DISSERTATION

PHENOTYPIC AND MOLECULAR CHARACTERISTICS OF FRANCISELLA TULARENSIS LEADING TO ENHANCED SURVEILLANCE TOOLS

Submitted by

Claudia Roser Molins Schneekloth

Department of Microbiology, Immunology and Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2007

COLORADO STATE UNIVERSITY

November 1, 2007

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CLAUDIA ROSER MOLINS SCHNEEKLOTH ENTITLED PHENOTYPIC AND MOLECULAR CHARACTERISTICS OF FRANCISELLA TULARENSIS LEADING TO ENHANCED SURVEILLANCE TOOLS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

COMMITTEE ON GRADUATE WORK

Jeannine M. Petersen

Kenneth F. Reardon

Co-Advisor: Herbert P. Schweizer

Advisor: John T. Belisle

Department Head: Carol D. Blair

ABSTRACT OF DISSERTATION

PHENOTYPIC AND MOLECULAR CHARACTERISTICS OF FRANCISELLA TULARENSIS LEADING TO ENHANCED SURVEILLANCE TOOLS

Francisella tularensis is divided into four subspecies (*tularensis*, *holarctica*, *mediasiatica*, and *novicida*), of which two, *tularensis* (type A) and *holarctica* (type B), are clinically relevant as the etiologic agents of tularemia. The bioterrorist attacks of 2001 and this bacterium's high infectivity prompted increased research funding for *F. tularensis* with specific emphasis on therapeutics, diagnostics, and vaccines for tularemia. The focus of the work presented in this dissertation is the development of tools for enhancing the identification of *F. tularensis* within clinical and environmental samples. The observation that *F. tularensis* is inhibited when co-plated with *Escherichia coli* led to the hypothesis that the inhibitory substance could be of use as a diagnostic reagent. Using genetic methods, the inhibitory substance was identified as aerobactin. Given aerobactin's lack of specificity for *Francisella*, its use as a diagnostic reagent was reconsidered and the focus of these findings shifted to understand the significance of this iron chelating molecule in relation to the survival of *F. tularensis* in the presence of siderophore-producing bacteria. Separate from the above studies, epidemiological and molecular analyses provided evidence for subpopulations (A.I and A.II) of the highly virulent *F. tularensis* subsp. type A and that these subpopulations differed in their resulting clinical manifestations, geographical location, and preferential vectors. Based

on these studies, we hypothesized that that genomic differences exist between A.I and A.II subpopulations and can be used to develop a diagnostic assay for differentiation of A.I and A.II. Additionally, it is possible that the genomic differences will lead to identification of virulence factors linked to the differential disease outcome associated with infections caused by the two subpopulations. Two PCR assays, one to identify subpopulations A.I and A.II as well as to discriminate from *F. tularensis* subspp. type B and *novicida* and another specific for subpopulation A.I, were developed based on genomic regions of difference identified by genomic suppression subtractive hybridization. In all, 18 genomic regions of difference were identified and verified among a panel of characterized *F. tularensis* subsp. type A isolates. Bioinformatic analyses revealed that genomic differences occurred in genes encoding hypothetical proteins, previously described proteins, and within intergenic regions. In silico analyses also identified products that could be associated with the differential clinical outcomes associated with these two *F. tularensis* subsp. type A subpopulations. This dissertation defines phenotypic and molecular characteristics of *F. tularensis* that provide new tools for improving the ability to identify and survey *F. tularensis* both within clinical samples and in the environment.

Claudia Roser Molins Schneekloth
Department of Microbiology,
Immunology and Pathology
Colorado State University
Fort Collins, Colorado 80523
Fall 2007

ACKNOWLEDGEMENTS

I would like to thank Drs. John T. Belisle, Jeannine M. Petersen, Herbert P. Schweizer, and Kenneth F. Reardon for their guidance and continued motivation while serving on my doctoral graduate committee. John, thank you for opening your laboratory to me and for believing in me and allowing me to pursue my ideas regardless of how crazy they may have seemed. Jeannine, thank you for keeping the door open for me at CDC and for your continued support and faith in me. To past committee members, Drs. May C. Chu, Claudia R. Gentry-Weeks, and Carol A. Ishimaru, I also thank you for the support you gave me during and after serving on my graduate committee. I would also like to thank the laboratory members of the Belisle, Dobos-Elder, and Schweizer laboratories at Colorado State University, as well as the members of the Division of Vector-Borne Infectious Diseases, Bacterial Disease Branch at the Centers for Disease Control and Prevention for their intellectual stimulation, motivation and friendship. It has been a true honor and pleasure learning from and working with all of you.

DEDICATION

For my Yayita

TABLE OF CONTENTS

Chapter IV.
Genomic Markers for Differentiation of *Francisella tularensis* type A Subpopulations

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AFLP	Amplified fragment-length polymorphism
Amp^R	Ampicillin resistant
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ATCC	American Type Culture Collection
BEADS	Battelle-PNNL Enhanced Biodetection Enabling Analyte Delivery System
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BSL3	Biosafety level 3
CDC	Centers for Disease Control and Prevention
CDS	Coding sequence
CFU	Colony-forming unit
CHAB	Cysteine heart agar with 9% chocolatized blood
CHAB-A	Cysteine heart agar with 9% chocolatized blood supplemented with antibiotics
DFA	Direct fluorescent antibody
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECL	Electrochemiluminescent assay
EDTA	Ethylene-diamine-tetra-acetic acid

ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FRET	Fluorescence resonance energy transfer
GC	Gonocoacal
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHA	Hand-held assay
IFA	Immunofluorescence assay
IFN-γ	Interferon-gamma
IPTG	isopropyl-beta-D-thiogalactopyranoside
IS	Insertion sequence
ISFtu	Insertion sequence element in *Francisella tularensis*
kb	Kilobase
kDa	Kilodalton
Kdo	keto-3-deoxy-D-manno-octulsonic acid
K_f	Formation constant
kg	Kilograms
LB	Luria-Bertani
LD_{50}	Lethal dose, 50%
LPS	Lipopolysaccharide
LRN	Laboratory response network
LVS	Live vaccine strain
M	Molar

mM	Millimolar
Mb	Megabases
mg	Milligrams
min	Minute
MLST	Multilocus sequence typing
MLVA	Multiple-locus variable-number tandem repeat analysis
NASBA	Nucleic acid sequence-based amplification
NCBI	National Center for Biotechnology Information
NFκ-B	Nuclear factor-kappa B
NO	Nitric oxide
NTPs	Nucleoside triphosphates
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PESM	Paired-end sequence mapping
PFGE	Pulsed-field gel electrophoresis
RDs	Regions of difference
rDNA	Ribosomal deoxyribonucleic acid
RAPD-PCR	Random amplified polymorphic PCR
RAPID	Ruggedized Advanced Pathogen Identification Device
RFLP	Restriction fragment length polymorphism
RT-PCR	Real-time PCR
SNP	Single nucleotide polymorphism

spp.	Multiple species
SSH	Suppression subtractive hybridization
SSTR	Short sequence tandem repeat
subsp.	Subspecies
subspp.	Multiple subspecies
TLR	Toll-like receptors
TN	Transposase-associated
TNF-α	Tumor necrosis factor-alpha
TRF	Time-resolved fluorescence
tRNA	Transfer ribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
µl	Microliter
µm	Micrometer
USSR	Union of Soviet Socialist Republics
VNTR	Variable number of tandem repeat
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

Chapter I

Literature Review Part I: General Introduction to *Francisella* Species

1.1 History, taxonomy and microbiology of *Francisella*

In 1954 Ohara reported on Yato-Byo (hare disease) in Japan and wrote: "Nevertheless, in our country, since about 1818, those who eat hare meat have often been found to suffer from poisoning" (120). While descriptions of tularemia-like disease in lemmings date back as far as 1653 (154), George McCoy who studied a plague-like disease that infected ground squirrels was the first to describe the disease tularemia in 1911 (108). In 1912, McCoy and Chapin reported on the identification and culturing of the etiologic agent of tularemia. In their studies, McCoy and Chapin differentiated tularemia from plague and defined rod-shaped bodies capable of being transmitted by fleas as the bacterium causing tularemia. The bacterium identified was named *Bacterium tularense* for its description in ground squirrels from Tulare County, California (109). The first confirmed human case and culture of *Francisella* occurred in 1914 when a 21 year-old male who worked as a meat cutter presented to the Cincinnati Hospital with oculoglandular tularemia (178).

Originally, *F. tularensis* was classified under the genus *Bacterium*, however, subsequent reclassification based on serology placed this bacterium under the genus *Pasturella* and temporarily under the genus *Brucella* (48). The proposal that this

bacterium be reclassified under the new genus *Francisella* occurred in 1947 by Dorofeev (123) and was accepted in 1959 by Olsufiev after comparative studies with other bacteria (122). The new genus was named *Francisella* in recognition of the contributions of Edward Francis who was a leader in early descriptions of tularemia (123).

Francisella is currently the only recognized genus in the family *Francisellaceae*. In 1994, Forsman *et al.* used 16S rDNA sequence analysis to place this bacterium under the γ-subclass of *Proteobacteria* and demonstrated that the obligate intracellular bacterium *Wolbachia persica* was the closest related organism to *Francisella* (59). *Francisella* is considered phylogenetically related to this bacterium as well as to other unclassified endosymbionts of ticks (9, 11, 156). The genus *Francisella* currently contains two species, *tularensis* and *philomiragia* (Figure 1.1), with the later previously classified under the genus *Yersinia.* Based on 16S rRNA sequencing, which revealed a 98% sequence similarity between *F. philomiragia* and *F. tularensis* (58) and on experimentally defined similar fatty acid profiles, *F. philomiragia* was officially classified under the genus *Francisella* (84).

F. tularensis is currently subdivided into four subspecies: *tularensis, holarctica*, *mediasiatica*, and *novicida* (Figure 1.1) (156). The separation of these subspecies is based on strain virulence, biochemical properties and geographic distribution (Table 1.1 and 1.2) (48, 156). In 1974, Jellison proposed the designations type A for *F. tularensis* subsp. *tularensis* and type B for *F. tularensis* subsp. *holarctica* and these designations are accepted and widely used today (154). In comparison to *F. tularensis* subspp. type A, type B, and *mediasiatica*, *F. tularensis* subsp. *novicida* exhibits differences in growth requirements, metabolic traits and virulence properties (Table 1.1 and 1.2). However,

based on DNA relatedness (75%) to the other *F. tularensis* species and almost identical 16S rRNA sequences the recent classification of this group of strains as a subspecies of *F. tularensis* is for the most part accepted (59, 79).

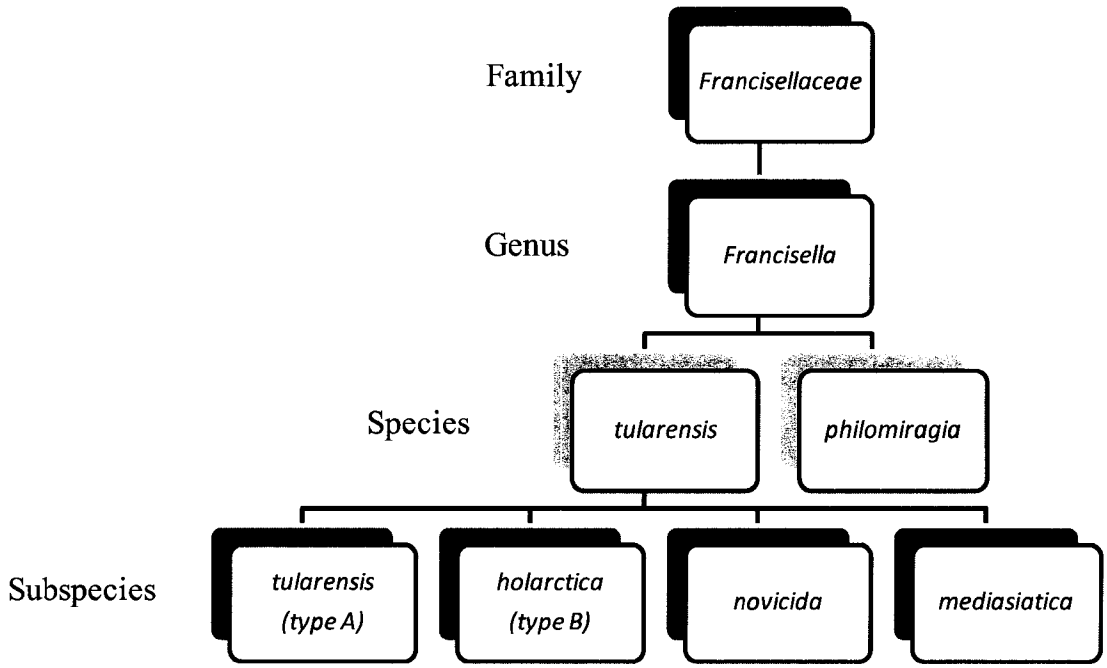


Figure 1.1 ***Francisellaceae*** **family.** The current classification for the *Francisellaceae* family.

Subsequent studies using microarrays (14), restriction fragment length polymorphism (RFLP) (167), and multi-locus variable number of tandem repeat (MLVA) analysis (86) verified the current classification scheme. Additional separation within the subspecies has been proposed (51, 93, 158), and recent identification of new *Francisella* strains in fish (118, 121) will likely change the current classification scheme for *Francisella* (9, 11).

Table 1.1 Summary of virulence and geographical distribution used to differentiate *Francisella* (48, 124, 156).

Species	Virulence	LD_{50} in humans (CFU)[a]	LD_{50} in rabbits (CFU)[b]	LD_{50} in mice (CFU)[c]	Geographic Distribution
Francisella tularensis					
tularensis	Highest	<10	$<10^1$	<10	North America
holarctica	High	$<10^3$	$>10^6$	<1	Northern Hemishpere
mediasiatica	High	nd[d]	$>10^6$	nd	Central Asia
novicida	Low	$>10^3$	$>10^6$	$<10^3$	Global
Francisella philomiragia	Low	nd	nd	nd	Northern Hemishpere

[a,b, c] LD_{50} is an estimation based on subcutaneous infection.

[d] Information has not been determined.

Table 1.2 Summary of biochemical characteristics used to differentiate *Francisella* (156).

Species	Cysteine required for growth	β-lactamase	Acid production from:				Citrulline ureidase pathway	Growth on MacConkey agar	Oxidase	Indole	Gelatin hydrolysis	H_2S slant, Triple sugar iron
			Maltose	Sucrose	D-glucose	Glycerol						
Francisella tularensis												
tularensis	+	+	+	-	+	+	+	-	-	-	-	-
holarctica	+	+	+	-	+	-	-	-	-	-	-	-
mediasiatica	+	-	-	-	-	+	+	-	-	-	-	-
novicida	-	+	m[a]	+	m	m	+	m	-	-	-	-
Francisella philomiragia	-	+	m	+	-	-	nd[b]	m	+	+	+	+

[a] The majority of strains can grow under these conditions.

[b] Information has not been determined.

F. tularensis is a gram-negative, capsulated short coccobacillus that occurs singly. This coccobacillus is a non-motile, non-spore forming facultative intracellular bacterium. *F. tularensis* is also fastidious, requiring enriched medium containing cysteine for growth, with the exception of *F. tularensis* subsp. *novicida* that does not require cysteine

(126). Optimal growth of this bacterium occurs at 35°C. Comparatively, *F. philomiragia* is more readily cultured due to its ability to grow without cysteine, and exhibits optimal growth at 25°C (156). Biochemical assays used to differentiate between *F. tularensis* and *F. philomiragia* include Kovacs oxidase reaction, hydrogen sulfide (H_2S) production on triple sugar iron agar, and gelatin hydrolysis, for which *F. tularensis* is negative for all tests and *F. philomiragia* is positive (Table 1.2) (156). During active growth, *F. tularensis* subspp. *tularensis*, *holarctica* and *mediasiatica* are small, at 0.2-0.7 x 0.2 μm in size and *F. tularensis* subsp. *novicida* and *F. philomiragia* are larger, at 0.7 x 1.7 μm. Colonies are smooth, convex, and white in appearance when grown on cysteine heart agar with 9% sheep blood (CHAB). Microaerophilic conditions are optimal for growth and colonies will reach their maximum size in 3 to 4 days (156).

1.2 Epidemiology and ecology of tularemia

Tularemia is a zoonotic disease commonly referred to as "rabbit fever", "hare fever", "deer fly fever" and "lemming fever" (113). Tularemia is primarily a disease of the Northern hemisphere, with only one case of *F. tularensis* subsp. *novicida* having been reported in the Southern hemisphere in Australia (179). In North America, tularemia cases are found in Canada, the United States, and Mexico, and are caused by both *F. tularensis* subspp. type A and type B, with the former limited to this region. In 2002, the Centers for Disease Control and Prevention (CDC) reported 1,368 tularemia cases of tularemia in the United States between 1990 and 2000 (an average of 124 cases per year). The current number of cases reported in the United States has declined dramatically from the thousands of cases in the 1930s (13). Between 1911 (when this disease was first described) and 1945 14,000 cases were reported in the United States (154). All states

except Hawaii have reported tularemia, with the highest prevalence of cases occurring in Arkansas, Missouri, South Dakota, Oklahoma, and Massachusetts (90).

In regions of Europe and Asia, *F. tularensis* subsp. type B is endemic. Tularemia cases have been reported from all European countries except Iceland and the British Isles, and only recent identification of *F. tularensis* in Portugal (34). An epidemiologic study of the human tularemia cases that occurred in Sweden in 2003 reported 698 cases (125). This is the highest incidence of tularemia for this region since 1967. The number of cases occurring today vary from year to year within endemic areas; however, studies such as that performed in Sweden report tularemia in areas not previously considered as endemic for this disease (125). Emergence of tularemia in Portugal is not unique to this region. In 1997, the emergence of tularemia in Spain was reported (128). Tularemia has also recently emerged in Kosovo. A tularemia outbreak occurred in Kosovo in 2000 that was associated with food and water resulting in 327 confirmed cases that were predominantly in the form of oropharyngeal tularemia (128, 142).

In Japan, the numbers of cases are low, with less than 10 cases reported annually since the 1960s. The epidemiology of this disease in Japan is understudied with little information being available for the associated strains. (154). *F. tularensis* subsp. *mediasiatica* is used to designate strains isolated from Central Asia. Limited research has been performed on these strains.

F. tularensis has one of the broadest and most diverse host ranges of any known bacterial pathogen. It is found in over 200 wildlife species, including mammals, birds, amphibians, fish, reptiles and even invertebrates (113). Small mammals, including lagomorphs, voles, squirrels, muskrats and various rodents serve as hosts for *F.*

tularensis. Ticks, biting flies, and mosquitoes are defined as arthropod vectors for *F. tularensis* (81, 113). The type of host and vector vary between regions, and data suggests that certain hosts and vectors are associated with specific subspp. and subpopulations of *F. tularensis* (51, 90, 113, 158). Further research is needed to better understand the associations between the hosts, and vectors of *F. tularensis,* with emphasis on maintenance and transmission of this disease and how this relates to the different subspecies of *F. tularensis*.

Two disease cycles, an aquatic and a terrestrial cycle, are described for *F. tularensis*. The aquatic cycle is primarily associated with beavers, voles, and muskrats who serve as hosts, with mosquitoes as possible vectors (1, 113). *F. tularensis* subsp. type B can be detected by PCR from streams, ponds and rivers supporting the aquatic cycle (154). In the terrestrial disease cycle, lagomorphs and rodents are the primary hosts, with ticks and flies serving as vectors (113). In addition to the known vectors and hosts of *F. tularensis*, studies have shown that *F. tularensis* subsp. type B can survive in mud and water for months (57). *F. tularensis* has also been shown to survive and grow within protozoa (1). Further studies are needed to better understand the interaction between the two disease cycles and whether all subsp. are associated with the two disease cycles.

There are many questions surrounding the long-term maintenance of *F. tularensis.* It is unknown whether hosts, including rodents, and lagomorphs can maintain this bacterium long-term since it causes an acute infection within these hosts. Whether *F. tularensis* is surviving as free-living or within protozoa in the environment is currently

unknown. These unanswered questions remain one of the most intriguing topics and areas of research for this bacterium.

1.3 Transmission and clinical manifestations of tularemia

All *Francisella* are documented as causing disease in humans (177); however, only *F. tularensis* subspp. type A and type B are of clinical relevance. In addition to *F. tularensis* subspp. type A and type B, *F. tularensis* subsp. *mediasiatica* is the only other subspecies that causes tularemia (156). *F. tularensis* subsp. *novicida* causes tularemia-like disease, and *F. philomiragia* is only rarely associated with septicemic and pneumonic disease in humans (79, 177).

Tularemia can be acquired through several routes. One common route of infection is the direct contact with infected animals, primarily the handling of infected lagomorphs and rodents. Cotton-tailed rabbits (*Sylvilagus* spp.) have commonly been associated with this route of transmission due to the handling of these animals by hunters. Transmission of tularemia to humans has also been associated with other infected animals including cats (139) and prairie dogs (7). In 2002, an outbreak of tularemia in wild-caught, black-tailed prairie dogs (*Cynomys ludovicianus*) occurred in a Texas facility that distributes these animals (129, 183). A 24-year old man who was a handler at the facility was serologically positive for *F. tularensis* and determined to have been recently exposed (7). Although tularemia can spread from animals to humans via direct and indirect routes, it is important to mention that the spread of tularemia by person-to-person contact has never been reported. This is intriguing given the highly infectious nature of this bacterium.

Bites from arthropod vectors are a second route of human infection. Ticks, biting flies, and mosquitoes are associated with human tularemia cases. Ticks (*Ixodidae*) are an important vector in the United States and are capable of transmitting tularemia (42, 51, 82). Among flies, horseflies (*Tabanus* spp. and *Chrysozona* spp.) and deerflies (*Chrysops* spp.), both of which belong to the family *Tabanidae* are also common vectors. In Sweden and Finland mosquitoes are also documented as vectors capable of spreading tularemia in these regions (43).

A third route of transmission is by inhalation of *F. tularensis*. It is estimated that as few as 25 organisms can cause disease when inhaled (150). A case-control study was performed during an outbreak of 15 tularemia cases, 11 of which were pneumonic cases that resulted in one fatal case (52). The outbreak occurred among professional landscapers on Martha's Vineyard in 2000 and the study found that lawn mowing and brush-cutting were risk factors associated with this outbreak. Thus, it was suggested that individuals had created aerosols by mowing over rabbits that had died from tularemia.

Ingestion of contaminated food and water with *F. tularensis* can also cause tularemia. Several outbreaks have been linked to ingestion of this organism in Europe, although it is a rare occurrence in the United States. A report describing 127 oropharyngeal tularemia cases by Luotonen *et al.* describes meat and strawberries as sources of infection (104). Drinking water is rarely associated with outbreaks, and only one such outbreak, connected to well-water, has been documented (164).

Tularemia is used to describe a disease with multiple clinical presentations. The different clinical manifestations are dependent upon the route of transmission and the subspecies involved. General tularemia symptoms include chills, fever, muscle pains,

headache, dry cough, and dermatologic manifestations (papular lesions and erythema nodosum). These symptoms are common to infections caused by both *F. tularensis* type A and type B (48). The incubation period is typically between 3 to 6 days with an abrupt onset of symptoms. In addition to these common symptoms, tularemia can be divided into 5 different clinical presentations: ulceroglandular/glandular, oropharyngeal, oculoglandular, typhoidal, and respiratory tularemia.

Ulceroglandular (glandular) tularemia is the most prevalent form of disease. It is commonly acquired by direct contact with infected animals or can be vector-borne. A papule develops at the site of infection that progresses into an ulcer. Lymph node enlargement is frequent, and dependent on the site of the primary lesion. Glandular tularemia is similar to ulceroglandular except that a primary lesion is not identified. *F. tularensis* type B is the primary cause of ulceroglandular tularemia that is likely due to their maintenance in the environment and their primary vertebrate hosts.

Oculoglandular tularemia is considered a form of ulceroglandular tularemia, but is specific to the conjunctiva (48, 163). Clinical manifestations of this form include unilateral conjunctivitis along with enlargement of preauricular lymph nodes. This form of tularemia, although rare, can be severe. It is usually caused by touching the eye after direct contact with an infected animal or contaminated water.

Oropharyngeal tularemia occurs when contaminated food or water is ingested. Patients develop an ulcerative tonsillitis or pharyngitis, with adjacent lymph node enlargement. If diagnosis is delayed, abscess formation can develop. Due to its similarity to other oropharyngeal infections, it can be speculated that many of these cases of tularemia are not diagnosed properly.

Typhoidal tularemia is the most difficult form of tularemia to diagnose. In addition to common tularemia symptoms, gastrointestinal symptoms and pulmonary infiltrates can occur. A prolonged high-fever and a relative bradycardia are the only symptoms specific to this form of tularemia. Although self-limiting when caused by *F. tularensis* subsp. type B, complications can occur including sepsis when caused by *F. tularensis* subsp. type A. With this form of tularemia other serious complications can occur including meningitis, endocarditis, and rhabdomyolysis (163). Several routes of infection can cause typhoidal tularemia.

Respiratory tularemia is the most acute form of the disease and occurs through inhalation of the organism. This form of tularemia results in a respiratory infection leading to necrotizing pneumonitis and severe shortness of breath. *F. tularensis* subsp. type A causes the severe pneumonic tularemia that is evident by radiology of the lungs. Hematogenous spread of the organism to the lungs has been reported (48, 163). Although less severe, pneumonic outbreaks involving *F. tularensis* subsp. type B have been reported with one such outbreak caused almost 700 respiratory cases in Sweden (32).

As described above, the most severe forms of tularemia are caused by the highly virulent *F. tularensis* subsp. type A. If untreated, severe complications, including lymph node suppuration, pneumonia, and septicemia can occur. Untreated cases have a case fatality rate of 30-60% (48). When treated promptly and appropriately, the case fatality rate is <2%, which demonstrates how critical an accurate diagnosis of this disease is for the outcome of the patient (38). Milder forms of tularemia, caused by *F. tularensis* subsp. type B are rarely fatal and in many cases are self-limiting.

1.4 Therapeutics for tularemia

As with most infectious diseases, effective treatment of tularemia is dependent on early diagnosis and appropriate administration of antibiotics. Aminoglycosides are currently recommended for treatment of tularemia and are bactericidal against *F. tularensis* (38, 154, 163). Parenteral antimicrobial therapy using streptomycin was shown to be effective against *F. tularensis* in the 1940s with an estimated cure rate of 97%, making this the drug of choice in the past (49). Today, the availability of streptomycin is limited due to its vestibular toxicity and the hypersensitivity reactions that this drug may cause while being handled. Thus, gentamicin is the first drug of choice for treating tularemia. Clinical studies have shown that relapses occur when using gentamicin, and data from rodent experiments suggests that gentamicin is not as effective as streptomycin (106). Regardless, gentamicin still has a cure rate of 88% (49). The drug regimen for gentamicin is 5 mg/kg given intravenously in two or three doses. A recent study by Hassoun *et al.* reports on the use of one dose of gentamicin daily (5mg/kg) for effective treatment of glandular tularemia (76). It should be noted that the study only included two patients with glandular tularemia and the effectiveness of this treatment on severe forms of tularemia is not known. Regardless of the number of doses given, patients should have their serum levels of gentamicin monitored during treatment to avoid oto- and nephrotoxicity. Treatment with gentamicin should continue for 7 to 14 days (38).

Alternative drugs for treatment of tularemia include tetracyclines and quinolones (38). Of the tetracyclines, doxycycline is the drug of choice for treatment of tularemia. The recommended regimen is 200 mg once daily for 10 days or 100 mg daily for three

weeks (38). An advantage of this drug when compared with gentamicin is that it can be administered orally. A disadvantage is that tetracyclines are bacteriostatic against *F. tularensis* and therefore relapse when using this drug can occur and has been documented in both human cases and mouse studies (38, 49, 144, 163).

Quinolones were also shown to be effective against *F. tularensis*, and exhibit bactericidal activity against this organism (87). As with tetracyclines, quinolones can be administered orally. Of the quinolones, ciprofloxacin is the most extensively studied for treatment of tularemia. A study conducted by Johansson *et al.* showed that unlike tetracyclines which are not recommended for use in children under the age of 8 years, ciprofloxacin administered for a median period of 4.5 days was effective in treating 10 of 12 children (ages 1-10) with ulceroglandular tularemia (85). Syrjälä *et al.* used orally administered ciprofloxacin to effectively treat 41 of 43 adults with ulceroglandular, pneumonic, or typhoidal tularemia infections (83). A recent study tested the effectiveness of several fluroquinolones in treatment against pneumonic tularemia in mice following an aerosol challenge with *F. tularensis* subsp. type A strain SCHU S4 (159). The fluroquinolones tested included gatifloxacin, moxifloxacin and ciprofloxacin. All three were found to be effective during treatment with a regimen of 100 mg/kg administered orally twice daily for 14 days. However, after treatment was stopped, 100% of the mice that were given ciprofloxacin relapsed and died. The mice given gatifloxacin and moxifloxacin had a significantly lower percentage of relapse, but in all cases the antibiotic activity was more bacteriostatic than bactericidal. The translation of this experiment to humans is unknown. Whether or not prolonging treatment with these antibiotics would decrease the rate of relapse is also uncertain, demonstrating the

difficulties and discrepancies between human clinical studies and studies performed using a mouse model. Ciprofloxacin has been used to treat patients during outbreaks of tularemia in Spain with mixed results (20, 127). Two reports document the effective use of levofloxacin to treat a 66-year-old man with oculoglandular tularemia (6) and two immunocompromised individuals with typhoidal tularemia (99). Data on the effectiveness of quinolones are primarily available for infections caused by *F. tularensis* subsp. type B, with little data available for the use of these drugs to treat infections caused by *F. tularensis* subsp. type A. In vitro studies testing *F. tularensis* subsp. type A strains suggest that similar responses will result in humans given their similar MIC values to those of *F. tularensis* subsp. type B strains (87). Further research to determine the effectiveness of these drugs for treatment of various tularemia infections in humans is needed. Ciprofloxacin and doxycycline have been recommended for the prophylactic use in the case of an attack with *F. tularensis* as a biological weapon (38).

Antibiotic resistance of *F. tularensis* to available drugs is not a major concern outside of having to treat a purposefully altered, drug resistant form of this bacterium. To date, no resistance to aminoglycosides, tetracyclines, or quinolones is documented for this bacterium. Erythromycin and rifampin resistance have been recognized with little concern since these drugs are not used for treatment of tularemia. Among the antibiotics that are not effective against *F. tularensis* are betalactams, co-trimoxazole and clindamycin (83, 163). Treatment efficacy of *F. tularensis* varies when using antibiotics common for treating gram negative bacterial infections. Minimal research has been done in this area and the reasons for the variations in treatment efficacy and for relapse are not well understood. It is likely that the unusual envelope structure of this bacterium is

linked to these findings. Specifically, the uptake and required translocation of certain antibiotics, such as those that inhibit protein synthesis, might be less efficient given the structural differences of this bacterium's cell wall and capsule (156). It has been shown that appropriate dosage and prolonged treatment of these antibiotics is essential in avoiding potential relapse (48).

1.5 History and significance of *F. tularensis* as a biowarfare agent

In 1969, President Richard Nixon gave the executive order to terminate the United States biological weapons development program, and signed the 1972 Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and their Destruction (124). Prior to the signing of this document, it was reported that in the 1950s and 1960s, the United States developed weapons that could disseminate *F. tularensis* aerosols and stockpiled this bacterium as one of several biological weapons (21). During this time, research was also performed using human volunteers in order to develop new antibiotics and vaccines against tularemia.

Properties of *F. tularensis* make it ideal for use as a biological weapon. Not only does this bacterium have one of the lowest infectious doses (as low as 10 cells), but it is easily disseminated, and can cause substantial illness and death if used as such (38). It was not only the United States that saw potential in this highly infectious bacterium as a biological weapon. The information provided by Ken Alibek, a scientist that worked for the former Soviet Union bioweapons program claims that the Soviet Union also weaponized and used *F. tularensis* as a biological weapon during World War II. He also revealed that the weaponized forms of *F. tularensis* strains developed were resistant to

both the antibiotics used to treat tularemia as well as to the only vaccine available, the live vaccine strain (LVS). *F. tularensis* subsp. type B strain LVS was developed in the United States from an attenuated *F. tularensis* subsp. type B that was already being used as a vaccine strain in the former USSR (41). The strain provided by the USSR to the United States resulted in two colony variant types (gray or blue) when plated onto peptone cysteine agar. The blue variant was found to be more protective and was further modified by lyophilization and several passages through mice before becoming the *F. tularensis* subsp. type B strain LVS used for research purposes today. *F. tularensis* subsp. type B strain LVS is not currently approved for use as a vaccine in the United States. Due to the minimal understanding of why this strain is attenuated, efforts continue in trying to identify the reasons for attenuation in this strain as well as in evaluating the possibilities of reversion into a more virulent form (174).

Between 1941 and 1942, 67,000 cases of tularemia occurred in the Soviet Union during World War II (154). Tularemia also occurred in high numbers during the Continuation War between Finland and the Soviet Union. It is uncertain if tularemia cases during these wars were due to the use of *Francisella* as a bioweapon, or more likely that the number of cases paralleled the increase in the rodent population resulting in greater contact between humans and rodents (154). In addition to the United States and the former Soviet Union, the Japanese also weaponized and studied this bacterium as part of their germ warfare research between the 1930s and mid 1940s (75).

The threat of *Francisella* as a bioweapon is uncertain. It is understood that the United States has destroyed all of its developed biological weapons and stockpiles, but it is uncertain whether all other countries that weaponized *F. tularensis* have done the

same. As important are the concerns surrounding the scientists that were directly involved in these biological programs. Where they are today, and what has happened to all of the data obtained through their research remains largely unknown.

Due to the past use and research of this bacterium as a biological weapon, and the properties that make this bacterium an attractive choice for such a purpose, the CDC has classified this bacterium as a Category A agent. Category A agents are classified based on the following criteria: their ease of dissemination, person-to-person transmission (not applicable to *Francisella*), potential for major public health impact, capability of causing public panic and social disruption, and the requirement for additional public health preparedness (31). It has been estimated by the World Health Organization (WHO) expert committee that if *F. tularensis* were disseminated as an aerosol in a biological attack over a city of five million inhabitants, that 250,000 incapacitating casualties with 19,000 deaths would occur. The CDC estimates that the total cost for such an attack would be $5.4 billion for every 100,000 persons exposed (89). After the terror attacks on the United States on September 11th, 2001, a renewed interest in researching biological agents has created funding opportunities for *Francisella* research that had not been available in the past. The research focus emphasizes on preparedness for such an event and has increased our knowledge of this bacterium tremendously over the last five years.

1.6 Host response to *F. tularensis* infection

1.6.1 Innate immune response

F. tularensis is a facultative intracellular pathogen that replicates primarily within macrophages, but has also been found within dendritic cells, hepatocytes (26), alveolar epithelial cell (74) and extracellularly in blood of mice (54, 103). The process by which

macrophages uptake this bacterium is novel (148). Uptake is mediated by complement receptor 3 (CD11b/CD18) and occurs via large, asymmetric pseudopod loops that extend from the macrophage and surround the bacterium. Using *F. tularensis* subsp. type B strain LVS, experiments have revealed that phagosomes containing this bacterium do not fuse with lysosomes in J774 macrophage cells (3). Instead, *F. tularensis* subsp. type B strain LVS enters a membrane-bound acidic vacuole in order to obtain necessary nutrients including iron (61). The necessity for an acidic vacuole agrees with the known mechanism used by *F. tularensis* for iron uptake, which is critical in this environment. Its rhizoferrin-like siderophore, the only iron uptake mechanism described thus far for this bacterium, functions optimally in acidic conditions (16). Several other studies have demonstrated escape of *F. tularensis* from the phagosome with replication within the cytosol (22, 68). Phagosomes containing *F. tularensis* cells accumulate late-endosome markers lamp-1, lamp-2 and CD63 (22, 68, 147). Decrease in lamp-1 and CD63 occurs 3 to 4 hours after infection coinciding with the presence of *F. tularensis* cells in the cytosol. Several virulence determinants have been identified as essential for macrophage replication, including proteins encoded for by the *Francisella* pathogenicity island (FPI) genes (115).

It was demonstrated that *F. tularensis* activates the inflammasome by triggering the release of IL-1β in several cell types, including macrophages (77), human monocytes (65), and both human and mouse dendritic cells (12, 98, 105). IL-18 has also been detected in the supernatant of infected macrophages and is dependent on ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and caspase-1 (a cysteine protease) (77, 105). It is unknown how these cell types sense *F. tularensis*,

although several sensors have been suggested, including human Nalp1 (78), class A scavenger receptor (132) and a mannose receptor (152). Death of the macrophage is estimated to occur between 5 to 24 hours after infection. This time depends on the multiplicity of infection as well as on the strain type. Although activation of the inflammasome is delayed by this bacterium, it plays an important role in innate immunity against *F. tularensis*, inducing the release of pro-inflammatory cytokines required for defense against this bacterium. Inhibitory properties by *F. tularensis* subsp. type B strain LVS during macrophage infection have been documented. Specifically, nuclear factor-kappa B (NFκB)-signal transduction is initially activated upon infection by this bacterium, but activation decreases after 4 to 5 hours (165), and Woolard *et al.* recently discovered that prostaglandin E_2 is produced by macrophages infected with *F. tularensis* subsp. type B strain LVS (180).

The role of toll-like receptors (TLR) in *F. tularensis* recognition is unclear at this point. Studies have demonstrated that *F. tularensis* lipopolysaccharide (LPS) does not interact with TLR-4 (18, 23, 24, 73, 146), although one study contradicts these findings (39). TLR-2 has been shown to be important in activation of several cell types (23, 88, 98), with specific recognition by TLR-2/TLR-6 (88). Research in this area is still needed to understand the specific involvement of TLRs in *F. tularensis* infection.

Neutrophils appear to be important during parenteral infection but not infection via the respiratory route (25, 107, 155). Infection of neutrophils by *F. tularensis* subsp. type B strain LVS does not result in respiratory burst. Further research is needed to better understand the importance of each cell type in combating and maintaining this

infection. Infection with *F. tularensis* results in dissemination of the bacterium to the spleen, liver, lungs, and lymph nodes.

Th-1-type cytokines, including interferon-gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α), seem to be important in control of primary infection. Animal models deficient in IFN-γ or TNF-α convert a sublethal *F. tularensis* subsp. type B strain LVS infection into a lethal one (18, 47, 96). IL-12, which regulates levels of IFN-γ, also appears to be important in clearing *F. tularensis* infection (44). TNF-α and IL-12 increase the levels of INF-γ that ultimately leads to nitric oxide (NO) production. The importance of NO is well documented in other intracellular infections (117, 151); however, the role of NO in killing *F. tularensis* subsp. type B strain LVS by murine macrophages is controversial (60, 62, 63, 101), and has been shown not to be necessary in controlling this infection (133). Lindgren *et al.* (100) obtained different results in which control of infections caused by clinical strains of *F. tularensis* subspp. type A and type B (as opposed to LVS) relied on NO. Other chemokines have also been identified during *F. tularensis* subsp. type B strain LVS infection, including MCP-1 (53, 102), and IL-8 (65). The mechanism by which murine alveolar macrophages control infection is not understood and will require further studies to better understand the mechanism of controlling *F. tularensis* infections and how these mechanisms differ between clinical strains and *F. tularensis* subsp. type B strain LVS.

1.6.2 Adaptive immune response

A role for both B cells and T cells has been identified in the adaptive immune response to *F. tularensis* infection. B cell and antibody involvement is apparent by the detection of IgM, IgG, and IgA in the serum of infected patients 6 to 10 days after onset,

with a peak in antibody levels 1 to 2 months after infection. Antibody detection is achievable for at least eleven years after infection occurs (91). The specific role for antibodies during infection is unclear and does not appear to be necessary in combating infection, as shown when infecting mice with *F. tularensis* subsp. type B strain LVS (182).

Mice intranasally infected with *F. tularensis* subsp. type A after vaccination with *F. tularensis* subsp. type B strain LVS survive; however, if mice are depleted of $CD4^+$ or $CD8^+$ cells, they do not survive (175, 181). Thus, adaptive immunity to *F. tularensis* appears to be primarily T cell mediated (45). Effector T cells become activated days after infection, but Chen *et al.* have suggested that *F. tularensis* subsp. type A may inhibit T cell mediated immunity (17). Multiple T cell populations are reported to be important during *F. tularensis* infection, including $CD4^+$, $CD8^+$, and $CD4^-CD8^-NK1.1^-$ (28, 29). Evidence in humans also suggests a role for γ/δ T cells (92, 135, 161). However, there are some reports that indicate T cell responses are not important for early protection. Specifically, studies using mice that are deficient in mature T cells and infected intradermally with *F. tularensis* subsp. type B strain LVS survive for one month before becoming consumed by the infection (46, 47, 182).

There are still many unanswered questions concerning both the innate and adaptive immune response against this pathogen. It is important to note that most of the studies regarding host innate and adaptive immunity against this pathogen have been conducted using *F. tularensis* subsp. type B strain LVS, and it will be necessary to show that these findings are pertinent to infections caused by *F. tularensis* strains that are

virulent in humans. Additionally, although the data gathered from experiments using mice models is informative, it may not necessarily translate to humans.

1.7 Evolution and genetics of *Francisella*

Based on DNA relatedness and 16S rRNA sequence analysis, the closest relatives to the *Francisellaceae* family are water-associated bacteria. These include *Piscirickettsia salmonis* (fish pathogen) with 84% sequence similarity, *Cycloclasticus pugetii*, *Thimicrospira nivea*, and *Caedibacter taeniospiralis* (87% similarity) (156). *Coxiella burnetti* and *Legionella* spp. are among the closest related human pathogens, but they are distant (156, 168).

Molecular subtyping methods, including PFGE (64, 158), RFLP (167), MLVA (50, 51, 86), and microarray studies (14) have been useful tools for understanding the evolution and diversity among *Francisella*; in particular the evolution of the four subspecies of *F. tularensis.* All methods show high relatedness between the different species and subspecies of this genus. Using MLVA studies, *F. tularensis* subsp. type A are found to possess higher genetic variability (at least in repeat regions) as compared to *F. tularensis* subsp. type B. This suggests that the more virulent *F. tularensis* subsp. type A is evolutionarily older than *F. tularensis* subsp. type B, with the latter having had a more recent global spread (86).

Svensson *et al.* analyzed unidirectional genomic deletions and single nucleotide variations in all four subspecies of *Francisella* (162). Their results propose an evolutionary scheme where *F. tularensis* subsp. *novicida* is evolutionarily the oldest and gave rise from the ancestral strain to both *F. tularensis* subspp. type A and type B. This

study also concluded that *F. tularensis* subsp. type A preceded *F. tularensis* subsp. type B.

Dempsey *et al.* recently conducted a study using paired-end sequence mapping (PESM) to compare *F. tularensis* subspp. type A and type B (36). Their findings suggest extensive insertion/deletion, translocation, and rearrangement events between the two subspecies with high conservation between *F. tularensis* subsp. type B strains. This is in agreement with previous studies (51, 86, 162), including a direct whole genome comparison between *F. tularensis* subsp. type A strain SCHU S4 and *F. tularensis* subsp. type B strain OSU18 by Petrosino *et al.* that found similar results (130).

Among the other *F. tularensis* subspecies, strains of *F. tularensis* subsp. *mediasiatica* were found to be similar to those of *F. tularensis* subsp. type A, but their place in the evolutionary scheme is still unclear. *F. tularensis* subsp. type B strains from Japan were found to be intermediates between *F. tularensis* subsp. type A and *F. tularensis* subsp. type B (86). Further studies are required in order to define the evolution of these strains.

Research indicates that homologous recombination events between different insertion sequence (IS) elements are likely the main mechanism for diversity between *F. tularensis* subspp. type A and type B (90). The number of IS elements contained within the various genomes differs, with a higher number of these elements present in *F. tularensis* subsp. type B (Table 1.3). Additionally, seven types of IS elements are identified within *F. tularensis* genomes that also vary among the different subspecies of *F. tularensis*. The genomic regions containing these elements likely account for differences between the subspecies but are not well characterized. These elements are

also responsible for all but two of the extensive rearrangements that occur between *F. tularensis* subsp. type B strain OSU18 and *F. tularensis* subsp. type A strain SCHU S4 (130).

It is hypothesized that genomic differences between *F. tularensis* subspp. type A and type B are due to the rapid gene decay of the evolutionarily younger *F. tularensis* subsp. type B that results from niche specialization (90). The evidence for this suggestion is based on *F. tularensis* subsp. type B containing 100 more pseudogenes than *F. tularensis* subsp. type A (Table 1.3) (130). It has also been proposed that *F. tularensis* subsp. type A is able to occupy more diverse niches as compared to *F. tularensis* subsp. type B due to its more intact genome (90). However, it is curious that *F. tularensis* subsp. type A has only been found in North America and has never been isolated directly from the environment. In contrast, *F. tularensis* subsp. type B has a broader geographical distribution and has been detected from several water sources in the environment (154).

To date, whole genome sequence data is available for three *F. tularensis* subsp. type A strains, two for *F. tularensis* subsp. type B strains, and three for *F. tularensis* subsp. *novicida* strains. Of these eight, the characterization of two of these are published (94, 130). The genome of *Francisella* is a circular genome with an approximate size of 1.9 Mb and has an overall G + C content of 32%. Table 1.3 summarizes the general properties of the genomes for *F. tularensis* subspp. *tularensis*, *holarctica*, and *novicida* (94, 130, 143). To date, only one native plasmid, pFNL10, has been identified within a *F. tularensis* subsp. *novicida*-like strain F6168 (134).

Table 1.3 Comparison of the general properties of the genomes of *F. tularensis* subspp. type A, type B, and *novicida* (94, 130, 143).

	F. tularensis subsp. *tularensis* strain SCHU S4	*F. tularensis* subsp. *holarctica* strain OSU18	*F. tularensis* subsp. *novicida* strain U112
Size	1,892,819 bp	1,895,727 bp	1,910,031 bp
G+C content (%)	32.9	32.2	32.5
No. of CDS[a]	1,804	1,934	1731[b]
No. of pseudogenes	201	325	14
No. of IS elements	71	109	26
No. of rRNA operons	3	3	nd
No. of tRNAs	38	38	nd
Other stable RNAs	1	7	nd

[a] Number of coding sequence published for strains SCHU S4 and OSU18.

[b] The number given is the number of protein coding genes published by Rohmenr *et al*. The number of protein coding regions for strains SCHU S4 and LVS are 1,445 and 1,380 resepectively.

It is evident that genomic differences are present between the different species and subspecies of *Francisella*. As more genomes become sequenced, more information will help reveal and define these variations. Also of interest will be additional data obtained from research aimed at describing proteome differences between the subspecies of *F. tularensis*. Such studies will allow greater insight into the consequences of the genomic variations.

1.8 Physiology and virulence determinants of *Francisella tularensis*

F. tularensis possesses unique structural characteristics among Gram-negative bacteria including the *F. tularensis* LPS, and capsule. The following section will discuss these unique characteristics as well as other physiological and virulence determinants of this pathogen.

1.8.1 Lipopolysaccharide

The LPS of *F. tularensis* is unique as compared to LPS of other Gram-negative bacteria, and differs slightly among the species and subspecies of *Francisella*. (71). The structure of *F. tularensis* lipid A was determined to be the same for two strains (1547-57 and LVS) of *F. tularensis* subsp. type B (131, 171) and for one strain (U112) of *F. tularensis* subsp. *novicida* (173). Compared to the lipid A of *Escherichia coli* the *Francisella* lipid A is an assymetrical tetraacylated product where the 6-linked reducing hexosamine is acylated similar to *E. coli* but the 1-linked non-reducing hexosamine is only singly acylated. Acylation occurs with longer fatty acids (C15 and C18) in comparison to the *E. coli* structure. Lipid A of *F. tularensis* subspp. type A and type B (but absent from *F. tularensis* subsp. type B strain LVS) has a single phosphate moiety linked to the reducing sugar of the glucosamine disaccharide (as opposed to two phosphate moieties in *E. coli*) (131). This phosphate moiety on the reducing glucosamine is further substituted with a α-linked galactosamine that is not found in *E. coli* (173). Lipid A is further modified in both subspecies with a mannose linked to the 4' position of the non-reducing glucosamine or by addition of an α-linked glucose at the 6' position. The core region of *Francisella* LPS is described for *F. tularensis* subsp. type B strain LVS (171) and for *F. tularensis* subsp. *novicida* strain U112 (170). The two subspecies differ from *E. coli* in that they both lack phosphate modifications, heptose (171), and contain a single 3-deoxy-D-manno-octulsonic acid (Kdo) residue. The core structures of *F. tularensis* subspp. type A and type B differ from *F. tularensis* subsp. *novicida* by an additional α-glucose residue present in *F. tularensis* subsp. *novicida*.

The O-antigen structure was also determined for two strains (15 and LVS) of *F. tularensis* subsp. type B (166, 172), two strains (SCHU S4 and OSU10) of *F. tularensis* subsp. type A (27, 136) and one strain (U112) of *F. tularensis* subsp. *novicida*. The O-antigen structure for *F. tularensis* subspp. type A and type B are identical, but differ from the two terminal residues found in subsp. *novicida*.

The unique LPS of *F. tularensis* equates to a lack of endotoxic properties such as those documented for other Gram-negative bacteria (146). Although *Francisella* LPS induces the production of TNF-α, interleukin-1 (IL-1) and NO, it is only at concentrations 100-1000 times more than that needed for similar induction by *E. coli* LPS (2). A recent study demonstrated that *F. tularensis* LPS can induce production of IL-6 and TNF-α in human B-cells, but not mouse B cells (140). Colony type variants for *F. tularensis* differing in virulence have been described (40) and variation in LPS has been proposed as the reason for phase changes (30).

1.8.2 Capsule

F. tularensis contains a capsule (80) that functions to protect the cell from killing by serum complement (145, 157). The capsule of *F. tularensis* subsp. type A strain SCHU S4 was found to contain carbohydrates, amino acids, and α-OH 14:0 and 16:0 fatty acids (80), and has unusually high lipid content as compared to capsules of other Gram-negative bacteria. Other characteristics of the capsule are long-chain saturated and monosaturated C_{18} to C_{26} acids, saturated $C_{10:0}$, $C_{14:0}$ and $C_{16:0}$, and two additional long-chain hydroxyl acids ($C_{10:0\ 3OH}$ and $C_{18:0\ 3\ OH}$) (156).

Unencapsulated, avirulent *F. tularensis* subsp. type B strain LVS has been shown to become encapsulated and virulent after growth in minimal medium, further supporting

its role in virulence (19). Weiss *et al.* recently used *in vivo* negative selection to determine *F. tularensis* subsp. *novicida* genes required for growth and survival within mice, and identified the capsule synthetic genes (176). Another study using *F. tularensis* subsp. type B strain LVS suggests that down-regulation of the capsule may occur inside the macrophage (68). Although there is some information on the chemical composition of the *F. tularensis* capsule, its precise molecular composition is not apparent. Moreover, it is unknown which biochemical entities participate in virulence. Given the importance of host-pathogen interactions and the apparent ability of *F. tularensis* to modulate the host response, elucidation of the capsule structure should be a priority for those studying the physiology of *F. tularensis*.

1.8.3 Pilus

Gil *et al.* demonstrated the presence of long pili on the surface of *F. tularensis* subsp. type B strain LVS (66), although the pili described on this bacterium's surface have not been identified in subsequent studies. It has been determined that *F. tularensis* subsp. type B LVS lacks type IV pilin since it is a *pilA* mutant (56). In a later study, Forslund *et al.* were unable to detect the long pili that had been previously described for *F. tularensis* subsp. type B strain LVS on the surface of pathogenic *F. tularensis* subsp. type B. Instead, they identified the presence of type IV pili in these strains and determined that their presence is required for spread of this bacterium from the initial sites of infection to the spleen (56). A type II secretion system, based on type IV pili has also been identified in *F. tularensis* (72). Differences in type II secretion genes are found between the *F. tularensis* subspecies, with components of this system shown to be important for virulence (55). In *F. tularensis* subsp. *novicida*, type IV pili have been

shown to be important in secretion of the anti-virulence factor PepO (66, 72). Although evidence exists for the presence of type IV pili in *F. tularensis*, there is little information on the major proteins responsible for pilin assembly. However, beyond these few studies the role of pili for physiology and virulence of *Francisella* is not well understood.

1.8.4 *Francisella* Pathogenicity Island (FPI)

Nano *et al.* identified an approximately 30 Kb pathogenicity island (Fig. 1.2) within *F. tularensis* that is required for intramacrophage growth (116). The whole genome sequence for *F. tularensis* subsp. type A strain SCHU S4, and *F. tularensis* subsp. type B strains LVS and OSU18 identified the presence of the FPI in duplicate (94) with only one copy found within *F. tularensis* subsp. *novicida* . The FPI consists of 16-19 genes that have operonic organization and are regulated by MglA (15) and iron concentration (37). The FPI differs between the *F. tularensis* subspecies with the most significant difference present in the region upstream of *iglB* (Figure 1.2).

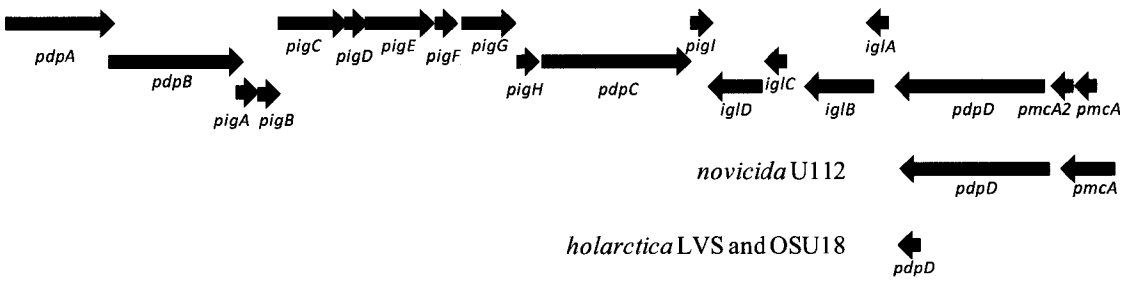


Figure 1.2 *Francisella* pathogenicity island. The genes present in the *Francisella* pathogenicity island are shown for *F. tularensis* subsp. type A strain SCHU S4 in blue. Variations in *F. tularensis* subsp. *novicida* are shown in red, and variations in *F. tularensis* subsp. type B are shown in green.

Studies using mutants in the various FPI genes have been performed and support the need for these genes in replication within macrophages (69, 70), amoebas (95), and for escape from the phagosome (149). Deletions of both *iglC* genes resulted in attenuation in *F. tularensis* subsp. type A SCHU S4 (169). However, beyond studies on *iglA*, *iglB*, *iglC* and *mglA* there is little direct information for the role of other FPI genes. In addition, a type VI secretion system has been proposed for *F. tularensis* (33). IglA and IglB that are encoded for by FPI and required for intracellular growth within macrophages in mice, are homologues to proteins of the type VI secretion system described for *Vibrio* (33, 137). In addition, genes including *pdpB* share homology with genes present in *Pseudomonas aeruginosa* (114) and *Vibrio cholera* (137) that also function in type VI secretion. Thus, bioinformatic analyses suggest a similar function for these genes in *F. tularensis*.

1.8.5 Other secretion systems

Type III and IV secretion systems that are commonly associated with pathogenic intracellular bacteria have not been identified in *F. tularensis* (35, 119, 153). However, homologues of genes involved in type I secretion, *tolC* and *ftlC*, are present within *F. tularensis*. Mutants in these genes result in an increased sensitivity of *F. tularensis* to drugs, detergents and toxic compounds (67).

1.8.6 Iron

As with most intracellular pathogens, iron concentration plays an essential role in the virulence of *F. tularensis*. Several studies have identified proteins that are induced under iron limitation and are associated with virulence (37, 97). In addition, a

rhizoferrin-like siderophore has been identified in *F. tularensis* (160). The current research on iron and *F. tularensis* is described in detail in Chapter 3.

1.8.7 Additional virulence determinants

Transposon mutagenesis and targeted knockouts of *F. tularensis* have identified several virulence determinants. The MinD homologue, and ValAB system were identified in *F. tularensis* subsp. *novicida* as being important for intramacrophage growth, specifically for resistance to oxidative killing and resistance to serum and deoxycholate, respectively (4, 5, 110, 111). Qin *et al.* identified transposon insertion mutants of *F. tularensis* subsp. type A that lack the ability to grow within HepG2 cells. These mutations were within *dsbB* (disulfide bond formation B) and *ggt* (gamma-glutamyl transferase) (138). A recently tested acid phosphatase mutant (*acpA*) was identified as unable to escape the phagosome, or survive and replicate within THP-1 macrophages (112). Older studies by Nano and collegues on *F. tularensis* subsp. *novicida* showed that a secreted acid phosphatase (AcpA) was potentially involved in the modulation of macrophage function (10, 112, 141). An *F. tularensis* subsp. type B strain LVS mutant of *sodB* (superoxide dismutase B) was shown to be less virulent in mice and to have be more sensitive to hydrogen peroxide (8). Mutants of *katG* in *F. tularensis* subsp. type B strain LVS and *F. tularensis* subsp. type A display different virulence phenotypes, with attenuation only in the *F. tularensis* subsp. type B strain LVS mutant (100).

1.9 Concluding remarks

A recurring theme throughout this introductory chapter is the differences between the subsp. in regards to their physiology, virulence, and associated clinical manifestations. A majority of the research conducted on *Francisella* has been performed using *F. tularensis* subsp. type B strain LVS or *F. tularensis* subsp. *novicida*. Both of these strains are not representative of the two primary *F. tularensis* subspp. type A and type B that cause infection in humans. Although research using these strains has been informative and in some cases translational to the virulent subsp., there are still numerous basic, unanswered questions for human virulent strains. For these reasons, we focused our research on data pertinent to the virulent *F. tularensis* subspecies and tried to highlight differences and similarities between all subspecies when relevant.

In addition, the current research in the study of *F. tularensis* focuses heavily on host immune responses and less on the ecology of this bacterium. The research presented in Chapter 3 looks to better understand environmental factors that may be relevant to the ecology of this bacterium by defining an inhibitory substance against *Francisella* and by comparing genomic differences between *F. tularensis* subsp. type A strains. The genomic differences identified in my doctoral research can further be exploited to develop tools that enhance the surveillance and isolation of virulent *F. tularensis* strains.

1.10 Literature cited

1. **Abd, H., T. Johansson, I. Golovliov, G. Sandstrom, and M. Forsman.** 2003. Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii.* Appl Environ Microbiol **69:**600-6.
2. **Ancuta, P., T. Pedron, R. Girard, G. Sandstrom, and R. Chaby.** 1996. Inability of the *Francisella tularensis* lipopolysaccharide to mimic or to antagonize the induction of cell activation by endotoxins. Infect Immun **64:**2041-6.
3. **Anthony, L. D., R. D. Burke, and F. E. Nano.** 1991. Growth of *Francisella* spp. in rodent macrophages. Infect Immun **59:**3291-6.
4. **Anthony, L. S., S. C. Cowley, K. E. Mdluli, and F. E. Nano.** 1994. Isolation of a *Francisella tularensis* mutant that is sensitive to serum and oxidative killing and is avirulent in mice: correlation with the loss of MinD homologue expression. FEMS Microbiol Lett **124:**157-65.
5. **Anthony, L. S., M. Z. Gu, S. C. Cowley, W. W. Leung, and F. E. Nano.** 1991. Transformation and allelic replacement in *Francisella* spp. J Gen Microbiol **137:**2697-703.
6. **Aranda, E. A.** 2001. Treatment of tularemia with levofloxacin. Clin Microbiol Infect **7:**167-8.
7. **Avashia, S. B., J. M. Petersen, C. M. Lindley, M. E. Schriefer, K. L. Gage, M. Cetron, T. A. DeMarcus, D. K. Kim, J. Buck, J. A. Montenieri, J. L. Lowell, M. F. Antolin, M. Y. Kosoy, L. G. Carter, M. C. Chu, K. A. Hendricks, D. T. Dennis, and J. L. Kool.** 2004. First reported prairie dog-to-human tularemia transmission, Texas, 2002. Emerg Infect Dis **10:**483-6.
8. **Bakshi, C. S., M. Malik, K. Regan, J. A. Melendez, D. W. Metzger, V. M. Pavlov, and T. J. Sellati.** 2006. Superoxide dismutase B gene (sodB)-deficient mutants of *Francisella tularensis* demonstrate hypersensitivity to oxidative stress and attenuated virulence. J Bacteriol **188:**6443-8.
9. **Barns, S. M., C. C. Grow, R. T. Okinaka, P. Keim, and C. R. Kuske.** 2005. Detection of diverse new *Francisella*-like bacteria in environmental samples. Appl Environ Microbiol **71:**5494-500.
10. **Baron, G. S., T. J. Reilly, and F. E. Nano.** 1999. The respiratory burst-inhibiting acid phosphatase AcpA is not essential for the intramacrophage growth or virulence of *Francisella novicida.* FEMS Microbiol Lett **176:**85-90.
11. **Beier, C. L., M. Horn, R. Michel, M. Schweikert, H. D. Gortz, and M. Wagner.** 2002. The genus *Caedibacter* comprises endosymbionts of *Paramecium* spp. related to the *Rickettsiales* (Alphaproteobacteria) and to *Francisella tularensis* (Gammaproteobacteria). Appl Environ Microbiol **68:**6043-50.
12. **Ben Nasr, A., J. Haithcoat, J. E. Masterson, J. S. Gunn, T. Eaves-Pyles, and G. R. Klimpel.** 2006. Critical role for serum opsonins and complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in phagocytosis of *Francisella tularensis* by human dendritic cells (DC): uptake of *Francisella* leads to activation of immature DC and intracellular survival of the bacteria. J Leukoc Biol **80:**774-86.

13. **Boyce, J. M.** 1975. Recent trends in the epidemiology of tularemia in the United States. J Infect Dis **131:**197-9.
14. **Broekhuijsen, M., P. Larsson, A. Johansson, M. Bystrom, U. Eriksson, E. Larsson, R. G. Prior, A. Sjostedt, R. W. Titball, and M. Forsman.** 2003. Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. J Clin Microbiol **41:**2924-31.
15. **Brotcke, A., D. S. Weiss, C. C. Kim, P. Chain, S. Malfatti, E. Garcia, and D. M. Monack.** 2006. Identification of MglA-regulated genes reveals novel virulence factors in *Francisella tularensis*. Infect Immun **74:**6642-55.
16. **Carrano, C. J., H. Drechsel, D. Kaiser, G. Jung, B. Matzanke, G. Winkelmann, N. Rochel, and A. M. Albrecht-Gary.** 1996. Coordination Chemistry of the Carboxylate Type Siderophore Rhizoferrin: The Iron(III) Complex and Its Metal Analogs. Inorg Chem **35:**6429-6436.
17. **Chen, W., R. Kuolee, J. W. Austin, H. Shen, Y. Che, and J. W. Conlan.** 2005. Low dose aerosol infection of mice with virulent type A *Francisella tularensis* induces severe thymus atrophy and CD4+CD8+ thymocyte depletion. Microb Pathog **39:**189-96.
18. **Chen, W., R. KuoLee, H. Shen, and J. W. Conlan.** 2004. Susceptibility of immunodeficient mice to aerosol and systemic infection with virulent strains of *Francisella tularensis*. Microb Pathog **36:**311-8.
19. **Cherwonogrodzky, J. W., M. H. Knodel, and M. R. Spence.** 1994. Increased encapsulation and virulence of *Francisella tularensis* live vaccine strain (LVS) by subculturing on synthetic medium. Vaccine **12:**773-5.
20. **Chocarro, A., A. Gonzalez, and I. Garcia.** 2000. Treatment of tularemia with ciprofloxacin. Clin Infect Dis **31:**623.
21. **Christopher, G. W., T. J. Cieslak, J. A. Pavlin, and E. M. Eitzen, Jr.** 1997. Biological warfare. A historical perspective. Jama **278:**412-7.
22. **Clemens, D. L., B. Y. Lee, and M. A. Horwitz.** 2004. Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. Infect Immun **72:**3204-17.
23. **Cole, L. E., K. L. Elkins, S. M. Michalek, N. Qureshi, L. J. Eaton, P. Rallabhandi, N. Cuesta, and S. N. Vogel.** 2006. Immunologic consequences of *Francisella tularensis* live vaccine strain infection: role of the innate immune response in infection and immunity. J Immunol **176:**6888-99.
24. **Collazo, C. M., A. Sher, A. I. Meierovics, and K. L. Elkins.** 2006. Myeloid differentiation factor-88 (MyD88) is essential for control of primary in vivo *Francisella tularensis* LVS infection, but not for control of intra-macrophage bacterial replication. Microbes Infect **8:**779-90.
25. **Conlan, J. W., R. KuoLee, H. Shen, and A. Webb.** 2002. Different host defences are required to protect mice from primary systemic vs pulmonary infection with the facultative intracellular bacterial pathogen, *Francisella tularensis* LVS. Microb Pathog **32:**127-34.

26. **Conlan, J. W., and R. J. North.** 1992. Early pathogenesis of infection in the liver with the facultative intracellular bacteria *Listeria monocytogenes, Francisella tularensis*, and *Salmonella typhimurium* involves lysis of infected hepatocytes by leukocytes. Infect Immun **60:**5164-71.
27. **Conlan, J. W., H. Shen, A. Webb, and M. B. Perry.** 2002. Mice vaccinated with the O-antigen of *Francisella tularensis* LVS lipopolysaccharide conjugated to bovine serum albumin develop varying degrees of protective immunity against systemic or aerosol challenge with virulent type A and type B strains of the pathogen. Vaccine **20:**3465-71.
28. **Cowley, S. C., and K. L. Elkins.** 2003. Multiple T cell subsets control *Francisella tularensis* LVS intracellular growth without stimulation through macrophage interferon gamma receptors. J Exp Med **198:**379-89.
29. **Cowley, S. C., E. Hamilton, J. A. Frelinger, J. Su, J. Forman, and K. L. Elkins.** 2005. CD4-CD8- T cells control intracellular bacterial infections both in vitro and in vivo. J Exp Med **202:**309-19.
30. **Cowley, S. C., S. V. Myltseva, and F. E. Nano.** 1996. Phase variation in *Francisella tularensis* affecting intracellular growth, lipopolysaccharide antigenicity and nitric oxide production. Mol Microbiol **20:**867-74.
31. **Cronquist, S. D.** 2004. Tularemia: the disease and the weapon. Dermatol Clin **22:**313-20, vi-vii.
32. **Dahlstrand, S., O. Ringertz, and B. Zetterberg.** 1971. Airborne tularemia in Sweden. Scand J Infect Dis **3:**7-16.
33. **de Bruin, O. M., J. S. Ludu, and F. E. Nano.** 2007. The *Francisella* pathogenicity island protein IglA localizes to the bacterial cytoplasm and is needed for intracellular growth. BMC Microbiol **7:**1.
34. **de Carvalho, I. L., R. Escudero, C. Garcia-Amil, H. Falcao, P. Anda, and M. S. Nuncio.** 2007. *Francisella tularensis,* Portugal. Emerg Infect Dis **13:**666-7.
35. **de Chastellier, C., and P. Berche.** 1994. Fate of *Listeria monocytogenes* in murine macrophages: evidence for simultaneous killing and survival of intracellular bacteria. Infect Immun **62:**543-53.
36. **Dempsey, M. P., J. Nietfeldt, J. Ravel, S. Hinrichs, R. Crawford, and A. K. Benson.** 2006. Paired-end sequence mapping detects extensive genomic rearrangement and translocation during divergence of *Francisella tularensis* subsp. *tularensis* and *Francisella tularensis* subsp. *holarctica* populations. J Bacteriol **188:**5904-14.
37. **Deng, K., R. J. Blick, W. Liu, and E. J. Hansen.** 2006. Identification of *Francisella tularensis* genes affected by iron limitation. Infect Immun **74:**4224-36.
38. **Dennis, D. T., T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, A. M. Friedlander, J. Hauer, M. Layton, S. R. Lillibridge, J. E. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, and K. Tonat.** 2001. Tularemia as a biological weapon: medical and public health management. Jama **285:**2763-73.

39. **Duenas, A. I., M. Aceves, A. Orduna, R. Diaz, M. Sanchez Crespo, and C. Garcia-Rodriguez.** 2006. *Francisella tularensis* LPS induces the production of cytokines in human monocytes and signals via Toll-like receptor 4 with much lower potency than *E. coli* LPS. Int Immunol **18:**785-95.
40. **Eigelsbach, H. T., W. Braun, and R. D. Herring.** 1951. Studies on the variation of *Bacterium tularense*. J Bacteriol **61:**557-69.
41. **Eigelsbach, H. T., and C. M. Downs.** 1961. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. J Immunol **87:**415-25.
42. **Eisen, L.** 2007. A call for renewed research on tick-borne *Francisella tularensis* in the Arkansas-Missouri primary national focus of tularemia in humans. J Med Entomol **44:**389-97.
43. **Eliasson, H., J. Lindback, J. P. Nuorti, M. Arneborn, J. Giesecke, and A. Tegnell.** 2002. The 2000 tularemia outbreak: a case-control study of risk factors in disease-endemic and emergent areas, Sweden. Emerg Infect Dis **8:**956-60.
44. **Elkins, K. L., A. Cooper, S. M. Colombini, S. C. Cowley, and T. L. Kieffer.** 2002. In vivo clearance of an intracellular bacterium, *Francisella tularensis* LVS, is dependent on the p40 subunit of interleukin-12 (IL-12) but not on IL-12 p70. Infect Immun **70:**1936-48.
45. **Elkins, K. L., S. C. Cowley, and C. M. Bosio.** 2007. Innate and adaptive immunity to *francisella*. Ann N Y Acad Sci **1105:**284-324.
46. **Elkins, K. L., T. Rhinehart-Jones, C. A. Nacy, R. K. Winegar, and A. H. Fortier.** 1993. T-cell-independent resistance to infection and generation of immunity to *Francisella tularensis*. Infect Immun **61:**823-9.
47. **Elkins, K. L., T. R. Rhinehart-Jones, S. J. Culkin, D. Yee, and R. K. Winegar.** 1996. Minimal requirements for murine resistance to infection with *Francisella tularensis* LVS. Infect Immun **64:**3288-93.
48. **Ellis, J., P. C. Oyston, M. Green, and R. W. Titball.** 2002. Tularemia. Clin Microbiol Rev **15:**631-46.
49. **Enderlin, G., L. Morales, R. F. Jacobs, and J. T. Cross.** 1994. Streptomycin and alternative agents for the treatment of tularemia: review of the literature. Clin Infect Dis **19:**42-7.
50. **Farlow, J., K. L. Smith, J. Wong, M. Abrams, M. Lytle, and P. Keim.** 2001. *Francisella tularensis* strain typing using multiple-locus, variable-number tandem repeat analysis. J Clin Microbiol **39:**3186-92.
51. **Farlow, J., D. M. Wagner, M. Dukerich, M. Stanley, M. Chu, K. Kubota, J. Petersen, and P. Keim.** 2005. *Francisella tularensis* in the United States. Emerg Infect Dis **11:**1835-41.
52. **Feldman, K. A., R. E. Enscore, S. L. Lathrop, B. T. Matyas, M. McGuill, M. E. Schriefer, D. Stiles-Enos, D. T. Dennis, L. R. Petersen, and E. B. Hayes.** 2001. An outbreak of primary pneumonic tularemia on Martha's Vineyard. N Engl J Med **345:**1601-6.
53. **Forestal, C. A., J. L. Benach, C. Carbonara, J. K. Italo, T. J. Lisinski, and M. B. Furie.** 2003. *Francisella tularensis* selectively induces proinflammatory changes in endothelial cells. J Immunol **171:**2563-70.

54. **Forestal, C. A., M. Malik, S. V. Catlett, A. G. Savitt, J. L. Benach, T. J. Sellati, and M. B. Furie.** 2007. *Francisella tularensis* has a significant extracellular phase in infected mice. J Infect Dis **196:**134-7.
55. **Forsberg, A., and T. Guina.** 2007. Type II secretion and Type IV pili of *Francisella*. Ann N Y Acad Sci **1105:**187-201.
56. **Forslund, A. L., K. Kuoppa, K. Svensson, E. Salomonsson, A. Johansson, M. Bystrom, P. C. Oyston, S. L. Michell, R. W. Titball, L. Noppa, E. Frithz-Lindsten, M. Forsman, and A. Forsberg.** 2006. Direct repeat-mediated deletion of a type IV pilin gene results in major virulence attenuation of *Francisella tularensis*. Mol Microbiol **59:**1818-30.
57. **Forsman, M., E. W. Henningson, E. Larsson, T. Johansson, and G. Sandstrom.** 2000. *Francisella tularensis* does not manifest virulence in viable but non-culturable state. FEMS Microbiol Ecol **31:**217-224.
58. **Forsman, M., K. Kuoppa, A. Sjostedt, and A. Tarnvik.** 1990. Use of RNA hybridization in the diagnosis of a case of ulceroglandular tularemia. Eur J Clin Microbiol Infect Dis **9:**784-5.
59. **Forsman, M., G. Sandstrom, and A. Sjostedt.** 1994. Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. Int J Syst Bacteriol **44:**38-46.
60. **Fortier, A. H., S. J. Green, T. Polsinelli, T. R. Jones, R. M. Crawford, D. A. Leiby, K. L. Elkins, M. S. Meltzer, and C. A. Nacy.** 1994. Life and death of an intracellular pathogen: *Francisella tularensis* and the macrophage. Immunol Ser **60:**349-61.
61. **Fortier, A. H., D. A. Leiby, R. B. Narayanan, E. Asafoadjei, R. M. Crawford, C. A. Nacy, and M. S. Meltzer.** 1995. Growth of *Francisella tularensis* LVS in macrophages: the acidic intracellular compartment provides essential iron required for growth. Infect Immun **63:**1478-83.
62. **Fortier, A. H., T. Polsinelli, S. J. Green, and C. A. Nacy.** 1992. Activation of macrophages for destruction of *Francisella tularensis:* identification of cytokines, effector cells, and effector molecules. Infect Immun **60:**817-25.
63. **Fortier, A. H., M. V. Slayter, R. Ziemba, M. S. Meltzer, and C. A. Nacy.** 1991. Live vaccine strain of *Francisella tularensis:* infection and immunity in mice. Infect Immun **59:**2922-8.
64. **Garcia Del Blanco, N., M. E. Dobson, A. I. Vela, V. A. De La Puente, C. B. Gutierrez, T. L. Hadfield, P. Kuhnert, J. Frey, L. Dominguez, and E. F. Rodriguez Ferri.** 2002. Genotyping of *Francisella tularensis* strains by pulsed-field gel electrophoresis, amplified fragment length polymorphism fingerprinting, and 16S rRNA gene sequencing. J Clin Microbiol **40:**2964-72.
65. **Gavrilin, M. A., I. J. Bouakl, N. L. Knatz, M. D. Duncan, M. W. Hall, J. S. Gunn, and M. D. Wewers.** 2006. Internalization and phagosome escape required for *Francisella* to induce human monocyte IL-1beta processing and release. Proc Natl Acad Sci U S A **103:**141-6.
66. **Gil, H., J. L. Benach, and D. G. Thanassi.** 2004. Presence of pili on the surface of *Francisella tularensis*. Infect Immun **72:**3042-7.

67. **Gil, H., G. J. Platz, C. A. Forestal, M. Monfett, C. S. Bakshi, T. J. Sellati, M. B. Furie, J. L. Benach, and D. G. Thanassi.** 2006. Deletion of TolC orthologs in *Francisella tularensis* identifies roles in multidrug resistance and virulence. Proc Natl Acad Sci U S A **103:**12897-902.
68. **Golovliov, I., V. Baranov, Z. Krocova, H. Kovarova, and A. Sjostedt.** 2003. An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. Infect Immun **71:**5940-50.
69. **Golovliov, I., A. Sjostedt, A. Mokrievich, and V. Pavlov.** 2003. A method for allelic replacement in *Francisella tularensis*. FEMS Microbiol Lett **222:**273-80.
70. **Gray, C. G., S. C. Cowley, K. K. Cheung, and F. E. Nano.** 2002. The identification of five genetic loci of *Francisella novicida* associated with intracellular growth. FEMS Microbiol Lett **215:**53-6.
71. **Gunn, J. S., and R. K. Ernst.** 2007. The structure and function of *francisella* lipopolysaccharide. Ann N Y Acad Sci **1105:**202-18.
72. **Hager, A. J., D. L. Bolton, M. R. Pelletier, M. J. Brittnacher, L. A. Gallagher, R. Kaul, S. J. Skerrett, S. I. Miller, and T. Guina.** 2006. Type IV pili-mediated secretion modulates *Francisella* virulence. Mol Microbiol **62:**227-37.
73. **Hajjar, A. M., M. D. Harvey, S. A. Shaffer, D. R. Goodlett, A. Sjostedt, H. Edebro, M. Forsman, M. Bystrom, M. Pelletier, C. B. Wilson, S. I. Miller, S. J. Skerrett, and R. K. Ernst.** 2006. Lack of in vitro and in vivo recognition of *Francisella tularensis* subspecies lipopolysaccharide by Toll-like receptors. Infect Immun **74:**6730-8.
74. **Hall, J. D., R. R. Craven, J. R. Fuller, R. J. Pickles, and T. H. Kawula.** 2007. *Francisella tularensis* replicates within alveolar type II epithelial cells in vitro and in vivo following inhalation. Infect Immun **75:**1034-9.
75. **Harris, S.** 1992. Japanese biological warfare research on humans: a case study of microbiology and ethics. Ann N Y Acad Sci **666:**21-52.
76. **Hassoun, A., R. Spera, and J. Dunkel.** 2006. Tularemia and once-daily gentamicin. Antimicrob Agents Chemother **50:**824.
77. **Henry, T., A. Brotcke, D. S. Weiss, L. J. Thompson, and D. M. Monack.** 2007. Type I interferon signaling is required for activation of the inflammasome during *Francisella* infection. J Exp Med **204:**987-94.
78. **Henry, T., and D. M. Monack.** 2007. Activation of the inflammasome upon *Francisella tularensis* infection: interplay of innate immune pathways and virulence factors. Cell Microbiol.
79. **Hollis, D. G., R. E. Weaver, A. G. Steigerwalt, J. D. Wenger, C. W. Moss, and D. J. Brenner.** 1989. *Francisella philomiragia* comb. nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup novicida (formerly *Francisella novicida*) associated with human disease. J Clin Microbiol **27:**1601-8.
80. **Hood, A. M.** 1977. Virulence factors of *Francisella tularensis*. J Hyg (Lond) **79:**47-60.
81. **Hopla, C. E.** 1974. The ecology of tularemia. Adv Vet Sci Comp Med **18:**25-53.
82. **Hopla, C. E.** 1953. Experimental studies on tick transmission of tularemia organisms. Am J Hyg **58:**101-18.

83. **Ikaheimo, I., H. Syrjala, J. Karhukorpi, R. Schildt, and M. Koskela.** 2000. In vitro antibiotic susceptibility of *Francisella tularensis* isolated from humans and animals. J Antimicrob Chemother **46:**287-90.
84. **Jensen, W. I., C. R. Owen, and W. L. Jellison.** 1969. *Yersinia philomiragia* sp. n., a new member of the *Pasteurella* group of bacteria, naturally pathogenic for the muskrat (*Ondatra zibethica*). J Bacteriol **100:**1237-41.
85. **Johansson, A., L. Berglund, L. Gothefors, A. Sjostedt, and A. Tarnvik.** 2000. Ciprofloxacin for treatment of tularemia in children. Pediatr Infect Dis J **19:**449-53.
86. **Johansson, A., J. Farlow, P. Larsson, M. Dukerich, E. Chambers, M. Bystrom, J. Fox, M. Chu, M. Forsman, A. Sjostedt, and P. Keim.** 2004. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J Bacteriol **186:**5808-18.
87. **Johansson, A., S. K. Urich, M. C. Chu, A. Sjostedt, and A. Tarnvik.** 2002. In vitro susceptibility to quinolones of *Francisella tularensis* subspecies *tularensis*. Scand J Infect Dis **34:**327-30.
88. **Katz, J., P. Zhang, M. Martin, S. N. Vogel, and S. M. Michalek.** 2006. Toll-like receptor 2 is required for inflammatory responses to *Francisella tularensis* LVS. Infect Immun **74:**2809-16.
89. **Kaufmann, A. F., M. I. Meltzer, and G. P. Schmid.** 1997. The economic impact of a bioterrorist attack: are prevention and postattack intervention programs justifiable? Emerg Infect Dis **3:**83-94.
90. **Keim, P., A. Johansson, and D. M. Wagner.** 2007. Molecular epidemiology, evolution, and ecology of *francisella*. Ann N Y Acad Sci **1105:**30-66.
91. **Koskela, P., and A. Salminen.** 1985. Humoral immunity against *Francisella tularensis* after natural infection. J Clin Microbiol **22:**973-9.
92. **Kroca, M., A. Tarnvik, and A. Sjostedt.** 2000. The proportion of circulating gammadelta T cells increases after the first week of onset of tularaemia and remains elevated for more than a year. Clin Exp Immunol **120:**280-4.
93. **Kudelina, R. I., and N. G. Olsufiev.** 1980. Sensitivity to macrolide antibiotics and lincomycin in *Francisella tularensis holarctica*. J Hyg Epidemiol Microbiol Immunol **24:**84-91.
94. **Larsson, P., P. C. Oyston, P. Chain, M. C. Chu, M. Duffield, H. H. Fuxelius, E. Garcia, G. Halltorp, D. Johansson, K. E. Isherwood, P. D. Karp, E. Larsson, Y. Liu, S. Michell, J. Prior, R. Prior, S. Malfatti, A. Sjostedt, K. Svensson, N. Thompson, L. Vergez, J. K. Wagg, B. W. Wren, L. E. Lindler, S. G. Andersson, M. Forsman, and R. W. Titball.** 2005. The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. Nat Genet **37:**153-9.
95. **Lauriano, C. M., J. R. Barker, S. S. Yoon, F. E. Nano, B. P. Arulanandam, D. J. Hassett, and K. E. Klose.** 2004. MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intraamoebae and intramacrophage survival. Proc Natl Acad Sci U S A **101:**4246-9.

96. **Leiby, D. A., A. H. Fortier, R. M. Crawford, R. D. Schreiber, and C. A. Nacy.** 1992. In vivo modulation of the murine immune response to *Francisella tularensis* LVS by administration of anticytokine antibodies. Infect Immun **60:**84-9.

97. **Lenco, J., M. Hubalek, P. Larsson, A. Fucikova, M. Brychta, A. Macela, and J. Stulik.** 2007. Proteomics analysis of the *Francisella tularensis* LVS response to iron restriction: induction of the *F. tularensis* pathogenicity island proteins IglABC. FEMS Microbiol Lett **269:**11-21.

98. **Li, H., S. Nookala, X. R. Bina, J. E. Bina, and F. Re.** 2006. Innate immune response to *Francisella tularensis* is mediated by TLR2 and caspase-1 activation. J Leukoc Biol **80:**766-73.

99. **Limaye, A. P., and C. J. Hooper.** 1999. Treatment of tularemia with fluoroquinolones: two cases and review. Clin Infect Dis **29:**922-4.

100. **Lindgren, H., H. Shen, C. Zingmark, I. Golovliov, W. Conlan, and A. Sjostedt.** 2007. Resistance of *Francisella tularensis* strains against reactive nitrogen and oxygen species with special reference to the role of KatG. Infect Immun **75:**1303-9.

101. **Lindgren, H., L. Stenman, A. Tarnvik, and A. Sjostedt.** 2005. The contribution of reactive nitrogen and oxygen species to the killing of *Francisella tularensis* LVS by murine macrophages. Microbes Infect **7:**467-75.

102. **Loegering, D. J., J. R. Drake, J. A. Banas, T. L. McNealy, D. G. Mc Arthur, L. M. Webster, and M. R. Lennartz.** 2006. *Francisella tularensis* LVS grown in macrophages has reduced ability to stimulate the secretion of inflammatory cytokines by macrophages in vitro. Microb Pathog **41:**218-25.

103. **Long, G. W., J. J. Oprandy, R. B. Narayanan, A. H. Fortier, K. R. Porter, and C. A. Nacy.** 1993. Detection of *Francisella tularensis* in blood by polymerase chain reaction. J Clin Microbiol **31:**152-4.

104. **Luotonen, J., H. Syrjala, K. Jokinen, S. Sutinen, and A. Salminen.** 1986. Tularemia in otolaryngologic practice. An analysis of 127 cases. Arch Otolaryngol Head Neck Surg **112:**77-80.

105. **Mariathasan, S., D. S. Weiss, V. M. Dixit, and D. M. Monack.** 2005. Innate immunity against *Francisella tularensis* is dependent on the ASC/caspase-1 axis. J Exp Med **202:**1043-9.

106. **Mason, W. L., H. T. Eigelsbach, S. F. Little, and J. H. Bates.** 1980. Treatment of tularemia, including pulmonary tularemia, with gentamicin. Am Rev Respir Dis **121:**39-45.

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

108. **McCoy, G. W.** 1911. A plague-like disease in rodents. Publ. Health Bull. 43:53-71. .

109. **McCoy, G. W., and C. W. Chapin.** 1912. Further observations on a plague-like disease of rodents with a preliminary note on the causative agent, *Bacterium tularense*. J Infect Dis **10:**61-72.

110. **McDonald, M. K., S. C. Cowley, and F. E. Nano.** 1997. Temperature-sensitive lesions in the *Francisella novicida valA* gene cloned into an *Escherichia coli msbA lpxK* mutant affecting deoxycholate resistance and lipopolysaccharide assembly at the restrictive temperature. J Bacteriol **179:**7638-43.
111. **Mdluli, K. E., L. S. Anthony, G. S. Baron, M. K. McDonald, S. V. Myltseva, and F. E. Nano.** 1994. Serum-sensitive mutation of *Francisella novicida*: association with an ABC transporter gene. Microbiology **140 (Pt 12):**3309-18.
112. **Mohapatra, N. P., A. Balagopal, S. Soni, L. S. Schlesinger, and J. S. Gunn.** 2007. AcpA is a *Francisella* acid phosphatase that affects intramacrophage survival and virulence. Infect Immun **75:**390-6.
113. **Morner, T.** 1992. The ecology of tularaemia. Rev Sci Tech **11:**1123-30.
114. **Mougous, J. D., M. E. Cuff, S. Raunser, A. Shen, M. Zhou, C. A. Gifford, A. L. Goodman, G. Joachimiak, C. L. Ordonez, S. Lory, T. Walz, A. Joachimiak, and J. J. Mekalanos.** 2006. A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. Science **312:**1526-30.
115. **Nano, F. E., and C. Schmerk.** 2007. The *francisella* pathogenicity island. Ann N Y Acad Sci **1105:**122-37.
116. **Nano, F. E., N. Zhang, S. C. Cowley, K. E. Klose, K. K. Cheung, M. J. Roberts, J. S. Ludu, G. W. Letendre, A. I. Meierovics, G. Stephens, and K. L. Elkins.** 2004. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. J Bacteriol **186:**6430-6.
117. **Nathan, C., and M. U. Shiloh.** 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc Natl Acad Sci U S A **97:**8841-8.
118. **Nylund, A., K. F. Ottem, K. Watanabe, E. Karlsbakk, and B. Krossoy.** 2006. *Francisella* sp. (Family *Francisellaceae*) causing mortality in Norwegian cod (*Gadus morhua*) farming. Arch Microbiol **185:**383-92.
119. **Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman.** 1996. Identification of a pathogenicity island required for *Salmonella* survival in host cells. Proc Natl Acad Sci U S A **93:**7800-4.
120. **Ohara, S.** 1954. Studies on yato-byo (Ohara's disease, tularemia in Japan). I. Jpn J Exp Med **24:**69-79.
121. **Olsen, A. B., J. Mikalsen, M. Rode, A. Alfjorden, E. Hoel, K. Straum-Lie, R. Haldorsen, and D. J. Colquhoun.** 2006. A novel systemic granulomatous inflammatory disease in farmed Atlantic cod, *Gadus morhua* L., associated with a bacterium belonging to the genus *Francisella*. J Fish Dis **29:**307-11.
122. **Olsufiev, N. G., O. S. Emelyanova, and T. N. Dunayeva.** 1959. Comparative study of strains of *B. tularense* in the old and new world and their taxonomy. J Hyg Epidemiol Microbiol Immunol **3:**138-49.
123. **Olsufjev, N. G.** 1970. Taxonomy and characteristic of the genus *Francisella* Dorofeev, 1947. J Hyg Epidemiol Microbiol Immunol **14:**67-74.
124. **Oyston, P. C., A. Sjostedt, and R. W. Titball.** 2004. Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*. Nat Rev Microbiol **2:**967-78.
125. **Payne, L., M. Arneborn, A. Tegnell, and J. Giesecke.** 2005. Endemic tularemia, Sweden, 2003. Emerg Infect Dis **11:**1440-2.

126. **Payne, M. P., and R. J. Morton.** 1992. Effect of culture media and incubation temperature on growth of selected strains of *Francisella tularensis*. J Vet Diagn Invest **4:**264-9.
127. **Perez-Castrillon, J. L., P. Bachiller-Luque, M. Martin-Luquero, F. J. Mena-Martin, and V. Herreros.** 2001. Tularemia epidemic in northwestern Spain: clinical description and therapeutic response. Clin Infect Dis **33:**573-6.
128. **Petersen, J. M., and M. E. Schriefer.** 2005. Tularemia: emergence/re-emergence. Vet Res **36:**455-67.
129. **Petersen, J. M., M. E. Schriefer, L. G. Carter, Y. Zhou, T. Sealy, D. Bawiec, B. Yockey, S. Urich, N. S. Zeidner, S. Avashia, J. L. Kool, J. Buck, C. Lindley, L. Celeda, J. A. Monteneiri, K. L. Gage, and M. C. Chu.** 2004. Laboratory analysis of tularemia in wild-trapped, commercially traded prairie dogs, Texas, 2002. Emerg Infect Dis **10:**419-25.
130. **Petrosino, J. F., Q. Xiang, S. E. Karpathy, H. Jiang, S. Yerrapragada, Y. Liu, J. Gioia, L. Hemphill, A. Gonzalez, T. M. Raghavan, A. Uzman, G. E. Fox, S. Highlander, M. Reichard, R. J. Morton, K. D. Clinkenbeard, and G. M. Weinstock.** 2006. Chromosome rearrangement and diversification of *Francisella tularensis* revealed by the type B (OSU18) genome sequence. J Bacteriol **188:**6977-85.
131. **Phillips, N. J., B. Schilling, M. K. McLendon, M. A. Apicella, and B. W. Gibson.** 2004. Novel modification of lipid A of *Francisella tularensis*. Infect Immun **72:**5340-8.
132. **Pierini, L. M.** 2006. Uptake of serum-opsonized *Francisella tularensis* by macrophages can be mediated by class A scavenger receptors. Cell Microbiol **8:**1361-70.
133. **Polsinelli, T., M. S. Meltzer, and A. H. Fortier.** 1994. Nitric oxide-independent killing of *Francisella tularensis* by IFN-gamma-stimulated murine alveolar macrophages. J Immunol **153:**1238-45.
134. **Pomerantsev, A. P., I. R. Golovliov, Y. Ohara, A. N. Mokrievich, M. Obuchi, A. Norqvist, K. Kuoppa, and V. M. Pavlov.** 2001. Genetic organization of the *Francisella* plasmid pFNL10. Plasmid **46:**210-22.
135. **Poquet, Y., M. Kroca, F. Halary, S. Stenmark, M. A. Peyrat, M. Bonneville, J. J. Fournie, and A. Sjostedt.** 1998. Expansion of Vgamma9 Vdelta2 T cells is triggered by *Francisella tularensis*-derived phosphoantigens in tularemia but not after tularemia vaccination. Infect Immun **66:**2107-14.
136. **Prior, J. L., R. G. Prior, P. G. Hitchen, H. Diaper, K. F. Griffin, H. R. Morris, A. Dell, and R. W. Titball.** 2003. Characterization of the O antigen gene cluster and structural analysis of the O antigen of *Francisella tularensis* subsp. *tularensis*. J Med Microbiol **52:**845-51.
137. **Pukatzki, S., A. T. Ma, D. Sturtevant, B. Krastins, D. Sarracino, W. C. Nelson, J. F. Heidelberg, and J. J. Mekalanos.** 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proc Natl Acad Sci U S A **103:**1528-33.
138. **Qin, A., and B. J. Mann.** 2006. Identification of transposon insertion mutants of *Francisella tularensis tularensis* strain Schu S4 deficient in intracellular replication in the hepatic cell line HepG2. BMC Microbiol **6:**69.

139. **Quenzer, R. W., S. R. Mostow, and J. K. Emerson.** 1977. Cat-bite tularemia. Jama **238:**1845.
140. **Rahhal, R. M., T. J. Vandenbush, M. K. McLendon, M. A. Apicella, and G. A. Bishop.** 2007. Differential effects of *Francisella tularensis* lipopolysaccharide on B lymphocytes. J Leukoc Biol.
141. **Reilly, T. J., G. S. Baron, F. E. Nano, and M. S. Kuhlenschmidt.** 1996. Characterization and sequencing of a respiratory burst-inhibiting acid phosphatase from *Francisella tularensis*. J Biol Chem **271:**10973-83.
142. **Reintjes, R., I. Dedushaj, A. Gjini, T. R. Jorgensen, B. Cotter, A. Lieftucht, F. D'Ancona, D. T. Dennis, M. A. Kosoy, G. Mulliqi-Osmani, R. Grunow, A. Kalaveshi, L. Gashi, and I. Humolli.** 2002. Tularemia outbreak investigation in Kosovo: case control and environmental studies. Emerg Infect Dis **8:**69-73.
143. **Rohmer, L., C. Fong, S. Abmayr, M. Wasnick, T. J. Larson Freeman, M. Radey, T. Guina, K. Svensson, H. S. Hayden, M. Jacobs, L. A. Gallagher, C. Manoil, R. K. Ernst, B. Drees, D. Buckley, E. Haugen, D. Bovee, Y. Zhou, J. Chang, R. Levy, R. Lim, W. Gillett, D. Guenthener, A. Kang, S. A. Shaffer, G. Taylor, J. Chen, B. Gallis, D. A. D'Argenio, M. Forsman, M. V. Olson, D. R. Goodlett, R. Kaul, S. I. Miller, and M. J. Brittnacher.** 2007. Comparison of *Francisella tularensis* genomes reveals evolutionary events associated with the emergence of human pathogenic strains. Genome Biol **8:**R102.
144. **Russell, P., S. M. Eley, M. J. Fulop, D. L. Bell, and R. W. Titball.** 1998. The efficacy of ciprofloxacin and doxycycline against experimental tularaemia. J Antimicrob Chemother **41:**461-5.
145. **Sandstrom, G., S. Lofgren, and A. Tarnvik.** 1988. A capsule-deficient mutant of *Francisella tularensis* LVS exhibits enhanced sensitivity to killing by serum but diminished sensitivity to killing by polymorphonuclear leukocytes. Infect Immun **56:**1194-202.
146. **Sandstrom, G., A. Sjostedt, T. Johansson, K. Kuoppa, and J. C. Williams.** 1992. Immunogenicity and toxicity of lipopolysaccharide from *Francisella tularensis* LVS. FEMS Microbiol Immunol **5:**201-10.
147. **Santic, M., M. Molmeret, and Y. Abu Kwaik.** 2005. Modulation of biogenesis of the *Francisella tularensis* subsp. *novicida*-containing phagosome in quiescent human macrophages and its maturation into a phagolysosome upon activation by IFN-gamma. Cell Microbiol **7:**957-67.
148. **Santic, M., M. Molmeret, K. E. Klose, and Y. Abu Kwaik.** 2006. *Francisella tularensis* travels a novel, twisted road within macrophages. Trends Microbiol **14:**37-44.
149. **Santic, M., M. Molmeret, K. E. Klose, S. Jones, and Y. A. Kwaik.** 2005. The *Francisella tularensis* pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. Cell Microbiol **7:**969-79.
150. **Saslaw, S., H. T. Eigelsbach, J. A. Prior, H. E. Wilson, and S. Carhart.** 1961. Tularemia vaccine study. II. Respiratory challenge. Arch Intern Med **107:**702-14.
151. **Schaible, U. E., H. L. Collins, and S. H. Kaufmann.** 1999. Confrontation between intracellular bacteria and the immune system. Adv Immunol **71:**267-377.

152. **Schulert, G. S., and L. A. Allen.** 2006. Differential infection of mononuclear phagocytes by *Francisella tularensis*: role of the macrophage mannose receptor. J Leukoc Biol **80:**563-71.
153. **Segal, G., M. Purcell, and H. A. Shuman.** 1998. Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the Legionella pneumophila genome. Proc Natl Acad Sci U S A **95:**1669-74.
154. **Sjostedt, A.** 2007. Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. Ann N Y Acad Sci **1105:**1-29.
155. **Sjostedt, A., J. W. Conlan, and R. J. North.** 1994. Neutrophils are critical for host defense against primary infection with the facultative intracellular bacterium *Francisella tularensis* in mice and participate in defense against reinfection. Infect Immun **62:**2779-83.
156. **Sjostedt, A. B.** 2005. *Francisella.* p.200-210. *In* D. J. Brenner, N. R. Krieg, J. T. Staley, and G. M. Garrity (ed.), The Proteobacteria, part B. Bergey's manual of systematic bacteriology, 2nd ed., vol. 2. Springer-Verlag, New York, N. Y.
157. **Sorokin, V. M., N. V. Pavlovich, and L. A. Prozorova.** 1996. *Francisella tularensis* resistance to bactericidal action of normal human serum. FEMS Immunol Med Microbiol **13:**249-52.
158. **Staples, J. E., K. A. Kubota, L. G. Chalcraft, P. S. Mead, and J. M. Petersen.** 2006. Epidemiologic and molecular analysis of human tularemia, United States, 1964-2004. Emerg Infect Dis **12:**1113-8.
159. **Steward, J., T. Piercy, M. S. Lever, A. J. Simpson, and T. J. Brooks.** 2006. Treatment of murine pneumonic *Francisella tularensis* infection with gatifloxacin, moxifloxacin or ciprofloxacin. Int J Antimicrob Agents **27:**439-43.
160. **Sullivan, J. T., E. F. Jeffery, J. D. Shannon, and G. Ramakrishnan.** 2006. Characterization of the siderophore of *Francisella tularensis* and role of fslA in siderophore production. J Bacteriol **188:**3785-95.
161. **Sumida, T., T. Maeda, H. Takahashi, S. Yoshida, F. Yonaha, A. Sakamoto, H. Tomioka, T. Koike, and S. Yoshida.** 1992. Predominant expansion of V gamma 9/V delta 2 T cells in a tularemia patient. Infect Immun **60:**2554-8.
162. **Svensson, K., P. Larsson, D. Johansson, M. Bystrom, M. Forsman, and A. Johansson.** 2005. Evolution of subspecies of *Francisella tularensis*. J Bacteriol **187:**3903-8.
163. **Tarnvik, A., and M. C. Chu.** 2007. New approaches to diagnosis and therapy of tularemia. Ann N Y Acad Sci **1105:**378-404.
164. **Tarnvik, A., G. Sandstrom, and A. Sjostedt.** 1997. Infrequent manifestations of tularaemia in Sweden. Scand J Infect Dis **29:**443-6.
165. **Telepnev, M., I. Golovliov, and A. Sjostedt.** 2005. *Francisella tularensis* LVS initially activates but subsequently down-regulates intracellular signaling and cytokine secretion in mouse monocytic and human peripheral blood mononuclear cells. Microb Pathog **38:**239-47.
166. **Thirumalapura, N. R., D. W. Goad, A. Mort, R. J. Morton, J. Clarke, and J. Malayer.** 2005. Structural analysis of the O-antigen of *Francisella tularensis* subspecies tularensis strain OSU 10. J Med Microbiol **54:**693-5.

167. **Thomas, R., A. Johansson, B. Neeson, K. Isherwood, A. Sjostedt, J. Ellis, and R. W. Titball.** 2003. Discrimination of human pathogenic subspecies of *Francisella tularensis* by using restriction fragment length polymorphism. J Clin Microbiol **41:**50-7.

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

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

170. **Vinogradov, E., and M. B. Perry.** 2004. Characterisation of the core part of the lipopolysaccharide O-antigen of *Francisella novicida* (U112). Carbohydr Res **339:**1643-8.

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

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

173. **Wang, X., A. A. Ribeiro, Z. Guan, S. C. McGrath, R. J. Cotter, and C. R. Raetz.** 2006. Structure and biosynthesis of free lipid A molecules that replace lipopolysaccharide in *Francisella tularensis* subsp. *novicida*. Biochemistry **45:**14427-40.

174. **Wayne Conlan, J., and P. C. Oyston.** 2007. Vaccines against *francisella* tularensis. Ann N Y Acad Sci **1105:**325-50.

175. **Wayne Conlan, J., H. Shen, R. Kuolee, X. Zhao, and W. Chen.** 2005. Aerosol-, but not intradermal-immunization with the live vaccine strain of *Francisella tularensis* protects mice against subsequent aerosol challenge with a highly virulent type A strain of the pathogen by an alphabeta T cell- and interferon gamma- dependent mechanism. Vaccine **23:**2477-85.

176. **Weiss, D. S., A. Brotcke, T. Henry, J. J. Margolis, K. Chan, and D. M. Monack.** 2007. In vivo negative selection screen identifies genes required for *Francisella* virulence. Proc Natl Acad Sci U S A **104:**6037-42.

177. **Wenger, J. D., D. G. Hollis, R. E. Weaver, C. N. Baker, G. R. Brown, D. J. Brenner, and C. V. Broome.** 1989. Infection caused by *Francisella philomiragia* (formerly *Yersinia philomiragia*). A newly recognized human pathogen. Ann Intern Med **110:**888-92.

178. **Wherry, W. B., and B. H. Lamb.** 2004. Infection of man with *Bacterium tularense*. 1914. J Infect Dis **189:**1321-9.

179. **Whipp, M. J., J. M. Davis, G. Lum, J. de Boer, Y. Zhou, S. W. Bearden, J. M. Petersen, M. C. Chu, and G. Hogg.** 2003. Characterization of a novicida-like subspecies of *Francisella tularensis* isolated in Australia. J Med Microbiol **52:**839-42.

180. **Woolard, M. D., J. E. Wilson, L. L. Hensley, L. A. Jania, T. H. Kawula, J. R. Drake, and J. A. Frelinger.** 2007. *Francisella tularensis*-infected macrophages release prostaglandin E2 that blocks T cell proliferation and promotes a Th2-like response. J Immunol **178:**2065-74.

181. **Wu, T. H., J. A. Hutt, K. A. Garrison, L. S. Berliba, Y. Zhou, and C. R. Lyons.** 2005. Intranasal vaccination induces protective immunity against intranasal infection with virulent *Francisella tularensis* biovar A. Infect Immun **73:**2644-54.

182. **Yee, D., T. R. Rhinehart-Jones, and K. L. Elkins.** 1996. Loss of either CD4+ or CD8+ T cells does not affect the magnitude of protective immunity to an intracellular pathogen, *Francisella tularensis* strain LVS. J Immunol **157:**5042-8.

183. **Zeidner, N. S., L. G. Carter, J. A. Monteneiri, J. M. Petersen, M. Schriefer, K. L. Gage, G. Hall, and M. C. Chu.** 2004. An outbreak of *Francisella tularensis* in captive prairie dogs: an immunohistochemical analysis. J Vet Diagn Invest **16:**150-2.

Chapter II

Literature Review Part II: Diagnostics of Bacterial Biothreat Agents with Emphasis on Molecular Diagnostics

2.1 Diagnosing bacterial infections

Accurate and rapid diagnosis is optimal for any infectious disease and is crucial when dealing with infections caused by highly virulent bacteria. The preferred diagnostic for any bacterial infection would be bacterium-specific and compatible with the type or site of infection and available resources. Several categories of diagnostics are currently used for infectious disease and include biochemical-based, immunological, culture-based, and nucleic acid-based assays.

Common, naturally occurring bacterial infections are presumptively diagnosed in the clinic, and later confirmed by culturing, or other forms of standardized diagnostics. Unlike naturally occurring infections, bacterial infections caused by the intentional release of a biothreat agent need to be diagnosed quickly and accurately in order for appropriate control measures to be taken. The CDC has classified several bacteria as category A, B, or C agents based on their biothreat level (Table 2.1) (2) .

In the United States, few infections are caused by these biothreat agents. Due to the infrequent number of cases, many physicians would not recognize these infections and would likely make presumptive diagnoses based on symptoms while overlooking

what could potentially be the first of many patients infected during an intentional release of such an agent (27). In the past, minimal research has been performed for developing diagnostics for biothreat agents. The anthrax attacks in 2001 prompted a significant effort for the development of rapid and accurate diagnostics for these biothreat agents. The research currently underway has given rise to numerous tools for use in both clinical applications, and environmental monitoring and surveillance of these agents and will continue to do so.

Table 2.1 CDC bioterrorism diseases/agents category list[a]

	Category A Agents	Category B Agents	Category C Agents
Bacteria	*Bacillus anthracis*	*Brucella* species	
	Yersinia pestis	Food safety threats (e.g., *Escherichia coli O157:H7*, *Salmonella* species, *Shigella*)	Emerging infectious diseases (e.g., multidrug resistant *Mycobacterium tuberculosis*)
	Francisella tularensis	*Burkholderia mallei* *Burkholderia pseudomallei* *Chlamydia psittaci* *Coxiella burnetii* *Rickettsia prowazekii*	
		Water safety threats (e.g., *Vibrio cholerae*)	
Viruses	Smallpox (Variola major)	Viral encephalitis (alphaviruses) (e.g., Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis)	Emerging infectious diseases (e.g., hantavirus and Nipah virus)
	Viral hemorrhagic fevers filoviruses (e.g., Ebola, Marburg) and arenaviruses (e.g., Lassa, Machupo)		
Toxins	Clostridium botulinum toxin	*Clostridium perfringens* epsilon toxin Ricin toxin Staphylococcal enterotoxin B	
Parasites		Water safety threats (e.g., *Cryptosporidium parvum*)	

[a] The lists provided for Category A through C are not all inclusive.

For most bacterial infections, the gold standard diagnostics are culture-based (Table 2.2). A culture-based diagnostic provides direct evidence of the causative agent, and has the advantage of not requiring new technologies or equipment, and can be performed in most clinical and research settings. However, for many bacteria, culturing can be difficult and is often not a rapid method for diagnosing an infection. Challenges particularly arise when dealing with fastidious bacteria that frequently require specialized media and specific growth conditions for culturing. If optimal conditions are not initially established, fastidious bacteria will not grow and a misdiagnosis or lack of a diagnostic confirmation can occur. Other bacteria, for example *Mycobacterium leprae*, are non-culturable on artificial media and instead must be diagnosed clinically by patients having one or more of the following: acid-fast bacilli on skin smears of biopsy material, thickened peripheral nerves, or hypopigmented or reddish patches with loss of sensation (11). Given the limitations of culturing, other methods have been developed that offer greater sensitivity. Among these methods are immunological assays and nucleic acid-based assays.

Table 2.2 Examples of detection methods accepted for biothreat agents in 2003 (72).

Bacterium	Standard Methods	Other Detection Methods[a]				
		HHA	ELISA	ECL	TRF	PCR
Bacillus anthriacis	culture, γ-phage, IFA[b]	x	x	x	x	x
Brucella species	culture, IFA	x	x			x
Burkholderia species	culture	x	x			x
Clostridium botulinum	bioassay	x	x			x
Coxiella burnetii	culture, serology, IFA	x	x			x
Escherichia coli O157:H7	culture, serology	x	x	x		x
Francisella tularensis	culture, IFA	x	x		x	x
Salmonella species	culture, serology	x	x			x
Shigella species	culture	x	x			x
Vibrio cholerae	culture, serology	x	x			x
Yersinia pestis	culture, IFA	x	x			x

[a] HHA, hand-held assay; ELISA, enzyme-linked immunosorbent assay; ECL, electrochemiluminescent assay; TRF, time-resolved fluorescence assay; x, assay available.

[b] IFA, immunofluorescence assay.

2.2 Detection and identification of bacterial biothreat agents

Early detection and diagnosis are critical for effective intervention and control of an attack with a biological agent. In 1999, a national laboratory response (LRN) network was established for monitoring biothreat agents (14). This network is responsible for leading the response after an attack and has appointed LRN laboratories that will contain, collect, ship, and test the agents under strict protocols. Detection of a biological threat involves the use of necessary technology for identification of an intentionally released biological agent before or simultaneous to exposure. Federal surveillance systems such as the environmental detection system (BioWatch), the syndromic surveillance and clinical case presentation system (BioSense), and National Biosurveillance Integration System should aid in providing advanced warning of a large-scale biological attack (27). Detection technologies both used as environmental monitors and within the laboratory

must account for altered or uncharacterized agents that may be within complex samples and must also minimize false positives. As with all diagnostics, detection and identification methods for biothreat agents must be sensitive and specific, and preferably capable of detecting multiple agents. Of high consideration are the platforms used, requiring that sample processing be simple and not deplete the system of the agent of interest in case further testing is required (59). Although several systems are in place for large-scale detection, there are still multiple challenges for detection of smaller scale biological attacks and for naturally occurring infections associated with these agents.

Unlike diagnoses made within clinical settings, a culture-based diagnosis for the detection of an attack with a biothreat agent is not sufficiently rapid (Table 2.3). In a clinical setting, it is common practice to presumptively diagnose a patient and provide treatment prior to diagnostic confirmation. In the case of a biological attack, this approach would hinder appropriate control, treatment and prophylaxis, and could result in mass casualties. However, it is possible that a culture-based diagnostic would be the method utilized by clinics if a patient were to present with an infection caused by one of these agents, especially if there is no knowledge of an attack. In the anthrax attacks of 2001, a 61 year old female presented to the hospital with a 3-day history of symptoms. A presumptive diagnosis of community-acquired pneumonia, congestive heart failure, or inhalational anthrax was made and was not confirmed as anthrax until a culture of *Bacillus anthracis* was obtained (44). For such cases, culturing, along with automated systems are available for biochemical profiling of bacterial biothreat pathogens and include the MicroLog System using the Dangerous Pathogen Database (BIOLOG, Hayward CA), Vitek (bioMérieux, Inc. Durham, NC) and API Series (bioMérieux, Inc.

Durham, NC) (59). Similar to biochemical screening, there is a fatty acid profiling system available, the FAME-GC system (MIDI, Inc., Newark, DE), which can also be used for bacterial identification. All biochemical and fatty acid profiling assays require a purified culture before being performed, and therefore, can require lengthy periods of time. These technologies are however beneficial for strain characterization in the later stages of investigation and suitable for routine clinical practices, particularly for confirmation of diagnosis.

Table 2.3 Limit detection and analysis time for commonly used detection assays for *Bacillus anthracis* (72).

Dectetion Method[a]	Detection Limit (CFU/ml)	Analysis Time (minus sample preparation time)
HHA	100,000	<15 min
ELISA	10,000	90-180 min
ECL	1,000	90-180 min
TRF	1,000	90-180 min
Microscopy[b]	10,000	5-120 min
PCR	1-100	<60 min
Culture	1-10	8-20 h

[a] HHA, hand-held assay; ELISA, enzyme-linked immunosorbent assay; ECL, electrochemiluminescent assay; TRF, time-relsoved fluorescent assay; PCR, polymerase chain reaction.
[b] Microscopy includes simple staining methods or fluorescence immunoassays.

Given the limitations of culturing, other diagnostic methods have been developed. These include immunological assays and nucleic acid-based assays. Immunologic assays have vast utility within clinical settings. Advantages to immunological assays are their ability to detect and identify an infection when culture-based assays fail, and they are

usually rapid once performed. These assays are a widely used form of diagnostics, with traditional assays relying on an immunological host response caused by an antigen that is subsequently detected using antibodies (71). Disadvantages to this approach include lack of sensitivity, and requirement of a strong immune response for detection when measuring antibodies. In some cases they lack specificity when compared to nucleic acid-based assays. Additionally, antibodies can be used to detect antigens directly. This method has the advantage of not requiring an immune response and is therefore a rapid diagnostic. Polyclonal, monoclonal, or recombinant antibodies can be used for antigen detection. The quality of antibodies or antigens used is crucial for these diagnostics, but as recombinant antibody technology becomes more widely applied, this will result in improvement of both sensitivity and specificity for the detection of antigens using antibodies, increasing their value as diagnostics.

The application of immunological assays for detection of biothreat bacterial agents does not differ from their use in clinical diagnostics (71). Several platforms are presently available and include biosensors, flow cytometry, microarrays, lateral flow diffusion devices, and assays that are combinations of the above (59). Detection is achieved using fluorescent, chemiluminescent, and electrochemiluminescent substrates and labels. Commercially available antibody-based detection systems for biothreat agents are available and include microarray-based assays, smart tickets and hand-held devices (variations of solid-support models and lateral flow diffusion apparatuses), and time resolved fluorescence (TRF)-based assays (59). Most of these target the detection of *Yersinia pestis*, *Bacillus anthracis*, *F. tularensis*, *Clostridium botulinum*, *E. coli* O157:H7, *Salmonella* species, *Brucella* species, and *V. cholera*, with some additionally

capable of detecting toxins. Peruski *et al.* developed TRF assays for detection of *F. tularensis*, *C. botulinum* A/B neurotoxin, and *Staphylococcus aureus* enterotoxin B (70). Although useful and in some cases mobile, these diagnostics have the drawback of depending on a robust antibody response before detection can occur and rely on collection of patient sera (14). To overcome the time constraint disadvantages with culture-based and some immunological diagnostics, nucleic acid-based diagnostics and identification methods are promising tools for detection of biothreat agents. Table 2.2 summarizes standard methods and other accepted detection methods for bacterial biothreat agents (72).

2.3 Nucleic acid-based diagnostics

Detection and characterization of microbial DNA/RNA is the basis for nucleic acid-based diagnostics. The discovery and implementation of four primary techniques has defined the foundation for the various detection and identification applications developed using nucleic acids. These discoveries include enzymatic DNA restriction, polymerase chain reaction (PCR), nucleic acid hybridization, and fluorescence-based methods (5). Nucleic acid-based diagnostics are gaining popularity and have become particularly important for the detection and identification of biothreat agents. Several of these technologies are discussed below.

2.3.1 PCR-based methods

In 1993, Dr. Mullis was awarded the Noble Prize in Chemistry for his role in the development of the PCR (79, 80). This award was in recognition of how PCR had reshaped many aspects of life sciences, and since its first introduction how PCR has revolutionized the methods by which identification of bacterial pathogens is carried out.

This relatively simple method has provided a degree of standardization among clinical laboratories not previously existing. FDA-approved and commercially existing PCR-based kits for the detection of bacterial pathogens are available for *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, and *Neisseria gonorrhea,* among others (75). Non-commercial PCR-based assays that are also available and clinically important include those for *Ehrlichia*, *Bordetella pertussis, Legionella pneumophila*, and *Helicobacter pylori* (75). PCR assays for most bacteria have been developed, but are not necessarily FDA-approved for use in clinical laboratories. An example of such an applied PCR method is amplification of bacterial 16S rDNA sequence. A disadvantage for PCR as a clinical diagnostic is the difficulties in obtaining results from complex samples, including sputum and feces that contain substances that inhibit PCR and therefore lowers the sensitivity of the assay (26). Many variants of standard PCR have been applied to microbial diagnostics, but the most frequently used are nested PCR, multiplex PCR and real-time PCR (RT-PCR).

An advancement in standard PCR technology came in 1992 when Higuchi *et al.* introduced RT-PCR (40). This powerful, highly sensitive (approximate detection limit from 10 to 100 cells) technique allows for specific identification of a pathogen within complex samples, and as with conventional PCR, has importance in the diagnosis of slow growing fastidious pathogens including those that are non-culturable. This technology is gel-free, making it rapid and possible to automate. RT-PCR can also be a quantitative assay. TaqMan probes, molecular beacons, and fluorescence resonance energy transfer (FRET) hybridization probes are common detection formats.

As with clinical samples, the use of both conventional PCR and RT-PCR are translational to detection and identification of biothreat agents. Given the advantages of RT-PCR over conventional PCR, RT-PCR is the more practical option for detecting biothreat agents. In addition, RT-PCR is adaptable to both portable and laboratory-based platforms. Several portable devices are available for detecting biothreat agents in the field and include the Ruggedized Advanced Pathogen Identification Device (RAPID) (Idaho Technology, Salt lake City, UT) (38, 93) and BioSeeq (Smiths Detection, Edgewood, MD) (25). Disadvantages of this method, and among all nucleic-acid based diagnostics are; efficiency of DNA extraction, degradation of DNA by nucleases, probe and primer reactivity, inherent PCR bias (variations in polymerase, buffers, thermocycler performance), and presence of reaction inhibitors.

The literature offers multiple primer sets as PCR formats for biothreat agents. Several RT-PCR methods have been developed and evaluated for detection of biothreat agents, including *B. anthracis* (43, 55). Multiplex PCR assays are particularly useful in detection of multiple organisms simultaneously. Several multiplex RT-PCR assays are described for use in detecting multiple biothreat agents (84, 94, 100). Although multiple primer sets and PCR formats are available for most bacterial biothreat agents, many have not been evaluated using human samples and therefore, further testing is required before they can be approved as diagnostics for these agents (59). It should be noted that for routine naturally occurring infections with an agent of biothreat concern, diagnostic confirmation with currently published PCR-based assays is common.

2.3.2 Other nucleic acid-based technologies for biothreat bacterial identification

Molecular subtyping methods are necessary in monitoring the environment for unnatural events involving biothreat agents, as well as for surveillance of these agents. For forensic purposes, molecular subtyping methods provide tools for "fingerprinting" strains that allow tracing and characterization of strains, both of which are useful for investigating an intentional release of a biothreat agent and during a natural outbreak. In addition, these tools function for taxonomy purposes and some can provide additional information useful for elucidating evolutionary relationships between strains. Several molecular subtyping tools are available, and each has advantages and disadvantages. Brief descriptions of common molecular subtyping methods are provided below.

Pulsed-field gel electrophoresis (PFGE). PFGE compares patterns of large genomic fragments after digestion with a restriction enzyme (63). Fragments are electrophoresed on an agarose gel and digest profiles are compared. Clonal strains have the same digest pattern and different strains produce distinct digest patterns. This method is useful for distinguishing strains at the subsp. level, and although used for isolate discrimination and comparison, caution should be taken given this methods inability to resolve small differences such as insertions, deletions, and mutations present between isolates. PFGE is commonly used for investigating bacterial foodborne outbreaks (6, 35) including foodborne pathogens that are on the list of bioterrorism diseases/agents and for typing of bacterial pathogens that are not foodborne related but are on the list of biothreat agents (includes *Y. pestis, Burkholderia mallei* and *F. tularensis*) (20, 34, 45, 86).

Restriction fragment length polymorphism (RFLP). RFLP is a subtyping method that provides information on specific genomic sequences using probes based on repetitive

elements that are capable of differentiating strains (62). Bacterial genomic DNA is digested using a restriction enzyme and electrophoresed on an agarose gel. The DNA is transferred to a blot and specific fragments are detected with a probe by DNA hybridization. This method is useful primarily for differentiation at the subsp. level but in some cases can differentiate between isolates. RFLP has been used for genotyping of *Yersinia pestis* strains isolated in the United States (45). Interestingly, when the resolving ability between PFGE and RFLP were compared for this bacterium, PFGE proved to be a more discriminating method. However, this is not inclusive to all bacteria. For example, RFLP analysis is the primary method for typing strains of *Mycobacterium tuberculosis* and serves as an excellent tool to track the epidemiology of disease associated with specific strains (18).

Amplified fragment-length polymorphism (AFLP). AFLP analysis is similar to RFLP but uses selective PCR amplification of genome fragments that are generated by restriction enzyme digestion (97). Bacterial DNA is digested using two restriction enzymes that cut with different frequencies. The resulting fragments are ligated to end-specific adaptor molecules. Selective PCR is performed using primers complementary to the adaptor sequences and which contain restriction site specific sequences. Electrophoresis is then performed and banding patterns are compared (65). Similar to PFGE, AFLP can differentiate between subspp. and to some extent between isolates. Differences are based on the comparison of amplified fragments and can be missed if differences are present in non-amplified fragments. AFLP has been used for evaluating genetic diversity among *F. tularensis* (34) and *B. anthracis* isolates (41).

Multiple locus variable number tandem repeat analysis (MLVA). MLVA assays are developed for the rapid genetic strain typing of *B. anthracis* (53, 56, 58), *Y. pestis* (1, 58, 76), *Brucella* (9) and *F. tularensis* (28, 47). MLVA uses oligonucleotide primers that flank tandem repeat regions found within bacterial genomes in a species-specific PCR amplification reaction. This assay is able to differentiate between strains within a species based on allelic variations. This technique is easily standardized and amenable to automation.

Multilocus sequence typing (MLST). MLST differentiates between subspp. and to some extent between isolates within a subsp. based on sequence polymorphisms that are contained within functional genes. Muliplex PCR is used to evaluate the gene regions of choice and sequences are compared between strains. This subtyping method was developed for use with *Burkholderia* and gave results similar to those obtained using PFGE (36).

Microarrays. Microarrays of nucleic acid, also known as "gene chips" offer a new platform for detection of pathogens (15). Microarrays are currently used extensively for multiple other purposes, including high throughput gene expression experiments, but are still in development for bacterial detection. The target nucleic acid hybridized onto an array can be either DNA or RNA. Several studies demonstrate the potential of this technique in combination with other technologies including PCR, for high throughput detection applications. Panicker *et al.* used gene-specific DNA microarrays coupled with multiplex PCR for the detection of pathogenic *Vibrio* in its natural environment (68), and Call *et al.* used microarrays to detect *E. coli* O157:H7 (16). The hybrid technology developed by Call and colleagues uses tiny probes each specific for a

pathogen and printed on a glass plate. Sensitivity of the assay is increased by concentrating the bio-threat markers that include whole cells and DNA, and PCR of nucleic acids prior to detection (17, 46). The system developed, Battelle-PNNL Enhanced Biodetection Enabling Analyte Delivery System (BEADS), is automated and can monitor environmental samples 24 hours a day sending results electronically without the presence of a technician. An electric field-driven assay has been used to detect concentrated bacteria that are lysed via dielectrophoresis. DNA released is denatured and amplified using strand displacement amplification (8) and is subsequently analyzed using an on-chip electric-field driven hybridization assay (99, 101). Additional microarray technologies that incorporate other methods are underway, but at the moment the limitations of microarrays alone, including sensitivity, reagent cost, and challenges with sample processing, will likely hinder its use as a valuable detection method in the near future (15). In addition to its potential use for microbial detection, microarrays are demonstrated as useful for typing of strains for epidemiological studies (33).

Single nucleotide polymorphisms (SNP) analysis. SNPs are DNA sequence variations that occur when a single nucleotide in the genome sequence is changed and are evolutionary stable markers used for bacterial identification and subtyping. Their application in subtyping of strains has proven valuable for *B. anthracis* (54, 69, 92) and SNP analysis of *F. tularensis* is underway (52). Subtyping using SNPs provides resolution capable of differentiating between strains, and as the number of whole genome sequences increases the application of SNP analyses for strain identification will likely increase.

Other nucleic acid-based technologies in development. Developing technologies for detection and identification of biothreat agents are numerous and are being explored among the different types of diagnostics (59). Among new nucleic acid-based detection and identification technologies is Mass Tag PCR, a multiplex diagnostic that combines PCR with mass spectrometry analysis (10).

Another technology is the isothermal amplification method, nucleic sequence-based amplification (NASBA), and has been reported as being useful for identification of foodborne pathogens (3, 4, 21, 78). In this method, RNA is amplified. Three enzymes, a reverse transcriptase, RNaseH, and T7 RNA are required along with dNTPs and NTPs. Oligonucleotide primers that are complimentary to the target RNA are included in the reaction, with one of the primers containing a recognition sequence for T7 RNA polymerase. The RNA transcripts formed proceed through the reaction to result in single stranded RNA. RNA is then electrophoresed or verified using probe hybridization. NASBA has been used successfully to detect bacteria in clinical and environmental samples and is described as a sensitive, specific and rapid method (59). It is also advantageous in its ability to specifically detect viable cells (21).

The miniaturized technology "Lab-on-a-chip" is rapidly advancing. This miniaturized, portable system is capable of sample preparation and detection in an automated format (32). Liu *et al.* recently developed a biochip that consists of microfluidic mixers, valves, pumps, channels, heaters, and DNA microarray sensors that is able to detect pathogens using milliliters of blood within 2.7 hours (60). Several companies are currently developing this technology and already have products on the market that are based on microfluidic "Lab-on-a-chip" technology including Agilent

Technologies (Palo Alto, CA), Affymetrix (Santa Clara, CA), and ACLARA Biosciences (Mountain View, CA) (32). This technology will likely have a strong impact on environmental microbial monitoring (particularly biothreat agents) given its rapid detection and a sensitivity level of a single cell.

Application of nucleic acid-based methods in the 2001 anthrax attacks. The use of genomic nucleic acid-based technologies in combination with conventional strain characterization techniques demonstrated to be useful in the aftermath investigation of the 2001 bioterrorism-associated anthrax outbreak (42, 77). Variable number of tandem repeats (VNTR) analysis was used to trace the strain of *B. anthracis* used in the attacks back to the Ames isolate and aided in the investigation of this attack (77). Many lessons were learned from the anthrax attacks, prompting a trend of research for the detection and rapid identification of biothreat agents.

2.4 Diagnostics for tularemia

Diagnosing tularemia can be challenging due to its infrequent occurrence and similarity in symptoms to other infections. In 2000, tularemia was reinstated as a notifiable infectious disease in the United States, and if suspected should be reported to public health authorities (19). A physician may suspect tularemia based on the symptoms presented by a patient, and the patient's history of exposure to vectors or hosts of *F. tularensis*, or contaminated water. A presumptive diagnosis can be made by evaluating elevated serum antibodies to *F. tularensis* antigen, or by positive detection of *F. tularensis* in clinical specimens using direct fluorescent antibody (DFA) testing. Several types of antibodies are commercially available for immunofluorescence detection of *F. tularensis* and include IgG3 and IgG2a (detect lipopolysaccharide) and IgG1 (detects

vegetative cells) (85). Although DFA is a rapid, specific test, it has a low sensitivity (10^6 cells/ml) (96). Diagnosis of tularemia has relied heavily in the past on the host's antibody response to this organism, with a tube agglutination assay for antibody detection first developed in 1961 by Saslaw *et al.* (82). In 1980, Brown *et al.* published on the development of a microagglutination method adapted from the tube agglutination assay (13). Both agglutination-based methods are highly sensitive and specific for *F. tularensis*. The limitation to this assay is that an antibody response does not appear until two weeks after infection (24). Enzyme-linked immunosorbent assays (ELISA) are also available for testing antibody responses to *F. tularensis,* but has limited utility since it does not differentiate between a current and past infection (87). Antigen (including *F. tularensis* LPS and outer membrane protein) can be detected from serum, urine, and organs including spleen, liver and lungs (85, 89). ELISA assays can also be used to test environmental samples (7, 37, 85).

Confirmation of tularemia is done either by isolation of *F. tularensis* from clinical specimens or by a four-fold (or higher) change in serum antibody titer to *F. tularensis* (88). Culturing *F. tularensis* from clinical specimens is difficult due to the fastidious nature of this bacterium and the requirement of a biosafety level 3 (BSL3) laboratory for its handling.

2.4.1 Culture-based diagnostics for tularemia

Culturing *F. tularensis* from clinical specimens can be challenging. The first culture of *F. tularensis* obtained by McCoy and Chapin in 1912 was achieved using Dorset's egg medium (64). This medium has been shown to maintain the viability of fastidious bacteria including *Neisseria meningitidis* and *Streptococcus pneumonia* (98),

and was the only medium from which McCoy and Chapin were able to obtain *F. tularensis* growth (64). Today, the medium used for optimal growth of *F. tularensis* from clinical specimens is cysteine supplemented agar media, such as enriched chocolate agar, CHAB, or gonococcal (GC) agar II with 1% isovitalex (88). For research purposes, Mueller Hinton II (MH II) agar supplemented with isovitalex and iron is commonly used due to its ease in preparation.

The recovery of *F. tularensis* from samples that are contaminated with other organisms can be difficult. Organisms including *Pseudomonas*, *Staphylococcus*, and *Proteus* species have been shown to dominate over *F. tularensis*, suppressing its growth and precluding its isolation (74). Past studies show a 30% recovery rate from animal carcasses (66) with this number increasing to 81% when CHAB supplemented with antibiotics (CHAB-A) was used (74). The antibiotics present in this medium include ampicillin, colistin, lincomycin, trimethoprim and amphotericin. The use of liquid culture to enrich for *F. tularensis* is not common practice given the slow rate at which this organism grows in culture (3 to 7 days) and its inability to out-compete other organisms. The use of antibiotics in culture medium is not thoroughly researched due to the risks involved in handling liquid cultures. Proper care and manipulation of samples presumed to contain *F. tularensis* is critical for isolation of this fastidious organism. Enhanced recovery is achieved when clinical specimens are immediately frozen and kept frozen during transportation (74).

If a culture is obtained from a clinical specimen or environmental sample, it can be used in biochemical assays for initial subsp. differentiation and for characterization of the isolate. At the CDC reference laboratory for tularemia the routine practice for

processing a sample suspected of containing *F. tularensis* is to perform both culture-based assays and serology on samples when possible. Culturing is performed by plating the sample onto both CHAB and CHAB-A. If characteristic growth of *F. tularensis* is obtained, the culture is verified by DFA, followed by profiling using a Microlog Microstation™ screen. Differentiation between *F. tularensis* subspp. type A and type B is done by results obtained from a glycerol reaction. In addition, cultures are inoculated into mice for isolation from other contaminating organisms. The microagglutination test is done on patient sera, both acute and convalescent, with a four-fold increase in titer considered positive for a tularemia infection. Positive sera and culture from a patient is not common with either one or the other resulting in a positive diagnosis (personal communication Sandy Urich, CDC, Fort Collins, CO) (73). The use of centrifugation-cell culture was shown by Fournier *et al.* to be effective in isolation of *F. tularensis* from an eschar biopsy that was culture-negative and may offer an alternative to mouse inoculation in the future (30). Several molecular subtyping methods are available for use in verification of results obtained by conventional methods and for strain characterization.

2.4.2 Molecular methods available for confirmation and strain characterization

Due to the fastidious nature *F. tularensis*, it is essential to have methods that are non-culture based for the identification of this organism. Non-culture based assays reduce the risk to laboratory personnel of handling this highly infectious bacterium and do not require a BSL-3 laboratory. Given these advantages, several molecular methods have been developed for identification of *F. tularensis* within both clinical and environmental samples as well as for species differentiation.

PCR-based methods.

The first PCR for *F. tularensis* was developed in 1993 by Long *et al.* and targets the *tul4* gene (61). The gene *tul4* encodes for a 17 kDa outer membrane lipoprotein and is conserved among *F. tularensis* strains (81). In 2001, Karhukorpi *et al.* successfully utilized this PCR assay to confirm a tularemia diagnosis of a 9-year old boy with ulceroglandular tularemia. Pus was taken from the boy's infected mosquito bite and used for culturing and DNA extraction. Although growth was obtained from the sample, it took two days, in comparison to 4 hours for a positive PCR result (51). Junhui *et al.* also developed a PCR assay targeting this gene (50). A nested PCR targeting *fopA* (outer membrane protein specific for *F. tularensis*) was developed that increased the sensitivity (as compared to the *tul4* assay) to 10^2 organisms/ml (31).

In 1994, Forsman *et al.* developed the first PCR assay that could distinguish strains at the genus, species and subspecies (*F. tularensis* subspp. type A from type B) levels by targeting the 16S rRNA gene (29). Sjöstedt *et al.* utilized this assay in combination with the *tul4* PCR assay to screen 40 wound swab samples from patients serologically positive for tularemia from an outbreak of the ulceroglandular form in Sweden (83). Of these, 73% were confirmed by PCR with 29 of 40 amplifying when using the *tul4* primer set and 20 of 40 amplifying when using the 16S rRNA primer set. A second PCR that could differentiate between subspp. was developed using long arbitrary primers that identified a region of heterogeneity that differentiated *F. tularensis* subspp. type A and type B (49). The first PCR assay that discriminated between all four subspecies was developed in 2003 by Broekhuijsen *et al.* (12). It was developed using a microarray library of *F. tularensis* subsp. type A strain SCHU S4 that was used to screen

27 strains representing all four subspecies. A region of difference was identified and targeted for this PCR assay.

Although a PCR assay that distinguishes different subspecies is not as important in the clinical setting, such an assay is valuable for epidemiological purposes and potentially treatment strategies for places where more than one subspecies is endemic. As described, several PCR assays are developed and useful in confirming diagnosis of tularemia infections caused by *F. tularensis* subsp. type B. However, they have yet to be used for infections caused by the more virulent *F. tularensis* subsp. type A (88). When summarizing the data of several studies directed at PCR assays to confirm human ulceroglandular tularemia cases, it is estimated that 25% of tularemia cases would not be diagnosed by PCR alone (23, 49, 83, 88). Thus, more accurate diagnostics would be beneficial for diagnosing tularemia.

The use of conventional PCR for confirming tularemia diagnoses has been shown to be effective. However, conventional PCR methods are gel-based and have a lower sensitivity and specificity when compare to RT-PCR assays. The first RT-PCR assay for *F. tularensis* detection was developed in 2000 by Higgins *et al.* who used the TaqMan™ System directed against the *fopA* gene (39). This assay has a detection limit of 1 pg of genomic DNA (<100 colony-forming units) and when tested against 21 other bacterial species had 100% specificity for *F. tularensis*. In 2003, Versage *et al.* developed an RT-PCR assays using TaqMan™ probes that targeted *ISFtu2*, the *23kDa* gene, *tul4* and *fopA* (95). A multitarget assay was developed using *ISFtu2*, *23kDa* and *tul4* that can differentiate between *F. tularensis* and *F. philomiragia*. This assay was also shown to be significantly more sensitive than culturing and capable of detecting one organism. When

tested on highly contaminated samples containing *F. tularensis*, all were positive. A third RT-PCR assay was also published in 2003 by Emanuel *et al.* (25) that targeted *tul4* and *fopA*. In addition to testing these two targets with a focus on detecting *F. tularensis* from tissue samples, they also compared the use of a conventional fluorescence-based PCR thermocycler to a portable hand-held device. Both instruments performed equally with a detection limit of approximately 25 genome equivalents. However, both were found to be less sensitive than culturing. Culturing, although taking 72 hours for results as compared to same day results using RT-PCR, was able to detect bacterial growth from liver and spleen a day before PCR.

Two RT-PCR methods were developed in 2006 by Tomaso *et al.* and Kugeler *et al.*, that specifically detect *F. tularensis* subsp. type A (57, 91). Both assays targeted the pathogenicity determinant protein D locus (*pdpD*) that is absent in *F. tularensis* subsp. type B (67). In addition to this assay, Kugeler *et al.* also developed an RT-PCR assay specific for *F. tularensis* subsp. type B that targets the junction between *ISFtu2* and the flanking 3' region that is only present in this subspecies.

Molecular subtyping methods.

Molecular subtyping of *F. tularensis* strains is important in the surveillance of this bacterium. Tularemia is currently not a major public health concern as a naturally occurring infection. Nevertheless, given the minimal understanding that we have of its potential and triggers and knowing that it has been weaponized in the past, justifies the development of tools for tracking this organism. In addition, in areas where both clinically relevant subspecies occur naturally, such as in North America, molecular

subtyping tools aid in investigating this organism's geographic spread and mode of transmission.

Several molecular methods that can discriminate between subspecies and even strains have been developed for *Francisella*. Initial typing techniques were solely PCR-based and used: extragenic palindromic element PCR, enterobacterial repetitive intergenic consensus sequence PCR, random amplified polymorphic DNA PCR (RAPD-PCR), and long primers RAPD-PCR (22, 49). None of these methods used alone were shown to be effective for differentiating strains. Moreover, they are complex, non-rapid, and lack reproducibility and inter-laboratory comparability. For subspecies discrimination, several techniques have been shown to be efficient; these include PFGE, AFLP, 16S rRNA gene sequencing, and RFLP. A study by Garcia del Blanco *et al.* compared PFGE, AFLP, and 16S rRNA sequencing using a panel of *F. philomiragia* and *F. tularensis* strains (34). This study found PFGE and AFLP as acceptable tools for subspecies differentiation. Although not ideal, these methods are also capable of distinguishing between strains. RFLP based on the insertion sequence (IS) elements ISFtu1 and ISFtu2 was found to be the most powerful tool for subspecies discrimination (90). In 2001, Johansson *et al.* demonstrated the use of short sequence tandem repeat (SSTR) loci as useful for discriminating between strains (48). Specifically, SSTR9 and SSTR16 present in *F. tularensis* genomes are highly discriminatory. Farlow *et al.* identified and used six variable-number tandem repeat (VNTR) loci in a MLVA analysis to discriminate between *F. tularensis* subspecies and strains (28). In 2004, Johansson *et al.* used 25 VNTR loci to perform MLVA analysis on 192 world-wide *F. tularensis* isolates (47). This analysis resulted in 120 genotypes that accurately discriminated the

different subspecies and further identified subpopulations A.I and A.II within the highly virulent *F. tularensis* subsp. type A.

The need for a strong discriminatory molecular subtyping tool for *F. tularensis* is currently driven from a forensic perspective. For such purposes, the method of choice will likely be SNP analysis given its power to discriminate among strains. Several groups are currently working towards a SNP analysis for *Francisella* (52). By far, the most powerful tool for strain differentiation is whole genome sequencing. As the cost of this method decreases, more and more strains will be sequenced and will likely change how strains are compared and classified.

It is important to separate the needs of molecular subtyping methods for clinical purposes and for research purposes. For research purposes, complex methods requiring advanced training and costly equipment is acceptable. However, in a clinical setting a cost-effective, quick, reliable assay is ideal. As discussed by Keim *et al.* simple PCR-based assays could be easily incorporated into the workflow of many clinical laboratories (52). Such an assay would be an RT-PCR assay that could quickly differentiate to the subpopulation level providing both clinical and epidemiology data with minimal exposure of laboratory personnel to this organism.

2.5 Rationale and objectives

The primary objective of this dissertation is to provide new tools that can be used for surveillance of *F. tularensis* given the need to better understand the ecology, epidemiology of outbreaks, and human illness associated with this bacterium. Specifically, this dissertation explores the identification of an inhibitory substance produced by *E. coli* against *F. tularensis* for use as a diagnostic reagent and also

identifies molecular markers that differ between *F. tularensis* subsp. type A subpopulations A.I and A.II and their use in diagnostic development. In addition to the diagnostic focus of this dissertation, the implications of the inhibitory substance on *Francisella* ecology and isolation from its natural environment along with the significance of the regions of difference between A.I, A.II and among the other *F. tularensis* subspecies are explored in relation to ecology and virulence. Thus, two separate hypotheses were developed and tested: 1) identification of a *F. tularensis* inhibitory substance produced by *E. coli* would lead to a novel diagnostic reagent; 2) genomic regions of difference identified between *F. tularensis* subsp. type A subpopulations (A.I and A.II) could be applied as diagnostics to differentiate these subpopulations and for comparative analyses to enhance understanding of the previously described phenotypic characteristics associated with these two subpopulations.

2.6 Literature Cited

1. **Adair, D. M., P. L. Worsham, K. K. Hill, A. M. Klevytska, P. J. Jackson, A. M. Friedlander, and P. Keim.** 2000. Diversity in a variable-number tandem repeat from *Yersinia pestis*. J Clin Microbiol **38:**1516-9.
2. **Atlas, R. M.** 2002. Bioterriorism: from threat to reality. Annu Rev Microbiol **56:**167-85.
3. **Baeumner, A. J., R. N. Cohen, V. Miksic, and J. Min.** 2003. RNA biosensor for the rapid detection of viable *Escherichia coli* in drinking water. Biosens Bioelectron **18:**405-13.
4. **Baeumner, A. J., B. Leonard, J. McElwee, and R. A. Montagna.** 2004. A rapid biosensor for viable *B. anthracis* spores. Anal Bioanal Chem **380:**15-23.
5. **Barken, K. B., J. A. Haagensen, and T. Tolker-Nielsen.** 2007. Advances in nucleic acid-based diagnostics of bacterial infections. Clin Chim Acta **384:**1-11.
6. **Barrett, T. J., P. Gerner-Smidt, and B. Swaminathan.** 2006. Interpretation of pulsed-field gel electrophoresis patterns in foodborne disease investigations and surveillance. Foodborne Pathog Dis **3:**20-31.
7. **Berdal, B. P., R. Mehl, H. Haaheim, M. Loksa, R. Grunow, J. Burans, C. Morgan, and H. Meyer.** 2000. Field detection of *Francisella tularensis*. Scand J Infect Dis **32:**287-91.
8. **Bergmann, J. S., and G. L. Woods.** 1998. Clinical evaluation of the BDProbeTec strand displacement amplification assay for rapid diagnosis of tuberculosis. J Clin Microbiol **36:**2766-8.
9. **Bricker, B. J., D. R. Ewalt, and S. M. Halling.** 2003. *Brucella* 'HOOF-Prints': strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). BMC Microbiol **3:**15.
10. **Briese, T., G. Palacios, M. Kokoris, O. Jabado, Z. Liu, N. Renwick, V. Kapoor, I. Casas, F. Pozo, R. Limberger, P. Perez-Brena, J. Ju, and W. I. Lipkin.** 2005. Diagnostic system for rapid and sensitive differential detection of pathogens. Emerg Infect Dis **11:**310-3.
11. **Britton, W. J., and D. N. Lockwood.** 2004. Leprosy. Lancet **363:**1209-19.
12. **Broekhuijsen, M., P. Larsson, A. Johansson, M. Bystrom, U. Eriksson, E. Larsson, R. G. Prior, A. Sjostedt, R. W. Titball, and M. Forsman.** 2003. Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. J Clin Microbiol **41:**2924-31.
13. **Brown, S. L., F. T. McKinney, G. C. Klein, and W. L. Jones.** 1980. Evaluation of a safranin-O-stained antigen microagglutination test for *francisella tularensis* antibodies. J Clin Microbiol **11:**146-8.
14. **Burnett, J. C., E. A. Henchal, A. L. Schmaljohn, and S. Bavari.** 2005. The evolving field of biodefence: therapeutic developments and diagnostics. Nat Rev Drug Discov **4:**281-97.
15. **Call, D. R.** 2005. Challenges and opportunities for pathogen detection using DNA microarrays. Crit Rev Microbiol **31:**91-9.

16. **Call, D. R., F. J. Brockman, and D. P. Chandler.** 2001. Detecting and genotyping *Escherichia coli* O157:H7 using multiplexed PCR and nucleic acid microarrays. Int J Food Microbiol **67:**71-80.
17. **Call, D. R., D. P. Chandler, and F. Brockman.** 2001. Fabrication of DNA microarrays using unmodified oligonucleotide probes. Biotechniques **30:**368-72, 374, 376 passim.
18. **Cave, M. D., M. Murray, and E. Nardell.** 2005. Molecular epidemiology of *Mycobacterium tuberculosis*. In Tuberculosis and the Tubercle Bacillus. (ed. S.T. Cole, K.D. Eisenach, D.N. McMurray and W.R. Jacobs Jr.) ASM Press Washington D.C.
19. **CDC.** 2002. Tularemia--United States, 1990-2000. MMWR Morb Mortal Wkly Rep **51:**181-4.
20. **Chantratita, N., M. Vesaratchavest, V. Wuthiekanun, R. Tiyawisutsri, T. Ulziitogtokh, E. Akcay, N. P. Day, and S. J. Peacock.** 2006. Pulsed-field gel electrophoresis as a discriminatory typing technique for the biothreat agent *burkholderia mallei*. Am J Trop Med Hyg **74:**345-7.
21. **Cook, N.** 2003. The use of NASBA for the detection of microbial pathogens in food and environmental samples. J Microbiol Methods **53:**165-74.
22. **de la Puente-Redondo, V. A., N. G. del Blanco, C. B. Gutierrez-Martin, F. J. Garcia-Pena, and E. F. Rodriguez Ferri.** 2000. Comparison of different PCR approaches for typing of *Francisella tularensis* strains. J Clin Microbiol **38:**1016-22.
23. **Eliasson, H., A. Sjostedt, and E. Back.** 2005. Clinical use of a diagnostic PCR for *Francisella tularensis* in patients with suspected ulceroglandular tularaemia. Scand J Infect Dis **37:**833-7.
24. **Elkins, K. L., S. C. Cowley, and C. M. Bosio.** 2007. Innate and adaptive immunity to *francisella*. Ann N Y Acad Sci **1105:**284-324.
25. **Emanuel, P. A., R. Bell, J. L. Dang, R. McClanahan, J. C. David, R. J. Burgess, J. Thompson, L. Collins, and T. Hadfield.** 2003. Detection of *Francisella tularensis* within infected mouse tissues by using a hand-held PCR thermocycler. J Clin Microbiol **41:**689-93.
26. **Espy, M. J., J. R. Uhl, L. M. Sloan, S. P. Buckwalter, M. F. Jones, E. A. Vetter, J. D. Yao, N. L. Wengenack, J. E. Rosenblatt, F. R. Cockerill, 3rd, and T. F. Smith.** 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev **19:**165-256.
27. **Estacio, P. L.** 2006. Surge capacity for health care systems: early detection, methodologies, and process. Acad Emerg Med **13:**1135-7.
28. **Farlow, J., K. L. Smith, J. Wong, M. Abrams, M. Lytle, and P. Keim.** 2001. *Francisella tularensis* strain typing using multiple-locus, variable-number tandem repeat analysis. J Clin Microbiol **39:**3186-92.
29. **Forsman, M., G. Sandstrom, and A. Sjostedt.** 1994. Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. Int J Syst Bacteriol **44:**38-46.

30. **Fournier, P. E., L. Bernabeu, B. Schubert, M. Mutillod, V. Roux, and D. Raoult.** 1998. Isolation of *Francisella tularensis* by centrifugation of shell vial cell culture from an inoculation eschar. J Clin Microbiol **36:**2782-3.
31. **Fulop, M., D. Leslie, and R. Titball.** 1996. A rapid, highly sensitive method for the detection of *Francisella tularensis* in clinical samples using the polymerase chain reaction. Am J Trop Med Hyg **54:**364-6.
32. **Galluzzi, L., M. Magnani, N. Saunders, C. Harms, and I. J. Bruce.** 2007. Current molecular techniques for the detection of microbial pathogens. Sci Prog **90:**29-50.
33. **Garaizar, J., A. Rementeria, and S. Porwollik.** 2006. DNA microarray technology: a new tool for the epidemiological typing of bacterial pathogens? FEMS Immunol Med Microbiol **47:**178-89.
34. **Garcia Del Blanco, N., M. E. Dobson, A. I. Vela, V. A. De La Puente, C. B. Gutierrez, T. L. Hadfield, P. Kuhnert, J. Frey, L. Dominguez, and E. F. Rodriguez Ferri.** 2002. Genotyping of *Francisella tularensis* strains by pulsed-field gel electrophoresis, amplified fragment length polymorphism fingerprinting, and 16S rRNA gene sequencing. J Clin Microbiol **40:**2964-72.
35. **Gerner-Smidt, P., K. Hise, J. Kincaid, S. Hunter, S. Rolando, E. Hyytia-Trees, E. M. Ribot, and B. Swaminathan.** 2006. PulseNet USA: a five-year update. Foodborne Pathog Dis **3:**9-19.
36. **Godoy, D., G. Randle, A. J. Simpson, D. M. Aanensen, T. L. Pitt, R. Kinoshita, and B. G. Spratt.** 2003. Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, *Burkholderia pseudomallei* and *Burkholderia mallei.* J Clin Microbiol **41:**2068-79.
37. **Grunow, R., W. Splettstoesser, S. McDonald, C. Otterbein, T. O'Brien, C. Morgan, J. Aldrich, E. Hofer, E. J. Finke, and H. Meyer.** 2000. Detection of *Francisella tularensis* in biological specimens using a capture enzyme-linked immunosorbent assay, an immunochromatographic handheld assay, and a PCR. Clin Diagn Lab Immunol **7:**86-90.
38. **Higgins, J. A., M. Cooper, L. Schroeder-Tucker, S. Black, D. Miller, J. S. Karns, E. Manthey, R. Breeze, and M. L. Perdue.** 2003. A field investigation of *Bacillus anthracis* contamination of U.S. Department of Agriculture and other Washington, D.C., buildings during the anthrax attack of October 2001. Appl Environ Microbiol **69:**593-9.
39. **Higgins, J. A., Z. Hubalek, J. Halouzka, K. L. Elkins, A. Sjostedt, M. Shipley, and M. S. Ibrahim.** 2000. Detection of *Francisella tularensis* in infected mammals and vectors using a probe-based polymerase chain reaction. Am J Trop Med Hyg **62:**310-8.
40. **Higuchi, R., G. Dollinger, P. S. Walsh, and R. Griffith.** 1992. Simultaneous amplification and detection of specific DNA sequences. Biotechnology (N Y) **10:**413-7.

41. **Hill, K. K., L. O. Ticknor, R. T. Okinaka, M. Asay, H. Blair, K. A. Bliss, M. Laker, P. E. Pardington, A. P. Richardson, M. Tonks, D. J. Beecher, J. D. Kemp, A. B. Kolsto, A. C. Wong, P. Keim, and P. J. Jackson.** 2004. Fluorescent amplified fragment length polymorphism analysis of *Bacillus anthracis,* Bacillus cereus, and Bacillus thuringiensis isolates. Appl Environ Microbiol **70:**1068-80.

42. **Hoffmaster, A. R., C. C. Fitzgerald, E. Ribot, L. W. Mayer, and T. Popovic.** 2002. Molecular subtyping of *Bacillus anthracis* and the 2001 bioterrorism-associated anthrax outbreak, United States. Emerg Infect Dis **8:**1111-6.

43. **Hoffmaster, A. R., R. F. Meyer, M. D. Bowen, C. K. Marston, R. S. Weyant, K. Thurman, S. L. Messenger, E. E. Minor, J. M. Winchell, M. V. Rassmussen, B. R. Newton, J. T. Parker, W. E. Morrill, N. McKinney, G. A. Barnett, J. J. Sejvar, J. A. Jernigan, B. A. Perkins, and T. Popovic.** 2002. Evaluation and validation of a real-time polymerase chain reaction assay for rapid identification of *Bacillus anthracis.* Emerg Infect Dis **8:**1178-82.

44. **Holtz, T. H., J. Ackelsberg, J. L. Kool, R. Rosselli, A. Marfin, T. Matte, S. T. Beatrice, M. B. Heller, D. Hewett, L. C. Moskin, M. L. Bunning, and M. Layton.** 2003. Isolated case of bioterrorism-related inhalational anthrax, New York City, 2001. Emerg Infect Dis **9:**689-96.

45. **Huang, X. Z., M. C. Chu, D. M. Engelthaler, and L. E. Lindler.** 2002. Genotyping of a homogeneous group of *Yersinia pestis* strains isolated in the United States. J Clin Microbiol **40:**1164-73.

46. **Ivnitski, D., D. J. O'Neil, A. Gattuso, R. Schlicht, M. Calidonna, and R. Fisher.** 2003. Nucleic acid approaches for detection and identification of biological warfare and infectious disease agents. Biotechniques **35:**862-9.

47. **Johansson, A., J. Farlow, P. Larsson, M. Dukerich, E. Chambers, M. Bystrom, J. Fox, M. Chu, M. Forsman, A. Sjostedt, and P. Keim.** 2004. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J Bacteriol **186:**5808-18.

48. **Johansson, A., I. Goransson, P. Larsson, and A. Sjostedt.** 2001. Extensive allelic variation among *Francisella tularensis* strains in a short-sequence tandem repeat region. J Clin Microbiol **39:**3140-6.

49. **Johansson, A., A. Ibrahim, I. Goransson, U. Eriksson, D. Gurycova, J. E. Clarridge, 3rd, and A. Sjostedt.** 2000. Evaluation of PCR-based methods for discrimination of *Francisella* species and subspecies and development of a specific PCR that distinguishes the two major subspecies of *Francisella tularensis.* J Clin Microbiol **38:**4180-5.

50. **Junhui, Z., Y. Ruifu, L. Jianchun, Z. Songle, C. Meiling, C. Fengxiang, and C. Hong.** 1996. Detection of *Francisella tularensis* by the polymerase chain reaction. J Med Microbiol **45:**477-82.

51. **Karhukorpi, E. K., and J. Karhukorpi.** 2001. Rapid laboratory diagnosis of ulceroglandular tularemia with polymerase chain reaction. Scand J Infect Dis **33:**383-5.

52. **Keim, P., A. Johansson, and D. M. Wagner.** 2007. Molecular epidemiology, evolution, and ecology of *francisella.* Ann N Y Acad Sci **1105:**30-66.

53. **Keim, P., L. B. Price, A. M. Klevytska, K. L. Smith, J. M. Schupp, R. Okinaka, P. J. Jackson, and M. E. Hugh-Jones.** 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J Bacteriol **182:**2928-36.
54. **Keim, P., M. N. Van Ert, T. Pearson, A. J. Vogler, L. Y. Huynh, and D. M. Wagner.** 2004. Anthrax molecular epidemiology and forensics: using the appropriate marker for different evolutionary scales. Infect Genet Evol **4:**205-13.
55. **King, D., V. Luna, A. Cannons, J. Cattani, and P. Amuso.** 2003. Performance assessment of three commercial assays for direct detection of *Bacillus anthracis* spores. J Clin Microbiol **41:**3454-5.
56. **Klevytska, A. M., L. B. Price, J. M. Schupp, P. L. Worsham, J. Wong, and P. Keim.** 2001. Identification and characterization of variable-number tandem repeats in the *Yersinia pestis* genome. J Clin Microbiol **39:**3179-85.
57. **Kugeler, K. J., R. Pappert, Y. Zhou, and J. M. Petersen.** 2006. Real-time PCR for *Francisella tularensis* types A and B. Emerg Infect Dis **12:**1799-801.
58. **Le Fleche, P., Y. Hauck, L. Onteniente, A. Prieur, F. Denoeud, V. Ramisse, P. Sylvestre, G. Benson, F. Ramisse, and G. Vergnaud.** 2001. A tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus anthracis*. BMC Microbiol **1:**2.
59. **Lim, D. V., J. M. Simpson, E. A. Kearns, and M. F. Kramer.** 2005. Current and developing technologies for monitoring agents of bioterrorism and biowarfare. Clin Microbiol Rev **18:**583-607.
60. **Liu, R. H., J. Yang, R. Lenigk, J. Bonanno, and P. Grodzinski.** 2004. Self-contained, fully integrated biochip for sample preparation, polymerase chain reaction amplification, and DNA microarray detection. Anal Chem **76:**1824-31.
61. **Long, G. W., J. J. Oprandy, R. B. Narayanan, A. H. Fortier, K. R. Porter, and C. A. Nacy.** 1993. Detection of *Francisella tularensis* in blood by polymerase chain reaction. J Clin Microbiol **31:**152-4.
62. **Mahillon, J., and M. Chandler.** 1998. Insertion sequences. Microbiol Mol Biol Rev **62:**725-74.
63. **Maslow, J. N., M. E. Mulligan, and R. D. Arbeit.** 1993. Molecular epidemiology: application of contemporary techniques to the typing of microorganisms. Clin Infect Dis **17:**153-62; quiz 163-4.
64. **McCoy, G. W., and C. W. Chapin.** 1912. Further observations on a plague-like disease of rodents with a preliminary note on the causative agent, *Bacterium tularense*. J Infect Dis **10:**61-72.
65. **Mendelson, T. C., and K. L. Shaw.** 2005. Use of AFLP markers in surveys of arthropod diversity. Methods Enzymol **395:**161-77.
66. **Morner, T., G. Sandstrom, R. Mattsson, and P. O. Nilsson.** 1988. Infections with *Francisella tularensis* biovar *palaearctica* in hares (*Lepus timidus, Lepus europaeus*) from Sweden. J Wildl Dis **24:**422-33.
67. **Nano, F. E., N. Zhang, S. C. Cowley, K. E. Klose, K. K. Cheung, M. J. Roberts, J. S. Ludu, G. W. Letendre, A. I. Meierovics, G. Stephens, and K. L. Elkins.** 2004. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. J Bacteriol **186:**6430-6.

68. **Panicker, G., D. R. Call, M. J. Krug, and A. K. Bej.** 2004. Detection of pathogenic *Vibrio* spp. in shellfish by using multiplex PCR and DNA microarrays. Appl Environ Microbiol **70:**7436-44.
69. **Pearson, T., J. D. Busch, J. Ravel, T. D. Read, S. D. Rhoton, J. M. U'Ren, T. S. Simonson, S. M. Kachur, R. R. Leadem, M. L. Cardon, M. N. Van Ert, L. Y. Huynh, C. M. Fraser, and P. Keim.** 2004. Phylogenetic discovery bias in *Bacillus anthracis* using single-nucleotide polymorphisms from whole-genome sequencing. Proc Natl Acad Sci U S A **101:**13536-41.
70. **Peruski, A. H., L. H. Johnson, 3rd, and L. F. Peruski, Jr.** 2002. Rapid and sensitive detection of biological warfare agents using time-resolved fluorescence assays. J Immunol Methods **263:**35-41.
71. **Peruski, A. H., and L. F. Peruski, Jr.** 2003. Immunological methods for detection and identification of infectious disease and biological warfare agents. Clin Diagn Lab Immunol **10:**506-13.
72. **Peruski, L. F., Jr., and A. H. Peruski.** 2003. Rapid diagnostic assays in the genomic biology era: detection and identification of infectious disease and biological weapon agents. Biotechniques **35:**840-6.
73. **Petersen, J. M., M. E. Schriefer, L. G. Carter, Y. Zhou, T. Sealy, D. Bawiec, B. Yockey, S. Urich, N. S. Zeidner, S. Avashia, J. L. Kool, J. Buck, C. Lindley, L. Celeda, J. A. Monteneiri, K. L. Gage, and M. C. Chu.** 2004. Laboratory analysis of tularemia in wild-trapped, commercially traded prairie dogs, Texas, 2002. Emerg Infect Dis **10:**419-25.
74. **Petersen, J. M., M. E. Schriefer, K. L. Gage, J. A. Montenieri, L. G. Carter, M. Stanley, and M. C. Chu.** 2004. Methods for enhanced culture recovery of *Francisella tularensis*. Appl Environ Microbiol **70:**3733-5.
75. **Pfaller, M. A.** 2001. Molecular approaches to diagnosing and managing infectious diseases: practicality and costs. Emerg Infect Dis **7:**312-8.
76. **Pourcel, C., F. Andre-Mazeaud, H. Neubauer, F. Ramisse, and G. Vergnaud.** 2004. Tandem repeats analysis for the high resolution phylogenetic analysis of *Yersinia pestis*. BMC Microbiol **4:**22.
77. **Read, T. D., S. L. Salzberg, M. Pop, M. Shumway, L. Umayam, L. Jiang, E. Holtzapple, J. D. Busch, K. L. Smith, J. M. Schupp, D. Solomon, P. Keim, and C. M. Fraser.** 2002. Comparative genome sequencing for discovery of novel polymorphisms in Bacillus anthracis. Science **296:**2028-33.
78. **Rodriguez-Lazaro, D., J. Lloyd, A. Herrewegh, J. Ikonomopoulos, M. D'Agostino, M. Pla, and N. Cook.** 2004. A molecular beacon-based real-time NASBA assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in water and milk. FEMS Microbiol Lett **237:**119-26.
79. **Saiki, R. K., T. L. Bugawan, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1986. Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. Nature **324:**163-6.
80. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230:**1350-4.

81. **Sandstrom, G., A. Sjostedt, T. Johansson, K. Kuoppa, and J. C. Williams.** 1992. Immunogenicity and toxicity of lipopolysaccharide from *Francisella tularensis* LVS. FEMS Microbiol Immunol **5:**201-10.
82. **Saslaw, S., and S. Carhart.** 1961. Studies with tularemia vaccines in volunteers. III. Serologic aspects following intracutaneous or respiratory challenge in both vaccinated and nonvaccinated volunteers. Am J Med Sci **241:**689-99.
83. **Sjostedt, A., U. Eriksson, L. Berglund, and A. Tarnvik.** 1997. Detection of *Francisella tularensis* in ulcers of patients with tularemia by PCR. J Clin Microbiol **35:**1045-8.
84. **Skottman, T., H. Piiparinen, H. Hyytiainen, V. Myllys, M. Skurnik, and S. Nikkari.** 2007. Simultaneous real-time PCR detection of *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis*. Eur J Clin Microbiol Infect Dis **26:**207-11.
85. **Splettstoesser, W. D., H. Tomaso, S. Al Dahouk, H. Neubauer, and P. Schuff-Werner.** 2005. Diagnostic procedures in tularaemia with special focus on molecular and immunological techniques. J Vet Med B Infect Dis Vet Public Health **52:**249-61.
86. **Staples, J. E., K. A. Kubota, L. G. Chalcraft, P. S. Mead, and J. M. Petersen.** 2006. Epidemiologic and molecular analysis of human tularemia, United States, 1964-2004. Emerg Infect Dis **12:**1113-8.
87. **Syrjala, H., P. Koskela, T. Ripatti, A. Salminen, and E. Herva.** 1986. Agglutination and ELISA methods in the diagnosis of tularemia in different clinical forms and severities of the disease. J Infect Dis **153:**142-5.
88. **Tarnvik, A., and M. C. Chu.** 2007. New approaches to diagnosis and therapy of tularemia. Ann N Y Acad Sci **1105:**378-404.
89. **Tarnvik, A., S. Lofgren, L. Ohlund, and G. Sandstrom.** 1987. Detection of antigen in urine of a patient with tularemia. Eur J Clin Microbiol **6:**318-9.
90. **Thomas, R., A. Johansson, B. Neeson, K. Isherwood, A. Sjostedt, J. Ellis, and R. W. Titball.** 2003. Discrimination of human pathogenic subspecies of *Francisella tularensis* by using restriction fragment length polymorphism. J Clin Microbiol **41:**50-7.
91. **Tomaso, H., H. C. Scholz, H. Neubauer, S. Al Dahouk, E. Seibold, O. Landt, M. Forsman, and W. D. Splettstoesser.** 2007. Real-time PCR using hybridization probes for the rapid and specific identification of *Francisella tularensis* subspecies *tularensis*. Mol Cell Probes **21:**12-6.
92. **Van Ert, M. N., W. R. Easterday, T. S. Simonson, J. M. U'Ren, T. Pearson, L. J. Kenefic, J. D. Busch, L. Y. Huynh, M. Dukerich, C. B. Trim, J. Beaudry, A. Welty-Bernard, T. Read, C. M. Fraser, J. Ravel, and P. Keim.** 2007. Strain-specific single-nucleotide polymorphism assays for the *Bacillus anthracis* Ames strain. J Clin Microbiol **45:**47-53.
93. **Van Kessel, J. S., J. S. Karns, and M. L. Perdue.** 2003. Using a portable real-time PCR assay to detect *Salmonella* in raw milk. J Food Prot **66:**1762-7.
94. **Varma-Basil, M., H. El-Hajj, S. A. Marras, M. H. Hazbon, J. M. Mann, N. D. Connell, F. R. Kramer, and D. Alland.** 2004. Molecular beacons for multiplex detection of four bacterial bioterrorism agents. Clin Chem **50:**1060-2.

95. **Versage, J. L., D. D. Severin, M. C. Chu, and J. M. Petersen.** 2003. Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. J Clin Microbiol **41:**5492-9.
96. **Vinogradov, E., and M. B. Perry.** 2004. Characterisation of the core part of the lipopolysaccharide O-antigen of *Francisella novicida* (U112). Carbohydr Res **339:**1643-8.
97. **Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and et al.** 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res **23:**4407-14.
98. **Wasas, A. D., R. E. Huebner, and K. P. Klugman.** 1999. Use of Dorset egg medium for maintenance and transport of *Neisseria meningitidis* and Haemophilus influenzae type b. J Clin Microbiol **37:**2045-6.
99. **Westin, L., C. Miller, D. Vollmer, D. Canter, R. Radtkey, M. Nerenberg, and J. P. O'Connell.** 2001. Antimicrobial resistance and bacterial identification utilizing a microelectronic chip array. J Clin Microbiol **39:**1097-104.
100. **Wilson, W. J., A. M. Erler, S. L. Nasarabadi, E. W. Skowronski, and P. M. Imbro.** 2005. A multiplexed PCR-coupled liquid bead array for the simultaneous detection of four biothreat agents. Mol Cell Probes **19:**137-44.
101. **Yang, J. M., J. Bell, Y. Huang, M. Tirado, D. Thomas, A. H. Forster, R. W. Haigis, P. D. Swanson, R. B. Wallace, B. Martinsons, and M. Krihak.** 2002. An integrated, stacked microlaboratory for biological agent detection with DNA and immunoassays. Biosens Bioelectron **17:**605-18.

Chapter III

Identification of an Inhibitory Substance against *F. tularensis* for Use as a Diagnostic Reagent

3.1 Introduction

3.1.1 Iron as a limiting growth factor for bacteria

Iron is an essential trace element for most bacterial species (39) and is considered a limiting element for bacterial growth due to its presence as an unusable form under aerobic conditions as well as its unavailability within living hosts. In aerobic environments, iron is readily oxidized to FeIII, forming insoluble compounds including oxide hydrates FeO(OH), carbonate $Fe_2(CO_3)_3$, and magnetite Fe_3O_4 [iron (II/III) oxide] (44). These compounds become soluble only at an acidic pH, requiring bacteria to have mechanisms in place for uptake of usable iron.

For most intracellular pathogens, iron availability is a limiting factor for the survival and growth within the host, as well as for pathogenesis (44). Within multicellular bacterial hosts, iron is reduced from FeII to FeIII ($FeII + H_2O_2 \rightarrow FeIII + OH^- + OH$), during which hydroxyl radicals are formed. Hydroxyl radicals have a toxic effect on macromolecules and it is therefore essential for multicellular organisms to have mechanisms to maintain iron homeostasis and protection against iron toxicity. Carrier proteins such as transferrin found in serum, lactoferrin found in lymph, and mucosal secretions, have a high affinity for FeIII and act to sequester iron, thus

preventing iron toxicity in the host and at the same time, leaving little if any free iron available for bacterial use (44).

Most bacteria require iron concentrations of 10^{-6} to 10^{-7} M to support molecular activities such as replication, signaling, glycolysis, electron transport, DNA synthesis, and defense against reactive-oxygen intermediates (29, 39, 44, 46). To ensure proper intracellular concentrations are maintained, bacteria have developed specialized mechanisms for iron uptake that vary among bacterial species (36, 46). These mechanisms include the use of ABC iron transporters, as well as the removal of iron from transferrin and/or lactoferrin as direct sources, and siderophores and hemophores for uptake of iron from indirect sources (29, 39).

3.1.2 Aerobactin: a siderophore produced by *E. coli*

E. coli requires iron for the synthesis of heme enzymes including cytochromes and hydroperoxidases, as well as for production of nitrogenase, ribonucleotide reductase and sulfur proteins. Due to its requirements for iron, *E. coli* has multiple iron acquisition and transport systems that it can use for the uptake of iron (9). One such mechanism used for uptake of iron from indirect sources is the production of the iron-binding siderophore aerobactin (47).

Aerobactin was first isolated from *Enterobacter aerogenes* 62-I (17) and has subsequently been found to be produced by an array of *Enterobacteriaceae,* including *Enterobacter cloacae* (42), *Shigella flexineri* (33), *Salmonella* (10), *Klebsiella pneumoniae* (28), *Yersinia* (40), *Erwinia carotovora* (21) as well as by non-enteric bacteria such as *Proteus* (8), *Staphylococcus* (25), and *Pseudomonas* (6). Aerobactin is a non-catechol dihydroxamate derivative of citric acid. It is a low-molecular weight

(564.54 Da) iron-chelating compound that is produced by some *E. coli* strains (45, 47). Its structure is diagramed in Figure 3.1. The affinity (K_f) of aerobactin for FeIII is 10^{23}, and it is stable over a wide pH range. Other siderophores, such as enterobactin, have a stronger affinity for FeIII, but are less stable (2).

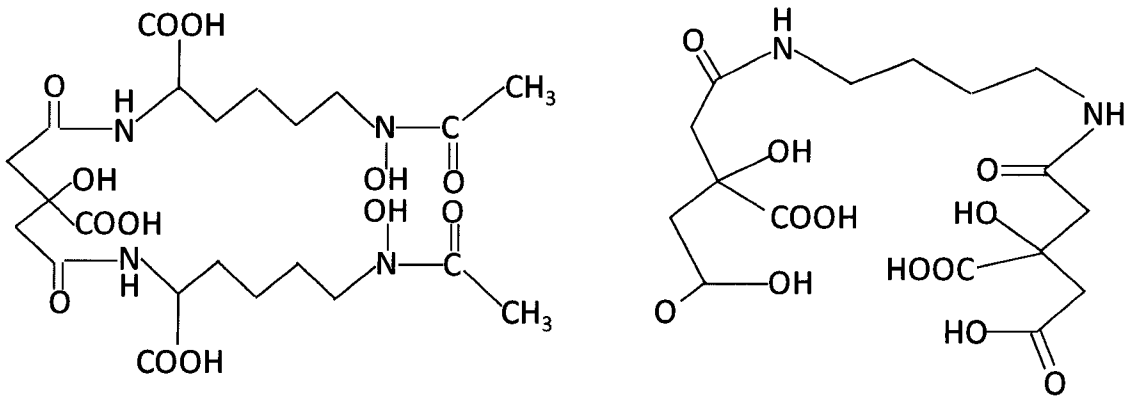


Figure 3.1 Structure of aerobactin and rhizoferrin. The structures for the siderophores aerobactin (left) and rhizoferrin (right) are shown.

The biosynthetic components for aerobactin production can be chromosomal or plasmid encoded (27), with the necessary genes for biosynthesis and transport of aerobactin clustered as an 8.3 kb operon that consists of five genes (*iucABCD* and *iutA*) (Figure 3.2).

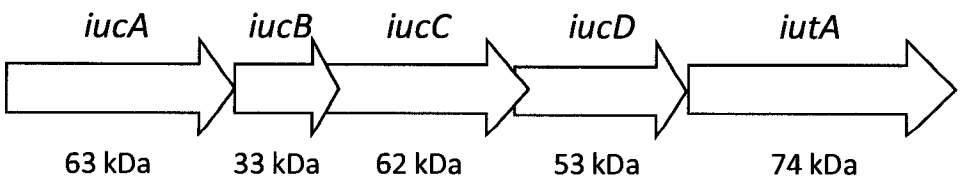


Figure 3.2 Aerobactin operon. The aerobactin operon consists of five genes, *iucABCD* and *iutA* (*iuc* stands for iron uptake chelate and *iut* stands for iron uptake transport). Regions encoding for IucABCD enzymes are represented as purple arrows and the region encoding for the receptor protein IutA is represented as an orange arrow.

Four genes, *iucABCD,* are involved directly with the biosynthesis of aerobactin, and one, *iutA*, encodes for the membrane receptor protein (13). All five aerobactin-associated genes are regulated by the product of the universal negative regulator *fur* (2, 5). The function for each of the five aerobactin-related proteins has been determined. IucA and IucC function as amide synthetases (14), IucB is an *N*-6-hydroxylysine acetyltransferase (13), IucD is a lysine *N*-6-monooxygenase (26), and IutA is an outer membrane receptor for iron loaded aerobactin (13). Aerobactin is assembled by nonribosomal, cytoplasmic peptide synthetases (44). Once assembled, aerobactin must be excreted from the cell. Little information is known about how this occurs, although it is thought that the molecule is too large to diffuse out freely. Once extracellular, aerobactin binds FeIII and is taken back into the cell through interaction with a specific membrane receptor IutA and inner membrane-associated TonB-ExbB-ExbD complex, which commonly interacts with the outer membrane receptor for siderophore uptake (12). The aerobactin-iron complex is taken up as a whole and transferred to the cytosol, where reduction of the bound FeIII occurs to form the usable FeII, which then causes release of the iron molecule from aerobactin. Unlike other siderophores, aerobactin is resistant to hydrolysis and is recycled after its release of iron (11).

3.1.3 *Francisella tularensis* and iron

F. tularensis is a facultative intracellular bacterium that replicates primarily within macrophages where its survival and growth is critical for causing disease within the host. It has been shown that *F. tularensis* acquires sufficient iron levels within the phagosome for virulence (16). Studies also show that *F. tularensis* subsp. type B strain LVS grown under iron-limiting conditions alters the composition of its cell envelope.

Increased virulence of this attenuated strain also occurs, suggesting that iron-depletion may up-regulate the expression of genes associated with virulence (3). Sequencing of the whole *F. tularensis* subsp. type A strain SCHU S4 genome identified iron-related genes *ftnA* (ferritin), *fumB* (fumarate hydratase class I), *acnA* (aconitate hydratase 1), *sodB* (superoxide dismutase), an ortholog of *iraB* (putative peptide transporter) , and *frgA* (Fur-regulated gene; siderophore biosynthetic protein) and their possible regulator (*fur*), but a definitive mechanism for iron uptake was not revealed. In addition, no genes encoding TonB an energy tranducer, as well as an outer-membrane receptor protein for uptake of iron-binding molecules were found.

Deng *et al.* focused on determining the genes affected by iron limitation (15). Their study identified two proteins, IglC (intracellular growth locus C) and PdpB (pathogenicity determinant protein B), which were previously described to be encompassed within the *Francisella* pathogenicity island (FPI) and as being iron-regulated. Further analyses revealed that the sequences directly upstream of these genes had homology to the consensus Fur box sequence, and that these genes were organized as operons *igl*ABCD, and *pdp*AB. Additionally, the *Francisella* iron-regulated gene (*fig*A) was discovered and found to contain an upstream sequence with high homology to the consensus Fur box sequence. The operon encompassing the *fig*A gene, described as *fig*ABCD, was determined to have the highest level of up-regulation when *Francisella* was grown under an iron-restricted environment. Mutants in *fig*A were shown to be important in what was speculated to be siderophore production. The genes identified by this study correlate in their expression levels to those of other well-documented iron-regulated genes.

Sullivan *et al.* identified and purified a siderophore produced by *F. tularensis* subsp. type A strain SCHU S4 and *F. tularensis* subsp. type B strain LVS. The siderophore was found to be structurally similar to rhizoferrin, a siderophore produced by the fungus *Rhizopus microsporus* (Figure 3.1) (22). Rhizoferrin is a polycarboxylate siderophore and is composed of two citric acid moieties linked by amide bonds to a putrescine residue. The carboxyl and hydroxyl groups of this molecule give rhizoferrin its highly electronegative properties that enable it to chelate ferric iron (FeIII).

The genes associated with the production of this siderophore were found to be in an operon named *fsl*ABCD (*Francisella* siderophore locus A, B C, and D). FslA was found to function similarly to the NIS synthetase and to IucA and IucB (aerobactin biosynthesis), FslB and FslD act as efflux transporters, and FslC resembles a diaminopimelate decarboxylase. These proteins have similar roles to those found for other siderophore production and transport proteins (12). Neither genes involved in the uptake of the siderophore-iron complex, nor homologues to proteins for the inner membrane-associated TonB-ExbB-ExbD complex have been identified in *Francisella*.

Lenco *et al.* recently confirmed the findings of Deng *et al.* through a proteomic analysis of the *F. tularensis* subsp. type B strain LVS response to iron-limitation and provided evidence for siderophore production by *F. tularensis* subsp. *novicida* (24). Although siderophore production has been identified in several *Francisella*, little is understood about how it functions. It is yet to be determined if other iron uptake mechanisms are also present in *Francisella*.

3.1.4 Project rationale

The observation that *F. tularensis* is inhibited when co-plated with *E. coli* was made during routine antibiotic susceptibility testing at the Diagnostic Reference Laboratory for tularemia at the CDC (Figure 3.3).

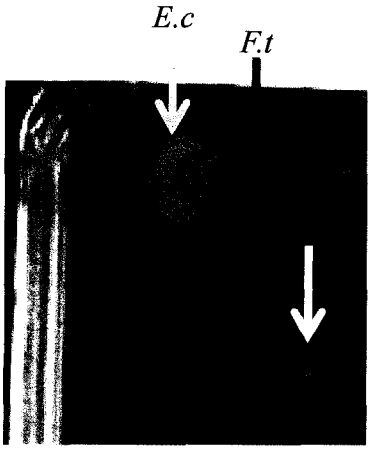


Figure 3.3 Initial observation that *F. tularensis* is inhibited by *E. coli*. The orange arrow is pointing to a spot of *E. coli* strain 25922 growth; red arrow is pointing to a spot of poor *F. tularensis* growth; white arrow is pointing to a standard spot of *F. tularensis* growth.

Based on this observation, we hypothesized that the substance produced by *E. coli* and that inhibits *Francisella* could be used as a reagent in the development of a rapid *Francisella* diagnostic that can differentiate between live and dead cells. To test our hypothesis, our first aim was to develop assays for use in characterizing the inhibition against *Francisella*. A genetic approach was then used to identify the inhibitory substance as the siderophore aerobactin.

3.2 Materials and Methods

3.2.1 Bacterial strains

The bacterial strains and fosmids used in this study are listed in Table 3.1. *E. coli* was cultured on Luria-Bertani (LB) agar plates for 24 h at 37°C and all *F. tularensis* strains were grown on CHAB medium prior to co-plating experiments, for 48 h at 35°C. Co-plating experiments with *E. coli* and *F. tularensis* were performed on MHII agar plates (Becton, Dickinson and Company, Sparks, MD) supplemented with 1 % isovitalex (Becton, Dickinson and Company). For selection of recombinant *E. coli* clones, LB agar plates were supplemented with chloramphenicol (12.5 μg/ml) and/or kanamycin (50 μg/ml).

3.2.2 Bioassays for *F. tularensis* growth inhibition

Co-plating spot assay. *F. tularensis* type B strain LVS was suspended in 0.85% saline to an absorbance (A_{600}) of 0.05 and an aliquot (400 μl) was spread using a sterile swab onto MHII agar supplemented with 1% isovitalex. Separately, *E. coli* was suspended in 0.85% saline to an absorbance (A_{600}) of 0.02 and 2-20 μl was spotted onto the *F. tularensis* spread plates. Co-cultures were incubated at 37°C for 48 h. Inhibition of *F. tularensis* growth was qualitative and determined by assaying for the zone of inhibition surrounding the *E. coli*. The same assay was performed using *F. tularensis* subsp. type A strain SCHU S4 except that a Biolog turbidimeter (Biolog, Inc., Hayward, CA) was used to measure the bacterial suspensions to a 70% transmittance for *F. tularensis* subsp. type A strain SCHU S4 and 90% transmittance for *E. coli* suspensions made in 0.85% saline.

Reversal of inhibition was performed by ferric chloride ($FeCl_3$) supplementation. Specifically, $FeCl_3$ (anhydrous) (Fisher Scientific, Pittsburg, PA) at a final concentration

of 10 μM was supplemented into MHII with 1% isovitalex agar plates. All assays were performed in triplicate.

Table 3.1 Bacterial strains and fosmids used in this study.

	Designation	Description	Relevant Characteristic[a]	Source or Reference
Strains				
Escherichia coli	ATCC 25922	Clinical Isolate	Iuc^+	CDC, Fort Collins, ATCC
	EPI300™*-T1*®	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 recA1endA1 araD139 Δ(ara, leu)7697galU galK λ- rpsL nupG trfA tonA	Iuc^-	Epicentre Technologies, Madison WI
Francisella tularensis subsp. *holarctica*	LVS	Vaccine strain, NDBR Lot 101	Iuc^S	CDC, Fort Collins
Francisella tularensis subsp. *tularensis*	SCHU S4	Clinical Isolate	Iuc^S	CDC, Fort Collins
Fosmids				
	pCC1FOS™	Copy control fosmid vector	*lacZ*, λcos, CmR, loxP, Bacteriophage P1	Epicentre Technologies, Madison WI
	pCC1FOS820	pCC1FOS™ containing a 40 Kb genomic fragments from *E. coli* ATCC 25922	Iuc^+, Cm^R	This study
	pCC1FOS820-1	pCC1FOS820 containing EZ::TN<KAN-2> Tnp insertion in *iucA*	Iuc^-, Cm^R, Km^R	This study
	pCC1FOS820-241	pCC1FOS820 containing EZ::TN<KAN-2> Tnp insertion in *iucB*	Iuc^-, Cm^R, Km^R	This study
	pCC1FOS820-49	pCC1FOS820 containing EZ::TN<KAN-2> Tnp insertion in *iucC*	Iuc^-, Cm^R, Km^R	This study

[a] Abbreviations: Iuc^+, Iuc^s and Iuc^- indicate aerobactin producing, aerobactin-sensitive, and non-aerobactin producing strains, respectively; *lacZ*, *E. coli* β-galactosidase-encoding gene; λcos, lambda packaging site(s); Cm^R, chloramphenicol reistance-encoding gene; *loxP*, Cre-recombinase target site; Km^R, kanamycin reistance-encoding gene.

Agar-plug transfer. *E. coli* strain 25922 suspended in 0.85% saline was spotted once (20 μl) onto the MHII agar with 1% isovitalex. Following 48 h of incubation at 37°C, agar plugs (0.7 x 1.5 mm) directly adjacent to the *E. coli* strain 25922 growth were aseptically

removed. *F. tularensis* subsp. type B strain LVS plates were prepared as described for the co-plating assay. Immediately after inoculation, an agar plug (0.7 x 1.5 mm) was aseptically removed and replaced with an agar plug from the *E. coli* strain 25922 plate. Plates were incubated at 37°C for 48 h and zones of *F. tularensis* subsp. type B strain LVS inhibition were scored as (+) for small inhibition zones < *E. coli* strain 25922 inhibition zone, (++) for larger inhibition zone ≥ *E. coli* strain 25922 inhibition zone, or (-) for no-inhibition. All assays were performed in triplicate. Figure 3.4 diagrams the methods for the agar-plug transfer assay.

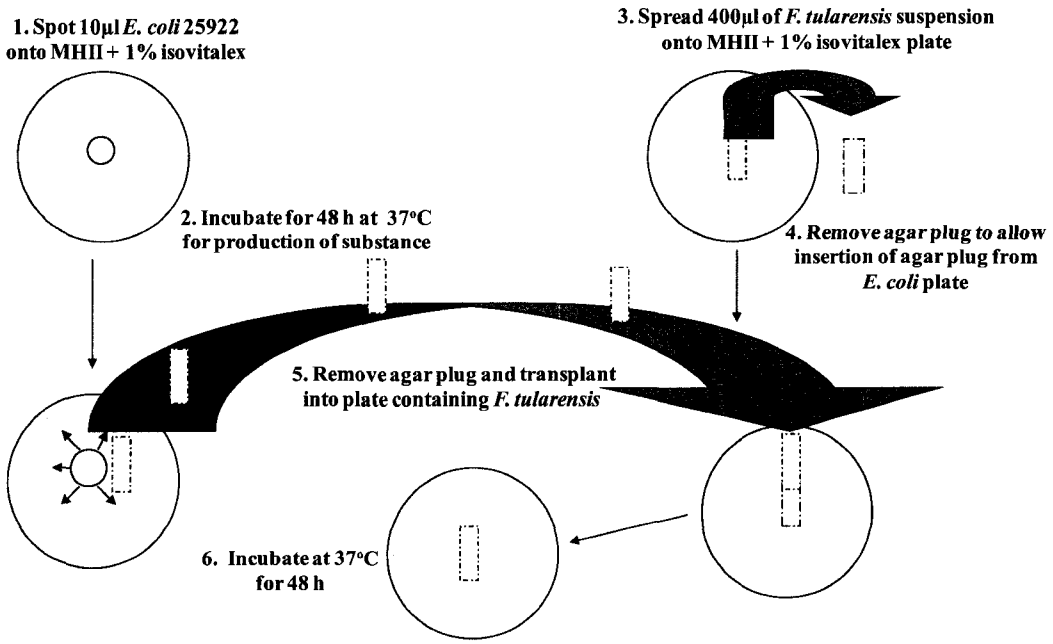


Figure 3.4 Diagram of agar-plug transfer assay. The agar-plug transfer assay was performed by first spotting *E. coli* onto an MHII + 1% isovitalex agar plate. The plate was incubated for 48 h to allow the inhibitory substance to be produced by *E. coli* and to diffuse through the medium. Another MHII + 1% isovitalex plate was spread with *Francisella* suspension. An agar plug was cut out of the agar plate. A similar sized agar plug was removed from the plate containing *E. coli* growth adjacent to the spot of growth and transferred into the well created on the plate spread with *Francisella*. The plate with *Francisella* and the transferred agar plug was incubated for 48 hours at 36°C.

Direct broth assay. A 1:20 dilution of a 0.05 absorbance (A_{600}) *F. tularensis* type B strain LVS was prepared in MH broth (Becton, Dickinson, and Company) supplemented with 1% isovitalex. *E. coli* was grown on MHII agar plates with 1% isovitalex as described for the agar-plug transfer assay. After growth at 37°C for 48 h, *E. coli* was scraped with a sterile swab to remove all cells, and any remaining growth was killed by exposing the agar plates to chloroform fumes for 10 min. Two agar plugs (0.7 x 1.5 mm) were then cut from the area of the plate where the *E. coli* had been spotted. Plugs were extracted with 600 µl of HEPES buffer (pH 8.0) (Sigma Chemicals, St. Louis, MO) at 37°C for 30 min. The supernatant was collected and designated *E. coli* agar-plug fluid. In 96-well plates, 50 µl of the agar-plug fluid was mixed with 175 µl of the *F. tularensis* subsp. type B strain LVS suspension. The negative control was 50 µl of *E. coli* agar-plug fluid mixed with 175 µl of MH broth supplemented with 1% isovitalex, and the positive control was 175 µl of the *F. tularensis* subsp. type B strain LVS suspension mixed with 50 µl of MH broth supplemented with 1% isovitalex. Inhibition of *F. tularensis* subsp. type B strain LVS was monitored over time by measuring absorbance at 600 nm using a Multiskan® Spectrum Microplate Spectrophotometer (ThermoLabsystems, Vantaa, Finland). Purified aerobactin (Biophore Research Products, Tübingen, Germany) was used in this assay to determine the inhibitory concentration of aerobactin. All assays were performed in triplicate.

Percent inhibition was calculated using the following formula:

$$\frac{[OD_{600nm} \text{ of } F.\ tularensis \text{ subsp. type B strain LVS growth (negative plug fluid)} - OD_{600nm} \text{ of } F.\ tularensis \text{ subsp. type B strain LVS growth } (E.\ coli \text{ plug fluid})]}{[OD_{600nm} \text{ of } F.\ tularensis \text{ subsp. type B strain LVS growth (negative plug fluid)}]} \times 100\%$$

As a control, negative plug fluid was obtained by isolating plugs from a MHII + 1% isovitalex plate in the absence of *E. coli* growth.

3.2.3 Growth phase dependency of *Francisella* for *E. coli* inhibition

The co-plating spot assay was performed as described above with the following modifications. After incubation of the freshly inoculated *F. tularensis* plates for 0, 4, 6, or 10 h at 37°C, *E. coli* was suspended in 0.85% saline to an absorbance (A_{600}) of 0.02 and 2-20 µl was spotted onto the *F. tularensis* lawns. Co-cultures were incubated at 37°C for 48 h. Inhibition of *F. tularensis* growth was qualitative and determined by assaying for the zone of inhibition surrounding the *E. coli*.

3.2.4 Genomic library construction and phenotype selection

Genomic DNA was isolated from *E. coli* strain 25922 using the DNeasy® Tissue Kit (Qiagen Inc., Valencia, CA) and concentrated using ethanol precipitation (38). DNA fragments of approximately 40 kb were cloned using the CopyControl ™ Fosmid Library Production Kit (Epicentre, Madison, WI) following the manufacturer's protocol, with a modification made for the size selection of the end-repaired DNA. The modification was initial electrophoresis of the end-repaired DNA through a 1% SeaKem® GTG® agarose (Cambrex Bio Science Rockland, Inc., Rockland, MD). A region of the gel below the 40 kb DNA fragments was removed and replaced with 1% SeaKem® LE agarose

(BioWhittaker Molecular Applications, Rockland, MD). Electrophoresis was continued and the 40 kb DNA fragments that migrated into the LE agarose were isolated according to the kit instructions. The 40 kb fragments were then ligated into the pCC1FOS™ fosmid. Recombinant fosmids were packaged into phage using the MaxPlax™ lambda packaging extracts (Epicentre), and the resulting phage library was used to transduct *E. coli* strain EPI300™-T1®.

Individual *E. coli* strain EPI300™-T1® transductants were grown in 96-well plates in 200 µl of LB broth at 37°C for 24 h. An aliquot (2 µl) of each transductant was spotted on MHII agar with 1% isovitalex spread with *F. tularensis* subsp. type B strain LVS as described for the co-plating spot assay, and incubated at 37°C for 48 h. Transductants able to inhibit *F. tularensis* subsp. type B strain LVS were identified by comparing inhibitory zones to those formed by *E. coli* strains 25922 and EPI300™-T1®.

3.2.5 Transposon mutagenesis

Fosmid DNA was isolated from transductants that inhibited growth of *F. tularensis* subsp. type B strain LVS using the Qiagen HiSpeed® Plasmid Midi Kit (Qiagen Inc., Valencia, CA). Transposon mutagenesis was performed *in vitro* using the EZ::TN<KAN-2>Tnp transposome system (Epicentre) (20), and mutagenized fosmids were electroporated into *E. coli* strain EPI300™-T1®. The resulting clones with a transposon insert were selected by growth on LB agar containing kanamycin. Kanamycin resistant clones were grown in LB broth in 96-well plates for 24 h and screened for *F. tularensis* subsp. type B strain LVS growth inhibition using the co-plating spot assay.

3.2.6 DNA sequencing and data analysis

Fosmid DNA was isolated using the Qiagen HiSpeed® Plasmid Midi Kit (Qiagen Inc., Valencia, CA) and concentrated by ethanol precipitation (38). To sequence the ends of fosmid insert, Epicentre's primer pair pCC1™ / pEpiFOS™ forward sequencing primer (5'-GGATGTGCTGCAAGGCGATTAAGTTGG-3') and pCC1™ / pEpiFOS™ reverse sequencing primer (5'-CTCGTATGTTGTGTGGAATTGTGAGC-3') was used. Epicentre's primer pair KAN-2 FP-1 forward primer (5'-ACCTA CAACAAAGCTCTCATCAACC-3') and KAN-2 RP-1 reverse primer (5' GCAATGTAACATCAGAGATTTTGAG-3') was used for sequencing directly from the two ends of the EZ::TN<KAN-2>Tnp transposon. DNA sequencing was performed by Davis Sequencing (Davis, CA) using Applied Biosystems Big Dye Terminator V3.0 sequencing chemistry. BLAST analysis (blastn) was performed using the NCBI website (http://www.ncbi.nlm.nih.gov/).

3.2.7 PCR amplification

The *iuc*A, *iuc*B, and *iuc*C genes were PCR amplified using either pCC1FOS280 fosmid DNA or *E. coli* strain 25922 genomic DNA as the template, and the following primer pairs, 5'-TTCACAGCGCAGTCCATATCTGCTG-3' and 5'-AACGCAAGTG GCCAGCAGGACAGTG-3' (*iuc*A), 5'-TCGTCATACTCCATCTCAGACAAC-3' and 5'-TGTCTCAACTACGTGCTGGAAAG-3' (*iuc*B), 5'-ATCTGATGTGACAATGCA GC-3' and 5'-TCGTCATCACTTCTTCACGG-3' (*iuc*C). PCR conditions consisted of an initial denaturation step at 95°C for 5 min , followed by 30 cycles including denaturation at 95°C for 1 min, annealing temperature of 65°C, 60°C, 52°C and extension times at 72°C for 2 min, 1 min 30 seconds, and 3 min for *iuc*A, *iuc*B, and *iuc*C

respectively. DNA was electrophoresed through agarose gels and extracted using the QIAquick® Gel Extraction Kit (Qiagen Inc., Valencia, CA) for use in electrophoresis comparisons and Southern blot analysis.

PCR amplification of the EZ::TN<KAN-2>Tnp transposon was performed using pCC1FOS820-11 fosmid DNA as the template and using primer pairs: EZTn-FP-1 forward primer 5'-CAAGGGGTGTTATGAGCCATATTC-3' and EZTn-RP-1 reverse primer 5'-CGGTTGATGAGAGCTTTGTTGTAGG-3'. PCR conditions included an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 51°C for 45 sec and extension at 72°C for 1 min.

3.2.8 Southern blot analysis

Fosmid DNA was digested with restriction endonucleases *Eco*RV and *DraI* (Invitrogen, Carlsbad, CA) to complete digestion (2 h at 37 °C) and electrophoresed in a 1% agarose gel. The DNA was transferred to Hybond-N^+ nylon membrane (Amersham Biosciences, Piscataway, NJ) according to standard procedures (38). AlkPhos Direct Labelling Reagents (Amersham Biosciences, Piscataway, NJ) were used to label the PCR amplified EZ::TN<KAN-2>Tnp transposon DNA to make the probe. Detection of the hybridized probe was done using CDP-Star™ Detection Reagent (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol followed by exposing the blot to Fuji Medical X-Ray Film (Fuji Medical Systems U.S.A., Inc., Stamford, CT).

3.2.9 Aerobactin purification

CM Sepharose™ Fast Flow (Amersham Biosciences) resin was washed three times with sterile milliQ water, followed by three washes of 20 mM potassium phosphate (pH 7.0). Plug fluid (200 µl) was dried by lyophilization and suspended in 200 µl of 20 mM potassium phosphate (pH 7.0). This suspension was added to 100 µl of CM Sepharose resin mixed gently for 30 min and centrifuged for 30 sec at 4,000 rpm. The supernatant was collected and 200 µl of 0.6 M NaCl was added. Mixing and centrifugation was repeated and the supernatant collected.

The inhibiting fraction was recovered from the CM Sepharose matrix and was applied to a Bio-Gel P-2 (BioRad) size exclusion column equilibrated in 20 mM ammonium bicarbonate. Chromatography was performed at a flow rate of 5-10 cm/hr with 20 mM ammonium bicarbonate as the elution buffer. Fractions of 1 ml were collected, dried in a Speed Vac and suspended in 100 µl of sterile milliQ water. Drying and resuspension of the fractions was repeated three times to ensure complete volatilization of the ammonium bicarbonate. The final products were suspended in 200 µl of sterile milliQ water and filter sterilized using a 13 mm, 0.2 µm Acrodisc syringe filter (HT Tuffryn membrane) (Pall Life Sciences, East Hills, NY).

3.2.10 Ferric perchlorate assay

The ferric perchlorate assay for measuring hydroxamate was modified for use in a 96-well plate (1). Briefly, 83 µl of reagent (5 mM $Fe(ClO_4)_3$ in 0.1 M $HClO_4$) was added to 17 µl of sample, and allowed to incubate at room temperature for 20 min. The absorbance was measured at 480nm and aerobactin concentration determined based on a comparison to standard curve generated using purified aerobactin.

3.3 Results

3.3.1 Bioassays for characterization of *Francisella* inhibition by *E. coli*

Co-plating of *F. tularensis* subsp. type B strain LVS and *F. tularensis* subsp. type A strain SCHU S4 with *E. coli* strain ATCC 25922 demonstrated that *E. coli* strain 25922 inhibited growth of these two strains of *F. tularensis*, as a zone of inhibition was clearly observed (Figure 3.5). Results using this assay were consistent.

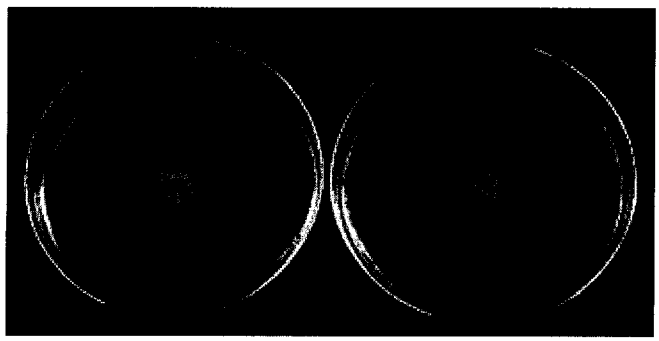


Figure 3.5 Co-plating of *E. coli* and *Francisella*. Co-plating spot assay resulting in the growth inhibition of *F. tularensis* subsp. type B strain LVS (right) and *F. tularensis* subsp. type A strain SCHU S4 (left) when co-plated as lawns with *E. coli* strain ATCC 25922 (which is at the center of each plate). The red-dashed line marks the upper and lower boundary of the inhibition zone.

The inhibitory substance produced by *E. coli* strain 25922 was easily diffusible in agar as shown by the transfer of agar plugs free of live *E. coli* cells. This suggested the substance was secreted and inhibition was independent of cell to cell contact between *E. coli* and *F. tularensis* (Figure 3.6).

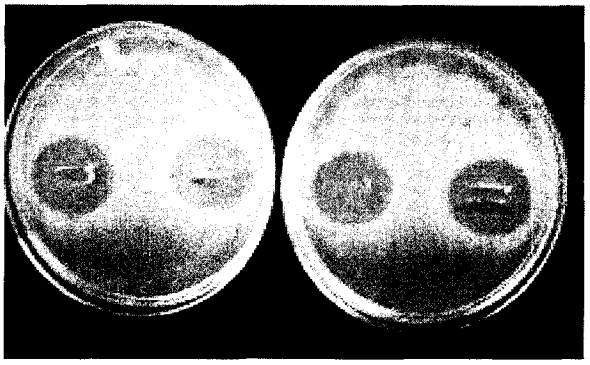


Figure 3.6 Agar-plug transfer assay. Agar-plug transfer assay: lawn is *F. tularensis* subsp. type B strain LVS, agar plugs are from areas surrounding *E. coli* strain 25922 spots (duplicate plates).

3.3.2 Growth phase dependent inhibition of *Francisella* by *E. coli*

Inhibition of growth appeared to be dependent on the growth phase of *F. tularensis* subsp. type B strain LVS. *F. tularensis* subsp. type B strain LVS growth for 0 h and 4 h prior to spotting *E. coli* strain 25922 resulted in zones of *F. tularensis* subsp. type B strain LVS growth inhibition. Inhibition was seen at 6 h growth but was less than the 0 h and 4 h growth time points (Figure 3.7A). The growth phase dependent inhibition was also observed using the absorbance bioassay when agar-plug fluid was added to cultures of *F. tularensis* subsp. type B strain LVS cultures grown for 0, 5, and 10 h. Inhibition occurred when *F. tularensis* subsp. type B strain LVS was grown for 0 and 5 h, and no inhibition was observed when it was grown for 10 h prior to coming in contact with the *E. coli* strain 25922 agar-plug fluid (Figure 3.7B). No *E. coli* strain 25922 contaminating growth occurred with either of these assays. These data also suggest that the substance produced by *E. coli* strain 25922 is secreted, and is inhibitory and not cidal.

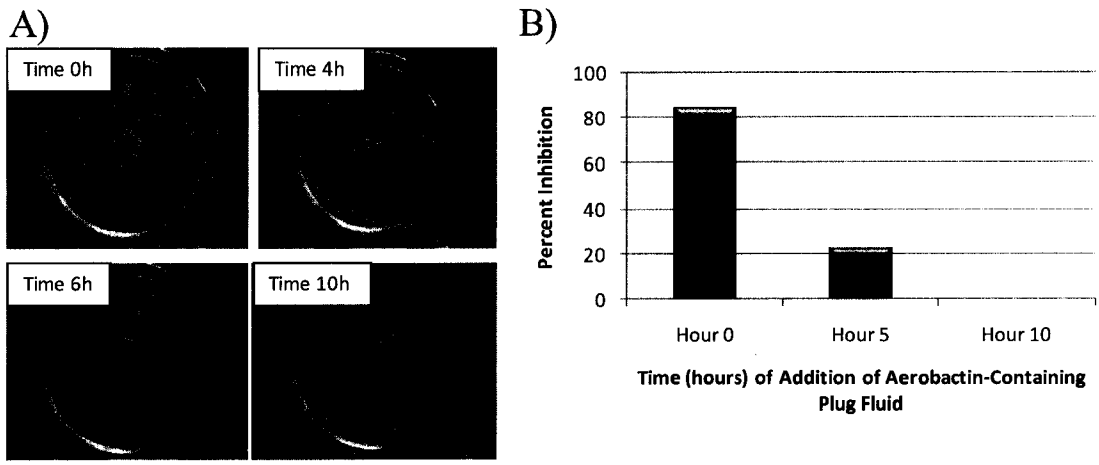


Figure 3.7 Inhibition of *F. tularensis* subsp. type B strain LVS by *E. coli* strain 25922. A) Inhibitory affect of spotting *E. coli* strain 25922 onto *F. tularensis* subsp. type B strain LVS lawns allowed to grow for 0, 4, 6 and 10 h. **B)** Percent Inhibition of *F. tularensis* subsp. type B strain LVS, measured using the absorbance bioassay, when aerobactin-containing agar-plug fluid is added after allowing *F. tularensis* subsp. type B strain LVS to grow for 0, 5 and 10 h.

3.3.3 Identification of a recombinant clone able to inhibit *F. tularensis* subsp. type B strain LVS growth

To identify the secreted *E. coli* strain 25922 product responsible for *F. tularensis* growth inhibition, a genetic approach was utilized that exploited the observation that *E. coli* strain EPI300^{TM}-T1 did not inhibit *F. tularensis* growth (Fig. 3.8). Phenotypic screening of *E. coli* strain EPI300^{TM}-T1 transduced with an *E. coli* strain 25922 genomic library identified a recombinant fosmid (pCC1FOS820) that conferred to *E. coli* strain EPI300^{TM}-T1 the ability to inhibit *F. tularensis* subsp. type B strain LVS growth (Figure 3.8). A total of 382 transductants were screened with three identified as conferring the ability to inhibit *F. tularensis*, of which only pCC1FOS820 was further analyzed.

pCC1FOS820 pCC1FOS820 Epi300™-T1

Figure 3.8 Identification of aerobactin as the *F. tularensis* growth inhibiting substance using a molecular approach. Phenotypic selection of inhibiting recombinant clone pCC1FOS820 using the co-plating spot assay (left), and comparison of phenotypes between the low-inhibiting host *E. coli* strain EPI300™-T1® and inhibiting recombinant clone pCC1FOS820 (right). All assays were performed using *F. tularensis* subsp. type B strain LVS.

Purification of the pCC1FOS820 fosmid and transformation back into *E. coli* strain EPI300™-T1 cells confirmed the inhibitory property was encoded by the fosmid. Using the direct broth assay, the percent inhibition of *F. tularensis* subsp. type B strain LVS by *E. coli* strain EPI300™-T1 containing pCC1FOS820 was determined to be 76.5% as compared to the percent inhibition shown by *E. coli* strain 25922 of 70.5% (Figure 3.7). Sequencing of the fosmid-insert DNA junctions of pCC1FOS820 revealed homology at both ends to sequences found within the pathogenicity island II carried by some *E. coli* strains (18). More specifically, one end was found to contain a sequence homologous to the gene *pap*A, and at the other end, a sequence homologous to a putative insertion sequence IS8-associated element found within the *E. coli* pathogenicity island II of strain Nissle 1917 (18). In *E. coli* strains documented as containing pathogenicity island II, this ~48 kb region identified as the size of the insert within pCC1FOS820, contained the genes essential for encoding aerobactin production and transport, along with approximately 31 additional genes (Figure 3.9).

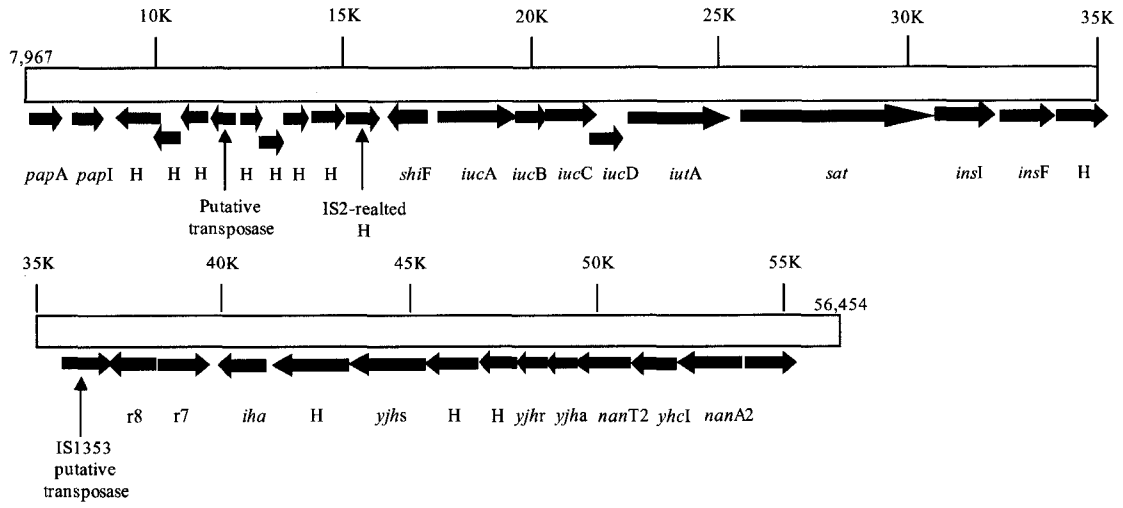


Figure 3.9 Predicted sequence map for cloned insert within pCC1FOS820. Sequence map of the *E. coli* strain 25922 DNA fragment cloned into *E. coli* strain EPI300™-T1® resulting in the clone designated pCC1FOS820. The cloned DNA fragment is approximately 48 kb in size with 35 genes present (green arrows), of which five genes constitute the aerobactin operon (red arrows).

3.3.4 Identification of genes responsible for inhibition of *F. tularensis* growth

Transposon mutagenesis of fosmid pCC1FOS820 resulted in 15 kanamycin resistant transformants out of 100 screened that did not inhibit growth of *F. tularensis* subsp. type B strain LVS. Co-plating *F. tularensis* subsp. type B strain LVS with the transposon mutants resulted in little to no inhibition of *F. tularensis* growth. A total of 188 mutagenized clones were screened and 30 of these were found to have lost the ability to inhibit *F. tularensis*. Of these 30, three (pCC1FOS820-11, pCC1FOS820-49, pCC1FOS820-241) were further analyzed and found to have 0% inhibition of *F. tularensis* subsp. type B strain LVS growth when tested using the direct broth assay (Fig. 3.10).

The three fosmids (pCC1FOS820-11, pCC1FOS820-41, and pCC1FOS820-241) were sequenced to determine the location of the transposon insertion (Fig 3.11). DNA sequencing outward from the EZ::TN<KAN-2>Tnp transposon revealed insertions occurred in genes essential for aerobactin biosynthesis. Insertions were present within *iucA* (clone pCC1FOS820-11), *iucB* (clone pCC1FOS820-49) and *iucC* (clone pCC1FOS820-241) (Figure 3.11).

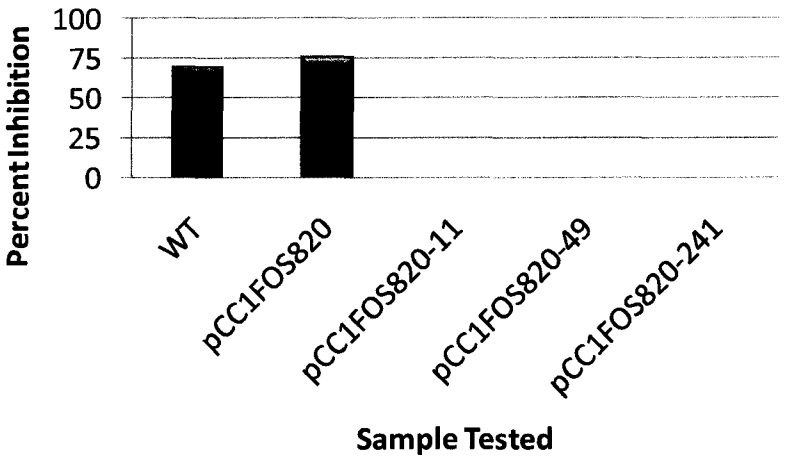


Figure 3.10 Percent inhibition of *E. coli* clone PCC1FOS820 and mutagenized clones. *F. tularensis* subsp. type B strain LVS growth inhibition by *E. coli* strain 25922 (70.5%), the recombinant clone pCC1FOS820 (76.5%) and mutagenized clones pCC1FOS820-11 (not detectable), pCC1FOS820-49 (not detectable), and pCC1FOS820-241 (not detectable).

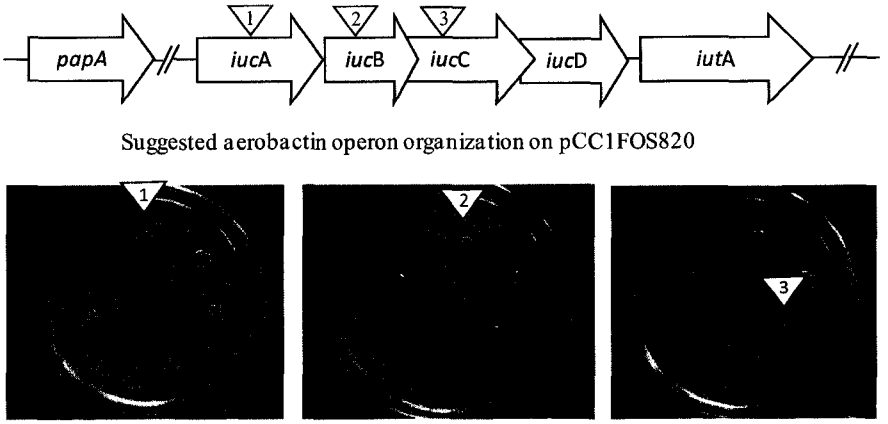


Figure 3.11 Transformants lacking the ability to inhibit *Francisella*. The locations of three transposon insertions leading to the loss of *F. tularensis* subsp. type B strain LVS growth inhibition are indicated by labeled triangles. The number 1 represents an insertion within *iucA* (clone pCC1FOS820-11), number 2 within *iucB* (clone pCC1FOS820-49) and number 3 within *iucC* (clone pCC1FOS820-241).

PCR amplification of the *iucA*, *iucB* and *iucC* genes from *E. coli* strain 25922 genomic DNA and the fosmid clone pCC1FOS820 DNA produced appropriately sized products of the three intact aerobactin genes, 1787 bp, 947 bp, and 1742 bp, corresponding to *iucA*, *iucB*, and *iucC* respectively. PCR amplification of these same genes from the mutagenized fosmids (pCC1FOS820-11, pCC1FOS820-49, and pCC1FOS820-241), confirmed the presence of a transposon (1 kb shift in product size) in each gene product (Figure 3.12).

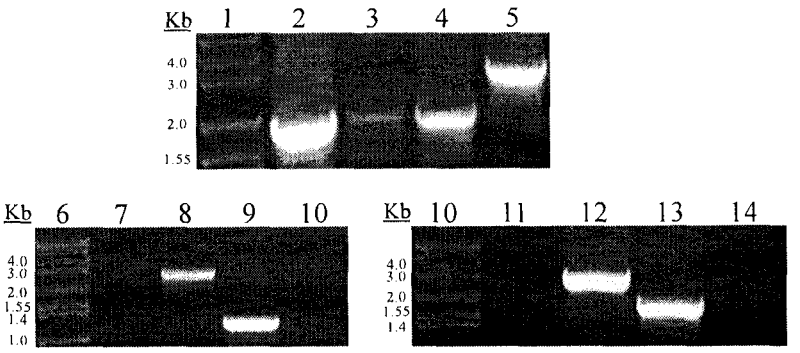


Figure 3.12 PCR amplification of mutagenized aerobactin genes. Lane 1: Hi-Lo DNA marker, **lane 2:** amplified *iucA* from *E. coli* strain 25922, **lane 3:** amplified *iucA* from *E. coli* strain EPI300^{TM}-T1, **lane 4:** *iucA* amplified from fosmid pCC1FOS820, **lane 5:** *iucA* amplified from fosmid pCC1FOS820-11, **lane 6:** Hi-Lo DNA marker, **lane 7:** blank, **lane 8:** *iucB* amplified from fosmid pCC1FOS820-49, **lane 9:** *iucB* amplified from *E. coli* strain 25922, **lane 10:** negative control, **lane 11:** Hi-Lo DNA marker, **lane 12:** *iucC* amplified from fosmid pCC1FOS820-241, **lane 13:** *iucC* amplified from *E. coli* strain 25922, **lane 14:** negative control.

Southern blot analysis of the mutagenized fosmids using the transposon as a probe, revealed that only one transposon was present within each of the three fosmids. These data suggest that inhibition of *F. tularensis* by *E. coli* strain 25922 was due to the production of aerobactin, and presumably sequestration of iron from *F. tularensis*.

3.3.5 Biochemical evidence of aerobactin involvement

To determine if the diffusible inhibitory substance present in the agar-plug fluid was aerobactin, chromatography for purification of aerobactin was applied to the *E. coli* agar-plug extracts (17). The supernatant fluid recovered from the CM Sepharose cation exchange matrix resulted in two fractions, CM1 and CM2. The first fraction, CM1, eluted with 20 mM potassium phosphate (pH 7.0) and was positive for inhibitory activity against *F. tularensis* subsp. type B strain LVS as measured by the direct broth bioassay. Fraction CM1 was positive in the ferric perchlorate assay and comparison with a standard curve indicated a hydroxamate concentration of 0.29 mM as compared to the crude agar-

plug fluid which contained 0.36 mM of hydroxamate (Figure 3.13). The second CM Sepharose fraction CM2, eluted with 0.6 M NaCl, was negative when tested in the absorbance bioassay. Size exclusion chromatography produced two fractions (fractions 2 and 3) out of six that were inhibitory against *F. tularensis* subsp. type B strain LVS and positive for hydroxamate showing concentrations of 0.21 mM and 0.16 mM for fractions 2 and 3 respectively by the ferric perchlorate assay (Figure 3.13).

Use of purified aerobactin to generate a standard curve for use in quantifying the results obtained from the ferric perchlorate assay showed that a minimum of 0.06 mM of aerobactin is needed to inhibit *F. tularensis* subsp. type B strain LVS growth, when performing the direct broth assay. The inhibitory effects of aerobactin were also overcome by supplementation of MHII agar with 10 μM ferric chloride. In comparison to medium not supplemented with ferric chloride, the zone of inhibition produced by *E. coli* strain 25922 was dramatically reduced (approximately 4-fold) on the supplemented medium (Figure 3.14). These biochemical observations support the genetic data and provide additional proof that the ability of *E. coli* strain 25922 to produce aerobactin results in the inhibition of *F. tularensis* growth when these two bacteria are co-cultured.

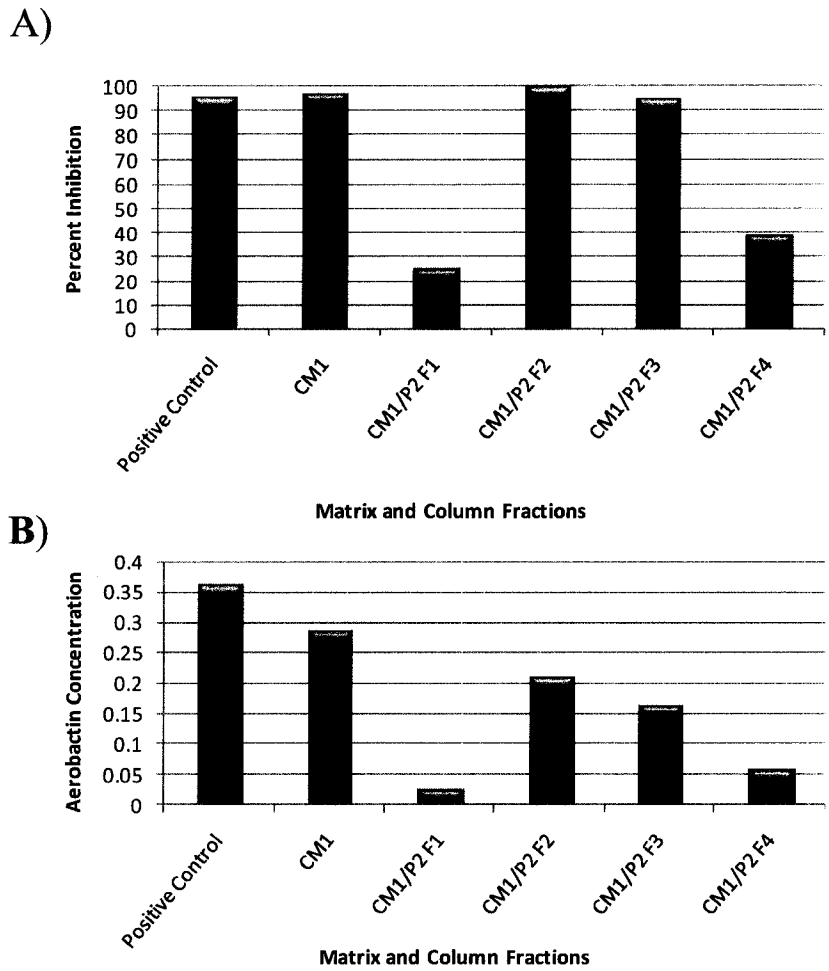


Figure 3.13 ***F. tularensis*** **subsp. type B strain LVS growth inhibition by partially purified aerobactin.** Correlation between amount of aerobactin present and the percent inhibition against *F. tularensis* subsp. type B strain LVS in column fractions from fluid collected in the first fraction recovered using CM Sepharose™ matrix (CM1) and the fractions collected from the first (CM1/P2 F1), second (CM1/P2 F2), third (CM1/P2 F3) and fourth (CM1/P2 F4) 1 ml fractions recovered after chromatography of CM1 fluid through Bio-Gel® P2 column. The positive control was *E. coli* strain 25922 agar-plug fluid extract. **A)** Percent *F. tularensis* subsp. type B strain LVS growth inhibition by matrix and column fractions. *F. tularensis* subsp. type B strain LVS growth inhibition by samples taken from the indicated fractions was determined using the absorbance assay. **B)** Estimated aerobactin concentrations (mM) in matrix and column fractions. A ferric perchlorate assay was used to track the presence of hydroxamate (indicative of the presence of aerobactin) in the same fractions.

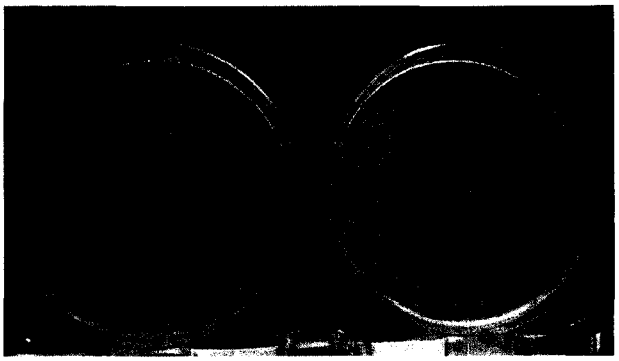


Figure 3.14 Reversal of inhibitory effect by *E. coli* strain 25922 on *F. tularensis* subsp. type b strain LVS by iron supplementation. *E. coli* strain 25922 (center) and *F. tularensis* subsp. type B strain LVS (lawns) were co-plated onto MHII with 1% isovitalex supplemented with either 10 μM $FeCl_3$ (right) or with no iron supplementation (left).

3.4 Discussion

In this study, we used a combination of genetic and biochemical methods to show that growth of *F. tularensis* is inhibited in the presence of aerobactin. Aerobactin is an iron-chelating molecule with specific ligands for Fe(III) that is produced by *E. coli* and other gram negative bacteria under iron-limiting conditions (30). Although aerobactin does not have the highest affinity for FeIII (K_f is 10^{23}) when compared to other siderophores produced by *E. coli* such as enterobactin (K_f is 10^{52}), its affinity for iron is sufficient to strip iron from transferrin, lactoferrin and ferritin (4, 19). Aerobactin is able to resist hydrolysis after it cycles iron into the cell and can also be recycled (31). These properties likely account for or enhance the accumulation of sufficient quantities of aerobactin in the agar medium. This makes it possible to extract this product from the agar, and causes inhibition of *F. tularensis*.

In *E. coli*, aerobactin is synthesized and transported by the gene products encoded in the aerobactin operon (*iucABCD* and *iutA*) (13). The aerobactin operon was contained in the fosmid insert which conferred the *F. tularensis* subsp. type B strain LVS growth

inhibiting phenotype to the non-inhibiting *E. coli* strain EPI300™-T1. Moreover, transposon insertions in three of the aerobactin biosynthetic genes *iucA, iucB*, and *iucC* resulted in the loss of the *F. tularensis* subsp. type B strain LVS growth inhibiting phenotype. The inhibition of *F. tularensis* when co-plated with *E. coli* strains 25922 and EPI300™-T1 transformed with the aerobactin operon appears to be a direct result of iron deprivation caused by the presence of aerobactin. It is possible that other *E. coli* products or activities could be attributing to the inhibition of *F. tularensis*, for example, products encoded for by the other genes present within the cloned fragment. In order to rule out the involvement of another product in the inhibition of *F. tularensis*, mutagenesis of each of the cloned genes within pCC1FOS820 would need to be tested for loss of *F. tularensis* inhibition. It is also unknown if either *E. coli* strains 25922 or EPI300 ™-T1 produce other siderophores and whether these are influencing the inhibition of *F. tularensis*.

Francisella also produces a siderophore that highly resembles rhizoferrin (41). Rhizoferrin, unlike aerobactin, is a polycarboxylate siderophore that is more efficient at a lower pH and has been determined to have a weaker affinity for iron when compared to aerobactin (7). *E. coli* has been determined to have the ability to use several types of exogenous siderophores, but has not been shown capable of using rhizoferrin, that is likely due to the lack of its specific receptor (22). Given that an outer membrane receptor for this rhizoferrin-like siderophore in *Francisella* has not been found, it would be interesting to see if this siderophore differs in its uptake mechanism from that of other bacteria, and additionally, if other bacteria are capable of using the *F. tularensis* siiderophore. If so, this would suggest an extensive competitive environment for *F. tularensis*. It may also provide new hypotheses as to why the two clinically relevant *F.*

tularensis subspecies are not found in the environment (outside of finding *F. tularensis* subsp. type B in water) as compared to the other subspecies of *F. tularensis.* The subspecies that are found in the environment, particularly *F. tularensis* subsp. *novicida*, have not undergone the extensive genome reduction as *F. tularensis* subspp. type A and type B have. *F. tularensis* subsp. *novicida* has few pseudogenes (a total of fourteen) implying the presence of a higher number of systems available for adaptability, which may include iron uptake mechanisms. *F. tularensis* subspp. type A and type B have a large number of pseudogenes, 201 and 325 respectively, which results in lower adaptability and the requirement of a specialized niche for survival (23, 35, 37). It would be interesting to compare the protein profiles for the different subspecies grown under iron-limited conditions as well as to see if the different subspecies vary in their ability to grow in the presence of siderophore-producing bacteria. Both Deng *et al.* (15) and Lenco *et al.* (24) have performed proteomic analyses of *F. tularensis* subsp. type B strain LVS, but a comparison across all subspecies has not been done. Whether *Francisella* can use exogenous siderophores is unknown and investigation of this question might provide some answers to the mysteries surrounding *Francisella* and its mechanisms for iron uptake.

The inability of *F. tularensis* to grow in the presence of an aerobactin-producing *E. coli* strain suggests the inability of *F. tularensis* to compete and thrive within an environment where aerobactin or other iron-chelator producing organisms with higher affinities for iron are present. In the laboratory, difficulties in culturing *F. tularensis* from environmental and animal samples have been encountered due to its slow growth rate *in vitro* and the overgrowth by other organisms. When attempting to culture *F.*

tularensis from tissues of animals thought to have had tularemia (PCR positive), three bacterial species - *Pseudomonas*, *Proteus* and *Staphylococcus* - were predominantly recovered while *F. tularensis* was not unless CHAB supplemented with antibiotics that inhibit these bacteria was used (34). All of these bacteria produce aerobactin, suggesting that recovery of *Francisella* species may be hindered by iron-limitation (6, 8, 25). When growing *F. tularensis in vitro*, supplementation with iron greatly enhances the growth of this bacterium. This suggests that iron is either required in greater quantities by this bacterium in comparison to levels required by other bacteria that grow on the same medium (without supplementation of iron), or more likely, the mechanisms for iron uptake used by *F. tularensis* are not as efficient as those present in these other bacteria. The information gained through this project does give us insight into the sensitivity of *F. tularensis* under iron limitation and could be useful for development of a medium that would enhance the culturing of this bacterium in the presence of other bacteria that may be inhibiting its growth. The addition of different iron sources and testing this hypothesis may greatly improve culturing of this bacterium. The finding that *F. tularensis* is more susceptible to aerobactin in its early growth stages also suggests an essential iron requirement in its initial growth phase. It would be interesting to evaluate gene expression profiles of *F. tularensis* under iron deprivation and normal growth conditions at different growth stages of this bacterium to determine which genes are expressed early on and how iron limitation affects the expression of these genes over time. It is possible that the reason why *F. tularensis* is not inhibited in later growth stages is due to the storage of iron during early growth stages that can be utilized later in the presence of aerobactin, or it may be that *F. tularensis* needs time to produce its own siderophore and

that in later growth stages enough of this molecule is present or able of being synthesized to compete with aerobactin or any iron-limiting environment. Measurement of *F. tularensis* siderophore production under iron-limiting ad normal growth conditions over time may provide input for answering this question. Additionally, experiments to evaluate the potential of aerobactin to inhibit across both *Francisella* species, as well as the four *F. tularensis* subspecies would be interesting.

An additional finding of this study was the production of aerobactin by *E. coli* strain ATCC 25922, a commonly used reference for various studies, including standardization of antibiotic susceptibility testing (32, 43). To our knowledge, this strain has not been documented as a siderophore-producing *E. coli*. Our studies show that when this bacterium is grown using a MHII-based medium aerobactin is actively synthesized. Additionally, this finding should be taken into account when choosing strains for genetic manipulation of *F. tularensis*, specifically for conjugative purposes.

Tularemia is an interesting disease that is typified by sudden outbreaks over a lengthy period and in which no predictors for the occurrence of disease are available (6). Whether *F. tularensis*' passive existence is due to an unknown reservoir or to other underlying environmental factors is yet to be determined. This bacterium has a broad-host range and is also known to be vector-borne. Thus, the lack of higher disease prevalence is paradoxical with the broad-host range and known virulence of *F. tularensis*. One limitation that may account for this paradox is the inability to accurately detect this bacterium in the environment. Thus, a better understanding of *F. tularensis* physiology and nutritional requirements should not only aid in the study of pathogenesis, but also allow for the development of tools to better assess the true prevalence of *F. tularensis*.

It is necessary to mention that identification of *Francisella* genes affected by iron limitation and the identification of the siderophore produced by *Francisella* were not known until well after this project was completed. Although we now know that *Francisella* produces a siderophore, the conditions for its production are not fully understood and alternate mechanisms for iron uptake for *Francisella* have not been described. *Francisella* may likely have a novel mechanism that is unrecognizable, and further research in this area is needed to better understand how *Francisella* acquires iron.

The primary purpose for this project was to test our hypothesis that the inhibitory substance produced by *E. coli* could be used as a reagent in a diagnostic test for *Francisella*. The approach taken was to characterize and identify the inhibitory substance instead of addressing our hypothesis directly. The initial question and concern should have been to ask if the inhibitory substance meets the criteria for use as a reagent in a *Francisella* diagnostic. To do so, specificity of the inhibitory substance for *Francisella* should have been evaluated. Addressing this question may have revealed that the inhibitory substance was likely not specific for *Francisella* and therefore does not meet the criteria for a diagnostic reagent. Even though we were unable to prove our hypothesis, the results and data gathered from this study generated interesting questions regarding the growth and survival of this bacterium in the environment and provides a hypothesis as to why we are unable to grow and culture this organism when it is present with other bacteria. Further work to better understand the strengths and weaknesses of this bacterium in relation to its environment are critical to understanding the camouflaged ability of this bacterium to unpredictably cause disease.

3.5 Literature Cited

1. **Atkin, C. L., J. B. Neilands, and H. J. Phaff.** 1970. Rhodotorulic acid from species of *Leucosporidium, Rhodosporidium, Rhodotorula, Sporidiobolus*, and *Sporobolomyces*, and a new alanine-containing ferrichrome from Cryptococcus melibiosum. J Bacteriol **103:**722-33.
2. **Bagg, A., and J. B. Neilands.** 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol Rev **51:**509-18.
3. **Bhatnagar, N. B., K. L. Elkins, and A. H. Fortier.** 1995. Heat stress alters the virulence of a rifampin-resistant mutant of *Francisella tularensis* LVS. Infect Immun **63:**154-9.
4. **Boukhalfa, H., and A. L. Crumbliss.** 2002. Chemical aspects of siderophore mediated iron transport. Biometals **15:**325-39.
5. **Braun, V., and R. Burkhardt.** 1982. Regulation of the ColV plasmid-determined iron (III)-aerobactin transport system in *Escherichia coli.* J Bacteriol **152:**223-31.
6. **Buyer, J. S., V. de Lorenzo, and J. B. Neilands.** 1991. Production of the siderophore aerobactin by a halophilic pseudomonad. Appl Environ Microbiol **57:**2246-50.
7. **Carrano, C. J., H. Drechsel, D. Kaiser, G. Jung, B. Matzanke, G. Winkelmann, N. Rochel, and A. M. Albrecht-Gary.** 1996. Coordination Chemistry of the Carboxylate Type Siderophore Rhizoferrin: The Iron(III) Complex and Its Metal Analogs. Inorg Chem **35:**6429-6436.
8. **Cercenado, E., F. Baquero, A. Delgado-Iribarren, and J. L. Martinez.** 1986. Epidemiology of aerobactin production in *Enterobacteriaceae*. Ann Inst Pasteur Microbiol **137B:**297-303.
9. **Clarke, T. E., L. W. Tari, and H. J. Vogel.** 2001. Structural biology of bacterial iron uptake systems. Curr Top Med Chem **1:**7-30.
10. **Colonna, B., M. Nicoletti, P. Visca, M. Casalino, P. Valenti, and F. Maimone.** 1985. Composite IS1 elements encoding hydroxamate-mediated iron uptake in FIme plasmids from epidemic *Salmonella* spp. J Bacteriol **162:**307-16.
11. **Crichton, R. R., and M. Charloteaux-Wauters.** 1987. Iron transport and storage. Eur J Biochem **164:**485-506.
12. **Crosa, J. H.** 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol Rev **53:**517-30.
13. **de Lorenzo, V., A. Bindereif, B. H. Paw, and J. B. Neilands.** 1986. Aerobactin biosynthesis and transport genes of plasmid ColV-K30 in *Escherichia coli* K-12. J Bacteriol **165:**570-8.
14. **de Lorenzo, V., and J. B. Neilands.** 1986. Characterization of *iucA* and *iucC* genes of the aerobactin system of plasmid ColV-K30 in Escherichia coli. J Bacteriol **167:**350-5.
15. **Deng, K., R. J. Blick, W. Liu, and E. J. Hansen.** 2006. Identification of *Francisella tularensis* genes affected by iron limitation. Infect Immun **74:**4224-36.

16. **Fortier, A. H., D. A. Leiby, R. B. Narayanan, E. Asafoadjei, R. M. Crawford, C. A. Nacy, and M. S. Meltzer.** 1995. Growth of *Francisella tularensis* LVS in macrophages: the acidic intracellular compartment provides essential iron required for growth. Infect Immun **63:**1478-83.
17. **Gibson, F., and D. I. Magrath.** 1969. The isolation and characterization of a hydroxamic acid (aerobactin) formed by *Aerobacter aerogenes* 62-I. Biochim Biophys Acta **192:**175-84.
18. **Grozdanov, L., C. Raasch, J. Schulze, U. Sonnenborn, G. Gottschalk, J. Hacker, and U. Dobrindt.** 2004. Analysis of the genome structure of the nonpathogenic probiotic *Escherichia coli* strain Nissle 1917. J Bacteriol **186:**5432-41.
19. **Harris, W. R., C. J. Carrano, and K. N. Raymond.** 1979. Coordination chemistry of microbial iron transport compounds. 16. Isolation, characterization, and formation constants of ferric aerobactin. J Am Chem Soc **101:**2722-2727.
20. **Hoffman, L. M., J. J. Jendrisak, R. J. Meis, I. Y. Goryshin, and S. W. Reznikof.** 2000. Transposome insertional mutagenesis and direct sequencing of microbial genomes. Genetica **108:**19-24.
21. **Ishimaru, C. A., and J. E. Loper.** 1992. High-affinity iron uptake systems present in *Erwinia carotovora* subsp. *carotovora* include the hydroxamate siderophore aerobactin. J Bacteriol **174:**2993-3003.
22. **Kuhn, S., V. Braun, and W. Koster.** 1996. Ferric rhizoferrin uptake into *Morganella morganii:* characterization of genes involved in the uptake of a polyhydroxycarboxylate siderophore. J Bacteriol **178:**496-504.
23. **Larsson, P., P. C. Oyston, P. Chain, M. C. Chu, M. Duffield, H. H. Fuxelius, E. Garcia, G. Halltorp, D. Johansson, K. E. Isherwood, P. D. Karp, E. Larsson, Y. Liu, S. Michell, J. Prior, R. Prior, S. Malfatti, A. Sjostedt, K. Svensson, N. Thompson, L. Vergez, J. K. Wagg, B. W. Wren, L. E. Lindler, S. G. Andersson, M. Forsman, and R. W. Titball.** 2005. The complete genome sequence of *Francisella tularensis,* the causative agent of tularemia. Nat Genet **37:**153-9.
24. **Lenco, J., M. Hubalek, P. Larsson, A. Fucikova, M. Brychta, A. Macela, and J. Stulik.** 2007. Proteomics analysis of the *Francisella tularensis* LVS response to iron restriction: induction of the *F. tularensis* pathogenicity island proteins IglABC. FEMS Microbiol Lett **269:**11-21.
25. **Lisiecki, P., B. Tkacz, M. Sobis, and J. Mikucki.** 1994. The occurrence of siderophores in staphylococci. Acta Microbiol Pol **43:**21-31.
26. **Martinez, J. L., M. Herrero, and V. de Lorenzo.** 1994. The organization of intercistronic regions of the aerobactin operon of pColV-K30 may account for the differential expression of the *iucABCD iutA* genes. J Mol Biol **238:**288-93.
27. **McDougall, S., and J. B. Neilands.** 1984. Plasmid- and chromosome-coded aerobactin synthesis in enteric bacteria: insertion sequences flank operon in plasmid-mediated systems. J Bacteriol **159:**300-5.
28. **Nassif, X., and P. J. Sansonetti.** 1986. Correlation of the virulence of *Klebsiella pneumoniae* K1 and K2 with the presence of a plasmid encoding aerobactin. Infect Immun **54:**603-8.

29. **Neilands, J. B.** 1981. Iron absorption and transport in microorganisms. Annu Rev Nutr **1:**27-46.
30. **Neilands, J. B.** 1981. Microbial iron compounds. Annu Rev Biochem **50:**715-31.
31. **O'Brien, I. G., G. B. Cox, and F. Gibson.** 1971. Enterochelin hydrolysis and iron metabolism in *Escherichia coli*. Biochim Biophys Acta **237:**537-49.
32. **Odland, B. A., M. E. Erwin, and R. N. Jones.** 2000. Quality control guidelines for disk diffusion and broth microdilution antimicrobial susceptibility tests with seven drugs for veterinary applications. J Clin Microbiol **38:**453-5.
33. **Payne, S. M., D. W. Niesel, S. S. Peixotto, and K. M. Lawlor.** 1983. Expression of hydroxamate and phenolate siderophores by *Shigella flexneri*. J Bacteriol **155:**949-55.
34. **Petersen, J. M., M. E. Schriefer, K. L. Gage, J. A. Montenieri, L. G. Carter, M. Stanley, and M. C. Chu.** 2004. Methods for enhanced culture recovery of *Francisella tularensis*. Appl Environ Microbiol **70:**3733-5.
35. **Petrosino, J. F., Q. Xiang, S. E. Karpathy, H. Jiang, S. Yerrapragada, Y. Liu, J. Gioia, L. Hemphill, A. Gonzalez, T. M. Raghavan, A. Uzman, G. E. Fox, S. Highlander, M. Reichard, R. J. Morton, K. D. Clinkenbeard, and G. M. Weinstock.** 2006. Chromosome rearrangement and diversification of *Francisella tularensis* revealed by the type B (OSU18) genome sequence. J Bacteriol **188:**6977-85.
36. **Ratledge, C., and L. G. Dover.** 2000. Iron metabolism in pathogenic bacteria. Annu Rev Microbiol **54:**881-941.
37. **Rohmer, L., C. Fong, S. Abmayr, M. Wasnick, T. J. Larson Freeman, M. Radey, T. Guina, K. Svensson, H. S. Hayden, M. Jacobs, L. A. Gallagher, C. Manoil, R. K. Ernst, B. Drees, D. Buckley, E. Haugen, D. Bovee, Y. Zhou, J. Chang, R. Levy, R. Lim, W. Gillett, D. Guenthener, A. Kang, S. A. Shaffer, G. Taylor, J. Chen, B. Gallis, D. A. D'Argenio, M. Forsman, M. V. Olson, D. R. Goodlett, R. Kaul, S. I. Miller, and M. J. Brittnacher.** 2007. Comparison of *Francisella tularensis* genomes reveals evolutionary events associated with the emergence of human pathogenic strains. Genome Biol **8:**R102.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.
39. **Schaible, U. E., and S. H. Kaufmann.** 2004. Iron and microbial infection. Nat Rev Microbiol **2:**946-53.
40. **Stuart, S. J., J. K. Prpic, and R. M. Robins-Browne.** 1986. Production of aerobactin by some species of the genus *Yersinia*. J Bacteriol **166:**1131-3.
41. **Sullivan, J. T., E. F. Jeffery, J. D. Shannon, and G. Ramakrishnan.** 2006. Characterization of the siderophore of *Francisella tularensis* and role of *fslA* in siderophore production. J Bacteriol **188:**3785-95.
42. **Van Tiel-Menkveld, G. J., J. M. Mentjox-Vervuurt, B. Oudega, and F. K. de Graaf.** 1982. Siderophore production by *Enterobacter cloacae* and a common receptor protein for the uptake of aerobactin and cloacin DF13. J Bacteriol **150:**490-7.

43. **Vila, J., M. Vargas, C. Casals, H. Urassa, H. Mshinda, D. Schellemberg, and J. Gascon.** 1999. Antimicrobial resistance of diarrheagenic *Escherichia coli* isolated from children under the age of 5 years from Ifakara, Tanzania. Antimicrob Agents Chemother **43:**3022-4.
44. **Wandersman, C., and P. Delepelaire.** 2004. Bacterial iron sources: from siderophores to hemophores. Annu Rev Microbiol **58:**611-47.
45. **Warner, P. J., P. H. Williams, A. Bindereif, and J. B. Neilands.** 1981. ColV plasmid-specific aerobactin synthesis by invasive strains of *Escherichia coli.* Infect Immun **33:**540-5.
46. **Weinberg, E. D.** 1978. Iron and infection. Microbiol Rev **42:**45-66.
47. **Williams, P. H.** 1979. Novel iron uptake system specified by ColV plasmids: an important component in the virulence of invasive strains of *Escherichia coli.* Infect Immun **26:**925-32.

Chapter IV

Genomic Markers for Differentiation of *F. tularensis* Type A Subpopulations

4.1 Introduction

F. tularensis is divided into four subspecies (*tularensis*, *holarctica*, *mediasiatica* and *novicida*) that differ with respect to geographic location, virulence, and biochemical properties (18). Two subspecies, *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B) are of clinical relevance, with *F. tularensis* subsp. type A causing the more severe forms of tularemia (7). Human tularemia infections caused by *F. tularensis* subsp. type A have only been documented in North America. Recent studies have further divided *F. tularensis* subsp. type A isolates into two subpopulations, A.I and A.II (8, 12, 19).

Separation of *F. tularensis* subsp. type A into two subpopulations was first reported in a 2004 study conducted by Johansson *et al.* in which 45 diverse *F. tularensis* subsp. type A strains grouped into two distinct clusters by MLVA using 25 loci (12). In 2005, Farlow *et al.* typed 161 North American *F. tularensis* isolates (*F. tularensis* subspp. type A, type B, and *novicida*) from the United States using a 24-marker system (8). Phylogenetic analyses resulted in four major genetic groups, one for *F. tularensis* subsp. type B, one for subsp. *novicida*, and two for *F. tularensis* subsp. type A corresponding to the two subpopulations A.I and A.II. Additionally, distinct geographic distribution of subpopulation A.I and A.II isolates was also reported. Subpopulation A.I

isolates were primarily found in the eastern and central United States (exceptions were isolates collected from Alaska, British Columbia and California), and subpopulation A.II isolates were primarily collected from the western United States (exceptions were isolates collected in Ontario and Texas).

The authors propose several abiotic and biotic factors as reasons for the differences in geographic distributions for subpopulation A.I and A.II isolates. Among these factors are known vectors and hosts for *F. tularensis* subsp. type A. Subpopulation A.I was found to be closely associated with the distribution of the vectors *Amblyomma americanum* (Lone Star tick) and *Dermacentor variabilis* (American dog tick), while subpopulation A.II was found in regions primarily containing the vectors *Dermacent andersoni* (Rocky Mountain wood tick) and *Chrysops discalis* (deer fly). Different rabbit hosts were also found to be associated with the two subpopulations, with *Sylvilagus floridanus* dominating areas where A.I subpopulation isolates cause tularemia and *Sylvilagus nuttallii* dominating areas where A.II subpopulation isolates cause tularemia. In addition, a significant difference was found between the elevation in areas from which A.I and A.II subpopulation isolates were recovered.

In 2006, Staples *et al.* conducted epidemiologic comparisons and molecular analyses on isolates associated with human tularemia that occurred within the United States from 1964-2004 (19). Through PFGE, this study also identified two subpopulations for *F. tularensis* subsp. type A isolates. The two main clusters of *F. tularensis* subsp. type A isolates correlated with the geographic location from which the isolates were collected. Isolates from one cluster were primarily isolated from states completely west of the 100th meridian and were named type A-west, while isolates from

the second cluster were primarily isolated from states transecting or east of the 100th meridian and were named type A-east. Additional epidemiological analyses revealed a significant difference in case fatality rates between infections caused by subpopulations A-west and A-east. A case fatality rate of 0% (0/55) was found for type A-west in comparison to a case fatality rate of 14% (15/106) found for type A-east infections. A significant difference was additionally found regarding the age between patients infected with type A-west and type A-east, with a median age of 33 years and 44 years respectively. When the anatomical sources of the isolates were examined, type A-east isolates were recovered more frequently from blood and lung while type A-west were recovered more frequently from lymph nodes. A difference in transmission was also found, with biting flies being associated with type A-west infections and not type A-east infections. Biting flies accounted for 55% of the arthropod-associated infections caused by type A-west. Comparative analysis of this study with the work by Farlow *et al.* demonstrates that subpopulation A.I is equivalent to type A-east and subpopulation A.II is equivalent to type A-west. A.I/A.II terminology will be used to describe *F. tularensis* subsp. type A subpopulations in this study.

Suppression subtractive hybridization (SSH) is a method used to perform whole genome comparisons. SSH was originally developed to study gene expression in eukaryotes and was later modified by Akopyants *et al.* for application in bacterial genomes for identification of strain-specific DNAs (1). SSH is particularly useful for comparisons when genomes of the strains being compared have not been fully sequenced. Past studies have utilized this technique to compare closely related strains that differ in virulence, thus identifying unique regions associated with virulence factors (25). In

addition to identification of virulence factors, unique sequences identified through SSH have been used for the development of diagnostic and differentiation assays (2, 26).

We hypothesized that genomic differences exist between A.I and A.II subpopulations, and can be used to develop a diagnostic assay for differentiation of subpopulations A.I and A.II. Additionally, it is possible that the genomic differences will lead to identification of virulence factors linked to the differential disease outcome associated with infections caused by the two subpopulations. This hypothesis was tested using SSH to identify regions of difference (RDs) between subpopulations A.I and A.II that were subsequently characterized using bioinformatic analyses.

4.2 Materials and Methods

4.2.1 Bacterial strains, culture conditions and DNA isolation

F. tularensis strains were grown from frozen stocks on CHAB at 35°C for 48 h, followed by subculture onto CHAB for 24 h at 35°C. Genomic DNA was isolated from each *F. tularensis* strain using the PureGene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions with minor modifications. Specifically, bacterial cells were harvested from 24 h agar plates and suspended in cell suspension buffer (100 mM Tris, 100 mM EDTA, at pH 8.0). The cell suspension was adjusted to a turbidity of 0.7 using a MicroScan turbidity meter (Dade Behring, Deerfield, IL) and 500 µl of the cell suspension was used for DNA isolation.

4.2.2 Suppression subtractive hybridization

Genomic SSH (Figure 4.1) was performed using the PCR-Select Bacterial Genome Subtraction Kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's instructions, with minor modifications. Specifically, tester and driver

DNA were digested with *Alu*I. In the first subtraction, DNA of *F. tularensis* subsp. type A strain MA00-2987 (A.I) was used as the tester and DNA of *F. tularensis* subsp. type A strain WY96-3418 (A.II) as the driver. The initial hybridization step was performed for 2.25 h at 58°C and the second hybridization was performed at 58°C overnight. For the reverse subtraction, DNA of *F. tularensis* subsp. type A WY96-3418 (A.II) minus DNA of *F. tularensis* subsp. type A MA00-2987 (A.I), the initial hybridization step was carried out for 2.25 h at 60°C and the second hybridization was performed at 60°C overnight. PCR amplification of control genes *dnaK*, *rpoH*, and 23S was conducted using the primer sets: dnaK-F and dnaK-R, 23S-F and 23S-R, and rpoH-F and rpoH-R (Table 4.1). Tester-specific fragments were amplified by both primary and nested PCR.

Table 4.1 Sequences of oligonucleotide primers.

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
dnaK-F	TATCATTGTGAAGATCTATACC	RD-12F	TCAATGGTGGACACAAGGTG
dnaK-R	AAGATGCTGGTAAAATAGCAG	RD-12R	TTAGCGCCTTGGTATGTAAC
23S-F	TAAATGGCGAACAGCCCATACC	RD-13F	TGGGGTAATTATACCTTCAGC
23S-R	ACGCCAGTAGTTGTGGAGTCG	RD-13R	AAATCCAAATATTGCCGATGC
rpoH-F	AGCTAGATCCTGAAGAGTTGC	RD-14F	AGATACAATGGTAAGTATGTC
rpoH-R	TAGTGTTCAAAGAAGAAACTGTC	RD-14R	AGCCAAAATACTATCAATTATG
RD-1F	TAGCAGAGTTGGATGATTATTC	RD-15F	TCATCAGCAGGGATACTAAC
RD-1R	TGTTTGAGCATCTCCTTCAAAG	RD-15R	ATAGCCAATAAATTTGATGATC
RD-2F	AGGTCCTAATATTTGCCAATC	RD-16F	TTTGGATACGGTTGGTATAG
RD-2R	TGAACCAATAGATCTAGAATTC	RD-16R	TCGAACTCCGTCATACCTTC
RD-3F	ATATATGGTGATGCTAAAGAC	RD-17F	AACATTGATTGAACAGTATTTG
RD-3R	TCTATTGAGATCTAAATCTGC	RD-17R	TATTTGTTGTTCTTGGTGTTG
RD-4F	TGGTCAAGAAATTAGACTTTTG	RDR-5F	AAATCAAGATGTTTTGCTATAAG
RD-4R	ATATTTATGTACTTGTGTTCAAG	RDR-5R	TATTTCTAAGTCTTTCCAAGC
RD-5F	TACTTGAGAATGATAGTAAATAC	RDR-14F	AAGAACTTCTCTGCTAAGATC
RD-5R	ATATTATCAAAATGTAATTTACTG	RDR-14R	TAGATACAATGGTAAGTATGTC
RD-6F	TGACCTGAGCCAGAGGTTAC	TN-1F	TGTCTTGGTGAAGAATGCTAAC
RD-6R	ACTTGCCAGCCTAATAATTAC	TN-1R	TACTTTACTATCATGAGTTTTATC
RD-7F	TAATTGGTATCAAATCTGGAG	TN-2F	TATGATTCAACAAGGTAGATAC
RD-7R	AGATAGCCACTCTCTTCAAG	TN-2R	TTTGTGCACAGAACCTAGAC
RD-8F	TAACGGAATCTATGAGTGTG	TN-3F	TCAGTTGGTTAGAGTACTATC
RD-8R	AAGAATCCTGCATATATCGC	TN-3R	TTGACCAACGAGTTTCACAC
RD-9F	TGGGGTAATTATACCTTCAGC	TN-4F	TATACTAAGGTGTATGTTTTAC
RD-9R	AAATCCAAATATTGCCGATGC	TN-4R	TTATCCCTTGTAAATCAAATAC
RD-10F	AAATCATTGATTGAGTCTATTC	TN-5F	TTGTACTTTTATTTGGTTACGA
RD-10R	TCCTCCTTGATAACCAACTG	TN-5R	AACTCCGTCATACCTTCTTC
RD-11F	AGAAATAATGTTTGAATCTTCC	RD-10J-F[a]	ATCATTGATTGGAAATCAAAAATTG
RD-11R	ACAGTACCATCACTCTTAAC	RD-10J-R	ATCCTGATATTCCTCCTAACACAA

[a] Base pair changes are indicated in bold.

Post-subtraction methods are diagramed in Figure 4.2. Nested PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI) and transformed into competent DH5α cells (Invitrogen, Carlsbad, CA). Transformants were screened on LB medium supplemented with ampicillin (100 μg/ml), X-gal, and IPTG. For each subtraction, 100 β-galactosidase$^-$/Amp^R clones were picked for a total of 200 clones. Clones were grown overnight in 5 ml of LB broth supplemented with ampicillin (100 μg/ml). Plasmid isolation was performed using the Qiagen mini prep kit (Qiagen Inc., Valencia, CA). The DNA was digested with *Bst*ZI and electrophoresed on a 1.5% agarose gel to check for the presence of an insert. Inserts were sequenced by Macromolecular Resources (Colorado State University, Fort Collins, Colorado) using SP6 and T7 primers and an ABI 3130 Genetic Analyzer. BLAST analysis (blastn) of the cloned fragment was performed using the NCBI website (http://www.ncbi.nlm.nih.gov/).

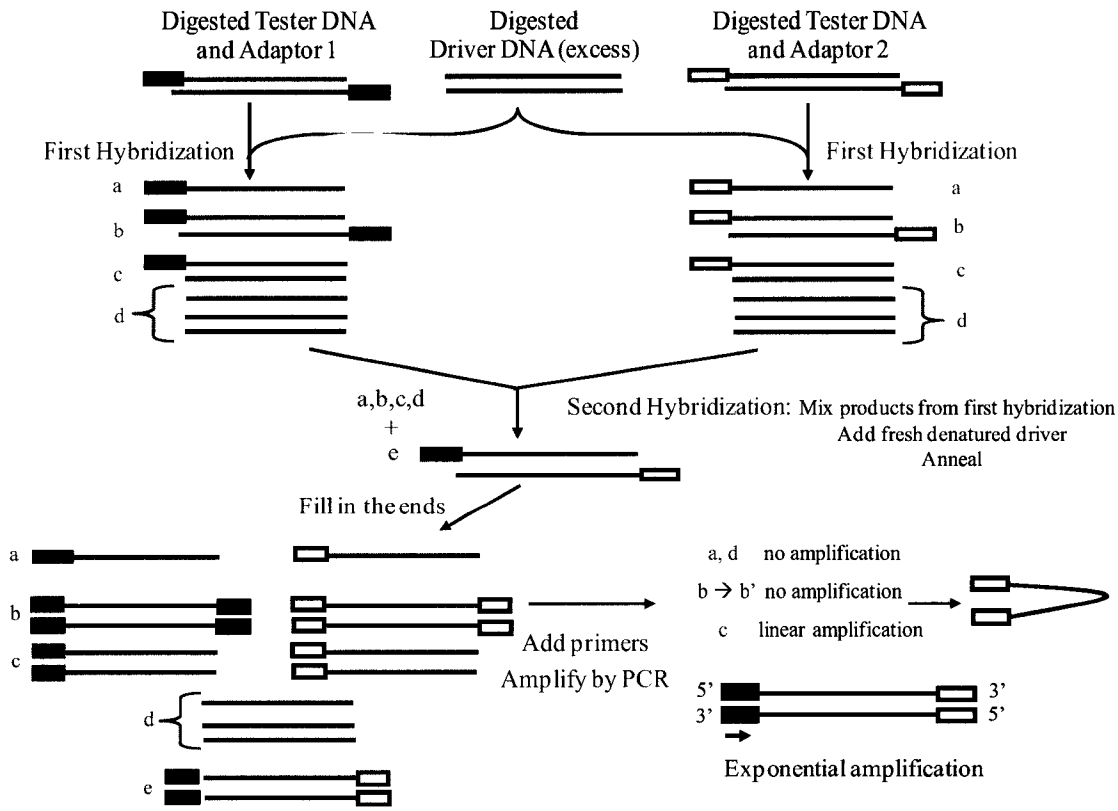


Fig 4.1 Suppression subtractive hybridization. The first step in suppression subtractive hybridization was to digest both tester and driver DNA with an enzyme that yields fragment sizes smaller than 1 kb. Tester DNA was then ligated in two separate reactions to adaptor 1 and adaptor 2. The first hybridization occurred when excess digested driver DNA was mixed in separate reactions with each of the tester-adaptor ligation reactions. The samples were heat denatured and allowed to anneal. The products from the first hybridization were mixed together and were not heat denatured. Fresh denatured driver DNA was added and allowed to anneal in a second hybridization. The ends of all products were filled in with DNA polymerase. PCR using primers that anneal to the adaptor ends exponentially amplified double stranded tester-specific DNA that had both adaptors. A second PCR was performed using nested primers for background reduction and further enrichment of tester-specific sequences.

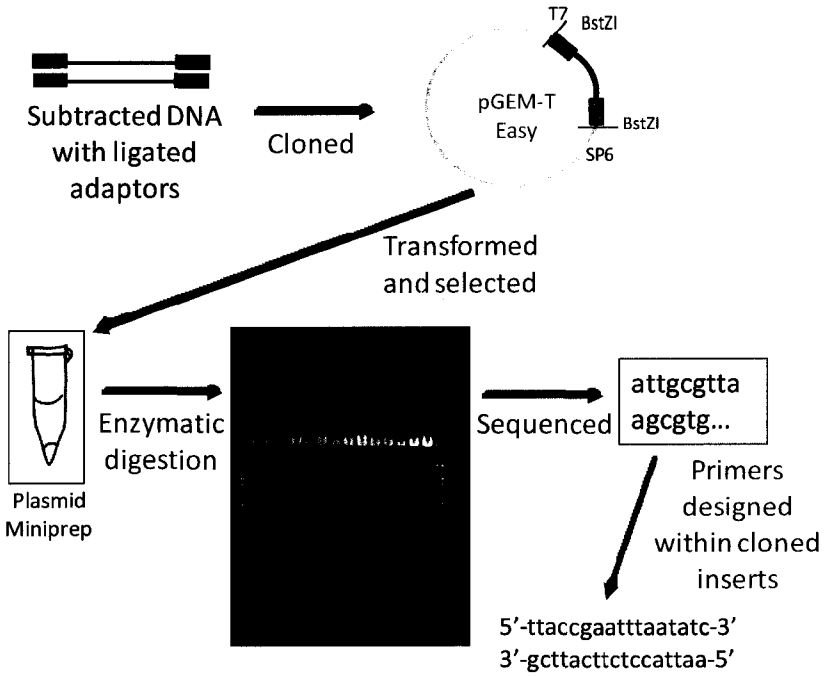


Figure 4.2 Post subtraction methods. Subtractive hybridization products were cloned into the vector pGEM-T Easy. Positive clones were selected using ampicillin resistance and blue/white screening. Cultures were grown and plasmid DNA was isolated. BstZI digests were performed and clones containing an insert were sequenced. Sequence data was used to design primers within the cloned insert.

4.2.3 PCR for validation of RDs and subpopulation A.I/A.II differentiation

Primers were designed within the cloned sequenced inserts (Table 4.1). For each PCR reaction, 50 ng of template DNA (see Table 4.3 for strains used) and 1.2 μM of each primer were used with PureTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK). PCR conditions consisted of 95°C for 5 min, followed by 30 cycles of 95°C for 45 sec, annealing temperature of 51-55°C for 30 sec, and 72°C for 45 sec. Positive (tester-specific fragments cloned into pGEM-T Easy vector) and negative (no DNA template) controls were included in each run. Amplified fragments were electrophoresed using a 1.5% agarose gel followed by staining with ethidium bromide and visualization using a Gel Doc XR imager (Bio-Rad Laboratories, Hercules, CA).

The multiplex assay was performed with PureTaq Ready-To-Go PCR beads, 2.5 mM $MgCl_2$ and 1 μM of each primer for RD-3 and RD-6 (Table 4.1). The PCR conditions were the same as above except for the annealing temperature of 53°C for 45 sec. The A.I-specific PCR was performed with primers RD-10J-F and RD-10J-R (Table 4.1) as above except for an annealing temperature of 55°C for 45 sec. PCR reactions for both assays were electrophoresed using precast 3.0% agarose gels with ethidium bromide (Bio-Rad Laboratories, Hercules, CA).

4.2.4 Genome mapping, and bioinformatic analysis of predicted proteins and genes

RDs were mapped using the whole genome sequences of *F. tularensis* subsp. type A strains SCHU S4 (AJ749949) and WY96-3418 (NC_009257), *F. tularensis* subsp. type B strains LVS (AM233362) and OSU18 (NC_008369), and *F. tularensis* subsp. *novicida* strain U112 (NC_008601) available through NCBI (http://www.ncbi.nlm.nih.gov/) with blastn analysis (http://www.ncbi.nlm.nih.gov/). Sequence alignments were also performed using LALIGN (www.ch.embnet.org/software/LALIGN_form.html) and ClustalW (www.ebi.ac.uk/clustalw/#). Protein homologues were found using blastp analysis ((http://www.ncbi.nlm.nih.gov/).

Bioinformatic programs including PSORT, a prediction program of protein subcellular localization (http://www.psort.org/psortb/index.html); SignalP, a signal peptidase cleavage site prediction program (http://www.cbs.dtu.dk/services/SignalP/); TMHMM program, for prediction of transmembrane helices in proteins (http://www.cbs.dtu.dk/services/TMHMM-2.0/); Pfam HMM search program that scans a sequence against the Pfam protein families database (http://www.sanger.ac.uk /Software/Pfam/search.shtml); and BPROM (http://www.softberry.com/berry.phtml

?topic=bprom& group=programs&subgroup=gfindb), a prediction program for gram-negative bacterial promoters were used to analyze proteins annotated within RDs and any homologues within all genomes available. Translation of DNA sequence to protein sequence for pseudogenes was performed using the ExPASy translate tool (http://www.expasy.org/tools/dna.html). Additional BLAST analyses were also performed on hypothetical proteins using blastn and blastp against the non-redundant database.

Junction regions, consisting of 30 nucleotides taken in both directions at each of the two RD junctions, were aligned using the local alignment method of the alignment program lalign.

4.3 Results

4.3.1 Subtractive hybridization

To generate DNA fragment sizes of $\leq$ 1kb, the restriction enzyme *Alu*I, a four-base, blunt-end restriction enzyme was used in place of *Rsa*I which generated DNA fragment sizes > 1 kb. The efficiency of subtraction was verified by PCR amplification of three control genes (23S, *rpoH*, and *dnaK*) present in both the tester and driver genomic DNA (Figure 4.2). A marked reduction of PCR product for two of the three control genes was considered efficient subtraction (Figure 4.3). The subtraction of subpopulation A.II from subpopulation A.I worked efficiently with an annealing temperature of 58°C, whereas the reverse subtraction (A.I from A.II) was most effective with 60°C as the annealing temperature.

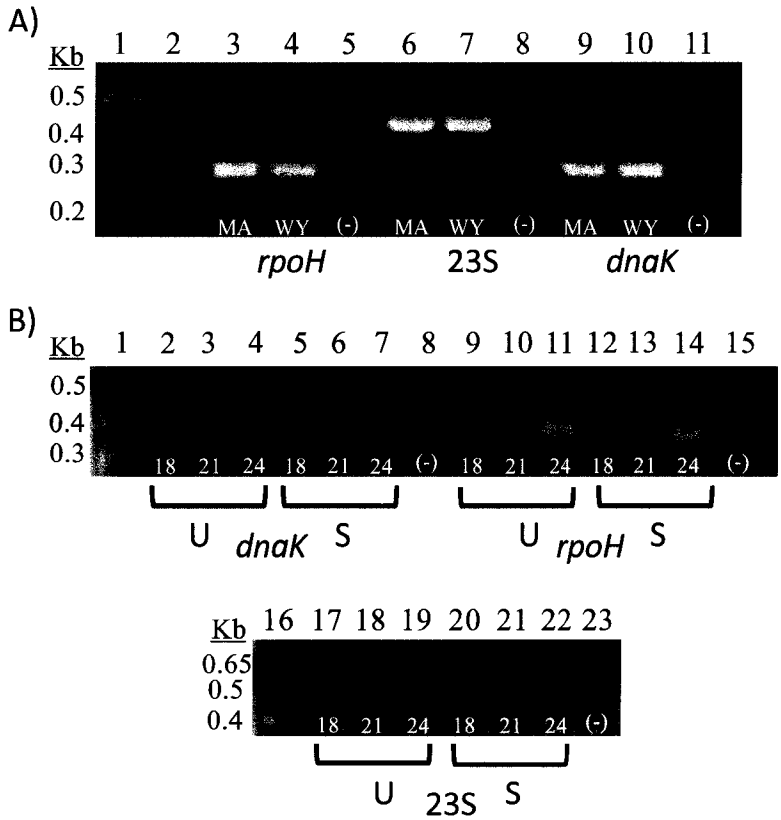


Figure 4.3 Subtraction efficiency using control genes *dnaK*, *rpoH* and 23S. A) PCR amplification of control genes using *F. tularensis* subsp. type A DNA from strains MA00-2987 DNA and WY96-3418, and no DNA for the negative PCR control. **Lane 1**: 1 kb molecular weight ladder, **lane 2:** blank, **lane 3**: MA00-2987 using primer set rpoH, **lane 4**: WY96-3418 using primer set rpoH, **lane 5**: negative PCR control using primer set rpoH, **lane 6**: MA00-2987 using primer set 23S, **lane 7**: WY96-3418 using primer set 23S, **lane 8**: negative PCR control using primer set 23S, **lane 9**: MA00-2987 using primer set dnaK, **lane 10**: WY96-3418 using primer set dnaK, **lane 11**: negative PCR control using primer set 23S. **B)** Subtraction hybridization controls for A.I minus A.II. **Lane 1**: 1 kb molecular weight ladder, **lane 2:** unsubtracted dnaK PCR at 18 cycles, **lane 3**: unsubtracted dnaK PCR at 21 cycles, **lane 4**: unsubtracted dnaK PCR at 24 cycles, **lane 5**: subtracted dnaK PCR at 18 cycles, **lane 6**: subtracted dnaK PCR at 21 cycles, **lane 7**: subtracted dnaK PCR at 24 cycles, **lane 8:**negative PCR control for dnaK, **lane 9**: unsubtracted rpoH PCR at 18 cycles, **lane 10**: unsubtracted rpoH PCR at 21 cycles, **lane 11**: unsubtracted rpoH PCR at 24 cycles, **lane 12**: subtracted rpoH PCR at 18 cycles, **lane 13**: subtracted rpoH PCR at 21 cycles, **lane 14**: subtracted rpoH PCR at 24 cycles, **lane 15**: negative PCR control for rpoH, **lane 16:** 1 kb molecular weight ladder, **lane 17**: unsubtracted 23S PCR at 18 cycles, **lane 18**: unsubtracted 23S PCR at 21 cycles, **lane 19**: unsubtracted 23S PCR at 24 cycles, **lane 20**: subtracted 23S PCR at 18 cycles, **lane 21**: subtracted 23S PCR at 21 cycles, **lane 22**: subtracted 23S PCR at 24 cycles, **lane 23**: negative PCR control for 23S. **U** represents unsubtracted PCR amplified samples and **S** represents subtracted PCR amplified samples.

Of the resulting β-galactosidase$^-$/Amp^R *E. coli* clones transformed with tester-specific DNA, 100 clones were randomly selected from both subtractions (A.I-A.II and A.II-A.I), and plasmid DNA recovered. To verify the presence of tester-specific DNA inserts, plasmids were digested with *Bst*ZI, and those with an insert (137 clones) were sequenced (Figure 4.2) and internal primers designed (Table 4.1). PCR with the internal primers and DNA from *F. tularensis* subsp. type A strains MA00-2987 (A.I) and WY96-3418 (A.II) identified 24 regions of difference (RD) (Table 4.2). In this work an RD is defined as a genomic region present in one subpopulation and absent from the other subpopulation.

Table 4.2 Summary of suppression subtractive hybridization experiments.

Number of β-galactosidase$^-$ /Amp^R clones digested		Number of Amp^R clones with insert screened by PCR		Number of RDs identified (minus IS-element associated)		Number of RDs panel verified (minus IS-element associated and repeated RDs)		Insert size range (bp)	RD size range (bp)	Genes with putative function associated with RDs (may include intergenic region)	Hypothetical proteins associated with RDs	RDs only within an intergenic regions	RDs associated with IS elements	RDs identified more than once
S1[a]	S2[b]	S1	S2	A.I	A.II	A.I	A.II							
100	100	77	60	9	10	5	8	202-768	15-1181	6	9	1	5	2

[a] S1 represents SSH A.I-A.II.

[b] S2 represents SSH A.II-A.I.

Of these 24 RDs, 12 (RD-2, RD-3, RD-7, RD-8, RD-11, RD-14, RD-16, RD-17, RDR-14, TN-1, TN-2, and TN-4) were present in *F. tularensis* subsp. type A strain MA00-2987 (A.I), and another 12 (RD-1, RD-4, RD-5, RD-6, RD-9, RD-10, RD-12, RD-13, RD-15, RDR-5, TN-3, and TN-5) were present in *F. tularensis* subsp. type A WY96-3418 (A.II). It should be noted that primers used to define RD-1, RD-2, RD-7, RD-8, RD-9, RD-11, RD-12, RD-14, RD-16, RD-17, RDR-14, TN-2, and TN-4 yielded PCR products from both subpopulation A.I and A.II genomic DNA, but with different

amplicon sizes. Thus, in some cases one or both of the internal primers fell within the RD present only in one subpopulation, resulting in the amplification of a product from only the subpopulation containing the RD (Figure 4.9) and in other cases the internal primers flanked the RD absent from one subpopulation (Figure 4.5) resulting in amplified product in both subpopulations that differ in size. In the cases where there was a difference in product size, both PCR products were cloned and sequenced.

4.3.2 Verification of regions of difference

To ensure that the 24 RDs represented conserved differences among either subpopulation A.I or A.II strains, nine A.I and nine A.II clinical strains (Table 4.3) from diverse geographical locations were PCR amplified using the internal primer sets (Table 4.1). All strains on the panel were identified as either subpopulation A.I or A.II by PFGE molecular subtyping. In addition to the subpopulation A.I and A.II panel of strains, DNA from three *F. tularensis* subsp. type B strains and three *F. tularensis* subsp. *novicida* strains were also PCR amplified.

Of the 24 RDs, 19 (RD-1 to RD-13, RDR-5, and TN-1 to TN-5) were conserved among the panel of nine A.I and nine A.II subpopulation strains (Tables 4.2). The five non-conserved RDs (RD-14, RD-15, RD-16, RD-17, and RDR-14) were dropped from further analysis. Figure 4.4 shows the results of an RD (RD-17) that did not panel verify.

Table 4.3 *Francisella* strains used in this study.

	CDC Accession Number	Anatomic Source[a]	State of Exposure
F. tularensis subsp. *tularensis* subpopulation A.I	MA00-2987[b]	Blood	Massachusetts
	AR99-3448	Exudate	Arkansas
	M002-1911	Node	Missouri
	NE03-1457	Pleural Fluid	Nebraska
	NY04-2565	Pleural Fluid	New York
	OK01-2528	Wound	Oklahoma
	SCHU S4	Ulcer	Ohio
	SD00-3147	Wound	South Dakota
	VA00-1000	Blood	Virginia
F. tularensis subsp. *tularensis* subpopulation A.II	WY96-3418[c]	Wound	Wyoming
	AZ01-4999	Wound	Arizona
	CA02-0099	Wound	California
	GA02-5453	Node	Wyoming
	ID04-2687	Unknown	Oregon
	UT02-1927	Wound	Utah
	WY01-3847	Blood	Wyoming
	WY01-3911	Unknown	Wyoming
	WY03-1228	Eye	Wyoming
F. tularensis subsp. *holarctica* (type B)	KY99-3387	Wound	Kentucky
	LVS	-	Russia
	OR96-0246	-	Oregon
F. tularensis subsp. *novicida*	GA99-3548	Node	Louisiana
	GA99-3549	Blood	California
	GA99-3550 (U112)	-	Utah

[a] Anatomic source given for human isolates.

[b] A.I strain used as both tester and driver for SSH.

[c] A.II strain used as both tester and driver for SSH.

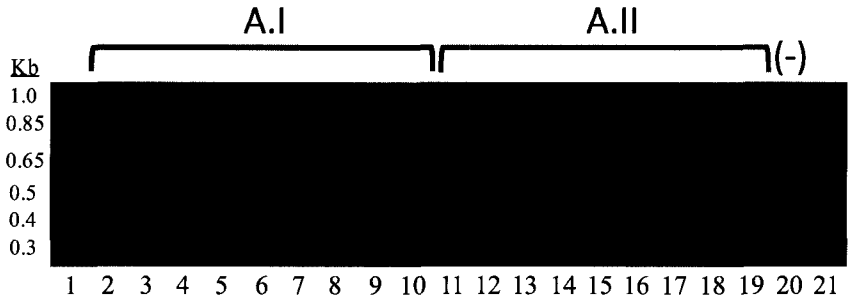


Figure 4.4 RD-17, a non-conserved RD. Panel verification of RD-17 illustrating an RD that was conserved among subpopulation A.I strains, but was not conserved among subpopulation A.II strains. **Lane 1**: 1 kb molecular weight ladder, **lane 2**: NE03-1457, **lane 3**: MA00-2987, **lane 4**: Schu S4, **lane 5**: OK01-2528, **lane 6**: SD00-3147, **lane 7**: NY04-2526, **lane 8**: VA00-1000, **lane 9**: M002-1911, **lane 10**: AR99-3448, **lane 11**: WY01-3847, **lane 12**: WY96-3418, **lane 13**: GA02-5453, **lane 14**: ID04-2687, **lane 15**: WY03-1228, **lane 16**: CA02-0099, **lane 17**: AZ01-4999, **lane 18**: UT02-1927, **lane 19**: WY01-3911, **lane 20**: negative control, **lane 21**: 1kb molecular weight ladder.

4.3.3 Mapping of the 19 regions of difference to the *F. tularensis* genome

To further verify the PCR results and to aid in selection of diagnostic markers, the RDs were mapped via BLAST analyses (blastn and blastp) to whole genome sequences of *F. tularensis* subsp. type A strains SCHU S4 (A.I) and WY96-3418 (A.II), *F. tularensis* subsp. type B strains LVS and OSU18 (type B), and *F. tularensis* subsp. *novicida* U112. Mapping RDs to the different *Francisella* genomes also helped resolve differences in annotation between genomes (Table 4.4).

One of the 19 verified RDs was identified more than once (RD-5/RDR-5), five were associated with insertion sequence (IS) elements (TN-1 through TN-5), 12 were gene/pseudogene-associated (RD-1,RD-2, RD-3, RD-4, RD-5, RD-6, RD-8, RD-9, RD-10, RD-11, RD-12, and RD-13), and one mapped to an intergenic region (RD-7) (Table 4.2).

Table 4.4 Mapped genome location of the 13 non-IS element associated conserved RDs.

RD	Size of mapped RD (bp)[a]	RD present within *F. tularensis*				Genome location of RD (bp)				
		A.I	A.II	B[b]	N[c]	SCHU S4 (A.I)	WY96-3418 (A.II)	LVS (type B)	OSU18 (type B)	U112 (*novicida*)
RD-1	380	-	+	+	+	-	1715859-1716238	153148-153527	152826-153205	155315-155697
RD-2	148	+	-	+	+	1822217-1822364	-	1808260-1808407	1807894-1808041	1836762-1836908
RD-3	1181	+	-	+	+	1210749-1211929	-	742203-743383	745833-747013	1242339-1243519
RD-4	674	-	+	+	+	-	855388-856061	1166893-1167576	1170844-1171527	917978-918657
RD-5	310	-	+	+	+	-	427831-428140	617645-617954	621331-621640	1478605-1478914
RD-6	138	-	+	+	+	-	1622469-1622606	1251432-1251569	1254923-1255060	276175-276312
RD-7	45	+	-	+	+	866395-866439	-	329314-329358	330896-330940	383249-383293
RD-8	264	+	-	+	+	100785-101048	-	1693922-1694185	1693435-1693698	1724735-1724472
RD-9	16	-	+	+	+	-	130959-130944	1748784-1748799	1748414-1748429	1780284-1780299
RD-10	33	-	+	+	+	-	1274365-1274397	309305-309337	310886-310918	351043-351075
RD-11	15	+	-	+	-	1089471-1089485	-	1069151-1069165	1073057-1073071	-
RD-12	179	-	+	-	+	-	1431642-1431464	-	-	661139-661317
RD-13	246	-	+	+	+	-	301503-301748	1619237-1619475	1618751-1618989	323557-323802

[a] The size corresponding to strains SCHU S4 (A.I) or WY96-3418 (A.II) of *F. tularensis* subsp. *tularensis* is given.

[b] B represents both *F. tularensis* subsp. *holarctica* (type B) genomes, strains LVS and OSU18.

[c] N represents *F. tularensis* subsp. *novicida* U112.

Of the non-IS element associated RDS, five (RD-2, RD-3, RD-7, RD-8, RD-11) were present in subpopulation A.I and eight (RD-1, RD-4, RD-5, RD-6, RD-9, RD-10, RD-12, and RD-13) were present in subpopulation A.II. The size of these 13 RDs ranged from 15 bp to 1181 bp (Tables 4.2, and 4.4). The predicted genes associated with these 13 RDs for subpopulations A.I and A.II are shown in Table 4.5 and for their homologues in other *F. tularensis* subspecies in Table 4.6.

Table 4.5 Predicted proteins associated with RDs in *F. tularensis* subsp. type A strains SCHU S4 (A.I) and WY96-3418 (A.II).

RD	Accession number(s) within RD in Schu S4	Identification of RD in Schu S4	Accession number(s) within RD in WY96-3418	Identification of RD in WY-96-3418
RD-1	-	-	FTW_1826	hypothetical protein
RD-2	FTT1733	hypothetical protein	-	-
RD-3	FTT1194c/ FTT1195c	conserved hypothetical lipoprotein, intergenic region, conserved hypothetical protein (pseudogene)	-	-
RD-4	-	-	FTW_0888/ FTW_0889	intergenic region, hypothetical protein, hypothetical protein
RD-5	-	-	FTW_0430/ FTW_0431	hypothetical protein, intergenic region, putative methyltransferase (pseudogene)
RD-6	-	-	FTW_1709	C4-dicarboxylate anaerobic carrier (pseudogene)
RD-7	-	intergenic region	-	-
RD-8	FTT0096	hypothetical protein (similarity to a putative biotin dependent carboxylase)	-	-
RD-9	-	-	FTW_0121	magnesium chelatase (pseudogene)
RD-10	-	-	FTW_1356	DNA processing protein DprA
RD-11	FTT1078c	conserved hypothetical protein (similarity to FrpA/C)	-	-
RD-12	-	-	FTW_2084	chitinase family 18 protein (pseudogene)
RD-13	-	-	FTW_0298	putative drug transporter (pseudogene)

Table 4.6 Predicted proteins associated with RDs and their homologues in other *F. tularensis* subspecies.

RD	Accession number(s) within RD in LVS	Identification of RD in LVS	Accession number(s) within RD in OSU18	Identification of RD in OSU18	Accession number(s) within RD in *novicida* U112	Identification of RD in *novicida* U112
RD-1	FTL_0147	hypothetical protein	FTH_0140	conserved hypothetical protein (pseudogene)	FTN_0142	hypothetical protein
RD-2	-	intergenic region	FTH_1802	hypothetical protein	FTN_1712	hypothetical protein
RD-3	FTL_0750/ FTL_0751	conserved hypothetical protein (pseudogene), intergenic region, conserved hypothetical lipoprotein	FTH_0752/ FTH_0753	conserved hypothetical protein (pseudogene), intergenic region, conserved hypothetical protein	FTN_1171/ FTN_1172	hypothetical protein, hypothetical protein
RD-4	FTL_1217	hypothetical protein, intergenic region	FTH_1195	conserved hypothetical protein, intergenic region	FTN_0864	intergenic region, hypothetical protein
RD-5	FTL_0628/ FTL_0629	methyltransferase (pseudogene), conserved hypothetical protein (pseudogene)	FTH_0631/ FTH_0632	probable methyltransferase, intergenic region, hypothetical protein	FTN_1399/ FTN_1400	hypothetical protein, intergenic region, S-adenosylmethionine-dependent methyltransferase
RD-6	FTL_1313	short-chain fatty acid transporter	FTH_1284	S-tranferase (pseudogene)	FTN_0269	C4-dicarboxylate anaerobic carrier
RD-7	-	intergenic region	-	intergenic region	-	intergenic region
RD-8	FTL_1759	hypothetical protein with similarity to a putative biotin dependent carboxylase	FTH_1699	hypothetical protein with similarity to a putative biotin dependent carboxylase	FTN_1615	hypothetical protein with similarity to a putative biotin dependent carboxylase
RD-9	FTL_1814	chelatase family protein (pseudogene)	FTH_1750	ComM (pseudogene)	FTN_1665	magnesium chelatase
RD-10	-	intergenic region	FTH_0322	nucleotide-binding DNA uptake protein (pseudogene)	FTN_0345	DNA uptake protein, SMF family
RD-11	FTL_1123	conserved hypothetical protein with similarity to FrpA/C	FTH_1097	hypothetical protein with similarity to FrpA/C	-	-
RD-12	-	-	-	-	FTN_0627	ChiA
RD-13	FTL_1685	major facilitator superfamily transport protein	FTH_1624	MFS family major facilitator transporter	FTN_0312	drug:H+ antiporter-1 (DHA1) family protein

4.3.4 Analysis of gene and intergenic differences between subpopulations A.I and A.II for the conserved, non-IS element associated RDs.

Bioinformatic analyses were performed using the software programs PSORT, SigP, TMHMM and Pfam to predict protein localization, signal sequences, transmembrane helix domains, and protein families for hypothetical proteins encoded within the RDs. When present, the corresponding homologues within other genomes were also analyzed using these programs. The RDs within intergenic regions were analyzed using the promoter prediction program BPROM. The analysis for each of the 13 conserved, non-IS element associated RDs is summarized below.

Detailed description of RD-1

RD-1 is present in subpopulation A.II and absent from subpopulation A.I (Figure 4.5). RD-1 was determined to fall within a gene predicted to encode for a hypothetical protein (FTW_1826) (Table 4.7). FTW_1826 is a 512 amino acid protein predicted to contain an N-terminal signal sequence, one transmembrane helix domain and to localize to the outer membrane. The absence of RD-1 in subpopulation A.I causes an in-frame deletion of 380 bp, deleting the N-terminal signal sequence and shifting the start codon for this hypothetical protein in subpopulation A.I. The subpopulation A.I homologue (FTT0267) is predicted to encode for a 372 amino acid protein (Figure 4.6). RD-1 is also present in *F. tularensis* subspp . type B and *novicida* (Figure 4.5). The signal sequence is conserved within *F. tularensis* subsp. type B strains OSU18, LVS and *F. tularensis* subsp. *novicida* U112, however, *F. tularensis* subsp. type B strain OSU18 is truncated to 335 amino acids as compared to both *F. tularensis* subsp. type B strain LVS (512 amino acids) and *F. tularensis* subsp. *novicida* strain U112 (513 amino acids) (Table 4.8).

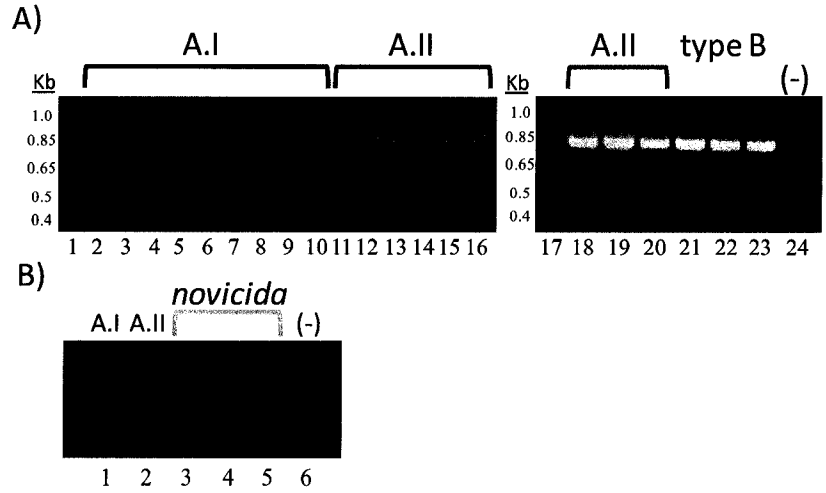


Figure 4.5 Agarose gel of PCR amplified *F. tularensis* strains using RD-1 primer set. A) PCR amplification of *F. tularensis* subpopulations A.I, A.II and *F. tularensis* subsp. type B strains. **Lane 1:** 1 kb molecular weight ladder, **lanes 2-10:** panel of A.I strains, **lanes 11-20:** panel of A.II strains, **lanes 21-23:** panel of type B strains, **lane 24:** negative control. **B)** PCR amplification of MA00-2987 (A.I), WY96-3418 (A.II) and panel of *novicida* strains.

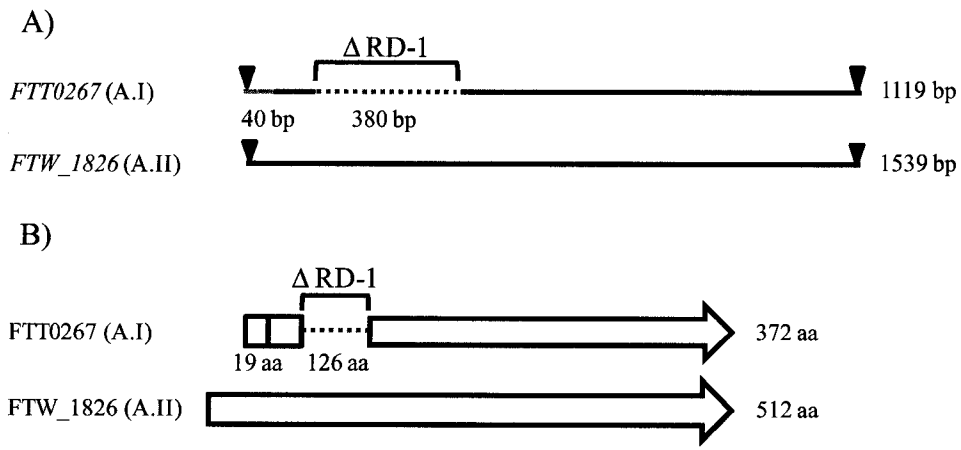


Figure 4.6 Alignment of subpopulations A.I and A.II illustrating the effects of RD-1. A) Nucleotide alignment of regions encoding for homologous proteins *FTT0267* in subpopulation A.I and *FTW_1826* in subpopulation A.II. Green line represents nucleotide sequence that is out of frame in comparison to the rest of the sequence; black line represents homologous sequence between subpopulations A.I and A.II; dashed red line represents the absence of RD-1 in subpopulation A.I; blue triangles represent start codons; red triangles represent stop codons. **B)** Amino acid sequence alignment of homologous proteins FTT0267 (A.I) and FTW_1826 (A.II) represented by arrows. Yellow represents amino acid sequence that differs between the two proteins. Dashed red line represents the amino acids that are absent from A.I. Blue represents homology between the two sequences.

Table 4.7 Summary of BLAST analyses for RD-1 gene *FTW_1826.*

BLAST Program	Description	ORF	E-value	Percent Identity	Percent Query coverage	Positives
blastn	*F. tularensis* subsp. *tularensis* WY96-3418	-	0	100	100	-
	F. tularensis subsp. *holarctica* LVS	-	0	99	100	-
	F. tularensis subsp. *novicida* U112	-	0	99	100	-
	F. tularensis subsp. *holarctica* OSU18	-	0	99	99	-
	F. tularensis subsp. *tularensis* FSC198	-	0	99	75	-
	F. tularensis subsp. *tularensis* FSC198	-	3.00E-39	98		-
	F. tularensis subsp. *tularensis* SCHU S4	-	0	99	75	-
	F. tularensis subsp. *tularensis* SCHU S4	-	3.00E-39	98		-
blastp	*F. tularensis* subsp. *tularensis* WY96-3418	FTW_1826	0	100	-	512/512
	F. tularensis subsp. *novicida* U112	FTN_0142	0	99	-	511/513
	F. tularensis subsp. *holarctica* LVS	FTL_0147	0	99	-	508/512
	F. tularensis subsp. *holarctica* 257	FTHG_00154	0	99	-	508/512
	F. tularensis subsp. *tularensis* SCHU S4	FTT0267	6.E-178	99	-	354/354
	F. tularensis subsp. *tularensis* FSC198	FTF0267	6.E-178	99	-	354/354
	F. tularensis subsp. *novicida* GA99-3548	FTDG_00036	5.E-115	52	-	324/461
	F. tularensis subsp. *novicida* U112	FTN_0444	6.E-114	51	-	323/461
	F. tularensis subsp. *tularensis* SCHU S4	FTT0918	7.E-114	51	-	319/461
	F. tularensis subsp. *novicida* GA99-3549	FTCG_01635	2.E-113	51	-	322/461
	F. tularensis subsp. *holarctica* 257	FTHG_00411	4.E-113	51	-	321/461
	F. tularensis subsp. *tularensis* WY96-3418	FTW_1261	8.E-113	51	-	320/461
	F. tularensis subsp. *holarctica* OSU18	FTH_0431	8.E-113	51	-	320/462
	F. tularensis subsp. *tularensis* FSC198	FTF0918	4.E-109	47	-	314/452
	F. tularensis subsp. *novicida* GA99-3548	FTDG_01243	3.E-109	48	-	345/509
	F. tularensis subsp. *novicida* U112	FTN_1686	4.E-109	47	-	345/509
	F. tularensis subsp. *novicida* GA99-3549	FTCG_01685	1.E-107	47	-	343/509
	F. tularensis subsp. *holarctica* LVS	FTL_1836	5.E-107	47	-	343/509
	F. tularensis subsp. *holarctica* OSU18	FTH_1772	5.E-107	47	-	343/510
	F. tularensis subsp. *tularensis* WY96-3418	FTW_0100	7.E-107	47	-	343/509
	F. tularensis subsp. *tularensis* SCHU S4	FTT0025c	1.E-106	47	-	343/509
	F. tularensis subsp. *tularensis* FSC198	FTF0025c	1.E-106	47	-	343/509
	F. tularensis subsp. *novicida* GA99-3548	FTDG_00655	7.E-100	46	-	310/477
	F. tularensis subsp. *holarctica* LVS	FTL_0439	2.E-93	48	-	297/464
	F. tularensis subsp. *novicida* GA99-3548	FTDG_00037	9.E-84	49	-	258/387
	F. tularensis subsp. *novicida* U112	FTN_0445	9.E-84	49	-	258/357
	F. tularensis subsp. *novicida* GA99-3549	FTCG_01636	9.E-84	42	-	308/516
	F. tularensis subsp. *tularensis* SCHU S4	FTT0919	1.E-83	49	-	258/357
	F. tularensis subsp. *tularensis* FSC198	FTF0919	1.E-83	49	-	258/357
	F. tularensis subsp. *holarctica* OSU18	FTH_0432	2.E-83	49	-	260/388
	F. tularensis subsp. *holarctica* 257	FTHG_00412	2.E-83	49	-	260/388
	F. tularensis subsp. *tularensis* WY96-3418	FTW_1260	2.E-83	48	-	258/387
	F. tularensis subsp. *holarctica* LVS	FTL_0867	3.E-82	45	-	267/423
	F. tularensis subsp. *holarctica* OSU18	FTH_0856	3.E-82	45	-	267/423
	F. tularensis subsp. *holarctica* 257	FTHG_00806	4.E-82	45	-	267/423
	F. tularensis subsp. *tularensis* SCHU S4	FTT0602c	6.E-82	45	-	266/423
	F. tularensis subsp. *tularensis* FSC198	FTF0602c	8.E-82	45	-	266/423
	F. tularensis subsp. *tularensis* WY96-3418	FTW_1127	1.E-81	45	-	266/423

Table 4.8 Summary of bioinformatic analyses for RD-1 associated proteins and truncated homologue of subpopulation A.I.

	F. tularensis Strains	ORF	Number of Amino Acids	Pseudogene	PSORT	SignalP Analysis	TMHMM Analysis	Pfam Analysis
Proteins associated with RD region	WY96-3418 (A.II)	FTW_1826	512	-	OM[a]	1(1-23)[b]	1(7-25)	NP[c]
	OSU18 (type B)	FTH_0140	335	+	U[d]	1(1-23)	1(7-25)	NP
	LVS (type B)	FTL_0147	512	-	OM	1 (1-23)	1 (7-25)	NP
	U112 (*novicida*)	FTN_0142	513	-	OM	1(1-23)	1(7-25)	NP
Homologue	SCHU (A.I)	FTT0267	372	-	U	NP	NP	NP

[a] OM represents proteins predicted to be localized to the outer membrane.

[b] Number of predictions followed in parenthesis by amino acid sequence postion.

[c] NP represents an analysis with no prediction.

[d] U represents unknown.

Detailed description of RD-2

RD-2 is present in subpopulation A.I and absent from subpopulation A.II (Figure 4.7). RD-2 is localized within the sequence encoding a hypothetical protein (FTT1733) predicted to be functional in subpopulation A.I with no homologue in subpopulation A.II (Table 4.9). The absence of RD-2 in subpopulation A.II results in a deletion of a 148 bp region (Figure 4.8). RD-2 is present in *F. tularensis* subspp. type B and *novicida* (Figure 4.7). The homologues of hypothetical protein FTT1733 are annotated as functional in OSU18 (FTH_1802) encoding a 103 amino acid protein (similar to subpopulation A.I) and *F. tularensis* subsp. *novicida* (FTN_1712) that encodes for a 151 amino acid protein predicted to localize to the cytoplasm (Table 4.10). In *F. tularensis* subsp. type B strain LVS, this region is annotated as falling within an intergenic region. It should be noted that nucleotide sequence comparison of this region revealed no sequence differences

between *F. tularensis* subsp. type B strains LVS and OSU18 and *F. tularensis* subsp. type A strain SCHU S4, thus differences in predicted proteins are due to annotation.

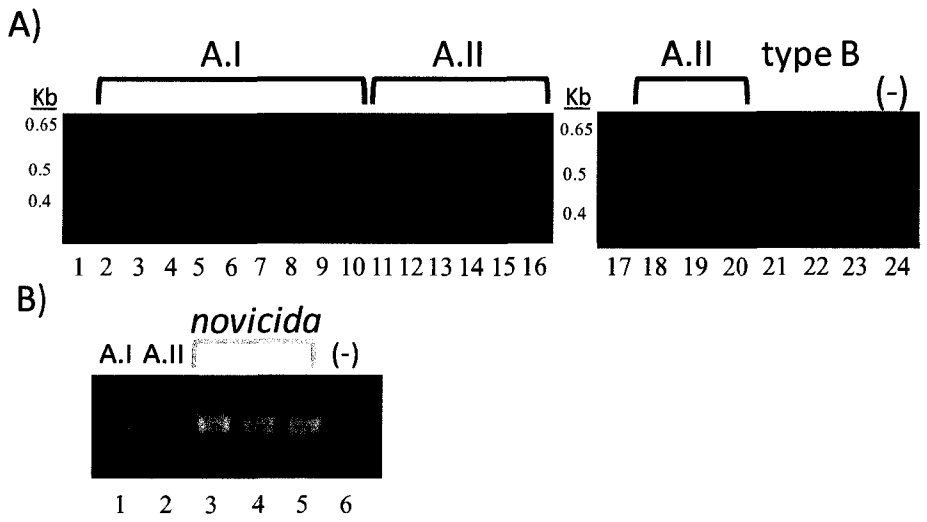


Figure 4.7 Agarose gel of PCR amplified *F. tularensis* strains using RD-2 primer set. A) PCR amplification of *F. tularensis* subpopulations A.I, A.II and *F. tularensis* subsp. type B strains. **Lane 1:** 1 kb molecular weight ladder, **lanes 2-10:** panel of A.I strains, **lanes 11-20:** panel of A.II strains, **lanes 21-23:** panel of type B strains, **lane 24:** negative control. **B)** PCR amplification of MA00-2987 (A.I), WY96-3418 (A.II) and panel of *novicida* strains.

Table 4.9 Summary of BLAST analyses for RD-2 gene *FTT1733*.

BLAST Program	Description	ORF	E-value	Percent Identity	Percent Query coverage	Positives
blastn	*F. tularensis* subsp. *tularensis* FSC198	-	1.E-161	100	100	-
	F. tularensis subsp. *tularensis* SCHU S4	-	1.E-161	100	100	-
	F. tularensis subsp. *holarctica* OSU18	-	2.E-158	99	100	-
	F. tularensis subsp. *holarctica* LVS	-	2.E-158	99	100	-
	F. tularensis subsp. *novicida* U112	-	2.E-153	98	99	-
	F. tularensis subsp. *tularensis* WY96-3418	-	2.E-48	100	56	-
	F. tularensis subsp. *tularensis* WY96-3418	-	1.E-26	100		-
blastp	*F. tularensis* subsp. *novicida* GA99-3548	FTDG_01270	2.E-48	97	-	101/103
	F. tularensis subsp. *novicida* U112	FTN_1712	2.E-48	97	-	101/103
	F. tularensis subsp. *tularensis* SCHU S4	FTT1733	5.E-48	100	-	103/103
	F. tularensis subsp. *tularensis* FSC198	FTF1733	5.E-48	100	-	103/103
	F. tularensis subsp. *holarctica* OSU18	FTH_1802	1.E-46	98	-	101/103
	F. tularensis subsp. *holarctica* 257	FTHG_01732	1.E-46	98	-	101/103
	Chromohalobacter salexigens DSM 3043	Csal_1301	3.E-10	36	-	34/94
	Aeromonas hydrophila subsp. *hydrophila* ATCC 7966	AHA_0046	4.E-05	32	-	33/101
	Moritella sp. PE36	PE36_05013	5.E-05	30	-	32/105

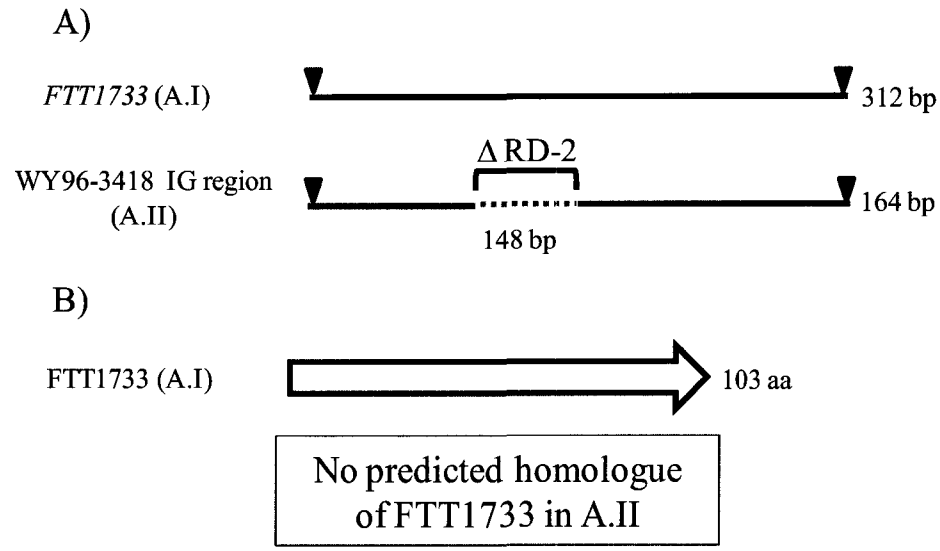


Figure 4.8 Alignment of subpopulations A.I and A.II illustrating the effects of RD-2. **A)** Nucleotide alignment of homologous gene-encoding region *FTT1733* in subpopulation A.I and intergenic region in subpopulation A.II. Black line represents homologous sequence; dashed red line represents the absence of RD-2 in subpopulation A.II; blue triangle represents start codon; red triangle represents stop codon. **B)** Illustration of amino acid sequence of hypothetical protein FTT1733 represented by an arrow. Pink represents no homology.

Table 4.10 Summary of bioinformatic analyses for RD-2 associated proteins.

	F. tularensis Strains	ORF	Number of Amino Acids	Pseudogene	PSORT	SignalP Analysis	TMHMM Analysis	Pfam Analysis
Proteins associated with RD region[a]	SCHU (A.I)	FTT1733	103	-	U[b]	NP	NP	NP
	OSU18 (type B)	FTH_1802	103	-	U	NP	NP	NP
	U112 (*novicida*)	FTN_1712	151	-	Cyto[c]	NP	NP	NP

[a] No homologues were found in LVS (type B) or WY96-3418 (A.II).

[b] U represents unknown.

[c] Cyto represents proteins predicted to be localized to the cytoplasm.

Detailed description of RD-3

RD-3 is present in subpopulation A.I and absent from subpopulation A.II (Figures 4.9). RD-3 was determined to be an 1181 bp region in subpopulation A.I that lies within a gene encoding for a conserved hypothetical lipoprotein (FTT1194c), the adjacent intergenic region, and a downstream pseudogene encoding a conserved hypothetical protein (FTT1195c) (Tables 4.11 and 4.12). Absence of RD-3 in subpopulation A.II results in one functionally annotated gene (FTW_1242) that is homologous to the two ends of the two coding sequences described for subpopulation A.I (Figure 4.10). The conserved hypothetical lipoprotein is predicted to contain a DUF403 (domain of unknown function) domain (Table 4.13). Homologue comparison between *F. tularensis* subspp. type B and *novicida* to subpopulations A.I and A.II reveals differences between all genomes; however RD-3 is present in *F. tularensis* subspp. type B and *novicida* (Figure 4.9).

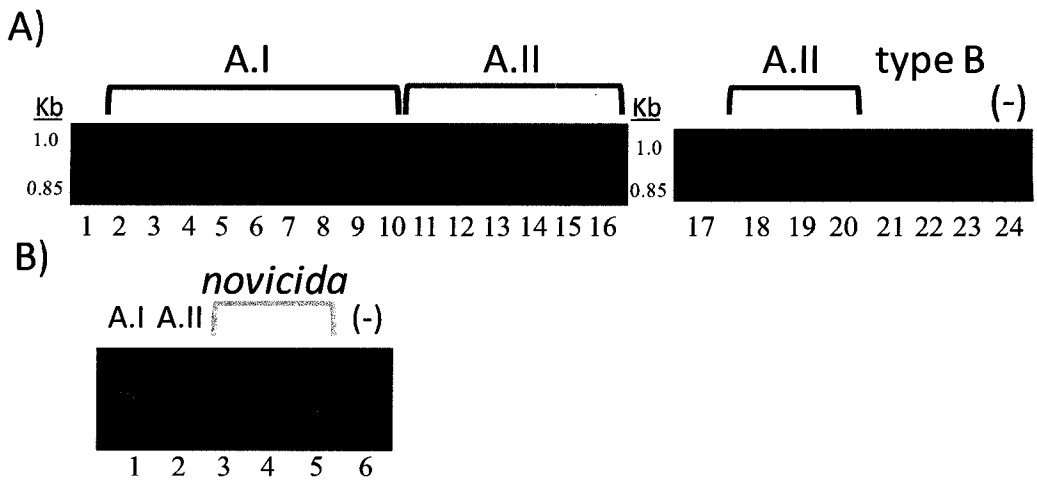


Figure 4.9 Agarose gel of PCR amplified *F. tularensis* strains using RD-3 primer set. A) PCR amplification of *F. tularensis* subpopulations A.I and A.II, and *F. tularensis* subsp. type B strains. **Lane 1:** 1 kb molecular weight ladder, **lanes 2-10:** panel of A.I strains, **lanes 11-20:** panel of A.II strains, **lanes 21-23:** panel of type B strains, **lane 24:** negative control. **B)** PCR amplification of MA00-2987 (A.I), WY96-3418 (A.II) and panel of *novicida* strains.

Table 4.11 Summary of BLAST analyses of RD-3 gene *FTT1194c*.

BLAST Program	Description	ORF	E-value	Percent Identity	Percent Query coverage	Positives
blastn	*F. tularensis* subsp. *tularensis* FSC198	-	0.E+00	100	100	-
	F. tularensis subsp. *tularensis* SCHU S4	-	0.E+00	100	100	-
	F. tularensis subsp. *novicida* U112	-	0.E+00	99	100	-
	F. tularensis subsp. *holarctica* OSU18	-	0.E+00	99	100	-
	F. tularensis subsp. *holarctica* LVS	-	0.E+00	99	100	-
	F. tularensis subsp. *tularensis* WY96-3418	-	3.E-146	100	30	-
blastp[a]	*F. tularensis* subsp. *novicida* U112	FTN_1171	5.E-163	100	-	308/308
	F. tularensis subsp. *novicida* GA99-3549	FTCG_00699	5.E-163	100	-	308/308
	F. tularensis subsp. *tularensis* SCHU S4	FTT1194c	5.E-163	100	-	308/308
	F. tularensis subsp. *tularensis* FSC198	FTF1194c	8.E-163	100	-	308/308
	F. tularensis subsp. *tularensis* FSC033	FTBG_00549	8.E-163	100	-	308/308
	F. tularensis subsp. *novicida* GA99-3548	FTDG_00744	1.E-160	99	-	306/308
	F. tularensis subsp. *holarctica* LVS	FTL_0751	5.E-159	98	-	304/308
	F. tularensis subsp. *holarctica* OSU18	FTH_0753	5.E-159	98	-	304/308
	F. tularensis subsp. *holarctica* 257	FTHG_00705	5.E-159	98	-	304/308
	F. tularensis subsp. *tularensis* WY96-3418	FTW_1242	2.00E-47	100	-	94/94

[a] Hits at least 100 more hypothetical proteins of unknown function with significant e-values.

Table 4.12 Summary of BLAST analysis for RD-3 pseudogene *FTT1195c*.

BLAST Program	Description	E-value	Percent Identity	Percent Query coverage
blastn	*F. tularensis* subsp. *tularensis* FSC198	0.E+00	100	100
	F. tularensis subsp. *tularensis* SCHU S4	0.E+00	100	100
	F. tularensis subsp. *holarctica* OSU18	0.E+00	98	100
	F. tularensis subsp. *holarctica* LVS	0.E+00	98	99
	F. tularensis subsp. novicida U112	0.E+00	98	100
	F. tularensis subsp. *tularensis* WY96-3418	0.E+00	99	67

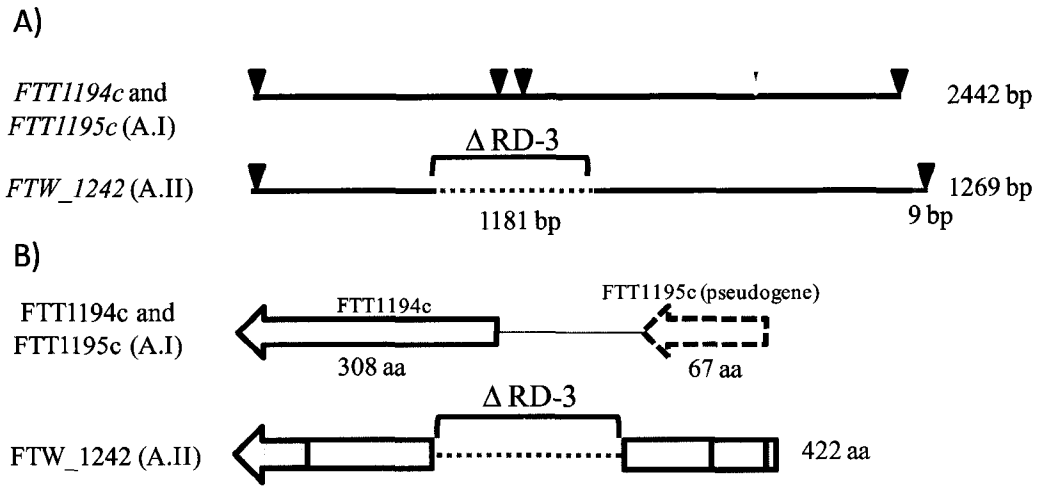


Figure 4.10 Alignment of subpopulations A.I and A.II illustrating the effects of RD-3. A) Nucleotide alignment of regions encoding for proteins *FTT1194c* and *FTT1195c* in subpopulation A.I with homologue *FTW_1242* in subpopulation A.II. Black line represents homologous sequence; green line represents sequence that is out of frame in comparison to the rest of the sequence; dashed red line represents absence of RD-3 in subpopulation A.II; blue triangles represent start codons; red triangles represent stop codons. **B)** Amino acid sequence alignment of subpopulation A.I proteins FTT1194c and FTT1195c (A.I) with FTW_1242 (A.II) with proteins predicted as functional represented as solid blue arrows and the pseudogene represented as a dashed blue arrow. Yellow represents amino acid sequence that differs between subpopulations A.I and A.II. Blue represents homology between the two sequences.

Table 4.13 Summary of bioinformatic analyses for RD-3 associated proteins and homologue in subpopulation A.II .

	F. tularensis Strains	ORF	Number of Amino Acids	Pseudogene	PSORT	SignalP Analysis	TMHMM Analysis	Pfam Analysis
Proteins associated with RD region	SCHU (A.I)	FTT1194c	308	-	Cyto[a]	NP[b]	NP	DUF403 (1-295)[c]
	SCHU (A.I)	FTT1195c	67	+	U[d]	NP	NP	DUF404(12-59)
	OSU18 (type B)	FTH_0752	160	+	Cyto	NP	NP	DUF404(12-159)
	OSU18 (type B)	FTH_0753	308	-	Cyto	NP	NP	DUF403(1-295)
	LVS (type B)	FTL_0750	317	+	U	NP	NP	DUF404 (1-175), DUF407 (200-307)
	LVS (type B)	FTL_0751	308	-	Cyto	NP	NP	DUF403 (1-295)
	U112 (*novicida*)	FTN_1171	328	-	Cyto	NP	NP	DUF403 (1-315)
	U112 (novicida)	FTN_1172	484	-	U	NP	NP	DUF404 (12-313), DUF407 (338-481)
Homologue	WY96-3418 (A.II)	FTW_1242	422	-	Cyto	NP	NP	DUF404(15-316)

[a] Cyto represents proteins predicted to be localized to the cytoplasm.

[b] NP represents an analysis with no prediction.

[c] Protein family domain followed in parenthesis by amino acid sequence postion.

[d] U represents unknown.

Detailed description of RD-4

RD-4 is present in subpopulation A.II and absent in subpopulation A.I (Figure 4.11). RD-4 is 674 bp and encompasses an intergenic region and two adjacent downstream regions encoding for hypothetical proteins (FTW_0888 and FTW_0889) (Table 4.14). FTW_0888 is predicted to have a transmembrane helix domain. The intergenic region is predicted to contain two putative promoter sequences. Both coding sequences as well as the intergenic region are absent in subpopulation A.I (Figure 4.12). In *F. tularensis* subspp. type B and *novicida*, there is only one hypothetical protein homologous to this subpopulation A.II region (FTL_1217, FTH_1195, for *F. tularensis* subsp. type B strains LVS and OSU18, and FTN_0864 for *F. tularensis* subsp. *novicida*). For all genomes, except *F. tularensis* subsp. type A strain SCHU S4, the upstream intergenic regions are predicted to contain two possible promoter positions (Table 4.15).

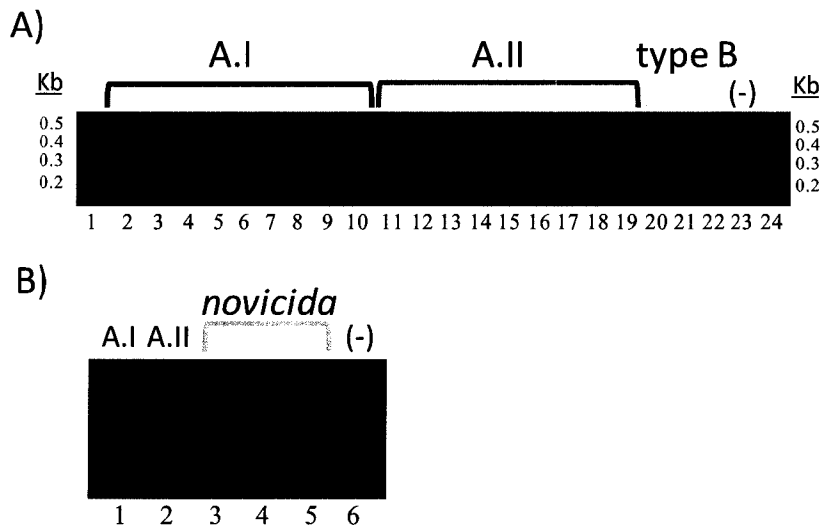


Figure 4.11 Agarose gel of PCR amplified *F. tularensis* strains using RD-4 primer set. A) PCR amplification of *F. tularensis* subpopulations A.I and A.II, and *F. tularensis* subsp. type B strains. **Lane 1:** 1 kb molecular weight ladder, **lanes 2-10:** panel of A.I strains, **lanes 11-20:** panel of A.II strains, **lanes 21-23:** panel of type B strains, **lane 24:** negative control. **B)** PCR amplification of MA00-2987 (A.I), WY96-3418 (A.II) and panel of *novicida* strains.

Table 4.14 Summary of BLAST analyses for RD-4 gene *FTW_0888*.

BLAST Program	Description	ORF	E-value	Percent Identity	Percent Query coverage	Positives
blastn	*F. tularensis* subsp. *tularensis* WY96-3418	-	2.E-70	100	100	-
	F. tularensis subsp. *holarctica* OSU18	-	2.E-70	100	100	-
	F. tularensis subsp. *holarctica* LVS	-	2.E-70	100	100	-
	F. tularensis subsp. *novicida* U112	-	2.E-65	97	100	-
	F. tularensis subsp. *tularensis* FSC 198	-	7.E-100	99	48	-
	F. tularensis subsp. *tularensis* SCHU S4	-	7.E-100	99	48	-
blastp	*F. tularensis* subsp. *tularensis* WY96-3418	FTW_0888	2.00E-12	100	-	48/48

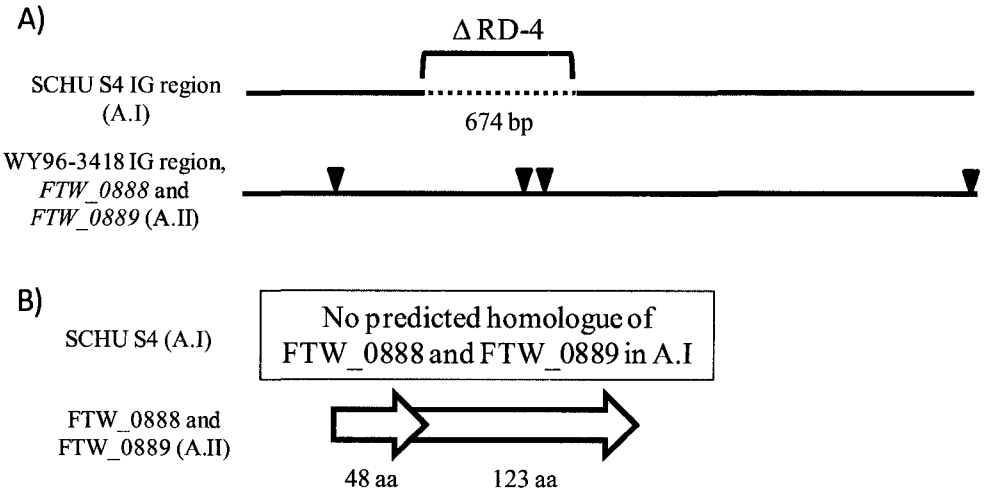


Figure 4.12 Alignment of subpopulations A.I and A.II illustrating the effects of RD-4. A) Nucleotide alignment of regions encoding for subpopulation A.I intergenic region and gene-encoding regions *FTW_0888* and *FTW_0889* in subpopulation A.II. Black line represents homologous nucleotide sequence between subpopulations A.I and A.II; dashed red line represents absence of RD-4 in subpopulation A.I; blue triangles represent start codons; red triangles represent stop codons. **B)** Arrows represent amino acid sequence of FTW_0888 and FTW_0889. Pink represents no homology.

Table 4.15 Summary of bioinformatic analyses for RD-4 associated proteins.

	F. tularensis Strains	ORF	Number of Amino Acids	Pseudogene	PSORT	SignalP Analysi	TMHMM Analysis	Pfam Analysis	BPROM
Proteins associated with RD region[a]	WY96-3418 (A.II)	FTW_0088	48	-	U[b]	NP[c]	1(12-34)[d]	NP	2 promoter pos. 82, 403
	WY96-3418 (A.II)	FTW_0089	123	-	U	NP	NP	NP	-
	OSU18 (type B)	FTH_1195	123	-	U	NP	NP	NP	2 promoter pos. 69, 518
	LVS (type B)	FTL_1217	123	-	U	NP	NP	NP	2 promoter pos. 69, 518
	U112 (*novicida*)	FTN_0864	123	-	U	NP	NP	NP	2 promoter pos. 64, 420

[a] No homologues were found in SCHU (A.I).

[b] U represents unknown.

[c] NP represents an analysis with no prediction.

[d] Number of predictions followed in parenthesis by amino acid sequence postion.

Detailed description of RD-5

RD-5 is present in subpopulation A.II and is absent from subpopulation A.I (Figure 4.13). RD-5 was predicted by BLAST analysis to span a region of 310 bp that encompasses the coding region of a hypothetical protein (FTW_0430), an intergenic region, and a downstream pseudogene for a methyltransferase (FTW_0431) predicted to be truncated after amino acid 122 (Table 4.16 and 4.17). Deletion of RD-5 in subpopulation A.I results in a loss at the C-terminus of the methyltransferase fragment (pseudogene) and a loss at the N-terminus of the hypothetical protein (Figure 4.14). Both of the coding sequences (FTT1429c and FTT1430c) in subpopulation A.I are annotated as pseudogenes. RD-5 is present in *F. tularensis* subspp. type B *novicida* (Figure 4.13). In *F. tularensis* subspp. type B (OSU18) and *novicida*, homologous to these coding regions, FTH_0631 and FTH_0632 in OSU18 and FTN_1399 and FTN_1400 in *F. tularensis* subsp. *novicida*, are annotated as functional. In *F. tularensis* subsp. type B strain LVS, both are annotated as pseudogenes (FTL_0628 and FTL_0629) (Table 4.18).

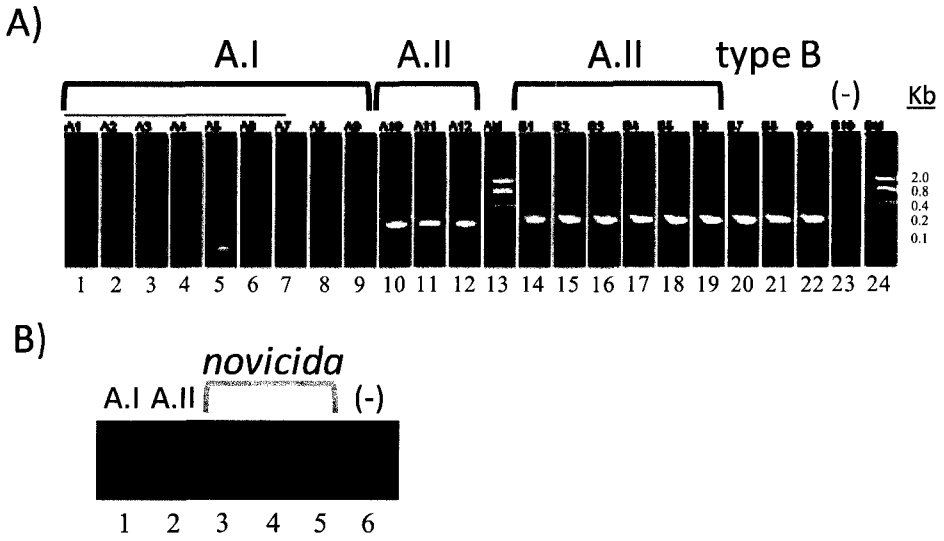


Figure 4.13 E-gel of PCR amplified *F. tularensis* strains using RD-5 primer set. A) PCR amplification of *F. tularensis* subpopulations A.I and A.II, and *F. tularensis* subsp. type B strains. **Lane 1-9:** panel of A.I strains, **lanes 10-19:** panel of A.II strains, **lanes 20-22:** panel of type B strains, **lane 23:** negative control, **lane 24:** molecular weight standard. **B)** PCR amplification of MA00-2987 (A.I), WY96-3418 (A.II) and panel of *novicida* strains.

Table 4.16 Summary of BLAST analyses for RD-5 gene *FTW_0430.*

BLAST Program	Description	ORF	E-value	Percent Identity	Percent Query coverage	Positives
blastn	*F. tularensis* subsp. *tularensis* WY96-3418	-	0.0E+00	100	100	-
	F. tularensis subsp. *holarctica* OSU18	-	0.0E+00	99	100	-
	F. tularensis subsp. *holarctica* LVS	-	0.0E+00	99	100	-
	F. tularensis subsp. *novicida* U112	-	0.0E+00	98	100	-
	F. tularensis subsp. *tularensis* FSC198	-	0.0E+00	99	91	-
	F. tularensis subsp. *tularensis* SCHU S4	-	0.0E+00	99	91	-
blastp	*F. tularensis* subsp. *tularensis* WY96-3418	FTW_0430	9.0E-117	100	-	238/238
	F. tularensis subsp. *novicida* U112	FTN_1399	5.0E-116	98	-	236/238
	F. tularensis subsp. *novicida* GA99-3549	FTCG_00911	8.0E-116	98	-	236/238
	F. tularensis subsp. *novicida* GA99-3548	FTDG_00963	8.0E-116	98	-	236/238
	F. tularensis subsp. *holarctica* OSU18	FTH_0632	2.0E-114	98	-	236/238
	F. tularensis subsp. *holarctica* 257	FTHG_00593	2.0E-114	98	-	236/239
	F. tularensis subsp. *tularensis* SCHU S4	FTT1429	3.0E-33	100	-	78/78

Table 4.17 Summary of BLAST analysis for RD-5 pseudogene *FTW_0431.*

BLAST Program	Description	E-value	Percent Identity	Percent Query coverage
blastn	*F. tularensis* subsp. *tularensis* WY96-3418	0.0E+00	100	100
	F. tularensis subsp. *holarctica* OSU18	0.0E+00	99	100
	F. tularensis subsp. *holarctica* LVS	0.0E+00	99	100
	F. tularensis subsp. *novicida* U112	0.0E+00	99	100
	F. tularensis subsp. *tularensis* FSC198	0.0E+00	99	65
	F. tularensis subsp. *tularensis* SCHU S4	0.0E+00	99	65

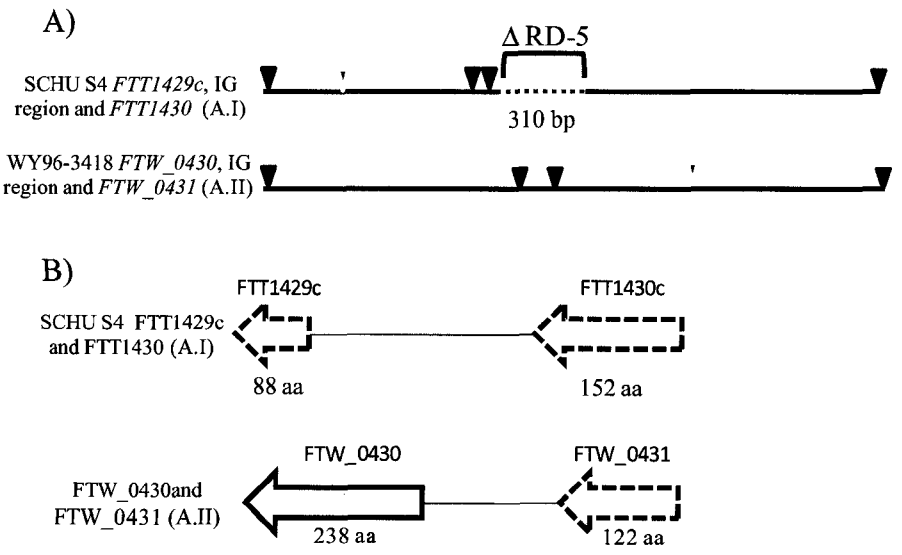


Figure 4.14 Alignment of subpopulations A.I and A.II illustrating the effects of RD-5. A) Nucleotide alignment of regions encoding for subpopulation A.I *FTT1429c*, intergenic region and *FTT1430c* and *FTW_0430*, intergenic region and *FTW_0431*. Red triangles represent stop codons; yellow triangles represent premature stop codons; blue triangles represent start codons; dashed red line represents absence of RD-5 in subpopulation A.I. **B)** Amino acid sequence alignment illustration of FTT1429c/FTT1430c and FTW_0430/FTW_0431 for which the protein predicted to be functional is depicted as a solid blue arrow and predicted pseudogenes are represented by dashed blue arrows. Blue represents homology between the two sequences.

Table 4.18 Summary of bioinformatic analyses for RD-5 associated proteins and homologues in subpopulation A.I.

	F. tularensis Strains	ORF	Number of Amino Acids	Pseudogene	PSORT	SignalP Analysis	TMHMM Analysis	Pfam Analysis
Proteins associated with RD region	WY96-3418 (A.II)	FTW_0430	238	-	U[a]	NP[b]	NP	NP
	WY96-3418 (A.II)	FTW_0431	122	+	U	NP	NP	methyltransf_4 (35-116)[c]
	OSU18 (type B)	FTH_0631	229	-	Cyto[d]	NP	NP	methyltransf_4 (35-229)
	OSU18 (type B)	FTH_0632	238	-	U	NP	NP	NP
	LVS (type B)	FTL_0628	229	+	Cyto	NP	NP	methyltransf_4 (35-229)
	LVS (type B)	FTL_0629	238	+	U	NP	NP	NP
	U112 (*novicida*)	FTN_1400	229	-	Cyto	NP	NP	methyltransf_4 (35-229)
	U112 (*novicida*)	FTN_1399	238	-	U	NP	NP	NP
Homologues	SCHU (A.I)	FTT1429c	88	+	U	NP	NP	NP
	SCHU (A.I)	FTT1430c	152	+	U	NP	NP	methyltransf_4 (35-152)

[a] U represents unknown.

[b] NP represents an analysis with no prediction.

[c] Protein family domain followed in parenthesis by amino acid sequence postion.

[d] Cyto represents proteins predicted to be localized to the cytoplasm.

Detailed description of RD-6

RD-6 is present in subpopulation A.II and absent from subpopulation A.I (Figure 4.15). By BLAST analysis, RD-6 mapped to the C4-dicarboxylate anaerobic carrier pseudogene (FTW_1709) in subpopulation A.II (Table 4.19). However, this RD was downstream of the premature stop codon that defined FTW_1709 as a pseudogene. The same premature stop codon is present in the subpopulation A.I homologue. Thus, even if the pseudogene resulted in a product it would not differ between subpopulations A.I and A. II (Figure 4.16). RD-6 is present in *F. tularensis* subspp. type B and *novicida* (Figure 4.15). This coding region is annotated as a pseudogene in *F. tularensis* subsp. type B strain OSU18 (FTH_1284) and as functional in strain LVS (FTL_1313). The homologue in *F. tularensis* subsp. *novicida* FTN_0269 is predicted functional (Table 4.20).

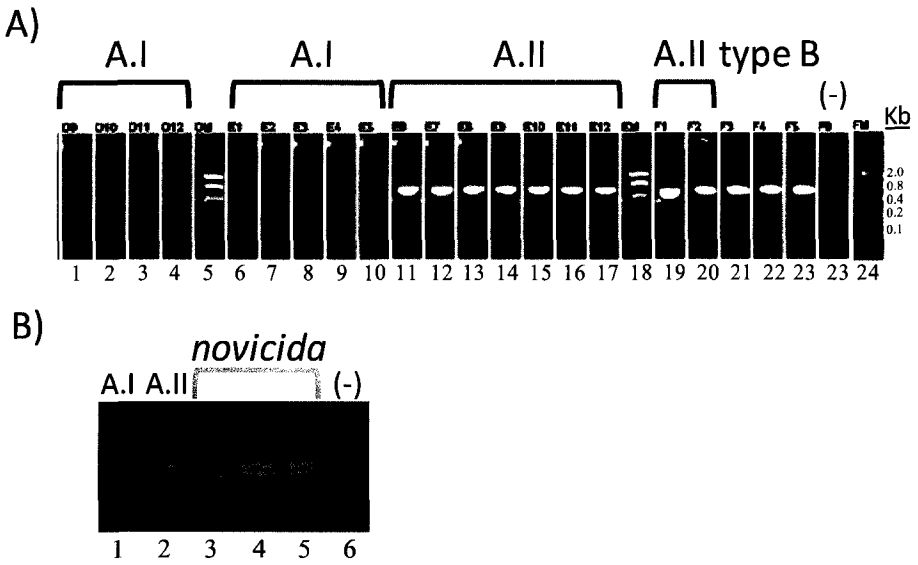


Figure 4.15 E-gel of PCR amplified *F. tularensis* strains using RD-6 primer set. A) PCR amplification of *F. tularensis* subpopulations A.I and A.II, and *F. tularensis* subsp. type B strains. **Lane 1-4:** panel of A.I strains, **lane 5:** molecular weight standard, **lanes 6-10:** panel of A.I strains, **lanes 11-17:** panel of A.II strains, **lane 18:** molecular weight standard, **lanes 19-20:** panel of A.II strains, **lanes 21-23:** panel of type B strains, **lane 24:** molecular weight standard. **B)** PCR amplification of MA00-2987 (A.I), WY96-3418 (A.II) and panel of *novicida* strains.

Table 4.19 Summary of BLAST analysis for RD-6 pseudogene *FTW_1709*.

BLAST Program	Description	E-value	Percent Identity	Percent Query coverage
blastn	*F. tularensis* subsp. *tularensis* WY96-3418	0.0E+00	100	100
	F. tularensis subsp. *holarctica* OSU18	0.0E+00	99	100
	F. tularensis subsp. *holarctica* LVS	0.0E+00	99	100
	F. tularensis subsp. *novicida* U112	0.0E+00	98	100
	F. tularensis subsp. *novicida* U112	1.0E-83	88	-
	F. tularensis subsp. *tularensis* FSC198	0.0E+00	99	91
	F. tularensis subsp. *tularensis* FSC198	7.0E-135	100	-
	F. tularensis subsp. *tularensis* SCHU S4	0.0E+00	99	91
	F. tularensis subsp. *tularensis* SCHU S4	7.00E-135	100	-

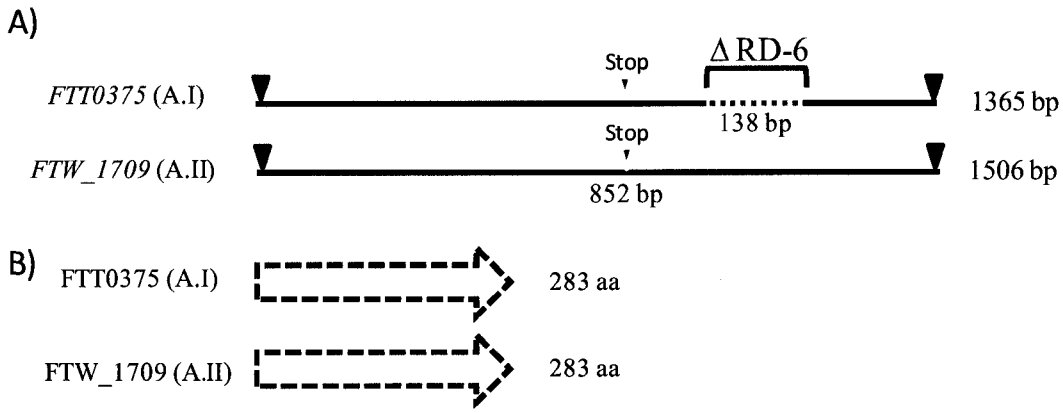


Figure 4.16 Alignment of subpopulations A.I and A.II illustrating RD-6. A) Nucleotide alignment of regions encoding for pseudogenes *FTT0375* in subpopulation A.I and *FTW_1709* in subpopulation A.II. Black line represents nucleotide sequence that is homologous between subpopulations A.I and A.II; dashed red line represents the absence of RD-6 in subpopulation A.I; blue triangles represent start codons; yellow triangles represent premature stop codons; red triangles represent stop codons. **B)** Amino acid sequence of pseudogenes FTT0375 (A.I) and FTW_1709 (A.II) represented as dashed blue arrows. Blue represents homology between the two sequences.

Table 4.20 Summary of bioinformatic analyses for RD-6 associated proteins and homologue in subpopulation A.I.

	F. tularensis Strain	ORF	Number of Amino Acids	Pseudogene	PSORT	SignalP Analysis	TMHMM Analysis	Pfam Analysis
Proteins associated with RD region	WY96-3418 (A.II)	FTW_1709	283	+	Cyto-Mem[a]	NP[b]	6(13-32, 103-122, 143-162, 172-194, 199-221, 225-247)[c]	DcuC (9-282)[d]
	OSU18 (type B)	FTH_1284	284	+	Cyto-Mem	NP	6(13-32, 103-122, 143-162, 172-194, 199-221, 225-247)	DcuC (9-284)
	LVS (type B)	FTL_1313	244	-	Cyto-Mem	NP	6(23-45,50-72, 93-112, 150-172, 192-211, 221-243)	DcuC (1-242)
	U112 (*novicida*)	FTN_0269	503	-	Cyto-Mem	NP	11(13-32, 103-122, 143-162, 172-194, 199-221, 225-247, 280-302, 307-329, 350-369, 454-476, 483-502)	DcuC (9-499)
Homologue	SCHU (A.I)	FTT0375	283	+	Cyto-Mem	NP	6(13-32, 103-122, 143-162, 172-194, 199-221, 225-247)	DcuC (9-282)

[a] Cyto-Mem represents proteins predicted to be localized to the cytoplasmic membrane.

[b] NP represents an analysis with no prediction.

[c] Number of predictions followed in parenthesis by amino acid sequence postion.

[d] Protein family domain followed in parenthesis by amino acid sequence postion.

Detailed description of RD-7

RD-7 is present in subpopulation A.I and absent from subpopulation A.II (Figure 4.17). Using BLAST analyses, RD-7 was found to map within a predicted promoter sequence of an intergenic region of subpopulation A.I upstream of a gene encoding an

arsenical pump membrane protein (ArsB). Deletion of RD-7 in subpopulation A.II removes the predicted promoter sequence and creates an alternative promoter (Figure 4.18). Promoter sequences similar to those in subpopulation A.I were predicted for *F. tularensis* subsp. type B strains OSU18 and LVS as well as for *F. tularensis* subsp. *novicida*. The ArsB homologues in *F. tularensis* subsp. type B strains OSU18 (FTH_0346) and LVS (FTL_0350) are annotated as pseudogenes whereas the homologue in *F. tularensis* subsp. *novicida* (FTN_0382) is predicted functional (Table 4.21).

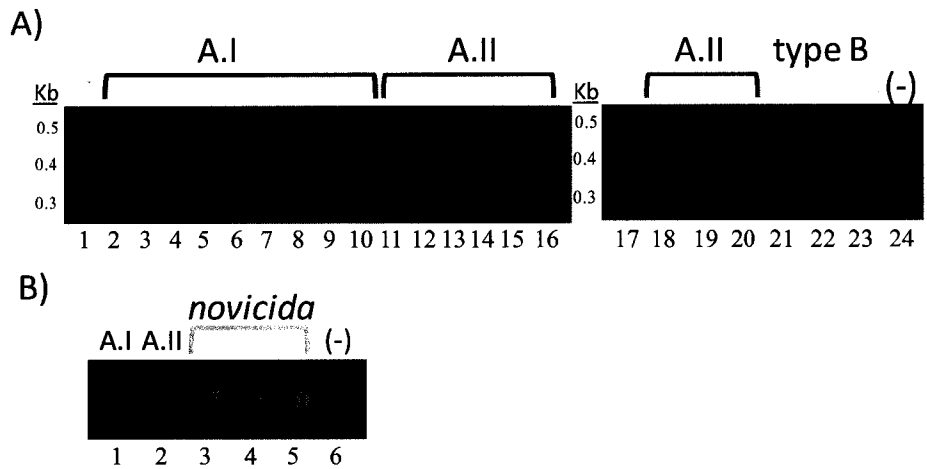


Figure 4.17 Agarose gel of PCR amplified *F. tularensis* strains using RD-7 primer set. A) PCR amplification of *F. tularensis* subpopulations A.I and A.II, and *F. tularensis* subsp. type B strains. **Lane 1:** 1 kb molecular weight ladder, **lanes 2-10:** panel of A.I strains, **lanes 11-20:** panel of A.II strains, **lanes 21-23:** panel of type B strains, **lane 24:** negative control. **B)** PCR amplification of MA00-2987 (A.I), WY96-3418 (A.II) and panel of *novicida* strains.

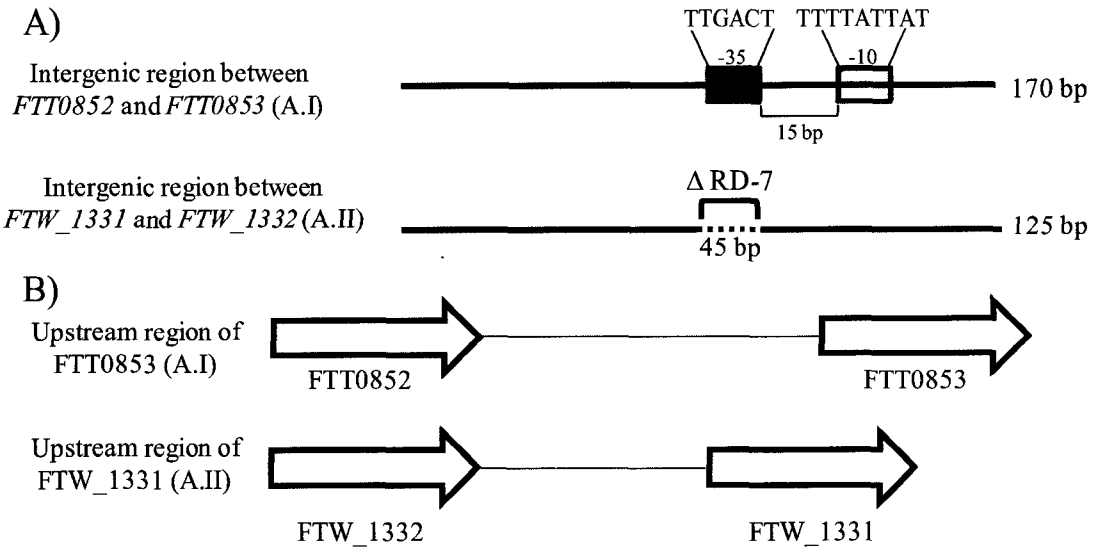


Figure 4.18 Alignment of subpopulations A.I and A.II illustrating the effects of RD-7. A) Nucleotide alignment of subpopulations A.I and A.II intergenic regions. Brown squares represent promoter sequence; dashed red line represents absence of RD-7 in subpopulation A.II. **B)** Illustration of the differences caused by the presence of RD-7 in subpopulation A.I and the effects of its absence in subpopulation A.II. Amino acid sequences adjacent to the intergenic regions for subpopulations A.I and A.II are represented as solid blue arrows. Blue represents homology between the two sequences.

Table 4.21 Summary of bioinformatic analyses for RD-7 associated proteins and homologue in subpopulation A.II.

	F. tularensis Strain	ORF	Number of Amino Acids	Pseudogene	PSORT	SigP Analysis	TMHMM Analysis	Pfam Analysis	BROM
Proteins associated with RD region		None[a]							
	Downstream CDS								
	SCHU (A.I)	FTT0853	412	-	Cyto-Mem[b]	NP[c]	12(5-22, 27-44, 59-81, 94-116, 131-153, 174-196, 218-240, 247-266, 281-303, 316-338, 353-375, 388-410)[d]	ArsB(5-411), CitMHS(8-362)[e]	1 promoter pos. 129
	OSU18 (type B)	FTH_0346	201	+	Cyto-Mem	NP	6(2-21, 25-46, 58-80, 95-117, 129-151, 171-193)	CitMHS(31-175)	1 promoter pos. 129
	LVS (type B)	FTL_0350	201	+	Cyto-Mem	NP	6(2-21, 25-46, 58-80, 95-117, 129-151, 171-193)	CitMHS(31-175)	1 promoter pos. 132
	U112 (*novicida*)	FTN_0382	412	-	Cyto-Mem	NP	12(4-21, 28-50, 60-82, 94-116, 131-153, 174-196, 220-242, 249-266, 281-303, 316-338, 353-375, 388-410)	ArsB(5-411), CitMHS(8-362)	1 promoter pos. 129
Homologue	WY96-3418 (A.II)	FTW_1331	412	-	Cyto-Mem	NP	12(5-22, 27-44, 59-81, 94-116, 131-153, 174-196, 218-240, 247-266, 281-303, 316-338, 353-375, 388-410)	ArsB(5-411), CitMHS(8-362)	1 promoter pos. 61

[a] RD-7 falls within an intergenic region therefore downstream genes were analyzed.

[b] Cyto-Mem represents proteins predicted to be localized to the cytoplasmic membrane.

[c] NP represents an analysis with no prediction.

[d] Number of predictions followed in parenthesis by amino acid sequence postion.

[e] Protein family domain followed in parenthesis by amino acid sequence postion.

Detailed description of RD-8

RD-8 is present in subpopulation A.I and absent from subpopulation A.II (Figure 4.19). In subpopulation A.I, RD-8 falls within a region encoding a hypothetical protein (FTT0096) (Table 4.22). FTT0096 is predicted to localize to the cytoplasm and to contain an ATP_GRASP 3 domain (ATP-binding domain) (Table 4.23). In subpopulation A.II, the absence of RD-8 results in an in-frame deletion of 88 amino acids (of 346 amino acids in subpopulation A.I), including a portion of the ATP_GRASP domain. Nevertheless, the truncated hypothetical protein in subpopulation A.II has been annotated as functional (Figure 4.20). Homologues within *F. tularensis* subsp. type B strains OSU18 (FTH_1699) and LVS (FTL_1759) and *F. tularensis* subsp. *novicida* (FTN_1615) are all predicted functional and to encode for a 426 amino acid protein (Table 4.23).

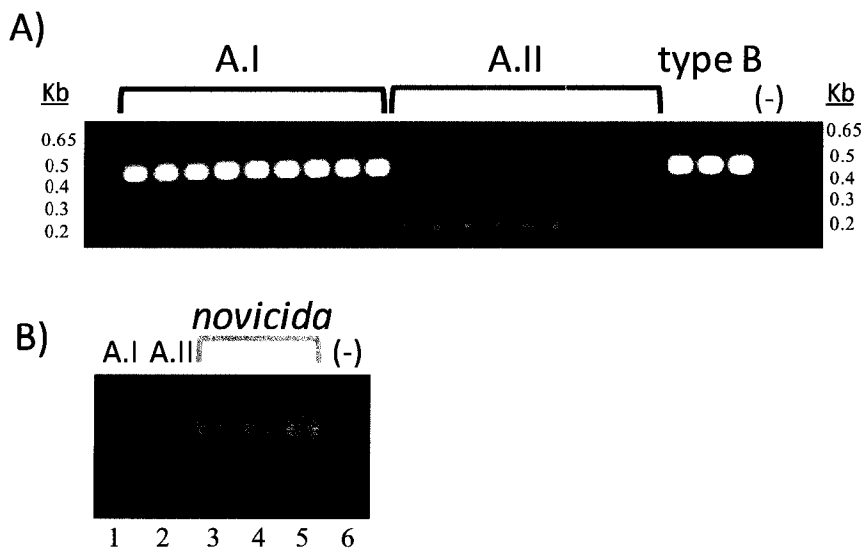


Figure 4.19 Agarose gel of PCR amplified *F. tularensis* strains using RD-8 primer set. A) PCR amplification of *F. tularensis* subpopulations A.I and A.II and *F. tularensis* subsp. type B strains. **Lane 1:** 1 kb molecular weight ladder, **lanes 2-10:** panel of A.I strains, **lanes 11-20:** panel of A.II strains, **lanes 21-23:** panel of type B strains, **lane 24:** negative control. **B)** PCR amplification of MA00-2987 (A.I), WY96-3418 (A.II) and panel of *novicida* strains.

Table 4.22 Summary of BLAST analyses for RD-8 gene *FTT0096*.

BLAST Program	Description	ORF	E-value	Percent Identity	Percent Query coverage	Positives
blastn	*F. tularensis* subsp. *tularensis* FSC198	-	0.0E+00	100	100	-
	F. tularensis subsp. *tularensis* SCHU S4	-	0.0E+00	100	100	-
	F. tularensis subsp. *holarctica* OSU18	-	0.0E+00	98	100	-
	F. tularensis subsp. *holarctica* LVS	-	0.0E+00	98	100	-
	F. tularensis subsp. *novicida* U112	-	0.0E+00	98	100	-
	F. tularensis subsp. *tularensis* WY96-3418	-	0.0E+00	99	75	-
	F. tularensis subsp. *tularensis* WY96-3418	-	0.0E+00	100		-
blastp	*F. tularensis* subsp. *tularensis* SCHU S4	FTT0096	0.0E+00	100	-	346/346
	F. tularensis subsp. *tularensis* FSC198	FTF0096	0.0E+00	100	-	346/346
	F. tularensis subsp. *novicida* U112	FTN_1615	0.0E+00	98	-	341/346
	F. tularensis subsp. *novicida* GA99-3549	FTCG_01476	0.0E+00	97	-	341/346
	F. tularensis subsp. *novicida* GA99-3548	FTCG_01476	0.0E+00	97	-	341/346
	F. tularensis subsp. *holarctica* LVS	FTL_1759	0.0E+00	96	-	336/346
	F. tularensis subsp. *holarctica* OSU18	FTH_1699	0.0E+00	96	-	336/346
	F. tularensis subsp. *holarctica* 257	FTHG_01632	0.0E+00	96	-	336/346
	F. tularensis subsp. *tularensis* WY96-3418	FTW_0178	7.0E-73	93	-	144/150
	F. tularensis subsp. *tularensis* WY96-3418	-	5.0E-62	100	-	121/121

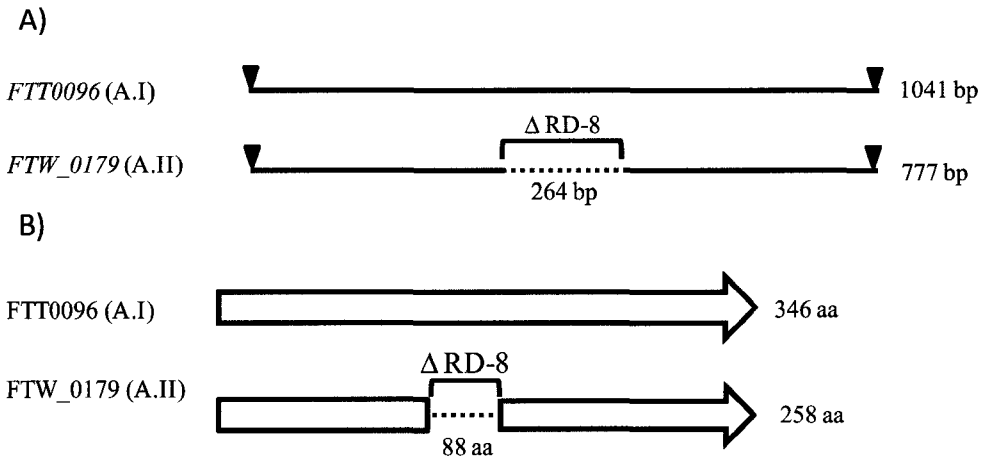


Figure 4.20 Alignment of subpopulations A.I and A.II illustrating the effects of RD-8. A) Nucleotide alignment of regions encoding hypothetical proteins *FTT0096* in subpopulation A.I and *FTW_0179* in subpopulation A.II. Black line represents homologous nucleotide sequence between subpopulations A.I and A.II; blue triangles represent start codons; red triangles represent stop codons; dashed red line represents the absence of RD-8 in subpopulation A.II. **B)** Amino acid sequence alignment of homologous proteins FTT0096 in subpopulation A.I and FTW_0179 in subpopulation A.II represented as solid blue arrows. Blue represents homology between the two sequences.

Table 4.23 Summary of bioinformatic analyses for RD-8 associated proteins and homologue in subpopulation A.II.

	F. tularensis Strain	ORF	Number of Amino Acids	Pseudogene	PSORT	SignalP Analysi	TMHMM Analysis	Pfam Analysis
Proteins associated with RD region	SCHU (A.I)	FTT0096	346	-	Cyto[a]	NP[b]	NP	ATP_GRASP 3 (68-255)[c]
	OSU18 (type B)	FTH_1699	426	-	Cyto	NP	NP	ATP_GRASP 3 (148-335)
	LVS (type B)	FTL_1759	426	-	Cyto	NP	NP	ATP_GRASP 3 (148-335)
	U112 (*novicida*)	FTN_1615	426	-	Cyto	NP	NP	ATP_GRASP 3 (148-335)
Homologue	WY96-3418 (A.II)	FTW_0179	258	-	Cyto	NP	NP	ATP_GRASP 3 (68-255)

[a] Cyto proteins predicted to be localized to the cytoplasm.

[b] NP represents an analysis with no prediction.

[c] Protein family domain followed in parenthesis by amino acid sequence postion.

Detailed description of RD-9

RD-9 is a small, 16 bp RD present in subpopulation A.II and absent from subpopulation A.I. The results obtained using primer set RD-9 for panel verification are shown in Figure 4.21. The amplicon size for subpopulation A.I is larger than subpopulation A.II implying that the RD is large and present in subpopulation A.I. After sequence analysis, it was determined that this discrepancy was due to mispriming. RD-9 lies within a magnesium chelatase pseudogene in subpopulation A.II (FTW_0121) (Table 4.24). Absence of RD-9 in subpopulation A.I results in deletion of 16 bp and introduction of a premature stop codon to give an 81 amino acid product. In subpopulation A.II the gene is truncated prematurely to give a protein of 355 amino acids. The genes in both subpopulations A.I and A.II are annotated as pseudogenes (Figure 4.22). Homologues in *F. tularensis* subsp. type B are annotated as pseudogenes (FTH_1750 in strain OSU18 and FTL_1814c in strain LVS). In *F. tularensis* subsp. *novicida*, a functional 502 amino acid magnesium chelatase is predicted (Table 4.25).

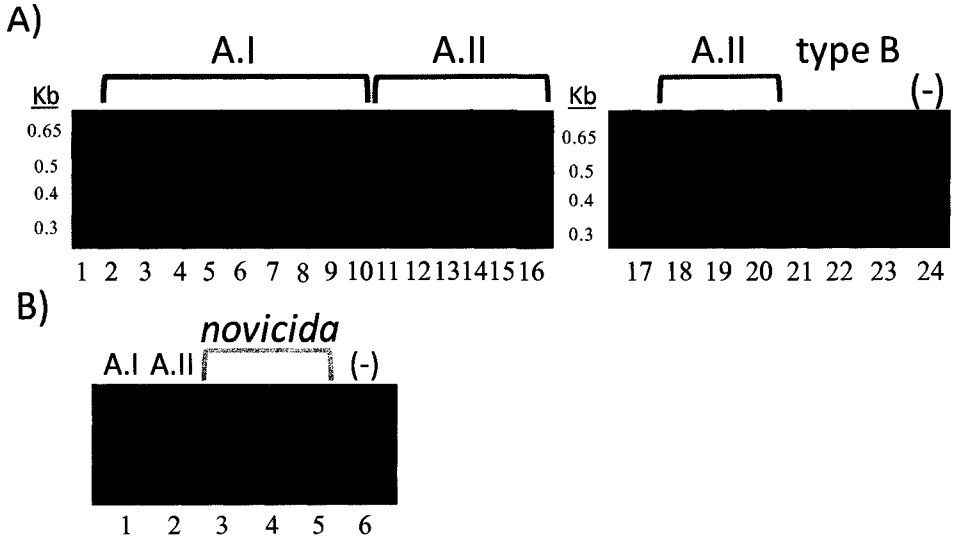


Figure 4.21 Agarose gel of PCR amplified *F. tularensis* strains using RD-9 primer set. A) PCR amplification of *F. tularensis* subpopulations A.I and A.II, and *F. tularensis* subsp. type B strains. **Lane 1:** 1 kb molecular weight ladder, **lanes 2-10:** panel of A.I strains, **lanes 11-20:** panel of A.II strains, **lanes 21-23:** panel of type B strains, **lane 24:** negative control. **B)** PCR amplification of MA00-2987 (A.I), WY96-3418 (A.II) and panel of *novicida* strains.

Table 4.24 Summary of BLAST analysis for RD-9 pseudogene *FTW_0121.*

BLAST Program	Description	E-value	Percent Identity	Percent Query coverage
blastn	*F. tularensis* subsp. *tularensis* WY96-3418	0.0E+00	100	100
	F. tularensis subsp. *tularensis* FSC198	0.0E+00	98	100
	F. tularensis subsp. *tularensis* SCHU S4	0.0E+00	98	100
	F. tularensis subsp. *novicida* U112	0.0E+00	98	100
	F. tularensis subsp. *holarctica* OSU18	0.0E+00	99	100
	F. tularensis subsp. *holarctica* OSU18	8.0E-30	100	-
	F. tularensis subsp. *holarctica* LVS	0.0E+00	99	100
	F. tularensis subsp. *holarctica* LVS	8.0E-30	100	-

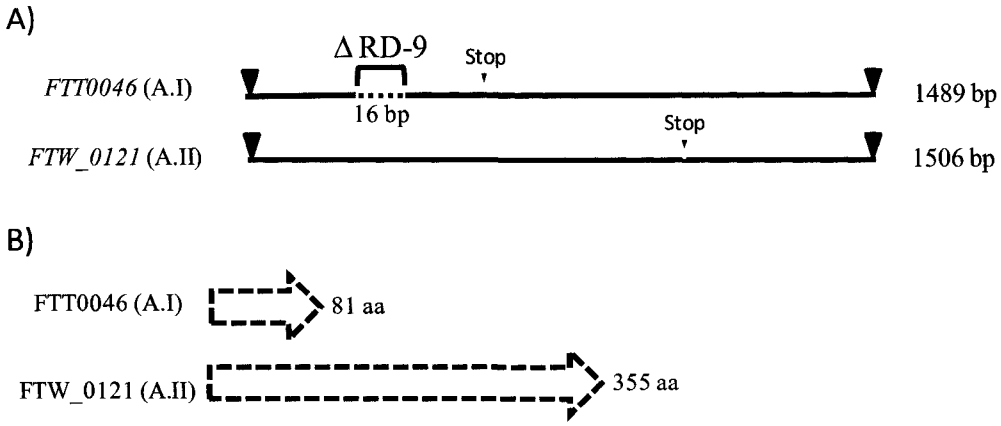


Figure 4.22 Alignment of subpopulations A.I and A.II illustrating the effects of RD-9. A) Nucleotide alignment of *FTT0046* in subpopulation A.I and *FTW_0121* in subpopulation A.II. Black line represents nucleotide sequence that is homologous between subpopulations A.I and A.II; blue triangles represent start codons; yellow triangles represent premature stop codons; red triangles represent stop codons; dashed red line represents absence of RD-9 in subpopulation A.I. **B)** Amino acid sequence alignment of homologous pseudogenes FTT0046 in subpopulation A.I and FTW_0121 in subpopulation A.II represented as dashed blue arrows. Blue represents homology between the two sequences.

Table 4.25 Summary of bioinformatic analyses for RD-9 associated proteins and homologue in subpopulation A.I.

	F. tularensis Strain	ORF	Number of Amino Acids	Pseudogene	PSORT	SignalP Analysis	TMHMM Analysis	Pfam Analysis
Proteins associated with RD region	WY96-3418 (A.II)	FTW_0121	355	+	Cyto[a]	NP[b]	NP	Mg_chelatase(190-354), AAA(213-228), AAA_5(213-231)[c]
	OSU18 (type B)	FTH_1750	25	+	U[d]	NP	NP	NP
	LVS (type B)	FTL_1814c	288	+	Cyto	NP	NP	Mg_chelatase(65-271), AAA(88-103), AAA_5(88-106)
	U112 (*novicida*)	FTN_1665	502	-	Cyto	NP	NP	Mg_chelatase(190-396), AAA(213-228), AAA_5(213-231)
Homologue	SCHU (A.I)	FTT0046	81	+	U	NP	NP	NP

[a] Cyto represents proteins predicted to be localized to the cytoplasm.

[b] NP represent an analysis with no prediction.

[c] Protein family domain followed in parenthesis by amino acid sequence postion.

[d] U represents unknown.

Detailed description of RD-10

RD-10 is present in subpopulation A.II and absent from subpopulation A.I (Figure 4.23). RD-10 falls within a region encoding a DNA processing protein DprA that in subpopulation A.II (FTW_1356) is annotated as functional (368 amino acids) (Table

4.26). The subpopulation A.I homologue to DprA is annotated as a pseudogene (229 amino acids) due to the introduction of a premature stop codon upstream of the RD-10 deletion (Figure 4.24). The homologues within *F. tularensis* subsp. type B are annotated as a pseudogene in strain OSU18 (FTH_0322) and as intergenic region within strain LVS. The homologue in *F. tularensis* subsp. *novicida* (FTN_0345) is predicted functional and to encode for a 368 amino acid protein (Table 4.27).

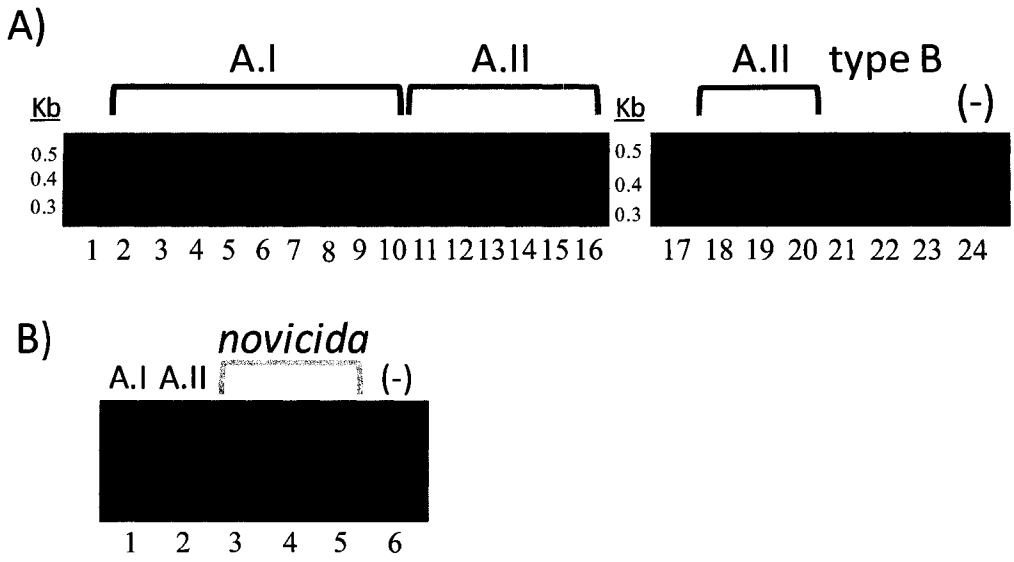


Figure 4.23 Agarose gel of PCR amplified *F. tularensis* strains using RD-10 primer set. A) PCR amplification of *F. tularensis* subpopulations A.I and A.II, and *F. tularensis* subsp. type B strains. **Lane 1:** 1 kb molecular weight ladder, **lanes 2-10:** panel of A.I strains, **lanes 11-20:** panel of A.II strains, **lanes 21-23:** panel of type B strains, **lane 24:** negative control. **B)** PCR amplification of MA00-2987 (A.I), WY96-3418 (A.II) and panel of *novicida* strains.

Table 4.26 Summary of BLAST analyses for RD-10 gene *FTW_1356*.

BLAST Program	Description	ORF	E-value	Percent Identity	Percent Query coverage	Positives
blastn[a]	*F. tularensis* subsp. *tularensis* WY96-3418	-	0.0E+00	100	100	-
	F. tularensis subsp. *holarctica* OSU18	-	0.0E+00	99	100	-
	F. tularensis subsp. *holarctica* LVS	-	0.0E+00	99	100	-
	F. tularensis subsp. *tularensis* FSC 198	-	0.0E+00	99	96	-
	F. tularensis subsp. *tularensis* SCHU S4	-	0.0E+00	99	96	-
	F. tularensis subsp. *novicida* U112	-	0.0E+00	98	100	-
blastp[a]	*F. tularensis* subsp. *tularensis* WY96-3418	FTW_1356	0.0E+00	100	-	368/368
	F. tularensis subsp. *novicida* GA99-3549	FTCG_01392	0.0E+00	98	-	365/368
	F. tularensis subsp. *novicida* U112	FTN_0345	0.0E+00	97	-	365/368
	F. tularensis subsp. *novicida* GA99-3548	FTDG_01583	0.0E+00	98	-	361/368
	F. tularensis subsp. *holarctica* 257	FTHG_00311	3.0E-119	97	-	243/245
	F. tularensis subsp. *tularensis* FSC033	FTBG_00195	3.0E-116	99	-	225/227

[a] Only *Francisella* hits are shown due to numerous significant hits.

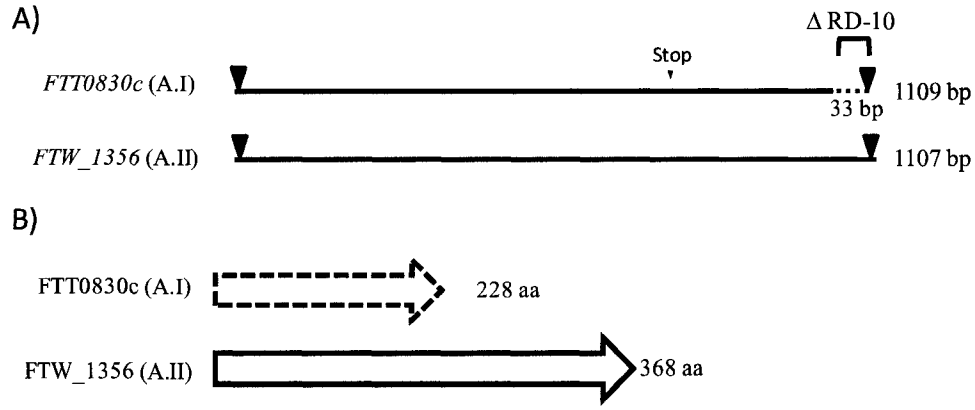


Figure 4.24 Alignment of subpopulations A.I and A.II illustrating RD-10. A) Nucleotide alignment of region encoding for pseudogene DprA in subpopulation A.I and its DprA homologue *FTW_1356* in subpopulation A.II. Black line represents nucleotide sequence that is homologous between subpopulations A.I and A.II; blue triangles represent start codons; yellow triangle represents a premature stop codon; dashed red line represents absence of RD-10 in subpopulation A.I. **B)** Amino acid sequence alignment of pseudogene FTT0839c in subpopulation A.I and its homologue FTW_1356 in subpopulation A.II for which the pseudogene is represented as a dashed blue arrow and the protein predicted as functional is represented as a solid blue arrow. Blue represents homology between the two sequences.

Table 4.27 Summary of bioinformatic analyses for RD-10 associated proteins and homologue in subpopulation A.II.

	F. tularensis Strain	ORF	Number of Amino Acids	Pseudogene	PSORT	SigP Analysis	TMHMM Analysis	Pfam Analysis
Proteins associated with RD region	WY96-3418 (A.I)	FTW_1356	368	-	U[a]	NP[b]	NP	SMF (72-281) [c]
	OSU18 (type B)	FTH_0322	80	+	U	NP	NP	NP
	U112 (*novicida*)	FTN_0345	368	-	U	NP	NP	SMF (72-281)
Homologue	SCHU (A.II)	FTT0830c	228	+	U	NP	NP	NP

[a] U represents unknown.

[b] NP represents an analysis with no prediction.

[c] Protein family domain followed in parenthesis by amino acid sequence postion.

Detailed description of RD-11

RD-11 is present in subpopulation A.I and absent from subpopulation A.II (Figure 4.25). By BLAST analysis, RD-11 was determined to be 15 bp and present within hypothetical protein (FTT1078c) (Table 4.28). Its absence in subpopulation A.II results in an in-frame deletion of 5 amino acids within FTT1078c that is predicted to be functional in subpopulation A.I (270 amino acids). Additionally, subpopulation A.II contains a non-RD associated frameshift that introduces a stop codon resulting in an annotated pseudogene encoding an 86 amino acid truncated protein (Figure 4.26). Functional homologues are found in *F. tularensis* subsp. type B strains OSU18 (FTH_1097) and LVS (FTL_1123) with only a weak homologue in *F. tularensis* subsp. *novicida* (Table 4.29).

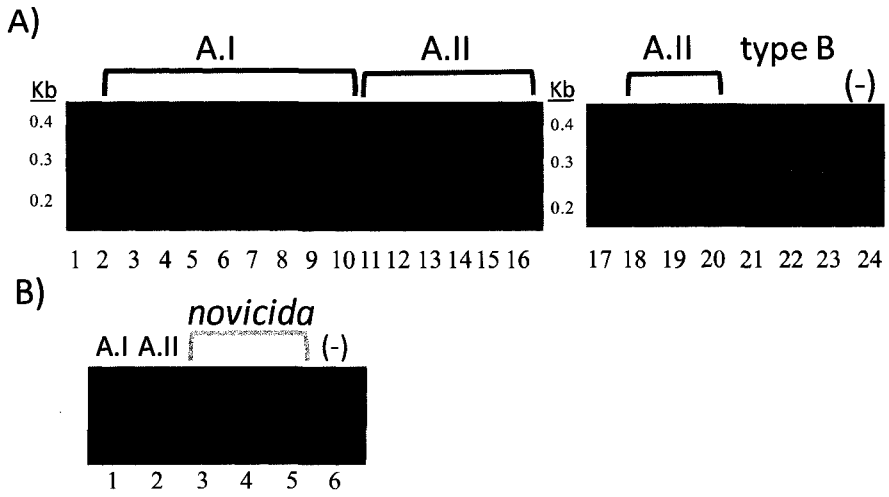


Figure 4.25 Agarose gel of PCR amplified *F. tularensis* strains using RD-11 primer set. A) PCR amplification of *F. tularensis* subpopulations A.I and A.II, and *F. tularensis* subsp. type B strains. **Lane 1:** 1 kb molecular weight ladder, **lanes 2-10:** panel of A.I strains, **lanes 11-20:** panel of A.II strains, **lanes 21-23:** panel of type B strains, **lane 24:** negative control. **B)** PCR amplification of MA00-2987 (A.I), WY96-3418 (A.II) and panel of *novicida* strains.

Table 4.28 Summary of BLAST analyses for RD-11 gene *FTW_1078c.*

BLAST Program	Description	ORF	E-value	Percent Identity	Percent Query coverage	Positives
blastn[a]	*F. tularensis* subsp. *tularensis* FSC 198	-	0.0E+00	100	100	-
	F. tularensis subsp. *tularensis* SCHU S4	-	0.0E+00	100	100	-
	F. tularensis subsp. *holarctica* OSU18	-	0.0E+00	98	100	-
	F. tularensis subsp. *tularensis* WY96-3418	-	0.0E+00	97	100	-
	F. tularensis subsp. *holarctica* LVS	-	0.0E+00	98	93	-
	F. tularensis subsp. *holarctica* LVS	-	5.0E-54	96		-
	F. tularensis subsp. *novicida* U112	-	9.0E-67	82	36	-
blastp[a]	*F. tularensis* subsp. *tularensis* SCHU S4	FTT1078c	7.0E-111	100	-	270/270
	F. tularensis subsp. *tularensis* FSC 198	FTF1078c	7.0E-111	100	-	270/270
	F. tularensis subsp. *tularensis* FSC033	FTBG_00438	7.0E-111	100	-	270/270
	F. tularensis subsp. *holarctica* OSU18	FTH_1097	2.0E-104	90	-	254/271
	F. tularensis subsp. *holarctica* LVS	FTL_1123	7.0E-88	97	-	205/207
	F. tularensis subsp. *novicida* U112	FTN_0793	4.0E-25	82	-	84/91

[a] Only *Francisella* hits are shown due to numerous significant hits.

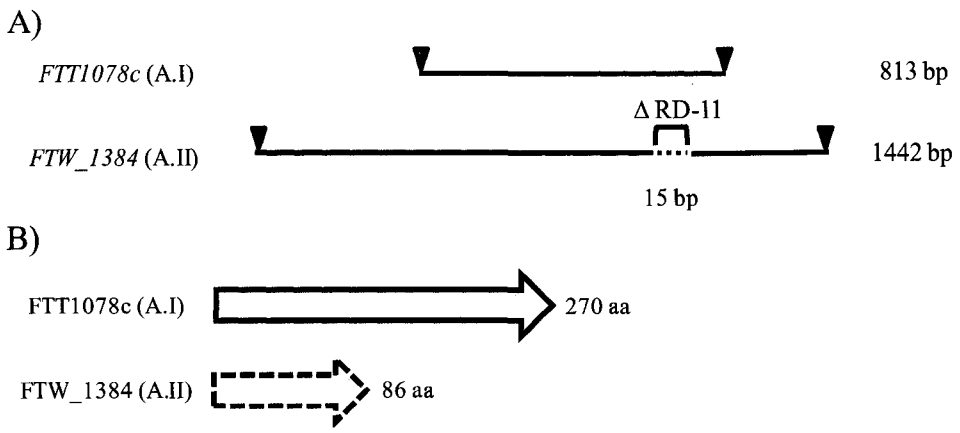


Figure 4.26 Alignment of subpopulations A.I and A.II illustrating RD-11. A) Nucleotide alignment of region encoding *FTT2078c* in subpopulation A.I and its homologue *FTW_1384* in subpopulation A.II. Black line represents nucleotide sequence that is homologous between subpopulations A.I and A.II; blue triangles represent start codons; red triangles represent stop codons; dashed red line represents the absence of RD-11 in subpopulation A.II. **B)** Amino acid sequence alignment of FTT1078c in subpopulation A.I represented by a pink arrow with solid blue outline and pseudogene FTW_1384 in subpopulation A.II represented by the dashed blue arrow. Pink represents no homology between the two protein sequences.

Table 4.29 Summary of bioinformatic analyses for RD-11 associated proteins and homologue in subpopulation A.II.

	F. tularensis Strain	ORF	Number of Amino Acids	Pseudogene	PSORT	SigP Analysis	TMHMM Analysis	Pfam Analysis
Proteins associated with RD region	SCHU (A.I)	FTT1078c	270	-	U[a]	NP[b]	NP	NP
	LVS (type B)	FTL_1123	300	-	U	NP	NP	NP
	OSU18 (type B)	FTH_1097	271	-	U	NP	NP	NP
Homologue	WY96-3418 (A.II)	FTW_1384	86	+	U	NP	NP	NP

[a] U represnets unknown.

[b] NP represents an analysis with no prediction.

Detailed description of RD-12

RD-12 is present in subpopulation A.II and is absent from subpopulation A.I (Figure 4.27). RD-12 falls within a chitinase family 18 protein pseudogene (*chiA*) in subpopulation A.II (Table 4.30). Absence of RD-12 in subpopulation A.I results in an in-

frame deletion of 44 amino acids, but this shortened product (760 amino acids) is annotated as functional. Subpopulation A.II contains a premature stop codon not present in subpopulation A.I and allows for a 198 amino acid protein (Figure 4.28). RD-12 is absent from *F. tularensis* subsp. type B although functional homologues are found in strains OSU18 (FTH_1471) and LVS (FTL_1521). RD-12 is present within *F. tularensis* subsp. *novicida* (FTN_0627) and is predicted functional, although it is not present among all *F. tularensis* subsp. *novicida* strains (Table 4.31).

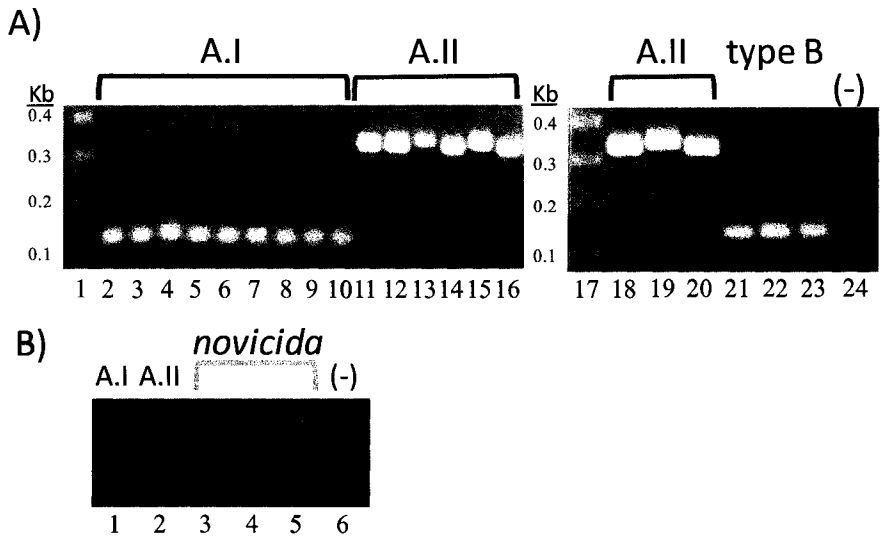


Figure 4.27 Agarose gel of PCR amplified *F. tularensis* strains using RD-12 primer set. A) PCR amplification of *F. tularensis* subpopulations A.I and A.II, and *F. tularensis* subsp. type B strains. **Lane 1:** 1 kb molecular weight ladder, **lanes 2-10:** panel of A.I strains, **lanes 11-20:** panel of A.II strains, **lanes 21-23:** panel of type B strains, **lane 24:** negative control. **B)** PCR amplification of MA00-2987 (A.I), WY96-3418 (A.II) and panel of *novicida* strains.

Table 4.30 Summary of BLAST analysis for RD-12 pseudogene *FTW_2084*.

BLAST Program	Description	E-value	Percent Identity	Percent Query coverage
blastn	*F. tularensis* subsp. *tularensis* WY96-3418	0.0E+00	100	100
	F. tularensis subsp. *holarctica* LVS	0.0E+00	98	95
	F. tularensis subsp. *holarctica* LVS	4.0E-99	94	-
	F. tularensis subsp. tularensis FSC198	0.0E+00	99	95
	F. tularensis subsp. tularensis FSC198	4.0E-99	94	-
	F. tularensis subsp. tularensis FSC198	1.0E-64	97	-
	F. tularensis subsp. *tularensis* SCHU S4	0.0E+00	99	95
	F. tularensis subsp. *tularensis* SCHU S4	4.0E-99	94	-
	F. tularensis subsp. *tularensis* SCHU S4	1.00E-64	97	-
	F. tularensis subsp. *novicida* U112	0.0E+00	98	100
	F. tularensis subsp. *novicida* U112	0.0E+00	99	-
	F. tularensis subsp. *holarctica* OSU18	0.0E+00	99	95
	F. tularensis subsp. *holarctica* OSU18	4.00E-99	94	-
	F. tularensis subsp. *holarctica* OSU18	3.00E-66	98	-
	F. tularensis subsp. *tularensis* VNTR locus Ft-M20	1.00E-64	97	14
	F. tularensis subsp. *tularensis* VNTR locus Ft-M20	6.00E-48	90	-
	F. tularensis subsp. *tularensis* VNTR locus Ft-M20	8.00E-22	98	-

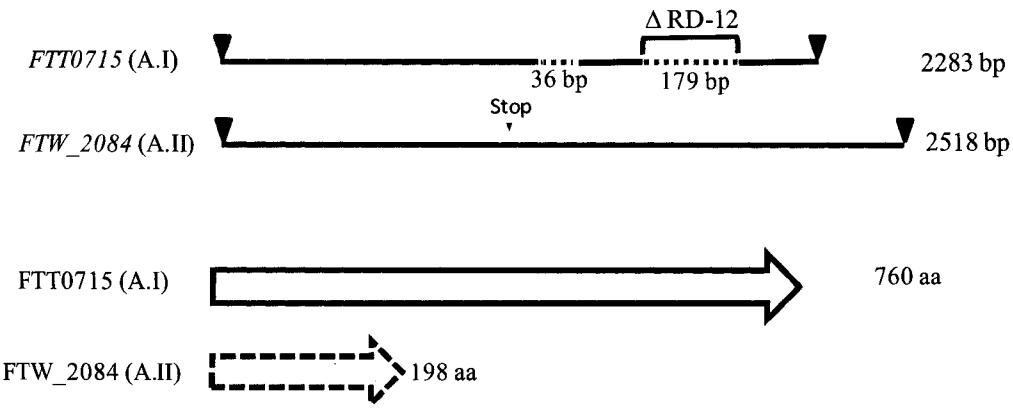


Figure 4.28 Alignment of subpopulations A.I and A.II illustrating RD-12. A) Nucleotide alignment of region encoding *FTT0715* in subpopulation A.I and its homologue *FTW_2084* in subpopulation A.II. Black line represents nucleotide sequence that is homologous between subpopulations A.I and A.II; blue triangles represent start codons; red triangles represent stop codons; yellow triangles represents a premature stop codon; dashed red line represents the absence of RD-11 in subpopulation A.I as well as another small region present in subpopulation A.II that is absent from subpopulation A.I. **B)** Amino acid sequence alignment of FTT0715 in subpopulation A.I and FTW_2084 in subpopulation A.II for which a protein predicted as functional is represented as a solid blue arrow and a protein predicted to be a pseudogene as a dashed blue arrow. Blue represents homology between the two sequences.

Table 4.31 Summary of bioinformatic analyses for RD-12 associated proteins and homologues in subpopulation A.I, and *F. tularensis* subsp. type B.

	F. tularensis Strain	ORF	Number of Amino Acids	Pseudogene	PSORT	SigP Analysis	TMHMM Analysis	Pfam Analysis
Proteins associated with RD region	WY96-3418 (A.II)	FTW_2084	198	+	U[a]	1(1-24)[b]	NP[c]	NP
	U112 (*novicida*)	FTN_0627	870	-	U	1(1-24)	NP	Glyco_hydro_18 (162-553); fn3 (570-642); CBM_5_12 (716-758, 782-821, 828-867)[d]
Homologues	SCHU (A.I)	FTT0715	760	-	U	1(1-24)	NP	Glyco_hydro_18 (162-553); fn3 (570-642); CBM_5_12 (672-711, 718-757)
	OSU18 (type B)	FTH_1471	760	-	U	1(1-24)	NP	Glyco_hydro_18 (162-553); fn3 (570-642); CBM_5_12 (672-711, 718-757)
	LVS (type B)	FTL_1521	764	-	U	1(1-24)	NP	Glyco_hydro_18 (162-553); fn3 (570-642); CBM_5_12 (676-715, 722-761)

[a] U represents unknown.

[b] Number of predictions followed in parenthesis by amino acid sequence postion.

[c] NP represents no predictions.

[d] Protein family domain followed in parenthesis by amino acid sequence postion.

Detailed description of RD-13

RD-13 is present in subpopulation A.II and absent from subpopulation A.I (Figure 4.29). RD-13 is within a putative drug transporter pseudogene (FTW_0298) in subpopulation A.II (Table 4.32). The encoded protein is truncated in both subpopulations A.II and A.I; however in subpopulation A.I the putative product is 57 amino acids compared to 210 amino acids in subpopulation A.II. RD-13 is downstream of the premature stop codon in subpopulation A.II and its deletion is downstream of the premature stop codon in subpopulation A.I (Figure 4.30). Functional homologues are present in *F. tularensis* subsp. type B strains OSU18 (FTH_1624) and LVS (FTL_1685) and *F. tularensis* subsp. *novicida* (FTN_0312) (Table 4.33).

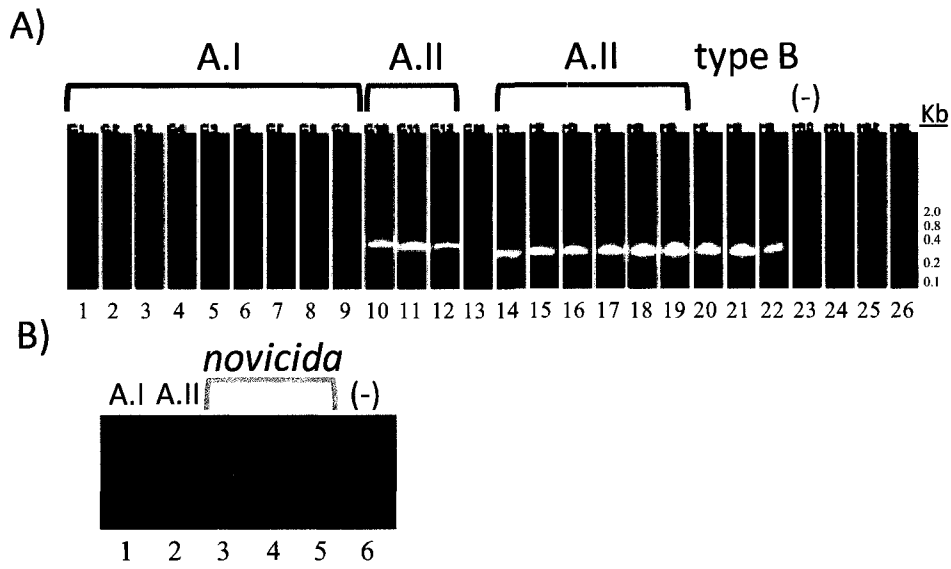


Figure 4.29 E-gel of PCR amplified *F. tularensis* strains using RD-13 primer set. A) PCR amplification of *F. tularensis* subpopulations A.I and A.II and *F. tularensis* subsp. type B strains. **Lane 1-9:** panel of A.I strains, **lanes10-12:** panel of A.II strains, **lane 13:** molecular weight standard, lanes 14-19: panel of A.II strains, **lanes 11-17:** panel of A.II strains, **lane 18:** molecular weight standard, **lanes 20-22:** panel of type B strains, **lane 23:** negative control, **lanes 24-25:** blank, **lane 26:** molecular weight standard. **B)** PCR amplification of MA00-2987 (A.I), WY96-3418 (A.II) and panel of *novicida* strains.

Table 4.32 Summary of blastn analysis for RD-13 pseudogene *FTW_0298.*

BLAST Program	Description	E-value	Percent Identity	Percent Query coverage
blastn	*F. tularensis* subsp. *tularensis* WY96-3418	0.0E+00	100	100
	F. tularensis subsp. *holarctica* OSU18	0.0E+00	98	100
	F. tularensis subsp. *holarctica* LVS	0.0E+00	98	100
	F. tularensis subsp. *novicida* U112	0.0E+00	98	100
	F. tularensis subsp. *tularensis* FSC198	0.0E+00	99	80
	F. tularensis subsp. *tularensis* FSC198	5.0E-76	99	-
	F. tularensis subsp. *tularensis* SCHU S4	0.0E+00	99	80
	F. tularensis subsp. *tularensis* SCHU S4	5.00E-76	99	-

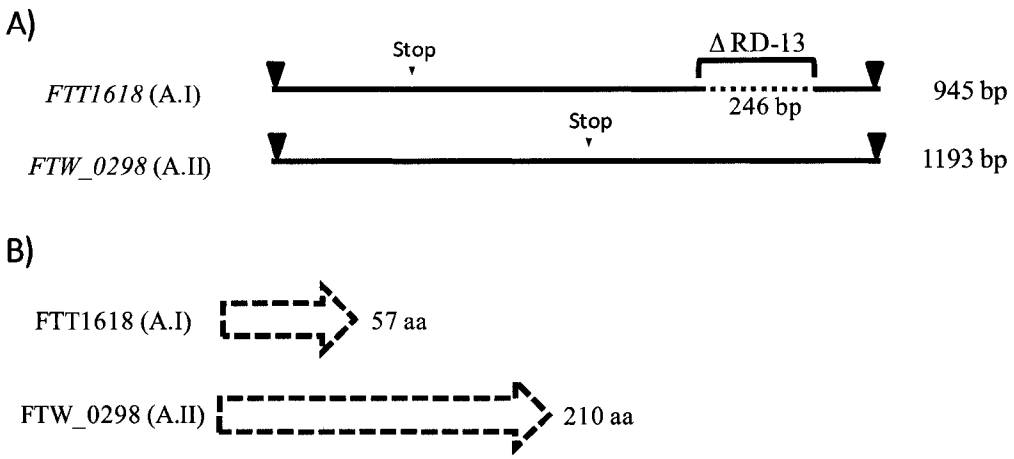


Figure 4.30 Alignment of subpopulations A.I and A.II illustrating RD-13. A) Nucleotide alignment of *FTT1618* in subpopulation A.I and its homologue *FTW_0298* in subpopulation A.II. Black line represents nucleotide sequence that is homologous between subpopulations A.I and A.II; blue triangles represent start codons, yellow triangles represent premature stop codons; red triangles represent stop codons; dashed red line represents the absence of RD-13 in subpopulation A.I. **B)** Amino acid sequence alignment of pseudogenes FTT1618 in subpopulation A.I and its pseudogene homologue FTW_0298 in subpopulation A.II represented by dashed blue arrows. Blue represents homology between the two sequences.

Table 4.33 Summary of bioinformatic analyses for RD-13 associated proteins and homologue in subpopulation A.I.

	F. tularensis strain	ORF	Number of Amino Acids	Pseudogene	PSORT	SignalP Analysis	TMHMM Analysis	Pfam Analysis
Proteins associated with RD region	WY96-3418 (A.I)	FTW_0298	210	+	Cyto-Mem[a]	1(1-29)[b]	6(13-30, 53-72, 79-101, 105-127, 140-162, 172-191)	MSF_1(19-190), sugar_tr(53-117)[c]
	OSU18 (type B)	FTH_1624	395	-	Cyto-Mem	1(1-29)	12(13-30, 53-72, 79-101, 105-127, 140-162, 172-194, 214-236, 251-269, 309-331, 344-366, 370-389)	MSF_1(19-377), sugar_tr(53-117)
	LVS (type B)	FTL_1685	395	-	Cyto-Mem	1(1-29)	12(13-30, 53-72, 79-101, 105-127, 140-162, 172-194, 214-236, 251-269, 282-304, 309-331, 344-366, 370-389)	MSF_1(19-377), sugar_tr(53-117)
	U112 (*novicida*)	FTN_0312	396	-	Cyto-Mem	1(1-29)	12(13-30, 53-72, 79-101, 105-127, 140-162, 172-194, 214-236, 251-269, 282-304, 309-331, 340-362, 372-390)	MSF_1(19-363), sugar_tr(53-117)
Homologue	SCHU (A.I)	FTT1618	57	+	U[d]	1(1-29)	1(13-30)	NP

[a] Cyto-Mem represents proteins predicted to be localized to the cytoplasmic membrane.

[b] Number of predictions followed in parenthesis by amino acid sequence postion.

[c] Protein family domain followed in parenthesis by amino acid sequence postion.

[d] U represents unknown.

A large number of the proteins encoded within the RDs were annotated as conserved hypothetical proteins. Thus additional BLAST analysis was performed to obtain potential insight into the function of the conserved hypothetical proteins. BLAST analysis of the nine RD-associated hypothetical proteins against the non-redundant database predicted similarity for two subpopulation A.I (strain SCHU S4) hypothetical proteins encoded by other bacterial genomes. Specifically, hypothetical protein FTT0096 (RD-8) shows weak similarity in a 251 amino acid region of a putative biotin dependent carboxylase of *Bacillus circulans* (*E*-value of 2e-8). Hypothetical protein FTT1078c (RD-11) showed similarity to several regions of the FrpA/C-related proteins of *N. meningitidis*. These regions of homology ranged in size from 157 to 186 amino acids and yielded *E*-values ranging from 4e-36 to 7e-33.

The analyses for the 5 conserved, IS element associated RDs are summarized below in Table 4.34.

Table 4.34 Summary of conserved, IS element associated RDs

Clone	Size of cloned insert (bp)	Size of mapped RD (bp)	RD present within *F. tularensis*				Mapped Location	Comments
			A.I	A.II	B[a]	N[b]		
TN-1	293	119	+	-	+	-	352550-352668, 381229-381347, and 1286318-1286436	Maps to region found in triplicate in the A.I genome with the RD falling between an ISFtu2 fragment and a hypothetical protein
TN-2	245	17	-	+	ND[c]	ND	744356-744373	Maps to intergenic region between ISFtu2 (FTW_0764) and Glutamate/Leucine/Phenylalanine/Valine dehydrogenase (FTW_0766)
TN-3	528	1155	-	+	+	+	792288-793441	In A.II, 477 bp of TN-3 fall within a putative retron reverse transcriptase pseudogene and 678 bp of the downstream intergenic region
TN-4	191	ND	+	-	+	ND	ND	-
TN-5	244	67	-	+	-	ND	ND	-

[a] B represents a panel of *F. tularensis* subsp. *holarctica* strains.

[b] N represents a panel of *F. tualrensis* subsp. *novicida* strains.

[c] ND represents not determined.

4.3.5 PCR assays for differentiation of subpopulation A.I from subpopulation A.II

Two regions of difference (RD-3 and RD-6) were targeted for development of a multiplex diagnostic PCR that could identify subpopulations A.I and A.II as well as discriminate from *F. tularensis* subspp. type B and *novicida.* The RD-3 primer set (Table 4.1) amplifies a product in subpopulation A.I (570 bp) and not subpopulation A.II, whereas the RD-6 primer set (Table 4.1) results in an amplicon for subpopulation A.II (396 bp) and not for subpopulation A.I. PCR was performed using DNA from a panel of *F. tularensis* subspp. type A (A.I and A.II), type B, and *novicida* strains (Table 4.3). Both of these primer sets amplify the corresponding regions in *F. tularensis* subspp. type B and *novicida* strains. However, when these two primer sets are used in a multiplex PCR, three different banding patterns are observed that distinguish subpopulation A.I from subpopulation A.II strains and further differentiate these two subpopulations from *F. tularensis* subspp. type B and *novicida* strains (Figure 4.31).

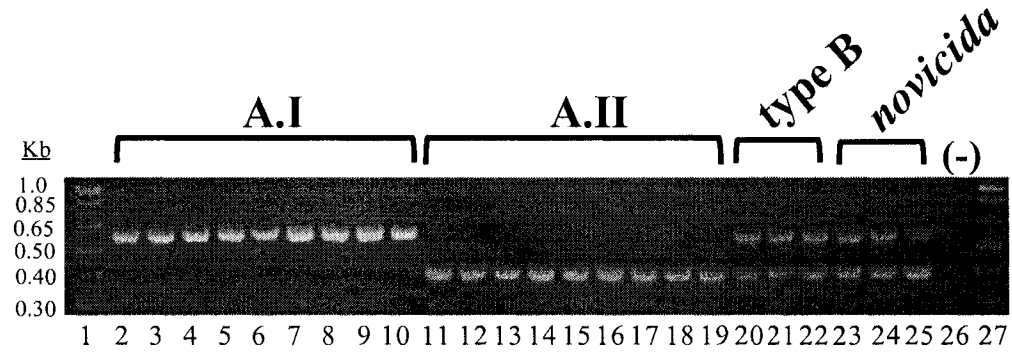


Figure 4.31 Multiplex PCR assay for differentiation of subpopulation A.I and A.II strains. PCR assay differentiating A.I from A.II using two RD primer sets: RD-3 and RD-6. **Lane 1**: 1 kb molecular weight ladder, **lane 2**: NE03-1457, **lane 3**: MA00-2987, **lane 4**: Schu S4, **lane 5**: OK01-2528, **lane 6**: SD00-3147, **lane 7**: NY04-2526, **lane 8**: VA00-1000, **lane 9**: M002-1911, **lane 10**: AR99-3448, **lane 11**: WY01-3847, **lane 12**: WY96-3418, **lane 13**: GA02-5453, **lane 14**: ID04-2687, **lane 15**: WY03-1228, **lane 16**: CA02-0099, **lane 17**: AZ01-4999, **lane 18**: UT02-1927, **lane 19**: WY01-3911, **lane 20**: KY99-3387, **lane 21**: LVS, **lane 22**: OR96-0246, **lane 23**: GA99-3448, **lane 24**: GA99-3449, **lane 25**: GA99-3550, **lane 26:** negative control, **lane 27**: 1 kb molecular weight ladder.

An A.I-specific PCR was also developed by targeting the junction region resulting from the deletion of RD-10 in subpopulation A.I. The forward primer RD-10J-F (Table 4.1) was designed with three base pair changes in order to ensure specificity for subpopulation A.I (Figure 4.32).

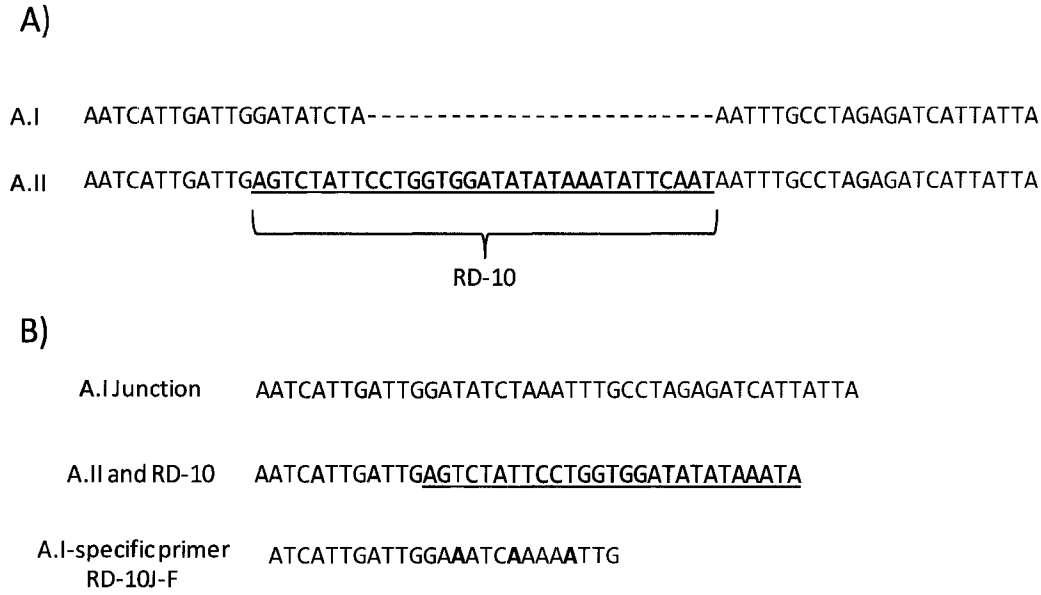


Figure 4.32 Subpopulation A.I-specific primer design. **A)** Alignment of subpopulations A.I and A.II showing the presence of RD-10 in subpopulation A.II and absence of RD-10 in subpopulation A.I. Red and blue represent the A.I junction region. The black letters in the A.II sequence represent nucleotides that differ from the A.I sequence. The underlined sequence represents RD-10. **B)** Subpopulation A.I junction is shown first. Red and blue represent the junction sequence present in A.I and where they meet represents where RD-10 is present in A.II and absent in A.I. A.II region with RD-10 is shown below. The black letters in the A.II sequence represent nucleotides that differ from the A.I sequence. The underlined sequence represents RD-10. The bottom sequence is primer RD-10J-F targeting the junction region and specific for A.I. Green letters show the nucleotides that were changed from thymine to adenine for specificity to subpopulation A.I.

The primer set for this junction was used to amplify DNA employing the panel of *F. tularensis* subspp. type A (A.I and A.II), type B, and *novicida* strains. All subpopulation A.I strains were amplified and no cross-reactivity was observed with *F. tularensis* subsp. type A subpopulation A.II, *F. tularensis* subspp. type B, or *F. tularensis* subsp. *novicida* strains. Thus, demonstrating use of this primer set as a diagnostic specific for subpopulation A.I (Figure 4.33).

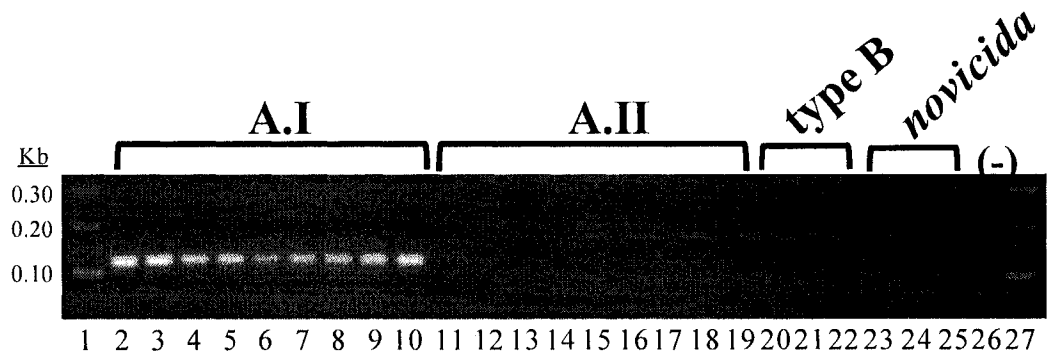
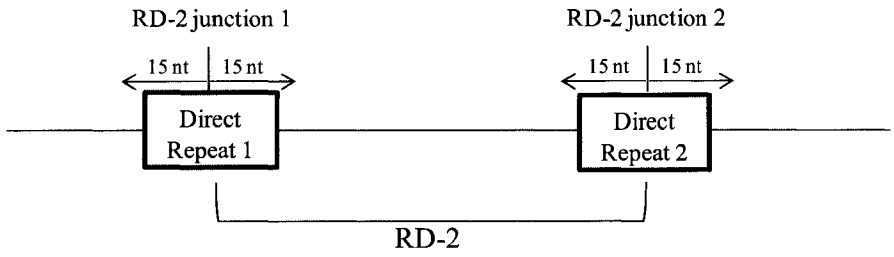


Figure 4.33 Subpopulation A.I-specific assay. An A.I-specific PCR assay was developed using primer primer set RD-10J-F and RD-10J-R. **Lane 1**: 1 kb molecular weight ladder, **lane 2**: NE03-1457, **lane 3**: MA00-2987, **lane 4**: Schu S4, **lane 5**: OK01-2528, **lane 6**: SD00-3147, **lane 7**: NY04-2526, **lane 8**: VA00-1000, **lane 9**: M002-1911, **lane 10**: AR99-3448, **lane 11**: WY01-3847, **lane 12**: WY96-3418, **lane 13**: GA02-5453, **lane 14**: ID04-2687, **lane 15**: WY03-1228, **lane 16**: CA02-0099, **lane 17**: AZ01-4999, **lane 18**: UT02-1927, **lane 19**: WY01-3911, **lane 20**: KY99-3387, **lane 21**: LVS, **lane 22**: OR96-0246, **lane 23**: GA99-3448, **lane 24**: GA99-3449, **lane 25**: GA99-3550, **lane 26:** negative control, **lane 27**: 1 kb molecular weight ladder.

4.3.6 RD junctions

To assess possible mechanisms by which RDs were deleted, bioinformatic (sequence alignments) analyses were performed to determine the homology of the junctions between the RDs and adjacent sequences. Figure 4.34 summarizes the results of these alignments for 13 conserved, non-IS associated RDs. As shown, there was considerable homology (68%-100%) for the left and right junctions of 11 of the 13 RDs. Further, these left and right junction regions were oriented as direct repeats, providing evidence that RD deletions occur through homologous recombination for 11 of the RDs.

A)



RD-2 junction 1 TGTGGCAAAAAATCATT

RD-2 junction 2 TCTGGCAAAAAATCAAT

88.2% identity over a 17 bp overlap

B)

RD	Junction Alignment	nt	% ID	RD	Junction Alignment	nt	% ID
RD-1	AAACAGATT ATACAGCTT	9	77.8	RD-8	GCTGTAATTATGCCAAGAGCA GCTGTAATTGAGTTATGTGCA	21	71.4
RD-3	TAATCATCTGC TTATCATCTGC	11	90.9	RD-9	Unable to align	-	-
RD-4	ATATAAAATCAAGATGT ATTTTAATTCAACAAGT	17	70.6	RD-10	TTAAATCATT TTCAATAATT	10	80.0
RD-5	ATCAAAATGT ATCAATATTT	10	80.0	RD-11	Unable to align	-	-
RD-6	TGGCTCAAATCCCGAAG TGGCTCAGGTCAAGCAG	17	70.6	RD-12	TGGTGGACACAAGGTGAAAATCC TGGTGGACACAAGGTAATAATCC	23	91.3
RD-7	TATTTTTATTCAAAA TATTTTTATTCAAAA	15	100.0	RD-13	GGTAATTATACCTTCAGCAATATTT GATAGGTGTTGCATCGGCAATATTT	25	68.0

Figure 4.34 Alignment of junction regions for the 13 conserved non-IS element associated RDs. A) Illustration of junction regions of RD-2 that consist of direct repeat 1 and direct repeat 2 on either side of RD-2. For each junction, 15 nucleotides (nt) were taken in both directions and designated a junction region. Junction regions were aligned as shown and the percent identity determined. For RD-2, the 30 nucleotides aligned from each junction region gave a percent identity of 88.2% over a 17 nucleotide region. **B)** Junction alignment results for RD-1 and RD-3 through RD-13. RD-9 and RD-11 were not aligned due to their limited size.

4.3.7 Screening of previously identified regions unique to type A

The literature was searched for regions identified as unique to *F. tularensis* subsp. type A and these were compared between the subpopulations A.I and A.II using the whole genome sequences for *F. tularensis* subsp. type A strains SCHU S4 (AJ749949) and WY96-3418 (NC_009257) by BLAST analyses (blastn and blastp). Forty-five non-IS element associated regions were screened. Table 4.35 summarizes 11 differences identified.

Table 4.35 Summary of differences found between subpopulations A.I and A.II by screening previously identified regions unique to *F. tularensis* subsp. type A

Region Screened	Presence of Sequence				Accession Number in SCHU S4	Accession Number in WY96-3418	Comments	Reference
	blastn		blastp					
	A.I	A.II	A.I	A.II				
FTT1068c	+	+	+	+	-	-	Hypothetical protein; 8 aa truncation in A.I	14
FTT1791	+	-	+	-	-	-	Hypothetical protein in A.I	14
FTT0432	+	+	+	-	-	-	Putative arginine decarboxylase in A.I	14
FTT0754c	+	+	+	-	-	-	Hypothetical membrane protein in A.I; 64 bp not present in A.II; 20 aa missing in A.II	14
FTT1122c	+	+	+	+	-	-	Hypothetical lipoprotein in A.I; 25 aa truncation in A.II at N-terminus	14
FTT1784c	+	+	+	-	-	-	Hypothetical protein in A.I	14
RD-1	+	+	+	-	FTT1579c	FTW_0348	Type III restriction enzyme	17
RD-2	+	+	+	+	FTT0126	FTW_0214	Oligopeptide transporter, subunit F, ABC transporter, ATP-binding protein in A.I; FTW_0214 has an N-terminal truncation compared to A.I	17
RD-5	+	+	+	-	FTT0523	FTW_1020 (pseudo)	Conserved hypothetical protein in A.I; FTW_1020 has a frameshift and is a pseudogene	17
RD-6	+	+	-	+	FTT1716/ FTT1361c	FTW_0533	Conserved hypothetical protein; both pseudogenes in A.I; one functional in A.II (FTW_0533 is upstream of *pdpD2*)	17
RD-7	+	+	-	+	FTT0427 (pseudo)	FTW_1647/ FTW_1648	Homoserine kinase; 159 bp missing in A.I	17

4.4 Discussion

Human infections caused by subpopulations A.I and A.II were previously shown to differ with respect to geographical location (Figure 4.35), modes of transmission, anatomical source of recovered isolates, and disease outcome (8, 19).

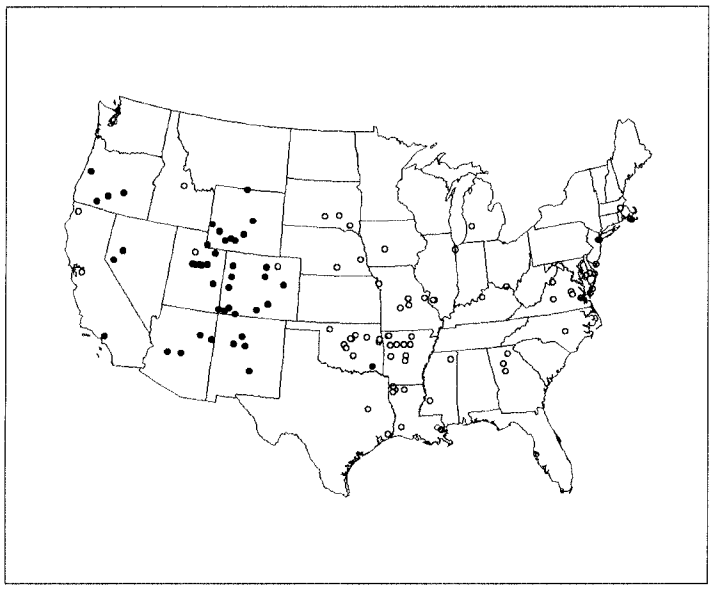


Figure 4.35 Geographical distribution of A.I and A.II strains. The map above, created by Kiersten Kugeler (CDC, Fort Collins, CO) illustrates the geographical distribution of A.I strains in orange and A.II strains in blue.

Molecular subtyping methods MLVA and PFGE resulted in two separate clusters representing the two *F. tularensis* subsp. type A subpopulations; however, the molecular differences between these two subpopulations have not been identified. In our study, SSH was used to identify RDs between the genomes of subpopulation A.I and A.II clinical isolates. The representative A.I subpopulation isolate used in this study, strain MA00-2987, was recovered from the blood of a human case of pneumonic tularemia on Martha's Vineyard in 2000. The genome sequence is not available for this subpopulation A.I strain. *F. tularensis* subsp. type A strain WY96-3418 was chosen as the A.II subpopulation representative. This isolate was recovered from a finger wound of

a human ulceroglandular case of tularemia in Wyoming in 1996. At the time this study was initiated, no whole genome sequence was available for a subpopulation A.II isolate. However, the genome sequence became available for *F. tularensis* subsp. type A strain WY96-3418 during the later course of this study and was used to verify and map the RDs present in this strain.

SSH performed in both directions (A.I-A.II and A.II-A.I) resulted in the identification of 24 RDs. For diagnostic development and identification of potential differences related to pathogenesis, it was critical to focus on analyses of RDs conserved among a panel of diverse strains. Of the 24 RDs identified, 13 were non-IS element associated and conserved across a panel of subpopulation A.I and A.II strains. Our analyses also identified five conserved RDs that are IS element associated, five RDs that were not conserved among the subpopulation A.I or A.II strains, and two RDs that were repeated (of which only one was conserved). The five conserved IS element-associated RDs were not candidates for diagnostic development because these elements are more likely to be associated with additional polymorphisms (9). Due to this reason, the focus of our study was on the 13 conserved, non-IS element associated RDs.

Based on the 13 conserved, non-IS element associated RDs, two different diagnostic PCR assays were developed. The 13 RDs were found to be present within at least one of the non-type A genomes and therefore, for the first PCR assay developed, a multiplex approach was used. The multiplex PCR assay developed is based on RD-3 and RD-6 and can distinguish between subpopulations A.I and A.II as well as differentiate these subpopulations from *F. tularensis* subspp. type B and *novicida* strains. In addition to the multiplex assay, an assay specific for subpopulation A.I was developed targeting

the junction region (representing the deleted RD in A.I) in subpopulation A.I. In several subpopulation A.I junction regions (RD-4, RD-9 and RD-10) nucleotide differences occurred and degenerate primers specific for subpopulation A.I could be designed. Thus, a PCR, based on the RD-10 junction, was developed. All subpopulation A.I isolates tested were recognized with this assay and no cross-reactivity was detected with *F. tularensis* subsp. type A subpopulation A.II, *F. tularensis* subspp. type B or *novicida* strains. An A.II-specific PCR can be developed using the same principle of targeting subpopulation A.II junction regions where RDs have been deleted. Currently, the only methods available for distinguishing these subpopulations are PFGE and MLVA, both of which are time consuming and labor intensive. The two PCR assays developed provide new diagnostic tools for identification of subpopulation A.I and A.II isolates. The conserved RDs identified here are also amenable for future development of a RT-PCR assay. Such an assay could be used in combination with existing *F. tularensis* RT-PCR assays (13, 23) so that strains could be identified at the subspecies and subpopulation level in a single run.

The RDs identified in this study were not restricted to the A.I or A.II subpopulations; they were also found in the genomes of either *F. tularensis* subspp. type B or *novicida*. Given the high similarity between the *F. tularensis* subspecies, a better approach for identification of unique subpopulation A.I and A.II RDs would have been to subtract a combined driver composed of a representative *F. tularensis* subspp. type B strain, *novicida* strain and subpopulation strain from either the subpopulation A.I or A.II representative strain. This approach has been shown to be successful in other SSH experiments (2). The results obtained were consistent with the study of Svensson *et al.*

that compared genomic differences between *F. tularensis* subspp. type A, type B, *novicida* and *mediasiatica* that did not find any unique RDs for one given subsp. However, the RDs identified by Svensson *et al.* were considerably larger (300 bp to ~11,500 bp) than those found in our study (20).

RDs, such as those found in this study, could result from genetic deletions or insertions. It is likely that direct repeat mediated homologous recombination was the primary mechanism leading to the subpopulation A.I and A.II RDs, since direct repeats such as those described for other deletions within the *F. tularensis genomes* (3) were observed flanking the RDs. The percent identities for the junction alignments ranged from 68% to 100% over a range of 9 to 25 nucleotides. These findings are similar to the 9- to 38-bp direct repeat motifs flanking RD regions described previously for *Francisella* (3). It is unclear whether homologous recombination was the mechanism for all RDs, in particular the smaller RDs, RD-9 and RD-11 that are 16 bp and 15 bp respectively. Alignments could not be performed for these RDs due to their size limitation. Short sequences are not ideal for RecA-mediated recombination and therefore short sequences may have been deleted by errors during chromosomal replication or by non-specific recombination events. Of the 13 non-IS associated RDs identified here, 12 were present in *F. tularensis* subsp. *novicida*, supporting the idea that at least these 12 RDs arose via a recombination mediated deletion since this subspecies is considered evolutionarily the oldest (20). RD-11 is not present in *F. tularensis* subsp. *novicida* and whether this RD is an insertion or a deletion is unclear.

Genome reduction is a phenomenon characteristic of multiple bacterial pathogens with narrowly restricted biological niches (4, 6, 27). Like other intracellular pathogens,

F. tularensis subspp. type A and type B contain large numbers of putative pseudogenes, 201 and 325 respectively (16). It is unknown whether these pseudogenes are expressed and if so whether these proteins are functional or if function is altered. All of the genes associated with the conserved RDs identified in this study were present and annotated as functional in the genome of *F. tularensis* subsp. *novicida,* also consistent with previous observations that this subsp. is evolutionarily oldest and an environmental strain (20). Interestingly, among the conserved RDs identified in this study, RDs were more commonly missing in subpopulation A.I as compared to subpopulation A.II, suggesting that the A.I subpopulation may have undergone greater gene reduction than subpopulation A.II and may have a more specialized niche as compared to subpopulation A.II. The comparison of complete genome sequences from multiple subpopulation A.I and A.II isolates will further elucidate the evolutionary connection between the subpopulations.

We hypothesized that identification of major genomic differences between subpopulations A.I and A.II via SSH would provide potential leads to explain differences in the disease outcomes associated with subpopulation A.I and A.II isolates. Differences in hypothetical proteins accounted for much of the genomic variability associated with the defined RDs. For example, an in-frame deletion leads to removal of an N-terminal signal sequence in a hypothetical protein in subpopulation A.I strains, with the resulting prediction that this protein is secreted in subpopulation A.II (FTW_1826), but not in subpopulation A.I (FTT0267). Interestingly, in *F. tularensis* subsp. type B (OSU18) this hypothetical protein is annotated as a pseudogene due to truncation by a premature stop codon resulting from a frameshift, and in *F. tularensis* subsp. *novicida,* it is predicted as

functional and secreted. Differences in secretion are already noted to correlate with virulence in *F. tularensis*. In particular, the secretion of PepO of *F. tularensis* subsp. *novicida* is linked to reduced virulence of this bacterium, and the more virulent *F. tularensis* subspp. type A and type B contain a mutation in the PepO coding sequence that abolishes secretion (10). Other hypothetical proteins are absent from either subpopulations A.I (FTW_0888 and FTW_0889) or A.II (FTT1733) strains, or are predicted to be pseudogenes (FTT1195c, FTT1429c, FTW_1356, FTW_1384, and FTW_2084). With respect to proteins that are assigned functionality by sequence homology, the protein FTT1078c that displays similarity to the Frp proteins in *Neisseria meningitides* and other repeat-in-toxin (RTX) proteins is predicted functional in subpopulation A.I and is annotated as a pseudogene in subpopulation A.II (21, 22). In *N. meningitides*, the iron-regulated *frp* genes (*frpA*, *frpC* and *frpC*-like) encode large secreted proteins of unknown biological activity (15). These proteins contain an RTX motif and are therefore thought to act as cytotoxins that may play important for virulence. The significance of this protein in subpopulation A.I is unclear, as the similarity observed occurs only over a 185 and 157 amino acid region of the FrpA and FrpC proteins of *N. meningitidis* (serogroup C). Nonetheless, Frp-like proteins are linked to virulence in a broad range of pathogenic Gram-negative bacteria (24), and the predicted protein in subpopulation A.I may be of interest for further study(24).

Differences between the *chiA* (chitinase gene) were also found between *F* subpopulations A.I and A.II. ChiA is predicted functional in subpopulation A.I and as a pseudogene in subpopulation A.II (regardless of the presence of RD-12 within A.II). Differences were also found among *chiA* and other predicted chitinase genes (including

chiB and a chitinase-binding protein gene) when compared across the genomes of *F. tularensis* subsp. type A (A.I and A.II), type B, and *novicida*. In *F. tularensis* subsp. type A subpopulation A.I, ChiA, ChiB and a truncated chitin-binding protein are annotated as functional, although ChiB lacks a signal sequence and is not predicted to be secreted. *F. tularensis* subsp. type A subpopulation A.II has one chitinase (non-ChiA/B) and a chitin-binding protein, both annotated as functional. *F. tularensis* subsp. type B contains three chitinases (ChiA and ChiB and a non-ChiA/B chitinase) and a chitin-binding protein (non-secreted) predicted functional; *F. tularensis* subsp. *novicida* has two chitinases annotated as functional (ChiA and ChiB) and a chitin-binding protein. This is of interest since previous studies demonstrate a role for chitinases in parasite transmission, during *in vivo* infections by *Legionella pneumophila*, and for environmental survival (5, 11). Thus, these proteins may have some role in determining the different host vectors between subpopulations A.I and A.II, as well as environmental survival or possibly having a role in modification of host environment leading to differential disease outcome.

The remaining conserved, non-IS element associated RDs not discussed in detail appear to be important for environmental survival and metabolism. These include a magnesium chelatase, several transporters, and a DNA uptake protein among others. Obvious links to pathogenesis were not found with these RDs, however, given the little information that is known about this bacterium and its maintenance within the environment, any of these RDs could potentially be important differences between subpopulations A.I and A.II contributing to its ability to survive and cause infection.

Additional findings are specific to *F. tularensis* subsp. *novicida*. Two genes associated with RD-3 and RD-9 (FTN_1172, a hypothetical protein, and FTN_1665, a

magnesium chelatase) are predicted functional (non-pseudogenes) only in *F. tularensis* subsp. *novicida* and one (FTN_0269, a C4-dicarboxylate anaerobic carrier) is predicted functional only in *F. tularensis* subsp. type B (strain LVS) and *novicida*. Further studies are necessary to determine if other functional homologues to these proteins are present in the other subspecies and to determine the importance of these proteins in *F. tularensis* subsp. *novicida*.

IS elements have been shown to be a source of genomic variability between similar bacterial strains and have been recognized as the cause for the numerous rearrangements between the *F. tularensis* subspp. type A and type B genomes (16). Given the difference in number and type of IS element-associated regions within the different *F. tularensis* subspecies (79 in type A subpopulation A.I strain SCHU S4, 109 in type B OSU18, and 26 in *F. tularensis* subsp. *novicida*), analyses of RDs associated with these elements may provide insight into the differences described for the different subspecies, including virulence factors. Five conserved, IS element-associated RDs were identified in this study. Mapping of these RDs was challenging given the numerous blastn hits obtained when analyzing the sequences and two RDs, TN-4 and TN-5 will require further sequence analysis for precise mapping. TN-1 through TN-3 are not within regions that have an obvious link to pathogenesis; however, further studies and detailed mapping and analysis of these and adjacent regions are needed to better address this question.

Previous studies comparing *F. tularensis* subspp. type A to type B identified RDs and ORFs unique or only functional in type A (3, 17). Screening of these regions using BLAST analyses revealed several differences between subpopulations A.I and A.II.

Nucleotide sequences for all regions were present in both subpopulations A.I and A.II with one exception, the sequence encoding hypothetical protein FTT1791 was found only in subpopulation A.I with no protein homologues in subpopulation A.II. The remaining differences observed were due to predicted pseudogenes for subpopulations A.I or A.II with its homologue in the other subpopulation predicted functional. Of particular interest are RD-6 and RD-7. RD-6, a conserved hypothetical protein found in duplicate in subpopulation A.I (both annotated as pseudogenes) was found in subpopulation A.II directly upstream of the virulence factor *pdpD2*, that encodes for the pathogenicity determinant protein D (14). RD-7 was found to be associated with a homoserine kinase that is predicted functional in subpopulation A.II with no apparent functional homologue in subpopulation A.I. Two membrane-associated hypothetical proteins were also among some of the differences identified. A hypothetical membrane protein (FTT0754c) and hypothetical lipoprotein (FTT1122c) are predicted functional in subpopulation A.I with no homologue in subpopulation A.II. Differences found in these regions were found by bioinformatic analyses and require panel verification to determine if they are conserved within the subpopulations. These RDs offer additional options for diagnostic development and targets for future studies.

The list of RDs identified in this study is not a complete list of RDs between subpopulation A.I and A.II. While further studies are required to determine the true biological relevance of any of the gene products encoded by the RDs, this study, along with future genome comparisons of subpopulations A.I and A.II, provide a basis for the development of hypotheses that can be experimentally tested.

4.5 Literature cited

1. **Akopyants, N. S., A. Fradkov, L. Diatchenko, J. E. Hill, P. D. Siebert, S. A. Lukyanov, E. D. Sverdlov, and D. E. Berg.** 1998. PCR-based subtractive hybridization and differences in gene content among strains of *Helicobacter pylori*. Proc Natl Acad Sci U S A **95:**13108-13.
2. **Bae, J. W., S. K. Rhee, Y. D. Nam, and Y. H. Park.** 2005. Generation of subspecies level-specific microbial diagnostic microarrays using genes amplified from subtractive suppression hybridization as microarray probes. Nucleic Acids Res **33:**e113.
3. **Broekhuijsen, M., P. Larsson, A. Johansson, M. Bystrom, U. Eriksson, E. Larsson, R. G. Prior, A. Sjostedt, R. W. Titball, and M. Forsman.** 2003. Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent F. tularensis subsp. tularensis. J Clin Microbiol **41:**2924-31.
4. **Cole, S. T., K. Eiglmeier, J. Parkhill, K. D. James, N. R. Thomson, P. R. Wheeler, N. Honore, T. Garnier, C. Churcher, D. Harris, K. Mungall, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. M. Davies, K. Devlin, S. Duthoy, T. Feltwell, A. Fraser, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, C. Lacroix, J. Maclean, S. Moule, L. Murphy, K. Oliver, M. A. Quail, M. A. Rajandream, K. M. Rutherford, S. Rutter, K. Seeger, S. Simon, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, K. Taylor, S. Whitehead, J. R. Woodward, and B. G. Barrell.** 2001. Massive gene decay in the leprosy bacillus. Nature **409:**1007-11.
5. **DebRoy, S., J. Dao, M. Soderberg, O. Rossier, and N. P. Cianciotto.** 2006. *Legionella pneumophila* type II secretome reveals unique exoproteins and a chitinase that promotes bacterial persistence in the lung. Proc Natl Acad Sci U S A **103:**19146-51.
6. **Dobrindt, U., and J. Hacker.** 2001. Whole genome plasticity in pathogenic bacteria. Curr Opin Microbiol **4:**550-7.
7. **Ellis, J., P. C. Oyston, M. Green, and R. W. Titball.** 2002. Tularemia. Clin Microbiol Rev **15:**631-46.
8. **Farlow, J., D. M. Wagner, M. Dukerich, M. Stanley, M. Chu, K. Kubota, J. Petersen, and P. Keim.** 2005. *Francisella tularensis* in the United States. Emerg Infect Dis **11:**1835-41.
9. **Gurtler, V., and B. C. Mayall.** 2001. Genomic approaches to typing, taxonomy and evolution of bacterial isolates. Int J Syst Evol Microbiol **51:**3-16.
10. **Hager, A. J., D. L. Bolton, M. R. Pelletier, M. J. Brittnacher, L. A. Gallagher, R. Kaul, S. J. Skerrett, S. I. Miller, and T. Guina.** 2006. Type IV pili-mediated secretion modulates *Francisella* virulence. Mol Microbiol **62:**227-37.
11. **Huber, M., E. Cabib, and L. H. Miller.** 1991. Malaria parasite chitinase and penetration of the mosquito peritrophic membrane. Proc Natl Acad Sci U S A **88:**2807-10.

12. **Johansson, A., J. Farlow, P. Larsson, M. Dukerich, E. Chambers, M. Bystrom, J. Fox, M. Chu, M. Forsman, A. Sjostedt, and P. Keim.** 2004. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J Bacteriol **186:**5808-18.
13. **Kugeler, K. J., R. Pappert, Y. Zhou, and J. M. Petersen.** 2006. Real-time PCR for *Francisella tularensis* types A and B. Emerg Infect Dis **12:**1799-801.
14. **Nano, F. E., N. Zhang, S. C. Cowley, K. E. Klose, K. K. Cheung, M. J. Roberts, J. S. Ludu, G. W. Letendre, A. I. Meierovics, G. Stephens, and K. L. Elkins.** 2004. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. J Bacteriol **186:**6430-6.
15. **Osicka, R., J. Kalmusova, P. Krizova, and P. Sebo.** 2001. *Neisseria meningitidis* RTX protein FrpC induces high levels of serum antibodies during invasive disease: polymorphism of frpC alleles and purification of recombinant FrpC. Infect Immun **69:**5509-19.
16. **Petrosino, J. F., Q. Xiang, S. E. Karpathy, H. Jiang, S. Yerrapragada, Y. Liu, J. Gioia, L. Hemphill, A. Gonzalez, T. M. Raghavan, A. Uzman, G. E. Fox, S. Highlander, M. Reichard, R. J. Morton, K. D. Clinkenbeard, and G. M. Weinstock.** 2006. Chromosome rearrangement and diversification of *Francisella tularensis* revealed by the type B (OSU18) genome sequence. J Bacteriol **188:**6977-85.
17. **Rohmer, L., C. Fong, S. Abmayr, M. Wasnick, T. J. Larson Freeman, M. Radey, T. Guina, K. Svensson, H. S. Hayden, M. Jacobs, L. A. Gallagher, C. Manoil, R. K. Ernst, B. Drees, D. Buckley, E. Haugen, D. Bovee, Y. Zhou, J. Chang, R. Levy, R. Lim, W. Gillett, D. Guenthener, A. Kang, S. A. Shaffer, G. Taylor, J. Chen, B. Gallis, D. A. D'Argenio, M. Forsman, M. V. Olson, D. R. Goodlett, R. Kaul, S. I. Miller, and M. J. Brittnacher.** 2007. Comparison of *Francisella tularensis* genomes reveals evolutionary events associated with the emergence of human pathogenic strains. Genome Biol **8:**R102.
18. **Sjostedt, A. B.** 2005. *Francisella.* p.200-210. *In* D. J. Brenner, N. R. Krieg, J. T. Staley, and G. M. Garrity (ed.), The Proteobacteria, part B. Bergey's manual of systematic bacteriology, 2nd ed., vol. 2. Springer-Verlag, New York, N. Y.
19. **Staples, J. E., K. A. Kubota, L. G. Chalcraft, P. S. Mead, and J. M. Petersen.** 2006. Epidemiologic and molecular analysis of human tularemia, United States, 1964-2004. Emerg Infect Dis **12:**1113-8.
20. **Svensson, K., P. Larsson, D. Johansson, M. Bystrom, M. Forsman, and A. Johansson.** 2005. Evolution of subspecies of *Francisella tularensis.* J Bacteriol **187:**3903-8.

21. **Tettelin, H., N. J. Saunders, J. Heidelberg, A. C. Jeffries, K. E. Nelson, J. A. Eisen, K. A. Ketchum, D. W. Hood, J. F. Peden, R. J. Dodson, W. C. Nelson, M. L. Gwinn, R. DeBoy, J. D. Peterson, E. K. Hickey, D. H. Haft, S. L. Salzberg, O. White, R. D. Fleischmann, B. A. Dougherty, T. Mason, A. Ciecko, D. S. Parksey, E. Blair, H. Cittone, E. B. Clark, M. D. Cotton, T. R. Utterback, H. Khouri, H. Qin, J. Vamathevan, J. Gill, V. Scarlato, V. Masignani, M. Pizza, G. Grandi, L. Sun, H. O. Smith, C. M. Fraser, E. R. Moxon, R. Rappuoli, and J. C. Venter.** 2000. Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. Science **287:**1809-15.

22. **Thompson, S. A., L. L. Wang, A. West, and P. F. Sparling.** 1993. *Neisseria meningitidis* produces iron-regulated proteins related to the RTX family of exoproteins. J Bacteriol **175:**811-8.

23. **Versage, J. L., D. D. Severin, M. C. Chu, and J. M. Petersen.** 2003. Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. J Clin Microbiol **41:**5492-9.

24. **Welch, R. A.** 1991. Pore-forming cytolysins of gram-negative bacteria. Mol Microbiol **5:**521-8.

25. **Winstanley, C.** 2002. Spot the difference: applications of subtractive hybridisation to the study of bacterial pathogens. J Med Microbiol **51:**459-67.

26. **Zhang, Q., U. Melcher, L. Zhou, F. Z. Najar, B. A. Roe, and J. Fletcher.** 2005. Genomic comparison of plant pathogenic and nonpathogenic *Serratia marcescens* strains by suppressive subtractive hybridization. Appl Environ Microbiol **71:**7716-23.

27. **Zomorodipour, A., and S. G. Andersson.** 1999. Obligate intracellular parasites: *Rickettsia prowazekii* and *Chlamydia trachomatis*. FEBS Lett **452:**11-5.

Chapter V

Final Discussion

F. tularensis is one of the most pathogenic bacteria known (6). The use of this pathogen as a potential bioweapon has renewed research interest for this bacterium (5, 6, 24). Recent identification of subpopulations within *F. tularensis* subsp. type A demonstrates the advances in molecular methods developed for typing *Francisella* strains (10, 33), but also brings forth numerous gaps in our understanding of this bacterium in regards to its evolution, ecology and pathogenesis. It was our goal through subtractive hybridization to identify genomic regions of difference between the two *F. tularensis* subsp. type A subpopulations (A.I and A.II) to facilitate target identification for diagnostic methods, as well as to identify genomic regions that may be related to the differential disease outcomes described for the two subpopulations (33). Through this work, we were able to develop the first PCR assay for differentiating A.I and A.II strains, and we described 18 regions of difference between the two subpopulations for which comparisons between the two subpopulations, *F. tularensis* subspp. type B, and *novicida* were also performed.

In a separate study, aerobactin produced by *E. coli* was identified as an inhibitory substance against *F. tularensis*. The relevance of this finding is unclear at this time, but does provoke interesting hypotheses for future work aiming to better understand this

bacterium's ecology. This includes the ability of *F. tularensis* to survive in the presence of other bacteria within clinical samples or possibly within the environment.

5.1 Relevance of RDs for future directions for *Francisella* detection and diagnostics

As discussed in Chapter 3, it was our goal to develop a PCR assay capable of differentiating A.I from A.II and to show proof of concept for the use of identified RDs in the development of a RT-PCR assay. A RT-PCR assay that can differentiate A.I from A.II would compliment the existing RT-PCR assays available for *Francisella* (19, 41). With the assays developed as part of this dissertation it is now possible to accurately differentiate A.I and A.II. Differentiation of A.I and A.II is important in the surveillance of these two strains in North America and a useful tool for investigating natural outbreaks. Additionally, in order to better understand the ecology and evolution of the highly virulent *F. tularensis* subsp. type A strains, it is necessary to have appropriate diagnostic tools that reliably identify these strains in an easy, affordable and convenient manner. In July 2007, an outbreak of 16 tularemia cases occurred in Utah Lake among 275 youths attending a camp. The outbreak was associated with deer-fly exposure and evidence of a rabbit die-off was reported. During the investigation of the outbreak, lead by a team of CDC scientists, deerflies and rabbit carcasses were collected and human samples were sent to CDC. Five of the human cases were culture confirmed as *F. tularensis* subsp. type A. PFGE was performed on the isolates and resulted in three typing as subpopulation A.I and two as subpopulation A.II. Real-time PCR was performed on bone marrow samples from the rabbit carcasses and 11 of 12 rabbits were positive for *F. tularensis* subsp. type A. The multiplex assay developed in this study was used to determine if the rabbits were infected with *F. tularensis* subsp. type A.I or A.II.

Due to the low sensitivity of conventional PCR, only six of the rabbit isolates were typed to the subpopulation level; however, four were positive for A.I and one was positive for A.II. These results demonstrate that both A.I and A.II isolates are found within this area and could have simultaneously caused this outbreak. This is the first evidence suggestive of two different isolate types causing a tularemia outbreak and opens up many new questions concerning the maintenance and transmission of *F. tularensis* subsp. *tularensis*.

As Chapter 2 discusses, there are several subtyping methods developed for *Francisella* (9, 11, 13, 17, 37). Among these, MLVA efficiently provides the strongest resolution between strains (9, 10, 16, 17), but is currently the only method available that will reliably provide this power of resolution. A single nucleotide polymorphism (SNP) analysis has not been developed for *F. tularensis*. Several groups are working on this technology and their efforts should provide additional means for subtyping *Francisella* strains (18). Moreover, as whole genome sequences becomes more readily available, it is not unlikely that every strain will be sequenced and that this will be the definitive method for understanding evolution as well as for forensic analyses. The current methods will remain as tools for quicker, more specific comparisons between strains, but ultimately, each strain will have its own complete "fingerprint".

Given the weaponization of *F. tularensis* subsp. type A in the past, and the little we know and understand about this bacterium's ecology, the need for rapid and accurate diagnostics for the detection of this bacterium in a clinical setting and the environmental is warranted. Furthermore, the potential use of this bacterium as a bioweapon enhances the need for surveillance and evolutionary characterization of *Francisella* strains, particularly that of *F. tularensis* subspp. type A and type B strains. Current clinical

diagnostics for tularemia use traditional methods such as culturing, immunofluorescence, and serology (36). Culturing this fastidious bacterium can be challenging and is not always possible. The introduction of CHAB-A medium has tremendously improved the ability to culture this bacterium from samples containing other bacteria, but even with this advancement, cultures are not always obtained (26).

The current diagnostics for tularemia are effective for diagnosing and confirming infections; however, most are not rapid methods and are labor intensive. The development of a defined diagnostic for both clinical and environmental detection of *F. tularensis*, mainly those that are rapid and handheld would be beneficial. Tetracore (Rockville, MD) and New Horizons Diagnostics Inc (Columbia, MD) have developed rapid lateral flow diagnostics for tularemia. The SMART™ Tularemia diagnostic developed by New Horizons Diagnostics Inc takes only 20 seconds and can be stored at room temperature. Although both companies offer rapid diagnostics for tularemia, neither of them have disclosed the target antigen used or have tested their product. Given the few number of tularemia cases that occur, particularly within the United States, testing any new diagnostics is difficult. In particular, the development of a defined rapid diagnostic that could be used to assay for *F. tularensis* in the environment and to directly test environmental samples would be useful. A similar format has been used successfully by Binax, Inc. (Scarborough, ME) to develop a rapid immunochromatographic diagnostic Now® *Strep* A for detection of *Streptococcus pyogenes* Group A antigen from throat samples. The test requires three simple steps and can be performed in five minutes. The advantages to this type of diagnostic are that it does not require a host antibody response, typically it is highly sensitive and specific (Now® *Strep* A has a sensitivity of 92% and

specificity of 100%, with an overall agreement of 98%), it is a standardized diagnostic from one laboratory to another, the sample is contained within the apparatus used, it does not require additional equipment, and necessitates only minimal training to perform (14). In addition, because the detection targets a specific product or enzyme produced of the bacterium, and it does not require the presence of whole cells, giving this format a much higher sensitivity as compared to other methods. Since it's 1986 FDA clearance for the Now® *Strep* A, Binax Inc. has developed several additional antigen-based diagnostics including NOW *Streptococcus pneumoniae* Antigen Test (7), and NOW *Legionella* Urinary Antigen Test (23), both that test urine samples. The difference in number of *Streptococcus* infections, as compared to those caused by *F. tularensis,* makes it necessary to have an assay like the Now® *Strep* A for clinics to use and allows for such diagnostics to be tested thoroughly and become FDA approved.

5.2 Relevance of current work for future directions in the study of disease ecology

The increase in *Francisella* research during the past five years has focused primarily on development of new detection methods, vaccines, as well studies on the host immune response, and more recently, studies on the pathogenesis of this organism. The amount of information known at this time is limited, but continues to increase. The current technologies and studies being performed are important, especially if the misuse of this agent were to occur or if tularemia were to become an emerging infectious disease. However, research is moving towards the development of stronger, more reliable antibiotics. Currently, there are several drugs available now that can be used to treat tularemia, all of which (including any new antibiotic developed) may or may not be useful against a strain that is engineered to be resistant to such drugs. Other studies are

focusing on the development of a new vaccine. If an attack with this agent were to occur, having an effective and defined vaccine that could be administered to all individuals would be of great importance not only for future protection but for panic control among the population.

Given that tularemia is not an infection with high priority (aside from bioterror) and that the interest in studying this disease is driven by its potential use as a biological weapon, it is unusual that a higher priority is not given to understanding the ecology of this bacterium. The consequences of releasing this agent into the environment are currently unknown, and the long-term problems associated with this are undefined. The possibility that this bacterium would continue to cause infections long after an attack in both the human and animal population is possible. Moreover, this bacterium is capable of infecting more hosts than any other known bacterium, and whether this bacterium is capable of re-emerging as the pathogen that it once was, or even as a greater potential with or without its use as a biological weapon is unknown.

Several questions surrounding the ecology of this bacterium have gone unanswered. In particular, it is surprising that we do not recover more isolates from the environment, and it is particularly interesting that *F. tularensis* subspp. type A or type B isolates have never been recovered outside of its host cells. Given the high virulence of this bacterium, it could be that *F. tularensis* subspp. type A and type B stops its own transmission by rapidly killing its host; however, this does not explain how subsequent outbreaks occur. This suggests that a reservoir exists and there must be reasons as to why it cannot maintain itself outside of a host if indeed this is the case. *F. tularensis* subsp. type B has been detected in the environment by PCR but it has never been cultured from

the environment. The inability to culture *F. tularensis* subspp. type A and type B from the environment could be attributed to its absence outside of host cells or it may be that a sufficient search has not been performed. *F. tularensis* subsp. type B has been associated extensively with natural waters, specifically with protozoa that are predicted to serve as reservoirs for this bacterium (1, 2). To date, this is not the case for *F. tularensis* subsp. type A and the reasons for this are unknown, but are of high importance in understanding this bacterium's ecology. If *F. tularensis* subspp. type A and type B strains cannot live outside of host cells, it would be of interest to investigate the differences in gene decay between these two virulent *F. tularensis* subspecies to understand why *F. tularensis* subsp. type B can be detected from water and *F. tularensis* subsp. type A cannot. Additionally, it is necessary to compare both *F. tularensis* subspp. type A and type B to the genomes of the environmental *F. tularensis* subspecies. This analysis has been done to a minimal extent by bioinformatics where the whole genomes of three strains were compared: the SCHU S4 strain of *F. tularensis* subsp. type A, the LVS strain of *F. tularensis* subsp. type B, and the U112 strain of *F. tularensis* subsp. *novicida* (29). It would be interesting to use a similar approach but on a much wider scale for more accurate comparisons, as well as to use a more relevant strain besides the LVS strain of *F. tularensis* subsp. type B.

As discussed in Chapter 4, genome reduction appears to be occurring in the pathogenic *F. tularensis* strains (21, 27). Of the 13 non-IS element associated RDs identified, 12 were present in *F. tularensis* susbp. *novicida*, supporting the idea that these 12 RDs arose via a recombination mediated deletion and suggests that genome decay is occurring within *F. tularensis* subsp. type A strains. Additionally, the genes annotated as

pseudogenes within *F. tularensis* subsp. type A were not annotated as such in *F. tularensis* subsp. *novicida* and the difference in number of pseudogenes between *F. tularensis* subspp. type A and *novicida* is large, with 201 and 14 annotated pseudogenes respectively. Further genome decay between these two subspecies is represented in several of their biochemical characteristics. For example, *F. tularensis* subsp. type A requires cysteine for growth and cannot utilize sucrose, whereas *F. tularensis* subsp. *novicida* does not require cysteine for growth and can utilize sucrose (32). All of these observations are consistent with the previous findings that *F. tularensis* subsp. *novicida* is evolutionarily the oldest and exhibits the lowest virulence among *F. tularensis* subspecies (35). It is quite possible that *F. tularensis* subsp. type A has lost the ability to survive outside of host cells due to gene decay.

Given that many of the RDs identified were associated with hypothetical proteins and pseudogenes, it would be interesting to try and determine the role (if any) of these as they relate to difference between the two subpopulations and between these and the other subspecies of *F. tularensis.* Pseudogenes are commonly defined as inactive sequences of DNA that have a similar sequence to known functional genes. Inactivation is usually due to the introduction of a premature stop codon within the coding sequence that result from frameshift mutations, or as shown in this study, deletions of DNA sequence. Pseudogenes are also considered to be evolutionarily related to normally functioning genes. Although pseudogenes are considered inactive and non-functional, this may not necessarily be the case and until pseudogenes are analyzed and tested for function (which may be altered compared to its related functioning gene), it cannot be discarded as non-

functional. In order to determine the function of pseudogenes and hypothetical proteins, a general approach, as diagramed in Figure 5.1 could be taken.

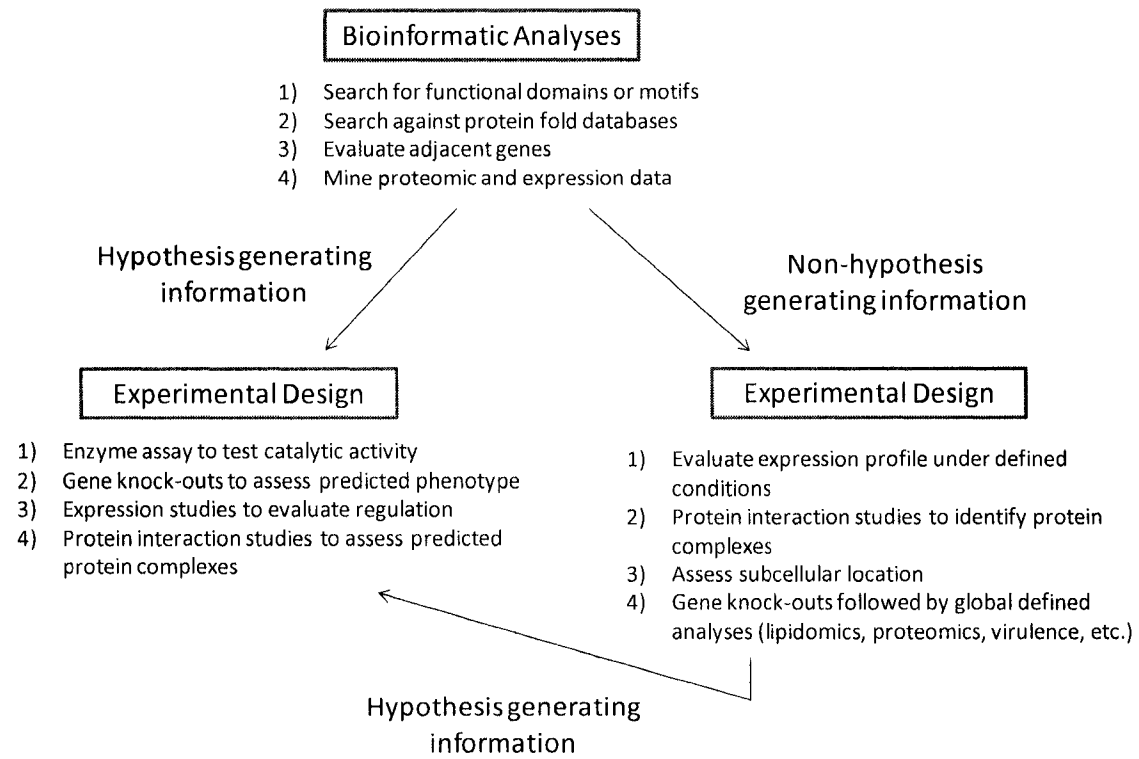


Figure 5.1 Approach for determining the function of hypothetical proteins and pseudogenes. The diagram above illustrates the approach that can be taken when trying to determine the function of a hypothetical protein or a pseudogene. The initial step is to perform thorough bioinformatic analyses of the genomic and predicted protein sequence for the region of interest. Depending on the data gathered, experimental designs for both hypothesis generating information and non-hypothesis generating information can be developed. If the bioinformatic analyses lead to non-hypothesis generating information, several experiments can be performed to gain more information so that a hypothesis can be generated and experiments to test these hypotheses can be carried out.

Keeping with the theme of gene decay and strict nutrient requirements the work described in Chapter 3 describes how *E. coli* can inhibit *F. tularensis* through its production of the siderophore aerobactin. It is unknown at this time if other siderophores would also inhibit *Francisella* and the results from this study lead to the question: are other organisms suppressing this bacterium in the environment by sequestering nutrients

such as iron. Little is understood about the iron requirements of *F. tularensis*. Fortier *et al.* (12) demonstrated using *F. tularensis* subsp. type B strain LVS (*F. tularensis* subsp. type B) that acidification of the endosome is essential for iron release from transferrin for survival of this bacterium within the macrophage. Sullivan *et al.* (34) showed the production of a siderophore structurally similar to rhizoferrin by both *F. tularensis* subsp. type B strain LVS and *F. tularensis* subsp. type A strain SCHU S4. It is interesting that rhizoferrin is a siderophore that is also produced by fungi that live at an optimal pH of 5 (3). Keeping in mind that no other iron uptake system is described for *Francisella*, it could be possible that pathogenic *F. tularensis* subspp. type A and type B strains (unlike the other *F. tularensis* species and subspecies) require an acidic environment for appropriate uptake of nutrients when under nutrient limitation. An approach to study this might include obtaining expression profiles of *F. tularensis* subsp. type A compared to the other subspecies of *F. tularensis* grown at different pH. A simpler experiment would be to measure the production of rhizoferrin at different pHs and compare the amounts produced among the other subspecies of *F. tularensis*. In addition, studies to test if *F. tularensis* can utilize other siderophores have not been performed. Interestingly, the bacterium *Morganella morganii* is found in the open environment and is also a component of the normal intestinal flora of humans, mammals and reptiles and although this organism does not produce rhizoferrin, it is known to uptake rhizoferrin and staphyloferrin A as a source of iron (20). A brief BLAST analysis of the nucleotide sequence for the described uptake system of *M. morganii* was performed against the *F. tularensis* database with no significant hits. It would be interesting to search for homologous rhizoferrin uptake systems that have already been described for other

bacteria to try to identify the uptake system used by *F. tularensis*. Furthermore, the search for other iron uptake mechanisms might be important for understanding the ecology and pathogenesis of this bacterium. Given the unique properties already identified for this bacterium, including LPS structure (15) and its novel macrophage entry mechanism (30), it would not be unexpected to discover a novel system of iron uptake by this bacterium.

5.3 Relevance of current work for future work in the study of pathogenesis

As discussed in Chapter 4, the differential characteristics described for subpopulations A.I and A.II, including geographical separation, preferential vectors, anatomical source of recovered isolates, and disease outcome, are intriguing given how closely related these two subpopulations are (9, 16, 33). The results obtained from our subtractive hybridization study did not reveal any obvious regions of difference associated with previously characterized genes linked to virulence. However, given how little we understand about this bacterium, one cannot eliminate the possibility that any of the products encoded for by the 18 RDs identified may be important for pathogenesis either alone, or in conjunction with other proteins. Each of these regions can be used to form new testable hypothesis for comparing A.I and A.II.

Staples *et al.* established that among the *F. tularensis* subsp. type A tularemia cases studied, A.I had a statistically higher case fatality ratio than A.II (14% compared to 0%) (33). One controversy of these is A.II has a lower case fatality than *F. tularensis* subsp. type B thus moving away from the *F. tularensis* dogma that all *F. tularensis* subsp. type A strains are more virulent than *F. tularensis* subsp. type B that has always been documented as less virulent and as causing milder forms of tularemia as compared

to *F. tularensis* subsp. type A (32). To better understand the virulence determinants and pathogenesis associated with the two *F. tularensis* subsp. type A subpopulations A.I and A.II, and between these and *F. tularensis* subsp. type B, experimental animal infection studies would be beneficial. Unfortunately, there is currently no optimal animal model for infection studies using *F. tularensis* subsp. type A strains (28). When studying infectious diseases using animal models, most models can not be utilized to answer all questions. Instead, the model selected needs to be done based on the question being asked. In the case of *F. tularensis* subsp. type A, with its high virulence the murine model does not provide the ability to appropriately assess differences in virulence among strains. Mouse models for type A studies using BALB/c and C57BL/6 mice were recently developed (4, 42). However, tularemia infections caused by *F. tularensis* type A and type B using these models gave similar results and infection by the two and cannot be differentiated. Additionally, mice are also susceptible to infection with both F. tularensis type B strain LVS and subsp. *novicida* that are generally not infectious to humans; therefore, questions concerning the translational value of data gained from these studies to humans need to be addressed. Only one study, by Twine *et al.*, has directly compared virulence between two type A strains using the mouse model. In their study, they were able to show subtle differences that were statistically significant through measurements of time to death by vaccinating mice with *F. tularensis* subsp. type B strain LVS prior to infection with *F. tularensis* subsp. type A strains (40). Using the approach of Twine *et al.*, or even trying to expand pathogenesis studies to a more suitable model such as the rabbit (this model is only susceptible to *F. tularensis* subsp. type A strains) to test several *F. tularensis* subsp. type A subpopulations (A.I and A.II) isolates, and *F. tularensis*

subsp. type B isolates would be ideal. Rabbits are natural transmitters of this infection (8). They have a slightly higher LD_{50} that may allow for increased accuracy in inoculation, and have been previously shown to develop symptoms that more closely resemble those described for human infections (28). Disadvantages to using rabbits include the limitations associated with an undeveloped model such as the lack of reagents to assess the host response.

Although advances in genetic tools such as allelic exchange strategies have progressed for studying *F. tularensis* subsp. type A strains (22, 38, 39), without an appropriate animal model in which non-manipulated and manipulated strains can be tested the limitations for the use of these tools are numerous. The same constraints apply to future studies on the 18 RDs we identified by subtractive hybridization. The lack of appropriate animal models for bacterial pathogenesis is not unique to *F. tularensis*. In some cases such as *Mycobacterium leprae* no animal model exists (31). However, in case of leprosy and other bacterial diseases there is the availability of human tissues that can be studied to assess host-pathogen interactions of the true disease states. Unfortunately this is not the case for tularemia. The diagnosis of disease is generally confirmed after treatment has been completed thus limiting the availability of useful clinical samples and low prevalence of the disease does not facilitate use of clinical samples. Consequently, a significant portion of data being generated in this field for *F. tularensis* type A results in the generation of lists of genes and proteins for which their significance cannot be appropriately assessed outside of the macrophage model.

5.4 Concluding remarks

As discussed in previous chapters, research on *Francisella* is primarily dominated by studies using *F. tularensis* subsp. type B strain vaccine strain LVS or *F. tularensis* subsp. *novicida* strains due the minimal safety requirements needed to work with these strains. A slight movement away from this is currently underway, and increasingly more studies using *F. tularensis* subsp. type A are being performed. However, there are still numerous studies and technologies being developed using strains that are not relevant to human infection. In addition, the data acquired from these studies is rarely tested in clinically relevant human strains and inferences are made that many times do not hold true for the virulent strains. As Chapter 1 describes, the subsp. of *F. tularensis* differ in almost every aspect studied, including virulence, ecology, structure, genetic makeup, and growth requirements (32). Given that the priority for researching *Francisella* is currently to be prepared for the potential use of this bacterium as a biological weapon, the focus for this research should be on enhancing understanding and describing the virulent subsp. The use of *F. tularensis* subsp. type B strain LVS and *F. tularensis* subsp. *novicida* are useful tools for identifying possible virulence determinants through comparisons between these strains and the clinically relevant strains and work with these strains should be limited to such studies.

The future and potential of this pathogen is unknown. Emergence of *F. tularensis* into new, non-endemic areas has occurred and will likely continue to occur (25). Appropriate diagnostics and surveillance methods for *F. tularensis* are essential in prevention and preparedness for both natural and provoked increases in this infection.

In closing, the work presented in this dissertation has provided tools for enhancing our ability to identify *F. tularensis* subsp. type A subpopulations A.I and A.II in a quick manner and provided proof-of-concept for RT-PCR development specific for A.I and A.II. In addition, the regions of difference identified between A.I and A.II and the discovery that aerobactin inhibits *Francisella* provoke multiple hypotheses that can be tested and will further enhance out knowledge of this bacterium.

5.3 Literature cited

1. **Abd, H., T. Johansson, I. Golovliov, G. Sandstrom, and M. Forsman.** 2003. Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. Appl Environ Microbiol **69:**600-6.
2. **Berdal, B. P., R. Mehl, H. Haaheim, M. Loksa, R. Grunow, J. Burans, C. Morgan, and H. Meyer.** 2000. Field detection of *Francisella tularensis*. Scand J Infect Dis **32:**287-91.
3. **Carrano, C. J., H. Drechsel, D. Kaiser, G. Jung, B. Matzanke, G. Winkelmann, N. Rochel, and A. M. Albrecht-Gary.** 1996. Coordination Chemistry of the Carboxylate Type Siderophore Rhizoferrin: The Iron(III) Complex and Its Metal Analogs. Inorg Chem **35:**6429-6436.
4. **Conlan, J. W., W. Chen, H. Shen, A. Webb, and R. KuoLee.** 2003. Experimental tularemia in mice challenged by aerosol or intradermally with virulent strains of *Francisella tularensis*: bacteriologic and histopathologic studies. Microb Pathog **34:**239-48.
5. **Cronquist, S. D.** 2004. Tularemia: the disease and the weapon. Dermatol Clin **22:**313-20, vi-vii.
6. **Dennis, D. T., T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, A. M. Friedlander, J. Hauer, M. Layton, S. R. Lillibridge, J. E. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, and K. Tonat.** 2001. Tularemia as a biological weapon: medical and public health management. Jama **285:**2763-73.
7. **Diederen, B. M., and M. F. Peeters.** 2007. Rapid diagnosis of pneumococcal pneumonia in adults using the Binax NOW *Streptococcus pneumoniae* urinary antigen test. Int J Infect Dis **11:**284-5.
8. **Ellis, J., P. C. Oyston, M. Green, and R. W. Titball.** 2002. Tularemia. Clin Microbiol Rev **15:**631-46.
9. **Farlow, J., K. L. Smith, J. Wong, M. Abrams, M. Lytle, and P. Keim.** 2001. *Francisella tularensis* strain typing using multiple-locus, variable-number tandem repeat analysis. J Clin Microbiol **39:**3186-92.
10. **Farlow, J., D. M. Wagner, M. Dukerich, M. Stanley, M. Chu, K. Kubota, J. Petersen, and P. Keim.** 2005. *Francisella tularensis* in the United States. Emerg Infect Dis **11:**1835-41.
11. **Forsman, M., G. Sandstrom, and A. Sjostedt.** 1994. Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. Int J Syst Bacteriol **44:**38-46.
12. **Fortier, A. H., D. A. Leiby, R. B. Narayanan, E. Asafoadjei, R. M. Crawford, C. A. Nacy, and M. S. Meltzer.** 1995. Growth of *Francisella tularensis* LVS in macrophages: the acidic intracellular compartment provides essential iron required for growth. Infect Immun **63:**1478-83.

13. **Garcia Del Blanco, N., M. E. Dobson, A. I. Vela, V. A. De La Puente, C. B. Gutierrez, T. L. Hadfield, P. Kuhnert, J. Frey, L. Dominguez, and E. F. Rodriguez Ferri.** 2002. Genotyping of *Francisella tularensis* strains by pulsed-field gel electrophoresis, amplified fragment length polymorphism fingerprinting, and 16S rRNA gene sequencing. J Clin Microbiol **40:**2964-72.
14. **Gerber, M. A., and S. T. Shulman.** 2004. Rapid diagnosis of pharyngitis caused by group A *streptococci*. Clin Microbiol Rev **17:**571-80, table of contents.
15. **Gunn, J. S., and R. K. Ernst.** 2007. The structure and function of *francisella* lipopolysaccharide. Ann N Y Acad Sci **1105:**202-18.
16. **Johansson, A., J. Farlow, P. Larsson, M. Dukerich, E. Chambers, M. Bystrom, J. Fox, M. Chu, M. Forsman, A. Sjostedt, and P. Keim.** 2004. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J Bacteriol **186:**5808-18.
17. **Johansson, A., I. Goransson, P. Larsson, and A. Sjostedt.** 2001. Extensive allelic variation among *Francisella tularensis* strains in a short-sequence tandem repeat region. J Clin Microbiol **39:**3140-6.
18. **Keim, P., A. Johansson, and D. M. Wagner.** 2007. Molecular epidemiology, evolution, and ecology of *francisella*. Ann N Y Acad Sci **1105:**30-66.
19. **Kugeler, K. J., R. Pappert, Y. Zhou, and J. M. Petersen.** 2006. Real-time PCR for Francisella tularensis types A and B. Emerg Infect Dis **12:**1799-801.
20. **Kuhn, S., V. Braun, and W. Koster.** 1996. Ferric rhizoferrin uptake into *Morganella morganii*: characterization of genes involved in the uptake of a polyhydroxycarboxylate siderophore. J Bacteriol **178:**496-504.
21. **Larsson, P., P. C. Oyston, P. Chain, M. C. Chu, M. Duffield, H. H. Fuxelius, E. Garcia, G. Halltorp, D. Johansson, K. E. Isherwood, P. D. Karp, E. Larsson, Y. Liu, S. Michell, J. Prior, R. Prior, S. Malfatti, A. Sjostedt, K. Svensson, N. Thompson, L. Vergez, J. K. Wagg, B. W. Wren, L. E. Lindler, S. G. Andersson, M. Forsman, and R. W. Titball.** 2005. The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. Nat Genet **37:**153-9.
22. **LoVullo, E. D., L. A. Sherrill, L. L. Perez, and M. S. Pavelka, Jr.** 2006. Genetic tools for highly pathogenic *Francisella tularensis* subsp. *tularensis*. Microbiology **152:**3425-35.
23. **Okada, C., F. Kura, A. Wada, H. Inagawa, G. H. Lee, and H. Matsushita.** 2002. Cross-reactivity and sensitivity of two *Legionella* urinary antigen kits, Biotest EIA and Binax NOW, to extracted antigens from various serogroups of *L. pneumophila* and other *Legionella* species. Microbiol Immunol **46:**51-4.
24. **Oyston, P. C., A. Sjostedt, and R. W. Titball.** 2004. Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*. Nat Rev Microbiol **2:**967-78.
25. **Petersen, J. M., and M. E. Schriefer.** 2005. Tularemia: emergence/re-emergence. Vet Res **36:**455-67.
26. **Petersen, J. M., M. E. Schriefer, K. L. Gage, J. A. Montenieri, L. G. Carter, M. Stanley, and M. C. Chu.** 2004. Methods for enhanced culture recovery of *Francisella tularensis*. Appl Environ Microbiol **70:**3733-5.

27. **Petrosino, J. F., Q. Xiang, S. E. Karpathy, H. Jiang, S. Yerrapragada, Y. Liu, J. Gioia, L. Hemphill, A. Gonzalez, T. M. Raghavan, A. Uzman, G. E. Fox, S. Highlander, M. Reichard, R. J. Morton, K. D. Clinkenbeard, and G. M. Weinstock.** 2006. Chromosome rearrangement and diversification of *Francisella tularensis* revealed by the type B (OSU18) genome sequence. J Bacteriol **188:**6977-85.

28. **Rick Lyons, C., and T. H. Wu.** 2007. Animal models of *Francisella tularensis* infection. Ann N Y Acad Sci **1105:**238-65.

29. **Rohmer, L., C. Fong, S. Abmayr, M. Wasnick, T. J. Larson Freeman, M. Radey, T. Guina, K. Svensson, H. S. Hayden, M. Jacobs, L. A. Gallagher, C. Manoil, R. K. Ernst, B. Drees, D. Buckley, E. Haugen, D. Bovee, Y. Zhou, J. Chang, R. Levy, R. Lim, W. Gillett, D. Guenthener, A. Kang, S. A. Shaffer, G. Taylor, J. Chen, B. Gallis, D. A. D'Argenio, M. Forsman, M. V. Olson, D. R. Goodlett, R. Kaul, S. I. Miller, and M. J. Brittnacher.** 2007. Comparison of *Francisella tularensis* genomes reveals evolutionary events associated with the emergence of human pathogenic strains. Genome Biol **8:**R102.

30. **Santic, M., M. Molmeret, K. E. Klose, and Y. Abu Kwaik.** 2006. *Francisella tularensis* travels a novel, twisted road within macrophages. Trends Microbiol **14:**37-44.

31. **Scollard, D. M., L. B. Adams, T. P. Gillis, J. L. Krahenbuhl, R. W. Truman, and D. L. Williams.** 2006. The continuing challenges of leprosy. Clin Microbiol Rev **19:**338-81.

32. **Sjostedt, A. B.** 2005. *Francisella.* p.200-210. *In* D. J. Brenner, N. R. Krieg, J. T. Staley, and G. M. Garrity (ed.), The *Proteobacteria*, part B. Bergey's manual of systematic bacteriology, 2nd ed., vol. 2. Springer-Verlag, New York, N. Y.

33. **Staples, J. E., K. A. Kubota, L. G. Chalcraft, P. S. Mead, and J. M. Petersen.** 2006. Epidemiologic and molecular analysis of human tularemia, United States, 1964-2004. Emerg Infect Dis **12:**1113-8.

34. **Sullivan, J. T., E. F. Jeffery, J. D. Shannon, and G. Ramakrishnan.** 2006. Characterization of the siderophore of *Francisella tularensis* and role of fslA in siderophore production. J Bacteriol **188:**3785-95.

35. **Svensson, K., P. Larsson, D. Johansson, M. Bystrom, M. Forsman, and A. Johansson.** 2005. Evolution of subspecies of *Francisella tularensis*. J Bacteriol **187:**3903-8.

36. **Tarnvik, A., and M. C. Chu.** 2007. New approaches to diagnosis and therapy of tularemia. Ann N Y Acad Sci **1105:**378-404.

37. **Thomas, R., A. Johansson, B. Neeson, K. Isherwood, A. Sjostedt, J. Ellis, and R. W. Titball.** 2003. Discrimination of human pathogenic subspecies of *Francisella tularensis* by using restriction fragment length polymorphism. J Clin Microbiol **41:**50-7

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

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

40. **Twine, S. M., H. Shen, J. F. Kelly, W. Chen, A. Sjostedt, and J. W. Conlan.** 2006. Virulence comparison in mice of distinct isolates of type A *Francisella tularensis.* Microb Pathog **40:**133-8.

41. **Versage, J. L., D. D. Severin, M. C. Chu, and J. M. Petersen.** 2003. Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. J Clin Microbiol **41:**5492-9.

42. **Wu, T. H., J. A. Hutt, K. A. Garrison, L. S. Berliba, Y. Zhou, and C. R. Lyons.** 2005. Intranasal vaccination induces protective immunity against intranasal infection with virulent *Francisella tularensis* biovar A. Infect Immun **73:**2644-54.