DISSERTATION

THE SURFACE PROTEOME OF *FRANCISELLA TULARENSIS*

Submitted by

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ABSTRACT

THE SURFACE PROTEOME OF *FRANCISELLA TULARENSIS*

The surface associated lipids, polysaccharides, and proteins of bacterial pathogens often have significant roles in environmental and host-pathogen interactions. Lipopolysaccharide and an O-antigen polysaccharide capsule are the best defined *Francisella tularensis* surface molecules, and are important virulence factors that also contribute to the phenotypic variability of *Francisella* species, subspecies, and populations. In contrast, little is known regarding the composition and contributions of surface proteins in the biology of *Francisella*, or what roles they have in the documented phenotypic variability of this genus. A sufficient understanding of the *Francisella* surface proteome has been hampered by the few surface proteins identified and the inherent difficulty of characterizing new surface proteins. Thus, the objective of this dissertation was to provide an enhanced definition of *F. tularensis* surface proteome and evaluate how surface proteins relate to aspects of *F. tularensis* physiology, specifically humoral immunity and phenotypic variability of subspecies and populations. Analyses of the *F. tularensis* live vaccine strain surface proteome resulted in the identification of 36 proteins, 28 of which were newly described to the surface of this bacterium. Bioinformatic comparisons of surface proteins to their homologs in other *Francisella* species, subspecies, and populations revealed numerous differences that may contribute variable phenotypes, including significant alterations in the ChiA chitinase (FTL_1521).

Given the utility of surface proteins in vaccines effective against other bacterial pathogens, the *F. tularensis* surface proteins recognized by three forms of vaccination were determined in a murine model. Immune serum derived from the most effective *F. tularensis* vaccine, *F. tularensis* live vaccine strain, recognized a small set of proteins, of which the majority of antigens were surface localized. In comparison, sera collected from mice vaccinated with two less effective subunit vaccines containing *F. tularensis* membrane and surface proteins recognized a much greater number of antigens. Although surface proteins were also recognized in response to subunit vaccinations, the majority of antigens were not surface associated. These data suggest that a targeted humoral response to a select set of identified surface proteins offers the greatest protective effect.

Finally, the research presented in this dissertation provided the first biochemical characterization of the surface localized ChiA chitinase that was predicted to contribute to the phenotypic variability of *Francisella* biotypes. Multiple chitinases are often produced by a single organism which synergistically depolymerize chitin in nature. Thus, biochemical evaluations were extended to other *F. tularensis* and *F. novicida* chitinases, ChiB (FTT_1768), ChiC (FTW_0313), and ChiD (FTT_0066), that were identified by *in silico* analyses of *Francisella* genomes. Differences were noted between the chitinase genes and chitinase activities of *Francisella* species, subspecies, and populations. The chitinase activities observed for *F. tularensis* strains were predominantly associated with whole cell lysates, while the chitinase activities of *F. novicida* localized to the culture supernatant. The overall level of chitinase activity differed among the subpopulations of *F. tularensis*, and between *F. tularensis* and *F. novicida*. Recombinant production of the putative chitinases and enzymatic evaluations revealed ChiA, ChiB, ChiC, and ChiD possessed dissimilar chitinase activities. These

biochemical studies coupled with bioinformatic analyses and the evaluation of *chiA* and *chiC* knockouts in *F. tularensis* A1 and A2 populations, respectively, provided a molecular basis to explain the differential chitinase activities observed among the species and subpopulations of *Francisella*.

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ABBREVIATIONS

A	Absorbance
Å	Angstrom
ABC	ATP-binding cassette
ACN	Acetonitrile
ATP	Adenosine-5'-triphosphate
AU	Absorbance units
BCA	Bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
CDC	Centers for Disease Control and Prevention
CDS	Protein coding sequences
CFU	Colony forming unit
CHAB	Cysteine heart agar supplemented with 9% heated sheep blood
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate
CL	Cardiolipin
CLDC	Cationic liposome DNA complexes
CM	Cytoplasmic membrane
CS	Culture supernatant
CV	Column volumes
Cyto	Cytosol
Da	Dalton
DNA	Deoxyribonucleic acid
DSA	*Datura stramonium agglutinin*
DTT	Dithiothreitol
FPI	*Francisella* Pathogenicity Island
g	Gram
GC	Guanine-cytosine
GH	Glycosyl hydrolase
h	Hour
HM	Host membrane
HRP	Horse radish peroxidase
Hsp	Heat shock family proteins
i.d.	Intradermally
IEF	Isoelectric focusing
IFN-γ	Interferon gamma
Ig	Immunoglobulin
Igl	Intracellular growth locus
IL	Interleukin
IM	Innermembrane
i.n.	Intranasally
INDEL	Insertion-deletion marker

i.p.	intraperitoneally
IPTG	Isopropyl-β-D-thiogalactopyranoside
i.v.	Intravenously
KDO	3-deoxy-D-mannose-octulosonic acid
l	Liter
kg	Kilogram
kV	Kilovolt
LB	Luria-Bertani
LC	Liquid chromatography
LC-Biotin	EZ-Link Sulfo-NHS-LC-Biotin
LPS	Lipopolysaccharide
LVS	Live vaccine strain
M	Molar
MAA	*Maakcia amurensis agglutinin*
Mb	Megabase
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Milimolar
MH	Mueller-Hinton
MMH	Modified Mueller-Hinton
MPF	Membrane protein fraction
MPLA	Monophosphoryl lipid A
MS	Mass spectrometry
NAD	Nicotinic acid dinucleotide
NC	Non-classical
NCBI	National Center for Biotechnology Information
NLR	Nod-like receptors
nm	Nanometer
NMR	Nuclear magnetic resonance
NT	Not tested
OD	Optical density
OM	Outer membrane
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAI	Pathogenicity island
PATRIC	Pathosystems Resource Integration Center
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pdp	Pathogenicity determining protein
PE	Phosphatidylethanolamie
Peri	Periplasm
PG	Phosphatidylglycerol
PNA	*Peanut agglutinin*
ref	Reference
RefSeq	Reference Sequence database
RML	Rocky Mountain Laboratories
RNA	Ribonucleic acid
s.c.	Subcutaneously

SDS ..... Sodium dodecyl sulfate
Sec ..... Generalized secretion system
SNA ..... *Sambucus nigra agglutinin*
SP ..... Signal peptide
SpI ..... Signal peptidase I
SpII ..... Signal peptidase II
Tat ..... Two-arginine translocation
TBS ..... Tris buffered saline
Th ..... T helper
TLR ..... Toll-like receptor
TNF-α ..... Tumor necrosis factor alpha
type A ..... *F. tularensis* subspecies *tularensis*
type B ..... *F. tularensis* subspecies *holarctica*
V ..... Volt
WCL ..... Whole cell lysate
Ω ..... Ohm
µF ..... Microfarad
µg ..... Microgram
µl ..... Microliter
µm ..... Micrometer
µM ..... Micromolar
2D ..... Two-dimensional

Chapter I

Literature Review Part I: The Genus *Francisella*

1.1 The history of tularemia

In 1911, a report emerged from U.S. Public Health Service researcher George W. McCoy describing a novel disease (tularemia) identified in the California ground squirrel (*Citellus beecheyi* Richardson) (117). The disease was characterized as having plague-like lesions, transmissibility to other mammals (rodents and monkeys), and multiple routes of transmission (subcutaneous, cutaneous, intranasal, and intraperitoneal). The following year McCoy and his colleague Charles W. Chapin isolated the causative agent, naming it *Bacterium tularense* (now *Francisella tularensis*) in recognition of Tulare County, California where the disease was first observed (118). McCoy and Chapin's work was the first to definitively characterize tularemia, but earlier reports from the U.S., Japan, Russia, and Norway described illnesses in both humans and animals with similar symptoms (185). Beyond these observations, human symptoms described in several historical texts led some to speculate that tularemia was responsible for disease outbreaks centered in the Eastern Mediterranean during the 2nd millennium BC, including the biblical plague of the Philistines and the Hittite plague (202, 203).

The ability of *F. tularensis* to cause disease in man was first recognized in 1914 by W. B. Wherry, B.H. Lamb, and D. T. Vail of Cincinnati Hospital, Cincinnati, Ohio (217). A 21 year old male presented physicians with an inflammation of the eye

attributed to about 10 discrete ulcers formed on the conjunctiva. Three days after presentation, swelling of the patient's neck and submaxillary lymph nodes was noted. Approximately one week later the inflammatory process had spread to the left lacrimal sac, resulting in abscess formation. Initial attempts to identify the causative agent failed because organisms were not observed microscopically nor were they culturable on standard diagnostic media of the era. The patient left the hospital still suffering from disease, before diagnosis was achieved, and did not provide additional samples for confirmatory tests. Nonetheless, scrapings of infected material were injected into guinea pigs where disease was subsequently observed and propagated. After numerous serial passages cultivation of the infectious agent was successful on the same coagulated egg-yolk media used by McCoy and Chapin, and it shared identical phenotypic properties to *F. tularensis*. (118). This study provided some of the first clues regarding the epidemiology and transmission of tularemia. The patient worked as a restaurant meat cutter, and a poorly characterized illness (market men's disease) was associated with men who butchered rabbit meat (84). Incidentally, a year before observing their patient, a hunter in the area communicated to physicians the occurrence of a large rabbit die-off. Wherry and colleagues speculated the two incidents were linked and would later isolate *F. tularensis* from a wild cotton tail rabbit (*Sylvilagus*) (56).

Some of the largest gains in understanding *F. tularensis* and its associated illness were made by Dr. Edward Francis. Francis began his study of *F. tularensis* in 1919 on a field trip to Utah, examining a human illness reported by Dr. R.A. Pearse that initiated with the bite of a deer fly (Tabanidae family of flies in the order Diptera), had a one to four week duration, and severity ranging from slight malaise to death (144). In 1919, Francis identified the agent by successfully culturing *F. tularensis* (59). In collaboration with North American, Japanese, and European investigators, Francis demonstrated numerous illnesses with unknown etiological agents resulted from *F.*

tularensis infection. Francis' works are currently recognized for providing the framework describing the bacteriology, symptomology, pathology, diagnosis, immunity, transmissibility, and epidemiology of *F. tularensis* (54, 56-58, 60).

The severity of *F. tularensis* infection, in addition to its ease of transmissibility and environmental stability, attracted the interest of several world governments for development as a biological weapon. The U.S., Japan, and former Soviet Union have all been involved in *F. tularensis* weaponization (41). Japanese scientists spearheaded biological weapons development, beginning in the early 1930's. Other nations were soon to follow, including the U.S. which began research in 1942 (72). The U.S. biological weapons program investigated the offensive potentials of *F. tularensis* and also developed defensive countermeasures. Throughout the 1950's and 1960's payload delivery systems were designed to disseminate *F. tularensis* aerosols (41). Vaccine and chemotherapeutic studies in humans were simultaneously conducted (41). Concern over these programs grew in the 1960's and many nations entered into disarmament agreements (31). An executive order given by U.S. President Richard M. Nixon in 1969 formally terminated offensive development of microorganisms in the U.S., and all stock piles were subsequently destroyed (31). The former Soviet Union continued development into the early 1990's and reportedly produced drug and vaccine resistant strains of *F. tularensis* (41, 216). Today at least 10 nations are thought to have active biological weapons programs, but the scope of *F. tularensis* development is unknown (2, 72).

In the late 1990's the Working Group on Civilian Biodefense lead by the U.S. Centers for Disease Control and Prevention (CDC) identified *F. tularensis* as one of the agents most likely to be used in a biological attack on the U.S. (41). The World Health Organization estimates the dispersal of 50 kilograms of *F. tularensis* in a metropolitan area of 5 million people would lead to 19,000 deaths and cause significant morbidity in

250,000 individuals (221). From an economic standpoint, a CDC report from 1997 predicted the cost of a *F. tularensis* attack to be 5.4 billion dollars for every 100,000 affected individuals (94). Although the production of large quantities of *F. tularensis* would require entities with considerable resources given that one liter of liquid media typically yields 5 grams (wet weight) of cells, these reports along with the terrorist attacks of September 2001, which included the intentional release of anthrax, raised questions about the capability of the U.S. to respond to biological threats (17). Prompted by these concerns government initiatives began to address biodefense preparedness including the development of new diagnostics, drugs, and vaccines effective against *F. tularensis* strains, including those with engineered resistances (41).

1.2 Taxonomic classification

The classification of *F. tularensis* by McCoy and Chapin was based on differences in hosts, clinical manifestations, and morphological characteristics setting it apart from other known pathogens (118). A decade after its discovery, serological studies indicated *F. tularensis* was a member of the genus *Pasteurella*, and later, studies provisionally placed *F. tularensis* in the genus *Brucella* (75, 185). However, these classifications did not reflect the uniqueness of the *Francisella* bacterium and were not accepted by all researchers, including Edward Francis who did not concur with the "*Pasteurella*" designation (85). In 1947 Dorofeev *et al*. proposed *F. tularensis* should be placed in its own genus, coining the genus name *Francisella* in honor of Dr. Francis (136). Dorofeev's proposal was later supported by DNA hybridization studies that definitively showed *F. tularensis* was distinct from the *Pasteurella* (162). The relationship of *Francisella* strains to other genera was clarified in 1994 by 16S ribosomal gene analysis that determined it belonged to the γ-proteobacteria class of bacteria, but had no close genetic relationships to any other known genus (51). *Francisella's* closest

relative is *Caedibacter taeniospiralis*, a parasite of the protozoan *Paramecium tetraurelia*, that shares 87% 16S rRNA sequence similarity (16). Analyses of the 16S sequence also provided genetic evidence for *F. tularensis* species and subspecies discriminations previously indicated by the differential virulence and metabolic characteristics of strains (51, 137).

Phenotypic observations and modern genetic typing have defined three species in *Francisella* (*F. novicida*, *F. philomiragia*, and *F. tularensis*) (Fig. 1.1) (96). Genetic comparisons of representative strains show a >91% genetic similarity between all species and >95% similarity between *F. tularensis* and *F. novicida* (25, 163). Of these, only *F. tularensis* is a clinically significant human pathogen and is divided into three subspecies, *tularensis* (type A), *holarctica* (type B), and *mediasiatica* (Fig. 1.1) (25, 193). *F. tularensis* type A and type B are responsible for most cases of tularemia whereas infection with *F. tularensis mediasiatica* is rare and is believed to cause a mild form of disease (25). Within subspecies *tularensis*, molecular typing data identified two populations, A1 and A2, each containing two subpopulations, A1a/A1b and A2a/A2b, respectively (Fig. 1.1) (100). *F. tularensis* A1 is differentiated from *F. tularensis* A2 by geographic distribution and virulence characteristics (100). Epidemiological evidence also indicates differences in virulence between *F. tularensis* A1 subpopulations that are supported by differential disease outcomes in a murine model (100, 129). A higher mortality in humans is associated with *F. tularensis* A1b compared to *F. tularensis* A1a, and *F. tularensis* A1b infected mice succumb to infection more rapidly than mice infected with *F. tularensis* A1a (100, 129). There are no known epidemiological or virulence differences between A2a and A2b subpopulations (149).

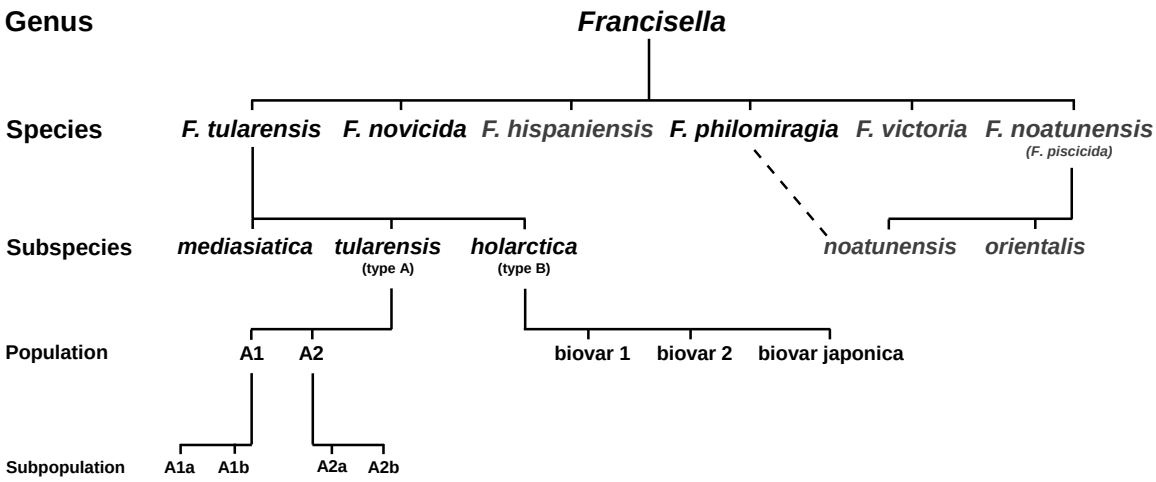

Fig. 1.1 Taxonomic organization of the genus *Francisella.* Species and subspecies in grey are not officially recognized according to the rules of the international code of bacterial nomenclature (101). The dashed line between *F. philomiragia* and *noatunensis* indicates *F. noatunensis* subspecies *noatunensis* is currently considered a subspecies of *F. philomiragia* (*F. philomiragia* subspecies *noatunensis*).

Classifications of species and subspecies in the genus *Francisella* are debated within the field of study. Proposals have been made to raise or lower the taxonomic ranking of several subspecies and species (23, 81, 88, 126, 139). Arguments for rearrangement are made from multiple premises, but largely based on genetic similarities. According to Wayne *et al*. (1987), bacterial strains are considered to be the same species if their respective DNA's reassociate at levels of 70% or greater and have

DNA melting temperatures that deviate <5% (211). This point of view does not directly take into consideration phenotypic differences such as virulence and metabolic capacity (88). Additionally, DNA reassociation based classification ignores divergent evolutionary patterns and dichotomous population structures many investigators consider important for discriminating species and subspecies (88).

The results of DNA reassociation experiments found *F. novicida* strains had 87 to 92% DNA relatedness to *F. tularensis* (75). According to the rules described by Wayne *et al*., *F. novicida* would be considered a subspecies of *F. tularensis*. However, the phenotype of *F. novicida* is substantially different from *F. tularensis*. It has unique nutritional requirements, functional metabolic pathways, geographic distributions, and perhaps most importantly, displays low virulence in humans (184). *F. novicida* also has an evolutionary history that shows divergence from *F. tularensis* (96). Thus, maintaining the current species classification of *F. novicida* is appropriate due to the relevant phenotypic features and evolutionary patterns.

The organization of the *F. tularensis* type B subspecies is also evolving. Olsufjev *et al*. proposed three unique *F. tularensis* type B populations (biovars I, II, and japonica) based on differential metabolic signatures and geographic distributions (Fig. 1.1) (138). *F. tularensis* type B biovar I differs from biovar II in that it is sensitive to erythromycin, presumably a result of a single insertion-deletion marker (INDEL) (149). Biovar japonica is similar to biovar I in that it is sensitive to erythromycin, but differs from biovar's I and II in that it is capable of glycerol fermentation and is found only in Japan (138). Surpassing the proposal of Olsufjev *et al*., multilocus variable-number tandem repeat, single nucleotide polymorphism, and INDEL analyses have differentiated *F. tularensis* type B into as many as 10 distinct biovars where differences in geographic distribution are noted (89, 104, 208). There is little genetic diversity between *F. tularensis* type B

biovars, no documented differences in virulence, and few other phenotypic variations, thus elevation of any of these biovars to its own subspecies is unlikely (97).

Genetic and biochemical analyses of a number of clinical and environmental isolates led investigators to propose an expanded taxonomy of the *Francisella* genus (81, 95, 126, 139). A human clinical isolate from Spain identified by Huber *et al*., was found to fall between *F. tularensis*/*F. novicida* and *F. philomiragia* phylogenetically, and was provisionally named *F. hispaniensis* (Fig. 1.1) (81). 16S rRNA gene sequence and DNA-DNA hybridization analyses of *F. philomiragia* subspecies *noatunensis* (also known as *F. psicidia*) have provided some evidence to support elevating its classification to the species level (*F. noatunensis*), that itself contains two subspecies (Fig. 1.1) (126, 139). Additionally, a number of recently identified isolates from both environmental and clinical samples display a high degree of 16S sequence similarity (between 87 and 99%) to characterized *Francisella* species and will likely expand the *Francisellaceae* family (13, 16, 95, 111, 126).

1.3 Epidemiology and transmission

Francisella is considered one of the most successful genera of microorganisms for its ability to occupy a wide variety of hosts (>300 currently documented), survive in many environments, and have a near global distribution (149). The clinically relevant subspecies, *F. tularensis* type A and type B, are phenotypically distinct from environmental species *F. novicida* and *F. philomiragia*. Further, these subspecies also display distinct characteristics that differentiate them from one another in terms of geographic distribution, vertebrate reservoirs, arthropod vector associations, and virulence (Table 1.1) (96, 100, 149, 185).

Table 1.1 Epidemiology of *F. tularensis*

			Vectors for human transmission			Common vertebrate reservoirs	
***F. tularensis* subspecies and populations**	**Geographic distribution**	**% mortality in humans**	**Flies**	**Hard ticks**	**Mosquitoes**	**Lagomorphs**	**Rodents**
A1a	Eastern U.S., California, Oregon, and Utah	4%	*Chrysops discalis*	*Amblyomma americanum* and *Dermacentor variabilis*	-	*Sylvilagus floridanus*	-
A1b	Eastern U.S., Alaska, California, Colorado, Idaho, and Oregon	24%	*Chrysops discalis*	*Amblyomma americanum* and *Dermacentor variabilis*	-	*Sylvilagus floridanus*	-
A2	Western U.S. and Alaska	0%	*Chrysops discalis*	*Dermacentor andersoni*	-	*Sylvilagus audubonii*	-
type B	North America, Europe, and Asia	7%	*Chrysops discalis*, *Chrysops relictus*, and *Haematopota pluvialis*	*Dermacentor variabilis*	*Aedes cinereus* and *Ochlerotatus excrucians*	*Lepus europaeus*, *Lepus timidis*, and *Lepus brachyurus*	*Arvicola terrestris*, *Clethrionomys* species, *Microtus arvalis*, *Mus musculus*, and *Ondatra zibethicus*

Data compiled from (96, 100, 149, 185).

In the U.S., tularemia was more common and lethal in the first half of the 20th century compared to the present day. There were 22,812 cases diagnosed between 1927 and 1948 with a fatality rate of 7.7% (170). Peak incidence occurred in 1939 with 2,291 cases reported (22). Currently, an average of 126 cases of tularemia are reported each year with 1,133 cases reported from 2000 to 2008 (1). The highest incidence of cases occurs from May thru July (1). Tularemia has been reported in every state except Hawaii, but most incidences are reported in Arkansas, Massachusetts, Missouri, Oklahoma, and South Dakota. Both *F. tularensis* type A and type B are present in the U.S., but can occupy distinct ecological niches (130). *F. tularensis* type B is found throughout the U.S. and is believed to occupy a greater spectrum of habitats compared to *F. tularensis* type A (149). Natural occurrence of *F. tularensis* type A is exclusive to

North America, and these strains appear to be more host adapted (149). *F. tularensis* A1 and A2 populations are observed primarily in eastern and western U.S., respectively (100). Computational modeling suggests *F. tularensis* A1 is associated with lower elevations and higher temperatures compared to *F. tularensis* A2, but did not find ecologies unique to the *F. tularensis* A1a/A1b or A2a/A2b subpopulations (130). Kugeler *et al*., reported the highest mortality from tularemia is associated with A1b (24%), which is significantly more than A1a (4%) (100). These investigators found no mortality associated with A2 infection, but did with *F. tularensis* type B infections (7%).

Tularemia is endemic to many Eurasian regions and has caused recent outbreaks in Spain, Kosovo, Ukraine, Germany, Sweden, Norway, Finland, Bulgaria, France, Austria, Turkey, and the Czech Republic (30, 92, 123, 183, 185, 194). *F. tularensis* type B strains are responsible for nearly all instances of tularemia in these regions (149). Populations of the three major *F. tularensis* type B biovars are found in different, and sometimes overlapping regions of Eurasia (113, 175). Similar to infection in the U.S., the number of tularemia cases in Europe and Asia has decreased recently. Early in the 20th century, up to 100,000 cases were reported in the former Soviet Union annually, compared to approximately 100 cases currently diagnosed in Russia each year (185, 194).

Transmission of *F. tularensis* to humans commonly occurs by the bite of an infected arthropod (97). Numerous species are naturally infected with *F. tularensis*, but only tabanid flies, mosquitoes, and hard-ticks represent important human vectors (Table 1.1) (148). The tabanid flies and mosquitoes transmit *F. tularensis* mechanically, with no evidence of long term survival in either arthropod type (148). In contrast, ticks maintain the bacteria in the gut and haemolymph (148). As reviewed by Petersen *et al*., transtadial transmission of *F. tularensis* from the larvae, to nymphs, and then to adult ticks has been demonstrated under laboratory conditions for *Amblyomma americanum*,

Dermacentor andersoni, and *Dermacentor variabilis* (148). Studies also indicate a slow proliferation of the bacteria in ticks, with higher titers in adult compared to larval ticks (77) . The suitability of ticks as reservoirs is not fully clear. Several reports indicate that *F. tularensis* can be transmitted transovarially, but these findings are disputed (148). Similarly, the fitness cost to infected ticks is not well described, and significant mortality has been noted in some studies but not others (148, 161).

A compilation of tularemia cases in the U.S. during the 1980's indicates 63% of infections reported in southwestern-central states are attributed to tick bites (195). Further, another survey found 74% of *F. tularensis* type A infections result from tick bites (188). Three tick species are suspected to be the most important for human transmission of *F. tularensis* type A strains. The geographic ranges of *A. americanum* and *D. variabilis* overlap with *F. tularensis* A1 populations, and *D. andersoni* coincides with the distribution of *F. tularensis* A2 populations (148). *D. variabilis* and *D. andersoni* are found naturally infected with *F. tularensis*, and *A. americanum* is known to maintain the bacteria experimentally (67, 148). It is not clear if there are differences in the tick host range between *F. tularensis* A1a/A1b and A2a/A2b subpopulations (148, 149). The geographic distribution and prevalence of *F. tularensis* type B is most closely associated with *D. variabilis* in the U.S. (148) Little is known regarding how the different *F. tularensis* subspecies and populations are maintained or transmitted by different tick species, but specific fitness costs between biotypes were identified in *D. variabilis* (161). Specifically, decreased survivorship was observed for *F. tularensis* A2 and type B, but not *F. tularensis* A1b infected nymphs.

Associations with small mammals represent the other important avenue of human acquired tularemia. In North America and Eurasia, cotton tail rabbits and hares (*Lepus*) are important hosts in the transmission of *F. tularensis* to man (96). In North America, the Eastern cottontail (*Sylvilagus floridanus*) is associated with *F. tularensis* A1

and the desert cottontail (*Sylvilagus audubonii*) with *F. tularensis* A2 (149). The brown hare (*Lepus europaeus*) is thought to be the major foci of tularemia in Central Europe (96). Rodents are the other major vertebrate foci of *F. tularensis*, particularly type B strains. A rise of rodent populations was directly attributed to an increased number of tularemia cases in Europe (185) . Hunting and processing of infected rodents can directly lead to tularemia, and rodents may contaminate food stocks as well as serve as the infection source of some arthropod vectors (185).

1.4 Disease manifestations

F. tularensis infections can initiate through the skin, mucosal membranes, lungs, and gastrointestinal tract. Infection is characterized by common symptoms including fever, sweats, chills, headache, body ache, nausea, vomiting, diarrhea, sore throat, and coughing. Disease onset is abrupt, usually within three to five days, but can be as rapid as one day or prolonged as 14 days post exposure (41). A study of 614 cases of human tularemia conducted by Dr. Francis delineated tularemia into four major clinical manifestations, ulceroglandular, glandular, oculoglandular, and typhoidal (56). Additional manifestations are now recognized such as oropharyngeal, gastrointestinal, and several other rare forms (41, 55).

The ulceroglandular form accounts for 80% of all diagnosed cases and occurs following the bite of an infected arthropod or handling of contaminated materials, typically infected meat (44). First, a papule appears at the site of infection at approximately the same time as the onset of general symptoms. Later, the papule becomes pustular and ulcerates concurrently with enlargement of regional lymph nodes (56). Active disease usually lasts two to three weeks, recovery can be lengthy, but mortality is rare even without treatment (48). Glandular tularemia is similar to the ulceroglandular type, except no primary lesion is observed at the site of infection. The

oculoglandular type occurs as a result of the bacterium entering though the eye and can be considered a variation of the ulceroglandular tularemia (44). Discrete lesions form on the conjunctiva of the eye, become pustular, and ulcerate. This form is rare, but is associated with a higher mortality rate (8 to 30%) (189). Consumption of contaminated food or water may result in oropharyngeal or gastrointestinal tularemia. The oropharyngeal type presents as a tonsillitis and is often accompanied by swelling of the cervical lymph nodes (44). The severity of the gastrointestinal form ranges from diarrhea to an acute fatal disease, and is related to the infectious dose (44). Typhoidal tularemia is characterized by a septicemia without the formation of a primary lesion or lymphadenopathy, and has high mortality (35%) without antibiotic treatment (44, 63).

Primary pneumonic tularemia results from inhalation of contaminated aerosols, and if caused by a *F. tularensis* type A strain, can result in 30 to 60% mortality if untreated (172). Outbreaks of pneumonic tularemia are commonly associated with lawn mowing and brush cutting, as these activities may aerosolize *F. tularensis* from environmental and animal reservoirs (41). Pneumonic tularemia is considered the most acute manifestation of infection, and was determined by the Working Group on Civilian Biodefense to be the most likely form encountered as a result of intentional release (41). Like other bacterial pneumonias, high fevers (as high as 104°F), headaches, and a nonproductive cough are dominant symptoms (198). A secondary pneumonic tularemia may also manifest. This form arises as a complication of other types of tularemia, most notably the typhoidal and ulceroglandular forms (198).

1.5 Treatment

Mortality associated with tularemia dramatically decreased in the antibiotic era. Currently in the U.S., <2% of *F. tularensis* cases are fatal with appropriate treatment (41). *F. tularensis* has no known resistances to aminoglycosides, tetracyclines,

chloramphenicol, or quinolones (193). Aminoglycosides are considered the first line antibiotic therapy. The first aminoglycoside discovered, streptomycin, was highly efficacious against tularemia (52). One to three grams of streptomycin dosed daily over seven to 10 days routinely cured 97% of adult human subjects (63, 198). For this reason, streptomycin was considered the drug of choice until the mid-1990's. However, because of concerns over high doses, toxicity, hypersensitivity reactions, and drug availability streptomycin was replaced by gentamicin as the drug of choice. Gentamicin treatment is slightly less effective than streptomycin, having an increased instance of relapse and diminished cure rate (86%) (45). The specifics of this change in effectiveness are not fully clear, both gentamicin and streptomycin are able to kill extracellular and intracellular bacteria, but could be impacted by the slower intracellular accumulation of gentamicin (114, 115). Nonetheless, the lower doses needed for gentamicin treatment (5 mg/kg daily) helps to abrogate some of the damaging toxic effects associated with streptomycin use (45, 193). Other aminoglycosides have also successfully treated tularemia, but had a decreased efficacy compared to gentamicin (45).

Second-line antibiotic treatments of tularemia include daily doses of tetracycline and chloramphenicol proving 88% and 77% effective, respectively (41, 45). Increased rates of relapse are associated with these drugs, presumably because both are bacteriostatic (45). More recently quinolones were shown to be highly efficacious treatments for tularemia, matching cure rates of aminoglycoside therapies (5, 107, 124, 176). Quinolones, particularly ciprofloxacin, may soon become the drug of choice against tularemia because they can be given orally, accumulate intracellulary, and are generally well tolerated (107).

1.6 Vaccines

Efforts to develop a tularemia vaccine have been underway since the early 1930's (53). The original formalinized killed vaccine preparation developed by Dr. Lee Foshay in 1932 provided limited protection against tularemia caused by *F. tularensis* type A strains in human and animal models (53, 90, 164). However, 30% of vaccinated humans contracted tularemia, and severe local and systemic side effects including necrosis at the injection site and high fever were noted (90). The extent of these symptoms required several Foshay vaccine participants to be hospitalized (90). The minimal success of the Foshay vaccine led Soviet researchers to investigate the effectiveness of live attenuated strains. One promising candidate was imported into the U.S. under the name "cutaneous tularemia live vaccine" in 1956 and is now referred to as the "live vaccine strain" (LVS) (200). Tests on U.S. military personnel determined *F. tularensis* LVS vaccination by scarification conferred significant levels of protection against high dose (1,000 CFU) s.c. and low dose (100 CFU) aerosol infection with the highly virulent *F. tularensis* type A strain SCHU S4 (172, 173). Subsequent works demonstrated increased efficacy when *F. tularensis* LVS vaccination was administered by aerosol, but a high percentage of volunteers developed illness and protection diminished over time (78). For these reasons the *F. tularensis* LVS vaccine lacks federal approval. Nonetheless, *F. tularensis* LVS vaccination has been the most widely used tularemia vaccine, and remains the comparative standard of all new tularemia vaccines.

Numerous attempts have been made to develop tularemia vaccines more effective than *F. tularensis* LVS. Because *F. tularensis* LVS is the most successful vaccine, a large focus has been placed on developing other live attenuated strains. To produce these strains, genetic modifications of one or more genes essential to *in vivo* propagation are necessary. Until recently, genetic manipulation of *F. tularensis* was

difficult because of a lack of genetic tools and restrictions of working with biosafety level three pathogens (109). In contrast, genetic manipulation of *F. novicida* was more readily accomplished because of available tools and its low virulence in humans (109). Thus, a large body of work accrued evaluating the efficacy of *F. novicida* mutants to generate a protective response (Appendix 1) (37, 91, 99, 127, 128, 142, 156, 182, 196, 215). Many *F. novicida* mutants offer protection to challenges with *F. novicida* species, but to date, have not been shown to protect against *F. tularensis* type A strains. More recently, attenuated *F. tularensis* strains (both type A and type B) were produced and evaluated as vaccine candidates (Appendix 1) (38, 50, 121, 125, 145, 154, 155, 167, 171, 181, 199, 204). These strains can protect against *F. tularensis* type A challenge in animal models, but efficacy is limited. The same issues of safety and efficacy experienced with *F. tularensis* LVS make the wide spread use and approval of attenuated strains questionable.

There have been several attempts to formulate a live tularemia vaccine using heterologous hosts stably producing *F. tularensis* antigens (Appendix 1) (61, 87, 186). Jia *et al*. showed vaccination with an attenuated *Listeria monocytogenes* strain producing the *Francisella* membrane protein IglC (intracellular growth locus) offered protection to *F. tularensis* type A aerosol challenge near the level of *F. tularensis* LVS (87). No other heterologous vaccine has been shown effective (146). Others have taken a novel approach for live vaccine generation by substituting, evolutionary conserved, critical genes for *in vivo* growth of *Francisella* with temperature sensitive orthologs (42). Investigators found three temperature sensitive *F. novicida* mutants were capable of eliciting protective immune responses against lethal *F. novicida* challenge. These strains survived at cool body sites but showed reduced presence compared to wild-type in the warmer internal organs.

To circumvent safety issues of live attenuated vaccines, a number of investigators have explored subunit vaccines (Appendix 1). Vaccines designed with one or a few antigenic *F. tularensis* proteins, lipids, and carbohydrates were relatively ineffective against *F. tularensis* type A challenge (39, 61, 66). Nonetheless, the most effective vaccine candidates known are lipopolysaccharide (LPS) and the surface proteins FopA (FTT_0583) and TUL4 (FTT_0901) (174). To date, the best protective response with a single antigen is obtained using LPS. *F. tularensis* LPS vaccination affords a high degree of protection to *F. tularensis* type B, and is marginally effective against *F. tularensis* type A infections (39). Several explanations were suggested for why these vaccines are not sufficient, including the correct antigens cocktails were not used, or the correct type of immune response was not stimulated. Better protection to *F. tularensis* type A infections was obtained using complex subunit preparations containing bacterial surface components. Administration of a cell wall fraction provided modest protection against virulent strains in humans (119). More recently, Huntley *et al*. found an outer membrane protein fraction provided moderate protection against a low dose *F. tularensis* type A aerosol challenge (40 CFU) in a murine model (82). Another successful subunit vaccine did not contain any *F. tularensis* derived molecules. This preparation consisted of detoxified *E. coli* LPS (representing a highly conserved Gram-negative epitope) and an outer membrane protein from *Neisseria meningitidis* (68). This formulation offered some protection in mice to an intratracheal *F. tularensis* type A challenge. Thus, in light of these successes the generation of an effective subunit vaccine containing bacterial surface components appears plausible.

1.7 Physiology of *Francisella*

Francisella cells are small (0.2 to 0.7 µm × 0.2 to 1.7 µm), Gram-negative staining coccobacilli. All are non-motile obligate aerobes that form distinct pale white colonies with a convex shape when grown on agar medium. On blood containing agar small zones of what looks like α-haemolysis are observed around colonies (141). A uniform coccoidal bacterial morphology is observed during logarithmic growth in liquid medium. However, when isolated from infected tissues or during stationary phase, bacteria are pleomorphic with some cells appearing elongated and filamentous. *F. tularensis* strains grow slowly in comparison to *F. novicid*a and *F. philomiragia*. All strains grow between 24°C and 39°C, with optimal growth at 37°C. Supplementation of the media with cysteine or cystine is essential for growth of most *F. tularensis* strains, but not *F. novicida* or *F. philomiragia*. This requirement is thought to be due to a disruption of the sulfate assimilation pathway, specifically the gene encoding adenylylsulfate kinase (FTT_1049) (103). This gene is intact in *F. novicida*, *F. philomiragia*, *F. tularensis* A2, and some strains of *F. tularensis* type B, but is disrupted in *F. tularensis* A1a and A1b populations.

Several biochemical features (Table 1.2) differentiate *Francisella* species and subspecies. *F. tularensis* and *F. novicida* are oxidase negative, while *F. philomiragia* is oxidase positive. *F. tularensis* type A and type B slowly catabolize D-glucose and maltose. *F. novicida* is able to degrade sucrose. *F. tularensis* type A has a functional citrulline ureidase pathway where *F. tularensis* type B does not. *F. tularensis* type A and type B biovar japonica ferment glycerol (184).

Table 1.2 Phenotypic and biochemical characteristics of *Francisella* biotypes

Characteristics	*F. tularensis* subspecies *tularensis* type A1 (SCHU S4)	*F. tularensis* subspecies *tularensis* type A2 (WY96-3418)	*F. tularensis* subspecies *holarctica* type B (LVS)	*F. tularensis* subspecies *mediasiatica* (FSC147)	*F. novicida* (U112)	*F. philomiragia* (ATCC25017)
Dimensions (µm)	0.2-0.7 × 0.2	0.2-0.7 × 0.2	0.2-0.7 × 0.2	0.2-0.7 × 0.2	0.7 × 1.7	0.7 × 1.7
Optimal growth temp.	37°C	37°C	37°C	37°C	37°C	25°C or 37°C
Cysteine requirement[A]	Yes	Yes	Yes	Yes	No	No
Glycerol fermentation[B]	Yes	Yes	No	Yes	Weak	No
D-glucose fermentation	Yes	Yes	Yes	No	Yes	Weak
Maltose fermentation	Yes	Yes	Yes	No	Weak	Yes
Sucrose fermentation	No	No	No	No	Yes	Yes
Citrulline ureidase	Yes	Yes	No	Yes	Yes	Unknown

Data compiled from (25, 163, 184).

[A]A few strains of *F. tularensis* (type A and type B) do not have a requirement for cysteine.

[B]Type B biovar japonica ferments glycerol.

1.7.1 Genomics and proteomics

All *Francisella* have a circular chromosome approximately 2 Mb in size with similar guanine-cytosine (GC) contents of 30 to 33% (Table 1.3). Although the overall genomic content of *Francisella* species is highly similar, significantly greater decay of open reading frames (ORF) has been identified in *F. tularensis* (approximately 225) compared to *F. novicida* (approximately 55) (25). Genomic rearrangements have also occurred between *F. tularensis* subspecies and populations (15, 25, 150). There are more transposable elements in *F. tularensis* type A and type B compared to *F. novicida* and *F. philomiragia* (approximately 80, 110, 20, and 10, respectively) that are implicated in the diminished coding capacity of *F. tularensis* (25). One plasmid has been isolated from *F. novicida* and two from *F. philomiragia* (106, 143). The necessity of these plasmids is not understood, and no plasmids have been identified in *F. tularensis*.

Table 1.3 Genomic characteristics of *Francisella* biotypes

Characteristics	*F. tularensis* subspecies *tularensis* type A1 (SCHU S4)	*F. tularensis* subspecies *tularensis* type A2 (WY96-3418)	*F. tularensis* subspecies *holarctica* type B (LVS)	*F. tularensis subspecies mediasiatica* (FSC147)	*F. novicida* (U112)	*F. philomiragia* (ATCC25017)
Genome size (Mb)	1.892	1.898	1.895	1.893	1.910	2.045
% GC	32.9	32.3	32.2	32.3	32.5	32.6
Predicted proteins	1603	1634	1754	1470	1719	1918
Predicted pseudogenes	101	186	213	263	14	7

Data compiled from (10, 25, 102, 184).

F. tularensis genomes encode between 1,600 and 1,750 proteins depending on the subspecies and population (Table 1.3). The differences in the predicted coding capacities are a result of subtle genetic alterations, and also arise from differences in the methods used for genome annotation (201). The small coding capacity is reflected in the decayed biosynthetic pathways of this genus, such as those for nucleic acid and amino acid synthesis (103). *F. tularensis* proteins are biased to contain amino acids from % GC poor codons, particularly isoleucine, tyrosine, asparagine, lysine, and phenylalanine (102). *F. tularensis* is unusual in that its genome is predicted to encode a greater proportion of envelope proteins (10% of all predicted ORFs) compared to typical proteobacteria species (6% of all predicted ORFs) (201). This suggests that envelope proteins play an especially significant role in the biology and pathogenicity of *F. tularensis*. As with other proteobacteria, approximately 30% of the gene products encoded by *Francisella* are annotated as hypothetical proteins with unknown function (201).

1.7.2 Lipids

Francisella species have unusually high cell wall lipid contents (70%) compared to other Gram-negative bacteria (about 25%) (3, 166). The majority of studies analyzing *Francisella* lipids characterize the fatty acid profiles of different strains (3, 75, 76, 81, 83,

140, 219). These analyses are useful for low resolution strain typing, and have also provided some insight about the lipid components of the cell wall. *Francisella* strains are all found to contain long-chain saturated and monounsaturated C_{18} – C_{26} fatty acids, abundant saturated even-chain acids ($C_{10:0}$, $C_{14:0}$, and $C_{16:0}$), and two long-chain hydroxy acids (3-OH $C_{16:0}$ and 3-OH $C_{18:0}$).

Few reports, with the exception of the lipid A component of LPS, are available describing lipid classes found in *Francisella* species, subspecies, and populations. Anderson *et al*. identified two major phospholipids in *F. tularensis* LVS, phosphatidylglycerol (PG) and phosphatidylethanolamie (PE), that constituted 24% and 76% of the total lipid, respectively (3). This analysis also identified trace amounts of two lysophospholipids, lyso-PE and lyso-PG, that increased in abundance with prolonged Bligh and Dyer extractions. No cardiolipin (CL) or phosphatidylserine, lipids commonly observed in other Gram-negative bacteria, was detected (62, 65). Analyses by Huber *et al*. identified additional lipid species in *F. tularensis*, *F. novicida*, *F. philomiragia*, and *F. noatunensis* (81). They describe the following polar lipid species in *F. tularensis* (type A, strain ATCC 6223^T), PE, CL, phosphatidylcholine, aminolipid, aminophospholipids, phospholipids, three unidentified phospholipids, and PG. The authors were unable to specifically characterize several lipid species they describe as unidentified aminophospholipids, aminophosphoglycolipids, and a lipid with highly hydrophobic chromatographic behavior. The *F. tularensis* lipid profile was identical to *F. novicida* (ATCC 15482^T). Polar lipid profiles differed between *F. tularensis* and *F. philomiragia*/*F. noatunensis*. Recently, the lipid profile of *F. novicida* U112 was analyzed using mass spectrometry (MS) (210). Seven lipids were identified in this study, CL, PG, PE, lyso-PE, phosphatidylcholine, and two species of lipid A. The investigators note the difference in lipid profiles between this and previous studies could be due to differences in growth state and culture medium.

1.8 Virulence factors

Bacteria use diverse and tightly regulated mechanisms to colonize and propagate in host niches. *F. tularensis* does not encode for classical virulence factors such as toxins or Type III and IV secretion systems, but has evolved different and equally effective components to cope with a changing host environment. A principle strategy of this bacterium is to avoid immune recognition. Specific examples include resistance to the action of human serum, modified structural components that do not elicit innate immune responses, and an intracellular lifecycle (168). Further, *F. tularensis* actively manipulates the host response for its advantage by suppressing inflammation, inhibiting phagosome maturation, and skewing cytokine responses (6, 20, 220).

1.8.1 Envelope

The envelope structure is the interface between a bacterium and the extracellular environment, and is composed of an array of macromolecules including proteins, lipids, sugars, and nucleic acids (19, 135). They are dynamic, and processes including membrane turn over, alterations in gene expression profiles, capsular formation, and retractable elements modify the envelope structure to allow bacteria to adapt to changing environments. Therefore the envelope is extremely important in pathogenesis. Bacteria use envelope components to hide from the immune system, attach to host cells, secrete effector molecules, and cope with physiological stresses. Hosts also take advantage of envelope components, using them to mount both innate and adaptive immune responses (43). In *F. tularensis*, the envelope's role in virulence is linked to the production of a capsule and unusual LPS (4, 69). A number of characterized and putative envelope proteins also impact pathogenesis (70, 120, 165, 204). Only a small number of proteins are specifically identified on the surface of the bacterium and will be discussed independently in Chapter II (110, 122, 131, 169).

1.8.1.1 Capsule

The report that first identified *F. tularensis* described what was believed to be a capsule that encompassed the surface of the coccobacilli (118). Subsequent studies also noted the presence of a capsule on both *F. tularensis* type A and type B strains (76, 168, 187). Capsule deficient *F. tularensis* are sensitive to the action of normal human serum via activation of the classical complement pathway (168, 187). Differences in virulence associated with capsular production between strains have also been noted (29). For example, certain lyophilized stocks of *F. tularensis* LVS that do not produce a capsule are 1,000 fold less virulent than the capsule producing wild-type (29). The capsule has also been a target of therapeutic development. Apicella *et al*. successfully used anti-capsule antibodies as a therapeutic to *F. tularensis* type B infection (4).

Biochemical evaluation of capsular material was initially performed by Hood *et al*. (76). The capsule was extracted using a hypertonic sodium chloride solution (76). Analysis of this material detected four sugars (mannose, rhamanose, and two unidentified dideoxy sugars), two fatty acids (α-OH 14:0 and 16:0), and amino acids (particularly aspartic acid and glutamic acid). Later, unpublished observations from Sorokin *et al*. found the capsule to consist of LPS (187). Whole genome sequencing of *F. tularensis* revealed two genes with homology to genes found in the poly-γ-glutamate capsule biosynthetic locus of *Bacillus anthracis* (*capABCD*) (103). The homologs, CapB (FTL_1416) and CapC (FTL_1415), are reported in *F. tularensis* LVS to share 38% and 29% amino acid similarity to *B. anthracis*, respectively (192). *In silico* analyses indicate CapB and CapC homologues are present in all subspecies and populations of *F. tularensis*. However, there is no evidence of a poly-γ-glutamate capsule, and if either of these genes are knocked out in *F. tularensis*, a capsule is still observed (4). Although the *F. tularensis* Cap homologs have an unknown function, when they are deleted the

coccobacillus is attenuated in animal models (4, 86, 192, 213). Recent MS and nuclear magnetic resonance (NMR) analyses indicate the capsule is composed of the O-antigen repeat core subunit of LPS (4). Mutational studies have found the genes of four glycosyltransferases involved in LPS biosynthesis, *wbtI* (FTT_1455), *wtbA1* (FTT_1464), *wtbM* (FTT_1450), and *wtbC* (FTT_1462) to be essential for capsular formation (4). These findings are in accordance with the effect of glycosyltransferase deletions on O-antigen capsule biosynthesis in other bacteria including *Escherichia coli* and *Salmonella enterica* (218).

1.8.1.2 Lipopolysaccharide

The most important component to the stability of the outer membrane and surface of Gram-negative bacteria is LPS, a complex macromolecule composed of a hydrophobic anchor, non-repeating core oligosaccharide, and a structurally diverse outer polysaccharide (157). Despite commonalities of LPS in Gram-negative bacteria, LPS is structurally diverse, and variations are noted between genera and species in all three major components. Anomalies in these structures are identified between *Francisella* species and subspecies (157).

Structural characterization of *F. tularensis*, *F. novicida*, and *F. philomiragia* lipid A has been accomplished using NMR, gas chromatography, MS, and chemical methods (151, 178, 205, 206, 209). *Francisella* lipid A is structurally distinct from other bacteria, and between *Francisella* species and subspecies. The dominant lipid A species is an asymmetric β-1,6 linked diglucosamine backbone containing four amide or ester linked long chain fatty acids (Fig. 1.2) (206). Between the three species, specific differences in lipid A structures are noted, and include mannose addition to the 4' position in *F. tularensis* and *F. novicida* and α-linked glucose to the 6' position of the non-reducing glucosamine in *F. novicida*. Additionally, a single phosphate moiety can be linked to the

1' reducing end of the lipid A core glucosamine in *F. tularensis* type B and *F. novicida*. In *F. novicida* this phosphate residue can be substituted with the positively charged sugar galactosamine (209). There is also evidence to suggest *F. tularensis* lipid A may be further modified by a hexosamine, but the exact nature of linkage is not clear (178). Differences in one or more of the acyl chains are apparent among species and subspecies. The number of carbons range between 14 and 20, and different chain lengths are noted in the same strain (69, 178).

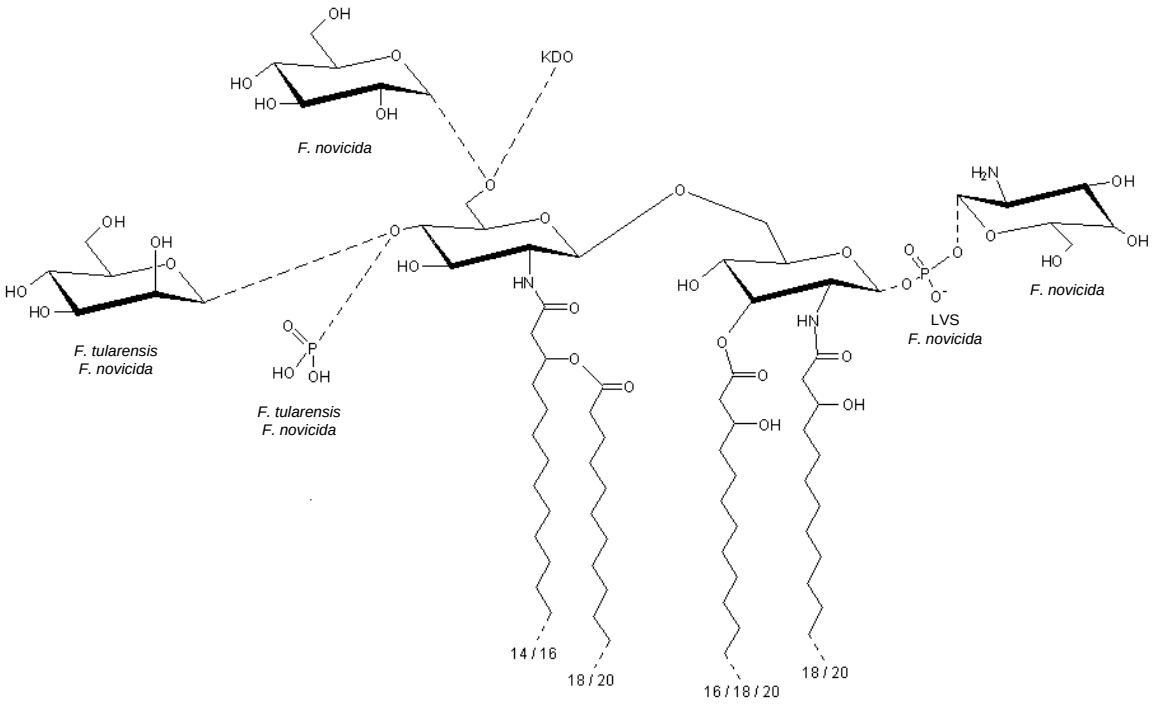

Fig. 1.2 Structure of *F. tularensis* and *F. novicida* tetraacylated lipid A. Dashed lines indicate various possible chemical substitutions. KDO is 3-deoxy-D-mannose-octulosonic acid. Data compiled from (151, 178, 205, 206, 209).

The core component of LPS bridges lipid A to the outer polysaccharide. Similar analytical techniques to those described above were used to determine the core structure of *F. tularensis* type B and *F. novicida* (69, 205, 206). Between these species the core structure is nearly identical. The inner core is composed of 3-deoxy-D-manno-octulosonic acid (KDO) linked to α-mannose which itself is linked to β-mannose. The outer core of both species contains a β-glucose linked (1,2) to the inner core α-mannose, and the inner core β-mannose is substituted with α-*N*-acetyl galactosamine and α-glucose. Structurally the outer core of *F. novicida* differs from *F. tularensis* in that the β-glucose linked to the α-mannose inner core is itself substituted with α-glucose.

Genome analyses of the O-antigen gene cluster suggest an identical repertoire of genes between *F. tularensis* type A and type B strains, and the O-antigen is identical between subspecies (69). The *F. tularensis* O-antigen polysaccharide structure was described in 1991 and is composed of a four sugar repeat of → 4)-2-acetamido-2-deoxy-D-galacturonamide-(1 → 4)- 2-acetamido-2-deoxy-D-galacturonamide-(1 →3)- 2-acetamido-2,6-dideoxy-D-glucose-(1→2)-4,6-dideoxy-4-formamido-D-glucose-(1→ (207). This structure differs in *F. novicida*. The adjacent 2-acetamido-2-deoxy-D-galacturonamide residues are conserved, but the other two sugars are replaced by a third 2-acetamido-2-deoxy-D-galacturonamide and 2,4-diacetamino-2,4,6-trideoxy-D-glucose (205).

The unusual structure of *F. tularensis* LPS, specifically lipid A, represents a key virulence factor. Lipid A is typically recognized by the TLR-4-MD2-CD14 complex with the aid of LPS binding protein and typically leads to an inflammatory immune response (157). However, *F. tularensis* lipid A is not efficiently recognized by TLR-4, does not bind to the LPS binding protein, and is approximately 1,000 fold less endotoxic than *E. coli* lipid A (205). The inertness of *F. tularensis* lipid A is thought to be due to absence of a phosphate at the 4' non-reducing glucosamine dimer and unusual acylation pattern

(tetraacylated with longer fatty acid chains) compared to bacteria such as *E. coli* that produce highly stimulatory lipid A (69). Production of altered LPS structures is also correlated to distinct changes in *F. tularensis* virulence and immunity (69). Most prominently, mutations in the O-antigen gene cluster that result in the loss of O-antigen, greatly increase both *F. tularensis* type A's and type B's sensitivity to serum, hinder intracellular replication, and attenuate the coccobacilli in animal models (160, 199).

1.8.1.3 Envelope proteins

Envelope virulence factors of *F. tularensis* include genes encoding for components of Type IV pili, secretion and translocation, LPS, and capsule production. A recent study by Salomonsson *et al*. identified genes of two envelope proteins *pilA* (FTT_0890) and FTT_0918, absent in the *F. tularensis* LVS genome, that encode a putative Type IV pilin and siderophore uptake protein, respectively (108, 165). When complemented into *F. tularensis* LVS, these genes restored virulence to levels indistinguishable from other *F. tularensis* type B isolates (165). PilA and FTT_0918 also impact virulence in *F. tularensis* type A strains (165, 204). In support of these data, attenuation is observed in animal models when two additional Type IV pili genes, *pilT* (FTT_0088) and *pilF* (FTT_1156), and a siderophore biosynthetic gene *flsA* (FTT_0029), are deleted (24, 108). The necessity for iron is well documented in intracellular pathogens, but the potential role of *pilA* is more ambiguous. *F. tularensis* lacking *pilA* is unable to disseminate like wild-type strains when introduced by the s.c. route of infection, but behave similarly when introduced by the i.p. route (165). In *Burkholderia pseudomallei* Type IV pili act as adhesions, allowing bacteria to attach to epithelial cells (47). Type IV pili may also participate in virulence in other ways such as facilitating biofilm formation, DNA uptake, and twitching motility (147).

A considerable number of ATP-binding cassette (ABC) transporters are routinely identified as virulence factors in both *in vitro* and *in vivo* genetic screens (120). ABC transporters are involved in both export and import of a variety of molecules including sugars, amino acids, inorganic and organic ions, lipids, and certain drugs (74). It is likely *F. tularensis'* transporters allow for adaptation to new niches and compensate for eroded biosynthetic pathways. For example, a knockout of the transporter *tolC* (FTL_1865) in *F. tularensis* LVS prevents the bacterium's ability to delay the early proinflammatory response (153). Interestingly, although there is no clinically relevant drug resistance to aminoglycosides, tetracycline, or chloramphenicol, transporters allowing for multidrug efflux of these drugs have been identified in *Francisella* genomes (18, 64). The Type II secretion system could also impact *F. tularensis* virulence. Several proteins shown to be secreted by this system in *F. novicida* such as chitinases ChiA (FTN_0627) and ChiB (FTN_1744), and M13 family metallopeptidase (FTN_1186), are reported to be significantly altered in specific *F. tularensis* subspecies and populations (15, 70, 103).

1.8.2 The *Francisella* pathogenicity island

Pathogenicity islands (PAIs) are gene clusters, 10 to 200 kb, that encode at least one gene important in virulence (180). These clusters are flanked by mobile genetic elements, often have differing GC contents compared to the core genome, and are thought to be transferred horizontally between species (180). The *Francisella* PAI (FPI) was identified by comparing the GC content adjacent to known virulence genes located in the *igl* operon to the core genome (132). One region, 17-kDa downstream of this operon, was found to have a GC content of 26.6%, 6.6% less than the core genome, and is surrounded by transposable genetic elements (133). The FPI is approximately 30 Kb, contains between 16 and 19 genes depending on the species and subspecies, and shares 97% sequence similarity between species (12). The FPI is duplicated in *F.*

tularensis (A1a, A1b, A2, type B, and *F. tularensis mediasiatica*), but not in species rarely pathogenic for humans (*F. novicida* and *F. philomiragia*) (12). Two predicted operons are encoded within the FPI, *igl* and pathogenicity determining protein (*pdp*) (132). Both operons are virulence factors specifically related to diminished phagosomal maturation and escape and cytosolic replication (12). Several FPI genes share some similarity to components of the Type VI secretion apparatus of *Vibrio cholerae* and represent another virulence factor linked to the envelope of *F. tularensis* (12). The two most conserved proteins of the Type VI secretion system, hypothetical protein VgrG (FTT_1347) and Hcp (FTT_1355), display a high degree of similarity to bacteriophage proteins and are encoded by all *F. tularensis* biotypes (12). VgrG resembles the spike complex of bacteriophage T4 which is capable of puncturing the outer membrane, and Hcp resembles the tail tube complex of phage lambda which polymerizes to form a 40 Å channel (9). Functional Type VI secretion was recently described in *F. novicida* (12).

Expression of FPI genes is tightly regulated by several protein transcription factors that are also essential for virulence. The most important of these are members of the stringent starvation family A gene family and include *mglA* (FTT_1275), *mglB* (FTT_1276), and *sspA* (FTT_0458). Deletion studies of *mglA* and *mglB* have found that both of these regulators are essential for virulence in animal and cellular models (14, 105). MglA forms a complex with SspA that in turn interacts with RNA polymerase to control expression of over 100 genes and upregulate expression of all FPI genes (26). *MglA* knockouts show decreased ability to escape from the phagosome and do not prevent phagosomal maturation (112). Less is known about the global regulatory impact of *mglB*, but its deletion decreases the production of several FPI proteins such as IglB (FTT_1713), PdpA (FTT_1699), and PdpB (FTT_1700) (179). Another regulator, FevR/PigR (FTT_0383), has been shown to directly interact with the MglA-SspA complex, and regulate many of the known target genes of this complex (26). Other

known regulators of the FPI include MigR (FTT_0694), and PmrA (FTT_1557), but little is known about their modes of action (146).

1.8.3 Chaperone proteins

Proteins involved in macromolecule assembly and fate are known virulence factors in *F. tularensis* (120, 212). Several heat shock family proteins (Hsp) involved in protein folding and chromosomal replication were the first chaperons implicated in *F. tularensis* virulence (46). The expression of Hsp generally increases in response to stresses such as temperature, pH , and oxidative stress (134). Hsp GroES (FTT_1695), GroEL (FTT_1696), and DnaK (FTT_1269) were all found upregulated in *F. tularensis* LVS when coccobacilli were exposed to hydrogen peroxide and a change of temperature from 37°C to 42°C (46). Chaperones are among the most highly upregulated genes during the bacterium's intracellular lifecycle. Wehrly *et al*. performed global transcriptional profiling of *F. tularensis* type A strains grown in murine bone marrow-derived macrophages finding that the previous three Hsp were upregulated intracellulary (212). This study also identified numerous other upregulated chaperones including: HtpG (FTT_0356), ClpP (FTT_0624), ClpX (FTT_0625), Lon (FTT_0626), HslU (FTT_0687), HslV (FTT_0688), HtpX (FTT_0862), DnaJ (FTT_1268), GrpE (FTT_1270), a DnaJ-like chaperone (FTT_1512), ClpB (FTT_1769), and Hsp (FTT_1794).

Screens of transposon mutant libraries led to the identification of seven chaperones important in virulence including the peptidases Lon (FTT_0626), CphB (FTT_0802), and PepP (FTT_0609), proteins participating in disulfide bond formation DsbA (FTT_1103) and DsbB (FTT_0107), and Hsp HptG (FTT_0356) and ClpB (FTT_1769) (120). DsbA and ClpB are the best understood of these chaperons. DsbA is a predicted periplasmic protein containing a disulfide bridged thioredoxin fold which acts as an oxidant for a dithiol in substrate proteins, and is kept oxidized by DsbB (73).

Proteins containing disulphide bonds are virulence factors for many bacteria and include toxins and adhesions, and disruption of their folding renders many of these products non-functional (73). In *F. tularensis*, DsbA mutants are attenuated for survival and replication in macrophages and in mice (154, 191). ClpB is a virulence factor in *L. monocytogenes* and *Salmonella* typhimirium, and participates in the stress response of numerous other bacteria (121). It is a member of the Clp family ATPases that functions by disaggregating highly complex proteins and reactivating them (223). ClpB was shown to be essential for replication of *F. tularensis* LVS in target organs (121). *F. tularensis* LVS ClpB mutants also show an increased sensitivity to oxidative stress and pH and are partially attenuated for intracellular survival (121).

1.8.4 Metabolic proteins

The success of a pathogen is dependent on acquisition or biosynthesis of key metabolites. The genome of *F. tularensis* contains approximately 350 genes predicted to be involved in 155 pathways of small molecule metabolism (103). *F. tularensis* genomes are considered to be highly decayed, as illustrated by the numerous pathway holes predicted to disrupt more than half of the reactions for *F. tularensis*' central metabolic network (103). Thus, many remaining metabolic genes are also important for virulence.

Amino acid and nucleotide metabolic pathways are mostly disrupted in *F. tularensis* type A and type B strains. *F. tularensis* SCHU S4 growth is supported by supplementation with 13 amino acids, but does not require aromatic amino acid supplementation (93, 103). Deletions of aromatic amino acid biosynthetic genes attenuate virulence of *F. tularensis* in cell culture and *in vivo*, a finding consistent with the limited pool of aromatic amino acids found intracellulary (120, 159). Additionally,

over 10 virulence genes involved in nucleotide biosynthesis are known, and deletions of these genes are also thought to interfere with intracellular survival (120).

1.9 Host-pathogen interactions

The ability of *F. tularensis* to survive and persist in the host is dependent on intrinsic factors of the bacterium that allow the organism to occupy a specific niche, and also the mechanisms and regulatory events of the host itself. *F. tularensis* strains survive both intracellulary and extracellulary within its many susceptible hosts (120). This dichotomy is presumably a result of highly governed metabolic regulatory events allowing for rapid, and dramatically different, molecular responses to changing niches. A number of genes involved in central metabolism, envelope biogenesis, secretion, and regulation were identified in *F. tularensis* that promote these changes and are critical to the success of the bacterium. Similarly, hosts have developed complex networks to counter infection that take into account the bacterium's different lifecycles and multiple colonization pathways.

1.9.1 Pathogenesis

Shortly after host entry, *F. tularensis* is either eliminated by the innate immune system, or invades host cells including dendritic cells, monocytes, lung and kidney epithelial cells, neutrophils, hepatocytes, fibroblasts, and most frequently macrophages (21, 71, 79). Entry into macrophages involves bacterial ligand interactions with several types of host cell receptors also known to mediate uptake of other intracellular bacterial pathogens. These include complement receptor 3, mannose receptor, nucleolin, and class A scavenger receptors (8, 11, 33, 152). However, the mechanism by which phagocytosis of *F. tularensis* is achieved is unique from the normal trigger and zipper mechanisms (120). Following ligand recognition, pseudopod loops surround and

internalize bacteria into large phagosomal compartments that dramatically shrink in size shortly after formation (6). The specific ligand or ligands involved are unknown, but are likely pre-formed surface carbohydrates (32). Within the phagosome bacterial effectors, namely the *igl* proteins IglA (FTT_1714), IglB (FTT_1713), and IglC (FTT_1712), alter normal phagosomal maturation (6). The *Francisella* containing phagosome acquires late endosomal markers and the vacuolar ATPase, but not cathepsin D, and does not fuse with lysosomes (32). Following phagosomal acidification, bacteria degrade the phagosomal membrane by an unknown mechanism and escape into the cytosol as rapidly as 30 to 60 minutes after uptake (146). Alternatively, the coccobacilli may stay in the phagosome for many hours, or escape and enter another vacuole-like structure following cytosolic replication (27). The final result of a cell infected with *F. tularensis* is death, initiated by the induction of Type I apoptosis and pyropoptosis (120). The bacteria then escape the initial host cell, disseminate to other susceptible cells locally, and then spread throughout the body. The primary sites of dissemination are lymph nodes, lungs, intestine, pleura, spleen, liver, and the kidneys (41, 198). Severe inflammation and necrosis may occur at these sites, which can eventually lead to death.

1.9.2 Innate immunity

Complement deposition is a principle innate immune response mounted against many Gram-negative pathogens to counter infections and slow or prevent dissemination (40). However, most wild-type strains of *F. tularensis* have limited susceptiblity to direct complement mediated killing because of capsular production (43). Complement can increase opsonization of the coccobacilli by polymorphonuclear leukocytes, monocytes, macrophages, dendritic cells, and neutrophils, but this might not be advantageous to the host (43). McCaffrey *et al*. found opsonized *F. tularensis* LVS ingested by neutrophils inhibited activation of the host cell and prevented the respiratory burst (116). Bacteria

were able to survive in these cells, thus neutrophil involvement may contribute to the dissemination of bacteria (116).

The initial cytokine response to *F. tularensis* is characterized by a delayed production of pro-inflammatory and Th-1 type cytokines by macrophages, dendritic cells, and especially natural killer cells (80, 146). These include tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin 12 (IL-12) (43). Production of cytokines occurs 72 to 96 and 24 to 48 hours post infection in *F. tularensis* type A and type B, respectively (98). Activation of macrophages by both IFN-γ and TNF-α is crucial for controlling infection with *F. tularensis* LVS, in large part by stimulating the production of nitric oxide (146). The role of IL-12 is multifaceted, and besides stimulating the production of IFN-γ, independently affects bacterial clearance (43). Cytokine production can be initiated through pattern recognition receptors, specifically TLR-2, but requires active synthesis from the coccobacilli (34).

Activation of the TLR-2/TLR-1 and TLR-2/TLR-6 heterodimer complexes is linked to the recognition of triacylated and diacylated lipoproteins, respectively (177). *F. tularensis* encodes both the prolipoprotein diacylglyceryltransferase (FTT_1228) required for diacylated lipoprotein synthesis and the apolipoprotein *N*-acyltransferase (FTT_0614) required for triacylated lipoprotein synthesis. To date, only two TLR-2/TLR-1 ligands have been identified, the surface and envelope associated lipoproteins TUL4 (FTT_0901) and FTT_1103, respectively (197). The TLR-2/TLR-6 heterodimer is also stimulated by *F. tularensis*, but the ligands are unknown (197). Other TLR's are likely activated by *F. tularensis*, but the specific receptors and their ligands are not defined. Collazo *et al*. demonstrated MyD88 (shared TLR pathway adaptor protein) knockout mice were more susceptible to *F. tularensis* LVS infection than TLR-2 deficient mice (36).

Intracellular Nod-like receptors (NLR) also participate in innate immunity against *F. tularensis* (214). Recognition of bacterial DNA by the NLR AIM2 leads to the formation of an intracellular protein complex called the inflammasome (49, 158). The inflammasome recruits and activates caspase-1, a cysteine protease that processes pro-forms of IL-1β and IL-18 into their active forms (28). Pro-IL-1β is not abundant in resting macrophages and monocytes, and its production is stimulated via MyD88 dependent TLR activation (80). In contrast, sufficient levels of pro-IL-18 are constitutively present in these cells (214). IL-1β and IL-18 are potent secreted pro-inflammatory cytokines that are important in establishing fever and up-regulate the production of IFN-γ, respectively (214).

1.9.3 Adaptive immunity

Before the introduction of streptomycin, tularemia was treated with marginal success using xenogeneic immune serum (53). It was also noted by Edward Francis, "One who has recovered from an attack of tularaemia need not fear a second attack, because he is then immune to the disease" (60). These and other early reports definitively established the importance of an adaptive response to *F. tularensis*. It is now clear that both the cell mediated and humoral responses work synergistically to provide protection. Like the innate response, bacterial surface components appear to be key targets and stimulators of adaptive immunity (174). .

The cell mediated response is considered to be the most important for controlling *F. tularensis* infection due to the intracellular lifecycle of the coccobacilli, and is effective against both *F. tularensis* type A and type B strains. The response is mediated primarily by $CD4^+$ and $CD8^+$ T cells. In mice, both $CD4^+$ and $CD8^+$ T cell populations are required to control *F. tularensis* type A infection, but either type offers protection against *F. tularensis* type B (43). The signatures of $CD4^+$ and $CD8^+$ T cell responses induced by *F.*

tularensis in murine models are similar in humans and are characterized by significant production of Th-1 type cytokines including IFN-γ and TNF-α (43). Therefore, T cell immunity against *F. tularensis* is strongly linked to macrophage activation. Only two *F. tularensis* T cell antigens are well described, the surface protein TUL4 (FTT_0901) and the predicted outer membrane protein FopB (FTT_1747) (169, 222).

Despite the necessity of cell mediated immunity, antibodies enhance immunity against *F. tularensis* (43). Specific IgG, IgM, and IgA antibodies are detectable in human patients about 2 weeks after infection (43). Titers of all three antibody isotypes peak 1 to 2 months after infection, but can be detected up to 11 years post infection (43). A large percentage of the specific antibody response is directed at LPS, and an unusual early LPS-specific antibody response has been shown to protect mice in a T cell independent manner (35, 43). Passive transfer of immune sera has also shown efficacy in humans and animals, but significant protection is not observed with *F. tularensis* type A strains (53, 190). Monoclonal antibodies reactive to two *F. tularensis* surface proteins, TUL4 (FTT_0901) and FopA (FTT_0583), and the capsule also provide a limited protective effect against *F. tularensis* infection (4, 174). The protective roles of specific antibodies through other types of mechanisms such as lysis, antibody-mediated cellular toxicity, and cooperation with T cells are not clear (43).

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Chapter II

Literature Review Part II: The Surface Proteome of *F. tularensis*

2.1 Introduction

The surface proteins of bacterial pathogens constitute a diverse group of structures and enzymes that are involved in numerous biotic and abiotic interactions including, adhesion, invasion of host cells, environmental sensing, nutrient processing and acquisition, and immune effectors (14, 53, 68). Further, bacterial surface proteins are often recognized by hosts and are considered the basis for the many rationally designed vaccines (12, 84). Given the diversity and importance of these proteins, the characterization of surface proteomes can provide valuable insights into pathogenesis, the niches bacterium exploit for survival, and vaccine development. *Francisella* species, subspecies, and populations are documented to survive in >300 species of animals (intracellulary and extracellulary) and identified in environmental samples, but have different niche requirements (11, 66, 75). It is likely that surface proteins mediate these interactions, and alterations in surface proteomes may contribute to the phenotypic variability of *Francisella* biotypes (19, 62). Although only 10 proteins are reported on the surface of *Francisella* species, several have documented roles in pathogenesis and immunity (5, 49, 62, 82, 90, 92). Further, experimental characterizations of the *F. tularensis* membrane proteome and secretome as well as bioinformatic predictions indicate a much large number of proteins are surface associated, and these too may play important roles in *F. tularensis* ecology and virulence (44, 48). This chapter discusses the known and candidate surface proteins of *Francisella* species, subspecies,

and populations, and their roles in the biology, pathogenesis, and phenotypic variation of this genus.

2.2 Bioinformatic signatures of *F. tularensis* surface proteins

To be surface associated, the proteins of Gram-negative bacteria must first bypass the outer membrane and integrate with cell surface components (41). The physiological processes that allow for these interactions are determined largely by a protein's structure. The two most common signatures that are used for the identification and support of surface protein classifications are signal peptides and β-barrels that enable protein translocation/secretion and integration into the outer membrane, respectively (94, 99). Bioinformatic analyses of *F. tularensis* genomes indicate >1/6th of the proteins are secreted or translocated (Appendix 2). In contrast, a much smaller number of proteins contain β-barrels (Appendix 2) (about 40). Of these approximately half contain a signal peptide (Appendix 2). Other methods to identify surface proteins *in silico* rely primarily on homology searches that compare a target sequence to proteins of known surface localization (101). These types of analyses indicate approximately 3% of the proteins encoded by *F. tularensis* could be surface associated.

2.3 Surface proteins of *F. tularensis*

Currently, 10 proteins have been specifically localized to the surface of *F. tularensis*. (60, 62, 67, 80). The first of these identified, FopA (FTT_0583), is a 43 kDa antigen that was identified in lithium chloride extracts (13). In addition to frequent identification in membrane preparations, the surface localization of this protein was confirmed by Melillo *et al*. using surface protein biotinylation (62). FopA contains a C-terminal OmpA-C like domain, and thus belongs to the OmpA family of proteins. OmpA homologs are among the most abundant types of surface proteins in other Gram-

negative bacteria and function as cell surface receptors, enhance conjugation frequencies, aid in host cell invasion, and participate in biofilm formation (87). FopA is highly conserved among *F. tularensis* genomes, is required for *F. novicida* growth in murine macrophages, and is described as a virulence factor in *F. tularensis* LVS (90, 92). Monoclonal antibodies to FopA can be administered therapeutically to provide a marginal protective effect against *F. tularensis* infection (82).

A 17 kDa lipoprotein, TUL4 (FTT_0901), was another presumed *F. tularensis* surface protein that was first identified in *N*-lauroyl sarcosinate insoluble protein fractions (13). It was initially considered an integral membrane lipoprotein protein based on the results of [^{3}H] palmitate radiolabeling and detergent extractions (86). However, subsequent studies clearly determined that TUL4 localized on the outer membrane (44, 47). Antibodies directed against this protein have some protective effect, and TUL4 stimulation of T cells leads to the production of important proinflammatory cytokines IFN-γ and TNF-α (26, 82, 85). The function of TUL4 is unknown for the coccobacilli, it is not required for virulence, and beyond Francisellaceae, bioinformatic similarity searches do not identify any closely related orthologs with known function (29).

Specific biotin labeling and subsequent enrichment was used to identify the remaining known surface proteins of *Francisella* species (60, 62). Using these methods, Melillo *et al*. found six proteins in addition to FopA (FTT_0583) on the surface of *F. tularensis* LVS (DnaK, FTT_1269; GroEL, FTT_1696; hypothetical protein FsaP, FTT_0119; IglA, FTT_1359; hypothetical protein VgrG, FTT_1347; and hypothetical protein Bfr, FTT_1441), but did not detect TUL4 (FTT_0901). Similarly, Ludu *et al*. determined the surface localization of the proteins IglB (FTN_1323) and IglC (FTN_1322) in *F. novicida* (60). Of these, bioinformatic analyses suggest only hypothetical protein FTT_0119 has a surface localization (60, 62). However, the identification of predicted cytosolic proteins on the surface is not altogether unexpected,

as these are increasingly being described on the surface of other pathogenic bacteria (69). Chaperones such as GroEL and DnaK have auxiliary functions while surface associated and are involved in processes such as immune modulation, cell adhesion, and host factor binding (9, 16). GroEL and DnaK are upregulated when coccobacilli are exposed to hydrogen peroxide, and GroEL was especially abundant in the *F. tularensis* LVS-containing phagosomes of macrophages indicating an important role for these proteins during intracellular survival (49).

The hypothetical surface protein FsaP (FTT_0119) is reported to contain a lysine motif LysM domain which may anchor it to peptidoglycan or act as a surface associated adhesion (6, 62). In a study by Melillo *et al*., *F. tularensis* LVS was shown to efficiently adhere to A549 human epithelial cells, and production of *F. tularensis* FsaP in *E. coli* allowed for epithelial cell interaction with the heterologous host (62). In contrast, *F. novicida* does not associate with lung epithelial cells. Bioinformatic analyses of the *F. novicida* FsaP homolog identified a single amino acid substitution in its signal peptide that was predicted to prevent protein translocation (62). In support of this prediction FsaP was not localized in the outer membrane of *F. novicida*. Thus, surface localization of FsaP represents a distinct difference in the surface proteomes of *F. tularensis* and *F. novicida*.

IglA (FTT_1714), IglB (FTT_1713), and IglC (FTT_1712) are surface proteins thought to be involved in Type VI secretion. All of these proteins have been identified as antigens and have important roles in pathogenesis (15, 45, 46, 81). IglA and IglB are conserved components of Type VI secretion systems and orthologs are identified in bacterial pathogens such as *V. cholerae*, *Pseudomonas aeruginosa*, and *Burkholderia mallei* (15). Together these proteins form a complex shown to enhance the intracellular growth and virulence *F. tularensis* LVS (15). IglC is unique to *Francisella* and is required for the coccobacilli to escape the phagosome and replicate in the cytosol of

macrophages (57, 81). Crystallization and structural analyses of IglC revealed an unusual structure that displayed little homology to any other protein (91). The predominant feature is a β-sandwich plate conformation composed of two units of anti-parallel β-strands that are sandwiched together by hydrophobic interactions. This conformation is unique to IglC (91). Mechanistically, the specific function of IglC is unknown, thus it is difficult to speculate on the structural significance of this protein. Another surface protein involved with Type VI secretion, hypothetical protein VgrG (FTT_1347) resembles the spike complex of bacteriophage T4 and is encoded by all *F. tularensis* biotypes (5). In *V. cholerae*, VgrG homologs form a trimeric complex that may puncture host membranes and also enzymatically cross-link host cell actin (77).

2.4 Gram-negative protein secretion and the secretome of *F. tularensis*

Considering the importance of translocation and secretion in surface protein localization and identification, it is necessary to briefly preface the specific mechanisms and structures that allow for protein export. Two evolutionary conserved pathways, the generalized secretion system (Sec) and two-arginine translocation (Tat), allow protein transport into or across the plasma membrane in Gram-negative bacteria (Fig. 2.1) (41). A number of more specialized “one-step” and “two-step” secretion systems are also known (Fig. 2.1) (25). One-step secretion systems export proteins directly from the cytoplasm beyond the outer membrane, and include Type I, III, VI, and VI systems (41). Two-step secretion systems rely on Sec or Tat machinery to move protein to the periplasm where Type II or V, and occasionally Type I, secretion systems shuttle proteins in or beyond the outer membrane (41).

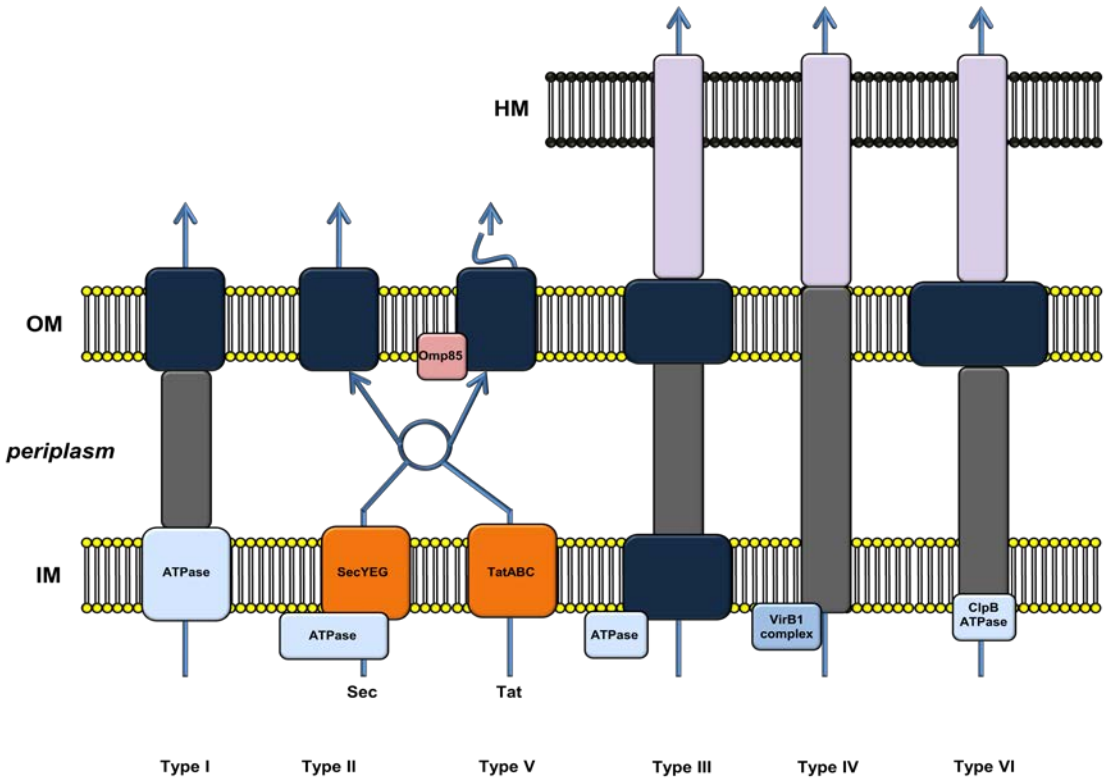


Fig. 2.1 Gram-negative secretion and translocation adapted from Tseng *et al*. (94). A simplified view of secretion and translocation systems of Gram-negative bacteria. HM, Host membrane; OM, outer membrane; IM, inner (cytoplasmic) membrane.

The specificity of secretion is largely dependent on the recognition of signal peptides. In the case of Sec secretion, hydrophobic N-terminal leader sequences of 20 to 30 amino acids are recognized on unfolded proteins (71). Signal sequence recognition is very similar between Sec and Tat systems except the Tat signal peptides

contain a motif of two arginines, have different charge properties, and are slightly less hydrophobic than Sec signal peptides (23). Most Type I signaling motifs are located at the C-terminus, but these sequences are not always highly conserved (41). The molecular features allowing for recognition of Type II secretion substrates are not completely characterized, but can involve the tertiary structure of the substrate and multiple contacts with the secretion complex (20). Type II protein secretion appears to require N-terminal secretion signals on the protein or even mRNA (24). Similarly, Type III secretion utilizes N-terminal signal peptides, but non-N-terminal signaling motifs are known to allow the secretion of several proteins (24). Analyses of protein substrates of Type IV secretion in *Legionella pneumophila* and *Agrobacterium tumefaciens* showed that Type IV signaling motifs reside at the C-terminus and consist of hydrophobic or positively charged amino acid clusters (2). However, other motifs and accessory proteins are shown to effect Type IV secretion of some proteins (2). In general, Type V secretins contain N-terminal cleavable secretion sequences for Sec translocation (94). Following Sec translocation of Type V secretins the C-terminal domain forms a β-barrel pore which allows for export of the passenger domain through the outer membrane (94). The events and signals of Type VI secretion that allow for substrate recognition and export are currently unclear (10).

The Sec system is complete and highly conserved in the *Francisella* genus, but no functional Tat system is apparent (19). In addition, three specialized secretion systems, Type I, Type II, and Type VI are identified (19, 93). Components of both Type III and Type IV systems are recognized, but are predicted to be non-functional (19). There is currently no evidence to support the presence of Type V autotransporter proteins (93). Few studies have identified substrates of *Francisella* protein secretion (Table 2.1). Two proteins have been shown to be secreted by the Type VI secretion system in *F. novicida*, a VgrG homolog (FTN_1312) and a hypothetical protein IglI

(FTN_1317) (5). Lee *et al*. characterized the protein secretomes of *F. tularensis* LVS and a *F. tularensis* type A clinical isolate grown *in vitro*, identifying 12 substrates (54). A similar study identified 21 and 26 proteins in culture supernatants (CS) of *F. tularensis* LVS and SCHU S4, respectively (48). Interestingly, only 17 of the 47 proteins in the latter study were predicted to contain signal peptide motifs. Along with chaperons containing no predicted signal peptides, other predicted cytosolic proteins were identified in these studies including glyceraldehyde-3-phosphate dehydrogenase (FTT_1368). Glyceraldehyde-3-phosphate dehydrogenase was the first assumed cytosolic protein identified on the surface of a bacterium, and was shown to interact with host plasminogen, fibronectin, and lysozyme as well as the cytoskeletal proteins myosin and actin (59, 70). *F. tularensis* is also capable of binding plasminogen to its surface which can then be digested into plasmin by a tissue plasminogen activator (21). Cell surface plasmin is an important virulence mechanism of other bacterial pathogens that allows these organisms to degrade the extracellular matrix surrounding host cells and enhances dissemination to distal sites (21). *F. tularensis* SCHU S4 was recently shown to bind activated plasmin to its surface whereas *F. tularensis* LVS did not (22). The authors of this study showed that plasmin coated *F. tularensi*s SCHU S4 was capable of degrading antibodies and reduced uptake of the bacteria by macrophages. The specific molecules that allow *F. tularensis* to bind to plasminogen/plasmin are unknown, but several *F. tularensis* membrane proteins have plasminogen binding affinities (21).

Table 2.1 Secreted proteins of *F. tularensis* and *F. novicida*

Strain (ref)	Growth conditions	Protein identified	Loci
F. tularensis LVS and *F. tularensis* (recent clinical isolate) (54)	Chamberlain broth, CS collected at 8, 16, and 24 hours post inoculation	Superoxide dismutase (Fe)	FTT_0068
		Succinyl-CoA synthetase, alpha subunit	FTT_0503
		Succinyl-CoA synthetase subunit beta	FTT_0504
		AhpC/TSA family protein	FTT_0557
		Peroxidase/catalase	FTT_0721
		Purine nucleoside phosphorylase	FTT_0766
		Molecular chaperone DnaK	*FTT_1269
		Glyceraldehyde-3-phosphate dehydrogenase	FTT_1368
		Malonyl CoA-acyl carrier protein transacylase	FTT_1374
		Hypothetical protein	*FTT_1441
		Co-chaperonin GroES	FTT_1695
		Chaperonin GroEL	*FTT_1696
F. novicida U112 (35)	Tryptic soy broth supplement with 0.1% cysteine, CS collected mid log phase	Hypothetical protein	FTN_0175
		Glycosyl hydrolase family chitinase	FTN_0627
		M13 family metallopeptidase	FTN_1186
		Hypothetical protein	FTN_1261
		Glycosy hydrolase family 3 protein	FTN_1474
		Chitin-binding protein	FTN_1485
		Chitinase	FTN_1744
F. novicida U112 (5)	TSAP broth, CS collected when growth reached an OD_{600} of 1.0	Hypothetical protein VgrG	*FTN_1312
		IglI	FTN_1318
F. tularensis LVS (48)	Chamberlain broth, CS harvested at late log phase	Pyrrolidone-carboxylate peptidase	FTL_0207
		Glutamate dehydrogenase	FTL_0269
		Glycine cleavage system aminomethyltransferase T	FTL_0477
		Hypothetical protein	*FTL_0617
		Histone-like protein HU form B	FTL_0895
		ATP-dependent protease peptidase subunit	FTL_0965
		AhpC/TSA family protein	FTL_1015
		Glyceraldehyde-3-phosphate dehydrogenase	FTL_1146
		Molecular chaperone DnaK	*FTL_1191
		D-ribulose-phosphate 3-epimerase	FTL_1432
		Purine nucleoside phosphorylase	FTL_1461
		Translation initiation inhibitor	FTL_1498
		Peroxidase/catalase	FTL_1504
		Glycerophosphoryl diester phosphodiesterase family protein	FTL_1511
		Putative periplasmic protease	FTL_1605
		Chaperonin GroEL	*FTL_1714
		50S ribosomal protein L11	FTL_1748
		Transcription antitermination protein nusG	FTL_1749
		Triosephosphate isomerase	FTL_1780

Table 2.1 Secreted proteins of *F. tularensis* and *F. novicida*, continued

Strain (ref)	Growth conditions	Protein identified	Loci
F. tularensis LVS (48)	Chamberlain broth, CS harvested at late log phase	Succinate dehydrogenase iron-sulfur subunit	FTL_1785
		Superoxide dismutase	FTL_1791
F. tularensis SCHU S4 (48)	Chamberlain broth, CS harvested at late log phase	Superoxide dismutase (Fe)	FTT_0068
		Triosephosphate isomerase	FTT_0080
		Acid phosphatase (precursor)	FTT_0221
		Ribosome recycling factor	FTT_0316
		3-dehydroquinate dehydratase	FTT_0471
		Hypothetical protein	FTT_0484
		Lactate dehydrogenase	FTT_0535
		AhpC/TSA family protein	FTT_0557
		Outer membrane associated protein	*FTT_0583
		Beta-lactamase	FTT_0611
		Hypothetical protein	FTT_0704
		Peroxidase/catalase	FTT_0721
		Glycerophosphoryl diester phosphodiesterase family protein	FTT_0726
		Purine nucleoside phosphorylase	FTT_0766
		D-ribulose-phosphate 3-epimerase	FTT_0789
		Fumarylacetoacetate hydrolase family protein	FTT_1023
		50S ribosomal protein L9	FTT_1060
		Transcriptional regulator	FTT_1075
		Molecular chaperone DnaK	*FTT_1269
		Translation initiation inhibitor	FTT_1338
		Glyceraldehyde-3-phosphate dehydrogenase	FTT_1368
		Acyl carrier protein	FTT_1376
		Hypothetical protein	*FTT_1441
		Co-chaperonin GroES	FTT_1695
		Chaperonin GroEL	*FTT_1696
		Outer membrane protein	FTT_1747

*Indicates the protein was previously identified as surface exposed.

Hager *et al*. confirmed the presence of a functional Type II secretion system in *F. novicida* and identified seven substrate proteins that included three chitinolytic proteins (FTN_0627, FTN_1485, and FTN_1744), a M13 family metallopeptidase (FTN_1186), a family 3 glycosyl hydrolase (FTN_1474), and two hypothetical proteins (FTN_0175 and FTN_1261) (35). A homolog of the *F. novicida* secreted chitinase, ChiA (FTN_0627), was identified in *F. tularensis* membrane fractions and was recognized as an antigen in

multiple studies (Appendix 3) (44). Genome analyses indicated the secreted chitinases differ between *F. tularensis* biotypes, and this variability might contribute to unique phenotypes and niche occupations (19, 64). Chitinases are similar to cellulases in that they are both glycosyl hydrolases that act on similar polymers, chitin (a polymer of β-1,4 linked *N*-acetyl-D-glucosamine) and cellulose (a polymer of β-1,4 linked D-glucose), respectively (65). Several bacteria that degrade cellulose assemble large cellulase-containing surface structures called cellulosomes (7, 83). Given the similarities of chitinases and cellulases and their substrates, the existence of analogous surface associated chitinosomes has been proposed (52).

Bacterial glycoproteins are typically translocated products that may be fully secreted or incorporated into surface structures such as an S layer or the outer membrane (39, 83). Balonova *et al*. used four lectins, *Sambucus nigra agglutinin* (SNA), *Maakcia amurensis agglutinin* (MAA), *Datura stramonium agglutinin* (DSA), and *Peanut agglutinin* (PNA) to identify and profile *F. tularensis* membrane glycoproteins (3). SNA preferentially recognizes sialic acid linked (2-6) to galactose; MAA recognizes sialic acid linked (2-3) to galactose; DSA recognizes structures with terminal galactose (1-4) linked with *N*-acetyl glucosamine; and PNA recognizes galactose (1-3) linked to *N*-acetyl galactosamine (3). Lectin profiling, in conjunction with hydrazide labeling, identified 20 putative glycoproteins and provided evidence of the glycan moieties synthesized by *F. tularensis*. Sixteen of the proteins identified showed affinity for SNA, six for MAA, two for DSA, and one for PNA. Four proteins (outer membrane protein FopA, FTH_1293; signal recognition particle receptor FtsY, FTH_1598; ABC superfamily ATP binding cassette transporter, FTH_1206; and succinate dehydrogenase, FTH_1721) had affinity for two or more of these lectins.

2.5 Analyses of *F. tularensis* membrane fractions

Membrane localization is a principle signature of many surface associated proteins, and analyses of membrane fractions by 2D-PAGE has provided insights to the possible composition of the *F. tularensis* surface proteome (33, 37, 44, 45, 72, 73, 89, 95, 97). 2D separation of *F. tularensis* membrane proteins was largely pioneered under the direction of Jiri Stulik and resulted in improved procedures for sample fractionation (carbonate extraction), solubilization (addition of the detergent ASB-14), and introduced narrow range isoelectric focusing (IEF) pH gradients that allowed for improved protein resolution (37, 43). Solubilization methods were optimized by Twine *et al*. who demonstrated that specific concentrations of ASB-14 allowed for the visualization of additional membrane proteins (95). The number of protein spots observed in sodium carbonate membrane preparations ranges from 300-800 when separated using pH 3-10 IEF strips and 200 spots using pH 6-11 IEF strips (43, 72, 73). Despite these findings, only 38 and 85 membrane proteins were identified in *F. tularensis* type A and type B, respectively (Appendix 4) (37, 45, 46, 72, 73, 89, 95).

Analyses of outer membrane preparations of *F. tularensis* SCHU S4 and LVS by Huntley *et al*. may provide a more realistic view of the surface proteome. In this study twelve proteins were identified by MS in conjunction with 2D-PAGE. Of these, only five were predicted by the authors bioinformatic analyses to be outer membrane proteins (peroxidase/catalase, FTT_0721; Type IV pilin multimeric outer membrane protein, FTT_1156; hypothetical protein, FTL_0439; outer membrane associated protein, FTT_0583; and peptidoglycan-associated lipoprotein, FTT_0842). The remaining proteins were predicted to be cytosolic (pyruvate dehydrogenase subunit E1, FTT_1485 and GroEL, FTT_1696), periplasmic (DnaK, FTT_1269), or could not be assigned a localization (F0F1 ATP synthase subunit beta, FTT_0064; lipoprotein, FTT_1103; OmpA family protein, FTT_0831; and TUL4, FTT_0901). Although MS characterizations were

not achieved, Huntley *et al*. identified several other surface protein candidates (hypothetical protein, FTT_1095; outer membrane efflux protein, FTT_1258; outer membrane protein, FTT_1573; outer membrane protein TolC precursor, FTT_1724; FKBP-type peptidyl-prolyl cis-trans isomerase family protein, FTT_1043; hypothetical protein FupA, FTT_0918; hypothetical protein FupB, FTT_0919; hypothetical protein, FTT_0025; lipoprotein, FTT_0904; and LamB/YcsF family protein, FTT_0223) by Western blot analyses of the outer membrane fraction.

Several of the identified outer membrane proteins (hypothetical protein, FTT_1095; outer membrane efflux protein, FTT_1258; and outer membrane protein TolC precursor, FTT_1724) share significant similarity to the *E. coli* protein TolC (44). TolC forms an outer membrane channel that is utilized by efflux pumps and Type I secretion systems to expel toxins, proteases, and lipases, and can also be involved in multidrug resistances (32, 76). In *F. tularensis* TolC homologs (FTT_1095 and FTT_1724) are involved in drug efflux, and deletion of either gene caused increased sensitivities to other small molecules including detergents and dyes (32). Deletion of FTT_1724 resulted in significant attenuation of *F. tularensis* in an i.d. murine model of infection (32). In this model, decreased bacterial burdens of *F. tularensis* Δ*FTT_1724* compared to wild-type were observed in the lungs, liver, and spleen (76). Additionally, *F. tularensis* Δ*FTT_1724* was hypercytotoxic to both murine and human macrophages and caused overproduction of several proinflammatory chemokines (76).

F. tularensis does not encode all of the genes classically associated with iron acquisition, such as *tonB* (63). Nonetheless, several of the possible surface exposed outer membrane proteins identified by Huntley *et al*. were documented to play a role in iron sequestering. The homolog of hypothetical protein FTT_0025 was upregulated in *F. novicida* during iron limiting conditions, co-transcribed with a siderophore operon, and had an impact on siderophore secretion or uptake (63). Hypothetical protein FupA

(FTT_0918) regulates both siderophore dependent and independent uptake of iron, and is a major virulence factor in *F. tularensis* (58, 79). The *fupA* gene is disrupted in *F. tularensis* LVS along with the adjacent gene encoding for hypothetical protein FupB (FTT_0919). This alteration of a possible surface protein is thought to be one of two mutations most responsible for *F. tularensis* LVS attenuation (79).

2.6 Type IV pili of *Francisella* species

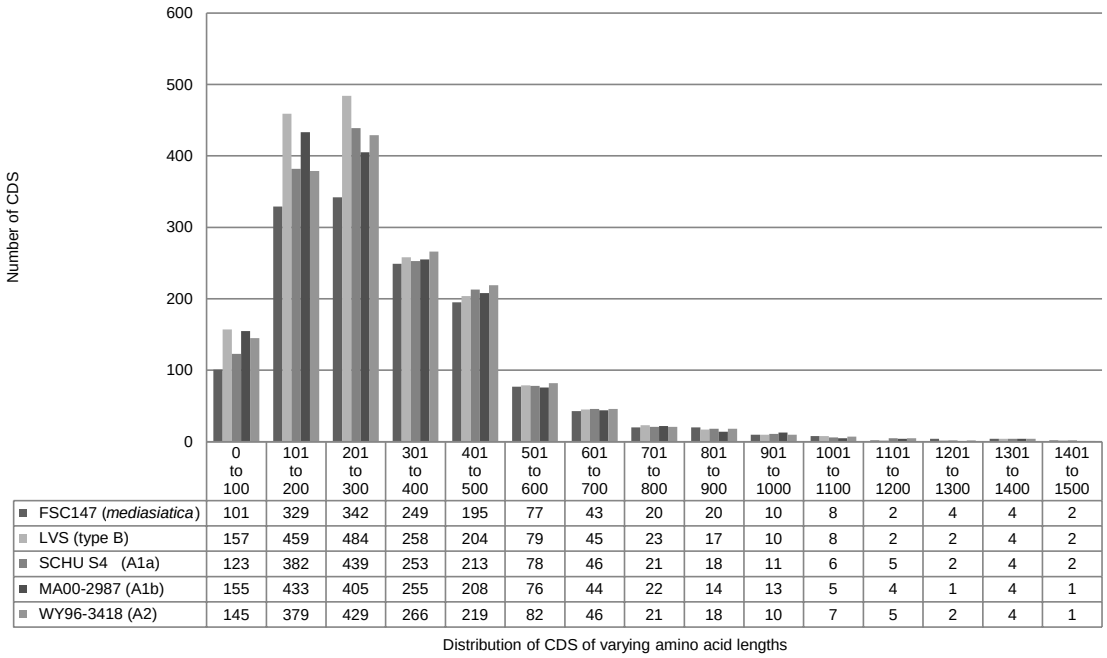
Type IV pili are filamentous fibers of protein observed on the surface of numerous pathogenic bacteria including enteropathogenic *E. coli*, *V. cholerae*, *P. aeruginosa*, and *Neisseria* species (17, 61). In these organisms Type IV pili are implicated in adhesion to host cells, biofilm formation, twitching motility, and natural transformation (1, 28, 40). Type IV pili or pili-like structures have been reported on the surface of *F. tularensis* LVS and *F. novicida* (18, 31, 102). The abundance, and potentially the structure, of *F. tularensis* LVS Type IV pili differ depending on the growth conditions used for cultivation (31). Bacteria grown on solid Mueller Hinton (MH) chocolate agar were shown to form short, think, horn-like structures (31). However, based on previous observations, it was speculated that these might be membrane protrusions (30, 31). The large protrusions were absent from bacteria grown in liquid media and were replaced by long thin fibers that frequently had polar localizations. *F. tularensis* growth in Chamberlains medium increased the proportion of cells producing Type IV pili compared to cells grown in MH broth. Fiber production was greatest after 16 hours of growth in both liquid media tested. *F. tularensis* LVS that is deficient of *pilF* (FTL_0828) or *pilT* (FTL_1770/FTL_1771, split gene) did not produce Type IV pili, but complementation of these genes to their respective knockouts restored surface fiber production (18). Similarly, Type IV pilus formation of *F. novicida* requires *pilF* and *pilT* (102). The gene encoding PilF is highly conserved in *F. tularensis* genomes (4, 8, 19,

51). In contrast *pilT* is split into two genes in most *F. tularensis* type B, but not *F. tularensis* type A strains (4, 8, 19, 51). Type IV pilus fibers have been observed microscopically when *F. novicida* PilA (FTN_0415) was produced in a pili negative *Neisseria gonorrhea* strain (78). However, there is no evidence to support that PilA itself is the major structural Type IV pilin subunit in *F. tularensis* (31). Complementation of *F. novicida pilA* in *N. gonorrhea* supported genetic transformation but did not allow for twitching motility (78). The *pilA* gene is conserved in all biotypes of *F. tularensis*, but is absent in the *F. tularensis* LVS strain (4, 8, 19, 51). *F. tularensis* lacking *pilA* is deficient in dissemination compared to wild-type strains via the subcutaneous route of infection (79).

2.7 Possible variations in the surface proteome of *Francisella* biotypes

The composition of the surface proteome of *F. tularensis* subspecies and populations is not only dependent on the repertoire of surface proteins encoded, but can be influenced by differences in non-surface proteins such as those involved in regulation or secretion. Although genetic comparisons of 1.1 million genomic nucleotide sites between 13 *F. tularensis* strains (two A1a, two A2, eight type B, and one subspecies *mediasiatica*) indicate that *F. tularensis* genomes are monomorphic, sharing >99.2% pair-wise average nucleotide identity, slight differences alter the coding capacity of *F. tularensis* biotypes. (50). Analyses of genome annotations obtained from the National Center for Biotechnology Information (NCBI) reference sequence database (RefSeq) using the pathosystems resource integration center database (PATRIC) identified variance in the number of protein coding sequences (CDS) between *F. tularensis* subspecies and populations (Fig. 2,2) (88). *F. tularensis* subspecies *mediasiatica* has the lowest coding capacity (1,406 CDS), followed by *F. tularensis* subspecies *tularensis* A1a (1,603 CDS), *F. tularensis* subspecies *tularensis* A2 (1,634 CDS), *F. tularensis*

subspecies *tularensis* A1b (1,640 CDS), and *F. tularensis* subspecies *holarctica* type B (1,704 CDS), respectively. The average length of proteins (in terms of the number of amino acids) also differs (Fig. 2.2). *F. tularensis* subspecies *mediasiatica* encodes on average the longest proteins and *F. tularensis* type B the shortest, 322 and 296 amino acids, respectively. The average length of *F. tularensis* A1a (310 amino acids) and A2 (309 amino acids) proteins is similar and *F. tularensis* A1b and type B proteins also have similar average amino acid lengths, 297 and 296, respectively. A comparison of the distributions of CDS of defined amino acid length identifies additional variance in subspecies and populations (88) (Fig. 2.2). In all *F. tularensis* subspecies and populations, the highest number of proteins range between 101-200 or 201-300 amino acids in length. *F. tularensis* A1a and A2 populations encode an average of 380 proteins with lengths of 101 to 200 amino acids and an average of 434 proteins with lengths of 201 to 300 amino acids. The number of proteins ranging from 101 to 200 amino acids are similar between *F. tularensis* A1b and type B, 433 and 459, respectively. *F. tularensis* A1b has fewer proteins (405) than *F. tularensis* type B (484) with lengths of 201 to 300 amino acids. *F. tularensis* subspecies *mediasiatica* has fewer proteins between 0-500 amino acids in length compared to *F. tularensis* type A and type B, but shares a similar number of proteins containing 501 amino acids or more.



	0 to 100	101 to 200	201 to 300	301 to 400	401 to 500	501 to 600	601 to 700	701 to 800	801 to 900	901 to 1000	1001 to 1100	1101 to 1200	1201 to 1300	1301 to 1400	1401 to 1500
FSC147 (*mediasiatica*)	101	329	342	249	195	77	43	20	20	10	8	2	4	4	2
LVS (type B)	157	459	484	258	204	79	45	23	17	10	8	2	2	4	2
SCHU S4 (A1a)	123	382	439	253	213	78	46	21	18	11	6	5	2	4	2
MA00-2987 (A1b)	155	433	405	255	208	76	44	22	14	13	5	4	1	4	1
WY96-3418 (A2)	145	379	429	266	219	82	46	21	18	10	7	5	2	4	1

Distribution of CDS of varying amino acid lengths

Fig. 2.2 Comparison of the distributions of *F. tularensis* CDS. The distributions of predicted CDS (RefSeq annotations), based on defined ranges of amino acid length, of *F. tularensis* subspecies and populations (A1a, A1b, A2, type B, and *mediasiatica*) are presented.

PATRIC analyses of *F. tularensis* genomes identified additional differences in CDS of twelve major classes of biosynthetic pathways that could alter surface protein composition including amino acid metabolism, biosynthesis of polyketides and nonribosomal peptides, biosynthesis of secondary metabolites, carbohydrate metabolism, energy metabolism, glycan biosynthesis and metabolism, lipid metabolism, metabolism of cofactors and vitamins, metabolism of other amino acids, nucleotide metabolism, translation, and xenobiotics biodegradation and metabolism (Table 2.2) (88). In each genome, the total number of CDS assigned to pathways differed, ranging from 1,336 in *F. tularensis* A1a to 1,383 in *F. tularensis* A1b. It should be noted that a

single protein can be assigned to a particular metabolic pathway more than once, and may also be assigned to more than one pathway.

Table 2.2 Metabolic pathways of *F. tularensis* subspecies and populations

Predicted pathway	SCHU S4 (A1a)	MA00-2987 (A1b)	WY96-3418 (A2)	LVS (type B)	FSC147 (*mediasiatica*)
Amino acid metabolism	210	226	204	215	219
Biosynthesis of polyketides and nonribosomal peptides	20	20	19	20	18
Biosynthesis of secondary metabolites	258	263	259	274	255
Carbohydrate metabolism	208	217	209	206	213
Energy metabolism	78	80	77	77	76
Glycan biosynthesis and metabolism	71	71	77	72	73
Lipid metabolism	85	82	85	81	83
Metabolism of cofactors and vitamins	126	130	123	123	124
Metabolism of other amino acids	44	44	45	42	46
Nucleotide metabolism	104	111	102	101	102
Translation	26	28	26	26	26
Xenobiotics biodegradation and metabolism	106	111	113	113	106

The number of CDS features assigned to particular physiological pathways of *F. tularensis* subspecies and populations (A1a, A1b, A2, type B, and *mediasiatica*). Pathway predictions were compiled from PATRIC using PATRIC CDS annotations.

Champion *et al*. prepared a comparative analysis of the predicted proteomes of *F. tularensis* type A and type B as well as *F. philomiragia* and *F. novicida*, and identified a number of genes present, absent, or disrupted between species and subspecies (19). Fourteen loci were uniquely found in *F. tularensis* type A and type B. Of these, seven are specific to the *F. tularensis* type A populations (hypothetical protein, FTT_0496; hypothetical protein, FTT_0677; adenosine deaminase, FTT_0939; hypothetical protein, FTT_1080; lipoprotein, FTT_1122; O-methyltransferase, FTT_1766; and hypothetical protein FTT_1791), one to *F. tularensis* A1 (hypothetical protein, FTT_1068), and one to *F. tularensis* type B (hypothetical protein, FTL_0776). The remaining five (hypothetical protein, FTT_0524; cold shock protein, FTT_1172; hypothetical protein, FTT_0755; hypothetical protein, FTT_1011; and hypothetical protein, FTT_1580) are shared by both *F. tularensis* subspecies, and are disrupted or absent in at least one of the less virulent

species (*F. novicida* and *F. philomiragia*). Eleven of these genes encode for products of unknown function, but three are predicted to be membrane proteins (FTT_1080, FTT_0755, and FTL_0776) and one a lipoprotein (FTT_1122). Sixty three genes are disrupted in *F. tularensis* type A or type B subspecies compared to *F. novicida*. Seven disrupted genes are conserved between both subspecies, 21 are specific to *F. tularensis* type A, and 35 specific to *F. tularensis* type B. Sixteen of these are predicted components of transport systems and 18 are annotated as hypothetical proteins and may have significant on the surface proteome. The majority of the remaining disrupted genes are predicted to be involved in amino acid or nucleic acid metabolism.

2.8 Differential production of *F. tularensis* surface proteins

Analyses of the *F. tularensis* proteome in response to various abiotic and biotic stresses identified differences in production of several likely surface proteins (45, 55, 56, 89, 96). As *F. tularensis* survives intracellulary in cells such as macrophages, multiple studies have evaluated the effects of reactive oxygen species on protein production (45, 55, 56, 96). Lenco *et al*. found 5 mM hydrogen peroxide added to liquid cultures of *F. tularensis* LVS and a *F. tularensis* LVS Δ*iglC* (FTL_0113/FTL_1159)_mutant had similar effects on both strains (56). Analyses by 2D-PAGE showed 19 spots were found to be more than 2-fold upregulated in the wild-type and mutant strains. A large number of these spots were chaperone proteins that were identified in membrane preparations, as secreted proteins, or surface exposed including HtpG (FTT_0356), GroES (FTT_1695), GroEL (FTT_1696), ClpB (FTT_1769), and Hsp (FTT_1794), This finding is consistent with reports of chaperone upregulation in response to oxidative pressure (27, 100). In contrast to a previous report, the surface protein IglC was not upregulated in *F. tularensis* LVS, a result later duplicated (33, 55). However, another study found IglC to be upregulated in *F. tularensis* type A strain FSC033 when grown in modified MH broth

supplemented with 5 mM hydrogen peroxide (96). Janovska *et al*. identified a single protein recognized by immune serum that appeared only after *F. tularensis* LVS was exposed to hydrogen peroxide (45). Two proteins were identified in the spot, FTT_1702 and the surface protein TUL4 (FTT_0901).

A study by Twine *et al*. evaluated the proteomic differences of *F. tularensis* grown *in vivo* by 2D-PAGE (96). BALB/c mice were infected i.d. with *F. tularensis* type A strain FSC033. Four days after infection, bacteria were isolated from the spleen using an immunomagnetic isolation protocol. *In vivo* regulation of proteins was then compared to bacteria grown in broth (also exposed to hydrogen peroxide). These analyses identified 78 differentially produced proteins. The abundance of 23 proteins increased while 32 decreased. Ten protein spots were unique to bacteria isolated *in vivo*, while 14 protein spots were specific to broth grown bacteria. The abundance of three proteins increased by more than 4-fold (family 18 chitinase ChiA, FTT_0715; peroxidase/catalase, FTT_0721; and hypothetical protein, FTT_1539), and all had bioinformatic signatures for surface associations and were identified in *F. tularensis* membrane fractions (44, 73).

DsbA acts as an oxidant for a dithiol in substrate proteins, and disulphide bond formation enables proper folding of the substrate protein that is often critical for its function (38). DsbA substrates are often surface associated and can include pili, adhesions, and components of secretion systems (38). Straskova *et al*. utilized 2D-PAGE to identify potential substrate proteins of the membrane associated thiol disulfide oxidoreductase (DsbA, FTL_1096) in *F. tularensis* LVS (89). Their approach centered on identifying proteins that accumulate in the membrane fraction by 2D-PAGE, given this is the subcellular localization most impacted by a DsbA knockout. As a secondary technique, the authors utilized quantitative MS analysis to validate their observations. Nine proteins were found to differentially accumulate in the mutant strain. Five of the

upregulated or unique proteins were annotated as hypothetical proteins (FTL_1579, FTL_1532,FTL_1306, FTL_0661, and FTL_0115). FTL_0115 is the *F. tularensis* LVS homolog of the Hcp protein associated with Type VI secretion (5). Two of the hypothetical proteins, FTL_1579 and FTL_1306, are important for *F. tularensis* survival in macrophages (48, 100). Secretion or production of hypothetical protein FTL_1579 differs between *F. tularensis* SCHU S4 and LVS. It is not secreted by *F. tularensis* LVS, indicating a possible difference in the respective surface proteomes (48). Two other proteins consistently accumulated in DsbA mutants, serine-type D-Ala-D-Ala carboxypeptidase (FTL_1060) and chitinase family 18 protein ChiA (FTL_1521). Two proteins containing signal peptides were less abundant in the membrane of the DsbA mutant, hypothetical protein (FTL_0694) and macrophage infectivity potentiator, fragment (FTL_1097).

Growth temperature is another factor that effects production of proteins likely associated with the surface of *F. tularensis* (34, 42). Temperature shifts from 37°C to 44°C caused the upregulation of surface associated (or likely surface associated) chaperones of *F. tularensis* LVS including GroEL (FTL_1714), GroES (FTL_1715), DnaK (FTL_1191), ClpB (FTL_0094) and HtpG (FTL_0267) (34). Numerous membrane proteins were also upregulated and had either predicted roles in transport or unknown functions. Several FPI genes, including *iglB* (FTL_1158), were downregulated in response to this temperature shift. Shifting growth from ambient temperature to 37°C had a similar effect on a number of chaperones and membrane proteins (42). One of the most highly upregulated proteins in response to this change was identified by Grall, *et al*. who determined a >10-fold increase in transcription of the gene encoding the homolog of the *F. novicida* secreted chitinase ChiB (FTN_1744) (34).

2.9 Research objectives

Surface proteins have documented roles in ecology, pathogenesis, immunity, and phenotypic variability of *Francisella* biotypes. Experimental evidence of membrane and secreted proteins, as well as bioinformatic signatures of surface proteins, led to the hypothesis that the number of *Francisella* surface associated proteins is much greater than what is currently identified. Therefore, a more comprehensive characterization of the *F. tularensis* surface proteome was a primary objective of the research in this dissertation. Comparisons of the identified surface proteins to those of known structure and function may reveal linked mechanisms important to the survival and virulence of *Francisella* biotypes. It is also likely that uncharacterized or novel types of surface proteins are present, and evaluation of these would not only further the study of *Francisella,* but provide insight into the physiological process of organisms that produce similar products. An additional hypothesis tested in this dissertation is that differences in previously unidentified surface proteins contribute to the phenotypic variability of *Francisella* species, subspecies, and populations. This hypothesis was supported by the biological consequences of known surface protein variations and altered coding capacities of *Francisella* biotypes (19, 62).

The identification of bacterial surface proteins has an added advantage that many are immunogens, and their presentation is required for the host to mount effective immune responses (84). Accordingly, the surface proteins naturally recognized during infection are often the most effective immunogens and serve as the basis for a number of subunit vaccines. Effective vaccinations with live attenuated strains of *F. tularensis* elicit a strong antibody response that recognizes a number of membrane proteins, and fractions containing these proteins can provide modest protection as a vaccine. Thus, it could be hypothesized that the protective antigens are likely surface associated. The determination of these antigens was an additional objective of this dissertation and is

significant given there is no approved prophylactic to *F. tularensis* infection because of issues of safety and efficacy with live attenuated vaccine strains.

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Chapter III

Expanded Characterization of the *F. tularensis* Surface Proteome

3.1 Introduction

Surface proteins are required for maintaining biological functions in bacteria and mediate interactions of pathogens with their hosts, vectors, and the environment (21). Despite the importance of this group of proteins, research to better understand the composition and activities of surface proteomes is limited because of numerous analytical challenges. The prediction of surface proteins using bioinformatic tools is often inaccurate, classifying known surface proteins as having intracellular or unknown localizations (10, 28, 30, 39). Many surface proteins are of low abundance, and although they may be enriched by subcellular fractionation, these procedures often contaminate the surface protein fraction (50). Insolubility and membrane associations are other characteristics adding to the difficulty of analyzing these proteins. Extraction of surface proteins from membranes can be achieved using organic solvents or detergents; however, these approaches also extract non-surface exposed membrane proteins and may adversely impact downstream analyses such as protein ionization for mass MS (4).

Chemical labeling of surface proteins with biotin offers a robust method to characterize bacterial surface proteomes, allowing for their specific labeling and enrichment (23). This approach has been applied to surface protein identification in Gram-negative and Gram-positive bacteria, and spirochetes (12, 33, 34). Additionally, cell surface biotinylation has proven effective in other intracellular Gram-

negative pathogens that share many commonalities, such as a small genome, a low % GC, and arthropod associations, with *F. tularensis*. In one such species, *Rickettsia parkeri*, specific biotinylation facilitated the identification of over 90 surface proteins (34, 49).

Despite having a larger coding capacity compared to a similar bacterium like *R. parkeri* (1.9 Mbp compared to an estimated 1.3 Mbp), only 10 proteins have been reported on the surface of *Francisella* species (22, 25, 26, 36). FopA (FTT_0583) and TUL4 (FTT_0901) were the first *F. tularensis* proteins described as surface exposed, but their classifications were based solely on membrane localization (26, 36). The surface association of FopA was confirmed by Melillo *et al*. utilizing chemical labeling with biotin, affinity chromatography, and protein identification by MS (25). Melillo *et al*. identified six additional surface proteins on *F. tularensis* LVS (DnaK, FTT_1269; GroEL, FTT_1696; hypothetical protein FsaP, FTT_0119; IglA, FTT_1359; hypothetical protein, FTT_1347; and hypothetical protein Bfr, FTT_1441), but did not detect TUL4. A similar study by Ludu *et al*. identified two *F. novicida* surface proteins, IglB (FTN_1323) and IglC (FTN_1322), that participate in Type VI secretion (1, 22).

The analysis by Melillo *et al*. of the enriched surface protein fraction identified only the most abundant proteins in the sample (25). Visualization of the total protein content of this sample using SDS-PAGE clearly showed that many more protein bands were present, that were not subject to further characterization (25). Similarly, the work of Ludu *et al*. only targeted specific proteins for identification (22). Based on these observations, and the much larger number of surface proteins in similar Gram-negative bacteria, it was hypothesized that the majority of *F. tularensis* surface proteins are unidentified. To test this hypothesis, we built upon the methods of Melillo *et al*., utilizing a similar surface labeling and purification methodology, but extended the scope of

protein identification with the intent of identifying the majority of proteins enriched after chemical labeling. These analyses identified 36 proteins on the surface of *F. tularensis* LVS, 28 of which are newly described to this localization. Further, due to the phenotypic variations documented for *F. tularensis* subspecies and populations, and the unknown relationships of these differences in regard to the surface proteome, experiments were performed to determine if surface protein labeling could be extended to other *F. tularensis* biotypes. Specific labeling of surface proteins was achieved for a *F. tularensis* type B clinical isolate and *F. tularensis* A1a, A1b, and A2 populations.

3.2 Materials and methods

3.2.1 Strains and culture conditions

F. tularensis strains (LVS, type B; KY99-3387, type B; SCHU S4, A1a; MA00-2987, A1b; WY96-3418, A2) were kindly provided by Dr. Jeannine Petersen (CDC, Fort Collins, CO). For identification of surface proteins, *F. tularensis* strains were grown on Cysteine Heart agar supplemented with 9% heated sheep blood (CHAB) for 44 to 52 h at 37°C. Cells were collected by scraping and then suspended in phosphate buffered saline, pH 7.4 (PBS), for surface labeling. To generate sufficient quantities of *F. tularensis* LVS to prepare a membrane protein fraction (MPF), *F. tularensis* LVS was inoculated directly from frozen stocks into MH broth supplemented with 1% Isovitalex (BD Biosciences, San Jose, CA) and incubated at 37°C on a platform shaker for 30 h. Subcultures were inoculated to fresh MH broth supplemented with 1% Isovitalex and harvested after 20 h of growth. Cells were collected by centrifugation at 4,500 × g and washed in PBS, pelleted and stored at -80°C.

3.2.2 Surface protein labeling and label localization

Suspensions of *F. tularensis* strains were collected by centrifugation at 4,500 × g, washed three times in PBS, and adjusted to an OD_{600} of 0.15-0.19 AU in PBS. An aliquot (80 µl) at 6.6 mg/ml of EZ-Link Sulfo-NHS-LC-Biotin (LC-Biotin) (Thermo/Pierce, Rockford, IL) was added for every 1 ml of the cell suspension. Labeling of proteins was conducted at room temperature for 1 h with gentle rocking. Labeled cells were collected as described above, washed once in Tris buffered saline, pH 7.4 (TBS), and twice in PBS. Biotin labeled cells were suspended in SDS-PAGE sample buffer (0.3 M Tris, 50% glycerol, 10% SDS, 25% β-mercaptoethanol, and trace bromophenol blue) for analysis by SDS-PAGE, or 1% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS) buffer (1% CHAPS in PBS) for affinity purification of labeled proteins.

F. tularensis LVS lysates from cells grown on CHAB, were prepared by suspending cells in breaking buffer (PBS, 1.2 µg/ml DNase I, 1.2 µg/ml RNase A, 1 µg/ml lysozyme), and one Complete EDTA-free protease inhibitor cocktail tablet (Roche Applied Science, Mannheim, Germany) per 50 ml buffer. Cell suspensions were lysed by nine repetitions of pulsed sonication using a 4710 series Ultrasonic Homogenizer (Cole and Palmer Instrument Company, Vernon Hills, IL) employing the following instrument parameters: % duty of 50, output of 5, 60 seconds pulsed, 60 seconds off. Unbroken cells and debris were removed from the lysate by centrifugation at 4,500 × g, 4°C, for 20 min. Lysates were labeled with LC-Biotin as described above.

3.2.3 Biotinylated protein purification

Surface labeled proteins were extracted for affinity purification by boiling cells suspended in CHAPS buffer for 15 min at 120°C with vigorous intermittent vortexing.

Extracts were added to immobilized streptavidin resin (Thermo/Pierce) pre-equilibrated in PBS and incubated overnight at 4°C with gentle shaking. Ten resin volumes of CHAPS buffer, followed by 10 resin volumes of PBS was used to wash the resin. Biotinylated proteins were eluted by boiling the resin in SDS-PAGE sample buffer for 10 min at 120°C.

3.2.4 Isolation of MPF

F. tularensis LVS cell pellets were thawed and suspended in breaking buffer to a final concentration of 1 g cells (wet weight) per 1 ml of buffer. Cells were placed on ice and lysed by sonication as described above. Unbroken cells were removed from the lysate by centrifugation at 4,500 × g at 4°C for 20 min. MPF was obtained by centrifugation of cell lysates at 100,000 × g for 4 h. Supernatants were removed; pellets were suspended in breaking buffer and dialyzed against 10 mM ammonium bicarbonate buffer using 3,500 Da molecular weight cut-off dialysis membranes (Spectrum Laboratories Incorporated, Rancho Dominguez, CA). MPF protein concentration was determined by the bicinchoninc assay (BCA) (Thermo/Pierce) (41).

3.2.5 SDS-PAGE

Aliquots of labeled proteins were applied to SDS-PAGE using 4-12% Bis-Tris SDS-Polyacrylamide gels (Invitrogen, Carlsbad, CA) under denaturing conditions (19). Detection and destaining of protein in polyacrylamide gels was accomplished by staining with Simply Blue (Invitrogen) according to the protocols of the manufacturer. Gel images were digitized using a HP Scanjet 4850 photo scanner (Hewlett-Packard Company, Palo Alto, CA).

3.2.6 Western blotting

Biotinylated surface proteins resolved by SDS-PAGE were transferred to nitrocellulose membranes by Western blot as described previously (42). Membranes were incubated in TBS containing 0.1% Tween 20 and 5% nonfat milk and were washed in TBS containing 0.1% Tween 20 (TBST). Nitrocellulose membranes were incubated in horse radish peroxidase (HRP) conjugated anti-biotin antibody (1:1,000) (Cell Signaling Technology, Danvers MA). Following incubation, membranes were again washed in TBST before being incubated with LumiGLO (Cell Signaling Technology). Chemiluminescence signals were visualized using CL-Exposure film (Thermo/Pierce). Western blot images were digitized using a GS-710 calibrated imaging densitometer (Bio-Rad, Hercules, CA).

3.2.7 Computer aided image analysis

Digitized images of anti-biotin Western blots were analyzed with ImageQuant TL software (GE Health Care Life Sciences, Piscataway, NJ), and the number of reactive protein bands in each sample determined based on densometric parameters.

3.2.8 Fractionation of MPF

An aliquot (400 µg) of MPF suspended in 50 µl of 0.2 M ammonium bicarbonate was digested with 10 µg sequencing grade trypsin (modified) (Roche Applied Science). MPF suspended with trypsin was incubated at 37°C for 4 h, followed by overnight digestion with an additional 10 µg of trypsin. Samples were dried under vacuum and reconstituted in HPLC grade H_2O. MPF was dried and reconstituted in H_2O an additional two times before suspension in 120 µl of 3% acetonitrile (ACN) containing

0.1% acetic acid. Insoluble material was removed from the sample by centrifugation at 16,000 × g for 10 min.

Peptides were separated by strong cation exchange chromatography using an Alliance 2695 separations model HPLC (Waters, Milford, MA) coupled with a PolyLC Polysulfoethyl A column (4.6 mm x 200 mm) (The Nest Group, Inc., Southborough, MA). Peptides were eluted with increasing gradient of potassium chloride (KCl) by mixing 10 mM potassium dihydrogen phosphate (KH_2PO_4), 25% ACN with 10 mM KH_2PO_4, 25% ACN, and 500 mM KCl. A fraction was collected every minute at a flow rate of 1 ml/min. Fractions were dried under vacuum and reconstituted in 100 µl of 0.1% trifluoroacetic acid, and desalted using OMIX C18 tips (Varian, Palo Alto, CA). Desalted samples were dried under vacuum and suspended in 11 µl of 3% ACN, 0.1% formic acid. Samples were sonicated for 5 min followed by centrifugation for 10 min at 16,000 × g. Each sample was transferred to clean autosampler vials in preparation for LC-MS/MS analyses.

3.2.9 Protein identification by MS

Affinity purified proteins were subjected to in-gel proteolytic digestion with trypsin or chymotrypsin in 0.2 M ammonium bicarbonate buffer at 25°C or 37°C, respectively. Peptides from enzymatic digestion were applied to a capillary C18 reversed phase column (0.74 x 50 mm) (Agilent Technologies, Santa Clara, CA) and eluted with an increasing linear gradient of ACN in 0.1% formic acid. Effluent was introduced directly into a ThermoFinnigan LTQ electrospray mass spectrometer (Thermo Scientific, San Jose, CA) or an Agilent 6520 quadrapole-time of flight mass spectrometer (Agilent Technologies).

Identification of affinity purified surface proteins was achieved using Sequest (Ver. 27, rev. 12), X tandem (Ver. 2006.04.01.2), and MASCOT (Matrix Science Ltd,

www.matrixscience.com) softwares. Scaffold version 2_01_02 software (Proteome Software Incorporated, Portland, OR) was used to validate peptide and protein identifications. Peptide identities were accepted only when a peptide identification probability of >95% was obtained as specified by the peptide prophet algorithm (16). Protein identifications were accepted if protein identification probabilities were >99%, as assigned by the protein prophet algorithm, and contained at least two unique peptides (27). Oxidation of methionine and the addition of LC-Biotin to lysine were considered for search analyses.

3.2.10 Bioinformatic analyses

Numerous bioinformatic approaches were employed to predict the subcellular localization of *F. tularensis* (accession number: NC_007880) proteins. Subcellular protein localizations were predicted with PSORTb (Ver. 3.0.0) (http://www.psort.org/psortb/index.html). Classical signal peptides were detected with SignalP (Ver. 3.0) (http://www.cbs.dtu.dk/services/SignalP/), and signal peptides and lipoproteins were predicted with LipoP (Ver. 1.0) (http://www.cbs.dtu.dk/services/LipoP/). Non-classically secreted proteins (those lacking N-terminal signal peptides) were predicted with the SecretomeP (Ver. 2.0) (http://www.cbs.dtu.dk/services/SecretomeP/) software.

3.3 Results

3.3.1 Bioinformatic predictions of *F. tularensis* surface proteins

In silico analyses of *F. tularensis* subspecies and populations (type B, A1a, A1b, and A2) were conducted to identify signatures of surface exposed proteins. SignalP software indicated an average of 289 proteins are translocated across the cytoplasmic membrane between all biotypes (Table 3.1). Further, analyses using the LipoP software

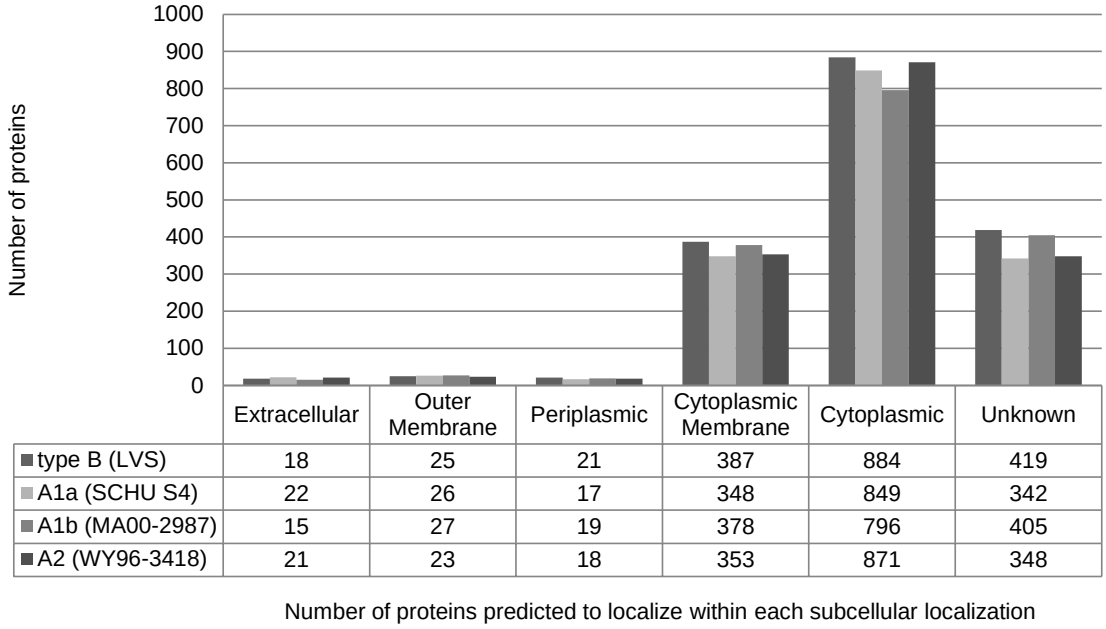
predicted between 52 to 57 proteins are processed by signal peptidase II, indicating a similar number of lipoproteins encoded in each *F. tularensis* subtype (Table 3.1).

Table 3.1 Comparative analysis of *F. tularensis* signal sequences

Strain	SignalP	LipoP (SpII)
LVS (type B)	294	52
SCHU S4 (A1a)	288	56
MA00-2987 (A1b)	284	57
WY96-3418 (A2)	291	55

The number of proteins predicted by SignalP v. 3.0 and LipoP v 1.0 softwares to contain signal peptides (SignalP) or be processed by signal peptidase II (LipoP). Analyses were performed using *F. tularensis* subspecies and populations (A1a, A1b, A2, type B) CDS annotated by RefSeq.

PSORTb was used to identify the outer membrane and extracellular proteins of *F. tularensis* subspecies and populations (Fig. 3.1). The number of proteins predicted slightly differed between each *F. tularensis* biotype at each location. *F. tularensis* type A1b encoded for the highest number of predicted outer membrane proteins, and *F. tularensis* A1a the most extracellular proteins. Based on these *in silico* analyses, *F. tularensis* subspecies and populations should possess between 42 and 48 potential surface (outer membrane and extracellular) proteins. Given that surface proteins are frequently assigned to other subcellular localizations [cytoplasmic, cytoplasmic membrane (CM), periplasmic, and unknown] by PSORTb, and surface protein composition can be influenced by intracellular processes, analyses were extended to identify proteomic differences between *F. tularensis* biotypes at other subcellular localizations (Fig 3.1). The total number of proteins predicted within the additional subcellular localizations were similar for all *F. tularensis* subspecies and populations, but *F. tularensis* type B was predicted to encode slightly more cytoplasmic, CM, periplasmic, and unknown localization proteins than *F. tularensis* A1a, A1b, and A2.



	Extracellular	Outer Membrane	Periplasmic	Cytoplasmic Membrane	Cytoplasmic	Unknown
type B (LVS)	18	25	21	387	884	419
A1a (SCHU S4)	22	26	17	348	849	342
A1b (MA00-2987)	15	27	19	378	796	405
A2 (WY96-3418)	21	23	18	353	871	348

Number of proteins predicted to localize within each subcellular localization

Fig. 3.1 Predicted subcellular localization of *F. tularensis* (type B, A1a, A1b, and A2) proteins. PSORTb v. 3.0 software was used to predict the subcellular localizations of *F. tularensis* proteins. The strain archetype used for the analyses is indicated in parentheses.

3.3.2 Biotinylation of surface exposed proteins of *F. tularensis* LVS

A membrane impermeable biotin label, LC-Biotin (35), was employed to characterize the surface proteome of *F. tularensis* LVS. Initial experiments to optimize surface labeling were performed with LC-Biotin concentrations in a range between 6.6 and 26.4 mg/ml, and incubation times of 10, 30, 60, and 120 min. This established that treatment of freshly harvested *F. tularensis* with 6.6 mg/ml LC-Biotin for 60 min provided optimal labeling (data not shown). After surface labeling of *F. tularensis* LVS, the proteins were extracted from the cells and detected by Western blot using a HRP-conjugated anti-biotin antibody as the probe (Fig. 3.2). This resulted in the detection of

17 distinct bands using the ImageQuant TL software. In contrast, Western blot analyses of an equivalent amount of *F. tularensis* LVS whole cell lysate (WCL) labeled with an equal amount of biotin resulted in a more complex banding pattern. Additionally, compared to surface labeled cells, the signature of biotinylation was more rapidly detected in WCL by Western blot, and exposure times of the X-ray film were reduced to 15 sec in contrast to the two min exposure of the surface labeled samples (Fig 3.2).

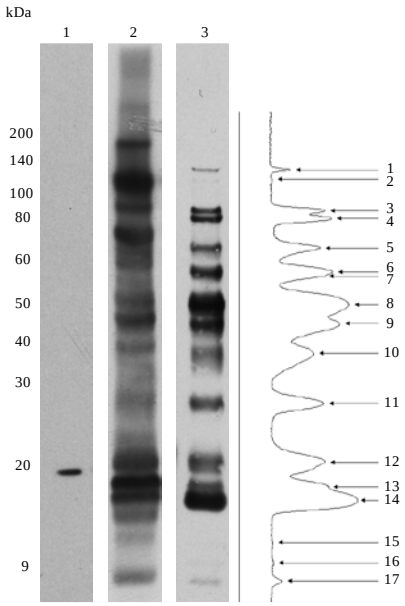


Fig. 3.2 Biotinylation of *F. tularensis* LVS surface exposed proteins. Anti-biotin Western blots were performed to detect biotinylated proteins after labeling *F. tularensis* LVS with LC-Biotin. Lane 1, WCL of *F. tularensis* LVS unlabeled control; Lane 2, labeled *F. tularensis* LVS WCL (15 sec exposure); and Lane 3, labeled *F. tularensis* LVS intact cells (2 min exposure) accompanied by densitometry analysis of reactive protein bands.

3.3.3 Surface protein purification

Identification of surface proteins was facilitated by purification of the biotin labeled products with immobilized streptavidin affinity chromatography. Anti-biotin

Western blot analysis of the purified material demonstrated the enrichment of several biotin labeled proteins and the presence of 23 anti-biotin reactive bands (Fig. 3.3). An aliquot of affinity purified material was resolved by SDS-PAGE, stained with Simply Blue, and the gel cut into nine slices based on molecular mass (Fig. 3.3). Gel slices were subjected to digestion with trypsin and chymotrypsin, and the resulting peptides analyzed by LC-MS/MS leading to the identification of 36 proteins (Tables 3.2 and 3.3).

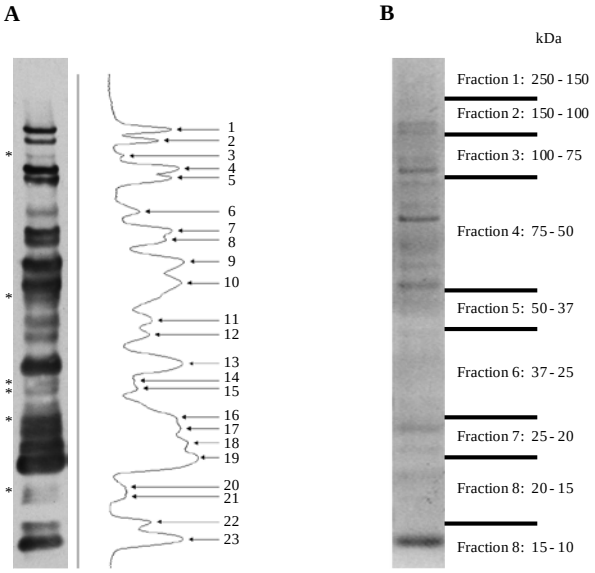


Fig. 3.3 Purified *F. tularensis* LVS LC-Biotin labeled surface proteins. Biotinylated surface proteins purified using immobilized streptavidin were separated by SDS-PAGE and analyzed by anti-biotin Western blot. (A) Anti-biotin Western blot of purified biotinylated surface proteins accompanied by densitometry analysis of reactive protein bands. (B) Simply Blue stained gel of affinity purified material. Gel fractions excised for LC-MS/MS analysis are denoted on the right by the molecular mass range. *indicates reactive protein bands only visualized after affinity purification.

Table 3.2 Identification and bioinformatic analyses of *F. tularensis* LVS surface proteins

Gene locus	Protein name	Signal Peptide[A]	PSORTb localization (score)[B]	Identified in MPF	Previously identified as membrane or surface protein and ref
FTL_0009	Outer membrane protein	SpI	Peri (9.84)	Yes	Yes (14, 31, 32)
FTL_0111	Intracellular growth locus, subunit A	NC (SP)	Un	Yes	*Yes (14)
FTL_0112	Intracellular growth locus, subunit B	-	Un	Yes	*Yes (22, 47)
FTL_0113	Intracellular growth locus, subunit C	-	Un	Yes	*Yes (9, 14, 22, 32)
FTL_0234	Elongation factor G	-	Un	Yes	No
FTL_0267	Heat shock protein 90	-	Cyto (9.26)	Yes	Yes (14)
FTL_0269	Glutamate dehydrogenase	-	OM (9.92)	Yes	Yes (32)
FTL_0325	OmpA family protein	SpII	Un	Yes	Yes (13, 14, 31, 45)
FTL_0336	Peptidoglycan-associated lipoprotein	SpII	OM (10.00)	Yes	Yes (6, 13, 31, 45)
FTL_0387	Aspartate aminotransferase	-	Un	Yes	No
FTL_0421	Lipoprotein	SpII	OM (10.00)	Yes	*Yes (6, 11, 13, 14, 31, 40)
FTL_0569	Hypothetical protein	SpI	Un	Yes	Yes (31)
FTL_0572	Hypothetical protein	SpI	Un	Yes	Yes (14, 32)
FTL_0617	Hypothetical protein	-	Un	Yes	*Yes (14, 25, 32, 47)
FTL_1015	AhpC/TSA family protein	NC (SP)	Un	Yes	Yes (14)
FTL_1026	50S ribosomal protein L9	-	Cyto (9.26)	Yes	No
FTL_1096	Lipoprotein	SpII	Un	Yes	Yes (13, 31, 32, 44, 45, 47)
FTL_1146	Glyceraldehyde-3-phosphate dehydrogenase	-	Cyto (9.26)	No	No
FTL_1191	Chaperone protein dnaK	NC (SP)	Cyto (9.26)	Yes	*Yes (13, 14, 24, 25, 47)
FTL_1225	Hypothetical protein	SpII	Un	No	No
FTL_1328	Outer membrane associated protein	SpI	OM (9.93)	Yes	*Yes (6-8, 13, 14, 25, 26, 45, 47)
FTL_1494	Hypothetical protein	SpI	Un	No	No
FTL_1504	Peroxidase/catalase	NC (SP)	Cyto (9.26)	Yes	Yes (13, 14, 31)
FTL_1521	Chitinase family 18 protein	SpI	Un	Yes[C]	Yes (13, 44, 47)
FTL_1527	Enolase (2-phosphoglycerate dehydratase)	-	Cyto (9.26)	Yes	No
FTL_1553	Succinyl-CoA synthetase subunit beta	-	Un	Yes	No
FTL_1579	Hypothetical protein	SpI	Un	No	Yes (44)
FTL_1592	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit	-	Un	Yes	Yes (14, 31)
FTL_1714	Chaperonin GroEL	-	Cyto (9.26)	Yes	*Yes (11, 13, 14, 25, 47)
FTL_1745	50S ribosomal protein L7/L12	-	Un	Yes	Yes (14)
FTL_1751	Elongation factor Tu	-	Cyto (9.26)	Yes	Yes (14)
FTL_1772	Aconitate hydratase	-	Cyto (9.26)	Yes	Yes (14, 32, 47)
FTL_1786	Succinate dehydrogenase, catalytic and NAD/flavoprotein subunit	-	CM (9.82)	Yes	Yes (14)
FTL_1789	Citrate synthase	-	Cyto (9.26)	No	No
FTL_1907	Cell division protein FtsZ	-	Un	Yes	Yes (14)
FTL_1912	30S ribosomal protein S1	-	Cyto (9.26)	Yes	Yes (47)

[A]Signal peptide prediction based on analyses by SignalP (Ver. 3.0), LipoP (Ver. 1.0), and SecretomeP (Ver. 2.0).
[B]Subcellular location and scores were obtained with PSORTb (Ver. 3.0.0).
[C]Indicates protein was found in MPF only by LC-MS/MS analysis of a protein spot.
*Indicates that the protein was previously characterized as surface exposed.
SpI, signal peptide cleaved by signal peptidase I; SpII, signal peptide cleaved by signal peptidase II; NC (SP), non-classical sec-translocation signal peptide; Peri, periplasmic; Cyto, cytosol; OM, outer membrane; CM, cytoplasmic membrane.

Table 3.3 MS/MS validation of proteins identified as surface exposed

Protein name	Locus	[A]Fraction	Predicted molecular mass	[B]Protein identification probability	Number of unique peptides	Percentage sequence coverage
50S ribosomal protein L7/L12	FTL_1745	10-15 kDa	12847.82	99.80%	2	22%
Lipoprotein	FTL_0421	15-20 kDa	15771.85	100.00%	3	22%
Hypothetical protein	FTL_0617	15-20 kDa	16809.36	100.00%	5	45%
50S ribosomal protein L9	FTL_1026	15-20 kDa	16087.49	99.80%	2	19%
Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit	FTL_1592	20-25 and 15-20 kDa	16403.98	100.00%	6	59%
Outer membrane protein	FTL_0009	20-25 kDa	19477.11	100.00%	5	34%
Intracellular growth locus, subunit A	FTL_0111	20-25 kDa	20890.88	99.80%	2	14%
Intracellular growth locus, subunit C	FTL_0113	20-25 kDa	22133.41	99.80%	2	13%
Peptidoglycan-associated lipoprotein	FTL_0336	20-25 kDa	23291.32	99.80%	2	11%
Hypothetical protein	FTL_0569	20-25 kDa	19785.57	99.80%	2	24%
AhpC/TSA family protein	FTL_1015	20-25 kDa	19668.22	100.00%	3	28%
Hypothetical protein	FTL_1494	20-25 kDa	18158.27	99.80%	2	18%
Hypothetical protein	FTL_1225	25-37 kDa	25699.94	99.80%	2	11%
Hypothetical protein	FTL_1579	25-37 kDa	25702.85	99.80%	2	11%
OmpA family protein	FTL_0325	37-50 kDa	46754.18	99.80%	2	6%
Aspartate aminotransferase	FTL_0387	37-50 kDa	44382.87	100.00%	4	14%
Glyceraldehyde-3-phosphate dehydrogenase	FTL_1146	37-50 kDa	35413.40	99.80%	2	8%
Outer membrane associated protein	FTL_1328	37-50 kDa	41259.65	100.00%	5	12%
Succinyl-CoA synthetase subunit beta	FTL_1553	37-50 kDa	41541.74	100.00%	4	17%
Citrate synthase	FTL_1789	37-50 kDa	46794.56	100.00%	3	10%
Cell division protein FtsZ	FTL_1907	37-50 kDa	39745.31	100.00%	4	15%
Elongation factor Tu	FTL_1751	50-75 and 37-50 kDa	43390.62	100.00%	7	31%
Intracellular growth locus, subunit B	FTL_0112	50-75 kDa	57917.61	100.00%	7	17%
Glutamate dehydrogenase	FTL_0269	50-75 kDa	49158.07	100.00%	3	11%
Hypothetical protein	FTL_0572	50-75 kDa	51977.34	100.00%	3	7%
Lipoprotein	FTL_1096	50-75 kDa	39545.19	100.00%	4	18%
Enolase (2-phosphoglycerate dehydratase)	FTL_1527	50-75 kDa	49511.19	99.80%	2	8%
Chaperonin GroEL	FTL_1714	50-75 kDa	57402.70	100.00%	8	20%
Succinate dehydrogenase, catalytic and NAD/flavoprotein subunit	FTL_1786	50-75 kDa	65860.61	99.90%	2	5%
30S ribosomal protein S1	FTL_1912	50-75 kDa	61669.35	100.00%	5	12%
Elongation factor G	FTL_0234	75-100 kDa	77730.37	100.00%	6	13%
Heat shock protein 90	FTL_0267	75-100 kDa	72371.65	100.00%	7	13%
Chaperone protein dnaK	FTL_1191	75-100 kDa	69182.22	100.00%	5	10%
Peroxidase/catalase	FTL_1504	75-100 kDa	81226.95	100.00%	3	6%
Chitinase family 18 protein	FTL_1521	75-100 kDa	83595.21	100.00%	3	5%
Aconitate hydratase	FTL_1772	100-150 kDa	102703.80	99.80%	2	3%

[A]Fraction indicates the SDS-PAGE based molecular mass fraction in which the respective surface protein was identified (Fig 3.3).
[B]Protein identification probabilities were calculated using Scaffold version 2_01_02 software.

A positive correlation was observed between the calculated molecular mass of each surface protein identified and the expected molecular mass based on SDS-PAGE migration (Table 3.3). Of the proteins identified, 27 were previously shown to be membrane associated or surface exposed (Table 3.2). Bioinformatic analyses revealed that 16 of the 36 proteins contained predicted signal peptides and four were defined as non-classical secreted proteins (Table 3.2). Further evaluation of the 36 surface proteins with PSORTb did not predict a subcellular location for nine of the 19 proteins not identified as secreted or translocated. An additional nine of these 19 proteins were predicted to be localized to the cytosol, and one (FTL_0269) was predicted as an outer membrane protein. The identification of non-membrane or non-secreted proteins was not unexpected since bioinformatic prediction and experimental detection of presumably cytosolic proteins in bacterial surface and membrane preparations is not uncommon (10, 30, 39).

3.3.4 Proteins identified in MPF

To evaluate the association of identified surface proteins to proteins contained within the *F. tularensis* LVS membrane, the MPF was digested with trypsin followed by 2D-LC, MS/MS, and protein identification. This resulted in the identification of 278 proteins with a high degree of confidence (Appendix 5). Of the 36 proteins identified by surface biotin labeling, 31 were detected in the MPF, indicating they were in high enough concentrations to be identified without further enrichment (Table 3.2). The validity of MPF protein identifications were supported by the identification of numerous identical proteins in independently prepared membrane fractions. Over 55% of previously identified membrane proteins were identified in MPF (Appendix 4) (11, 14, 15, 31, 32, 44, 45).

3.3.5 Surface proteomes of *F. tularensis* subspecies and populations

The genetic differences that exist between *F. tularensis* subspecies and populations could alter the surface proteome and contribute to the documented phenotypic variability of *F. tularensis* biotypes (Appendix 6). Thus the application of surface protein labeling was evaluated in other *F. tularensis* subspecies and populations (type B, A1a, A1b, and A2). Following surface labeling, the proteins were extracted from the cells and detected by anti-biotin Western blot (Fig. 3.4). The repertoire of protein bands observed by this analysis was highly similar between all strains, but differences were noted in the apparent abundance or labeling efficiencies of surface proteins. *F. tularensis* A2 had a consistently weaker reactive profile compared to *F. tularensis* type A1 and type B (Fig. 3.4).

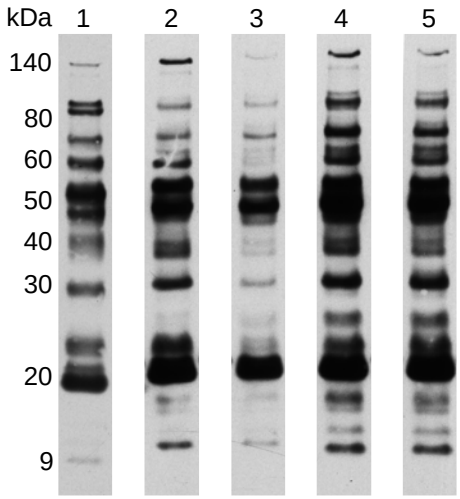


Fig. 3.4 Surface proteins of *F. tularensis* subspecies and populations. Anti-biotin Western blot of surface labeled *F. tularensis* lysates. Lane 1, *F. tularensis* LVS (type B); Lane 2, *F. tularensis* KY99-3387 (type B); Lane 3, *F. tularensis* WY96-3418 (A2); Lane 4, *F. tularensis* SCHU S4 (A1a); Lane 5, *F. tularensis* MA00-2987 (A1b). Unlabeled controls of each lysate were evaluated and a single reactive band that resolved at 19 kDa was observed for each *F. tularensis* subspecies and population.

3.4 Discussion

Prior to this work only 10 proteins were identified on the surface of *F. tularensis* or *F. novicida* (22, 25, 26, 36). However, experimental characterization of surface proteomes of other Gram-negative pathogens, and bioinformatics predictions, suggested many more proteins exist on the surface of *F. tularensis*. The methodology employed here of protein labeling with membrane impermeable *N*-hydroxysuccinimide esters of biotin identified over five times the number of surface proteins previously described in the most complete surface proteome characterization of *F. tularensis* (25). A total of 36 surface exposed proteins were identified in this study, and of these, 28 were newly established *Francisella* surface proteins. A strong correlation was observed between the newly identified surface proteins and studies that characterized *F. tularensis* proteins that could be surface localized. Specifically, 19 of the surface proteins identified here were also observed in membrane fractions (Table 3.1). Bioinformatic analyses provided additional support that the proteins identified were surface exposed. Seventeen of the 36 surface proteins were predicted to be secreted or membrane associated.

Bioinformatic approaches failed to predict a subcellular location for nine of the surface proteins, while another 10 were predicted to localize to the cytosol. The experimental identification of known or predicted cytosolic proteins in bacterial membranes and surface subcellular fractions is not uncommon. There is a growing list of presumed intracellular proteins identified on the surface of pathogenic bacteria that appear to be multifunctional based on their subcellular location (29). We identified glycolytic enzymes, 2-phosphoglycerate dehydratase (FTL_1527) and glyceraldehyde 3-phosphate dehydrogenase (FTL_1146), as being surface exposed. This is consistent with findings in both Gram-negative and Gram-positive pathogens where these proteins were demonstrated to participate in bacterial adhesion and were identified on cell surfaces despite lacking signal peptides (30, 39). Likewise, elongation factor Tu

(FTL_1751) was observed as surface associated, and homologues of this gene product in *Lactobacillus, Mycoplasma,* and *Pseudomonas* species are described as surface associated and act as adhesions and plasminogen ligands (10, 18). The bacterial GroEL and DnaK chaperones or stress response proteins are also known to interact with the innate immune response and enhance antigen presentation (43). Although they are cytosolic functioning chaperones, they have been noted as surface exposed in other bacteria (37). Thus, the surface presentation of GroEL (FTL_1714) and DnaK (FTL_1191) in *F. tularensis* was not unexpected. Recently, a study by Koneca *et al*. identified 22 predicted cytosolic proteins that were expelled into the CS by *F. tularensis* LVS and SCHU S4 (17). The authors ruled out the possibility that cell lysis contributes to the pool of culture filtrate proteins. Of the predicted cytosolic proteins identified, four were found in the current studies on the surface of *F. tularensis* LVS including GroEL (FTL_1714), glutamate dehydrogenase (FTL_0269), glyceraldehyde-3-phosphate dehydrogenase (FTL_1146), and hypothetical protein (FTL_0617).

The mechanism that allows for cytosolic proteins to reach the bacterial surface is not clear, but likely involves bacterial secretion. Thus far, four translocation and secretion systems are described or predicted to be functional in *F. tularensis* (Sec, Type I secretion, Type II secretion, and Type VI secretion) (5). While the less well characterized Type VI secretion could be hypothesized for the surface localization of predicted cytosolic proteins, the emerging hypothesis that non-traditional conserved signal peptide like motifs on selected cytosolic proteins allow for Sec mediated translocation is another explanation for the surface presence of cytosolic proteins that have similar localization profiles across bacterial species (3).

Another possibility to explain the identification of predicted cytosolic proteins as surface structures is that the *N*-hydroxysuccinimide biotin labeling reagent was able to cross both the outer and inner membranes and label the most abundant cytosolic

proteins. However, the protein profile obtained from cell surface labeling was markedly different from that obtained by *N*-hydroxysuccinimide biotin labeling of a WCL. Alternatively, cytosolic contaminants may have been co-purified with biotinylated surface proteins. This possibility could have been explored further by increasing salt gradients during washing to reduce non-specific interactions and monitor contaminants, or by identifying the covalently linked biotin tag on specific peptides. Attempts to perform the latter were largely unsuccessful, with only a few biotinylated peptides detected by MS (data not shown). However, detection was only performed on biotinylated surface peptides obtained after in-gel proteolytic digestion. It is possible that this procedure could be optimized by digesting biotinylated surface proteins in-solution and specifically purifying and analyzing the labeled peptides.

An intriguing hypothesis is that differences in surface proteomes contribute to the variable phenotypes of *Francisella* species, subspecies, and populations. A specific example of a difference in surface proteome composition is potentially the surface associated chitinase ChiA (FTL_1521). In *F. tularensis* A1 the homolog of *chiA* is intact, known to express, and its product is associated with virulence in a murine model (20, 46). The *chiA* gene is truncated in *F. tularensis* A2 and is predicted to be a pseudogene (2). In addition, ChiA is a documented *F. tularensis* antigen, thus differences in the surface presentation of this protein between *F. tularensis* biotypes could also impact immune recognition and be an important consideration in areas such as vaccine design. Another aspect of *F. tularensis*' surface proteins that warrants further investigation is how the proteome could change in response to different stimuli. Differential surface protein compositions are critical to the success of some pathogens. The best examples of this are the reciprocally regulated surface proteins OspA and OspC of *Borrelia burgdorferi* involved in attachment to the mid-gut of ticks and dissemination to vertebrate hosts, respectively (38). A similar type of surface proteome remodeling may be used by

F. tularensis to cope with the changing environments. Evidence for this includes the upregulation of the oxidative stress related surface proteins KatG (FTL_1504) and AhpC/TSA family protein (FTL_1015), in macrophages (48).

In conclusion, the work presented in this chapter provides the most complete characterization of *F. tularensis* surface proteins to date, and confirmed the hypothesis that the majority of these proteins were previously unknown to localize at the surface. Of the identified proteins, several may have unique roles in virulence and the phenotypic variations observed between *F. tularensis* subspecies and populations. The surface proteome identification strategy utilized here can be used as a platform to evaluate these differences, and this methodology may be applied to the investigation of how the *F. tularensis* surface proteome could adapt to changing environments and stresses, such as the transition from ticks to vertebrates and survival of the bacterium intracellulary.

3.5 Literature cited

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Chapter IV

Surface Protein Antigens of *F. tularensis*

4.1 Introduction

Presently, there is no approved vaccine available in the United States for the prevention of tularemia (35). However, an attenuated *F. tularensis* type B strain, LVS, was previously developed for human use (41, 42). This vaccine provides a significant level of protection against virulent *F. tularensis* infection in humans and animals, is more effective than other reported vaccines, but is no longer used due to issues of safety and sustained efficacy (29, 34, 54, 55). Although T cell mediated responses are an aspect of protection afforded by *F. tularensis* LVS vaccination, an important role for humoral immunity has been documented (6, 12, 15, 18). Early studies provided evidence that humans could be treated using immune sera (15, 18). Similarly, Drabick *et al*. demonstrated that passive transfer of immune sera could protect mice against a *F. tularensis* LVS infection that was 10,000 times higher than the LD_{50} (12). The protective effect of this serum could be abrogated by preabsorption with a *F. tularensis* LVS lysate, implicating antibodies in protection (12). To emphasize the importance of antibodies, specific IgG, IgM, and IgA were detectable in human patients for at least 1.5 years after *F. tularensis* LVS vaccination (15). Additionally, mice depleted of CD4+ and CD8+ T cells are known to retain a high degree of immunity against reinfection with *F. tularensis* LVS (10).

Many antigens, including proteins and polysaccharides, are recognized by specific antibodies after *F. tularensis* LVS infection or vaccination (8, 26). Several of

these were previously tested as vaccines, but displayed little to no protective efficacy against a challenge with virulent *F. tularensis* (Appendix 1). Two surface proteins, outer membrane associated protein FopA (FTT_0583) and lipoprotein TUL4 (FTT_0901), when presented individually in a heterologous host or with immune-stimulating complexes, failed to offer any substantial protection in murine models (19, 21, 46). However, marginal protection was recently observed when FopA and TUL4 monoclonal antibodies were administered therapeutically (43). The best protective effects observed with a single antigen were obtained using the surface molecule LPS (7-9, 13, 19). Cole *et al*. demonstrated that vaccination with LPS elicits the production of specific antibodies from B-1a B cells that are capable of providing lasting protective immunity against *F. tularensis* LVS (8). Unfortunately, antibodies to LPS do not provide complete protection to infection with *F. tularensis* type A (9, 43) . Significant protection to *F. tularensis* type B infection was also obtained using anti-capsule monoclonal antibodies that recognizes the O-antigen repeat of LPS, but it is unknown if the protective effect extends to *F. tularensis* type A infection (2).

As reported in this chapter, and previously by Huntley *et al*., mice vaccinated with *F. tularensis* membrane preparations admixed with adjuvant are modestly protected against *F. tularensis* type A (25). However, these vaccines do not produce the same level of protective immunity as *F. tularensis* LVS. It is possible that a subunit vaccine based on a membrane preparation lacks the immunogens required for full protection against virulent *F. tularensis*. Alternatively, membrane preparations could possess the correct set of immunogens, but their presentation in the context of an adjuvant elicits a different antigen response compared to *F. tularensis* LVS vaccination.

Although many different molecules may be recognized as antigens, only a portion of these can serve as protective immunogens (38). Immunogen identification is facilitated by identifying bacterial components recognized during active infections, and

often these are surface proteins (3, 44). This premise is best demonstrated by the use of surface proteins in effective vaccines against organisms such as serogroup B *N. meningitidis*, *Chlamydia pneumonia*, *Streptococcus pyogenes* and *Streptococcus agalactiae* (20, 31, 39, 50). Based on this evidence, and the known protective effect of *F. tularensis* membrane proteins, it was hypothesized that surface proteins would be recognized by immune sera from mice vaccinated with *F. tularensis* LVS. Accordingly, immune sera from *F. tularensis* LVS vaccinated mice were used to identify antigenic surface proteins that could also be immunogens. Further, immune sera from two adjuvanted subunit vaccinations containing MPF, a fraction that contains the majority of surface proteins, were evaluated and compared to *F. tularensis* LVS immune sera to assess changes in the humoral response (in the context of surface proteins) associated with decreased efficacy. The adjuvants, cationic liposome DNA complex (CLDC) and monophosphoryl lipid A (MPLA), were chosen because of their ability to stimulate differing effector responses (Th1/Th2 and Th1 type responses, respectively) and their use in humans (32). Presented here, 10 surface proteins were recognized as antigens by *F. tularensis* LVS vaccination. Sera from both subunit vaccinations also recognized surface proteins, but the breadth of the humoral responses were expanded to a much larger number of proteins, most of which are not surface associated.

4.2 Materials and methods

4.2.1 Strains and culture conditions

To generate sufficient quantities of MPF for immunoproteomic analyses, *F. tularensis* LVS was inoculated directly from frozen stocks into MH broth supplemented with 1% Isovitalex (BD Biosciences) and incubated at 37°C on a platform shaker for 30 h. Subcultures were inoculated to fresh MH broth supplemented with 1% Isovitalex and harvested after 20 h of growth. Cells were collected by centrifugation at 4,500 × g and

washed in PBS, pelleted and stored at -80°C. Production of stocks for vaccination or infection was achieved by culturing *F. tularensis* strains in modified MH broth supplemented with 0.14% $CaCl_2$, 0.21% $MgCl_2$ hexahydrate, 0.1% glucose, 0.025% ferric pyrophosphate and 2% Isovitalex at 37°C with constant shaking overnight (MMH). Aliquots of 1 ml were frozen at -80ºC and thawed prior to use (4). Frozen stocks were titered by enumerating viable bacteria from serial dilutions plated on modified MMH agar (5, 16).

4.2.2 Mice

Specific-pathogen-free, 6 to 8 week old male Balb/c mice (wild-type) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in sterile microisolater cages in the biosafety level three facility at the National Institute of Allergy and Infectious Diseases/Rocky Mountain Laboratories (RML) (Hamilton, MT). All mice were provided sterile water and food *ad libitum* and all research involving animals was conducted in accordance with Animal Care and Use guidelines and approved by the Animal Care and Use Committee at RML

4.2.3 Immunization of mice

In two independent experiments mice were immunized s.c. with *F. tularensis* LVS (n = 10 or 5), MPF mixed with CLDC (n = 10) (provided by Dr. Jeff Fairman, Juvaris Biotherapeutic, Burlingame, CA), or MPLA (n = 10) (Invivogen, San Diego, CA). CLDC and MPLA were prepared per manufacturers' instructions. MPF was serially diluted in 5% dextrose/water for addition to CLDC, and in PBS, for addition to MPLA. Each mouse was injected s.c. with 10 µg MPF in adjuvant in a total of 200 µl. Mice were immunized with MPF twice, two weeks apart. *F. tularensis* LVS was serially diluted in PBS and 200 CFU, in 200 µl, and injected s.c. into each mouse. Actual inoculum concentration was

confirmed by plating a portion of the inoculums, incubating plates at 37°C, and enumerating colonies. Unvaccinated mice served as negative controls. All mice were bled for serum 12 days after the final injection. Blood was collected via the tail vein into microtainer tubes (BD Biosciences). Blood was subsequently allowed to coagulate at room temperature for 30 min, centrifuged at 9.6 × g to separate cells from serum, and stored at -80°C.

4.2.4 Infection of mice with *F. tularensis* SCHU S4

Mice were infected i.n. with approximately 10 CFU of *F. tularensis* SCHU S4 30 days after the last vaccination. Just prior to infection, mice were anesthetized by injection of 100 µl of 12.5 mg/ml ketamine + 3.8 mg/ml xylazine i.p. Approximately 10 CFU in 25 µl was administered into the nares of each mouse. Actual inoculum concentration was confirmed by plating a portion of the inoculum, incubating plates at 37°C, and enumerating colonies. This dose routinely resulted in a mean-time-to-death of five days in naïve Balb/c mice. Statistical analyses of survival were performed using the log-rank (Mantel-Cox) test.

4.2.5 Isolation of MPF

F. tularensis LVS cell pellets were thawed and suspended in breaking buffer to a final concentration of 1 g cells (wet weight) per 1 ml of buffer. Cells were placed on ice and lysed by nine repetitions of pulsed sonication using a 4710 series Ultrasonic Homogenizer (Cole and Palmer Instrument Company) employing the following instrument parameters: % duty of 50, output of 5, 60 seconds pulsed, 60 seconds off. Unbroken cells and debris were removed from the lysate by centrifugation at 4,500 × g, 4°C, for 20 min. MPF was obtained by centrifugation of cell lysates at 100,000 × g for 4 h. Supernatants were removed, pellets suspended in breaking buffer, and dialyzed

against 10 mM ammonium bicarbonate buffer using 3,500 Da molecular weight cut-off dialysis membranes (Spectrum Laboratories Incorporated). MPF protein concentration was determined by the BCA assay (Thermo/Pierce) (47).

4.2.6 SDS-PAGE and IEF

Aliquots of MPF were applied to SDS-PAGE using 4-12% Bis-Tris SDS-Polyacrylamide gels (Invitrogen) under denaturing conditions (30). 2D-PAGE) was performed with 300 µg aliquots of MPF. First, MPF was precipitated with three volumes of acetone and 13.3% trichloroacetic acid and incubated overnight at -20°C. The precipitate was collected by centrifugation at 4,000 × g, 4°C, for 20 min, and was washed twice with 1 ml of cold acetone. The protein pellet was air dried and suspended in 22.5 µl of 50 mM Tris, pH 7.1, 8 M urea, 2 M thiourea, 100 mM dithiothreitol (DTT), 4% (w/v) CHAPS, 1% (w/v) ASB-14, 7% pH 4-7 ampholytes, and 0.3% pH 3-10 ampholytes, sonicated for 30 min, and incubated for 4 h at room temperature. Samples were diluted with 130 µl of 8 M urea, 2 M thiourea, 20 mM DTT, 4% (w/v) CHAPS, 1% (w/v) ASB-14, 0.7% pH 4-7 ampholytes, and 0.3% pH 3-10 ampholytes and centrifuged for 30 min at 13,000 × g to remove any insoluble material. One third of the sample was applied to an Immobiline dry strip (GE Healthcare Life Sciences) with either pH gradients of 4-7 or 6-11, following the manufacturer's instructions.

IEF was performed using a Multiphore II unit (GE Healthcare Life Sciences) at 50 V, 100 V, 150 V, 200 V, 250 V, 300 V sequentially for 6 min followed by 500 V for 12 min and 3000 V for 5 h. The focused Immobiline strips were rinsed in ultrapure H_2O and incubated in 0.375 M Tris, pH 7.1, 6 M urea, 2% SDS, 30% glycerol, 1% DTT, and trace bromophenol blue for 15 min at room temperature. Immobiline strips were again rinsed in ultrapure H_2O and incubated in 0.375 M Tris, pH 7.1, 6 M urea, 2% SDS, 30%

glycerol, 2.5% iodoacetamide, and trace bromophenol blue before a final rinsing in ultrapure H_2O. Proteins were resolved in the second dimension by SDS-PAGE.

Detection and destaining of protein in polyacrylamide gels was accomplished by staining with the Pierce Silver Stain Kit (Thermo/Pierce) according to the protocols of the manufacturer. Gel images were digitized using a HP Scanjet 4850 photo scanner (Hewlett-Packard Company).

4.2.7 Western blotting

MPF resolved by SDS-PAGE was transferred to nitrocellulose membranes by Western blot as previously described by Sonnenberg *et al*. with the following modifications (48). Membranes were incubated in TBS containing 0.1% Tween 20 and 5% nonfat milk and were washed in TBS containing 0.1% Tween 20 (TBST). Nitrocellulose membranes were incubated with sera pooled from vaccinated or control mice (1:200). Alternatively, membranes were incubated with anti-*Francisella* LPS (1:250) monoclonal antibodies (Immunoprecise, Victoria, British Columbia, Canada). Following incubation, membranes were again washed in TBST before being incubated with alkaline phosphatase conjugated goat-anti-mouse IgG or IgM (1:5,000) to detect primary antibodies. A final TBST wash was performed and antibody reactive proteins were detected using BCIP/NBT SigmaFAST tablets (Sigma-Aldrich, St. Louis, MO). Western blot images were digitized using a HP Scanjet 4850 photo scanner.

4.2.8 Computer aided image analysis

The digitized images of 2D-Western blots were compared to Pierce Silver Stain Kit stained 2D-protein gels using Delta2D gel analysis software (DECODON, Greifswald Germany), and areas of immunoreactivity were correlated with specific spots or areas of stained gels.

4.2.9 Protein identification by MS

Protein spots were subjected to in-gel proteolytic digestion with trypsin in 0.2 M ammonium bicarbonate buffer at 37°C. Peptides from enzymatic digestion were applied to a capillary C18 reversed phase column (0.74 x 50 mm) (Agilent Technologies) and eluted with an increasing linear gradient of ACN in 0.1 % formic acid. Effluent was introduced directly into an Agilent 6520 quadrapole-time of flight mass spectrometer (Agilent Technologies).

Proteins present in the MPF were identified using Mass Hunter Qualitative Analysis (Ver. B.02.00) (Agilent Technologies) and MASCOT softwares (Matrix Science Ltd). Spectral data were searched against the γ-proteobacteria NCBI non-redundant protein database. A peptide tolerance of 20 ppm, MS/MS tolerance of 0.01 Da, and 1 missed cleavage were allowed. Oxidation of methionine and carbamidomethyl modification of cysteine was considered in the search analyses. Protein and peptide identification was validated with Scaffold version 2_01_02 (Proteome Software Incorporated). Peptide identities were accepted only when a peptide identification probability of >95% was obtained as specified by the peptide prophet algorithm (27). Protein identifications were accepted if protein identification probabilities were >99.0%, as assigned by the protein prophet algorithm, and contained at least two unique peptides (33).

4.3 Results

4.3.1 Protective immunity of MPF vaccination

A comparative evaluation of the protective immune response generated by *F. tularensis* LVS *versus* the MPF based subunit vaccines was performed by challenging vaccinated mice with *F. tularensis* strain SCHU S4 30 days after the last vaccination in two independent experiments. As expected, mice vaccinated with *F. tularensis* LVS

were well protected against a low dose (10 CFU) i.n. challenge (Fig. 4.1). Animals vaccinated with either of the MPF formulations showed moderate protection as compared to unvaccinated controls. Specifically, those receiving MPF+MPLA showed the greatest protection, with 40-60% of the animals surviving infection (Fig. 4.1). Vaccination with MPF+CLDC resulted in 0-30% survival (Fig. 4.1). No statistical differences in survival rates were determined between identical vaccine groups in the two experiments.

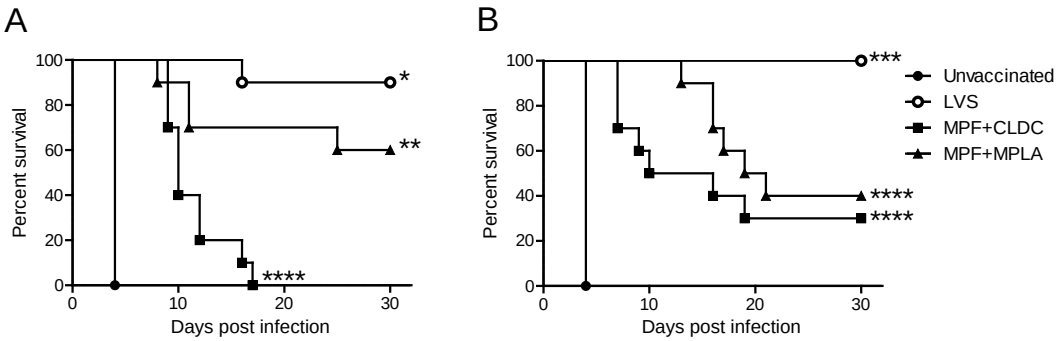

Fig 4.1 Survival of mice following a low dose i.n. challenge with *F. tularensis* SCHU S4. Mice were immunized s.c. with *F. tularensis* LVS (n = 10, experiment A or n = 5, experiment B), MPF+MPLA (n = 10), or MPF+CLDC (n = 10) in two independent experiments (A and B). Differences in survival of groups in the same experiment were evaluated using the Mantel Cox log rank test. * = significantly greater than all other groups except MPF+CLDC ($p<0.0002$). ** = significantly greater than all other groups except *F. tularensis* LVS ($p<0.0001$). *** = significantly greater than all other groups ($p<0.04$). **** = significantly greater than unvaccinated controls ($P<0.0002$).

4.3.2 Live attenuated and subunit vaccines recognize membrane proteins

Immune sera from LVS and MPF vaccinations were tested by 1D-Western blot to determine the presence of specific antibodies to MPF (Fig. 4.2). Vaccination with MPF and adjuvant produces a strong antibody response to proteins of both high and low

molecular masses. In contrast to MPF vaccination, most proteins recognized by *F. tularensis* LVS vaccination were resolved at molecular masses over 50 kDa. The extent of the number of bands recognized by *F. tularensis* LVS immune serum was much less compared to both MPF vaccinations. All immune sera blots were developed until non-specific reactivity was observed with their respective naïve control serums (data not shown). In all cases control serum reactivity was rare and weak. As expected, LPS was readily detectable in the sample and was the primary epitope recognized by IgM antibody isotypes (Fig 4.2B) (24, 25).

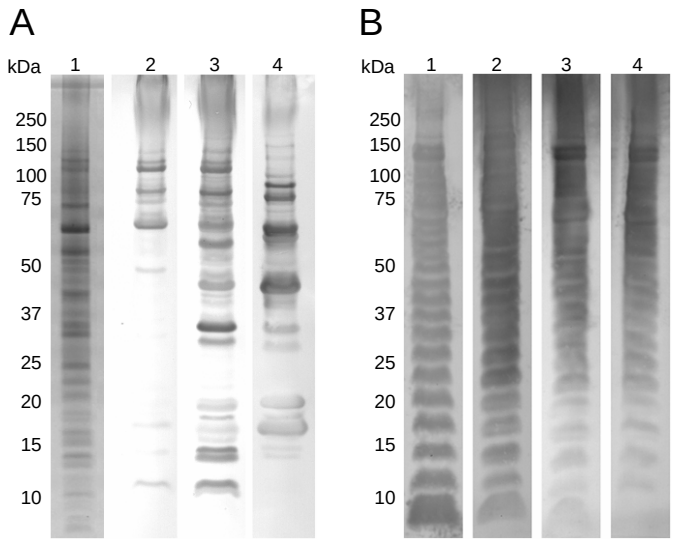

Fig. 4.2 Immune sera response directed against MPF from various forms of vaccination. A) Western blot analysis to determine immune serum reactivity (IgG) to MPF. Silver stained MPF (Lane 1); MPF probed with *F. tularensis* LVS anti-serum (Lane 2); MPF probed with MPF+MPLA anti-serum (Lane 3); MPF probed with MPF+CLDC anti-serum (Lane 4). B) Western blot analysis to determine immune serum reactivity (IgM) to MPF. MPF probed with anti-LPS antibody (Lane 1); MPF probed with *F. tularensis* LVS anti-serum (Lane 2); MPF probed with MPF+MPLA anti-serum (Lane 3); MPF probed with MPF+CLDC anti-serum (Lane 4).

4.3.3 2D-separation of *F. tularensis* MPF

2D-PAGE analysis of the MPF revealed 295 and 271 individual protein spots when pH 4-7 and pH 6-11 IPG strips were used, respectively (Fig. 4.3A and 3B). However, only proteins with higher isoelectric points were well resolved on basic IPG strips (Fig. 4.3B). Numbered spots were subject to MS analyses for protein identification (Table 4.1). Identifications were successful for 52 individual spots corresponding to 48 proteins. Multiple proteins were identified in 15 of the spots (spot numbers 8, 13, 15, 16, 17, 22, 29, 58, 59, 60, 61, 62, 63, 64, and 72). Of these, the proteins identified in spots 8, 13, and 16 were recognized by immune serum from other studies (26, 51). At least one protein was previously identified as immune reactive in spots 15, 17, 22, 29, 60, 61, 62, and 63 (26, 51). Until now, the proteins located in spots 58, 59, 64 and 72 have not been shown to react to immune serum.

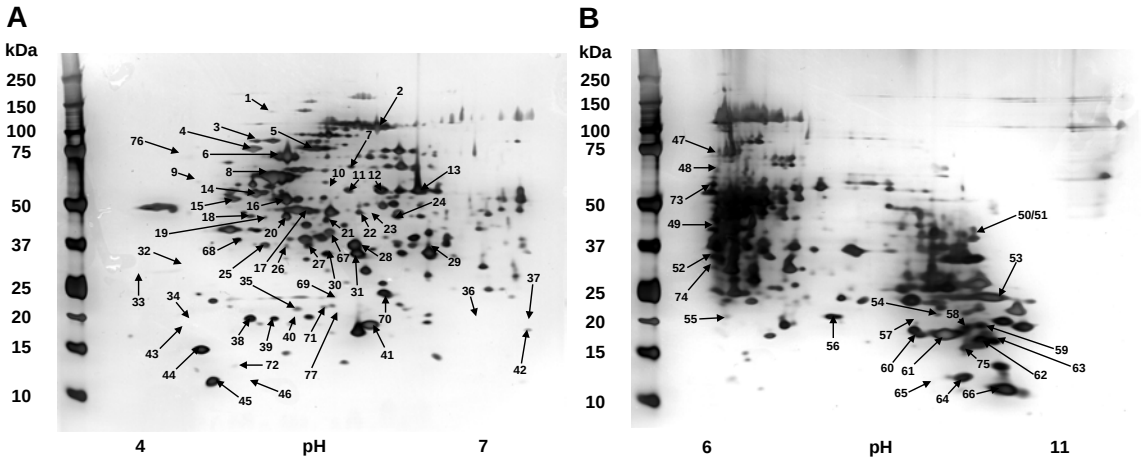

Fig 4.3 2D-PAGE analysis of MPF. Three hundred micrograms of MPF was separated in the pH range of 4-7 (A) or 6-11 (B). Representatives of silver stained gels from numerous technical replicates are shown. The numbered arrows indicate spots taken for LC-MS/MS analysis.

Table 4.1 MPF spot identification by MS

Spot number	Protein name	Locus	Predicted molecular mass (Da)	Sera reactivity	Protein identification probability	No. of unique peptides	% sequence coverage
1	Could not be identified with confidence	-	-	X, Y	-	-	-
2	Pyruvate dehydrogenase, E1 component	FTL_0309	100268.34	X, Y, Z	100.00%	24	27%
3	Chitinase family 18 protein	*FTL_1521	83595.21	X, Y, Z	100.00%	6	8%
4	Chaperone protein dnaK	*FTL_1191	69182.22	X, Y, Z	100.00%	22	41%
5	Peroxidase/catalase	*FTL_1504	81226.95	X, Z	100.00%	5	6%
6	Dihydrolipoamide acetyltransferase	FTL_0310	56798.85	X, Y, Z	100.00%	14	29%
7	Aspartyl-tRNA synthetase	FTL_0020	66805.23	X	100.00%	4	5%
8	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex	FTL_1783	52717.94	X, Y, Z	100.00%	10	26%
	Chaperonin GroEL	*FTL_1714	57402.70		100.00%	39	79%
9	Could not be identified with confidence	-	-	X, Y, Z	-	-	-
10	Could not be identified with confidence	-	-	X, Y	-	-	-
11	Hypothetical protein	*FTL_0572	51977.34	X, Y	100.00%	8	16%
12	Hypothetical protein	*FTL_0572	51977.34	X, Y	100.00%	17	36%
13	Hypothetical protein	*FTL_0572	51977.34	X, Y	100.00%	8	17%
	Dihydrolipoamide dehydrogenase	FTL_0311	50527.53		100.00%	10	22%
14	F0F1 ATP synthase subunit alpha	FTL_1797	55536.23	X, Y	100.00%	22	37%
15	Enolase (2-phosphoglycerate dehydratase)	*FTL_1527	49511.19	X	100.00%	5	11%
	F0F1 ATP synthase subunit alpha	FTL_1797	55536.23		100.00%	9	17%
16	F0F1 ATP synthase subunit beta	FTL_1795	49864.94	X	100.00%	22	60%
	Outer membrane associated protein	*FTL_1328	41259.65		100.00%	8	18%
17	Elongation factor Tu	*FTL_1751	43390.62	X, Y, Z	100.00%	16	49%
	Aspartate aminotransferase	*FTL_0387	44382.87		99.80%	2	4%
18	Outer membrane associated protein	*FTL_1328	41259.65	X	100.00%	6	13%
19	Outer membrane associated protein	*FTL_1328	41259.65	X, Y	100.00%	6	13%
20	Outer membrane associated protein	*FTL_1328	41259.65	X, Y	100.00%	13	29%
21	Outer membrane associated protein	*FTL_1328	41259.65	X, Y	100.00%	5	10%
22	Glutamate-1-semialdehyde-2,1-aminomutase	FTL_1283	47064.12	X	99.80%	2	4%
	OmpA family protein	*FTL_0325	46754.18		100.00%	8	21%
	Cell division protein FtsA	FTL_1908	44804.05		100.00%	8	17%
23	Could not be identified with confidence	-	-	X	-	-	-
24	NADH dehydrogenase I, F subunit	FTL_1825	46282.70	X	100.00%	6	14%
25	Could not be identified with confidence	-	-	X, Y	-	-	-
26	Outer membrane associated protein	*FTL_1328	41259.65	X, Y	100.00%	4	10%
27	Outer membrane associated protein	*FTL_1328	41259.65	X, Y	100.00%	7	20%
28	Outer membrane associated protein	*FTL_1328	41259.65	X, Y, Z	100.00%	8	20%
29	Universal stress protein	FTL_0166	30220.68	X, Y	100.00%	5	19%
	Outer membrane associated protein	*FTL_1328	41259.65		100.00%	6	12%
30	Periplasmic solute binding family protein	FTL_1936	33814.86	X, Y	100.00%	6	21%

Table 4.1 MPF spot identification by MS, continued

Spot number	Protein name	Locus	Predicted molecular mass (Da)	Sera reactivity	Protein identification probability	No. of unique peptides	% sequence coverage
31	Periplasmic solute binding family protein	FTL_1936	33814.86	X, Y	100.00%	9	24%
32	Could not be identified with confidence	-	-	X, Y	-	-	-
33	Hypothetical protein	FTL_0929	26874.96	X, Y	99.80%	2	11%
34	Outer membrane protein	*FTL_0009	19477.11	X, Y	100.00%	4	29%
35	AhpC/TSA family protein	*FTL_1015	19668.22	X, Y	100.00%	6	35%
36	Could not be identified with confidence	-	-	X, Y	-	-	-
37	Could not be identified with confidence	-	-	X	-	-	-
38	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit	*FTL_1592	16403.98	X, Y, Z	100.00%	7	62%
39	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit	*FTL_1592	16403.98	X, Y	100.00%	7	69%
40	Could not be identified with confidence	-	-	X	-	-	-
41	50S ribosomal protein L9	*FTL_1026	16087.49	X, Z	100.00%	7	39%
42	F0F1 ATP synthase subunit epsilon	FTL_1794	15736.95	X	100.00%	6	44%
43	Could not be identified with confidence	-	-	X	-	-	-
44	50S ribosomal protein L7/L12	*FTL_1745	12847.82	X, Y, Z	100.00%	6	43%
45	Hypothetical protein	FTL_0105	13728.82	X, Y, Z	100.00%	5	42%
46	Could not be identified with confidence	-	-	X, Y, Z	-	-	-
47	Could not be identified with confidence	-	-	X, Y	-	-	-
48	Could not be identified with confidence	-	-	X, Y	-	-	-
49	Could not be identified with confidence	-	-	X	-	-	-
50 & 51	F0F1 ATP synthase subunit B	FTL_1799	17383.26	X	100.00%	3	19%
52	Could not be identified with confidence	-	-	X, Y	-	-	-
53	50S ribosomal protein L3	FTL_0236	22307.33	X, Y	100.00%	5	29%
54	Could not be identified with confidence	-	-	X, Y	-	-	-
55	Could not be identified with confidence	-	-	X, Y	-	-	-
56	Hypothetical protein	FTL_0571	22451.65	X, Y	100.00%	7	0%
57	Could not be identified with confidence	-	-	X, Y	-	-	-
58	50S ribosomal protein L15	FTL_0255	15095.61	X, Y	100.00%	2	11%
	50S ribosomal protein L11	FTL_1748	15269.89		100.00%	5	27%
59	50S ribosomal protein L15	FTL_0255	15095.61	X, Y	100.00%	5	30%
	50S ribosomal protein L5	FTL_0248	19996.34		99.80%	2	9%
	50S ribosomal protein L11	FTL_1748	15269.89		100.00%	7	38%
60	Hypothetical protein	FTL_0850	20453.33	X, Y	100.00%	2	7%
	50S ribosomal protein L15	FTL_0255	15095.61		100.00%	4	26%
	Lipoprotein	*FTL_0421	15771.85		100.00%	6	46%
61	Lipoprotein	*FTL_0421	15771.85	X, Y	100.00%	8	55%
	50S ribosomal protein L10	FTL_1746	18731.70		100.00%	5	29%
62	Lipoprotein	*FTL_0421	15771.85	X, Y	100.00%	3	24%
	50S ribosomal protein L11	FTL_1748	15269.89		100.00%	3	22%
	50S ribosomal protein L10	FTL_1746	18731.70		100.00%	5	32%

Table 4.1 MPF spot identification by MS, continued

Spot number	Protein name	Locus	Predicted molecular mass (Da)	Sera reactivity	Protein identification probability	No. of unique peptides	% sequence coverage
63	Lipoprotein	*FTL_0421	15771.85	X, Y	100.00%	2	17%
	50S ribosomal protein L11	FTL_1748	15269.89		100.00%	7	38%
	50S ribosomal protein L10	FTL_1746	18731.70		100.00%	5	32%
64	50S ribosomal protein L25	FTL_0950	10903.65	X, Y	100.00%	3	22%
	Preprotein translocase family protein	FTL_0847	12881.27		100.00%	2	14%
65	Could not be identified with confidence	-	-	X	-	-	-
66	Could not be identified with confidence	-	-	X	-	-	-
67	Glutamine synthetase	FTL_1899	38256.94	Y	100.00%	6	13%
68	DNA-directed RNA polymerase subunit alpha	FTL_0261	35357.51	Y	100.00%	2	5%
69	Could not be identified with confidence	-	-	Y	-	-	-
70	Intracellular growth locus, subunit C	*FTL_0113	22133.41	Y	100.00%	7	38%
71	LemA-like protein	FTL_0361	21986.26	Y, Z	100.00%	7	32%
72	Hypothetical protein	FTL_0104	14595.73	Y, Z	100.00%	3	24%
	Thioredoxin	FTL_0611	12022.97		100.00%	4	43%
73	Could not be identified with confidence	-	-	Y	-	-	-
74	Could not be identified with confidence	-	-	Y	-	-	-
75	30S ribosomal protein S8	FTL_0250	14410.78	Y	100.00%	5	42%
76	Could not be identified with confidence	-	-	Z	-	-	-
77	Could not be identified with confidence	-	-	Z	-	-	-

X, Y, and Z designate proteins reactive to sera from mice vaccinated with MPF-CLDC, MPF-MPLA, and *F. tularensis* LVS, respectively. Protein probabilities of correct identification, number of unique peptides, and % sequence coverage were determined by Scaffold software.
*Indicates proteins previously identified as surface exposed in Chapter III. Loci are from the *F. tularensis* LVS genome.

4.3.4 Sera recognition of surface proteins by *F. tularensis* LVS vaccinated mice

To address the immunological relevance of surface exposed proteins, we assessed whether vaccination of mice with *F. tularensis* LVS elicited a humoral response to the surface proteins. However, direct 2D-PAGE and 2D-Western blot analyses of chemically labeled surface proteins (described in Chapter III) was not possible due to the low abundance of recoverable surface proteins and the potential interference of LC-Biotin. Therefore, 2D-Western blot analyses were performed using

the MPF as a surface protein surrogate, as the majority of surface proteins are contained in this fraction (described Chapter III). Pooled sera from mice vaccinated with *F. tularensis* LVS recognized 18 MPF protein spots. Reactivity was observed only to proteins that resolved in the pH range of 4-7, and like the 1D-Western blot analyses, the majority of dominant immunogens displayed a mass of greater than 50 kDa (Fig. 4.4). From these 18 immune reactive spots 17 proteins were identified by MS (Table 4.1). These included, pyruvate dehydrogenase, E1 component (FTL_0309), chitinase family 18 protein (FTL_1521), chaperone protein dnaK (FTL_1191), peroxidase/catalase (FTL_1504), dihydrolipoamide acetyltransferase (FTL_0310), dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (FTL_1783), chaperonin GroEL (FTL_1714), elongation factor Tu (FTL_1751), aspartate aminotransferase (FTL_0387), outer membrane associated protein (FTL_1328), acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit (FTL_1592), 50S ribosomal protein L9 (FTL_1026), 50S ribosomal protein L7/L12 (FTL_1745), hypothetical protein (FTL_0105), LemA-like protein (FTL_0361), thioredoxin (FTL_0611), and hypothetical protein (FTL_0104). Three of the immune-reactive protein spots (spot numbers 8, 17, and 72) each provided two protein identifications, and four of the immune-reactive protein spots (spot numbers 9, 46, 76, and 77) could not be identified, likely due to low abundance. Of these 17 proteins that were reactive to *F. tularensis* LVS immune sera, 10 were shown in Chapter III to be surface exposed (Table 4.1).

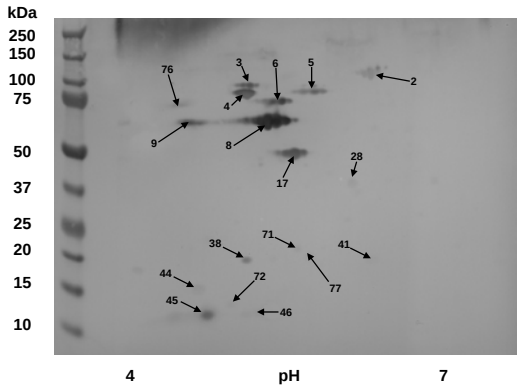

Fig. 4.4 2D-Western blot showing immunoreactive proteins contained in MPF in a pH range of 4-7 as a result of *F. tularensis* LVS vaccination. The numbered arrows correspond to the spots labeled in Fig. 4.3 and the protein identifications presented in Table 4.1.

4.3.5 Altered antigen profiles are associated with differential protection

The inability of *F. tularensis* subunit vaccines to induce protective immunity to the same level as *F. tularensis* LVS vaccination suggests that either the appropriate antigens are not present in the subunit vaccines or different types of immunity are induced (19, 21, 25). To assess whether the surface proteins recognized after *F. tularensis* LVS vaccination were also antigens when presented as a subunit vaccine, mice were vaccinated s.c. with MPF admixed with the adjuvant MPLA or CLDC, and the serological responses evaluated.

Based on the reactivity of immune sera from MPF vaccinated animals, the antigen profiles of the MPF+CLDC and MPF+MPLA vaccines were similar to one another (Fig. 4.5), but strikingly different from the antigen profile obtained by *F. tularensis* LVS vaccination. Specifically, animals vaccinated with MPF+CLDC displayed reactivity to a total of 66 protein spots, and sera from MPF-MPLA vaccinated animals recognized 57 protein spots (Table 4.1). A large number of these (48 protein spots) were recognized by both MPF-CLDC and MPF-MPLA vaccinated mice. However, it

should be noted that several of the shared antigenic spots were more reactive with the MPF-CLDC sera than with sera of MPF-MPLA vaccinated animals (e.g. spots numbered 9, 19, 21, 25, 26, 27, 57, and 58) (Fig. 4.5A-D). In sharp contrast to mice vaccinated with *F. tularensis* LVS, those that received the MPF subunit vaccines strongly recognized both high and low molecular mass proteins over a broad pH range, not just those with an acidic to neutral pH. All but two of the protein spots (spot numbers 76 and 77) recognized by the *F. tularensis* LVS vaccinated animals, reacted with sera from animals vaccinated with at least one of the MPF-adjuvant preparations.

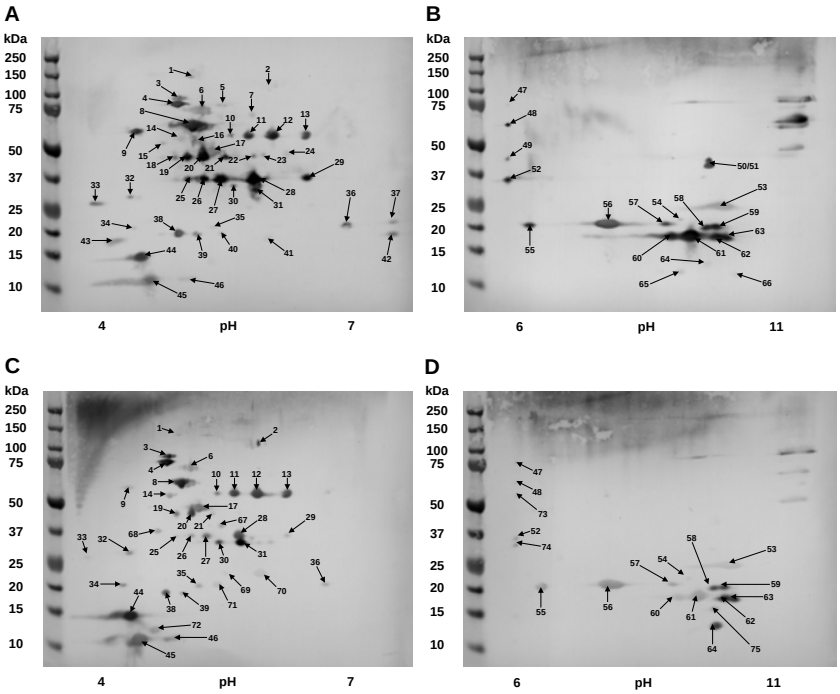

Fig. 4.5 2D-Western blot showing immunoreactive proteins contained in MPF as a result of MPF+CLDC and MPF+MPLA vaccination. A) MPF resolved in a pH range of 4-7 evaluated using MPF+CLDC immune sera. B) MPF resolved in a pH range of 6-11 evaluated using MPF+CLDC immune sera. C) MPF resolved in a pH range of 4-7 evaluated using MPF+MPLA immune sera. D) MPF resolved in a pH range of 6-11 evaluated using MPF+MPLA immune sera. The numbered arrows correspond to the spots labeled in Fig. 4.3 and the protein identifications presented in Table 4.1.

Protein identification was successful for 52 of the 75 protein spots reactive to sera from MPF+CLDC or MPF+MPLA vaccinated mice, and resulted in the identification of 48 individual proteins (Table 4.1). The 17 proteins identified in evaluation of sera from *F. tularensis* LVS vaccinated animals were also recognized after vaccination with MPF + adjuvant (Fig. 4.6A and Table 4.1). Considerable overlap was noted in the profile of proteins reactive to sera from MPF+MPLA and MPF+CLDC vaccinated mice, but sera from each of these groups also recognized a small subset of unique proteins (Fig. 4.6A and Table 4.1). Seventeen of the 48 immunoreactive proteins were found on the surface of *F. tularensis* LVS (described in Chapter III) (Fig. 4.6B and Table 4.1). These data demonstrated that the humoral responses to proteins of the membrane fraction are expanded after vaccination with the MPF based subunit vaccines compared with live *F. tularensis* LVS.

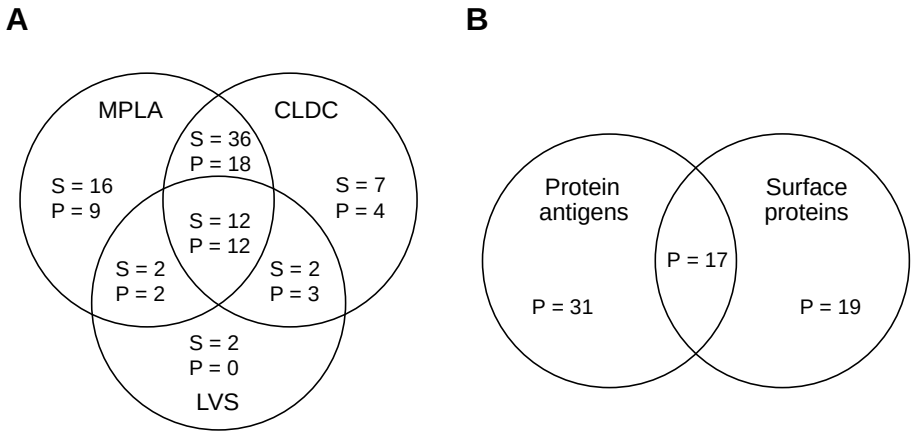

Fig. 4.6 The relationships of antigenic and surface proteins. A) The relationship of antigenic spots (S) and proteins (P) recognized by immune sera of mice vaccinated with *F. tularensis* LVS, MPF+CLDC and MPF+MPLA. B) The relationship of proteins identified as antigenic in this study compared to proteins described on the surface of *F. tularensis* LVS in Chapter III.

4.4 Discussion

An efficient humoral immune response is important for control and clearance of *F. tularensis* infections (12, 14). However, the repertoire of bacterial products that serve as protective immunogens is unknown. In many pathogens, naturally recognized surface proteins are immunogens, and these serve as the basis of rationally designed vaccines (3, 44). Presented here, a strong correlation was observed between proteins identified on the surface of *F. tularensis* LVS and those recognized as antigens by immune sera from *F. tularensis* LVS vaccinated mice. Specifically, 10 of the 17 antigenic proteins were surface exposed, strongly suggesting at least some of these are immunogens. The less protective MPF vaccinations also reacted to surface proteins, but the breadth of the immune responses was expanded to recognize a greater number of protein antigens, the majority of which were not identified as surface exposed. Of the antigens successfully identified after MPF vaccinations, 17 of 48 were surface associated.

This dichotomy in the breadth of the humoral response to vaccination of mice with live *F. tularensis* LVS *versus* that with a subunit vaccine is noted in previous studies (17). The evaluation of microarrays consisting of 1,741 recombinant *F. tularensis* proteins by Eyles *et al*. revealed that mice vaccinated with adjuvant mixed with killed *F. tularensis* LVS generated a much broader humoral response than mice vaccinated with live *F. tularensis* LVS (17). A comparison of the data presented here to the study by Eyles *et al*. showed that 16 of the top 48 antigens detected using the microarray platform were recognized as major antigens of the MPF. Additionally, 11 of the antigens defined by Eyles *et al*. were characterized as surface products in the experiments presented in Chapter III. These combined data demonstrate a relatively strong correlation in the major antigens of *F. tularensis* LVS regardless of the screening platform and the vaccine preparation. The importance of *F. tularensis* surface proteins as antigens, and potential

immunogens, is further underscored by the observation that all but one of the surface exposed proteins identified in Chapter III (FTL_0387) were classified as antigens in previous studies (17, 22, 23, 26, 37, 49, 51, 52).

In addition to recognizing a limited number of predominantly surface proteins, another possibility to explain the increased efficacy of *F. tularensis* LVS compared to MPF vaccinations is functional differences of the antibodies produced. Agglutinating antibodies aid in opsonization by phagocytes and restrict movement of some bacteria (1). Anderson *et al*. determined that mice vaccinated with *F. tularensis* LVS produced high titers of agglutinating antibodies 30 days post vaccination which correlated with the control and clearance of *F. tularensis* SCHU S4 infection (1). Ninety days post vaccination, the titer of agglutinating antibodies decreased and mice were unable to control *F. tularensis* SCHU S4 replication in the lung. Certain antibody subtypes have also been implicated in protection against *F. tularensis*, particularly those that are efficient at complement fixation (IgM, IgG3, and IgG1) (28). Thus, assessing the production of agglutinating antibodies and antibody subtyping could provide insight into the protective effect of MPF vaccinations.

An alternative hypothesis to explain the diminished efficacy of subunit vaccination is that different quantities of antibodies to particular antigens were produced. Consistent with previous reports, LPS was recognized only by IgM antibodies of *F. tularensis* LVS and MPF vaccinated mice (24, 25). However, Western blot analyses revealed differences in LPS reactivity between the different vaccinations used here. The reactivity to LPS appeared stronger in *F. tularensis* LVS immune sera at lower molecular masses compared to MPF vaccinated mice. Given that IgM is the most efficient agglutinating antibody this could be of particular significance (28). Western blot profiling of IgG responses revealed additional differences in the level of reactivity to some proteins. In general, IgG recognition by *F. tularensis* LVS immune sera was limited in

contrast to MPF seras. Overproduction of antibodies can be associated with immunosuppression (53). Therefore, along with the expanded recognition of proteins antigens, greater quantities of antibodies produced could account for the reduced efficacy of MPF vaccinations.

The protective effect exerted by *F. tularensis* LVS to *F. tularensis* type A infection is critically dependent on cell mediated responses, and it is possible that differences in the cell mediated effector mechanisms stimulated by the vaccines tested account for differences in the protective efficacies observed (15). In mice vaccinated with *F. tularensis* LVS, IFN-γ production by re-stimulated *F. tularensis* LVS-specific T cells is one of the most important mediators of protection upon infection (36, 45). These cells must recognize specific antigens to impart their effects, and the expanded humoral recognition of MPF vaccinations may also coincide with expanded presentation of non-protective epitopes to T cells, resulting in diminished T cell recognition of *F. tularensis* (11). Nonetheless, like the humoral response to *F. tularensis* LVS vaccination, T cell stimulation is at least partially dependent on surface structures. The only two well described *Francisella* T cell antigens, TUL4 (FTL_0421) and FopB (FTL_0009), are both surface proteins also recognized by the humoral response (40, 56).

The work presented here confirms the hypothesis that surface proteins would be recognized during *F. tularensis* LVS immunization, and correlates decreased efficacy of MPF subunit vaccinations to expanded antibody recognition of non-surface proteins. The antigenic surface proteins identified are noted in numerous *F. tularensis* vaccine studies conducted in both animals and humans and may be protective immunogens. This hypothesis is supported by the successful therapeutic use of monoclonal antibodies and immune recognition (both B and T cells) of several *F. tularensis* surface proteins (43). Thus, in addition to the humoral response, subunit vaccines containing surface proteins may also activate the cell mediated response that is critical for protective

immunity. Further study is needed to determine which of the other surface proteins may act as protective immunogens and how they can be combined with adjuvants to elicit appropriate effector responses required for optimal protection.

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Chapter V

Differential Chitinase Activity and Production within *Francisella* Species, Subspecies, and Populations

5.1 Introduction

The second most abundant polysaccharide found in nature is chitin, a biopolymer composed of repeating units of β-1,4 linked *N*-acetyl-D-glucosamine (30). Chitin is synthesized by many eukaryotes and is an essential component of arthropod cuticles, insect peritrophic membranes, and fungal cell walls. The abundance of this biopolymer allows it to serve as a major environmental reservoir of carbon and nitrogen, and it is especially concentrated in marine environments. Therefore, many bacteria have evolved chitinases to depolymerize chitin into metabolically accessible subunits. However, chitinases are not unique to prokaryotes and can be found in vertebrates, arthropods, plants, protozoa, and fungi (19, 33). All chitinases possess one or more glycosyl hydrolase (GH) superfamily domains of the 18, 19, 20 or 48 superfamilies responsible for their enzymatic activities (7, 10). Non-enzymatic domains commonly found in chitinases include those that facilitate adherence to chitin or potentially the depolymerization of the polysaccharide such as *N*-acetylglucosamine-binding protein A, carbohydrate binding, and fibronectin type 3 domains (3, 18, 45). The enzymatic activities of individual chitinases differ based on whether they act as endo- or exochitinases. Endochitinases catalyze the hydrolysis of the β-1,4 glycosidic bonds at internal sites of the chitin polymer. In contrast, exochitinases progressively release single sugars (*N*-acetylglucosaminidase activity), or disaccharides (chitobiosidase activity) from the non-reducing end of the polymer (7). The form of hydrolyzing activity,

organization of functional domains, primary amino acid sequence, and protein folds are all used to distinguish and subgroup individual chitinases (6, 7).

Chitinases are especially abundant in the γ-proteobacteria, including both saprophytic and pathogenic species, likely due to the association of this bacterial class with marine environments (5, 8, 9, 12, 23, 27, 38). However, the involvement of chitinases in bacterial infections or vector transmission has received limited evaluation. A study by Kirn *et al.* found that a chitin binding protein of *V. cholerae* allowed for binding to glycan moieties on the surface of human epithelial cells (18). In *L. pneumophila*, a chitinase was shown to enhance bacterial survival in the lungs of mice, but the specific mechanism remains unknown (8). Adding to the potential importance of chitinases in human disease is the observation that chitinases enable vector based transmission of the protist *Plasmodium falciparum* (the etiological agent of malaria) by degrading the peritrophic membrane that surrounds the mosquito blood meal (43).

F. tularensis and *F. novicida* genomes are known to encode for two putative chitinases, ChiA (FTT_0715) and ChiB (FTT_1768) (22, 34). Both of these chitinases are secreted in *F. novicida* where they likely function synergistically to attach to and depolymerize chitin (12, 26). In *F. tularensis,* ChiA is identified in membrane preparations, has a possible association in virulence, and was shown in Chapter III to be a surface associated protein (39, 42). *In silico* analyses of ChiA identified differences in this protein between *F. tularensis* subspecies and populations that could alter its presentation on the surface of some biotypes (28). Although ChiB was not identified as a surface protein in *F. tularensis* LVS, the secretion of this protein in *F. novicida* suggests there could be a surface interaction. The failure to identify ChiB on the surface of *F. tularensis* LVS localization may be a result of regulation of gene expression or alterations in secretion pathways between *Francisella* species and populations. *In silico* analyses of ChiB also indicated differences between *F. tularensis* biotypes that could

influence the surface localization of this protein (4). Therefore, it was hypothesized that chitinases represent an enzymatic class of surface proteins that contribute to the phenotypic variation of *F. tularensis* subspecies and populations.

In this study, chitinase production and activity was evaluated across the *F. tularensis* subspecies and populations (A1a, A1b, A2, and type B) and compared to that of *F. novicida.* This included two previously undescribed chitinase gene products (ChiC and ChiD) as well as the previously studied ChiA and ChiB (12, 26). Despite the similarity among *Francisella* genomes, variability was observed between chitinase genes and gene products of the *Francisella* species and *F. tularensis* subspecies and populations. These differences correlated to the chitinase activity of individual species and populations, and individual chitinases presented unambiguous variability in activity.

5.2 Materials and methods

5.2.1 Bacterial strains and growth conditions

Francisella strains (Table 5.1) were grown from frozen stocks on CHAB at 35°C for 48 h, followed by subculture onto CHAB for 24 h at 35°C. *Francisella* liquid cultures were grown using a 24 h CHAB subculture to inoculate modified MMH broth (1, 28). Liquid cultures of *Francisella* were incubated at 35°C overnight with shaking at 160 rpm. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth, with shaking at 160 rpm or on LB agar. Appropriate antibiotics were added to the media when needed.

Table 5.1 *F. tularensis and F. novicida* strains

Francisella strain[a]	Species	Subspecies	Population	Source	Geographic origin
OK01-2528	*tularensis*	A	A1a[b]	Human	Oklahoma
MO02-4195	*tularensis*	A	A1a[b]	Human	Missouri
SCHU S4	*tularensis*	A	A1a[b]	Human	Ohio
MA00-2987	*tularensis*	A	A1b[b]	Human	Massachusetts
MD00-2970	*tularensis*	A	A1b[b]	Human	Maryland
WY96-3418	*tularensis*	A	A2a[b]	Human	Wyoming
NM99-1823	*tularensis*	A	A2b[b]	Human	New Mexico
MI00-1730	*tularensis*	B	-	Human	Michigan
KY99-3387	*tularensis*	B	B.II[c]	Human	Kentucky
LVS	*tularensis*	B	B.Br.LVS[c]	Rat	Russia
GA99-3550 (U112)	*novicida*	-	-	Water	Utah
GA99-3548	*novicida*	-	-	Human	Louisiana

[a]CDC accession number.
[b]Classification based on Kugeler *et al*. (20).
[c]Classification based on Vogler *et al*. (44).

5.2.2 PCR and cloning of chitinase genes

PCR amplification of chitinase genes was performed using 30 to 75 ng of genomic DNA, 2.5 units of PrimeSTAR™ HS DNA Polymerase (Takara Bio Inc., Otsu, Shiga, Japan), 1x PrimeSTAR Buffer (Mg^{2+} plus), 200 µM of each dNTP, and 1 µM of each primer in a final reaction volume of 50 µl. Primers (Appendix 7) were designed using VectorNTI advance 11.0 software (Invitrogen) and included specific restriction enzyme sites for use in downstream cloning. PCR conditions were 98°C for 2 min, followed by 30 cycles of 98°C for 10 sec, 45°C to 65°C for 5 sec and 72°C for 2 min 30 sec. Amplicons were cloned using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) following the manufacturer's instructions. Engineered restriction enzyme sites were used to isolate the cloned chitinase gene from TOPO® and ligation into the pET23b-(+) expression vector (Novagen, San Diego, CA). All clones were sequence verified by the

Proteomics and Metabolomics Facility at Colorado State University using the primers listed in Appendix 7.

5.2.3 Construction of *chiA* and *chiC* knockouts and complemented strains

Constructs for in-frame deletions of *chiA* (FTMG_00598) in *F. tularensis* strain MA00-2987 (A1b) and *chiC* (FTW_0313) in *F. tularensis* strain WY96-3418 (A2) were made using the *sacB*-based allelic exchange vector pMP590 (24). Specifically, constructs pMP590-Δ*chiA* and pMP590-Δ*chiC* (Table 5.2) were created by were created by PCR amplification of 477 to 941 bp DNA fragments that encompassed both flanking regions and 65 to 120 bp 65 to 120 bp of the 5' and 3' regions *chiA* and *chiC*, followed by ligation of the corresponding amplicons into the multiple cloning site of pMP590 (primers used are listed in Appendix 7). Electroporation was used to transform competent *Francisella* cells with the pMP590-*chiA* or pMP590-*chiC* constructs (24). Plasmid DNA (0.1-1.0 µg) was added to 100 µl of competent cells and electroporation was performed using a Bio-Rad Gene Pulsar (Bio-Rad) with the following parameters: 2.0 kV, 25 µF, 200 Ω. Electroporated cells were incubated in 1 ml of MMH broth for 3 h at 35°C while shaking (160 rpm). Transformants were selected by plating on MMH agar containing 5 µg/ml of kanamycin and incubating at 35°C for 2 to 4 days. Individual clones were transferred to MMH plates containing 8% sucrose to select for homologous recombination events. Genomic DNA was isolated from the knockout strains, the genomic fragment representing the region of homologous recombination was amplified by PCR and the sequence verified.

Table 5.2 Plasmids used for chitinase production and knockouts

Plasmid	Description	Source
pCR®-Blunt II-TOPO®	Km^R, Zeo^R, cloning vector	Invitrogen
pET23b-(+)	Ap^R, Histidine tagged, expression vector	Novagen
pET23b-(+)_FTT_0715	A1a/A1b *chiA* expression vector	This study
pET23b-(+)_FTH_1471	type B *chiA* expression vector	This study
pET23b-(+)_FTN_0627	*F. novicida chiA* expression vector	This study
pET23b-(+)_FTT_1768	A1a/A1b *chiB* expression vector	This study
pET23b-(+)_FTH_0088	type B *chiB* expression vector	This study
pET23b-(+)_FTN_1744	*F. novicida chiB* expression vector	This study
pET23b-(+)_FTT_1592	A1a/A1b *chiC* (GH18 fragment) expression vector	This study
pET23b-(+)_FTW_0313	A2 *chiC* expression vector	This study
pET23b-(+)_FTH_1579*	type B *chiC* (no signal peptide) expression vector	This study
pET23b-(+)_FTT_0066	A1a/A1b *chiD* expression vector	This study
pET23b-(+)_FTW_0142	A2 *chiD* expression vector	This study
pET23b-(+)_FTH_1730	type B *chiD* expression vector	This study
pET23b-(+)_FTN_1644	*F. novicida chiD* expression vector	This study
pMP590	*sacB*-based allelic exchange vector	LoVullo(25)
pMP529	*Francisella* shuttle/integration vector	LoVullo(25)
pMP590-Δ*chiA*	allelic exchange vector for *chiA* knockout	This study
pMP590-Δ*chiC*	allelic exchange vector for *chiC* knockout	This study
pMP529-*chiA*	*chiA* integration vector	This study
pMP529-*chiC*	*chiC* integration vector	This study

*Attempts to produce protein with the signal peptide failed.

FTT, FTW, FTH and FTN loci correspond to those listed in Appendix 7.

Deletion mutants were complemented by cloning the full length target genes, *chiA* (FTMG_00598) and *chiC* (FTW_0313) into the shuttle vector pMP529 to create pMP529-*chiA* and pMP529-*chiC*. Electroporation of the deletion mutants with the complementing plasmids was performed as described for wild-type *F. tularensis*. To select for transformants, electroporated cells were plated onto MMH plates containing 200 µg/ml of hygromycin and incubated for 2 to 4 days at 35°C. Complementation was confirmed by PCR amplification, sequencing, and Western blot.

5.2.4 Recombinant chitinase production and purification

To produce recombinant chitinases, expression vectors possessing individual chitinase genes (Table 5.2) were transformed into chemically competent *E. coli* BL21* (DE3) pLysS cells (Invitrogen). Recombinant clones were grown in 2 L of LB broth with 100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol at 37°C for 3 h while shaking at 160 rpm prior to addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were grown for an additional 4 h and the cells harvested by centrifugation at 3,500 × g. Cells were suspended in 10 ml of breaking buffer [PBS, pH 7.4, 1.2 µg/ml DNase I, 1.2 µg/ml RNase A, 1 µg/ml lysozyme and one Complete EDTA-free protease inhibitor cocktail tablet (Roche Applied Sciences) per 50 ml of buffer], placed on ice and lysed by probe sonication using a Vibra Cell VCX750 sonicator (Sonics and Materials, INC, Newton, CT). Sonication was performed at an amplitude setting of 30 with seven 60 sec pulses and a 60 sec pause between the pulse cycles. Unbroken cells and debris were removed by centrifugation at 12,000 × g, and the clarified lysate was applied to a 0.8 × 0.4 mm Poly-Prep (Bio-Rad) column pre-packed with 1.5 ml His-Resin (EMD Chemicals, Gibbstown, NJ) pre-equilibrated in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Unbound proteins were eluted with 15 column volumes (CV) of binding buffer, followed sequentially with 10 CV of wash buffer A (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and 23 CV of wash buffer B (40 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), and 20 CV of 10 mM Tris-HCl, pH 8.0). The bound recombinant protein was eluted with 5 ml of elution buffer (0.5 M imidazole, 10 mM Tris-HCl, pH 8.0). All purification steps were performed at 4°C. The eluent of purified protein was dialyzed at 4°C against 10 mM ammonium bicarbonate using a 3,500 Da molecular mass cut off dialysis membrane and concentrated using a 10,000 Da molecular mass cut off Amicon Ultra Centrifugal Filter Unit (Millipore, Billerica, MA).

Protein concentrations were determined using the BCA assay (36). Samples were aliquoted and frozen at -80°C until further use.

5.2.5 Assays for chitinase activity

To assess secreted chitinase activity of *Francisella* strains, the cells were grown in MHH broth (10 ml) overnight. Immediately prior to harvesting the CS, an aliquot (2 ml) of each culture was serially diluted on CHAB agar in duplicate. The agar plates were incubated for 48 hrs at 35°C and colonies enumerated. The CS was separated from the bacterial cells by centrifugation at 5,000 × g for 15 min, concentrated using a 10,000 Da molecular mass cut off Amicon Ultra Centrifugal Filter Unit, and brought to a volume of 500 µl with PBS, pH 7.4. An aliquot of the processed CS corresponding to approximately 6.0×10^8 CFU of the original 10 ml culture was assayed against three chitin analogs (*p*-nitrophenyl *N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl *N*,*N′*-diacetyl-β-D-chitobioside and *p*-nitrophenyl β-D-*N*,*N′*,*N″*-triacetylchitotriose) provided in the Colorimetric Chitinase Assay (Sigma-Aldrich), and according to the manufacturer's instructions, except activity was assessed after incubation of the enzyme reaction for 16 h at 35°C. Release of *p*-nitrophenol was measured at an absorbance of 405 nm using a Multiskan EX spectrophotometer with Ascent v2.6 software (Thermo-Fisher, Waltham, MA).

To evaluate cell associated chitinase activity, *Francisella* strains were grown on CHAB agar for 24 h at 35°C. The cells were scraped from the agar and suspended in PBS to a turbidity of 0.6-0.7 measured using a Microscan turbidity meter (Siemens Healthcare Diagnostics, Deerfield, IL). To ensure consistency and to normalize enzymatic activity between each culture, the number of CFUs in the cell suspension was determined. An aliquot (300 µl) of the cell suspension was subjected to seven freeze-thaw cycles using a dry ice-ethanol bath and a 37°C heat block. This process allowed

for the lysis of *F. tularensis* without generating hazardous aerosols. Unbroken cells and the WCL were separated by centrifugation at 17,000 × g for 15 min. An aliquot of the WCL corresponding to approximately 1.6×10^8 CFU of the original cell suspension was applied to the Colorimetric Chitinase Assay (Sigma-Aldrich) and chitinase activity measured as described for the CS. All chitinase activity was reported as the average of chitinase activity of the three replicate cultures from each strain of *F. tularensis* or *F. novicida*. Background activity from substrate alone was subtracted from all the activity values reported.

The enzymatic activity of individual recombinant chitinases (ChiA, ChiB, ChiC or ChiD) produced in *E. coli* was determined using a variation of the Colorimetric Chitinase Assay. An aliquot (10 µl) of the recombinant chitinases at 1 µg/ml in 0.1 M sodium phosphate buffer, pH 5.9, was added to 90 µl of the appropriate chitin analog at a concentration of 267 µM in 0.1 M sodium phosphate, pH 5.9. Separate assays were conducted at ambient temperature and 37°C. The release of *p*-nitrophenol was determined at various times (0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, 360, 420, 480, and 540 sec) during the enzymatic reaction. Three technical replicates were used for each time point and activity was reported as the average of these replicates. Enzymatic activity of the individual recombinant chitinases was confirmed using a glycol chitin substrate as described by DebRoy *et al*. (8).

5.2.6 Antibody production, SDS-PAGE, and Western blotting

Anti-sera were generated in rabbits to histidine tagged products of *F. tularensis* A1a/A1b ChiA (FTT_0715), *F. tularensis* A1a/A1b ChiB (FTT_1768), and *F. tularensis* A2 ChiC (FTW_0313). All anti-sera were produced by SDIX (Windham, ME).

Recombinant proteins (0.1 µg) and WCLs (15 µg) of *F. tularensis* or *F. novicida* strains were separated by SDS-PAGE using NuPAGE 4-12% Bis-Tris SDS-

polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes by electro-blotting (37). The nitrocellulose membranes were incubated in PBS, pH 7.4 containing 5% nonfat milk for 1 h and washed in PBS. Incubation of the primary antibody [anti-chitinase serum diluted 1:500 in PBS with 0.1% $Tween_{20}$ + 5% nonfat dry milk] with the nitrocellulose membranes was performed at ambient temperature with gentle shaking for 3 h, followed by washing with PBS, pH 7.4. The secondary antibody, goat anti-rabbit IgG (1:5,000) (Calbiochem, Darmstadt, Germany), was applied for 1 h at room temperature with gentle shaking. A final series of PBS washes were performed and antibody reactive proteins were detected using a solution of BCIP/NBT made with SigmaFAST tablets (Sigma-Aldrich). Western blot and SDS-PAGE images were digitized using a HPScanjet 4850 photo scanner (Hewlett-Packard Company).

5.2.7 Bioinformatic analyses of *Francisella* chitinases

DNA and protein similarity searches were performed using BLASTN and BLASTP respectively, against the non-redundant and whole-genome shotgun reads databases of NCBI. To predict similarity to other known products, the largest product from each chitinase class (ChiA, ChiB, ChiC, and ChiD) of *Francisella* was used for BLAST similarity searches. If multiple products were predicted to be the same size, BLAST searches were performed using the *F. novicida* U112 chitinase. *Francisella* genomes that were available in GenBank and used in this study were: *F. tularensis* subsp. *tularensis* A1b strain MA00-2987 (accession number *ABRI00000000*), *F. tularensis* subsp. *tularensis* A1a strain SCHU S4 (accession number AJ749949), *F. tularensis* subsp. *tularensis* A2 strain WY96-3418 (accession number CP000608), *F. tularensis* subsp. *holarctica* OSU18 (accession number CP000437), *F. tularensis* subsp. *holarctica* LVS (accession number AM233362), and *F. novicida* strain GA99-3550/U112 (accession number CP000439). DNA and protein sequence alignments

were performed using LALIGN (www.ch.embnet.org/software/LALIGN_form.html) and ClustalW (www.ebi.ac.uk/clustalw/#). Conserved domains within the individual chitinase sequences of *F. tularensis* were identified using the NCBI Conserved Domain search software (concise display) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (25). The presence of GH18, GH19, GH20, and GH48 domains were also identified in *Francisella* genomes using the UniProt database (www.uniprot.org). Signal peptide predictions were accomplished using the SignalP 3.0 software (http://www.cbs.dtu.dk/services/SignalP).

5.3 Results

5.3.1 *In vitro* chitinase activity of *F. tularensis* and *F. novicida*

To determine the chitinase activity of *Francisella* grown *in vitro*, endochitinase, chitobiosidase and *N*-acetylglucosaminidase activities were evaluated using a panel of 12 *Francisella* strains (Table 5.1) representing *F. tularensis* (A1a, A1b, A2, and type B) and *F. novicida* (Fig. 5.1). Comparisons were normalized to the cell numbers used to generate the WCL and CS. Endochitinase activity differed between the *Francisella* species and populations (Fig. 5.1A and B). The *F. tularensis* A2 strains showed the highest level of endochitinase activity in the WCL (avg. A_{405} of 0.27), but displayed minimal activity in the CS (Fig. 5.1A and B). Endochitinase activity was detectible in the WCL of *F. tularensis* A1a and A1b strains; however, this activity was barely above background. The WCL of *F. tularensis* type B strains displayed approximately half of the endochitinase activity of the *F. tularensis* A2 strains (avg. A_{405} of 0.13). In contrast, the endochitinase activity of *F. novicida* strains was observed in the WCL and CS, with the CS possessing the greatest activity (avg. A_{405} of 0.57) and the WCL activity (avg. A_{405} of 0.11) similar to that of *F. tularensis* type B strains. No chitobiosidase or *N*-acetylglucosaminidase activities were detected in the WCL of *F. tularensis* or *F. novicida*

strains (data not shown). However, both types of exochitinase activities were detectable in the CS of *F. novicida* (Fig. 5.1C and D). These data provide strong evidence of differential production of chitinase among the *Francisella* species and populations, and suggest underlying differences in their chitinase genetic profiles and subcellular localizations.

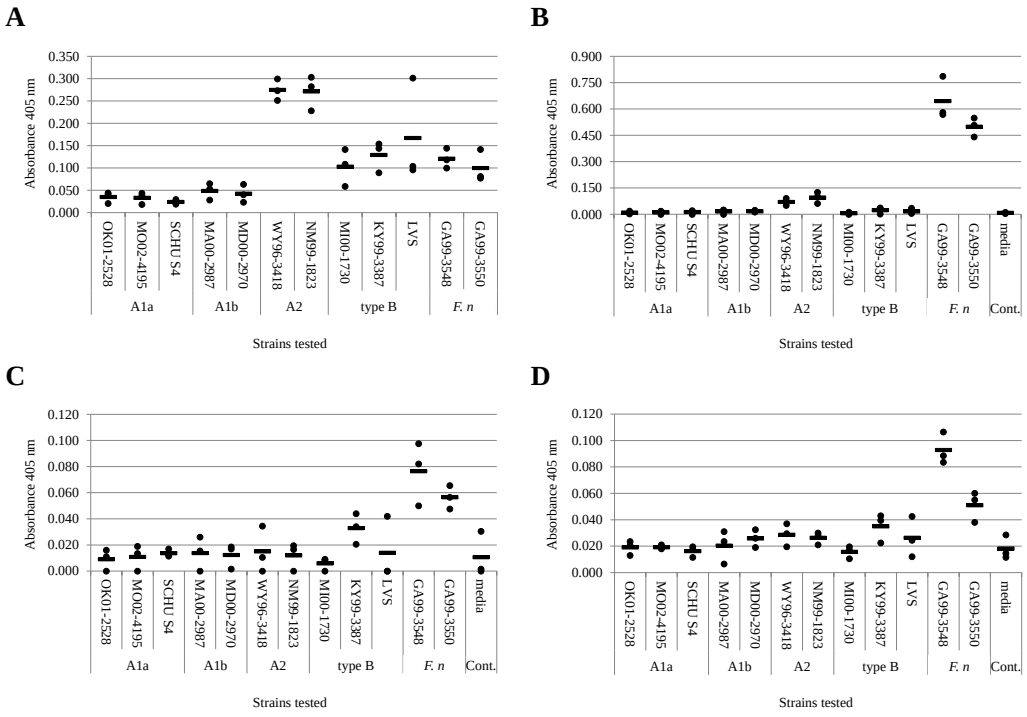

Fig. 5.1 *In vitro* chitinase activities of *F. tularensis* and *F. novicida* strains. A panel of 12 characterized strains (three A1a, two A1b, two A2, three type B, and two *F. novicida*) were evaluated for endochitinase, chitobiosidase, and *N*-acetylglucosaminidase activity. (A) endochitinase activities of WCL, (B) endochitinase activities of CS, (C) chitobiosidase activities of CS, (D) *N*-acetylglucosaminidase activities of CS. The average absorbance at 405 nm from three biological replicates (two technical replicates) are reported as (•) with the average of all replicates indicated by (▬). Little to no exochitinase activity was detected in WCL. Background measurements of the chitin analogs were subtracted from the data reported.

5.3.2 *F. tularensis* and *F. novicida* encode four putative chitinase genes

To more completely evaluate the genetic differences existing between *Francisella* species and populations with respect to chitinase genes, a comprehensive bioinformatics analysis was performed. Genes encoding for *chiA* and *chiB* were previously described in *F. novicida* and *F. tularensis* (12, 22). Domain searches of the *chiA* and *chiB* gene products found they possessed a GH18 domain (PfamID: PF00704), a characteristic domain of bacterial chitinases. Thus, a search for chitinase genes was performed by BLAST analyses using the previously described *Francisella* ChiA and ChiB and their GH18 domains. These and additional search analyses identified a total of four chitinase genes, *chiA*, *chiB*, *chiC*, and *chiD* in *F. tularensis* and *F. novicida* (Fig. 5.2). The *chiA* gene product shared the greatest similarity with a probable chitinase of *Polysphondylium pallidum* (*E* value 6×10^{-51}). The *Francisella* ChiB most closely resembled a chitinase from *Saccharophagus degradans* (*E* value 2×10^{-11}), and the ChiC displayed the greatest similarity to a hypothetical chitinase of *Photobacterium damselae* (*E* value 2×10^{-22}). *F. tularensis* and *F. novicida* ChiD showed limited similarity to other known chitinases, but most closely resembled a chitinase from *Lactococcus lactis* (*E* value 7×10^{-8}). However, a specific type of GH18 domain, ChiD-like domain (cd02871), previously identified in *Bacillus circulans* resembles the GH18 of the *Francisella* ChiD (*E* value 2.65×10^{-79}).

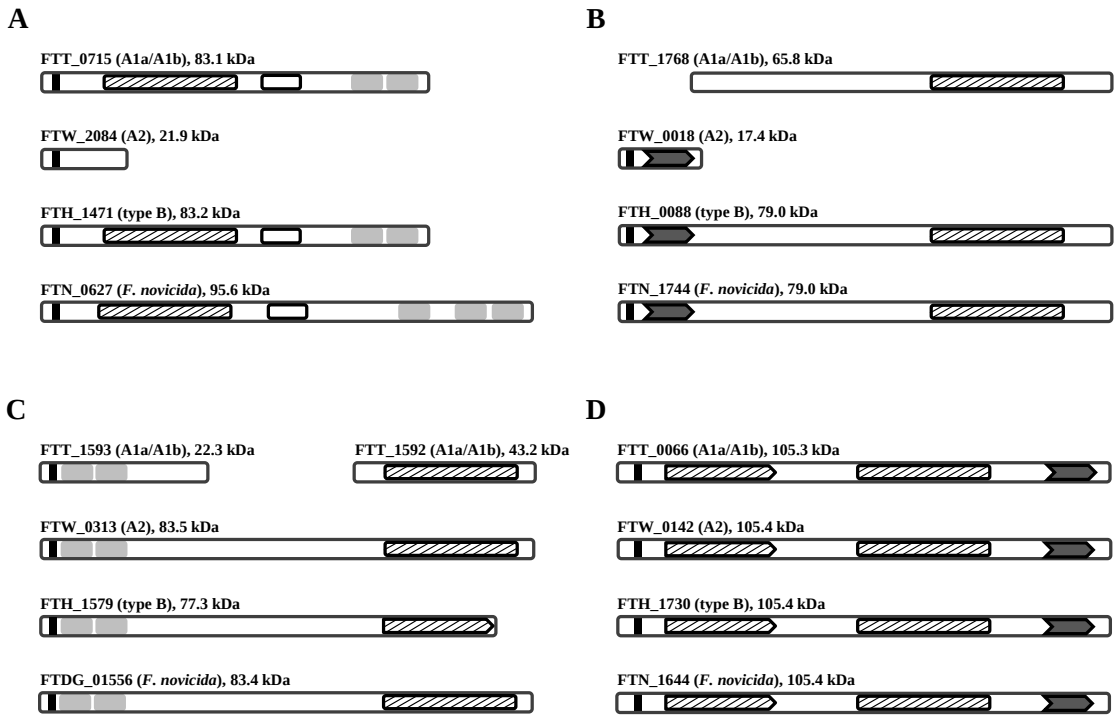

Fig. 5.2 Domain features of *F. tularensis* (A1a, A1b, A2, and type B) and *F. novicida* chitinases. The relative positions of conserved domains were identified in the chitinase gene products of *F. tularensis* and *F. novicida*. Solid black boxes indicate the predicted signal peptide cleavage site, striped boxes indicate the location and completeness of the GH18 domain (truncated GH18 domains appear as a pentagon), open boxes indicate the position of fibronectin type 3 domains, grey boxes indicate carbohydrate binding domains and dark grey chevrons represent incomplete *N*-acetylglucosamine-binding protein A domains. (A) *chiA* gene products, (B) *chiB* gene products, (C) *chiC* gene products, (D) *chiD* gene products. A *chiC* gene was identified in the genome of *F. novicida* GA99-3548 and GA99-3549 but not *F. novicida* U112.

Differences in the predicted presence, functionality, and subcellular localizations of these four genes were observed among *F. tularensis* populations (A1a, A1b, A2, and type B), and *F. novicida*. *F. tularensis* A1a and A1b possessed genes for *chiA*, *chiB*, *chiC*, and *chiD* with an altered *chiB* and *chiC*. Specifically the *chiC* of *F. tularensis* A1a possessed a point mutation causing a premature stop codon and two predicted ORFs annotated as FTT_1592 and FTT_1593 and encoding products of 387 and 207 amino acids, respectively. The *F. tularensis* A1a FTT_1592 ORF encodes for the C-terminal portion of ChiC that includes a complete GH18 domain. This was found to be identical in the *F. tularensis* A1b genome (MA00-2987); however, it is currently annotated as one reading frame (FTMG_01551). The ChiB of the *F. tularensis* A1a and A1b populations contained a 124 amino acid N-terminal truncation that removed a partial *N*-acetylglucosamine-binding protein A domain and the conserved signal peptide for translocation. *F. tularensis* A2 appeared to encode a functional *chiC* and *chiD*. *F. tularensis* type B was predicted to have all four chitinase genes, but the *chiC* product possessed a 58 amino acid C-terminal truncation that appeared to impact the essential GH18 domain. *F. novicida* strain U112 was predicted to only lack *chiC*. Two additional *F. novicida* genomes became available during the course of this study, the genome for strain GA99-3548 (accession number ABAH00000000) and strain GA99-3549 (accession number AAYF00000000). Both of these *F. novicida* strains possessed a *chiC*. In addition to the overall absence or presence of the four chitinases, the domain structure between ChiA, ChiB, ChiC, and ChiD differed. A more comprehensive bioinformatic analysis of the *Francisella* chitinases can be found in Appendix 8.

The *in silico* analyses of the putative chitinases predicted that each of the *Francisella* species, subspecies and populations possessed two or more functional chitinases. When applied to the differential endochitinase activities observed with the WCL of *Francisella* species, subspecies and populations (Fig. 5.1) this bioinformatics

data led to several hypotheses. 1) Enzyme kinetics of the individual chitinases or different levels of chitinase production in the various *Francisella* strains significantly influence the overall endochitinase activity; 2) the strong chitinase activity of the *F. tularensis* A2 population is attributable to ChiC or ChiC in combination with ChiD; and 3) the moderate endochitinase activity of *F. tularensis* type B and *F. novicida* is a result of activity from ChiA, ChiB, ChiD or a combination of these proteins.

5.3.3 Differential endochitinase activities of ChiA, ChiB, ChiC, and ChiD

To establish the level of endochitinase enzyme activity associated with individual gene products, *F. tularensis* and *F. novicida* chitinases predicted to be functional (those with a complete or partially altered GH18 domain) were produced as recombinant proteins in *E. coli* and assayed for endochitinase, chitobiosidase, and *N*-acetylglucosaminidase function. The ChiA, ChiB, and ChiC proteins possessing a complete GH18 domain were positive for endochitinase activity (Fig. 5.3). The *F. tularensis* type B ChiC was found to be negative for all chitinase activity; a result that correlates with the 58 amino acid C-terminal deletion that truncates the GH18 domain of this protein. The greatest endochitinase activity over a nine min assay was observed for the ChiB product of *F. tularensis* A1a/A1b and type B, and *F. novicida*. The full length ChiC of *F. tularensis* A2 was slightly less active than the intact ChiB proteins. Interestingly, the recombinant *F. tularensis* A1a/A1b C-terminal ChiC fragment (FTT_1592) was active, but at decreased levels compared to the full length *F. tularensis* A2 ChiC. The ChiA of *F. tularensis* A1a/A1b and type B, and *F. novicida* yielded modest endochitinase activity (A_{405} of 0.40, 0.38 and 0.40, respectively), and none of the ChiD proteins were found to possess chitinase activity. Only ChiA and ChiC chitinases displayed minimal measurable activity to the chitobiosidase analog, and *N*-acetylglucosaminidase activity was not observed for any chitinase enzyme (Fig. 5.3).

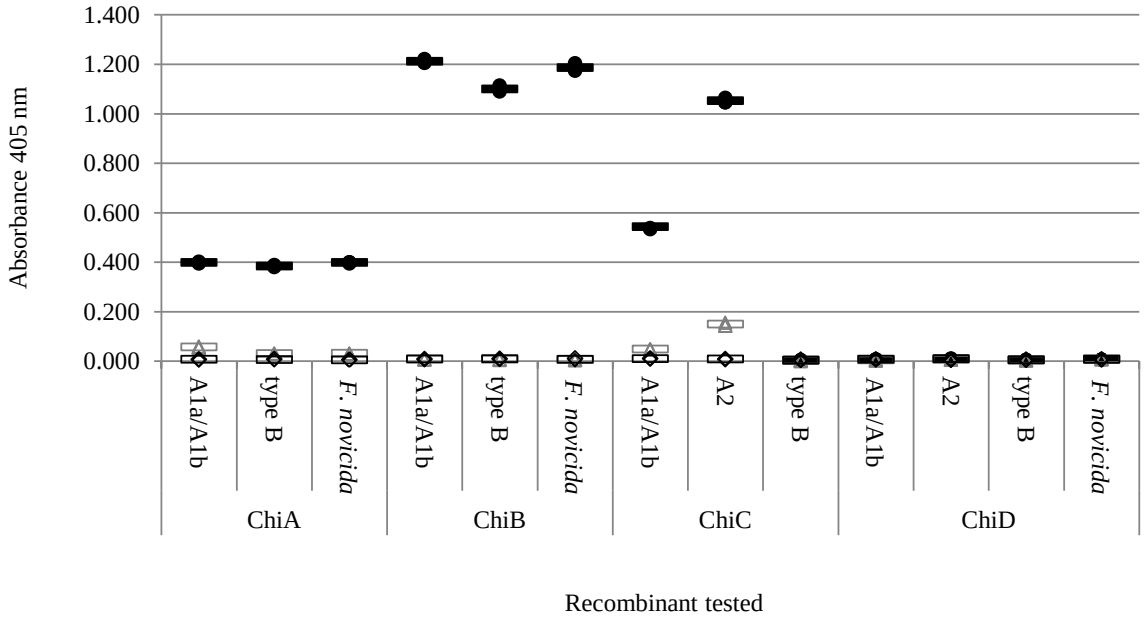

Fig. 5.3 Chitinase activity and substrate specificities of *F. tularensis* (A1a, A1b, A2, and type B) and *F. novicida* recombinant chitinases. *F. tularensis* and *F. novicida* chitinases with complete or partial GH18 domains were produced as recombinants in a heterologous system and assayed for nine minutes to determine their specificities to analogs capable of distinguishing between (•) endochitinase, (∆) chitobiosidase and (◊) *N*-acetylglucosaminidase activities. Absorbance at 405 nm was determined for three technical replicates of each enzyme at 37°C. The average of the technical replicates is reported as a bar of corresponding color and shading. Background measurements of the chitin analogs were subtracted from the data reported.

The kinetics of endochitinase activity were evaluated to further to define differential enzyme activities (Fig. 5.4). The kinetic profiles of the ChiA proteins of *F. tularensis* A1a/A1b and type B, and *F. novicida* were similar to one another (Fig. 5.4A), but these chitinases yielded a much slower rate of catalysis than ChiB or ChiC (Fig. 5.4D). The ChiB recombinant proteins of *F. tularensis* A1a/A1b and type B, and *F. novicida* all presented similar kinetic profiles, and these recombinant products provided the greatest activity over the nine min endochitinase assay (Fig. 5.4B). Although the maximum release of *p*-nitrophenol from the endochitinase substrate by ChiC of *F. tularensis* A2 was slightly less than that observed with the most active ChiB protein, the ChiC protein had a dramatically higher rate of catalysis, where the activity plateaued after 90 sec. The *F. tularensis* A1a/A1b C-terminal ChiC fragment (FTT_1592) containing the intact GH18 domain again presented significantly less activity than the full length ChiC of *F. tularensis* A2, but its activity also plateaued at 90 sec. (Fig. 5.4C). To test whether the form of the recombinant protein influenced activity, each chitinase was also produced as a recombinant product without predicted signal peptides or without histidine-tags, and assayed for chitinase activity. The relative endochitinase activities between ChiA, ChiB, ChiC, and ChiD did not change with these other recombinant forms (data not shown). Kinetic evaluations were also conducted at ambient temperature, and although total activities were lower at this temperature compared to 37°C, the relative endochitinase activities did not differ between ChiA, ChiB, ChiC, and ChiD (data not shown).

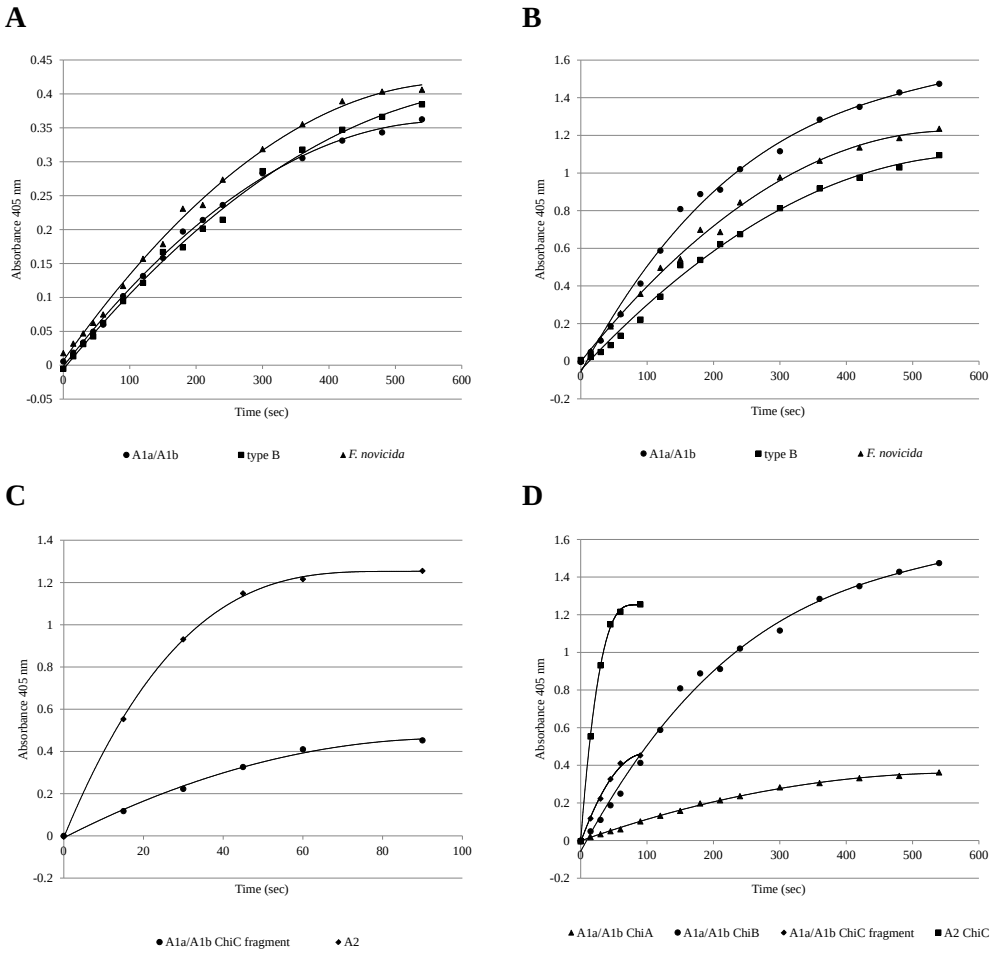

Fig. 5.4 Comparative endochitinase kinetics of *F. tularensis* (A1a, A1b, A2, and type B) and *F. novicida* recombinant chitinases. Recombinants that tested positive for endochitinase activity (Fig. 3) were assayed with an endochitinase analog to determine the relative activity of each functional enzyme at 37°C. The average absorbance at 405 nm of three technical replicates is reported for time points 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, 360, 420, 480 and 540 sec, except for ChiC where activity plateaued at 90 sec. Kinetics of (A) ChiA recombinant proteins, (B) ChiB recombinant proteins, (C) ChiC recombinant proteins and (D) relative comparison of all functional chitinases. Background measurements of the endochitinase analog were subtracted from the data reported.

To validate the activity observed with the small chitin analogues in the colorimetric assay, a second assay with glycol chitin (a soluble polymeric chitin) as the substrate was performed (8). The presence or absence of chitinase activity for each of the recombinant chitinases was the same as that observed using the colorimetric assay (Fig. 5.5). To account for the neutral pI of the ChiD products, the glycol chitin assay was also performed at a neutral pH, and no activity was observed (data not shown).

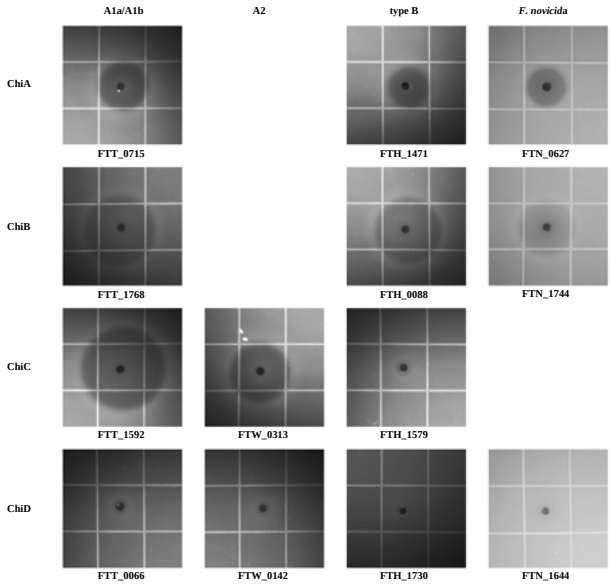

Fig. 5.5 Degradation of glycol chitin by *F. tularensis* (A1a, A1a, A2, and type B) and *F. novicida* recombinant chitinases. 10 µg of recombinant chitinases were assayed for their capacity to hydrolyze glycol chitin. A zone of clearance around the well where the chitinase is added indicates positive chitinase activity. For positive and negative controls, 10 µg of *Trichoderma viride* chitinase and 10 mM ammonium bicarbonate were used, respectively (data not shown).

5.3.4 Production of individual chitinases by *F. tularensis* and *F. novicida*

Western blot analyses of WCL from *in vitro* grown *F. tularensis* and *F. novicida* were performed with antiserum generated against the recombinant chitinases (Fig. 5.6).

This evaluation was performed to determine the relative production of individual chitinase proteins shown to possess endochitinase activity (Fig. 5.3). Antiserum specificity was confirmed using the individual recombinant chitinases (data not shown). A protein band of the correct molecular mass (83 kDa) reactive to anti-ChiA antiserum was detected in WCL of *F. tularensis* A1a/A1b and type B, but as predicted by bioinformatics, not to *F. tularensis* A2. A ChiA product of about 125 kDa was detected in *F. novicida* WCL. This was greater than the predicted mass of 95.5 kDa. The ChiB antiserum recognized products at a predicted mass of 79 kDa in the WCL of *F. tularensis* type B and *F. novicida*, but did not identify a product in the WCL of *F. tularensis* A1a, A1b, or A2. A protein band corresponding to ChiC was only detected in *F. tularensis* A2. Products corresponding to the *F. tularensis* A1a and A1b ChiC fragments or the inactive *F. tularensis* type B ChiC were not detected.

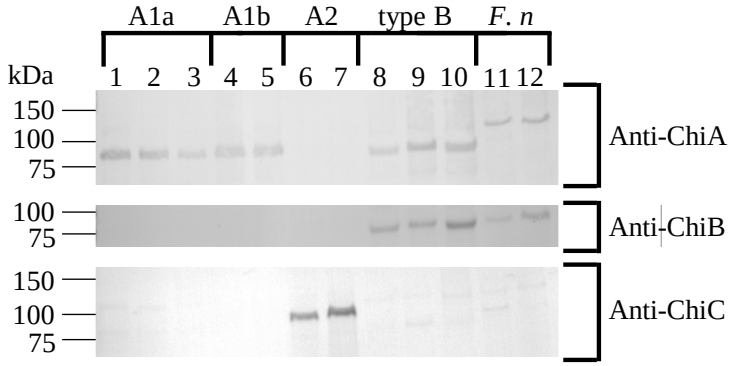

Fig. 5.6 Chitinases produced *in vitro* by *F. tularensis* (A1a, A1b, A2 and type B) and *F. novicida*. Western blots of WCL (15 µg) of each *Francisella* strain using anti-ChiA antiserum, anti-ChiB antiserum and anti-ChiC antiserum. Lanes 1-3, A1a strains OK01-2528, MO02-4195 and SCHU S4, respectively; Lanes 4-5, A1b strains MA00-2987 and MD00-2970, respectively; Lanes 6-7, A2 strains NM99-1823 and WY96-3418, respectively; Lanes 8-10, type B strains KY99-3387, LVS and MI00-1730; Lanes 11-12, *F. novicida* strains GA99-3548 and U112.

5.3.5 Analysis of *chiA* and *chiC* knockouts of *F. tularensis*

To demonstrate that the observed endochitinase activity of *F. tularensis* A2 was attributable solely to ChiC, a *chiC* knockout (Δ*chiC)* and the corresponding complement were generated in *F. tularensis* A2 strain WY96-3418 and evaluated for chitinase activity (Fig. 5.7B). The Δ*chiC* strain displayed minimal to no endochitinase activity (A_{405} of 0.005). When this mutant was complemented with *chiC*, endochitinase activity was restored (A_{405} of 1.088) to a level greater than that of the wild-type *F. tularensis* A2 strain (A_{405} of 0.239). Western blot analysis using anti-ChiC antiserum verified the production of ChiC in WCL of the wild-type *F. tularensis* A2 and the complemented Δ*chiC* strains, and confirmed its absence in WCL of the Δ*chiC* mutant (Fig. 5.7D). Additionally, to determine whether the low endochitinase activity observed for *F. tularensis* A1a and A1b strains was provided by ChiA and not the fragmented ChiC, a *chiA* knockout (Δ*chiA)* in A1b strain MA00-2987 and the corresponding complemented strain were generated (Fig. 5.7A and D). The endochitinase activity of the wild-type strain was low (A_{405} of 0.054), but was reduced further to an A_{405} of 0.004 in the Δ*chiA* mutant and restored to a level moderately higher (A_{405} of 0.097) than the wild-type *F. tularensis* A1b strain when the Δ*chiA* strain was complemented with *chiA*. Western blot analyses using anti-ChiA antiserum verified the production of ChiA in WCL of the wild-type *F. tularensis* A1b and the complemented Δ*chiA* strains, and demonstrated its absence in the Δ*chiA* mutant (Fig. 5.7D). These results indicate that the ChiC fragment of *F. tularensis* A1b is not active under the conditions tested and the low endochitinase activity observed is due to ChiA, a protein that yields modest endochitinase activity as a purified recombinant product.

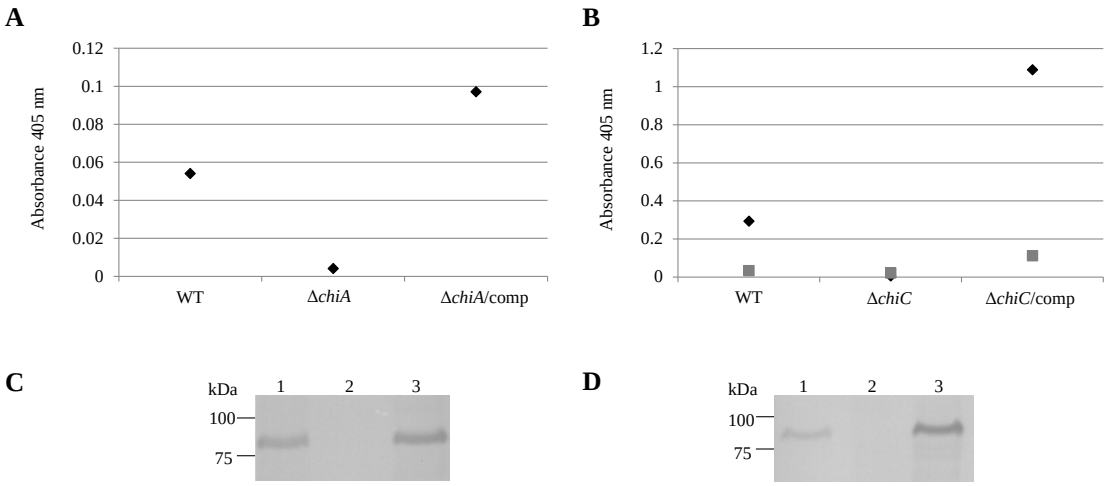

Fig. 5.7 Analysis of *chiA* and *chiC* chitinase knockouts in MA00-2987 (A1b) and WY96-3418 (A2), respectively. Endochitinase (♦) and chitobiosidase (■) activity of WCL from (A) wild-type strain MA00-2987, Δ*chiA* MA00-2987, and Δ*chiA*/comp MA00-2987; and (B) wild-type strain WY96-3418, Δ*chiC* WY96-3418, and Δ*chiC*/comp WY96-3418. Western blot with anti-ChiA (C) against WCL from Lane 1, wild-type strain MA00-2987; Lane 2, Δ*chiA* MA00-2987; and Lane 3, Δ*chiA*/comp MA00-2987. Western blot with anti-ChiC (D) against WCL from Lane 1, wild-type strain WY96-3418; Lane 2, Δ*chiC* WY96-3418; and Lane 3, Δ*chiC*/comp WY96-3418. For (A) and (B), background measurements of the chitin analogs were subtracted from the data reported.

5.4 Discussion

The genomes of *F. tularensis* and *F. novicida* are highly similar with >95% minimum percent identity, and the genomes of *F. tularensis* strains are considered monomorphic with pair-wise average nucleotide identity of >99.2% (4, 21). Despite this, genetic differences do exist among the *Francisella* species, subspecies, and populations, and these are reflected in the documented phenotypic variations among members of this genus (35). However, it is unknown how alterations in *Francisella's*

surface proteome contribute to this variability. This study determined how genetic alterations in a surface exposed chitinase, ChiA, as well as other chitinases that bare bioinformatic signatures of surface proteins, dictate unique *F. tularensis* and *F. novicida* phenotypes. Differences in endochitinase activity were detected between *Francisella* species, and even between *F. tularensis* subspecies and populations. A detailed comparison of *F. tularensis* A1a, A1b, A2, and type B strains and *F. novicida,* coupled with evaluation of the two previously identified chitinase genes (*chiA* and *chiB)* and two newly identified putative chitinase genes (*chiC* and *chiD)* provided a molecular basis for the observed chitinase phenotypes. Table 5.3 provides a summary of the chitinase gene products for each of the *Francisella* species and populations.

Table 5.3 Summary of *Francisella* chitinase bioinformatics and activities

Francisella biotype	Chitinase class	Predicted pseudogene [a]	Recombinant activity	*In vitro* production
A1a/A1b	ChiA	No	+	+
	ChiB	No [b]	+	-
	ChiC	Yes/Yes [c]	-/+	-
	ChiD	No	-	NT [d]
A2	ChiA	Yes [d]	NT	-
	ChiB	Yes [e]	NT	-
	ChiC	No	+	+
	ChiD	No	-	NT
type B	ChiA	No	+	+
	ChiB	No	+	+
	ChiC	No [f]	-	-
	ChiD	No	-	NT
F. novicida	ChiA	No	+	+
	ChiB	No	+	+
	ChiC	No [g]	NT	-
	ChiD	No	-	NT

[a] The pseudogene designation is based on existing notations made to the annotated genome sequences.

[b] *F. tularensis* A1a/A1b ChiB has an N-terminal truncation resulting in deletion of the signal peptide and the *N* -acetylglucosamine-binding protein A domain, but does not impact the GH18 domain.

[c] *F. tularensis* A1a/A1b*chiC* contains a point mutation that causes a premature stop codon and results in two predicted open reading frames. One reading frame (FTT_1592c) encodes an unaltered GH18 domain.

[d] *F. tularensis* A2 *chiA* is truncated due to a mutation resulting in a premature stop codon; GH18 domain is missing.

[e] *F. tularensis* A2 *chiB* is truncated due to a mutation resulting in a premature stop codon; GH18 domain is missing.

[f] *F. tularensis* type B ChiC contains a C-terminal truncation of that impacts the GH18 domain, but its gene is not annotated as a pseudogene.

[g] *F. novicida* strain GA99-3550 did not encode *chiC* . However, *F. novicida* GA99-3548 encoded a *chiC* whose product was predicted functional.

NT designates not tested.

Among the four chitinases, ChiD was not a factor in defining the variable chitinase activity among *Francisella* species, subspecies, and populations. The *chiD* gene was ubiquitous in all strains evaluated, but the purified ChiD protein was the only putative *F. tularensis* and *F. novicida* chitinase that failed to display any form of chitinase activity. Examination of the active chitinases (ChiA, ChiB, and ChiC) revealed that the single active chitinase in *F. tularensis* A2 was ChiC, and this protein accounted for the relatively robust endochitinase activity of the *F. tularensis* A2 strains. In contrast, *F. tularensis* A1 strains had multiple predicted functional chitinases (ChiA, ChiB, and ChiC), but barely detectible enzymatic activity. This weak activity was a result of ChiA, since neither ChiB nor the ChiC fragment were detected in the WCL of *F. tularensis* A1 strains grown *in vitro*. When compared to the *F. tularensis* A1 and A2 populations, *F. tularensis* type B had a dissimilar chitinase profile comprised of a predicted functional ChiA and ChiB. These differences between the chitinase profiles of type A and type B were not surprising given the number of phenotypic variations that exists among these two subspecies (4, 16, 29, 32). The low endochitinase activity of purified *F. tularensis* ChiA proteins and the dominant activity of the ChiB protein support the conclusion that the robust endochitinase activity observed for *F. tularensis* type B is attributable to ChiB. Unlike *F. tularensis*, *F. novicida* displayed high chitinase activity in the CS with considerably less activity detected in the WCL. Additionally, the total chitinase activity of this species was markedly higher than that of *F. tularensis*. Analysis of *F. novicida* chitinases revealed a functional ChiA and ChiB in *F. novicida* strain U112. As previously shown, *chiC* was absent from this strain (34); however, when *F. novicida* genome GA99-3548 and GA99-3549 became available, a *chiC* nearly identical to the *F. tularensis* A2 *chiC* was identified. The presence of *chiC* in *F. novicida* GA99-3548 might account for the consistently higher levels of chitinase activity of this strain as compared to the *F. novicida* U112.

As noted, the differential chitinase phenotypes of the *Francisella* species and populations were not a simple reflection of the number of active chitinases produced, but were also a result of the variable activity among the predicted chitinases. All the putative *F. tularensis* and *F. novicida* chitinases contained GH18 family domains. Interestingly, the *chiC* fragments identified in *F. tularensis* A1a and A1b were annotated as pseudogenes, but the C-terminal fragment containing an intact GH18 domain was active as a recombinant product. On the contrary, the *chiC* of *F. tularensis* type B was annotated as a functional gene; however, its GH18 domain was truncated and displayed no activity as a recombinant protein. The inactivity of ChiD is also likely a result of a non-functional GH18 domain. Functional GH18 domains contain a conserved catalytic motif of "DXDXE" where the glutamate residue acts as an acid critical for catalysis (13). Examination of this motif in the ChiD products (487-NFDLS-491) revealed the glutamate was replaced by a neutral serine residue. This along with a non-conserved aspartic acid to asparagine substitution likely inactivated this catalytic domain in ChiD.

Beyond GH18 domains, the other regions of the individual *F. tularensis* chitinases were hypothesized to contribute to their variable activity. A fibronectin type 3 domain, was present only in the ChiA proteins and was positioned between the GH18 and carbohydrate binding domains, a common observation in other chitinases (40). This domain is thought to serve as a linker that adjusts the relative position of the chitinase catalytic and carbohydrate binding domains (40). Fibronectin type 3 domains of cellulases are also known to help disorganize polymers of cellulose. Thus, it may play a similar role in chitin depolymerization (15, 45). Tandem bacterial (Type 3) carbohydrate binding domains were identified in ChiA and ChiC. These domains have been shown to localize chitinases to their substrate and aid in chitin depolymerization (14, 41). The carbohydrate binding domains were not essential for the chitinolytic activity, as demonstrated with the C-terminal ChiC fragment of *F. tularensis* A1 strains; however, a

comparison of activity from this C-terminal fragment and the intact ChiC of *F. tularensis* A2 strains demonstrated that the presence of the non-enzymatic carbohydrate binding domains enhanced activity. A second binding domain, *N*-acetylglucosamine-binding protein A domain, was identified in ChiB and ChiD but in a truncated form. This domain was previously described in a surface associated chitin binding protein of *V. cholerae* and enhanced attachment of this bacterium to both chitin and human epithelial cells (18). The linkage of these bioinformatic analyses with the production of individual chitinases and their differential activities provide a molecular basis to explain the overall chitinase activity of the different *Francisella* species and populations. It also suggests that each of these proteins identified as chitinases may act on different substrates or provide different biological functions.

Putative signal peptides were identified in all of the chitinases (except *F. tularensis* A1a/A1b ChiB and ChiC GH18 containing fragment). Despite this, *F. tularensis* endochitinase activity was dominant in the WCL, in contrast to *F. novicida* where activity was focused in the CS. *F. novicida* chitinase (ChiA and ChiB) secretion is dependent on a Type II secretion system and in particular four “pili” proteins [PilA (FTN_0415), PilB (FTN_1115), PilC (FTN_1116) and PilQ (FTN_1137)] are known to be essential for chitinase secretion (12). Homologues of these are encoded by all *Francisella* biotypes analyzed in this study, except *F. tularensis* LVS that lacks *pilA* (11). Thus, the absence of chitinase secretion in *F. tularensis* is hypothesized to result from differences in the expression and production of secretion machinery between *F. novicida* and *F. tularensis.* Alternatively, with the larger number of pseudogenes in *F. tularensis* as compared to *F. novicida*, it is possible that one or more unidentified products essential for secretion are absent from *F. tularensis* (4, 34).

F. tularensis type A strains are considered more virulent than *F. tularensis* type B strains, and recent studies revealed differences among the virulence of *F. tularensis* type

A populations (A1a, A1b, and A2) (20, 29). The ChiA protein is highly over produced (>20 times) in mice infected with an *F. tularensis* A1a strain FSC033, but a knockout of *chiA* in *F. tularensis* A1 strain SCHU S4 revealed no difference in virulence as compared to the wild-type strain. These data suggest that *F. tularensis* A1a produces ChiA during *in vivo* growth for a function not directly linked to pathogenesis. To date, no studies have been conducted to decipher the potential roles of ChiB and ChiC in *F. tularensis* virulence. The virulence differences that exist between *F. tularensis* A1, A2, and type B, however, provide a justification for the study of ChiB and ChiC mutants in animal models. Additionally, ecological niche modeling predicts that *F. tularensis* A1 and A2 populations occupy distinct habitats within the United States, and therefore, the identified differences in chitinases between these two populations may be required for maintenance and survival (31). Unlike *F. tularensis*, *F. novicida* is regarded as an environmental organism and only rarely causes infections within humans (17). The more complete repertoire and higher *in vitro* activities of *F. novicida* chitinases within the CS may reflect its ecological niche and utilization of chitin for nutrients.

These studies validated the hypothesis that genetic permutations of chitinases contribute to the phenotypic variability of *Francisella* species, subspecies, and populations, and link these differences to the surface proteome. Although only one surface associated chitinase was identified in Chapter III, truncation and inactivation of this chitinase in *F. tularensis* A2 represents a distinct difference in the surface proteomes of *F. tularensis* populations. It is also possible that the other chitinases alter the *F. tularensis* surface proteome, as they have signal peptides and are secreted in *F. novicida* (12). The observed variability in structure and function of the specific chitinases provides a foundation to investigate the role of these enzymes in the growth and survival of pathogenic *F. tularensis* subspecies and populations, and environmental strains of *F. novicida*.

5.5 Literature cited

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Chapter VI

Final Discussion and Future Directions

6.1 Introduction

Bacteria are the most physiologically diverse organisms on Earth, and are involved in seemingly infinite types of biological, physical, and chemical interactions. At the center of these complex interactions are proteins, whose roles may best be summarized as “the heart of all living processes” (79). *Francisella* biotypes encode a limited number of proteins compared to many other bacteria, yet these coccobacilli persist in diverse environments including water, soil, and over 300 different species of animals (7, 60). As described within this dissertation it is recognized that bacterial surface proteins influence niche occupations and host pathogen interactions, but prior to the characterization of the *F. tularensis* surface proteome described in Chapter III, only 10 proteins were localized to the surface of *F. tularensis* and *F. novicida* (46, 50, 53, 65). Nevertheless, the majority of these 10 previously identified proteins were documented to have roles in virulence, phenotypic variation, and immunity, emphasizing the importance of the *F. tularensis* surface proteome (3, 39, 50, 67, 75). In light of bioinformatic predictions and evidence regarding the surface proteomes of similar bacterial pathogens it was hypothesized that the majority of *F. tularensis* surface proteins were still unknown. The following discussion addresses the characterization of the *F. tularensis* LVS surface proteome, and provides insight into how these data were used to more completely understand the humoral response to effective *F. tularensis* vaccinations and the role of

surface associated chitinases in the phenotypic variability of *Francisella* species, subspecies, and populations.

6.2 The surface proteome of *F. tularensis* LVS

The characterization of the *F. tularensis* LVS surface proteome in Chapter III was a method development project that provided a platform amenable to the characterization of the surface proteomes of *F. tularensis* subspecies and populations and allowed for the evaluation of the hypothesis that the majority of *F. tularensis* surface proteins were unknown. This methodology was shown as a viable technique for surface proteome characterizations of *F. tularensis* A1a, A1b, and A2 populations. Further, the localization of eight previously identified surface proteins was confirmed and 28 new surface proteins were identified. The identification of additional *F. tularensis* surface proteins is significant given their roles in niche occupation, as immune targets, and in phenotypic variability of other bacterial pathogens (3, 39, 50, 67, 75). Importantly, the discovery of these proteins allowed for additional experiments to be conceived and conducted that examined unknown properties of the *F. tularensis* surface proteome. These experiments included determining the surface proteins targeted by the humoral responses of effective *F. tularensis* vaccination and biochemical characterizations of surface associated chitinases that were predicted to alter the phenotypes of *Francisella* biotypes

Characterizations of bacterial surface proteomes are a significant technical challenge due to issues such as inaccurate bioinformatic predictions, low abundance, hydrophobicity, and outer membrane associations (9, 57, 91). Given these obstacles, no single method is currently sufficient for positive identifications. In this dissertation, a surface protein biotinylation strategy was used as the primary tool for surface protein identification and was supported by bioinformatics, membrane protein identifications, and comparisons to previously reported membrane and secreted proteins. Although the

combination of these data provided a high degree of confidence for accurate surface protein identifications, other complementary techniques not addressed in Chapter III could have been beneficial. Perhaps the most useful of these would have been 2D-PAGE / Western blot and cryo-immunogold electron microscopy (14, 58). In the former, the combination of this procedure with MS could provide an unambiguous definition of the proteins labeled with biotin. Cryo-immunogold electron microscopy using anti-biotin probes, in contrast, could determine that biotinylation of proteins was specific to the cell surface. However, the usefulness of this technique could be compromised by the presence of naturally biotinylated cytosolic biotin carrier proteins produced by *F. tularensis*. The utility of cryo-immunogold electron microscopy characterization of surface proteins could be enhanced if monoclonal antibodies to specific proteins were used as probes, but this requires extensive resources that limit the practicality of this methodology.

Many of the proteins identified in Chapter III have physiological roles not fully understood in *F. tularensis*, but homologs in other bacteria provide insight to their functions. In addition to FopA (FTL_0583), two newly identified surface proteins; OmpA family protein (FTL_0325) and peptidoglycan-associated lipoprotein (FTL_0336) were predicted to contain OmpA_C-like domains. OmpA-like proteins often act as cell surface receptors, enhance conjugation frequency, participate in biofilm formation, and are host cell invasins in other Gram-negative pathogens (73). The *F. tularensis* OmpA family protein shares significant similarity with OmpA/MotB like proteins that form multi-protein complexes that are ion channels and the stator of the flagella motor of *E. coli*. (37). The peptidoglycan-associated lipoprotein of *F. tularensis* may act as a cell surface receptor, and although it remains to be demonstrated for living bacteria, this protein was recently shown to interact with plasminogen (13). Coating of the bacterial surface with plasminogen or the activated form of plasminogen, plasmin, could be an important

strategy used by *F. tularensis* for dissemination, host cell invasion, or to avoidance of recognition (13).

The close association of *F. tularensis* with phagocytic cells, particularly macrophages, suggests that this coccobacillus evolved mechanisms to efficiently cope with oxidative pressures. Antioxidant proteins produced by intracellular pathogens are often conserved and localized in CS (74, 78). However, in addition to being identified in the CS, two oxidative stress proteins, peroxidase/catalase (FTL_1504) and AhpC/TSA family protein (FTL_1015), were found on the surface of *F. tularensis* LVS (38). Peroxidase/catalase is perhaps the best understood of these oxidative stress proteins, where in pathogens, including *Mycobacterium tuberculosis* and *Salmonella enterica* serovar Typhimurium, it is an important virulence factor that detoxifies hydrogen peroxide produced by macrophages (28, 54). In contrast, AhpC/TSA family protein is a putative disulfide oxidoreductase that is predicted to detoxify reactive sulfur species (11, 48). Both of these antioxidant proteins are upregulated by *F. tularensis* growing in macrophages, and peroxidase/catalase knockouts show increased sensitivity to hydrogen peroxide (11, 45, 88). Surface localization of these proteins in *F. tularensis* may provide an additional barrier to reactive intermediates by concentrating antioxidant potential directly around the coccobacilli. A third surface protein, NAD/flavoprotein subunit protein has a putative role in oxidative protection. This protein contains a Pyr_redox superfamily domain identified in NADH peroxidases, but its mitigation of the oxidative stress response has not been determined in *Francisella* (48).

An increasing number of proteins with defined functions are now known to have additional roles depending on their subcellular localizations or differing environmental conditions (56). Future evaluations of the *F. tularensis* surface proteome should consider the enzymatic and structural contributions of multifunctional proteins. The production of these so called “moonlighting proteins” may be an important strategy used

by bacteria such as *F. tularensis* to cope with diverse and changing environments given extensive genomic decay. Proteins involved in central metabolism or chaperones are among the most intensively evaluated multifunctional proteins. While surface localized, many of these proteins are involved in immune modulation, cell adhesion, and host factor binding (4, 8, 33, 89). Not unexpectedly, proteins including glyceraldehyde-3-phosphate dehydrogenase (FTT_1368), GroEL (FTT_1696), and DnaK (FTT_1269) were identified on the surface of *F. tularensis* LVS where they may play analogous moonlighting roles. It is now recognized that a much broader spectrum of proteins have multiple functions and include proteins with known roles in transcription, translation, signal transduction, proteolysis, DNA binding, and cell wall biosynthesis. (29, 31). Of the additional types of multifunctional proteins, the most prominent representatives detected on the surface of *F. tularensis* were involved in translation. These included two elongation factors and three ribosomal proteins. Elongation factors are surface associated in other bacteria such as *Lactobacillus, Mycoplasma,* and *Pseudomonas* species and act as adhesions and bind host factors (24, 40). The role for surface associated ribosomal proteins is unclear, but while localized in the cytosol a variety of alternative functions including regulation of ribosome synthesis and cell stress responses are described (86).

A portion of surface proteins identified in Chapter III were classified as hypothetical proteins with no known function. The characterization or at least partial definition of these proteins will be a necessary step to develop a sufficient understanding of the physiological processes governed by the *F. tularensis* surface proteome. The importance of hypothetical proteins in *F. tularensis* biology is emphasized by the recently described virulence factors, hypothetical proteins FTT_1103 and FTT_0918 (FupA) (61, 64). The homolog of hypothetical protein FTT_1103 was identified on the surface of *F. tularensis* LVS, and when this gene was disrupted in *F. tularensis* SCHU S4, the

bacteria were greatly attenuated in mice and showed deficiencies in HepG2 cells for intracellular replication and phagosomal escape (61). Siderophore dependent and independent uptake of iron in *F. tularensis* is mediated by hypothetical protein FTT_0918 (44). Although the homolog of this protein was not surface localized in *F. tularensis* LVS, likely due to a C-terminal truncation, in *F. tularensis* SCHU S4 it is an outer membrane protein that is likely surface associated (32). In addition to identifying new virulence factors, unique structural conformations may be observed in hypothetical surface proteins and link to unique functions. The surface protein IglC (FTT_1712) was recently annotated as a hypothetical protein and described to form a unique β-sandwich plate conformation (76). The precise mechanism of IglC has yet to be determined, but this protein is essential for bacteria to escape the phagosome (43, 66). Additionally, several of the hypothetical surface proteins identified in Chapter III are conserved in other species. Therefore further investigation in *F. tularensis* could support physiological evaluations of a wide array of organisms.

Another objective of the expanded characterization the *F. tularensis* LVS surface proteome was to identify proteins that might contribute to the phenotypic variability of *F. tularensis* biotypes. A comparison of the CDS (the numbers of amino acids) between *F. tularensis* subspecies and populations identified numerous differences in surface proteins (Appendix 6). Of the 38 known *F. tularensis* surface proteins, 22 differ in size compared to *F. tularensis* LVS in at least one other *F. tularensis* subspecies or population. This includes the surface chitinase, ChiA (FTT_0715), which was shown in Chapter V to contribute to differential chitinase activities of *F. tularensis* biotypes. Extensive variations were noted in several other surface proteins. Outer membrane protein (FTL_0009), glutamate dehydrogenase (FTL_0269), 50S ribosomal protein L7/L12 (FTL_1745), and succinate dehydrogenase, catalytic and NAD/flavoprotein subunit (FTL_1786) are substantially truncated in *F. tularensis* A1b. The gene encoding

hypothetical protein (FTL_1225) is highly eroded in *F. tularensis* A2. Glyceraldehyde-3-phosphate dehydrogenase (FTL_1146) and elongation factor Tu (FTL_1751) are fused with adjacent genes in *F. tularensis mediasiatica* and *F. tularensis* A1b, respectively. These changes could alter the function of the specific surface protein, and also impact downstream pathways. Further, single nucleotide polymorphisms and novel CDS may contribute to variability *of F. tularensis* surface protein function. The impact of single nucleotide polymorphisms in altering the function of *Francisella* surface proteins is already recognized. A single amino acid substitution in hypothetical protein FsaP (FTL_1658) of *F. novicida* disrupts its translocation by Sec and abrogates *F. novicida* attachment to human A549 epithelial cells (50).

6.3 The immunoreactive surface proteins of *F. tularensis*

Bacterial molecules naturally recognized by hosts during infection are often surface proteins with immunogenic properties (5, 69). This observation was confirmed by the use of surface proteins in vaccines effective against pathogens including serogroup B *N. meningitidis*, *C. pneumonia*, *S. pyogenes* and *S. agalactiae* (20, 47, 62, 81). Given the role of surface proteins in vaccinology, and that there is no licensed vaccine for *F. tularensis*, the surface proteins recognized by the humoral response to vaccination with live *F. tularensis* LVS (the most effective *F. tularensis* vaccine) were determined in Chapter IV. Using MPF as a surface protein surrogate, *F. tularensis* LVS immune sera recognized a total of 17 antigens, 10 of which were localized to the surface in Chapter III. Similarly, proteins recognized by the host after immunization with marginally effective adjuvanted MPF preparations were determined. Surface proteins were recognized, but the humoral response targeted many more antigens compared to *F. tularensis* LVS vaccination, and the majority of these were not surface associated. Therefore, expanded humoral recognition to non-surface proteins does not correlate to

enhanced protection. These data suggest that the select set of surface proteins recognized by *F. tularensis* LVS are protective surface protein immunogens.

Vaccines designed with surface proteins typically rely on the humoral immune response to initiate antibody dependent mechanisms such as complement or phagocytosis mediated killing by macrophages and neutrophils (35). Thus, experiments to determine the ability of the *F. tularensis* surface protein antigens to stimulate these mechanisms will be required. However, there may be additional protective properties of antibodies that must be considered. In the case of *F. tularensis* it is particularly important to evaluate how antibodies control bacteria with an intracellular lifecycle. Antibodies could target bacteria during an extracellular phase, and in addition to the classic extracellular killing mechanisms, they may block or inhibit cell surface invasins or bacterial secretion systems, attenuating the pathogen (51). Antibodies may also have a direct impact on intracellular bacteria. An antibody able to neutralize the listeriolysin O toxin of *L. monocytogenes* was found to accumulate in *L. monocytogenes* infected macrophages where it attenuated growth of this bacteria (17). Further, the production of some proinflammatory cytokines can be influenced by antibodies (42).

Immunity to *F. tularensis* infection is most effective when both the humoral and cell mediated responses are elicited (18, 35, 68). Thus, new tularemia vaccines will need to contain the components needed to activate both arms of adaptive immunity. In addition to *F. tularensis* surface proteins identified as being antigens recognized by the humoral response, they also stimulate cell mediated immunity. TUL4 (FTT_0901) and outer membrane protein FopB (FTT_1747) were both shown to be surface associated in Chapter III, and are the only well described T cell antigens (65, 72, 90). T cell recognition of these proteins leads to the production of IFN-γ and TNF-α that elicit important effector mechanisms to control *F. tularensis* infections (18). Both IFN-γ and TNF-α production is interlinked with the activation of macrophages and their ability to kill

intracellular bacteria (18). However, macrophages have little effect on non-opsonized *F. tularensis* (34, 35). Therefore, the production of opsonizing antibodies has a synergistic relationship with cell mediated killing. A protective response may involve innate immune mechanisms that work independently or that support the adaptive response. For example, IFN-γ production by T cells and natural killer cells can be driven by activation of TLR-2 (1, 35). The surface proteins TUL4 and lipoprotein (FTT_1103) are the only two *F. tularensis* TLR-2 ligands. These observations clearly show the importance of *F. tularensis* surface proteins in immunity, and how they function to bridge immune responses.

Surface proteins to be included in an effective subunit vaccine against tularemia must consider several factors relevant to the biology of *F. tularensis*. 1) As discussed above, the surface proteins of *F. tularensis* interact with the host in a variety of ways to produce differing types of immune responses. It is likely that multiple surface protein immunogens are required for protection. This could explain the limited effectiveness of previously evaluated vaccines that contained only one or two surface proteins (19, 21, 71). 2) Protection afforded by *F. tularensis* LVS vaccination to infection with *F. tularensis* type A strains indicates a number of surface protein immunogens are conserved between *F. tularensis* subspecies. Nonetheless, the inclusion of *F. tularensis* subspecies and population specific immunogens could have a significant positive impact on vaccine efficacy. In particular, the repertoire of *F. tularensis* type A1 surface protein immunogens should be identified due to the more severe disease outcomes associated with this population. Such studies could be accomplished using the methodologies described in Chapters III and IV. 3) A subunit vaccine should contain proteins produced by bacteria *in vivo*. *F. tularensis* surface proteomes could vary *in vivo* compared to bacteria grown *in vitro*. The abundances of particular proteins may differ, and the *in vivo* surface proteome of *F. tularensis* may contain proteins not produced or surface localized

in bacteria grown on laboratory medium, or vice versa. As a possible example, transcriptional analyses suggest that production of the surface associated, acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit (FTL_1592), could be diminished *in vivo* (23). Although this protein is a frequently identified antigen, its decreased presence on *in vivo* bacteria may correlate to poor immunogenicity. Additional transcriptional analyses as well as *in vivo* surface protein and antigen profiling could identify new immunogens, and help to better delineate immunogen candidates.

6.4 Differential chitinases of *F. tularensis* and *F. novicida*

The surface associated chitinase ChiA (FTT_0715) of *F. tularensis*, along with other putative chitinases that bare signatures of surface localization, ChiB (FTT_1768), ChiC (FTT_1592 / FTT_1593), and ChiD (FTT_0066), were shown in Chapter V to contribute to the phenotypic variability of *Francisella* species, subspecies, and populations. Specifically, the greatest chitinase activities were associated with *F. novicida* which can encode as many as three functional chitinases. Compared to *F. novicida*, chitinase activities of *F. tularensis* strains were substantially lower. In *F. tularensis* the highest activity was detected in *F. tularensis* A2 populations that produce a single, but highly active chitinase, ChiC. Little activity was detected in *F. tularensis* type A1 strains that encode a functional ChiA and ChiB, but these strains only produced a weakly active ChiA *in vitro*. The chitinase activities of *F. tularensis* type B strains was greater than *F. tularensis* A1, less than *F. tularensis* A2, and were linked to the production of ChiA and ChiB..

The enzymatic action of the *F. tularensis* and *F. novicida* chitinases is a result of GH18 domains identified in all functional chitinases. However, despite a similar enzymatic domain, disparate kinetics and substrate specificities between ChiA, ChiB, ChiC, and ChiD were detected. Differences in enzymatic activities of GH18 domains are

common, and in accordance with the benefits of a single organism's ability to produce multiple chitinases with dissimilar activities to synergistically process chitin into monomers or small oligosaccharides (16, 27, 77). Enzymatic variability is also attributed to the different crystalline configurations and molecular associations of chitin found in nature (52). Surprisingly, although ChiD was predicted to be a functional chitinase containing an intact GH18 domain, it was inactive in all *Francisella* biotypes against the chitin analogs evaluated in Chapter V. Closer bioinformatic inspection of the predicted active site, 487-NFDLS-491, identified amino acid substitutions from the expected motif of DXDXE, where X usually indicates aromatic or aliphatic amino acid (30). Glutamate-491 to serine and aspartic acid-487 to asparagine substitutions are likely responsible for inactivation of the ChiD GH18 domain (30). The identification of apparently inactivated GH18 chitinases is not unusual, and the primary role of these chitinase-like proteins is hypothesized to be chitin binding (55). It could be informative to mutate the active site of ChiD to a highly conserved "DIDLE" motif found in ChiD homologs to determine if this protein has chitinolytic potential. If chitinase activity is restored, it would likely differ from other *Francisella* chitinases given that ChiD's typically function as *N*-acetylglucosaminidases (2). This form of chitinase activity could allow *Francisella* to survive in novel niches, and the loss of ChiD enzymatic activity could provide insight on the evolutionary history of *Francisella*. Nevertheless, *chiD* is present in all *Francisella* genomes suggesting it has an important function.

The *F. tularensis* chitinases contained several non-enzymatic accessory domains including *N*-acetylglucosamine-binding protein A, carbohydrate binding, and fibronectin type 3 domains that putatively facilitate adherence to or the depolymerization of chitin (6, 36, 87). Bioinformatic analyses of *Francisella* genomes also identified two non-chitinases with accessory domains that may contribute to the activity or function of chitinases. A chitin binding protein (FTT_0816) was identified in *F. tularensis* and *F.*

novicida genomes and contained a chitin_bind_3 superfamily domain. This domain was recently shown to enzymatically introduce chain breaks in crystalline chitin by a newly described mechanism (85). However, the specifics of catalysis are not well understood (85). The chitin_bind_3 domain shares structural similarities with GH61 domains that are found in fungi, thus proteins containing this domain may eventually be classified as chitinases (26). Another non-chitinase protein, hypothetical protein (FTT_1577), was identified that contained putative chitin binding domains similar to those identified in the functional *Francisella* chitinases.

Although multiple chitinolytic proteins are encoded, not all are needed in every *F. tularensis* subspecies and population. Genes encoding *chiB* and *chiC* are altered in *F. tularensis* A1 and their products were not detectable during *in vitro* growth. The proposed GH61-like "chitinase" of *F. tularensis* A1 (FTT_0816) is truncated by nearly 300 amino acids. *F. tularensis* A2 *chiA* and *chiB* are truncated and do not encode the enzymatic GH18 domain, while the ChiC GH18 domain is partially truncated in *F. tularensis* type B, rendering the enzyme inactive. The erosion of multiple chitinases in *F. tularensis* genomes may coincide with the phenomena of genomic reduction that is observed in many intracellular pathogens (10). Genomic reduction of *F. tularensis* chitinases is best illustrated by examining the genome of *F. philomiragia*. Other types of GH domains act as chitinases, and *F. philomiragia* encodes for two putative chitinases with GH19 and GH20 domains (16). There are remnants of this putative GH20 chitinase in *F. tularensis* and *F. novicida* genomes (FTL_0869), but the gene is extensively truncated compared to *F. philomiragia* (79, 79, and 813 amino acids, respectively). A putative GH19 chitinase is not present in either *F. tularensis* or *F. novicida*. The more complete repertoire of chitinases in *F. philomiragia* is not unexpected given the ties of this bacterium to aquatic environments where chitin is abundant and is frequently used as a nutrient source (70). However, the reduction of chitinolytic genes is also apparent

in *F. philomiragia*. The gene encoding for the putative GH20 chitinase is split in *F. philomiragia* ATCC 25017 but not *F. philomiragia* ATCC 25015.

It is difficult to speculate whether the different chitinase repertoires confer an advantage to their respective *F. tularensis* populations. In most cases, the chitinases from each class were shown in Chapter V to have similar kinetics regardless of the *F. tularensis* or *F. novicida* parent strain. Based on this observation, it could be argued that the chitinases did not evolve differently between *Francisella* populations to act on unique substrates. However, only a small number of chitin analogs were tested in Chapter V, thus differences in kinetics may be detectable using other substrates. The ChiB and ChiC chitinases of *F. tularensis* A1 displayed the greatest differences in activity compared to their respective archetype chitinases, possibly indicating some degree of enzyme differentiation or specialization. These two chitinases were not detectable in *F. tularensis* A1 strains *in vitro,* but may be produced under differing circumstances. Therefore, closer evaluations of *F. tularensis* A1 ChiA and ChiB substrate specificities and studies to evaluate the regulators of chitinase expression could provide useful information for determining the biological roles of these enzymes. Given the synergistic activities of chitinases, an alternative explanation for the differing chitinase repertoires is that multiple types of chitinases are required to process a particular substrate. In this regard, different chitinase combinations might expand or retract substrate specificities.

One of the most striking observations resulting from *in vitro* growth of *F. tularensis* and *F. novicida* was the differential localization of chitinase activities between these two species. All of the chitinases identified in this study, except those with N-terminal truncations, were identified with signal peptides. However, activity of *F. tularensis* chitinases was associated with WCL, whereas the chitinase activity in *F. novicida* was detected in CS. The identification of secreted chitinases is consistent with

the utilization of chitin for nutrients. Bacteria do not synthesize chitin and are incapable of internalizing large polymers of chitin, thus many chitinases process chitin into short oligosaccharides extracellulary (52). In accordance with the function of secreted chitinases, *F. novicida* forms biofilms on chitin and can use chitin as a sole source of carbon (49). An intracellular role of *F. tularensis* chitinases is more ambiguous. Intracellular chitinolytic enzymes are identified in the periplasm of Gram-negative bacteria, but these are usually involved in the processing of chitibiose into *N*-acetylglucosamine (82). When tested against a chitibiose-like analog, the *Francisella* chitinases were inactive. Intracellular localization of *F. tularensis* chitinases could be an artifact defective secretion involving the secretion apparatus or regulatory components of secretion. Multiple genes whose products are predicted to be involved with secretion, transport, and regulation are differentially altered in *F. tularensis* subspecies and populations compared to *F. novicida* (12). A final possibility is that subtle changes of *F. tularensis* chitinase structures make the proteins incompatible for secretion. Studies that investigate the subcellular localizations of *F. tularensis* chitinases could provide useful information to help determine an unknown role or substrate for these enzymes.

Given that 1) ChiA was the most highly upregulated protein in a murine infection model; 2) ChiB was the most highly upregulated protein in response to a temperature shift from 37°C to 44°C; 3) the repertoire of chitinases differs between *Francisella* species, subspecies, and populations; and 4) that virulence and vector survival roles have been described for the chitinases of human pathogens, it is likely that chitinases play important and possibly dichotomous roles in the biology *Francisella* biotypes (12, 23, 83, 84). In addition to the upregulation of ChiB, increased production of ChiA may be associated with a temperature up-shift. *In vivo* upregulation of ChiA was determined by examining bacteria isolated from the spleen of mice collected in a latter stage of infection when the potential for fever is high (84). Thus, the increased production of

these chitinases could be related to the colonization of a new host before the animal succumbs to infection. Often the next host is a biting arthropod, and after ingestion of the *Francisella*-containing blood meal, the food bolus is typically surrounded by peritrophic membrane containing chitin (41, 59). Accordingly, the modification of the peritrophic membrane by chitinases is an important aspect to the transmission cycle of the malaria parasite *P. falciparum* (41, 83). Another explanation for the presence of at least one functional chitinase in each *Francisella* species, subspecies, and population is this bacterium's association with ciliates and amoeba that synthesize chitin (22, 25, 80). Chitin synthesis in protozoans is thought to occur either at the cell surface or possibly in specialized vesicles called chitosomes (63). In this scenario chitinases may be analogous to proteins such as plasmin that degrade components of the extracellular matrix and enhance the invasiveness of some bacterial pathogens (15). Further, the chitosome may represent a niche exploitable by *F. tularensis*, where chitin and its precursors may be processed by chitinases for nutrients.

6.5 Conclusions

In conclusion, three aspects of *F. tularensis* biology that are directly related to the bacterium's surface proteins were evaluated in this dissertation. An expanded description of the surface proteome provided experimental evidence for the surface localization of 36 proteins, 28 of which are newly identified to the surface of *F. tularensis*. This work also identified the surface protein antigens recognized by the most effective tularemia vaccine and correlated expanded antigen recognition of non-surface proteins to decreased efficacy of subunit vaccines. The identification of naturally recognized surface protein antigens supports future studies seeking to develop a safe and effective subunit vaccine. Finally, the first biochemical characterization of the surface exposed *F. tularensis* chitinase, ChiA, was performed. Given the synergistic action of chitinases,

and their likely surface associations, biochemical analyses were extended to other putative chitinases identified in *F. tularensis* subspecies and populations and *F. novicida*. Genetic differences in chitinases between *Francisella* biotypes translated into biochemically distinct phenotypes. The variations in chitinase repertoires and their activities may be relevant to the different niche occupations and virulence characteristics of *Francisella* species, subspecies, and populations.

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Appendix 1

Francisella vaccines, adapted from Pechous *et al*.

***Francisella* vaccines, adapted from Pechous *et al*. (146)**

Subunit vaccine

Model	Antigen	Adjuvant	Vaccination route (dose µg)	Challenge (dose, CFU)	Boost	Prot.	ref
C3H/HeN	Ethanol-inactivated LVS	Freund's adjuvant	i.p. (20)	SCHU S4, i.n. (40)	No	Yes	(82)
C3H/HeN	LVS LPS	Freund's adjuvant	i.p. (20)	SCHU S4, i.n. (40)	No	No	(82)
C3H/HeN	Outer membrane proteins	Freund's adjuvant	i.p. (20)	SCHU S4, i.n. (40)	No	Yes	(82)
BALB/c	LPS (smooth)	Freund's adjuvant	s.c. (50)	LVS, i.p. (2.5×10^6)	Yes	Yes	(61)
BALB/c	Outer membrane preparation	Freund's adjuvant	s.c. (100)	LVS, i.p. (2.5×10^6)	Yes	Yes	(61)
BALB/c	LVS LPS	Bovine serum albumin	s.c. (20)	type B no. 108, i.d. (80)	No	Yes	(39)
BALB/c	LVS LPS	Bovine serum albumin	s.c. (20)	type B no. 108, Aerosol (30)	No	No	(39)
BALB/c	LVS LPS	Bovine serum albumin	s.c. (20)	type A no. 33, i.d. (60)	No	No	(39)
BALB/c	LVS LPS	Bovine serum albumin	s.c. (20)	type A no. 33, Aerosol (50)	No	No	(39)

Live attenuated vaccines

Species or subspecies	Model	Mutated locus	Vaccination route (dose, CFU)	Challenge (dose, CFU)	Boost	Prot.	ref
F. novicida	BALB/c	None (wt)	i.d. (100)	FSC033, Aerosol (10)	No	No	(182)
	C57BL/6	FTN_1645	i.n. (149)	U112, i.n. (25)	No	Yes	(99)
	BALB/c	FTN_0178	i.p. (3.3×10^6)	U112, i.p. (170)	No	No	(156)
	BALB/c	FTN_1700	i.p. (6.95×10^3)	U112, i.p. (170)	No	Yes	(156)
	BALB/c	FTN_1700	i.p. (6.95×10^3)	SCHU S4, i.p. (100)	No	No	(156)
	BALB/c	FTN_1465	i.n. (10^6)	U112, i.n. (10^6)	No	Yes	(127)
	BALB/c	FTN_1465	i.n. (10^6)	SCHU S4, i.n. (100)	No	No	(127)
	BALB/c	FTN_1608	i.n. (6×10^5)	U112, i.n. (6×10^7)	No	Yes	(196)
	BALB/c	FTN_0714	i.n. (6×10^7)	U112, i.n. (6×10^7)	No	Yes	(196)
	BALB/c	FTN_1310	i.n. (6×10^7)	U112, i.n. (6×10^7)	No	Yes	(196)
	BALB/c	FTN_0337	i.n. (6×10^5)	U112, i.n. (6×10^7)	No	Yes	(196)
	BALB/c	FTN_0020	i.n. (6×10^3)	U112, i.n. (8×10^5)	No	Yes	(196)
	BALB/c	FTN_1322	i.n. (10^6)	U112, i.n. (10^3)	No	Yes	(142)
	BALB/c	FTN_1322	i.n. (10^6)	U112, i.n. (10^4)	No	Yes	(142)
	BALB/c	FTN_1322	i.n. (10^6)	U112, i.n. (10^5)	No	No	(142)
	BALB/c	FTN_1323	i.n. (10^6)	U112, i.n. (10^3)	No	Yes	(37)
	BALB/c	FTN_1323	i.n. (10^6)	LVS, i.n. (3×10^4)	No	Yes	(37)
	BALB/c	FTN_1323	Oral (10^3)	SCHU S4, i.n. (52)	Yes	No	(37)
	C57BL/6	FTN_1290	Aerosol (10^5)	U112, Aerosol ($35\text{-}38 \times 10^4$)	No	No	(215)
	C57BL/6	FTN_1290	Aerosol (10^5)	U112, Aerosol ($35\text{-}38 \times 10^4$)	No	No	(215)
	BALB/c	FTN_0090 FTN_1556 FTN_1061 FTN_0954	i.n. (10^3)	U112, i.n. (10^6)	No	Yes	(128)
	BALB/c	FTN_0090 FTN_1556 FTN_1061 FTN_0954	i.n. (10^6)	U112, i.n. (10^6)	No	Yes	(128)
	BALB/c	FTN_0090 FTN_1061 FTN_0954	i.n. (10^3)	U112, i.n. (10^3)	No	No	(128)

Live attenuated vaccines, continued

Species or subspecies	Model	Mutated locus	Vaccination route (dose, CFU)	Challenge (dose, CFU)	Boost	Prot.	ref
F. novicida	BALB/c	FTN_1061 FTN_0954	i.n. (10^3)	U112, i.n. (10^3)	No	No	(128)
	BALB/c	FTN_0546	s.c. (400-500)	U112, s.c. (660)	No	Yes	(91)
	C57BL/6	FTN_0546	s.c. (400-500)	U112, s.c. (660)	No	Yes	(91)
	BALB/c	FTN_0546	Aerosol (100)	U112, i.n. (50)	No	Yes	(91)
F. tularensis subsp. *tularensis* (type A)	C57BL/6	FTT_0107	i.n. (6.8 x 10^3)	SCHU S4, i.n. (13- 1.3 x 10^4)	No	No	(154)
	BALB/c	FTT_0893 FTT_0894	i.n. (10^4)	SCHU S4, i.n. (100)	No	No	(145)
	BALB/c	FTT_0893 FTT_0894	i.n. (10^4)	SCHU S4, i.n. (2 x 10^3)	No	No	(145)
	BALB/c	FTT_0893 FTT_0894	i.n. (10^4)	SCHU S4, i.d. (100)	No	Yes	(145)
	BALB/c	FTT_0893 FTT_0894	i.n. (10^4)	SCHU S4, i.d. (2 x 10^3)	No	Yes	(145)
	BALB/c	FTT_0893 FTT_0894	i.n. (10^4)	SCHU S4, i.n. (100)	Yes	Yes	(145)
	BALB/c	FTT_0893 FTT_0894	i.n. (10^4)	SCHU S4, i.n. (2 x 10^3)	Yes	No	(145)
	BALB/c	FTT_0893 FTT_0894	i.n. (10^4)	SCHU S4, i.d. (100)	Yes	Yes	(145)
	BALB/c	FTT_0893 FTT_0894	i.n. (10^4)	SCHU S4, i.d. (2 x 10^3)	Yes	Yes	(145)
	BALB/c	FTT_0918	i.d. (10^5)	FSC033, i.d. (500)	No	Yes	(204)
	BALB/c	FTT_0918	i.d. (10^5)	FSC033, Aerosol (10)	No	Yes	(204)
	BALB/c	FTT_1357 FTT_1712	i.d. (10^6-10^7)	FSC033, i.d. (500)	No	No	(204)
	BALB/c	FTT_1357 FTT_1712	i.d. (10^6-10^7)	FSC033, Aerosol, (10)	No	No	(204)
	BALB/c	FTT_1459 FTT_1460 FTT_1461	s.c. (10^5)	SCHU S4, s.c. (100)	No	No	(199)
	BALB/c	FTT_1103	i.n. (1.6 x 10^8)	SCHU S4, i.n. (1 x 10^3)	No	Yes	(155)
	C57BL/6	FTT_1103	i.n. (1.6 x 10^8)	SCHU S4, i.n. (1 x 10^3)	No	Yes	(155)
	BALB/c	FTT_0805	s.c. (10^4)	SCHU S4, s.c. (10^3)	No	Yes	(125)
F. tularensis subsp. *holarctica* (type B)	BALB/c	None (wt)	i.n. (200)	SCHU S4, i.n. (100)	No	Yes	(145)
	BALB/c	None (wt)	i.n. (200)	SCHU S4, i.n. (2 x 10^3)	No	No	(145)
	BALB/c	None (wt)	i.n. (200)	SCHU S4, i.d. (100)	Yes	Yes	(145)
	BALB/c	None (wt)	i.n. (200)	SCHU S4, i.d. (2 x 10^3)	Yes	Yes	(145)
	BALB/c	FTL_0094	i.p. (13)	LVS, i.p. (1 x 10^4)	No	Yes	(121)
	BALB/c	FTL_0094	i.p. (1.3 x 10^3)	LVS, i.p. (1 x 10^4)	No	Yes	(121)
	BALB/c	FTL_0395 FTL_0396	i.n. (10^6)	SCHU S4, i.n. (100)	No	No	(145)
	BALB/c	FTL_0395 FTL_0396	i.n. (10^6)	SCHU S4, i.n. (2 x 10^4)	No	No	(145)
	BALB/c	FTL_0395 FTL_0396	i.n. (10^6)	SCHU S4, i.d. (100)	No	No	(145)
	BALB/c	FTL_0395 FTL_0396	i.n. (10^6)	SCHU S4, i.d. (2 x 10^4)	No	No	(145)
	BALB/c	FTL_0395 FTL_0396	i.n. (10^6)	SCHU S4, i.n. (100)	Yes	Yes	(145)
	BALB/c	FTL_0395 FTL_0396	i.n. (10^6)	SCHU S4, i.n. (2 x 10^4)	Yes	No	(145)

Live attenuated vaccines, continued

Species or subspecies	Model	Mutated locus	Vaccination route (dose, CFU)	Challenge (dose, CFU)	Boost	Prot.	ref
F. tularensis subsp. *holarctica* (type B)	BALB/c	FTL_0395 FTL_0396	i.n. (10^6)	SCHU S4, i.d. (100)	Yes	Yes	(145)
	BALB/c	FTL_0395 FTL_0396	i.n. (10^6)	SCHU S4, i.d. (2×10^4)	Yes	Yes	(145)
	BALB/c	FTL_0552	i.n. (10^5)	SCHU, i.n. (200)	No	Yes	(167)
	C57BL/6	FTL_0552	i.n. (10^5)	SCHU, i.n. (200)	No	Yes	(167)
	C3H/HeN	FTL_0421	i.d. (10^5)	LVS, i.d. (10^7)	No	Yes	(50)
	BALB/c	FTL_1071	i.p. (2.2×10^7)	LVS, i.p. (2.8×10^5)	No	Yes	(171)
	BALB/c	FTL_1478	i.p. (3.6×10^7)	LVS, i.p. (2.8×10^5)	No	Yes	(171)
	C57BL/6	FTL_1793	i.n. (5×10^4)	SCHU S4, i.n. (14)	No	Yes	(7)
	C57BL/6	FTL_1793	i.n. (500)	LVS S4, i.n. (104)	Yes	Yes	(7)
	C57BL/6	FTL_1793	i.n. (500)	SCHU S4, i.n. (104)	Yes	No	(7)
	BALB/c	FTL_0592	i.d. (1.5×10^8)	FSC 108, i.d. 17	Yes	Yes	(181)
	BALB/c	FTL_0592	i.d. (1.5×10^8)	SCHU S4, i.d. 10	Yes	Yes	(181)
	BALB/c	FTL_1416	i.n. (1×10^5)	SCHU S4, Aerosol (10x LD_{50})	No	Yes	(86)
	BALB/c	FTL_1416	i.d. (1×10^6)	SCHU S4, Aerosol (10x LD_{50})	No	No	(86)
	BALB/c	FTL_1416	i.n. (1×10^5)	LVS, i.n. (4×10^3)	No	Yes	(86)
	BALB/c	FTL_1416	i.d. (1×10^6)	LVS, i.n. (4×10^3)	No	Yes	(86)

Heterologous host vaccines producing *Francisella* antigens

Model	Antigen	Host	Vaccination route (dose, CFU)	Challenge (dose, CFU)	Boost	Prot.	ref
BALB/c	FTT_1357, FTT_1712	*Listeria monocytogenes* Δ*actA*	i.d. (10^6-10^7)	SCHU S4, Aerosol (1 [10 LD_{50}])	Yes	Yes	(87)
BALB/c	FTT_1357, FTT_1712	*Listeria monocytogenes* Δ*actA*	i.d. (10^6-10^7)	LVS, i.n. (4.4×10^3)	Yes	Yes	(87)
BALB/c	FTT_0721	*Listeria monocytogenes* Δ*actA*	i.d. (10^6-10^7)	SCHU S4, Aerosol (1 [10 LD_{50}])	Yes	No	(87)
BALB/c	FTT_0721	*Listeria monocytogenes* Δ*actA*	i.d. (10^6-10^7)	LVS, i.n. (4.4×10^3)	Yes	No	(87)
BALB/c	FTT_0221	*Listeria monocytogenes* Δ*actA*	i.d. (10^6-10^7)	LVS, i.n. (4.4×10^3)	Yes	No	(87)
BALB/c	FTT_1441	*Listeria monocytogenes* Δ*actA*	i.d. (10^6-10^7)	LVS, i.n. (4.4×10^3)	Yes	No	(87)
BALB/c	FTT_1269	*Listeria monocytogenes* Δ*actA*	i.d. (10^6-10^7)	LVS, i.n. (4.4×10^3)	Yes	No	(87)
BALB/c	FTT_1696	*Listeria monocytogenes* Δ*actA*	i.d. (10^6-10^7)	LVS, i.n. (4.4×10^3)	Yes	No	(87)
BALB/c	FTT_0997	*Listeria monocytogenes* Δ*actA*	i.d. (10^6-10^7)	LVS, i.n. (4.4×10^3)	Yes	No	(87)
BALB/cj	FTT_0901	*Salmonella enterica* serovar Typhimirium Δ*asd* Δ*cya* Δ*crp*	i.p. (5×10^3)	LVS, i.v. (160-230)	No	No	(186)

Heterologous host vaccines producing *Francisella* antigens, continued

Model	Antigen	Host	Vaccination route (dose, CFU)	Challenge (dose, CFU)	Boost	Prot.	ref
BALB/c	FTT_0583	*Salmonella enterica* serovar Typhimirium Δ*aroA*	i.v. (10^7)	LVS, i.p. (10^1-2.5 x 10^6)	Yes	No	(61)

Prot. = protection, i.d. = intradermally, i. n. = intranasally, i.p. = intraperitoneally, i.v = intravenously, s.c. = subcutaneously.
References correspond to those of Chapter I.

Appendix 2

F. tularensis LVS proteins containing predicted signal peptides and beta barrels

***F. tularensis* LVS proteins containing predicted beta barrels and signal peptides**

Protein	Locus
Hypothetical protein	FTL_0147
TolB protein precursor	FTL_0334
Hypothetical protein	FTL_0439
Soluble lytic murein transglycosylase	FTL_0466
Hypothetical protein	FTL_0469
Hypothetical protein	FTL_0574
FAD-binding family protein	FTL_0645
VacJ lipoprotein	FTL_0765
Hypothetical protein	FTL_0823
Hypothetical protein	FTL_0867
Hypothetical protein	FTL_0994
Hypothetical protein	FTL_1107
Outer membrane associated protein	FTL_1328
Chitinase family 18 protein	FTL_1521
Putrescine-binding periplasmic protein	FTL_1582
Hypothetical protein	FTL_1646
Hypothetical protein	FTL_1689
Hypothetical protein	FTL_1694
Hypothetical protein	FTL_1836
Hypothetical protein	FTL_1852
Outer membrane lipoprotein	FTL_1897

***F. tularensis* LVS proteins containing predicted beta barrels, but lacking signal peptides**

Protein	Locus
Hypothetical protein	FTL_0116
Acetyltransferase	FTL_0455
Glycogen branching enzyme	FTL_0483
Hypothetical protein	FTL_0752
Lipoprotein	FTL_0784
Lipoprotein	FTL_0841
Hypothetical protein	FTL_0862
Hypothetical protein	FTL_0998
Hypothetical protein	FTL_1002
Hypothetical protein	FTL_1005
Hypothetical protein	FTL_1162
Hypothetical protein	FTL_1327
Lipoprotein	FTL_1372
Organic solvent tolerance protein	FTL_1374
FAD-binding family protein	FTL_1489
Hypothetical protein	FTL_1629
16S rRNA processing protein RimM	FTL_1737
2-oxoglutarate dehydrogenase E1 component	FTL_1784
Hypothetical protein	FTL_1840
Outer membrane associated protein, fragment	FTL_1876

***F. tularensis* LVS proteins containing predicted signal peptides, but not beta barrels**

Protein	Locus
Peptidase	FTL_0008
Outer membrane protein	FTL_0009
Phosphate acetyltransferase	FTL_0016
Acid phosphatase	FTL_0031
Hypothetical protein	FTL_0035
Hypothetical protein	FTL_0039
Chorismate mutase	FTL_0043
Aromatic amino acid HAAP transporter	FTL_0058

***F. tularensis* LVS proteins containing predicted signal peptides, but not beta barrels, continued**

Protein	Locus
Major facilitator transporter	FTL_0063
Outer membrane lipoprotein	FTL_0069
Hypothetical protein	FTL_0073
Acetyltransferase protein	FTL_0088
Chitinase	FTL_0093
Voltage-gated ClC-type chloride channel clcA	FTL_0101
Hypothetical protein	FTL_0102
Hypothetical protein	FTL_0103
Hypothetical protein	FTL_0104
Hypothetical protein	FTL_0105
Hypothetical protein	FTL_0108
Hypothetical protein	FTL_0115
Hypothetical protein	FTL_0124
CrcB family protein	FTL_0141
Hypothetical protein	FTL_0142
Hypothetical protein	FTL_0143
ABC transporter, membrane protein	FTL_0145
Lipoprotein releasing system, subunit B, outer membrane lipoprotein	FTL_0150
UDP-N-acetylmuramate--alanine ligase	FTL_0172
Hypothetical protein	FTL_0174
Inner-membrane protein	FTL_0178
Cyclohexadienyl dehydratase precursor, pseuodogene	FTL_0187
Cytochrome O ubiquinol oxidase subunit II	FTL_0191
Hypothetical protein	FTL_0199
TPR repeat-containing protein	FTL_0204
TPR repeat-containing protein	FTL_0205
Hypothetical protein	FTL_0206
Pyrrolidone-carboxylate peptidase	FTL_0207
Glutamate_gamma-aminobutyrate anti-porter	FTL_0273
Mercuric reductase protein	FTL_0277
Hypothetical protein	FTL_0279
Aromatic amino acid HAAP transporter	FTL_0283
Hypothetical protein	FTL_0286
Hypothetical protein	FTL_0287
Aromatic amino acid HAAP transporter	FTL_0291
Hypothetical protein	FTL_0297
Hypothetical protein	FTL_0298
Hypothetical protein	FTL_0300
Hypothetical protein	FTL_0317
Lipoprotein	FTL_0318
OmpA family protein	FTL_0325
CDP-alcohol phosphatidyltransferase	FTL_0329
Hypothetical protein	FTL_0330
TolR protein	FTL_0332
Peptidoglycan-associated lipoprotein	FTL_0336
Hypothetical protein	FTL_0347
4-hydroxybenzoate polyprenyltransferase	FTL_0355
Type IV pili fiber building block protein	FTL_0359
Hypothetical protein	FTL_0371
Glycerol-3-phosphate dehydrogenase	FTL_0372
Hypothetical protein	FTL_0373
Methionine sulfoxide reductase B	FTL_0379
Superoxide dismuate _Cu-Zn_ precusor	FTL_0380
Amino acid permease	FTL_0382
Cation transporter	FTL_0388

***F. tularensis* LVS proteins containing predicted signal peptides, but not beta barrels, continued**

Protein	Locus
Type IV pili fiber building block protein	FTL_0392
Hypothetical protein	FTL_0411
Lipoprotein	FTL_0421
Hypothetical protein	FTL_0423
Lipoprotein	FTL_0424
Hypothetical protein	FTL_0434
Hypothetical protein	FTL_0448
Hypothetical protein	FTL_0451
Hypothetical protein	FTL_0464
Lipoprotein releasing system, subunit C,putative membrane protein	FTL_0474
Outer membrane lipoprotein	FTL_0491
Hypothetical protein	FTL_0493
UDP-N-acetylmuramate_L-alanyl-gamma-D-glutamyl- me so-diaminopimelate ligase	FTL_0508
Hypothetical protein	FTL_0514
ABC transporter, periplasmic protein	FTL_0515
Hypothetical protein	FTL_0523
Hypothetical protein	FTL_0532
Outer membrane protein	FTL_0535
Outer membrane protein OmpH	FTL_0536
Hypothetical protein	FTL_0559
Hypothetical protein	FTL_0560
Hypothetical protein	FTL_0569
Hypothetical protein	FTL_0571
Hypothetical protein	FTL_0572
Hypothetical protein	FTL_0573
UDP-glucose 4-epimerase	FTL_0594
NADH oxidase	FTL_0634
Hypothetical protein	FTL_0638
Lipoprotein	FTL_0642
Hypothetical protein	FTL_0647
Hypothetical protein	FTL_0656
Cell division protein	FTL_0659
Hypothetical protein	FTL_0661
Hypothetical protein	FTL_0665
Polyamine transporter, subunit I, ABC transporter, membrane protein	FTL_0679
Hypothetical protein	FTL_0684
Outer membrane efflux protein	FTL_0686
Major facilitator transporter	FTL_0688
Hypothetical protein	FTL_0694
Hypothetical protein	FTL_0698
Lipoprotein	FTL_0700
FAD binding family protein	FTL_0701
Hypothetical protein	FTL_0702
Hypothetical protein	FTL_0706
2-octaprenyl-6-methoxyphenyl hydroxylase	FTL_0726
Hypothetical protein	FTL_0737
Hypothetical protein	FTL_0738
Oxidoreductase, short-chain dehydrogenase family protein	FTL_0743
Lipoprotein	FTL_0764
Gamma-glutamyltranspeptidase	FTL_0766
Type IV pili associated protein	FTL_0797
Type IV pili glycosylation protein	FTL_0798
Type IV pili lipoprotein	FTL_0799
Type IV pilin multimeric outer membrane protein	FTL_0800
Amino acid transporter family protein	FTL_0806

***F. tularensis* LVS proteins containing predicted signal peptides, but not beta barrels, continued**

Protein	Locus
Hypothetical protein	FTL_0814
Hypothetical protein	FTL_0822
Hypothetical protein	FTL_0845
Preprotein translocase family protein	FTL_0847
Preprotein translocase subunit SecD	FTL_0848
Hypothetical protein	FTL_0850
Major facilitator transporter	FTL_0865
DNA_RNA endonuclease family protein	FTL_0878
Beta-lactamase	FTL_0879
Hypothetical protein	FTL_0881
Apolipoprotein N-acyltransferase	FTL_0882
Hypothetical protein	FTL_0896
SPFH domain-containing protein_band 7 family protein	FTL_0904
Hypothetical protein	FTL_0919
Proton-dependent oligopeptide transporter	FTL_0924
Hypothetical protein	FTL_0951
Hypothetical protein	FTL_0954
Beta-lactamase class A	FTL_0957
Hypothetical protein	FTL_0958
Proton-dependent oligopeptide transport _POT_ family protein	FTL_0963
Hypothetical protein	FTL_0988
Lipoprotein	FTL_0990
Hypothetical protein	FTL_0997
Hypothetical protein	FTL_1000
Hypothetical protein	FTL_1001
Hypothetical protein	FTL_1021
Hypothetical protein	FTL_1037
Hypothetical protein	FTL_1040
FKBP-type peptidyl-prolyl cis-trans isomerase family protein	FTL_1042
Lipoprotein	FTL_1045
D-alanyl-D-alanine carboxypeptidase_penicillin binding protein_ family protein	FTL_1046
D-alanyl-D-alanine carboxypeptidase_penicillin binding protein_ family protein	FTL_1060
Hypothetical protein	FTL_1064
Amino acid permease	FTL_1069
Hypothetical protein	FTL_1080
Choloylglycine hydrolase family protein	FTL_1089
Lipoprotein	FTL_1096
Macrophage infectivity potentiator, fragment	FTL_1097
Hypothetical protein	FTL_1101
Hypothetical protein	FTL_1110
Hypothetical protein	FTL_1133
Hypothetical protein	FTL_1161
Hypothetical protein	FTL_1170
Hypothetical protein	FTL_1178
Membrane-bound lytic murein transglycosylase A _MLT_ family protein	FTL_1189
ZIP metal transporter family protein	FTL_1201
Hypothetical protein	FTL_1202
Hypothetical protein	FTL_1209
Lipoprotein	FTL_1211
Hypothetical protein	FTL_1213
Hypothetical protein	FTL_1225
Hypothetical protein	FTL_1238
Hypothetical protein	FTL_1247
Glutathione reductase	FTL_1248
Major facilitator transporter	FTL_1278

***F. tularensis* LVS proteins containing predicted signal peptides, but not beta barrels, continued**

Protein	Locus
Short-chain dehydrogenase_oxidoreductase	FTL_1280
Hypothetical protein	FTL_1297
Hypothetical protein	FTL_1299
Virulence factor MviN	FTL_1305
Hypothetical protein	FTL_1306
CTP synthetase	FTL_1311
Hypothetical protein	FTL_1353
Hypothetical protein	FTL_1365
Hypothetical protein	FTL_1384
Hypothetical protein	FTL_1385
L-aspartate oxidase	FTL_1388
ABC transporter, ATP-binding and membrane protein	FTL_1428
Hypothetical protein	FTL_1438
Beta-lactamase superfamily hydrolase	FTL_1440
Major facilitator transporter	FTL_1450
Hypothetical protein	FTL_1459
Hypothetical protein	FTL_1472
Type IV pili fiber building block protein	FTL_1475
Permease YjgP_YjgQ family protein	FTL_1481
Hypothetical protein	FTL_1494
Cysteine_glutathione ABC transporter membrane_ATP-binding component	FTL_1495
Cysteine_glutathione ABC transporter membrane_ATP-binding component	FTL_1496
C4-dicarboxylate transport protein	FTL_1497
3-oxoacyl_acyl-carrier protein_ reductase	FTL_1507
D-alanyl-D-alanine carboxypeptidase_D-alanyl-D-alanine-endopeptidase	FTL_1509
Glycerophosphoryl diester phosphodiesterase family protein	FTL_1511
Serine transporter	FTL_1524
Major facilitator transporter	FTL_1528
Hypothetical protein	FTL_1532
Hypothetical protein	FTL_1540
Hypothetical protein	FTL_1548
Lipoprotein	FTL_1550
Hypothetical protein	FTL_1552
Hypothetical protein	FTL_1555
Hypothetical protein	FTL_1556
Major facilitator transporter	FTL_1567
Phospholipase D family protein	FTL_1570
Thioredoxin reductase	FTL_1571
Major facilitator transporter	FTL_1573
Hypothetical protein	FTL_1574
Hypothetical protein	FTL_1575
Hypothetical protein	FTL_1578
Hypothetical protein	FTL_1579
Lipoprotein	FTL_1581
Peptidyl-prolyl cis-trans isomerase _PPIase	FTL_1596
Organic solvent tolerance protein	FTL_1597
Putative periplasmic protease	FTL_1605
Cell division protein FtsW	FTL_1613
Hypothetical protein	FTL_1618
Major facilitator transporter	FTL_1624
Hypothetical protein	FTL_1628
Hypothetical protein	FTL_1633
Chitinase, fragment	FTL_1635
Lipoprotein	FTL_1637
Amino acid transporter protein, fragment	FTL_1640

***F. tularensis* LVS proteins containing predicted signal peptides, but not beta barrels, continued**

Protein	Locus
Hypothetical protein	FTL_1654
Hypothetical protein	FTL_1658
Nucleoside permease NUP family protein	FTL_1661
Disulfide bond formation protein	FTL_1670
RND efflux transporter	FTL_1671
Hypothetical protein	FTL_1678
Major facilitator transporter	FTL_1685
Hypothetical protein	FTL_1692
Hypothetical protein	FTL_1693
Hypothetical protein	FTL_1696
Hypothetical protein	FTL_1699
Amino acid transporter	FTL_1703
Lipoprotein releasing system, subunit A, outer membrane lipoproteins carrier	FTL_1706
Potassium uptake protein	FTL_1708
Hypothetical protein	FTL_1709
Lipoprotein	FTL_1724
Eflux protein	FTL_1725
Sensor histidine kinase	FTL_1762
Hypothetical protein	FTL_1776
Succinate dehydrogenase, cytochrome b556	FTL_1788
Major facilitator superfamily tranporter	FTL_1790
Hypothetical protein	FTL_1793
F0F1 ATP synthase subunit C	FTL_1800
NADH dehydrogenase I, L subunit	FTL_1819
NADH dehydrogenase I, J subunit	FTL_1821
NADH dehydrogenase I, A subunit	FTL_1830
Hypothetical protein	FTL_1837
RND efflux membrane fusion protein	FTL_1845
Hypothetical protein	FTL_1848
Lipoprotein	FTL_1853
Outer membrane protein tolC precursor	FTL_1865
Protease yegQ	FTL_1867
Multidrug resistance protein, membrane located	FTL_1868
Na_H_ antiporter	FTL_1869
Amino acid transporter	FTL_1873
Aromatic amino acid HAAP transporter	FTL_1875
Potassium-transporting ATPase C chain	FTL_1880
Hypothetical protein	FTL_1896
Cell division protein FtsQ	FTL_1909
Putative acyltransferase	FTL_1915
Competence-related protein	FTL_1916
Periplasmic solute binding family protein	FTL_1936
Outer membrane lipoprotein	FTL_1939
Peptide methionine sulfoxide reductase msrA	FTL_1960

Beta barrel and signal peptide predictions were accomplished using the BOMP and SignalP 3.0 softwares (Hidden Markov model), respectively.

Appendix 3

F. tularensis protein antigens detected by Western blot and 2D-PAGE

F. tularensis protein antigens detected by Western blot and 2D-PAGE

Loci[A]	Protein identified	ref
FTT_0037	NADH dehydrogenase subunit G	(97)
FTT_0049	Transcription elongation factor NusA	(96)
FTT_0062	F0F1 ATP synthase subunit alpha	(74, 97, 98)
FTT_0064	F0F1 ATP synthase subunit beta	(74, 97)
FTT_0071	Citrate synthase	(36, 74)
FTT_0074	Succinate dehydrogenase, catalytic and NAD/flavoprotein subunit	(45, 74, 98)
FTT_0077	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex	(45, 46, 97, 98)
FTT_0086	Hypothetical protein	(98)
FTT_0087	Aconitate hydratase	(45, 46, 97)
FTT_0119	Hypothetical protein	(96)
FTT_0137	Elongation factor Tu	(36, 37, 43, 45, 96-98)
FTT_0141	50S ribosomal protein L1	(98)
FTT_0143	50S ribosomal protein L7/L12	(36, 45, 74, 96, 98)
FTT_0183	30S ribosomal protein S1	(74, 97, 98)
FTT_0188	Cell division protein FtsZ	(45, 74, 97, 98)
FTT_0189	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	(97)
FTT_0209	Periplasmic solute binding family protein	(96-98)
FTT_0245	Universal stress protein	(74)
FTT_0296	Pyrrolidone-carboxylate peptidase	(36)
FTT_0314	Elongation factor Ts	(36, 96, 98)
FTT_0323	Elongation factor G	(97, 98)
FTT_0342	30S ribosomal protein S5	(98)
FTT_0350	DNA-directed RNA polymerase subunit alpha	(96, 97)
FTT_0356	Heat shock protein 90	(36, 45, 74)
FTT_0373	Nucleoside diphosphate kinase	(36, 98)
FTT_0380	NAD(P)-specific glutamate dehydrogenase	(74)
FTT_0471	3-dehydroquinate dehydratase	(37, 43)
FTT_0472	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit	(37, 43, 45, 74, 97, 98)
FTT_0473	Acetyl-CoA carboxylase, biotin carboxylase subunit	(36)
FTT_0503	Succinyl-CoA synthetase, alpha subunit	(36, 98)
FTT_0504	Succinyl-CoA synthetase subunit beta	(46)
FTT_0510	DNA gyrase subunit B	(97)
FTT_0511	Pyridoxal biosynthesis lyase PdxS	(96, 97)
FTT_0535	Lactate dehydrogenase	(36)
FTT_0557	AhpC/TSA family protein	(45)
FTT_0580	Hypothetical protein	(97)
FTT_0583	Outer membrane associated protein	(36, 45, 96-98)
FTT_0627	Histone-like protein HU form B	(45)
FTT_0630	Host factor I for bacteriophage Q beta replication	(36)
FTT_0715	Chitinase family 18 protein	(36, 97, 98)
FTT_0721	Peroxidase/catalase	(36, 45, 46, 74, 96-98)
FTT_0726	Glycerophosphoryl diester phosphodiesterase family protein	(45)
FTT_0766	Purine nucleoside phosphorylase	(74)
FTT_0817	Threonyl-tRNA synthetase	(98)
FTT_0831	OmpA family protein	(45, 97)
FTT_0863	LemA-like protein	(45, 97, 98)
FTT_0901	Lipoprotein	(37, 43, 45, 98)
FTT_0918	Hypothetical protein	(46, 96, 98)
FTT_0975	Hypothetical protein	(36)
FTT_1043	FKBP-type peptidyl-prolyl cis-trans isomerase family protein	(45, 98)
FTT_1060	50S ribosomal protein L9	(36, 97, 98)
FTT_1101	4Fe-4S ferredoxin (electron transport) family protein	(45)
FTT_1103	Conserved hypothetical lipoprotein	(97, 98)

***F. tularensis* protein antigens detected by Western blot and 2D-PAGE, continued**

Loci[A]	Protein identified	ref
FTT_1156	Type IV pilin multimeric outer membrane protein	(97)
FTT_1201	Oxidoreductase, short-chain dehydrogenase family protein	(36)
FTT_1269	Molecular chaperone DnaK	(36, 37, 43, 45, 46, 74, 96-98)
FTT_1270	Heat shock protein GrpE	(36)
FTT_1281	Sigma-54 modulation protein	(36)
FTT_1303	Hypothetical protein	(97)
FTT_1313	Transcriptional elongation factor	(98)
FTT_1317	Inosine-5-monophosphate dehydrogenase	(45)
FTT_1368	Glyceraldehyde-3-phosphate dehydrogenase	(36, 74)
FTT_1369	Transketolase	(36)
FTT_1373	3-oxoacyl-[acyl carrier protein] synthase III	(97)
FTT_1374	Malonyl CoA-acyl carrier protein transacylase	(36, 97)
FTT_1377	3-oxoacyl-[acyl-carrier-protein] synthase II	(74)
FTT_1389	3-methyl-2-oxobutanoate hydroxymethyltransferase	(97)
FTT_1390	Pantoate-beta-alanine ligase	(98)
FTT_1402	Hypothetical protein	(45)
FTT_1441	Hypothetical protein	(36, 45, 74, 97)
FTT_1460	UDP-glucose/GDP-mannose dehydrogenase	(74, 98)
FTT_1483	Dihydrolipoamide dehydrogenase	(45)
FTT_1484	Dihydrolipoamide acetyltransferase	(45, 46, 97)
FTT_1485	Pyruvate dehydrogenase subunit E1	(46)
FTT_1498	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	(36, 45)
FTT_1526	Isocitrate dehydrogenase	(36, 45)
FTT_1530	Fusion product of 3-hydroxacyl-CoA dehydrogenase and acyl-CoA-binding protein	(97)
FTT_1531	Acetyl-CoA acetyltransferase	(45)
FTT_1540	Hypothetical protein	(97)
FTT_1539	Hypothetical protein	(36, 45, 98)
FTT_1572	Outer membrane protein OmpH	(36)
FTT_1591	Lipoprotein	(45)
FTT_1616	Cysteinyl-tRNA synthetase	(98)
FTT_1676	Hypothetical protein	(45, 46)
FTT_1695	Co-chaperonin GroES	(36, 37, 43)
FTT_1696	Chaperonin GroEL	(36, 37, 43, 45, 74, 96-98)
FTT_1702	Hypothetical protein	(45)
FTT_1712 FTT_1357	Intracellular growth locus, subunit C	(36, 37, 43, 45, 74, 98)
FTT_1713 FTT_1358	Intracellular growth locus, subunit B	(46, 97)
FTT_1714 FTT_1359	Intracellular growth locus, subunit A	(45)
FTT_1747	Outer membrane protein	(45)
FTT_1749	Preprotein translocase subunit SecB	(45)
FTT_1752	Single-strand binding protein	(36)
FTT_1769	ClpB protein	(36, 45, 97)
FTT_1778	Hypothetical membrane protein	(97, 98)

[A]FTT loci correspond to the predicted coding protein sequence tags of *F. tularensis* strain SCHU S4.
References correspond to those of Chapter II.

Appendix 4

F. tularensis proteins identified in membrane fractions by 2D-PAGE

F. tularensis proteins identified in membrane fractions by 2D-PAGE

Membrane proteins identified in *F. tularensis* type A

Loci[A]	Protein identified	ref
FTT_0018	Secretion protein	(73)
FTT_0077	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex	(46, 73)
FTT_0087	Aconitate hydratase	(46, 73)
FTT_0142	50S ribosomal protein L10	(73)
FTT_0209	Periplasmic solute binding family protein	(73)
FTT_0245	Universal stress protein	(73)
FTT_0365	Phenol hydroxylase	(73)
FTT_0373	Nucleoside diphosphate kinase	(73)
FTT_0380	Glutamate dehydrogenase	(73)
FTT_0503	Succinyl-CoA synthetase, alpha subunit	(73)
FTT_0504	Succinyl-CoA synthetase subunit beta	(46)
FTT_0634	SPFH domain-containing protein/band 7 family protein	(73)
FTT_0721	Peroxidase/catalase	(46)
FTT_0726	Glycerophosphoryl diester phosphodiesterase family protein	(73)
FTT_0903	Hypothetical protein	(73)
FTT_0918	Hypothetical protein	(46)
FTT_1043	FKBP-type peptidyl-prolyl cis-trans isomerase family protein	(73)
FTT_1103	Lipoprotein	(73)
FTT_1157	Type IV pili lipoprotein.	(73)
FTT_1260	Hypothetical protein	(73)
FTT_1269	Molecular chaperone DnaK	(46)
FTT_1346	Hypothetical protein	(73)
FTT_1354	Hypothetical protein	(73)
FTT_1441	Hypothetical protein	(73)
FTT_1483	Dihydrolipoamide dehydrogenase	(73)
FTT_1484	Dihydrolipoamide acetyltransferase	(46, 73)
FTT_1485	Pyruvate dehydrogenase subunit E1	(46)
FTT_1539	Hypothetical protein	(73)
FTT_1572	Outer membrane protein OmpH	(73)
FTT_1591	Lipoprotein	(73)
FTT_1651	Hypothetical protein	(73)
FTT_1666	3-hydroxyisobutyrate dehydrogenase	(73)
FTT_1676	Hypothetical protein	(46, 73)
FTT_1701	Hypothetical protein	(73)
FTT_1712 FTT_1357	Intracellular growth locus, subunit C	(73)
FTT_1713	Intracellular growth locus, subunit B	(46)
FTT_1714 FTT_1359	Intracellular growth locus, subunit A	(73)
FTT_1747	Outer membrane protein	(73)

Membrane proteins identified in *F. tularensis* type B

FTT_0033	NADH dehydrogenase I	(73)
FTT_0074	Succinate dehydrogenase, catalytic and NAD/flavoprotein subunit	(45)
FTT_0077	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex	(45, 73)
FTT_0087	Aconitate hydratase	(45, 73)
FTT_0101	Hypothetical protein	(72)
FTT_0120	Signal recognition particle receptor FtsY	(73)
FTT_0137	Elongation factor Tu	(45)
FTT_0142	50S ribosomal protein L10	(73)
FTT_0143	50S ribosomal protein L7/L12	(45)
FTT_0166	Hypothetical protein	(73)
FTT_0188	Cell division protein FtsZ	(45)

Membrane proteins identified in *F. tularensis* type B, continued

Loci[A]	Protein identified	ref
FTT_0198	Outer membrane lipoprotein	(72)
FTT_0245	Universal stress protein	(73)
FTT_0276	Cyclohexadienyl dehydratase	(73)
FTT_0296	Pyrrolidone-carboxylate peptidase	(72)
FTT_0356	Heat shock protein 90	(45)
FTT_0365	Phenol hydroxylase	(73)
FTT_0369	Hypothetical protein	(72, 89)
FTT_0373	Nucleoside diphosphate kinase	(73)
FTT_0380	Glutamate dehydrogenase	(73)
FTT_0472	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit	(45, 72)
FTT_0490	Phospholipase D family protein	(89)
FTT_0503	Succinyl-CoA synthetase, alpha subunit	(73)
FTT_0557	AhpC/TSA family protein	(45)
FTT_0558	Short chain dehydrogenase	(73)
FTT_0562	Polyamine transporter, ABC transporter,ATP-binding protein	(72)
FTT_0583	Outer membrane associated protein	(45, 72, 95)
FTT_0627	Histone-like protein HU form B	(45)
FTT_0634	SPFH domain-containing protein/band 7 family protein	(72, 73)
FTT_0704	Hypothetical protein	(89)
FTT_0721	Peroxidase/catalase	(45, 72)
FTT_0726	Glycerophosphoryl diester phosphodiesterase family protein	(45, 72, 73)
FTT_0746	Hypothetical protein	(72)
FTT_0757	UTP--glucose-1-phosphate uridylyltransferase	(73)
FTT_0825	Hypothetical protein	(95)
FTT_0831	OmpA family protein	(45, 72)
FTT_0842	Peptidoglycan-associated lipoprotein	(72, 95)
FTT_0863	LemA-like protein	(45, 95)
FTT_0879	Superoxide dismuate (Cu-Zn) precusor	(95)
FTT_0900	Hypothetical protein	(72)
FTT_0901	Lipoprotein	(45, 72)
FTT_0904	Lipoprotein	(37)
FTT_0972	ABC transporter, ATP-binding protein	(72)
FTT_0991	Lipoprotein	(72)
FTT_1029	D-alanyl-D-alanine carboxypeptidase (penicillin binding protein) family protein	(72, 89)
FTT_1040	Lipoprotein	(72)
FTT_1043	FKBP-type peptidyl-prolyl cis-trans isomerase family protein	(45, 72, 73)
FTT_1101	4Fe-4S ferredoxin (electron transport) family protein	(45)
FTT_1103	Lipoprotein	(72, 73, 89, 95)
FTT_1234	Choloylglycine hydrolase family protein	(72)
FTT_1249	Cell entry (mce) related family protein	(72)
FTT_1250	Hypothetical protein	(73, 89)
FTT_1257	HlyD family secretion protein	(72)
FTT_1258	Outer membrane efflux protein	(72)
FTT_1269	Molecular chaperone DnaK	(45)
FTT_1317	Inosine-5-monophosphate dehydrogenase	(45, 72)
FTT_1346	Hypothetical protein	(73, 95)
FTT_1354	Hypothetical protein	(73)
FTT_1355	Hypothetical protein	(73)
FTT_0209	Periplasmic solute binding family protein	(73, 95)
FTT_1402	Hypothetical protein	(45, 89)
FTT_1416	Lipoprotein	(95)
FTT_1441	Hypothetical protein	(45, 73)
FTT_1483	Dihydrolipoamide dehydrogenase	(45, 73)
FTT_1484	Dihydrolipoamide acetyltransferase	(45, 72, 73)
FTT_1498	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	(45)

Membrane proteins identified in *F. tularensis* type B, continued

Loci[A]	Protein identified	ref
FTT_1526	Isocitrate dehydrogenase	(45)
FTT_1531	Acetyl-CoA acetyltransferase	(45)
FTT_1539	Hypothetical protein	(45, 73)
FTT_1540	Hypothetical protein	(72)
FTT_1567	Hypothetical protein	(95)
FTT_1572	Outer membrane protein OmpH	(72)
FTT_1591	Lipoprotein	(45, 72, 73)
FTT_1676	Hypothetical protein	(45, 73)
FTT_1696	Chaperonin GroEL	(37, 45)
FTT_1701	Hypothetical protein	(73)
FTT_1702 FTT_1347	Hypothetical protein	(45)
FTT_1710 FTT_1355	Hypothetical protein	(73)
FTT_1712 FTT_1357	Intracellular growth locus, subunit C	(45, 73)
FTT_1714 FTT_1359	Intracellular growth locus, subunit A	(45, 73)
FTT_1747	Outer membrane protein	(45, 72, 73)
FTT_1749	Preprotein translocase subunit SecB	(45)
FTT_1769	ClpB protein	(45)
FTT_1778	Hypothetical protein	(95)
FTT_1794	Heat shock protein	(73)

[A]FTT loci correspond to the predicted coding protein sequence tags of *F. tularensis* strain SCHU S4.
References correspond to those of Chapter II.

Appendix 5

Proteins identified in *F. tularensis* LVS MPF

Proteins identified in MPF by 2D LC-MS/MS

Protein name	Locus	Predicted molecular mass	Protein identification probability	Number of unique peptides	Percentage sequence coverage
Chromosomal replication initiator protein dnaA	FTL_0001	55839.60	99.80%	2	6%
DNA polymerase III, beta chain	FTL_0002	41674.84	100.00%	3	9%
Outer membrane protein	FTL_0009	19477.11	100.00%	3	24%
Recombinase A protein	FTL_0012	38833.58	100.00%	2	8%
Phosphate acetyltransferase	FTL_0016	77167.56	99.80%	2	4%
Aspartyl-tRNA synthetase	FTL_0020	66805.23	100.00%	6	13%
Carbamoyl phosphate synthase large subunit	FTL_0029	120723.72	99.80%	2	2%
Hypothetical protein	FTL_0039	15326.41	99.80%	2	19%
GTP-binding protein LepA	FTL_0071	65573.59	100.00%	5	13%
Hypothetical protein	FTL_0073	37517.73	100.00%	5	16%
Acetyltransferase protein	FTL_0087	27857.30	99.80%	2	9%
Acetyltransferase protein	FTL_0088	28838.19	100.00%	2	12%
ClpB protein	FTL_0094	96047.04	100.00%	10	15%
Hypothetical protein	FTL_0097	13793.84	99.80%	2	19%
Hypothetical protein	FTL_0104	14595.73	99.80%	2	26%
Hypothetical protein	FTL_0105	13728.82	100.00%	3	35%
Intracellular growth locus, subunit A	FTL_0111	20890.88	99.80%	2	14%
Intracellular growth locus, subunit B	FTL_0112	57917.61	100.00%	11	27%
Intracellular growth locus, subunit C	FTL_0113	22133.41	100.00%	6	37%
Intracellular growth locus, subunit D	FTL_0114	46401.43	100.00%	4	11%
Hypothetical protein	FTL_0116	155908.31	100.00%	6	6%
Hypothetical protein	FTL_0120	55363.07	99.80%	2	4%
Hypothetical protein	FTL_0125	127540.09	100.00%	10	12%
Hypothetical protein	FTL_0126	95339.57	100.00%	4	6%
Ferrous iron transport protein	FTL_0133	81459.74	100.00%	5	10%
Lipopolysaccharide protein	FTL_0137	38744.36	100.00%	6	22%
ABC transporter, ATP-binding protein	FTL_0146	49453.15	100.00%	4	13%
Universal stress protein	FTL_0166	30220.68	99.80%	2	14%
Inner-membrane protein	FTL_0178	61952.44	100.00%	4	10%
Acyltransferase	FTL_0180	36125.96	99.80%	2	8%
Cytochrome d terminal oxidase, polypeptide subunit I	FTL_0189	64275.75	100.00%	4	14%
Cytochrome O ubiquinol oxidase subunit I	FTL_0192	76200.11	100.00%	3	6%
Hypothetical protein	FTL_0199	15640.67	99.80%	2	16%
Pyrrolidone-carboxylate peptidase	FTL_0207	24215.13	100.00%	3	19%
30S ribosomal protein S2	FTL_0224	26421.69	100.00%	8	36%
Elongation factor Ts	FTL_0225	30959.65	100.00%	8	34%
30S ribosomal protein S7	FTL_0233	17807.64	100.00%	3	23%
Elongation factor G	FTL_0234	77730.37	100.00%	9	15%
30S ribosomal protein S10	FTL_0235	11895.96	100.00%	4	31%
50S ribosomal protein L3	FTL_0236	22307.33	100.00%	3	20%
50S ribosomal protein L4	FTL_0237	22553.16	100.00%	5	17%
50S ribosomal protein L23	FTL_0238	11135.83	100.00%	3	40%
50S ribosomal protein L2	FTL_0239	30401.42	100.00%	4	19%
30S ribosomal protein S3	FTL_0242	24877.10	100.00%	3	17%
50S ribosomal protein L16	FTL_0243	15710.48	100.00%	5	38%
50S ribosomal protein L14	FTL_0246	13235.48	100.00%	2	30%
50S ribosomal protein L24	FTL_0247	11476.34	100.00%	5	50%
50S ribosomal protein L5	FTL_0248	19996.34	100.00%	8	51%
30S ribosomal protein S8	FTL_0250	14410.78	100.00%	5	45%
50S ribosomal protein L6	FTL_0251	19075.03	99.90%	2	17%
50S ribosomal protein L18	FTL_0252	13036.06	99.80%	2	23%

Proteins identified in MPF by 2D LC-MS/MS, continued

Protein name	Locus	Predicted molecular mass	Protein identification probability	Number of unique peptides	Percentage sequence coverage
50S ribosomal protein L30	FTL_0254	6871.10	99.80%	2	25%
50S ribosomal protein L15	FTL_0255	15095.61	100.00%	4	32%
30S ribosomal protein S13	FTL_0258	13377.65	100.00%	3	20%
30S ribosomal protein S4	FTL_0260	23236.78	100.00%	6	27%
DNA-directed RNA polymerase subunit alpha	FTL_0261	35357.51	100.00%	3	14%
50S ribosomal protein L17	FTL_0262	16784.34	100.00%	7	53%
Heat shock protein 90	FTL_0267	72371.65	100.00%	7	15%
Glutamate dehydrogenase	FTL_0269	49158.07	100.00%	3	10%
Heat shock protein, hsp40	FTL_0281	33669.83	100.00%	3	13%
DNA mismatch repair protein MutS	FTL_0294	95812.74	99.80%	2	3%
Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	FTL_0295	35436.02	100.00%	10	32%
Pyruvate dehydrogenase, E1 component	FTL_0309	100268.34	100.00%	17	18%
Dihydrolipoamide acetyltransferase	FTL_0310	56798.85	100.00%	8	19%
Hypothetical protein	FTL_0317	12214.60	100.00%	4	55%
OmpA family protein	FTL_0325	46754.18	100.00%	4	13%
Hypothetical protein	FTL_0333	34654.68	99.80%	2	7%
Peptidoglycan-associated lipoprotein	FTL_0336	23291.32	100.00%	3	18%
Ribonuclease PH	FTL_0357	25440.65	99.80%	2	12%
Hypothetical protein	FTL_0358	12702.64	99.80%	2	21%
LemA-like protein	FTL_0361	21986.26	99.90%	2	16%
Aspartate aminotransferase	FTL_0387	44382.87	99.90%	2	5%
2-polyprenylphenol 6-hydroxylase	FTL_0407	63814.61	100.00%	6	11%
GTP-binding protein EngA	FTL_0414	52468.74	99.80%	2	5%
Lipoprotein	FTL_0421	15771.85	100.00%	3	30%
Hypothetical protein	FTL_0423	19303.26	99.80%	2	16%
Chromosome partition protein B	FTL_0428	34592.91	99.80%	2	9%
Isoleucyl-tRNA synthetase	FTL_0436	106971.95	100.00%	3	5%
Malate dehydrogenase	FTL_0438	67344.46	100.00%	3	9%
Hypothetical protein	FTL_0439	58445.63	99.80%	2	5%
DNA topoisomerase IV subunit A	FTL_0462	83930.88	100.00%	6	9%
Soluble lytic murein transglycosylase	FTL_0466	76931.27	99.80%	2	3%
Lysine decarboxylase, inducible	FTL_0476	81935.00	100.00%	3	6%
Glycine dehydrogenase subunit 2	FTL_0480	52781.98	99.80%	2	3%
DNA gyrase, subunit A	FTL_0533	97102.92	100.00%	5	6%
UDP-N-acetylglucosamine acyltransferase	FTL_0539	28124.64	100.00%	5	27%
Two-component response regulator	FTL_0552	25519.67	100.00%	3	21%
Ribonuclease R	FTL_0556	85994.62	100.00%	7	14%
Hypothetical protein	FTL_0569	19785.57	100.00%	2	14%
Hypothetical protein	FTL_0571	22451.65	100.00%	3	22%
Hypothetical protein	FTL_0572	51977.34	100.00%	3	9%
Hypothetical protein	FTL_0574	52201.14	99.80%	2	6%
Fusion product of 3-hydroxacyl-CoA dehydrogenase and acyl-CoA-binding protein	FTL_0584	100624.28	100.00%	7	11%
Acyl-CoA dehydrogenase	FTL_0585	83333.70	100.00%	7	14%
Hypothetical protein	FTL_0589	33263.61	99.80%	2	9%
dTDP-glucose 4,6-dehydratase	FTL_0592	65709.53	100.00%	4	9%
Galactosyl transferase	FTL_0593	23778.16	99.80%	2	7%
UDP-glucose 4-epimerase	FTL_0594	29955.57	100.00%	4	19%
Galacturonosyl transferase	FTL_0595	41599.47	100.00%	5	13%
UDP-glucose/GDP-mannose dehydrogenase	FTL_0596	48862.13	100.00%	2	5%
Glycosyltransferase	FTL_0604	32975.90	100.00%	3	12%

Proteins identified in MPF by 2D LC-MS/MS, continued

Protein name	Locus	Predicted molecular mass	Protein identification probability	Number of unique peptides	Percentage sequence coverage
Transcription termination factor Rho	FTL_0610	47129.05	100.00%	4	12%
DNA-directed RNA polymerase subunit alpha	FTL_0616	35074.14	100.00%	5	15%
Hypothetical protein	FTL_0617	16809.36	100.00%	3	31%
ABC transporter, ATP-binding protein	FTL_0623	34827.65	100.00%	4	15%
Glycosyl transferase	FTL_0625	35582.32	99.80%	2	11%
Hypothetical protein	FTL_0655	40009.17	100.00%	4	15%
Hypothetical protein	FTL_0656	44513.74	99.80%	2	7%
Pantothenate kinase	FTL_0671	27922.49	99.80%	2	5%
Pantoate-beta-alanine ligase	FTL_0673	29685.32	100.00%	5	20%
Hypothetical protein	FTL_0675	27475.54	99.80%	2	11%
Polyamine transporter, ABC transporter, ATP-binding protein	FTL_0681	42296.53	99.80%	2	8%
Outer membrane efflux protein	FTL_0686	54535.43	100.00%	5	19%
HlyD family secretion protein	FTL_0687	37853.27	99.80%	2	7%
Cell entry (mce) related family protein	FTL_0695	26945.01	100.00%	4	19%
Ribonuclease E	FTL_0717	95948.52	100.00%	14	25%
Type IV pilin multimeric outer membrane protein	FTL_0800	64493.72	100.00%	3	6%
Shikimate kinase I	FTL_0801	19752.84	100.00%	3	14%
Bifunctional proline dehydrogenase,pyrroline-5-carboxylate dehydrogenase	FTL_0805	150007.35	100.00%	19	18%
Type IV pili nucleotide binding protein, ABC transporter, ATP-binding protein	FTL_0828	67091.35	100.00%	5	12%
Glycerophosphoryl diester phosphodiesterase	FTL_0829	29669.29	99.80%	2	12%
Cyanophycin synthetase	FTL_0831	103993.16	100.00%	3	5%
Rhodanese-like family protein	FTL_0834	27864.42	99.80%	2	11%
D-methionine transport protein, ABC transporter, ATP-binding subunit	FTL_0838	39193.40	100.00%	8	34%
Preprotein translocase family protein	FTL_0847	12881.27	100.00%	3	33%
Preprotein translocase subunit SecD	FTL_0848	69654.94	100.00%	9	23%
Major facilitator transporter	FTL_0865	49728.62	100.00%	3	7%
Peptidase, M24 family protein	FTL_0877	68759.17	100.00%	2	6%
Trigger factor	FTL_0891	49570.86	100.00%	5	13%
ATP-dependent Clp protease subunit P	FTL_0892	22150.49	99.80%	2	13%
DNA-binding, ATP-dependent protease La	FTL_0894	86222.64	100.00%	9	14%
Hypothetical protein	FTL_0896	54765.05	100.00%	3	9%
Protease, GTP-binding subunit	FTL_0899	50111.49	99.80%	2	7%
SPFH domain, band 7 family protein	FTL_0903	40421.33	100.00%	4	12%
SPFH domain, band 7 family protein	FTL_0904	34589.21	100.00%	8	28%
Ketol-acid reductoisomerase	FTL_0916	37878.69	99.80%	2	6%
Glutaredoxin 2	FTL_0923	25150.96	99.80%	2	14%
Ferritin-like protein	FTL_0926	19073.63	100.00%	3	33%
Aldolase/adducin class II family protein	FTL_0939	26480.33	99.80%	2	13%
Ribose-phosphate pyrophosphokinase	FTL_0949	34909.35	99.80%	2	8%
50S ribosomal protein L25	FTL_0950	10903.65	99.80%	2	34%
Hypothetical protein	FTL_0951	46805.03	100.00%	3	11%
ATP-dependent protease ATP-binding subunit	FTL_0964	51237.15	99.90%	2	5%
Lactate dehydrogenase	FTL_0987	34076.74	100.00%	4	13%
Haloacid dehalogenase	FTL_0995	21938.39	99.80%	2	15%
Hypothetical protein	FTL_1013	27667.18	100.00%	3	12%
AhpC/TSA family protein	FTL_1015	19668.22	100.00%	3	25%
30S ribosomal protein S6	FTL_1024	13054.09	99.90%	2	28%
50S ribosomal protein L9	FTL_1026	16087.49	100.00%	7	56%
Ribosomal large subunit pseudouridine synthase B	FTL_1030	31171.86	100.00%	3	11%

Proteins identified in MPF by 2D LC-MS/MS, continued

Protein name	Locus	Predicted molecular mass	Protein identification probability	Number of unique peptides	Percentage sequence coverage
FKBP-type peptidyl-prolyl cis-trans isomerase family protein	FTL_1042	29357.52	100.00%	6	27%
D-alanyl-D-alanine carboxypeptidase (penicillin binding protein) family protein	FTL_1046	51339.76	100.00%	3	11%
30S ribosomal protein S21	FTL_1047	7840.30	99.80%	2	15%
RNA polymerase sigma-70 factor	FTL_1050	67646.46	99.80%	2	4%
D-alanyl-D-alanine carboxypeptidase (penicillin binding protein) family protein	FTL_1060	48033.60	100.00%	3	14%
Hypothetical protein	FTL_1064	31675.76	100.00%	3	16%
Hypothetical protein	FTL_1067	69415.81	100.00%	3	7%
Bifunctional GMP synthase/glutamine amidotransferase protein	FTL_1071	57698.31	99.80%	2	5%
1-deoxy-D-xylulose-5-phosphate synthase	FTL_1072	67385.50	100.00%	3	10%
Lipoprotein	FTL_1096	39545.19	100.00%	6	27%
Macrophage infectivity potentiator, fragment	FTL_1097	10524.01	99.80%	2	37%
Hypothetical protein	FTL_1100	15476.99	99.80%	2	19%
3-oxoacyl-[acyl-carrier-protein] synthase II	FTL_1137	44020.95	100.00%	3	10%
3-oxoacyl-(acyl-carrier-protein) reductase	FTL_1139	26357.24	100.00%	3	20%
Putative glycerol-3-phosphate acyltransferase PlsX	FTL_1142	37840.30	100.00%	4	22%
Pyruvate kinase	FTL_1148	51776.46	100.00%	5	13%
Fructose-1,6-bisphosphate aldolase	FTL_1149	38159.39	99.80%	2	7%
Hypothetical protein	FTL_1164	44640.56	100.00%	5	14%
30S ribosomal protein S9	FTL_1186	14736.01	99.80%	2	22%
50S ribosomal protein L13	FTL_1187	15937.61	100.00%	3	30%
Chaperone protein dnaK	FTL_1191	69182.22	100.00%	3	6%
Heat shock protein DnaJ	FTL_1192	41480.80	100.00%	5	14%
Phenylalanyl-tRNA synthetase subunit alpha	FTL_1197	38505.40	99.80%	2	7%
Phenylalanyl-tRNA synthetase, beta subunit	FTL_1198	88165.30	100.00%	2	3%
Hypothetical protein	FTL_1202	36896.57	100.00%	3	13%
Lipoprotein	FTL_1211	21033.65	100.00%	3	22%
Hypothetical protein	FTL_1213	73307.99	100.00%	4	10%
Hypothetical protein	FTL_1219	47072.58	99.90%	2	5%
Signal recognition particle protein, Ffh	FTL_1239	50311.00	100.00%	9	30%
2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase/dihydropteroate synthase	FTL_1265	48246.88	99.80%	2	4%
Biotin synthase	FTL_1272	34906.21	100.00%	4	17%
Dethiobiotin synthetase	FTL_1275	24502.30	99.90%	2	13%
Glutathione synthetase	FTL_1284	37159.94	99.90%	2	8%
23S rRNA m(2)G2445 methyltransferase	FTL_1287	83481.52	100.00%	3	6%
Acetyl-CoA carboxylase beta subunit	FTL_1309	33386.81	100.00%	6	14%
Outer membrane associated protein	FTL_1328	41259.65	100.00%	7	19%
Hypothetical protein	FTL_1341	15407.51	99.80%	2	16%
UTP--glucose-1-phosphate uridylyltransferase	FTL_1357	32148.15	100.00%	3	7%
Cation-efflux family protein	FTL_1358	42813.45	100.00%	4	15%
Hypothetical protein	FTL_1363	36704.83	100.00%	3	9%
Hypothetical protein	FTL_1364	26430.83	100.00%	4	24%
Hypothetical protein	FTL_1384	13313.44	99.80%	2	18%
Cold-shock DEAD-box protein A	FTL_1392	64008.24	100.00%	14	31%
50S ribosomal protein L20	FTL_1404	13348.57	100.00%	3	27%
Threonyl-tRNA synthetase	FTL_1407	72381.56	100.00%	2	3%
Hypothetical protein	FTL_1414	44264.38	100.00%	3	7%
Capsule biosynthesis protein capB	FTL_1416	44579.71	100.00%	4	12%

Proteins identified in MPF by 2D LC-MS/MS, continued

Protein name	Locus	Predicted molecular mass	Protein identification probability	Number of unique peptides	Percentage sequence coverage
Cyanophycinase	FTL_1419	29302.93	99.80%	2	9%
Carbohydrate/purine kinase pfkB family protein	FTL_1420	40313.98	99.80%	2	5%
Hypothetical protein	FTL_1426	26495.63	100.00%	3	16%
Enoyl-[acyl-carrier-protein] reductase (NADH)	FTL_1442	27775.17	99.80%	2	8%
50S ribosomal protein L21	FTL_1453	11561.63	100.00%	4	34%
Preprotein translocase subunit SecA	FTL_1458	103590.04	100.00%	5	8%
ATP-dependent metalloprotease	FTL_1464	70752.29	100.00%	4	8%
Inosine-5-monophosphate dehydrogenase	FTL_1478	52091.72	100.00%	3	8%
Cytosol aminopeptidase	FTL_1479	51988.90	100.00%	2	6%
FAD-binding family protein	FTL_1489	114567.82	100.00%	6	8%
Deoxyguanosinetriphosphate triphosphohydrolase	FTL_1503	50409.72	99.80%	2	6%
Peroxidase/catalase	FTL_1504	81226.95	100.00%	5	10%
Glycerophosphoryl diester phosphodiesterase family protein	FTL_1511	39076.81	100.00%	4	17%
2-amino-3-ketobutyrate coenzyme A ligase	FTL_1522	43952.33	99.80%	2	9%
Enolase (2-phosphoglycerate dehydratase)	FTL_1527	49511.19	100.00%	6	22%
Polynucleotide phosphorylase/polyadenylase	FTL_1537	75501.74	100.00%	8	15%
Penicillin binding protein (peptidoglycan synthetase)	FTL_1539	62733.93	100.00%	4	8%
Hypothetical protein	FTL_1542	78582.16	100.00%	6	11%
Glutamine amidotransferase subunit PdxT	FTL_1545	19960.97	99.90%	2	13%
DNA gyrase subunit B	FTL_1547	89740.80	100.00%	2	3%
Hypothetical protein	FTL_1552	69884.07	100.00%	12	26%
Succinyl-CoA synthetase subunit beta	FTL_1553	41541.74	100.00%	4	12%
Acetyl-CoA carboxylase, biotin carboxylase subunit	FTL_1591	50050.70	100.00%	6	17%
Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit	FTL_1592	16403.98	100.00%	4	48%
Putative periplasmic protease	FTL_1605	38067.40	100.00%	7	18%
Dolichyl-phosphate-mannose-protein mannosyltransferase family protein	FTL_1609	68148.00	99.80%	2	4%
Glycosyl transferase, group 2 family protein	FTL_1611	35935.44	100.00%	4	15%
Phosphoenolpyruvate carboxykinase	FTL_1616	60476.89	100.00%	3	9%
Hypothetical protein	FTL_1639	29381.81	99.90%	2	9%
Hypothetical protein	FTL_1658	49321.86	100.00%	6	18%
Lipid A transport protein, ABC transporter, ATP-binding and membrane protein	FTL_1668	66676.73	100.00%	6	12%
RND efflux transporter	FTL_1671	50088.80	99.80%	2	5%
AcrB/AcrD/AcrF family transporter	FTL_1672	112503.15	100.00%	3	4%
Hypothetical protein	FTL_1678	38458.69	100.00%	3	11%
Cell division protein	FTL_1705	92016.36	100.00%	7	10%
Chaperonin GroEL	FTL_1714	57402.70	100.00%	16	46%
Co-chaperonin GroES	FTL_1715	10271.87	100.00%	2	24%
Hypothetical protein	FTL_1723	23828.44	100.00%	3	17%
S-adenosylmethionine synthetase	FTL_1739	42103.04	99.80%	2	7%
DNA-directed RNA polymerase, beta subunit	FTL_1743	157387.04	100.00%	26	24%
DNA-directed RNA polymerase subunit beta	FTL_1744	151335.66	100.00%	19	19%
50S ribosomal protein L7/L12	FTL_1745	12847.82	99.80%	2	20%
50S ribosomal protein L10	FTL_1746	18731.70	100.00%	5	42%
50S ribosomal protein L1	FTL_1747	24641.67	100.00%	11	54%
50S ribosomal protein L11	FTL_1748	15269.89	100.00%	3	16%
Transcription antitermination protein nusG	FTL_1749	19981.99	100.00%	3	21%
Elongation factor Tu	FTL_1751	43390.62	100.00%	13	38%

Proteins identified in MPF by 2D LC-MS/MS, continued

Protein name	Locus	Predicted molecular mass	Protein identification probability	Number of unique peptides	Percentage sequence coverage
Anaerobic glycerol-3-phosphate dehydrogenase	FTL_1756	57775.19	100.00%	11	26%
Sensor histidine kinase	FTL_1762	54752.94	100.00%	3	7%
Aconitate hydratase	FTL_1772	102703.80	100.00%	13	24%
Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex	FTL_1783	52717.94	100.00%	9	29%
Alpha-ketoglutarate decarboxylase	FTL_1784	105679.39	100.00%	18	24%
Succinate dehydrogenase iron-sulfur subunit	FTL_1785	26565.80	100.00%	5	24%
Succinate dehydrogenase, catalytic and NAD/flavoprotein subunit	FTL_1786	65860.61	100.00%	7	15%
F0F1 ATP synthase subunit epsilon	FTL_1794	15736.95	100.00%	3	30%
F0F1 ATP synthase subunit beta	FTL_1795	49864.94	100.00%	10	37%
F0F1 ATP synthase subunit gamma	FTL_1796	33235.28	100.00%	4	18%
F0F1 ATP synthase subunit alpha	FTL_1797	55536.23	100.00%	10	23%
F0F1 ATP synthase subunit delta	FTL_1798	19202.29	99.90%	2	20%
F0F1 ATP synthase subunit B	FTL_1799	17383.26	100.00%	7	45%
Translation initiation factor IF-2	FTL_1809	92422.06	100.00%	10	20%
Transcription elongation factor NusA	FTL_1810	55178.89	100.00%	6	15%
Hypothetical protein	FTL_1811	16546.95	100.00%	4	35%
NADH dehydrogenase subunit G	FTL_1824	87340.84	100.00%	9	17%
NADH dehydrogenase I, F subunit	FTL_1825	46282.70	100.00%	3	9%
NADH dehydrogenase subunit D	FTL_1827	47585.74	100.00%	6	16%
NADH dehydrogenase I	FTL_1828	24988.46	99.80%	2	11%
Amidophosphoribosyltransferase	FTL_1861	55417.57	100.00%	4	8%
Outer membrane protein tolC precursor	FTL_1865	57243.63	100.00%	3	11%
Protein-L-isoaspartate O-methyltransferase	FTL_1866	23203.94	99.80%	2	11%
GTPase ObgE	FTL_1874	36887.99	99.80%	2	10%
Glutamine synthetase	FTL_1899	38256.94	99.80%	2	9%
Cell division protein FtsZ	FTL_1907	39745.31	100.00%	11	41%
Cell division protein FtsA	FTL_1908	44804.05	100.00%	7	20%
30S ribosomal protein S1	FTL_1912	61669.35	100.00%	9	22%
Adenylosuccinate synthetase	FTL_1930	46879.73	100.00%	2	8%
ABC transporter, ATP-binding protein	FTL_1935	25593.76	99.80%	2	12%
Periplasmic solute binding family protein	FTL_1936	33814.86	100.00%	7	40%
Heat shock protein	FTL_1957	16739.78	99.80%	2	15%
Bifunctional indole-3-glycerol phosphate synthase/phosphoribosylanthranilate isomerase	FTL_1958	51323.54	100.00%	3	11%
Anthranilate synthase component I	FTL_1966	58066.29	100.00%	4	11%

Protein identifications were accomplished using Scaffold version 2_01_02 software. Identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides as assigned by the protein prophet algorithm.

Appendix 6

The similarity of *F. tularensis* LVS surface proteins to homologs of other *Francisella* species, subspecies, and populations

The similarity of *F. tularensis* LVS surface proteins to homologous of other *Francisella* species, subspecies, and populations

Protein name	*F. tularensis* LVS (type B) loci / protein size (# amino acids)	*F. tularensis* OSU18 (type B) loci / protein size (# amino acids)	*F. tularensis* Schu S4 (A1a) loci / protein size (# amino acids)	*F. tularensis* MA00-2897 (A1b) loci / protein size (# amino acids)	*F. tularensis* WY96-3418 (A2) loci / protein size (# amino acids)	*F. novicida* U112 loci / protein size (# amino acids)	*F. tularensismediasiatica* loci / proteinsize (# amino acids)
Outer membrane protein	FTL_0009 / 179	**FTH_0009 / 191**	**FTT_1747 / 191**	***FTMG_01241 / 79**	**FTW_1985 / 191**	FTN_0119 / 179	FTM_1727 / 179
Intracellular growth locus, subunit A	FTL_0111 / 184	**FTH_0103 / 196**	**FTT_1359 / 196**	**FTMG_01711 / 196**	FTW_0535 / 184	FTN_1324 / 184	FTM_1711 / 184
Intracellular growth locus, subunit B	FTL_0112 / 506	**FTH_0104 / 514**	**FTT_1358 / 514**	**FTMG_01710 / 514**	FTW_0536 / 506	FTN_1323 / 506	FTM_1710 / 506
Intracellular growth locus, subunit C	FTL_0113 / 209	**FTH_0105 / 211**	**FTT_1357 / 211**	**FTMG_01636 / 211**	**FTW_0537 / 211**	FTN_1322 / 209	FTM_1709 / 209
Hypothetical protein	FTL_0123 / 164	FTH_0115 / 164	FTT_1347 / 164	FTMG_01699 / 164	FTW_0547 / 164	FTN_1312 / 164	FTM_1699 / 164
Elongation factor G	FTL_0234 / 704	FTH_0229 / 704	FTT_0323 / 704	***FTMG_01100 / 273**	FTW_1759 / 704	FTN_0237 / 704	FTM_1529 / 704
Heat shock protein 90	FTL_0267 / 628	***Gene absent**	FTT_0356 / 628	*FTMG_01718 / 628	FTW_1729 / 628	FTN_0266 / 628	FTM_0300 / 628
Glutamate dehydrogenase	FTL_0269 / 449	FTH_0268 / 449	FTT_0380 / 449	**FTMG_00268 / 186**	FTW_0766 / 449	FTN_1532 / 449	FTM_1499 / 449
OmpA family protein	FTL_0325 / 417	FTH_0323 / 417	FTT_0831 / 417	FTMG_00712 / 417	FTW_1355 / 417	FTN_0346 / 417	FTM_0423 / 417
Peptidoglycan-associated lipoprotein	FTL_0336 / 207	FTH_0334 / 207	FTT_0842 / 207	FTMG_00723 / 207	FTW_1344 / 207	FTN_0357 / 207	FTM_0434 / 207
Aspartate aminotransferase	FTL_0387 / 396	FTH_0378 / 396	FTT_0884 / 396	FTMG_00763 / 396	FTW_1295 / 396	FTN_0410 / 396	FTM_0473 / 396
Lipoprotein	FTL_0421 / 149	FTH_0414 / 149	FTT_0901 / 149	FTMG_00011 / 149	FTW_1278 / 149	FTN_0427 / 149	FTM_0489 / 149
Hypothetical protein	FTL_0569 / 178	FTH_0570 / 178	FTT_1542 / 178	FTMG_00937 / 178	FTW_0387 / 178	FTN_1451 / 178	**FTM_0356 / 179**
Hypothetical protein	FTL_0572 / 476	FTH_0573 / 476	FTT_1539 / 476	FTMG_00934 / 476	FTW_0390 / 476	FTN_1448 / 476	FTM_0359 / 476
Hypothetical protein	FTL_0617 / 146	**FTH_0620 / 161**	**FTT_1441 / 161**	**FTMG_00833 / 161**	**FTW_0445 / 157**	FTN_1410 / 146	FTM_1475 / 146
AhpC/TSA family protein	FTL_1015 / 174	FTH_0990 / 174	FTT_0557 / 174	FTMG_00439 / 174	FTW_0983 / 174	FTN_0958 / 174	FTM_1128 / 174
50S ribosomal protein L9	FTL_1026 / 151	**FTH_1002 / 154**	FTT_1060 / 151	**FTMG_00172 / 154**	FTW_0970 / 151	FTN_0949 / 151	FTM_0893 / 151
Lipoprotein	FTL_1096 / 373	FTH_1071 / 373	**FTT_1103 / 365**	**FTMG_00217 / 365**	FTW_1411 / 373	FTN_0771 / 373	**FTM_1247 / 373**
Glyceraldehyde-3-phosphate dehydrogenase	FTL_1146 / 333	**FTH_1121 / 352**	**FTT_1368 / 348**	**FTMG_01648 / 348**	**FTW_0523 / 348**	FTN_1332 / 333	FTM_0654 / 1009
Chaperone protein dnaK	FTL_1191 / 642	FTH_1167 / 642	FTT_1269 / 642	FTMG_01748 / 642	FTW_0571 / 642	FTN_1284 / 642	FTM_1061 / 642
Hypothetical protein	FTL_1225 / 229	FTH_1203 / 229	FTT_0975 / 229	FTMG_00087 / 229	**FTW_0879 / 77**	FTN_0855 / 229	FTM_0973 / 229
Outer membrane associated protein	FTL_1328 / 392	**FTH_1293 / 393**	**FTT_0583 / 393**	FTMG_00466 / 392	**FTW_1428 / 393**	FTN_0756 / 392	FTM_1261 / 392
Hypothetical protein	FTL_1494 / 169	FTH_1449 / 169	FTT_1334 / 169	FTMG_01519 / 169	FTW_1500 / 169	FTN_0643 / 169	FTM_1348 / 169
Peroxidase/catalase	FTL_1504 / 728	**FTH_1458 / 741**	**FTT_0721 / 741**	**FTMG_00604 / 741**	**FTW_1518 / 741**	**FTN_0633 / 739**	**FTM_1361 / 741**
Chitinase family 18 protein	FTL_1521 / 764	**FTH_1471 / 760**	**FTT_0715 / 760**	**FTMG_00598 / 760**	**FTW_2084 / 198**	**FTN_0627 / 870**	**FTM_1367 / 776**
Enolase (2-phosphoglycerate dehydratase)	FTL_1527 / 456	FTH_1477 / 456	FTT_0709 / 456	FTMG_00592 / 456	FTW_1532 / 456	FTN_0621 / 456	FTM_1373 / 456
Succinyl-CoA synthetase subunit beta	FTL_1553 / 387	FTH_1504 / 387	FTT_0504 / 387	FTMG_00391 / 387	FTW_1561 / 387	FTN_0594 / 387	FTM_1400 / 387
Hypothetical protein	FTL_1579 / 239	FTH_1525 / 239	FTT_0484 / 239	FTMG_00372 / 239	FTW_1586 / 239	FTN_0575 / 239	FTM_1419 / 239
Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit	FTL_1592 / 157	FTH_1538 / 157	FTT_0472 / 157	FTMG_00360 / 157	FTW_1598 / 157	**FTN_0563 / 160**	FTM_1431 / 157
Hypothetical protein	FTL_1658 / 438	FTH_1599 / 438	FTT_0119 / 438	FTMG_01378 / 438	FTW_0204 / 438	FTN_1596 / 438	**FTM_0181 / 439**
Chaperonin GroEL	FTL_1714 / 544	FTH_1651 / 544	FTT_1696 / 544	FTMG_01472 / 544	FTW_0264 / 544	FTN_1538 / 544	FTM_0235 / 544
50S ribosomal protein L7/L12	FTL_1745 / 125	FTH_1685 / 125	FTT_0143 / 125	***FTMG_01581 and FTMG_01582 / 38 and 62**	FTW_0233 / 125	FTN_1569 / 125	**FTM_0208 / 126**
Elongation factor Tu	FTL_1751 / 394	FTH_1691 / 394	FTT_0137 / 394	***FTMG_01128 / 605**	FTW_0227 / 394	FTN_1576 / 394	FTM_0201 / 394
Aconitate hydratase	FTL_1772 / 937	FTH_1708 / 937	FTT_0087 / 937	FTMG_01346 / 937	FTW_0164 / 937	FTN_1623 / 937	FTM_0152 / 937
Succinate dehydrogenase, catalytic and NAD/flavoprotein subunit	FTL_1786 / 597	FTH_1722 / 597	FTT_0074 / 597	**FTMG_01333 / 509**	FTW_0150 / 597	FTN_1637 / 597	FTM_0138 / 597
Citrate synthase	FTL_1789 / 419	**FTH_1725 / 424**	**FTT_0071 / 424**	**FTMG_01330 / 424**	**FTW_0147 / 421**	FTN_1640 / 419	FTM_0135 / 419
Cell division protein FtsZ	FTL_1907 / 381	FTH_1830 / 381	FTT_0188 / 381	FTMG_00967 / 381	FTW_1903 / 381	FTN_0164 / 381	FTM_1653 / 381
30S ribosomal protein S1	FTL_1912 / 556	**FTH_1835 / 574**	FTT_0183 / 556	FTMG_01621 / 556	FTW_1908 / 556	FTN_0159 / 556	FTM_1658 / 556

Protein sequence information was derived from GenBank or the Broad Institute databases. Sequences obtained from the latter are denotted by *.

Loci in bold differ in protein size (number of amino acids) compared to *F. tularensis* LVS.

Appendix 7

Primers used to construct recombinant chitinases and chitinase knockouts

Primers used to generate histidine tagged chitinase recombinants

Chitinase class	Loci[a]	Amplicon size	Forward primer (5'-3')[b]	Reverse Primer (5'-3')[b]
ChiA	FTT_0715	2292	GCT AGC ATG AAC AAA ACA AAA TTA GTC TCA GTA G	GTC GAC TTG TTT TTC CCA AAC ATT AC
	FTH_1471	2292	GCT AGC ATG AAC AAA ACA AAA TTA GTC TCA GTA G	GTC GAC TTG TTT TTC CCA AAC ATT AC
	FTN_0627	2622	GCT AGC ATG AAC AAA ACA AAA TTA	GTC GAC TTG TTT TTC CCA AAC
ChiB	FTT_1768	1830	GCT AGC ATG CCA TAT TCT GAT ACC C	CTC GAG TTT ATC ATT TAT AGG ATA A
	FTH_0088	2202	GCT AGC ATG AAA TAC AAA AAG TTA TTA	CTC GAG TTT ATC ATT TAT AGG ATA A
	FTN_1744	2202	GCT AGC ATG AAA TAC AAA AAG TTA TT	CTC GAG TTT ATC ATT TAT AGG ATA AA
ChiC	FTT_1592	1173	ACT AGT GTG ACT GGA TAT AAA GCT ATC	CTC GAG TTT AGA AGT ATA CTT TTC TTG AG
	FTW_0313	2298	ACT AGT ATG AAA AAA ATG AAA TTA ATC TCA TC	CTC GAG TTT AGA AGT ATA CTT TTC TTG AG
	FTH_1579	2049	ACT AGT ACT ACT ATT AAA TCA GCA TCA TC	CTC GAG TCG ACT TCC ATG CC
ChiD	FTT_0066	2853	GCT AGC ATG AGA AAA CTT TTT ATA A	CTC GAG TTT ACT ATC TAT TTT TGT CCA
	FTT_0142	2853	GCT AGC ATG AGA AAA CTT TTT ATA A	CTC GAG TTT ACT ATC TAT TTT TGT CCA
	FTT_1730	2853	GCT AGC ATG AGA AAA CTT TTT ATA A	CTC GAG TTT ACT ATC TAT TTT TGT CCA
	FTN_1644	2853	GCT AGC ATG AGA AAA CTT TTT ATA A	CTC GAG TTT ACT ATC TAT TTT TGT CCA

[a] FTT corresponds to A1a/A1b strains; FTW corresponds to A2 strains; FTH corresponds to type B strains; FTN corresponds to *F. novicida* strains.

[b] The *NheI* (GCT AGC), *SpeI* (ACT AGT), *SalI* (GTC GAC) and *XhoI* (CTC GAG) restriction sites are underlined.

Internal sequencing primers (5'-3')

Loci[a]	Primer 1	Primer 2	Primer 3	Primer 4
FTT_0715	GTA TGG AAA ACT TTG CTA AGC AGT	GTT GCA TTG CTA CTT TAC CGT ACT TGT TAA	-	-
FTH_1471	GTA TGG AAA ACT TTG CTA AGC AGT	GTT GCA TTG CTA CTT TAC CGT ACT TGT TAA	-	-
FTN_0627	GTA TGG AAA ACT TTG CTA AGC AGT	GTT GCA TTG CTA CTT TAC CGT ACT TGT TAA	CGC TAG ATC AAT GAC TGT AGC TAG TG	-
FTT_1768	GAG CCT ATA ACT TTA GGC AAG GG	GAT AAA GTT GCA GGA CCA CTT CT	TTT ACG AAA CAC GGA AAC CGA A	-
FTH_0088	GAG CCT ATA ACT TTA GGC AAG GG	GGG TGA GTT TAC AAT TAA GCC AG	-	-
FTN_1744	GGG TGA GTT TAC AAT TAA GCC AG	ACA ACT GGC TTA CCT CAA ACT AT	-	-
FTT_1592	-	-	-	-
FTT_1593	-	-	-	-
FTW_0313	GTT ATT GCT GAA GTG AAA GAT GCT A	AGC AAC AGT CTC GCC AGC TGA G	GAC GTT AGA TGG TTC ACC AAA TCC T	CTG CAG AAG ATG TGG CTC CTT AC
FTH_1579	GTT ATT GCT GAA GTG AAA GAT GCT A	GAC GTT AGA TGG TTC ACC AAA TCC T	-	-
FTT_0066	TAA TTG CTG CTG AAC CAG AAG T	CAT GGG CCA GTT GTT GCT GG	GTA GTT CTG ATA TGC CTA AGA ATG AT	GGA TAC ATT CCA AAT GGA CTA TAT GG
FTW_0142	TAA TTG CTG CTG AAC CAG AAG T	CAT GGG CCA GTT GTT GCT GG	GTA GTT CTG ATA TGC CTA AGA ATG AT	GGA TAC ATT CCA AAT GGA CTA TAT GG
FTH_1730	TAA TTG CTG CTG AAC CAG AAG T	CAT GGG CCA GTT GTT GCT GG	GTA GTT CTG ATA TGC CTA AGA ATG AT	GGA TAC ATT CCA AAT GGA CTA TAT GG
FTN_1644	TAA TTG CTG CTG AAC CAG AAG T	CAT GGG CCA GTT GTT GCT GG	GTA GTT CTG ATA TGC CTA AGA ATG AT	GGA TAC ATT CCA AAT GGA CTA TAT GG

[a] FTT corresponds to A1a/A1b strains; FTW corresponds to A2 strains; FTH corresponds to type B strains; FTN corresponds to *F. novicida* strains.

Primers used for constructing knockouts and complemented knockouts

Construct	Fragment amplified		Amplicon size (bp)	Forward primer (5'-3')[b]	Reverse Primer (5'-3')[b]
pMP590- Δ*chiA*	*chiA*	upstream	562	TTT GAC TAA GGA TCC ATA TTA AC	TCT AGC A GT CGA C AT GAT CC
	chiA	downstream	941	AAG TAG CTG CAG ACC AAG GC	AGT TA G GAT CC A ATT ACT AAT AG
pMP590- Δ*chiC* [a]	*chiC*	upstream	477	ATG AGT ACG CGT ATG ATG CTG	ATC CTC GTC GAC AAC ACG G
	chiC	downstream	608	TAA CAC A GT CGA C AT TTA TGG	AGG AA A CGC GT C ATA CTA TC
pMP529- *chiA*	*chiA*		2918	AAT ACG CGT TCA TGT GAG CTC CTT TAA GC	TAT ACG CGT TAT CTT CAT AAA TTA TGC GC
pMP529- *chiC* [a]	*chiC*		2298	ACG CGT ATG AAA AAA ATG AAA TTA ATC TCA TC	ACG CGT TTT AGA AGT ATA CTT TTC TTG AG

[a]Primers designed using complement sequence.

[b]The *BamHI* (GGA TCC), *SalI* (GTC GAC), *PstI* (CTG CAG) and *MluI* (ACG CGT) restriction sites are underlined.

Appendix 8

Bioinformatic analyses of *F. tularensis* and *F. novicida* chitinases

ChiA: Homologues of *F. novicida* ChiA were identified in *F. tularensis* A1a, A1b, A2 and type B. The *F. tularensis* A1a/A1b and type B, and *F. novicida chiA* genes were predicted to encode proteins of 760, 760 and 870 amino acids respectively (Fig. 5.2A). The *F. tularensis* A2 *chiA* gene is annotated as a pseudogene and is predicted to encode a truncated product of 198 amino acids. All the predicted intact ChiA proteins contained a full length GH18 domain (PfamID: PF00704). Three accessory domains, fibronectin type 3 domain (Pfam ID: PF00041) and two tandem carbohydrate binding domains (PfamID: PF02839), were identified in the ChiA sequences of *F. tularensis* A1a, A1b, and type B, and *F. novicida*. The *F. novicida* ChiA, however, possessed a third carbohydrate binding domain that partially accounts for its larger size. All accessory domains are located C-terminal to the GH18 domain. All *chiA* gene products were predicted to possess an N-terminal signal peptide suggesting they are translocated across the CM.

ChiB: The genomes of *F. tularensis* A1a, A1b, and type B, and *F. novicida* possessed the *chiB* gene encoding chitinases of 606, 730 and 730 amino acids, respectively, and that contained an intact GH18 domain. *F. tularensis* A2 displayed an altered *chiB* gene resulting in a product with a 566 amino acid truncation that accounts for the absence of the GH18 domain (Fig. 5.2B). A partial *N*-acetylglucosamine-binding protein A domain (conserved domain (cdd) ID: PRK13211) was identified at the N-terminus of all predicted ChiB proteins except that of the *F. tularensis* A1a/ A1b where a 124 amino acid N-terminal truncation occurs. This truncation also encompassed the signal peptide conserved in the other ChiB sequences of *F. tularensis* A2 and type B, and *F. novicida*.

ChiC: The third chitinase (ChiC) identified in *F. tularensis* A1a, A1b, A2 and type B, and *F. novicida* differed significantly when compared across the subtypes. *F. tularensis* A2 and *F. novicida* strains GA99-3548 and GA99-3549 encode *chiC* with

predicted full length products of 762 amino acids. The *F. tularensis* type B ChiC contained a C-terminal truncation of 58 amino acids that impacts the GH18 domain. Most interestingly, the *F. tularensis* A1a/A1b *chiC* gene possessed a point mutation that causes a premature stop codon and two predicted ORFs. In *F. tularensis* A1a these two reading frames were annotated as FTT_1592 and FTT_1593 and encode products of 387 and 207 amino acids, respectively. The *F. tularensis* A1a FTT_1592 ORF encodes for the C-terminal portion of ChiC that includes a complete GH18 domain (Fig. 5.2C). The ChiC proteins also possess two tandem carbohydrate binding domains (PfamID: PF02839). The position of these domains is directly after the N-terminal signal sequence, a location that is in sharp contrast to those of the ChiA proteins.

ChiD: ChiD was highly conserved among all *Francisella* species and populations (Fig. 5.2D). All were predicted to contain a full length C-terminal GH18 domain, and an incomplete second GH18 domain. A partial *N*-acetylglucosamine-binding protein A domain was also predicted at the C-terminus. It should be noted that the shotgun genome sequence used by others to assemble the *F. tularensis* A1b genome found several alterations in *chiD*, including a mutation leading to a premature stop codon. However, our conventional sequencing of *chiD* amplified by PCR from *F. tularensis* A1b strain MA00-2987 found it to be identical to the *F. tularensis* A1a *chiD*.