Surface Polysaccharides of *Francisella tularensis*: Further Characterization, Role in Virulence, and Application to Novel Vaccine Strategies

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Abstract

Francisella tularensis is a Gram-negative, zoonotic bacterium that causes tularemia in animals and humans. The two subspecies *tularensis* (Type A) and *holarctica* (Type B) are considered Tier I Select Agents due to the bioweapon potential of these subspecies. Type A strains, considered the more virulent of the subspecies, are highly infective producing respiratory tularemia with inhalation of as few as 10 cells. Due to classification as a Select Agent, a vast amount of *F. tularensis* research has occurred in the last two decades after the September 11th terrorism attack and the use of *Bacillus anthracis* spores in a biological attack on the United States Postal Services in 2001. This research has uncovered many of the various virulence factors of *F. tularensis* including an intracellular nature, the unique lipopolysaccharide produced, and a genetic pathogenicity island. This dissertation aims to further characterize outer surface antigens of *F. tularensis* subspecies in regards to virulence, biofilm formation, and role in vaccine development. In addition, this dissertation will also investigate the use of a novel vaccine delivery vehicle, alginate microencapsulation, in increasing the efficacy of these mutant strains.

F. novicida is a subspecies of *F. tularensis* and usually classified as being non-encapsulated. However, *F. novicida* has a similar capsule glycosylation locus as *F. tularensis* and could produce a similar capsule-like complex that has previously been described for the *F. tularensis* LVS strain. I was able to isolate and characterize this CLC of *F. novicida*, which contained a heterogenous mixture of proteins and possible glycosylated proteins. A mutant with a multi-gene interruption within the glycosylation locus (*F. novicida*Δ1212-1218) produced significantly less carbohydrate than the parent strain, was attenuated in the mouse model, and was partially protective when used to immunize mice against a virulent challenge. Biofilms of *F. novicida* were also characterized in regards to biofilm formation in various growth media and biofilm formation of strains lacking the O-antigen of the lipopolysaccharide (LPS). In general, *F. novicida* produced the greatest amount of biofilm in a brain heart infusion (BHI) broth, compared to other media. Loss of the O-antigen led to increased biofilm production when grown in BHI and decreased or similar biofilm production as the wildtype when grown in other media. This highlights the need to carefully select the growth medium when assessing biofilm formation of *Francisella* strains in the future.

A final study of this dissertation characterized the use of alginate microspheres as a vaccine vehicle for an attenuated *F. tularensis* type A O-antigen deficient strain. O-antigen deficient strains of *F. tularensis* are highly attenuated *in vivo* and would be a safe choice for a vaccine candidate. However, these strains produce less than ideal protection against virulent challenge when used to immunize mice, possibly due to a lack of persistence in the host. In an attempt to increase persistence, we encapsulated an O-antigen deficient strain within sodium alginate microspheres and used those microspheres to immunize mice. The immunized mice produced a higher level of antibody response than mice immunized with a non-encapsulated version. However, this immunization only partially protected mice from a virulent challenge and did not match the protection afforded by the former Live Vaccine Strain (LVS). In part the deficiency in protection appears to be due to a lack of a robust cellular immune response in mice immunized with the alginate microspheres.

In summary, this dissertation focuses on the various extracellular polysaccharides of *F. tularensis*: the glycosylation of CLC, the O-antigen, and the biofilm. Each polysaccharide plays a role in the virulence and pathogenesis of *F. tularensis*. Glycosylation of the CLC and the O-antigen are important virulence factors in mammalian disease, and mutants lacking either (not type A strains) are attenuated in the mouse model. Both also appear to play a role in the formation of the *F. tularensis* biofilm in a manner dependent on the environment or culture medium used. Each of these extracellular polysaccharides contribute to the lifecycle of *Francisella*.

Public Abstract

Francisella tularensis is a highly infectious bacterial pathogen that can cause disease in a wide array of animals and in humans. *F. tularensis* is also considered a potential weapon of bioterrorism and the development of an effective vaccine is a critical area of research. One strategy of developing a tularemia vaccine includes mutating a strain of *F. tularensis* to reduce expression of extracellular components that include polysaccharides. Strains that cannot express these components are usually unable to produce clinical signs in the host and may provide protection against fully virulent *F. tularensis* strains. The work presented in this dissertation will focus on characterizing the polysaccharide extracellular components of *F. tularensis* and developing a novel vaccine vehicle to increase protection from strains that do not cause disease.

Dedication

To my loving husband and best friend Nicholas Catanzaro who supported every single endeavor I attempted and has been my rock throughout this program.

And to my parents, John and Tina Freudenberger, who made me take Advanced Placement Physics, Spanish instead of French, and helped convince me that an in-state veterinary program was the way to go. Obviously, you both knew what you were talking about.

And to every single animal in my life now and previously.

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First, I would like to acknowledge my family and friends who have supported me all of these years through both good and bad times. I do not know how I would have accomplished this endeavor alone, without my family and friends.

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Attributions

Chapter 2: Glycosylation of a Capsule-Like Complex (CLC) by *Francisella novicida* is Required for Virulence and Partial Protective Immunity in Mice

KF conceived and performed most of the experiments, wrote the initial draft of the manuscript, contributed to revisions, and read/edited the final draft. AC helped with genetic analysis, contributed to that area of the manuscript, and read/edited the final draft. NM contributed to generating the complemented mutant, contributed to that area of the manuscript, and read/edited the final draft. TC performed the clinical pathology and read/edited the final draft. TI conceived the experiments, revised the manuscript, and carried out editing the final draft. The manuscript was published in "Frontiers in Microbiology" on May 30, 2017.

Chapter 3: Surface Antigens Affect the Ability of *Francisella novicida* to Produce a Biofilm

KF conceived and performed all experiments in the chapter and wrote the chapter. Portions of the chapter are submitted for publication in Scientific Reports in conjunction with work by AC.

Chapter 4: Alginate Microencapsulation of an Attenuated O-Antigen Mutant of *Francisella tularensis* LVS as a Model for a Vaccine Delivery Vehicle

KF conceived and performed the majority of the experiments, wrote the initial draft of the manuscript, contributed to revisions, and read/edited the final draft. AM helped with technical aspects of some experiments and read/edited the final draft. KL performed the clinical pathology and read/edited the final draft. CA performed the IPA analysis. TI revised the manuscript, and carried out editing the final draft.

Chapter 5: Deciphering the Capsules of *Francisella tularensis*

KF wrote the review, contributed to revisions, and read/edited the final draft. TI helped revise the review.

Chapter 1: Introduction & Literature Review

1.1. History of *Francisella*

At the turn of the twentieth century, an outbreak of plague occurred in California. This outbreak caused over one hundred deaths within urban centers such as San Francisco and Los Angeles (1). By 1904, sanitation and rat control efforts were able to temporarily eradicate the disease until the devastating earthquake and fire of 1906 renewed spread of the disease in San Francisco (1). During this same period large groups of California ground squirrels (*Spermophilus beecheyi*) in Contra Costa County were dying from plague and incidents of human plague in these rural areas were reported (2). The United States (U. S.) Public Health and Marine-Hospital Service conducted a study of the extent of squirrel plague by testing squirrels for *Yersinia pestis* (at the time *Bacillus pestis*) (2). During this study, Dr. George W. McCoy observed a disease characteristic of plague in several ground squirrels that could not be contributed to *Yersinia pestis* infection (2). The etiological agent of this plague-like disease was isolated soon after and designated *Bacterium tularense* after Tulare County where the bacterium was first discovered (3). McCoy and Chapin observed that this new bacterial species was infectious for a variety of species and postulated that human infection was possible (3). In 1914, a putative case of tularemia in a meat cutter was reported from Cincinnati, Ohio (4). More cases of tularemia in Utah were later reported by Dr. Edward Francis (5). *Bacterium tularense* was eventually renamed *Francisella tularensis* in honor of the work carried out with this bacterium by Dr. Francis (6). *F. tularensis* is now recognized as a zoonotic pathogen that causes the severe illness tularemia by as few as ten organisms and is classified as a potential weapon of bioterrorism (6, 7).

1.2. The Family Francisellaceae

1.2.1. Taxonomy

Francisella is the sole genus within the family Francisellaceae (8). *Francisella* are small, Gram negative coccobacilli and divided into four recognized species: *F. tularensis*, *F. hispaniensis*, *F. noatunensis,* and *F. philomaragia* (9). *F. tularensis* is further divided into four sub-species: *tularensis* (Type A), *holarctica* (Type B), *mediasiatica*, and *novicida*. Subspecies

novicida was formerly considered a separate species from *F. tularensis* though has been reclassified as a subspecies (10-12). However, the debate on the classification of *F. tularensis* subspecies *novicida* (hereon referred to as *F. novicida*) continues. *F. tularensis* causes tularemia in both humans and animals. Type A infection causes the more severe manifestation of tularemia while Type B infection causes milder disease (13). *F. philomaragia* and *F. novicida* are significantly less virulent, but can potentially cause disease in immunocompromised humans (9). *F. noatunensis* is purely a fish pathogen (9). Other species within the genus *Francisella* exist and ultimate classification of species is still in flux (8, 14-16).

1.2.2. Bacterial Features and Genetics

Francisellaceae are small, nonmotile, microaerophilic, gram-negative coccobacilli (8). When grown *in vitro*, *F. tularensis* strains require media supplemented with cysteine for growth. *F. novicida*, however, does not require cysteine for growth, but will grow better if cysteine is supplemented (17). In general, all *Francisella* species are slow growing with the exact conditions and generation time varying between species. *F. tularensis* subspecies grow optimally at 37°C with 6% CO_2, whereas environmental *Francisella* species such as *philomargia* may grow better at 22-25°C (8, 16).

Francisella spp. have a single chromosome that is less than 2 Mbp in size and a generally low GC content of 33% or less (8, 16). Species within the family *Francisella* also are approximately 85% or greater genetically similar (16). Genetic similarity between subspecies is even greater with *F. tularensis* subspecies *tularensis* (approximately 95% genetic similarity Type A strains and *F. novicida* strains) (8, 16). However, whereas *F. tularensis* subspecies *tularensis* is considered a highly important human pathogen, *F. novicida* is not. A pathogenicity island (discussed further in section 1.6.1) with considerably low GC content is present in all *Francisella* species, including environmental isolates, and is duplicated in the two most virulent *tularensis* subspecies (*tularensis* and *holarctica*) (16, 18, 19).

1.2.3. Geographic Distribution and Ecology

All naturally occurring *Francisella* species are exclusively found in the Northern Hemisphere (20). Type A strains are mainly confined to North America with Type A.1. occupying the eastern half and Type A.II occupying the western half of the continent (21). Type B strains, however, are found across all continents of the Northern hemisphere with a higher

incident in the Nordic countries, such as Sweden (13). Sweden has an annual incident rate of 3 to 4 cases of tularemia per 100,000 people (21). *F. novicida* appears to occupy a water niche and may be a murine pathogen due to its high virulence in mice compared to other animals (21). Subspecies *mediasiatica* is confined to Central Asia, particularly Kazakhstan and Turkmenistan (21).

Many *Francisella* species are associated with vectors and a diverse range of environments or host species. *F. tularensis* is often found in arthropod vectors such as the American dog tick *Dermacentor variabili* and the Lone Star tick *Amblyomma americanun* in the United States, and various biting flies and mosquitoes in other parts of the Northern Hemisphere (22). Ticks are frequently implicated in the transmission of *F. tularensis* to humans in the U.S. The incidence of tick-borne tularemia is higher in the U.S. than in European countries possibly because the *Dermacentor* tick species in the U.S. have a higher affinity for humans than those present in Europe (22). Rabbits and other lagomorphs act as a reservoir for the bacterium, with arthropods functioning as intermediate vectors of transmission between mammalian species (22, 23).

However, direct infection from infected mammalian species to humans is possible (24, 25). As previously stated, *F. tularensis* can infect numerous different hosts and tularemia can result in a number of those species (26). This host range for *F. tularensis* is of particular concern when considering human encroachment into untouched environments and the interaction of domestic animals and wildlife. The increasing number of natural human cases of tularemia may indicate that *F. tularensis* is a re-emerging zoonotic pathogen (27, 28).

1.3. Clinical Manifestations of Tularemia

1.3.1. Disease in Humans

Dr. McCoy first described the disease and associated lesions of *F. tularensis* infection of ground squirrels as “plague-like” (2, 3). Evidence later suggested that Dr. McCoy and a lab assistant may have become infected with *F. tularensis* why investigating the illness among the ground squirrels (29). Tularemia is the clinical manifestation of *F. tularensis* infection. Other synonymous names include Deer-Fly Fever (30), Ohara’s Disease (31), and Rabbit Fever.

Tularemia is generally characterized by acute onset of flu-like symptoms and formation of granulomatous lesions at various tissue locations (8, 32). However, the severity of clinical

signs depends on the infectious strain, the infectious dose, and the route of infection. *F. tularensis* Type A.I. strains are considered the most virulent, followed by Type B strains and Type A.II. strains (33). Inhalation of 10 cells of a Type A.1. strain can lead to pneumonic tularemia that is associated with 30% mortality if left untreated (32). Conversely, Type B infections may be under diagnosed because these lead to less severe clinical signs and frequently present as nonspecific flu-like symptoms that resolve with time (33). *F. novicida* strains usually only causes disease in immunocompromised individuals (9).

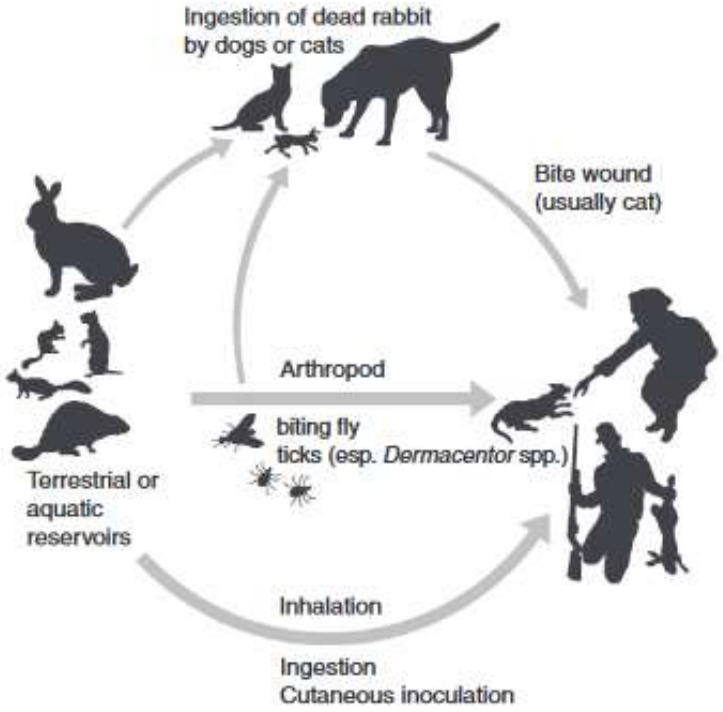

Figure 1.1. Zoonotic transmission of *Francisella tularensis*.
F. tularensis affects a large range of species. Common routes of infection in humans include arthropod transmission, inhalation of cells, cutaneous inoculation or ingestion of infected animal tissues, or bite wounds from infected cats. Taken from Sykes *et al*. 2014 (34).

Routes of infection (Figure 1.1) include direct contact with infectious material, ingestion of infected tissues, airborne transmission, and vector borne transmission (33, 34). The route of infection determines the specific manifestation of tularemia. Major forms of tularemia include: ulceroglandular, glandular, oculoglandular, oropharyngeal, and pneumonic (33). The ulceroglandular form occurs from inoculation through the skin by direct contact or by vector transmission and results in an ulcer forming at the site of inoculation (35). The oculoglandular

form results from infection of the eye and leads to conjunctivitis. Both ulceroglandular and oculoglandular forms can spread to regional lymph nodes resulting in lymphadenopathy if left untreated (33). Glandular disease happens when no distinct ulcer forms and lymphadenitis follows non-specific symptoms such as fever, chills, and malaise (33). The oropharyngeal form results from ingestion of infected material that leads to pharyngitis or lymphadenitis (33). Inhalation of *F. tularensis* leads to pneumonic tularemia with symptoms that depend on the strain involved in the infection (35). Following effective antibiotic treatment of tularemia, case fatality rates fall to 2% (33). *F. tularensis* is susceptible to a wide range of antibiotics such as streptomycin, doxycycline, and gentamycin (26).

1.3.2. Disease in Small Animals

Dogs and cats can become infected with *F. tularensis* via ingestion of infected reservoir species such as rabbits or by vector borne transmission (Figure 1.1) (36-38). The extent of clinical disease varies greatly between the two (36-38). Compared to other species, dogs are surprisingly resistant to *F. tularensis* infection and any clinical signs are milder (38, 39). A large proportion of dogs in *F. tularensis* endemic regions are seropositive indicating that subclinical tularemia in dogs may be common (37, 38) In comparison, cats are less resistant and the infection can be rapidly fatal in untreated cats with clinical symptoms (37, 40-45).

The disease in cats is similar to that in humans. Cats will present as febrile and lethargic with lymphadenopathy, possible ulcerations or abscesses at the site of infection, and various organomegalies (37). Acutely ill cats with suspected tularemia often have a history of contact with or hunting of lagomorphs or rodents (34). Thrombocytopenia in cats seems to be the only consistent laboratory abnormality described (34) and titers to *Francisella* may be negative due to the acute nature of the disease (34, 38). Culture and isolation of the bacterium is the gold standard of diagnosis. However, the fastidious nature of *Francisella* and the extended growth time necessary require that cats with suspected tularemia be treated based on clinical suspicion prior to definitive diagnosis (34, 37, 38). Special precautions should be taken to prevent spread of *F. tularensis* to personnel and other patients. Cat bite wounds have been documented as a source of infection to humans (46-48).

1.4. Weaponized Tularemia

During the Battle of Stalingrad, a turning point in World War II, Nazi forces were significantly defeated both in combat and from illness. Dr. Kenneth Alibek, a former United Soviet Socialist Republics (U.S.S.R.) biowarfare expert, claimed that the Soviets purposefully released *F. tularensis* against the Nazi troops (49). U.S.S.R. reports and a better understanding of the transmission of *F. tularensis* cast doubts on this claim and suggest that the occurrence of a natural outbreak of tularemia was more probable (50). The U.S. and U.S.S.R. established programs to weaponize biological agents such as *F. tularensis*, but these programs were later terminated (20, 51). Following the termination of the program, the U.S. and other countries ratified the Biological Weapons Convention that outlawed the development, production, and stockpiling of biological weapons.

Public information on the U.S. biological warfare programs before its termination indicate that *F. tularensis* was a major area of research, and by the late 1950s was the number one agent considered as a potential bioweapon (51). A 1970 World Health Organization (WHO) report stated that release of 50 kg of aerosolized *F. tularensis* in a city of five million people had the potential to cause a quarter of a million causalities and significant economic damages (52). Stockpiles of aerosolized *F. tularensis* were kept by both the U.S. and U.S.S.R. governments throughout parts of the Cold War and were weaponized to various degrees (20, 51). In the U.S. possible antibiotic resistant strains were created, and in the U.S.S.R. a weaponized vaccine-resistant strain was created (49, 51). However, much information about the U.S. biological warfare program remains confidential.

The concern surrounding weaponized tularemia and biowarfare in general escalated after the 2001 Amerithrax anthrax attacks that followed the September 11th terrorist attacks (20). Congressional and executive acts such as “Public Health Security and Bioterrorism Preparedness and Response Act of 2002” were passed to heighten the control and security of potential agents of biological warfare (53). The Center for Disease Control and Prevention (CDC) and the Department of Health and Human Services (HHS) characterize a Tier I Select Agent as a biological agent with “the greatest risk of deliberate misuse with the most significant potential for mass casualties or devastating effects to the economy, critical infrastructure, or public confidence” (53).

F. tularensis Types A and B are considered Tier I Select Agents for their potential use as bioweapons because of their level of virulence, ease of dispersal, persistence in the environment, and non-specific clinical signs (54, 55). *F. novicida* is not considered a significant threat to immunocompetent individuals and is not considered a Select Agent (20).

1.5. Pathogenesis and Survival of *Francisella*

1.5.1. Intracellular Pathogen

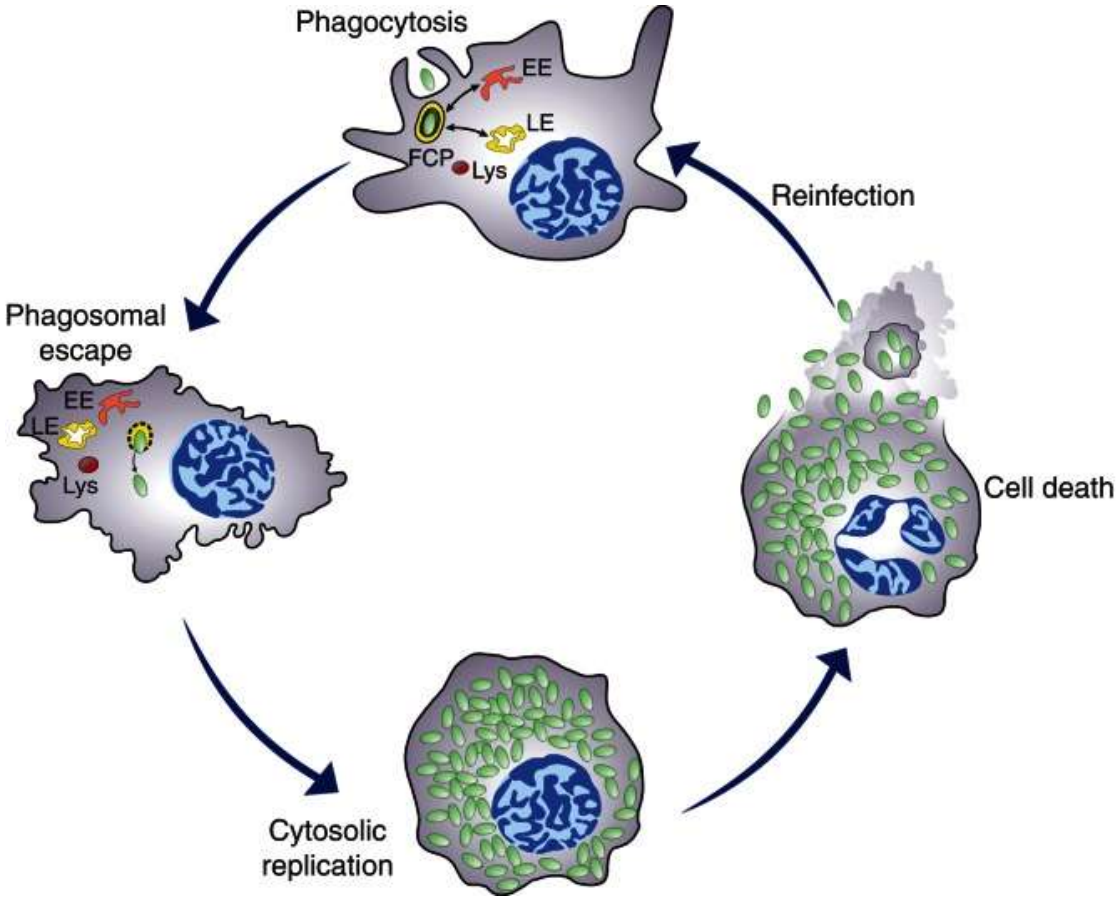

Figure 1.2. Intracellular survival and replication of *F. tularensis*
Within the host, *Francisella* bacterial cells are phagocytized by pseudopod loops and contained intracellularly within the phagosome. Prior to formation of the phagolysosome, *F. tularensis* cells disrupt the phagosome membrane and escape into the cytosol. Unheeded replication eventually leads to cell death and release of bacteria extracellularly. *Taken from*: J Celli and TC Zahrt (56).

Francisella tularensis is a facultative intracellular pathogen (Figure 1.2) with the ability to live extracellularly and environmentally (13). Disease occurs due to both the intracellular and extracellular stages. Within the host, replication occurs intracellularly within phagocytic cells

such as macrophages, neutrophils, and dendritic (DN) cells (57, 58). *F. tularensis* cells are engulfed by macrophages via asymmetric pseudopod loops (59) and sequestered intracellularly (Figure 1.2) within *Francisella*-containing phagosomes (FCP) (56). Normally this phagosome would process into a phagolysosome where an oxidative burst would destroy contained bacterial cells. However, *Francisella* is able to modify this pathway, prevent fusion of the phagolysosome, and disrupt the phagosomal membrane to escape into the cytosol of the host cell (60). Ledvina *et al.* (61) showed that *Francisella* secretes a mimic of a host protein (OpiA) that is able to alter phosphatidylinositol-3-phosphate (PI(3)P) levels and delay the phagolysosome pathway sufficiently enough to escape into the host's cytosol. This host protein mimic OpiA is secreted by a secretion system encoded on the *Francisella* Pathogenicity Island (FPI). Genes encoded on the FPI are essential to the ability of *F. tularensis* to not only infect phagocytic cells, but effectively escape the phagosome and replicate in the cytosol (18, 62). Escape of *F. tularensis* into the cytosol leads to uninhibited bacterial replication, eventual death of the host cell, and the efflux of bacterial cells extracellularly when the host cell ruptures (56). After escape, *F. tularensis* disseminates throughout the body during the extracellular stage. This stage results in a significant bacteremia in the mouse model and is postulated to be responsible for the dissemination of the organism to locations distal to the site of infection in the mouse and in other species (63).

1.5.2. Host Innate Immune Response

Infection with *Francisella* is typically characterized as the temporary subversion of the host immune system, unregulated growth of the bacterium, and an eventual "cytokine storm" that leads to most of the clinical signs (64). Prior to the "cytokine storm," *Francisella* resist the effects of extracellular bactericidal components such as serum antibodies and complement proteins. Numerous studies have shown that wild-type *Francisella* species are highly resistant to the bactericidal effects of serum (65-70). Resistance to serum is often attributed to capsule production and this was initially the hypothesis for serum resistance by *F. tularensis*. However, multiple surface antigens contribute to the ability of *F. tularensis* to avoid complement-mediated lysis. Serum resistance is typically attributed to the unusual LPS, in particular the O-antigen (O-Ag) of *F. tularensis*. Interruption of O-Ag production significantly increases the sensitivity of *Francisella* to the bactericidal effects of serum (65-67). O-Ag mutants are usually highly attenuated *in vivo*, which has been attributed to their inability to survive in serum (65). Though *Francisella* is resistant to complement when the O-Ag is intact, bacterial cells still bind

complement proteins such as C3 (68). C3-opsonization is important for uptake of *F. tularensis* cells and binds all *Francisella* strains, but more so to the complement sensitive strains (59, 68, 71). Strains that are resistant to complement-mediated lysis bind factor H and quickly convert C3 to the inactive iC3b form (72). This inactivation allows *Francisella* to prevent binding of the membrane attack complex and aids in uptake of the bacterial cells by phagocytic cells where *Francisella* begins its intracellular stage (72). In addition to avoiding complement-mediated lysis, *Francisella* is able to avoid early recognition by the innate immune system. *Francisella* LPS is not recognized by the traditional Toll-like receptor (TLR) TLR4 and thus does not induce an LPS-mediated immune response (73, 74).

With the ability to initially evade the host immune system, *Francisella* does not stimulate production of pro-inflammatory cytokines until after significant bacterial growth has occurred (7). Pro-inflammatory cytokines are detected approximately two to three days post-infection in the mouse model (7). Andersson *et al*. (75) evaluated the timeline of cytokine production following intranasal infection with *F. tularensis* in mice. INF-γ and TNF-α were not detectable until approximately days two to four post-infection, and levels of IL-6 and IL-1β were not detectable until three days post-infection (75). This delayed cytokine upregulation does not help survival and other studies have shown that stimulating production of pro-inflammatory cytokines immediately following infection with *F. tularensis* increases survival of the mice (76). Cytokines, particularly those that stimulate a Th_1 immune response, are essential to survival after challenge. Mice that are deficient in either INF-γ or TNF-α are highly susceptible to LVS infection (77, 78). Treating *Francisella* infected macrophages with exogenous INF-γ inhibits *Francisella* growth and may reduce the ability of *F. tularensis* to escape the phagosome (79). INF-γ stimulates the production of bactericidal reactive oxygen species (ROS) and reactive nitrogen species (RNS), two mechanisms aimed at combating intracellular pathogens. Lindgren *et al*. (80) showed that mice deficient in production of ROS or RNS had significantly lower lethal doses of *F. tularensis* LVS than mice able to produce these reactive species.

Francisella tularensis infects a number of cells of the innate immune system including macrophages, DN, neutrophils, and many non-phagocytic cells (7). In addition to evading the host immune system to prevent production of pro-inflammatory cells, *Francisella* also appears to be able to stimulate production of anti-inflammatory cytokines in these cells. Shirey *et al*. (81) describes activated macrophage population that are produced after intraperitoneal infection with

F. tularensis. This population had a reduced expression of pro-inflammatory markers and an up-regulation of anti-inflammatory markers such as IL-4 and TFG-β (81). In addition, mice unable to produce active IL-4 showed increased survival compared to wildtype mice when given a lethal dose of LVS (81). DN infected with *Francisella* do not produce pro-inflammatory cytokines and are refractory to inflammatory stimuli such as *Escherichia coli* LPS, which may be attributed to production of TGF-β (82).

1.5.3. Host Adaptive Immune Response

Development of a tularemia vaccine is dependent on understanding how the adaptive immune system responds and remembers a *Francisella* infection. *Francisella* is an intracellular pathogen and thus many vaccine studies have focused on developing a Th_1, cellular-mediated immune response. However, due to the significant extracellular phase of *Francisella*, an antibody response may also aid in providing full, protective immunity. The exact amount of necessary antibody response to protect against *F. tularensis* is debatable. Many vaccine studies have shown partial protection against challenge after immunization with *Francisella* LPS or other purified protein conjugates (83-85). Kaur *et al*. (85) produced an adenovirus-vector vaccine expressing an immunodominant surface protein from *Francisella* called Tul4 that has been shown to be important for antibody-mediated protection. Three boosters of this Tul4-expressing vector conferred approximately 60% protection in mice challenged with a lethal dose of LVS (85). However, a majority of these studies were completed in the *F. tularensis* LVS strain and a similar approach with a Type A strain may be ineffective. Type A strains, unlike LVS, are able to bind the host protease plasmin, degrade opsonizing antibodies, and avoid antibody-mediated uptake or lysis (86). This difference between Type A strains and LVS brings into question the significance of any protective antibody response in the face of *Francisella* infection.

In general, a T-cell immune response is necessary for protection against subsequent challenge with *F. tularensis*. Mice deficient in both $CD4^+$ and $CD8^+$ are unable to effectively clear a sublethal *Francisella* challenge, but mice deficient in one population or the other population are able to effectively clear a sublethal challenge (87, 88). Further studies indicate the importance of INF-γ and IL-17A produced by $CD4^+$, $CD8^+$, and DN T-cells to activate macrophages and inhibit *F. tularensis* intracellular growth (78, 89). Cell culture studies of LVS-immune T-cells and macrophages demonstrate the importance of T-cell-produced INF-γ and TNF-α for controlling *Francisella* infections, separating out the importance of these cytokines

for the innate and adaptive immune system (78, 89). The exact role and importance of the various populations of the adaptive immune system is still be investigated.

1.5.4. Biofilm Lifestyle

In addition to the lifestyles required upon mammalian infection, *Francisella* must survive environmentally or within vectors such as biting insects or ticks. These niches are significantly different in respects to intra- and extra-cellular niches within the mammalian host. Factors such as temperature and nutrients are different between the external environment, vectors, and mammals. As such, *Francisella* species have developed various strategies to live in these niches. One such strategy may be the ability to form a biofilm (90). Biofilms are living bacteria surviving in a matrix of various bacterial molecules that adhere to a surface and provide protection against environmental stresses. The majority of biofilm research in *Francisella* has been accomplished in the *F. novicida* strain, a strain usually implicated as a water-colonizing bacterium (90). More recently work has been completed that identify factors that contribute to biofilm formation by Type A and Type B strains (90, 91).

F. novicida has been shown to form biofilms on a variety of surfaces, including plastic dishes, test tubes, and chitin surfaces (91, 92). The environmental *Francisella* species *philomirargia* can actively form biofilms in the presence of the aquatic organism *Acanthamoeba castellanii* (93). Mahajan *et al*. (94) successfully grew *F. tularensis* LVS as a biofilm in natural water and showed that *Culex* mosquito larvae would readily feed off of the biofilm. These findings help support the hypothesis that *Francisella* biofilm production is related to environmental or vector-related survival.

Ongoing research is attempting to elucidate important molecular mechanisms of the *Francisella* biofilm. Possible mechanisms contributing to biofilm formation include pilin production, extracellular carbohydrate production, outer membrane vesicles, quorum sensing, and chitinases. Pilin production is an important first step in adhesion for other bacteria to produce biofilms. However, Type IV pili proteins do not appear to contribute to the *Francisella* biofilm, as strains deficient in production of Type IV pili do not have an alteration in biofilm production (95). Chitinases produced by *F. novicida* contribute to this specie's ability to produce a biofilm by providing a carbon source in nutrient-limited environments; the lack of a functioning chitinase prohibits biofilm formation in these environments (91, 92, 96). *F. novicida* biofilm formation is also dependent on QseC, an orphan response regulator (95). *Francisella*

QseC is a homolog to one of the *E. coli* quorum sensing system components that regulates biofilm formation through flagellar motility and a secretion system (90). A second component to this system has not yet been identified; however, deletion of the gene responsible for QseC significantly impaired biofilm formation (95). The downstream effects of QseC, and if those effects are directly related to biofilm formation, are still being determined.

Bacterial surfaces carbohydrates may play a role in biofilm formation due to their direct relation with the extracellular environment. These various extracellular carbohydrates, discussed in detail later, are differentially regulated based on the temperature and culture medium (97-100), and may influence the ability of various *F. tularensis* strains to form biofilms. Interestingly, deletion of the gene responsible for QseC creates a serum-sensitive strain of *Francisella* with alterations in the O-Ag of the LPS (101). The effects of the O-Ag on biofilm formation in *Francisella* are further explored later in this dissertation.

1.6. *Francisella* Virulence Factors

1.6.1. *Francisella* Pathogenicity Island

The FPI is a segment of chromosomal DNA containing approximately 17 ORFs spanning 33-kb that is essential for *F. tularensis* to survive intracellularly (18, 102). This region is essential for intracellular survival and growth (18). Transposon mutagenesis studies of *F. novicida* identified several genes required for intracellular growth within an intracellular growth locus, *iglABCD* (103). Interruption of one *igl* gene compromised the ability of *F. novicida* to grow within macrophages *in vitro* (103). One of the most prominent proteins produced during intramacrophage infection by *Francisella* is encoded by *iglC* in this locus (57, 103). Further bioinformatic studies identified an area of unusually low G/C content that spanned the intracellular growth locus and is present in all *Francisella* species (18), but is duplicated in Type A and Type B subspecies (19). Insertion sequence elements are adjacent to the FPI and may be responsible for this duplication (104). Due to the duplication, many mutagenesis studies of the FPI have been conducted in *F. novicida*.

Extensive numbers of studies have examined the critical role the FPI plays in intracellular survival and replication (61, 102, 105-110). In general, disruption of a gene in the FPI leads to defects in intracellular growth and virulence in mice (111). However, disruption of certain genes in the FPI (*pdpC, pdpE, pdpD,* and *anmK*) have not altered the ability of *Francisella* to grow

intracellularly (112) and the disruption of a *pdpE* did not affect the mutant's virulence in mice (111). In addition to the actual proteins of the FPI, there are six known proteins that regulate FPI expression (111). The macrophage growth locus protein MglA is a positive major regulator of the FPI (19, 113). Disruption of *mglA* also reduces the ability of *Francisella* to grow intracellularly (103). The pathways affected by the FPI and its regulators are still being determined. The FPI encoded Type VI secretion system (T6SS) is an ongoing area of investigation.

1.6.2. Type VI Secretion System

The unique *Francisella* Type VI secretion system (T6SS) is encoded on the FPI (61, 110, 114). T6SSs are found in many pathogenic Gram-negative bacteria and act to inject bacterial molecules across the bacterial cell wall into host cells for the purpose of killing or hijacking the host cells. These systems are frequently characterized as being similar to the contractile injection systems (CISs) of phages (115). Determination of the presence of a T6SS system usually requires finding homologs to the T6SS of *Vibro cholerae*. *Francisella* contains homologs of two proteins essential to the structure of the *V. cholerae* system: IglA and IglB (116). Clemens *et al.* (117) showed through cyro-electron microscopy (cyroEM) that IglA and IglB of *F. novicida* interdigitate and assemble sheaths resembling the phage-like tail of T6SSs. The intramacrophage environment stimulates assembly of the structure (117). Clemens *et al.* proposes that the interlaced IglA and IglB sheath forms around the IglC tube (Figure 1.3) (117). Additional studies showed that truncation of IglA or IglB interfered with the function of the T6SS and inhibited secretion and the ability of *F. novicida* to replicate within macrophages (117). Brodmann *et al.* showed the assembly, contraction, and disassembly of the T6SS at the poles of *Francisella* and the similarity of the mechanism to the T6SS found in *V. cholerae* (118). Figure 1.3 shows the proposed structure of the *Francisella* T6SS. As stated previously, IglA and IglB form a sheath around IglC that forms a central rigid tube that may dissemble into the host cell's cytosol following secretion of effector molecules (115, 117). There is a central spike composed of VgrG that in *Francisella* is unusually short (110). PdpA, another FPI encoded protein, often co-immunoprecipitates with VgrG and may cap VgrG to complete the central spike (110).

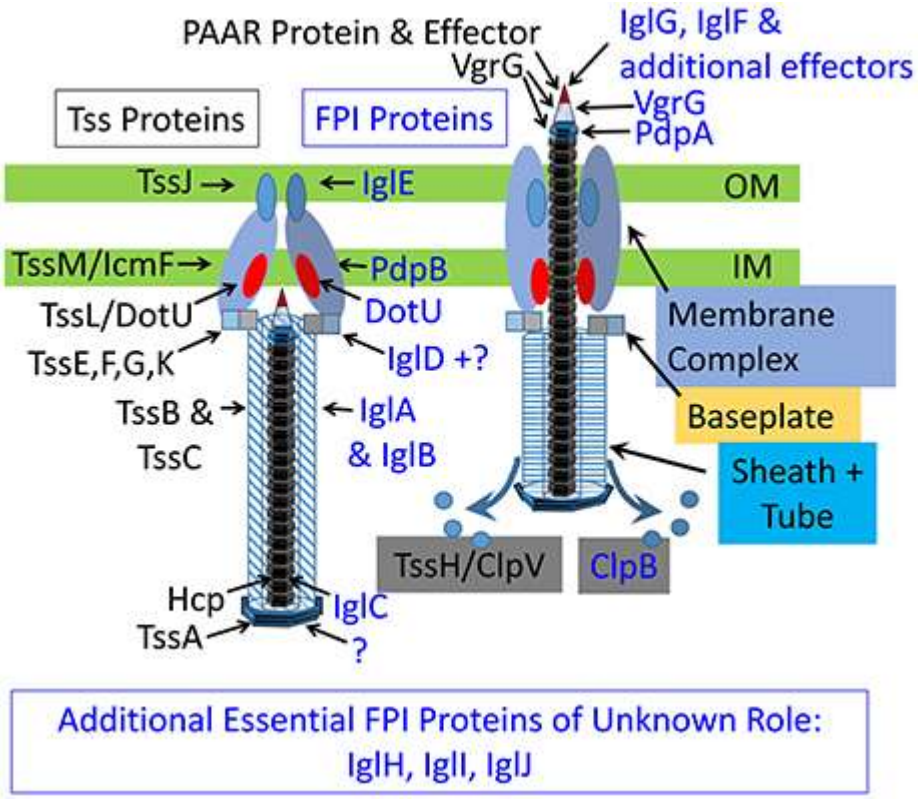

Figure 1.3. T6SS of *Francisella*
The proposed *Francisella* T6SS is shown with proteins forming the structure labeled in blue (FPI Proteins) and the proteins of a classical T6SS shown in black (Tss proteins). The FPI encodes for the proteins forming the T6SS though 3 FPI proteins (IglH, IglI, and IglJ) are unaccounted for in relation to the T6SS. The T6SS in shown in the extended form on the left and the contracted form on the right. The T6SS mechanism develops on the poles of *Francisella* and is proposed to pierce the phagosome, facilitate escape into the host cell's cytosol, and release bacterial molecules into the cytosol. *Taken from:* Clemens, Lee, and Horwitz (115).

The proposed purposes of the *Francisella* T6SS are to pierce the phagosome membrane and to secrete effector proteins into the host's cytosol. Several FPI encoded proteins (IglC, VgrG, PdpA, PdpC, and PdpD) have been identified as secreted via the T6SS (19, 107, 110, 114, 115, 118). In addition to FPI encoded proteins, several proteins outside of the FPI are also secreted (61). Proteins such as IglC, VgrG, and PdpA structurally make up the T6SS and mutagenesis of the responsible genes leads to the abolishment of the secretion system. However, mutagenesis of genes responsible for secreted proteins, such as OpiA or OpiB, do not lead to disruption of the secretion system and therefore these proteins are considered effector proteins (61, 110). Secreted OpiA may contribute to arresting phagosome maturation by affecting host phosphoinositides (PIs) that coat the FCP (61). Ledvina *et al*. (61) proposes that T6SS secreted

OpiA is a phosphatidylinositol-3-kinase that binds and phosphorylates PI on the outside of the FCP, resulting in a delay of phagosome maturation and *Francisella* escaping into the cytosol (61, 119). This research was completed in *F. novicida* and whether that model holds for the more virulent subspecies has yet to be determined.

1.6.3. Lipopolysaccharide

Lipopolysaccharide is the defining characteristic of Gram-negative bacteria and is often implicated as an endotoxin. LPS is composed of three main components: Lipid A, a non-repeating core sugar, and the repeating O-antigen (O-Ag) units. The *Francisella* LPS, though still an important virulence factor, is atypical compared to other Gram-negative LPSs, such as that from *E. coli*. Unlike the LPS of *E. coli*, the LPS from *Francisella* species is generally considered nontoxic (120-123). The low endotoxic activity of the *Francisella* LPS is most likely due to its unusual structure.

Generally, Lipid A molecules of Gram negative bacteria consist of 6 acyl fatty acid chains anchored to the bacterial membrane and two glycosamine sugar units, each phosphorylated (124). This structure of Lipid A allows for interaction with the TLR4 pathway and a potent stimulation of the innate immune system through NF-κB gene transcription. *Francisella* does not stimulate the innate immune system through TLR4 and the basis for this lack of interaction appears to be the asymmetrical structure of the Lipid A (73). *Francisella* species' Lipid A has only 4 acyl fatty acid chains that vary in length depending on growth conditions from 16 to 18 carbons, is phosphorylated once, and contains an additional galactosamine unit (125). The reduction in the acyl fatty acid chains, reduction in phosphorylation, and the additional galactosamine unit affects the overall charge of the Lipid A molecule and the ability of that molecule to interact with MD-2 on the TLR4 pathway (122).

Francisella only contains one 3-deoxy-D-manno-2-octulosonic acid (Kdo) unit linking the core region to the Lipid A compared to the usual two Kdo units of other Gram negatives (122). *Francisella* LPS is initially created with two Kdo units and a novel Kdo hydrolase system removes the second Kdo unit prior to addition of the O-Ag (126). Mutation of the Kdo hydrolase system leads to attenuation of *Francisella* in the mouse model (74). This Kdo hydrolase mutant *kdhAB* activates the TLR2 signaling pathway, possibly by changing the overall charge of the bacterial surface and allowing TLR-2 to interact with lipoproteins that would otherwise be

hidden (74). Therefore, *Francisella*'s unusual core oligosaccharide may play a hole in evading the host immune system.

Prior to compositional studies of the O-Ag, certain *Francisella* species were shown to phase vary between blue and grey colonies (127-129). Blue colonies were usually large, smooth, and appeared as white to blue opaque colonies under oblique light (129). Grey colonies appeared smaller, rough around the edges, and grey in coloration (129). This visible phenotype correlated with the ability of the strain to cause mortality in mice. Strains consisting of only blue colonies caused more severe disease than those containing mixed colony morphologies or those of just grey colonies (127, 129). Studies of grey strains showed that these strains were poorly immunogenic (130), survived poorly in macrophages (130), and contained a structurally different O-Ag compared to the virulent blue strains (127, 128, 130). This phase variation has been reported in strains of subspecies *tularensis* and *holarctica* (127-130).

Differences in the O-Ag between *F. tularensis* and *F. novicida* also exist. *F. tularensis* Type A and Type B subspecies contain identical O-Ag molecules (131). Type A and Type B strains contain an O-Ag of repeating β-D-Qui4NFm—α-D-GalNAcAN—α-D-GalNAcAN—β-D-QuiNAc (131). *F. novicida*'s O-Ag is antigenically distinct containing an O-Ag of repeating α-D-GalNAcAN—α-D-GalNAcAN—α-D-GalNAcAN— β-D-Qui2NAc4NAc (132). These differences in structure between *F. tularensis* subspecies and *F. novicida* lead to differing activities of the LPS. *F. novicida* LPS has been shown to induce a more potent inflammatory response *in vivo* in mice than *F. tularensis* subspecies (120). The O-Ag genetic locus has been identified in both *F. tularensis* and *F. novicida* and mutations in this locus can lead to attenuation of the bacteria *in vivo* (65, 70, 133, 134). These O-Ag mutants are highly sensitive to killing by normal serum, indicating the O-Ag plays a role in serum resistance (65, 133, 134).

1.6.4. Capsules

The presence or absence of a *Francisella* capsule has been a controversial subject for many years. Capsules are found in many bacterial species and are usually implicated in host evasion. Generally, bacterial capsules contain high molecular weight polysaccharides adhering to the outer bacterial surface. These capsules can be ideal targets for vaccine development, and many successful vaccines have been developed that can stimulate capsule-specific antibodies (135). Determination of the presence and composition of any *Francisella* capsule could prove useful in vaccine development.

Hood reported the first physical description and chemical characteristics of the *Francisella* capsule in 1977 (136). Electron microscopy of SchuS4 cells showed the presence of an electron transparent halo around bacterial cells and extraction of this material identified mannose, rhamnose, and dideoxy sugars that differed from the cell wall composition (136). In 1994 Cherwonogrodzky (137) identified an electron-dense capsule surround LVS cells after multiple passages in Chamberlain's Defined Medium (CDM). Eventual characterization of the *Francisella* capsules led to the identification of two independent capsules: the O-Ag capsule (138) and the Capsule-like Complex (CLC) (97).

Apicella *et al.* (138) identified a monoclonal antibody (11B7) that bound to a molecule on the surface of *F. tularensis*. Further characterization identified that 11B7 was binding to a capsular polysaccharide composed of repeating O-Ag subunits (138). This O-Ag repeating subunit was distinct from the LPS O-Ag in that there was no identified Lipid A or core sugar attached, the sugar did not partition with LPS O-Ag into Triton X114, and the material reacted with the 11B7 mAb (138). This O-antigen capsule can be grouped into the Group 4 capsules described by Whitfield due to its similarity to the *E. coli* group 4 LPS O-Ag capsules and the presence of acetamido sugars in the composition (138). Barker *et al.* (139) used metabolic labeling to identify a lipid attached to the O-Ag capsular polysaccharide that may anchor the material to the bacterial cell wall. The lipid identified contained 3-OH-fatty acids usually found in Lipid A molecules, but the makeup and ratios of the 3-OH-fatty acids distinguished this lipid as different from the *Francisella* LPS Lipid A molecule (139).

Distinct from the O-Ag capsule is the CLC material that contains a heterogenous mixture of proteins and glycoproteins identified independently by Bandara *et al.* (97) and Zarrella *et al*. (100). The CLC appears as an electron dense material when visualized by electron microscopy and is upregulated by passage in defined medium, similar to that described by Cherwondogronzky *et al*. (98, 137, 140). The carbohydrate component of the isolated material is composed of glucose, galactose, and mannose making this polysaccharide component chemically distinct from the O-Ag (97). In addition to the carbohydrate, CLC extractions contain a large amount of protein (97). There is diffuse banding between 150 to 250kDa on electrophoretic preparations that stains with glycoprotein stains and supports the thought that the CLC contains glycosylated proteins. This material separated into lower sized bands when run through a GelFree fractionation unit indicating that the diffuse banding may be a collection of closely

associated smaller proteins (141). The fractionated sections revealed a prominent 45kDa protein that may be FopA, a virulence factor previously characterized as being glycosylated (141). Further characterization of the proteins found in the CLC support similarities of the CLC to previously described outer membrane vesicles and tubes (OMV/T) (98). Approximately 60% of the proteins isolated in CLC extracts from LVS match previously isolated OMV/T proteins from *F. novicida* (98). These proteins could be contaminants of OMV/Ts in the CLC preparation, or the CLC and OMV/T may be related. A definitive link between OMV/T and the CLC has not been established.

A glycosylation locus from FTT0789 to FTT0800 was identified and is conserved across all *Francisella* species (97, 142, 143). Disruption of multiple genes within the locus leads to the abolishment of CLC in LVS (97) and SchuS4 (98). A similar mutant was constructed in *F. novicida* and led to reduction in the carbohydrate component of the CLC, but did not lead to a reduction in the protein component as found with LVS mutants (99). These findings were similar to a newly constructed SchuS4 glycosylation mutant (141). Both the CLC-deficient LVS strain and *F. novicida* strain were attenuated in the mouse model and protective to partially protective, respectfully, against a subsequent intranasal challenge with a virulent strain (97). However, the CLC-deficient Type A strain was not attenuated or protective in the mouse model (98). These differences may highlight the differences in importance of the CLC for the highly virulent strains compared to the less virulent strains. More in-depth information on the capsules and carbohydrates of *Francisella* species is further discussed throughout this dissertation.

1.6.5. Glycosylation

Protein glycosylation is highly linked to virulence in *F. tularensis* with loss of glycosylation machinery usually attenuating the bacterium (97, 99, 143, 144). PilA, a Type IV pilin protein, was one of the first *Francisella* proteins to be confirmed as glycosylated and loss of the encoding gene leads to attenuation of the strain (145, 146). O-linked glycosylation of PilA leads to the addition of a pentasaccharide to serine attachments sites (147). The protein responsible for glycosylating PilA, known as PglA, is conserved across all species of *Francisella* (147). Balonova *et al*. (148) further determined possible *Francisella* glycosylated proteins using lectin affinity studies. Disruption of the O-Ag genetic locus or the previously characterized PglA interfered with glycosylation of PilA and putative lipoprotein FTH_0069 in addition to abolishing the O-Ag (143). Interruption of the CLC glycosylation locus resulted in the loss of

glycosylation of DsbA with no effect on the O-Ag (142). PilA, FTH_0069, and DsbA all appear to be glycosylated with the same HexNAc-HexNAc-Hex-Hex-HexNAc sugar and a possible phosphate moiety (144). A possible common glycosylation pathway may exist that uses machinery from both the O-Ag locus and the CLC glycosylation locus. The exact purpose of glycosylation in the pathogenesis of *Francisella* species is still unknown.

1.7. Tularemia Vaccines

No vaccine is currently licensed in the U.S. for the prevention of tularemia. However, vaccine development has been a major research focus over the past few decades. Various attenuated modified live vaccines, killed vaccines, and subunit vaccines have been developed with some possibly showing promise as an effective vaccine. The goal is the production of a stable vaccine, which is also safe in the immunocompromised population and protects against a *Francisella* Type A infection, specifically the pneumonic form. Unfortunately, all developed tularemia vaccines remain unlicensed for a variety of reasons, such as the lack of suitable animal models, questionable retention of virulence, and instability of the *Francisella* genome (149).

1.7.1. The "Foshay" Vaccine

The earliest vaccine developed to prevent disease due to *F. tularensis* is the killed, whole cell vaccine known as the "Foshay" vaccine, named for the person who developed the vaccine (150). A virulent culture of *F. tularensis* cells were killed in phenolized liquid and administered in 3 doses subcutaneously over a period of 6 days (150). Though the vaccine was tolerated well, the protection provided was questionable and inadequate against the pneumonic form of tularemia (150, 151). Approximately 31% of individuals working with a highly virulent strain of *F. tularensis* still developed tularemia and required antibiotic treatment (151). This incidence rate was similar to the estimated incidence of tularemia in unvaccinated individuals in a similar environment (151). *F. tularensis* is an intracellular pathogen and it is generally accepted that vaccines against intracellular pathogens must induce an appropriate cellular immune response. Possibly, the "Foshay" vaccine may not induce that appropriate cellular immune response as the vaccine consists of killed cells, or the processing removed antigens necessary to stimulate the appropriate immune response (150, 151). Though killed vaccines are stable and generally safer, current research has focused more on the creation of modified live vaccines or subunit vaccines

that can stimulate the proper cellular immune response; killed vaccines are normally inadequate at stimulating a cellular immune reaction.

1.7.2. The Live Vaccine Strain (LVS)

The Live Vaccine Strain (*F. tularensis* LVS) is derived from *F. tularensis* Type B in response to the failure of killed vaccines to prevent tularemia. In the 1950s, the Soviet Union attempted to create a live attenuated strain of *F. tularensis* through repeated passage of the bacterium in guinea pigs and on artificial medium (152, 153). This repeated passage produced a strain that retained some virulence for mice, but did not cause clinical disease in healthy individuals (152). Two colony phenotypes, blue and grey, were isolated at Fort Detrick from vials of the viable cutaneous vaccine strain gifted from the Societ Union to the U.S. (152, 154). The blue colony was more virulent and immunogenic for animal models compared to the grey colony (154). The variance in virulence and immunogenicity are now known to be due to phase variation of *F. tularensis*; the grey colony is the result of loss of the LPS O-Ag. The blue colony variant was designated the "Live Vaccine Strain" or LVS (154). Saslaw *et al*. (155) used LVS to immunize a group of inmates at Ohio State Penitentiary via scarification. The inmates were then subjected to a respiratory challenge of approximately 10 to 50 cells of *F. tularensis* Type A SchuS4 strain (155). Clinical disease occurred in 3 of the 18 LVS-immunized individuals, compared to 8 out of 10 of the non-immunized individuals after challenge (155). However, a respiratory challenge of 50 or less *F. tularensis* cells is considered a low dose challenge. LVS immunization through scarification did not afford protection to volunteers when the respiratory challenge dose increased to 1,000 colony forming units (CFU) (153). Humans immunized with LVS through aerosolization of the strain protected against a greater challenge dose than scarification; however, the LVS aerosolization dose necessary for protection could also produce a mild clinical disease (156).

1.7.3. Subunit Vaccines

Effective subunit vaccines against tularemia are desirable due to the safety of a subunit vaccine compared to a modified live vaccine, for which residual virulence, phase changes, or genetic instability could put a recipient at risk. However, the development of such a subunit vaccine has so far been unsuccessful. Subunit vaccine development has been centered around the highly immunogenic antigens of *F. tularensis*, such as the LPS or other surface molecules (83,

157). Success has been variable and highly depends on the virulence of the challenge strain. Mice vaccinated with LPS subcutaneously were successfully protected against a high dose intraperitoneal challenge with LVS. However, Conlan *et al*. (83) reported variable protection in mice vaccinated with LPS. Mice vaccinated with LPS were protected against an intradermal low-dose Type B challenge, but were not protected by a low-dose Type B aerosol challenge or any Type A challenge (83). Protection against LVS or another Type B strain does not necessary correlate with protection against a Type A challenge, which is also true for vaccine types other than the sub-unit vaccine. Other molecules such as GroE (158), Tul4 (85), and outer membrane proteins (159) offer variable protection against challenge. Immunization with FopA, an outer membrane protein, was able to protect mice against a virulent LVS challenge but showed no protection against a challenge with Type A strain SchuS4 (159).

Mice immunized with outer membrane proteins (OMP) and subsequently challenged with SchuS4 had an overall survival of 50% (160). However, these mice were challenged with a very low dose of SchuS4 (40 CFU, IN) (160). An increase in the challenge dose would possibly reduce the protection of this immunization. Post *et al.* demonstrated that mice who were vaccinated with a SchuS4 outer membrane vaccine encapsulated in poly-l-glycolic acid (PLGA) survived an LVS challenge, but were not protected against a SchuS4 challenge (161). However, when these PLGA immunized mice were also boostered with LVS they survived the virulent SchuS4 challenge (161). This demonstrated that a subunit vaccine may be ineffective against a Type A and that the addition of a live attenuated strain may be necessary to develop the required cell mediated immunity for protection. These surviving mice produced high antibody levels to FopA and other outer membrane proteins, including a hypothetical membrane protein and two lipoproteins (161).

In general, these sub-unit vaccines may not be highly protective due to use of the incorrect antigen necessary for protection or a lack of intra-cellular infection possibly necessary to induce proper immunity to protect against virulent Type A challenges. Most recent vaccine studies focus on creation of live attenuated mutants in the hopes of creating the proper immune response.

1.7.4. Other Modified Live Vaccines

Numerous tularemia vaccine attempts center around creation of an attenuated strain of *F. tularensis* capable of providing protection against lethal challenge while also remaining safe for

all persons. Jia *et al*. (162) reviews the extensive list of attenuated *F. tularensis* mutants and notes that creation of attenuated mutants safer than LVS is relatively easy, but on the contrary creation of an attenuated mutant that also affords protection against a Type A respiratory dose has proven to be exceedingly difficult. Many of the attenuated mutants reviewed require immunization boosters to provide any level of protection. *F. tularensis* attenuated mutants that have deletions in genes affecting LPS O-antigen production usually require booster immunization to provide protection (65, 127, 134). O-antigen mutants are highly serum sensitive (65, 67, 69) because the O-antigen plays a role in complement-mediated serum resistance. Therefore, the bacteria are most likely cleared too quickly *in vivo* to produce an effective immune response.

Nonetheless, modified live vaccines have the most promise as potential vaccine candidates. Modified live vaccines usually have greater ability to stimulate a cellular immune response, which is usually necessary to protect against an intracellular pathogen. However, though there are many promising candidates outlined by Jia *et al* (162) a number of concerns about these strains still persist, including concern that a single deletion mutant in the Type A SchuS4 strain is not adequate to ensure safety and that no reversion to virulence will occur upon immunization with the strain.

1.8. Alginate Microencapsulation

1.8.1. Alginate and Calcium Reaction

Alginate is a biomaterial used commonly in a diverse range of applications including medicine, food science, and molecular gastronomy. Alginate is also relatively inexpensive and relatively inert *in vivo* (163), making the polymer a favorite in biomedical applications such as drug delivery and tissue engineering. Alginate is an anionic polymer capable of binding to multiple divalent cations to form a cross-linked structure (164). This copolymer is formed of blocks of alternating mannuronate (M) and guluronate (G) residues with only the G blocks participating in the ionic crosslinking with cations (164). A viscous solution of alginate can be introduced in droplet form to a solution containing a divalent cation, such as calcium. Upon introduction to the calcium solution, the alginate and calcium will cross link and trap materials within an alginate bead (164). Chelating agents such as EDTA or citrate can be used to disassociate the cross-linking bonds and dissolve the alginate beads (Figure 1.4) (164).

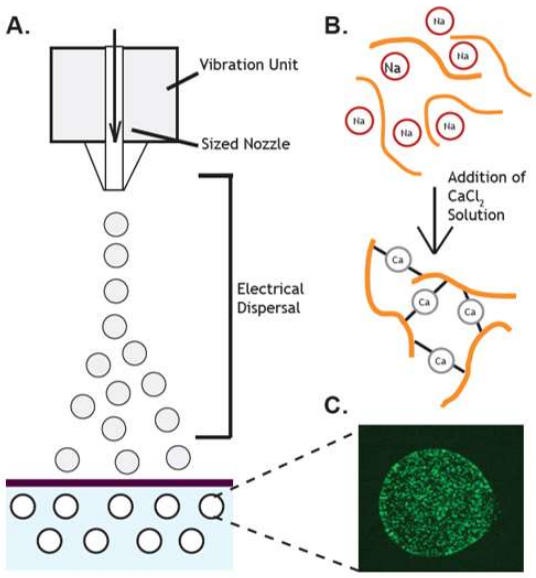

Figure 1.4. Production of alginate beads using a Buchi Encapsulator.
A homogenous viscous solution of sodium alginate and antigen can be extruded through the Buchi unit to produce droplets of a size dependent on the nozzle that disperse into a calcium chloride solution (a). Upon introduction into the calcium chloride solution, the sodium ions are displaced by the divalent calcium ion that cross-links between alginate strands (b). This creates a bead that traps whatever was added into the original solution such as GFP-expressing bacterial cells (c).

1.8.2. Current uses of Alginate Microencapsulation of Live Cells

The majority of alginate microencapsulation of live cells centers around probiotic development (165, 166). Alginate is a breathable polymer that is able to expand or contract depending on the tonicity of the suspension solution. This allows possible nutrient substrates to enter the beads and metabolized products to leave while blocking entrance to larger objects such as the host's immune system cells (Figure 1.5) (165). Alginate beads have served as effective barriers between probiotic bacteria such as *Lactobacillus* and harmful environments, such as the stomach (166). *L. acidophilus* encapsulated in alginate was protected from stimulated gastric conditions including the acidic pH and addition of bile (166). Multiple other studies have shown how alginate microencapsulation improves survival of bacterial cells in gastric environments compared to non-encapsulated cells (167-169)

In addition to protection of probiotics, alginate microspheres have shown some utility as a vaccine delivery system. Alginate is considered a safe polymer and has shown minimal to no toxic effects after oral or parental administration (164). The lack of toxicity makes alginate an ideal substance to serve as an inert delivery vehicle. Previously, a vaccine strain of *Brucella*

abortus was encapsulated in alginate beads and used to immunize mice prior to a virulent challenge (170). Mice immunized with an attenuated, encapsulated vaccine strain effectively produced an antibody response and were better protected against a virulent challenge than those who were given the vaccine strain non-encapsulated (170). This alginate encapsulation strategy has been shown effective for other *Brucella* species (171-173). Theoretically, alginate encapsulation should provide a protected environment to a highly attenuated *F. tularensis* strain, but with sustained release that could improve the immune response without the need for booster vaccines. The effect of alginate encapsulation on an attenuated strain of *Francisella* will be further discussed in this dissertation.

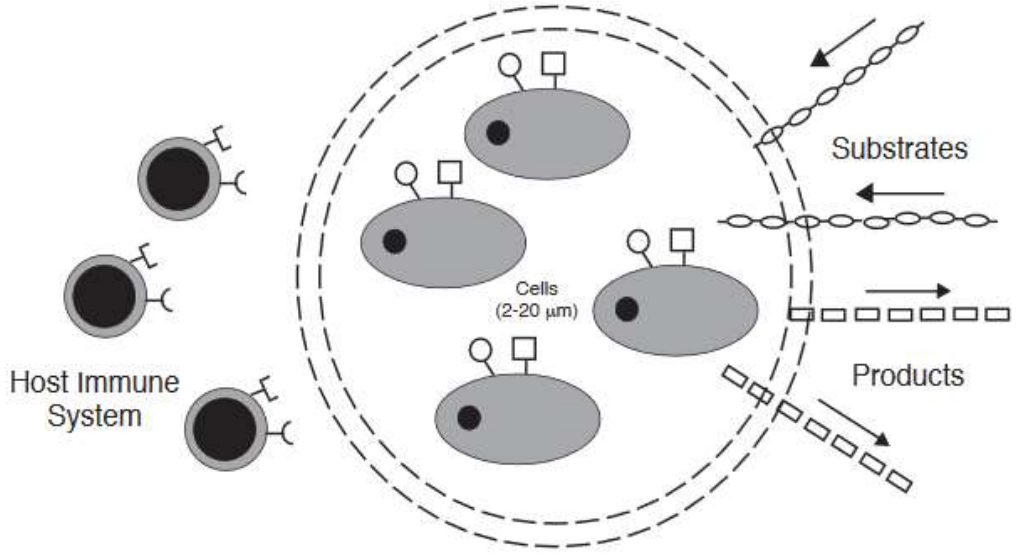

Figure 1.5. Microencapsulation of Live Cells
Alginate microencapsulation of live cells protects the cells from the host immune system while allowing the passage of essential nutrients and other substrates through the permeable alginate capsule. Taken from Kailasapathy *et al*. (165).

1.9. References

1. Anderson ET. 1978. Plague in the continental United States, 1900-76. Public Health Rep 93:297-301.
2. McCoy GW. 1910. Plague among ground squirrels in America. J Hyg (Lond) 10:589-601.
3. McCoy GW, Chapin CW. 1912. Further observations on a plague-like disease of rodents with a preliminary note on the causative agent, *bacterium tularense.* J Infect Dis 10:61-72.

4. Wherry WB, Lamb BH. 1914. Infection of man with *Bacterium Tularense*. J Infect Dis 15:331-340.
5. Francis E. 1922. Tularemia Francis 1921: A new disease of man. J Am Med Assoc 78(14):1015-1018.
6. Philip CB, Owen CR. 1961. Comments on the nomenclature of the causative agent of tularemia. Int B Bact Nomencl T 11:67-72.
7. Cowley SC, Elkins KL. 2011. Immunity to *Francisella*. Front Microbiol 2:26.
8. Sjostedt A. 2005. Family III. Francisellaceae, p 199-209. *In* Brenner DJ, Krieg NR, Garrity GM, Staley JT (ed), Bergey's Manual of Systematic Bacteriology: Volume 2: The Proteobacteria, 2 ed, vol 2. Springer.
9. Sjodin A, Svensson K, Ohrman C, Ahlinder J, Lindgren P, Duodu S, Johansson A, Colquhoun DJ, Larsson P, Forsman M. 2012. Genome characterisation of the genus *Francisella* reveals insight into similar evolutionary paths in pathogens of mammals and fish. BMC Genomics 13:268.
10. Busse HJ, Huber B, Anda P, Escudero R, Scholz HC, Seibold E, Splettstoesser WD, Kampfer P. 2010. Objections to the transfer of *Francisella novicida* to the subspecies rank of *Francisella tularensis* - response to Johansson et al. Int J Syst Evol Microbiol 60:1718-20.
11. Johansson A, Celli J, Conlan W, Elkins KL, Forsman M, Keim PS, Larsson P, Manoil C, Nano FE, Petersen JM, Sjostedt A. 2010. Objections to the transfer of *Francisella novicida* to the subspecies rank of *Francisella tularensis*. Int J Syst Evol Microbiol 60:1717-8; author reply 1718-20.
12. Huber B, Escudero R, Busse HJ, Seibold E, Scholz HC, Anda P, Kampfer P, Splettstoesser WD. 2010. Description of *Francisella hispaniensis* sp. nov., isolated from human blood, reclassification of *Francisella novicida* (Larson et al. 1955) Olsufiev et al. 1959 as *Francisella tularensis* subsp. *novicida* comb. nov. and emended description of the genus *Francisella*. Int J Syst Evol Microbiol 60:1887-96.
13. Santic M, Al-Khodor S, Abu Kwaik Y. 2010. Cell biology and molecular ecology of *Francisella tularensis*. Cell Microbiol 12:129-39.
14. Qu PH, Chen SY, Scholz HC, Busse HJ, Gu Q, Kampfer P, Foster JT, Glaeser SP, Chen C, Yang ZC. 2013. *Francisella guangzhouensis* sp. nov., isolated from air-conditioning systems. Int J Syst Evol Microbiol 63:3628-35.
15. Brevik OJ, Ottem KF, Kamaishi T, Watanabe K, Nylund A. 2011. Francisella halioticida sp. nov., a pathogen of farmed giant abalone (Haliotis gigantea) in Japan. J Appl Microbiol 111:1044-56.
16. Colquhoun DJ, Larsson P, Duodu S, Forsman M. 2014. The Family *Francisellaceae*, p 288-314. *In* Rosenberg E (ed), The Prokaryotes - Gammaproteobacteria. Springer, Verlag.
17. Bernard K, Tessier S, Winstanley J, Chang D, Borczyk A. 1994. Early recognition of atypical Francisella tularensis strains lacking a cysteine requirement. J Clin Microbiol 32:551-3.
18. Nano FE, Zhang N, Cowley SC, Klose KE, Cheung KK, Roberts MJ, Ludu JS, Letendre GW, Meierovics AI, Stephens G, Elkins KL. 2004. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. J Bacteriol 186:6430-6.
19. Nano FE, Schmerk C. 2007. The *Francisella* pathogenicity island. Ann N Y Acad Sci 1105:122-37.

20. McLendon MK, Apicella MA, Allen LA. 2006. *Francisella tularensis*: taxonomy, genetics, and Immunopathogenesis of a potential agent of biowarfare. Annu Rev Microbiol 60:167-85.
21. Keim P, Johansson A, Wagner DM. 2007. Molecular epidemiology, evolution, and ecology of *Francisella*. Ann N Y Acad Sci 1105:30-66.
22. Petersen JM, Mead PS, Schriefer ME. 2009. *Francisella tularensis*: an arthropod-borne pathogen. Vet Res 40:7.
23. Centers for Disease C, Prevention. 2005. Tularemia transmitted by insect bites--Wyoming, 2001-2003. MMWR Morb Mortal Wkly Rep 54:170-3.
24. Centers for Disease C, Prevention. 2005. Tularemia associated with a hamster bite--Colorado, 2004. MMWR Morb Mortal Wkly Rep 53:1202-3.
25. Feldman KA, Stiles-Enos D, Julian K, Matyas BT, Telford SR, 3rd, Chu MC, Petersen LR, Hayes EB. 2003. Tularemia on Martha's Vineyard: seroprevalence and occupational risk. Emerg Infect Dis 9:350-4.
26. Feldman RA, Koehler RE. 1973. Tularemia. JAMA 226:189.
27. Zargar A, Maurin M, Mostafavi E. 2015. Tularemia, a re-emerging infectious disease in Iran and neighboring countrie. Epidemiol Health 37:e2015011.
28. Faber M, Heuner K, Jacob D, Grunow R. 2018. Tularemia in Germany-A Re-emerging Zoonosis. Front Cell Infect Microbiol 8:40.
29. Francis E. 1925. Tularemia. JAMA 84:1243-1250.
30. Pearse RA. 1911. Insect bites. Northwest Med 3:81-82.
31. Ohara S. 1954. Studies on yato-byo (Ohara's disease, tularemia in Japan). I. Jpn J Exp Med 24:69-79.
32. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Layton M, Lillibridge SR, McDade JE, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Tonat K, Working Group on Civilian B. 2001. Tularemia as a biological weapon: medical and public health management. JAMA 285:2763-73.
33. Meibom KL, Charbit A. 2010. The unraveling panoply of *Francisella tularensis* virulence attributes. Curr Opin Microbiol 13:11-7.
34. Sykes JE, Chomel BB. 2014. Chapter 56 - Tularemia, p 537-545. *In* Sykes JE (ed), Canine and Feline Infectious Diseases. Elsevier, St. Louis, Missouri.
35. Sjostedt A. 2007. Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. Ann N Y Acad Sci 1105:1-29.
36. Feldman KA. 2003. Tularemia. J Am Vet Med Assoc 222:725-30.
37. Ridgway MD, Taboada J. 2015. Tularemia, p 1022-1024. *In* Côté E (ed), Clinical Veterinary Advisor. Elsevier, Missouri.
38. Greene CE. 2012. Tularemia, p 476-482. *In* Greene CE (ed), Infectious Diseases of the Dog and Cat, 4 ed. Saunder Elsevier, St. Louis.
39. Meinkoth KR, Morton RJ, Meinkoth JH. 2004. Naturally occurring tularemia in a dog. J Am Vet Med Assoc 225:545-7, 538.
40. Centers for Disease C. 1982. Tularemia associated with domestic cats--Georgia, New Mexico. MMWR Morb Mortal Wkly Rep 31:39-41.
41. Baldwin CJ, Panciera RJ, Morton RJ, Cowell AK, Waurzyniak BJ. 1991. Acute tularemia in three domestic cats. J Am Vet Med Assoc 199:1602-5.
42. Liles WC, Burger RJ. 1993. Tularemia from domestic cats. West J Med 158:619-22.

43. Woods JP, Crystal MA, Morton RJ, Panciera RJ. 1998. Tularemia in two cats. J Am Vet Med Assoc 212:81-3.
44. DeBey BM, Andrews GA, Chard-Bergstrom C, Cox L. 2002. Immunohistochemical demonstration of Francisella tularensis in lesions of cats with tularemia. J Vet Diagn Invest 14:162-4.
45. Inzana TJ, Glindemann GE, Snider G, Gardner S, Crofton L, Byrne B, Harper J. 2004. Characterization of a wild-type strain of *Francisella tularensis* isolated from a cat. J Vet Diagn Invest 16:374-81.
46. Quenzer RW, Mostow SR, Emerson JK. 1977. Cat-bite tularemia. JAMA 238:1845.
47. von Schroeder HP, McDougall EP. 1993. Ulceroglandular and pulmonary tularemia: a case resulting from a cat bite to the hand. J Hand Surg Am 18:132-4.
48. Arav-Boger R. 2000. Cat-bite tularemia in a seventeen-year-old girl treated with ciprofloxacin. Pediatr Infect Dis J 19:583-4.
49. Alibek K, Handelman S. 2000. Biohazard: The Chilling True Story of the Largest Covert Biological Weapons Program in the World - Told from Inside by the Man Who Ran It. Dell Publishing, New York.
50. Croddy E, Krcalova S. 2001. Tularemia, biological warfare, and the battle for Stalingrad (1942-1943). Mil Med 166:837-8.
51. Hudspeth J. 2008. Tularemia (*Francisella tularensis*), p 43-58. *In* Zubay GL (ed), Agents of Bioterrorism: Pathogens and Their Weaponization. Columbia University Press, New York.
52. World Health Organization. 1970. Health aspects of chemical and biological weapons. Report of a WHO Group of Consultants, Geneva,.
53. Services DoHaH. 2005. Federal Register. Services DoHaH,
54. Centers for Disease C, Prevention DoH, Human S. 2017. Possession, Use, and Transfer of Select Agents and Toxins; Biennial Review of the List of Select Agents and Toxins and Enhanced Biosafety Requirements. Final rule. Fed Regist 82:6278-94.
55. Oyston PC, Sjostedt A, Titball RW. 2004. Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*. Nat Rev Microbiol 2:967-78.
56. Celli J, Zahrt TC. 2013. Mechanisms of Francisella tularensis intracellular pathogenesis. Cold Spring Harb Perspect Med 3:a010314.
57. Golovliov I, Ericsson M, Sandstrom G, Tarnvik A, Sjostedt A. 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.
58. Jones BD, Faron M, Rasmussen JA, Fletcher JR. 2014. Uncovering the components of the *Francisella tularensis* virulence stealth strategy. Front Cell Infect Microbiol 4:32.
59. Clemens DL, Lee BY, Horwitz MA. 2005. *Francisella tularensis* enters macrophages via a novel process involving pseudopod loops. Infect Immun 73:5892-902.
60. Clemens DL, Lee BY, Horwitz MA. 2009. Francisella tularensis phagosomal escape does not require acidification of the phagosome. Infect Immun 77:1757-73.
61. Ledvina HE, Kelly KA, Eshraghi A, Plemel RL, Peterson SB, Lee B, Steele S, Adler M, Kawula TH, Merz AJ, Skerrett SJ, Celli J, Mougous JD. 2018. A Phosphatidylinositol 3-Kinase Effector Alters Phagosomal Maturation to Promote Intracellular Growth of Francisella. Cell Host Microbe 24:285-295 e8.
62. Lauriano CM, Barker JR, Yoon SS, Nano FE, Arulanandam BP, Hassett DJ, Klose KE. 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.
63. Forestal CA, Malik M, Catlett SV, Savitt AG, Benach JL, Sellati TJ, Furie MB. 2007. Francisella tularensis has a significant extracellular phase in infected mice. J Infect Dis 196:134-7.
64. Mares CA, Ojeda SS, Morris EG, Li Q, Teale JM. 2008. Initial delay in the immune response to *Francisella tularensis* is followed by hypercytokinemia characteristic of severe sepsis and correlating with upregulation and release of damage-associated molecular patterns. Infect Immun 76:3001-10.
65. Li J, Ryder C, Mandal M, Ahmed F, Azadi P, Snyder DS, Pechous RD, Zahrt T, Inzana TJ. 2007. Attenuation and protective efficacy of an O-antigen-deficient mutant of *Francisella tularensis* LVS. Microbiology 153:3141-53.
66. Sandstrom G, Lofgren S, Tarnvik A. 1988. A Capsule-Deficient Mutant of *Francisella-Tularensis* Lvs Exhibits Enhanced Sensitivity to Killing by Serum but Diminished Sensitivity to Killing by Polymorphonuclear Leukocytes. Infection and Immunity 56:1194-1202.
67. Sorokin VM, Pavlovich NV, Prozorova LA. 1996. *Francisella tularensis* resistance to bactericidal action of normal human serum. FEMS Immunol Med Microbiol 13:249-52.
68. Clay CD, Soni S, Gunn JS, Schlesinger LS. 2008. Evasion of complement-mediated lysis and complement C3 deposition are regulated by Francisella tularensis lipopolysaccharide O antigen. J Immunol 181:5568-78.
69. Rasmussen JA, Post DM, Gibson BW, Lindemann SR, Apicella MA, Meyerholz DK, Jones BD. 2014. *Francisella tularensis* Schu S4 lipopolysaccharide core sugar and O-antigen mutants are attenuated in a mouse model of tularemia. Infect Immun 82:1523-39.
70. Rasmussen JA, Fletcher JR, Long ME, Allen LA, Jones BD. 2015. Characterization of *Francisella tularensis* Schu S4 mutants identified from a transposon library screened for O-antigen and capsule deficiencies. Front Microbiol 6:338.
71. Barker JH, McCaffrey RL, Baman NK, Allen LA, Weiss JP, Nauseef WM. 2009. The role of complement opsonization in interactions between *F. tularensis* subsp. *novicida* and human neutrophils. Microbes Infect 11:762-9.
72. Ben Nasr A, Klimpel GR. 2008. Subversion of complement activation at the bacterial surface promotes serum resistance and opsonophagocytosis of Francisella tularensis. J Leukoc Biol 84:77-85.
73. Hajjar AM, Harvey MD, Shaffer SA, Goodlett DR, Sjostedt A, Edebro H, Forsman M, Bystrom M, Pelletier M, Wilson CB, Miller SI, Skerrett SJ, Ernst RK. 2006. Lack of in vitro and in vivo recognition of Francisella tularensis subspecies lipopolysaccharide by Toll-like receptors. Infect Immun 74:6730-8.
74. Okan NA, Chalabaev S, Kim TH, Fink A, Ross RA, Kasper DL. 2013. Kdo hydrolase is required for Francisella tularensis virulence and evasion of TLR2-mediated innate immunity. MBio 4:e00638-12.
75. Andersson H, Hartmanova B, Kuolee R, Ryden P, Conlan W, Chen W, Sjostedt A. 2006. Transcriptional profiling of host responses in mouse lungs following aerosol infection with type A Francisella tularensis. J Med Microbiol 55:263-71.
76. Pyles RB, Jezek GE, Eaves-Pyles TD. 2010. Toll-like receptor 3 agonist protection against experimental Francisella tularensis respiratory tract infection. Infect Immun 78:1700-10.

77. Elkins KL, Cowley SC, Bosio CM. 2007. Innate and adaptive immunity to *Francisella*. Ann N Y Acad Sci 1105:284-324.
78. Cowley SC, Sedgwick JD, Elkins KL. 2007. Differential requirements by CD4+ and CD8+ T cells for soluble and membrane TNF in control of Francisella tularensis live vaccine strain intramacrophage growth. J Immunol 179:7709-19.
79. Lindgren H, Golovliov I, Baranov V, Ernst RK, Telepnev M, Sjostedt A. 2004. Factors affecting the escape of Francisella tularensis from the phagolysosome. J Med Microbiol 53:953-8.
80. Lindgren H, Stenmark S, Chen W, Tarnvik A, Sjostedt A. 2004. Distinct roles of reactive nitrogen and oxygen species to control infection with the facultative intracellular bacterium Francisella tularensis. Infect Immun 72:7172-82.
81. Shirey KA, Cole LE, Keegan AD, Vogel SN. 2008. Francisella tularensis live vaccine strain induces macrophage alternative activation as a survival mechanism. J Immunol 181:4159-67.
82. Bosio CM, Bielefeldt-Ohmann H, Belisle JT. 2007. Active suppression of the pulmonary immune response by Francisella tularensis Schu4. J Immunol 178:4538-47.
83. Conlan JW, Shen H, Webb A, Perry MB. 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.
84. Dreisbach VC, Cowley S, Elkins KL. 2000. Purified lipopolysaccharide from Francisella tularensis live vaccine strain (LVS) induces protective immunity against LVS infection that requires B cells and gamma interferon. Infect Immun 68:1988-96.
85. Kaur R, Chen S, Arevalo MT, Xu Q, Chen Y, Zeng M. 2012. Protective immunity against tularemia provided by an adenovirus-vectored vaccine expressing Tul4 of Francisella tularensis. Clin Vaccine Immunol 19:359-64.
86. Crane DD, Warner SL, Bosio CM. 2009. A novel role for plasmin-mediated degradation of opsonizing antibody in the evasion of host immunity by virulent, but not attenuated, Francisella tularensis. J Immunol 183:4593-600.
87. Conlan JW, Sjostedt A, North RJ. 1994. CD4+ and CD8+ T-cell-dependent and -independent host defense mechanisms can operate to control and resolve primary and secondary Francisella tularensis LVS infection in mice. Infect Immun 62:5603-7.
88. Yee D, Rhinehart-Jones TR, Elkins KL. 1996. Loss of either CD4+ or CD8+ T cells does not affect the magnitude of protective immunity to an intracellular pathogen, Francisella tularensis strain LVS. J Immunol 157:5042-8.
89. Cowley SC, Meierovics AI, Frelinger JA, Iwakura Y, Elkins KL. 2010. Lung CD4-CD8-double-negative T cells are prominent producers of IL-17A and IFN-gamma during primary respiratory murine infection with Francisella tularensis live vaccine strain. J Immunol 184:5791-801.
90. van Hoek ML. 2013. Biofilms: an advancement in our understanding of *Francisella* species. Virulence 4:833-46.
91. Margolis JJ, El-Etr S, Joubert LM, Moore E, Robison R, Rasley A, Spormann AM, Monack DM. 2010. Contributions of *Francisella tularensis* subsp. *novicida* chitinases and Sec secretion system to biofilm formation on chitin. Appl Environ Microbiol 76:596-608.

92. Chung MC, Dean S, Marakasova ES, Nwabueze AO, van Hoek ML. 2014. Chitinases are negative regulators of Francisella novicida biofilms. PLoS One 9:e93119.
93. Verhoeven AB, Durham-Colleran MW, Pierson T, Boswell WT, Van Hoek ML. 2010. *Francisella philomiragia* biofilm formation and interaction with the aquatic protist *Acanthamoeba castellanii*. Biol Bull 219:178-88.
94. Mahajan UV, Gravgaard J, Turnbull M, Jacobs DB, McNealy TL. 2011. Larval exposure to Francisella tularensis LVS affects fitness of the mosquito Culex quinquefasciatus. FEMS Microbiol Ecol 78:520-30.
95. Durham-Colleran MW, Verhoeven AB, van Hoek ML. 2010. Francisella novicida forms in vitro biofilms mediated by an orphan response regulator. Microb Ecol 59:457-65.
96. Chandler JC, Molins CR, Petersen JM, Belisle JT. 2011. Differential chitinase activity and production within Francisella species, subspecies, and subpopulations. J Bacteriol 193:3265-75.
97. Bandara AB, Champion AE, Wang X, Berg G, Apicella MA, McLendon M, Azadi P, Snyder DS, Inzana TJ. 2011. Isolation and mutagenesis of a capsule-like complex (CLC) from *Francisella tularensis*, and contribution of the CLC to *F. tularensis* virulence in mice. PLoS One 6:e19003.
98. Champion A, Mohapatra N, Bandara A, Twine S, Inzana TJ. 2015. The Capsule-Like Complex (CLC) of Type A *Francisella tularensis* may be Derived from Outer Membrane Vesicles, but Glycosylation of the CLC is not Required for Virulence and is not Protective., abstr 115th General Meeting of the American Society for Microbiology, New Orleans, LA., May 30-June 2, 2015,. American Society for Microbiology,
99. Freudenberger Catanzaro KC, Champion AE, Mohapatra N, Cecere T, Inzana TJ. 2017. Glycosylation of a Capsule-Like Complex (CLC) by Francisella novicida Is Required for Virulence and Partial Protective Immunity in Mice. Front Microbiol 8:935.
100. Zarrella TM, Singh A, Bitsaktsis C, Rahman T, Sahay B, Feustel PJ, Gosselin EJ, Sellati TJ, Hazlett KR. 2011. Host-adaptation of *Francisella tularensis* alters the bacterium's surface-carbohydrates to hinder effectors of innate and adaptive immunity. PLoS One 6:e22335.
101. Mokrievich AN, Kondakova AN, Valade E, Platonov ME, Vakhrameeva GM, Shaikhutdinova RZ, Mironova RI, Blaha D, Bakhteeva IV, Titareva GM, Kravchenko TB, Kombarova TI, Vidal D, Pavlov VM, Lindner B, Dyatlov IA, Knirel YA. 2010. Biological properties and structure of the lipopolysaccharide of a vaccine strain of Francisella tularensis generated by inactivation of a quorum sensing system gene qseC. Biochemistry (Mosc) 75:443-51.
102. Santic M, Molmeret M, Barker JR, Klose KE, Dekanic A, Doric M, Abu Kwaik Y. 2007. A *Francisella tularensis* pathogenicity island protein essential for bacterial proliferation within the host cell cytosol. Cell Microbiol 9:2391-403.
103. Gray CG, Cowley SC, Cheung KK, Nano FE. 2002. The identification of five genetic loci of Francisella novicida associated with intracellular growth. FEMS Microbiol Lett 215:53-6.
104. Larsson P, Elfsmark D, Svensson K, Wikstrom P, Forsman M, Brettin T, Keim P, Johansson A. 2009. Molecular evolutionary consequences of niche restriction in Francisella tularensis, a facultative intracellular pathogen. PLoS Pathog 5:e1000472.

105. Ludu JS, de Bruin OM, Duplantis BN, Schmerk CL, Chou AY, Elkins KL, Nano FE. 2008. The Francisella pathogenicity island protein PdpD is required for full virulence and associates with homologues of the type VI secretion system. J Bacteriol 190:4584-95.
106. Cong Y, Yu JJ, Guentzel MN, Berton MT, Seshu J, Klose KE, Arulanandam BP. 2009. Vaccination with a defined *Francisella tularensis* subsp. *novicida* pathogenicity island mutant (DeltaiglB) induces protective immunity against homotypic and heterotypic challenge. Vaccine 27:5554-61.
107. Broms JE, Meyer L, Sun K, Lavander M, Sjostedt A. 2012. Unique substrates secreted by the type VI secretion system of Francisella tularensis during intramacrophage infection. PLoS One 7:e50473.
108. Wrench AP, Gardner CL, Gonzalez CF, Lorca GL. 2013. Identification of a small molecule that modifies MglA/SspA interaction and impairs intramacrophage survival of Francisella tularensis. PLoS One 8:e54498.
109. Nguyen JQ, Gilley RP, Zogaj X, Rodriguez SA, Klose KE. 2014. Lipidation of the FPI protein IglE contributes to Francisella tularensis ssp. novicida intramacrophage replication and virulence. Pathog Dis 72:10-8.
110. Eshraghi A, Kim J, Walls AC, Ledvina HE, Miller CN, Ramsey KM, Whitney JC, Radey MC, Peterson SB, Ruhland BR, Tran BQ, Goo YA, Goodlett DR, Dove SL, Celli J, Veesler D, Mougous JD. 2016. Secreted Effectors Encoded within and outside of the Francisella Pathogenicity Island Promote Intramacrophage Growth. Cell Host Microbe 20:573-583.
111. Broms JE, Sjostedt A, Lavander M. 2010. The Role of the Francisella Tularensis Pathogenicity Island in Type VI Secretion, Intracellular Survival, and Modulation of Host Cell Signaling. Front Microbiol 1:136.
112. Ahlund MK, Ryden P, Sjostedt A, Stoven S. 2010. Directed screen of Francisella novicida virulence determinants using Drosophila melanogaster. Infect Immun 78:3118-28.
113. Baron GS, Nano FE. 1998. MglA and MglB are required for the intramacrophage growth of Francisella novicida. Mol Microbiol 29:247-59.
114. Barker JR, Chong A, Wehrly TD, Yu JJ, Rodriguez SA, Liu J, Celli J, Arulanandam BP, Klose KE. 2009. The Francisella tularensis pathogenicity island encodes a secretion system that is required for phagosome escape and virulence. Mol Microbiol 74:1459-70.
115. Clemens DL, Lee BY, Horwitz MA. 2018. The Francisella Type VI Secretion System. Front Cell Infect Microbiol 8:121.
116. Broms JE, Lavander M, Sjostedt A. 2009. A conserved alpha-helix essential for a type VI secretion-like system of Francisella tularensis. J Bacteriol 191:2431-46.
117. Clemens DL, Ge P, Lee BY, Horwitz MA, Zhou ZH. 2015. Atomic structure of T6SS reveals interlaced array essential to function. Cell 160:940-951.
118. Brodmann M, Dreier RF, Broz P, Basler M. 2017. Francisella requires dynamic type VI secretion system and ClpB to deliver effectors for phagosomal escape. Nat Commun 8:15853.
119. Jaggavarapu S, Weiss DS. 2018. Bacterial Prison Break: A Host Protein Mimic Paves the Way. Cell Host Microbe 24:189-191.
120. Kieffer TL, Cowley S, Nano FE, Elkins KL. 2003. *Francisella novicida* LPS has greater immunobiological activity in mice than *F. tularensis* LPS, and contributes to *F. novicida* murine pathogenesis. Microbes Infect 5:397-403.

121. Gunn JS, Ernst RK. 2007. The structure and function of *Francisella* lipopolysaccharide. Ann N Y Acad Sci 1105:202-18.
122. Okan NA, Kasper DL. 2013. The atypical lipopolysaccharide of *Francisella*. Carbohydr Res 378:79-83.
123. Barker JH, Weiss J, Apicella MA, Nauseef WM. 2006. Basis for the failure of Francisella tularensis lipopolysaccharide to prime human polymorphonuclear leukocytes. Infect Immun 74:3277-84.
124. Raetz CR. 1990. Biochemistry of endotoxins. Annu Rev Biochem 59:129-70.
125. Schilling B, McLendon MK, Phillips NJ, Apicella MA, Gibson BW. 2007. Characterization of lipid A acylation patterns in Francisella tularensis, Francisella novicida, and Francisella philomiragia using multiple-stage mass spectrometry and matrix-assisted laser desorption/ionization on an intermediate vacuum source linear ion trap. Anal Chem 79:1034-42.
126. Chalabaev S, Kim TH, Ross R, Derian A, Kasper DL. 2010. 3-Deoxy-D-manno-octulosonic acid (Kdo) hydrolase identified in Francisella tularensis, Helicobacter pylori, and Legionella pneumophila. J Biol Chem 285:34330-6.
127. Hartley G, Taylor R, Prior J, Newstead S, Hitchen PG, Morris HR, Dell A, Titball RW. 2006. Grey variants of the live vaccine strain of Francisella tularensis lack lipopolysaccharide O-antigen, show reduced ability to survive in macrophages and do not induce protective immunity in mice. Vaccine 24:989-96.
128. Soni S, Ernst RK, Muszynski A, Mohapatra NP, Perry MB, Vinogradov E, Carlson RW, Gunn JS. 2010. Francisella tularensis blue-gray phase variation involves structural modifications of lipopolysaccharide o-antigen, core and lipid a and affects intramacrophage survival and vaccine efficacy. Front Microbiol 1:129.
129. Eigelsbach HT, Braun W, Herring RD. 1951. Studies on the variation of Bacterium tularense. J Bacteriol 61:557-69.
130. Cowley SC, Myltseva SV, Nano FE. 1996. Phase variation in Francisella tularensis affecting intracellular growth, lipopolysaccharide antigenicity and nitric oxide production. Mol Microbiol 20:867-74.
131. Vinogradov E, Perry MB, Conlan JW. 2002. Structural analysis of *Francisella tularensis* lipopolysaccharide. Eur J Biochem 269:6112-8.
132. Vinogradov E, Conlan WJ, Gunn JS, Perry MB. 2004. Characterization of the lipopolysaccharide O-antigen of Francisella novicida (U112). Carbohydr Res 339:649-54.
133. Raynaud C, Meibom KL, Lety MA, Dubail I, Candela T, Frapy E, Charbit A. 2007. Role of the wbt locus of *Francisella tularensis* in lipopolysaccharide O-antigen biogenesis and pathogenicity. Infect Immun 75:536-41.
134. Twine SM, Vinogradov E, Lindgren H, Sjostedt A, Conlan JW. 2012. Roles for wbtC, wbtI, and kdtA Genes in Lipopolysaccharide Biosynthesis, Protein Glycosylation, Virulence, and Immunogenicity in Francisella tularensis2 Strain SCHU S4. Pathogens 1:12-29.
135. Lesinski GB, Westerink MA. 2001. Vaccines against polysaccharide antigens. Curr Drug Targets Infect Disord 1:325-34.
136. Hood AM. 1977. Virulence factors of *Francisella tularensis*. J Hyg (Lond) 79:47-60.
137. Cherwonogrodzky JW, Knodel MH, Spence MR. 1994. Increased encapsulation and virulence of *Francisella tularensis* live vaccine strain (LVS) by subculturing on synthetic medium. Vaccine 12:773-5.

138. Apicella MA, Post DM, Fowler AC, Jones BD, Rasmussen JA, Hunt JR, Imagawa S, Choudhury B, Inzana TJ, Maier TM, Frank DW, Zahrt TC, Chaloner K, Jennings MP, McLendon MK, Gibson BW. 2010. Identification, characterization and immunogenicity of an O-antigen capsular polysaccharide of *Francisella tularensis*. PLoS One 5:e11060.
139. Barker JH, Kaufman JW, Apicella MA, Weiss JP. 2016. Evidence Suggesting That Francisella tularensis O-Antigen Capsule Contains a Lipid A-Like Molecule That Is Structurally Distinct from the More Abundant Free Lipid A. PLoS One 11:e0157842.
140. Bandara AB, Lawrence ML, Veit HP, Inzana TJ. 2003. Association of *Actinobacillus pleuropneumoniae* capsular polysaccharide with virulence in pigs. Infect Immun 71:3320-3328.
141. Champion AE, Bandara AB, Mohapatra N, Fulton KM, Twine SM, Inzana TJ. 2018. Further Characterization of the Capsule-Like Complex (CLC) Produced by Francisella tularensis Subspecies tularensis: Protective Efficacy and Similarity to Outer Membrane Vesicles. Front Cell Infect Microbiol 8:182.
142. Thomas RM, Twine SM, Fulton KM, Tessier L, Kilmury SL, Ding W, Harmer N, Michell SL, Oyston PC, Titball RW, Prior JL. 2011. Glycosylation of DsbA in *Francisella tularensis* subsp. *tularensis*. J Bacteriol 193:5498-509.
143. Balonova L, Mann BF, Cerveny L, Alley WR, Jr., Chovancova E, Forslund AL, Salomonsson EN, Forsberg A, Damborsky J, Novotny MV, Hernychova L, Stulik J. 2012. Characterization of protein glycosylation in *Francisella tularensis* subsp. *holarctica*: identification of a novel glycosylated lipoprotein required for virulence. Mol Cell Proteomics 11:M111 015016.
144. Dankova V, Balonova L, Link M, Straskova A, Sheshko V, Stulik J. 2016. Inactivation of Francisella tularensis Gene Encoding Putative ABC Transporter Has a Pleiotropic Effect upon Production of Various Glycoconjugates. J Proteome Res 15:510-24.
145. Forslund AL, Kuoppa K, Svensson K, Salomonsson E, Johansson A, Bystrom M, Oyston PC, Michell SL, Titball RW, Noppa L, Frithz-Lindsten E, Forsman M, Forsberg A. 2006. Direct repeat-mediated deletion of a type IV pilin gene results in major virulence attenuation of *Francisella tularensis*. Mol Microbiol 59:1818-30.
146. Forslund AL, Salomonsson EN, Golovliov I, Kuoppa K, Michell S, Titball R, Oyston P, Noppa L, Sjostedt A, Forsberg A. 2010. The type IV pilin, PilA, is required for full virulence of *Francisella tularensis* subspecies *tularensis*. BMC Microbiol 10:227.
147. Egge-Jacobsen W, Salomonsson EN, Aas FE, Forslund AL, Winther-Larsen HC, Maier J, Macellaro A, Kuoppa K, Oyston PC, Titball RW, Thomas RM, Forsberg A, Prior JL, Koomey M. 2011. O-linked glycosylation of the PilA pilin protein of *Francisella tularensis*: identification of the endogenous protein-targeting oligosaccharyltransferase and characterization of the native oligosaccharide. J Bacteriol 193:5487-97.
148. Balonova L, Hernychova L, Mann BF, Link M, Bilkova Z, Novotny MV, Stulik J. 2010. Multimethodological approach to identification of glycoproteins from the proteome of *Francisella tularensis*, an intracellular microorganism. J Proteome Res 9:1995-2005.
149. Oyston PC. 2009. *Francisella tularensis* vaccines. Vaccine 27 Suppl 4:D48-51.
150. Foshay L, Hesselbrock WH, Wittenberg HJ, Rodenberg AH. 1942. Vaccine Prophylaxis against Tularemia in Man. Am J Public Health Nations Health 32:1131-45.
151. Kadull PJ, Reames HR, Coriell LL, Foshay L. 1950. Studies on tularemia. V. Immunization of man. J Immunol 65:425-35.

152. Tigertt WD. 1962. Soviet viable *Pasteurella tularensis* vaccines. A review of selected articles. Bacteriol Rev 26:354-73.
153. Pechous RD, McCarthy TR, Zahrt TC. 2009. Working toward the future: insights into *Francisella tularensis* pathogenesis and vaccine development. Microbiol Mol Biol Rev 73:684-711.
154. Eigelsbach HT, Downs CM. 1961. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. J Immunol 87:415-25.
155. Saslaw S, Eigelsbach HT, Prior JA, Wilson HE, Carhart S. 1961. Tularemia vaccine study. II. Respiratory challenge. Arch Intern Med 107:702-14.
156. Hornick RB, Eigelsbach HT. 1966. Aerogenic immunization of man with live Tularemia vaccine. Bacteriol Rev 30:532-8.
157. Wayne Conlan J, Oyston PC. 2007. Vaccines against *Francisella tularensis*. Ann N Y Acad Sci 1105:325-50.
158. Ericsson M, Golovliov I, Sandstrom G, Tarnvik A, Sjostedt A. 1997. Characterization of the nucleotide sequence of the groE operon encoding heat shock proteins chaperone-60 and -10 of Francisella tularensis and determination of the T-cell response to the proteins in individuals vaccinated with F. tularensis. Infect Immun 65:1824-9.
159. Hickey AJ, Hazlett KR, Kirimanjeswara GS, Metzger DW. 2011. Identification of *Francisella tularensis* outer membrane protein A (FopA) as a protective antigen for tularemia. Vaccine 29:6941-7.
160. Huntley JF, Conley PG, Rasko DA, Hagman KE, Apicella MA, Norgard MV. 2008. Native outer membrane proteins protect mice against pulmonary challenge with virulent type A *Francisella tularensis*. Infect Immun 76:3664-71.
161. Post DMB, Slutter B, Schilling B, Chande AT, Rasmussen JA, Jones BD, D'Souza AK, Reinders LM, Harty JT, Gibson BW, Apicella MA. 2017. Characterization of Inner and Outer Membrane Proteins from *Francisella tularensis* Strains LVS and Schu S4 and Identification of Potential Subunit Vaccine Candidates. MBio 8.
162. Jia Q, Horwitz MA. 2018. Live Attenuated Tularemia Vaccines for Protection Against Respiratory Challenge With Virulent *F. tularensis* subsp. *tularensis*. Front Cell Infect Microbiol 8.
163. Orive G, Ponce S, Hernandez RM, Gascon AR, Igartua M, Pedraz JL. 2002. Biocompatibility of microcapsules for cell immobilization elaborated with different type of alginates. Biomaterials 23:3825-31.
164. Lee KY, Mooney DJ. 2012. Alginate: properties and biomedical applications. Prog Polym Sci 37:106-126.
165. Kailasapathy K. 2002. Microencapsulation of probiotic bacteria: technology and potential applications. Curr Issues Intest Microbiol 3:39-48.
166. Chandramouli V, Kailasapathy K, Peiris P, Jones M. 2004. An improved method of microencapsulation and its evaluation to protect Lactobacillus spp. in simulated gastric conditions. J Microbiol Methods 56:27-35.
167. Sultana K, Godward G, Reynolds N, Arumugaswamy R, Peiris P, Kailasapathy K. 2000. Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. Int J Food Microbiol 62:47-55.

168. Su R, Zhu XL, Fan DD, Mi Y, Yang CY, Jia X. 2011. Encapsulation of probiotic Bifidobacterium longum BIOMA 5920 with alginate-human-like collagen and evaluation of survival in simulated gastrointestinal conditions. Int J Biol Macromol 49:979-84.
169. Trabelsi I, Bejar W, Ayadi D, Chouayekh H, Kammoun R, Bejar S, Ben Salah R. 2013. Encapsulation in alginate and alginate coated-chitosan improved the survival of newly probiotic in oxgall and gastric juice. Int J Biol Macromol 61:36-42.
170. Arenas-Gamboa AM, Ficht TA, Kahl-McDonagh MM, Gomez G, Rice-Ficht AC. 2009. The Brucella abortus S19 DeltavjbR live vaccine candidate is safer than S19 and confers protection against wild-type challenge in BALB/c mice when delivered in a sustained-release vehicle. Infect Immun 77:877-84.
171. Arenas-Gamboa AM, Ficht TA, Kahl-McDonagh MM, Rice-Ficht AC. 2008. Immunization with a single dose of a microencapsulated *Brucella melitensis* mutant enhances protection against wild-type challenge. Infect Immun 76:2448-55.
172. Arenas-Gamboa AM, Ficht TA, Davis DS, Elzer PH, Kahl-McDonagh M, Wong-Gonzalez A, Rice-Ficht AC. 2009. Oral vaccination with microencapsuled strain 19 vaccine confers enhanced protection against *Brucella abortus* strain 2308 challenge in red deer (Cervus elaphus elaphus). J Wildl Dis 45:1021-9.
173. Arenas-Gamboa AM, Ficht TA, Davis DS, Elzer PH, Wong-Gonzalez A, Rice-Ficht AC. 2009. Enhanced immune response of red deer (Cervus elaphus) to live rb51 vaccine strain using composite microspheres. J Wildl Dis 45:165-73.

Chapter 2: Glycosylation of a Capsule-like Complex (CLC) by *Francisella novicida* is Required for Virulence and Partial Protective Immunity in Mice

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2.1. Abstract

Francisella tularensis is a gram-negative bacterium and the etiologic agent of tularemia. *F. tularensis* may appear encapsulated when examined by transmission electron microscopy, which is due to production of an extracellular capsule-like complex (CLC) when the bacterium is grown under specific environmental conditions (Bandara et al. PLOS ONE, 6(4): e19003, 2011). Deletion of two glycosylation genes in the live vaccine strain (LVS) results in loss of apparent CLC and attenuation of LVS in mice. In contrast, *F. novicida*, which is also highly virulent for mice, is reported to be nonencapsulated. However, the *F. novicida* genome contains a putative polysaccharide locus with homology to the CLC glycosylation locus in *F. tularensis*. Following passage of *F. novicida* in Chamberlain's defined medium, an electron dense material surrounding *F. novicida,* similar to the *F. tularensis* CLC, was evident. Extraction with urea effectively removed the CLC, and compositional analysis indicated the extract contained galactose, glucose, mannose, and multiple proteins, similar to those found in the *F. tularensis* CLC. The same glycosylation genes deleted in LVS were targeted for deleted in *F. novicida* by allelic exchange using the same mutagenesis vector used for mutagenesis of LVS. However, this mutation also resulted in the loss of 5 additional genes immediately upstream of the targeted mutation (all within the glycosylation locus), resulting in strain *F. novicida*Δ1212-1218. The passed mutant *F. novicida*Δ1212-1218 was CLC-deficient and the CLC contained significantly less carbohydrate than the passed parent strain. The mutant was severely attenuated in BALB/c mice inoculated intranasally, as determined by the lower number of *F. novicida*Δ1212-1218 recovered in tissues compared to the parent, and by clearance of the mutant by 10-14 days post-challenge. Mice vaccinated intranasally with *F. novicida*Δ1212-1218 were partially protected against challenge with the parent, produced significantly reduced levels of inflammatory cytokines, and their spleens contained only areas of lymphoid hyperplasia, whereas control mice challenged with the parent exhibited hypercytokinemia and splenic necrosis. Therefore, *F. novicida* is capable of producing a CLC similar to that of *F. tularensis*, and glycosylation of the CLC contributed to *F. novicida* virulence and immunoprotection.

2.2. Introduction

The gram-negative, intracellular bacterium *Francisella tularensis* causes the disease tularemia in numerous animal species and humans (1). Humans can acquire *F. tularensis* through the bite of an arthropod vector such as a tick, the inhalation of aerosolized bacteria, the handling of an infected carcass, or laboratory exposure (2, 3). Manifestations of tularemia in humans depend on the route of inoculation and the infectious strain. *F. tularensis* subspecies *tularensis* (Type A) is responsible for more severe disease, including pneumonic tularemia and potential mortality, whereas subspecies *holarctica* (Type B) causes less severe disease (4). Type A isolates are found exclusively in North America and as few as 10 bacterial cells can cause human disease (4, 5). Both subspecies *tularensis* and *holarctica* are considered Tier I Select Agents by the Center for Disease Control and Prevention (CDC) due to the low infectious dose, the high level of virulence, and the ease of dispersal (6). The live vaccine strain (LVS) was derived from *F. tularensis* subspecies *holarctica* and is exempt from Select Agent regulations (6). However, LVS is no longer used as a vaccine candidate due to strain instability and potential virulence for immunocompromised individuals (7).

Relatively little is known about factors that contribute to the virulence of *F. tularensis*, though the lipopolysaccharide (LPS) is the most well characterized surface antigen. However, unlike the LPS of enteric gram-negative bacteria, the LPS of *F. tularensis* does not signal through, and is not an agonist of, toll-like receptor 4 (TLR-4) and has little to no endotoxic activity (8). Loss of the LPS O-antigen severely attenuates *F. tularensis* in the mouse model and provides some protection against challenge with Type B strains, but not against Type A strains (9-11). *F. tularensis* strains have also been reported to be encapsulated, based on identification of an electron dense material surrounding the cells by transmission electron microscopy (TEM) (1, 12-14). Enhanced encapsulation increases the virulence of LVS for mice (12). The capsule observed as an electron-dense substance around *F. tularensis* Type A and Type B strains is referred to as a large molecular size capsule-like complex (CLC) and can be identified using negative staining TEM (13). The CLC appears to be glycoprotein in nature and is distinct from *F. tularensis* LPS or the O-antigen capsular polysaccharide, which is only visible around the cells when bound to labeled, specific antibodies, and is distinct from the CLC (13, 15). Daily passage of *F. tularensis* in Chamberlain's defined medium (CDM) broth, followed by culture on

CDM agar (CDMA) at about 30°C results in enhanced expression of the CLC. In contrast, no detectable CLC is produced in culture supernatant or around the cells when *F. tularensis* is grown in broth to mid-log phase (13). Deletion of two glycosyltransferase genes in a glycosylation locus distinct from the O-antigen locus in LVS results in the loss of detectable CLC and attenuation of LVS in mice (13). Immunization with this deletion mutant (LVSΔ1422-23) also protects mice against lethal challenge with virulent LVS (13).

F. tularensis subspecies *novicida* (referred to here as *F. novicida*) retains a high genetic similarity to the more virulent subspecies, is highly virulent in mice, but is only considered virulent for compromised humans (16). Unlike subspecies *tularensis* and *holarctica*, *F. novicida* is reported to be nonencapsulated, (1, 17, 18). *F. novicida* does not produce the O-antigen capsule described for LVS (15). However, we have identified a locus in *F. novicida* (FTN_1200-FTN_1221) with substantial homology to the locus involved in CLC glycosylation of *F. tularensis* (FT0789-FT0806). Therefore, due to the large degree of genetic similarity between *F. novicida* and *F. tularensis* (approximately 97% (19)), and that *F. novicida* is as virulent as *F. tularensis* for mice, which is the most common animal model for studying tularemia, clarifying the presence and role of the CLC in *F. novicida* will aid in clarifying its role as a virulence factor in *Francisella* spp. In this investigation we showed that when *F. novicida* was grown under conditions that enhance expression of the CLC in *F. tularensis* (13), a negatively-stained electron dense material was also detected surrounding *F. novicida*. A *F. novicida* mutant was generated that lacked multiple genes within the glycosylation locus (FTN_1212-1218). This mutant CLC-deficient, attenuated in a mouse model of tularemia, and the live mutant provided limited protection to mice against challenge with wild-type *F. novicida*.

2.3. Methods and Materials

2.3.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2.1. *Francisella* strains were grown on Chamberlain's defined medium agar (CDMA; (20)) or brain heart infusion agar (BD) containing 0.1% cysteine (BHI-C) at 37˚C with 6% CO_2. Broth cultures of *Francisella* strains were grown in Chamberlain's defined medium broth (CDMB) or BHI-C broth with shaking (175-200 rpm) at 37˚C, unless otherwise indicated. Type A strain TI0902 was grown in the CDC-certified BSL-3 laboratory at the Center for Molecular Medicine and

Infectious Diseases (CMMID). The BSL-3 laboratory is located within the Infectious Disease unit at CMMID and is comprised of two BSL-3 rooms with over 2000 sq. ft. All the equipment necessary to grow type A *F. tularensis* in isolation are available. For enhanced expression of any potential CLC, all *Francisella* strains were subcultured daily in CDMB for 10 days (identified by extension name _P10), and then grown on CDMA for 5 days at 30-32˚C with approximately 6% CO_2, as described (13). *Escherichia coli* strains were grown at 37˚C in Luria-Bertani (LB) broth or on LB agar (BD, Franklin Lakes, N.J.). Antibiotics included for growth of recombinant *F. novicida* and *E. coli* strains were 20 and 50 µg/ml of kanamycin, or 250 and 100 µg/ml of hygromycin, respectively.

Strain/Plasmid	**Description**	**Source**
F. novicida	U112 wild type strain	Dr. Karen Elkins
*F. novicida*_P10	*F. novicida* subcultured ten times in CDMB	This work
*F. novicida*Δ1212-1218	*F. novicida* with a deletion spanning FTN_1212 through FTN_1218; insertion of kanamycin resistance cassette	This work
*F. novicida*Δ1212-1218_P10	*F. novicida*Δ1212-1218 subcultured ten times in CDMB	This work
tnfn1_pw060323p05q162	*F. novicida* strain with a T20 transposon insertion in FTN_1212	(21)
tnfn1_pw060323p03q152	*F. novicida* strain with a T20 transposon insertion in FTN_1213	(21)
tnfn1_pw060328p06q149	*F. novicida* strain with a T20 transposon insertion in FTN_1214	(21)
tnfn1_pw060323p05q110	*F. novicida* strain with a T20 transposon insertion in FTN_1215	(21)
tnfn1_pw060420p04q184	*F. novicida* strain with a T20 transposon insertion in FTN_1216	(21)
tnfn1_pw060418p03q107	*F. novicida* strain with a T20 transposon insertion in FTN_1217	(21)
tnfn1_pw060323p07q127	*F. novicida* strain with a T20 transposon insertion in FTN_1218	(21)
Type A *F. tularensis* strain TI0902	Type A wildtype strain	(22)
LVS	*F. tularensis* subspecies *holarctica* live vaccine strain	Dr. May Chu
LVS_P10	LVS subcultured ten times in CDMB	(13)
E. coli DH5α	Genotype: F^- Φ80*lac*ZΔM15Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-,m_k^+) *pho*A*sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Invitrogen

E. coli Top10	Genotype: F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
pSC-1423/1422K	*Francisella* suicide vector containing flanking regions to FTL_1423-22 with kanamycin resistance	(13)
pMP822	Hygromycin resistant *E. coli*/*Francisella* shuttle vector with a *blab* promoter	(23)
pMP_FTN1212-1213	pMP822 with FTN1212-1213 inserted downstream of the *blab* promoter	This work

Table 2.1. Bacterial strains used in this study

2.3.2. Blast analysis of the *F.* novicida genome

The presence of a putative glycosylation locus distinct from the O-antigen locus in *F. novicida* was searched for using BLAST (24) using the *F. tularensis* Type A SchuS4 glycosylation locus (FTT_0789 to FTT_0800) for comparison.

2.3.3. Mutagenesis and complementation of *F. novicida*

Suicide plasmid pSC-1423/1422K, which was used for mutagenesis of LVS, was also used for mutagenesis of *F. novicida* by allelic exchange, targeting FTN_1212 and FTN_1213 (13). Plasmid DNA was purified from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA). The chemical transformation protocol used to introduce pSC-1423/1422K into *F. novicida* was based on a combination of previously described methods (25, 26). An overnight culture of *F. novicida* grown on CDMA was suspended in 1 ml of phosphate buffered saline (PBS), pH 7.6. Approximately 100 ng of the plasmid DNA and 100 µl of the bacteria in PBS were added to 1 ml of transformation buffer (0.04% L-arginine, 0.04% L-aspartic acid, 0.02% L-histidine, 0.04% DL-methionine, 0.004% spermine phosphate, 1.58% sodium chloride, 0.294% CaCl2, and 0.6% Trizma base). This suspension was incubated at 37˚C with slow agitation (100 rpm) for 1 hr, 2 ml of CDMB was added, and incubation continued for 4 to 6 hours with shaking at 200 rpm. Various concentrations of the culture were inoculated to selective BHI-C agar and incubated at 37˚C in 6% CO_2 for up to five days. Kanamycin-resistant colonies were screened for the correct insertion by PCR, and one mutant was confirmed to contain a deletion spanning at least FTN_1212 and FTN_1213 by a second round of PCR and by reverse transcriptase PCR (RT-PCR). To our surprise, subsequent analysis by PCR and RT-PCR indicated that in addition to FTN_1212-1213, the additional open reading frames FTN_1214-1218, which all reside within

this putative glycosylation locus, were also absent. Therefore, the mutant was named *F. novicida*Δ1212-1218.

However, subsequent analysis by PCR and RT-PCR indicated that in addition to FTN_1212-1213, the additional open reading frames FTN_1214-1218, which all reside within this putative glycosylation locus, were also absent. Therefore, the mutant was named *F. novicida*Δ1212-1218.

2.3.4. PCR and DNA-sequencing

A typical PCR reaction consisted of 1x PCR HIFI SuperMix (Invitrogen), 0.02 µg genomic DNA as template, and 0.4 µM of each oligonucleotide primer in 50 µl of reaction mixture. The PCR cycling parameters used were 94 ºC for 2 min followed by 35 cycles of 94 ºC for 30 s, 52 ºC for 30 s, and 68 ºC for 2 min, and an additional extension for 5 min at 68 ºC. For sequencing of the putative CLC glycosylation locus the forward primer was FTN_1219_F and the reverse primer was FTN_1210_R (Table S1). Amplicons from LVS, *F. novicida* U112, and the mutant strain were sequenced using FTN_1219_forward and FTN_1210_reverse primers at the Biocomplexity Institute at Virginia Tech using the ABI 3730 sequencer. Sequence files were analyzed using NCBI nucleotide BLAST online program (24).

2.3.5. Reverse transcriptase-PCR (RT-PCR) of *F. novicida*Δ1212-1213 and *F. novicida*Δ1212-1213[1212-1213+]

RNA was isolated from *F. novicida* and *F. novicida*Δ1212-1218 using the RNeasy Mini Kit (Qiagen). cDNA was generated using the SuperScript III First-Strand (ThermoFisher) synthesis system (Invitrogen) following the manufacturer's instructions. Expression of genes within the glycosylation locus was determined by RT-PCR. The primers used to amplify cDNA of genes FTN_1211 to FTN_1221 for RT-PCR are listed in Table A.1.

2.3.6. Purification of the CLC

F. novicida was grown in CDMB to just past mid-log phase, subcultured consecutively in fresh medium 10 times daily, and then grown on CDMA for 5 days at 32˚C with approximately 7% CO_2 to obtain strain *F. novicida*_P10. The strain was then extracted with 0.5% phenol as previously described (13) and by a modified method using urea (27). Briefly, the bacteria were

scraped off 10 agar plates and suspended in approximately 100 ml of 1 M urea. This suspension was incubated at room temperature for 15 minutes and sedimented by centrifugation at 10,000 x *g* for 15 minutes. The supernatant was subjected to ultracentrifugation at 40,000 x *g* for 4 hours to overnight, and the subsequent supernatant was dialyzed through a membrane with a pore size of 50,000 kDa in 10 mM HEPES and 0.1% sodium dodecyl sulfate (SDS) twice, followed by dialysis in 10 mM HEPES once. Sodium acetate (30 mM final) was added to the dialyzed liquid, followed by addition of 3-5 volumes of 95% ethanol, and the mixture incubated overnight at -20˚C to precipitate any large molecular size material. The precipitate was sedimented by centrifugation at 10,000 x *g* for 30 minutes and resuspended in approximately 25 ml of buffer containing 50 mM Trizma base, 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 0.05% sodium azide. Ten µl of RiboShredder™ RNase Blend (Epicentre, Madison, WI) and 25 µg/ml of DNase were added, the solution incubated at 37˚C overnight, and the mixture extensively dialyzed through a 50,000 kDa membrane in 4 L of distilled water (4 to 5 changes). Any large molecular size material in the retentate was precipitated with ethanol, as above, and lyophilized. Any putative CLC from other strains and mutants that were subcultured in CDMB was extracted in the same manner.

2.3.7. TEM

Francisella strains were subcultured in CDMB and finally on CDMA, as described above, to enhance expression of CLC, or grown in BHI-C broth with shaking to minimize CLC production. TEM of the bacteria was carried out as previously described (13). Briefly, the bacteria were gently suspended and fixed in 0.1 M sodium cacodylate buffer with 2.5% glutaraldehyde and incubated with end-over-end rotation at room temperature for 2 hours. The suspension was allowed to adhere to formvar-coated grids for 5 minutes and then stained with 0.5% uranyl acetate. The grids were briefly washed with distilled water, dried, and the bacteria viewed on a Jeol JEM-1400 electron microscope. Type Astrain TI0902 was subcultured in CDMB in the BSL-3 lab as described above, fixed in 0.1 M sodium cacodylate buffer with 2.5% glutaraldehyde, and incubated with end-over-end rotation at room temperature for 2 hours before being stored at 4°C for 5 days. To ensure complete loss of bacterial viability, a sample was inoculated onto CDMA and allowed to incubate for 5 days at 37°C before the fixed cells were removed from the BSL-3 laboratory. The cells in the suspension were then allowed to adhere to

formvar-coated grids and stained as described above and examined using a Jeol JEM-1400 electron microscope.

2.3.8. Compositional Analysis of the CLC

The carbohydrate composition of the *F. novicida*_P10 extract was determined by combined gas chromatography-mass spectrometry (GC/MS) at the University of Georgia Complex Carbohydrate Research Center, as previously described (13, 28).
Crude urea extracts were used to compare the relative amounts of protein and/or carbohydrate present in the putative CLC from *F. novicida*_P10, *F. novicida*Δ1212-1218_P10, and each transposon (Tn) mutant with single mutations in FTN_1212, FTN_1213, FTN_1214, FTN_1215, FTM_1216, FTN_1217, FTN_1218 that were subcultured on CDM 10 times to enhance surface expression of CLC. Colonies from individual plates were suspended in 4 ml of 1 M urea and incubated at room temperature for 15 min. The bacteria were sedimented by centrifugation and the supernatant was carefully removed for further analysis. Pelleted cells were used to determine the wet weight of bacteria for each crude extract. The amount of protein and carbohydrate/g of bacterial wet weight of the 1 M urea extract was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA), and anthrone assay (29), respectively.

2.3.9. Electrophoretic Profile of the CLC

Electrophoretic profiles were resolved on NuPAGE® Novex® 4-12% Bis-Tris Protein Gels (Life Technologies) by electrophoresis at a constant voltage of 200 v for approximately 40 minutes. Gels were stained with the Pierce Silver Stain Kit (Thermo Scientific), or 0.25% Stains-All (Sigma-Aldrich, St. Louis, MO) (13), or both.

2.3.10. Virulence and Protective Efficacy of *F. novicida*Δ1212-1213 in Mice

Female BALB/c mice 6-8 weeks old (Charles River Laboratories, Wilmington, MA) were housed in an AALAC-accredited ABSL-2 facility. Groups of 7 to 8 mice were used to assess the virulence of *F. novicida*Δ1212-1218 compared to the parent strain. Mice were anesthetized with 3-4% isofluorane and inoculated intranasally (IN) with 50, 100, 1000, or 10,000 CFU of *F. novicida*Δ1212-1218/mouse, or 1000 CFU of *F. novicida* (>100 X the LD_{50})/mouse (30-32), determined spectrophotometrically, in 20 µl of PBS. All inoculation doses

were confirmed by viable plate count on BHI-C agar. Mice were monitored daily, scored for health status, and weighed daily after inoculations. Moribund mice were euthanized with excess CO_2 and lungs, liver, and spleen were collected. Some mice were euthanized at 1, 3, 6, 10, and 14 days post-challenge. The number of bacteria in the liver, lungs, and spleen were determined by viable plate count of weighed, homogenized tissues.

Groups of four mice each were immunized IN with various doses of *F. novicida*Δ1212-1218 (50, 100, 1000, or 10,000 CFU/mouse) in 20 µl of PBS or PBS alone. Six weeks after immunization the mice were challenged IN with 1000 CFU of *F. novicida*. All mice were monitored for 14 days and then euthanized. Any animals that became moribund prior to day 14 post-challenge were euthanized immediately. Tissues were harvested from all mice and cultured to determine bacterial numbers from each inoculation group.

2.3.11. Histopathology of Spleen Samples

Spleen samples were sent to the histopathology laboratory at the Virginia-Maryland College of Veterinary Medicine (VMCVM) for preparation and staining. Briefly, sections of spleen were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E), read with an Olympus BX43 microscope, and photomicrographs were taken with an Olympus DP73 digital camera and cellSens software.

2.3.12. Cytokine Analysis

Spleen tissues from the challenged mice were lysed using the Bio-Plex Cell Lysis Kit according to the manufacturer's instructions (Bio-Rad). The Pierce™ BCA Protein Assay Kit (Thermo Scientific) was used to determine the total protein level of each lysate for standardization of samples. Lysates were analyzed in triplicate using the Bio-Plex Pro™ Mouse Cytokine Th1/Th2 Assay (Bio-Rad, Hercules, CA) following the manufacturer's instructions.

2.3.13. Statistical Analyses

Student's T-test was used to evaluate significant differences in the carbohydrate and protein composition of the putative CLC from *F. novicida*_P10 and *F. novicida*Δ1212-1218_P10. The carbohydrate content of subcultured *F. novicida* transposon mutants

corresponding to each gene affected in the *F. novicida*Δ1212-1218 strain was compared to the subcultured parent strain using One-Way ANOVA. The Mantel-Cox log-rank test was used to compare the survival curves of the control and immunized mice following challenge. Multiple T-tests using the Holm-Sidak method for correcting multiple comparisons was used to assess differences in bacterial load in mice inoculated with *F. novicida* or *F. novicida*Δ1212-1218. One-way ANOVA was also used to evaluate significance in bacterial loads and cytokine levels of immunized mice compared to control mice. Tukey's post hoc test was used after the completion of the one-way ANOVA to identify specific differences between the bacterial loads and cytokine levels of inoculation groups. Statistical analyses were determined using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA).

2.4. Results

2.4.1. Extraction of putative CLC from *F. novicida*

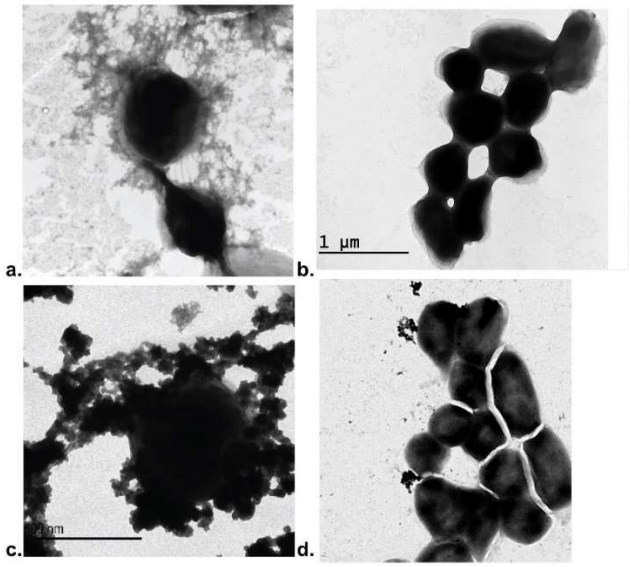

Figure 2.1. Electron micrographs of *Francisella* subspecies and glycosyltransferase mutants.
Bacterial cells were negatively stained with uranyl acetate and examined by TEM. *Francisella* strains were subcultured in CDMB (a, c, and d) for ten consecutive days and then grown for five days at 32˚C on CDMA for 5 days to enhance CLC expression. *F. novicida* grown in BHI-C broth with shaking to mid-log phase did not express this electron dense material (b). *F. novicida* cells grown to enhance CLC expression (c) were surrounded by a similar electron dense material as that produced by *F. tularensis* Type A grown to enhance CLC expression (a). The glycosyltransferase mutant *F. novicida*Δ1212-1218_P10 (d) produced little of the electron dense material, but what material was present was not closely associated with the cell surface. Micrographs shown are representative of most fields. Scale bars represent 500 nm.

Following serial passage of *F. novicida* strain U112 in CDMB and growth on CDMA for 5 days at 32ºC, aggregates of an electron dense material were identified around the bacteria following negative staining and TEM (Figure 2.1c). The electron dense material surrounding *F. novicida*_P10 was similar to the aggregated material observed surrounding *F. tularensis* Type A strain TI0902_P10 (Figure 2.1a) and *F. tularensis* Type B strain LVS_P10 (13). As reported for LVS (13), *F. novicida* cells that were grown to mid-log phase in BHI-C broth at 37°C did not produce a visible electron dense material (Figure 2.1b).

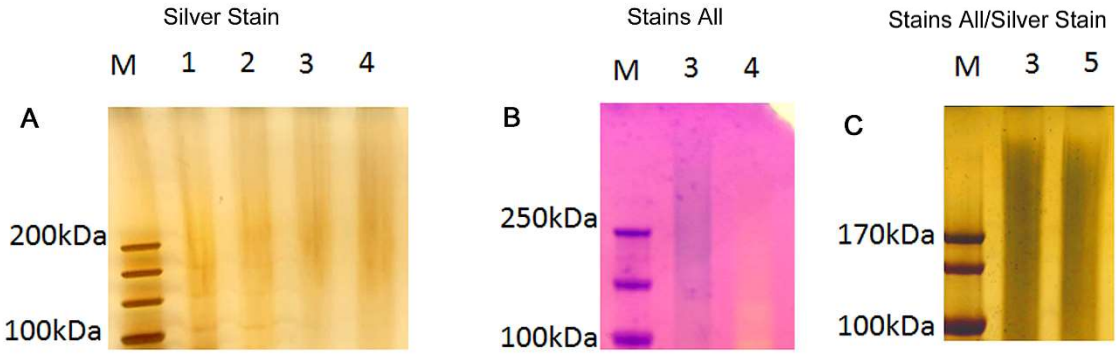

Figure 2.2. Electrophoretic profile of large molecular size *F. novicida* extracts.
Extracts from F. novicida were separated by electrophoresis through a 4-12% SDS-PAGE gel. The gels were subsequently stained by silver stain (a), Stains-All (b), and Stains-All/silver (c, d). LVS CLC was used for comparison. Analysis of the profiles focused on material greater than 100 kDa. Lanes: M, molecular size standards; 1, LVS crude CLC in 1 M urea; 2, F. novicida crude extract in 1 M urea; 3 F. novicida soluble fraction extract (urea extracted); 4, F. novicida insoluble fraction extract (urea extracted); 5 F. novicida soluble fraction extract (phenol extracted).

The putative CLC from *F. novicida*_P10 was extracted as described in methods using 1 M urea in place of 0.5% phenol (13). The use of urea improved solubility and diminished CLC aggregation for type A and B strains (27) (submitted), which was problematic with the CLC extracted with phenol (13). The electrophoretic profiles of *F. novicida*_P10 soluble fraction extracts using urea versus phenol were similar (Figure 2.2c) and were similar to the profile of the CLC from LVS_P10, although the extracts stained poorly with only silver stain (Figure 2.2a). Of particular note were the similarities of the large molecular size band from the *F. novicida*_P10 extract (Figure 2.2a, lanes 2 and 3) and the *F. tularensis* LVS_P10 CLC (Figure 2.2a, lane 1). As previously shown with the CLC from LVS and type A strains (13, 27), a wide variety of proteins were isolated from both the crude and the enzyme-digested *F. novicida*_P10 extracts. The putative CLC proteins could be further divided into soluble and insoluble portions

based on their solubility in water, but the large molecular size smear (~250 kDa) was present in only the soluble portion of the *F. novicida*_P10 extract and was highly visible using the cationic dye Stains-all (Figure 2.2b). Selective staining with Stains-All further supported that the material at ~250 kDa contained carbohydrate (stained blue), which is likely why the material stained poorly with only silver stain and appeared as a smear in the gels. GC-MS indicated that the carbohydrate portion of the putative CLC was composed of glucose and galactose in equal amounts, and less of mannose, which is identical to the glycoses identified in the LVS CLC (13).

2.4.2. Mutagenesis of the putative CLC glycosylation locus and reduction of the carbohydrate content in CLC extracts

***F. tularensis* subsp. *novicida* ORF**	**Size (bp)**	**Protein Product[a]**	***F. tularensis* subsp. *holarctica* LVS ORF**	***F. tularensis* subsp. *tularensis* SchuS4 ORF**	**% Sequence Identity to Type A**
FTN_1221	669	D-ribulose-phosphate 3-epimerase	FTL_1432	FTT_0789	98%
FTN_1220	1395	Glycosyltransferase	FTL_1431	FTT_0790	97%
FTN_1219	1020	UDP-glucose 4-epimerase	FTL_1430	FTT_0791	97%
FTN_1218	1230	Glycosyltransferase	FTL_1429	FTT_0792	97%
FTN_1217	1689	ATP-binding membrane transporter	FTL_1428	FTT_0793	98%
Not present	1287	Hypothetical protein	FTL_1427	FTT_0794	NA[b]
Not present	684	Hypothetical protein	FTL_1426	FTT_0795	NA
Not present	762	Hypothetical protein	FTL_1425	FTT_0796	NA
FTN_1216	744	Hypothetical protein with methyltransferase domain	Not present	Not present	NA
FTN_1215	1161	Capsule polysaccharide export protein	Not present	Not present	NA
FTN_1214	960	Galactosyltransferase	FTL_1424	FTT_0797	98%
FTN_1213	1008	Galactosyltransferase	FTL_1423	FTT_0798	97%
FTN_1212	1014	Mannosyltransferase	FTL_1422	FTT_0799	96%
FTN_1211	663	Haloacid dehalogenase	FTL_1421	FTT_0800	98%

Table 2.2. CLC glycosylation locus in *F. tularensis* subspecies.
[a]F. novicida homologs to the F. tularensis Type A and LVS CLC glycosylation locus. Two genes are present in F. novicida that are not found in the F. tularensis locus. There are three genes in the F. tularensis CLC glycosylation locus that are not present in the F. novicida locus: FTT0794, FTT0795, and FTT0796; all three genes encode for hypothetical proteins.
[b]NA-Not applicable.

A DNA region in the genome of *F. novicida* U112 with homology to the LVS CLC glycosylation locus (13) was identified by BLAST analysis (Table 2.2). The glycosylation locus in *F. tularensis* is comprised of 12 open reading frames (ORF), whereas the *F. novicida* locus contained 11 ORFs (Figure 2.3B). The *F. novicida* locus did not contain homologs to FTL_1425, FTL_1426, and FTL_1427, but did contain two alternate genes (FTN_1215 [*kpsC*] and FTN_1216) that are not found in *F. tularensis*. *F. novicida* did contain homologs to two genes required for CLC glycosylation in LVS (13) and glycosylation of DsbA in type A *F. tularensis* (33). These two genes (FTN_1212 and FTN_1213) were targeted for deletion in *F. novicida* to determine if they were also responsible for CLC glycosylation in *F. novicida*. Two genes were mutated because we previously showed that deletion of one gene (of two separate genes) in this locus failed to adequately delete glycosylation (13).

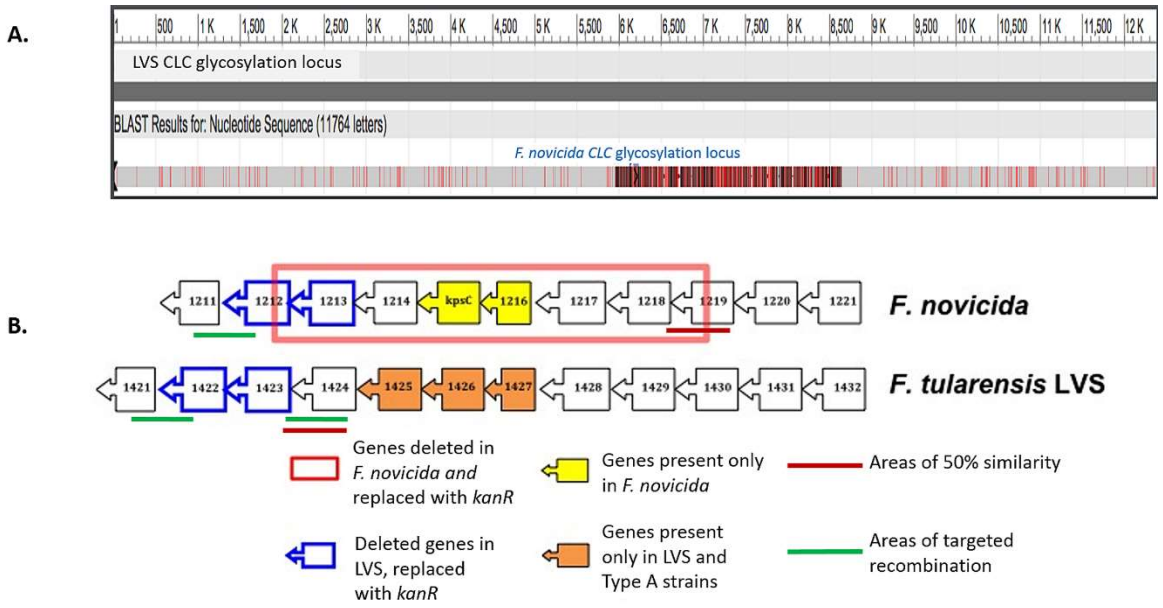

Figure 2.3. Comparison of the CLC glycosylation loci of LVS and *F. novicida*.
A. Needleman-Wunsch alignment of the glycosylation loci of LVS with F. novicida showing the regions of dissimilarity in red. B. Cartoon of the LVS and F. novicida CLC glycosylation loci showing the region deleted in F. novicidaΔ1212-1218 (red box), genes unique to F. novicida and LVS (yellow and orange boxes, respectively), areas targeted for recombination using the same recombination vector used for LVS (green lines), areas of 51% identity where recombination actually occurred in F. novicidaΔ1212-1218, and genes replaced with kanR gene in LVS and homologous genes targeted for deletion in F. novicida (blue boxes).

FTN_1212 and FTN_1213 were successfully deleted from *F. novicida* using allelic exchange with suicide plasmid pSC-1422/23K, as determined by amplification of the kanamycin resistance cassette and failure to amplify the targeted region by PCR or RT-PCR. However, RT-

PCR of FTN_1212 and FTN_1213 and the remaining genes in this locus indicated that ORFs FTN_1212 through FTN_1218 were not expressed, but cDNA was amplified from the first (upstream) three genes in the locus: FTN_1219 to FTN_1221 and the gene downstream of the targeted mutation, FTN_1211, indicating the mutation was not polar and was restricted to the glycosylation locus (Figure 2.4B). Each of these genes in the parent were expressed normally (Figure 2.4A). Furthermore, PCR of each of the individual genes within the locus amplified FTN_1211 and FTN_1219-1221 but failed to amplify DNA from FTN_1212-1218, indicating that the allelic exchange mutation affected 7 genes rather than only the two target genes (Figure 2.5).

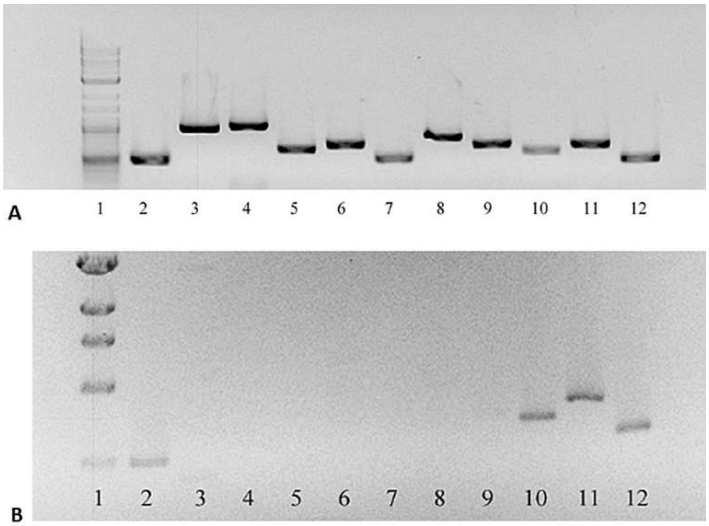

Figure 2.4. RT-PCR of genes in the glycosylation locus of *F. novicida*Δ1212-1218 compared to the *F. novicida* parent.
RNA was isolated from both *F. novicida* and *F. novicida*Δ1212-1218 and converted to cDNA. Primers to each gene in the glycosylation locus were used to amplify gene products. Products were successfully amplified for the entire glycosylation locus in *F. novicida* (A). Gene products were not amplified from FTN_1212 through FTN_1218 in *F. novicida*Δ1212-1218 (B). The absence of amplification of genes downstream of the deletion in *F. novicida*Δ1212-1218 indicated that the mutation induced a polar effect within the glycosylation locus. Gene products from FTN_1219 through FTN_1221, also within the glycosylation locus, in *F. novicida*Δ1212-1218 were amplified. Lanes: 1, 1-kb ladder; 2-12, transcription products from genes FTN_1211-1221, respectively.

This mutant was named *F. novicida*Δ1212-1218. DNA sequencing of the entire locus showed that 100-bp of FTN_1219, all of FTN_1218-1213, and 210-bp of FTN_1212 were deleted and replaced with the kanamycin resistance gene (Figure 2.3B), confirming that FTN_1212-1218 were not functional (data not shown). Needleman-Wunsch global alignment of

the glycosylation locus of LVS compared to *F. novicida* indicated there was 96-99% identity in 9 out of 12 genes present in the two loci (Figure 2.3A). Three LVS genes (FTL_1425-27) were missing in *F. novicida*, while two additional genes (*kpsC* and FTN_1216) were present exclusively in the locus of *F. novicida* (Figure 2.3A). DNA sequence analysis of the entire *F. novicida* glycosylation locus identified 1,019 nucleotides spanning FTN_1218 and FTN_1219 that shared 51% identity with the targeted region in FTL_1424 that flanks the LVS mutagenesis vector (Figure 2.3B, red line) where a recombination event occurred (not shown).

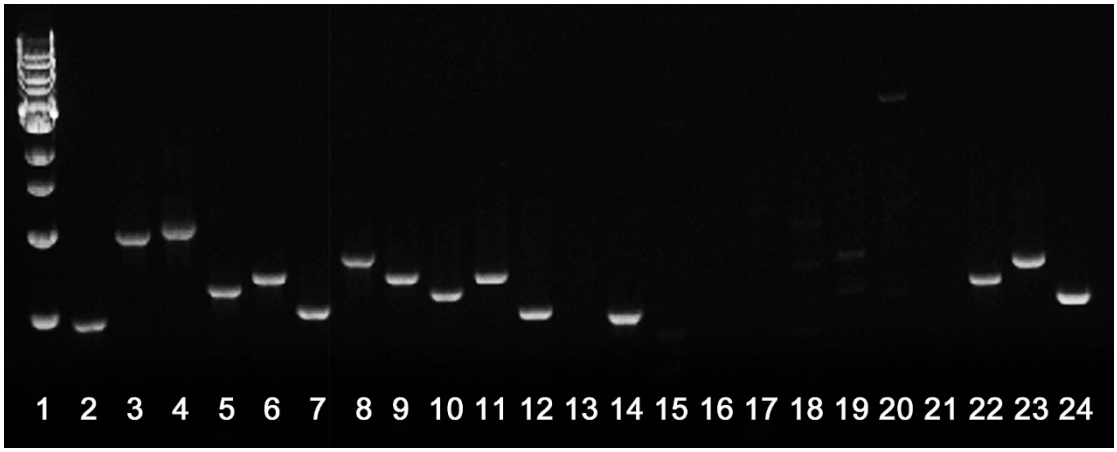

Figure 2.5. PCR of each open reading frame within the putative CLC glycosylation locus. Lanes 2-12 represent PCR amplification products from parent strain *F. novicida* U112. Lanes 14-24 represent PCR amplification products from mutant *F. novicida*Δ1212-1218. Lanes: 1, molecular size standards; 2 and 14, FTN_1211; 3 and 15, FTN_1212; 4 and 16, FTN_1213; 5 and 17, FTN_1214; 6 and 18, FTN_ 1215; 7 and 19, FTN_1216; 8 and 20, FTN_1217; 9 and 21, FTN_1218; 10 and 22, FTN_1219; 11 and 23, FTN_1220; 12 and 24, FTN_1221; 13, blank.

*F. novicida*Δ1212-1218 and each of the 7 *F. novicida* transposon (TN) mutants with a single TN-interrupted gene in FTN_1212-1218 were subcultured ten times in CDMB to enhance any CLC material they were capable of making. The majority of *F. novicida*Δ1212-1218_P10 cells lacked any surface material around them, but some small amounts of putative CLC was observed by TEM surrounding *F. novicida*Δ1212-1218_P10. In contrast, the majority of the subcultured, *F. novicida*Δ1212-1218_P10 cells lacked any surface material around them. Furthermore, what surface material was present was not closely associated to the cells (Figure 2.1d), as it was on the surface of *F. novicida*_P10 (Figure 2.1c). The amount of protein in the extract from *F. novicida*Δ1212-1218_P10 was not significantly different from that found on *F. novicida*_P10, but the carbohydrate content of the putative CLC extract from *F. novicida*Δ1212-

1218_P10 was significantly lower ($p = 0.02$) (Figure 2.6), even though the LPS was not affected. However, the amount of carbohydrate present in the CLC from each of the subcultured isogenic TN mutants was not significantly different ($p > 0.05$) from that of the subcultured parent strain (Table A.2). Therefore, mutagenesis of a single gene within this glycosylation locus was inadequate to significantly affect CLC glycosylation, as previously reported (13).

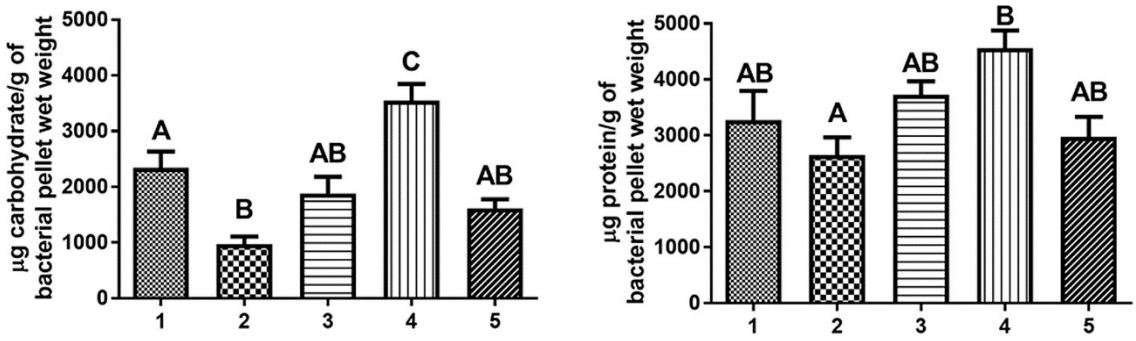

Figure 2.6. Carbohydrate and protein content of urea extracts from *F. novicida* and LVS strains.
All strains were subcultured in CDMB and grown on CDMA at 32°C to enhance putative CLC production. The material was extracted using 1 M urea, and the crude CLC extracts were analyzed for carbohydrate content by the anthrone assay and for protein content by the BCA assay. The carbohydrate content of the crude extract from *F. novicida*Δ1212-1218_P10 contained significantly less carbohydrate than that of *F. novicida*_P10 ($p = 0.02$). The carbohydrate content of the CLC extract from *F. novicida*Δ1212-1218[1212-1213+]_P10 was not significantly different from that of *F. novicida*_P10 ($p = 0.69$) or *F. novicida*Δ1212-1218_P10 ($p = 0.15$). The protein content between subcultured strains of *F. novicida* or between strains of LVS was not statistically different. Lanes: 1, *F. novicida*_P10; 2, *F. novicida*Δ1212-1218_P10; 3, *F. novicida* Δ1212-1218[1212-1213+];4, LVS_P10; 4, LVSΔ1422-1423_P10. Different letters above the bars indicate significant differences between the means when one-way ANOVA and Tukey's post-hoc were performed ($p < 0.05$). Similar uppercase letters indicate that there is no significant difference between the means. The value of a bar marked with “A” is significantly different from the values of bars marked with “B” or C". The values of bars marked “AB" are not statistically different from the values of bars marked with "A" or with "B," but are statistically different from a bar marked with "C."

2.4.3. Attenuation of *F. novicida*Δ1212-1218 in mice

Female BALB/c mice were inoculated IN with *F. novicida* or *F. novicida*Δ1212-1218 to determine if the lack of glycosylation affected virulence. All mice challenged with up to 10,000 CFU of *F. novicida*Δ1212-1218 IN survived the study duration of 14 days with minimal to no clinical signs (Figure 2.7d). Some groups of mice were inoculated with 1,000 CFU of *F.*

novicida or *F. novicida*Δ1212-1218 IN and humanely euthanized at days 1, 3, 6, 10, and 14. The bacterial burden in the lungs (Figure 2.7a), liver (Figure 2.7b), and spleen (Figure 2.7c) increased significantly from days 1 to 3 in mice infected with *F. novicida* compared to mice challenged with *F. novicida*Δ1212-1218 ($p = 0.0021$, 0.0005, and < 0.0001, respectively). Mice infected with *F. novicida* did not survive beyond 3 days post-challenge. Bacterial burdens on day 1 in the lungs, liver, and spleen of mice infected with *F. novicida*Δ1212-1218 were below the level of detection. Bacterial burdens in the lungs (Figure 2.7a) and spleen (Figure 2.7b) were detected on day 3, but were significantly lower ($p = 0.0018$ and > 0.001 respectively) than bacterial numbers detected on day 3 in mice infected with *F. novicida*. *F. novicida*Δ1212-1218 was detected in the liver on day 6, but not at other time points (Figure 2.7c). *F. novicida*Δ1212-1218 was undetectable in any organs of the mice by day 14 post-challenge.

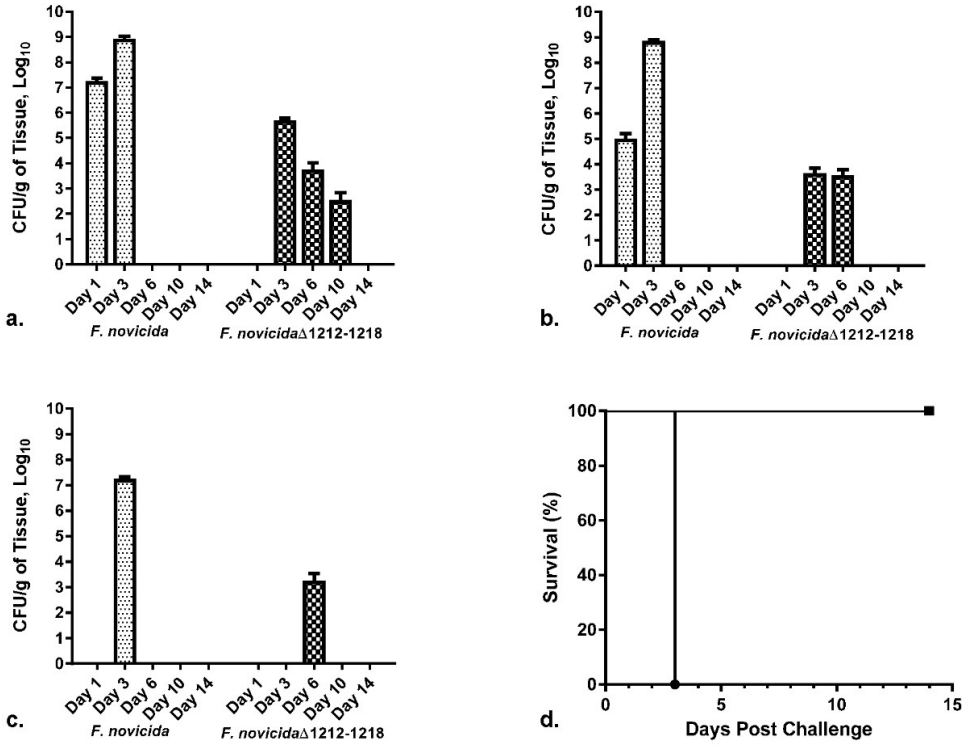

Figure 2.7. Attenuation of *F. novicida*Δ1212-1218 in mice.
Mice were inoculated with 1000 CFU of wild-type *F. novicida* and *F. novicida*Δ1212-1218 IN, followed by euthanasia on days 1, 3, 6, 10, and 14. The bacterial burden in the lungs (a), spleen (b), and liver (c) were determined by viable plate counts. The bars represent the mean bacterial burden ± the SEM for *F. novicida* (dotted bar) and *F. novicida*Δ1212-1218 (checkered bar). All mice inoculated with *F. novicida* (●) needed to be euthanized by day 3. All mice inoculated with *F. novicida*Δ1212-1218 cleared the bacterial infection by day 10-14. *F. novicida*Δ1212-1218 (■)

was attenuated in mice IN at a dose of at least 10,000 CFU and infection caused minimal to no signs of clinical illness (d).

Therefore, *F. novicida*Δ1212-1218 was unable to multiply and disseminate in mice as efficiently as *F. novicida* and was significantly attenuated following challenge by the IN route of infection compared to *F. novicida* (p =0.0183). Mice inoculated with 10,000 CFU of *F. novicida*Δ1212-1218 also had significantly less weight loss than mice inoculated with 1,000 CFU of *F. novicida* at 1, 3, and 4 days post-inoculation ($p < 0.005$) (Figure A.1).

2.4.4. Protective efficacy of *F. novicida*Δ1212-1218 against challenge with the parent strain

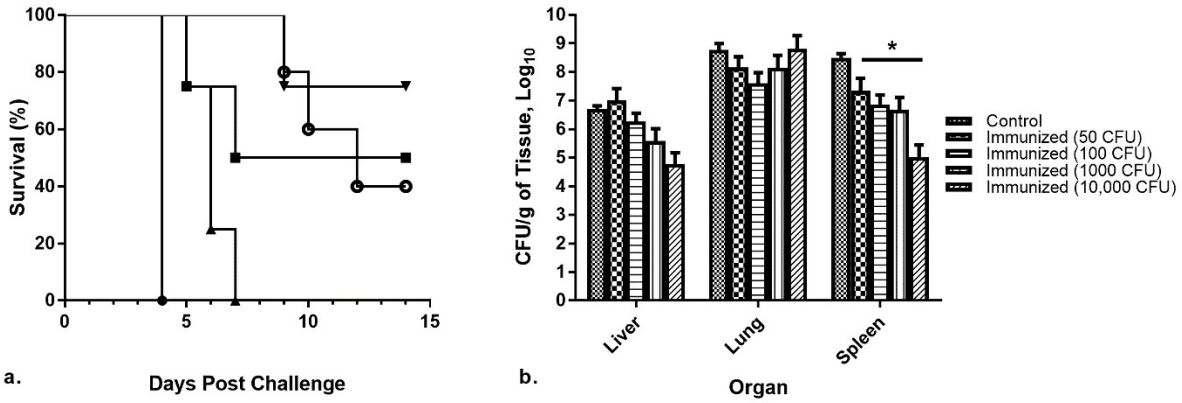

Figure 2.8. Protection of mice immunized with *F. novicida*Δ1212-1218 against IN challenge with wildtype *F. novicida*.
(a) BALB/c mice were immunized IN with varying doses of *F. novicida*Δ1212-1218 (50 CFU, ■; 100 CFU, ▲; 1000 CFU, ▼; and 10,000 CFU, ○) or with PBS (●). Six weeks after immunization the mice were challenged IN with 1000 CFU of *F. novicida* U112. Control mice immunized with PBS needed to be euthanized by day 4. Some of the mice in each group immunized with 50, 1000, or 10,000 CFU of *F. novicida*Δ1212-1218 survived until day 14 post-challenge, which was statistically significant ($p < 0.001$) compared to the control group. (b) Bacterial numbers in the liver, lungs, and spleen were determined after euthanasia. All groups of immunized mice had significantly lower bacterial burdens in the spleen compared to control mice ($p < 0.0001$, *).

BALB/c mice were immunized with PBS or variable doses of *F. novicida*Δ1212-1218 (50, 100, 1,000, and 10,000 CFU) IN. Six weeks after immunization the mice were challenged IN with 1,000 CFU of *F. novicida*, monitored for two weeks, and the survival of each group recorded (Figure 2.8a). All control mice became moribund between days 3 to 4 and were euthanized. All groups of immunized mice developed clinical symptoms following challenge with the parent strain and no group had 100% survival by day 14. Mice that developed more

severe clinical symptoms, including hunched appearance and closed eyes, did not recover and were euthanized when the mice became moribund. Mice that experienced milder symptoms including slightly ruffled fur and some weight loss recovered by day 14, which included some mice in groups immunized with 50, 1,000, and 10,000 CFU of *F. novicida*Δ1212-1218; all surviving mice showed no clinical symptoms by day 14. All mice immunized with 100 CFU of *F. novicida* Δ1212-1218 needed to be euthanized by day 7 post-challenge.

Lungs, liver, and spleen were collected from each mouse post-mortem to determine the bacterial burden of *F. novicida* U112 in each organ (Figure 2.8b). No difference in bacterial burden was found in the liver and lungs of immunized mice compared to control mice, but immunized mice had significantly lower bacterial burdens in the spleen compared to control mice ($p < 0.0001$ for all immunized groups compared to control). Overall, immunized mice were partially protected from respiratory challenge with *F. novicida* U112, and the protection was dose dependent.

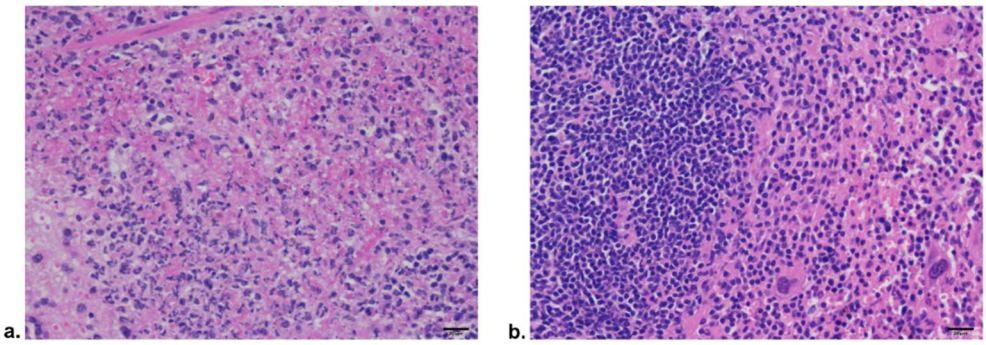

Figure 2.9. Histopathology of spleens of immunized mice and control mice after challenge with *F. novicida*.
There is necrosis and fibrin accumulation surrounded by neutrophils and cellular debris within the red pulp of spleens of control mice (a). In contrast, there is moderate to marked lymphoid hyperplasia characterized by nodular aggregates of lymphocytes, but no evidence of necrosis or neutrophilic inflammation in the spleens of immunized mice (b). H&E stain, bar = 20 µm.

Severe multifocal to coalescing fibrino-necrotizing splenitis was evident following histopathological examination of post-mortem spleens from control mice challenged with *F. novicida* (Figure 2.9a). However, there was lymphoid hyperplasia and extramedullary hematopoiesis in the spleens of mice immunized with *F. novicida*Δ1212-1218 and then challenged with *F. novicida* (Figure 2.9b). There was no evidence of necrosis or neutrophilic inflammation in the spleens of immunized mice, unlike the spleens of control mice.

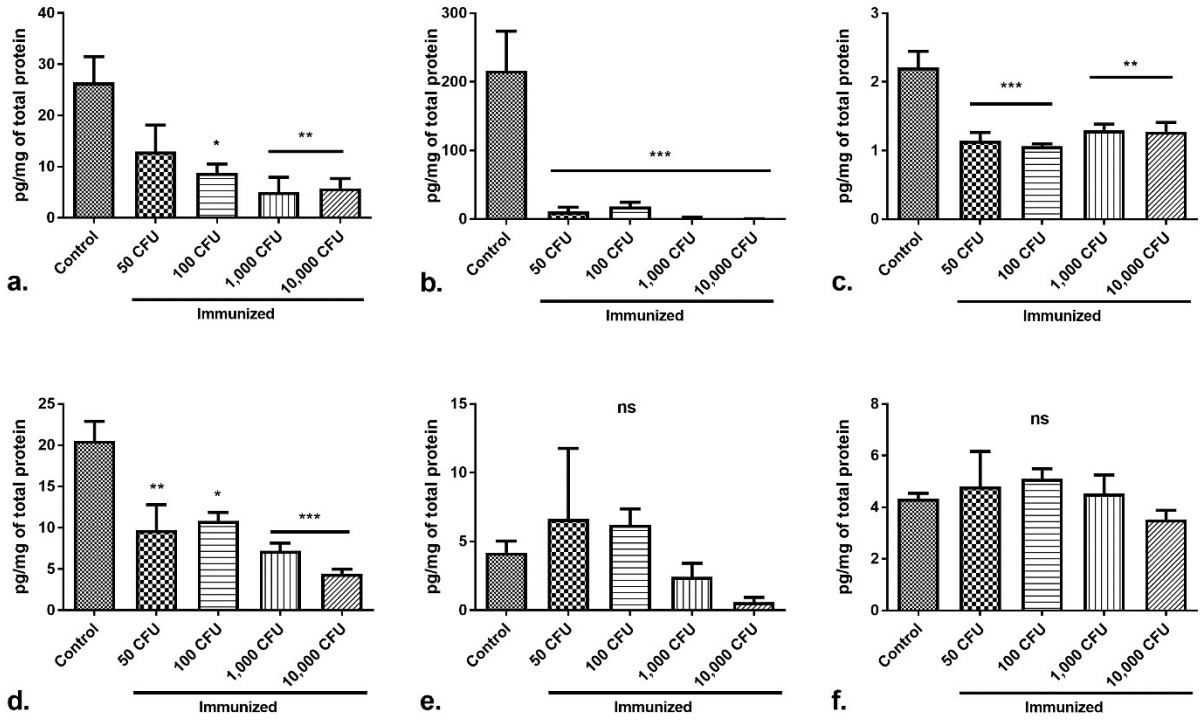

Figure 2.10. Levels of splenic cytokines in control and immunized mice after challenge. Spleens were harvested after euthanasia and lysed as described in Methods to analyze the levels of splenic cytokines/g of tissue. Cytokine levels were determined by Bio-Plex cytokine assay and reported as μg/mg of the total protein, determined by BCA. Levels of TNF (a), INF-γ (b), IL-4 (c), and IL-10 (d)/g of tissue were significantly lower in mice immunized with *F. novicida*Δ1212-1218 than control mice. Levels of IL-12 (p70) (e) and IL-2 (f) were not significantly different between groups. $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

Spleen lysate cytokine levels were determined as pg of cytokine/mg of total protein $\pm$ the standard error of the mean. Levels of TNF-α, INF-γ, IL-4, and IL-10 were significantly higher in the spleens of challenged control mice than in the spleens of challenged immunized mice (Figure 2.10a-d) (*p*-values for cytokine levels of the group immunized with 10,000 CFU compared to the control mice were $p < 0.01$, < 0.001, < 0.01, and < 0.001 respectively*).* There was no significant difference in the levels of IL-12(p70) and IL-2 produced by the spleens of control and immunized mice (Figure 2.10e-f) ($p \geq 0.325$), although there was detectably less IL-12(p70) made by mice immunized with 10,000 CFU of the mutant than controls (Figure 2.10e). GM-CSF levels were only detected in control mice and not in immunized mice, and IL-5 was not detected in any group. Overall, control mice exhibited hypercytokinemia compared to immunized mice.

2.5. Discussion

F. novicida is reported to be nonencapsulated, whereas *F. tularensis* subspecies *tularensis* and *holarctica* are described as encapsulated (34-36). The presence of a capsule around *F. tularensis* subspecies *tularensis* and *holarctica* is based on the observation of an electron dense material surrounding the cells by electron microscopy (37, 38). Nevertheless, such "encapsulation" is only observed following specific growth conditions. Cherwonogrodsky *et al.* (37) reported that subculture of LVS in defined broth medium, such as CDMB, followed by culture for several days on defined medium agar (CDMA), increased the amount of electron dense material surrounding the bacterial cells, and enhanced the virulence of these cells in mice. Previously, we used this serial passage method (adding to it lowering the culture temperature to 32°C) to confirm that the electron dense material around LVS and type A strains is enhanced during such growth conditions, and that it consists predominately of protein, but also carbohydrate; it is now referred to as CLC (13). *F. novicida* may have been described as nonencapsulated because it was not grown under the proper conditions to make a similar electron dense material visible. It has not been determined what component(s) of CDM enhance(s) expression of the CLC. However, spermine is present in the CDM and has been shown to induce extensive changes in gene expression, enabling *F. tularensis* to recognize its eukaryotic host environment (39). Furthermore, mutagenesis of the gene putatively responsible for the spermine response attenuated both the LVS and Type A strain SchuS4 *in vivo* (40). Lowering the temperature to 32°C may also simulate environmental conditions more closely related to other hosts, such as ticks. Therefore, CDM may simulate signaling by the host environment for *F. tularensis* more closely than other media.

Passage of *F. novicida* in CDMB, followed by growth at 32˚C on CDMA, enhanced surface expression of an electron dense material, which was not present around *F. novicida* cells grown to mid-log phase in shaking broth. This electron dense material appeared similar to the material surrounding both LVS (13) and *F. tularensis* subspecies *tularensis*. The *F. novicida* extracellular material was composed of an array of proteins and a relatively small portion of carbohydrate (consisting of glucose, galactose, and mannose), similar to the CLC of LVS (13). The electrophoretic protein profile of the *F. novicida*_P10 extract was also similar to the profile of the CLC from LVS and contained components of similar high molecular size. Differential

staining further supported that this large molecular size material was glycosylated. Therefore, *F. novicida* did produce a CLC similar to the CLC of the more virulent subspecies.

A genetic locus with homology to the CLC glycosylation locus of LVS and Type A strains was also identified in *F. novicida* (13, 33). Deletion of two glycosyltransferases (FTL_1423 and FTL_1422; a mannosyltransferase and galactosyltransferase, respectively) eliminated expression of the CLC in LVS. However, deletion of only one of the genes in this locus did not significantly affect glycosylation (13). We have previously reported that deletion of only one glycosyltransferase gene from the capsule locus of *Actinobacillus pleuropneumoniae* did not completely eliminate capsule expression (41). Furthermore, there was no significant difference in the carbohydrate content of the CLC from 7 *F. novicida* TN mutants with one gene inactivated within the glycosylation locus and the parent strain. Therefore, the two genes homologous to those deleted in LVS were targeted for deletion (FTN_1212 and FTN_1213) using the same vector used for mutation of LVS. To our surprise, the allelic exchange process in *F. novicida* also resulted in loss of upstream genes FTN_1214-1218, but not downstream gene FTN_1211.

It is not clear why so many genes were affected by this allelic exchange procedure, but substantial identity (51%) between base pairs at FTN_1219 (the upstream region deleted in *F. novicida*) and FTL_1424 (the upstream gene in the LVS mutagenesis vector) was identified that may have accounted for the recombination event involving a vector with highly similar DNA of another subspecies. Nonetheless, all of the affected genes were within the glycosylation locus, and deletion of the additional genes should only affect glycosylation of proteins and the CLC.

The content of carbohydrate in crude CLC extracts of the subcultured mutant was significantly reduced compared to the subcultured parent, even though the LPS content was not affected in the mutant. However, the mutations did not significantly affect CLC protein content. Nonetheless, the mutation reduced both the amount of CLC observed, and the association of the CLC proteins with the *F. novicida* cell surface. When observed by electron microscopy, what little CLC was present around *F. novicida*Δ1212-1218_P10 was less adherent, scattered, and away from the bacterial cells, whereas the CLC of wild-type *F. novicida*_P10 covered the cells and was more directly associated with the bacterial surface. In *Haemophilus influenzae,* the adhesin HMW1 is glycosylated and this glycosylation is necessary to tether the protein to the bacterial surface: removal of the glycan disassociates the protein from the bacterium (42).

Therefore, the carbohydrate component of the CLC may also contribute to close association of the CLC proteins to the bacterial surface and contribute to the aggregation of these proteins, as observed by electron microscopy. Enhanced expression of CLC may also promote binding of the bacteria to phagocytic cells and promote bacterial uptake.

Protein glycosylation appears to be widespread in *Francisella* species. Identical glycans have been identified that modify proteins that include PilA (43), DsbA (33, 44), and FTH_0069 (44) in *F. tularensis* subspecies *holarctica* and *tularensis*. The glycan modifying these proteins is a hexasaccharide and the synthesis of this glycan has been linked to genes present in the putative CLC glycosylation locus of subspecies *tularensis* (33). A multi-method approach of lectin enrichment, lectin blotting, and glycoprotein-specific staining was also used to identify up to 15 putative glycoproteins in *F. tularensis* subspecies *holarctica* extracts (45). All of these 15 putative glycoproteins have homologs in *F. novicida* and some of the proteins are putative outer membrane proteins such as FopA and TUL4. Nevertheless, other than PilA, DsbA, and FTH_0069, no other *Francisella* proteins have been definitively confirmed as glycoproteins.

The differences noted by electron microscopy and virulence between LVS and *F. novicida* mutants lacking the same glycosyltransferases are minimal but may be related to other mutations in LVS. There are multiple mutations in LVS that may contribute to the bacterium's attenuation in immunocompetent individuals, including multiple hypothetical proteins, outer membrane proteins, metabolism proteins, and more (46). LVS also contains a truncated version of PilA, which has been shown to be necessary for full virulence in both subspecies *holarctica* (47) and *tularensis* (48). In contrast, strain U112 is a wildtype strain of *F. tularensis* and is as virulent for mice as *F. tularensis* type A. In contrast, mice challenged IN with up to 1000 times the LD_{50} of *F. novicida*Δ1212-1218 only developed subclinical infections. During a time-course comparing an *F. novicida*Δ1212-1218 infection with that of an equal dose of the parent, *F. novicida*Δ1212-1218 did not proliferate in the tissues to the same extent as the wild-type strain and was below detection level in tissues until day three post-challenge, whereas bacterial numbers of *F. novicida* U112 were one or more logs greater. *F. novicida*Δ1212-1218 was also unable to disseminate throughout the tissues as effectively as the parent. Therefore, protein glycosylation of the CLC played an important role in the ability of *F. novicida* to disseminate in the mouse, as previously described for LVS (13). The observation that enhanced expression of CLC increases the virulence of *F. tularensis* (37) and our current and previous findings that

inability to glycosylate and fully produce CLC attenuates the bacteria (13), indicates that the CLC contributes to *F. tularensis* virulence. The mechanism responsible for enhancing virulence is unknown but may be related to resistance to innate host defenses, promoting macrophage uptake, or promoting escape from the phagosome to the cytosol (49).

It was not practical to attempt complementation of 7 genes in *F. novicida*Δ1212-1218. Nonetheless, the mutant was transformed with shuttle vector pFNLTP6 (13) containing the genes targeted for deletion. In data not shown this partially complemented strain contained more carbohydrate than the mutant, less than the parent, but was not significantly different from either. Mice inoculated with 1,000 CFU or more of the mutant expressing FTN_1212 and FTN_1213 demonstrated clinical symptoms that included ruffled fur, reduced activity, and weight loss, but the mice survived for 14 days, unlike mice challenged with the parent. Mice inoculated with 10,000 CFU of this partially complemented mutant had significantly more weight loss than mice inoculated with 10,000 CFU of *F. novicida*Δ1212-1218 ($p < 0.005$) (data not shown). Thus, although as expected complementation of *F. novicida*Δ1212-1218 with only genes FTN_1212 and FTN_1213 did not restore full virulence to the mutant, these results showed that full glycosylation in *F. novicida* required more than 1 gene, and partial glycosylation and virulence could be restored despite the lack of several genes. Synthesis of a carbohydrate polymer requires many genes, including transferases, polymerase, phosphorylases, and proteins for export and transport of the polymer across membranes. FTN_1212-1214 and FTN_1218 are putative glycosyltransferases. FTN_1216 encodes a hypothetical protein of yet unknown function. FTN_1215 may encode a protein responsible for capsule export and FTN_1217 for an ATP-binding membrane transporter; both are involved in polysaccharide export. Transport and synthesis of other polysaccharides in *F. tularensis*, such as LPS and O-antigen capsule, likely also utilize membrane transporters and transferases (the LPS core also contains mannose) that could substitute for some of the missing proteins, enabling partial restoration of CLC glycosylation.

Immunization with *F. novicida*Δ1212-1218 provided partial protection to mice against IN challenge with the parent strain. This incomplete protection may be due to the inability of *F. novicida*Δ1212-1218 to effectively disseminate throughout the host and persist long enough to induce a more protective immune response, or the missing carbohydrate may be an important antigen for protective immunity. *F. tularensis* and *F. novicida* are facultative intracellular

pathogens, and a robust cellular immune response is necessary to effectively clear the infection (50-52). Immunodominant antigens such as the O-antigen provide partial protection against aerosolized challenge with virulent Type B strains, but not Type A strains (53). Immunization of mice with recombinant FopA enclosed in liposomes can afford partial protection against challenge with LVS, but not against SchuS4 (54). These studies suggest that humoral immunity may play an ancillary role in protection against *F. tularensis*. The disruption of the CLC glycan may result in instability of surface antigens surrounding the bacterium resulting in the loss of important antigenic epitopes necessary to stimulate supportive humoral immunity.

In mice, outward clinical signs of tularemia are delayed 2-3 days following infection with *Francisella*, but the disease quickly progresses and becomes lethal (35, 55, 56). The pathology of tularemia is largely due to tissue damage following severe inflammation and hypercytokinemia that occurs after *Francisella* has replicated extensively (56-59). In this study, control mice infected with *F. novicida* did not show clinical signs until approximately 48 hours after infection and became moribund between 72-96 hours post infection. Disease in the challenged, immunized mice did not advance as rapidly as in the control mice, and the infection in mice challenged with *F. novicida*Δ1212-1218 was subclinical. Sharma *et al*. (57, 59) reported that mice challenged with *F. novicida* IN become severely septic and hypercytokinemic, whereas mice challenged with a *F. novicida* mutant lacking a 58-kDa protein produce lower levels of pro-inflammatory cytokines and do not succumb to disease. Mice immunized with *F. novicida*Δ1212-1218 and then challenged with the parent also exhibit reduced levels of pro-inflammatory cytokines, such as TNF-α, compared to control mice (57). The level of tissue destruction in the lungs correlates with the levels of pro-inflammatory cytokines; the hypercytokinemic control mice exhibit more severe tissue destruction than the immunized mice after challenge (57). Our results were similar to those of Sharma *et al.* (57) in regard to differences in cytokine responses between immunized and control mice. Immunized mice from both studies produced significantly less TNF-α, IL-10, and GM-CSF after challenge than control mice after challenge. The spike in TNF-α that precedes morbidity may be more detrimental to the host and contribute to sepsis instead of effectively controlling the bacterial infection (56-59). Bakshi *et al.* showed that immunization with an attenuated LVS stain, $sodB_{FT}$, results in a more controlled release of pro-inflammatory cytokines after challenge than unimmunized control mice (60). Higher production of pro-inflammatory cytokines in mice correlates with greater amounts of inflammation and tissue destruction; a

delayed response in cytokine production followed by massive up-regulation is a sign of severe tularemia (3, 60). In our study, unlike control mice, mice inoculated with the mutant were not hypercytokinemic after challenge and the spleens of these mice did not exhibit the same level of inflammation and tissue destruction as the spleens of mice inoculated with the parent. Although immunization of mice with the mutant was not fully protective, the partial protection afforded may be attributed to a more regulated immune response in immunized mice than in control mice after challenge. Interferon gamma (IFN-γ) has been implicated as essential for controlling the replication of *F. tularensis* during the initial infection. Both Sharma *et al.* (57, 59) and Bakshi *et al*. (61) reported that immunized mice surviving a lethal challenge exhibit a significant increase in IFN-γ compared to controls after infection, followed by a return to baseline levels. In this study, the spleens of immunized mice did not contain significantly higher levels of IFN-γ than control mice. The lack of enhanced expression of IFN-γ in mice immunized with *F. novicida*Δ1212-1218 may explain the lack of full protection following challenge with the parent. The LD_{50} of *F. novicida* for mice by the respiratory route is <10 CFUs (30-32). Therefore, it was not possible to compare the immune response of mice immunized with the mutant to that of a sublethal dose of the parent.

In summary, we have shown that, as for *F. tularensis, F. novicida* produced a CLC when grown under conditions that enhanced expression of the electron dense CLC in LVS. The mixture of glycosylated proteins in the *F. tularensis* CLC have been partially identified (manuscript in preparation). Furthermore, the use of *F. novicida* and the immense transposon library available may further aid in identifying these proteins and the role of CLC in pathogenesis. Loss of surface protein glycosylation and the deficiency of surface-associated CLC on the bacteria attenuated *F. novicida* and LVS in a mouse model, and such mutants conferred partial protective immunity against challenge with virulent strains. Mutagenesis of these glycosyltransferase genes in a Type A strain of *F. tularensis* is in progress to determine if such a mutant would also be attenuated and induce protective immunity against type A challenge.

2.6. References

1. Sjostedt A. 2005. Family III. Francisellaceae, p 199-209. *In* Brenner DJ, Krieg NR, Garrity GM, Staley JT (ed), Bergey's Manual of Systematic Bacteriology: Volume 2: The Proteobacteria, 2 ed, vol 2. Springer.

2. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Layton M, Lillibridge SR, McDade JE, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Tonat K, Working Group on Civilian B. 2001. Tularemia as a biological weapon: medical and public health management. JAMA 285:2763-73.
3. Metzger DW, Bakshi CS, Kirimanjeswara G. 2007. Mucosal immunopathogenesis of *Francisella tularensis*. Ann N Y Acad Sci 1105:266-83.
4. Sjostedt A. 2007. Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. Ann N Y Acad Sci 1105:1-29.
5. Saslaw S, Eigelsbach HT, Prior JA, Wilson HE, Carhart S. 1961. Tularemia vaccine study. II. Respiratory challenge. Arch Intern Med 107:702-14.
6. Services CfDClPaDoHaH. 2012. Possession, Use, and Transfer of Select Agents and Toxins; Biennial Review; Final Rule.
7. Wayne Conlan J, Oyston PC. 2007. Vaccines against *Francisella tularensis*. Ann N Y Acad Sci 1105:325-50.
8. Gunn JS, Ernst RK. 2007. The structure and function of *Francisella* lipopolysaccharide. Ann N Y Acad Sci 1105:202-18.
9. Li J, Ryder C, Mandal M, Ahmed F, Azadi P, Snyder DS, Pechous RD, Zahrt T, Inzana TJ. 2007. Attenuation and protective efficacy of an O-antigen-deficient mutant of *Francisella tularensis* LVS. Microbiology 153:3141-53.
10. Raynaud C, Meibom KL, Lety MA, Dubail I, Candela T, Frapy E, Charbit A. 2007. Role of the wbt locus of *Francisella tularensis* in lipopolysaccharide O-antigen biogenesis and pathogenicity. Infect Immun 75:536-41.
11. Modise T, Ryder C, Mane SP, Bandara AB, Jensen RV, Inzana TJ. 2012. Genomic comparison between a virulent type A1 strain of *Francisella tularensis* and its attenuated O-antigen mutant. J Bacteriol 194:2775-6.
12. Cherwonogrodzky JW, Knodel MH, Spence MR. 1994. Increased encapsulation and virulence of *Francisella tularensis* live vaccine strain (LVS) by subculturing on synthetic medium. Vaccine 12:773-5.
13. Bandara AB, Champion AE, Wang X, Berg G, Apicella MA, McLendon M, Azadi P, Snyder DS, Inzana TJ. 2011. Isolation and mutagenesis of a capsule-like complex (CLC) from *Francisella tularensis*, and contribution of the CLC to *F. tularensis* virulence in mice. PLoS One 6:e19003.
14. Sandstrom G, Lofgren S, Tarnvik A. 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.
15. Apicella MA, Post DM, Fowler AC, Jones BD, Rasmussen JA, Hunt JR, Imagawa S, Choudhury B, Inzana TJ, Maier TM, Frank DW, Zahrt TC, Chaloner K, Jennings MP, McLendon MK, Gibson BW. 2010. Identification, characterization and immunogenicity of an O-antigen capsular polysaccharide of *Francisella tularensis*. PLoS One 5:e11060.
16. Keim P, Johansson A, Wagner DM. 2007. Molecular epidemiology, evolution, and ecology of *Francisella*. Ann N Y Acad Sci 1105:30-66.
17. Elkins KL, Cowley SC, Bosio CM. 2007. Innate and adaptive immunity to *Francisella*. Ann N Y Acad Sci 1105:284-324.

18. Barker JH, McCaffrey RL, Baman NK, Allen LA, Weiss JP, Nauseef WM. 2009. The role of complement opsonization in interactions between *F. tularensis* subsp. *novicida* and human neutrophils. Microbes Infect 11:762-9.
19. Kingry LC, Petersen JM. 2014. Comparative review of *Francisella tularensis* and *Francisella novicida*. Front Cell Infect Microbiol 4:35.
20. Chamberlain RE. 1965. Evaluation of Live Tularemia Vaccine Prepared in a Chemically Defined Medium. Appl Microbiol 13:232-5.
21. Gallagher LA, Ramage E, Jacobs MA, Kaul R, Brittnacher M, Manoil C. 2007. A comprehensive transposon mutant library of Francisella novicida, a bioweapon surrogate. Proc Natl Acad Sci U S A 104:1009-14.
22. Inzana TJ, Glindemann GE, Snider G, Gardner S, Crofton L, Byrne B, Harper J. 2004. Characterization of a wildtype strain of *Francisella tularensis* isolated from a cat. J Vet Diagn Invest 16:374-381.
23. LoVullo ED, Sherrill LA, Pavelka MS, Jr. 2009. Improved shuttle vectors for *Francisella tularensis* genetics. FEMS Microbiol Lett 291:95-102.
24. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403-10.
25. Frank DW, Zahrt TC. 2007. Genetics and genetic manipulation in *Francisella tularensis*. Ann N Y Acad Sci 1105:67-97.
26. Gallagher LA, McKevitt M, Ramage ER, Manoil C. 2008. Genetic dissection of the *Francisella novicida* restriction barrier. J Bacteriol 190:7830-7.
27. Champion A, Mohapatra N, Bandara A, Twine S, Inzana TJ. 2015. The Capsule-Like Complex (CLC) of Type A *Francisella tularensis* may be Derived from Outer Membrane Vesicles, but Glycosylation of the CLC is not Required for Virulence and is not Protective., abstr 115th General Meeting of the American Society for Microbiology, New Orleans, LA., May 30-June 2, 2015,. American Society for Microbiology,
28. Merkle RK, Poppe I. 1994. Carbohydrate composition analysis of glycoconjugates by gas-liquid chromatography/mass spectrometry. Methods Enzymol 230:1-15.
29. Scott TA, Melvin EH. 1953. Determination of Dextran with Anthrone. Anal Chem 25:1656-1661.
30. Cong Y, Yu JJ, Guentzel MN, Berton MT, Seshu J, Klose KE, Arulanandam BP. 2009. Vaccination with a defined *Francisella tularensis* subsp. *novicida* pathogenicity island mutant (DeltaiglB) induces protective immunity against homotypic and heterotypic challenge. Vaccine 27:5554-61.
31. Lauriano CM, Barker JR, Yoon SS, Nano FE, Arulanandam BP, Hassett DJ, Klose KE. 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.
32. Kieffer TL, Cowley S, Nano FE, Elkins KL. 2003. *Francisella novicida* LPS has greater immunobiological activity in mice than *F. tularensis* LPS, and contributes to *F. novicida* murine pathogenesis. Microbes Infect 5:397-403.
33. Thomas RM, Twine SM, Fulton KM, Tessier L, Kilmury SL, Ding W, Harmer N, Michell SL, Oyston PC, Titball RW, Prior JL. 2011. Glycosylation of DsbA in *Francisella tularensis* subsp. *tularensis*. J Bacteriol 193:5498-509.

34. Barker JH, McCaffrey RL, Baman NK, Allen LA, Weiss JP, Nauseef WM. 2009. The role of complement opsonization in interactions between *F. tularensis* subsp. *novicida* and human neutrophils. Microbes Infect 11:762-9.
35. Elkins KL, Cowley SC, Bosio CM. 2007. Innate and adaptive immunity to *Francisella*. Ann NY Acad Sci 1105:284-324.
36. Sjöstedt A. 2005. Family III. *Francisellaceae*, *fam. nov.*, p 199-210. *In* D. J. Brenner NRK, and J. T. Staley (ed), Bergey's Manual of Systematic Bacteriology second edition, vol Two. Springer, New York.
37. Cherwonogrodzky JW, Knodel MH, Spence MR. 1994. Increased encapsulation and virulence of *Francisella tularensis* live vaccine strain (LVS) by subculturing on synthetic medium. Vaccine 12:773-775.
38. Sandström G, Löfgren S, Tärnvik A. 1988. A capsule-deficient mutant of *Francisella tularensis* LVS exhibits enhanced sensitivity to killing but diminished sensitivity to killing by polymorphonuclear leukocytes. Infect Immun 56:1194-1202.
39. Carlson PE, Jr., Horzempa J, O'Dee DM, Robinson CM, Neophytou P, Labrinidis A, Nau GJ. 2009. Global transcriptional response to spermine, a component of the intramacrophage environment, reveals regulation of Francisella gene expression through insertion sequence elements. J Bacteriol 191:6855-64.
40. Russo BC, Horzempa J, O'Dee DM, Schmitt DM, Brown MJ, Carlson PE, Jr., Xavier RJ, Nau GJ. 2011. A *Francisella tularensis* locus required for spermine responsiveness is necessary for virulence. Infect Immun 79:3665-76.
41. Bandara AB, Lawrence ML, Veit HP, Inzana TJ. 2003. Association of *Actinobacillus pleuropneumoniae* capsular polysaccharide with virulence in pigs. Infect Immun 71:3320-3328.
42. Grass S, Buscher AZ, Swords WE, Apicella MA, Barenkamp SJ, Ozchlewski N, St Geme JW, 3rd. 2003. The *Haemophilus influenzae* HMW1 adhesin is glycosylated in a process that requires HMW1C and phosphoglucomutase, an enzyme involved in lipooligosaccharide biosynthesis. Mol Microbiol 48:737-51.
43. Egge-Jacobsen W, Salomonsson EN, Aas FE, Forslund AL, Winther-Larsen HC, Maier J, Macellaro A, Kuoppa K, Oyston PC, Titball RW, Thomas RM, Forsberg A, Prior JL, Koomey M. 2011. O-linked glycosylation of the PilA pilin protein of *Francisella tularensis*: identification of the endogenous protein-targeting oligosaccharyltransferase and characterization of the native oligosaccharide. J Bacteriol 193:5487-97.
44. Balonova L, Mann BF, Cerveny L, Alley WR, Jr., Chovancova E, Forslund AL, Salomonsson EN, Forsberg A, Damborsky J, Novotny MV, Hernychova L, Stulik J. 2012. Characterization of protein glycosylation in *Francisella tularensis* subsp. *holarctica*: identification of a novel glycosylated lipoprotein required for virulence. Mol Cell Proteomics 11:M111 015016.
45. Balonova L, Hernychova L, Mann BF, Link M, Bilkova Z, Novotny MV, Stulik J. 2010. Multimethodological approach to identification of glycoproteins from the proteome of *Francisella tularensis*, an intracellular microorganism. J Proteome Res 9:1995-2005.
46. Rohmer L, Brittnacher M, Svensson K, Buckley D, Haugen E, Zhou Y, Chang J, Levy R, Hayden H, Forsman M, Olson M, Johansson A, Kaul R, Miller SI. 2006. Potential source of *Francisella tularensis* live vaccine strain attenuation determined by genome comparison. Infect Immun 74:6895-906.

47. Forslund AL, Kuoppa K, Svensson K, Salomonsson E, Johansson A, Bystrom M, Oyston PC, Michell SL, Titball RW, Noppa L, Frithz-Lindsten E, Forsman M, Forsberg A. 2006. Direct repeat-mediated deletion of a type IV pilin gene results in major virulence attenuation of *Francisella tularensis*. Mol Microbiol 59:1818-30.
48. Forslund AL, Salomonsson EN, Golovliov I, Kuoppa K, Michell S, Titball R, Oyston P, Noppa L, Sjostedt A, Forsberg A. 2010. The type IV pilin, PilA, is required for full virulence of *Francisella tularensis* subspecies *tularensis*. BMC Microbiol 10:227.
49. Golovliov I, Baranov V, Krocova Z, Kovarova H, Sjostedt A. 2003. An attenuated strain of the facultative intracellular bacterium Francisella tularensis can escape the phagosome of monocytic cells. Infect Immun 71:5940-50.
50. Baron SD, Singh R, Metzger DW. 2007. Inactivated *Francisella tularensis* live vaccine strain protects against respiratory tularemia by intranasal vaccination in an immunoglobulin A-dependent fashion. Infect Immun 75:2152-62.
51. Shen H, Harris G, Chen W, Sjostedt A, Ryden P, Conlan W. 2010. Molecular immune responses to aerosol challenge with *Francisella tularensis* in mice inoculated with live vaccine candidates of varying efficacy. PLoS One 5:e13349.
52. Pechous RD, McCarthy TR, Zahrt TC. 2009. Working toward the future: insights into *Francisella tularensis* pathogenesis and vaccine development. Microbiol Mol Biol Rev 73:684-711.
53. Conlan JW, Shen H, Webb A, Perry MB. 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.
54. Hickey AJ, Hazlett KR, Kirimanjeswara GS, Metzger DW. 2011. Identification of *Francisella tularensis* outer membrane protein A (FopA) as a protective antigen for tularemia. Vaccine 29:6941-7.
55. Cowley SC, Elkins KL. 2011. Immunity to *Francisella*. Front Microbiol 2:26.
56. Mares CA, Ojeda SS, Morris EG, Li Q, Teale JM. 2008. Initial delay in the immune response to *Francisella tularensis* is followed by hypercytokinemia characteristic of severe sepsis and correlating with upregulation and release of damage-associated molecular patterns. Infect Immun 76:3001-10.
57. Sharma J, Li Q, Mishra BB, Georges MJ, Teale JM. 2009. Vaccination with an attenuated strain of *Francisella novicida* prevents T-cell depletion and protects mice infected with the wild-type strain from severe sepsis. Infect Immun 77:4314-26.
58. Sharma J, Mares CA, Li Q, Morris EG, Teale JM. 2011. Features of sepsis caused by pulmonary infection with *Francisella tularensis* Type A strain. Microb Pathog 51:39-47.
59. Sharma J, Li Q, Mishra BB, Pena C, Teale JM. 2009. Lethal pulmonary infection with *Francisella novicida* is associated with severe sepsis. J Leukoc Biol 86:491-504.
60. Bakshi CS, Malik M, Mahawar M, Kirimanjeswara GS, Hazlett KR, Palmer LE, Furie MB, Singh R, Melendez JA, Sellati TJ, Metzger DW. 2008. An improved vaccine for prevention of respiratory tularemia caused by *Francisella tularensis* SchuS4 strain. Vaccine 26:5276-88.
61. Bakshi CS, Malik M, Regan K, Melendez JA, Metzger DW, Pavlov VM, Sellati TJ. 2006. Superoxide dismutase B gene (sodB)-deficient mutants of *Francisella tularensis* demonstrate hypersensitivity to oxidative stress and attenuated virulence. J Bacteriol 188:6443-8.

Chapter 3: The Effect of Growth Medium and Surface Antigens on Biofilm Formation by *Francisella novicida*

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3.1. Abstract

Francisella tularensis is a zoonotic pathogen capable of causing fatal disease in humans and other species. The general ecology of *F. tularensis* suggests that arthropods serve as both a reservoir, and as a vector that can transmit the bacterium to mammalian species. However, recent research within the past few decades suggest that *Francisella* species and outbreaks of the disease are highly associated with waterways with some species exclusively aquatic pathogens. *F. tularensis* is capable of producing a biofilm *in vitro*. Waterways may serve as an environmental reservoir for *Francisella* species, and it is possible that *Francisella* survives such conditions as a biofilm. This chapter examines the effect of different growth conditions and differential expression of surface-associated antigens on the ability of *F. tularensis* subspecies *novicida* to produce a biofilm. Statistically significant differences where observed between *F. novicida* biofilm when the bacteria were grown in different media. Crystal violet staining of *F. novicida* biofilm after growth in different media was statistically higher in brain heart infusion broth with 0.1% cysteine (BHI-C) and tryptic soy broth with 0.1% cysteine compared to other commonly used *Francisella* growth media. *F. novicida* strains with transposon insertions in the O-antigen locus were also assayed for biofilm growth in different media in comparison to the wildtype strain. When grown in Mueller-Hinton broth supplemented with 0.1% cysteine there was no statistical difference in biofilm formation; whereas some strains grown in BHI-C produced more biofilm than the wildtype. However, a majority of the O-antigen-deficient strains produced less biofilm than the wildtype when grown in Chamberlain's defined medium broth. In general, this research suggests that more care must be taken when assaying *Francisella* species for biofilm formation as different growth media and cell surface phase variation of the cells can cause differences in the biofilm produced.

3.2. Introduction

Francisella tularensis is a Gram-negative pathogen able to infect a variety of host species including humans, lagomorphs, and various biting insects (1). Highly virulent *F. tularensis* species are able to cause disease following inhalation of as few as 10 bacterial cells (2). The disease *F. tularensis* causes, tularemia, is potentially fatal if left untreated in humans and is variably fatal for other mammalian species (3-5). *Francisella* species are distributed across the Northern Hemisphere with the most virulent subspecies *tularensis* (Type A) centered in North America, and a lesser virulent subspecies *holarctica* (Type B) covering the entire Northern hemisphere (1). Disease outbreaks of tularemia are mainly linked to increased disease in resident lagomorphs (5-7), vector-borne transmission (8, 9), water-associated infection (10), or a combination of multiple factors (11). Though discovery of *F. tularensis* dates to the early 20th century, it is possible that tularemia is a re-emerging disease in parts of the world (11, 12). Therefore, much research has focused on development of a potential tularemia vaccine. This is in part also due to the status of *F. tularensis* as a Tier I Select Agent of biowarfare (13).

In general, the ecology of *F. tularensis* species is complex, in part due to the vast array of affected species and vectors, but also possibly due to the ability of *Francisella* species to survive and replicate in numerous environments (1). *Francisella tularensis* is an obligate intracellular pathogen able to replicate in phagocytic cells (14) and cause bacteremia in a host (15). Additionally, *F. tularensis* subspecies are increasingly found associated with waterways (10, 11, 16) and these waterways are postulated as an environmental reservoir of *F. tularensis* (17). Outbreaks in Sweden, occurring mainly during the summer months, are highly associated with mosquito bites and with the patient having traveled near a body of water (10). Broman *et al.* (10) demonstrated molecular identification of a diverse group of *F. tularensis* organisms in water samples from bodies of water located in endemic areas during both outbreak and non-outbreak years. Thus, *F. tularensis* subspecies are capable of surviving in the environment and water sources may serve as a reservoir for infection of arthropod vectors, such as biting flies and mosquitos.

F. tularensis is a highly fastidious bacterium that requires specific growth media to survive in a laboratory setting, requirements that are difficult to find outside of a host. Van Hoek (17) postulates this survival of *F. tularensis* in the environment may be accomplished through

the ability of *Francisella* species to form a biofilm. Biofilms are colonies of bacterial cells housed in an extracellular matrix capable of protecting the bacterium from the surrounding environment. Within the last decade researchers have shown that *F. tularensis* subspecies are capable of producing a biofilm *in vitro* (18, 19). Further research has also shown an interaction between amoebae or mosquitos with *Francisella* biofilms (20, 21).

F. tularensis subspecies *novicida* (hereto referred as *F. novicida*) is the predominant *Francisella* strain that has been studied for biofilm formation. *F. novicida* is less fastidious than other *F. tularensis* subspecies and is not subject to the heightened biosecurity regulations of those subspecies since this subspecies is not considered a Select Agent (13). *F. novicida* is considered an environmental subspecies and only causes disease in immunocompromised individuals (22), though mice are highly susceptible to disease. Cases of tularemia contributed to *F. novicida* are often associated with water such as a near-drowning incident (23) or contaminated ice (24). These characteristics of water-association, ability to manipulate at a lower biosecurity, and a relative ease of genetic manipulation make *F. novicida* an ideal model organism to study *Francisella* biofilm formation.

Research of *Francisella* biofilms is relatively new in comparison to other areas of *Francisella* research, such as the host immune response. A large portion of the biofilm research focuses on regulation through an orphan quorum sensing regulator (18), production of chitinase enzymes (19, 25, 26), and contribution of pilin proteins (17). *Francisella* species produce other surface-associated components that could contribute to biofilm formation including a unique lipopolysaccharide (LPS), the O-antigen capsule (27), the glycosylated Capsule-like Complex (CLC) (28, 29), and outer membrane vesicles and tubes (OMV/T) (30-32). *Francisella* also alters expression of various surface-associated factors when cultured in host-adapted versus non-host-adapted media (33-35). The presence and differential expression of these surface-associated antigens may contribute to the ability of *Francisella* species to form a biofilm. This chapter examines the effect of different growth media and differential expression of surface-associated antigens in the development of an *F. novicida* biofilm. The differences examine suggest that more care must be taken when assessing *Francisella* species for the capability to form a biofilm.

3.3. Methods and Materials

3.3.1. Bacterial strains and growth conditions

Strain Name Abbreviation	Strain Name	Description	Source
F. novicida	*F. tularensis* subspecies *novicida* U112	U112 wild type strain	Dr. Karen Elkins
WbtA	tnfn1_pw060323p06q123	Transposon mutant with insertion disrupting the *wbtA* gene	(36)
WbtD	tnfn1_pw060419p04q192	Transposon mutant with insertion disrupting the *wbtD* gene	(36)
WbtE	tnfn1_pw060328p03q164	Transposon mutant with insertion disrupting the *wbtE* gene	(36)
WbtF	tnfn1_pw060323p06q161	Transposon mutant with insertion disrupting the *wbtF* gene	(36)
WbtG	tnfn1_pw060323p03q189	Transposon mutant with insertion disrupting the *wbtG* gene	(36)
WbtH	tnfn1_pw060323p06q119	Transposon mutant with insertion disrupting the *wbtH* gene	(36)
WbtN	tnfn1_pw060323p06q136	Transposon mutant with insertion disrupting the *wbtN* gene	(36)
WbtO	tnfn1_pw060323p03q182	Transposon mutant with insertion disrupting the *wbtO* gene	(36)
WbtP	tnfn1_pw060328p05q149	Transposon mutant with insertion disrupting the *wbtP* gene	(36)
WbtQ	tnfn1_pw060419p04q158	Transposon mutant with insertion disrupting the *wbtQ* gene	(36)
Wzx	tnfn1_pw060328p01q189	Transposon mutant with insertion disrupting the *wzx* gene	(36)
Wzy	tnfn1_pw060328p05q111	Transposon mutant with insertion disrupting the *wzy* gene	(36)
"Strain Name Abbreivation" is the way the strain will be referred to throughout the paper. "Strain Name" is the formal name given by the strain source.			

Table 3.1. Bacterial strains used in this study.

The bacterial strains used in this study are described in Table 3.1. Strains were grown on Chamberlain's defined medium agar (37) with 1.5% glucose (CDMA), brain heart infusion agar (BD, Franklin Lakes, N.J.) with 0.1% cysteine (BHI-C), tryptic soy broth agar (BD, Franklin Lakes, N. J.) with 0.1% cysteine (TSB-C), or Mueller Hinton agar (BD, Franklin Lakes, N.J.) with 0.1% cysteine (MH-C) at 37°C with 6% CO_2 unless otherwise indicated. Broth cultures were grown in Chamberlain's defined medium broth with 1.5% glucose (CDMB), BHI-C broth, TSB-C broth, or MH-C broth with shaking (175-200 rpm) at 37°C unless otherwise indicated. Kanamycin at 20 μg/ml was added to the growth media for recombinant strains of *F. novicida*.

All experiments were carried out in a biosafety level 2 (BSL-2) facility in an approved biosafety cabinet.

3.3.2. Crystal violent detection of biofilm growth

F. novicida strains were grown for biofilm production as previously described (18). In brief, an overnight suspension of bacteria was diluted in the appropriate broth medium and 200 µl was added to wells of a 96-well round bottom polystyrene microtiter plate (Nunc, Rochester, N.Y.). Bacterial strains were inoculated into replicate groups of five wells. Fresh medium was used as a negative control. The wells were incubated for 5 days at 37°C and then biofilms were assessed via staining with crystal violet (CV). Prior to the addition of CV, all media and freely suspended cells were removed by aspiration from the wells. A 100 µl aliquot of 0.1% CV was added to each well and incubated at room temperature for approximately 15 minutes. The wells were then washed with 200µl of sterile water three times to remove excess CV. After washing, the adherent CV was solubilized with 95% ethanol and the absorbance at 560 nm was determined. CV biofilm results are reported as either the optical density (OD) value at 560 nm or as a percent of the wildtype CV OD value at 560 nm depending on the experimental conditions. Experimental conditions include growth of *F. novicida* as a biofilm in different media and growth of *F. novicida* transposon mutants compared to the wild-type.

3.3.3. Bacterial attachment assay

Overnight stationary cultures of broth-grown *F. novicida* strains (200 µl) were added to wells of 96-well round-bottom polystyrene microtiter plates in groups of five. Fresh sterile medium was added as a negative control. The OD at 650 nm was determined before incubation to account for cell density. The plate was incubated at 37°C for 6 hours to allow bacterial cells to attach to the wells. After 6 hours, freely suspended bacteria and media were removed by aspiration from each well. CV staining was performed on each well as described above. Attachment results were determined as the ratio of the OD_{560nm} to OD_{650nm}.

3.3.4. Microbial adhesion to hexadecane assay

Microbial adhesion to hexadecane (MATH) was used to determine the hydrophobicity of different *F. novicida* strains. MATH was performed as previously described (38). Briefly, an equal amount of hexadecane was added to a suspension of bacterial cells and vortexed at full speed for approximately 2 minutes. The suspension was then allowed to settle for 15 minutes. The absorbance at 650nm was then determined for the aqueous phase and compared to the

absorbance at 650nm of the bacterial suspension prior to hexadecane addition. The results were calculated as the percent of the starting culture that had partitioned into the hydrophobic layer, as determined by the absorbances at 650 nm for the aqueous phases. This assay was used to determine the effect of growth in different media on the cell surface hydrophobicity of *F. novicida*.

3.3.5. Auto-aggregation assay

Cultures of *F. novicida* strains were grown to stationary phase and resuspended at an OD_{650nm} of 0.300 in phosphate buffered saline, pH 7.2 (PBS). Bacterial suspensions were incubated at room temperature and the absorbance at 650 nm was determined from the upper 1 ml of the suspension at specific timepoints. The results were calculated as the ratio of the absorbance of the suspension at a specific timepoint compared to the absorbance of the suspension at the start of the experiment (percent of auto-aggregation). These results were used to determine the effect of different growth media of *F. novicida* auto-aggregation.

3.3.6. Rapid isolation and analysis of lipopolysaccharide

Lipopolysaccharide (LPS) purification from *F. novicida* and selected transposon mutants with disruptions within the O-antigen genetic locus was performed as previously described (39). Electrophoretic profiles of purified LPS were resolved on NuPAGE Novex 4-12% Bis-Tris Protein Gels (Life Technologies) with electrophoresis at a constant voltage of 200V for approximately 40 minutes. The gels were stained with either the Pierce Silver Stain Kit (Thermo Scientific) or the Emerald Q300 fluorescent glycoprotein stain.

3.3.7. Serum complement bactericidal assay

Mid-log cultures of *F. novicida* strains were diluted to a concentration of approximately $1x10^4$ colony forming units (CFU)/ml. In microcentrifuge tubes, 10 µl of the diluted cell culture was added to 10 µl or 20 µl (10% or 20%, respectively) of complement-active guinea pig serum and PBS supplemented with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PCM) was added to bring the volume to 100 µl. The tubes were incubated at 37°C for 1 hour. A viable plate count was performed in duplicate on an aliquot of each suspension prior to incubation and after 1 hour of incubation. The results are reported as the percent survival of cells after incubation in complement-active serum. Serum-resistant wildtype *F. novicida* and serum-sensitive *F. tularensis* LVS $WbtI_{G191V}$ were used as controls.

A microtiter plate complement bactericidal assay was performed to determine serum sensitivity of strains in 25% or 50% complement active guinea pig serum as previously described, with some modifications (40). In brief, 10 µl of a diluted cell culture were added to a 96-well microtiter plate in triplicate. Either 0 µl, 12.5 µl, or 25 µl (0%, 25%, 50%, respectively) of complement-active guinea pig serum and an appropriate amount of PCM were added to each well to bring the volume to 50 µl. The plate was incubated at 37°C for 1 hour and then 150 µl of BHI-C was added to each well. At 24 hours, the absorbance of each well at 650 nm was determined. Growth in wells with 25% or 50% complement-active guinea pig serum were compared to the growth in wells with the 0% guinea pig serum to determine growth or no growth. Growth in the well was considered positive when the absorbance was at least two times that of the blank well. Wells with an absorbance of less than half of the 0% complement-active serum well for the same strain were considered to have partial growth. Wildtype *F. novicida* and *F. tularensis* LVS $WbtI_{G191V}$ were used as controls.

3.3.8. Statistical analysis

One-way ANOVA tests were used to determine statistical significance in biofilm formation, auto-aggregation, attachment, cell surface hydrophobicity, and survival in bactericidal complement serum. Tukey's *post-hoc* test or Dunney's multiple comparison test was used after completion of the one-way ANOVA to identify specific differences between results, if indicated. Statistical analyses were determined using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA).

3.4. Results

3.4.1. Different growth media altered the expression of the *F. novicida* biofilm

Extracellular surface properties can alter interactions with environmental surfaces and can contribute to biofilm formation (41). The extracellular surface components of *Francisella* have been shown to be altered by growth of the bacterium in different growth media (28, 29, 31, 33, 42). Previous reports have demonstrated that extracellular carbohydrate production, such as glycoses on the CLC or the O-antigen capsule, is enhanced in media such as BHI-C and CMDB (31, 33). *F. novicida* produces a CLC that containing glycosylated protein aggregates and is upregulated by grown in CDMB (29). Glycosylation may make the bacterial extracellular surface more hydrophobic and alter the biofilm formation of *F. novicida*. We were interested in

determining how biofilm formation by *F. novicida* grown in different media may reflect these bacterial surface changes. *F. novicida* was grown in four different broth media and tested for biofilm formation (Figure 3.1.a), attachment to microtiter plates (Figure 3.1.b), hydrophobicity (Figure 3.1.c), and auto-aggregation (Figure 3.1.d) to better elucidate the contributions of the growth medium to biofilm formation.

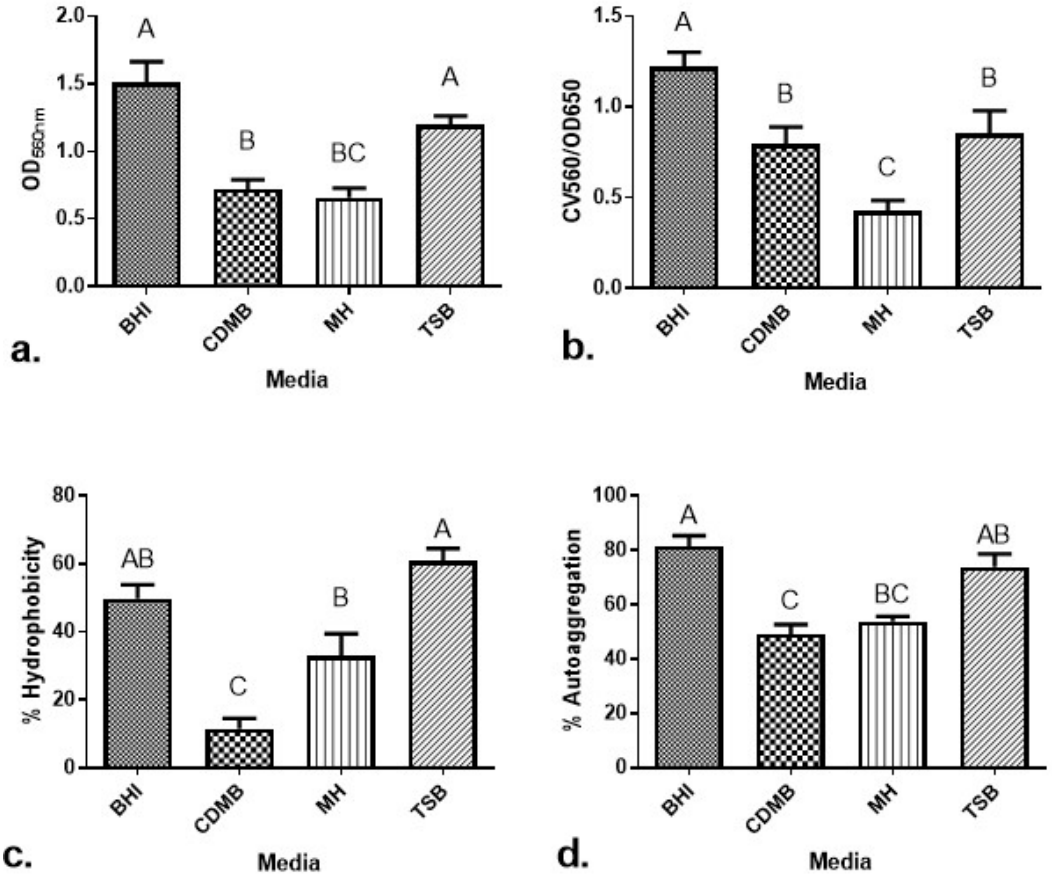

Figure 3.1. Effect of growth medium on parameters of biofilm formation.
Biofilm formation determined by CV assay (a), attachment to microtiter plates (b), hydrophobicity of the cell surface determined by partitioning of cells into hexadecane (c), and auto-aggregation (d) were determined for *F. novicida* grown in four different growth media. Each bar represents the mean of the replicates with error bars representing the standard error of the mean. Identical uppercase letters indicate that there is no significant difference between the means. The value of a bar marked with "A" is significantly different from the values of bars marked with "B" or "c." The values of bars marked "AB" are not statistically different from the values of bars marked with "A" or with "B," but are statistically different from a bar marked with "C."

Growth medium had a statistically significant effect on biofilm formation ($p < 0.0001$), attachment ($p < 0.0001$), hydrophobicity ($p < 0.0001$), and auto-aggregation ($p = 0.0006$). Figure

3.1 illustrates these differences (bars that are not statistically different have the same alphabetical label). In general, *F. novicida* grown in BHI-C created a more robust biofilm (Figure 3.1a), more cells attached to microtiter plates (Figure 3.1b), was more hydrophobic (Figure 3.1c), and auto-aggregated faster (Figure 3.1d) than *F. novicida* grown in either MH-C or CDMB. TSB-grown *F. novicida* only differed from BHI-C grown *F. novicida* in the amount of attachment to the microtiter plates (Figure 3.1d) and was otherwise similar in regards to the other parameters. *F. novicida* grown in CDMB or MH-C were similar in biofilm formation and auto-aggregation (Figure 3.1a, d). However, bacteria grown in CDMB were the least hydrophilic compared to bacteria grown in any of the other growth media (Figure 3.1c, d).

In general, bacteria grown in more complex media out performed bacteria grown in the defined nutrient medium in all parameters. However, biofilms from *F. novicida* grown in MH-C were comparable in size and texture to biofilms from *F. novicida* grown in defined medium. These results indicate a strong correlation between growth media and biofilm formation, or factors contributing to biofilm formation, for *F. novicida*.

3.4.2. Expression of the O-antigen influences biofilm formation by *F. novicida* in certain media

Growth in different media changes the expression of the LPS O-antigen for more virulent *F. tularensis* subspecies (33). *F. tularensis* LVS grown in host-adapted media such as BHI-C produced longer O-antigen chains compared to LVS grown in non-host-adapted media such as MH-C (33). Changes to the O-antigen chain length in different media has not been shown in *F. novicida*. *F. novicida* also contains an O-antigen that is immunogenically and structurally different from the O-antigen of more virulent subspecies (43). Regardless of these changes, the O-antigen is an important component of the extracellular bacterial surface and may influence the ability of the bacterium to produce biofilm. Biofilm production of transposon mutants with a single interruption in the O-antigen locus was compared to the wildtype (Figure 3.2). These comparisons were completed with the strains grown in BHI-C, CDMB, and MH-C due to the previously determined differences in biofilm formation, attachment, hydrophobicity, and auto-aggregation of the wildtype grown in these media.

The ability of an O-antigen mutant to produce biofilm compared to the wildtype was altered dramatically depending on the media used for growth. There was no statistical differences in the amount of biofilm formed compared to wildtype *F. novicida* when the O-antigen mutants were grown in MH-C (ANOVA $p = 0.1038$, Figure 3.2c). In contrast, the majority of the O-

antigen mutants produced significantly less biofilm than the wildtype when the bacteria were grown in CDMB (ANOVA $p < 0.0001$, Figure 3.2b). *F. novicida* transposon mutants WbtN (Dunnett's $p = 0.4280$) and WbtQ (Dunnett's $p = 0.1409$) were not statistically different in the amount of biofilm formed by the wildtype in CDMB according to the post-hoc comparison test (Figure 3.2a).

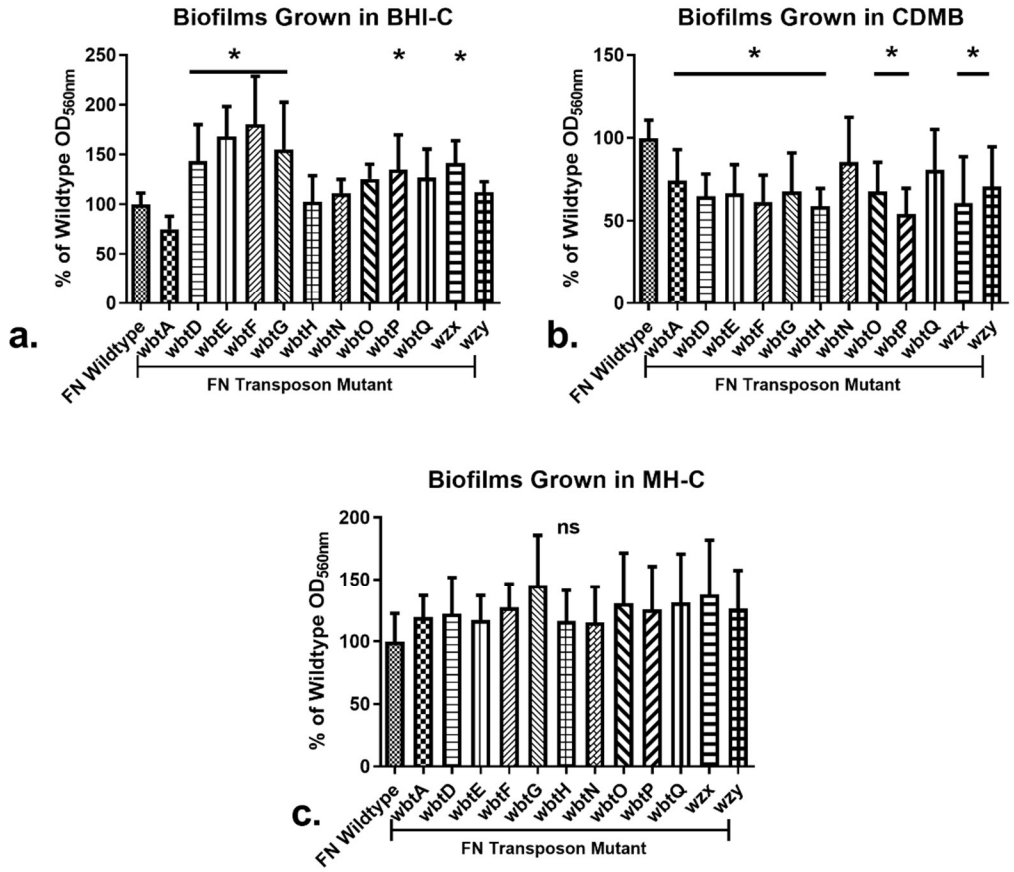

Figure 3.2. Biofilm formation by *F. novicida* O-antigen mutants compared to the parent strain when grown in different media.
Biofilm formation was assessed for transposon mutants containing disrupted genes associated with O-antigen production. Strains were grown in microtiter plates statically for 5 days, and then stained with CV to determine the amount of biofilm formed. Some O-antigen mutants produced significantly more biofilm than the wildtype in BHI-C (b). The O-antigen mutants, in general, produced significantly less biofilm than the wildtype when grown in CDMB (b). However, no significant difference was found when the mutants and wildtype were grown in MH-C (c). Biofilm formation results are represented as the percent of absorbance to the wildtype. Each bar represents the mean of all replicates and the error bars represent the standard error of the mean. Means were compared to the wildtype *F. novicida* with an "*" representing a $p < 0.05$ and signifying the amount of biofilm formed was statistically different from the wildtype.

F. novicida O-antigen mutants grown in BHI-C varied considerably in biofilm formation. However, some of the strains (WbtD, WbtE, WbtF, WbtG, WbtP, and Wzx) produced significantly more biofilm compared to the wildtype (ANOVA $p < 0.0001$, Figure 3.2a), as determined by Dunnett's multiple comparison post hoc test.

3.4.3. Loss of O-antigen expression did not correlate with increased serum sensitivity for *F. novicida*

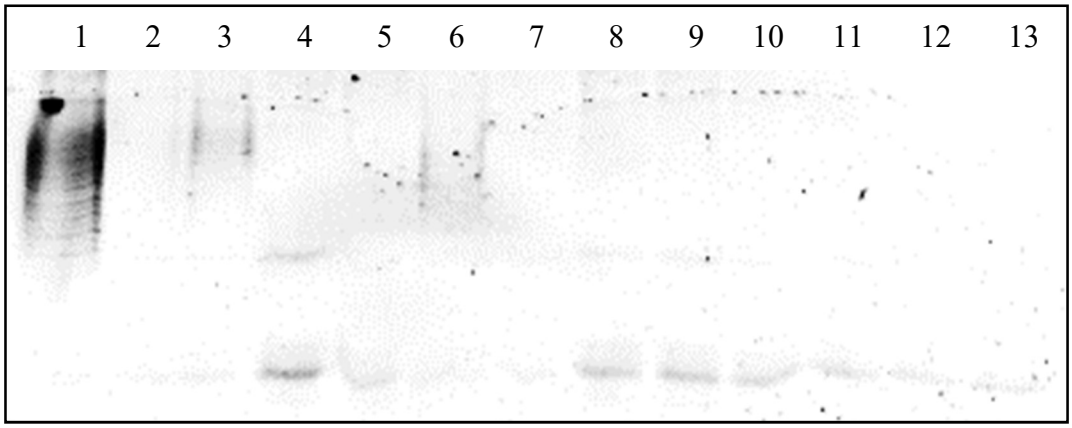

Figure 3.3. Electrophoretic profile of LPS extracts from strains of *F. novicida* with disruptions within the O-antigen locus.
Rapid phenol-water extractions of LPS from *F. novicida* strains resolved on a 4-12% bis-tris pre-cast gel before staining with Emerald 300Q glycoprotein stain. *F. novicida* wildtype (1st lane) clearly shows a characteristic ladder pattern of the O-antigen. All of the transposon mutants lack that ladder. Lanes and strain or mutated gene: 1, *F. novicida*; 2, *wbtA*; 3, *wbtD*; 4, *wbtE*; 5, *wbtF*; 6, *wbtG*; 7, *wbtH*; 8, *wbtN*; 9, *wbtO*; 10, *wbtP*; 11, *wbtQ*; 12, *wzx*; 13, *wzy*.

A select number of *F. novicida* O-antigen transposon mutants were assayed to determine if loss of the O-antigen led to increased serum sensitivity, which occurs when the more virulent *F. tularensis* subspecies loss O-antigen. The strains chosen included WbtA, WbtF, WbtH, and WbtN. None of these chosen strains exhibited increased serum sensitivity compared to wild-type *F. novicida* in 10% or 20% serum. In comparison, LVS $WbtI_{G191V}$ was significantly serum-sensitive and all cells were killed in less than an hour when incubated in 10% or greater complement-active guinea pig serum. These discrepancies in phenotype led us to question whether the interrupted genes actually affected the production of the O-antigen. Rapid extracts of LPS from each O-antigen mutant followed by electrophoretic analysis indicated that each *F. novicida* O-antigen transposon mutant lacked the characteristic O-antigen ladder (Figure 3.3).

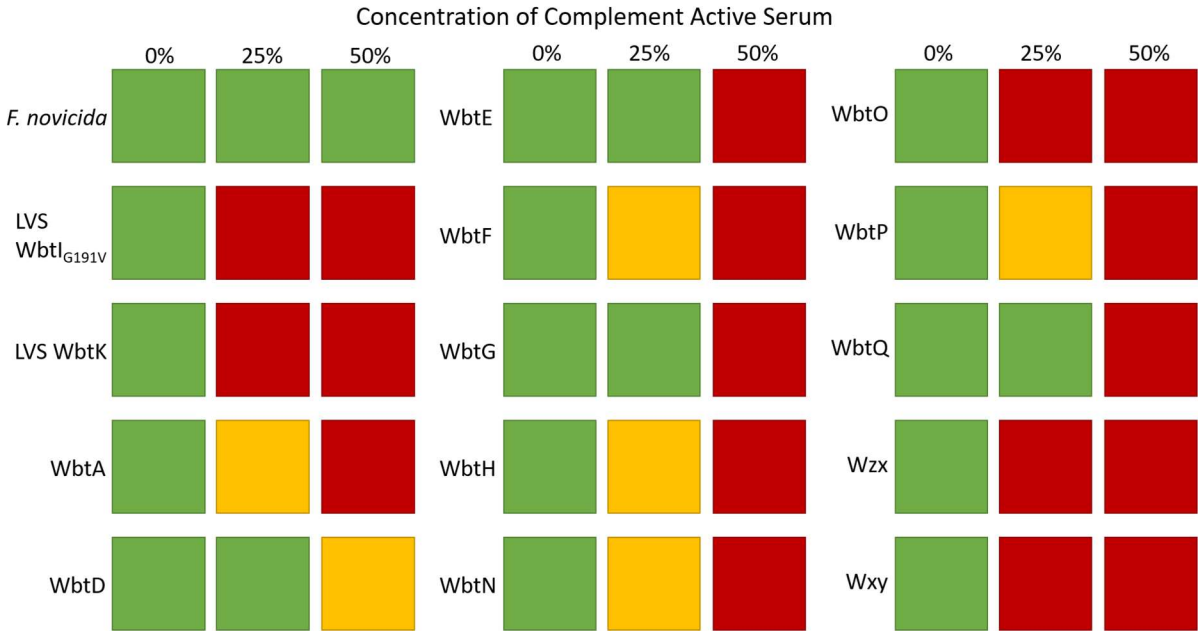

Figure 3.4. Ability of *F. novicida* O-antigen deficient strains to grow in different concentrations of complement-active serum.
Francisella strains or mutants were grown to mid-log phase, diluted, and added to a microtiter plate with 0 to 50% complement-active guinea pig serum in PCM to a final volume of 50 µl. The microtiter plate was incubated for one hour, 150 µl of BHI-C was added, and the plate was incubated overnight for growth. Absorbance of each well was determined and compared to blank media wells and the 0% serum concentration well for that strain. Absorbance twice the blank medium well was considered positive for growth. An absorbance less than 50% the absorbance of the 0% complement-active serum concentration well was considered to have only partial growth. Strains and the growth in different concentrations of complement active serum are displayed above with green signifying growth, yellow signifying partial growth, and red signifying no growth.

A follow-up microtiter plate assay was completed to qualitatively determine the effect of 25% and 50% complement-active serum on each of the *F. novicida* O-antigen mutants. This assay was able to determine growth, partial growth, or no growth of the strain under these conditions and was compared with serum-sensitive LVS $WbtI_{G191V}$ as a control. Previously assayed WbtA, WbtF, WbtH, and WbtN mutants that had 100% survival in up to 20% complement active serum, were able to partially grow in 25% complement-active serum, but did not survive in 50% complement-active serum. Similar results were obtained with the other *F. novicida* O-antigen transposon mutants; WbtO, wzx, and wxy appeared to be the most serum-sensitive. This qualitative screening test confirmed that *F. novicida* deficient in O-antigen may be more serum-resistant than Type A or B strains lacking O-antigen. However, loss of the O-antigen still resulted in serum sensitivity at higher concentrations of complement-active serum.

3.5. Discussion

Biofilm formation by *Francisella* species is an emerging area of study for the ecology and pathogenesis of *Francisella*. More of the *Francisella* subspecies that sare associated with the environment, such as *F. philomergia* and *F. novicida*, have been shown to create biofilms in various conditions (17-19, 21, 25, 26). The more virulent *Francisella tularensis* Type A and Type B strains have also been shown to create a biofilm, and do so under such conditions that may be relevant to the ecology of bacterium (20). Prior studies have sought to further the understanding and the importance of the *Francisella* biofilm. Though these studies have improved the understanding of how certain gene products, such as chitnases (25, 26), cyclic di-GMP machinery (19), and a response regulator (18) affect biofilm formation, one important factor has been overlooked: growth media.

F. tularensis has been shown to alter the expression of gene products, such as outer membrane proteins and carbohydrates, based on the selection of growth media. *F. tularensis* grown in BHI-C most closely resembles bacteria grown extracellularly in the presence of macrophages, leading to the theory that BHI-C-grown *F. tularensis* are host-adapted (33, 35). In comparison, *F. tularensis* grown in MH-C or CDMB appear to have a dramatically altered proteome compared to host-adapted bacteria (34). Alterations in outer membrane carbohydrate components have also been demonstrated following sequential passage in CDMB, leading to expression of the CLC in both Type A, Type B, and *F. novicida* strains (28, 29, 31). More high molecular weight surface carbohydrate and longer O-antigen capsular polymers are present on bacteria grown as host-adapted bacteria in BHI-C than grown as non-host adapted in MH-C (33). The differential expression of surface components by planktonic *Francisella* grown in various media elicits the question of whether *Francisella* grown as a biofilm in various media will exhibit differential expression of biofilm formation.

Four types of media frequently used for the cultivation of *Francisella* species were used and different aspects of biofilm formation were assessed for *F. novicida* grown in these media. *F. novicida* grown in either BHI-C or TSB-C produced the most biofilm, as determined by crystal violet staining, attached and auto-aggregated at a greater efficiency, and were the most hydrophobic. Hazlett *et al*. (35) has shown that *F. tularensis* grown in BHI or TSB have similarly expressed proteomes that differ from bacteria grown in CDMB or MH-C. This

similarity in proteomes may contribute to the similarities seen here with biofilm formation between BHI-C and TSB-C grown *F. novicida.* The differences as compared to CDMB or MH-C illustrate the concept that the same strain of *F. novicida* alters components, such as surface proteins or carbohydrates, when grown in various media.

Growth in BHI broth stimulates upregulation of MglA, a homologue to a starvation response regulator in *E. coli* (35) and lack of environmental nutrients or starvation can be cues to bacteria to start forming a biofilm. Hazlett *et al*. (35) showed that growth of *F. tularensis* in BHI-C leads to a host-adapted phenotype that is both MglA-dependent and MglA-independent. The cells produce longer polymers of O-antigen, more O-antigen capsule carbohydrate, and a high molecular weight carbohydrate greater than 250 kDa in size. MglA has also been shown to coprecipitate with PmrA, an orphan response regulator that is homologous to the quorum sensing regulatory protein QseB in *E. coli* (44, 45). Mutation of the PmrA gene in *F. novicida* reduces the amount of biofilm formed when grown in TSB-C broth (18). The upregulation of MglA in BHI-C and TSB-C broth may lead to the upregulation of PmrA and subsequently an increase in the formation of biofilm when *F. novicida* is grown in these media. Growth in both BHI-C and TSB-C led to an increase in the amount of biofilm formed and other biofilm parameters, which lends support to this hypothesis. Conversely, media such as MH-C that reduces expression of MglA (33, 35), resulted in less biofilm formed compared to BHI-C and TSB-C. Further assays in gene and protein regulation would be necessary to confirm this theory.

Another surface component, the CLC, has been shown to be differentially regulated by growth in and on different media. Champion *et al*. (31) recently confirmed that CLC can be recovered from cells grown on BHI-C agar and CDMA, but not on cells grown on MH-C agar. Both CDMA and BHI-C agar favor production and cell association of the CLC (31). *F. novicida* produces a similar, but not identical, CLC to LVS when grown on CDMA, and planktonic growth in broth results in no cell-associated CLC (29). Alterations in surface antigen production, such as CLC or the O-antigen, may expose or obscure surface antigens that accelerate attachment and auto-aggregation in the aqueous environment. Determining the contribution of each of these surface antigens to biofilm formation is difficult since multiple other components may be unidentified or alter biofilm appearance based on the growth media. For example, increased expression of the CLC in *F. novicida* negatively impacts biofilm production in CDMB (data not shown). However, the CLC glycosylation mutant of *F. novicida* (*F. novicida*Δ1212-1218) did

not have an altered expression of biofilm compared to the wildtype when grown in CDMB (data not shown, (46). The CLC is not a monomorphic capsule, but instead an aggregate of secreted proteins, both glycosylated and non-glycosylated, and possibly outer membrane vesicles and tubes (OMV/T) (28, 29, 31). These data may indicate that glycosylation of the CLC does not significantly contribute to the *F. novicida* biofilm because the glycosylation mutant is not altered in the amount of biofilm formed. In contrast, the CLC proteins, OMV/T expression, or cell-association of the CLC may play a larger role in biofilm formation by *F. novicida*. However, this mutant and the CDMB-passed *F. novicida* strain were not tested in all of the media described in this study. In this study, *F. novicida* grown in both CDMB (enhances CLC production) and MH-C (reduces CLC production) produced a similar amount of biofilm material (Figure 3.1a), but differed in regards to attachment and hydrophobicity. These findings suggest that CLC production may not play a role in biofilm bulk, as assayed by crystal violet staining, but may play a role in the ability of *F. novicida* to initiate biofilm formation.

Another factor contributing to the differential production of biofilm when *F. novicida* was grown in various media is alteration of the O-antigen of *F. novicida*. The *F. tularensis* O-antigen has long been shown to play a key role in pathogenesis and virulence as part of the LPS and part of the O-antigen capsule (27, 33, 47, 48). However, an O-antigen capsule surrounding *F. novicida* has not been reported. The initial discovery of the O-antigen capsule was made possible using monoclonal antibody 11B7 that binds specifically to the *F. tularensis* O-antigen capsule polymer and not to the *F. tularensis* LPS O-antigen (27). There are significant differences between the structure of the *F. novicida* and *F. tularensis* O-antigen; whereas all Type A and Type B *F. tularensis* strains share an identical O-antigen repeating tetrasaccharide (43, 49). Therefore, a monoclonal antibody specific for the *F. tularensis* O-antigen capsule may not bind a possible *F. novicida* O-antigen capsule due to differences in the O-antigen composition between these subspecies. As evidence, the *F. tularensis* LPS specific monoclonal antibody FB11 that binds to the terminal end of the LPS O-antigen (50, 51) will bind the *F. tularensis* O-antigen capsule, but does not cross-react with *F. novicida* whole cells (27). This O-antigen capsule, as previously mentioned, is produced in higher quantities by bacteria grown in BHI-C compared to bacteria grown in MH-C.

Due to the significance of the O-antigen and the possibility of an *F. novicida* O-antigen capsule, *F. novicida* transposon mutants with insertions in the O-antigen locus were screened for

biofilm production following growth in BHI-C, CDMB, and MH-C. Similar to other *F. tularensis* strains lacking O-antigen (48, 52), *F. novicida* O-antigen deficient strains were more serum sensitive than the wildtype counterpart. However, serum sensitivity was not observed until the concentration of complement-active serum was greater than 20% and thus was not observed in the quantitative assay. Serum-sensitive *F. tularensis* strains appear to be more sensitive to complement-active serum compared to *F. novicida*.

All of the O-antigen deficient strains, when grown in MH-C broth under biofilm-forming conditions, were not statistically different from the wildtype in biofilm formation. MH-C is a medium high in casamino acids and free from iron, and is not considered to mimic the intra- or extracellular environment of the host (35). *F. tularensis* strains grown in MH-C produce a shorter O-antigen chain compared to bacteria grown in BHI-C and produce less O-antigen capsular carbohydrate through pathways that are dependent and independent of the regulatory protein MglA (33). The reduction in O-antigen chains and O-antigen capsule may account for the increase in the ability of antibodies to bind to *Francisella* surface proteins (33). Though this phenotypic variation has not been confirmed in *F. novicida*. *F. novicida* contains the same MglA regulatory protein as the more virulent *F. tularensis* subspecies, and may exhibit a similar phenotype as these subspecies when grown in MH-C. Therefore, growth of the bacteria in MH-C may lead to a blunted O-antigen polymer and a decrease in any possible O-antigen capsule, leading to a more exposed bacterial surface. A strain deficient in O-antigen production, that presumably has more bacterial surface exposed may mimic the phenotype of the wildtype with a shorter O-antigen polymer. Growth on MH-C has also been shown to decrease expression of a high molecular weight carbohydrate (33) and results in an undetectable production of CLC for *F. tularensis* subspecies (31), reducing the possibility that these surface antigens contributed to the effects seen in the CV biofilm assay.

Conversely, strains deficient in O-antigen had statistically altered biofilm formation compared to the wildtype when grown in BHI-C or in CDMB. Approximately half of the O-antigen deficient strains produced statistically more biofilm when grown in BHI-C compared to the wildtype; and the majority of the O-antigen deficient strains produced statistically less biofilm when grown in CDMB compared to the wildtype *F. novicida*. The loss of the O-antigen on BHI-C grown *F. novicida* may represent increased exposure to surface antigens responsible for biofilm formation steps such as attachment causing an acceleration in biofilm formation and

the ability to produce more mass compared to the wildtype at a similar timeline. The loss of the O-antigen on CDMB-grown *F. novicida* could reduce biofilm production in a medium that already diminishes biofilm mass compared to other media. This change in a surface antigen may further impair the ability of *F. novicida* to produce biofilms in this medium.

Overall, the role of growth medium in biofilm production should not be overlooked. Of the *Francisella* papers published examining biofilm, media used for biofilm cultivation include: TSB-C (17, 18, 21, 26), MH-C (25, 53), CDMB (19, 25), peptone-yeast extract-glucose (PYG) (21), and BHI-C (in this study). Statistical differences in biofilm formation by *F. novicida* when grown in different media reflect the importance of medium choice on biofilm assays. Which medium represents the most likely environment for *Francisella* biofilm formation is unknown. Certain media, such as BHI-C, have been labeled as inducing a host-adapted phenotype from *F. tularensis* and exhibit a starvation response to the low amino acid and low iron environment of the medium. Determining the location of a *Francisella* biofilm is critical in determining the most appropriate *in vitro* medium. Until such a location can be confirmed, the effect of medium should not be overlooked when assaying different strains for biofilm proficiency. As shown here, the same strain with an interruption in a single gene, such as *wbtF*, has a different biofilm phenotype that is medium dependent. Further investigation is warranted in determining the reason specific media affect the formation of biofilm in *Francisella* species.

3.6. References

1. Santic M, Al-Khodor S, Abu Kwaik Y. 2010. Cell biology and molecular ecology of *Francisella tularensis*. Cell Microbiol 12:129-39.
2. Keim P, Johansson A, Wagner DM. 2007. Molecular epidemiology, evolution, and ecology of *Francisella*. Ann N Y Acad Sci 1105:30-66.
3. Baldwin CJ, Panciera RJ, Morton RJ, Cowell AK, Waurzyniak BJ. 1991. Acute tularemia in three domestic cats. J Am Vet Med Assoc 199:1602-5.
4. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Layton M, Lillibridge SR, McDade JE, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Tonat K, Working Group on Civilian B. 2001. Tularemia as a biological weapon: medical and public health management. JAMA 285:2763-73.
5. Matyas BT, Nieder HS, Telford SR, 3rd. 2007. Pneumonic tularemia on Martha's Vineyard: clinical, epidemiologic, and ecological characteristics. Ann N Y Acad Sci 1105:351-77.

6. Feldman KA, Enscore RE, Lathrop SL, Matyas BT, McGuill M, Schriefer ME, Stiles-Enos D, Dennis DT, Petersen LR, Hayes EB. 2001. An outbreak of primary pneumonic tularemia on Martha's Vineyard. N Engl J Med 345:1601-6.
7. Feldman KA, Stiles-Enos D, Julian K, Matyas BT, Telford SR, 3rd, Chu MC, Petersen LR, Hayes EB. 2003. Tularemia on Martha's Vineyard: seroprevalence and occupational risk. Emerg Infect Dis 9:350-4.
8. Centers for Disease C, Prevention. 2005. Tularemia transmitted by insect bites--Wyoming, 2001-2003. MMWR Morb Mortal Wkly Rep 54:170-3.
9. Petersen JM, Mead PS, Schriefer ME. 2009. *Francisella tularensis*: an arthropod-borne pathogen. Vet Res 40:7.
10. Broman T, Thelaus J, Andersson AC, Backman S, Wikstrom P, Larsson E, Granberg M, Karlsson L, Back E, Eliasson H, Mattsson R, Sjostedt A, Forsman M. 2011. Molecular Detection of Persistent Francisella tularensis Subspecies holarctica in Natural Waters. Int J Microbiol 2011.
11. Faber M, Heuner K, Jacob D, Grunow R. 2018. Tularemia in Germany-A Re-emerging Zoonosis. Front Cell Infect Microbiol 8:40.
12. Zargar A, Maurin M, Mostafavi E. 2015. Tularemia, a re-emerging infectious disease in Iran and neighboring countrie. Epidemiol Health 37:e2015011.
13. Centers for Disease C, Prevention DoH, Human S. 2017. Possession, Use, and Transfer of Select Agents and Toxins; Biennial Review of the List of Select Agents and Toxins and Enhanced Biosafety Requirements. Final rule. Fed Regist 82:6278-94.
14. Clemens DL, Lee BY, Horwitz MA. 2005. *Francisella tularensis* enters macrophages via a novel process involving pseudopod loops. Infect Immun 73:5892-902.
15. Forestal CA, Malik M, Catlett SV, Savitt AG, Benach JL, Sellati TJ, Furie MB. 2007. Francisella tularensis has a significant extracellular phase in infected mice. J Infect Dis 196:134-7.
16. Anda P, Segura del Pozo J, Diaz Garcia JM, Escudero R, Garcia Pena FJ, Lopez Velasco MC, Sellek RE, Jimenez Chillaron MR, Sanchez Serrano LP, Martinez Navarro JF. 2001. Waterborne outbreak of tularemia associated with crayfish fishing. Emerg Infect Dis 7:575-82.
17. van Hoek ML. 2013. Biofilms: an advancement in our understanding of *Francisella* species. Virulence 4:833-46.
18. Durham-Colleran MW, Verhoeven AB, van Hoek ML. 2010. Francisella novicida forms in vitro biofilms mediated by an orphan response regulator. Microb Ecol 59:457-65.
19. Zogaj X, Wyatt GC, Klose KE. 2012. Cyclic di-GMP stimulates biofilm formation and inhibits virulence of *Francisella novicida*. Infect Immun 80:4239-47.
20. Mahajan UV, Gravgaard J, Turnbull M, Jacobs DB, McNealy TL. 2011. Larval exposure to Francisella tularensis LVS affects fitness of the mosquito Culex quinquefasciatus. FEMS Microbiol Ecol 78:520-30.
21. Verhoeven AB, Durham-Colleran MW, Pierson T, Boswell WT, Van Hoek ML. 2010. *Francisella philomiragia* biofilm formation and interaction with the aquatic protist *Acanthamoeba castellanii*. Biol Bull 219:178-88.
22. Kingry LC, Petersen JM. 2014. Comparative review of *Francisella tularensis* and *Francisella novicida*. Front Cell Infect Microbiol 4:35.

23. Brett M, Doppalapudi A, Respicio-Kingry LB, Myers D, Husband B, Pollard K, Mead P, Petersen JM, Whitener CJ. 2012. Francisella novicida bacteremia after a near-drowning accident. J Clin Microbiol 50:2826-9.
24. Brett ME, Respicio-Kingry LB, Yendell S, Ratard R, Hand J, Balsamo G, Scott-Waldron C, O'Neal C, Kidwell D, Yockey B, Singh P, Carpenter J, Hill V, Petersen JM, Mead P. 2014. Outbreak of Francisella novicida bacteremia among inmates at a louisiana correctional facility. Clin Infect Dis 59:826-33.
25. Margolis JJ, El-Etr S, Joubert LM, Moore E, Robison R, Rasley A, Spormann AM, Monack DM. 2010. Contributions of *Francisella tularensis* subsp. *novicida* chitinases and Sec secretion system to biofilm formation on chitin. Appl Environ Microbiol 76:596-608.
26. Chung MC, Dean S, Marakasova ES, Nwabueze AO, van Hoek ML. 2014. Chitinases are negative regulators of Francisella novicida biofilms. PLoS One 9:e93119.
27. Apicella MA, Post DM, Fowler AC, Jones BD, Rasmussen JA, Hunt JR, Imagawa S, Choudhury B, Inzana TJ, Maier TM, Frank DW, Zahrt TC, Chaloner K, Jennings MP, McLendon MK, Gibson BW. 2010. Identification, characterization and immunogenicity of an O-antigen capsular polysaccharide of *Francisella tularensis*. PLoS One 5:e11060.
28. Bandara AB, Champion AE, Wang X, Berg G, Apicella MA, McLendon M, Azadi P, Snyder DS, Inzana TJ. 2011. Isolation and mutagenesis of a capsule-like complex (CLC) from *Francisella tularensis*, and contribution of the CLC to *F. tularensis* virulence in mice. PLoS One 6:e19003.
29. Freudenberger Catanzaro KC, Champion AE, Mohapatra N, Cecere T, Inzana TJ. 2017. Glycosylation of a Capsule-Like Complex (CLC) by Francisella novicida Is Required for Virulence and Partial Protective Immunity in Mice. Front Microbiol 8:935.
30. Sampath V, McCaig WD, Thanassi DG. 2018. Amino acid deprivation and central carbon metabolism regulate the production of outer membrane vesicles and tubes by *Francisella*. Mol Microbiol 107:523-541.
31. Champion A, Mohapatra N, Bandara A, Twine S, Inzana TJ. 2015. The Capsule-Like Complex (CLC) of Type A *Francisella tularensis* may be Derived from Outer Membrane Vesicles, but Glycosylation of the CLC is not Required for Virulence and is not Protective., abstr 115th General Meeting of the American Society for Microbiology, New Orleans, LA., May 30-June 2, 2015,. American Society for Microbiology,
32. McCaig WD, Koller A, Thanassi DG. 2013. Production of outer membrane vesicles and outer membrane tubes by *Francisella novicida*. J Bacteriol 195:1120-32.
33. Zarrella TM, Singh A, Bitsaktsis C, Rahman T, Sahay B, Feustel PJ, Gosselin EJ, Sellati TJ, Hazlett KR. 2011. Host-adaptation of *Francisella tularensis* alters the bacterium's surface-carbohydrates to hinder effectors of innate and adaptive immunity. PLoS One 6:e22335.
34. Twine SM, Mykytczuk NC, Petit MD, Shen H, Sjostedt A, Wayne Conlan J, Kelly JF. 2006. In vivo proteomic analysis of the intracellular bacterial pathogen, *Francisella tularensis*, isolated from mouse spleen. Biochem Biophys Res Commun 345:1621-33.
35. Hazlett KR, Caldon SD, McArthur DG, Cirillo KA, Kirimanjeswara GS, Magguilli ML, Malik M, Shah A, Broderick S, Golovliov I, Metzger DW, Rajan K, Sellati TJ, Loegering DJ. 2008. Adaptation of Francisella tularensis to the mammalian environment is governed by cues which can be mimicked in vitro. Infect Immun 76:4479-88.

36. Gallagher LA, Ramage E, Jacobs MA, Kaul R, Brittnacher M, Manoil C. 2007. A comprehensive transposon mutant library of Francisella novicida, a bioweapon surrogate. Proc Natl Acad Sci U S A 104:1009-14.
37. Chamberlain RE. 1965. Evaluation of Live Tularemia Vaccine Prepared in a Chemically Defined Medium. Appl Microbiol 13:232-5.
38. Rosenberg M. 2017. Adhesion to hydrocarbons and microbial hydrophobicity-do the MATH. FEMS Microbiol Lett 364.
39. Inzana TJ. 1983. Electrophoretic heterogeneity and interstrain variation of the lipopolysaccharide of Haemophilus influenzae. J Infect Dis 148:492-9.
40. Jang MS, Sahastrabuddhe S, Yun CH, Han SH, Yang JS. 2016. Serum bactericidal assay for the evaluation of typhoid vaccine using a semi-automated colony-counting method. Microb Pathog 97:19-26.
41. Dunne WM, Jr. 2002. Bacterial adhesion: seen any good biofilms lately? Clin Microbiol Rev 15:155-66.
42. Cherwonogrodzky JW, Knodel MH, Spence MR. 1994. Increased encapsulation and virulence of *Francisella tularensis* live vaccine strain (LVS) by subculturing on synthetic medium. Vaccine 12:773-5.
43. Vinogradov E, Conlan WJ, Gunn JS, Perry MB. 2004. Characterization of the lipopolysaccharide O-antigen of Francisella novicida (U112). Carbohydr Res 339:649-54.
44. Bell BL, Mohapatra NP, Gunn JS. 2010. Regulation of virulence gene transcripts by the Francisella novicida orphan response regulator PmrA: role of phosphorylation and evidence of MglA/SspA interaction. Infect Immun 78:2189-98.
45. Mohapatra NP, Soni S, Bell BL, Warren R, Ernst RK, Muszynski A, Carlson RW, Gunn JS. 2007. Identification of an orphan response regulator required for the virulence of *Francisella* spp. and transcription of pathogenicity island genes. Infect Immun 75:3305-14.
46. Inzana TJ, Champion AE, Freudenberger Catanzaro KC. Loss of O-antigen and deglycosylation of the capsule-like complex promote biofilm formation in *Francisella tularensis*, p. *In* (ed),
47. Barker JH, Kaufman JW, Apicella MA, Weiss JP. 2016. Evidence Suggesting That Francisella tularensis O-Antigen Capsule Contains a Lipid A-Like Molecule That Is Structurally Distinct from the More Abundant Free Lipid A. PLoS One 11:e0157842.
48. Rasmussen JA, Fletcher JR, Long ME, Allen LA, Jones BD. 2015. Characterization of *Francisella tularensis* Schu S4 mutants identified from a transposon library screened for O-antigen and capsule deficiencies. Front Microbiol 6:338.
49. Vinogradov E, Perry MB, Conlan JW. 2002. Structural analysis of *Francisella tularensis* lipopolysaccharide. Eur J Biochem 269:6112-8.
50. Lu Z, Rynkiewicz MJ, Yang CY, Madico G, Perkins HM, Wang Q, Costello CE, Zaia J, Seaton BA, Sharon J. 2013. The binding sites of monoclonal antibodies to the non-reducing end of *Francisella tularensis* O-antigen accommodate mainly the terminal saccharide. Immunology 140:374-89.
51. Roche MI, Lu Z, Hui JH, Sharon J. 2011. Characterization of monoclonal antibodies to terminal and internal O-antigen epitopes of *Francisella tularensis* lipopolysaccharide. Hybridoma (Larchmt) 30:19-28.

52. Li J, Ryder C, Mandal M, Ahmed F, Azadi P, Snyder DS, Pechous RD, Zahrt T, Inzana TJ. 2007. Attenuation and protective efficacy of an O-antigen-deficient mutant of *Francisella tularensis* LVS. Microbiology 153:3141-53.
53. Soto E, Halliday-Simmonds I, Francis S, Kearney MT, Hansen JD. 2015. Biofilm formation of *Francisella noatunensis* subsp. *orientalis*. Vet Microbiol 181:313-7.

Chapter 4: Alginate Microencapsulation of an Attenuated O-Antigen Mutant of *Francisella tularensis* LVS as a Model for a Vaccine Delivery Vehicle

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4.1. Abstract

Francisella tularensis is the etiologic agent of tularemia and a Tier I Select Agent. Subspecies *tularensis* (Type A) is the most virulent of the four subspecies and inhalation of as few as 10 cells can cause severe disease in humans. Due to its niche as a facultative intracellular pathogen, a successful tularemia vaccine must induce a robust cellular immune response, which is best achieved by a live, attenuated strain. *F. tularensis* strains lacking the lipopolysaccharide (LPS) O-antigen are highly attenuated, but do not persist in the host long enough to induce protective immunity. Increasing the persistence of an O-antigen mutant may help stimulate the appropriate and protective immune response. Alginate encapsulation is frequently used with probiotics to increase persistence of the cells within the gastrointestinal system. We used alginate to encapsulate the LVS O-antigen mutant WbtIG191V. Encapsulation with alginate followed by a Poly-L-Lysine/Alginate coating increased the survival of WbtIG191V in complement-active serum. In addition, BALB/c mice immunized intraperitoneally (IP) with WbtIG191V and purified lipopolysaccharide (LPS) in Alginate/Poly-L-Lysine/Alginate microcapsules and challenged intranasally with LVS had a higher percent survival than mock immunized mice. However, the percent survival was similar to mice immunized IP with freely suspended WbtIG191V and purified LPS. Alginate encapsulation did increase antibody titers compared to the similar non-encapsulated suspension. These data suggest that alginate encapsulation does provide a slow-release vehicle for bacterial deposits as evidenced by the increased antibody titer and increased persistence in serum compared to freely suspended cells. Though the alginate vehicle did increase persistence, this increased persistence did not increase the efficacy of the immune response to *F. tularensis* when compared to the immune response induced by a similar formulation freely suspended in solution. An analysis of the immune response in mice that survived revealed that mice vaccinated with the alginate beads upregulated cell-mediated immune pathways to a lesser extent than LVS-vaccinated mice. In general, this vehicle may be more effective for pathogens that require antibody-mediated immune responses alone.

4.2. Introduction

Francisella tularensis is a gram negative, coccobacillus that causes the zoonotic disease tularemia. *F. tularensis* species are considered Tier I Select Agents due to the potential use of this bacterium as a possible bioterrorist weapon (1). This classification is due to the degree of virulence, low infectious dose, and ease of aerosol dispersal of the bacterium (1, 2). No current vaccine to prevent tularemia exists as the designated Live Vaccine Strain (LVS) is no longer licensed (3). This strain was developed from serial passages of a wildtype *F. tularensis* subspecies *holarctica* (Type B) strain in the mid-20th century and is attenuated compared to wild-type strains (4). However, LVS is still virulent in immunocompromised populations, is genetically unstable, and has questionable protective efficacy especially in regard to respiratory challenge (5-7). These concerns have regulated LVS to the status of laboratory model strain for *F. tularensis* and the standard of comparison for potential tularemia vaccines. Any possible new tularemia vaccine candidate must be more efficacious than LVS, especially against respiratory challenges, and must be safer in immunocompromised individuals (3).

Deletion of the lipopolysaccharide (LPS) O-antigen attenuates *F. tularensis* substantially (8-14). O-antigen mutants are serum sensitive compared to the wildtype and do not persist long in the host (10, 12, 14, 15). A genetically stable strain lacking the O-antigen would most likely be unable to revert to a virulent state *in vivo* and would be a safe vaccine candidate for immunocompromised individuals. However, attenuated O-antigen-lacking strains are inadequately protective against virulent challenges. Li et al. (14) created the O-antigen mutant $WbtI_{G191V}$, a point mutant and grey variant of the parent strain LVS. The point mutation is found in the gene *wbtI* that encodes a sugar transferase protein WbtI that is involved in elongation of the polysaccharide O-antigen portion of the LPS. $WbtI_{G191V}$ is sensitive to killing in 10% fresh serum in under 60 min., and unable to persistent in the host to establish an active infection (14). When used to immunize mice $WbtI_{G191V}$ is protective against low-dose intraperitoneal challenge, but ineffective against a high dose challenge (14) . Twine et al. (16) found similar results of partial and route dependent protection with LVS mutants lacking WbtC or kdtA. In attenuating LVS, the loss of the O-antigen may also hinder the ability of the host to develop a fully protective immune response against *F. tularensis* infection due to a lack of persistence of the bacteria *in vivo* and loss of the highly immunostimulating O-antigen.

Strategies exist to improve the efficacy of partially protective vaccine candidates. A common strategy are booster vaccines. Protection of $WbtI_{G191V}$ against a wild-type challenge may be greater when the immunization is boostered (14) compared to a single dose immunization (16). However, the booster immunization still only provides incomplete protection indicating that a more novel approach may be necessary. Microencapsulation of live bacteria with alginate was used for *Brucella* species (17-19) to increase host immuno-protection to highly attenuated strains. Attenuated *Brucella melitensis* in alginate microspheres induced a cell-mediated and antibody-mediated immune response greater than freely suspended cells, which was effective at reducing the bacterial burden in mouse tissues (17).

Alginate is a carbohydrate polymer that crosslinks when introduced to a solution of calcium forming a stable bead. Mixing antigens with alginate results in trapping the antigen within the polymerized alginate upon introduction of calcium. This technique is commonly used to protect probiotics for passage through acidic environments of the gastrointestinal track (20). *Lactobacillus* spp. encapsulated in alginate can survive in simulated gastric juices for extended periods of time compared to non-encapsulated *Lactobacillus* cells (21-23). In theory, the alginate barrier isolates cells within the microcapsule from the surrounding environment, increasing persistence. This same theory could be applied to the host immune system. Used as a vaccine vehicle, the alginate would prevent the immediate clearance of vaccine particles, allowing the vaccine to be slowly released into that environment.

Applying this principle to the attenuated strain $WbtI_{G191V}$, we predicted that alginate microencapsulation would increase the persistence of $WbtI_{G191V}$ *in vivo*. This increased persistence should act like a continuous booster and help stimulate an appropriate and protective immune response to protect the host against a virulent, intranasal challenge.

4.3. Methods and Materials

4.3.1. Bacterial strains and growth conditions.

The bacterial strains used in this study include the attenuated O-antigen mutant *F. tularensis* LVS $WbtI_{G191V}$ (14), the mouse-virulent parent strain *F. tularensis* LVS, and a strain of *F. tularensis* LVS expressing green fluorescent protein (GFP). *Francisella* strains were grown on chocolate brain heart infusion agar (BD) containing 0.1% cysteine (CBHI-C) at 37˚C with 5% CO_2. Broth cultures of *Francisella* strains were grown in brain heart infusion broth containing

0.1% cysteine (BHI-C) with shaking (175-200 rpm) at 37˚C. The GFP-expressing LVS strain required the addition of 10 µg/ml of kanamycin in growth medium.

4.3.2. Encapsulation of *Francisella* strains in alginate beads.

A 48-hour culture of *F. tularensis* was scraped off CBHI-C agar plates and suspended in morpholinepropanesulfonic acid buffer (MOPS: 10mM MOPS, 0.85% NaCl, pH 7.4). The bacteria were pelleted by centrifugation at 10,000x*g*, the supernatant was removed, and fresh MOPS was added to resuspend the pellet. This washing step was then repeated once. The final volume was adjusted appropriately to produce a suspension of $1x10^9$ CFU/ml determined spectrophotometrically using a Klett meter. This suspension was then diluted appropriately into 1.8% isotonic sodium alginate (Buchi, Swizterland) to obtain a final concentration between $1x10^6$ and $1x10^8$ CFU/ml in 1.2% alginate for nozzle sizes of 80-120 um or 1.5% alginate for nozzle sizes 150-200 um. Purified *F. tularensis* LVS LPS was included at a concentration of 100 ug/ml in the alginate-bacterial suspension of specific bead preparations. Alginate beads contained *F. tularensis* with or without LPS were produced as previously described with modification (17). Briefly, the alginate-bacterial suspension was loaded into a syringe and connected to the Buchi Encapsulator B-395 Pro (Buchi, Swizterland). See Table 4.1 for the encapsulation parameters for the various nozzle sizes. The encapsulator extruded the suspension through the attached nozzle into sterile calcium chloride buffer ($CaCl_2$: 100mM $CaCl_2$, 10mM MOPS pH 7.4) and slowly stirred for 15 to 30 minutes. $CaCl_2$ buffer was removed and only a small amount of liquid remained to prevent the beads from sticking. The beads were then washed twice with MOPS buffer for 5 minutes each. If necessary, a portion of the beads were then removed, diluted, and cultured to determine bacterial numbers, and for use in survival and animal studies. Beads removed at this point were designated as alginate beads.

Nozzle Size (um)	**Final Alginate Concentration (%)**	**Flow Rate (ml/min)**	**Frequency (Hertz)**	**Electrode (Volts)**
80	1.2	1.20	2700	450
120	1.2	1.40	2500	500
150	1.5	2.50	1500	750
200	1.5	4.00	1200	1100

Table 4.1. Parameters for production of alginate beads containing $1x10^8$ or fewer CFU/ml of *F. tularensis* bacteria.

Beads were then incubated with slow stirring in poly-L-lysine solution (PLL: 0.05% PLL molecular weight 30-70kDa (Sigma P2636) in $CaCl_2$ buffer, filter sterilized) for 15 minutes to create the coating layer around the alginate bead. Following incubation, beads were washed twice in MOPS buffer for 5 minutes each. A 0.03% solution of sodium alginate was added and the beads were incubated with slow stirring for 5 minutes to create the final shell. Completed beads were washed in MOPS buffer twice before use. These final beads were designated as Alginate-PLL-Alginate (APA) beads. Bead formulations can be found in Table 2.

Bead Designation	**Bead Core**	**Bead Coating**	**Purpose**
Alginate LVS GFP Beads	*F. tularensis* LVS GFP strain, $1x10^8$ CFU/ml	None	Microscopy
Alginate WbtI Beads	*F. tularensis* LVS WbtI strain	None	Microscopy, Bacterial viability, *in vivo*
APA WbtI Beads	*F. tularensis* LVS WbtI strain	PLL and Alginate	Microscopy, Bacterial viability, *in vivo*
APA WbtI and LPS Beads	*F. tularensis* LVS WbtI strain and 100ug/ml LVS LPS	PLL and Alginate	*In vivo*

Table 4.2. Characteristics of beads produced throughout the study.

4.3.3. Assessment of bacterial concentration within alginate beads.

Initially, the number of $WbtI_{G191V}$ bacterial cells that could be accommodated within the alginate beads was determined. Approximately 250 ul of alginate beads in 250 ul of MOPS buffer were added to 9.5 ml of solubilization solution (50mM sodium citrate, 0.45% NaCl, 10mM MOPS, pH 7.4). The suspension was incubated at 37°C with shaking (150 rpm) for 10 minutes until the beads were dissolved. The suspension was serially diluted and the concentration of bacteria within the beads was determined by viable plate count. A portion of the original alginate-bacterial suspension was used as a control to determine the starting bacterial concentration before encapsulation in $CaCl_2$. The encapsulation efficiency of a particular sample was calculated as the final bacterial concentration of 1ml of beads divided by the starting bacterial concentration in 1ml of alginate multiplied by 100.

4.3.4. Characterization of alginate beads.

Alginate beads were assessed via light microscopy to determine the sphere morphology and the shape from different preparations. For each preparation variation in the size of 30 beads were determined. The diameter of each bead was measured on an Olympus BX41 microscope using the Olympus DP Controller software (Olympus, Waltham, Massachusetts) and the mean values were determined.

4.3.5. Bacterial survival in complement active serum.

Alginate beads and APA beads containing $WbtI_{G191V}$ were incubated in 10% complement-active guinea pig serum for 0 hours, 1 hour, 24 hours, and 48 hours. Freely suspended $WbtI_{G191V}$ and freely suspended LVS cells were used as positive and negative controls. Aliquots of the alginate beads, freely suspended $WbtI_{G191V}$, and freely suspended LVS were harvested at each time point and subjected to dissolution and viable plate count as described above. CFUs were determined for each time point and compared to the CFU count from 0 hours.

APA beads cannot be dissolved via sodium citrate due to the covalent crosslinking of PLL and alginate. Instead, these beads were washed and suspended in BHI-C broth to determine if bacteria grew or did not grow. Alginate beads, freely suspended $WbtI_{G191V}$, and freely suspended LVS also underwent this procedure.

4.3.6. Immunization and challenge of mice.

An equal number of female and male BALB/c mice 6-8 weeks old (Charles River Laboratories, Wilmington, MA) were housed in an AALAC-accredited ABSL-2 facility. Groups of 2-4 mice were used to assess the protective efficacy of APA beads containing $WbtI_{G191V}$ with or without purified LVS LPS against an intranasal (IN) virulent challenge. Mice were immunized with either (i) PBS, (ii) $1x10^7$ CFU of $WbtI_{G191V}$, (iii) $1x10^7$ CFU of $WbtI_{G191V}$ with 10 µg of purified LPS, (iv) $1x10^7$ CFU of $WbtI_{G191V}$ in APA beads, or (v) $1x10^7$ CFU of $WbtI_{G191V}$ and 10 µg of purified LPS in APA beads intraperitoneally (IP), subcutaneously (SQ), or orally. A 25-guage needle was used for the IP and SQ immunizations and a curved 18-guage feeding needle was used for all oral immunizations. Mice were weighed and monitored for the presence of clinical signs twice daily after immunizations for 2 weeks. Mice were cheek bled 4 weeks after the immunization. Collected blood was incubated at room temperature for approximately 15 minutes and was centrifuged at 10,000 x *g* to remove the clot. Serum was collected and stored to determine *Francisella* specific antibody titers. Six weeks after immunizations, mice were

anesthetized with 3-4% isoflurane and inoculated IN with $1x10^6$ CFU of LVS. All mice were monitored for 2 weeks and any mouse that became moribund was euthanized immediately with excess CO_2. Mice that survived for the full 2 weeks were euthanized. Liver, lung, and spleen tissues were harvested from all euthanized mice. Tissue samples were homogenized in PBS containing 1 mM $CaCl_2$ and 2 mM $MgCl_2$. The homogenized tissue suspension was diluted serially ten-fold and bacterial concentration in weighed tissue was determined by viable plate count.

4.3.7. Assessment of *Francisella* titer after immunization.

The relative level of *Francisella* LVS specific antibodies in collected sera was measured by an enzyme-linked immunosorbent assay (ELISA). An overnight plate culture of LVS was resuspended in PBS at a concentration of $1x10^9$ CFU/ml and heat-inactivated at 65°C for 1 hour. Heat-inactivated LVS was diluted in carbonate buffer (50 mM, pH 9.6) to a concentration of $1x10^8$ CFU/ml. Ninety-six well Immunlon plates (Thermo Scientific) were coated with 100 µl of the heat-inactivated LVS in carbonate buffer and incubated at 4°C overnight. The plates were washed 3 times with PBS containing 0.05% Tween 20 (PBST) and then blocked with 1% non-fat dry milk (NFDM) at 37°C for 1 hour. The plates were washed once with PBST and serum samples were added in duplicates starting at a dilution of 1:100 followed by two-fold serial dilutions down the column. Plates were incubated at 37°C for 1 hour and then washed 3 times with PBST. Aliquots of 100 µl of horseradish peroxidase-conjugated anti-mouse antibodies diluted in NFDM at a concentration of 1:1000 were added to the plate and incubated for 1 hour at 37°C. The plate was washed an additional 3 times with PBST and then developed with the TMB Substrate Kit (Thermo Fisher) per the manufacturer's instructions. The color reaction was terminated with 2M H_2SO_4 and optical density was measured at 450 nm with a plate reader. Antibody titers were defined as the reciprocal of the highest dilution of immune serum with an OD value that was 0.1 greater than the mean OD of serum from non-immunized mice.

4.3.8. Innate and adaptive immune response profile of lung tissues.

Fresh frozen lung tissue from mice that survived challenge were homogenized as previously described. Total RNA was isolated from each homogenized sample using the RNeasy Mini Kit (Qiagen) as described by the manufacturer and then pooled proportionately by immunization group. The RT^2 First Strand Kit was used to create cDNA per the manufacturer's instructions (Qiagen). Synthesized cDNA was used as the template for the RT^2 Profile PCR

Array for Mouse Adaptive and Innate Immune Response (Qiagen) and run per the manufacturer's instructions on the ABI 7500. Lung tissue from mice that had not been immunized or challenged were used to determine basal levels of each gene product. Ingenuity Pathway Analysis (IPA, Qiagen) was used to analyze the cellular immune pathway up- and down-regulation from each group.

4.3.9. Histopathology of lung, liver, and spleen samples.

Sections of liver, lung, and spleen were fixed in 10% neutral buffered formalin at the time of necropsy for each mouse. The samples went sent to the histopathology laboratory at the Virginia-Maryland College of Veterinary Medicine (VMCVM) for processing and staining. Briefly, fixed samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) stain.

4.3.10. Statistical analyses.

Two-way ANOVAs followed by Tukey's *post-hoc* test were used to evaluate differences in characteristics (*i.e.* bead size, bacterial concentration, and efficiency) of produced alginate beads based on the different production parameters such as nozzle size and bacterial starting concentration. The Mantel-Cox log-rank test was used to compare the survival curves of the control and immunized mice following challenge. Kruskal-Wallis one-way ANOVA was used to evaluate the presence of significant differences in antibody titers post-immunization and bacterial loads after challenge of the various mouse groups. Dunn's multiple comparisons test was used after completion of the previous test to identify specific statistical differences between immunized mouse groups and the control sham-immunized mice. Statistical analyses were determined using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA).

4.4. Results

4.4.1. Sodium alginate bead characteristics

Prior to determining the optimal for immunization challenges, a GFP-expressing LVS strain was used to visualize the presence of viable bacterial cells within the beads after production. This LVS strain was successfully encapsulated in alginate using an 80 um nozzle on the Buchi Encapsulator B-395 Pro (Figure 4.1. Alginate bead containing GFP-expressing LVS.. The spheres were all less than 200 um in diameter and relatively spherical; though occasional

spheres exhibited a more tear drop shape. This quick assay showed that *F. tularensis* could be encapsulated in alginate effectively and using nozzle sizes smaller than previous reports (17, 18).

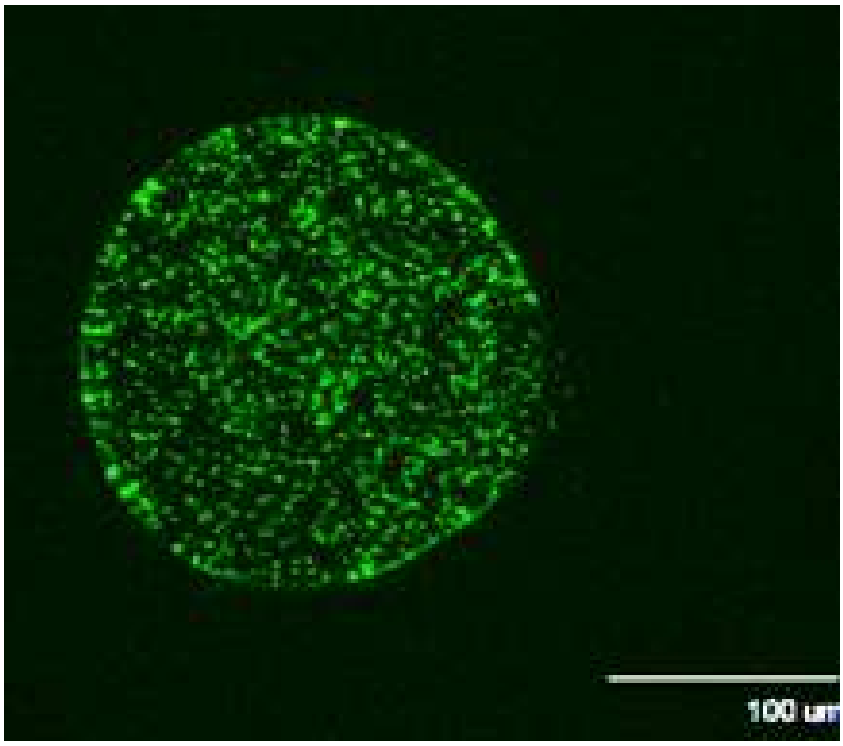


Figure 4.1. Alginate bead containing GFP-expressing LVS.
Alginate beads were created that contained LVS expressing the fluorescent protein GFP. Fluorescent microscopy shows the viable bacterial cells inside the capsule in a generally spherical shape.

To optimize production, different variations of alginate beads were created to assess how additional structures to the beads, such as a coating or content, contribute to bead formation and survival of the encapsulated bacteria. The beads were created with and without a PLL and alginate coating (Table 4.2). Alginate beads that were coated with PLL followed by a final layer of alginate were designated APA beads. Beads were also produced with varying levels of bacterial cell concentrations. Previous protocols for live cell encapsulation using the Buchi Encapsulator B-395 Pro limited the starting cell concentration to approximately $1x10^6$ cells/ml (17). Per the manual, a final concentration less than $1x10^{10}$ non-human cells/ml was recommended (24). Log increases in starting cell concentration were tested to determine if a final concentration of $1x10^{10}$ LVS cells/ml could be reached and how an increase in cell concentration affected bead characteristics.

In general, all capsules were spherical in shape (Figure 4.1) with some exhibiting a tear drop form. Cells that received the PLL and alginate coating no longer exhibited the tear drop shape. Using a starting concentration of $1x10^9$ cells/ml resulted in the formation of rafts of alginate on the surface of the polymerization solution. This represented the cell concentration at which beads no longer formed, possibly due to an inadequate ratio of cells to alginate. At this point, all further assays were conducted with less than $1x10^9$ cells/ml in the starting solution.

Diameter was measured on over 30 beads from each microencapsulation run to assess the contribution of starting cell concentration and nozzle size to the bead diameter. Mean diameter of the beads was significantly affected by the nozzle size ($p < 0.0001$), starting bacterial concentration ($p = 0.0032$), and the interaction between the two ($p = 0.0019$) (Figure 4.2). The average size of beads across all starting bacterial concentrations produced with the 80 µm nozzle was approximately 190 µm in diameter, with the use of a starting concentration $1x10^6$ cells/ml resulting in a statistically significant size difference compared to using the highest concentration $1x10^8$ cells/ml ($p = 0.0148$). Use of the 200 µm nozzle increased the bead diameter to approximately 550 µm, more than double the size of beads produced with the 80 µm nozzle.

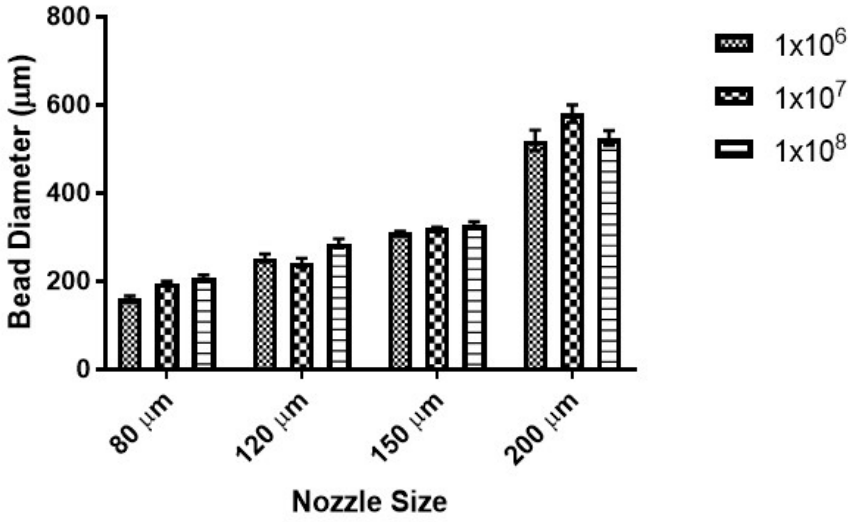


Figure 4.2. Effect of production parameters on bead size.
Bead diameter was assessed for 30 beads produced using nozzle sizes from 80 µm to 200 µm and starting bacterial concentrations of $1x10^6$ to $1x10^8$ CFU/ml. Bead sizes statistically differed due to nozzle sizes ($p < 0.0001$) and due to starting bead concentration ($p = 0.0032$). The interaction between nozzle size and starting concentration was statistically important ($p = 0.0019$). Differences between starting cell concentration within nozzle groups is signified as: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

Though statistical significance was found between the starting cell concentration, the main determinant of bead size was the nozzle size. In general, the diameter measurements are rough estimates on a comparatively small sample. The use of a particle size analyzer would dramatically increase the sample size and may reduce the significance of starting cell concentration as the average sizes within groups were within 25 to 50 um of each other. A particle size analyzer was not used in this case due to the lack of a machine within a biosecurity level 2 space.

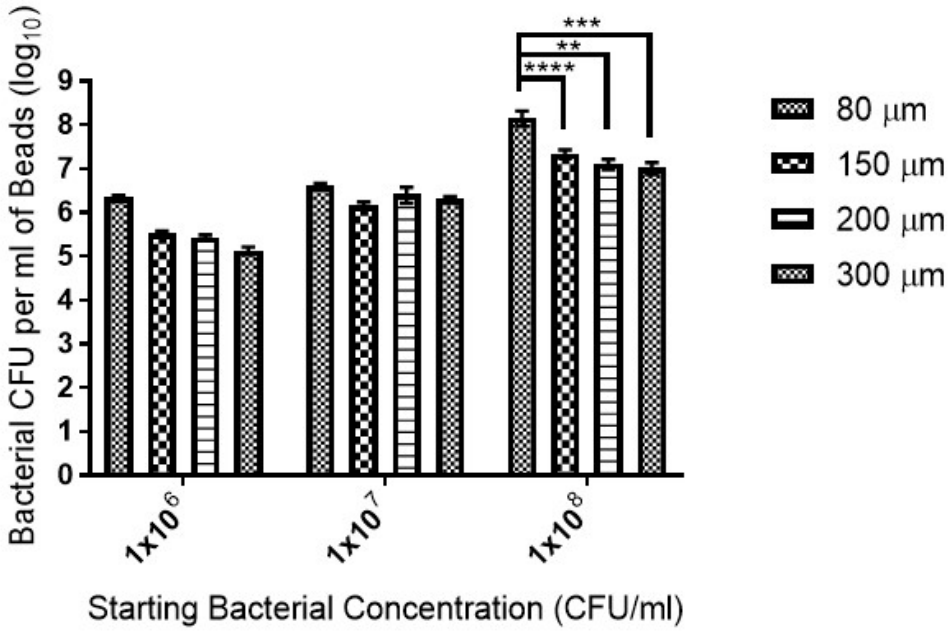


Figure 4.3. Bead bacterial concentration is determined by the bacterial starting concentration.
Viable bacterial concentration in a milliliter of beads was determined by dissolution of beads in citrate solution and viable plate count. Bacterial concentration within beads was statistically significant depending on the starting bacterial concentration ($p = 0.0097$) and was statistically affected by the interaction of starting concentration and nozzle size ($p = 0.0206$). Nozzle size alone did not statistically affect the bacterial concentration of beads ($p = 0.0646$). Differences between nozzle sizes within groups is signified as: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

Dissolution of alginate beads by a citrate solution was performed to determine the concentration of viable bacterial cells per milliliter of alginate beads (Figure 4.3). This assay was only performed on alginate beads and not APA beads due to the covalent crosslinking between alginate and PLL that prevents dissolution. APA beads were predicted to have the same concentration of viable bacterial cells as their non-coated counterpart. Starting bacterial

concentration significantly affected the final bacterial concentration ($p = 0.0097$), and the interaction between nozzle size and starting bacterial concentration significantly affected the final bacterial concentration per milliliter of alginate beads ($p = 0.0206$). No statistical differences were found comparing final bacterial concentration and nozzle size when the starting concentration was $1x10^6$ or $1x10^7$ CFU/ml ($p > 0.9998$). When bacterial starting concentrations were increased to $1x10^8$ CFU/ml, the final bacterial CFU per milliliter of beads decreased with the increased nozzle size (Figure 4.3). When a starting concentration $1x10^8$ CFU/ml was used in conjunction with the 80 μm nozzle, the highest final concentration of bacteria within the beads was achieved.

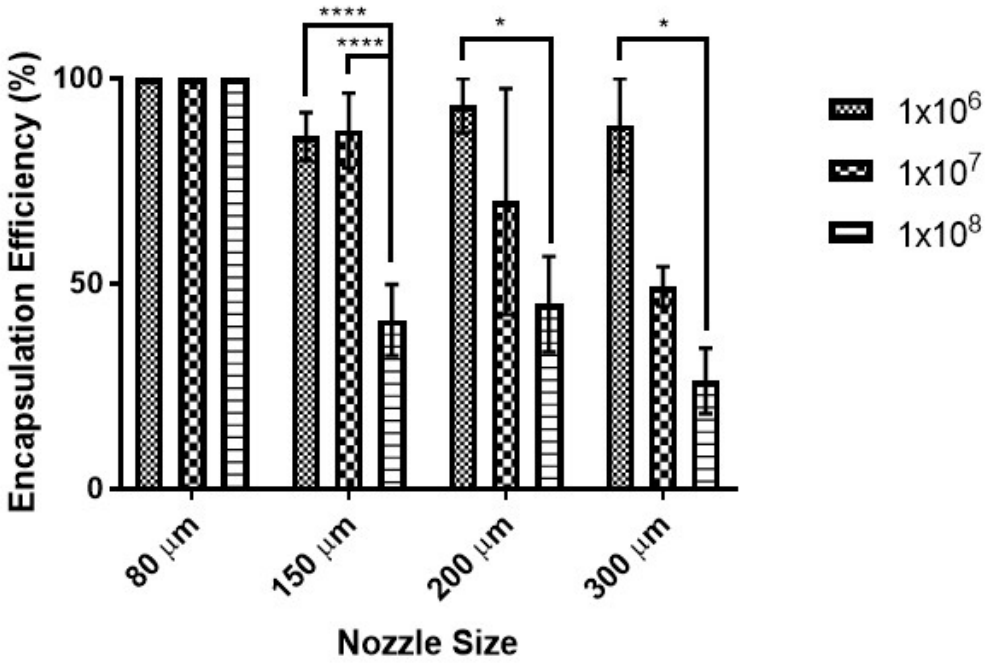


Figure 4.4. Encapsulation efficiency significantly decreases with increasing starting bacterial concentration.
Encapsulation efficiency is defined as the percentage of bacterial cells that become incorporated into the final alginate bead. Encapsulation efficiency decreased with increasing starting bacterial concentrations. Differences between starting bacterial concentrations within nozzle sizes is signified as: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

The encapsulation efficiency of each bead production run was classified as the proportion of bacterial cells released from alginate beads compared to the bacterial cells present in the starting alginate solution as a percent. Encapsulation efficiency was compared between all starting bacterial concentrations, the nozzle size, and the interaction of starting bacterial concentration with the nozzle size (Figure 4.4). Encapsulation efficiency numbers greater than

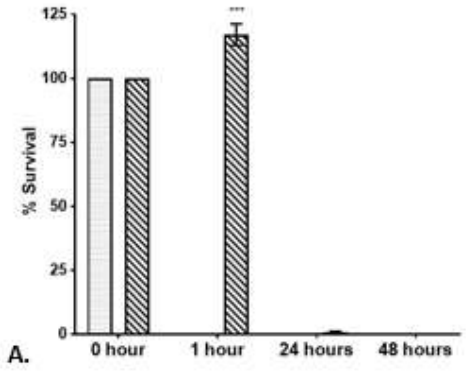
100% were classified as approximately 100% efficiency. Nozzle size ($p = 0.0005$) and starting bacterial concentration ($p < 0.0001$) statistically affected the encapsulation efficiency of the specific parameters. However, the interaction between nozzle size and bacterial concentration was not statistically significant ($p = 0.0943$). In general, as the starting bacterial concentration increased the encapsulation efficiency would decrease with a smaller proportion of cells becoming entrapped in the alginate beads. This mirrors the drop in final bacterial concentration of beads when using $1x10^8$ CFU/ml as the starting concentration (Figure 4.3). The encapsulation efficiency stayed at approximately 100% for the smallest nozzle and was most variable for the largest nozzle size tested.

For all immunization studies, beads were created using the 80 μm nozzle and a starting concentration of $1x10^8$ CFU/ml. This combination produced one of the most efficient encapsulation rates and the highest concentration of bacterial cells within the final bead. Previous studies have shown that the LVS O-antigen deficient strain $WbtI_{G191V}$ is highly attenuated and up to $2.8x10^7$ CFU given IP does not result in death of mice (14). Mice in this study only received 100 μl of beads containing $WbtI_{G191V}$ IP. With the 80 μm nozzle and starting concentration of $1x10^8$ CFU/ml, any inoculation dose would be less than $2.8x10^7$ CFU and should be safe. A portion of mice that received $2.8x10^7$ CFU IP in the aforementioned study did exhibit clinical signs that resolved by 5 days post infection (PI).

4.4.2. Encapsulation protects the serum sensitive O-antigen deficient $WbtI_{G191V}$ from complement mediated lysis

The O-antigen mutant $WbtI_{G191V}$ is serum-sensitive and can be killed in approximately 1% of pre-colostral calf serum (14). Encapsulation in alginate should create a barrier protecting the cells from complement proteins in the environment and increase survival time of $WbtI_{G191V}$. To test this hypothesis, $WbtI_{G191V}$ was encapsulated in alginate beads and in APA beads and then incubated in complement-active guinea pig serum. Survival of $WbtI_{G191V}$ in these bead formulations was compared to freely suspended $WbtI_{G191V}$ and LVS as negative and positive controls, respectively. Due to the inability to dissolve APA beads, viable plate counts could not be completed for APA beads. Instead of viable plate counts, the presence or absence of bacterial growth in broth from washed beads was used to assess survival of $WbtI_{G191V}$ in APA beads after incubation in complement active guinea pig serum.

Encapsulation with both the alginate beads and the APA beads increased survival of $WbtI_{G191V}$ in complement-active serum compared to the freely suspended $WbtI_{G191V}$ (Figure 4.5). Alginate encapsulation marginally increased survival of the bacteria ($p < 0.001$ at 1-hour post-incubation, Figure 4.5A). A minimal amount of $WbtI_{G191V}$ growth was recorded following the 24-hour incubation of alginate beads., but was not statistically significant compared to freely suspended $WbtI_{G191V}$ ($p = 0.128$). The addition of the PLL and alginate coating around the alginate beads enhanced survival of $WbtI_{G191V}$ to levels similar to serum-resistant LVS (Figure 4.5B).



B.

	Incubation Time		
	1 Hour	*24 Hours*	*48 Hours*
LVS	+	+	+
$WbtI_{G191V}$	-	-	-
Alginate Encapsulated $WbtI_{G191V}$	+	-	-
APA Encapsulated $WbtI_{G191V}$	+	+	+

Figure 4.5. Encapsulation increases survival of serum-sensitive $WbtI_{G191V}$ incubated in complement-active serum.
$WbtI_{G191V}$ was encapsulated in alginate or APA beads and then incubated in complement-active serum for 1 hour, 24 hours, and 48 hours. A: Viable plate counts were used to determine the percent survival of $WbtI_{G191V}$ freely suspended in solution (dotted bar) compared to $WbtI_{G191V}$ encapsulated in alginate beads (diagonal line bar). Encapsulated $WbtI_{G191V}$ survived statically more than freely suspended $WbtI_{G191V}$ at 1 hour ($p < 0.001$, ***). Viable $WbtI_{G191V}$ cells were detected after 24 hours of incubation in complement-active serum; however, the difference was not statistically different from freely suspended $WbtI_{G191V}$. B: The presence and absence of growth after incubation of the bacteria in complement-active serum for specified amounts of time was determined for washed alginate and APA beads containing $WbtI_{G191V}$. Bacterial growth resulted only after one hour incubation of the bacteria encapsulated in alginate beads in complement-active serum. $WbtI_{G191V}$ within APA capsules grew after 48 hours of incubation in complement-active serum similarly to serum-resistant LVS, indicating an increase in protection with the additional coating around the beads.

4.4.3. Immunization with microencapsulated $WbtI_{G191V}$ intraperitoneally stimulates higher antibody production

Mice were immunized with $WbtI_{G191V}$ within beads or freely suspended in solution to assess anti-*Francisella* antibody production. Only APA beads and not alginate beads were used for immunization as $WbtI_{G191V}$ survived longer *in vitro* in complement-active serum within APA

beads, and preliminary immunization and challenge studies showed no difference in survival when mice were immunized with alginate beads containing $WbtI_{G191V}$ compared to mock-immunized mice (data not shown). The effect of the route of immunization on antibody production was also assessed. A low dose of LVS given SQ was used as a benchmark for antibody production. LVS was not given IP as no safe low dose exists for this route with LVS. All mice were monitored for clinical signs (*i.e.* weight loss, ruffled fur, swollen eyes, hunched back) and any moribund mice were euthanized humanely. All mice immunized with $WbtI_{G191V}$ within beads or alone did not show any clinical signs. No swelling or vaccine induced reactions were noted at any site of inoculation. Mice immunized with LVS SQ did exhibit ruffled fur and weight loss, but recovered in approximately 10 days.

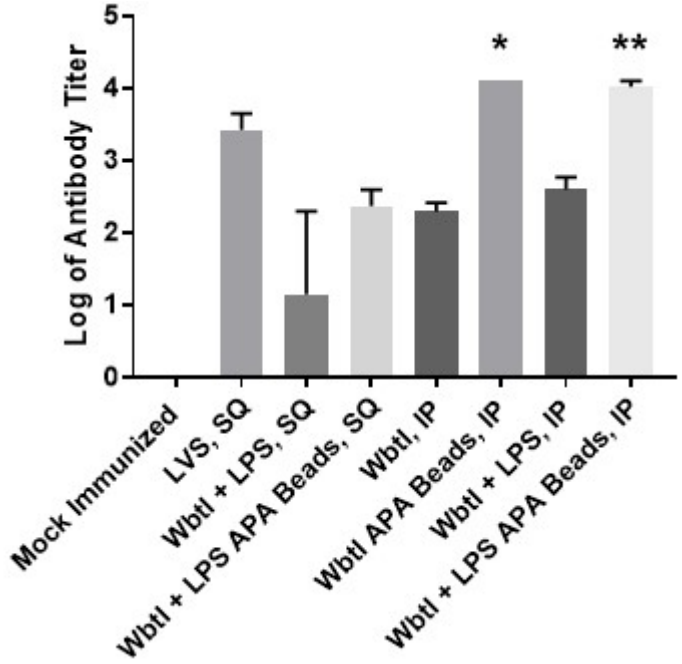


Figure 4.6. Anti-LVS antibody titers in immunized mice.
Mice were immunized with the described formulations either SQ or IP. Serum samples were taken post-immunization to determine antibody titers against *F. tularensis* LVS cells. The titer was determined to be the reciprocal of the dilution with an OD_{450nm} that was 0.100 greater than the OD_{450nm} of mock-immunized mice. Bars represent the mean titer with error bars representing the standard error of the mean. Antibody titer differences compared to mock-immunized mice are represented as: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

Although all mice immunized with some form of *Francisella* produced an anti-*Francisella* antibody response, only mice immunized with $WbtI_{G191V}$ with or without purified LPS in APA beads via the IP route produced antibody levels significantly higher than mock

immunized mice (Figure 4.6). Immunization with similar formulations of APA beads via the SQ route did not lead to antibody titers significantly higher than the mock-immunized mice and produced titers lower than mice immunized with LVS SQ.

4.4.4. Encapsulated $WbtI_{G191V}$ with purified LPS protects against a lethal respiratory challenge.

Immunized mice were challenged with a high dose of virulent LVS IN to determine the protective efficacy of the immunization. The IN route was chosen for challenge because this route is a more likely route of infection naturally than IP and, though the lethal dose required is higher than IP, this route is still highly virulent and results in a similar systemic infection (25). The LD_{50} of this strain of LVS is approximately 200 CFUs IN and approximately 40 CFUs IP (26). Mice were challenged 6 weeks post-immunization with approximately $1x10^6$ CFU or 5000 x LD_{50} of virulent LVS IN. Clinical signs were monitored and survival time post-challenge was determined for mice immunized SQ and IP (Figure 4.7).

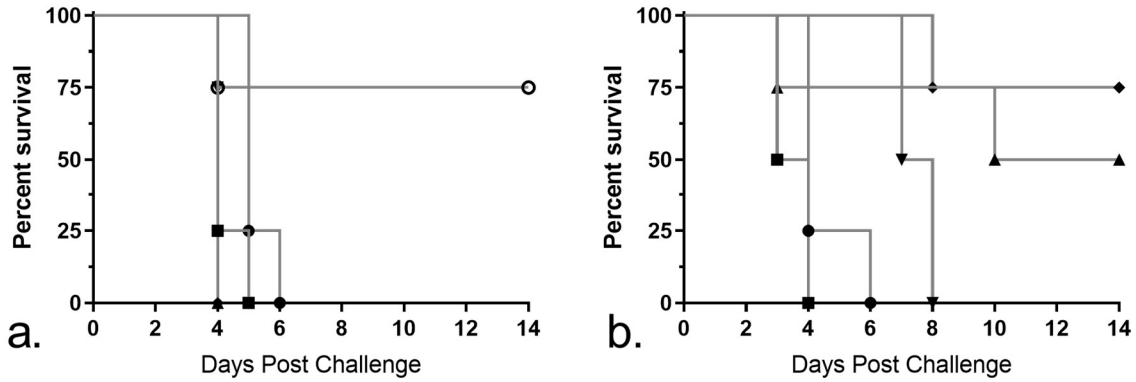


Figure 4.7. Survival of mice immunized intraperitoneally and challenged with virulent LVS intranasally.
Mice were immunized SQ (a) or IP (b) with either $WbtI_{G191V}$ (●), $WbtI_{G191V}$ + purified LPS (▲), APA-encapsulated $WbtI_{G191V}$ (▼), APA encapsulated $WbtI_{G191V}$ + purified LPS (♦), LVS (**o**), or PBS (■). Six weeks post-immunization, mice were challenge with a high dose of virulent LVS IN. Survival post-challenge was monitored and the curves were determined to be statistically significantly different (p = 0.0029 and 0.0014, respectfully). Of the SQ-immunized mice, only mice that were immunized with LVS survived the challenge period. Of the IP-immunized mice, only mice that were immunized with $WbtI_{G191V}$ and purified LPS, either encapsulated or freely suspended in solution, survived until the end of the study. Survival was 75% and 50%, respectively.

Of the SQ-immunized mice, only those were immunized with a low dose of LVS survived until the end of the study (Figure 4.7a). Mice immunized with $WbtI_{G191V}$, encapsulated

or freely suspended and with or without purified LPS, did not survive any longer than mock-immunized mice. The mean survival time was 4-5 days for all groups other than the LVS SQ-immunized group. The survival proportion of mice immunized with LVS via the SQ route was 75%.

Mice that were immunized IP with $WbtI_{G191V}$ had better survival times than those given the same immunization SQ (Figure 4.7b). Mice immunized IP with $WbtI_{G191V}$ and purified LPS in APA beads produced the highest survival proportion at 75%. The same combination freely suspended in solution resulted in 50% survival. $WbtI_{G191V}$ encapsulated in APA beads did not survive the study period, but did increase the mean survival time from 3.5 days for mock-infected mice to 7.5 days. $WbtI_{G191V}$ alone had a mean survival time of 4 days, similar to mice that were mock-immunized.

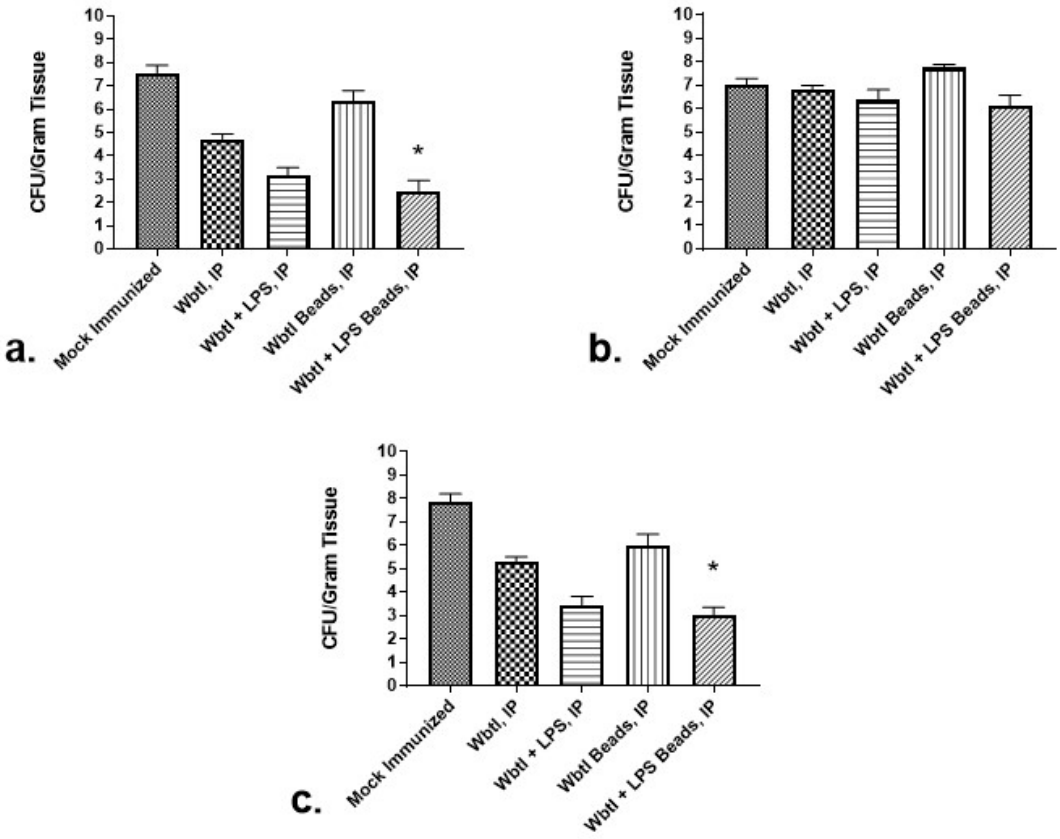


Figure 4.8. Tissue bacterial load of mice immunized intraperitoneally and then challenged with virulent LVS intranasally.
BALB/c mice were immunized with different formulations and then challenged with $1x10^6$ CFU/ml of virulent LVS intranasally. All beads were APA beads. At necropsy, tissue samples were harvested from the liver (a), lung (b), and spleen (c). Bacterial numbers in these tissues were determined by viable plate count. Significant differences in bacterial load in a gram of tissue are indicated by: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

Bacterial burdens were assessed in the liver, lung, and spleen of mice following necropsy to assess protection of the various immunizations. No mice, regardless of the immunization formulation, were able to clear all bacteria from any of the aforementioned organs, including the mice that survived to the end of the study. Mice immunized SQ with any of the $WbtI_{G191V}$ immunization forms had bacterial burdens similar to mice that were mock-immunized, which mirrored the similar survival percentages of these groups compared to the mock-infected groups. These bacterial burdens exceeded $1x10^7$ CFU/gram of tissue in the liver, lung, and spleen for these groups. However, mice immunized SQ with LVS had significantly lower bacterial burdens than any of the other SQ-immunized groups (Liver: 42 ± 31 CFU/gram of tissue, Spleen: 267 ± 165 CFU/gram of tissue, and Lung: $4.2 \times 10^5 \pm 3.6 \times 10^5$ CFU/gram of tissue).

Bacterial burdens were also determined for mice immunized IP (Figure 4.8). Mice that were immunized with $WbtI_{G191V}$ trended lower in bacterial burden within the liver and spleen compared to mock-immunized mice (Figure 4.8a, c). Mice with longer survival times tended to have lower bacterial burdens within the liver and spleen. Only mice immunized with APA beads containing $WbtI_{G191V}$ and purified LPS had statistically lower bacterial burdens than the mock immunized mice within the liver ($p = 0.0245$) and spleen ($p = 0.0348$). No other immunized group was significantly lower in bacteria burden in the liver and spleen than the mock-immunized mice. There was no statistical difference in bacterial burden in the lungs within the IP-immunized groups. Mice immunized with LVS via SQ, overall, had the lowest bacterial burdens at the end of the study compared to any other group.

4.4.5. Mice immunized with LVS had a greater cellular immune response following challenge than mice immunized with $WbtI_{G191V}$ beads

Overall, immunization with LVS SQ and $WbtI_{G191V}$ with purified LPS within APA beads IP produced similar survival rates (75% for each group) and had statistically lower bacterial burdens than the mock-immunized mice. However, the bacterial burdens for the LVS group were statistically lower than those in the $WbtI_{G191V}$ group. Due to the smaller sample size of mice within each group (n = 4), mice immunized IP with $WbtI_{G191V}$ and purified LPS freely suspended were similar in survival (50%) as the previously mentioned groups. In order to differentiate the immune response produced within these groups, RNA was isolated from fresh frozen lung from mice surviving challenge within these groups and assayed to determine differences in gene expression related to the cellular immune response between the surviving groups.

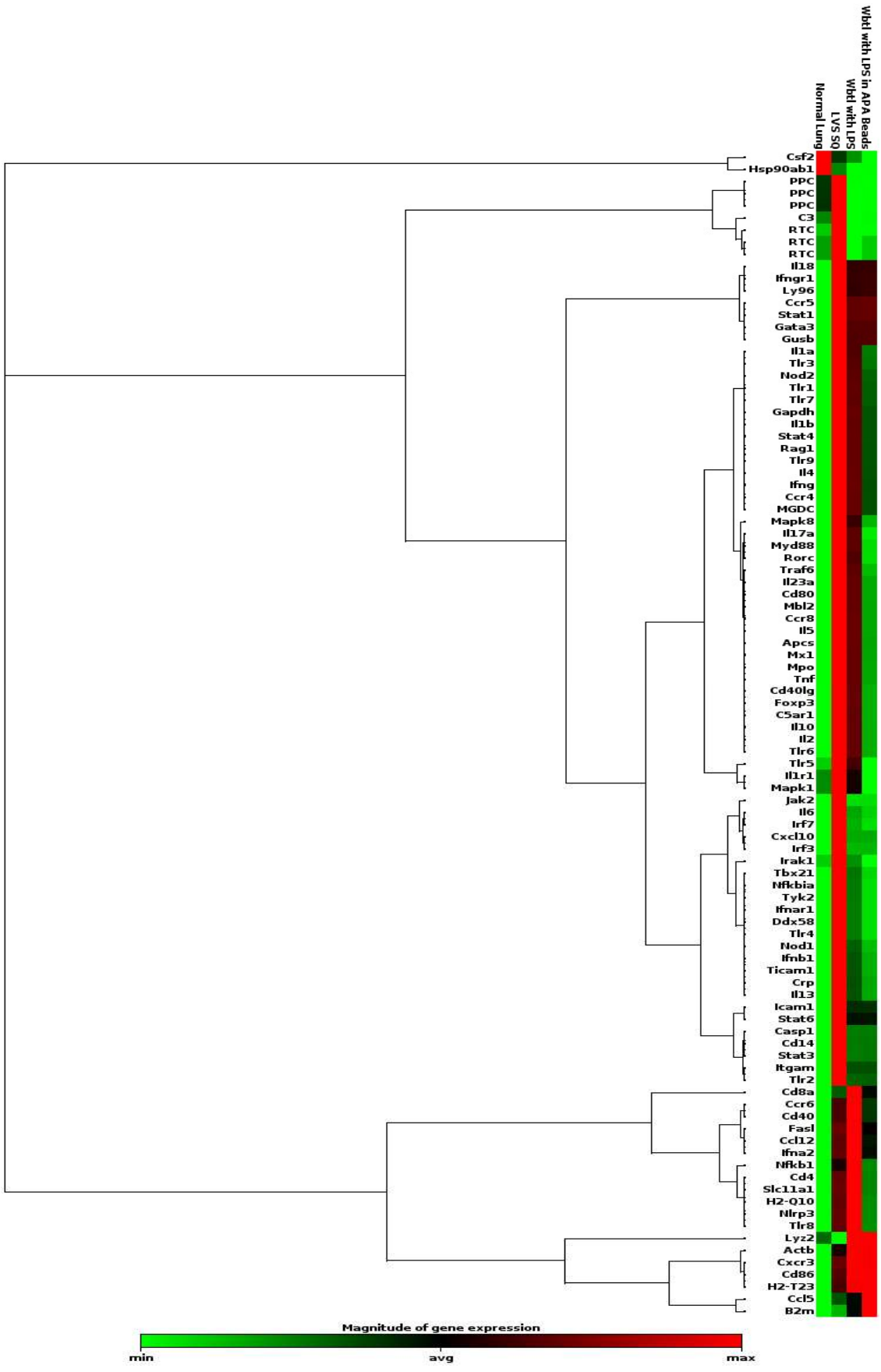

Normal Lung
LVS SG
Wbtl with LPS
Wbtl with LPS in APA Beads
Csf2
Hsp90ab1
PPC
PPC
PPC
C3
RTC
RTC
RTC
Il18
Ifngr1
Ly96
Ccr5
Stat1
Gata3
Gusb
Il1a
Tlr3
Nod2
Tlr1
Tlr7
Gapdh
Il1b
Stat4
Rag1
Tlr9
Il4
Ifng
Ccr4
MGDC
Mapk8
Il17a
Myd88
Rorc
Traf6
Il23a
Cd80
Mbl2
Ccr8
Il5
Apcs
Mx1
Mpo
Tnf
Cd40lg
Foxp3
C5ar1
Il10
Il2
Tlr6
Tlr5
Il1r1
Mapk1
Jak2
Il6
Irf7
Cxcl10
Irf3
Irak1
Tbx21
Nfkbia
Tyk2
Ifnar1
Ddx58
Tlr4
Nod1
Ifnb1
Ticam1
Crp
Il13
Icam1
Stat6
Casp1
Cd14
Stat3
Itgam
Tlr2
Cd8a
Ccr6
Cd40
Fasl
Ccl12
Ifna2
Nfkb1
Cd4
Slc11a1
H2-Q10
Nlrp3
Tlr8
Lyz2
Actb
Cxcr3
Cd86
H2-T23
Ccl5
B2m
Magnitude of gene expression
min
avg
max

Figure 4.9. Innate and adaptive immune response gene expression within lung tissue after challenge with *Francisella tularensis*.
Mice were immunized with LVS SQ, WbtI and LPS IP, or WbtI and LPS within APA beads IP, and then challenged with a high dose of LVS IN. RNA was isolated from the lungs of mice that survived challenge and analyzed with an RT^2 PCR Profiler for the mouse innate and adaptive immune response compared to normal lung tissue. The heat map above shows expression levels of each gene with green, which indicates minimal expression and red, which indicates maximum expression for each group and then grouped by trends in expression.

An RT^2 PCR Profiler Assay was used that targeted 85 genes related to the innate and adaptive immune system and several housekeeping and control genes. A comparison of the normalized gene expression between the groups showed a greater increase in innate and adaptive immune responses of the mice vaccinated with LVS SQ and subsequently challenged IN with LVS compared to other vaccination groups (Figure 4.9).

Further IPA analysis was completed to determine specific pathways affected between the immunization groups. In general, LVS-immunized mice expressed a higher level of cellular immunity following challenge and the mice immunized with $WbtI_{G191V}$ and LPS within APA beads expressed a comparatively lower level of cellular immunity. The cellular immune response of the group immunized with $WbtI_{G191V}$ and LPS free in suspension fell between those two groups. Of particular note were the levels of INFγ and IL-6 expression. INFγ was upregulated in all immunization groups post-challenge, but minimally upregulated in mice immunized with $WbtI_{G191V}$ and LPS in APA beads compared to the higher upregulation in the other immunization groups. IL-6 expression was also upregulated greatest in LVS-immunized mice. This IL-6 signaling pathway was down-regulated in mice immunized with $WbtI_{G191V}$ and LPS in APA beads. LVS-immunized mice had the highest level of INFγ and IL-6 upregulation post-challenge, which correlates with LVS-immunized mice having the lowest lung bacterial burden at the end of the study.

4.5. Discussion

An efficacious vaccine for tularemia does not exist. The previous vaccine LVS inadequately protects against respiratory challenges with virulent Type A strains and remains partially virulent in immunocompromised individuals (27-29). Developing an effective vaccine requires creating an attenuated strain of *F. tularensis* that remains safe and stimulates effective cellular and antibody-mediated immunity to protect against the most severe route: respiratory.

Numerous attenuated strains of *F. tularensis* have been created (14, 27, 29-31). However, the vaccine efficacy of these strains is lacking and safety in immunocompromised patients is questionable.

Disruption of the O-antigen of the *Francisella* LPS has consistently produced strains that are attenuated and are highly serum sensitive (9, 10, 12, 14, 32). We theorize that an O-antigen deficient mutant of *F. tularensis* would be a highly safe vaccine as no spontaneous mutation would restore the O-antigen in a mutant with one or more genes in the O-antigen locus deleted. In this study we used an O-antigen deficient mutant of LVS with a point mutation in the *wbtI* gene, $WbtI_{G191V}$, as a model. $WbtI_{G191V}$ has been shown to be attenuated to at least $2.7x10^7$ CFU given IP, approximately 200,000 times the LD_{50} of LVS given IP (14). $WbtI_{G191V}$ was cleared by day 8 in mice given $1x10^5$ CFU IN (14). Mice boostered twice intradermally (ID) with the O-antigen deficient strain and challenged with $25xLD_{50}$ of LVS IP were fully protected (14). However, protection decreased drastically to 20% when the challenge dose of LVS was increased 10 times (14). In order to reduce the need for a booster and increase persistence of the bacterium *in vivo* the novel vaccine vehicle, alginate encapsulation, was chosen.

Brucella species have previously been successfully encapsulated and, when used for immunization, highly protective against virulent challenges (17-19, 33). Arenas-Gamboa *et al*. demonstrated that an attenuated strain of *Brucella melitensis* encapsulated in APA beads was released over a one-month period *in vitro* with an initial burst release during the first few days (17). *In vivo*, this encapsulated strain stimulated a higher level of INFγ and antibody production compared to the non-encapsulated strain, which in theory may be due to the increased persistence of the bacterium *in vivo* within the beads (17). As immunity to *Francisella* requires an efficient cellular immune response, this novel vaccine delivery method was attempted with $WbtI_{G191V}$ to increase persistence of the bacterium *in vivo* and increase the cellular immune response.

A sustained release vehicle should negate the need for a booster and potentially increase vaccine efficacy of a strain that is weakly protective. A composite microsphere encapsulating the Hepatitis B vaccine induces an immune response comparable to the multi-dose booster series (34). Antibody levels resulting from immunization with the alginate composite microsphere equaled or exceeded the antibody titers from the traditional two booster Hepatitis B vaccine (34). A similar result was observed in this study. *F. tularensis* specific antibody titers for mice

immunized with the alginate beads exceeded the titers of those mice immunized with the freely suspended bacterial cells.

The alginate encapsulation procedure produces environments that are generally buffered and amendable to living cells compared to other forms of micro- or nanoencapsulation such as liposomal procedures (20, 35). Poly-lactic-co-glycolic acid (PGLA) has been used to create nanoparticles containing *F. tularensis* outer membrane proteins (36). This technique can easily be used to nano-encapsulate non-living components, but creates a toxic environment during preparation for living cells. Alginate polymerization is an ionic reaction that does not expose cells to extreme environments. This environment was desirable as we wanted to use a live attenuated strain to better stimulate a cell-mediated immune response.

F. tularensis was amendable to microencapsulation in sodium alginate as outlined in this paper. A GFP-labeled LVS strain was used to visually confirm the presence of viable encapsulated bacteria. Therefore, alginate encapsulation with or without the addition of a PLL and alginate coating could effectively encapsulate live *F. tularensis* cells. Further optimization studies confirmed that the Buchi Encapsulator could be used to encapsulate *F. tularensis* cells efficiently with lower concentrations of cells ($<1x10^7$ CFU/ml) and that an increase in starting cell concentration decreased the encapsulation efficiency. We were unable to encapsulate cell concentrations exceeding $1x10^8$ CFU/ml. This phenomenon may be due to a decrease in the ionic interactions of calcium and alginate as the proportion of bacterial cells to alginate increases.

The main goal of using an alginate vehicle was to enhance persistence of an O-antigen-deficient *F. tularensis* strain *in vivo* by providing protection against clearance mechanisms such as complement-mediated lysis. Encapsulation solely with alginate was only mildly effective at increasing survival of $WbtI_{G191V}$. However, the addition of the PLL and alginate coating to create an APA bead appeared to dramatically increase survival. Alginate polymers are porous environments that can expand and contract with changes in tonicity. This expansion and contraction may allow complement proteins access to the encapsulated bacteria, resulting in the inefficient protection seen here with $WbtI_{G191V}$ encapsulated solely in alginate. The addition of a covalently linked coating such as PLL stabilizes the beads and prevents the expansion and contraction (37). APA beads should still be porous and allow molecules into and out of the bead in order to provide a livable environment (37). We showed here that the addition of a coating increased persistence of $WbtI_{G191V}$ suspended in complement active serum. This increased

persistence *in vitro* and the increased antibody response against the encapsulated immunization suggest that $WbtI_{G191V}$ encapsulated in APA should be able to persistent *in vivo* more efficiently than the non-encapsulated cells.

F. tularensis is an intracellular pathogen and cell-mediated immunity is highly important for protection. However, various studies have also emphasized the importance of antibody production against *Francisella* for adequate protection (3). Cole *et al.* showed that mice immunized with purified LPS produced a significant antibody response that was protective against a virulent challenge and that the protection lasted for months past the immunization (38). In this study, antibody production did not correlate strongly with successful protection. $WbtI_{G191V}$ with purified LPS encapsulated in APA beads produced a significant antibody response following IP immunization and 75% of these mice survived a virulent IN challenge. However, a similar formulation without the purified LPS that also produced a significant antibody response following IP immunization did not confer protection against a virulent challenge. In comparison, LVS immunization SQ produced a smaller, non-statistically significant antibody response in mice and 75% of these mice survived a virulent IN challenge. Though mice with higher anti-*Francisella* antibody titers tended to survive longer post-challenge in this study, an exact correlation between antibody titer and protection cannot be made. The presence of antibodies to *Francisella* may still be a contributing factor for protection. Stenmark *et al.* showed that mice immunized with LVS given immune serum prior to challenge were not protected any better than mice solely immunized with LVS; but that naïve mice and B-cell deficient mice administered immune serum prior to challenge did survive lethal doses of LVS (39). In addition, vaccination with outer membrane proteins contained in PGLA nanospheres induced significant antibody titers and protected mice against a virulent LVS challenge, but did not protect mice against challenge with SchuS4 (36). However, when mice immunized with the PGLA nanospheres were then boosted with live LVS the protection against the SchuS4 challenge increased (36). A true infection, which is somewhat mimicked by repeated immunization with LVS, may be necessary for a tularemia vaccine to induce the necessary cell-mediated immune response and not just the antibody-mediated response. LVS is considered virulent in mice and will produce a more robust infection than $WbtI_{G191V}$. Even encapsulated, $WbtI_{G191V}$ may be too attenuated to persist long enough to induce the necessary cell-mediated immune response, but does help stimulate antibody-mediated immunity.

Alginate encapsulation may also be impeding the development of the cell-mediated immune response as seen in this study with the lowest cell-mediated immune response found in the alginate encapsulated immunization. The *Brucella abortus* vaccine, RB51, when encapsulated in alginate stimulates an effective Th1 immune response in deer that was statistically elevated compared to RB51 alone (19). Ajdary *et al*. also found that alginate encapsulation of the *Mycobacterium tuberculosis* vaccine Bacille Calmette-Guerin (BCG) increased the cell-mediated immune response and specifically the production of INF-γ (40). The opposite was seen here with $WbtI_{G191V}$ alginate encapsulation as the APA form of the immunization produced the lowest expression of INF-γ associated pathways.

In conclusion, this study demonstrates that alginate encapsulation of an attenuated *F. tularensis* strain can protect the bacterium from the host immune system and stimulate an antibody-mediated immune response. However, the alginate encapsulation appeared to dampen the ability of the live attenuated strain to induce a cell-mediated immune response. The addition of a protein conjugate that stimulates a Th1 response to the outer shell of the alginate microsphere, or the use of an attenuated strain that is not cleared as quickly, could increase the ability of this immunization to induce a cell-mediated immune response.

4.6. References

1. Services CfDCaPaDoHaH. 2012. Possession, Use, and Transfer of Select Agents and Toxins; Biennial review; Final Rule.
2. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Layton M, Lillibridge SR, McDade JE, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Tonat K, Working Group on Civilian B. 2001. Tularemia as a biological weapon: medical and public health management. JAMA 285:2763-73.
3. Jia Q, Horwitz MA. 2018. Live Attenuated Tularemia Vaccines for Protection Against Respiratory Challenge With Virulent *F. tularensis* subsp. *tularensis*. Front Cell Infect Microbiol 8.
4. Tigertt WD. 1962. Soviet viable *Pasteurella tularensis* vaccines. A review of selected articles. Bacteriol Rev 26:354-73.
5. Saslaw S, Eigelsbach HT, Wilson HE, Prior JA, Carhart S. 1961. Tularemia vaccine study. I. Intracutaneous challenge. Arch Intern Med 107:689-701.
6. Saslaw S, Eigelsbach HT, Prior JA, Wilson HE, Carhart S. 1961. Tularemia vaccine study. II. Respiratory challenge. Arch Intern Med 107:702-14.
7. Eigelsbach HT, Downs CM. 1961. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. J Immunol 87:415-25.

8. Hood AM. 1977. Virulence factors of *Francisella tularensis*. J Hyg (Lond) 79:47-60.
9. Sandstrom G, Lofgren S, Tarnvik A. 1988. A Capsule-Deficient Mutant of *Francisella-Tularensis* Lvs Exhibits Enhanced Sensitivity to Killing by Serum but Diminished Sensitivity to Killing by Polymorphonuclear Leukocytes. Infection and Immunity 56:1194-1202.
10. Sorokin VM, Pavlovich NV, Prozorova LA. 1996. *Francisella tularensis* resistance to bactericidal action of normal human serum. FEMS Immunol Med Microbiol 13:249-52.
11. Conlan JW, Shen H, Webb A, Perry MB. 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.
12. Hartley G, Taylor R, Prior J, Newstead S, Hitchen PG, Morris HR, Dell A, Titball RW. 2006. Grey variants of the live vaccine strain of Francisella tularensis lack lipopolysaccharide O-antigen, show reduced ability to survive in macrophages and do not induce protective immunity in mice. Vaccine 24:989-96.
13. Raynaud C, Meibom KL, Lety MA, Dubail I, Candela T, Frapy E, Charbit A. 2007. Role of the wbt locus of *Francisella tularensis* in lipopolysaccharide O-antigen biogenesis and pathogenicity. Infect Immun 75:536-41.
14. Li J, Ryder C, Mandal M, Ahmed F, Azadi P, Snyder DS, Pechous RD, Zahrt T, Inzana TJ. 2007. Attenuation and protective efficacy of an O-antigen-deficient mutant of *Francisella tularensis* LVS. Microbiology 153:3141-53.
15. Rasmussen JA, Post DM, Gibson BW, Lindemann SR, Apicella MA, Meyerholz DK, Jones BD. 2014. *Francisella tularensis* Schu S4 lipopolysaccharide core sugar and O-antigen mutants are attenuated in a mouse model of tularemia. Infect Immun 82:1523-39.
16. Twine SM, Vinogradov E, Lindgren H, Sjostedt A, Conlan JW. 2012. Roles for wbtC, wbtI, and kdtA Genes in Lipopolysaccharide Biosynthesis, Protein Glycosylation, Virulence, and Immunogenicity in Francisella tularensis2 Strain SCHU S4. Pathogens 1:12-29.
17. Arenas-Gamboa AM, Ficht TA, Kahl-McDonagh MM, Rice-Ficht AC. 2008. Immunization with a single dose of a microencapsulated *Brucella melitensis* mutant enhances protection against wild-type challenge. Infect Immun 76:2448-55.
18. Arenas-Gamboa AM, Ficht TA, Davis DS, Elzer PH, Kahl-McDonagh M, Wong-Gonzalez A, Rice-Ficht AC. 2009. Oral vaccination with microencapsuled strain 19 vaccine confers enhanced protection against *Brucella abortus* strain 2308 challenge in red deer (Cervus elaphus elaphus). J Wildl Dis 45:1021-9.
19. Arenas-Gamboa AM, Ficht TA, Davis DS, Elzer PH, Wong-Gonzalez A, Rice-Ficht AC. 2009. Enhanced immune response of red deer (Cervus elaphus) to live rb51 vaccine strain using composite microspheres. J Wildl Dis 45:165-73.
20. Kailasapathy K. 2002. Microencapsulation of probiotic bacteria: technology and potential applications. Curr Issues Intest Microbiol 3:39-48.
21. Sultana K, Godward G, Reynolds N, Arumugaswamy R, Peiris P, Kailasapathy K. 2000. Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. Int J Food Microbiol 62:47-55.
22. Chandramouli V, Kailasapathy K, Peiris P, Jones M. 2004. An improved method of microencapsulation and its evaluation to protect Lactobacillus spp. in simulated gastric conditions. J Microbiol Methods 56:27-35.

23. Ding WK, Shah NP. 2009. An improved method of microencapsulation of probiotic bacteria for their stability in acidic and bile conditions during storage. J Food Sci 74:M53-61.
24. Buchi. Operation Manual Encapsulator B-395 Pro, p 61-65.
25. Conlan JW, Chen W, Shen H, Webb A, KuoLee R. 2003. Experimental tularemia in mice challenged by aerosol or intradermally with virulent strains of Francisella tularensis: bacteriologic and histopathologic studies. Microb Pathog 34:239-48.
26. Bandara AB, Champion AE, Wang X, Berg G, Apicella MA, McLendon M, Azadi P, Snyder DS, Inzana TJ. 2011. Isolation and mutagenesis of a capsule-like complex (CLC) from *Francisella tularensis*, and contribution of the CLC to *F. tularensis* virulence in mice. PLoS One 6:e19003.
27. Conlan JW. 2004. Vaccines against Francisella tularensis--past, present and future. Expert Rev Vaccines 3:307-14.
28. Rohmer L, Brittnacher M, Svensson K, Buckley D, Haugen E, Zhou Y, Chang J, Levy R, Hayden H, Forsman M, Olson M, Johansson A, Kaul R, Miller SI. 2006. Potential source of *Francisella tularensis* live vaccine strain attenuation determined by genome comparison. Infect Immun 74:6895-906.
29. Wayne Conlan J, Oyston PC. 2007. Vaccines against *Francisella tularensis*. Ann N Y Acad Sci 1105:325-50.
30. Twine S, Bystrom M, Chen W, Forsman M, Golovliov I, Johansson A, Kelly J, Lindgren H, Svensson K, Zingmark C, Conlan W, Sjostedt A. 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.
31. Baron SD, Singh R, Metzger DW. 2007. Inactivated *Francisella tularensis* live vaccine strain protects against respiratory tularemia by intranasal vaccination in an immunoglobulin A-dependent fashion. Infect Immun 75:2152-62.
32. Sebastian S, Dillon ST, Lynch JG, Blalock LT, Balon E, Lee KT, Comstock LE, Conlan JW, Rubin EJ, Tzianabos AO, Kasper DL. 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.
33. Arenas-Gamboa AM, Ficht TA, Kahl-McDonagh MM, Gomez G, Rice-Ficht AC. 2009. The Brucella abortus S19 DeltavjbR live vaccine candidate is safer than S19 and confers protection against wild-type challenge in BALB/c mice when delivered in a sustained-release vehicle. Infect Immun 77:877-84.
34. Zheng X, Huang Y, Zheng C, Dong S, Liang W. 2010. Alginate-chitosan-PLGA composite microspheres enabling single-shot hepatitis B vaccination. AAPS J 12:519-24.
35. Khanna O, Larson JC, Moya ML, Opara EC, Brey EM. 2012. Generation of alginate microspheres for biomedical applications. J Vis Exp doi:10.3791/3388.
36. Post DMB, Slutter B, Schilling B, Chande AT, Rasmussen JA, Jones BD, D'Souza AK, Reinders LM, Harty JT, Gibson BW, Apicella MA. 2017. Characterization of Inner and Outer Membrane Proteins from *Francisella tularensis* Strains LVS and Schu S4 and Identification of Potential Subunit Vaccine Candidates. MBio 8.
37. Islam MA, Yun CH, Choi YJ, Cho CS. 2010. Microencapsulation of live probiotic bacteria. J Microbiol Biotechnol 20:1367-77.
38. Cole LE, Yang Y, Elkins KL, Fernandez ET, Qureshi N, Shlomchik MJ, Herzenberg LA, Herzenberg LA, Vogel SN. 2009. Antigen-specific B-1a antibodies induced by

Francisella tularensis LPS provide long-term protection against F. tularensis LVS challenge. Proc Natl Acad Sci U S A 106:4343-8.
39. Stenmark S, Lindgren H, Tarnvik A, Sjostedt A. 2003. Specific antibodies contribute to the host protection against strains of *Francisella tularensis* subspecies *holarctica*. Microb Pathog 35:73-80.
40. Ajdary S, Dobakhti F, Taghikhani M, Riazi-Rad F, Rafiei S, Rafiee-Tehrani M. 2007. Oral administration of BCG encapsulated in alginate microspheres induces strong Th1 response in BALB/c mice. Vaccine 25:4595-601.

Chapter 5: The *Francisella tularensis* Polysaccharides: A Search for the Real Capsule

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5.1. Abstract

Francisella tularensis is a Tier I Select Agent that causes a "plague-like" disease in humans. Extensive research during the 21st century has uncovered various virulence factors of the bacterium while attempting to create an efficacious vaccine. At least two such virulence factors are described as the capsules of *Francisella*: the O-antigen capsule and the Capsule-like Complex (CLC). These two separate capsules aid in immune avoidance and have been described as the single capsule of *Francisella*. However, these capsules are separate entities that differ in composition and genetic basis. The O-antigen capsule contains a polysaccharide nearly identical to the lipopolysaccharide (LPS) O-antigen; whereas the CLC is a heterogenous complex of glycoproteins, proteins, and possibly outer membrane vesicles and tubes (OMV/Ts). In this review, the current understanding of these two capsules is summarized and the historical references to "capsules" of *F. tularensis* clarified. A significant amount of research has explored the composition of each capsule and the genes involved in synthesis of the polysaccharide portion of each capsule. Areas of future research include further research of the molecular pathways responsible for each capsule and further elucidating the role that each capsule plays in virulence.

5.2. Introduction

Francisella tularensis is the gram negative, facultative intracellular bacterium responsible for the zoonotic "plague-like" disease tularemia (1). Tularemia is characterized by an acute onset of flu-like symptoms and granulomatous lesions of various tissues including the lungs, lymph nodes, and spleen (1, 2). The various forms of tularemia – pneumonic, glandular, ulceroglandular, oculoglandular, oropharyngeal, and typhoidal – are highly dependent on the route of infection. The pneumonic form is caused by inhalation of bacterial cells and is considered the most serious (2). Arthropod bites, contact with infected tissues, laboratory exposure, and aerosolization are all considered routes of infection for *F. tularensis* (2). Additionally, *F. tularensis* infects numerous animals and invertebrates, which have the potential to become sources of infection for humans (3-5).

The disease severity of tularemia is also dependent on the infecting strain. *F. tularensis* subspecies *tularensis* (Type A) causes more severe disease that is associated with possible mortality (2, 6). Type A1 strains leading to pneumonic tularemia are associated with 30% mortality if the patient is not treated with antibiotics (2). Type A2 strains are less severe, similarly to subspecies *holarctica* (Type B) (2, 6). Geographically, Type B strains are found across the Northern Hemisphere whereas Type A strains are concentrated in specifically in North America (6).

The Centers for Disease Control and Prevention (CDC) consider both *F. tularensis* subspecies *holarctica* and *tularensis* Tier I Select Agents for their potential use as bioweapons. The World Health Organization estimated that a 10-kilogram aerosolized dispersal of *F. tularensis* could lead to 50,000 infections and a possible 4,000 deaths (2, 7). *F. tularensis* has been deemed a potential bioweapon because of the level of virulence, ease of aerosolized dispersal, persistence in the environment, and the non-specific clinical signs (7, 8). Subspecies *novicida* (hereto referred as *F. novicida*) is not considered a significant threat to immunocompetent individuals and is not considered a Select Agent (9). No approved vaccine currently exists to prevent tularemia as the Live Vaccine Strain (LVS) is no longer considered safe and efficacious due to genetic instability, possible retained virulence, and a lack of protection against pneumonic infection (10).

Since the resurgence of tularemia research following the Amerithrax anthrax attack in 2001, many of the virulence properties of the bacterium have been identified while attempting to develop an effective vaccine (11). Well characterized virulence factors include the *Francisella* pathogenicity island (FPI) that is essential for intracellular replication (12-14) and the unusual lipopolysaccharide (LPS) involved in evasion of host defenses (10, 15-17). In addition to those virulence factors, *Francisella tularensis* also produces two different capsules: an electron transparent O-antigen capsule (18) and an electron dense capsule-like complex (CLC) (19, 20). Unfortunately, the common use of the phrase "capsule" of *Francisella* causes confusion when assessing previous literature on the subject as the identity and characterization of capsule, as it relates to *F. tularensis*, was not definitively established until 2011. This review aims to define both capsules and to discuss the significance of these capsules and other polysaccharides of *F. tularensis* in relation to the pathogenesis and virulence of the bacterium.

5.2.1. The Electron Dense Versus Electron Transparent Capsule

F. tularensis was first described by Dr. George W. McCoy as causing a "plague-like" disease of the squirrels in Tulare County, California in 1910 (21). Since that discovery, work on creating an effective vaccine commenced. Many efforts were taken to find an immunogenic antigen conserved across *F. tularensis* strains to serve as a safe, subunit vaccine. Immunodiffusion assays compared several strains and determined that a conserved extracellular polysaccharide existed between strains (22). Over a decade later, A. M. Hood (23) reported the first description and isolation of a capsule from *F. tularensis* SchuS4 in 1977. The capsule was described as being electron transparent and containing the sugars mannose, rhamnose, and dideoxy sugars (23). The broth grown culture and negatively stained bacteria were surrounded by a transparent halo against a stained background when viewed by electron microscopy (Figure 5.1a). However, in 1994, Cherwonogrodzky (20) reported the presence of an electron dense capsule surrounding *F. tularensis* LVS and that repeated subculture of the bacterium on the synthetic medium Chamberlain's Defined Medium (CDM) increased the presence of this electron dense material (Figure 5.1b). Physically, this second capsule also differed from the halo-like appearance of the electron transparent capsule in that this capsule was more irregular, branching, and extended further from the bacterial cell wall. These earlier reports that are based on physical appearance and partial chemical analysis set the groundwork for further chemical and molecular characterization of these two separate capsules of *F. tularensis*: the electron-

transparent O-antigen capsule and the electron-dense Capsule-Like Complex (CLC). Figure 5.1 shows early and recent representative examples of the basic appearance of these two capsule types; Figure 5.1A and 5.1C the O-antigen capsule and Figure 5.1B and 5.1D the CLC.

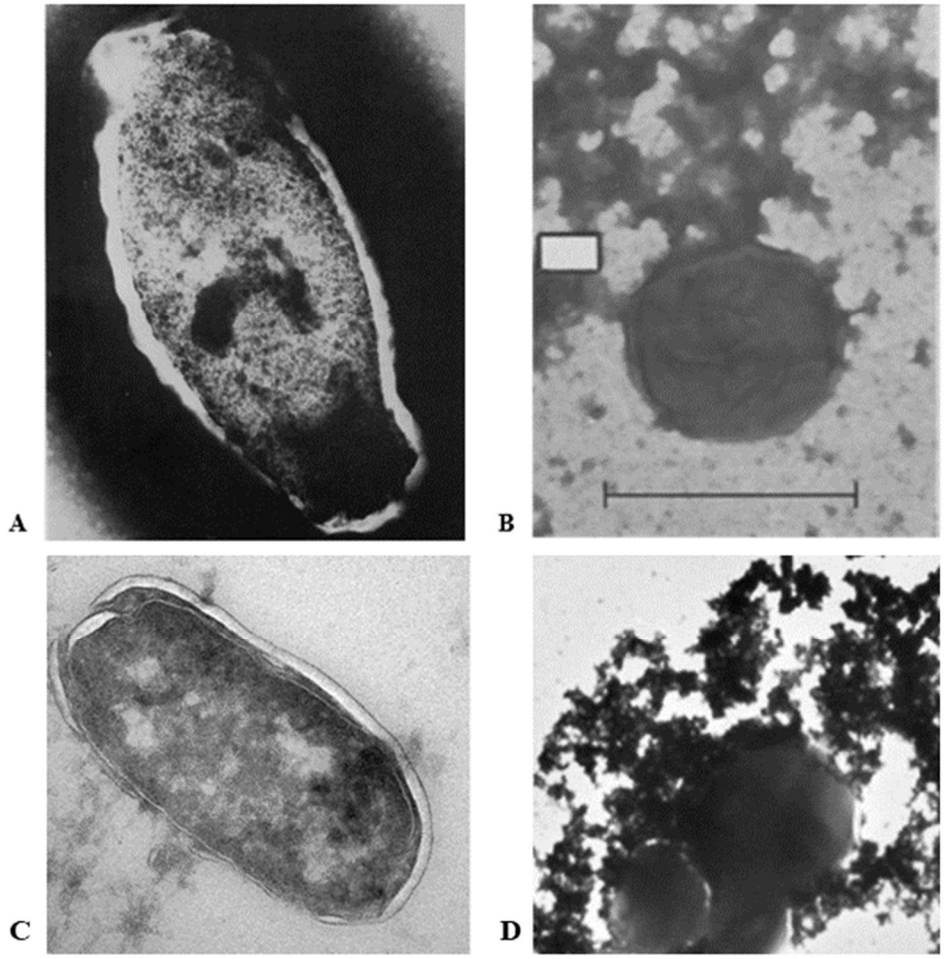

Figure 5.1. Microscopic difference between the O-antigen capsule and the Capsule-Like Complex (CLC) of *Francisella*.
Both the O-antigen capsule and the CLC can be seen surrounding F. tularensis cells using electron microscopy. The O-antigen capsule will present as an electron transparent halo closely surrounding each cell (a, c); whereas the CLC will present as an electron-dense material variably extending out from the cell (b, d). Pictograph "a" adapted from Hood (23), "b" adapted from Cherwonogrodzky (20), "c" adapted from Apicella *et al*. (18), and "d" adapted from Champion *et al.* (24) With permission.

5.3. The O-Antigen Capsule

5.3.1. Discovery

The initial basis for classifying *F. tularensis* as being encapsulated was the demonstration of a change in colony phenotype after serial passage (25, 26) or application of various chemical mutagens (23, 27). Virulent *F. tularensis* grown on supplemented blood agar exhibits a small,

smooth, white or blue opaque colony phenotype (Blue colonies) (25, 26, 28). Serial passage or chemical mutagenesis spontaneously leads to the appearance of *F. tularensis* colonies that appear larger, rougher, and grey (Grey colonies) that, based on many reports, are attenuated (25, 26, 28). Eigelsbach *et al.* used oblique lighting to classify colonies as blue or grey in heterogenous cultures and showed that prolong growth of a blue isolate led to increased numbers of spontaneously growing grey colonies (26). Mice are also more severely affected following inoculation with the blue colonies than with the grey colonies (26). This effect of apparent phase variation has been shown repeatedly and can be attributed, in most cases, to the loss of the O-antigen on the lipopolysaccharide (LPS) (10, 29). Prior to that determination, this phenotypic change was used to delineate a change in surface polysaccharide components between strains. It is worthwhile to note that the O-antigen locus is bordered up and down stream by the transposase and psuedotransposase IS sequences *isftu2* and *isftu1*, which may contribute to loss of O-antigen expression (30).

Characterization of these strains via electron microscopy documented the presence of an electron transparent ring around the blue, virulent strains (Figure 5.1a) and the lack of such a ring around the avirulent grey strains (23). The presence of this electron transparent ring correlated with the colony phenotypes and the ability of *Francisella* to resistant the bactericidal effects of serum. Grey strains that lacked this electron transparent capsule were sensitive to the bactericidal effects of serum whereas the blue strains expressing O-antigen were serum resistant (23, 27, 31). A crude extract of this capsular material revealed the presence of mannose, rhamnose, dideoxy sugars, and fatty acids which differentiated the extracted material from components in the bacterial cell wall (23). Based on these findings, *Francisella tularensis* has been listed as an encapsulated organism for years, but further isolation and characterization of this capsule was only recently completed in 2010 by Apicella *et al* (18).

Further characterization of the O-antigen capsule was made possible by the production of monoclonal antibodies against a crude capsular extract prepared in a similar manner to Hood's capsular extraction (18). Screening of these monoclonal antibodies against *F. tularensis* extracts identified a material that (1) had a high molecular weight (>100 kDa) and a diffusely migrating band, (2) was protease resistant, and (3) precipitated in ethanol, hallmarks of a possible capsular polysaccharide. Monoclonal antibody (MAb) 11B7 bound to this diffusely migrating high molecular size band in six *F. tularensis* strains that appeared to fit those aforementioned

characteristics (18). MAb 11B7 also circumferentially labels *F. tularensis* cells, appearing to bind to a cell surface associated antigen that is electron-transparent, and the antigen could only be identified by electron microscopy when labelled with MAb 11B7 (18). This material was effectively isolated from the crude extract through proteinase digestion, phenol extraction, Triton X-114 treatment, and chromatography (18). Compositional analysis of the material and reaction with an anti-LPS MAb (FB11) indicated that this capsular polysaccharide was highly similar to the LPS O-antigen (18). Further, *F. tularensis* mutants with disruptions in O-antigen synthesis genes did not produce this O-antigen capsule or the O-antigen of the LPS (18). Apicella *et al*. (18) concluded that this extracellular carbohydrate was a Group 4 capsular polysaccharide or an O-antigen capsule.

5.3.2. Composition and Structure

The O-antigen capsule appears to be conserved amoung *F. tularensis* strains, and is composed of a 792Da repeating unit identical to the subunit of the LPS O-Antigen (18). Mass spectrometry and NMR analysis was used to show that the carbohydrate is a repeating tetrasacchraide of 4)-α-D-Gal-NAcAN-(1->4)- α-D-GalNAcAN-(1->3)-β-D-QuiNAc-(1->2)-β-D-Qui4NFm-(1- units (18), which is identical to the chemical structure of LPS O-antigen (15). However, the O-antigen capsule is not fully identical to the LPS O-antigen immunologically. The LPS O-antigen MAb FB11 binds both the O-antigen capsule and the LPS O-antigen; whereas the MAb 11B7 only binds the O-antigen capsule indicating there are difference in the epitopes of these two components (18). This difference in binding helps distinguish strains that produce LPS O-antigen and not the O-antigen capsule. Further differences between the O-antigen capsule and the LPS exist. For example, 3-deoxy-*D-manno*-2-octulosonic acid (KDO), a portion of the LPS core, was not detected in any capsular sample (18).

Additional metabolic labeling studies with [C^{14}] sodium acetate identified a Lipid A-like molecule bound to the O-antigen capsule carbohydrate (32). This lipid is covalently bound to the O-antigen capsular polymers and can be separated from the carbohydrate by treatment with a mild acid (32). The fatty acids present include three 3-OH fatty acids and one non-hydroxylated fatty acid and are grossly similar to the fatty acids present in the *F. tularensis* LPS Lipid A structure (32). The ratio of the hydroxylated fatty acids differs between this Lipid A-like structure, the LPS Lipid A, and free Lipid A. Both LPS Lipid A and free Lipid A have a ratio of greater than 5 to 1 of 3-OH-18:0 to 3-OH-16:0 fatty acids compared to less than 3 to 1 for the

Lipid-A like structure (32). The most prominent non-hydroxylated fatty acid in the Lipid A-like structure is a C14:0 lipid compared to the C16:0 fatty acid found in the LPS Lipid A and free Lipid A (32). Further structural analysis of this lipid A-like structure has been hampered by the inability to purify larger quantities of the material.

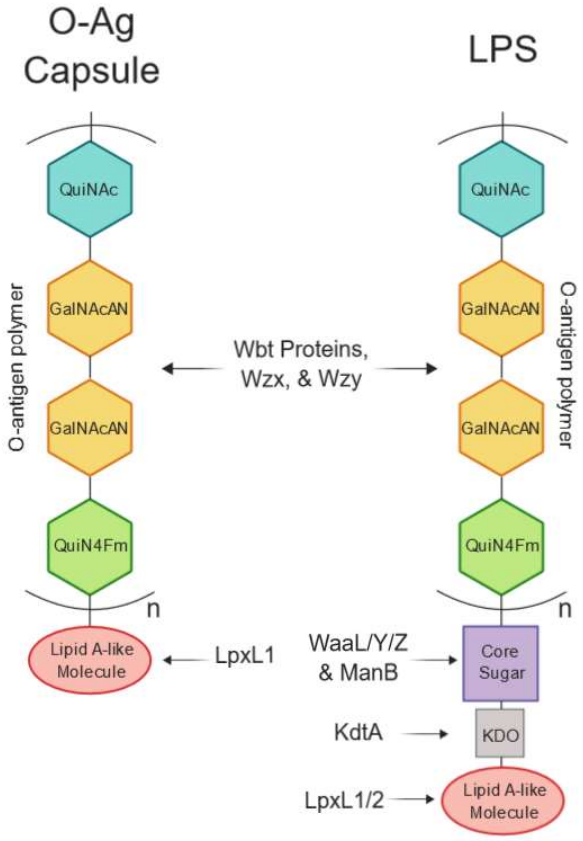

Figure 5.2. Structure of the *Francisella tularensis* O-antigen capsule compared to the lipopolysaccharide.
The polymers of the O-antigen of the *F. tularensis* LPS and O-antigen capsule share the same tetrasaccharide repeating units (4)-α-D-Gal-NAcAN-(1->4)- α-D-GalNAcAN-(1->3)-β-D-QuiNAc-(1->2)-β-D-Qui4NFm-(1-) that are produced by the Wbt proteins, Wzx, and Wzy. Interruption of these proteins results in the loss of O-antigen laddering, the O-antigen capsule, and O-antigen protein glycosylation. The Lipid A molecules, both of the O-antigen capsule and LPS, are assembled with Lpx proteins, but differ in composition. Mutation of LpxL1 results in the loss of a Lipid A subspecies of the O-antigen capsule, but does not affect expression of the O-antigen capsule. *F. tularensis* strains with interruptions of core sugar proteins WaaL, WaaY, WaaZ, and ManB lack LPS O-antigen and either lack or contain a modified O-antigen capsule indicating some role in O-antigen capsule assembly.

5.3.3. Genetic Machinery

In addition to structure, the O-antigen capsule and the LPS O-antigen share a similar biosynthetic pathway (Figure 5.2). The full biosynthetic pathway necessary for synthesis,

assembly, and transport of the exported mature capsule has not been definitively defined, but general characteristics exist. In general, O-antigen glycosyltransferases (FTT1451c to FTT1464c, Table 5.1) are responsible for the formation of the repeating tetrasaccharide of the O-antigen for both the O-antigen capsule and LPS. Interruptions or deletions of the responsible genes in LVS, including *wbtI*, *wbtA1*, *wbtM*, and *wbtC*, results in loss of O-antigen capsule expression and a loss of O-antigen laddering on the LPS (18). Interruption of the gene *wzy*, responsible for an O-antigen polymerase, also disrupts the O-antigen capsule and LPS formation (33). Therefore, production of the O-antigen repeating unit appears to be through the same biosynthetic pathway regardless of the final product (*i.e.* O-antigen capsule, LPS, or glycosylated protein).

However, there appear to be differences in subsequent steps that differentiate the O-antigen capsule and the O-antigen of LPS. Waa proteins WaaY and WaaZ appear to be responsible for the assembly of the core sugar onto Lipid A with WaaL mediating ligation of the O-antigen subunit to the core sugars on the Lipid A (34). Disruption of the responsible genes eliminates expression of the O-antigen on the LPS and, in the case of WaaZ and WaaY, leads to a modified or truncated core sugar (33, 34). The O-antigen capsule is similarly disrupted in strains lacking WaaY or WaaL (33-35) indicating that a similar pathway may be used to ligate and export the capsule. How these proteins specifically contribute to final production of O-antigen capsule has not been determined. Proteins responsible for core sugar assembly and ligation likely play a role in producing the final, exported O-antigen capsule, but do not play a role in assembly of the O-antigen subunits. These proteins may play a role in ligating the final O-antigen chain to a Lipid A-like molecule prior to exporting the capsule.

The late acyltransferase LpxL1 may also play a role in the export of a final O-antigen capsule (32). *F. tularensis* Lpx genes appear to be differentially controlled by temperature and have distinct fatty acid selectivity that accounts for the ability of *F. tularensis* to produce a host of Lipid A molecules (36). Barker *et al*. (32) notes that the interruption of LpxL1 by McLendon *et al.* (36) leads to a reduction of a Lipid A subspecies with remarkable similarities to the Lipid A-like molecule of the O-antigen capsule. Mutation of the LpxL1 gene leads to the loss of the LPS O-antigen, but does not affect the O-antigen capsule (18) The O-antigen capsule Lipid A-like molecule appears to require the LpxL1 gene for production. However, production of this Lipid A-like molecule does not appear to be necessary for full expression of the O-antigen capsule or transport of the O-antigen capsule.

Table 5.1. Proposed genes involved in O-antigen Capsule and CLC synthesis

Subsp. *tularensis* SchuS4 ORF	Subsp. *holarctica* LVS ORF	Gene Name	Predicted Protein Product	Role in CLC or O-Antigen Capsule Production	Mutation Study[a]
FTT_0789	FTL_1432	*rpe*	D-ribulose-phosphate 3-epimerase	CLC glycosylation	
FTT_0790	FTL_1431		Glycosyltransferase	CLC glycosylation	
FTT_0791	FTL_1430	*galE*	UDP-glucose 4-epimerase	CLC glycosylation	(37)
FTT_0792	FTL_1429		Glycosyltransferase	CLC glycosylation	
FTT_0793	FTL_1428		ATP-binding membrane transporter	CLC glycosylation	
FTT_0794	FTL_1427		Hypothetical protein	CLC glycosylation	
FTT_0795	FTL_1426		Hypothetical protein	CLC glycosylation	
FTT_0796	FTL_1425		Hypothetical protein	CLC glycosylation	
FTT_0797	FTL_1424		Galactosyltransferase	CLC glycosylation	
FTT_0798	FTL_1423		Galactosyltransferase	CLC glycosylation	(19, 37)
FTT_0799	FTL_1422		Mannosyltransferase	CLC glycosylation	(19)
FTT_0800	FTL_1421		Haloacid dehalogenase	CLC glycosylation	
FTT_1236	FTL_0708	*waaY*	Hypothetical protein	O-antigen capsule assembly	(33-35)
FTT_1237	FTL_0707	*waaZ*	Glycosyl transferase family protein	O-antigen capsule assembly	(33, 34)
FTT_1238	FTL_0706	*waaL*	Hypothetical protein	O-antigen capsule assembly	(33-35)
FTT_1447c	FTL_0609	*manB*	Phophomannomutase	O-antigen capsule assembly	(33, 34)
FTT_1450c	FTL_0606	*wbtM*	dTDP-D-glucose 4,6-dehydratase	O-antigen production	(18)
FTT_1451c	FTL_0605	*wbtL*	Glucose-1-phosphate thymidylyltransferase	O-antigen production	
FTT_1452c	FTL_0604	*wbtK*	Glycosyltransferase	O-antigen production	(18)
FTT_1453c	FTL_0603	*wzx*	O-antigen flippase	O-antigen production	(33, 34)
FTT_1454c	FTL_0602	*wbtJ*	O-antigen protein	O-antigen production	
FTT_1455c	FTL_0601	*wbtI*	Sugar transamine/ perosamine synthetase	O-antigen production	(18)
FTT_1456c	FTL_0600	*wbtH*	Glutamine amidotransferase/ asparagine synthase	O-antigen production	
FTT_1457c	FTL_0599	*wbtG*	Hypothetical protein	O-antigen production	
FTT_1458c	FTL_0598	*wzy*	Membrane protein/O-antigen protein	O-antigen production	
FTT_1459c	FTL_0597	*wbtF*	NAD dependent epimerase	O-antigen production	
FTT_1460c	FTL_0596	*wbtE*	UDP-glucose/GDP-mannose dehydrogenase	O-antigen production	
FTT_1461c	FTL_0595	*wbtD*	Galacturonosyl transferase	O-antigen production	
FTT_1462c	FTL_0594	*wbtC*	UDP-glucose 4-epimerase	O-antigen production	(18)
FTT_1463c	FTL_0593	*wbtA2*	Galactosyl transferase	O-antigen production	
FTT_1464c	FTL_0592	*wbtA1*	dTDP-glucose 4,6-dehydratase	O-antigen production	(18)
FTT_0232c	FTL_0179	*lpxL1*	LPS fatty acid acyltransferase	Lipid A-like molecule production	(32)

[a] References listed have analyzed production of the O-antigen capsule or CLC by strains with mutations in the specified genes.

5.3.4. Role in Virulence

The O-antigen of LPS has long been understood to play a significant role in pathogenesis of *Francisella*. Lack of O-antigen leads to serum sensitivity and a loss of virulence for *F.*

tularensis strains (10, 16, 33, 38, 39). All mutants lacking the O-antigen had growth defects in cell culture and were attenuated in the mouse model (18, 32, 34). Strains with mutations in *waaY* and *waaL* interrupted strains lack both the LPS O-antigen and the O-antigen capsule are more sensitive to complement-mediated lysis with serum, are phagocytized more readily, and are unable to replicate as efficiently as wildtype *F. tularensis* (35). These strains remain capable of disseminating to the liver and spleen after intranasal inoculation and are lethal to mice, but the mean time to death increases significantly compared to infection by the wildtype (34). Histopathology of tissue samples from mice infected with *waaY* and *waaL* mutants also had a greater amount of gross inflammation, including necrosis, compared to those infected with the wildtype strain (34). Therefore, the O-antigen of both the LPS and the O-antigen capsule may function as an immune-avoidance mechanism preventing the host from mounting inflammatory defenses.

However, the contribution of the O-antigen capsule compared to the O-antigen of the LPS to virulence and immune-avoidance has not been elucidated. Both O-antigen components share similar biosynthetic pathways and studies examining virulence utilize genetic knockouts or interruptions at shared points in the pathway. Differential growth studies have demonstrated that *F. tularensis* grown in Brain Heart Infusion (BHI) broth is similar to host-adapted, or macrophage grown, *F. tularensis* (40-42). Studies of the O-antigen capsule of *F. tularensis* grown in different media indicate, through antibody binding, that BHI-grown *F. tularensis* produce the greatest amount of O-antigen capsule and are the least accessible to antibodies directed at outer membrane components (42). When grown in Mueller-Hinton (MH) broth, which results in bacteria that are phenotypically distinct from macrophage-grown *F. tularensis*, less O-antigen capsule is produced and there is an increase in binding of antibodies to outer membrane components (42). BHI-grown *F. tularensis* also stimulate a significantly lower TNF and IL-1β response from macrophages compared to MH-grown *F. tularensis* where the TLR2 ligand lipoprotein Tul4A is readily accessible (42). Collectively, these results suggest that the O-antigen capsule has a role in immune-avoidance by preventing access of immune molecules to surface components of *Francisella*.

5.4. The Capsule-like Complex

5.4.1. Discovery

Cherwonogrodzky *et al*. (20) investigated the effect of subculturing the live vaccine strains (LVS) on its virulence as there were reports that this treatment restored virulence of this attenuated strain. Subculturing *F. tularensis* on a mildly acidic and defined medium, such as Chamberlain's Definied Medium (CDM), both decreased the lethal dose in mice and increased the amount of negatively staining material surrounding the cells, as observed by electron microscopy (Figure 5.1. Microscopic difference between the O-antigen capsule and the Capsule-Like Complex (CLC) of *Francisella*. (20). Multiple passages in CDM greatly increased the material and the mucoid appearance of the colonies on agar suggesting this was a capsular polysaccharide that was upregulated under these less than ideal growth conditions (20). This capsule was thought to be a similar capsule to what has been described by others (23, 43). Clemens *et al.* (44, 45) described a similar fibrinous structure surrounding intracellular *F. tularensis* within phagosomes suggesting this possible capsule was necessary for intracellular survival.

Around the same time Apicella *et al.* (18) described the structure of the O-antigen capsule, Bandara *et al.* (19) isolated a separate capsule-like material from *F. tularensis* LVS. This material was produced more heavily around LVS cells after multiple subcultures in CDM broth, followed by growth for 5 days on CDM agar at 32°C with 7% CO_2 (19). Interruption of genes within a novel glycosylation locus (FTT_0789 to FTT_0800, Table 5.1) abolished production of the CLC (19). This material was different from the O-antigen capsule in appearance, carbohydrate composition, and genetic machinery. Termed the Capsule-like Complex or CLC, the isolated material contained the sugars mannose, galactose, and glucose and could be isolated from strains completely lacking the O-antigen both on the LPS and as a capsule (19). The CLC was therefore determined to be a separate entity from the O-antigen capsule.

5.4.2. Composition and Structure

The CLC is a heterogenous complex of protein and carbohydrate (19) whose expression is enhanced by passaged on chemically defined medium and growth at a lower temperature (19, 20, 24). The material was first isolated from O-antigen mutants of *F. tularensis* LVS serially subcultured on defined medium so there was no contamination by LPS O-antigen (19). The CLC was initially extracted using phenol followed by purification steps including enzymatic digestion,

ethanol precipitation, and ultracentrifugation (19). Electrophoretic separation of the material revealed a large array of various weighted proteins and the presence of a high molecular weight (HWM) carbohydrate with no evidence of LPS or O-antigen contamination of the extracts (19). The CLC extracts did not react with the O-antigen capsule monoclonal antibody 11B7 and an identical extract could be isolated from the O-antigen negative strain LVS $WbtI_{G197V}$ further differentiating the two materials (19, 24). The proteins present were Proteinase K resistant and approximately one tenth of the material was a combination of mannose, glucose, and galactose carbohydrate residues (19). However, once sedimented by ultracentrifugation or concentration by ultrafiltration, the CLC became highly insoluble and could not be resolubilized, preventing further resolution of the components (19). Bandara *et al.* (19) postulated that the complex material contained multiple glycoproteins and a HMW carbohydrate. Similar to the O-antigen capsule, the CLC appears to be conserved between *Francisella* Type A and Type B strains as both LVS and SchuS4 have similar to identical CLC electrophoretic profiles (24).

To improve solubility of the CLC, alternative extraction mediums were examined, resulting in a similar extraction protocol utilizing urea in place of phenol and no proteinase K. To further improve solubility, the detergent Triton X-114 was used for some extractions. These modifications resulted in similar extract profiles that were more water-soluble and amendable to further analysis (24). Size fractionation of this urea extracted material using a GelFree fractionation unit revealed that the CLC is mainly composed of proteins or glycoproteins less than 150kDa in weight and that the HMW band of greater than 150kDa was no longer present (24). As a result, Champion *et al*. (24) postulated that the previously observed HMW band was an aggregate of glycoproteins and proteins that disassociated during size fractionation. Based on mass spectrometry analysis, the CLC is composed of mainly acidic or hydrophobic amino acids that likely contribute to the aggregation and insolubility of this complex prior to fractionation (24). Analysis of in-gel digests of these proposed aggregates prior to fractionation indicated the presence of a 420-Da subunit that appears to be glycan in nature (24).

Following fractionation of the CLC, a 45-kDa protein was the most reactive to CLC hyperimmune serum (24). This protein is similar in weight to a 45kDa protein described by Huntley *et al*. (46) that was isolated from outer membrane proteins and is highly immunogenic. This unidentified protein could be FopA, a highly immunogenic outer membrane protein that has been shown to be glycosylated (47). FopA has also been confirmed as present in Outer

Membrane Vesicles and Tubes (OMV/Ts) produced by *F. novicida* (48). Further electron microscopic analysis and mass spectrometry suggests that the CLC may in part be or contain OMV/Ts as it shares a gross similar appearance (24). All total 68 proteins have been identified in LVS CLC extracts, as determined by nano-liquid chromatography-mass spectrometry of trypsin digests; 56 proteins were identified in the insoluble portions of the extract and 12 proteins were identified within the soluble portions (24). Howevere, many of the proteins were heavily glycosylated and could not be identified (24). Of these identified CLC proteins, 8 of the 12 soluble proteins and 38 of the 56 insoluble proteins are present in *F. novicida* OMV/Ts (48, 49). Of the identified CLC proteins, 22 have not been identified in *Francisella* OMV/Ts. However, the protein composition of OMV/Ts is based on *F. novicida* OMV/Ts and not Type A or Type B OMV/Ts, which have not yet been characterized. Isolation and identification of proteins within Type A or B OMV/Ts is necessary to determine the exact extent of overlap between the CLC and the OMV/Ts. At this time, definitive determination of whether the CLC is purely enhanced expression or over-production of OMV/T or if OMV/Ts are a separate entity that have associated with the CLC cannot be made.

A second putative glycan fragment was observed in soluble portions of the CLC that contains the HMW carbohydrate (24). This hexasaccharide is composed of monosaccharide units of 203-223-203-162-162-203 Da with 203 Da possibly representing HexNAc, 162 Da possibly representing an undetermined hexose, and 223 Da representing an unknown glycan unit (24). An associated peptide fragment was unable to be effectively identified within this extract. This second glycan entity resembles the hexasaccharide motiety that is *O*-linked to the protein DsbA and has been shown to require the same glycosylation locus as the CLC (37).

The HMW band of the CLC may be the same carbohydrate smear described by Zarrella *et al.* (42). This HMW carbohydrate is a diffusely stained region that extends above 225-kDa, is protease-resistant, and is labeled with both carbohydrate and protein stains (42). The profile of the HMW carbohydrate greatly resembles the profile of the CLC prior to fractionation; however, confirmation that the HMW and CLC are the same entity has not been determined.

5.4.3. Genetic Machinery

Two known glycosylation or polysaccharide biosynthesis loci exist within the *F. tularensis* genome. One of these two loci is the previously mentioned O-antigen locus involved in production of O-antigen subunits for LPS and the O-antigen capsule. The second locus spans

from FTT_0789 to FTT_0800 (Table 5.1) and contains proteins with predicted functions of glycosylation. Homologs of this gene cluster have been identified in multiple subspecies of *F. tularensis* supporting the hypothesis that this is a conserved mechanism with a possible role in virulence (19, 24, 37, 50). Interruption of two genes within the locus, FTT_0791 and FTT_0798, in SchuS4 interrupted glycosylation of the protein DsbA, an essential virulence factor and possible lipoprotein, but did not affect O-antigen production (37). Conversely, interruption of the O-antigen locus did not affect the presence of the glycoform on DsbA, verifying that these loci are involved in two separate glycosylation processes (37). The specific glycosylation moiety observed in this study is similar to the hexasaccharide that has been found in soluble portions of the CLC (37).

An LVS strain with a double deletion targeting the homologous genes FTT_0789 and FTT_0790 was created specifically to study the effect of this glycosylation locus on the CLC (19). Deletion of these two glycosyltransferases significantly reduced the carbohydrate present in CLC extracts, and the CLC was no longer evident around the cells by electron microscopy (19). A similar mutation in SchuS4 resulted in the same effect with the level of protein in the CLC remaining similar to the parent (24). CLC observed in these mutants was significantly lower in concentration than the parent and did not associate closely with the bacterial cells (51). This genetic locus appears to function primarily, if not exclusively, in glycosylation modifications of *F. tularensis* CLC proteins.

The genes required for exportation of the CLC have not been defined. Genes have been identified in *F. novicida* that dramatically decrease OMV/T production or export (48, 52). These genes possibly encode for proteins involved in carbon metabolism, lipoic acid biosynthesis, and a cytoplasmic membrane protein (52). Each of these genes (FTN_0337/*fumA*, FTN_1333/*tkA*, FTN_0908, and FTN_1037) have homologs in the more virulent *F. tularensis* subspecies that are greater than 94% identical. The proposed function of these genes is based on bioinformatic analysis and has not been confirmed experimentally. If the CLC is in part OMV/Ts, then mutagenesis of these genes theoretically should reduce the production of the CLC. Analysis of CLC production of these mutants has not been conducted.

5.4.4. Role in Virulence

Within the phagosome, *Francisella* cells produce a fibrillar coating encircling the bacterial cells that appear to bud off within the phagosome and is shed prior to escaping into the

cytosol (44, 45). By electron microscopy, this material appears similar grossly to the CLC of bacterial cells grown on solid defined medium. Confirmation that this material is the CLC has not been determined, but genetic CLC glycosylation knockouts shed a light on the role of the CLC as a virulence factor. Due to the heterogeneous nature of the CLC, a genetic knockout abolishing the entire presence of the CLC would be difficult. Removing glycosylation of the CLC, though, is possible as previously mentioned by targeting two glycosyltransferases within the CLC glycosylation locus. Interruption of CLC glycosylation attenuates LVS in the mouse model (19). Nonetheless, *in vitro* this glycosylation mutant LVSΔ1423/1422 was not sensitive to complement-mediated killing and was able to replicate within a macrophage cell line (19). However, immunization with this attenuated mutant or with LVS CLC conjugated to an immunogenic protein were highly protective against a high-dose LVS challenge (19, 24).

Unfortunately, the same results were not obtained with the more virulent Type A strain. Type A CLC immunization did not protect mice against a high dose intranasal challenge with *F. tularensis* SchuS4, nor was strain ScuS4 attenuated following mutagenesis of the same genes that attenuated LVS (SchuS4Δ0789/799), although a similar loss of CLC glycosylation occurred (24). Subunit vaccines against tularemia do not provide adequate protection against a virulent high dose challenge as the subunit vaccine may not stimulate the needed cellular mediated immunity necessary for protection (53). Attenuated live strains are more desirable for inducing protective T-cell immunity due to the intracellular lifestyle of *Francisella*. This variation in attenuation of virulence highlights the difference between *F. tularensis* LVS and SchuS4 from pathogenesis studies in mice. Although LVS is still virulent for mice, the strain is of low virulence for humans and is less virulent in mice than *F. tularensis* SchuS4. Due to this discrepancy in virulence, interrupting glycosylation in SchuS4 may not be substantial enough to render the strain attenuated in the mouse model. Further testing of these strains in a more relevant animal model such as the Fischer 344 rat, which is less sensitive to *F. tularensis* than the mouse (54) may be warranted.

5.5. Effect of Growth Conditions

F. tularensis is able to persist in a vast range of niches in the host, which include intracellular, the extracellular milieu, and the environmental. As such, *Francisella* has developed mechanisms to sense changes in the environment and alter gene expression to adapt to

nutritionally variable conditions. The ability of *F. tularensis* to adapt to the host may in part be due to recognizing changes in the amino acid and free iron concentrations within the host. *Francisella* would possibly encounter environments deprived of amino acids or free iron extracellularly within the host (41, 42, 52). Production of extracellular carbohydrates and OMV/Ts directly correlate with the concentration of free amino acids present (Figure 5.3. Effect of Growth Conditions on the Extracellular Carbohydrates of *Francisella.*

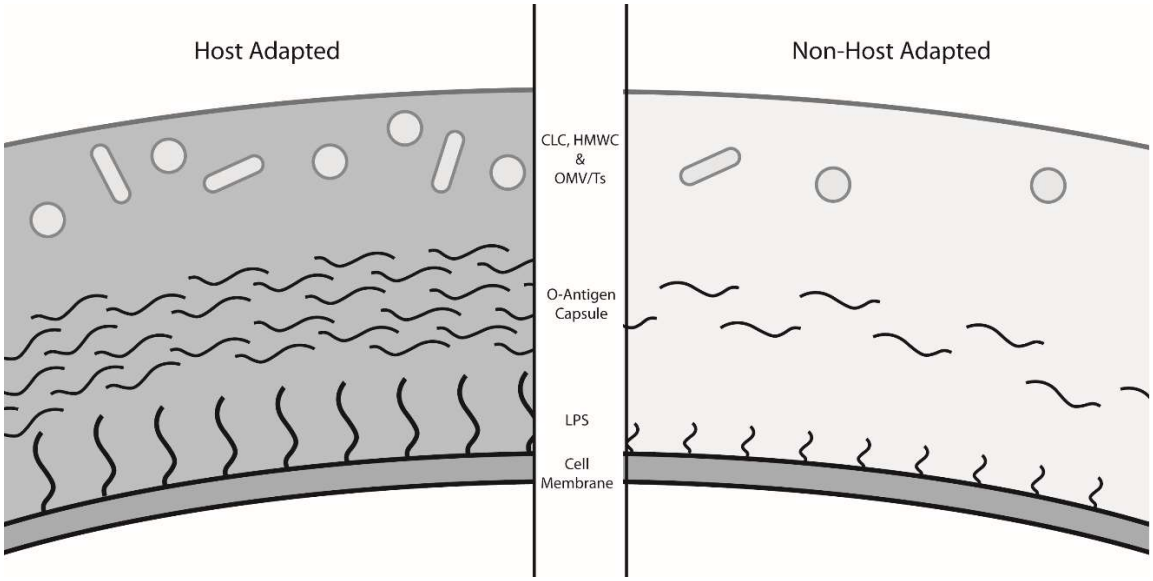

Figure 5.3. Effect of Growth Conditions on the Extracellular Carbohydrates of *Francisella.* *Francisella* responds to many environmental cues including free amino acid concentration. The availability of free amino acids for bacterial cells signals a change in the extracellular carbohydrate profile of *F. tularensis* including the LPS, the O-antigen Capsule, the CLC, the HMW Carbohydrate (HMWC), and the OMV/Ts. Host adapted *Francisella*, bacteria that live extracellularly within the mammalian host, live in an environment of low free amino acids, similarly to the culture medium BHI. These host-adapted *Francisella* cells produce longer O-antigen polymers on the LPS, produce more O-antigen capsule, produce a higher concentration of OMV/Ts, and produce greater amount of CLC or HMW compared to non-host adapted bacteria, or those grown in amino acid rich environments such as the culture medias TS or MH. *Francisella* grown in these conditions produce far fewer OMV/Ts, less O-antigen capsule, shorter O-antigen polymers on the LPS, and less CLC.

Culture of *F. tularensis* in BHI, or "host adapted" *F. tularensis,* best resembles *F. tularensis* found *in vivo* than other medias (40-42). These BHI-grown *F. tularensis* cells produce longer LPS O-antigen polymers, a higher concentration of O-antigen capsule, and produce the HMW carbohydrate that is not present in MH grown bacteria (42). BHI-grown *F. tularensis* also bound fewer antibodies targeting outer membrane components than MH-grown *F. tularensis* (42). These results indicate that the outer membrane is not easily accessible in host adapted *F.*

tularensis most likely due to upregulation of extracellular capsules, fitting with the hypothesis that the *F. tularensis* capsules contribute to the ability of the bacteria to evade the immune response. Supplementing BHI media with amino acids or using a medium containing high concentrations of amino acids such as Mueller Hinton represses this host adapted phenotype (41, 42, 55).

Expression of the CLC is similarly affected by the growth medium. BHI- and CDM-grown *F. tularensis* produce greater amounts of CLC than MH-grown *F. tularensis* (24). Possibly this difference in CLC production is related to differences in amino acid concentration of the media; but was not directly assessed. The production of OMV/Ts, which may be related to the CLC, has been shown to be affected directly by the concentration of free amino acids. Medias that are deprived of amino acids, such as BHI, lead to increased production of OMV/Ts, which are repressed by the addition of amino acids (52).

5.6. Polysaccharide Differences of *F. novicida*

The preceding information until this point has focused on *F. tularensis* Type A and Type B subspecies. Currently and debatably, *F. novicida* is considered a subspecies of *F. tularensis* and can be referred to as *F. tularensis* subspecies *novicida*. However, phenotypic and molecular differences exist between *F. novicida* and the other *F. tularensis* subspecies that possibly support reclassification of *F. novicida* as a separate species (56, 57). These differences also exist when comparing the capsules and polysaccharides produced by the bacterium.

F. novicida has been reported to be unencapsulated (58). Recently, we showed that *F. novicida* produces a CLC similar to the more virulent *F. tularensis* when grown similarly on chemically defined medium (19, 50). The carbohydrate contains similar sugars of mannose, glucose, and galactose, and interruption of the *F. novicida* putative glycosylation locus (FTN_1211 to FTN_1221) led to the abolishment of that carbohydrate and reduction in the visual presence of the CLC by electron microscopy (50). This glycosylation mutant was attenuated in the mouse model similarly to the LVS glycosylation mutant and provided partial protection against a virulent intranasal challenge (50). Production and glycosylation of the CLC appears to be a conserved mechanism of *Francisella* species. However, further analysis of the protein component of the *F. novicida* CLC is necessary to establish if similar proteins are present to

those in the more virulent subspecies. Analysis of *F. novicida* CLC proteins would also allow for a more direct comparison between the CLC and previously characterized *F. novicida* OMV/T proteins.

Though the presence of a CLC produced by *F. novicida* has been confirmed (50), confirmation of an *F. novicida* O-antigen capsule has yet to be shown. MAb 11B7 to the Type A/B O-antigen capsule did not react with *F. novicida* strain U112, indication that *F. novicida* does not produce an O-antigen capsule with an identical structure to the Type A and B strains (18). However, *F. novicida* U112 also does not react with LPS O-antigen MAb FB11, or other antibodies, that binds both the LPS O-antigen and the O-antigen capsule of Type A and Type B strains (18). Compositional analysis of *F. novicida* LPS indicates that, though Type A and B strains contain an identical repeating O-antigen tetrasaccharide subunit, the *F. novicida* O-antigen is antigenically different (15, 59). These structural differences have already been shown to affect the immunostimulatory properties of *F. novicida in vivo* compared to Type A and B strains (56, 57, 60). Therefore, it is possible that *F. novicida* produces an *F. novicida* specific O-antigen capsule, but it would not react with antibodies to Type A/B O-antigen due to differences in their compositional structure. *F. novicida* contains a similar, though not identical, O-antigen locus as Type A and Type B strains (59, 61, 62) that could theoretically produce an O-antigen capsule.

5.7. Conclusion

Collectively, *F. tularensis* produces two separate extracellular polysaccharides that function similarly to a traditional capsule. The O-antigen capsule is composed of a polysaccharide similar to the O-antigen of the LPS and shares a similar biosynthetic pathway. Strains that lack the O-antigen, both the capsule and the LPS, are typically serum complement sensitive and are attenuated *in vivo*. The CLC is a heterogenous group of proteins that appear to be glycosylated by either a repeating 420-Da glycan subunit or a previously described hexassacharide associated with glycosylation. The locus responsible for the glycosylation of the CLC represents a distinct locus separate from the O-antigen glycosylation locus. The exact nature of the CLC has not been defined, but may be, at least in part, composed of OMV/Ts as the appearance and the proteins isolated from OMV/Ts are highly similar to the proteins isolated from CLC extracts. The HMW carbohydrate present in host-adapted *F. tularensis* may represent

the high molecular size band of the CLC or could present a third extracellular capsule-like component. Expression both the O-antigen and the CLC appear to be affected by growth conditions that may be associated with the overall pathogenesis of *F. tularensis in vivo*. However, the exact role that either capsule plays in the virulence of *F. tularensis* has not been fully elucidated as there are overlaps between the functions of the O-antigen capsule and the LPS, and the functions of the CLC and OMV/Ts. Further studies are necessary to delineate the contributions of each capsule to the pathogenesis and virulence of *F. tularensis*.

5.8. References

1. Sjostedt A. 2005. Family III. Francisellaceae, p 199-209. *In* Brenner DJ, Krieg NR, Garrity GM, Staley JT (ed), Bergey's Manual of Systematic Bacteriology: Volume 2: The Proteobacteria, 2 ed, vol 2. Springer.
2. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Layton M, Lillibridge SR, McDade JE, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Tonat K, Working Group on Civilian B. 2001. Tularemia as a biological weapon: medical and public health management. JAMA 285:2763-73.
3. Francis E. 1925. Tularemia. JAMA 84:1243-1250.
4. Centers for Disease C, Prevention. 2005. Tularemia transmitted by insect bites--Wyoming, 2001-2003. MMWR Morb Mortal Wkly Rep 54:170-3.
5. Centers for Disease C, Prevention. 2005. Tularemia associated with a hamster bite--Colorado, 2004. MMWR Morb Mortal Wkly Rep 53:1202-3.
6. Keim P, Johansson A, Wagner DM. 2007. Molecular epidemiology, evolution, and ecology of *Francisella*. Ann N Y Acad Sci 1105:30-66.
7. Oyston PC, Sjostedt A, Titball RW. 2004. Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*. Nat Rev Microbiol 2:967-78.
8. Centers for Disease C, Prevention DoH, Human S. 2017. Possession, Use, and Transfer of Select Agents and Toxins; Biennial Review of the List of Select Agents and Toxins and Enhanced Biosafety Requirements. Final rule. Fed Regist 82:6278-94.
9. McLendon MK, Apicella MA, Allen LA. 2006. *Francisella tularensis*: taxonomy, genetics, and Immunopathogenesis of a potential agent of biowarfare. Annu Rev Microbiol 60:167-85.
10. Hartley G, Taylor R, Prior J, Newstead S, Hitchen PG, Morris HR, Dell A, Titball RW. 2006. Grey variants of the live vaccine strain of Francisella tularensis lack lipopolysaccharide O-antigen, show reduced ability to survive in macrophages and do not induce protective immunity in mice. Vaccine 24:989-96.
11. Jia Q, Horwitz MA. 2018. Live Attenuated Tularemia Vaccines for Protection Against Respiratory Challenge With Virulent *F. tularensis* subsp. *tularensis*. Front Cell Infect Microbiol 8.

12. Nano FE, Zhang N, Cowley SC, Klose KE, Cheung KK, Roberts MJ, Ludu JS, Letendre GW, Meierovics AI, Stephens G, Elkins KL. 2004. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. J Bacteriol 186:6430-6.
13. Santic M, Molmeret M, Barker JR, Klose KE, Dekanic A, Doric M, Abu Kwaik Y. 2007. A *Francisella tularensis* pathogenicity island protein essential for bacterial proliferation within the host cell cytosol. Cell Microbiol 9:2391-403.
14. Nano FE, Schmerk C. 2007. The *Francisella* pathogenicity island. Ann N Y Acad Sci 1105:122-37.
15. Vinogradov E, Perry MB, Conlan JW. 2002. Structural analysis of *Francisella tularensis* lipopolysaccharide. Eur J Biochem 269:6112-8.
16. Li J, Ryder C, Mandal M, Ahmed F, Azadi P, Snyder DS, Pechous RD, Zahrt T, Inzana TJ. 2007. Attenuation and protective efficacy of an O-antigen-deficient mutant of *Francisella tularensis* LVS. Microbiology 153:3141-53.
17. Clay CD, Soni S, Gunn JS, Schlesinger LS. 2008. Evasion of complement-mediated lysis and complement C3 deposition are regulated by Francisella tularensis lipopolysaccharide O antigen. J Immunol 181:5568-78.
18. Apicella MA, Post DM, Fowler AC, Jones BD, Rasmussen JA, Hunt JR, Imagawa S, Choudhury B, Inzana TJ, Maier TM, Frank DW, Zahrt TC, Chaloner K, Jennings MP, McLendon MK, Gibson BW. 2010. Identification, characterization and immunogenicity of an O-antigen capsular polysaccharide of *Francisella tularensis*. PLoS One 5:e11060.
19. Bandara AB, Champion AE, Wang X, Berg G, Apicella MA, McLendon M, Azadi P, Snyder DS, Inzana TJ. 2011. Isolation and mutagenesis of a capsule-like complex (CLC) from *Francisella tularensis*, and contribution of the CLC to *F. tularensis* virulence in mice. PLoS One 6:e19003.
20. Cherwonogrodzky JW, Knodel MH, Spence MR. 1994. Increased encapsulation and virulence of *Francisella tularensis* live vaccine strain (LVS) by subculturing on synthetic medium. Vaccine 12:773-5.
21. McCoy GW. 1910. Plague among ground squirrels in America. J Hyg (Lond) 10:589-601.
22. Carlisle HN, Hinchliffe V, Saslaw S. 1962. Immunodiffusion studies with Pasteurella tularensis antigen-rabbit antibody systems. J Immunol 89:638-44.
23. Hood AM. 1977. Virulence factors of *Francisella tularensis*. J Hyg (Lond) 79:47-60.
24. Champion AE, Bandara AB, Mohapatra N, Fulton KM, Twine SM, Inzana TJ. 2018. Further Characterization of the Capsule-Like Complex (CLC) Produced by Francisella tularensis Subspecies tularensis: Protective Efficacy and Similarity to Outer Membrane Vesicles. Front Cell Infect Microbiol 8:182.
25. Tigertt WD. 1962. Soviet viable *Pasteurella tularensis* vaccines. A review of selected articles. Bacteriol Rev 26:354-73.
26. Eigelsbach HT, Braun W, Herring RD. 1951. Studies on the variation of Bacterium tularense. J Bacteriol 61:557-69.
27. Sandstrom G, Lofgren S, Tarnvik A. 1988. A Capsule-Deficient Mutant of *Francisella-Tularensis* Lvs Exhibits Enhanced Sensitivity to Killing by Serum but Diminished Sensitivity to Killing by Polymorphonuclear Leukocytes. Infection and Immunity 56:1194-1202.

28. Eigelsbach HT, Downs CM. 1961. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. J Immunol 87:415-25.
29. Cowley SC, Myltseva SV, Nano FE. 1996. Phase variation in Francisella tularensis affecting intracellular growth, lipopolysaccharide antigenicity and nitric oxide production. Mol Microbiol 20:867-74.
30. Titball R, Petrosino J. 2007. *Francisella tularensis* genomics and proteomics. Ann N Y Acad Sci 1105:98-121.
31. Sorokin VM, Pavlovich NV, Prozorova LA. 1996. *Francisella tularensis* resistance to bactericidal action of normal human serum. FEMS Immunol Med Microbiol 13:249-52.
32. Barker JH, Kaufman JW, Apicella MA, Weiss JP. 2016. Evidence Suggesting That Francisella tularensis O-Antigen Capsule Contains a Lipid A-Like Molecule That Is Structurally Distinct from the More Abundant Free Lipid A. PLoS One 11:e0157842.
33. Rasmussen JA, Fletcher JR, Long ME, Allen LA, Jones BD. 2015. Characterization of *Francisella tularensis* Schu S4 mutants identified from a transposon library screened for O-antigen and capsule deficiencies. Front Microbiol 6:338.
34. Rasmussen JA, Post DM, Gibson BW, Lindemann SR, Apicella MA, Meyerholz DK, Jones BD. 2014. *Francisella tularensis* Schu S4 lipopolysaccharide core sugar and O-antigen mutants are attenuated in a mouse model of tularemia. Infect Immun 82:1523-39.
35. Lindemann SR, Peng K, Long ME, Hunt JR, Apicella MA, Monack DM, Allen LA, Jones BD. 2011. *Francisella tularensis* Schu S4 O-antigen and capsule biosynthesis gene mutants induce early cell death in human macrophages. Infect Immun 79:581-94.
36. McLendon MK, Schilling B, Hunt JR, Apicella MA, Gibson BW. 2007. Identification of LpxL, a late acyltransferase of *Francisella tularensis*. Infect Immun 75:5518-31.
37. Thomas RM, Twine SM, Fulton KM, Tessier L, Kilmury SL, Ding W, Harmer N, Michell SL, Oyston PC, Titball RW, Prior JL. 2011. Glycosylation of DsbA in *Francisella tularensis* subsp. *tularensis*. J Bacteriol 193:5498-509.
38. Sebastian S, Dillon ST, Lynch JG, Blalock LT, Balon E, Lee KT, Comstock LE, Conlan JW, Rubin EJ, Tzianabos AO, Kasper DL. 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.
39. Twine SM, Vinogradov E, Lindgren H, Sjostedt A, Conlan JW. 2012. Roles for wbtC, wbtI, and kdtA Genes in Lipopolysaccharide Biosynthesis, Protein Glycosylation, Virulence, and Immunogenicity in Francisella tularensis2 Strain SCHU S4. Pathogens 1:12-29.
40. Twine SM, Mykytczuk NC, Petit MD, Shen H, Sjostedt A, Wayne Conlan J, Kelly JF. 2006. In vivo proteomic analysis of the intracellular bacterial pathogen, *Francisella tularensis*, isolated from mouse spleen. Biochem Biophys Res Commun 345:1621-33.
41. Hazlett KR, Caldon SD, McArthur DG, Cirillo KA, Kirimanjeswara GS, Magguilli ML, Malik M, Shah A, Broderick S, Golovliov I, Metzger DW, Rajan K, Sellati TJ, Loegering DJ. 2008. Adaptation of Francisella tularensis to the mammalian environment is governed by cues which can be mimicked in vitro. Infect Immun 76:4479-88.
42. Zarrella TM, Singh A, Bitsaktsis C, Rahman T, Sahay B, Feustel PJ, Gosselin EJ, Sellati TJ, Hazlett KR. 2011. Host-adaptation of *Francisella tularensis* alters the bacterium's surface-carbohydrates to hinder effectors of innate and adaptive immunity. PLoS One 6:e22335.

43. Sandstrom G, Lofgren S, Tarnvik A. 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.
44. Clemens DL, Lee BY, Horwitz MA. 2005. *Francisella tularensis* enters macrophages via a novel process involving pseudopod loops. Infect Immun 73:5892-902.
45. Clemens DL, Horwitz MA. 2007. Uptake and intracellular fate of Francisella tularensis in human macrophages. Ann N Y Acad Sci 1105:160-86.
46. Huntley JF, Conley PG, Rasko DA, Hagman KE, Apicella MA, Norgard MV. 2008. Native outer membrane proteins protect mice against pulmonary challenge with virulent type A *Francisella tularensis*. Infect Immun 76:3664-71.
47. Hickey AJ, Hazlett KR, Kirimanjeswara GS, Metzger DW. 2011. Identification of *Francisella tularensis* outer membrane protein A (FopA) as a protective antigen for tularemia. Vaccine 29:6941-7.
48. McCaig WD, Koller A, Thanassi DG. 2013. Production of outer membrane vesicles and outer membrane tubes by *Francisella novicida*. J Bacteriol 195:1120-32.
49. Pierson T, Matrakas D, Taylor YU, Manyam G, Morozov VN, Zhou W, van Hoek ML. 2011. Proteomic characterization and functional analysis of outer membrane vesicles of *Francisella novicida* suggests possible role in virulence and use as a vaccine. J Proteome Res 10:954-67.
50. Freudenberger Catanzaro KC, Champion AE, Mohapatra N, Cecere T, Inzana TJ. 2017. Glycosylation of a Capsule-Like Complex (CLC) by Francisella novicida Is Required for Virulence and Partial Protective Immunity in Mice. Front Microbiol 8:935.
51. Champion A, Mohapatra N, Bandara A, Twine S, Inzana TJ. 2015. The Capsule-Like Complex (CLC) of Type A *Francisella tularensis* may be Derived from Outer Membrane Vesicles, but Glycosylation of the CLC is not Required for Virulence and is not Protective., abstr 115th General Meeting of the American Society for Microbiology, New Orleans, LA., May 30-June 2, 2015,. American Society for Microbiology,
52. Sampath V, McCaig WD, Thanassi DG. 2018. Amino acid deprivation and central carbon metabolism regulate the production of outer membrane vesicles and tubes by *Francisella*. Mol Microbiol 107:523-541.
53. Oyston PC. 2009. *Francisella tularensis* vaccines. Vaccine 27 Suppl 4:D48-51.
54. Ray HJ, Chu P, Wu TH, Lyons CR, Murthy AK, Guentzel MN, Klose KE, Arulanandam BP. 2010. The Fischer 344 rat reflects human susceptibility to *francisella* pulmonary challenge and provides a new platform for virulence and protection studies. PLoS One 5:e9952.
55. Holland KM, Rosa SJ, Kristjansdottir K, Wolfgeher D, Franz BJ, Zarrella TM, Kumar S, Sunagar R, Singh A, Bakshi CS, Namjoshi P, Barry EM, Sellati TJ, Kron SJ, Gosselin EJ, Reed DS, Hazlett KRO. 2017. Differential Growth of *Francisella tularensis*, Which Alters Expression of Virulence Factors, Dominant Antigens, and Surface-Carbohydrate Synthases, Governs the Apparent Virulence of Ft SchuS4 to Immunized Animals. Front Microbiol 8:1158.
56. Johansson A, Celli J, Conlan W, Elkins KL, Forsman M, Keim PS, Larsson P, Manoil C, Nano FE, Petersen JM, Sjostedt A. 2010. Objections to the transfer of *Francisella novicida* to the subspecies rank of *Francisella tularensis*. Int J Syst Evol Microbiol 60:1717-8; author reply 1718-20.

57. Kingry LC, Petersen JM. 2014. Comparative review of *Francisella tularensis* and *Francisella novicida*. Front Cell Infect Microbiol 4:35.
58. Colquhoun DJ, Larsson P, Duodu S, Forsman M. 2014. The Family Francisellaceae, p 287-314. *In* Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (ed), The Prokaryotes: Gammaproteobacteria doi:10.1007/978-3-642-38922-1_236. Springer Berlin Heidelberg, Berlin, Heidelberg.
59. Vinogradov E, Conlan WJ, Gunn JS, Perry MB. 2004. Characterization of the lipopolysaccharide O-antigen of Francisella novicida (U112). Carbohydr Res 339:649-54.
60. Kieffer TL, Cowley S, Nano FE, Elkins KL. 2003. *Francisella novicida* LPS has greater immunobiological activity in mice than *F. tularensis* LPS, and contributes to *F. novicida* murine pathogenesis. Microbes Infect 5:397-403.
61. Gunn JS, Ernst RK. 2007. The structure and function of *Francisella* lipopolysaccharide. Ann N Y Acad Sci 1105:202-18.
62. Raynaud C, Meibom KL, Lety MA, Dubail I, Candela T, Frapy E, Charbit A. 2007. Role of the wbt locus of *Francisella tularensis* in lipopolysaccharide O-antigen biogenesis and pathogenicity. Infect Immun 75:536-41.

Appendix A: Supplemental Figures

A.1. Supplemental Reference Material

Primer	Sequence (5' → 3')
FTN_1211_F	GAGTTTGGTTCAACTATGCTTGG
FTN_1212_F_BamHI	CGCGGATCCATGCCAAAATTATTAATAGATACGCGC
FTN_1213_F_Xbal	CTAGTCTAGAATCGATTGTTGTTTCAAGTTTTGATAATGATTA AAAATAATAGGAGTTAAAAATGTACAATCTTAATTATAAGCA GCTAATATCTATAATC
FTN_1214_F	GGCTCTACTGATAACTCTCTGG
FTN_1215_F	GTTCTAACTGGATGAGAGGGATG
FTN_1216_F	CCGGAGATGGTTTCAGGTAAA
FTN_1217_F	AAGAAGTACACCGACACTTATCC
FTN_1218_F	GCTATCGATTGCTACGGCTAAA
FTN_1219_F	GAGATTATCAAGTTGTGGTGGTAGA
FTN_1220_F	CTAAAGCTGAGGCTGCTAAGT
FTN_1221_F	CTTCTATACTCTCTGCCGATCTTG
FTN_1211_R	CTCCTAGCTAAATACTTACCCTCAAA
FTN_1212_R_XmaI	TCCCCCCGGGCTATAATAAATTAAGCTTTTTAAGCTCATCTT GGAC
FTN_1213_R_XmaI	TCCCCCCGGGTTAACTTCTAGTAATTCTTTTTTGTTTGAGTGAG
FTN_1214_R	CTACTTAATAACCCTGCTGAATCAA
FTN_1215_R	CTTCAAAGCCTAACTGTGATGTC
FTN_1216_R	CCAATCTCTATCTTAGGCTTCCAT
FTN_1217_R	GTGCCGTATAGAAGCCCATTA
FTN_1218_R	TGATCGCCATCTCCAACTATAAC
FTN_1219_R	CTCTCACTCCCGTACCATCTAT
FTN_1220_R	ACTTGCGCGTAACCAGTAATA
FTN_1221_R	GGATTTACGCCACCATCTATCT

Table A.1. Oligonucleotide primers used for RT-PCR.

Strain	Transposon Insertion Location	Carbohydrate of Crude Urea Extract (µg/gram of bacterial wet weight) (n = 3)[a]
F. novicida	N/A	4078 ± 415
tnfn1_pw060323p05q162	FTN_1212	3329 ± 596
tnfn1_pw060323p03q152	FTN_1213	2866 ± 613
tnfn1_pw060328p06q149	FTN_1214	3831 ± 544
tnfn1_pw060323p05q110	FTN_1215	3532 ± 545
tnfn1_pw060420p04q184	FTN_1216	3392 ± 641
tnfn1_pw060418p03q107	FTN_1218	3171 ± 393
tnfn1_pw060323p07q127	FTN_1219	3597 ± 192

Table A.2. Carbohydrate Content of Passed *F. novicida* Transposon Mutants

[a] Comparison of carbohydrate content of urea extracts of *F. novicida*_P10 and passed *F. novicida* transposon mutants was done with a One-Way ANOVA. No significant differences were found (p-value > 0.05).

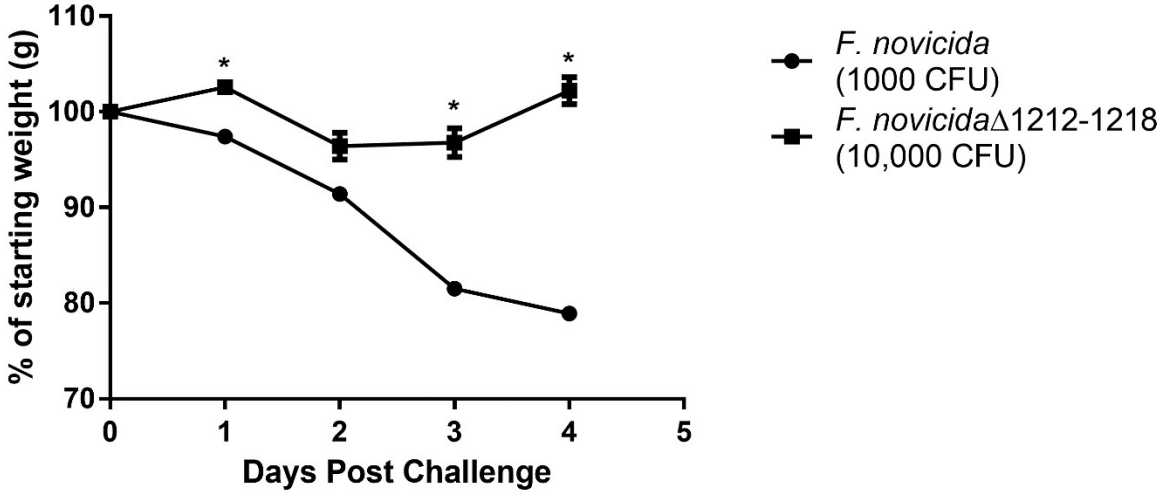

Figure A.1. Weight loss of mice challenged with *F. novicida* or *F. novicida*Δ1212-1218.
The percentage of weight loss based on starting weight was determined for each group of mice challenged with *F. novicida* or *F. novicida*Δ1212-1218. Multiple t-tests using the Holm-Sidak method for correction were used to determine statistical differences between groups at specific time points. Mice inoculated with *F. novicida* (●) were euthanized by four days post challenge. Mice inoculated with *F. novicida*Δ1212-1218 (■) had significantly less weight loss at 1, 3, and 4 days post challenge ($p < 0.005$, *).