THESIS

MINING THE *FRANCISELLA TULARENSIS* PROTEOME FOR VACCINE CANDIDATES

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ABSTRACT

MINING THE *FRANCISELLA TULARENSIS* PROTEOME FOR VACCINE CANDIDATES

Based on methodologies developed for the identification of T cell antigen of other intracellular bacterial pathogens, a proteomic approach was applied for the elucidation of T cell antigens of *Francisella tularensis* (Covert, 2001). Specifically, subcellular components (membrane and soluble) of *F. tularensis* LVS were resolved by size using preparative SDS-PAGE and fractions collected using a whole gel elution technique. A total of 16 soluble and 19 membrane-sized fractions were produced, each of which were assessed for antigen reactivity based on the ability to elicit IFN-γ from splenocytes of *F. tularensis* LVS-infected mice. Of these 35 preparative SDS-PAGE fractions, seven yielded a dominant T cell response. These seven fractions were further investigated using tandem mass spectrometry (MS/MS) to identify individual proteins in each immunodominant fraction. A total of 40 and 31 proteins were identified with greater than 95% confidence from the immunodominant membrane and soluble fractions, respectively. Further, MS/MS analysis of different protein quantities (2.5 μg to 10 μg) allowed for identification of the most abundant proteins in each fraction, thus focusing the number of possible proteins to nine proteins of interest. These data provide the basis for production of recombinant proteins and further immunological evaluations to select suitable candidates for inclusion in a subunit vaccine against tularemia.

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DEDICATION

To Marcia, thank you.

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LIST OF SYMBOLS

A……...Absobance
ACV…… ……..…Activating variant
APC……..……....Antigen presenting cell
B. tularense……...……..Bacterium *tularense*
BCA……..Bicinchoninic acid
BCR…….……B cell receptor
BHI…….……..……...…Brain heart infusion
BWC…….Prohibition of Production, Development, and Stockpiling of Bacteriological (Biological) and Toxic Weapons and on their Destruction
BSA……...……..Bovine serum albumin
°C…….……Degrees Celsius
CDC…… ……...Center for Disease Control and Prevention
CF…… ……Culture filtrate
CFU…….……..Colony forming units
CHAB……Cysteine heart agar with 9% chocolatized sheep blood
CMI…….Cell-mediated immunity
CMEM……...Complete minimum essential medium
CS...…….……Culture supernatant
CTL……..Cytotoxic T cell
DC…….Dendritic cell
EF……. ...……Elongation factor
ELISA…….Enzyme-linked immunosorbent assay
FCV……..... *Francisella* containing vacuoles
FDA ……...US Food and Drug Administration
GAS…… ……...Glycerol Alanine Salts
h…… …….Hour
HBSS…… ……..Hank's balanced salt solution
HPLC…….High-powered liquid chromatography
LC-MS…..…..…..…….……Liquid chromatography mass spectrometry
IEDB……..Immune epitope database
IND……Investigative new drug
IFN-γ …….Interferon gamma
IP…….Intraperitoneal
Kdo ……2-keto-3-deoxy-D-*manno*-octulosonic acid
LPS……..Lipopolysaccharide
LVS……..Live vaccine strain
µg…….Micrograms
min……..Minute
MH…….Mueller-Hinton
MHC…….Major histocompatabilty complex
mm ……Millimeter
MS/MS……Tandem mass spectrometry
MW…….Molecular weight

NCBI ……..National Center for Biotechnology Information
NIAID ……National Institute of Allergy and Infectious Diseases
O.D…….Optical density
OM ……..Outer membrane
O/N…….Overnight
OPS…….O-polysaccharide
OPS-TT……..O-polysaccharide tetanus toxoid
p.s.i……Pounds per square inch
PBS……Phosphate buffered saline
PCR …….Polymerase chain reaction
RBC ……Red blood cell
RT…….Room temperature
SDS-PAGE……Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIL……..Stable isotope labeling
TCR……T cell receptor
TEM…….Transmission electron microscopy
T4P……..Type IV pili
T6SS……..Type VI secretion system
TF…….Trigger factor
TFA…….Trifluoroacetic acid
TLR……Toll-like receptor
USAMRIID ……...United States Army Medical Research Institute of Infectious Diseases
V……..Volt

CHAPTER 1

INRODUCTION TO *FRANCISELLA TULARENSIS*

1.1 Tularemia

Tularemia is a resulting disease through infection by the bacterium *Francisella tularensis*, against which, there is no licensed vaccine (36, 91). Delayed treatment can result in debilitating illness and in some cases, death (48). Cutaneous infection with less than 10 colony forming units (CFU) of the subspecies *tularensis* make this bacterium one of the most infectious pathogens in humans and it can infect over 250 mammalian species (110, 140, 141). Water-borne transmission from *F. tularensis* subspecies *holarctica* is frequently reported, whereas this has yet to be shown as a reservoir for the subspecies *tularensis* (14, 15, 19, 47). Human-to-human transmission has not been documented, but *F. tularensis* can be transmitted to humans via insect vectors, inhalation, consumption, or direct contact with the bacterium from other infected mammals (47, 110, 123). *F. tularensis* has been previously weaponized and has the potential of being used as a biowarfare agent (36, 42, 44). This potential threat has increased research on tularemia, and thus increased the number of laboratory personnel working closely with this bacterium (36). While a vaccine for tularemia may not be widely used, it could be effectively administered to specific sections of our society such as the military, first responders, and laboratory workers. Additionally, the development of an effective vaccine could minimize, or eliminate the need for rapid therapeutic intervention in high-risk populations. The only vaccine against tularemia is not licensed for human use and the US Food and Drug Administration (FDA) has revoked its investigative new drug (IND) status due to critical knowledge gaps in mechanism of attenuation, ability to cause virulence in mice, immunogenic variability, and inability to establish levels of herd immunity (38, 48, 52, 55, 110, 140). The current vaccine

needs to be updated and improved to provide more consistent protection against infection by *F. tularensis* (36, 144).

F. tularensis was initially isolated in 1911 in Tulare County, California, by Dr. George McCoy *et al.* (109) from ground squirrels with a plague-like illness. Originally suspected to be caused by *Yersinia pestis,* this study established that *Bacterium tularense* (original spelling) was instead, the cause of the disease (109). The bacterium was grouped in the family *Pasturella*, and later, Dr. Edward Francis was able to link it to diseases named deer-fly fever, rabbit fever, and rancher's fever. In 1921, due to Dr. Francis' continual research efforts, *Pasturella tularensis* was re-named, *Francisella tularensis*, in his honor (133).

Francisella tularensis is an aerobic, Gram-negative, intracellular pathogen usually found as single pleomorphic cells, that commonly exhibit a coccobacilli-shape (160). It is part of the family *Francisellaceae,* genus *Francisella* and species *tularensis*. *F. tularensis* can further divided into three subspecies (Fig. 1.1). Of these, subsp. *tularensis* and subsp. *holarctica*, are associated most with the human disease whereas, subsp. *mediasiatica* is less likely to cause human disease. Predominantly found in North America, subsp. *tularensis* (Type A) is classified as a Category A agent by National Institute of Allergy and Infectious Diseases (NIAID) and the Center for Disease Control and Prevention (CDC) (122). *Francisella tularensis* subsp. *holartica* (Type B) is mainly found in Europe, North America, Siberia, and the Far East and is of similar virulence to that of subspecies *mediasiatica* encompassing most central Asian strains (26, 52, 54, 56, 90, 96). The live vaccine strain for *F. tularensis* is derived from *F. tularensis* subsp. *holarctica* and can cause lethal infections in some mouse strains (122). *F. novicida*, and *F. philomiragia* are opportunistic pathogens in immunocompromised humans and otherwise infrequent cause of disease in humans, but do produce high mortality in mice (21, 90, 96).

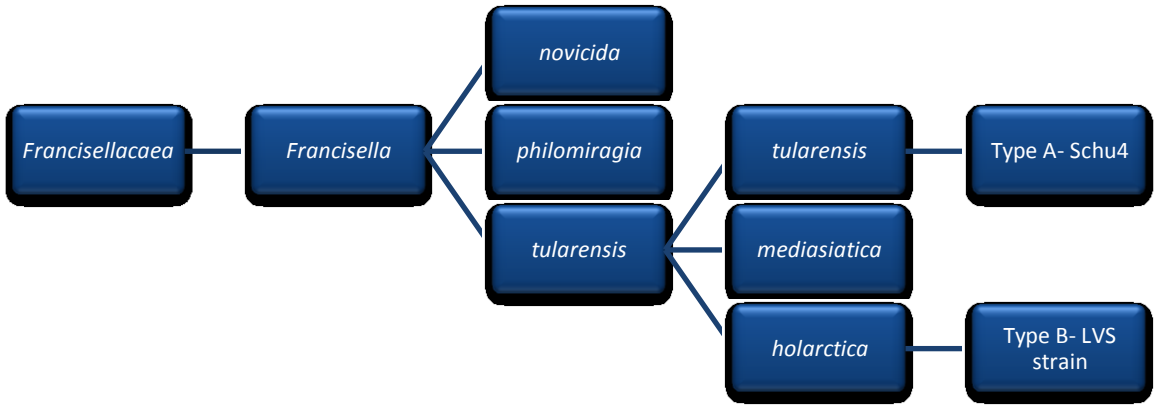

Figure 1.1 Phylogeny of *Francisella*caea. Figure adapted from Pathosystems Resource Integration Center (PATRIC): http://patricbrc.vbi.vt.edu/portal/portal/patric/Taxon?cType=taxon&cId=262

The greatest risk of contracting the disease lies with those who come in close contact with infected animals and vectors, such as hunters, lawn-care workers, farmers, laboratory-workers, veterinarians, and hikers (124, 152). A highly publicized account of tularemia brought this disease back to the forefront of human medicine in the United States in 2001, when an outbreak occurred in Martha's Vineyard, Massachusetts. Fifteen confirmed cases resulting in one death from "lawnmower tularemia", as it was later termed, took place after a lawn-care worker operating a brush-mower, drove over a rabbit, possibly causing the bacteria to become aerosolized and inhaled by the workers in the vicinity (62).

Outside of natural infection, many countries, including The United States and Japan, considered weaponizing tularemia during the 1930's and 1940's (77). During World War II, there was speculation that Russia purposely deployed *F. tularensis* as a bioweapon, although they reported these cases as a natural outbreak (4, 77, 95). During the 1972 Convention on the Prohibition of Production, Development, and Stockpiling of Bacteriological (Biological) and Toxic Weapons and on Their Destruction (BWC), that included over 100 nations, the termination

of offensive programs involving biological weapons and toxins was ratified (30). After the cessation of offensive biological weapons research and development, the U.S. focused on defensive research against possible tularemia attacks. Recent estimations of the economic impact of a bioterrorist attack involving tularemia is 3.8 to 5.4 billion U.S. dollars for every 100,000 persons infected (95). Vaccine research for the prevention of tularemia has the potential to thwart loss of life and minimize economic loss in the event of an attack or due to natural exposure.

1.2 Clinical manifestations and disease process

Clinical forms of tularemia can vary greatly, depending on the mode of transmission. Table 1.1 explains the different modes of transmission and risk factors for each clinical form of the disease.

Table 1.1 Modes of transmission and risk factors of tularemia

Clinical forms	Mode of Transmission	Risk Factor
Pneumonic/ Respiratory	Inhalation of contaminated dust (157)	Farmers may become infected with this form of tularemia due to handling of hay contaminated with infected mouse excrement (157)
Oculoglandular	Infection via the conjunctiva of the eye	Touching the eye after contact with contaminated substance (150)
Oropharyngeal	Ingestion	Contaminated food or water (83)
Typhoidal	Unknown route of infection (110, 123)	
Ulceroglandular	Bite from a recently-infected vector (161)	Flea, deer tick, rabbit tick, mosquito, and biting flies (161)
Glandular	Direct contact with infected tissue	Hunters occasionally contract the disease after skinning and cleaning wild hare

Respiratory tularemia, caused by *F. tularensis* subsp. *tularensis,* is the most lethal form of the disease, with as few as 25 organisms causing infection (110, 159). According to McCrumb, without treatment 40 - 60% of patients who developed respiratory tularemia, died of

the disease (110, 129). Ulceroglandular tularemia is the most frequently reported form of the disease; where 12-15% of all *F. tularensis* infected individuals develop pneumonia, and only 3% of non-treated non-respiratory cases result in death (60, 110).

Disease pathogenesis begins with the entrance of the pathogen into the host, through one of the routes listed in Table 1.1. The incubation period is between 3-6 days (60, 87) after which, flu-like symptoms usually occur suddenly and include fever, chills, malaise, lymphadenopathy, and headache (87). The bacterium is spread via the lymphatics to regional lymph nodes and can result in plague-like bubos on the infected individual (90, 151). From the nodes, the bacterium disseminates throughout the body and localizes mainly to the spleen, liver, and lymph nodes, but can also be found in the kidneys, intestine, and central nervous system, leading to death within 5-10 days (53, 56).

1.2.1 Initial stages of infection

One reason for *F. tularensis'* virulence is in its evasion of host immunity via escape from the phagosome and quiet replication within the cytosol of the phagocytic cell. During conventional phagocytosis, a bacterium can be taken up by a macrophage into a phagosome and can be killed quickly when the lysosome fuses with the phagosome. In reference to *F. tularensis,* once the bacterium is taken up in the macrophage, either by the pseudopodal loops or through conventional phagocytosis, it does not cause an NO oxidative burst. Instead it enters a vacuole, which does not bind with a lysosome, thus allowing time for *F. tularensis* to disrupt the phagosomal membrane and escape into the host cell cytoplasm where replication ensues (33). It was shown by Checroun *et al.* that *F. tularensis* could escape the phagosome as early as 20 min and re-enter the endocytic pathway to accumulate and form *Francisella* containing vacuoles (FCV) where it can thrive from 4 - 20 h (28). Goloviov *et al.* showed that IglC production (23-

kDa) was important in replication within the macrophage, for without the *igl*C gene, *F. tularensis* had impaired replication and was attenuated in the mouse model (72).

A unique feature recently uncovered by Santic *et al.* (138) is the mechanism by which the macrophage can engulf *F. tularensis* with asymmetric pseudopod loops. Clemens *et al.* (32) determined that this unique macrophage uptake required active complement component C3 in serum. The authors determined that formalin-killed *F. tularensis* was taken up by macrophages through the pseudopod loops, thus indicating the molecules that trigger the uptake into macrophages are pre-formed and do not require metabolic activity by the bacteria (32). In addition, they showed that heat-treated and protease-treated *F. tularensis* did not affect the formation of pseudopod loops for the uptake of the bacteria. This is important in vaccine development, because it shows that the molecules that trigger the uptake are heat resistant and protease resistant, thus indicating lipopolysaccharides as a possible ligand-mediating uptake mechanism (32). The LPS of *F. tularensis* is discussed further in section 1.6.

1.3 Treatment

Current treatment of tularemia is limited to antibiotic therapy, which is most effective during the initial onset of the disease. Up until 1995, streptomycin, an aminoglycoside, was the preferred antibiotic due to higher success rate of around 97% without relapse (57, 132). Gentamicin, another protein synthesis-inhibiting aminoglycoside, has a slightly lower success rate of 86% and relapse rate of about 6%, but with fewer side-effects and thus has become the preferred method of treatment for tularemia (78). Tetracycline had an 88% success rate and chloramphenicol had a 77% success rate and both work by inhibiting protein synthesis. However, they have been shown to have higher relapse rates of 12% and 21% respectively, and are best used only for long-term therapy (47, 48, 57). Fluoroquinolones, such as ciprofloxacin

and norfloxacin, work by inhibiting DNA synthesis and have also recently been shown to work well in treatment of tularemia without relapse (158). Among the drug therapies used during a 1997 tularemia outbreak in Spain, ciprofloxacin was shown to be a very good alternative to aminoglycosides and tetracycline therapy (126).

Due to the generic flu-like symptoms, tularemia is typically not recognized until the patient is hospitalized, and although there are treatment options for tularemia, delay in these treatments could lead to relapse, increased severity of disease and possible death (47, 71). Thus, there is also a need for more advanced diagnostics that decrease the time from initial symptoms to the identification of *F. tularensis* as the etiological agent of disease. This would dramatically influence the administration of effective antibiotics.

1.3.1 Diagnostics

Prior to treatment of the disease, one must know what is being treated and this is done through a variety of diagnostic methods. In 1965, White *et al.* suggested the most "satisfactory" way to diagnose *P. tularensis* infection was through bacteriological culture (169). This method requires special media, poses risk to the laboratory workers and takes time for growth (169). To achieve a quicker result, White *et al.* applied a technique by Coons and Kaplan, using a fluorescein-tagged antibody detection technique (original spelling) (39, 169). The bacterium can be successfully identified from the blood, ulcers, conjunctival exudates, pleural fluid, sputum, gastric aspirates, and pharyngeal exudates using the fluorescein-tagged antibody technique, antigen detection, PCR, or enzyme-linked immunoassays (16, 18, 48, 94, 156). Whereas these are the quickest ways to detect the pathogen, due to the hazards posed by this pathogen, only specific state and national laboratories are allowed to run these tests (48, 94). For retroactive studies or autopsies serology can be used to diagnose the cause of outbreak or death (48). This

serum antibody titer test needs to have a 4-fold change in titer, which takes at least 10 days, by which time it may be too late for treatment of the patient (18, 48).

1.4 Prevention

"The key event that triggers the immune response is the immune system sensing a vaccine or microbe (128)." This statement is the basis for the studies included in this thesis. One of the key virulence factors of *F. tularensis* is its ability to evade the immune system. It infects the host cells and quietly replicates, suppressing the innate immune response or inflammatory response long enough to replicate to a point where it is difficult for the body to clear the infection (162). Only when we can uncover key antigens that trigger active killing or suppression of the *F. tularensis* infection and illicit a strong memory immune response for these antigens, will we be able to say that we have created a successful vaccine candidate for tularemia.

Vaccine research of *F. tularensis* began in the 1930's in Russia. By the 1940's they had attenuated *Francisella tularensis* subsp. *holartica* by multiple passages of the bacterium through river rats (52, 163). In 1956, United States Army Medical Research Institute of Infectious Diseases (USAMRIID) received the attenuated strain during a medical exchange and refined it into the live vaccine strain (LVS) (1, 52). Eigelsbach *et al.* noted, when developing the live attenuated vaccine, two colony phenotypes were apparent when viewed with an oblique light-source (52). These colonies were the virulent, blue colonies or less virulent, gray colonies (52). For the vaccine to be effective, a ratio of at least 20% blue colonies to 80% gray colonies had to comprise the LVS inoculums (52).

Eigelsbach *et al.* stated that the rate at which population phenotypes change in *B. tularense* strongly correlates with the pH and the inoculums size, especially when incubated in

liquid media, without shaking, for a prolonged timeframe (51). Further studies into immune response and growth conditions of *Francisella* have uncovered another virulence variant, ACV (activating variant). The ACV variant, which arose from high density cultures, has uniquely been shown by Carlson *et al*. to induce increased pro-inflammatory cytokines such as TNF-α, IL-1β, IL-12p40, reiterating the fact that culture conditions must be optimal for *Francisella,* in order to have the most beneficial vaccine (23).

Experimental models for studying potential vaccine candidates and the pathogenesis of tularemia include: non-human primates, rats, mice, guinea pigs, rabbits, and in the past, humans (107). In a retrospective study at Fort Detrick, Maryland, LVS vaccination decreased the incidence of respiratory tularemia in humans from 5.7 to 0.27 cases per 1,000 subjects (22). The incidence of ulceroglandular tularemia in the same population remained unchanged, but the symptoms were less severe in those receiving the vaccine (22). Inmates at the Maryland House of Correction were used in a study conducted on the efficacy of an aerosolized route for LVS vaccination versus that of a cutaneous route (87). The investigators of this study came to the conclusion that inhalation of aerosolized LVS was not as protective as the parenteral injection method, but that inhalation should not be disregarded as a means for vaccination (22, 87). Whereas the human model was the most efficient for targeting new therapeutics and vaccines, the ability to extrapolate data from other animal models has allowed scientists to move away from human volunteers as models (107).

1.4.1 Live vaccines

The three types of vaccines commonly utilized to protect against infectious diseases consist of living microorganisms, killed microorganisms, or subunits of microorganisms (Table 1.2). The original live, attenuated *Francisella* vaccine (LVS) produced long-lasting humoral and

cellular responses, and required few doses to be effective. However, there is a risk that LVS might revert to a virulent form in the host due to its unknown basis of attenuation (136). This type of vaccine cannot be given to immune-compromised individuals or children, and there is a risk of variable protection from batch-to-batch, due to the organism's ability to randomly switch phenotypes and thus change immunogenicity (136). The LVS vaccine does not confer complete protection, but did drastically decrease the amount of respiratory, but not ulceroglandular-type, infections in laboratory personnel after its introduction (22). When compared to killed bacteria, Tärnvik suggests that the live bacteria are processed more efficiently by phagocytic cells and thus more of the immunogen circulates in the body for a more robust immune response (159).

Bakshi *et al.* created a mutant strain of *F. tularensis* LVS, in an attempt to attenuate it further and genetically define the mutation for use as a safer more efficacious vaccine over LVS. *F. tularensis* contains superoxide dismutases (SODs) that play an important role during phagocytosis to cause dismutation of superoxide radicals created during respiratory burst. Without the iron-containing SOD, the mutated *F. tularensis* LVS ($sodB_{Ft}$), became more sensitive to oxidative stress. The vaccination of mice with $sodB_{Ft}$ had a significant, protective, humoral, immune response against SchuS4, requiring both $CD4^+$ and $CD8^+$ T cells for protection. This was shown by depletion of $CD4^+$ and $CD8^+$ T cells. Bakshi *et al.* showed the $sodB_{Ft}$ mutant as a vaccine candidate against respiratory tularemia with enhanced protective efficacy over LVS (11).

Table 1.2 Comparison of vaccines

Advantage / Disadvantage (61, 136)	**Subunit**	**Killed**	**Live**
Reversion Risk	No	No	Yes
Specific Antigen Target	Yes	No	No
Multiple Doses	Yes	Yes	Fewer
Variable Production	No	Yes	Yes
Targeted Immune Response via Adjuvant	Yes	Yes/No	No
Demographic	All	All	Healthy Only

1.4.2 Killed vaccines

Vaccines containing killed bacteria do not have a risk of reversion, and also have fewer possible side-effects than vaccines containing live bacterial cells (136). There are some drawbacks to using killed vaccines, such as a requirement of multiple doses for continual protection, as well as the cost of manufacturing the vaccine , and the variable immunogenicity that is possible because of the methods used to kill the bacterium (136). A "killed-vaccine" for tularemia was tested in the early 1930's, but its efficacy was questionable, and some patients incurred severe local reactions at the site of vaccination (64, 140, 141). The *F. tularensis* "killed-vaccine" tested by Foshay, needed to be re-administered multiple times throughout the year for the patient to benefit (64). The immunized patients that were not fully protected by the vaccine however, suffered less severe symptoms from subsequent infection (64). In another study, a control group of non-human primates received the vaccine and had similar infectivity rates as the challenged group (40).

1.4.3 Subunit vaccines

Subunit vaccines normally consist of important pathogen genes, proteins or polysaccharides that are presented to the immune system. Unlike killed vaccines, a protein subunit vaccine is delivered in an immune-stimulating adjuvant formulation, that can stimulate long-lasting cellular and humoral immunity (76, 136), and it is safer than a live vaccine, because it poses no risk for reversion when introduced into a host. Further, specific immune responses can be targeted depending on the choice of adjuvant. However, there is a possibility for adverse reactions to the adjuvant, and the vaccination may require multiple doses for full protection (135). *F. tularensis* proteins with the molecular masses of 61, 37, 32, and 17 kDA have been shown in previous studies to stimulate T cell proliferation in human subjects. Unfortunately,

when used in protective studies, these proteins and others, failed to demonstrate complete protection in the mouse or human model (137, 146, 154, 155). In another study, a proven T cell reactive protein, Tul4, was shown to only induce a minimal protective response in mice through subcutaneous immunization (70). Subunit vaccines containing outer membrane proteins have been shown by Huntley *et al.* to be substantially protective against virulent type A *F. tularensis,* thus showing, at the very least, proof of concept for the use of a subunit vaccine (89).

An alternative to outer membrane isolated from whole cells is the outer membrane vesicles (OMV) secreted by *F. tularensis* LVS. The OMV can contain protein components from the outer membrane, periplasm and cytoplasm as well as cellular enzymes, which can aid in the pathogenic virulence in multiple ways (101). In study by Pierson *et al.*, OMVs were isolated from *F. novicida* and administered in their natively-folded state, as an intranasal vaccine, resulting in a significant survival rate when compared to the naïve mice (127).

1.5 Immunology of tularemia

The immune response can be broken down into innate and adaptive immunity. The innate immune system is the first line of defense against the pathogen, non-specific, without a memory response, and informs the adaptive response about the type of pathogens it encounters via cytokine secretions (43). In *F. tularensis,* the innate system has been shown to be helpful in the primary line of defense against tularemia, however, it alone does not provide for long term protection of infected mice (36). The adaptive immune system evolved from innate immune mechanisms and it does not work properly without interaction with the innate processes (43).

The initiation of the adaptive immune response requires the interaction of antigen presenting cells (APCs) with T or B cells, through recognition of antigen-bound receptors by specific T and B cell receptors (2). When a foreign antigen is engulfed by an APC, it is

processed and displayed on the surface of the APC by a MHC class II molecule (2). If the antigen escapes to the cytosol of the APC or if a nucleated cell is invaded, it will be processed by a proteosome in the cytosol and displayed on the surface bound to an MHC class I molecule (2). These interactions, are regulated by cytokines and can result in either a humoral or cellular immune response over the course of 2-3 days (2).

1.5.1 Humoral immunity to *F. tularensis*

The humoral response involves antibody production and is mediated by B-lymphocytes that have B cell receptors (BCR's) which can bind directly to epitopes on antigens (2). During a humoral response, the B cell receptors on the B cell can bind epitopes on invading pathogens, then engulf and process them through an MHCII, for display of the bound antigen onto its surface (2). A circulating T4-lymphocyte, with a complementary T cell receptor and CD4+ molecule, can recognize this activated, surface MHCII-bound antigen and begin releasing interleukins that either aid in, or block, B cell differentiation into memory B cells or antibody-secreting plasma cells (2). Humoral immunity can play a part in protection against *Francisella*, as indicated by a rise in antibody levels in human volunteers after vaccination with *F. tularensis* LVS (166).

1.5.2 Cellular immunity to *F. tularensis*

The cellular response is mediated by T-lymphocytes with T cell receptors (TCR's) that only recognize major histocompatibility complex (MHC)-bound antigens presented on APCs (2). In a cellular response, $CD4^+$ T cells play an important role and recognize exogenous antigens and $CD8^+$ T cells recognize endogenously-processed peptides via MHCI (6). In order to produce a $CD4^+$ T cell response, the APCs are first primed by engulfing the antigen, and forming a complex of peptides and MHCII molecule for presentation at the surface of the phagocyte (108).

In T cell-rich areas of the lymphatics, primed APCs must come into contact with the naïve T cell (128). Once antigen-specific T cells bind to the antigen displayed by MHCII, they are activated and travel back to the lymph nodes where clonal expansion of effector an memory T cells ensues (128). It is important in vaccine development, to maximize the number of recruited naïve T cells to encounter the antigen, thus improving the memory response (128). After the initial infection, obsolete, primed or effector T cells apoptose, while other memory T cells continue to circulate in the body as sentinels (128). A secondary memory response is initiated, after the same pathogen invades the host again. This normally involves swift recognition of the antigen by $CD4^+$ memory Th1 cells, which differentiate into Th1 cells that produce IFN-γ to help in the induction of $CD8^+$ cytotoxic T cells (CTLs) to kill the infected cells (6, 93, 128). CMI is primarily directed at microbes that survive in phagocytes and microbes that infect non-phagocytic cells, whereas humoral immunity is directed to extracellular microbes.

IFN-γ is an important cytokine in the protection against *F. tularensis*. As shown by Anthony *et al.,* when he exposed C57BL/6 mice exposed to *F. tularensis* after treatment with a monoclonal antibody to decrease the IFN-γ levels resulting in an increased susceptibility to tularemia, and at times a 15-fold increase in the amount of *F. tularensis* was recovered from the antibody-treated animals as compared to the controls (6). In a study by Sjöstedt *et al.*, it was shown that after stimulation with multiple *F. tularensis* protein antigens, $CD4^+$ T cells, not $CD8^+$ T cells, proliferated and produced IFN-γ. However, if $CD8^+$ T cells were stimulated with antigen for three days or if they were supplemented with IL-2, they would proliferate and produce IFN-γ, thus demonstrating the ability of $CD8^+$ T cells to produce IFN-γ, but only in response to initial $CD4^+$ T cell proliferation (145). In a study by Chen *et al.,* it was shown that the thymus, which produces T cells, is severely atrophied when an aerosol infection by *F. tularensis* Type A is

initiated, suggesting a delayed primary immune response when compared to other pathogens (29).

1.5.3 Protective immunity to *F. tularensis*

Some organisms predominantly elicit either humoral or cellular immune responses, but protection against most infectious diseases require a combination of the two, depending on various factors such as: inoculum size, route of infection, status of host immunity (35). Since *F. tularensis* is an intracellular pathogen, it is commonly thought to rely solely on cell-mediated immunity (75, 159). However, there have been multiple studies revealing the involvement of both arms of the adaptive immune response in combating *F. tularensis*. When using subimmunogenic doses, *F. tularensis* LVS LPS has surprisingly been shown to elicit a specific, strong and long-lived anti-*Francisella* resistance, that appears to be dependent on IFN-γ and B-cells (50, 165). In a study by Sebastian *et al.*, a combination of humoral and cellular response was invoked using components of *F. tularensis* (143). For the cell-mediated response, they immunized mice with *F. tularensis* LVS::*wbt*A, an O-polysaccharide (OPS)–negative mutant derived from the live vaccine strain of *F. tularensis*. To induce the humoral antibody response to the OPS, which is a key virulence determinant for type A organisms, an OPS–tetanus toxoid (OPS-TT) glycoconjugate was used for immunization (143). Mice received two doses of both vaccines, and after one month were challenged with a lethal dose of *F. tularensis* LVS. Mice that were given only one vaccine, either OPS-TT or the LVS::*wbt*A, succumbed to the infection within 5-7 days, whereas the mice receiving the combination vaccine didn't show any signs of illness and survived. Thus showing that a two-armed approach to the immune response can be more effective in vaccine development than a single-armed approach (143).

1.6 *F. tularensis* physiology and how it relates to vaccine development

F. tularensis is a Gram-negative bacterium, which mainly consists of an asymmetric outer membrane (OM), periplasmic space, cytoplasmic membrane, cytosolic region, Type IV pili (T4P), secretory systems and capsule (111) (Figure 1.2). The lipid bi-layer of the OM is composed of an outer leaflet that is dominated by lipopolysaccharides (LPS) (Figure 1.2) (86, 164). The inner leaflet of the OM is composed of phospholipids and lipoproteins that link the

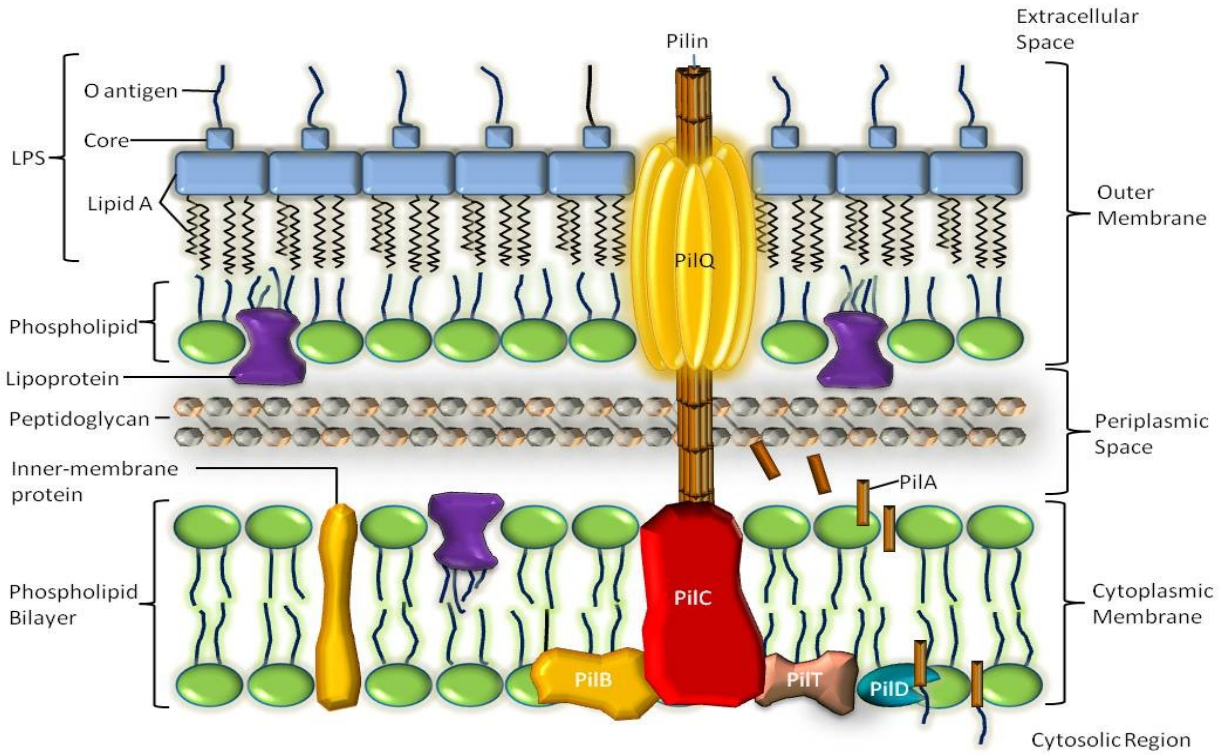

Figure 1.2 Schematic drawing of the cellular envelope of *F. tularensis*. The outer membrane consists of a asymmetric phospholipid and lipid A bilayer. Lipopolysaccharide makes up the outer membrane. Capsule, exterior to the OM is not shown. The peptidoglycan is interior to the OM and exterior to the cytoplasmic membrane which encases the cytosol. The T4P complex is made up of PilQ in the OM and PilB, PilC, PilT and PilD in the CM. PilA is cleaved by PilD and translocated through the inner membrane to form a pilin filament for secretion through PilQ protein to the surface of the bacterium. Figure adapted from Salomonsson *et al* (118) and White *et al.* (168).

OM with the thin peptidoglycan located in the periplasm and encapsulate the cytoplasmic membrane (164), and additional proteins are associated with the outer membrane (88). The capsule surrounds *F. tularensis* protecting it from serum-mediated lysis and is composed of the repeating polymer of the *F. tularensis* O-antigen subunit (7, 67). Independent of capsule, long fibers of pili can be found interspersed at polar locations on the cells (67). Components of each structure can contribute to the virulence of the bacterium as discussed in the following sections.

1.6.1 LPS of *F. tularensis*

LPS is a heat-stable, non-proteinaceous, non-secreted product present on the surface of Gram-negative bacteria and is usually involved in the stimulation of the immune system (164). The LPS of *F. tularensis* differs from other Gram-negative bacteria, in that it is a poor inducer of the immune response, does not have free phosphate moieties, exhibits low endotoxin activity, and thus is considered fairly non-toxic (5, 73, 136). Even though it is a poor stimulator of the innate immune system, several studies have indicated that antibody-mediated protection developed to the LPS of *F. tularensis* LVS, can aid in the defense against tularemia (65, 107, 159). Mice immunized intraperitoneally (IP) with the lipopolysaccharides (LPS) of *F. tularensis* LVS were protected against an IP challenge of attenuated *F. tularensis*, however, they were not protected against the virulent SchuS4 strain (65, 165).

The structure of *F. tularensis* LPS consists of lipid A, a short core oligosaccharide, and an O-antigen polysaccharide (OPS) side chain (164, 165). The basic structure of lipid A is consistent among the *F. tularensis* subspecies with some substitutions between species (73). The lipid A structure does not have phosphates on the 4-carbon of the non-reducing glucosamine residue and the adjacent reducing glucosamine lacks a free phosphate on the 1-carbon. This may contribute to the low endotoxicity of *F. tularensis* lipid A as demonstrated by lipid A

dephosphorylation in Salmonella, resulting in low endotoxic activity (165). It is also possible that the cause of the weak endotoxicity of LPS in *F. tularensis,* as compared to highly endotoxic *E. coli,* is caused by the longer chain fatty acids of the *F. tularensis* lipid A, and the lack of acetylation on the three carbon of the non-reducing glucosamine (165).

As in other Gram-negative bacteria, glucosamine-based, lipid A, aids in structural integrity of the membrane by acting as the hydrophobic anchor of LPS into the outer membrane portion of the bacteria (66, 130). Unlike most Gram-negative bacterium, the lipid A portion of the *F. tularensis* LPS is not involved in activation of the innate immune system through recognition by human host cells expressing Toll-like receptor 4 (TLR4) pattern-recognition receptors (3). The lack of phosphorylation on the non-reducing glucosamine of lipid A has been explored with respect to the biosynthetic process. *F. tularensis* initially creates a 4′ phosphate, then uses the 4′ phosphatase LpxF, encoded by *lpxF,* to remove this group, thus enabling *F. tularensis* to avoid the host immune system (167). Wang *et al.* created a mutant lacking *lpxF,* thus allowing a phosphate group to be left on the lipid A, resulting in the attenuation of the bacterium (167). Therefore, the use of 4′ phosphatase LpxF mutant has been suggested as a potential vaccine candidate, since it can be recognized by the immune system and at the same time is attenuated (167).

The core of LPS connects lipid A with the OPS and provides OM stability (92, 165). The core does not contain any phosphate substituents and can be broken down into an outer region which is distal from lipid A, and the inner region which is directly linked to the lipid A via a single 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo) (92). Unlike most Gram negative bacteria, where synthesis of LPS consists of two or three Kdo moieties, *F. tularensis* only attach one Kdo unit to lipid A during LPS synthesis (175). In *F.tularensis,* the inner region consists of

mannose linked to Kdo instead of the heptose commonly found in other Gram-negative bacteria (165). It appears that *F. tularensis* creates two Kdo units, but expresses Kdo hydrolase to eliminate the other unit. Mutations in the genes that encode for Kdo hydrolase could affect the OM assembly and pathogenesis by decreasing the stability of the OM, leading to the attenuation of the bacterium and its possible use as a vaccine candidate *(175).*

The surface polysaccharide, O antigen (O Ag) linked to the core of LPS is composed of a repeating carbohydrate tetramer and is identical in *F. tularensis ssp. tularensis* and *ssp. holarctica* (31, 38, 142). O Ag enables *F. tulernsis* to be resistant to serum-mediated lysis, as shown by the serum-susceptibility of a strain of *F. tularensis* with an O Ag mutation (31, 73). The O Ag mutation also resulted in decreased survival time of the bacterium within macrophages. Deactivation of the *wbtA*-encoded dehydratase of the O Ag in *F. tularensis* LVS (LVS *wbtA* mutant) results in attenuation of the bacterium in the mouse model. The attenuated strain has been used in vaccine testing with some conferred protection (142).

1.6.2 Capsule of *F. tularensis*

The capsule protects *F. tularensis* from being killed via serum complement and can be thick or thin depending on if it is Type A or Type B *F. tularensis,* respectively (111). The capsule of *F. tularensis* has been shown to increase the virulence of the bacterium and appears to be highly conserved among 14 type A and type B strains as shown by Apicella *et al.*(7). CapB, CapC and CapA are part of a complex that is responsible for the biosynthesis of capsule (173). Removal of *capB* results in a >100-fold increase in attenuation of *F. tularensis Schu4* in mice, thus indicating that the *capBCA* locus is essential for *F. tularensis* survival within the host. Further studies by Zhang *et al.* indicate the locus may facilitates escape from the phagosome and can halt phagosomal maturation, thus aiding in virulence (173).

Hood *et al.* demonstrated the virulence of capsule by inoculating guinea-pigs with modified *F. tularensis,* in which the capsule removed, resulting in avirulence towards most guinea-pigs tested (86). The capsular physiochemical analysis indicated that it is extremely similar to the composition of the LPS O-antigen with 50% lipid composition (7, 86). Upon mass spectrometry (MS) analysis, Kdo and lipid A were not shown to be a component in the capsular antigen. The studies done by Apicella *et al.* indicate that a purified capsule of O-antigen, free of LPS or other bacterial components, have the potential to protect against *F. tularensis* subsp. *holarctica* infection based on the active immunization by the capsule of *F. tularensis*, resulting in capsule-specific antibody production (7, 37).

1.6.3 Surface and outer membrane proteins and membrane vesicles

During invasion of the host by *F. tularensis*, outer membrane proteins or surface-bound proteins would potentially be the first structures encountered by the immune system (88). Thus, identification and an understanding of these proteins would be important in vaccine development (88). Some of the membrane proteins of *F. tularensis* were isolated in work done by Huntley *et al.*, including previously identified FopA, Tul4 (88, 117, 147). Using a 2DE immunoblot Huntley *et al.* identified 15 outer membrane proteins, with five as inducing antibody production during murine infection (KatG, PilQ, OmpA, FopA and Tul4-A) (88). The identification of surface proteins can facilitate the further understanding of host-pathogen interactions.

Another structure, called pilin, has been identified on the surface *F. tularensis.* Pilin are long, thin structures located polarly in bundles, and have been noted to be involved in biofilm formation, host cell attachment, DNA uptake and twitching motility in other bacteria (67, 111). The long surface fibers characteristic of Type IV pili (T4P) are encoded by the *pilA* gene (67). A protein complex of PilA, PilB, PilC, PilD, PilT, PilQ make up the T4P system in *F. tularensis*

(118) (Figure 1.2). PilQ is a pore-forming secretin located in the OM and facilitates secretion of PilA-derived multimeric pilus fiber (118). In *F. tularensis* subspecies *holarctica,* T4P has been uncovered as an important factor in the virulence and spread of *F. tularensis* from initial site of infection (63). In the study by Forslund *et al,* mice were infected with either a *pilA* deletion strain FSC354 or a pilin-expressing strain FSC352. After 11 days the mice without the *pilA* gene, were able to clear infection from the spleen, whereas the mice infected with FSC352 succumbed to the infection after 6 days (63).

Adherence to mucosal membranes is also important for the molecular pathogenesis of infection (113). It was shown by Melilli *et al.,* that expression of FspA, an outer membrane protein of *F tularensis,* aids in its adherence to A549 human lung cells (113). Of note, both *F. tularensis* LVS and the *F. novicida* express FspA, but *F. novicida* does not transport it to the outer membrane, so the ability of *F. novicida* to adhere to A549 lung tissue is greatly reduced when compared to *F. tularensis* LVS. When mice were infected with *F. tularensis,* antibodies to the FspA protein were recovered (113). Since FspA appears to aid in the virulence of *F. tularensis* and there is a recognizable immune response to it, it would be a likely vaccine candidate for a subunit vaccine.

1.6.4 Secreted proteins of *F. tularensis* and secretion systems

F. tularensis secretion systems were first described by Hagar *et al.,* in which components of a Type IV pili homologue are indicated as a mechanism for protein secretion (74) A protease, PepO, is secreted via the T4SS and plays a role in conversion of pre-endothelin into a robust vasoconstrictor to confine the bacterial infection to the skin (74). The gene encoding this protease, *pepO,* is mutated in the Type A and Type B agents, thus allowing for better evasion of the immune system and increased virulence through evolution (74)

The outer membrane (OM), which guards the bacterium from the environment, is asymmetric and can secrete OMVs (34). The OMVs can encase immunogens and have been shown in previous studies to be potential vaccine candidates (34). It is suggested that the *Francisella* pathogenicity island (FPI), made up of 33-kb gene cluster, encodes for the Type VI secretion system (T6SS) (20). Membrane puncturing and core stabilizing proteins, VgrG and DotU respectively, are encoded by the FPI (20). Without these two proteins, *Francisella* is unable to escape the phagosome, has decreased ability to multiply and thus has increased attenuation of the bacterium within the mouse (20).

1.7 MS identification of antigens

Mass spectrometers are used to further identify the protein through mass of the peptides derived by digestion of the protein, and their fragmentation profiles (49). Once the peptides are separated and fragmented by tandem MS (MS/MS), computer programs such as SEQUEST, Mascot, or X!Tandem are used to match spectra to previously identified protein sequences derived from genome sequencing data (114). MS is not quantitative, due to the vast physicochemical properties exhibited by proteolytic peptides such as size, charge, hydrophobicity, etc.; therefore one cannot directly quantify each peptide, and differences in mass spectrometric response will result (12). Comparison of individual peptides between experiments is required for accurate quantification (12).

Label-free and stable isotope labeling are two of the platforms used to quantify proteins (174). Stable isotope labeling (SIL) requires a specific mass tag to be incorporated into proteins or peptides metabolically, chemically, enzymatically, or through spiked synthetic peptide standards that can be recognized by a mass spectrometer (12). The theory behind stable-isotope labeling is that peptides with or without labeling behave the same during MS analysis, therefore

quantification can be achieved by comparing relative signal intensities of mass differences recognized by the mass spectrometer (12). A few of the different types of stable isotope labeling are: Isotope-coded affinity tags (ICATs), stable isotope labeling by amino acids in cell culture (SILAC), isobaric tag for relative and absolute quantification (iTRAQ) (174, 177). The benefit of SIL is its powerful, unbiased method for accurately determining changes in the levels of proteins and post-translational modifications (174, 177). However, for quantification of levels of post-translational modifications the presence and signatures of modified and unmodified peptides would be information required prior to the analysis of SIL based data. Some drawbacks to SIL are complex data analysis due to unequal incorporation of label into the peptide, and chemical cost for labeling higher sample concentration (12, 177). Label-free methods compare two or more experiments via direct mass spectrometric signal intensity for a peptide or via calculations of frequency for protein-matching spectra as indicator for the relative abundance of the protein within a sample (12, 177). The label-free strategy, has high reproducibility, requires less time and money due to omission of labeling reagents, and has no limit on the number of experiments that can be compared. While this is a useful method for proteomic expression profiling of samples, where large numbers of changes between samples occur, this method can be biased in the quantification of individual proteins because it assumes that each peptide in a protein has equal and linear MS response (12, 174).

Two of the more commonly used techniques of label-free quantification are spectral count-based LC-MS/MS and peak intensity-based comparative LC-MS (12, 174). Relative quantification by peak intensity is generally calculated by direct comparison of multiple analysis of ion intensity changes as denoted by peak heights in chromatography (177). The theory behind this is that the ion with a specific m/z (mass charge ratio) has a specific intensity at a specific

time. As the peptide in the sample increases, so does the peak area, thus correlating linearly to the concentration of protein in the sample (177). This type of quantification is geared towards clinical biomarker discovery, because of the high sample throughput required (177). When compared to spectral count, the peak intensity method is more accurate in reporting changes in protein abundances, but requires complex computer programs (121). The spectral count refers back to observation that the more a specific protein is seen in a sample, the more MS/MS spectra are collected for peptides of that protein (12). Therefore, relative abundance can be quantified by comparison of spectra between experiments (12, 177). An advantage of quantification by spectral count over peak-intensity is that peptides common between datasets are not required for the protein ratio calculations, thus allowing for comparison of greater percentages of proteins and greater sensitivity (121).

1.8 Objective of the thesis research

The work presented in this thesis investigated the immunogenicity of predicted *F. tularensis* LVS proteins. The number of proteins predicted to be encoded on the *F. tularensis* LVS genome, which is based off the information from the Bioproject database located within the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/bioproject/16421), is 1754. From these 1754 *F. tularensis* LVS proteins, we have narrowed our focus to nine proteins that were present and relatively abundant in fractions that elicited significant IFN-γ responses from splenocytes of *F. tularensis* infected mice. The advantage of this research was the ability to narrow down the number of proteins for future studies as well as reinforce previously determined immunogenic proteins of *F. tularensis* LVS.

The research was performed, based on the hypothesis that combining immunological assays for *F. tularensis* fractions containing multiple proteins, with MS/MS analysis of these same fractions, would lead to the identification of a defined set of antigens. The question of whether or not a subunit vaccine is a viable preventative measure lies in the discovery of an immunodominant antigen of *Francisella*. Can specific *F. tularensis* LVS protein fractions stimulate a memory T cell response and will it be strong enough to protect humans from virulent Type A tularemia? Although the available *F. tularensis* LVS proteins were limited and there was some overlap during protein separation, mining the LVS proteome yielded positive results, in so doing the number of proteins of interest were narrowed from 1754 to nine.

CHAPTER 2

MATERIALS AND METHODS

2.1 Conditions for growth of *F. tularensis*

An aliquot of lyophilized *F. tularensis* strain LVS was received from The Center for Disease Control and Prevention (CDC, Fort Collins, CO). Brain Heart Infusion (BHI) broth, 500 µl, was added to rehydrate the *F. tularensis* LVS and mixed well. Cysteine heart agar powder (Difco Cat. No. 0047-17-6) along with 9% sheep blood (Colorado Serum Co. 4950 York St. Denver, CO 80216) was used to create cysteine heart agar with chocolate sheep blood (CHAB) agar plates on which to grow *F. tularensis*. CHAB agar was inoculated with 50 µl of the *F. tularensis* LVS suspension and incubated for 48 h at 37°C. Single colonies were picked and inoculated to fresh CHAB plates and incubated for 48 h at 37°C until the colonies reached a size of about 2-4 mm. With a sterile loop, colonies were collected to resuspend in cryovials containing 1 ml BHI broth and 10% glycerol. The stocks were frozen at -80°C.

Multiple growth media's were evaluated to establish growth curves of *F. tularensis* and to visualize by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein contamination of the broth media. After multiple trials using Chamberlain's, Magar Traub, Mueller Hinton (MH) (Difco Cat. No. 275730) and Glycerol Alanine Salts (GAS) media, MH with an addition of 1% IsoVitaleX was chosen as the broth for batch growth of *F. tularensis* (25, 81, 153). The growth curve served as a good reference point for determining optical density (O.D.), which correlated with specific points on the growth curve, such as mid-log phase (Fig. 3.2).

A sterile wooden stick was used to transfer an inoculum of the *F. tularensis* LVS stock onto CHAB agar plates that were then incubated for 48 h at 37°C. MH broth with IsoVitalex (75 ml) was inoculated with a partial loopful of *F. tularensis* LVS colonies and incubated at 37°C with shaking at 150 RPM for 30 h until mid-log phase growth (~O.D. 1.0 A at 550 nm) was achieved. The culture was aseptically subcultured at a 1:50 ratio into 1 L of MH with IsoVitaleX, and grown an additional 24 h in a shaking incubator at 37°C until mid-log phase (O.D. ~1.0 A at 550 nm). Batch growth was repeated until about 28 L of culture were collected. An aliquot of each 1L culture was plated on LB agar and CHAB agar for quality control.

2.2 Preparation of cell fractions

The cells from the 28 L of culture were harvested using high speed centrifugation, at 10,000 x g, 4°C, for 20 min (Sorvall SLC-6000 rotor) to separate culture supernatant (CS) from the bacterial cells. The CS was decanted into a 4 L Winchester jug with 0.02% sodium azide to inhibit Gram-negative bacterial growth and stored at -20°C. The pellet of *F. tularensis* LVS cells was suspended in about 12 ml phosphate buffered saline (PBS), pH 7.4, and centrifuged in Oakridge tubes at 10,000 x g, 4°C, for 20 min. The supernatant containing PBS was carefully decanted. The mass of the pellet was recorded and frozen at -80°C. In total, 127.4 g of *F. tularensis* LVS cell pellet was collected from around 28 L of *F. tularensis* growth.

Each Oakridge tube containing a frozen cell pellet of *F. tularensis* LVS was thawed on ice and suspended in 4 ml of breaking buffer, containing 1 EDTA-free protease inhibitor cocktail tablet (Roche Cat. No. 11873580-001) per 50 ml PBS, pH 7.4, 0.06 µg/ml DNase, 0.06 µg/ml RNase, and 1 µg/ml lysozyme. An aliquot of the supernatant was carefully removed to analyze cell integrity by light microscopy. The cell suspension was centrifuged at 10,000 x g, 4°C, for 20 min. The newly formed pellet was suspended in 4 ml breaking buffer, sonicated on ice, 60

sec on and 60 sec off, with 3 repetitions. In order to visually compare the degree of cell fragmentation pre- and post-sonication, samples were Gram-stained and viewed under a microscope.

The cell lysate was centrifuged at 3,000 x g, for 20 min, to remove unbroken cells. The supernatant was transferred to pre-weighed ultracentrifuge tubes, and centrifuged for 4 h, 4°C, at 100,000 x g (SW28 rotor). The supernatant containing cytosolic and periplasmic proteins ("soluble fraction") was removed from the pellet containing membrane proteins ("membrane fraction") and the membrane pellet was suspended in 10 ml breaking buffer, and transferred with a pipette into 50ml conical tubes. The membrane and soluble fractions were frozen at -80°C (85, 103).

Soluble and membrane fractions were thawed on ice. Dialysis tubing (3,500 MWCO) was prepared by boiling in dH_2O for 7 min. Soluble and membrane fractions were added separately to the prepared dialysis tubing, and dialyzed against 10 mM ammonium bicarbonate for 24 h, at 4°C, with three buffer changes. After 24 h, the dialyzed material was collected into separate 50 ml conical tubes and aliquots of 100 µl were set aside for quality control. The remaining material was frozen in 5 mg aliquots (5.6 µg/µl soluble and 14.4 µg/µl membrane) at -80°C, and lyophilized. The protein content of each fraction was measured using bicinchoninic acid (BCA) protein assay kit (Pierce Cat. No. 23255) as stated in manufacturer's instructions (148). SDS-PAGE and silver-staining were performed on each fraction (102).

2.3 SDS-PAGE polyacrylamide gels

An aliquot (8 µl) of each sample protein fraction (5 µg) was mixed with 2 µl of 5X Laemmli loading buffer (63 mM Tris HCl, 10% glycerol, 2% SDS, 2.5% β–mercaptoethanol, 0.0025% bromophenol blue, pH 6.8) (102). The samples were boiled for 5 min, at 100°C, and

centrifuged briefly in a microfuge (100). Pre-stained markers (Bio-Rad Cat. No. 161-0374) and the denatured samples were loaded on a 10-20% Novex tris-glycine gel (Invitrogen Cat. No. EC61355BOX EC6135). Electrophoresis was performed in 1X running buffer (25 mM Tris HCl, 192 mM Glycine, 0.1% SDS at pH 8.3) at 125 V. Electrophoresis was terminated once the dye front reached the bottom edge of the gel. Gels were then silver-stained for visualization of resolved proteins.

2.4 Whole gel electroelution of proteins

Lyophilized protein fractions, from section 2.2, were dissolved with less than 1 ml of dH_2O, and 5X Laemmli loading buffer (200 µl), to a concentration of 5 µg/µl. Samples were boiled for 5 min, at 95°C, and centrifuged for 30 sec, at 10,000 x g. Pre-made PROTEAN II 10-20% tris-glycine gel was set up in PROTEAN II xi-Cell as directed by manufacturer (Bio-Rad Cat No. 165-1931). Samples (5 mg) were loaded each gel and run initially for 30 min at 30 mA (800 V), immediately after which, the power was increased to 60 mA (800 V) for ~3 h until the dye-front reached the bottom of the gel. Proteins were eluted from gel using the Whole Gel Eluter (Bio-Rad Cat. No. 165-1251) with 1X tris-CAPS buffer, 9.4 pH, operated at 250 mA for 1.25 h. Anode and cathode were reversed for 20 sec to loosen proteins from the membrane, and 30 fractions (about 2.5 ml each) were eluted into harvesting chamber using a vacuum pump. An aliquot (200 µl) of each fraction was dried in a Savant SpeedVac for about 3 h. Samples were suspended with 40 µl Millipore dH_2O. An aliquot of each sample was run on a 1.0 mm x 15 well 10-20% tris-Glycine gel (Invitrogen Cat. No. EC61355BOX) for 1.5 hr at 400 mA (200 V) thereafter, proteins were visualized by staining with silver nitrate. Protein concentration of each sample was assessed using the BCA kit (Pierce Cat. No. 23225) (148).

A total of five preparative SDS-PAGE runs were performed for the membrane and soluble protein preparations. Final Whole Gel Eluter fractions were pooled accordingly based on protein size as observed with the silver-stained SDS-PAGE gels. This resulted in a total of 16 soluble protein fractions, and 19 membrane protein fractions. The pooled fractions were lyophilized, sterilized with 24,000 Gy of γ-irradiation, and stored at -80°C.

2.5 T cell assay

T cell assays were performed by Emily Kampf, in the laboratory of Catherine Bosio, using following protocol. C57/BL6 mice were infected via intranasal inoculation with a sublethal dose (5,000 CFU) of *F. tularensis* LVS. After 40 days, spleens, from five infected C57/BL6 mice and three uninfected mice, were harvested. Spleens were placed in separate 15 ml conical tubes (BD Cat. No. 352097), on ice, with ~3 ml Hank's balanced salt solution (HBSS) (Gibco No. 14025). Spleens were decanted into 70 μm nylon cell strainer (BD Cat. No. 52350) over an open tissue-culture dish (60 x 15 mm BD Cat. No. 353002). Spleens were mechanically processed by forcing them through a strainer with the plunger end of a syringe (BD Cat. No. 301603). The strainer was rinsed 3 times using 5 ml HBSS. All cells were transferred back into the original 15 ml conical tube, and ~8 ml fresh HBSS was added. The spleens were centrifuged at 1,000 x g, for 5 min, at 4°C. Supernatants were decanted to waste. The cell pellets were suspended with 3 ml NH_4Cl, and allowed to react for 3 min at room temperature (RT) to lyse red blood cells (RBC). HBSS was added to each tube up to 10 ml, and the splenocytes collected by centrifugation at 1,000 x g, for 5 min, at 4°C. The final cell pellet was suspended in 1 ml complete minimum essential medium (CMEM, Gibco Cat. No. 11095) per spleen. All non-infected controls were pooled into one 15 ml conical tube and all infected spleens were pooled into a separate 15 ml conical tube. Splenocytes were counted under microscope with a

hemocytometer counting chamber (Brightline Cat. No. 1492, Hausser Scientific) at a 1:30 dilution of cells to live/dead trypan-blue stain for a total of 1 $x10^8$ live cells / ml (infected mice) and 2.3 x 10^7 live cells / ml (control mice). Cells were diluted with CMEM to a concentration of 2 x 10^6 cells / ml and 100 µl aliquots of the splenocytes were placed in tissue-grade, 96-well, U-bottom plates, with low evaporative lids (BD Cat. No. 353077). The antigen fractions (10 µl at 0.2 µg/µl) were added to appropriate wells. A whole membrane antigen (final concentration of 0.01 µg/µl), supplied by Dr. Bosio, was used as a previously proven, positive control (137, 145). Antigen was not added to negative control, standard or blank wells. An additional 100 µl CMEM was added to each well for a final antigen concentration of 0.01 µg/µl. The prepared, 96-well plates were incubated at 37°C, with 5% CO_2, for 3 days. After 3 days, plates were removed from incubator and 150 µl of the supernatant was transferred, via pipette, into a fresh 96-well plate. The new plate was wrapped with parafilm and frozen at -80°C.

2.6 ELISA

Murine IFN-γ ELISA kit (R&D Cat. No. DY 485) was utilized to assay IFN-γ. The IFN-γ standard from the kit was diluted 1:30. This primary standard was serially diluted at a 1:1 ratio until seven standards were created (1:30, 1:60, 1:120, 1:240, 1:480, 1:960, and 1:1920). Reagent diluent was created to a final concentration of 0.1% bovine serum albumin (BSA Invitrogen) and 0.05% tween20 in Tris buffered saline (20 mM Tris Base, 150 mM NaCl) at a pH of 7.3 and filtered through a 0.2 µm filter.

Capture antibody (50 µl / well) (rat anti-mouse IFN-γ diluted 1:180 in PBS from kit) was used to coat a 96-well ELISA plate (NUNC maxisorp Apogent Cat. No. 80040LE 0903) overnight at 20°C and the plate was washed using a plate washer. The plate was blocked with 1% BSA in PBS, 0.05% NaN_3, for 1 hr at room temperature (RT), then washed with the plate

washer. Each standard (50 µl), and supernatants (50 µl) from antigen-stimulated splenocytes were added to the wells. The plates were covered in parafilm, incubated at RT for 2 h, and washed with the plate washer. Detection antibody (biotinylated goat anti-mouse IFN-γ diluted 1:180 in reagent diluent, from kit) was added (50 µl) to each well, covered with parafilm and incubated for 1 h at RT, and the plate washed with the plate washer. Streptavidin-HRP (50 µl of a 1:200 dilution) was added to each well. The plate was covered in parafilm and incubated at RT in the dark for 20 min, and washed with the plate washer. Color reagents A (H_20_2) and B (tetramethylbezidine) were equilibrated to RT, and 50 µl (1:1 mixture of color reagent A and color reagent B) was added to each well, and incubated at RT, in the dark, for 20 min. Stop solution (2N H_2SO_4) (50 µl) was added and mixed gently. The plate was analyzed at 450 nm on a microplate reader and the data was plotted against a standard curve of IFN-γ. The standard error was within 10% of the mean values.

2.7 In-gel Digestion of Proteins

Preparative SDS-PAGE fractions (10 µg) were resolved on SDS-PAGE using 10-20% Novex tris-glycine gel (Invitrogen Cat. No. EC61355BOX EC6135) in 1X running buffer (25 mM Tris HCl, 192 mM Glycine, 0.1% SDS at pH 8.3) at 125 V for 90 min. The gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad) and the proteins of interest were excised with a razor blade. Each excised gel section was cut into small pieces and placed in a de-plasticized eppendorf tube. De-plasticizing of tubes was done by filling with 60% acetonitrile and 0.1% trifluoroacetic acid (TFA), followed by mixing, incubation at RT for 1 hr and decanting. The de-plasticizing step was repeated and tubes were dried with the Savant Speed-Vac. The gel pieces were de-stained with the de-stain solution (60% acetonitrile in 0.2 M ammonium bicarbonate, 0.1% TFA) at 37°C, for 30 min. The solution was discarded and the de-

staining step was repeated until the stain was completely gone. The gel pieces were dried in the Savant Speed-Vac. To prepare the samples for MS/MS, each tube containing a gel piece was filled, until the gel was covered, with a modified trypsin solution (25 µg of trypsin in 300 µl of 0.2 M ammonium bicarbonate). The samples were incubated at RT until all the trypsin solution was absorbed, after which, 0.2 M ammonium bicarbonate was added in 10-15 µl increments until the gel was completely rehydrated and slightly covered in solution. The samples were incubated overnight (O/N) at 37°C. The reaction was stopped by adding 10% TFA (at 0.1 the digest volume). The supernatant was collected and put in new de-plasticized tube. Extraction solution (60% acetonitrile, 0.1% TFA) was added (100 µl) to each sample, the samples were mixed by vortexing and incubated at 37°C, for 40 min, with one vortex at 20 min. The extract was centrifuged, the supernatant collected, and added to the previously collected supernatant. This collection step was repeated. The collected samples were dried in a Savant Speed-Vac until 1-2 µl of liquid was left in the tube. 10 µl buffer A (5% acetonitrile, 0.1% acetic acid) was added to each sample and mixed, followed by centrifugation for 5 min. Supernatant was transferred, via pipette, into autosampler vials and stored at -20°C until analysis by MS/MS (82, 134, 149).

2.8 In-Solution Digestion of Proteins

Trypsin solution (10 µl) (25 µg trypsin and 300 µl 0.2 M ammonium bicarbonate) was added to dried antigen sample (10 µg), and allowed to digest overnight at 37°C. After 24 h, 10% TFA (3 µl) was added to halt trypsin reaction. The sample was dried in a Speed-Vac savant for 10-15 min. The sample was stored at -70°C until ready for MS/MS analysis. Once ready for analysis, 10 µl of buffer A was added to the sample and mixed well. Sample was centrifuged for 5 min, at around 1,200 RPM and the supernatant (10 µl) was transferred to an autosampler vial.

2.9 LC-MS/MS

The peptides obtained via trypsin digestion, were resolved on a 0.2 × 50-mm C_{18} reversed phase HPLC column, (Agilent Technologies, Santa Clara, CA) using an Agilent 1100 capillary HPLC solvent delivery system at a flow rate of 5 µl/min with an increasing gradient of acetonitrile. The eluent was guided directly into the linear trap quadrupole electrospray ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA). The operating conditions for the electrospray needle of the mass spectrometer were 4 kV, with a sheath gas flow of nitrogen at 30 p.s.i., and a heated capillary temperature of 200°C. Individual peptide ions were subjected to data-dependent MS/MS fragmentation. Specifically, after a full MS scan the five most dominant ions were selected for fragmentation. Dynamic exclusion was used to ensure multiple ions were analyzed, as each precursor ion was chosen a maximum of two times for MS/MS fragmentation and was placed on the dynamic exclusion list for 1 min. MS/MS data were analyzed by SEQUEST software (Thermo Fisher Scientific, San Jose, CA; Version 27, Revision 12) against *F. tularensis* protein database (1754 entries) for protein identification (114). The percent confidence of protein identification was determined by statistical analysis of the turbo SEQUEST search data using Scaffold (version Scaffold_3.3.1, Proteome Software Inc., Portland, OR) software. Valid peptide and protein identifications were based on a two-peptide minimum per protein, a protein probability of 99.0%, and peptide threshold of 95%, as specified by the Peptide and Protein Prophet algorithms (46, 97, 119).

CHAPTER 3

RESULTS

3.1 Batch growth

F. novicida and the live vaccine strain of *F. tularensis holarctica*, LVS, are often used as models for tularemia in biosafety level 2 laboratory research This study involved the LVS strain as it's model. To define the best growth medium to evaluate protein antigens of *F. tularensis*, a number of different media were investigated as described in the material and methods. It was found that MH, supplemented with 1% IsoVitaleX, was most consistent as a liquid growth media and did not appear to have interfering banding patterns when run on an SDS-PAGE gel (Fig. 3.1A). IsoVitaleX provides essential nutrients such as glucose, iron, cysteine, glutamine, adenine, thiamine pyrophosphate, guanine, and p-aminobenzoic acid (9).

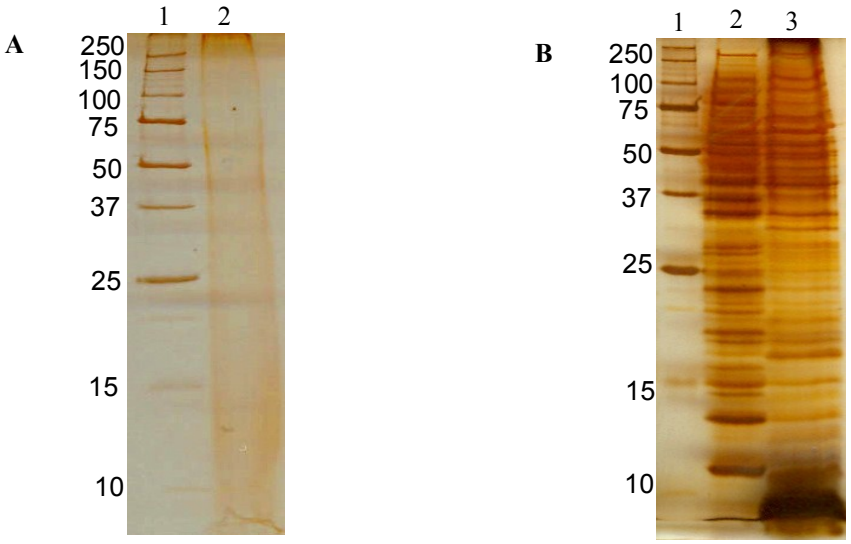

Figure 3.1 Silver-stained *F. tularensis* protein preparations resolved in 15% acrylamide gels by SDS-PAGE. A) Lane 1; molecular mass marker (Bio-rad #161-0374). Lane 2, Mueller-Hinton broth only. B) Lane 1; molecular mass marker (Bio-rad #161-0374). Lane 2, *F. tularensis* soluble fraction, Lane 3, *F. tularensis* membrane fraction.

In order to ensure enough proteins were available for experimentation, 30 L of *F. tularensis* LVS were grown in MH broth supplemented with 1% IsoVitalex. Rate of growth was quantified through measuring absorbance at 550 nm over a three to four day period. Data from three separate runs were plotted and a polynomial trendline was graphed to illustrate the averaged growth curve (Fig 3.2).

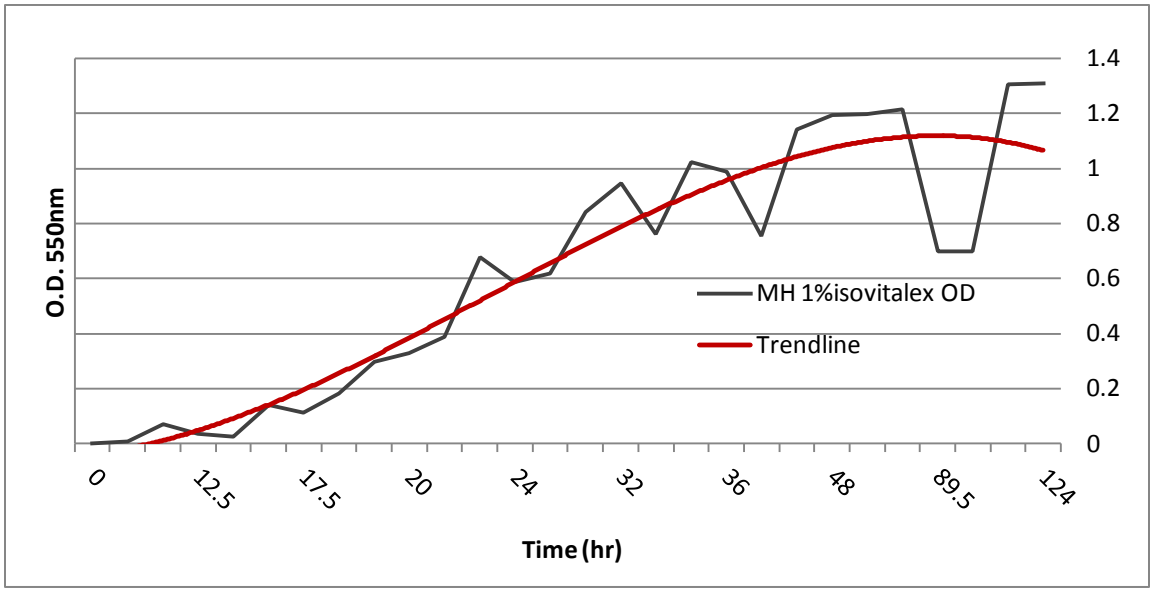

Figure 3.2 Growth curve of *F. tularensis* LVS in MH with 1% IsoVitalex. A two-degree polynomial trendline is displayed in red to indicate the average of three separate growth curves for *F. tularensis* LVS. The data was obtained using a spectrophotometer at 550 nm to estimate the rate of growth of *F. tularensis* LVS.

As for a solid medium, *Francisella* grows well on CHAB agar, as this provides the essential iron source for *Francisella* to grow. *F. tularensis* is unable to grow on LB agar due to lack of cysteine (10, 68). This knowledge was exploited to check for contamination, through simultaneously plating 1 ml of the 1 L batches of *Francisella* onto LB and CHAB plates. All LB plates with any growth were traced back to the original 1 L batch growth and those contaminated batches were removed from further use in the experiment, leaving 27 L of viable *F. tularensis*. Temperature variation was used to differentiate species and subspecies of *Francisella,* such as

novicida versus LVS, which prefers growth at 28°C versus 37°C, respectively (10, 56). Thus, *F. tularensis* LVS was always grown at a constant 37°C. The 1 L batches of *F. tularensis* were grown to mid-log phase, at which time the cells were harvested and the approximate 5 g of pelleted bacteria per liter were frozen at -80°C, until ready for use. Of the 27 L of batch growth, a total of 128 g of *F. tularensis* LVS was obtained.

3.2 Protein fractionation

The thawed cells were lysed through suspension in breaking buffer and probe sonication. The bacteria were determined to be sufficiently lysed through microscopic analysis of the Gram-stained samples pre- and post-sonication. The lysate was separated by centrifugation into crude membrane and soluble fractions. To visualize the separation of these two fractions, they were analyzed by SDS-PAGE (Figure 3.1B). The banding patterns representing the different molecular masses of proteins appeared to be slightly different between the two samples, suggesting there was at least partial uniqueness between the soluble and membrane fractions. Interference by the proteins from MH broth during growth of *F. tularensis* was deemed to not be a major contributor, due to its lack of banding patterns when analyzed by SDS-PAGE (Figure 3.1A).

The membrane and soluble proteins were separated by molecular weight via preparative SDS-PAGE, 5 mg at a time, and eluted via whole-gel elution. After elution, the SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad) to ensure the electroelution was complete. A total of 28 fractions were collected for each elution. Each fraction was run on an SDS-PAGE gel and silver-stained to visualize the separation of proteins and for later use in determination of protein pooling (Fig. 3.3 and Fig 3.4).

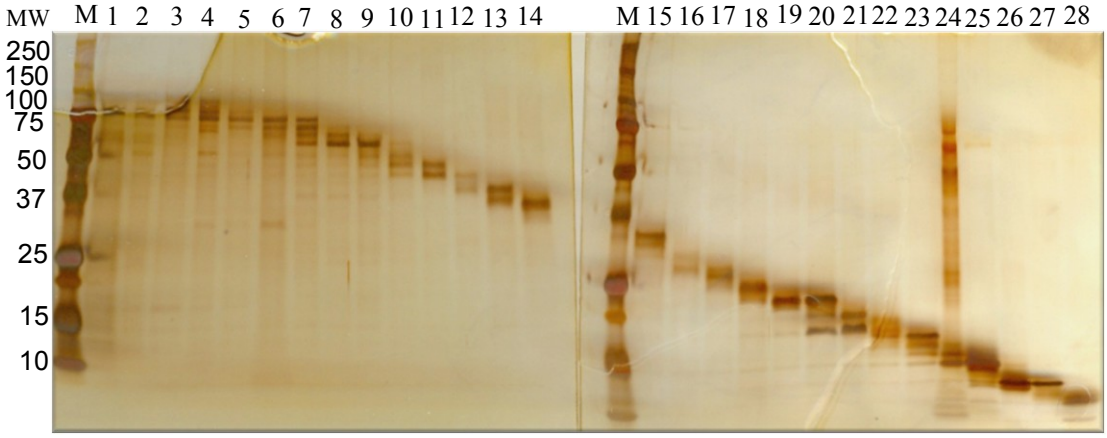

Figure 3.3 Soluble proteins (lane 1-28) eluted after separation by SDS-PAGE. Lane M (marker) (Bio-rad #161-0374). Each numbered lane contains proteins at a specific MW (~10 kDa to ~150 kDa) that were combined with complimentary proteins from each additional run (total of five gels processed). The combination of the proteins can be seen in Figure 3.6. 15% acrylimide gel and silver stain. (Contamination in lane 24; sample not further analyzed).

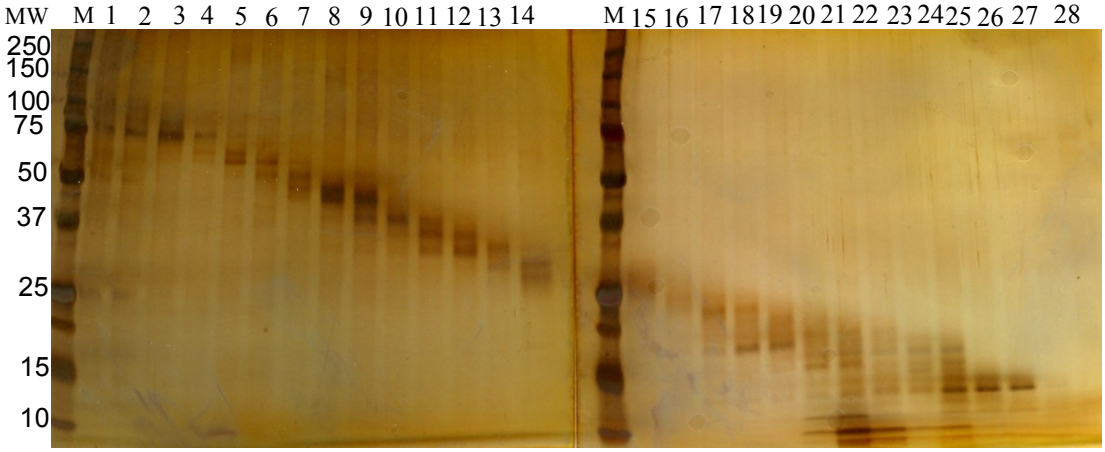

Figure 3.4 Membrane proteins (lane 1-28) eluted after separation by SDS-PAGE. Lane M (marker) (Bio-rad #161-0374). Each numbered lane contains proteins at a specific MW (~10 kDa to ~150 kDa) that were combined with complimentary proteins from each additional run (total of five gels processed). The combination of the proteins can be seen in Figure 3.5. 15% acrylimide gel and silver stain.

This process was repeated five times until the entire stock of membrane and soluble proteins were separated, eluted and visualized. Since separation is not precise, there was overlap of

neighboring proteins in every sample. Also, due to some leakage during elution, final quantities varied between the five runs. All lanes from the five gels for the membrane or soluble fraction were visually compared and the corresponding protein fractions with similar MW's were pooled together into 19 membrane and 16 soluble fractions (Fig 3.5 and 3.6). These protein samples were concentrated and sent for γ-irradiation, then used in the IFN-γ assay.

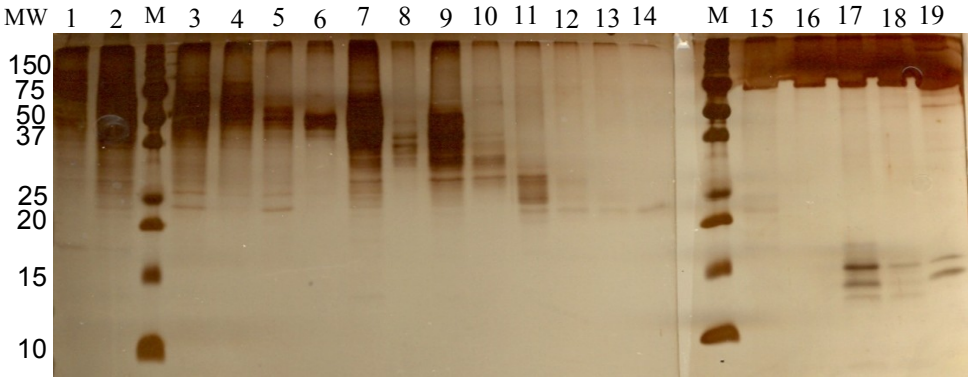

Figure 3.5 Membrane proteins (lane 1-19) pooled from five whole gel elutions after SDS-PAGE. Proteins range from ~10 kDa to ~150 kDA. Measurement of proteins by lane M (marker) (Bio-rad #161-0374). 15% acrylimide gel and silver stain.

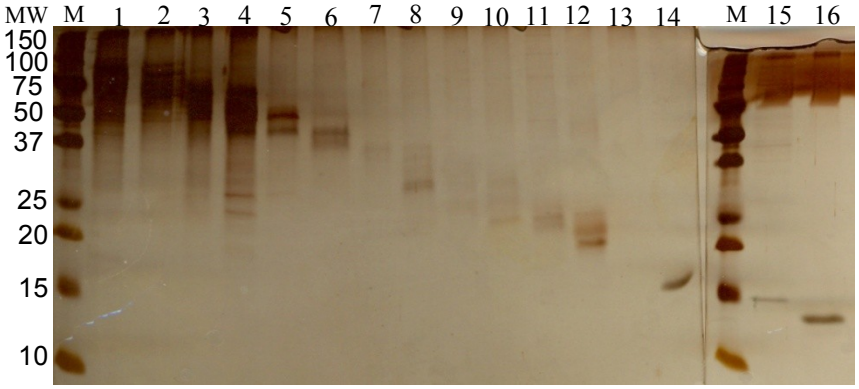

Figure 3.6 Soluble proteins (lane 1-16) pooled from five whole gel elutions after SDS-PAGE. Proteins range from ~10 kDa to ~150 kDA. Measurement of proteins by lane M (marker) (Bio-rad #161-0374). 15% acrylimide gel and silver stain.

3.3 Immunological activity

The data presented and described in this section was provided from the laboratory of Dr. Catherine Bosio as a collaborative process. C57/BL6 mice were intranasally-innoculated with live *F. tularensis* LVS and allowed to clear the infection over a 40 day period. After 40 days, the spleens of five infected and three uninfected mice were removed and processed in order to cultivate the splenocytes. Normal murine spleens can contain macrophages, dendritic cells, B-cells, reticular cells, $CD4^+$ T-cells, $CD8^+$ T-cells, hematopoietic cells, plasma cells, plasmablasts and erythrocytes (24). Complete composition of the splenocytes were not taken into account in the current study. Non-infected murine splenocytes and infected murine splenocytes were compared for their reaction to each antigen. The splenocytes were combined with the size-fractionated membrane (19 samples) and soluble proteins (16 samples) and placed in 96-well plates in triplicate. Whole *F. tularensis* membrane (0.01 μg/μl) was used as the positive control, while unstimulated splenocytes represented the negative control. The wells were covered with CMEM and incubated for three days to allow for immune response to occur from processing and presentation of the antigen to the immune T cells. After a three-day incubation period, IFN-γ secretion was quantified by an ELISA. The amount of IFN-γ (pg/ml) produced by splenocytes of non-infected mice were subtracted from the IFN-γ (pg/ml) produced by splenocytes from infected mice tested against the same fraction (41). The test was run in triplicate and error bars on the accompanying graphs (Fig 3.7 and Fig. 3.8) represent the standard deviation of each triplicate sample. Seven fractions (membrane fractions 1, 2, 7 and soluble fractions 1, 3, 4, 5) were found to induce significant IFN-γ responses (p-value of <0.05) when compared to three-fold greater than the average response of all membrane (314 pg/ml) or soluble (317 pg/ml)

fractions (41). The seven were chosen for further identification of protein composition by biochemical analysis through MS/MS (Fig. 3.7 and Fig. 3.8) (41).

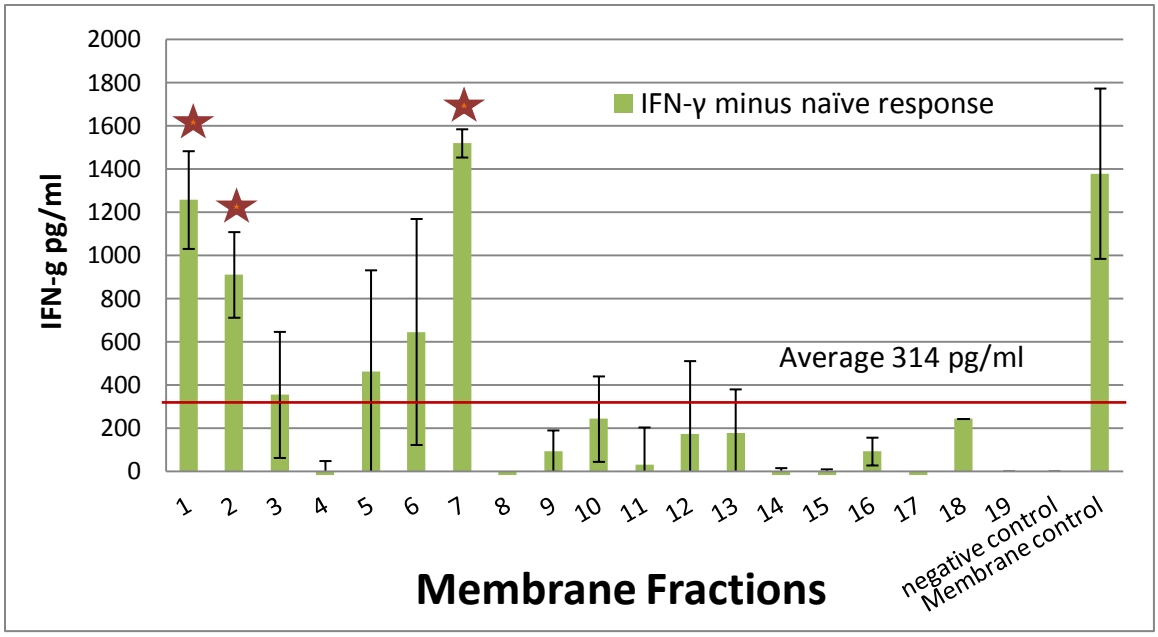

Figure 3.7 IFN-γ production by T cells from LVS-infected and non-infected control murine splenocytes that were stimulated by 0.01 µg/µl pooled membrane proteins of *F. tularensis*. Error bars reflect the standard deviation within the triplicate samples. Average of all samples are indicated by a red line at 314 pg/ml. Stars indicate protein fractions that had a p-value of <0.05 that were further analyzed through MS/MS analysis.

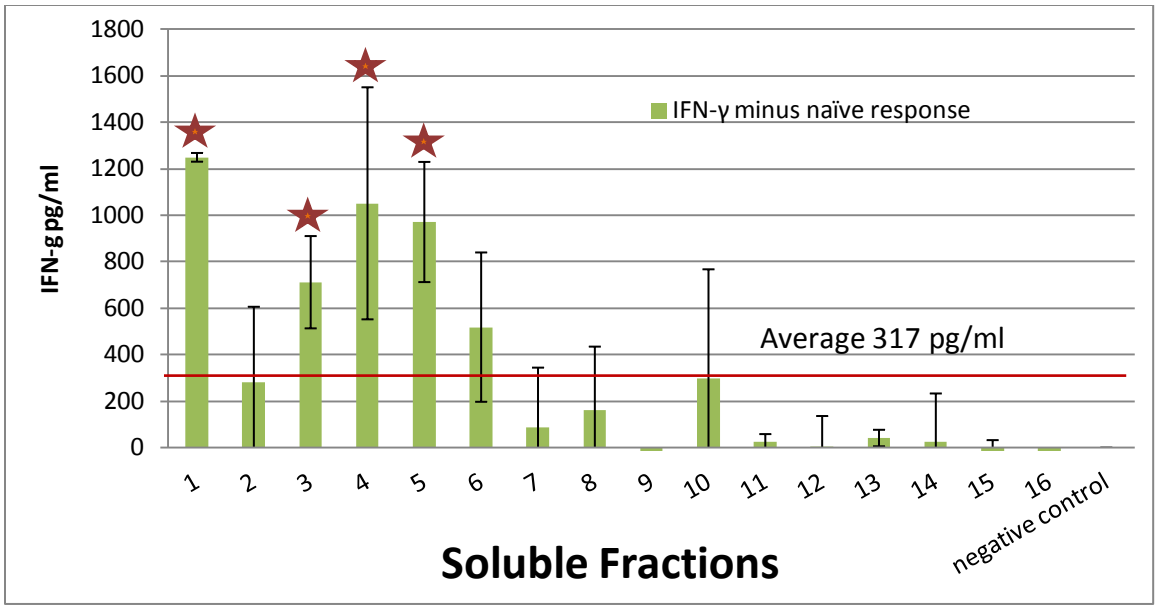

Figure 3.8 IFN-γ production by T cells from LVS-infected and non-infected control murine splenocytes stimulated by 0.01 µg/µl pooled soluble proteins of *F. tularensis* that were separated by MW. Error bars reflect the standard deviation within the triplicate samples. Average of all samples are indicated by a red line at 317 pg/ml Stars indicate protein fractions that have a p-value of <0.05 and were further analyzed through MS/MS analysis.

3.4 MS/MS Analysis of Reactive Fractions

Information gathered from the T cell assay via the ELISA readings indicated four soluble and three membrane fractions to have a three-fold higher than the average IFN-γ response (error taken into account) of the soluble (950 pg/ml) or membrane fractions (943 pg/ml) (see Fig. 3.7 and Fig. 3.8). This is in line with another *F. tularensis* immune assay in which T cells were stimulated with 0.01 µg/µl antigens to produce >400 pg/ml IFN- γ (125). An aliquot (10 µg) of each of these seven fractions was resolved by SDS-PAGE. The resulting Coomassie Brilliant Blue-stained bands were excised, digested with trypsin and analyzed via LC-MS/MS for identification of specific proteins. This attempt to perform MS/MS analysis on in-gel digests provided poor results. Another attempt at LC-MS/MS analysis was done using in-solution digestion at 10 µg per sample. Upon analysis of this 10 µg data, (data not shown) it was

determined that there was an overabundance of proteins per the 10 µg samples. Specifically a total of 3 to 26 proteins were identified in each fraction. Thus, for each fraction in solution, trypsin digestions were performed on 2.5 µg, 5 µg, and 10 µg of each fraction to compare the amount of proteins for differing concentrations. This approach was designed to allow for analysis of an optimal protein amount and to help determine the most abundant proteins for each active fraction. Additionally 5 µg of the non-INF-γ inducing fractions were analyzed by resolution trypsin digestion and LC-MS/MS (Table 3.1).

The identification of proteins based on the LC-MS/MS data was accomplished using the SEQUEST software and statistical validation via the Scaffold software package (Proteome Software Incorporated, Portland, OR). As assigned by the Protein Prophet algorithm, only proteins that had a 99% or greater confidence value and contained two or more unique peptides, were accepted (119). Peptide identification was accepted when the probability based on the Peptide Prophet algorithm was >95% (97). The parameters chosen in this study were set by Jeff Chandler during a similar study which defines the parameters for MS/MS analysis for the highest probability of correct peptide assignment and allows for ease of comparison between future research groups (97). In general, 2.5 µg samples showed between one to 13 proteins per sample with an average of four proteins per sample. The 5 µg samples allowed for identification of one to 14 proteins per sample with an average of eight proteins per sample, and the 10 µg samples allowed for the identification of three to 26 proteins in each sample with an average of 15 proteins per sample (Fig 3.9).

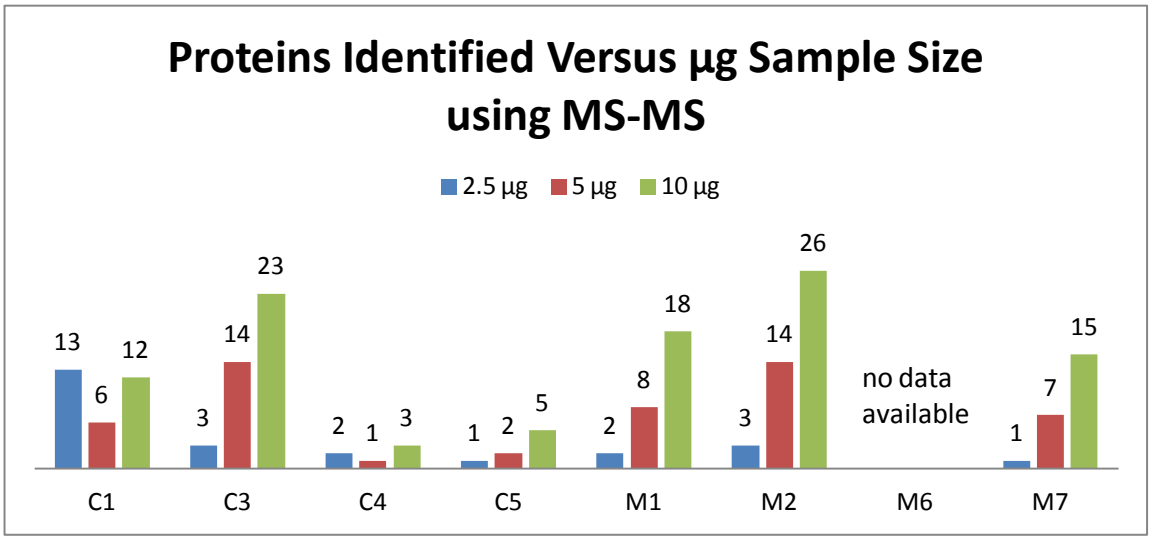

Figure 3.9 Number of proteins in each sample found after MS/MS analysis. See Table 3.2 for specific proteins in each sample.

The LC-MS/MS performed on the 5 µg samples for seven T cell reactive fractions was used to define the putative T cell antigens. This resulted in a list of 32 proteins (Table 3.1 and Appendix 1). In order to determine whether the proteins were unique to active fractions, an LC-MS/MS analysis was performed on trypsin digests (5 µg) of the fractions that failed to stimulate strong T cell response. The identification of proteins in these fractions (data not shown) showed that five of the 32 proteins were also present in the non-active fractions (Table 3.1). Thus, the number of putative T cell antigens was narrowed to 27. Of the 27 proteins, nine appeared to be relatively abundant. However another protein, EF-Tu (FTL_1751), that was observed in non-active fractions, was abundant based on the criteria described below and is a know antigen (79). Thus Ef-Tu was included in the list of dominant potential antigens. The relative abundance of the proteins was defined as the ability to be detected not only in the 5 µg, but also in the 2.5 µg sample size (Table 3.2). The 10 abundant proteins are listed in Table 3.1 and 3.2. Of these, five were found in both the soluble and the membrane fractions at greater than 95% probability,

indicating protein abundance in each fraction as a possible cause of the overlap (Table 3.2). It is also possible that incomplete separation of membrane and soluble fractions caused the like-proteins in both fractions as indicated by three of the 10 proteins not being found as abundant proteins. In a similar murine study done by Dr. Jeff Chandler to identify the immunogenic surface proteome of *F. tularensis,* seven proteins matched those also found in the current study (Table 3.1) (27). However, three of the seven proteins were not found in the membrane fraction of the current study, which could be due to the abundance of those proteins or incomplete fractionation. Complete separation of the membrane fraction from the soluble fraction was not verified through enzymatic assays as done by other studies, but could be a good tool for future work, prior to extensive fractionation (116, 120).

Table 3.1 Protein designation, name and percent confidence of identification of protein in 5 μg sample, viewing only proteins with >95% peptide identification probability, as well as a >99% protein identification probability when containing at least two unique peptides.

Locus Tag	Accession Numbers	Protein name	Protein molecular Mass (Da)	Percent [a] confidence for identification of protein:		
				in IFN-γ inducing fractions		in non-IFN-γ inducing fractions
				Soluble	Membrane	
FTL_1772	gi|89257037	*Aconitate hydratase	102688	0%	100%	0%
FTL_0309	gi|89255740	Pyruvate dehydrogenase, E1 component [c]	100254	0%	100%	0%
FTL_0094	gi|89255534	*ClpB protein [b]	96033	100%	91%	0%
FTL_1504	gi|89256792	Peroxidase/catalase [b,c]	81212	65%	100%	0%
FTL_0234	gi|89255668	*Elongation factor G (EF-G)	77712	100%	99%	0%
FTL_0267	gi|89255700	*Chaperone Hsp90, heat shock protein HtpG	72357	100%	91%	0%
FTL_1191	gi|89256512	*Chaperone protein dnaK (heat shock protein family 70 protein) [b,c]	69166	100%	100%	0%
FTL_0768	gi|89256151	GTP binding translational elongation factor Tu and G family protein	67619	0%	100%	0%
FTL_1912	gi|89257160	30S ribosomal protein S1	61538	100%	100%	83%[m]
FTL_0112	gi|89255549	Intracellular growth locus, subunit B	57902	100%	100%	0%
FTL_1714	gi|89256983	*Chaperone protein, GroEL [b,c]	57385	100%	100%	0%
FTL_1797	gi|89257060	ATP synthase alpha chain	55520	100%	100%	0%
FTL_1810	gi|89257073	N utilization substance protein A	55162	0%	100%	0%
FTL_1783	gi|89257046	*Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex [c]	52701	100%	93%	0%
FTL_1478	gi|89256767	inosine-5-monophosphate dehydrogenase	52092	100%	0%	87%[s]
FTL_1479	gi|89256768	Cytosol aminopeptidase	51973	100%	0%	0%
FTL_0572	gi|89255970	Hypothetical protein FTL_0572	51961	0%	100%	0%
FTL_0311	gi|89255742	dihydrolipoamide dehydrogenase	50529	100%	0%	0%
FTL_1334	gi|89256638	L-serine dehydratase 1	50011	100%	0%	0%

Locus Tag	Accession Numbers	Protein name	Protein molecular Mass (Da) (from smallest to largest)	Percent [a] confidence for identification of protein:		
				in IFN-γ inducing fractions		in non-IFN-γ inducing fractions
				Soluble	Membrane	
FTL_1591	gi|89256868	Acetyl-CoA carboxylase, biotin carboxylase subunit	50052	100%	0%	0%
FTL_1795	gi|89257058	ATP synthase beta chain	49865	0%	100%	0%
FTL_0891	gi|89256248	*Trigger factor (TF) protein (peptidyl-prolyl cis/trans isomerase)	49554	100%	93%	0%
FTL_0269	gi|89255702	*NAD(P)-specific glutamate dehydrogenase	49141	100%	100%	0%
FTL_0283	gi|89255715	aromatic amino acid transporter of the HAAAP family	43518	0%	100%	0%
FTL_1751	gi|89257020	*Elongation factor Tu (EF-Tu) [b,c]	43372	100%	100%	100%[m]
FTL_1553	gi|89256837	Succinyl-CoA synthetase beta chain	41542	100%	0%	0%
FTL_1096	gi|89256426	Lipoprotein	41542	0%	100%	0%
FTL_1328	gi|89256632	Outer membrane associated protein [c]	41242	92%	100%	100%[m]
FTL_1410	gi|89256703	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentape ptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	40698	100%	0%	0%
FTL_1511	gi|89256798	glycerophosphoryl diester phosphodiesterase family protein	39077	0%	100%	0%
FTL_1907	gi|89257155	Cell division protein	39727	0%	100%	0%
FTL_0617	gi|89256014	hypothetical protein FTL_0617 [b]	16792	0%	99%	100%[sm]

a) The percent confidence of protein identification as determined by statistical analyses of the SEQUEST search data using Peptide Prophet and Protein Prophet.
b) Previously recognized as immunoreactive with human sera (79, 80, 104).
c) Previously recognized as immunoreactive with mouse sera (27).
*** Denotes abundant proteins as defined by not only being located in the 5 µg sample, but also in 2.5 µg sample.**
s) found in soluble fraction only.
m) found in membrane fraction only.

Table 3.2 Relative abundance of proteins and the fractions in which they were identified. Protein location extrapolated from Scaffold software (99% protein probability, 2 minimum peptides, and 95% minimum peptide probability) originating from 2.5 µg, 5 µg, 10 µg soluble and membrane fractions run on LC-MS/MS.

Protein	s1			s3			s4			s5			m1			m2			m7			Totals	MW
Locus Tag	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10		kDa
FTL_1784	x																					1	106
*FTL_1772													x	x	x			x				4	103
FTL_0309	x		x											x	x			x				5	100
*FTL_0094	x	x	x			x									x							5	96
FTL_0588	x		x												x							3	82
FTL_1504	x		x														x	x				4	81
*FTL_0234	x	x	x											x	x			x				6	78
FTL_1537															x							1	76
*FTL_0267	x	x	x		x	x								x	x			x				8	72
FTL_1407															x			x				2	72
FTL_1464																		x				1	71
*FTL_1191	x	x	x		x	x						x	x	x	x	x	x	x				12	69
FTL_0768																	x					1	68
FTL_0020	x		x			x									x			x				5	67
FTL_0438			x			x																2	67
FTL_0828																		x				1	67
FTL_1786															x			x				2	66
FTL_0407																x						1	64
FTL_1947																		x				1	63
FTL_1912		x	x		x	x								x	x		x	x				8	62
FTL_0484						x																1	60
FTL_0112					x	x								x	x		x	x			x	7	58
FTL_1490						x																1	58
FTL_0310															x							1	57
*FTL_1714	x		x	x	x	x	x								x	x	x	x		x	x	12	57
FTL_1797					x	x												x		x	x	5	56
FTL_0525						x																1	55
FTL_1810																	x	x				2	55
*FTL_1783	x	x	x	x		x									x							6	53
FTL_1479					x																	1	52
FTL_0572																	x	x			x	3	52
FTL_1478					x	x																2	52
FTL_0311					x	x																2	51

Protein	s1			s3			s4			s5			m1			m2			m7			Totals	MW
Locus Tag	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10		kDa
FTL_0453						x																1	50
*FTL_0891				x	x	x												x				4	50
FTL_1334					x	x																2	50
FTL_1527																		x			x	2	50
FTL_1591					x	x			x													3	50
FTL_1795																	x	x			x	3	50
*FTL_0269					x	x	x	x	x			x					x				x	8	49
FTL_1658																		x				1	49
FTL_1908																		x			x	2	45
FTL_0283														x								1	44
*FTL_1751	x				x	x			x	x	x	x			x		x	x	x	x	x	13	43
FTL_1553											x	x									x	3	42
FTL_1410					x																	1	41
FTL_1328																	x	x		x	x	4	41
FTL_1096																	x	x		x	x	4	40
FTL_1907												x						x		x	x	4	40
FTL_1511																	x			x	x	3	39
FTL_1146																					x	1	35
FTL_0538	x																					1	18
FTL_0617														x	x							2	17

(s1, s3, s4, s5) soluble fractions.
(m1, m2, m7) from membrane fractions.
Items in red are also seen on Table 3.1.
(Totals) the number of times each protein is identified regardless of location.
(*) represent proteins of relative abundance as defined by the ability to be detected in not only the 5 µg, but also the 2.5 µg sample size.

CHAPTER 4

DISCUSSION

At this time, there is not an approved vaccine for tularemia in humans (36, 91). The previously-used vaccine, made from *F. tularensis* LVS, is no longer approved for human use due to the unclear source of attenuation and its variable immunogenicity (140). The search for a safe and effective vaccine is ongoing and is the basis of this study. Subunit vaccines employ specialized immunogenic proteins, that have the potential to illicit a better immune response, have fewer side-effects, and thus to be a more cost-effective than killed-vaccines (52). Because these vaccines are only part of the whole organism, they do not run the risk of reverting to a pathological phenotype like the live-vaccines. They can consist of, but are not limited to, one or more of the following components of the original bacteria: DNA, carbohydrate, or protein. Generally speaking, cellular immunity is used in protection against intracellular pathogens, and humoral immunity is best suited for protection against extracellular pathogens (99). However, it has been suggested that, for a vaccine against tularemia to be most effective, both arms of the adaptive immune response, humoral as well as cellular immunity, must be evoked (99, 131, 143). The humoral response can be observed through antibody production via response of B cells. Cellular immunity is associated with T cell activation, via antigen presentation of peptides on the surface of APC cell. The objective of the current study was to identify immunodominant antigens of *F. tularensis,* via measurement of the cellular immune response that could be used in a vaccine against tularemia.

Previously published techniques for isolating immunodominant antigens of *Mycobacterium tuberculosis* were modified and used as a template for the current study (41). A few of the limitations for using this method are incomplete resolution during SDS-PAGE, the

possibility of poor membrane and soluble fraction separation and LC-MS/MS analysis by the Scaffold program. We separated the intracellular pathogen, *F. tularensis* LVS, into membrane and soluble fractions, then into 28 membrane and 28 soluble protein fractions, based on resolution of molecular mass using preparative SDS-PAGE and whole gel elution (41). The 28 fractions were visually compared, and based on five SDS-PAGE gels, the fractions were combined into 19 membrane and 16 soluble fractions, to limit the samples for the T cell assay.

Mice (C57/B6) were inoculated with a sub-lethal dose of *F. tularensis* LVS by Emily Kampf and Dr. Bosio and the T cell-producing spleens were removed for use in the research presented here within. The composition of the spleens was not assessed, nor were T cells isolated for this study. While some antigen-containing fractions elicited a significant IFN-γ response, had the spleens been depleted of interfering components, such as B cells, the resulting IFN-γ response may have been more robust. Wolfe *et al.* showed that macrophages producing IL-10 decreased the amount of IFN-γ produced by IFN-γ-producing splenocytes (171). In a study by Wijesuriya *et al.* it was found that B cells could interfere with the production of IFN-γ by T cells in tumor-bearing mice (170). The method used in the current study, for evaluating cellular immune response in spleen populations, is a proven method (139) and if the IFN-γ response had been more robust, focusing the data to a manageable list may have been less successful.

The previously-exposed splenocytes react with familiar protein antigens from each fraction to trigger the production and release of IFN-γ, whereas the non-infected splenocyte (murine control) should not release IFN-γ (17). Thus, in this study, the desired cellular immune response from the T cells, after incubation with the prepared soluble and membrane protein "antigens", would be the production of IFN-γ. Production of this cytokine, as discussed earlier,

is one of the key indicators of the immune system recognizing a previously encountered foreign body. Quantification of lymphocyte population and cytokine production in the spleen, via flow cytometry, could also be used to study the immune response (139). However in this study, an ELISA test measuring the production of IFN-γ was used to quantify the immunological response.

From the ELISA results, the chosen fractions of interest were statistically relevant (p-value <0.05) to 3-fold that of the average IFN-γ (pg/ml) response for all 16 soluble or 19 membrane fractions tested. Each of the fractions were analyzed via LC-MS/MS to identify which proteins were in each fraction, and since each fraction contained multiple proteins, some of which may or may not stimulate an immune response, further analysis was required. Each fraction was thus compared to itself at three different concentrations (2.5 μg, 5 μg, 10 μg) to identify the most abundant proteins in each fraction. This step further narrowed the focus of possible immune-stimulating antigens from 1754 to 32.

One way to test for relative abundance is through a technique called spectral counting. Spectral counting uses the number of times a peptide is identified by MS/MS in a single experiment, compares it to multiple experiments and transforms it into a measure for relative peptide abundance (97, 115). Table 4.1 lists all 32 proteins in this study that were found in the 5 μg IFN-γ–producing fractions, with a short description. Of the 32 proteins, only 10 proteins are detected in both 5 μg and 2.5 μg sample size, thus indicating their relative abundance as compared to the rest of the proteins. These 10 proteins have been identified in Table 4.1 by an asterisk (FTL_1751, FTL_0269, FTL_0891, FTL_1783, FTL_1714, FTL_1191, FTL_0267, FTL_0234, FTL_0094, FTL_1772).

Table 4.1 Locus tag and description of the 32 unique vaccine candidates with the highest IFN-γ response as determined by LC- MS/MS with 5 μg sample.

Locus Tag	Description
FTL_1772	*Aconitate hydratase- aconitase activity
FTL_0309	Pyruvate dehydrogenase, E1 component- Thiamine pyrophosphate (TPP) family, E1 of E. coli PDC-like subfamily, TPP-binding module; composed of proteins similar to the E1 component of the Escherichia coli pyruvate dehydrogenase multienzyme complex (PDC)
FTL_0094	*ClpB protein
FTL_1504	Peroxidase/catalase
FTL_0234	*Elongation factor G (EF-G)
FTL_0267	*Chaperone Hsp90, heat shock protein HtpG
FTL_1191	*Chaperone protein dnaK (heat shock protein family 70 protein)
FTL_0768	GTP binding translational elongation factor Tu and G family protein
FTL_1912	30S ribosomal protein S1- RPS1 is a component of the small ribosomal subunit thought to be involved in the recognition and binding of mRNA's during translation initiation.
FTL_0112	Intracellular growth locus, subunit B
FTL_1714	*Chaperone protein, GroEL- GroEL_like type I chaperonin. Chaperonins are involved in productive folding of proteins. They share a common general morphology, a double toroid of 2 stacked rings, each composed of 7-9 subunits
FTL_1797	ATP synthase alpha chain
FTL_1810	N utilization substance protein A
FTL_1783	*dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex
FTL_1478	Inosine-5-monophosphate dehydrogenase
FTL_1479	Cytosol aminopeptidase
FTL_0572	Hypothetical protein FTL_0572
FTL_0311	dihydrolipoamide dehydrogenase
FTL_1334	L-serine dehydratase 1
FTL_1591	Acetyl-CoA carboxylase, biotin carboxylase subunit
FTL_1795	ATP synthase beta chain
FTL_0891	*Trigger factor (TF) protein (peptidyl-prolyl cis/trans isomerase)- molecular chaperone
FTL_0269	*NAD(P)-specific glutamate dehydrogenase
FTL_0283	aromatic amino acid HAAP transporter - amino acid permease
FTL_1751	*Elongation factor Tu (EF-Tu) (b)-EF-Tu subfamily. "This subfamily includes orthologs of translation elongation factor EF-Tu in bacteria, mitochondria, and chloroplasts. It is one of several GTP-binding translation factors found in the larger family of GTP-binding elongation factors"
FTL_1553	Succinyl-CoA synthetase subunit beta (sucC)- "catalyzes the interconversion of succinyl-CoA and succinate"
FTL_1096	Lipoprotein- "DsbA family, Com1-like subfamily; composed of proteins similar to Com1, a 27-kDa outer membrane-associated immunoreactive protein"

FTL_1328	Outer membrane associated protein- OmpA family (fopA). "The Pfam entry also includes MotB and related proteins which are not included in the Prosite family"
FTL_1410	undecaprenyldiphospho-muramoylpentapeptide beta-N- acetylglucosaminyl-transferase- "involved in cell wall formation; inner membrane-associated; last step of peptidoglycan synthesis"
FTL_1511	glycerophosphoryl diester phosphodiesterase family protein
FTL_1907	Cell division protein- "FtsZ is a GTPase that is similar to the eukaryotic tubulins and is essential for cell division in prokaryotes. FtsZ is capable of polymerizing in a GTP-driven process into structures similar to those formed by tubulin."
FTL_0617	hypothetical protein FTL_0617- "Bacterioferritin (cytochrome b1) [Inorganic ion transport and metabolism]"

Extrapolated from http://www.ncbi.nlm.nih.gov/protein
* denotes relatively abundant proteins.

In a study for the identification of immunogenic *F. tularensis* surface proteins by Dr. Jeff Chandler, four of these ten relatively abundant proteins were identified to be immunoreactive with mouse sera, as noted on Table 3.1 (FTL_1191, FTL_1714, FTL_1783, FTL_1751) (27). Of these four proteins, only one in the current study was found in only the soluble fraction at >95% probability, whereas three proteins were found in both soluble and membrane fractions (FTL_1191, FTL_1714, FTL_1751), leading to concerns of separation and actual location of the protein in current study (Table 3.1).

The separation of membrane from soluble fractions was accomplished via centrifugation after lysis of bacterial cells, subsequently each fraction was further separated by MW via SDS-PAGE. These two techniques allowed for possible incomplete separation on both the membrane-soluble interface and between the MW fractionations. This may account for some overlap in the immunogenic proteins of these subcellular fractions, as well as adjacent MW fractions. Theoretically, if proteins overlap from one group to another, there should also be overlapping IFN-γ responses between groups. Multiple proteins were also observed in each immune fraction. Thus, deciphering which protein specifically elicited the immune response becomes less clear.

For example, in Table 3.2, NAD(P)-specific glutamate dehydrogenase (FTL_0269) is found in five of the seven active fractions (soluble 3, soluble 4, soluble 5, membrane 2, and membrane 7). This could suggest either FTL_0269 is the immunogen of all five fractions. Alternatively, the presence of this protein could be coincidental and reactivity is due to some other protein in each fraction.

EF-Tu is another protein identified within the study, and is typically found to be in the cytoplasm, however, Barel *et al.* only reported finding it the membrane region of *F. tularensis* (13, 88). In the current study, EF-Tu (FTL_1751) was found in both membrane and soluble fraction, thus, the data from Barel *et al.* reinforces the idea of incomplete separation of the proteins within this study (13). Since this protein was also found in both the IFN-γ-inducing and the non-IFN-γ-inducing fraction, the question arises as to why it has previously been shown to illicit and immune response, but has conflicting results in the current study (13). It is possible there are products within the non- IFN-γ-inducing fractions, that inhibited the in vitro T cell assay; or there is not enough antigen in the non-inducing fraction to react with the primed T cells. Whereas, the proteins were analyzed by the Scaffold program at 95% peptide probability and 99% protein probability with 2 unique peptides, this was chosen to set a standard between studies within the Belisle lab and could be altered. If in fact this is changed in any way, there is a chance that other proteins will appear more dominant in each fraction and the results could change slightly. In a study of it's function, Barel *et al*. found that protein EF-Tu played an important role in *F. tularensis* infectivity as an LPS ligand facilitating the adhesion and entry of *F. tularensis* into human monocytes via surface nucleolin receptor (13). Thus, EF-Tu would make a good candidate for therapeutic interference of *F. tularensis* infections in hopes of blocking entry into the cell (13). Because Barel *et al.* found the EF-Tu protein to have

involvement in the virulence of *F. tularensis,* the argument is strengthened for the immunogenicity of the EF-Tu protein antigen within the current study, regardless of their location in the non-inducing fractions.

Chaperone, heat shock, or surface proteins would be good immunogenic protein candidates, because they are necessary to obtain entry into the cell or for survival of the bacterium within the cell (27, 58, 59). The intracellular compartment where *F. tularensis* resides is very hostile, and the upregulation of stress genes is necessary for continued life and proliferation (59, 105). Through 2-D PAGE, Lenco *et al.* identified proteins that were upregulated within the macrophage in response to oxidative pressure (105). Some of the proteins found in the Lenco *et al.* study overlap with proteins found during the current study (Hsp90 (FTL_0267), GroEL (FTL_1714), DnaK (FTL_1191), ClpB (FTL_0094)), reinforcing these as possible vaccine candidates (105). Other proteins from the current study may be beneficial candidates for vaccine developement, due to their requirement for entry or survival of the bacterium within the cell (EF-G (FTL_0234), TF (FTL_0891), NAD(P)-specific glutamate dehydrogenase (FTL_0269), dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (FTL_1783), and aconitate hydratase (FTL_1772)). While these proteins were not found in the study by Lenco *et al.*, the majority of these proteins have been confirmed by previous studies as immunogenic for *F.tularensis* and identified in the current study (8, 11, 13, 69, 106, 112, 172, 176, 178).

There is additional evidence that proteins involved in the virulence of *F. tularensis* are likely vaccine candidates or immunogens. For example, in *E. coli,* Zolkiewski *et al.* demonstrated cooperation of DnaK and ClpB within the macrophage, to disaggregate and reactivate strongly aggregated proteins, in order to allow *E. coli* to survive heat stress (178).

Later, Meibom *et al.* demonstrated ClpB protein of *F. tularensis* to contribute to intracellular multiplication as well as to be essential for replication and disease proliferation in target organs (112). Bakshi *et al.* showed DnaK, and GroEL upregulation during oxidative stress, by intranasal inoculation of mice with *F. tularensis* LVS or $sodB_{Ft}$, followed by intranasal challenge with Schu4 after 14 days (11). This upregulation was more abundant in the $sodB_{Ft}$ mutant, an attenuated form, than wild type *F. tularensis* LVS, thus indicating it's possible use as an attenuated vaccine strain (11).

The protein dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (FTL_1783), found in the soluble and membrane fraction of *F.tularensis* during the current study, has also been identified as an immunoreactive protein in intracellular pathogen *Bartonella henselae,* the causative agent of cat-scratch fever (69). Gilmore *et al.* detected the protein with antiserum from experimentally infected mice (69). In a study by Litwin *et al.,* this protein was also shown to be cross-reactive in *F. tularensis* among other intracellular pathogens (106). This leads to positive reassurance that the FTL_1783 protein, found in this study, could be a realistic protein to use for future vaccine studies against *F. tularensis.*

Whereas outer membrane protein, FopA, was not found in the current study as an immunogenic antigen, Hickey et al. have incorporated it into a recombinant subunit vaccine. They showed that a single outer membrane protein (FopA) within a subunit vaccine was enough to provide protection and clearance of *F. tularensis* LVS (84). The FopA protein however, was not enough to provide complete protection against the *F. tularensis* Schu4 strain, but speculated the use of multiple proteins could increase the protective ability of their vaccine (84). They were also able to show passive transfer protection to naïve mice with of FopA-immune serum, thus

also demonstrating humoral immunity playing a role in protection (84). Procedures extrapolated from Hickey *et al.* could be used in conjunction with the protein data that has been identified within the current study to move forward in creation of a subunit vaccine.

Another way to vaccinate against tularemia could be through the use of a DNA vaccine. While the subunit vaccine has an expected MHCII antigen presentation, due to exogenous protein uptake by APCs, DNA vaccines would mostly display antigens through MHCI presentation, due to the proteins being translated from within the APCs. Also, since *F. tularensis* gets taken up into the APC's and escape to the cytosol for replication, a DNA vaccine may mimic a response closer to the real infection than a non-replicating subunit vaccine. A vaccine study done by Yang *et al.* in 2005, on intracellular pathogen, *Brucella melitensis* gives insight into the possibility of DNA vaccines as a quick way to screen for potential antigens (172). Yang *et al.* states that the DNA vaccine is more cost-effective, easier to develop and induces long-lasting immunity as compared to the traditional protein subunit vaccine (172). They were able to identify two genes, the chaperone protein, trigger factor (TF) and the periplasmic protein, bp26 (172). When the genes were introduced as DNA vaccines, they caused a reduction in splenic colonization by >0.6 log over the control, which was an arbitrary number Yang *et al.* set to distinguish satisfactory protection (172). When used together as a vaccine they induced a mix of humoral and cellular immune response (172). TF is a potential vaccine candidate for intracellular pathogen *F. tularensis,* as shown by it's ability to induce IFN-γ in both the soluble and membrane fractions of this study. Even though it does not show any sequence homology to the *Brucella melentisis*, this does show proof of principle for testing the *F. tularensis* antigens uncovered in this study for possible use in DNA vaccines.

A future assay to determine good candidates could include prediction of novel T cell epitopes to facilitate vaccine design, as described by Kim *et al*. (98). The general idea behind the use of T-cell epitopes in vaccine design, is for a vaccine to induce memory T cell population capable of recognizing the pathogen (98). While the possible vaccine has to contain antigens that harbor T cell epitopes from the specific pathogen, it does not have to be made of individual epitopes (98). These epitopes can be screened by using the tools found on the immune epitope database (IEDB- http://www.immuneepitope.org/). Once the antigens are identified, they can be checked for expression by the pathogen and for immunogenicity during infection. This can be done by challenging human T cells obtained after an infection. These chosen antigens then can be tested for conservation within the species. If these antigens appear to stimulate the immune response, a large scale manufacturing of these antigens for clinical trials could be a next step. Animal immunization could be performed to test for a protective response and then on to human trials. However, since this pathogen is so infectious, we will need to find a better way to check humans for immunity. A study by Pascalis *et al.,* compared an in vivo murine assay with the in vitro functional activity of immune lymphocytes derived from vaccinated mice, and relative gene expression in immune lymphocytes to find the correlates of protection against *F. tularensis* (45). They define correlates as "a measurement that detects relevant biological functions critical for, and statistically related to, protection against an infectious disease" (45). By extrapolating the experiments from their study into the current study, it would be possible to get a better grasp on the specific antigens that would be best suited for a possible future human study.

At a minimum, the data here represents a starting point for further protein analysis by limiting the possible antigens to a manageable number, bringing us one step closer to finding an effective vaccine against tularemia.

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APPENDIX A

SEQUEST data from LC-MS/MS scan of soluble *F. tularensis* fractions

Protein accession number: gi|89257160
Protein name: 30S ribosomal protein S1 [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220604 – S1 (5μg)
Protein molecular weight (Da): 61653
Number of total spectra: 2
Number of unique spectra: 2
Number of unique peptides: 2
Amino Acid Coverage: 3.42%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
98.00%	QLSEDPFK	K	N	91.80%	0.945	0.0941	2.51	963.48	434	441
98.00%	SESFIPVSSLK	K	N	75.10%	0.836	0.0005	1.92	1193.64	44	54

Protein accession number: gi|89257037
Protein name: aconitate hydratase [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220604 - S1 (5μg)
Protein molecular weight (Da): 102688.3
Number of total spectra: 2
Number of unique spectra: 2
Number of unique peptides: 2
Amino Acid Coverage: 2.56%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
98.20%	PFVKTSLAPGSQVVTQYL	K	E	80.50%	1.59	0.251	0	1935.06	467	484
98.20%	VILAGK	L	E	75.50%	1.12	0.157	0	600.41	769	774

Protein accession number: gi|89255700
Protein name: Chaperone Hsp90, heat shock protein HtpG [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220604 - S1 (5μg)
Protein molecular weight (Da): 72357.1
Number of total spectra: 11
Number of unique spectra: 9
Number of unique peptides: 6
Amino Acid Coverage: 10.5%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	AAANNPQLEAFK	K	K	95.00%	1.22	0.471	5.19	1273.65	441	452
100.00%	FLESLTGDK	K	S	95.00%	1.69	0.376	1.92	1009.52	104	112
100.00%	FWDSFGQVLK	K	E	89.40%	0.775	0.0043	2.38	1226.62	375	384
100.00%	GDIDLDKFETPENK	K	E	95.00%	2.03	0.355	10.3	1620.78	487	500
100.00%	KYTFETEVDK	K	L	95.00%	1.22	0.183	3.89	1259.62	5	14
100.00%	QTVSLADYISR	K	M	95.00%	1.24	0.206	3.28	1252.65	413	423

Protein accession number: gi|89256512
Protein name: Chaperone protein dnaK (heat shock protein family 70 protein) [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220604 - S1 (5μg)
Protein molecular weight (Da): 69165.5

Number of total spectra: 41
Number of unique spectra: 24
Number of unique peptides: 11
Amino Acid Coverage: 17.1%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	ALEEAFAPIAQK	K	A	95.00%	1.26	0.475	4.44	1287.69	592	603
100.00%	EEAFAPIAQK	L	A	95.00%	1.19	0.438	0	1103.57	594	603
100.00%	FDLADIPPAPR	R	G	70.70%	0	0	1.82	1211.64	459	469
100.00%	FESLVSDLVMR	K	S	95.00%	1.64	0.32	4.16	1295.67	307	317
100.00%	FHDLVTAR	K	N	95.00%	1.21	0.374	1.04	958.51	531	538
100.00%	GILNVSAK	N	D	87.00%	1.27	0.3	0	801.48	484	491
100.00%	KEEDVVDADFEDVEDDKK	K	-	95.00%	2.82	0.671	8.66	2124.95	625	642
100.00%	MAPPQVSAEVLR	K	K	95.00%	1.44	0.524	6.8	1313.69	112	123
100.00%	NTADNLIHSSR	R	K	95.00%	0	0	3.37	1227.61	539	549
100.00%	SSSGLSEEDIEK	K	M	95.00%	0.935	0	2.89	1280.59	505	516
100.00%	YLIDEFK	N	K	95.00%	1.6	0.403	0	927.48	241	247

Protein accession number: gi|89255534
Protein name: ClpB protein [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220604 - S1 (5μg)
Protein molecular weight (Da): 96032.7

Number of total spectra: 6
Number of unique spectra: 4
Number of unique peptides: 4
Amino Acid Coverage: 5.24%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	GLEELWK	K	A	85.40%	1.54	0.232	0	874.47	457	463
100.00%	LADAGFDPVFGAR	K	P	95.00%	1.17	0.386	0	1335.67	804	816
100.00%	NNPVLIGEPGVGK	K	T	95.00%	1.49	0.214	4.07	1293.72	200	212
100.00%	VDDAIVFEPLNK	R	E	80.70%	0	0	2.07	1359.72	758	769

Protein accession number: gi|89257046
Protein name: dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220604 - S1 (5μg)
Protein molecular weight (Da): 52700.5
Number of total spectra: 2
Number of unique spectra: 2
Number of unique peptides: 2
Amino Acid Coverage: 4.09%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.50%	GLVVPVLR	R	D	89.00%	1.32	0.247	1.68	852.57	362	369
99.50%	SLAELEADVLDK	K	A	95.00%	1.62	0.393	4.64	1302.68	375	386

Protein accession number: gi|89255668
Protein name: elongation factor G (EF-G) [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220604 - S1 (5μg)
Protein molecular weight (Da): 77712.4
Number of total spectra: 3
Number of unique spectra: 3
Number of unique peptides: 3
Amino Acid Coverage: 3.84%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	ANVVPVQL	K	N	93.30%	1.07	0.339	0	839.5	164	171
100.00%	FVDEVVGGVVPK	K	E	95.00%	1.29	0.494	8.22	1244.69	536	547
100.00%	GVIDLIR	K	M	95.00%	1.52	0.0619	5.85	785.49	181	187

Protein accession number: gi|89257160
Protein name: 30S ribosomal protein S1 [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220609 – S3 (5μg)
Protein molecular weight (Da): 61653
Number of total spectra: 2
Number of unique spectra: 2
Number of unique peptides: 2
Amino Acid Coverage: 4.5%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.80%	SESFIPVSSLK	K	N	95.00%	1.08	0.38	1.68	1193.64	44	54
99.80%	VEQMTPTTLGDLIK	K	E	95.00%	1.88	0.333	4.27	1561.82	537	550

Protein accession number: gi|89257060
Protein name: ATP synthase alpha chain [*Francisella tularensis* subsp. *holarctica*]
Number of total spectra: 3
Number of unique spectra: 2

Fraction MS/MS sample name: 8220609 – S3 (5μg) | Number of unique peptides: 2
Protein molecular weight (Da): 55520.1 | Amino Acid Coverage: 4.87%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.80%	ILEVPVGEALLGR	R	V	95.00%	1.61	0.447	4.17	1365.81	94	106
99.80%	VVDALGNPIDGK	R	G	95.00%	0.987	0.333	6.6	1197.65	107	118

Protein accession number: gi|89255700 | Number of total spectra: 2
Protein name: Chaperone Hsp90, heat shock protein HtpG [*Francisella tularensis* subsp. *holarctica*] | Number of unique spectra: 2
Fraction MS/MS sample name: 8220609 – S3 (5μg) | Number of unique peptides: 2
Protein molecular weight (Da): 72357.1 | Amino Acid Coverage: 6.05%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.90%	FWDSFGQVLK	K	E	95.00%	0.955	0.309	3	1226.62	375	384
99.90%	QTVSLADYISRMKESQDTIYYITSDSYK	K	A	95.00%	1.39	0.353	0	3305.59	413	440

Protein accession number: gi|89256512 | Number of total spectra: 6
Protein name: Chaperone protein dnaK (heat shock protein family 70 protein) [*Francisella tularensis* subsp. *holarctica*] | Number of unique spectra: 5
Fraction MS/MS sample name: 8220609 – S3 (5μg) | Number of unique peptides: 3
Protein molecular weight (Da): 69165.5 | Amino Acid Coverage: 6.39%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	ALEEAFAPIAQK	K	A	95.00%	0.976	0.405	3.89	1287.69	592	603
100.00%	FDLADIPPAPR	R	G	95.00%	0.849	0.0946	5.72	1211.64	459	469
100.00%	KEEDVVDADFEDVEDDKK	K	-	95.00%	3.76	0.624	11.1	2124.95	625	642

Protein accession number: gi|89256983 | Number of total spectra: 33

Protein name: Chaperone protein, groEL [*Francisella tularensis* subsp. *holarctica*] Number of unique spectra: 25

Fraction MS/MS sample name: 8220609 – S3 (5μg) Number of unique peptides: 15

Protein molecular weight (Da): 57385.4 Amino Acid Coverage: 36.2%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	AAVEEGIVAGGGVALIR	R	A	95.00%	2	0.484	4.92	1581.9	405	421
100.00%	ALDGLTGENDDQNHGIALLR	K	K	95.00%	0	0	6.46	2122.05	425	444
100.00%	AVTAGMNPMDLK	K	R	95.00%	0	0	3.51	1263.61	106	117
100.00%	DLLPILEGVSK	R	S	84.90%	0	0	2.04	1183.69	232	242
100.00%	DTYGDMVEMGILDPTK	N	V	87.60%	1.44	0.278	0	1816.8	483	498
100.00%	GFEDELDVVEGMQFDR	K	G	93.30%	0.945	0.189	3.24	1885.83	182	197
100.00%	LEETNMEHLGTASR	K	V	95.00%	0	0	4.34	1603.74	309	322
100.00%	LSGGVAVIK	K	V	95.00%	1.73	0.312	2.59	843.53	372	380
100.00%	LTEGLK	L	A	92.50%	1.44	0.21	0	660.39	100	105
100.00%	MITTEAMIGEIK	L	E	95.00%	2.19	0.421	0	1352.68	514	525
100.00%	PILEGVSK	L	S	83.10%	1.11	0.198	0	842.5	235	242
100.00%	QIVSNAGGESSVVVNQVK	R	A	95.00%	1.39	0.438	6.75	1814.96	453	470
100.00%	QVLFSDEAR	K	A	77.30%	1.09	0.313	0	1064.54	5	13
100.00%	SIEQVGTISANSDATVGK	K	L	95.00%	1.73	0.59	0.495	1776.9	143	160
100.00%	TADVAGDGTTTATVLAQALL TEGLK	K	A	86.10%	0	0	2.08	2417.28	81	105

Protein accession number: gi|89256768 Number of total spectra: 2

Protein name: cytosol aminopeptidase [*Francisella tularensis* subsp. *holarctica*] Number of unique spectra: 2

Fraction MS/MS sample name: 8220609 – S3 (5μg) Number of unique peptides: 2

Protein molecular weight (Da): 51973 Amino Acid Coverage: 7.72%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.80%	GGNEGDAPIVLVGK	K	G	95.00%	1.65	0.372	3.59	1325.71	237	250
99.80%	QAAGMDSMKMDMGGVAAVMG TMK	K	A	95.00%	1.91	0.274	0	2318.01	263	285

Protein accession number: gi|89257020 Number of total spectra: 18

Protein name: elongation factor Tu (EF-Tu) [*Francisella tularensis* subsp. *holarctica*] Number of unique spectra: 14

Fraction MS/MS sample name: 8220609 – S3 (5μg) | Number of unique peptides: 9
Protein molecular weight (Da): 43372.2 | Amino Acid Coverage: 27.7%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	GEAGDNVGILVR	R	G	95.00%	1.35	0.36	8.36	1199.64	269	280
100.00%	GITINTSHVEYESPNR	R	H	95.00%	2	0.371	2.39	1816.88	60	75
100.00%	GVVNVGDEVEVVGIRPTQK	R	T	95.00%	1.46	0.316	1.85	1995.09	235	253
100.00%	HTPFFK	R	G	88.80%	1.5	0.22	0.699	776.41	320	325
100.00%	IVELVQAMDDYIPAPER	K	D	95.00%	0.984	0.101	3.66	1958.99	189	205
100.00%	IVVFLNK	K	C	77.00%	1.25	0.0857	1.92	832.53	131	137
100.00%	MTITLINPIAMDEGLR	K	F	95.00%	1.09	0.358	2.28	1787.94	359	374
100.00%	PQFYFR	R	T	95.00%	1.52	0.401	0	857.43	329	334
100.00%	TTVTGVEMFR	K	K	95.00%	1.04	0.268	3.68	1140.57	254	263

Protein accession number: gi|89255549 | Number of total spectra: 7
Protein name: intracellular growth locus, subunit B [*Francisella tularensis* subsp. *holarctica*] | Number of unique spectra: 6
Fraction MS/MS sample name: 8220609 – S3 (5μg) | Number of unique peptides: 4
Protein molecular weight (Da): 57901.5 | Amino Acid Coverage: 7.71%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	ALEQEWLK	R	V	95.00%	0.941	0.125	2.72	1016.54	99	106
100.00%	KEELQYDFER	K	N	95.00%	0	0	2.82	1356.64	127	136
100.00%	NIDFDVSDDASK	K	V	95.00%	0	0	3.3	1325.59	28	39
100.00%	SIISNDEFR	N	A	84.80%	1.15	0.321	0	1080.53	90	98

Protein accession number: gi|89255702 | Number of total spectra: 3
Protein name: NAD(P)-specific glutamate dehydrogenase [*Francisella tularensis* subsp. *holarctica*] | Number of unique spectra: 3
Fraction MS/MS sample name: 8220609 – S3 (5μg) | Number of unique peptides: 3
Protein molecular weight (Da): 49141 | Amino Acid Coverage: 8.91%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	EVFSTLKPALEHNPK	K	Y	94.60%	0	0	2.55	1709.92	30	44
100.00%	GFVHDPEGITTDEK	K	I	95.00%	1.71	0.428	6.24	1544.72	267	280
100.00%	LSWSAEEVESK	R	L	95.00%	1.03	0.293	2.85	1264.61	392	402

Protein accession number: gi|89256248 | Number of total spectra: 2
Protein name: trigger factor (TF) protein (peptidyl-prolyl cis/trans isomerase) [*Francisella tularensis* subsp. *holarctica*] | Number of unique spectra: 2

Fraction MS/MS sample name: 8220609 - S3 (5μg) | Number of unique peptides: 2
Protein molecular weight (Da): 49553.8 | Amino Acid Coverage: 4.8%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
98.80%	IEVQKPVVELTDK	K	E	95.00%	1.97	0.315	4.82	1497.85	122	134
98.80%	VTIDFVGK	K	K	75.40%	0.777	0.198	1.77	878.5	163	170

Protein accession number: gi|89255702 | Number of total spectra: 5
Protein name: NAD(P)-specific glutamate dehydrogenase [*Francisella tularensis* subsp. *holarctica*] | Number of unique spectra: 3
Fraction MS/MS sample name: 8220612 – S4 (5μg) | Number of unique peptides: 2
Protein molecular weight (Da): 49141 | Amino Acid Coverage: 6.01%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.80%	FLGFEQVFK	K	N	95.00%	1.25	0.375	3.52	1114.59	108	116
99.80%	HIGPDIDVPAGDIGVGGK	R	E	95.00%	1.67	0.394	3.01	1716.89	157	174

Protein accession number: gi|89257020 | Number of total spectra: 9
Protein name: elongation factor Tu (EF-Tu) [*Francisella tularensis* subsp. *holarctica*] | Number of unique spectra: 8
Fraction MS/MS sample name: 8220615 – S5 (5μg) | Number of unique peptides: 4
Protein molecular weight (Da): 43372.2 | Amino Acid Coverage: 11.2%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	GEAGDNVGILVR	R	G	95.00%	1.42	0.265	7.1	1199.64	269	280
100.00%	INPIAMDEGLR	L	F	95.00%	1.51	0.305	0	1244.63	364	374
100.00%	TTVTGVEMFR	K	K	95.00%	1.05	0.332	2.52	1140.57	254	263
100.00%	VGDEVEVVGIR	N	P	95.00%	1.78	0.473	0	1171.63	239	249

APPENDIX B

SEQUEST data from LC-MS/MS scan of membrane *F. tularensis* fractions

Protein accession number: gi|89256512
Protein name: Chaperone protein dnaK (heat shock protein family 70 protein) [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220620 – M1 (5μg)
Protein molecular weight (Da): 69165.5

Number of total spectra:11
Number of unique spectra: 6
Number of unique peptides: 5
AA Coverage: 8.57%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	ALEEAFAPIAQK	K	A	95.00%	1.28	0.317	5.43	1287.69	592	603
100.00%	FDLADIPPAPR	R	G	95.00%	0.951	0.259	6.15	1211.64	459	469
100.00%	FHDLVTAR	K	N	95.00%	1.64	0.35	2	958.51	531	538
100.00%	IIGIDL	K	G	95.00%	1.43	0.229	0	643.4	4	9
100.00%	KEEDVVDADFEDVEDDKK	K	-	95.00%	1.84	0.167	6.68	2124.95	625	642

Protein accession number: gi|89255740
Protein name: pyruvate dehydrogenase, E1 component [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220620 – M1 (5μg)
Protein molecular weight (Da): 100253.9

Number of total spectra: 4
Number of unique spectra: 4
Number of unique peptides: 4
AA Coverage: 3.36%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	HIVPITVDESR	K	T	95.00%	0.82	0.295	5.48	1265.69	520	530
100.00%	ITAEQLENFR	R	K	93.20%	0.928	0.148	8.51	1220.63	158	167
100.00%	TFGMEGLFR	R	Q	95.00%	0.81	0.371	5	1057.51	531	539

Protein accession number: gi|89255700
Protein name: Chaperone Hsp90, heat shock protein HtpG [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220620 – M1 (5μg)
Protein molecular weight (Da): 72357.1

Number of total spectra: 3
Number of unique spectra: 3
Number of unique peptides: 2
AA Coverage: 3.82%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.80%	FWDSFGQVLK	K	E	95.00%	1.29	0.33	3.68	1226.62	375	384
99.80%	GDIDLDKFETPENK	K	E	95.00%	1.9	0.322	0.824	1620.78	487	500

Protein accession number: gi|89256014
Protein name: hypothetical protein FTL_0617 [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220620 – M1 (5μg)
Protein molecular weight (Da): 16792.1
Number of total spectra: 3
Number of unique spectra: 2
Number of unique peptides: 2
AA Coverage: 13.7%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.80%	ILELEMSGIVR	K	Y	95.00%	0.91	0.41	1.85	1275.7	18	28
99.80%	SIILEEYAR	K	K	80.10%	1.07	0.11	1.92	1093.59	117	125

Protein accession number: gi|89255668
Protein name: elongation factor G (EF-G) [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220620 – M1 (5μg)
Protein molecular weight (Da): 77712.4
Number of total spectra: 2
Number of unique spectra: 2
Number of unique peptides: 2
AA Coverage: 4.12%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.10%	FVDEVVGGVVPK	K	E	95.00%	0	0	4.4	1244.69	536	547
99.10%	YLEGGELSEDEIHQGLR	K	A	81.40%	0	0	1.82	1944.93	237	253

Protein accession number: gi|89255549,gi|89256480
Protein name: intracellular growth locus, subunit B [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220620 – M1 (5μg)
Protein molecular weight (Da): 57901.5
Number of total spectra: 2
Number of unique spectra: 2
Number of unique peptides: 2
AA Coverage: 3.76%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
98.90%	DLSEITHIK	K	S	77.20%	1.03	0.0517	1.72	1055.57	209	217
98.90%	SFEALLEHPR	K	Y	95.00%	0.975	0.345	5.38	1198.62	218	227

Protein accession number: gi|89257160
Protein name: 30S ribosomal protein S1 [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220620 – M1 (5μg)
Protein molecular weight (Da): 61653
Number of total spectra: 2
Number of unique spectra: 2
Number of unique peptides: 2
AA Coverage: 3.06%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
98.60%	ISLGIK	R	Q	72.00%	1.07	0.243	0.201	630.42	255	260
98.60%	SESFIPVSSLK	K	N	95.00%	1.06	0.336	2.26	1193.64	44	54

Protein accession number: gi|89256983
Protein name: Chaperone protein, groEL [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220623 – M2 (5μg)
Protein molecular weight (Da): 57385.4

Number of total spectra: 15
Number of unique spectra: 13
Number of unique peptides: 10
AA Coverage: 27.2%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	ALDGLTGENDDQNHGIALLR	K	K	95.00%	0	0	7.36	2122.05	425	444
100.00%	AVTAGMNPMDLK	K	R	95.00%	1.26	0.304	4.96	1247.61	106	117
100.00%	DLLPILEGVSK	R	S	95.00%	0.893	0.424	3	1183.69	232	242
100.00%	EAAPAMPMGGGMGGMPGMM	K	-	95.00%	1.46	0.349	-0.532	1860.68	526	544
100.00%	EIELEDKFENMGAQIVK	K	E	91.00%	1.47	0.232	1.32	1993	59	75
100.00%	LEETNMEHLGTASR	K	V	86.50%	0	0	1.96	1603.74	309	322
100.00%	LSGGVAVIK	K	V	83.90%	1.42	0.225	1.17	843.53	372	380
100.00%	MITTEAMIGEIK	L	E	95.00%	1.82	0.426	0	1352.68	514	525
100.00%	QVLFSDEAR	K	A	92.70%	0	0	2.25	1064.54	5	13
100.00%	TADVAGDGTTTATVLAQALLTEGLK	K	A	95.00%	0	0	8.19	2417.28	81	105

Protein accession number: gi|89256512
Protein name: Chaperone protein dnaK (heat shock protein family 70 protein) [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220623 – M2 (5μg)
Protein molecular weight (Da): 69165.5

Number of total spectra:16
Number of unique spectra: 13
Number of unique peptides: 8
AA Coverage: 17.4%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	ALEEAFAPIAQK	K	A	95.00%	1.02	0.269	4.38	1287.69	592	603
100.00%	FDLADIPPAPR	R	G	95.00%	0	0	5.23	1211.64	459	469
100.00%	FESLVSDLVMR	K	S	95.00%	1.55	0.383	4.72	1295.67	307	317
100.00%	FHDLVTAR	K	N	95.00%	2	0.476	2.04	958.51	531	538
100.00%	KEEDVVDADFEDVEDDKK	K	-	95.00%	3.16	0.425	9.33	2124.95	625	642
100.00%	MAPPQVSAEVLR	K	K	95.00%	0	0	2.89	1297.69	112	123
100.00%	SDITEVLLVGGQTR	K	M	95.00%	0.568	0.219	6.12	1487.81	334	347
100.00%	TAEDYLGEPVTEAVITVPAYFNDSQR	K	Q	95.00%	0.994	0.109	4.47	2885.39	128	153

Protein accession number: gi|89257160
Protein name: 30S ribosomal protein S1 [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220623 – M2 (5μg)
Protein molecular weight (Da): 61653
Number of total spectra: 10
Number of unique spectra: 9
Number of unique peptides: 6
AA Coverage: 15.3%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	AFENNETVLGK	K	I	95.00%	1.29	0.414	4.42	1221.61	101	111
100.00%	AVSIGQEVEVIVLELDADNHR	K	I	76.50%	0	0	1.68	2306.2	321	341
100.00%	EGIEGLVHTSEMDWTNK	K	N	95.00%	1.51	0.187	2.28	1945.9	298	314
100.00%	GGYTMDVEGLR	R	A	95.00%	0.99	0.33	4.37	1197.56	118	128
100.00%	IIEATVVSIDK	K	E	95.00%	2.11	0.58	2.15	1187.69	23	33
100.00%	VEQMTPTTLGDLIK	K	E	85.40%	1.03	0.0678	1.92	1545.82	537	550

Protein accession number: gi|89255549
Protein name: intracellular growth locus, subunit B [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220623 – M2 (5μg)
Protein molecular weight (Da): 57901.5
Number of total spectra: 10
Number of unique spectra: 8
Number of unique peptides: 5
AA Coverage: 9.49%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	ALEQEWLK	R	V	93.80%	1.16	0.182	2.54	1016.54	99	106
100.00%	DLSEITHIK	K	S	95.00%	0.964	0	2.77	1055.57	209	217
100.00%	KEELQYDFER	K	N	95.00%	0.859	0.133	3.05	1356.64	127	136
100.00%	NIDFDVSDDASK	K	V	95.00%	1.4	0.297	5.72	1325.59	28	39
100.00%	SIISNDEFR	N	A	95.00%	1.19	0.453	0	1080.53	90	98

Protein accession number: gi|89257020
Protein name: elongation factor Tu (EF-Tu) [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220623 – M2 (5μg)
Protein molecular weight (Da): 43372.2
Number of total spectra: 8
Number of unique spectra: 7
Number of unique peptides:4
AA Coverage: 15.7%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	GVVNVGDEVEVVGIRPTQK	R	T	95.00%	1.51	0.487	2.06	1995.09	235	253
100.00%	IVELVQAMDDYIPAPER	K	D	93.80%	1.02	0.205	3.62	1958.99	189	205
100.00%	MTITLINPIAMDEGLR	K	F	95.00%	1.82	0.344	9.51	1787.94	359	374
100.00%	TTVTGVEMFR	K	K	95.00%	1.1	0.273	4.6	1140.57	254	263

Protein accession number: gi|89256792
Protein name: Peroxidase/catalase [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220623 – M2 (5μg)
Protein molecular weight (Da): 81211.6
Number of total spectra: 2
Number of unique spectra: 2
Number of unique peptides: 2
AA Coverage: 4.81%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.90%	WTASPVDLIFGSNSELK	K	A	95.00%	1.82	0.453	2.13	1863.95	676	692
99.90%	YTQEFYNNPEEFKEEFAK	K	A	95.00%	1.33	0.268	4.96	2313.03	379	396

Protein accession number: gi|89255970
Protein name: hypothetical protein FTL_0572 [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220623 – M2 (5μg)
Protein molecular weight (Da): 51961.4
Number of total spectra: 3
Number of unique spectra: 3
Number of unique peptides: 2
AA Coverage: 5.25%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.80%	ANLDIVGLK	K	T	95.00%	2.12	0.462	2.96	942.56	393	401
99.80%	SLTTGFGNLSGLLPIK	K	T	95.00%	0	0	4.5	1617.92	216	231

Protein accession number: gi|89256632
Protein name: outer membrane associated protein [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220623 – M2 (5μg)
Protein molecular weight (Da): 41242.4
Number of total spectra: 3
Number of unique spectra: 3
Number of unique peptides: 2
AA Coverage: 5.36%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.80%	GFGYNDTLGGIHK	K	S	95.00%	0	0	4.5	1378.68	360	372
99.80%	YVLPAGIK	K	Q	95.00%	1.22	0.215	2.68	860.53	244	251

Protein accession number: gi|89255702
Protein name: NAD(P)-specific glutamate dehydrogenase [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220623 – M2 (5μg)
Protein molecular weight (Da): 49141
Number of total spectra: 2
Number of unique spectra: 2
Number of unique peptides: 2
AA Coverage: 8.91%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.70%	AANAGGVAVSGLEMSQN SARLSWSAEEVESK	K	L	92.90%	0	0	2.27	3135.5	372	402
99.70%	FLGFEQVFK	K	N	95.00%	0	0	3.52	1114.59	108	116

Protein accession number: gi|89257020
Protein name: elongation factor Tu (EF-Tu) [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220630 - M7 (5μg)
Protein molecular weight (Da): 43372.2
Number of total spectra: 41
Number of unique spectra: 36
Number of unique peptides: 14
AA Coverage: 41.6%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
100.00%	ELLDQYEFPGDDTPVIMGSALR	R	A	95.00%	1.55	0.371	8.66	2466.19	156	177
100.00%	GEAGDNVGILVR	R	G	95.00%	1.33	0.34	3.48	1199.64	269	280
100.00%	GITINTSHVEYESPNR	R	H	95.00%	2.45	0.433	6.27	1816.88	60	75
100.00%	GVVNVGDEVEVVGIR	R	P	95.00%	1.65	0.474	0	1540.83	235	249
100.00%	GVVNVGDEVEVVGIRPTQK	R	T	95.00%	0.966	0.371	2.43	1995.09	235	253
100.00%	HTPFFK	R	G	95.00%	1.24	0.335	0.523	776.41	320	325
100.00%	INPIAMDEGLR	L	F	95.00%	1.5	0.378	0	1228.64	364	374
100.00%	IVELVQAMDDYIPAPER	K	D	95.00%	0	0	5.36	1958.99	189	205
100.00%	IVVFLNK	K	C	77.50%	1.4	0.229	0	832.53	131	137
100.00%	MTITLINPIAMDEGLR	K	F	95.00%	0	0	6.55	1787.94	359	374
100.00%	PQFYFR	R	T	95.00%	1.22	0.418	0	857.43	329	334
100.00%	TTDITGAVELPEGVEMVMPGDNVK	R	M	93.20%	0	0	2.34	2502.21	335	358
100.00%	TTLTAAITK	K	V	94.70%	1.14	0.265	1.92	919.55	26	34
100.00%	TTVTGVEMFR	K	K	95.00%	1.35	0.51	4.39	1140.57	254	263

Protein accession number: gi|89257155
Protein name: cell division protein [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220630- M7 (5μg)
Protein molecular weight (Da): 39727.4
Number of total spectra: 3
Number of unique spectra: 3
Number of unique peptides: 3
AA Coverage: 10.8%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.90%	EAAEAAISSPLLEDINLDGAK	R	G	95.00%	0	0	3.28	2127.08	238	258
99.90%	ILQIGTNLTK	N	G	77.50%	0.923	0.417	0	1100.67	57	66
99.90%	VTVVVTGIEK	K	V	95.00%	1.51	0.526	1.82	1044.63	308	317

Protein accession number: gi|89257060
Protein name: ATP synthase alpha chain [*Francisella tularensis* subsp. *holarctica]*
Fraction MS/MS sample name: 8220630- M7 (5μg)
Protein molecular weight (Da): 55520.1
Number of total spectra: 2
Number of unique spectra: 2
Number of unique peptides: 2
AA Coverage: 4.87%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.80%	ILEVPVGEALLGR	R	V	95.00%	1.67	0.417	5	1365.81	94	106
99.80%	VVDALGNPIDGK	R	G	95.00%	1.6	0.543	5.07	1197.65	107	118

Protein accession number: gi|89256983
Protein name: Chaperone protein, groEL [*Francisella tularensis* subsp. *holarctica*]
Fraction MS/MS sample name: 8220630- M7 (5μg)
Protein molecular weight (Da): 57385.4
Number of total spectra: 2
Number of unique spectra: 2
Number of unique peptides: 2
AA Coverage: 5.7%

Protein identification probability	Peptide sequence	Previous AA	Next AA	Peptide identification probability	SEQUEST XCorr score	SEQUEST DCn score	X! Tandem -log(e) score	Calculated Peptide Mass (AMU)	Peptide start index	Peptide stop index
99.80%	ALDGLTGENDDQNHGIALLR	K	K	95.00%	0	0	2.62	2122.05	425	444
99.80%	DLLPILEGVSK	R	S	95.00%	0.612	0.118	3.52	1183.69	232	242