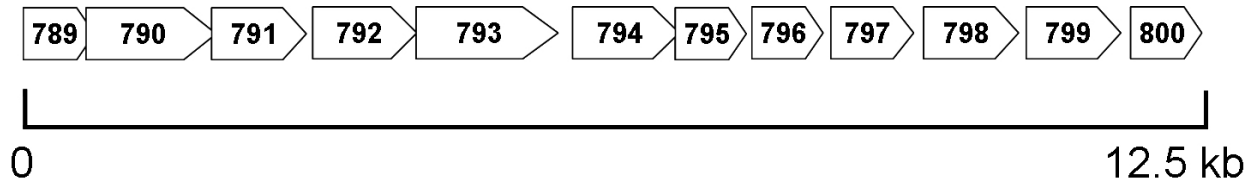
Figure 1.3. Putative *Francisella tularensis* exopolysaccharide locus in type A and type B stains. ORFs FTT0793, FTT0797 and FTT0798 were selected as the mutagenesis targets.

SPECIFIC AIMS:

♦ **To develop a new site-directed mutagenesis system in *Francisella tularensis*.**

♦ **Investigate the role of the putative exopolysaccharide locus (FTT0789−FTT0801) with this method.**

Table 1.2. Gene products and product similarity of the locus FTT0789-FTT0800

ORF name	Size	Product	Similarity
FTT0789	669 bp	D-ribulose-phosphate 3-epimerase	Similar to Ribulose-phosphate 3-epimerase (225 aa); 65.76% identity in 222 aa.
FTT0790	1395 bp	Glycosyltransferase	Similar to putative glycosyltransferase from *Lactobacillus rhamnosus* (466 aa); 33.33% identity in 399 aa overlap
FTT0791	1020 bp	UDP-glucose 4-epimerase	
FTT0792	1230 bp	Glycosyltransferase	Similar to Glycosyltransferase WbpZ from *Pseudomonas aeruginosa* (381 aa); 38.72% identity in 408 aa overlap.
FTT0793	1683 bp	ATP-binding membrane transporter	Similar to ATP-binding protein of ABC transporter from *Synechococcus elongatus* (*Thermosynechococcus elongatus*) (610 aa); 35.15% identity in 566 aa overlap.
FTT0794	1287 bp	Phosphoserine phosphatase	Similar to phosphoserine phosphatase from *Helicobacter hepaticus* (199 aa); 55.38% identity in 195 aa overlap (18-212:4-198) and to hypothetical protein from *Helicobacter hepaticus* (213 aa); 57.21% identity in 208 aa overlap (221-428:6-213). Seems to be two genes in some organisms.
FTT0795	684 bp	SAM-dependent methyltransferase	Similar to hypothetical protein cfa from *Helicobacter hepaticus* (210 aa); 44.66% identity in 206 aa overlap.
FTT0796	762 bp	Lipooligosaccharide cholinephosphotransferase	

ORF name	Size	Product	Similarity
FTT0797	960 bp	galactosyl transferase	Similar to galactosyl transferase from *Streptococcus pneumoniae* (323 aa); 40.17% identity in 234 aa overlap.
FTT0798	1008 bp	galactosyl transferase	Similar to galactosyl transferase from *Streptococcus pneumoniae* (323 aa); 34.45% identity in 238 aa overlap.
FTT0799	1014 bp	second mannosyl transferase	Similar to second mannosyl transferase from *Salmonella enterica* (336 aa); 35.45% identity in 330 aa overlap.
FTT0800	663 bp	haloacid dehalogenase	Similar to hydrolase, haloacid dehalogenase-like family from *Brucella suis* (234 aa); 33.48% identity in 218 aa overlap.

CHAPTER 2

Material and Methods

2.1. Bacterial strains and plasmids. The bacterial strains, plasmids and primers used in this work and their sources are listed in **Table 2.1**. *F. tularensis* LVS, which was obtained as a vaccine vial from Dr. May Chu, Centers for Disease Control, was subcultured to chocolate agar, and the cells were suspended in sterile skim milk and stored at -80 °C.

Table 2.1. Bacterial strains, plasmids, and DNA primers used in this study

Strain, plasmid or primer	Characteristics[*]	Reference or source
Bacterial strains		
E. coli DH5α	F⁻ φ80d *lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ⁻ *thi-1 gyrA96 relA1*	Invitrogen
Solopack competent cell	F⁻ φ80d T1ʳ T5ʳ *lacZ*ΔM15 *recA endA*	Stratagene
F. tularensis LVS	subsp. *holarctica* live vaccine strain	Dr. May Chu, CDC
F. tularensis LVS WbtIG191V P17	LPS O-antigen mutant with enhanced extracellular polysaccharide production.	Dr. Inzana's Lab
F. tularensis LVS ΔFTT0793	Kmʳ, *FTT0793*, which may encode for an ABC transporter, were replaced by a kanamycin resistance gene by allelic exchange.	This study
F. tularensis LVS ΔFTT0798-0799	Kmʳ, *FTT0798* and *FTT0799*, which may encode for galactosyl transferase and mannosyl transferase, were replaced by a kanamycin resistance gene by allelic exchange.	This study
Plasmids		

Strain, plasmid or primer	Characteristics[*]	Reference or source
pSC-A	Cloning vector; Amp^r Stp^r	Stratagene
pUC19	Cloning vector; Amp^r	Invitrogen
pUC4K	Kanamycin gene donor; Km^r	
pFNLTP6	Complementation shuttle vector; Km^r, Amp^r	(64)
pSC-FR	2.6 kb PCR fused flanking regions of target gene *FTT0793* cloned into pSC-A as a intermediate plasmid Amp^r Stp^r	This study
pSC-0793	1.6 kb kanamycin resistant gene digested from pUC4K cloned into pSC-FR to make the suicide mutagenesis vector; Amp^r, Km^r	This study
pSC-FR2	2.5 kb PCR fused flanking regions of target gene *FTT0798-0799* cloned into pSC-A as a intermediate plasmid Amp^r Stp^r	This study
pSC-798-799	1.6 kb kanamycin resistant gene digested from pUC4K cloned into pSC-FR2 to make the suicide mutagenesis vector; Amp^r, Km^r	This study
DNA primers		
0792_forward_SpeI	5'- GG**ACTAGT**TTTATACATGAGCTATC -3'; In bold is *Spe*I restriction site; PCR primer for *FTT0793* knockout	This study
0793_Reverse_StuI	5'- GA**AGGCCT**ACAAAAGGGATAACAGCTC -3'; In bold is *Stu*I restriction site; PCR primer for *FTT0793* knockout	This study
0793_Forward_StuI	5'-GAGCTGTTATCCCTTTTGT**AGGCCT**TCCAATAGCACATAGACTTTCTACT -3'; In bold is *Stu*I restriction site; PCR primer for FTT0793 knockout	This study

Strain, plasmid or primer	Characteristics*	Reference or source
0794_reverse_SalI	5'- CGC**GTCGAC**AAAGTTCTTAGCACGTA -3'; In bold is *Sal*I restriction site; PCR primer for *FTT0793* knockout	This study
FtABC_INSF	5'- GG**ACTAGT**AAGTATTTCATTGGTATTTT -3'; In bold is *Spe*I restriction site; forward PCR primer for *FTT0793* gene check.	This study
FtABC_INSR	5'- GG**CCCGGG**AGAACTAACAAAGTAAC -3'; In bold is *Sma*I restriction site; reverse PCR primer for *FTT0793* gene check.	This study
Kan_CHK_F	5'- AAGTTGGGTAACGCCAGGGTTTTCC -3'; forward PCR primer for kanamycin resistant gene amplification.	This study
Kan_CHK_R	5'- ATTAGGCACCCCAGGCTTTACACTT -3'; reverse PCR primer for kanamycin resistant gene amplification	This study
iglC_F	N/A; forward PCR primer for *iglC* gene amplification.	This study
iglC_R	N/A; reverse PCR primer for *iglC* gene amplification.	This study
FTT0797_F_SalI	5'-GC**GTCGAC**TATCAAAGTTGCACCTAG-3' In bold is *Sal*I restriction site; PCR primer for *FTT0798-0799* knockout.	This study
FTT0798_R_StuI	5'-GA**AGGCCT**CTAGCTAATGCCTGCCCA-3' In bold is *Stu*I restriction site; PCR primer for *FTT0798-0799* knockout.	This study
FTT0799_F_StuI	5'-GGATCGTTTTGCTAAATC**AGGCCT**TCAGAAGTT TTACAGGAAATAGCA-3' In bold is *Stu*I restriction site; PCR primer for *FTT0798-0799* knockout.	This study

Strain, plasmid or primer	Characteristics[*]		Reference or source
FTT0800_R	5'-ATGTAATCCAACACTCAGATGCAAA-3' primer for *FTT0798-0799* knockout	PCR	This study
0799_CHK_F	5'-AATAGATACGCGCTGGCAAGGC-3' Forward PCR primer for *FTT0799* gene check.		This study

[*]Amp[r], Km[r], Stp[r], and Cm[r], resistance genes to ampicillin, kanamycin, streptomycin and chloramphenicol, respectively.

2.2. Cultivation of bacteria. *Escherichia coli* DH5α (Invitrogen™, Carlsbad, CA) and StrataClone™ Solopack® (Stratagene™, La Jolla, CA) competent cells were grown in Luria–Bertani (LB) medium (Becton, Dickinson and Company, Franklin Lakes, NJ) at 37°C containing, as appropriate, 100 µg ampicillin (Amp) ml^{-1}, or 50 µg kanamycin (Km) ml^{-1} for selection of recombinant strains. *E. coli* DH5α. and Solopack® competent cell frozen stocks were made by resuspending bacteria in LB broth containing 30% glycerol; stocks were kept at -80°C. *F. tularensis* strains were grown in Difco™ Brain Heart Infusion broth (Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 0.15% L-cysteine hydrochloride monohydrate (Sigma-Aldrich, St. Louis, MO) (BHI-C) at 37°C. They were also cultured in Trypticase Soy Broth (TSB) (Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 0.15% L-cysteine hydrochloride monohydrate (TSB-C). For culture on agar plates, 5% (v/v) sheep blood was added to BHI-C agar (BHI-BC), and the cultures were incubated at 37°C in 5% CO_2 or a candle jar, unless otherwise stated. Frozen stocks were made by resuspending bacteria cells in 15% (w/v) sterile skim milk; stocks were kept at -80°C. Congo red agar plates were made by adding congo red dye to BHI-C to a final concentration of 75 µg/ml in BHI-C. All LVS related experiments were carried out under Biosafety level-2 conditions in a class II biological safety cabinet.

2.3. Preparation of *Escherichia coli* DH5α chemically competent cell. Five-10 ml of *E. coli* DH5α overnight culture was diluted to a volume of 100 ml to an OD_{600} of approximately 0.025, which was then incubated at 37ºC with shaking until the OD_{600} reached 0.5. The cells were chilled on ice for several minutes, and then harvested by centrifugation for 10 min at 2230 RCF at 4 ºC. The cells were then washed with 20 ml ice cold 0.1 M $MgCl_2$, followed by sedimenting the cells at 2230 RCF. Cells were washed again with 20 ml ice cold 0.1 M $CaCl_2$. After holding stationary for 20 min, the cells were harvested at 2230 RCF at 4ºC. The collected cells were resuspended in 4.24 ml 0.1 M $CaCl_2$ plus 0.76 ml cold glycerol. Fifty μl aliquots were distributed into 1.5 ml microcentrifuge tubes. Competent cells were stored at -80ºC.

2.4. DNA manipulations. All DNA manipulations, including plasmid purifications, restriction digestions, ligations and gel electrophoresis were performed according to the established protocols (81). Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs (New England Biolabs, Ipswich, MA). QIAprep® Spin Miniprep and QIAquick® Gel Extraction Kits (Qiagen, Valencia, CA) were used to prepare *E. coli* plasmid DNA as recommended by the manufacturer. Low melting point agarose was obtained from Sigma™ (Sigma-Aldrich, St. Louis, MO). The PUREGENE™ DNA Isolation Kit (Gentra Systems, Minneapolis, MN) was used to purify genomic DNA from *F. tularensis*. The StrataClone™ PCR cloning kit (Stratagene™, La Jolla, CA) was used for cloning. DNA sequencing was performed at core laboratory facilities at the Virginia Bioinformatics Institute (VBI), Virginia Tech.

2.5. Transformation

2.5.1. Cryotransformation for *F. tularensis* LVS. *F. tularensis* strains were grown on

BHI-BC chocolate agar for 48 h, suspended in 0.2 M KCl. Bacteria were harvested by centrifugation and washed with 0.2 M KCl twice. After the last wash, the bacteria were suspended in 0.2 M KCl at approximately 4×10^{10} colony forming units (CFU) ml^{-1}. The concentration was adjusted to 1×10^{11} CFU/ml in KCl (for *F. tularensis*, OD at 600 nm of $1 = \sim 1 \times 10^{9}$ CFU/ml) and 25 µl of cell suspension (approximately 2.5×10^{9} CFU) was added to 25 µl transformation buffer (0.2 M MgSO$_4$, 0.1 M Tris acetate buffer pH 7.5), and 2-4 µg DNA (suicide vector) was added to the cell suspension. The mixture was incubated at room temperature for 10 min, then incubated in liquid nitrogen for 5 min, and finally incubated at room temperature for 10 min to allow cells to thaw (73). The cells were then cultured on BHI-C chocolate agar without selection and incubated at 37˚C overnight. After recovery incubation, the cells were scraped into 1 ml of phosphate buffered saline, pH 7.4 (PBS). One µl was cultured onto chocolate agar with appropriate antibiotic, and the plates were incubated at 37ºC, in 5% CO$_2$ until colonies appeared (usually 3-5 days).

2.5.2 Electroporation for *F. tularensis* LVS. Bacteria were grown from frozen stock cultures onto BHI-C at 37˚C for 2 days in a candle jar. Two full loops of bacteria were inoculated into 100 ml TSB-C (Trypticase Soy Broth w/ 0.15% cysteine) and incubated with shaking at 37°C overnight. The cells were diluted to a final densitometric concentration of 30-40 Klett units in 100 ml TSB-C, and shaken at 200 rpm at 37˚C until the density reached 100-110 Klett units. The cells were chilled on ice for 30 min, then 40 ml of culture was subjected to centrifugation at 9,000 RCF for 10 min, and resuspended in 40 ml of 500 mM sucrose. The cells were washed with the sucrose wash buffer for 2-3 times. After washing, the bacteria were resuspended in 4 ml of sucrose buffer, and 1 ml was aliquot into each of 4 microtubes. The cells were harvested again by centrifugation for 5 min at 9,000 RCF for 1 min, and resuspend into 60 µl of sucrose buffer. Approximately 200 ng DNA was added to 60 µl of washed cell suspension. The mixture

was transferred into a 1 mm cuvette and kept on ice for 10 min. Electroporation was performed at 1.6 kv, 25 µF and 400 Ω using an Electro Cell manipulator ECM630 (BTX, Holliston, MA) (55, 64, 76). The cells were suspended in 1 ml of TSB-C, allowed to recover for 6 hrs at 37°C with shaking at 225 rpm, and 0.5 - 1µl was spread onto BHI-BC agar containing kanamycin.

2.6. Construction of mutagenesis vectors. Suicide vectors for site-directed mutagenesis were constructed containing the following elements: the pSC-A plasmid (**Fig 2.1**) backbone which contains an origin that can replicate in *E. coli* but not in *Francisella*; a kanamycin resistance gene (Tn903 kanamycin resistance gene) from pUC4K, which confers kanamycin resistance in *F. tularensis*; and the upstream and downstream flanking regions of the target gene. A region approximately 1.3 kb upstream (primer1 1 and 2) and a region downstream (Primer 3 and 4) of the target gene (Gene B as shown in Fig 2) were initially amplified by PCR (42). The N-terminal and C-terminal flanking regions were then ligated by a fusion PCR. The PCR ligated flanking region was TA cloned into pSC-A as an intermediate plasmid. The kanamycin gene was obtained by digesting pUC4K with *Pvu*II. The ~ 1.6 kb fragment was extracted and purified using a QIAquick® Gel Extraction Kit (QIAGEN, Valencia, CA). The purified kanamycin gene was then inserted into the *Stu*I-digested intermediate plasmid resulting in the final mutagenesis plasmid (**Fig 2.2**).
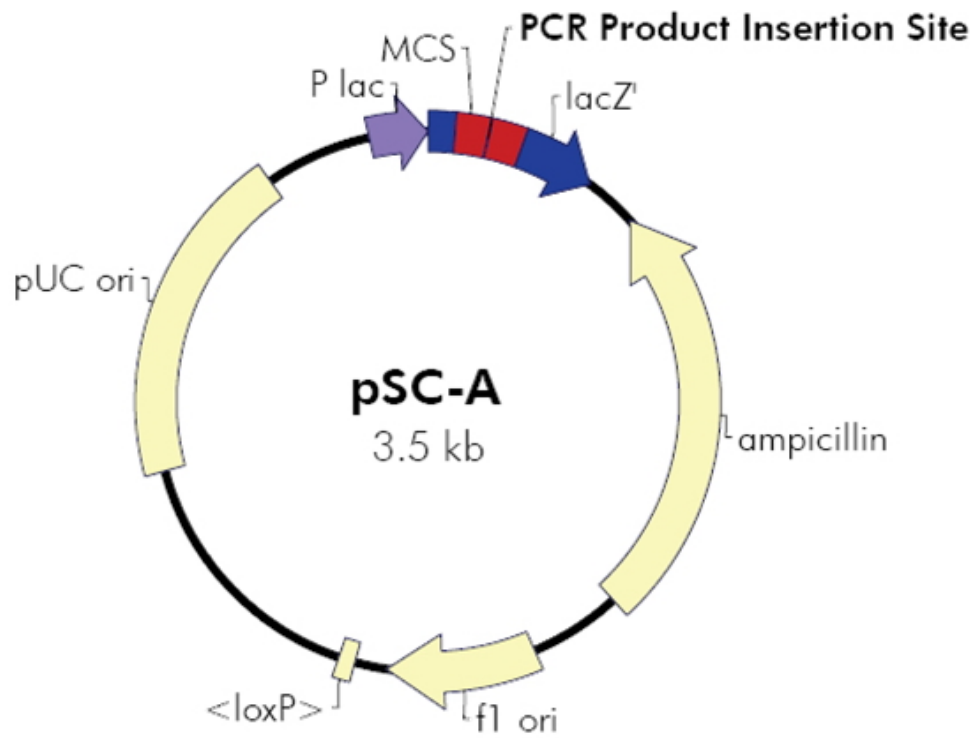
Figure 2.1. Diagram of pSC-A. The pSC-A PCR cloning vector used as the backbone of the mutagenesis plasmid.
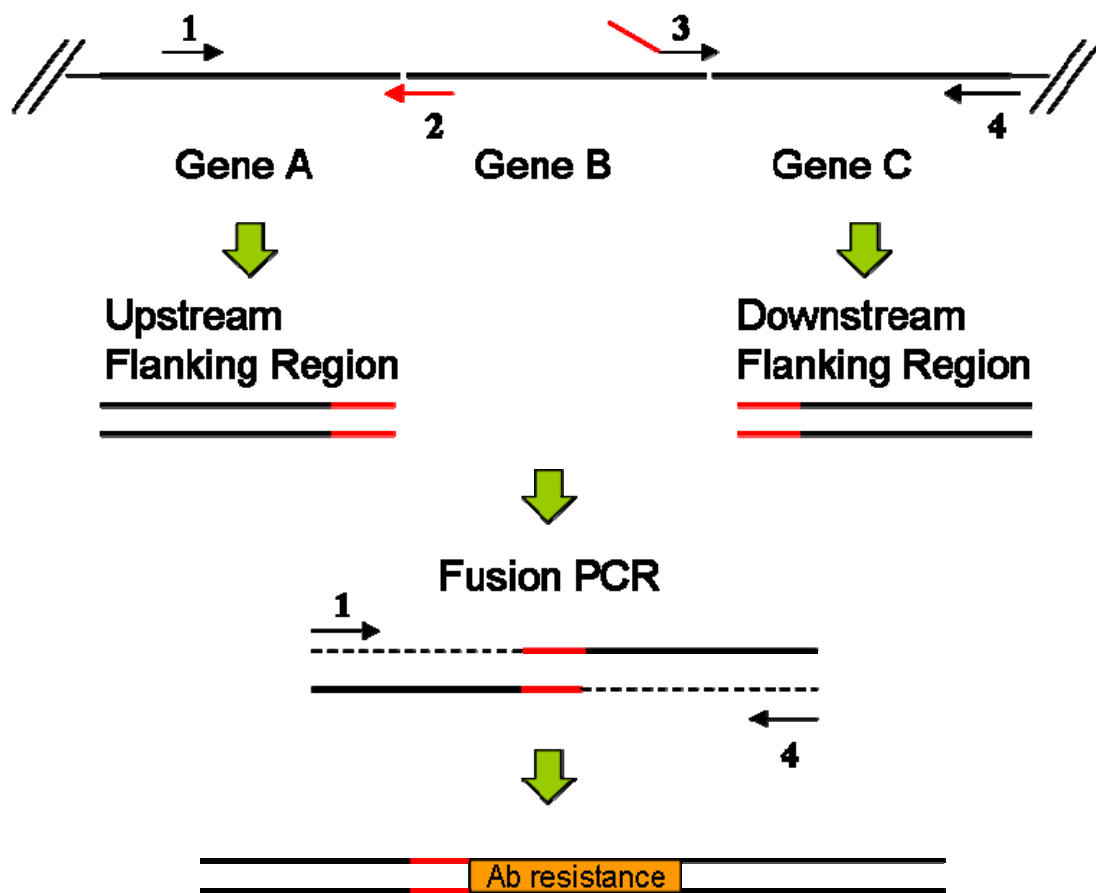
Figure 2.2. Processes for making the mutagenesis plasmid. A region upstream and a region downstream of gene B were amplified by PCR. The sequences in red are complimentary to each other. The two flanking regions were then ligated by a second fusion PCR. The product of the fusion PCR was TA cloned into pSC-A, followed by insertion of an antibiotic resistance cassette.

2.7. Mutagenesis. After creation of the mutagenesis plasmid, it was transformed into *F. tularensis* by cryotransformation. A shuttle vector pFNLTP6 (**Fig 2.3**) (64) was used as a positive control to confirm the transformation process was successful. The mutagenesis plasmid is a suicide vector in *F. tularensis*; the plasmid can only confer the antibiotic resistance if it integrates into the bacterial genome. If a double-crossover event occurs, the original gene (Gene B as shown in **Fig 2.4**.) will be replaced by the antibiotic

resistance gene (**Fig 2.4**).
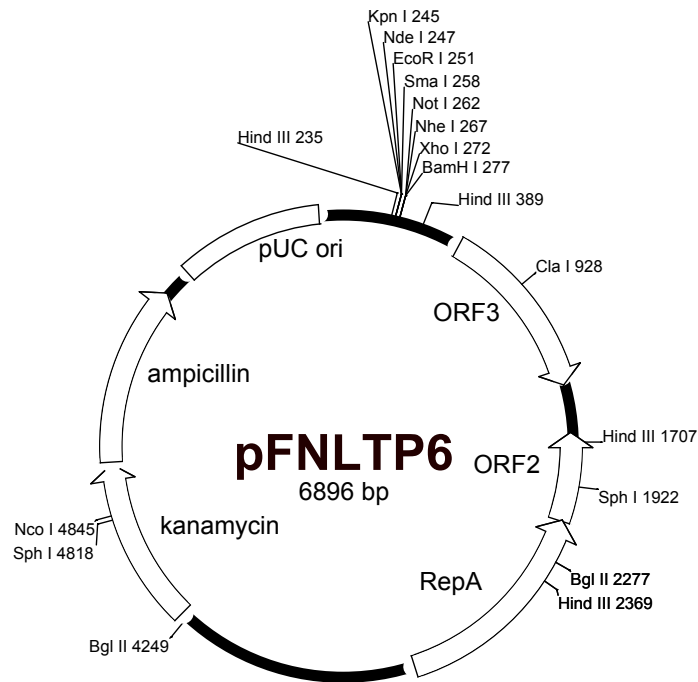
Figure 2.3. The pFNLTP6: a shuttle vector for *F. tularensis*. pFNLTP6 can replicate in *E. coli* and *F. tularensis*, and can be used as an expression vector in *F. tularensis*. In this study, pFNLTP6 was used as a positive control to confirm the transformation protocol successfully introduced plasmid into *F. tularensis*.
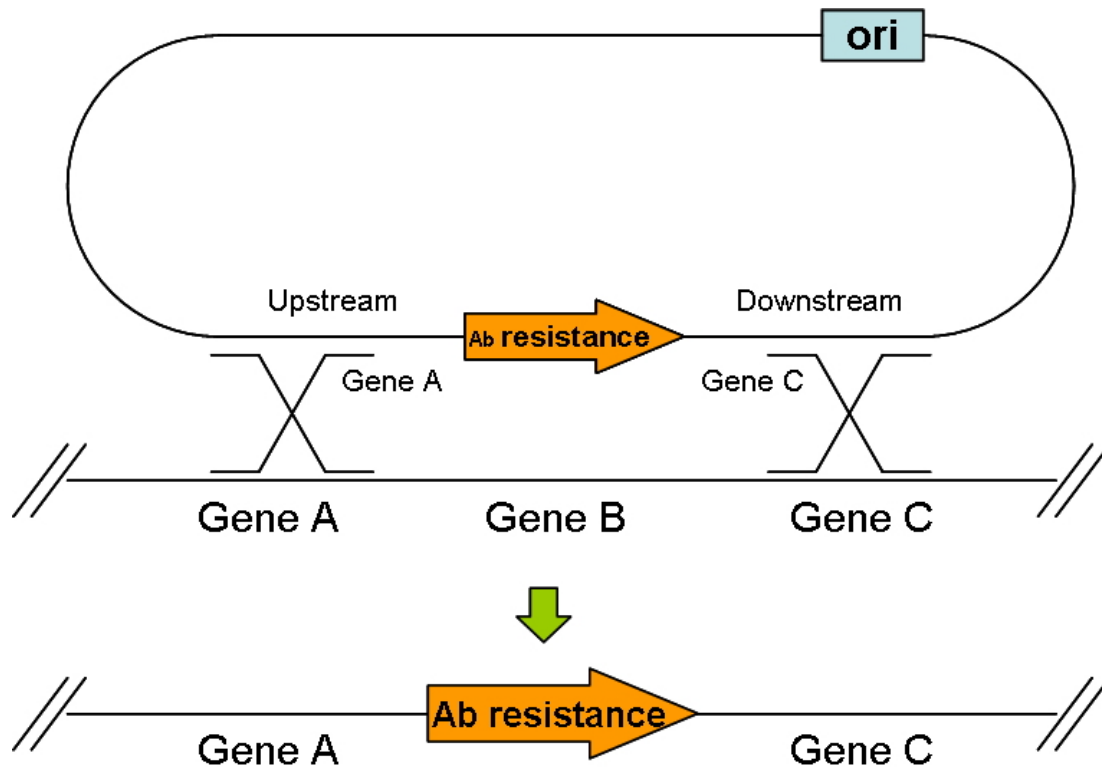
Figure 2.4. Strategy for making the allelic replacement using a suicide vector. The mutagenesis plasmid was introduced into *F. tularensis*. If a double-crossover event occurs, the target gene will be replaced by the antibiotic resistance gene.

2.8. Isolation of allelic exchange mutants. To select for mutants, the transformants were grown on BHI-BC agar supplemented with 8 μg/ml kanamycin until colonies appeared (3-5 days). A few colonies were then streaked for purity on fresh plates with antibiotics selection. The real mutants should grow well and faster on fresh antibiotic plates than spontaneous mutants. Then mutanted genomic DNA was extracted for PCR screening and DNA sequencing.

2.9 Congo red dye up-take. Congo red is a dye that can bind to neutral glycoses, including the core oligosaccharide of LPS. LVS O-antigen mutants and *F. tulaensis* subsp. *novicida* take up more dye because they lack specific dideoxy sugars present in the

O-antigen of *F. tularensis*, which appears to prevent the dye from binding to the neutral sugars in the core (62). Parent and mutants were grown on BHI-C agar containing 75 mg Congo Red L^{-1} for 3 -5 days at 37°C in 5% CO_2 prior to visual examination.

2.10. Adsorption of hyperimmune serum and enzyme linked immunosorbent assay (ELISA). 100 ml of strain LVS or a mutant was grown in BHI-C broth to mid-log phase. The culture was divided in two and the cells washed with PBS twice. The pellets were obtained by centrifugation at 12,100 RCF for 10 min, and one was held at 4°C. 1 ml of hyperimmune rabbit serum to LVS was added to the other pellet and the mixture was incubated at 4°C with vortexing about once an hour for 6 hours. After overnight incubation at 4°C, the serum was collected by centrifugation and added to the second pellet. The 4°C incubation and vortexing for 4-6 h was repeated, and the serum was then collected by centrifugation and sterilized through a 0.22 μm filter. The adsorbed serum was used as the source of primary antibodies in ELISA.

Francisella LPS and capsule were used as antigens and were purified as described below. Wells were inoculated with 100 μl of each antigen (10 μg/ml) and each antigen was tested in triplicate. Background absorbance was inhibited with blocking buffer containing 2% skim milk in PBS supplemented with 0.1% Tween 20. The absorbed and unabsorbed sera were serially diluted from 1:10-1:640 in 100 μl of PBS buffer supplemented with 2 mM $MgCl_2$ and 2% skim milk. A 1:5000 dilution of goat anti-rabbit IgG conjugated to HRP was used as the secondary antibody. Color was developed with the TMB Substrate Kit (Pierce, Rockford, IL), the reaction stopped in 5-10 min by addition of 1 M H_2SO_4 and the absorbance was determined at 450 nm using a microplate reader (VMax System, Molecular Devices Corporation, Sunnyvale, CA).

2.11. Extraction of LPS. LPS was extracted by a mini-aqueous phenol extraction

method as described (52). Briefly, bacteria grown in BHIC broth were harvested at 8000 RCF for 10 min. The cells were suspended in 400 µl distilled water and transferred to glass, screw-cap vials containing a small stir bar. Four hundred µl of 90% phenol was added to the vials and the mixture stirred at 65˚C for 15 min, and transferred to ice. The samples were transferred to microcentrifuge tubes and the phases separated by centrifugation at 9,000 RCF for 15 min at 4˚C. The aqueous (top) phase was removed and stored, 400 µl of distilled water was added to the vials containing the phenol phase, and the extraction and centrifugation repeated. The aqueous phase was added to the previous phase and sodium chloride was added to a final concentration of 0.5 M, followed by addition of 10 volumes 95% ethanol. The tubes were incubated several hours to overnight at -20˚C followed by centrifugation at 1500 RCF for 10 min at 4˚C. Insoluble material suspended in 90 µl of distilled water, plus 10 µl of 5 M NaCl was add to each tube, and 1 ml of 95% ethanol was added. The mixture was incubated at -20˚C overnight, followed by centrifugation for 5 min. The pellets were suspended in 50 µl of distilled water and stored at -20˚C.

2.12. Polyacrylamide gel electrophoresis and western blotting. The LPS electrophoretic profile was resolved by SDS-PAGE using Novex® 16 % Pre-Cast Tricine Gels (Invitrogen™, Carlsbad, CA). The Gels were transferred to a nitrocellulose membrane using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA), and the blots were blocked with murine mAb (Chemicon International, Temecula, CA) to *F. tularensis* O-antigen at 1:4000 dilution. Anti-murine IgG (heay and light chain) coupled to horseradish peroxidase (HRP; Jackson ImmunoResearch Labs, West Grove, PA) were used as secondary conjugates at 1:2000 dilution, and color was developed with the Protein Detector TMB Western Blotting Kit (KPL, Gaithersburg, MD).

2.13. Serum bactericidal assay. The bactericidal activity of 10% guinea pig serum, which contains no antibody to LVS or the mutant strain as previously described (53). The bactericidal effect of guinea pig serum was abrogated by incubation of serum at 56°C for 30 min. Heat-inactivated serum was used in the control tubes.

2.14. Virulence and immunoprotection studies in mice. To assess virulence, groups of 2 or 3 BALB/c mice 6–8 weeks old (Jackson Laboratory, Bar Harbor, ME) were challenged intraperitoneally (IP) with 5 times the LD_{50} of LVS (~ 1000 CFU per mouse) in 100 μl of PBS. All inoculation doses were confirmed by viable counts on BHI-BC agar. Animals exposed to LVS or mutant strains were maintained and cared for in an accredited ABSL-2 facility. Critically ill or surviving mice were humanely euthanatized using excess carbon dioxide.

2.15. Electron microscopy. For negative staining, *F. tularensis* strains LVS, LVS WbtIG191V P17, ΔFTT0793 mutant and ΔFTT0798-0799 transferase mutant were grown on CDMG agar for 5 days. Cells were scraped into sodium cacodylate buffer with 3% gluteraldehyde and turned end over end for 2 hours. The cells were then washed and resuspended in sodium cacodylate buffer. Cells were adhered to formvar-coated grids, stained with 0.5% uranyl acetate and viewed by a JEOL 100 CX-II (JEOL, Tokyo, Japan) transmission electron microscope.

CHAPTER 3

Results

3.1 Creation of a *F. tularensis* site-directed mutation by allelic replacement.

3.1.1 Creation of a ΔFTT0793 ABC transporter mutant (Δ0793 mutant).

3.1.1.1. Construction of FTT0793 gene mutagenesis vector.

A suicide vector for site-directed mutagenesis was constructed based on the PCR cloning vector pSC-A, which is unable to replicate in *F. tularensis*. First, regions approximately 1.3 kb upstream (primers FTT0792_forward_SpeI and FTT0793_Reverse_StuI) and downstream (FTT0793_forward_StuI and FTT0794_reverse_SalI) of the FTT0793 gene were PCR amplified (**Fig 3.1A**). The N-terminal and C-terminal flanking regions were then ligated by fusion PCR (**Fig 3.1B**). PCR conditions are listed in **Table 3.1**. The PCR reaction 1 mixture consisted of 0.5 U SpeedStar HS DNA polymerase (Takara USA, Madison, WI), 0.02 μg genomic DNA as template, 200 μM dNTP mix (Takara USA, Madison, WI), and 0.5 μM of each oligonucleotide primer in 50 μl of reaction mixture. The PCR reaction 2 mixture consisted of 0.5 U SpeedStar HS (hot start) DNA polymerase, 1 μl of each flanking region amplified from the previous PCR as template, 200 μM of dNTP mix, and 0.5 μM of each oligonucleotide primer in 50 μl. PCR primers (FTT0792_forward_SpeI and FTT0794_reverse_SalI) for the fusion PCR were added after the initial 15 cycles. The ligated fragment was then TA cloned into pSC-A. The resulting plasmid was designated pSC-FR (**Fig 3.1C**). The newly constructed plasmid was then confirmed by restriction digestion, and then digested with *Stu*I, which was designed into the reverse and forward primer (FTT0793_forward_StuI and FTT0793_Reverse_StuI) of the upstream and downstream flanking regions, respectively. A kanamycin resistance gene was used as a selectable marker. The antibiotic resistance gene was excised from pUC4K as a 1.6 kb *Pvu*II fragment and then ligated to the *Stu*I-digested pSC-FR plasmid. Antibiotic resistant transformants were obtained by

selection on LB plates containing 50 μg/ml kanamycin. The new plasmid was confirmed by restriction digestion and was designated as pSC-0793 (**Fig 3.1D**).

Table 3.1. PCR protocols

PCR protocols:	
PCR 1: Flanking regions amplification	**PCR 2: Fusion PCR**
Step 1: 95˚C 30s	Step 1: 95˚C 30s
Step 2: 65˚C 30s	Step 2: 65˚C 30s
Step 3: go to step 1 for 30 cycles	Step 3: go to step 1 for 15 cycles
Step 4: 4˚C Hold	Add primers 1 and 4 to PCR mix
	Step 1: 95˚C 30s
	Step 2: 65˚C 1 min
	Step 3: go to step 1 for 20 cycles
	Step 4: 4˚C Hold
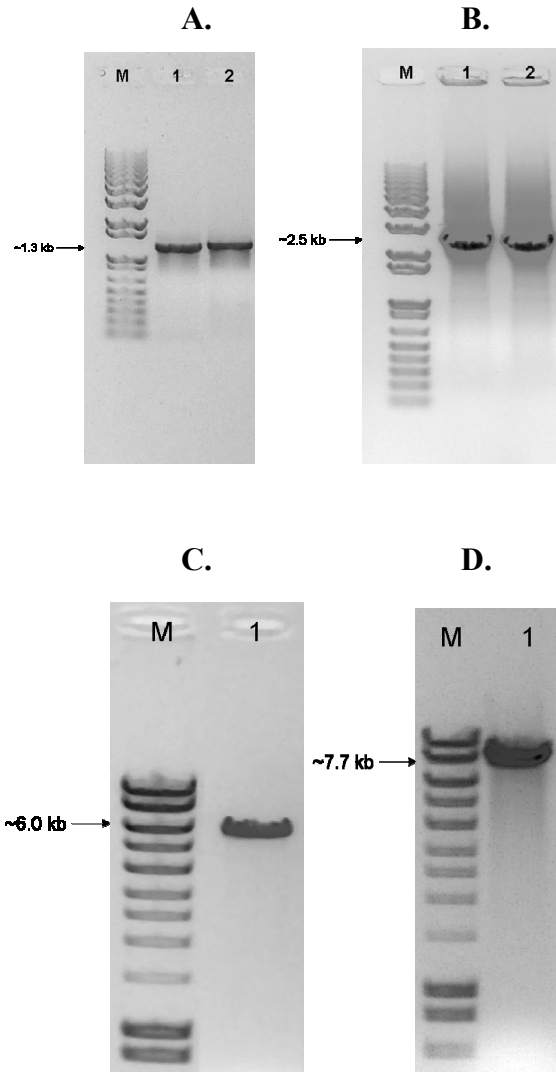
Figure 3.1. **Creation of the mutagenesis plasmid for deletion of FTT0793.** Fused flanking regions were first cloned into a PCR cloning vector and then an antibiotic resistance gene was inserted between them. A: PCR-amplified FTT0793 upstream and downstream flanking regions of about 1.3 kb each. B: the two flanking regions were ligated by a fusion PCR. C: the ligated flanking regions were inserted into a PCR cloning vector pSC-A, which resulted in the intermediate plasmid pSC-FR. D. The kan resistance gene obtained from pUC4K was inserted in the *Stu*I site of the ligated flanking regions. The new plasmid was designated as pSC-0793, which is the mutagenesis plasmid.

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3.1.1.2. Selection of the Δ0793 mutant

The suicide vector pSC-0793 was transformed into *F. tularensis* LVS by cyrotransformation. A shuttle vector pFNLTP6 (64) was used as a positive control to make sure the transformation process was successful. pFNLTP6 can be easily introduced into *F. tularensis* by either cryotransformation or electroporation with high efficiency (data not shown). This plasmid can also be used as an expression vector in *F. tularensis* (62). Transformants were first recovered on BHI-BC chocolate agar plates without selection overnight, and then spread on BHI-BC agar plates supplemented with 8 μg/ml kanamycin. The plates were then incubated in a candle jar at 37˚C. Tiny colonies were observed on the fourth day of incubation. On the fifth day, a total of 45 colonies were picked and subcultured on fresh kan plates. After overnight incubation, only 4/45 colonies grew well. They were selected as mutant candidates for further tests and the genomic DNA was extracted.

3.1.1.3. Mutant confirmation

To analyze the results of the recombination event by PCR, four different pairs of primers were used: 0792_forward_SpeI and 0794_reverse_SalI are specific for the entire mutagenesis region; FtABC_INSF and FtABC_INSR are specific for segment of the FTT0793 gene, Kan_CHK_F and Kan_CHK_R are specific for the kan resistance cassette. In addition, a fourth pair of primers iglC_F and iglC_R, which are specific for the *igl*C gene was used to eliminate contaminants. PCRs were then performed using these pairs of primers and the mutant genomic DNA as templates.

Step 1. Primers Kan_CHK_F and Kan_CHK_R were used. The ~1.3 kb kan fragment could be amplified from the genomic DNA of all 4 mutants selected (**Fig 3.2 left panel,**

lane 1-4.). Primers FtABC_INSF and FtABC_INSR were then used, and no product was amplified from PCR reactions with genomic DNA from any of the mutants as templates (**Fig 3.2 right panel, lanes 6-9.**). The results indicated FTT0793 no longer existed in the genomes of the selected mutants.
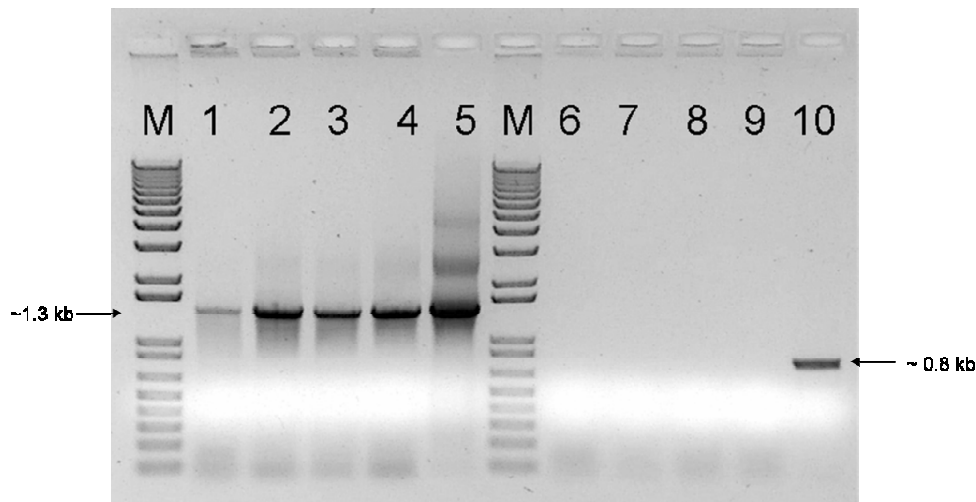
Figure 3.2. Confirmation of Δ0793 in each mutant, step 1. Lane 1-4: using primers Kan_CHK_F and Kan_CHK_R, kan cassette fragment (~1.3 kb) could be amplified from genomic DNA of all four mutants; lane 5: pSC-0793 was used as a positive control; lane 6-9: FTT0793 could not be amplified from the mutants; lane 10: LVS genomic DNA was used as a positive control for amplification of the ~0.8 kb FTT0793 fragment.

Step 2. The entire mutagenesis region was amplified by PCR with primer 0792_forward_SpeI and 0794_reverse_SalI from the mutants and the parent strain (**Fig 3.3A**). The amplified fragments were then purified and used as templates for another PCR. In the second PCR, primers Kan_CHK_F and Kan_CHK_R for amplification of the mutated fragments and primers FtABC_INSF and FtABC_INSR for amplification of the parent strain fragment were used. As expected, ~1.3 kb bands were amplified from the mutants and an approximately 0.8 kb band was amplified from parent strain (**Fig 3.3B**). The results supported that the amplification of the kan resistance cassette from the mutant

candidates' genomic DNA were not from false priming.
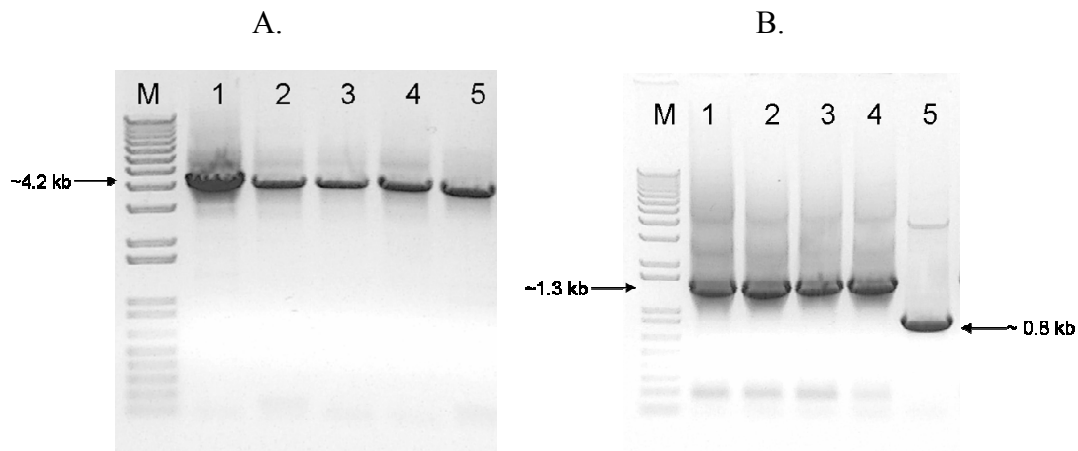
Figure 3.3. Δ0793 mutant confirmation, step 2. A. Lanes 1-4: the entire mutagenesis region could be amplified from genomic DNA of the mutants (~4.2 kb); lane 5, LVS genomic DNA was used as a positive control. B. Lanes 1-4: The kan resistance cassette could be amplified with PCR products from the previous experiment; lane 5, control DNA from previous experiment showing amplification of FTT0793.

Step 3.

Another pair of primers specific for the *iglC* gene (sequences not shown), which is a *Francisella*-specific gene, was used. The expected ~0.6 kb band was observed from all of the mutants (**Fig 3.4**). These results confirmed the transformants were not contaminants.
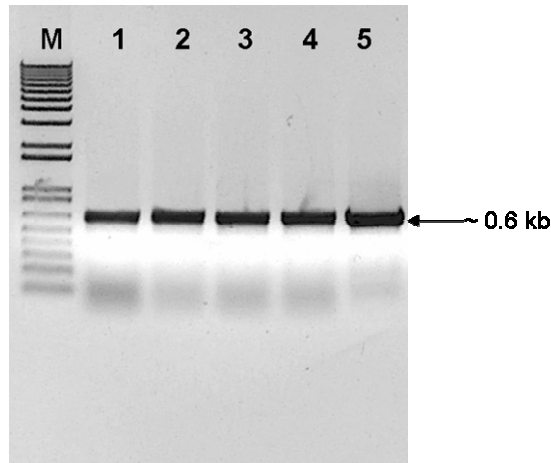
Figure 3.4. Δ0793 mutant confirmation, step 3. The *iglC* gene could be amplified from all mutants (lanes 1-4, ~0.6 kb) and the parent strain LVS (lane 5).

3.1.2. Creation of a ΔFTT0798-0799 transferase mutant (Δ0798-0799 mutant)

3.1.2.1. Creation of FTT0798-0799 mutagenesis vector

In case the activity of the ABC transporter in the pseudo LPS locus was compensated by other *Francisella* ATPases, a galactosyl-mannosyl transferase mutant was also created. The procedure for making the transferase mutant followed the one used to make the Δ0793 mutant. For this mutant, the *F. tularensis* LVS WbtIG191V P17 strain was used as the parent strain. This strain has enhanced capsule production, which may facilitate identification of capsule loss if the mutated transferases are required for encapsulation. A region approximately 1.3 kb upstream (primer FTT0797_F_SalI and FTT0798_R_StuI) of the FTT0798 gene and a 1.3 kb region downstream (FTT0799_F_StuI and FTT0800_R) of the FTT0799 gene were PCR-amplified and then ligated by fusion PCR. In the fusion PCR, Taq polymerase was used instead of the SpeedStar HS polymerase. PCR conditions are listed in **Table 3.2**. PCR reaction 1 mixture consisted of 0.5 U Phusion Hi-Fi DNA polymerase (Finnzymes, Espoo, Finland), 0.02 µg genomic DNA as template, 200 µM dNTP mix (New England Biolabs, Ipswich, MA), 0.5 µM of each oligonucleotide primer in 50 µl of reaction mixture. The PCR reaction 2 mixture

consisted of 1U Taq DNA polymerase (Eppendorf, Westbury, NY), 1 µl of each flanking region amplified from the previous PCR as template, 200 µM dNTP mix (New England Biolabs, Ipswich, MA), and 0.5 µM of each oligonucleotide primer in 50 µl. PCR primers (FTT0797_F_SalI and FTT0800_R) for the fusion PCR were added after the initial 15 cycles. The ligated flanking regions was also cloned into pSC-A, resulting in the intermediate mutagenesis plasmid pSC-FR2. The mutagenesis plasmid was completed by inserting the kan resistance gene into pSC-FR2, which was designated pSC-798-799.

Table 3.2. PCR protocols

PCR protocols:	
PCR 1: Flanking regions amplification	**PCR 2: Fusion PCR**
Step 1: 98 ˚C 30 s	Step 1: 95 ˚C 1 min
Step 2: 98 ˚C 10 s	Step 2: 95 ˚C 30 s
Step 3: 64 ˚C 40 s	Step 3: 56 ˚C 40 s
Step 4: 72 ˚C 1 min 15 s	Step 4: 68 ˚C 1 min 10 s
Step 5 go to step 2 for 30 cycles	Step 5 go to step 2 for 15 cycles
Step 6: 72 ˚C 7 min	Step 6: 68 ˚C 10 min
Step 7 Hold at 4˚C	Add PCR primers
	Step 1: 95 ˚C 1 min
	Step 2: 95 ˚C 30 s
	Step 3: 56 ˚C 40 s
	Step 4: 68 ˚C 2 min
	Step 5 go to step 2 for 20 cycles
	Step 6: 68 ˚C 10 min
	Step 7 Hold at 4˚C

3.1.2.2. Selection of the Δ0798-799 mutant.

The same selection procedure was used that was described for selection of the Δ0793 mutants. In total, 5 mutant colonies were obtained. Their genomic DNA was extracted for further testing.

3.1.2.3. Confirmation of mutagenesis.

Confirmation of the mutagenesis was as described for confirmation of the Δ0793 mutant, but with some modification, as described below.

Step 1. Primers Kan_CHK_F and Kan_CHK_R were used. The kan resistance cassette was amplified from genomic DNA templates from all 5 mutant colonies. Primers Kan_CHK_F and FTT0800_R were then used in a second PCR. An approximately 2.8 kb product (kan resistance cassette plus the downstream flanking region) could be amplified from all the mutant colonies (**Fig 3.5**). The results indicated the kan resistant gene was integrated into the bacterial genome in place of FTT0798 and FTT0799, and was not a result of false priming.

A.

B.
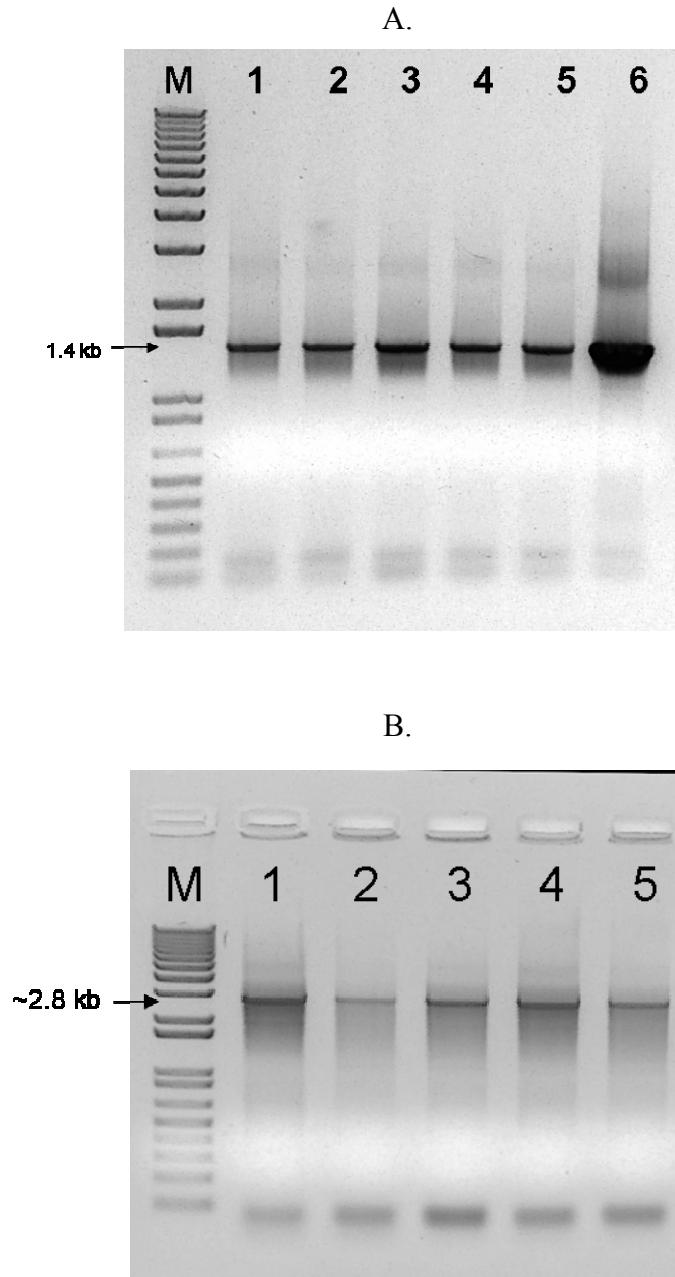
Figure 3.5. Confirmation of Δ0798-0799 mutation, step 1. A. Lane M, 1 kb plus DNA ladder. Lanes: 1-5, kan resistance gene amplified from the 5 mutant colonies; 6, kan resistant gene amplified from Δ0793 mutant as a control. B. The kan resistance gene plus the downstream flanking region (~2.8 kb) was amplified from all 5 mutant candidates.

Step 2. A primer 0799_CHK_F was specifically designed to amplify the region targeted

for deletion and was used with FTT0800_R. Mutant genomic DNA and parent strain genomic DNA were used as templates. If the target region was deleted during the expected double cross-over event, no product would be amplified, whereas an approximately 2.1 kb product should be amplified from the parent strain. As expected, no major product was amplified from genomic DNA of the mutants or the mutagenesis plasmid. An ~2.1 kb product was amplified from the parent strain (**Fig 3.6**). The results indicated the target region was deleted from the genomes of the mutants, and that the deletion resulted from a double cross-over event.
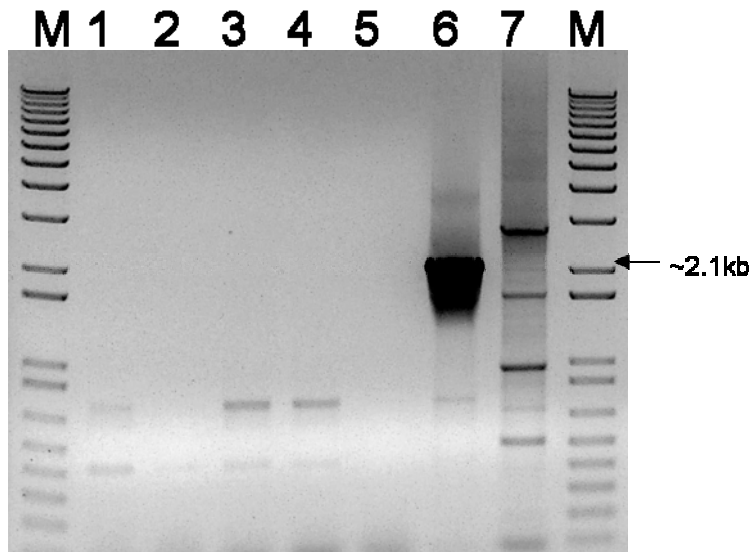
Figure 3.6. Confirmation of Δ0798-0799 mutation, **step 2.** Lane M, 1 kb plus DNA ladder. Lanes 1-5, no major product was amplified. Miscellaneous bands may be due to false priming. Lane 6, parent strain genomic DNA used as template showing amplification of ~2.1 kb band. Lane 7, mutagenesis plasmid used as template. No correct product. Small sized extra bands may be due to false priming.

Step 3.

The *iglC* gene could be amplified from each mutant (**Fig 3.7**), and DNA sequencing of the region (not shown) was used to confirm the DNA was from *F. tularensis* and were mutants.
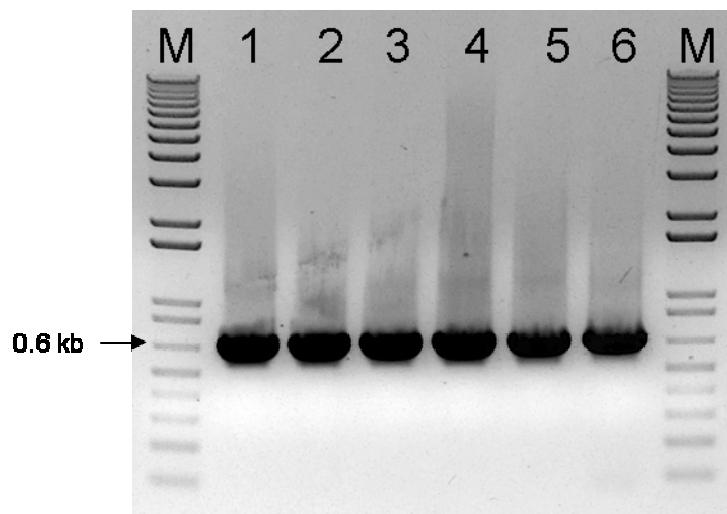
Figure 3.7. Confirmation of Δ0798-0799 mutation, step 3. The *iglC* gene could be amplified from each mutant (lanes 1-5, ~0.6 kb) and the parent strain (lane 6).

3.2. Mutant phenotypic tests

3.2.1. Congo red up-take assay.

Congo red is a dye that can bind to neutral glycoses in the carbohydrate region of the LPS or capsule. Type A and B *F. tularensis* strains do not take up this dye well, presumably due to the type and amount of dideoxy sugars in the O-antigen. In contrast, the LVS O-antigen mutant strain WbtIG191V and *F. tularensis* subsp. *novicida* take up more Congo Red than the type A or B wildtype strains because the O-antigen is missing or is distinct in composition (62). Mutant Δ0793 was indistinguishable from the parent strain (**Fig 3.8**), indicating it was not an O-antigen mutant. Mutant Δ0798-0799 was created from WbtIG191V P17 strain, and as expected the double mutant appeared similar to the original mutant on Congo red agar **(Fig 3.8E).**
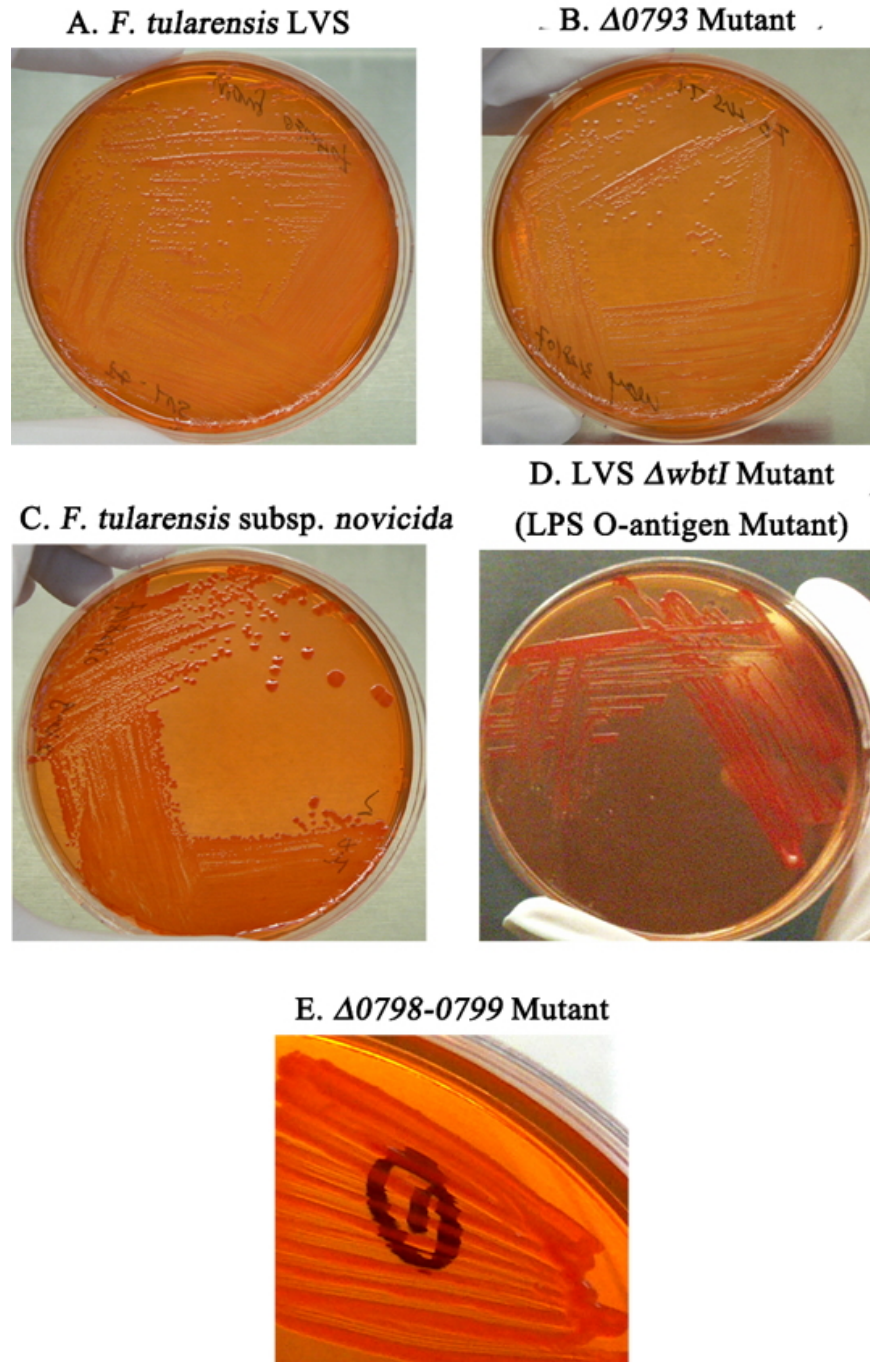
Figure 3.8. Congo red up-take assay. A. *F. tularensis* LVS forms colorless to light pink colonies on Congo red agar. B. The Δ0793 mutant looked similar to the LVS parent strain when grown on the same media. C. *F. tularensis* subsp. *novicida* forms dark red colonies on Congo red agar. D. O-antigen mutant strain WbtIG191V can also form red colonies. E. double mutant Δ0798-0799 takes up Congo red as does WbtiG191V during the growth on Congo red agar.

3.2.2. Serum bactericidal assay

Parent strain LVS and mutant strain Δ0793 were completely resistant to the bactericidal action of 10% guinea pig serum. However, approximately 95% of O-antigen mutant WbtIG191V was killed within 1 hour incubation in the same serum. The serum bactericidal effect on mutant strain WbtIG191V was abrogated by incubation of serum at 56˚C for 30 min. These results supported that Δ0793 mutant was not an O-antigen mutant (**Fig 3.9**). Similar results were obtained when 10% human serum was used (data not shown).

No bactericidal assay was performed with Δ0798-0799 mutant, because its parent strain is already serum susceptible.

Bactericidal Assay
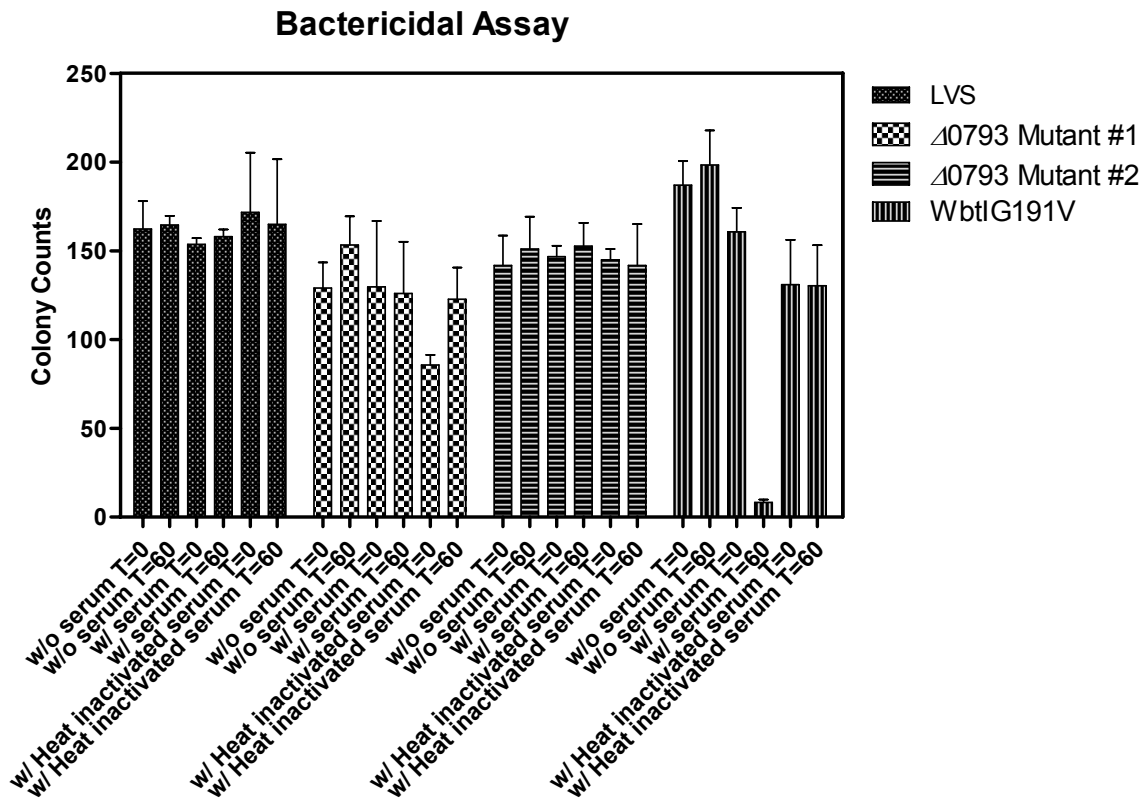
Figure 3.9. Serum bactericidal assay for Δ0793 mutant. Bacteria were incubated in microcentrifuge tubes at 5 x 10^2 CFU/100 μl. Before and after 1hour incubation 20 μl was plated. The bactericidal effect of guinea pig serum was abrogated by incubation of serum at 56˚C for 30 min. Results shown are from a single experiment of each strain tested in triplicate. Strains and symbols: LVS, (▨); Δ0793 mutant #1, (▩); Δ0793 mutant #2, (▤); WbtIG191V, (▥). Error bar = Standard deviation.

3.2.3. Virulence studies in mice

Mice were challenged with mutant Δ0793 to determine if there was any loss of bacterial virulence (**Fig 3.10**). Mutant Δ0793 was injected into BALB/c mice IP, which is the most severe challenge route. The mouse IP LD_{50} for our strain of LVS was previously determined to be about 120 CFU (54). For this study, mice were inoculated with bacterial numbers that were about 10 times the LD_{50} of LVS. All challenged mice showed clinical symptoms, such as ruffled fur, and inactivity within 3 days of inoculation. The two mice

challenged with LVS died between 4 and 5 days IP. Of the three mice challenged with Δ0793 mutant #1, two died on the sixth day postchallenge, and the other became ill but later recovered. The three mice challenged with Δ0793 mutant #2 died between 4 and 6 days postchallenge. Although only a small number of animals were used, there did not appear to be any apparent attenuation of the Δ0793 mutant for mice.

Virulence assays were not performed with mutant Δ0798-0799 because the parent strain is already attenuated (62).
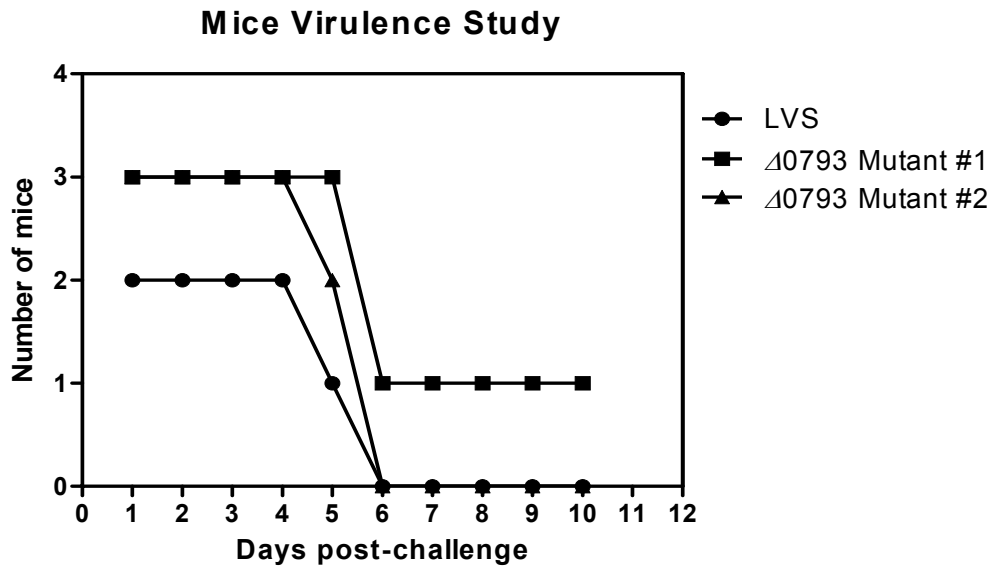
Figure 3.10. Virulence of Δ0793 mutants in mice following IP inoculation. One group of 2 mice each was inoculated with 10^4 CFU of LVS IP. Two groups of three were inoculated with 10^4 CFU of Δ0793 mutants IP. Strains and symbols: LVS, (–●–); Δ0793 mutant #1, (–■–); Δ0793 mutant #2, (–▲–).

3.2.4. Western blotting

The LPS from LVS, mutant strains Δ0793 and WbtIG191V were extracted using the LPS

micro-extraction method (52). To confirm whether the LPS is still present in mutant Δ0793, western blots were performed with murine mAb to *F. tularensis* O-antigen as the primary antibody and anti-murine IgG and IgM coupled to horseradish peroxidase as secondary conjugates. The western blot results showed that the Δ0793 mutant had a similar LPS profile as the LVS parent strain (**Fig 3.11**), while the O-antigen mutant WbtIG191V strain showed no ladder pattern. The western blot analysis also confirmed that Δ0793 was not an O-antigen mutant.
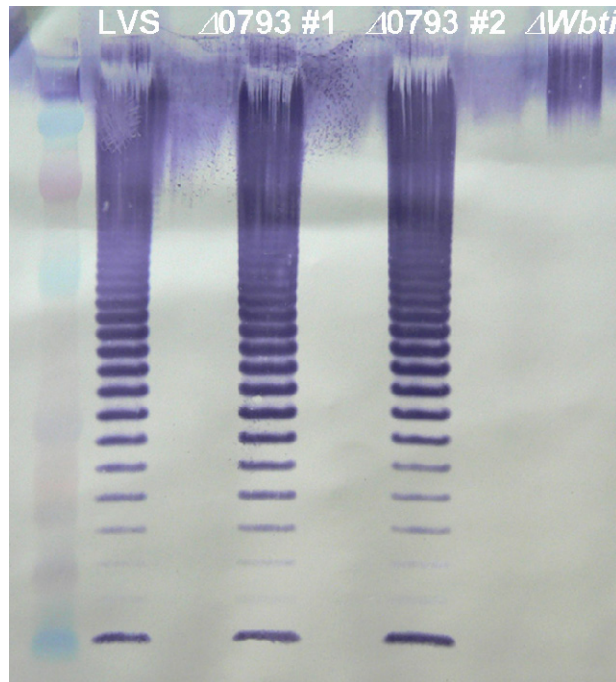
Figure 3.11. LPS Western blot. Extracted LPS was separated by SDS-PAGE and transferred to nitrocellulose and developed as described in Material and Methods. The Δ0793 mutant has a similar LPS profile to that of the LVS parent strain. The ladder-like pattern of LPS was absent from O-antigen mutant WbtIG191V.

For mutant Δ0798-0799, core LPS biosynthesis was not affected by deletion of the two transferase genes, as determined by western blotting with whole cell antiserum, which shows low molecular size LPS in both the parent and double mutant (data not shown). A

western blot of Δ0798-0799 membrane proteins showed that two protein bands were missing from mutant Δ0798-0799 compared with parent strain WbtIG191V P17 (**Fig 3.12** by Dr. Abey Bandara). Thus far it has not been determined if the missing protein bands are associated with the deleted transferase genes or if the missing protein products may cause phenotypic changes in the mutant.
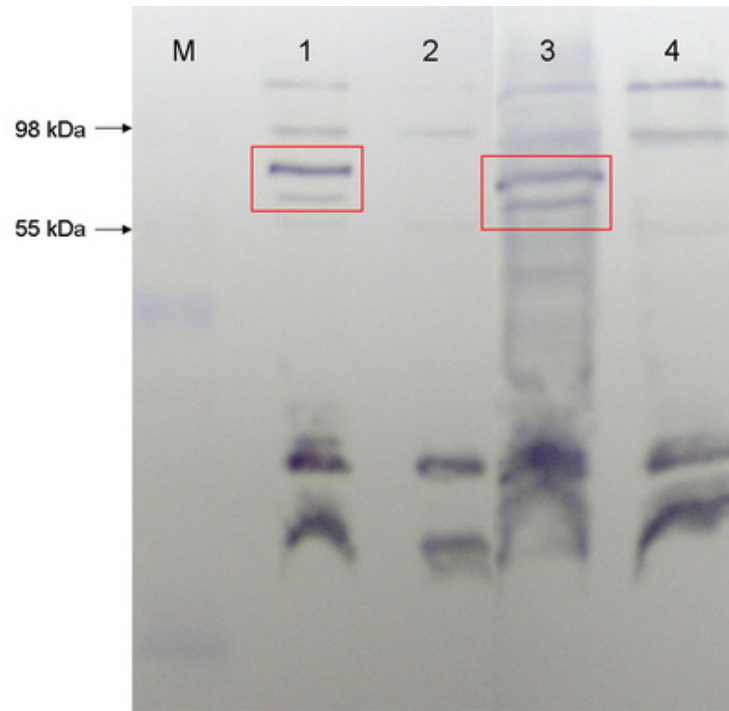
Figure 3.12. Total protein western blot of Δ0798-0799 and mutant WbtI191GV P17. Boiled whole cells were separated by SDS-PAGE and transferred to nitrocellulose, blotted with rabbit sera raised against *F. tularensis*, and developed as described in Material and Methods. Lane M, protein ladder. Lanes 1 and 3, Strain WbtIG191V extracts 2 and 8 µl respectively. Lanes 2 and 4: Strain Δ0798-0799 extracts 2 and 8 µl respectively. The two bands in the red box were missing from Δ0798-0799 mutant. Western blotting was performed by Dr. Bandara.

3.2.5. Hyper immune serum

ELISAs were used to determine whether mutant Δ0793 could still transport capsule across the outer membrane. Antibody-depleted sera were prepared by thorough absorption of hyperimmune rabbit sera with LVS or mutant Δ0793. The absorbed sera

were later used as the source of primary antibody in the ELISA. Unabsorbed hyperimmune serum was used as a control. The absorbed and unabsorbed sera were serially diluted from 1:10-1:640 in 100 μl of PBS ELISA buffer. LPS incubated with unabsorbed serum had OD values of 2.85 at a1:10 dilution. When the LPS was incubated with serum absorbed with Δ0793 mutant the initial OD value was lower than the OD using unabsorbed serum, There was no significant difference (*P* = 0.249, student's *t* test) in the reactivity of serum adsorbed with LVS and serum adsorbed with Δ0793 (**Fig 3.13**). There results indicated there was no difference in expression of LPS on the parent or mutant strain, and that normal LPS was present on mutant Δ0793.
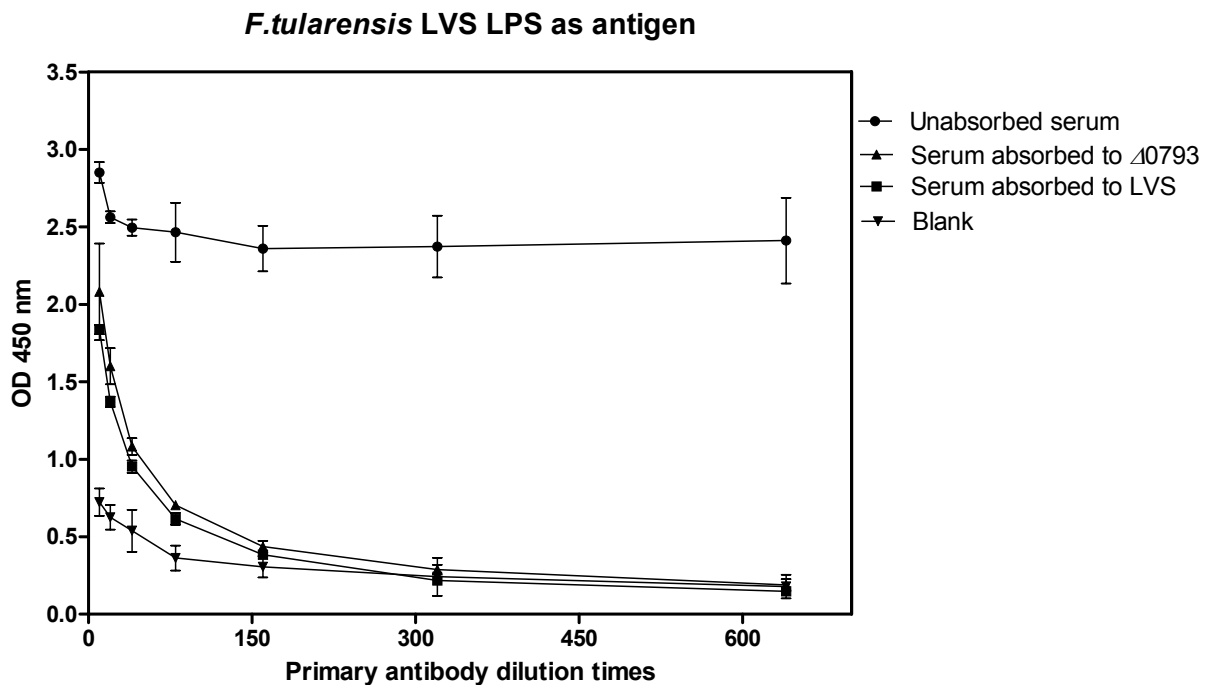
Figure 3.13. Reactivity of adsorbed and unadsorbed sera to *F. tularensis* LPS. Purified *F. tularensis* LPS (1 μg/well) was incubated with unabsorbed serum and hyperimmune sera absorbed to LVS or mutant Δ0793. Symbols: Unabsorbed serum, (–•–); serum absorbed to Δ0793 mutant, (–▲–); serum absorbed to parent strain LVS, (–■–); blank, (–▼–). Each point represents the mean of three experiments. Error bar = Standard deviation.

Purified capsule from *F. tularensis* was also incubated with unadsorbed sera and sera adsorbed with LVS or the mutants (**Fig 3.14**). Again, there was no significant difference (P =0.595, student's *t* test) between the reactivity of each adsorbed serum. If mutant Δ0793 no longer produced extracellular capsule, then the reactivity of serum adsorbed with Δ0793 should have been significantly higher than sera adsorbed with LVS. However, the reactivity of whole cell antiserum was relatively weak, indicating that even a small amount of capsule expression could remove most of the antibody reactivity. Nonetheless, the results could not demonstrate that extracellular capsule production by Δ0793 mutant was compromised.
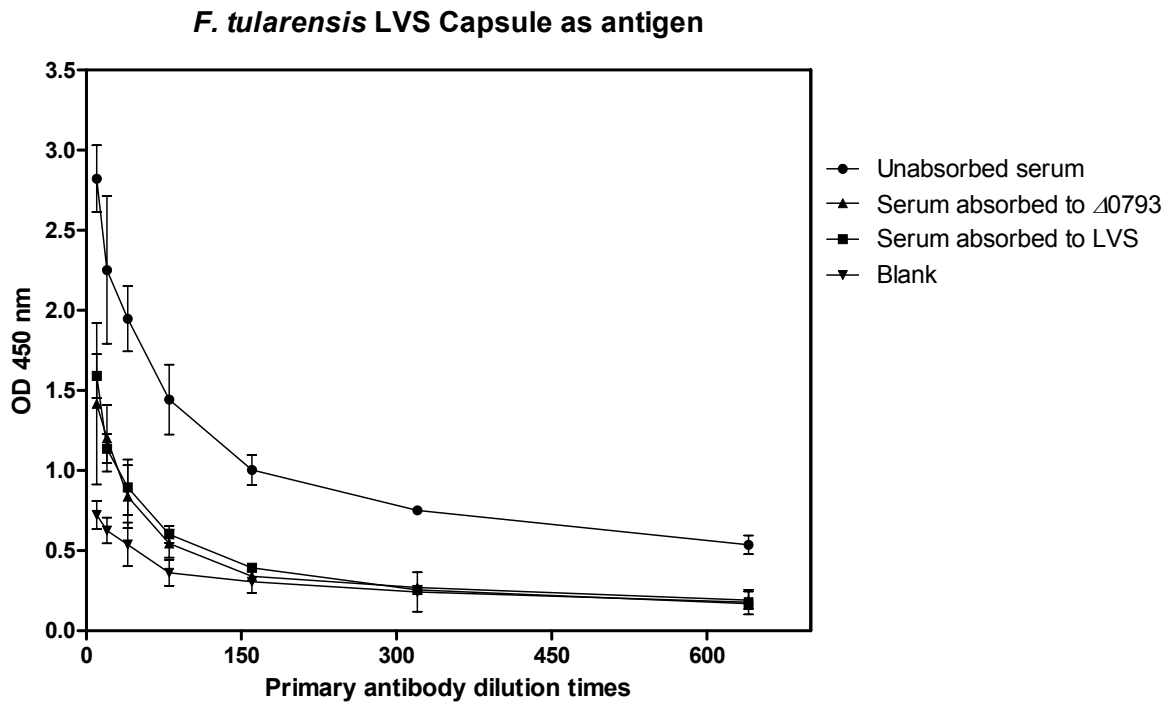
Figure 3.14. Reactivity of adsorbed and unadsorbed sera to *F. tularensis* capsule. Extracted *F. tularensis* capsule (1 µg/well) was incubated with unadsorbed serum and hyperimmune sera absorbed to LVS or mutant Δ0793. Symbols: Unabsorbed serum, (—●—); serum absorbed to Δ0793 mutant, (—▲—); serum absorbed to parent strain LVS, (—■—); blank, (—▼—). Each point represents the mean of three experiments. Error bar = Standard deviation.

3.2.6. Preliminary Alcian Blue silver stain

A preliminary Alcian Blue silver stain was performed with the exopolysaccharide extracted from *F. tualrensis* LVS, Δ0793 mutants and a control strain of *Actinobacillus pleuropneumoniae*. The LPS from LVS was not stained on the gel, however, a typical LPS ladder pattern was detected by western blotting (data not shown). Capsular polysaccharide extracted from *A. peuropneumoniae* could be observed on the gel. Attempts to isolate capsule from the two mutant strains resulted in no apparent capsule from one of the strains (lane 4), as determined by western blotting. Extracelluar carbohydrates in addition to LPS from *F. tularensis* LVS could be clearly observed on the gel. The results of this preliminary study may indicate the export of the capsule was affected in the Δ0793 mutant strain.
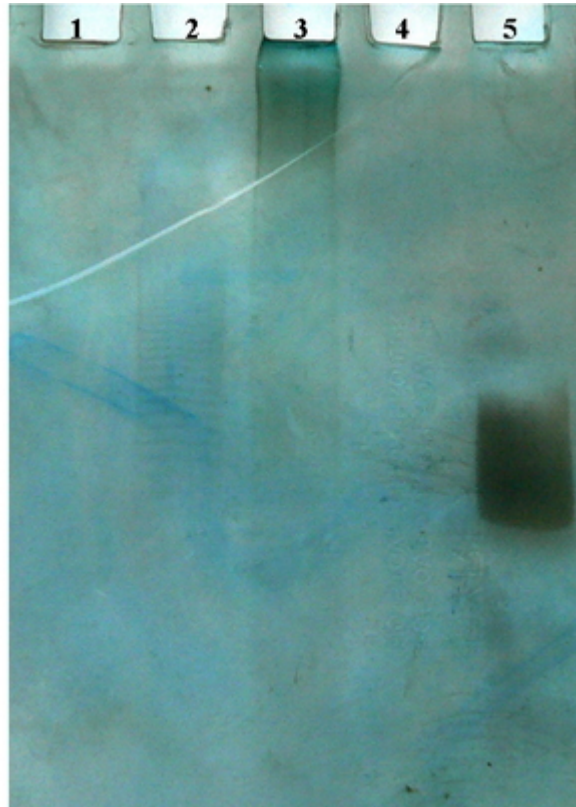
Figure 3.15. Preliminary Alcian Blue silver stain of extracellular carbohydrates. Lane 1, LPS extracted from *F. tularensis* LVS. Lane 2, capsular polysaccharide extracted from *A. pleuropneumoniae.* Lane 3 and 4, capsule extracted from Δ0793 mutants. Lane 5, capsule extracted from *F. tualrensis* LVS. This gel was run by Anna Champion.

3.2.7. Electron microscopy

Δ0793 mutant. Negative staining and electron microscopy of each *F. tularensis* strain demonstrated an electron dense layer encircling the cells (**Fig 3.16**). The layer appears thicker on LVS and the WbtIG191V strain than on the Δ0793 mutant. Thus, production of extracellular material by mutant Δ0793 may be compromised to some extent. However, due to the large number of other ATPases present in the *F. tularensis* genome, it is feasible that another ATPase can compensate for capsule transportation in Δ0793 mutant, resulting in this mutant strain still being capable of transporting some capsule.
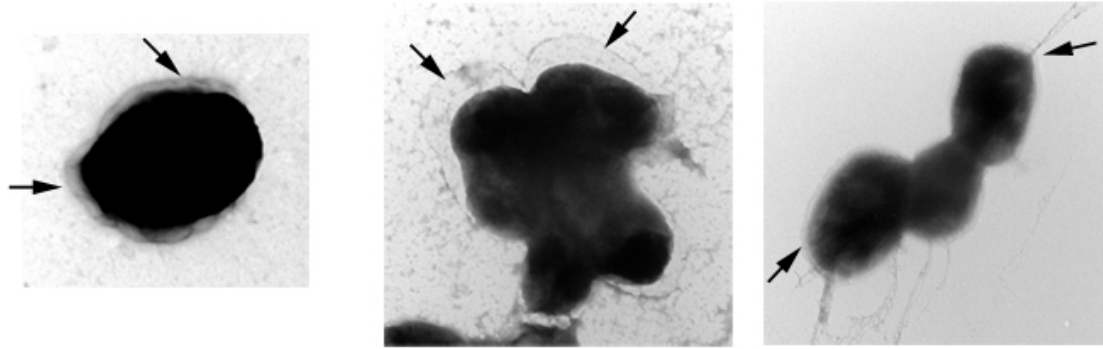
F. tularensis LVS   WbtIG191V strain   Δ0793 Mutant

Figure 3.16. Electron micrograph showing an extracellular layer encircling the *F. tularensis* bacterial surface. The extracellular layer looked thicker in the parent strain LVS and WbtIG191V strain than that in the Δ0793 mutant. Magnification, ×31500.

Δ0798-0799 mutant. LPS mutant strain WbtIG191V P17 is enhanced for capsule production, which facilitates visual comparison between the parent strain and potential mutants. The difference between the parent strain and the mutant strain is clear from electron microscopy. The amount of the extracelllular material encircling the bacteria was absent in mutant Δ0798-0799, but easily visible in parent strain WbtIG191V P17 (**Fig 3.17**).
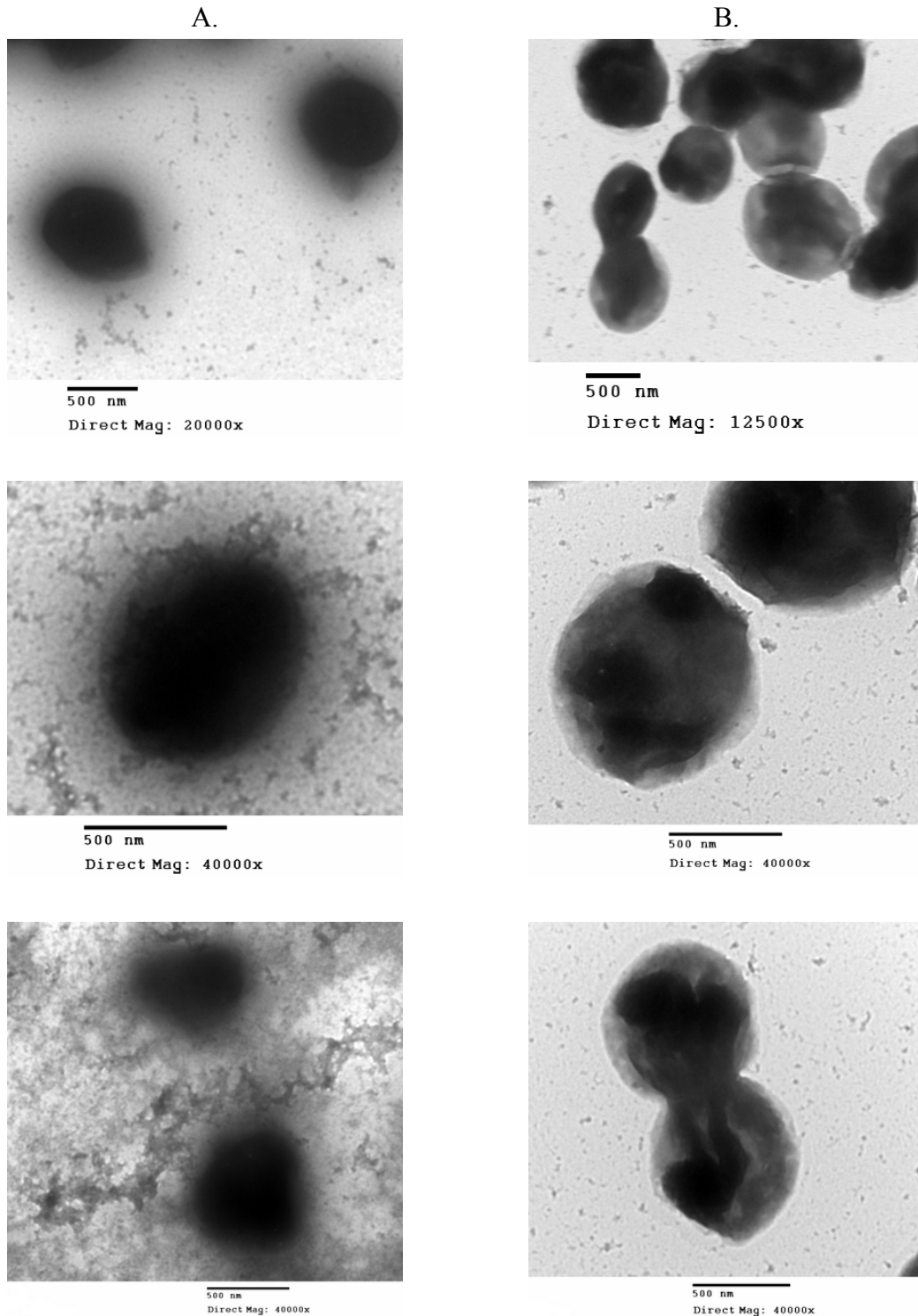
Figure 3.17. Electron micrograph of parent strain mutant WbtIG191V P17 and galactosyl-mannosyl transferase mutant ΔFTT0798-0799. Panel A. the parent strain has an electron dense layer surrounding the cells. Panel B. The transferase mutant ΔFTT0798-0799 had no such layer surrounding the cells. **Bar** = 500 nm. EM Pictures were taken by Anna Champion.

CHAPTER 4

Conclusion

The virulence factors and mechanisms that contribute to *F. tularensis* pathogenicity are not well characterized. An ideal approach to study these virulence factors and mechanisms is to mutate the putative genes that encode these virulence factors and study the corresponding phenotypic changes of the mutants. However, the lack of methods to generate mutations in *F. tularensis* types A and B strains has been a major draw back in this approach. With the development of genetic tools, some methods have proven feasible to make mutations in *F. tularensis* type A and type B stains. Prior to this study, a suicide mutagenesis vector pPV had been developed to make site-directed gene deletion in both type A and type B strains using a *sacB* gene replacement protocol (42, 62, 105). Other plasmids of similar design have also been developed. This study was inspired by the work that created the pPV plasmid, which was constructed based on the commonly used pUC19 plasmid backbone (42) and containing no fragments originating from *F. tularensis*.

Our study demonstrated a method that is able to create site-directed mutations in *F. tularensis* LVS. This method is novel compared to currently published vectors. The constructed mutagenesis plasmid allows the creation of mutants through allelic replacement, and is based on the commercial pSC-A vector. Other published mutagenesis vectors were modified from the pPV plasmid and also use a *sacB* cartridge (62, 105). The pSC-A derived mutagenesis plasmids do not require the use of *sacB*, which may be problematic due to the high spontaneous mutation rate to sucrose resistance in the presence of *sacB*. Spontaneous mutants that grow in the presence of sucrose while *sacB* is present occurs at frequencies ranging from 0 to 30% (63). The pSC-A plasmid is commercially available as a PCR cloning vector, which simplifies plasmid construction. In addition, the cryotransformation method can deliver higher transformation efficiency relative to conjugation. Furthermore, all the mutant candidates obtained in this study resulted from a double crossover event. No cointegration of the mutagenesis plasmid was

detected, whereas cointergration of the pPV plasmid is the first step in the mutagenesis procedure (42, 62). However, the use of the pSC-A plasmid does have some disadvantages, such as the requirement for antibiotic resistance-marked mutations, which may make it inconvenient to use with the type A strains. This method is also not very convenient for making multiple deletions of genes in separate location. The use of different antibiotic resistance genes is required in order to make multiple mutations when the target genes are located in different regions of the genome.

Initially, we used electroporation to introduce DNA into *F. tularensis*. However, this method became problematic when higher concentrations of DNA were used. The success of electroporation depended greatly on the purity of the plasmid, particularly the presence of salt, which caused arcing in the cuvette and a greater proportion of dead bacteria. Therefore, cryotransformation was adopted, which allowed the use of high concentrations of DNA, and appeared to be required for successful transformation. The disadvantage of cryotransformation is that the efficiency is more than 10-fold lower than electroporation; moreover the use of liquid nitrogen is required.

In pathogenic microorganisms the surface polysaccharides, including O-antigen polysaccharide and/or capsular polysaccharide, are considered key virulence determinants. They cover the surface of the organism and confers resistance to host-mediated defense mechanisms, such as opsonization and complement-mediated lysis (25). Thus far, the capsule of *Francisella* has not been characterized. A previous study identified a putative carbohydrate gene cluster (FTT0789−FTT0801) in *F. tularensis* type A and type B strains, which could encode a polysaccharide distinct from the lipopolysaccharide O-antigen (59). The thesis was focused on mutagenesis of this cluster. FTT0793 contained homology to an ABC transporter in this locus and therefore was suspected of being involved in exopolysaccharide or capsule export. A similar type of

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ABC transporter is located in the capsule export region of *Haemophilus influenza* type b, and mutagenesis of this gene results in mutants that synthesis, but cannot export capsule (96). The advantage of such a mutant is that the host can still make an immune response to the intracellular antigen, but if the capsule is required for virulence the mutant should be attenuated. ORFs FTT0798 and FTT0799 may encode for a galactosyl transferase and a mannosyl transferase respectively, which would transfer these sugars to the carbohydrate capsule chain. Therefore, a mutation in these genes would result in no capsule production. Therefore, these genes were targeted and were successfully deleted. In subsequent phenotypic tests of these mutants, we could not detect any significant differences in capsule production between mutant Δ0793 and parent strain LVS. The results from the Congo red up-take assay and LPS western blots showed the mutant has intact LPS, which indicated the FTT0793 gene did not affect LPS biosynthesis. The results of the bactericidal assay also indicated that, the mutant was not serum-sensitive, and no obvious attenuation of virulence for mice was observed. The difference noted was that the mutant strain demonstrated less extracellular material encircling the bacteria compared to the parent strain. However, the parent LVS had not been passed *in vitro* and was therefore not enhanced for encapsulation. Attempts to isolate capsule from the two mutant strains resulted in no apparent capsule from one of the strains in the Alcian Blue silver stain, as determined by western blotting. Nonetheless, it was not conclusive that there either mutant lacked any capsule on the surface. One reason that the mutants did not show obvious phenotypic changes may be due to compensation of the mutated transporter by any of a large number of other ABC transporters present in the genome. However, the transportation of capsule may have been compromised to some extent resulting in the layer encircling the cells appearing thinner than that of the parent strain.

In order to more clearly identify loss of capsule in mutants containing deletions in ORFs 0797-0798, *F. tularensis* LVS WbtIG191V P17 strain was used as the parent strain.

This strain is enhanced for capsule production, which would simplify determining if capsule production is affected in the mutant. When creating the mutagenesis plasmid for the transferases genes FTT0798-0799, the kan resistance gene was first inserted in an orientation different from the orientation of the flanking regions. This plasmid was confirmed to have the same structure as the one used to make Δ0793 mutant, except for the kan resistance gene orientation. No mutant was obtained when this plasmid was transformed into *F. tularensis* LVS. However, when the orientation of the kan gene was reversed, deletion of genes FTT0798-0799 was successful. These results suggest that the kan resistance gene may not be expressed well with its endogenous promoter. An orientation which allows read-through expression of the antibiotic resistance gene from an upstream *Francisella* promoter may be required. Therefore, inclusion of a strong *Francisella* promoter, such as the *groEL* promoter upstream of the antibiotic resistance gene may increase the probability of obtaining mutants. Bactericidal and virulence assays were not performed on this mutant, because the parent strain is an LPS O-antigen mutant and is already serum-sensitive and attenuated. Therefore, electron microscopy was used to clarify the loss of capsule in the mutant. In contrast to parent strain WbtIG191V P17, no detectable electron dense material was visible on the surface of the Δ0798-0799 mutant strain. Other than capsule, western blots of whole cell lysates demonstrated two protein bands that were missing from the Δ0798-0799 mutant compared with its parent strain WbtI191GV P17. Although we have not confirmed the missing two bands associated with the products of the two transferase genes, it is clear that the protein expression pattern is different between the mutant strain and the parent strain. These results indicated that the carbohydrate locus identified is responsible for capsule production, and that these two transferase genes are involved in the biosynthesis of this capsule. In order to confirm the nature of the mutation and the role of capsule in virulence, a plasmid is being constructed with a normal copy of the transferase genes to complement the mutation in trans, and attempts are underway to make a similar

transferase mutant in a virulent type A strain, respectively.

In summary, a plasmid and method for generating allelic exchange mutants is reported, which should be useful for generating additional mutants of *F. tularensis* to clarify the roles of specific genes.

Based on this study, the Cre-LoxP system may also be used as a potential tool to make unmarked mutants. LoxP sites could be added to the both ends of the antibiotic resistance gene by designing primers to amplify the LoxP sequence. A temperature-sensitive expression vector could also be used to produce Cre recombinase, although *Francisella* promoter may be required for recombinase expression.

REFERENCES

1.   Health Aspects of Chemical and Biological Weapon, p. 1970:105-107. World Health Organization, Geneva, Switzerland.

2.   1998. Summary of notifiable disease. United States 1997, p. 71-80. Centers for Disease Control and Prevention.

3.   **Alibek, K.** 1999. Biohazard, p. 29-38. Random House, New York, NY.

4.   **Ancuta, P., T. Pedron, R. Girard, G. Sandstrom, and R. Chaby.** 1996. Inability of the *Francisella tularensis* lipopolysaccharide to mimic or to antagonize the induction of cell activation by endotoxins. Infect Immun **64:**2041-6.

5.   **Anthony, L. S., M. Z. Gu, S. C. Cowley, W. W. Leung, and F. E. Nano.** 1991. Transformation and allelic replacement in *Francisella* spp. J Gen Microbiol **137:**2697-703.

6.   **Bakshi, C. S., M. Malik, K. Regan, J. A. Melendez, D. W. Metzger, V. M. Pavlov, and T. J. Sellati.** 2006. Superoxide dismutase B gene (*sodB*)-deficient mutants of *Francisella tularensis* demonstrate hypersensitivity to oxidative stress and attenuated virulence. J Bacteriol **188:**6443-8.

7.   **Baron, G. S., S. V. Myltseva, and F. E. Nano.** 1995. Electroporation of *Francisella tularensis*. Methods Mol Biol **47:**149-54.

8.   **Broekhuijsen, M., P. Larsson, A. Johansson, M. Bystrom, U. Eriksson, E. Larsson, R. G. Prior, A. Sjostedt, R. W. Titball, and M. Forsman.** 2003. Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. t*ularensis*. J Clin Microbiol **41:**2924-31.

9.   **Burke, D. S.** 1977. Immunization against tularemia: analysis of the effectiveness

of live *Francisella tularensis* vaccine in prevention of laboratory-acquired tularemia. J Infect Dis **135:**55-60.

10. **Chamberlain, R. E.** 1965. Evaluation of Live tularemia vaccine prepared in a chemically defined medium. Appl Microbiol **13:**232-5.

11. **Chen, W., R. Kuolee, H. Shen, M. Busa, and J. W. Conlan.** 2005. Toll-like receptor 4 (TLR4) plays a relatively minor role in murine defense against primary intradermal infection with *Francisella tularensis* LVS. Immunol Lett **97:**151-4.

12. **Cherwonogrodzky, J. W., M. H. Knodel, and M. R. Spence.** 1994. Increased encapsulation and virulence of *Francisella tularensis* live vaccine strain (LVS) by subculturing on synthetic medium. Vaccine **12:**773-5.

13. **Christenson, B.** 1984. An outbreak of tularemia in the northern part of central Sweden. Scand J Infect Dis **16:**285-90.

14. **Christopher, G. W., T. J. Cieslak, J. A. Pavlin, and E. M. Eitzen, Jr.** 1997. Biological warfare. A historical perspective. Jama **278:**412-7.

15. **Clemens, D. L., and M. A. Horwitz.** 2007. Uptake and intracellular fate of *Francisella tularensis* in human macrophages. Ann N Y Acad Sci.

16. **Clemens, D. L., B. Y. Lee, and M. A. Horwitz.** 2004. Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. Infect Immun **72:**3204-17.

17. **Cole, L. E., K. L. Elkins, S. M. Michalek, N. Qureshi, L. J. Eaton, P. Rallabhandi, N. Cuesta, and S. N. Vogel.** 2006. Immunologic consequences of *Francisella tularensis* live vaccine strain infection: role of the innate immune response in infection and immunity. J Immunol **176:**6888-99.

18. **Conlan, J. W., and R. J. North.** 1992. Early pathogenesis of infection in the liver with the facultative intracellular bacteria *Listeria monocytogenes, Francisella tularensis*, and *Salmonella typhimurium* involves lysis of infected

hepatocytes by leukocytes. Infect Immun **60:**5164-71.

19. **Conlan, J. W., and P. Oyston.** 2007. Vaccines against *Francisella tularensis*. Ann N Y Acad Sci.

20. **Conlan, J. W., H. Shen, A. Webb, and M. B. Perry.** 2002. Mice vaccinated with the O-antigen of *Francisella tularensis* LVS lipopolysaccharide conjugated to bovine serum albumin develop varying degrees of protective immunity against systemic or aerosol challenge with virulent type A and type B strains of the pathogen. Vaccine **20:**3465-71.

21. **Dahlstrand, S., O. Ringertz, and B. Zetterberg.** 1971. Airborne tularemia in Sweden. Scand J Infect Dis **3:**7-16.

22. **Darling, R. G., C. L. Catlett, K. D. Huebner, and D. G. Jarrett.** 2002. Threats in bioterrorism. I: CDC category A agents. Emerg Med Clin North Am **20:**273-309.

23. **Dennis, D. T., T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, A. M. Friedlander, J. Hauer, M. Layton, S. R. Lillibridge, J. E. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, and K. Tonat.** 2001. Tularemia as a biological weapon: medical and public health management. Jama **285:**2763-73.

24. **Dienst, F. T., Jr.** 1963. Tularemia: a perusal of three hundred thirty-nine cases. J La State Med Soc **115:**114-27.

25. **Ellis, J., P. C. Oyston, M. Green, and R. W. Titball.** 2002. Tularemia. Clin Microbiol Rev **15:**631-46.

26. **Evans, M. E., D. W. Gregory, W. Schaffner, and Z. A. McGee.** 1985. Tularemia: a 30-year experience with 88 cases. Medicine (Baltimore) **64:**251-69.

27. **Farlow, J., D. M. Wagner, M. Dukerich, M. Stanley, M. Chu, K. Kubota, J. Petersen, and P. Keim.** 2005. *Francisella tularensis* in the United States. Emerg Infect Dis **11:**1835-41.

28. **Feldman, K. A., R. E. Enscore, S. L. Lathrop, B. T. Matyas, M. McGuill, M. E. Schriefer, D. Stiles-Enos, D. T. Dennis, L. R. Petersen, and E. B. Hayes.** 2001. An outbreak of primary pneumonic tularemia on Martha's Vineyard. N Engl J Med **345:**1601-6.

29. **Forslund, A. L., K. Kuoppa, K. Svensson, E. Salomonsson, A. Johansson, M. Bystrom, P. C. Oyston, S. L. Michell, R. W. Titball, L. Noppa, E. Frithz-Lindsten, M. Forsman, and A. Forsberg.** 2006. Direct repeat-mediated deletion of a type IV pilin gene results in major virulence attenuation of Francisella tularensis. Mol Microbiol **59:**1818-30.

30. **Foshay, L.** 1950. Tularemia. Ann. Rev. Microbiol. **4:**313-330.

31. **Foshay, L., Hesselbrock, W. H., Wittenberg, H. J., and Rodenberg, A. H.** 1942. Vaccine prophylaxis against tularemia in man. Am J Public Health **32:**1131-1145.

32. **Fulop, M., R. Manchee, and R. Titball.** 1995. Role of lipopolysaccharide and a major outer membrane protein from *Francisella tularensis* in the induction of immunity against tularemia. Vaccine **13:**1220-5.

33. **Fulop, M., P. Mastroeni, M. Green, and R. W. Titball.** 2001. Role of antibody to lipopolysaccharide in protection against low- and high-virulence strains of *Francisella tularensis*. Vaccine **19:**4465-72.

34. **Gallagher, L. A., E. Ramage, M. A. Jacobs, R. Kaul, M. Brittnacher, and C. Manoil.** 2007. A comprehensive transposon mutant library of *Francisella novicida*, a bioweapon surrogate. Proc Natl Acad Sci U S A **104:**1009-14.

35. **Gay, P., D. Le Coq, M. Steinmetz, T. Berkelman, and C. I. Kado.** 1985. Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. J Bacteriol **164:**918-21.

36. **Geisbert, T. W., P. B. Jahrling, and J. W. Ezzell, Jr.** 1993. Use of immunoelectron microscopy to demonstrate *Francisella tularensis*. J Clin

Microbiol **31:**1936-9.

37. **Geissler, E.** 2005. Alibek, tularaemia, and the battle of stalingrad. CBWCB **September/December 2005:**10-15.

38. **Gil, H., J. L. Benach, and D. G. Thanassi.** 2004. Presence of pili on the surface of *Francisella tularensis*. Infect Immun **72:**3042-7.

39. **Gil, H., G. J. Platz, C. A. Forestal, M. Monfett, C. S. Bakshi, T. J. Sellati, M. B. Furie, J. L. Benach, and D. G. Thanassi.** 2006. Deletion of TolC orthologs in *Francisella tularensis* identifies roles in multidrug resistance and virulence. Proc Natl Acad Sci U S A **103:**12897-902.

40. **Golovliov, I., V. Baranov, Z. Krocova, H. Kovarova, and A. Sjostedt.** 2003. An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. Infect Immun **71:**5940-50.

41. **Golovliov, I., M. Ericsson, G. Sandstrom, A. Tarnvik, and A. Sjostedt.** 1997. Identification of proteins of Francisella tularensis induced during growth in macrophages and cloning of the gene encoding a prominently induced 23-kilodalton protein. Infect Immun **65:**2183-9.

42. **Golovliov, I., A. Sjostedt, A. Mokrievich, and V. Pavlov.** 2003. A method for allelic replacement in *Francisella tularensis*. FEMS Microbiol Lett **222:**273-80.

43. **Gordon, M., Donaldson, D. M., and Wright G. G.** 1964. Immunization of mice with irradiated *Pasteurella tularensis*. FEMS Microbiol Lett **114:**435-440.

44. **Gray, C. G., S. C. Cowley, K. K. Cheung, and F. E. Nano.** 2002. The identification of five genetic loci of *Francisella novicida* associated with intracellular growth. FEMS Microbiol Lett **215:**53-6.

45. **Griffin, K. F., P. C. Oyston, and R. W. Titball.** 2007. *Francisella tularensis* vaccines. FEMS Immunol Med Microbiol **49:**315-23.

46. **Hajjar, A. M., M. D. Harvey, S. A. Shaffer, D. R. Goodlett, A. Sjostedt, H. Edebro, M. Forsman, M. Bystrom, M. Pelletier, C. B. Wilson, S. I. Miller, S. J.**

Skerrett, and R. K. Ernst. 2006. Lack of in vitro and in vivo recognition of *Francisella tularensis* subspecies lipopolysaccharide by Toll-like receptors. Infect Immun **74:**6730-8.

47. **Hall, J. D., R. R. Craven, J. R. Fuller, R. J. Pickles, and T. H. Kawula.** 2007. *Francisella tularensis* replicates within alveolar type II epithelial cells in vitro and in vivo following inhalation. Infect Immun **75:**1034-9.

48. **Harris, S.** 1992. Japanese biological warfare research on humans: a case study of microbiology and ethics. Ann N Y Acad Sci **666:**21-52.

49. **Holmes RK, J. M.** 1996. Genetics: Exchange of Genetic Information. *In* e. Baron's Medical Microbiology (Baron S et al (ed.), 4th ed. Univ of Texas Medical Branch.

50. **Hood, A. M.** 1977. Virulence factors of *Francisella tularensis*. J. Hyg. (Lond). **79:**47-60.

51. **Hopla, C. F., and A. K. Hopla.** 1994. Tularemia, p. 113-126. *In* G. W. Beran and J. H. Steele (ed.), Handbook of Zoonoses, 2nd ed. ed. CRC Press, Inc., Boca Raton, Fl.

52. **Inzana, T. J.** 1983. Electrophoretic heterogeneity and interstrain variation of the lipopolysaccharide of *Haemophilus influenzae*. J Infect Dis **148:**492-9.

53. **Inzana, T. J., and P. Anderson.** 1985. Serum factor-dependent resistance of *Haemophilus influenzae* type b to antibody to lipopolysaccharide. J. Infect. Dis. **151:**869-877.

54. **Inzana, T. J., G. E. Glindemann, G. Snider, S. Gardner, L. Crofton, B. Byrne, and J. Harper.** 2004. Characterization of a wild-type strain of *Francisella tularensis* isolated from a cat. J Vet Diagn Invest **16:**374-81.

55. **Kawula, T. H., J. D. Hall, J. R. Fuller, and R. R. Craven.** 2004. Use of transposon-transposase complexes to create stable insertion mutant strains of *Francisella tularensis* LVS. Appl Environ Microbiol **70:**6901-4.

56. **Khatenever, L. M.** 1943. The allergic diagnosis specific prophylaxis and vaccine therapy of tularemia, p. 62-67. *In* E. B. Balsky, Kochergin, I. G. and Porin, V. V. (ed.), Microbiology and Epidemiology. Mdedical Publications Ltd., London.

57. **Lai, X. H., I. Golovliov, and A. Sjostedt.** 2001. *Francisella tularensis* induces cytopathogenicity and apoptosis in murine macrophages via a mechanism that requires intracellular bacterial multiplication. Infect Immun **69:**4691-4.

58. **Lai, X. H., and A. Sjostedt.** 2003. Delineation of the molecular mechanisms of *Francisella tularensis*-induced apoptosis in murine macrophages. Infect Immun **71:**4642-6.

59. **Larsson, P., P. C. Oyston, P. Chain, M. C. Chu, M. Duffield, H. H. Fuxelius, E. Garcia, G. Halltorp, D. Johansson, K. E. Isherwood, P. D. Karp, E. Larsson, Y. Liu, S. Michell, J. Prior, R. Prior, S. Malfatti, A. Sjostedt, K. Svensson, N. Thompson, L. Vergez, J. K. Wagg, B. W. Wren, L. E. Lindler, S. G. Andersson, M. Forsman, and R. W. Titball.** 2005. The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. Nat Genet **37:**153-9.

60. **Lauriano, C. M., J. R. Barker, F. E. Nano, B. P. Arulanandam, and K. E. Klose.** 2003. Allelic exchange in *Francisella tularensis* using PCR products. FEMS Microbiol Lett **229:**195-202.

61. **Lauriano, C. M., J. R. Barker, S. S. Yoon, F. E. Nano, B. P. Arulanandam, D. J. Hassett, and K. E. Klose.** 2004. MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intraamoebae and intramacrophage survival. Proc Natl Acad Sci U S A **101:**4246-9.

62. **Li, J., C. Ryder, M. Mandal, F. Ahmed, P. Azadi, D. S. Snyder, R. D. Pechous, T. Zahrt, and T. J. Inzana.** 2007. Attenuation and protective efficacy of an O-antigen-deficient mutant of *Francisella tularensis* LVS. Microbiology **153:**3141-53.

63.   **LoVullo, E. D., L. A. Sherrill, L. L. Perez, and M. S. Pavelka, Jr.** 2006. Genetic tools for highly pathogenic *Francisella tularensis* subsp. *tularensis*. Microbiology **152:**3425-35.

64.   **Maier, T. M., A. Havig, M. Casey, F. E. Nano, D. W. Frank, and T. C. Zahrt.** 2004. Construction and characterization of a highly efficient Francisella shuttle plasmid. Appl Environ Microbiol **70:**7511-9.

65.   **Maier, T. M., R. Pechous, M. Casey, T. C. Zahrt, and D. W. Frank.** 2006. In vivo Himar1-based transposon mutagenesis of *Francisella tularensis*. Appl Environ Microbiol **72:**1878-85.

66.   **McCaffrey, R. L., and L. A. Allen.** 2006. *Francisella tularensis* LVS evades killing by human neutrophils via inhibition of the respiratory burst and phagosome escape. J Leukoc Biol **80:**1224-30.

67.   **McCoy G.W., C. C. W.** 1912. Further observation on a plague-like disease of rodents with a preliminary note on the causative agent, *Bcterium tularense.* J Infect Dis**:**61-72.

68.   **McCrumb, F. R.** 1961. Aerosol Infection of Man with *Pasteurella Tularensis*. Bacteriol Rev **25:**262-7.

69.   **Mohapatra, N. P., A. Balagopal, S. Soni, L. S. Schlesinger, and J. S. Gunn.** 2007. AcpA is a *Francisella* acid phosphatase that affects intramacrophage survival and virulence. Infect Immun **75:**390-6.

70.   **Nano, F. E., N. Zhang, S. C. Cowley, K. E. Klose, K. K. Cheung, M. J. Roberts, J. S. Ludu, G. W. Letendre, A. I. Meierovics, G. Stephens, and K. L. Elkins.** 2004. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. J Bacteriol **186:**6430-6.

71.   **Ottem, K. F., A. Nylund, E. Karlsbakk, A. Friis-Moller, and B. Krossoy.** 2007. Characterization of *Francisella* sp., GM2212, the first *Francisella* isolate from marine fish, Atlantic cod (Gadus morhua). Arch Microbiol **187:**343-50.

72. **Oyston, P. C., A. Sjostedt, and R. W. Titball.** 2004. Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*. Nat Rev Microbiol **2:**967-78.

73. **Pavlov, V. M., A. N. Mokrievich, and K. Volkovoy.** 1996. Cryptic plasmid pFNL10 from *Francisella novicida*-like F6168: the base of plasmid vectors for *Francisella tularensis*. FEMS Immunol Med Microbiol **13:**253-56.

74. **Penn, R. L.** 2005. *Francisella tularensis* (Tularemia), p. 2674-2685. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Mandell, Douglas, and Bennett's Principals and Practice of Infectious Diseases, 6th ed, vol. 2. Elsevier Churchill Livingstone, Philadelphia.

75. **Plotkin, S. A.** 2003. Vaccines, vaccination, and vaccinology. J. Infect. Dis. **187:**1349-1359.

76. **Qin, A., and B. J. Mann.** 2006. Identification of transposon insertion mutants of *Francisella tularensis* tularensis strain Schu S4 deficient in intracellular replication in the hepatic cell line HepG2. BMC Microbiol **6:**69.

77. **Raynaud, C., K. L. Meibom, M. A. Lety, I. Dubail, T. Candela, E. Frapy, and A. Charbit.** 2007. Role of the wbt locus of *Francisella tularensis* in lipopolysaccharide O-antigen biogenesis and pathogenicity. Infect Immun **75:**536-41.

78. **Reintjes, R., I. Dedushaj, A. Gjini, T. R. Jorgensen, B. Cotter, A. Lieftucht, F. D'Ancona, D. T. Dennis, M. A. Kosoy, G. Mulliqi-Osmani, R. Grunow, A. Kalaveshi, L. Gashi, and I. Humolli.** 2002. Tularemia outbreak investigation in Kosovo: case control and environmental studies. Emerg Infect Dis **8:**69-73.

79. **Reyrat, J. M., V. Pelicic, B. Gicquel, and R. Rappuoli.** 1998. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. Infect Immun **66:**4011-7.

80. **Rubin, E. J., B. J. Akerley, V. N. Novik, D. J. Lampe, R. N. Husson, and J. J. Mekalanos.** 1999. In vivo transposition of mariner-based elements in enteric

bacteria and mycobacteria. Proc Natl Acad Sci U S A **96:**1645-50.

81. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. ed, vol. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

82. **Sandstrom, G., S. Lofgren, and A. Tarnvik.** 1988. A capsule-deficient mutant of *Francisella tularensis* LVS exhibits enhanced sensitivity to killing by serum but diminished sensitivity to killing by polymorphonuclear leukocytes. Infect Immun **56:**1194-202.

83. **Sandstrom, G., A. Sjostedt, T. Johansson, K. Kuoppa, and J. C. Williams.** 1992. Immunogenicity and toxicity of lipopolysaccharide from *Francisella tularensis* LVS. FEMS Microbiol Immunol **5:**201-10.

84. **Santic, M., M. Molmeret, and Y. Abu Kwaik.** 2005. Modulation of biogenesis of the *Francisella tularensis* subsp. *novicida*-containing phagosome in quiescent human macrophages and its maturation into a phagolysosome upon activation by IFN-gamma. Cell Microbiol **7:**957-67.

85. **Santic, M., M. Molmeret, K. E. Klose, S. Jones, and Y. A. Kwaik.** 2005. The *Francisella tularensis* pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. Cell Microbiol **7:**969-79.

86. **Saslaw, S., H. T. Eigelsbach, J. A. Prior, H. E. Wilson, and S. Carhart.** 1961. Tularemia vaccine study. II. Respiratory challenge. Arch Intern Med **107:**702-14.

87. **Saslaw, S., H. T. Eigelsbach, H. E. Wilson, J. A. Prior, and S. Carhart.** 1961. Tularemia vaccine study. I. Intracutaneous challenge. Arch Intern Med **107:**689-701.

88. **Sebastian, S., S. T. Dillon, J. G. Lynch, L. T. Blalock, E. Balon, K. T. Lee, L. E. Comstock, J. W. Conlan, E. J. Rubin, A. O. Tzianabos, and D. L. Kasper.** 2007. A defined O-antigen polysaccharide mutant of *Francisella tularensis* live

vaccine strain has attenuated virulence while retaining its protective capacity. Infect Immun **75:**2591-602.

89. **Shen, H., W. Chen, and J. W. Conlan.** 2004. Mice sublethally infected with *Francisella novicida* U112 develop only marginal protective immunity against systemic or aerosol challenge with virulent type A or B strains of *F. tularensis*. Microb Pathog **37:**107-10.

90. **Sjöstedt, A.** 2005. Family III. *Francisellaceae, fam. nov.*. p. 199-210. *In* N. R. K. D.J. Brenner, J.T. Staley (ed.), Bergey's Manual of Systematic Bacteriology second edition, vol. two. Springer, New York.

91. **Sjostedt, A.** 2007. Tularemia: History, epidemiology, pathogen physiology, and clinical manifestations. Ann N Y Acad Sci.

92. **Sjostedt, A.** 2003. Virulence determinants and protective antigens of *Francisella tularensis*. Curr Opin Microbiol **6:**66-71.

93. **Sjostedt, A., A. Tarnvik, and G. Sandstrom.** 1996. *Francisella tularensis*: host-parasite interaction. FEMS Immunol Med Microbiol **13:**181-4.

94. **Sorokin, V. M., N. V. Pavlovich, and L. A. Prozorova.** 1996. *Francisella tularensis* resistance to bactericidal action of normal human serum. FEMS Immunol Med Microbiol **13:**249-52.

95. **Su, J., J. Yang, D. Zhao, T. H. Kawula, J. A. Banas, and J. R. Zhang.** 2007. Genome-wide identification of *Francisella tularensis* virulence determinants. Infect Immun **75:**3089-101.

96. **Sukupolvi-Petty, S., S. Grass, and J. W. St Geme, 3rd.** 2006. The *Haemophilus influenzae* Type b *hcsA* and *hcsB* gene products facilitate transport of capsular polysaccharide across the outer membrane and are essential for virulence. J Bacteriol **188:**3870-7.

97. **Tarnvik, A.** 1989. Nature of protective immunity to *Francisella tularensis*. Rev Infect Dis **11:**440-51.

98.   **Telepnev, M., I. Golovliov, T. Grundstrom, A. Tarnvik, and A. Sjostedt.** 2003. *Francisella tularensis* inhibits Toll-like receptor-mediated activation of intracellular signalling and secretion of TNF-alpha and IL-1 from murine macrophages. Cell Microbiol **5:**41-51.

99.   **Tempel, R., X. H. Lai, L. Crosa, B. Kozlowicz, and F. Heffron.** 2006. Attenuated *Francisella novicida* transposon mutants protect mice against wild-type challenge. Infect Immun **74:**5095-105.

100. **Thomas, R. M., R. W. Titball, P. C. Oyston, K. Griffin, E. Waters, P. G. Hitchen, S. L. Michell, I. D. Grice, J. C. Wilson, and J. L. Prior.** 2007. The immunologically distinct O antigens from *Francisella tularensis* subspecies *tularensis* and *Francisella novicida* are both virulence determinants and protective antigens. Infect Immun **75:**371-8.

101. **Timoney, J. F., J. H. Gillespie, F. W. Scott, and J. E. Barlough.** 1988. The genus *Fraancisella*, p. 132-135. *In* J. F. Timoney, J. H. Gillespie, F. W. Scott, and J. E. Barlough (ed.), hagan and Bruner's Microbiology and Infectious Diseases of Demestic Animals, 8th ed. ed. Cornell University Press, Ithaca.

102. **Titball, R. W., A. Johansson, and M. Forsman.** 2003. Will the enigma of *Francisella tularensis* virulence soon be solved? Trends Microbiol **11:**118-23.

103. **Titball, R. W., and J. F. Petrosino.** 2007. *Francisella tularensis* genomics and proteomics. Ann N Y Acad Sci.

104. **Titball, R. W., and A. Sjostedt.** 2002. *Francisella tularensis*: an overview. ASM News **69:**558-563.

105. **Twine, S., M. Bystrom, W. Chen, M. Forsman, I. Golovliov, A. Johansson, J. Kelly, H. Lindgren, K. Svensson, C. Zingmark, W. Conlan, and A. Sjostedt.** 2005. A mutant of *Francisella tularensis* strain SCHU S4 lacking the ability to express a 58-kilodalton protein is attenuated for virulence and is an effective live vaccine. Infect Immun **73:**8345-52.

106.   **Vinogradov, E., M. B. Perry, and J. W. Conlan.** 2002. Structural analysis of *Francisella tularensis* lipopolysaccharide. Eur J Biochem **269:**6112-8.

107.   **Vinogradov, E. V., A. S. Shashkov, Y. A. Knirel, N. K. Kochetkov, N. V. Tochtamysheva, S. F. Averin, O. V. Goncharova, and V. S. Khlebnikov.** 1991. Structure of the O-antigen of *Francisella tularensis* strain 15. Carbohydr Res **214:**289-97.

108.   **Wherry, W. B. B. H. L.** 1914. Infection of man with *Bacterium tularense*. J. Infect. Dis.**:**331-340.

109.   **Zahrt, T. C., and D. W. Frank.** 2007. Genetics and genetic manipulation in *Francisella Tularensis*. Ann N Y Acad Sci.

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