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Exploring the genetic basis of intracellular pathogenesis in Francisella tularensis

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EXPLORING THE GENETIC BASIS OF INTRACELLULAR PATHOGENESIS IN *FRANCISELLA TULARENSIS*

by

Stephen Robert Lindemann

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Microbiology
in the Graduate College of
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Thesis Supervisor: Associate Professor Bradley D. Jones

ABSTRACT

Francisella tularensis is the etiological agent of tularemia, a severe and potentially fatal disease in humans. It is extremely infectious by the aerosol route, being thought to cause disease in humans with an infectious dose as small as one to ten organisms, which led to its weaponization by several nations and classification as a category A select agent by the Centers for Disease Control and Prevention. An intracellular pathogen, relatively little is known about the mechanisms by which *Francisella* is capable of successfully modulating host cell processes to escape its phagosome and replicate within the cytosol and what genes beyond the *Francisella* pathogenicity island are required. Furthermore, in the context of aerosol exposure, it is unknown what cells *F. tularensis* initially interacts with and the overall contribution of those interactions to inhalational tularemia. I initiated this study by generating an *in vitro* model system to study interactions of *F. tularensis* with epithelial cell lines in tissue culture. Utilizing this system, I determined that *F. tularensis* LVS was capable of adherence to human epithelial cell lines of alveolar (A549), bronchial airway (HBE), and cervical carcinoma (HEp-2) origin. Furthermore, LVS was capable of invading these cell lines and growing productively within them. In order to detect genes important for virulence in this system, I generated a ~15,000 member transposon library in virulent strain Schu S4 that was could be screened in a high-throughput manner by transposon site hybridization. As uptake in the *in vitro* epithelial cell line system was relatively inefficient, I screened this library through human primary macrophages. Results of the screen implicated 207 genes as negatively selected in the human macrophage model. Of these, I generated mutants in genes residing in a locus of the *Francisella* chromosome, *FTT1236*, *FTT1237*, and *FTT1238*, to determine their virulence phenotypes. Mutants in these genes demonstrated significant vulnerability to complement-

mediated lysis as compared with wild type Schu S4. Analysis of purified LPS and capsule from these mutants further showed that they had marked defects in O-antigen and capsular polysaccharide biosynthesis. Complementation of these mutants restored surface polysaccharide biosynthesis and further determined that *FTT1236* and *FTT1237* compose an operon, as a mutation in *FTT1236* is polar onto *FTT1237*. Characterization of the intracellular defect of these mutants in the absence of active complement demonstrated that they were taken up more efficiently by primary human macrophages than wild type Schu S4 and were capable of phagosomal escape but exhibited reduced intracellular growth. Microscopic analysis of macrophages infected with mutant bacteria revealed that, as early as 16 hpi, these macrophages exhibited signs of cell death. In contrast, cells infected with Schu S4 exhibited a healthy, spread morphology as late as 32 h, despite significantly more extensive *F. tularensis* cytosolic replication. Quantitation of cell death by the release of lactate dehydrogenase, signifying membrane permeability, confirmed that mutants in *FTT1236*, *FTT1237*, and *FTT1238* induced early cell death in infected macrophages as compared with wild type Schu S4. Together, this work contributes to our understanding of the factors, such as O-antigen and capsule, required for and genes involved in *Francisella*'s lifecycle as an intracellular pathogen.

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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To Terryn, especially, and also to Grace, Greta, Zeke and Elias, for your faith, hope, and love, expressed to me daily and also to many, many others: the work herein is as much yours as mine, as you have carried my burdens and refreshed my heart

The more I study nature, the more I stand amazed at the work of the Creator.

Louis Pasteur, as quoted in the *Literary Review*, Oct. 18, 1902

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ABSTRACT

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mediated lysis as compared with wild type Schu S4. Analysis of purified LPS and capsule from these mutants further showed that they had marked defects in O-antigen and capsular polysaccharide biosynthesis. Complementation of these mutants restored surface polysaccharide biosynthesis and further determined that *FTT1236* and *FTT1237* compose an operon, as a mutation in *FTT1236* is polar onto *FTT1237*. Characterization of the intracellular defect of these mutants in the absence of active complement demonstrated that they were taken up more efficiently by primary human macrophages than wild type Schu S4 and were capable of phagosomal escape but exhibited reduced intracellular growth. Microscopic analysis of macrophages infected with mutant bacteria revealed that, as early as 16 hpi, these macrophages exhibited signs of cell death. In contrast, cells infected with Schu S4 exhibited a healthy, spread morphology as late as 32 h, despite significantly more extensive *F. tularensis* cytosolic replication. Quantitation of cell death by the release of lactate dehydrogenase, signifying membrane permeability, confirmed that mutants in *FTT1236*, *FTT1237*, and *FTT1238* induced early cell death in infected macrophages as compared with wild type Schu S4. Together, this work contributes to our understanding of the factors, such as O-antigen and capsule, required for and genes involved in *Francisella*'s lifecycle as an intracellular pathogen.

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ASC ..... apoptosis-associated speck-like protein

BSL-3 ..... biosafety level 3

CDM ..... Chamberlain's defined medium

CFU ..... colony-forming unit

CR3 ..... complement receptor 3

EEA-1 ..... early endosome antigen-1

FCP ..... *Francisella*-containing phagosome

FPI ..... *Francisella* pathogenicity island

GFP ..... green fluorescent protein

HUVEC ..... human umbilical vein endothelial cell

hpi ..... hours post infection

IL ..... interleukin

LBP ..... lipopolysaccharide binding protein

LPS ..... lipopolysaccharide

LVS ..... live vaccine strain

lamp-1 ..... lysosome associated membrane protein-1

MDM ..... monocyte-derived macrophage

MOI ..... multiplicity of infection

MR ..... mannose receptor

MMH ..... modified Mueller-Hinton

NLR ..... NOD-like receptor

O-Ag ..... O-antigen

ORF ..... open reading frame

PAMP ..... pathogen-associated molecular pattern

PCR ..... polymerase chain reaction

PRR .....pattern recognition receptor

SRA .....scavenger receptor A

TGF-β ..... transforming growth factor-beta

TLR .....Toll-like receptor

TNF-α .....tumor necrosis factor-alpha

TraSH .....transposon site hybridization

CHAPTER I

INTRODUCTION

Francisella tularensis

First isolated in 1911 as the cause of a fatal, plague-like infection of ground squirrels in Tulare County, California (152), *Francisella tularensis* is a non-motile, Gram negative, pleiomorphic coccobacillus and the etiological agent of tularemia (240). A facultative intracellular pathogen requiring supplementation of most media with nutrients such as glucose, cysteine, and blood or its components, initial *in vitro* culture of *F. tularensis* from the infected rodents proved difficult and inconsistent (152). The first case of human tularemia was described by Wherry and Lamb in 1914 with a prescient warning: "…this recently discovered disease of rodents is sufficiently virulent…to warrant the presumption that it may some day take its place along *B.* [*Yersinia*] *pestis* as a menace to man" (240). A mere five years later, Edward Francis, for whom the genus *Francisella* is named, first described a human outbreak of this disease in Utah as "deer-fly fever" and documented the first human fatality (82), as well as experimentally proved its vector-borne transmission from infected to healthy animals as had been previously observed by McCoy and Chapin (83, 152). Francis also first demonstrated the extreme infectivity of the organism for humans, becoming accidently infected himself at least twice (and, apocryphally, as many as four times) over the course of his investigation of tularemia, as were all of his coauthors (133). Laboratory-acquired infection remained a significant threat to laboratorians until the introduction of an effective vaccine, which drastically reduced incidence of both ulceroglandular and typhoidal laboratory-acquired tularemia (29, 171, 203). Though an uncommon disease in the United States (189) and worldwide (62, 67), *F. tularensis* remains widely studied, in large part, for the

same reasons that it was studied by Francis and his contemporaries: it is capable, with a very small infectious dose, of causing a serious, and sometimes fatal, disease in humans.

F. tularensis has a remarkably broad host range and is capable of infecting over 250 known species from across the entire phylogenetic tree, including amoebae, insects, fish, birds, small mammals (such as rodents and lagomorph), and primates (30, 144, 168, 175, 183, 242). Humans are thought to be accidental hosts in the *F. tularensis* lifecycle, and the bacterium is thought to be maintained in the environment predominantly in arthropod vectors, such as ticks, and amplified in animal hosts such as rodents and lagomorphs (62, 76). Human tularemia can present clinically in a variety of forms depending upon the route of inoculation, though most involve generalized symptoms of disease such as fever, headaches, myalgia, and malaise. Typically characterized by ulceration at the site of infection and local lymphadenopathy, cutaneous ulceroglandular tularemia (mostly due to arthopod bite or contact with infected animals or their products) is the most common (~80% of cases) and least severe form of human tularemia. Oculoglandular and oropharyngeal cases are also observed with similar symptoms (62, 76, 217, 225), the latter mostly from contaminated water supplies (2, 44), and outbreaks of oropharyngeal tularemia frequently occur during wars or other disruptions in social order (200). Pneumonic and typhoidal tularemia are the most severe forms of the disease, and case fatality rates for the most severe forms of untreated pneumonic tularemia are estimated to be between 30% and 60%, which is especially significant in that the infectious dose by inhalation is thought to be as low as one (121) to ten (102, 153) organisms. Despite its extreme infectivity via the lungs, inhalational tularemia is rare (a notable exception being the outbreak on Martha's Vineyard in 2000, where more than 60% of cases were presumed to be inhalational) (150); however, secondary

pneumonic or typhoidal tularemia may follow local glandular infection by hematogenous spread (62, 225). Though there have been recent reports of significant numbers of *Francisella* observed outside of cells in the bloodstreams of infected mice (10, 78), dissemination is generally thought to occur intracellularly within macrophages (142), as PCR- or serologically-confirmed tularemia in humans frequently goes undetected by blood culture (117).

The severity of human disease depends greatly upon the subspecies of *Francisella* contracted. *F. tularensis* has two major biovars: type B (*F. tularensis* ssp. *holarctica*), which is found throughout the entire Northern hemisphere, and type A (*F. tularensis* ssp. *tularensis*), which is found exclusively within North America and typically causes a more severe disease in humans than type B isolates. In contrast with type A tularemia, infections with type B strains of *Francisella* are rarely fatal. These biovars are further subdivided into clades which exhibit marked differences in virulence phenotypes. Strain Schu S4 is the best-studied member of this subspecies and is classified as an A1a strain, which exhibits intermediate pathogenicity between type A1b and A2 clades of biovar A (163). Significant research effort to date has focused on the virulence properties of *F. novicida*, which generally does not cause disease in immunocompetent humans (127), and the *F. tularensis* live vaccine strain (LVS), an attenuated *holarctica* strain generated in the Soviet Union and imported into the United States in 1956 (231), due to the relative ease and safety of working with these strains as compared with fully virulent *F. tularensis* strains. While both of these strains are generally avirulent in humans, they do retain the ability to grow intracellularly in some human cells and cell lines *in vitro* and are virulent in the mouse model of infection (though LVS does not appear to be as virulent as wild type *holarctica* strains). These properties have made them attractive models for studying *F. tularensis* infection. However, in humans, the live vaccine provides

protection against cutaneous type A infection at low doses but fails to protect against aerosol exposure (41), which, coupled with its undefined mechanism of attenuation, has caused it to remain unlicensed by the U.S. Food and Drug Administration (173). There is currently no vaccine against *F. tularensis* licensed for general use in the United States (68).

Due to its extreme virulence and ease of aerosol dissemination, several nations have weaponized *F. tularensis.* Among other agents, Japan tested *Francisella* in its germ warfare units in occupied Manchuria during World War II (107). The Soviet Union (3) and the United States both developed offensive bioweapons programs that included aerosol dissemination of *F. tularensis* as well as defensive countermeasures against such attacks (46, 62). The U.S. Centers for Disease Control and Prevention estimated that an attack with *F. tularensis* would kill 6,188 people and cost society $5.4 billion (1997 dollars) per every 100,000 people exposed (124), underscoring the rationale for CDC's classification of this organism as a Category A select agent and potential agent of bioterrorism and providing a powerful motivation to continue to study the pathogenesis of the organism.

Intracellular Pathogenesis

The hallmark of *F. tularensis*' ability to cause disease is its ability to replicate extensively within an intracellular environment. Alveolar macrophages are generally thought to be the primary targets of inhalational *Francisella* infection. Dissemination of the bacteria is also thought to be mediated by macrophage migration from the site of infection to distal organs, such as the spleen, liver, and kidneys, through the lymphatic system (153, 170, 172). *F. tularensis* has been observed to infect other cell types as well (see Chapter II for further discussion). Further, it is thought that the ability to proliferate within macrophages constitutes a key component in the virulence of the organism, as

many strains that are incapable of growth within macrophages are also attenuated in the mouse model (14, 156, 179). In contrast, epithelial cell interactions with *F. tularensis* have remained relatively unstudied, though it is expected that some mutants with defects in their ability to modulate macrophage biology to their advantage may also be incapable of parasitizing other cell types as well.

Macrophages have been observed to take up *F. tularensis* by a novel "looping" mechanism, in which a pseudopod loop forms a spacious vacuole around a single organism which then progressively tightens into a more typical phagosome (49). This looping phagocytosis requires neither exogenously expressed protein nor viability of the organism, but, interestingly, treatment of bacteria with periodate and lysine to oxidize and cross-link surface polysaccharides such as lipopolysaccharide (LPS) O-antigen and capsule resulted in the majority of bacteria being taken up through traditional phagocytosis (49). Uptake of *F. tularensis* is greatly enhanced by opsonization with active complement factor C3 and blockade of complement receptor 3 (CR3) dramatically reduces uptake of opsonized bacteria (9, 48, 213). In the absence of complement opsonization, *F. tularensis* is internalized by macrophages through the mannose receptor (MR) and is competitively blocked with mannan (9, 213) and ablated by antagonistic blockade of MR (213). Further, scavenger receptor A (SRA) has been shown to be important in the uptake of *Francisella* by macrophage-like cell lines and primary macrophages (186) and surface-expressed nucleolin has been implicated in uptake of LVS into THP-1 human macrophage-like cells (11). It is unknown what contribution the route of uptake makes to the eventual intracellular fate of *F. tularensis*.

Once phagocytosed by a macrophage, *F. tularensis* initially occupies a phagosome that resembles an early endosome, transiently acquiring early

endosomal antigen-1 (EEA-1) (51, 58) and Rab5 (222) (Fig. I.1). Importantly, *F. tularensis* avoids the oxidative burst in macrophages and neutrophils, subverting a major mechanism of the innate immune system to control microbial infection, and alters typical intracellular trafficking to avoid the hostile lysosomal environment (5, 151, 209). In macrophages, *Francisella*-containing phagosomes (FCPs) appear at first to mature normally, acquiring proteins characteristic of late endosomes such as CD63 (51) and lysosome-associated membrane protein-1 (lamp-1) (25, 51, 94, 213). In contrast to typical pathways of endosome maturation, however, association of FCPs with lamp-1 is transitory and FCPs do not acquire the lysosomal acid hydrolase cathepsin D (25, 51). Increasing lamp-1 negativity correlates with breach of the phagosomal membrane and escape of the bacterium into the cytosol (45, 153), which occurs approximately one to four hours post infection (hpi) (37, 51, 94). Kinetic variability in phagosome escape is likely due to differences in cell species and type as well as the *Francisella* culture conditions utilized, as significant diversity exists in the conditions various laboratories have employed to study phagosome escape. While some controversy exists (45, 206, 207), it appears that the FCP is not significantly acidified and that transient acidification of the FCP is not required for escape of *Francisella* into the cytosol. There is agreement that inhibition of the vacuolar ATPase, which is required for the energetically-intensive transport of protons into the phagosome (112), with bafilomycin A retards *Francisella* replication in the cytosol of infected macrophages (45, 50). Once within the cytosol, *Francisella* is capable of rapid growth (48, 153); in type A strain Schu S4, the doubling time is estimated to be as little as 60 minutes (45).

Extensive replication of *Francisella* ultimately leads to cell death and the release of bacteria from the cell, occurring approximately 24 hours post infection in J774A.1 murine macrophage-like cells infected with LVS and *F. novicida* U112

(6, 95). This process requires active cytosolic growth of *Francisella* and, at least in some cases, appears to be dependent upon a threshold number of intracytosolic bacteria; inhibition of bacterial uptake, internalization of killed LVS, or killing of replicating intracellular bacteria with ciprofloxacin 12 hpi resulted in no significant induction of cell death (131). Induction of cell death is a tightly regulated process that is frequently modulated and exploited by bacterial pathogens, such as *Brucella* (109), *Coxiella* (235), *Yersinia*, and many others (130). To date, the four generally recognized pathways by which cells are known to die are pyroptosis, apoptosis, oncosis, and autophagy; the route by which a cell dies has profound consequences for both pathogen and host and may be salutary for either depending upon the strategies employed by the bacteria to parasitize the host. In pyroptosis, recognition of intracellular bacteria by NOD-like receptors (NLRs) in concert with adaptor molecules (like apoptosis-associated speck-like protein, ASC) results in recruitment and cascading activation of caspase-1, which leads to the development of a multimeric inflammasome that converts pro-forms of proinflammatory cytokines IL-1β and IL-18 to their mature forms. These proinflammatory signals generally serve to recruit and activate other cells of the immune system and typically improve the host's ability to clear the bacterial infection. Pyroptotic cell death is characterized by membrane swelling and rupture along with the DNA fragmentation and nuclear condensation commonly observed in apoptosis (130, 212). In contrast, apopotosis is generally a silent event immunologically and can be induced either intrinsically by mitochondrial outer membrane permeablization through caspase-9 or extrinsically by ligation of Fas through caspase-8. Ultimately, executioner caspase-3 is cleaved, which leads inevitably to cell shrinkage, nuclear condensation, DNA fragmentation and membrane blebbing. This results in the formation of apoptotic bodies which can be engulfed and digested by macrophages without proinflammatory activation

(130, 243). Cell death can also be triggered independently of caspase activation. Oncosis, which is characterized by cell swelling, loss of membrane integrity and rupture, is increasingly being perceived as a caspase-independent form of programmed cell death that can be activated by bacterial pathogens, especially at high multiplicities of infection (MOIs) and results in extensive inflammation (73, 130). Autophagy is a conserved cell death pathway activated by cellular stress, such as amino acid starvation, and serves to recycle cellular macromolecules by engulfing damaged organelles in a double membrane-bound vesicle that is fused with lysosomes. Autophagy does not stimulate inflammation, as cellular contents remain within the cell (59, 130). The term *necrosis*, previously used to refer to oncotic cell death, is now being used by pathologists to describe a postmortem cell condition in which cells have equilibrated with their surroundings but does not define the mechanism of death (130).

There are significant discrepancies in the type of cell death observed both *in vitro* and *in vivo*, and the mechanism of *Francisella*-mediated cell death may depend upon the bacterial strain, the cell type investigated, or both. *F. novicida* and LVS have been observed to trigger the inflammasome, inducing rapid, caspase-1 mediated, pyroptotic cell death in infected thioglycollate-elicited macrophages and concomitant release of proinflammatory cytokines IL-1β and IL-18 (148); however, this method requires extensive preactivation of macrophages with killed *F. novicida* which may not be representative of typical macrophages within the mouse. Activation of the inflammasome by *Francisella*, as determined by the release of proinflammatory cytokines, depends upon escape from the phagosome to the cytosol (88) but not upon intracytosolic replication (214). In contrast to those of normal C57BL/6 mice, macrophages isolated from caspase-$1^{-/-}$ (110, 238) or $ASC^{-/-}$ mice (238) do not exhibit significant cytotoxicity 10 hpi with *F. novicida*. Furthermore, caspase-1- and ASC-deficient

mice infected with *F. novicida* show >1000-fold increases in bacterial burdens in the spleen, liver and lungs as compared to wild type mice and succumbed to infection faster (148). Recently, absent in melanoma 2 (AIM2), a cytosolic NOD-like receptor (NLR) known to recognize cytosolic DNA, has been shown to be required for activation of the inflammasome in mice infected with *F. novicida* (71, 120), and *AIM2*$^{-/-}$ mice are significantly less able to control *F. novicida* infection, resulting in elevated organ burdens (120). Mice infected with LVS exhibit localized, microgranulomatous activation of caspase-1 within their spleens and livers that colocalizes with *Francisella* antigens (241). Taken together, these results suggest avirulent strains of *Francisella* elicit a significant proinflammatory response in infected macrophages and induce pyroptosis-mediated cell death both *in vitro* and *in vivo*.

In contrast to the caspase-1 activation observed in avirulent strains, the livers and spleens of mice infected with virulent type A strain KU49 exhibit extensive caspase-3 activation and necrosis with widespread dissemination of *Francisella* antigens, suggesting that virulent strains avoid pyroptosis and, therefore, leukocyte recruitment and containment within microgranulomas (241). Some have observed that LVS- infected J774A.1 cells (132) and U112-infected RAW 264.7 cells (194) exhibit caspase-3 mediated, apoptotic cell death, which may result from an observed deficiency in J774A.1 and RAW 264.7 cells to activate the inflammasome (111). Interestingly, a recently described LVS mutant in *tolC,* which is hypertoxic to macrophages as determined by lactate dehydrogenase (LDH) release, exhibits reduced growth within primary murine macrophages (91) and appears to induce a more rapid, caspase-3-mediated apoptosis than does wild type LVS. Under the conditions employed in this study, neither LVS nor the mutant appears to significantly activate caspase-1 or trigger IL-1β release. In human macrophages, however, this mutant exhibits

increased cytotoxicity concomitant with IL-1β release, suggesting caspase-1 involvement (188). Further, one research group has reported induction of autophagy in primary murine macrophages by *F. tularensis* at late time points after infection of murine macrophages (37), an observation that has not been duplicated in human macrophages. All told, these data suggest that cell death phenotypes are dependent upon the *Francisella* strain, the species (mouse or human) and type of cell (i.e. primary or immortalized) studied, making the cross-experiment comparison of data difficult and careful design of *Francisella*-modulated cell death experiments increasingly important.

Virulence Genetics

Despite the fact that *Francisella tularensis* is capable of causing potentially lethal human disease with an infectious dose possibly as small as one organism by subverting host mechanisms of intracellular trafficking and microbial killing, much of the molecular and genetic basis for its successful intracellular lifestyle remains mysterious. While genomic analysis of *F. tularensis* Schu S4 has revealed a genome adapting to intracellular pathogenesis with a corresponding loss of biosynthetic capacity, it has identified few canonical virulence factors in the *F. tularensis* armament. Of the 1,804 annotated open reading frames (ORFs) in the ~1.9 megabase Schu S4 genome, none appear to encode exotoxins similar to those found in other pathogenic bacteria. Furthermore, no homology to type III, IV or V secretion systems, frequently utilized by pathogenic bacteria to deliver effector proteins and/or DNA into host cells, has been found in the Schu S4 genome (134) or any other *Francisella* genome sequenced to date (17, 36, 185, 190, 202); however, the chromosome appears to contain genes involved in pilus biogenesis and type II secretion (79, 90, 105) as well as several homologs of proteins involved in type I secretion (134).

The majority of genes implicated in virulence to date lie within a 33.9-kb region of the *Francisella* chromosome termed the *Francisella* pathogenicity island (FPI), which is present in singlet in the *F. novicida* chromosome but duplicated in *F. tularensis* strains (134, 166, 202). This island encodes approximately 17 genes within two apparent operons, one containing *pdpABCE, vgrG, dotU,* and *iglEFGHIJ* and the other containing *pdpD* and *iglABCD* (165). Among the first virulence genes identified in *Francisella* and the first gene of the pathogenicity island to be characterized was *iglC*, which was determined to be upregulated during intracellular growth (95). Further, random mutants in the *pdpAiglABCD* operon of *F. novicida* had been found to be unable to grow within macrophages in early studies (98). Recently suggested to encode a type VI secretion system (13), most genes of the FPI are required for the escape of the bacterium from the phagosome as well as alteration of intracellular trafficking; the majority of FPI mutants described to date remain trapped within phagolysosomes and are consequently unable to grow within cells and are therefore avirulent in mice (13, 25, 60, 136, 140, 210, 211, 238). The requirement for an intact FPI appears to be central to the virulence of *Francisella*, as mutants in FPI genes are attenuated in amoeba (136) and insect cells (199) in similar fashion. Salient exceptions to the rule are *pdpD*, which appears to be involved in mouse virulence but is dispensable for intracellular growth (143), and *iglD*, which appears to be important for intracytosolic growth but not for escape from the phagosome (208). It should be noted that the majority of work with mutants in the FPI has been performed in *F. novicida*, as until recently construction of site-directed mutants was complicated by the relative genetic intractability of *F. tularensis* strains as well as the presence of duplication in the *F. tularensis* chromosome (201). Further, random transposon insertions in a single copy of FPI genes tend to have very mild, if any, observable defects in virulence and are rarely identified in

high-throughput screens of *F. tularensis* mutants through cells (145, 191, 223). Therefore, it will be necessary in the future to confirm that the FPI functions in *F. tularensis* in an analogous way to *F. novicida* by constructing double mutants to inactivate both alleles.

Francisella's ability to parasitize such a broad range of disparate hosts as well as its persistence in the environment suggests that it is able to modulate its gene expression to adapt to varying conditions. Interestingly, *Francisella's* does not appear to encode many proteins with predicted regulatory function; for example, while some organisms possess in excess of thirty two-component regulatory systems (135), *Francisella* possesses just two predicted orphan histidine kinases and two orphan response regulators. However, the first major *Francisella* virulence gene identified, *mglA*, (15) was determined to be incapable of intramacrophage growth due to regulation, exerting its attenuating effect predominantly by silencing gene expression of the FPI (136). Having homology to stringent starvation protein A (SspA), MglA has since been found to form a heterodimer with SspA and interact with RNA polymerase but not DNA (35) in a manner dependent upon the synthesis of ppGpp, a signal of starvation stress (34). Further, MglA and SspA are known to interact with a third protein, FevR, which, as expected, is also required for phagosome escape and intracellular growth (23, 25). The regulon of MglA has been determined to encompass ~100 genes, most of which lie outside the FPI (24, 99), and the regulon of FevR is identical to that of MglA except in the regulation of *fevR* itself (23). A putative two-component response regulator, PmrA, has also been identified to regulate the FPI as well as several genes distal to this region and is thought to interact with MglA and SspA (18, 160). Regulation of one operon on the FPI is also thought to be mediated by *hfq* (157), opening the possibility that small RNAs may contribute to FPI regulation as well. Finally, the FPI is known to be

upregulated under conditions of iron starvation (61, 138), possibly as a mechanism to detect intraphagosomal localization, where *Francisella* exhibits maximal intracellular FPI expression (45). Mutagenesis of an LVS strain bearing a transcriptional reporter within *iglB* allowed the identification of a fifth regulator of the FPI, MigR, which, like PmrA, is thought to exert its influence on FPI expression through misregulation of *fevR* (25, 26). Due to the large size of the regulons of these FPI regulators, it is likely that their mechanism is primarily global in nature and responsive to general stress conditions, possibly through ppGpp-mediated signaling.

As *F. novicida* and LVS both contain functional FPIs and are avirulent in humans, it is clear that the FPI is necessary, but not sufficient, to cause tularemia in humans. At present, only a handful of virulence genes outside the FPI have been identified and characterized. Some of these mutants have been implicated in iron acquisition (141, 233), biosynthetic pathways (114, 193, 214), and pilus biogenesis (32, 80, 244). Acid phosphatases, specifically *acpA*, have been implicated in *F. novicida* virulence (162) but Schu S4 mutants in *acpA* are not attenuated for virulence (43). Moreover, genes required for virulence in *Francisella* strains that are attenuated in humans may not be essential in fully virulent type A strains such as Schu S4; it is assumed that the avirulent strains may lack some virulence genes present in virulent *Francisella* strains or have defects, either arising naturally or intentionally, that make them unable to cause disease in immunocompetent humans. This may exaggerate the impact of mutations in genes that are dispensable for virulence in type A strains like Schu S4 and lead to false implication of genes as involved in virulence. Finally, in many cases, the function of the mutated gene's product remains unknown and cannot be predicted guessed by homology (84, 192), making it difficult to determine what role the gene plays in the overall virulence of the organism.

Evasion of Innate Immunity

Lacking the cytotoxic armament used by bacterial pathogens such as *Pseudomonas aeruginosa* (234), *Bacillus anthacis* (232), and *Yersinia pestis* (236) to prevent being killed by macrophages and other leukocytes of the innate immune system, *F. tularensis* must accomplish by stealth what it cannot achieve by force. Similar to many other intracellular bacterial pathogens, such as *Brucella* (63) and *Coxiella* (89), the ability of *F. tularensis* to avoid activation of macrophages (21) and dendritic cells (22) is central to its virulence strategy (153). Macrophages and dendritic cells form a key line of defense against invading microbes and form the interface between the innate and adaptive immune systems; by activating upon recognition of pathogen-associated molecular patterns (PAMPs), these cells respond by secreting proinflammatory cytokines and presenting peptide antigens of the pathogens to T cells, stimulating clonal expansion of B and T cells able to respond to the infection (195). *Francisella* is able to prevent its recognition as a pathogen, delaying and blunting an effective host response and permitting undetected dissemination to tissues distal to the original site of infection.

One major means by which *Francisella* evades recognition by cells of the innate immune system is avoidance of binding to proteins designed to detect PAMPs, such as the membrane-bound Toll-like receptors (TLRs) (129) and cytosolic NOD-like receptors (149). Toll-like receptor 4 (TLR4), in concert with CD14, MD-2, and lipopolysaccharide binding protein (LBP), stimulates activation of macrophages upon recognition of bacterial LPS, a major component of the outer membrane of Gram-negative organisms and among the most important activators of the innate immune system (19). LPS is composed of a lipid A moiety that secures it to the Gram-negative outer membrane upon which a core polysaccharide and O-polysaccharide are assembled (100, 153, 154). LPS is a known virulence factor in many pathogenic bacteria that functions in part to

protect against damage from serum complement components and antimicrobial peptides as well as mask bacterial surface antigens. In comparison to the highly stimulatory LPS of bacteria such as *Escherichia coli*, the LPS of *Francisella* strains is almost biologically inert (205), and fails to stimulate J774A.1 cells and human monocytes (65, 226). *Francisella* produces a noncanonical lipid A that is tetraacylated with longer-chain fatty acids in comparison to the hexaacylated lipid A of *E. coli* (154). This lack of TLR4 signaling in response to *Francisella* LPS is likely because the lipid A of *F. tularensis* does not bind to LBP and therefore is not recognized by TLR4 (12). Furthermore, TLR4 does not confer a significant survival advantage against *Francisella* in mouse aerosol infections with type A *F. tularensis* (39) or in dermal challenges with LVS (40), highlighting the biological consequences of *Francisella*'s ability to avoid the innate immune response by failing to stimulate TLR4. It appears, instead, that the majority of the innate immune response to *Francisella* is mediated by TLR2 signaling in response to *Francisella* lipoproteins (123, 229).

Francisella also seems to be able to actively suppress proinflammatory signaling from infected macrophages, which likely contributes to its ability to cause disease. LVS is able to prevent the secretion of proinflammatory cytokines TNF-α and IL-1β in murine and human macrophages (20, 226, 227). This suppression of activation appears to involve modulation of macrophage signaling pathways and requires escape from the phagosome and/or a functional type VI secretion system. For example, a mutant in *iglC*, which is incapable of escape from the phagosome, induces secretion of proinflammatory cytokines (226). Further, Schu S4 has been observed to actively prevent stimulation of the innate immune system in the lung and induce transient release of the anti-inflammatory cytokine TGF-β (21). The mechanisms by which *F. tularensis* is able to interfere with activation of the innate immune system are poorly understood.

Thesis

The general aim of this thesis is to further elucidate the genetic basis for *Francisella'*s ability to successfully invade and grow within both macrophages and epithelial cells, which in turn will aid comprehension of the mechanisms by which this intracellular bacterial pathogen is capable of causing a fulminant and potentially lethal disease in humans. Herein I describe the development of an *in vitro* model system to determine whether *F. tularensis* is capable of invading and growing within cell lines of epitheliod origin to begin to test the hypothesis that these cells are important in *Francisella*'s ability to cause disease in humans. Concurrent with the development of this *in vitro* system, I developed a near-saturating random mutant library in Schu S4, a virulent strain of *F. tularensis,* with a transposon capable of being used for negative selection via transposon site hybridization. This library was screened in a high-throughput manner through human primary macrophages to detect genes potentially involved in intramacrophage growth relevant to human tularemia and these genes are reported. Following this screen, I then generated site-directed mutants in genes residing in a poorly-studied locus of the *F. tularensis* chromosome, *FTT1235-FTT1238*. Several mutants in genes in this locus, *FTT1236*, *FTT1237*, and *FTT1238* were then characterized for their biosynthesis of surface polysaccharides and potential contribution to intramacrophage growth. These studies contribute to a better overall comprehension of the host-pathogen interactions that are potentially important in human tularemia as well as the genetic basis of *F. tularensis* intracellular pathogenesis, and, specifically, the role of LPS O-antigen and capsular polysaccharide in infection of macrophages.

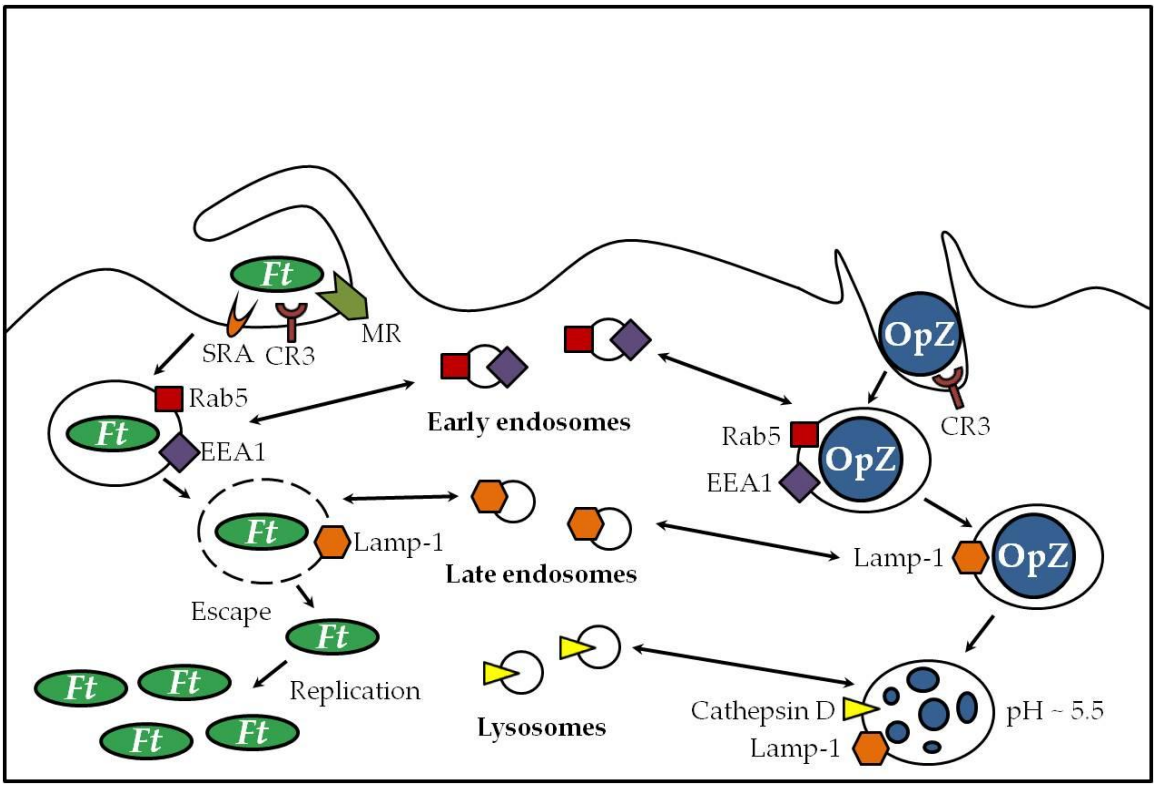

Figure I.1. Uptake and intracellular trafficking of *Francisella tularensis* in macrophages. *F. tularensis* (green) is internalized by a looping phagocytotic mechanism that involves complement receptor 3 (CR3), mannose receptor (MR), and scavenger receptor A (SRA). The *Francisella*-containing phagosome transiently acquires early endosomal markers Rab5 and EEA1, and then associates with late endosomal marker lamp-1. *Francisella* then arrests the maturation of the phagosome and prevents acquisition of cathepsin D and other lysosomal markers. Diminution of *F. tularensis* colocalization with lamp-1 correlates with escape from the phagosome and occurs one to four hours post infection. *Francisella* then goes on to replicate within the cytosol. In contrast, a particle such as opsonized zymosan (OpZ, blue) which is taken up by a CR3-mediated route inhabits a phagosome that matures completely into a phagolysosome, wherein the particle is degraded.

CHAPTER II

ADHERENCE AND INVASION OF *FRANCISELLA TULARENSIS* LVS TO NONPHAGOCYTIC CELLS: DEVELOPMENT OF A MODEL SYSTEM TO STUDY HOST-PATHOGEN INTERACTIONS

Introduction

While much was known about the interaction of *F. tularensis* with phagocytes of the innate immune system such as macrophages, at the inception of this work evidence of *Francisella* interaction with nonphagocytic cells was cryptic. Early studies of *Francisella* interactions with nonphagocytic cells reported growth of the bacterium in chicken embryos (27, 64), HeLa cells (219), mouse fibroblasts, bovine kidney and human amniotic cells (158); however, these studies focused more upon culture conditions for vaccine generation than the basic mechanisms for intracellular growth within nonphagocytic cells, assuming these interactions to be artificial (158). Consequently, the prevailing model for early pathogenesis in pneumonic tularemia was that inhaled aerosolized bacteria reached the alveolar space (121), were initially phagocytosed by resident alveolar macrophages, replicated intracellularly, and disseminated to distal organs such as the liver and spleen within these macrophages (68). In the lung, and specifically the alveolus, however, the majority of the surface area is composed of epithelial cells, and thus the first interaction experienced by an inhaled bacterium is likely to be with a nonmacrophage. By the time I undertook this study, evidence had begun to accumulate that *Francisella* interactions with nonphagocytic cells may be significant *in vivo* to the genesis and progress of disease and therefore be an important subject for continued study. Examination of the livers of experimentally infected mice showed significant invasion of hepatocytes, which was exacerbated under neutropenic conditions (53), and

extensive growth within hepatocytes (147). Recent data suggests that hepatocytes harbor the bacterium in *Francisella*-containing early hepatic lesions (196). Furthermore, in intranasal infections of mice, LVS has been found to replicate within the alveolar type II cells of the lung (58). *In vitro*, *Francisella* was determined to be protected from extracellular gentamicin in HUVEC cells (77) and to grow within HepG2 cells (191). Despite the building body of evidence that *F. tularensis* can enter and productively infect nonphagocytic cells, which may be significant *in vivo*, this process has not been thoroughly investigated, especially utilizing human-derived cells of airway origin which are likely relevant to the development of inhalational tularemia.

Significant challenges exist to the internalization of an extracellular bacterium by a nonphagocytic cell. Intracellular pathogens have established a variety of mechanisms to gain entry into and grow within these cells. Typically, the first stage in this process is adherence to the plasma membrane of the nonphagocytic cell, which provides for prolonged interaction. Following adherence to the host cell, bacteria are known to be internalized by one of two generally uptake mechanisms, termed the "trigger" and "zipper" mechanisms (55). The trigger mechanism, employed by bacteria such as *Salmonella* (81, 86) and *Shigella* (167), involves type III-secretion system-mediated injection of effectors into the nonphagocyte which induce dysregulation of the actin cytoskeleton, generate large scale microfilament remodeling and stimulate macropinocytosis, which results in engulfment and uptake of the bacterium (97, 187). The zipper mechanism, utilized by organisms such as *Yersinia* (70) and *Listeria* (137), employs ligation of receptors on the cell surface by bacterial factors and subsequent internalization through a process that resembles receptor-mediated endocytosis (54, 187). Both mechanisms require significant rearrangement of elements of the cytoskeleton for successful uptake, and

disruption of filamentous actin with cytochalasins (28) and, occasionally, inhibition of microtubule function with nocodazole (87) are known to prevent bacterial invasion into nonphagocytic cells. Prior to this work, the mechanisms of *Francisella* internalization into and growth within nonphagocytes were unknown.

I hypothesized that *Francisella* would productively invade and grow within nonphagocytic cells *in vitro*, as exemplified by its ability to cause pneumonic disease in humans at such a small infectious dose as well as the likelihood that the first contact of an inhaled bacterium would be with the epithelium. In this chapter I detail the genesis of a system to quantify and visualize the interactions of *F. tularensis* LVS with human cell lines in tissue culture, including those of airway origin. I present evidence that *Francisella* is capable of selectively adhering to human cell lines. I further show that *F. tularensis* LVS is capable of invasion of and rampant growth within these nonphagocytic cells and that both the microfilament and microtubule cytoskeletons of the host cell are involved in uptake of the organism, as inhibition of either decreases uptake of LVS. This work furthers our understanding of *Francisella'*s ability to parasitize epithelial cell lines a model system for the study of these interactions which may be important for the comprehension of early events in pneumonic tularemia.

Materials and Methods

Bacterial strains and growth conditions

F. tularensis LVS was grown in modified Mueller-Hinton broth without supplemented $MgCl_2$ and $CaCl_2$ (8). Briefly, Mueller-Hinton broth was modified by the addition of 1% glucose, 0.25% ferric pyrophosphate, and 2% IsoVitaleX and adjusted by addition of 1M NaOH to pH 6.8. *F. tularensis* strains were also grown in broth culture in Chamberlain's defined medium (33). For routine

culture, *F. tularensis* LVS was inoculated into fresh broth at ~1x10^5 CFU/ml and grown at 37°C with shaking (225 rpm) to the mid-log phase of growth (OD_{600} ~ 0.3-0.5). In order to generate a relatively translucent solid agar for genetic experiments, varying concentrations (0.25%-1%) of defibrinated sheep's blood (Colorado Serum Company, Denver, CO) were added to modified Mueller-Hinton agar and tested for colony growth. Routinely, bacteria were plated on modified Mueller-Hinton agar with a 1% sheep blood supplement, which resulted in colonies that could be seen and counted in less than 48 hours. Bacteria were also grown on cystine heart agar with 9% sheep blood plates (184).

For invasion assays, *S. enterica* serovar Typhimurium strain SL1344 was grown in Lennox broth (Gibco/BRL) statically at 37°C to OD_{600} ~ 0.4. All chemicals were purchased from Fisher Chemical, Fair Lawn, NJ.

Tissue culture cell cultivation

Human bronchial epithelial cells (HBE), human cervical epithelial adenocarcinoma cells (HEp-2), and human type-2-like alveolar carcinoma cells (A549) were obtained from ATCC (Manassas, VA). HBE and HEp-2 cells were routinely cultured in MEM with the addition of 10% FBS and 2 mM L-glutamine. A549 cells were cultured in DMEM/F12 (with 2.483 g/L sodium bicarbonate and pyridoxine hydrochloride), 10% FBS and 2mM L-glutamine. All cell culture components were purchased from Invitrogen, Carlsbad, CA. For adherence and invasion assays, 1 ml of cell suspension was seeded in a 24-well plate (Costar) at a concentration 10^5 cells/ml and allowed to adhere overnight prior to use. For confocal microscopy, 12mm circular coverslips were coated in 0.5 mg/ml bovine tendon type 1 collagen (Worthington Biochemicals, Lakewood, NJ), placed into a well and allowed to dry before addition of tissue culture cells.

Cell infections

Bacterial concentrations in broth culture were estimated from absorbance (OD_{600} = 1.0 ~ $2.5x10^9$ CFU/ml). To initiate an experiment, tissue culture wells were seeded with 10^5 cells and allowed to adhere to the wells overnight. The following day, each well was inoculated with one ml of prewarmed (37°C) bacterial suspension (10^7 CFU) in MEM with 10% FBS and 2 mM L-glutamine, resulting in an MOI of ~100. Tissue culture plates were centrifuged for 4 min at 600 x *g* to synchronize infection and incubated at 37°C in a 5% CO_2 environment. To determine the effect of cytoskeletal inhibitors on *F. tularensis* LVS entry into tissue culture cells, cells were treated for 30 min prior to infection with either 2 μg/ml cytochalasin D (Calbiochem, San Diego, CA), an inhibitor of actin polymerization, or 10 μg/ml nocodazole, an inhibitor of microtubule polymerization (Calbiochem) in MEM with 10% FBS and 2 mM L-glutamine. For invasion assays conducted in the presence of cytoskeletal inhibitors, the monolayers were lysed, and plated, after 1 hour of incubation.

Enumeration of bacteria in cell infections

For adherence assays, after 1 hour of incubation of *F. tularensis* with tissue culture cells, unattached bacteria were removed by washing three times with PBS for adherence assays. The cell monolayers were lysed with 1% saponin in PBS and dilutions were plated to enumerate the bacteria.

For invasion assays, the bacteria were allowed to interact with the cells for 4 hours before washing three times to remove extracellular bacteria. Cell culture media containing 10 μg/ml gentamicin (Sigma-Aldrich Co., St. Louis, MO) was added to each well and the plate was incubated at 37°C in 5% CO_2 for one hour to kill extracellular bacteria. Gentamicin treatment reduced the cell counts of extracellular LVS more than 10,000-fold. After gentamicin treatment, wells were washed three times with PBS to remove the antibiotic and then the cells in each

well were lysed with 1% saponin in PBS for 5 min at 37°C. For intracellular growth experiments, after the gentamicin treatment, the antibiotic was removed by washing three times with cell culture media, fresh media was added and the plates were incubated at 37°C in 5% CO_2. To determine intracellular growth, bacteria were collected at intervals from selected wells by the lysis of the cells in 1% saponin. Lysates were diluted in PBS and plated in triplicate on modified Mueller Hinton agar with 1% sheep blood plates.

Confocal microscopy

To prepare samples for confocal microscopy, coverslips were placed into the wells of a 24-well tissue culture dish and cells were seeded onto the coverslips. Bacteria were added to the wells and then washed as described above. Subsequently, cells infected with *F. tularensis* were fixed for 15 min in 3.7% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS at room temperature. After fixation, coverslips were treated with 0.2% Tween-20 in PBS (Fisher Chemical) for 15 min to permeablized cells. Bacteria were labeled for indirect immunofluorescence using primary rabbit anti-LVS polyclonal antibody (Becton-Dickenson Biosciences, San Jose, CA) and goat anti-rabbit IgG conjugated to Alexa 488 (Invitrogen) as a secondary antibody. Cells were also labeled with rhodamine phalloidin (Invitrogen) to visualize the actin cytoskeleton and mounted with VectaShield (Vector Laboratories, Burlingame, CA). Samples were examined using a BioRad Radiance 2100MP multiphoton confocal microscope. Captured images were processed with ImageJ (http://rsb.info.nih.gov/ij/).

Results

Adherence of *F. tularensis* LVS to epithelial cell lines *in vitro*

Before *F. tularensis* can invade and grow within an epithelial cell, it must adhere long enough to allow its invasive process to engage. The presence of long, thin fimbrial structures resembling type IV pili observed by others in *Francisella* when grown in Mueller-Hinton (MMH) broth or Chamberlain's defined medium (CDM) but not after growth on Mueller-Hinton agar (MHA) (90) suggested that *Francisella* may encode factors designed to promote its adherence to host cells. In many bacteria, pili are important adhesins that specifically bind to host cell moieties and are thought to be a virulence factor in *Francisella* (14). This observed variability suggested that growth conditions might affect expression of *F. tularensis* LVS pili. Based on this published work, I investigated whether *F. tularensis* LVS possessed the ability to specifically adhere to tissue culture cells and whether growth in different media conditions might alter this adherence. I grew *F. tularensis* LVS in four different conditions (MMH with or without 150 mM NaCl, CDM broth or on Cystine Heart Agar (CHA-B)) and assessed the ability of the bacteria to adhere to HEp-2 human cervical adenocarcinoma cells, SV40-transformed human bronchial epithelial cells (HBE), or A549 human alveolar type II-like carcinoma cells. In all instances, I was able to detect adherence of bacteria to the epithelial cells, regardless of cell type or growth conditions. Adherence, defined as retention in the well after washing in triplicate, of LVS grown to mid-log phase in MMH broth with 150 mM NaCl was 0.9% $\pm$ 0.04% for HEp-2 cells. Adherence of the bacteria grown under the same conditions was 0.5% $\pm$ 0.01% for HBE cells and 0.5% $\pm$ 0.08% for A549 cells (Fig. II.1). I obtained similar results regardless of whether the bacteria were grown in MMH broth, with or without added NaCl, in CDM or on CHA-B plates. This

suggests that neither the specific broth employed nor whether the bacteria were cultured in broth or on solid phase media were important to adherence of LVS, which may in turn imply that pilus formation as observed by Gil, *et al.* was not important for adherence to cells in tissue culture under these conditions.

To more fully characterize *F. tularensis* adherence to tissue culture cells and to ensure that retained bacteria were specifically bound to cells and not nonspecifically adherent to the tissue culture plastic of the wells, I performed bacterial adherence assays and examined the interactions between mid-log phase bacteria and tissue culture cells using confocal microscopy. I routinely observed bacteria, detected by fluorescent antibody binding, attached to the surfaces of HEp-2, HBE or A549 cells (Fig. II.2). Importantly, the bacteria appeared to specifically associate with the cells; I observed very few organisms bound to the glass surface of the coverslip. These observations provide complementing evidence that the quantitative adherence assay is measuring specific adherence. As far as I could discern, the distribution of the adherent bacteria was random on the surfaces of each of the different tissue culture cell types used in the assay. Additionally, the numbers of bacteria that I observed on the epithelial cells were generally consistent with results obtained using the quantitative adherence assay (0.5%-0.9% adherence is equivalent to 0.5-0.9 bacteria per cell).

I also examined whether growth state affected the levels of adherence to tissue culture cells, examining early (OD_{600} ~ 0.1-0.3 absorbance units, Abs) mid-, and late logarithmic (~0.8-1.0 Abs) and stationary (> 1.2 Abs) broth cultures. However, the density to which *F. tularensis* was grown in broth culture did not seem to play a significant role in the ability of the bacteria to bind to tissue culture cells (data not shown).

Invasion of epithelial cell lines by *F. tularensis* LVS

While examining 1 hour adherence assays by immunofluorescence confocal microscopy, it became apparent that some bacteria were located within HEp-2, HBE, and A549 cells. In preliminary observations I did not detect visible actin rearrangements as part of the host cell interactions, in contrast to bacterial pathogens such as *Salmonella*, *Shigella* and enteropathogenic *E. coli*. As the phenomenon of *F. tularensis* invasion into nonphagocytic cells was largely unreported, I performed work to characterize the process in more detail.

In order to quantitatively assess the level at which *F. tularensis* was able to invade tissue culture cells, I adapted a gentamicin protection assay that is used to study *Francisella*-macrophage interactions and that has been commonly used by us, and others, to study *Salmonella* invasion (95, 118, 140, 191). After allowing bacteria to interact with the cell monolayers for 4 h and then treating with gentamicin, substantial numbers of bacteria were consistently recovered from each of the three cell types. As a control, I confirmed that treatment of LVS with gentamicin in the absence of eukaryotic cells killed the bacteria to the limit of detection (< 20 CFU/ml). To determine if the percent invasion increased with exposure time, I infected HEp-2, HBE, and A549 cells with LVS and allowed to interact with the cell monolayer for 1, 2, 3, or 4 h prior to gentamicin treatment. *F. tularensis* invasion, as measured by organisms protected from gentamicin killing, steadily increased in each of the cell types with increasing incubation time (data not shown). At the four hour time point, I recovered anywhere from ~5 x 10^3- 10^4 CFU from the tissue culture cells and the number of viable bacteria recovered did not significantly vary based upon the cell line I used (Fig. II.3*a*). I did not study time points beyond 4 h because intracellular experiments with LVS by our group and by others (37) suggest that there is detectable intracellular

growth after 4 hours that would obscure the quantitative invasion numbers (data not shown). Due to these results, I used 4-hour assays as the standard incubation time for the experiments described here.

To independently confirm the results of the quantitative invasion assay, I visualized *F. tularensis* interactions with tissue culture cell lines using confocal microscopy. My primary goal for this set of experiments was to confirm that the organisms were physically located within the host cells. As seen in Fig. II.4 (a, c, and e), bacteria were clearly associated with the tissue culture cells with few, if any, organisms adhering to the surface of the glass slide. Analyzing the stacks of images, I could demonstrate that the cell-associated bacteria were intracellular; vertical digital slices (Fig. II.4b, d, and f) of the areas indicated by the white lines in the above panels show that the bacteria (stained green with Alexa 488) are enclosed within the cortical actin belt associated with the plasma membrane (stained red with rhodamine phalloidin). As seen in Fig II.4, virtually identical results were obtained for each of the epithelial cell lines tested.

In order to preliminarily determine what cellular mechanisms might be required for the internalization of *F. tularensis* LVS, I treated HEp-2 tissue culture cells with 2 μg/ml cytochalasin D to inhibit actin filament polymerization or 10 μg/ml nocodazole to inhibit microtubule polymerization and performed 1-hour viable cell counts to determine the impact upon invasion. Treatment of HEp-2 cells with cytochalasin D almost completely abrogated gentamicin protection of bacteria in HEp-2 infections, with ~2% of the normalized invasion to untreated cells (Fig. II.3b). Treatment of cells with nocodazole exhibited a milder effect than that of cytochalasin D, reducing invasion of LVS into treated HEp-2 cells to ~25% of that exhibited in untreated cells. *Salmonella enterica* serovar Typhimurium was included as a control for efficacy of cytoskeletal disruption, as *Salmonella* invasion is known to be dependent upon a functioning actin

cytoskeleton but is not inhibited by microtubule disruption (81, 85, 93, 119). As expected, *S. enterica* serovar Typhimurium invasion was reduced about 100-fold by treatment with cytochalasin D but treatment with nocodazole did not significantly reduce invasion of *Salmonella* into HEp-2 cells (Fig. II.3b). These data indicate that both the actin and microtubule cytoskeletons are important, likely to different extents, for invasion of LVS into HEp-2 cells. It is worth noting that the relative levels of invasion in the presence of cytoskeletal inhibitors are similar to those observed in *Brucella* invasion studies (103).

Intracellular growth of LVS in epithelial cell lines

To determine whether *F. tularensis* LVS invasion into human epithelial cell lines was followed by bacterial replication within these tissue culture cells, I examined samples by confocal microscopy 8 h and 24 h post-infection. At 8 hours post-infection, groups of dividing bacteria were consistently observed within the epithelial cells (data not shown). At 24 h post-infection, I consistently observed significant bacterial growth in each of the three tissue culture cell lines (Fig. II.5). In the majority of instances, the bacteria were clumped together as microcolonies growing within the cytoplasmic space of the cell. At 24 h, these microcolonies typically surrounded the nucleus of the cell, which was apparent for its lack of bacteria. The bacteria filled the cytosol, apparently completely occupying the entire non-nuclear volume of the cell. Occasionally, the intracellular bacteria displayed unusual immunostaining patterns that were not consistently observed (Fig. II.5b); in these cases, I determined that bacteria were occupying the entire cytosolic space by staining both the DNA of cell and the bacteria with nucleic acid stain TO-PRO3 (Fig. II.6). In some instances, I observed lysed eukaryotic cells with visible gaps in their cortical actin staining in concert with bacteria apparently exiting from the cell (data not shown). I did not observe any significant evidence of actin-based cell-to-cell spread of LVS as is

exhibited by *Listeria*, *Shigella*, and *Rickettsia* (55), although bacteria apparently released from infected cells were frequently observed associated with and inside neighboring cells (Fig II.6).

To quantify intracellular growth, I performed an intracellular growth curve in HEp-2, HBE, and A549 cells. Viable cell counts of well lysates showed that the bacteria began to multiply at ~5 hours post-infection and continued at a steady rate up to the 25 hour time point. Within this 20 hour time frame, the bacterial load increased ~1000-fold in each of the three cell types. I performed these experiments in the presence of low concentrations of gentamicin to eliminate the possibility that the bacteria were lysing the host cell and growing extracellularly (data not shown) and also, as in the invasion assays, after a 1 h gentamicin shock treatment (Fig. II.7). In both cases, I recovered no bacteria from gentamicin-treated wells lacking cells, indicating that extracellular growth is not responsible for this increase in bacterial number. This rate of growth is comparable to growth of the organisms in MMH broth and similar to that observed in macrophage studies. After 24 hours in tissue culture, I readily observed cells and cell debris detached from the surface, yet the cell monolayer remained largely intact. Taken together, these data demonstrate that *F. tularensis* LVS is able to parasitize human cell lines in culture, capably invading and replicating within epithelial cells and underscoring a basis for studying potentially important interactions of *Francisella* with nonphagocytes *in vivo*.

Discussion

In this chapter, I have initially characterized the ability of *F. tularensis* LVS to specifically adhere to nonphagocytic cells *in vitro*. In the process of examining the ability of *F. tularensis* to adhere to tissue culture cells, I observed that the bacteria were found localized within human cell lines in significant numbers. I then subsequently demonstrated that the entry process was actin-dependent and,

at least partially, microtubule-dependent as inhibitors of both of these cellular processes significantly inhibited *F. tularensis* entry. I have also showed that following entry into tissue culture cells, *F. tularensis* persists and grows substantially in nonphagocytic tissue culture cells. I further observed that *F. tularensis* was able to grow as fast within the tissue culture cells as it could in optimized MMH broth and that the infection progressed to completely fill the entire cytosolic volume of the cell, a level of intracellular growth of similar or greater extent as observed within macrophages (94).

The work described here contributes to the emerging picture of *F. tularensis* pathogenesis and is consistent with other research findings. Conlan and North performed mouse experiments in which they demonstrated that *F. tularensis* could enter into and grow within hepatocytes (53). My observations that *F. tularensis* efficiently adheres to, enters, and replicates within various nonphagocytic cells lines is additional evidence that *F. tularensis* interactions with many cell types is likely to be an important aspect of its virulence strategy. Since I completed this work, other studies have confirmed that *F. tularensis* is capable of adhering to, invading, surviving and replicating within nonphagocytic cells both *in vivo* and *in vitro* and that these interactions are likely to contribute significantly to the pathogenicity of the organism. Hall, *et al.*, also showed *F. tularensis* LVS is capable of invading alveolar type II cells *in vitro* and *in vivo*, detecting replicating bacteria in these epithelial cells of mice infected intranasally (106). Further research of the interactions between *Francisella* and mouse epithelial cell line TC-1 confirmed that uptake is dependent upon the actin and microtubule cytoskeletons, as well as diminished by broad inhibition of phosphatidylinositol 3-kinase and tyrosine kinases (58). Entry into nonphagocytic cells is typically an active process for the microorganism as the bacteria cannot depend upon an innate cellular internalization mechanism to

enter the host cell. In some cases, however, bacteria express ligands that commandeer normal cell uptake mechanisms. Internalization of killed *Francisella* was found to be as efficient as that of live, suggesting that the process is host-cell driven and responsive to a preformed ligand on the bacterial surface (58). While it is likely that *F. tularensis* enters cells by a ligand-receptor mechanism, the nature of the putative ligand and putative receptor remain unknown. Recently, two genes have been implicated as important for growth have been discovered in epithelial cells, *ripA* (84) and *FTT1103* (192). Mutants in these genes are unable to grow in either macrophages or epithelial cells. However, it is interesting to note that the genetic requirements for growth in epithelial cells and macrophages do not seem to be identical; our group has determined that mutants in pyrimidine biosynthesis pathway genes (214) as well as in *migR* (25) have macrophage-specific defects in replication. Mutants capable of growth within epithelial cells but not primary macrophages have been used to demonstrate that nonmacrophage growth alone is sufficient for pathogenesis in mice; a Schu S4 mutant in *pyrB* (122) as well as *pyrF*, a different gene in pyrimidine biosynthesis exhibiting the same macrophage-specific growth phenotype, retains its virulence in the mouse model (114). These data suggest that interactions of *F. tularensis* with epithelial cells are important to the overall virulence of the organism and are worthy of continued study.

My findings support the idea that *F. tularensis* interactions with nonphagocytic cells may be an important aspect of pathogenesis. In addition to confirming that *F. tularensis* possesses the ability to invade into nonphagocytic cells, the development of this *in vitro* model will allow comparisons of *F. tularensis* intracellular growth within phagocytic and nonphagocytic cells. While it is likely that growth mechanisms in each of these cell types will be shown to overlap significantly, it is also beginning to be appreciated that *Francisella*

uniquely interacts with each cell type (phagocytic and nonphagocytic), potentially due to differences in the entry pathway and/or differences in environmental signaling. Therefore, these different interactions will require identification and characterization of the virulence factors required in each intracellular environment and may further elucidate the unique requirements for survival of intracellular bacterial pathogens like *F. tularensis* in the phagocytic cell as compared to the nonphagocytic cell.

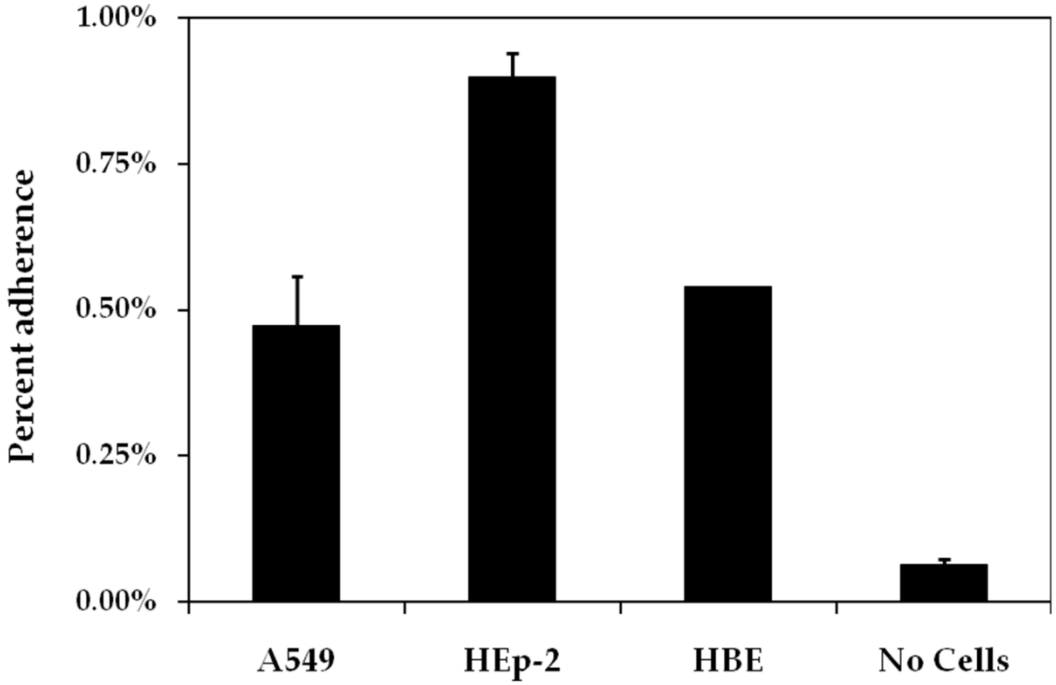

Figure II.1. Quantitative *in vitro* adherence to epithelial cell lines. Tissue culture cells (A549, HEp-2, or HBE cells) were infected with *F. tularensis* LVS at an MOI of 100 (10^7 bacteria to 10^5 cells) and incubated for 1 hour. Each bar represents the percent of the inoculum (10^7) remaining in the well after the final wash step. Error bars represent one standard deviation; all means are significantly different from the no cell control ($P < 0.001$). The experiment was performed three separate times.

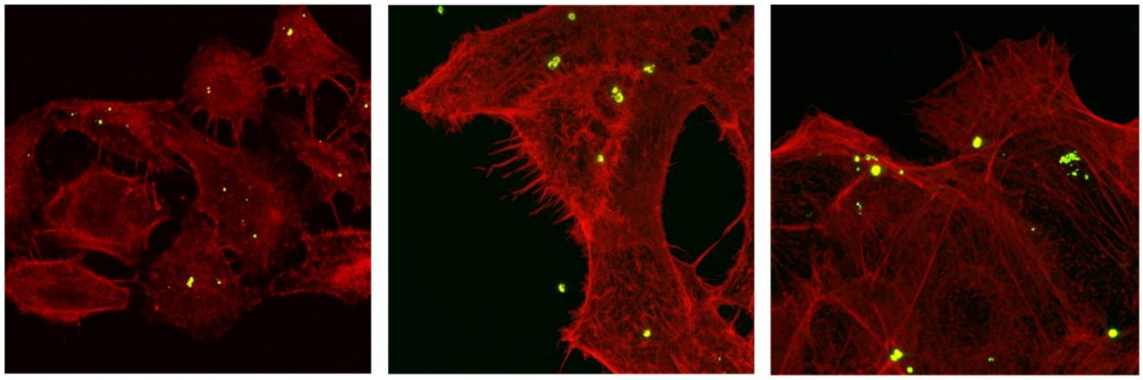

Figure II.2. Confocal microscopy of *F. tularensis* LVS adherence to A549, HEp-2, and HBE cells. Images were taken 1 h post-infection and are Z-projections of stacks of images. *F. tularensis* LVS (green) was detected with rabbit *Francisella tularensis* antiserum and goat anti-rabbit IgG conjugated to Alexa 488; F-actin stained with rhodamine phalloidin (red). Scale bars represent 10 μm.

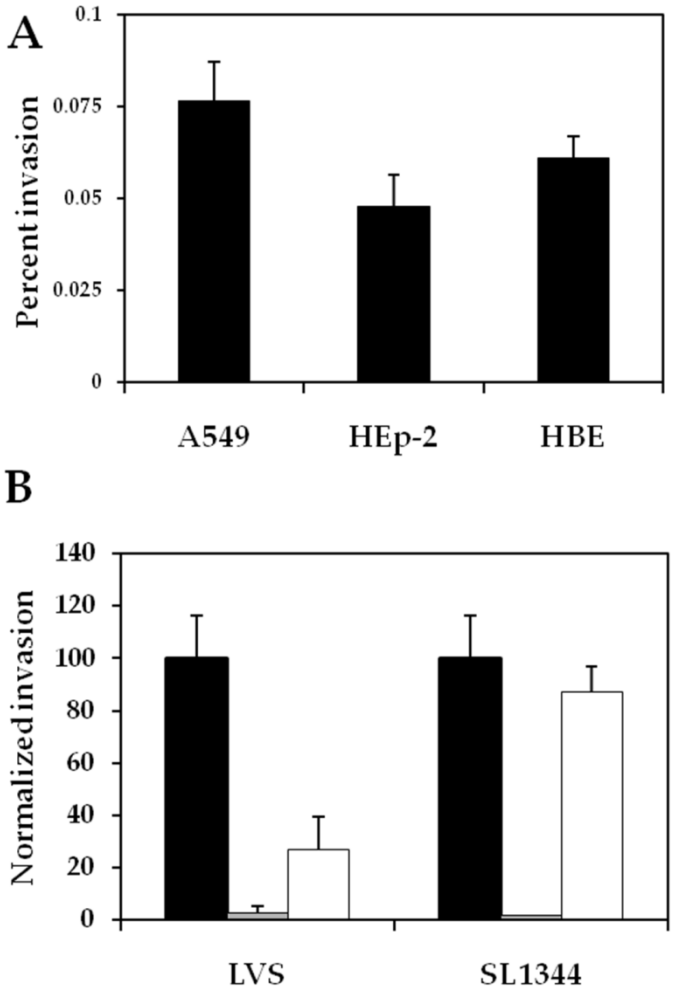

Figure II.3. Quantitative uptake of *F. tularensis* LVS into epithelial cell lines and inhibition by cytoskeletal disruption. A. Quantitative invasion of *F. tularensis* LVS for three different nonphagocytic cell lines. Bars represent percent of the inoculum (10^7) surviving treatment with 10 μg/mL gentamicin for 1 h following a 4 hour infection. Each error bar represents one standard deviation; differences between cell types are not statistically significant. B. Inhibition of invasion by disruption of cytoskeletal function. The bars represent the number of organisms that were protected from gentamicin treatment after 1 hour of incubation, normalized to the number of internalized bacteria in untreated cells. Cytochalasin D (gray) inhibits entry requiring actin polymerization and nocodazole (white) inhibits entry using microtubule polymerization. *Salmonella enterica* serovar Typhimurium (SL1344) is included as an invasive organism that is inhibited by actin disruption but not by microtubule disruption. Each error bar represents one standard deviation, inhibition of LVS invasion by both cytochalasin D and nocodazole are significantly different from the no inhibitor control ($P < .05$). Data shown are representative of several experiments.

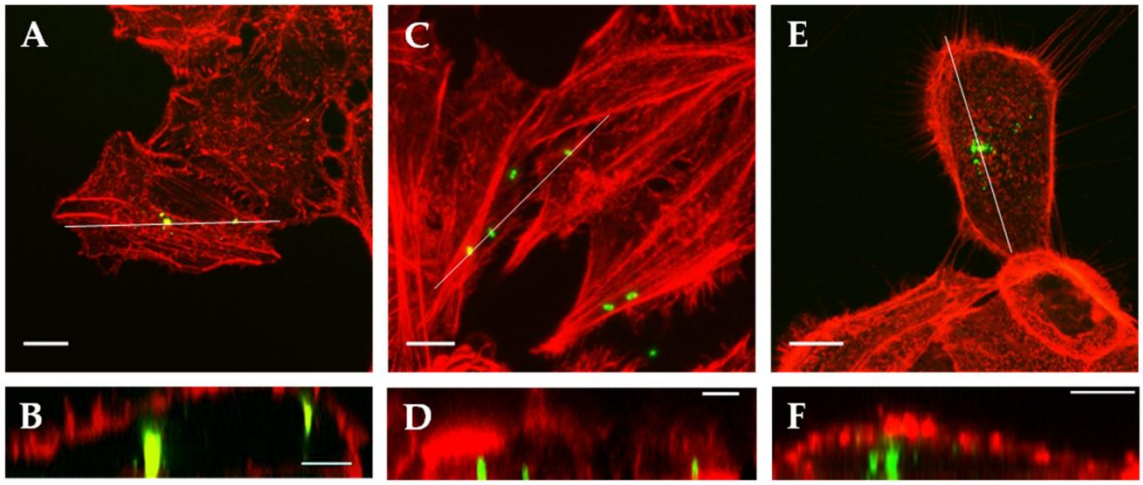

Figure II.4. Confocal microscopy of *F. tularensis* LVS invasion into A549, HEp-2, and HBE nonphagocytic tissue culture cells. Panels A (A549), C (HEp-2), and E (HBE) are Z-projections of stacks of images of a 4 hour invasion assay and show bacteria (green) that are located beneath the membrane of the infected nonphagocytic cells. Panels B, D, and F are selected slices through the Z-projection stacks above along the indicated lines. Bacteria are immunolabeled green with primary rabbit *Francisella tularensis* antiserum and secondary goat anti-rabbit IgG conjugated to Alexa 488. Cellular F-actin is stained red with rhodamine phalloidin. Scale bars represent 10 μm (A, C, E) and 5 μm (B, D, F).

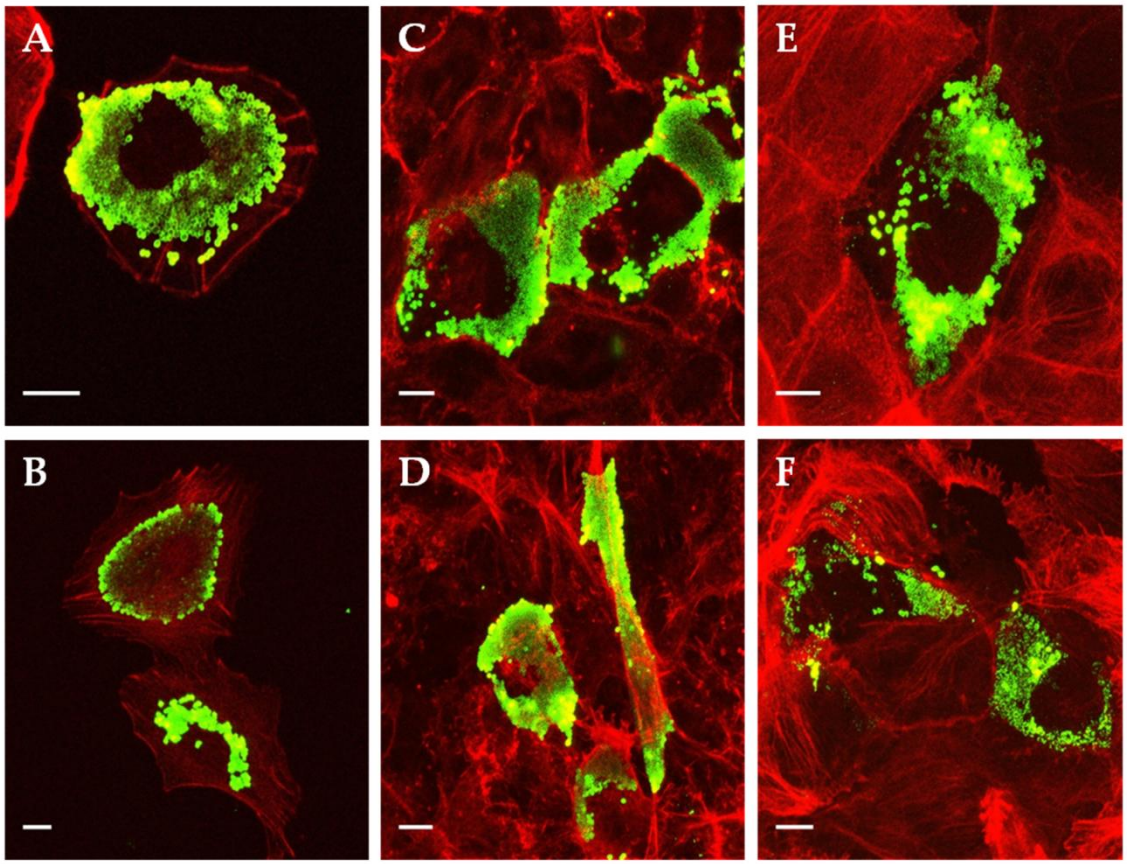

Figure II.5. Confocal microscopy of *F. tularensis* LVS growth within A549, HEp-2, or HBE cells. Bacteria have replicated extensively within the cytoplasm of each infected cell (A549, A,B; HEp-2, C, D; HBE, E, F); the nuclei of infected cells appear to exclude LVS replication. The images are Z-projections of stacks of images and were taken 25 hours post-infection. Bacteria are immunolabeled green with rabbit *Francisella tularensis* antiserum and goat anti-rabbit IgG conjugated to Alexa 488 and cellular F-actin is stained red with rhodamine phalloidin. Scale bars represent 10 μm.

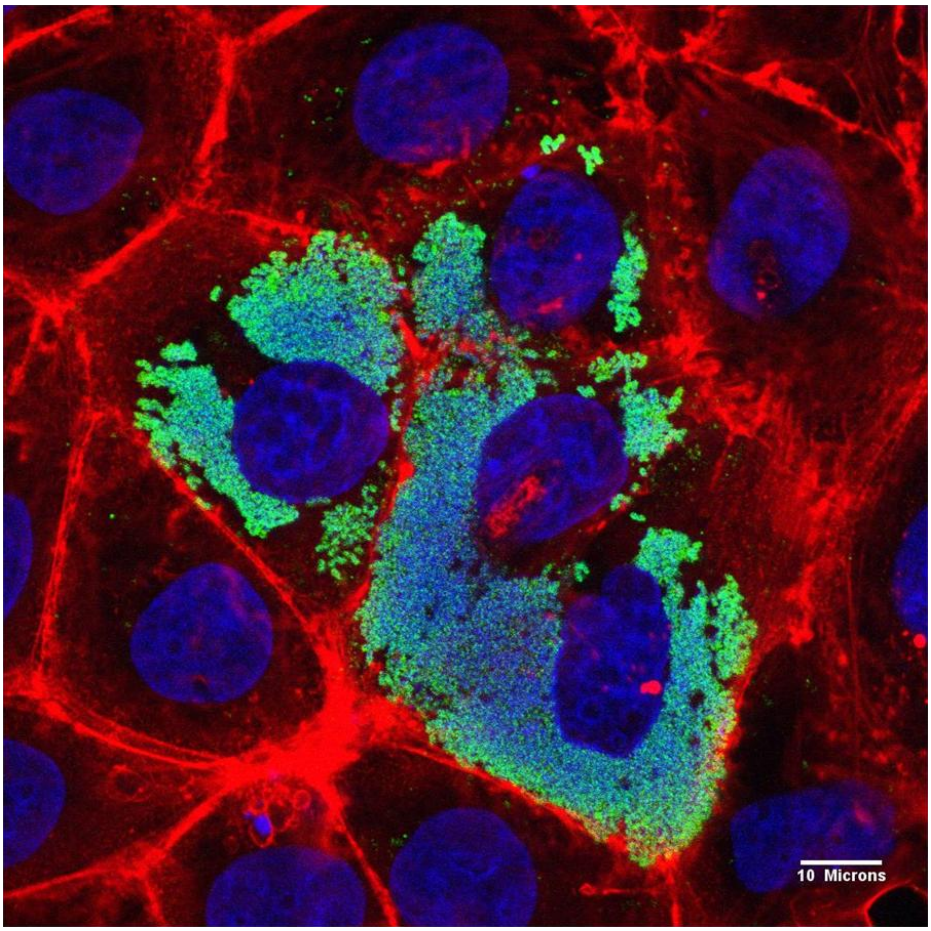

Figure II.6. Intracellular growth of *F. tularensis* LVS within human bronchial epithelial cells. Cells were fixed at 24 hours post-infection, at which time *Francisella* antigens (green) and DNA (blue cytosolic staining) are seen throughout the cytosol of infected cells, exhibiting the extent to which *Francisella* has occupied the intracellular volume. This image is a ~1 μm slice through the middle of the HBE monolayer. Consequently, bacterial antigens can be seen in the middle of neighboring cells; whether these represent live bacteria or components thereof is unknown. Bacteria are immunolabeled green with rabbit *Francisella tularensis* antiserum and goat anti-rabbit IgG conjugated to Alexa 488 and cellular F-actin is stained red with rhodamine phalloidin. DNA is stained with TO-PRO-3.

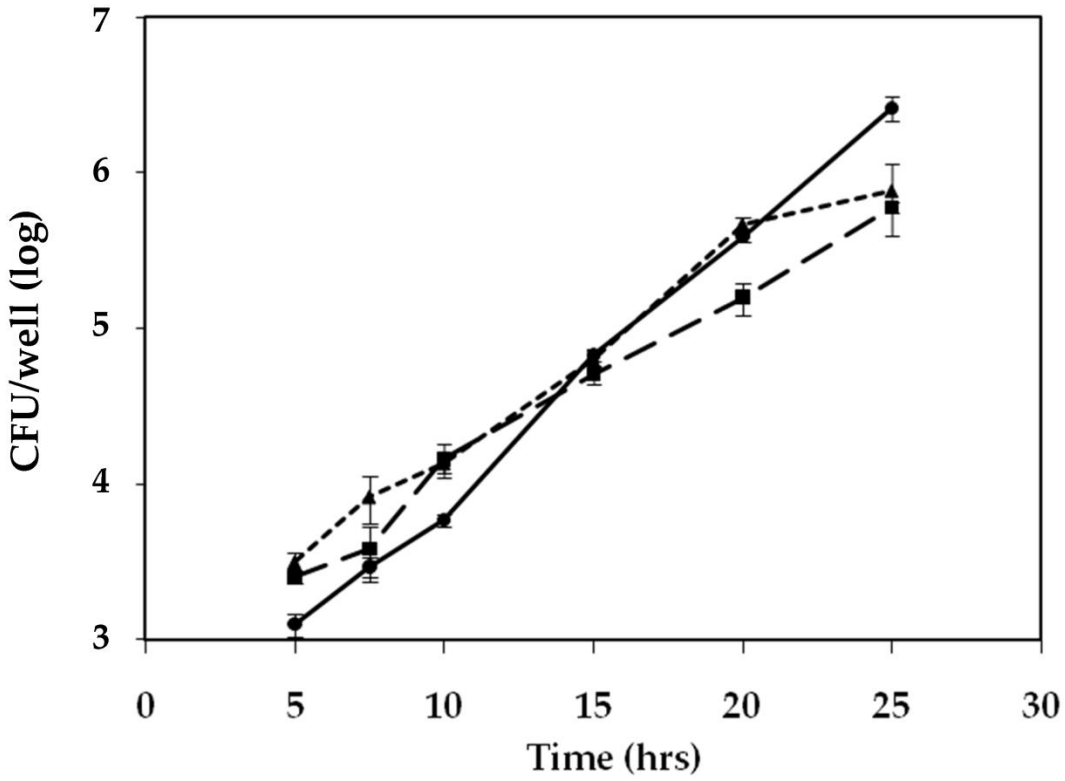

Figure II.7. *F. tularensis* LVS intracellular growth curve within epithelial cell lines. A549 (triangle symbols), HEp-2 (circle symbols) and HBE (square symbols) cells were infected at an MOI of 100 (10^7 CFU to 10^5 cells) for one hour followed by a one hour treatment with 10 μg/mL gentamicin. Samples were collected at time points of 5 h, 7.5 h, 10 h, 15 h, 20 h, and 25 h post-infection. Bacterial replication was continuous after 5 h. post-infection in all cell lines, with a doubling time of ~104 min. Error bars represent one standard deviation. Data are from a representative experiment of three.

CHAPTER III
IDENTIFICATION OF GENES REQUIRED FOR INTRAMACROPHAGE SURVIVAL AND GROWTH BY NEGATIVE SELECTION

Introduction

Francisella tularensis contains two major biovars that cause human tularemia: type B (*F. tularensis* ssp. *holarctica*), which is found throughout the entire Northern hemisphere, and type A (*F. tularensis* ssp. *tularensis*), which is found exclusively within North America and typically causes a more severe disease in humans than type B isolates (225). Significant research effort to date has focused on the virulence properties of the non-human pathogen *F. novicida* and the *F. tularensis* live vaccine strain (LVS), an attenuated *holarctica* strain generated in the Soviet Union in the 1950s, due to the relative ease and safety of working with these strains as compared with fully virulent *F. tularensis* strains. While both of these strains are generally avirulent in humans, they do retain the ability to grow in some human cells and cell lines intracellularly *in vitro* and are virulent in the mouse model of infection (though LVS does not appear to be as virulent as wild type *holarctica* strains) (153, 174, 179). Both of these properties have made them attractive models for studying human *F. tularensis* infection.

To date, several random transposon mutant libraries have been generated in *F. novicida* and LVS and screened through a variety of model systems in an attempt to understand *Francisella*'s virulence. *F. novicida* libraries have been screened through mouse macrophage-like cell line RAW 264.7 (228), *Drosophila melanogaster* (1), and the mouse intraperitoneal (238) and inhalational (128) model systems, as well as utilized to search for mutants incapable of forming biofilms (66). Transposon libraries in LVS have been employed to identify genes important for virulence in mouse macrophage-like cell line J774A.1 (145) and in

intranasal infection of mice (223) as well to detect mutants in genes that are involved in inhibition of the respiratory burst in neutrophils (214), are differentially regulated or required for growth on CDM (4, 26, 146), or are involved in regulation of the FPI (25).

Although many large random mutational studies have been performed in avirulent strains of *Francisella*, few such studies have been performed in virulent strains. The largest study to date was performed by Qin and Mann, who generated a ~700 member Tn*5* (EZ-TN) mutant library in the type A strain *F. tularensis* Schu S4 and screened each clone individually through hepatocyte-like cell line HepG2 to detect mutants defective in intracellular growth (191). Interestingly, among the mutants detected in this screen was in the *F. tularensis* Schu S4 ORF *FTT1236*. This mutant was also determined to be defective in growth within J774 cells. A library of approximately fifty Tn*phoA* clones has also been generated to detect genes that code for proteins with exposure to the periplasm (92). Until this work, no near-saturating random mutant library in a virulent *Francisella* strain had been screened in a high-throughput manner.

Consequently, in this chapter I describe the generation of a near-saturating transposon mutant library in virulent strain Schu S4 that is capable of being screened in a high-throughput way by transposon site hybridization, a relatively new microarray-based system. This is significant as recent survival studies suggest that there are fundamental differences between the disease processes and virulence genetics of avirulent strains as compared with that of virulent strains of *F. tularensis*. First, it appears that the mechanism by which cell death is induced *in vivo* varies between strains. Schu S4 is known to induce caspase-3 mediated cell death in the livers and spleens of infected mice, which results in widespread necrosis and dispersion of *Francisella* antigens throughout the liver; in contrast, the livers and spleens of LVS-infected mice exhibit caspase-

1 activation and containment of necrosis and *Francisella* antigens to microgranulomas (241). Second, some genes implicated in virulence in human-attenuated strains have been found to contribute little to pathogenicity in wild type strains; for example, *acpA* has been implicated as important for blockade of the respiratory burst and virulence in macrophages in *F. novicida* (155, 159, 161, 162) but has been shown to be dispensable in Schu S4 (43) and mutation of *tolC* has been suggested to result in hypercytopathogenicity in LVS (188) but its contribution to virulence in Schu S4 appears to be marginal (122). Taken together, these data suggest that the previously attempted high-throughput screens of random mutants in avirulent *Francisella* strains may be incomplete, in that they fail to detect virulence genes that are important in human-virulent strains but are nonfunctional in these avirulent strains. Furthermore, they may even be misleading in that they implicate genes which might be necessary in the attenuated strains for a virulence in a certain model system which is not active in the human-virulent strains or whose role in virulence is detectable in attenuated strains only because their virulence is markedly reduced globally.

Transposon site hybridization, or TraSH, is a method that utilizes promoters which can be selectively activated *in vitro* reading out from the transposon into the neighboring DNA into which the transposon has inserted (239). This method has been previously used in *F. novicida* to identify genes required for virulence in mice in an intraperitoneal inoculation model (238). However, this system was not immediately portable into Schu S4. First, this system employed the much more efficient transformation of *F. novicida* by electroporation, which is not safe for use within the biosafety level 3 (BSL-3) containment required for virulent *F. tularensis*. Further, this system employed transformation of a purified transposon-transposase complex (125) into *F. novicida* which is activated in the presence of magnesium ions. In my hands and

others', this system was inefficient and expensive even when utilizing electroporation, but the magnesium-containing buffers required for cryotransformation as required by BSL-3 safety protocols made use of this system impossible. Consequently, in this chapter I describe the effort by me and others to modify a previously-existing temperature-sensitive transposon delivery system (26) to generate mutants capable of use in transposon-site hybridization.

Upon the inception of the project, I initially intended to use this library to detect genes that were important for uptake and growth in epithelial cells. However, the inefficiency of internalization of LVS and also Schu S4 (data not shown) by epithelial cells made the high-throughput screen prohibitively difficult, as the size of the experiment required to achieve a statistically significant evaluation of the whole ~15,000-member library was extremely high. Efforts to increase the uptake of *Francisella* into epithelial cells by varying growth conditions and phase failed. I further attempted to isolate organisms cultured intracellularly in macrophages, reasoning that exposure to the intracellular environment might be required for expression of genes required for efficient uptake into epithelial cells; this too failed to increase uptake measurably (data not shown). Consequently, I decided to utilize a different model system relevant to human tularemia, the primary human macrophage, which I discuss in this chapter.

Materials and Methods

Bacterial strains and growth conditions

All *Francisella* strains studied in this work are isogenic with *F. tularensis* ssp. *tularensis* Schu S4. Bacteria were routinely cultured on modified Mueller-Hinton plates (Acumedia, Lansing, MI) or in modified Mueller-Hinton broth supplemented with 150 mM NaCl (MMH-Na). Where necessary to maintain plasmids, 25 μg/ml spectinomycin (Spec) or 25 μg/ml kanamycin (Kan) were

added to the media. *Escherichia coli* DH12S and DH5α λ pir were utilized in plasmid construction and were routinely cultured on Lennox agar and broth supplemented with 50μg Spec or 50μg Kan as appropriate. All work with Schu S4 was performed within the Carver College of Medicine Biosafety Level 3 Core Facility and all experimental protocols were reviewed for safety by the BSL-3 Oversight Committee of the University of Iowa Carver College of Medicine. Recombinant DNA work with Schu S4 was approved by the Institutional Biosafety Committee.

Library construction

Plasmid pBDJ314 was generated through the addition of T7 promoters, pointing away from transposon sequences, to pBDJ303, the construction of which has been previously described by our lab (26). Custom oligonucleotides (Integrated DNA Technologies, Coralville, IA) were designed and the single-stranded oligonucleotides annealed to form a double-stranded fragment with overhanging ends compatible with restriction sites of pBB303 at either end of the mini-Tn5 transposable element (KpnI/EcoRI and SphI). Insertion of the promoters was confirmed by restriction digestion and sequencing. T7 promoter activity from either end of the mini-Tn5 element was confirmed by digesting pBDJ314 with EagI and performing *in vitro* transcription using T7 RNA polymerase, which yielded the expected 1.0 kb and 1.6 kb RNA products from the respective T7 promoters (data not shown). Plasmid pBDJ314 was next fused with plasmid pMKM219 as described previously (26) to generate a temperature-sensitive *F. tularensis*/*E. coli* shuttle vector and named pSL103. Plasmid pSL103 was cryotransformed into *F. tularensis* Schu S4, selected on MMH + 0.5% sheep's blood (MMH-B 0.5) + 25 μg/ml Spec plates at 32°C and one colony was inoculated overnight into MMH-Na broth shaking at 32°C and grown overnight to an OD_{600} ~ 0.15. This culture was diluted and plated on MMH-B 0.5 + 25 μg/ml

Kan plates and incubated at 40°C to select for transposon insertions in the *Francisella* chromosome. Colonies were counted, swabbed into subpools of 100-200 independent mutants , swabbed into MMH-N + 25 μg/ml Kan broth and shaken overnight at 40°C to cure any remaining plasmid and to minimize the possibility of double transposon insertion mutants. These subpools were combined into pools of ~1,000 colonies, normalized by OD_{600} and frozen at -80°C in MMH-N broth + 0.5M sucrose and 10% glycerol.

Macrophage isolation and culture

Heparinized venous blood was drawn from healthy adult volunteers using a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa, and all subjects provided informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated as described previously (213, 214). Briefly, PBMCs were isolated from venous blood by centrifugation on Ficoll-Hypaque (GE Healthcare, Piscataway, NJ), washed twice in HEPES-buffered RPMI 1640 with L-glutamine (RPMI; Lonza, Walkersville, MD), seeded into Teflon jars at 2 x 10^6 cells/ml and allowed to differentiate into monocyte-derived macrophages (MDMs) for 5-7 days in RPMI 1640 + 20% autologous serum at 37°C.

Transposon site hybridization

MDMs were plated overnight into 6-well dishes (Costar, Corning, NY) at a density of 3 x 10^5 MDMs/well. All fifteen pools comprising our T7-capable mutant library were grown overnight in MMH-N broth to mid-log phase (OD_{600} ~0.3-0.6), normalized by OD_{600}, and combined. Genomic DNA (gDNA) was purified from this input pool using the DNeasy Blood and Tissue Kit (QIAgen, Alameda, CA) according to the manufacturer's instructions. The library was pelleted by centrifugation, washed in HBSS with calcium and magnesium (Gibco) opsonized by shaking incubation at 37°C in 50% normal human serum

(NH) for thirty minutes, washed in HBSS (Gibco) to remove calcium and magnesium ions, resuspended in RPMI + 2.5% normal human serum, and plated to determine the experimental MOI. MDMs were asynchonously infected at an MOI ~ 100 in 1 ml/well of RPMI + 2.5% normal human serum for 1 h, washed with phosphate-buffered saline (PBS; Gibco) to remove unbound bacteria, and incubated for 23 h in fresh RPMI 1640 + 2.5% serum. After 1 h, one well was routinely lysed in 1% saponin (Acros, Morris Plains, NJ) in PBS to determine the number of retained bacteria, which averaged 3.6×10^6 CFU/ml, representing approximately 20-fold coverage of the library/well (data not shown). MDMs were then lysed in 1% saponin in PBS, and a sample was taken from each well and plated to determine the extent of bacterial replication, which averaged 8.5×10^8 CFU/ml, approximately 200-fold growth from the 1h time point (data not shown). Lysates of each well were then pelleted by centrifugation, washed in MMH-N broth to remove the saponin, and inoculated into MMH-N broth and shaken for 12 h until the cultures reached mid-log phase. These cultures were then harvested for gDNA as described above, comprising the output pools.

Each DNA sample was divided in two and digested separately with BfaI and RsaI (NEB, Ipswich, MA). The digested DNA was used as the template for *in vitro* transcription with the AmpliScribe T7-*Flash* transcription kit (Epicentre, Madison, WI) following the manufacturer's protocol, except that 2 μg of digested DNA was used, and the reaction was allowed to proceed for 12-16 h. Purified RNA was used in a reverse transcription reaction using SuperScript II (-) (Invitrogen, Carlsbad, CA) and random hexamers as primers. cDNA was labeled with amino-allyl dUTP by using the Klenow enzyme (NEB, Ipswich, MA). The ssDNA containing amino-allyl dUTP from the mouse output or the library input pools were labeled with Cy5 and Cy3 respectively, before hybridization to our *Francisella* microarray as described (237). All raw data sets are freely available for

download from the GEO database. Normalized data were downloaded from the Stanford Microarray Database according to the median $\log_2$ Cy5/Cy3 (logRAT2N). Filters for feature quality, including a Cy3 net median intensity of ≥150 and regression correlation of >0.6, were applied. To compare data from separate macrophage infection experiments, each experimental sample was zero-transformed to the input/input control. Features (spots) missing values for ≥30% of the arrays were removed from the data set. The data sets were analyzed with the SAM program, by using the two-class analysis option to identify features that consistently deviated from the input and samples across all arrays with a false discovery rate of 3.9%.

Results

In order to achieve a comprehensive view of the *F. tularensis* genes required for intracellular survival and growth within human primary macrophages, I first generated a library of ~15,000 transposon mutants in Schu S4, collecting them into pools of 1,000 and subpools of 200. This library represents approximately eightfold coverage of the 1,804 annotated open reading frames of the Schu S4 genome. Adequate representation of the genome in the library was confirmed by analysis of the entire library grown *in vitro*, the input pooled genomic DNA (data not shown). This library was used *in toto* for infections of human monocyte-derived macrophages (MDMs). At 24 hpi, these macrophages were lysed and each replicate was reinoculated into MMH broth and grown to mid-log phase for purification of genomic DNA. I and Kaitian Peng then compared bacteria surviving and replicating within MDMs with bacteria in the input sample taken at the time of infection. A rigorous statistical program, Significance Analysis of Microarrays (SAM), was used to analyze the microarray data. We identified 207 genes as present in the input pool but absent from the MDMs after infection (comprehensively listed in Table III.1).

Utilizing the classification scheme proposed by Larsson, *et al.*, in their annotation of the Schu S4 genome (134), with the addition of two categories for secretion and stress response genes, genes implicated in this screen were categorized and are summarized in Figure III.1. Of note, the largest category and representing 24.2% of the genes identified were annotated as hypothetical proteins, as no sufficient annotation could be found. A large percentage of the Schu S4 genome cannot, at present, be annotated due to *Francisella*'s extreme phylogenetic distance from more well-known organisms (134). Further, as *Francisella*'s intracellular life cycle and mechanisms of virulence are largely novel, it is expected that homologous genes to those required for *Francisella* virulence will not be found in other organisms described to date.

As is commonly observed in high-throughput screening of mutants in *Francisella*, genes presumably involved in protein synthesis and various aspects of metabolism accounted for approximately 45% of the genes implicated in our study. We had expected these genes to be negatively selected since they encode proteins that are known to be required for *Francisella* growth and replication in its intracellular niche. It is further likely that some metabolic genes implicated in our screen are necessary for growth within macrophages but not when grown in rich MMH broth. For example, it is likely that *pyrH* is identified by this screen due to the same defect in growth within primary macrophages observed for other mutants in pyrimidine biosynthesis that is not present during growth in rich media (114, 213). Further, several genes detected in our screen are generally thought to encode proteins essential for bacterial survival, including genes composing structural components of ribosomes like *rplD* and *rpsC*. While others have detected these genes and others similar in function in high-throughput screens (223, 238), it is likely that the transposon insertions either significantly alter the expression of these genes by affecting promoter or enhancer regions or

result in truncations that encode partially-functional products, thereby decrease the growth rate in broth culture, macrophages, or both. The negative selection technique I used here also identified genes involved in cell envelope biosynthesis, especially LPS O-antigen biogenesis, such as those lying within the *wbt* operon. While not surprising, as fresh normal human serum was used to opsonize the bacteria for uptake by macrophages and mutants in O-antigen are frequently vulnerable to the lytic effects of complement, mutants with altered surface polysaccharide synthesis have been shown to exhibit various defects in intramacrophage growth (56, 108, 139, 198, 230), which is discussed in detail in Chapter IV.

Notably, the mutants in genes of the *Francisella* pathogenicity island (FPI) did not appear to be selected against in this screen. These genes, while central to intracellular pathogenesis, are not frequently identified in high-throughput screens conducted in *F. tularensis* strains due to their duplication within the genome. Su, *et al.*, detected mutants in *iglA*, *iglB*, and *iglC* in intranasally-infected mice by signature-tagged mutagenesis (223). When examined more closely, however, a transposon mutant in *iglC* resulted in a mere sixfold defect in competition with wild type and represented the least significant virulence phenotype observed by this group. Further, a mutational analysis in LVS has shown that one copy of *iglC* is dispensable for intracellular pathogenesis (96, 214). These data suggest that mutants in a single copy of the FPI genes have, at most, a small defect in intracellular growth. The fact that these genes were not implicated in virulence in my screen suggests that the negative selection described in this chapter robustly and sensitively identifies genes in which mutants are likely to have significant virulence defects.

Discussion

My application of transposon site hybridization to identify Schu S4 genes required for growth in human primary macrophages is the first near-saturating mutant library screened in a virulent strain of *Francisella* and has successfully identified several genes that, prior to this study, were not known to be involved in *Francisella* virulence. At the same time, this screen also confirms several genes implicated by other high-throughput screens as important for virulence in varying strains of *Francisella* and under varying experimental conditions (Table III.1). While there is significant overlap between the set of genes obtained through my screen and others, approximately 25% of the genes reported in this chapter have been independently detected in other high-throughput screens. Transposon site hybridization is somewhat unique, however, in the manner in which position information is obtained; as *in vitro* transcription from the ends of the inserted transposon reads into the neighboring genes, open reading frames proximal to the site of insertion can be inadvertently implicated as important in the model system of interest, creating a bystander effect. Further, transposon insertions into operons are likely to be polar mutations; hence a gene may be erroneously identified as important for virulence as an insertion into one gene may be exerting an indirect effect on the expression of a distal virulence gene. Once these limitations are factored in and neighboring genes and putative operons are considered, the overlap of the genes detected by this study and those of other studies broadens. It is likely that some of the lab-to-lab divergence in the genes implicated by high-throughput techniques as required for virulence are dependent upon many unknown variables particular to the *Francisella* strain and cell lines chosen; a meta-analysis of high-throughput random mutant screens utilizing a variety of *Francisella* strains and model systems showed that 81% of the genes implicated in virulence were detected in only one study of the nine

examined (156). Consequently, in view of the apparently divergent mechanisms of pathogenesis and virulence genetics of various *Francisella* strains, in order to generate results significant to the investigation of human tularemia, I chose to screen a near-saturating library of human-virulent type A1a strain Schu S4 in primary human macrophages. This screen identified 207 genes as potentially involved in survival and growth within these macrophages. The selection, mutagenesis and characterization of genes implicated by the screen as contributing to virulence are described in the following chapter.

The power of TraSH is that, utilizing one library, several different model systems can be screened and genes required for those specific models can be elucidated. Thus, increasing iterations of TraSH performed under different conditions (i.e. route of infection, animal host, cell type, etc.) will allow the determination of which genes are specifically required under certain conditions and which participate more globally in virulence functions. Consequently, screening of the Schu S4 TraSH library through ticks, intranasally through mice, and in epithelial cells, among others, will be useful for the further determination of genes important for *Francisella*'s pathogenic lifestyle and may uncover the virulence genetics required by *Francisella* to successfully parasitize such a wide variety of disparate hosts.

Table III.1. Schu S4 genes negatively selected in primary human macrophages

Schu S4 gene designation	Gene name	Annotation (Schu S4)
FTT0001	*dnaA*	chromosomal replication initiator protein dnaA
FTT0020	*gatA*	Glutamyl-tRNA(Gln) amidotransferase subunit A
FTT0037	*nuoG*	NADH dehydrogenase I, G subunit
FTT0046		chelatase family protein, pseudogene
FTT0050	*infB*	translation initiation factor IF-2
FTT0076	*sucA*	2-oxoglutarate dehydrogenase E1 component
FTT0077*	*sucB*	dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex
FTT0095		hypothetical protein
FTT0113*	*deoB*	Phosphopentomutase
FTT0119		hypothetical membrane protein
FTT0132	*glpA*	anaerobic glycerol-3-phosphate dehydrogenase
FTT0137	*tufA*	elongation factor Tu (EF-Tu)
FTT0141	*rplA*	50S ribosomal protein L1
FTT0143	*rplL*	50S ribosomal protein L7/L12
FTT0145*	*rpoC*	DNA-directed RNA polymerase, beta subunit
FTT0148		fatty acid desaturase
FTT0153	*rplS*	50S ribosomal protein L19
FTT0159c		hypothetical membrane protein
FTT0167	*hemA*	Glutamyl-tRNA reductase
FTT0183c*	*rpsA*	30S ribosomal protein S1
FTT0188	*ftsZ*	cell division protein
FTT0190c	*dnaX*	DNA polymerase III, gamma/tau subunits
FTT0206c		dienelactone hydrolase family protein, pseudogene
FTT0209c*		periplasmic solute binding family protein
FTT0213	*rbn*	tRNA processing ribonuclease BN
FTT0215	*priA*	Primosomal protein N
FTT0234c*		conserved hypothetical protein
FTT0255c		hypothetical protein
FTT0278c	*cydB*	cytochrome d terminal oxidase, polypeptide subunit II
FTT0279c*	*cydA*	cytochrome d terminal oxidase, polypeptide subunit I
FTT0291*		conserved hypothetical protein
FTT0313	*rpsB*	30S ribosomal protein S2
FTT0315*	*pyrH*	uridylate kinase
FTT0321	*rpsL*	30S ribosomal protein S12
FTT0323	*fusA*	elongation factor G (EF-G)
FTT0324	*rpsJ*	30S ribosomal protein S10
FTT0325	*rplC*	50S ribosomal protein L3
FTT0326*	*rplD*	50S ribosomal protein L4
FTT0327*	*rplW*	50S ribosomal protein L23
FTT0328*	*rplB*	50S ribosomal protein L2
FTT0329	*rpsS*	30S ribosomal protein S19
FTT0332	*rplP*	50S ribosomal protein L16
FTT0333*	*rpmC*	50S ribosomal protein L29
FTT0334	*rpsQ*	30S ribosomal protein S17
FTT0335	*rplN*	50S ribosomal protein L14
FTT0336	*rplX*	50S ribosomal protein L24
FTT0337	*rplE*	50S ribosomal protein L5

Table III.1. Continued

Schu S4 gene designation	Gene name	Annotation (Schu S4)
FTT0338	*rpsN*	30S ribosomal protein S14
FTT0339	*rpsH*	30S ribosomal protein S8
FTT0340	*rplF*	50S ribosomal protein L6
FTT0341	*rplR*	50S ribosomal protein L18
FTT0344*	*rplO*	50S ribosomal protein L15
FTT0347	*rpsM*	30S ribosomal protein S13
FTT0348	*rpsK*	30S ribosomal protein S11
FTT0349	*rpsD*	30S ribosomal protein S4
FTT0350	*rpoA1*	DNA-directed RNA polymerase, alpha subunit
FTT0358		overlaps conserved hypothetical protein, pseudogene
FTT0361c		amino acid transporter
FTT0416*	*glgA*	glycogen synthase
FTT0438	*mpl*	UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase
FTT0447c		conserved hypothetical membrane protein
FTT0479c	*perM*	PerM family protein
FTT0503c*	*sucD*	Succinyl-CoA synthetase, alpha subunit
FTT0504c*	*sucC*	Succinyl-CoA synthetase beta chain
FTT0535c	*mdh*	lactate dehydrogenase
FTT0559c	*cmk*	cytidylate kinase
FTT0563	*potH*	polyamine transporter, subunit H, ABC transporter, membrane protein
FTT0567c		conserved hypothetical membrane protein, pseudogene
FTT0583*	*fopA*	outer membrane associated protein
FTT0588*	*aroA*	3-phosphoshikimate 1-carboxyvinyltransferase
FTT0593c		hypothetical membrane protein
FTT0626*		DNA-binding, ATP-dependent protease La
FTT0628		conserved hypothetical protein
FTT0642	*ilvH*	acetolactate synthase, small subunit
FTT0650c	*grxB*	Glutaredoxin 2
FTT0678c		hypothetical lipoprotein
FTT0680c	*pth*	Peptidyl-tRNA hydrolase
FTT0685c*		potassium channel protein
FTT0698	*rpsO*	30S ribosomal protein S15
FTT0710	*ftsB*	cell division protein
FTT0711	*ispD*	2-C-methyl-D-erythitol 4-phosphate cytidylyltransferase
FTT0712c*	*sdaC1*	serine transporter
FTT0713c*	*tdh*	L-threonine 3-dehydrogenase
FTT0723c		Short-chain dehydrogenase/reductase family protein
FTT0737c		hypothetical membrane protein
FTT0754c*		hypothetical membrane protein
FTT0763c	*yjjV*	TatD related DNAse family protein
FTT0767c		hypothetical protein
FTT0775c	*bcr2*	major facilitator superfamily (MFS) transport protein, pseudogene

Table III.1. Continued

Schu S4 gene designation	Gene name	Annotation (Schu S4)
FTT0793*		ABC transporter, ATP-binding and membrane protein
FTT0794		conserved hypothetical protein
FTT0795		conserved hypothetical protein
FTT0807*		conserved hypothetical membrane protein
FTT0817	*thr*	Threonyl-tRNA synthetase
FTT0830c		Smf protein DNA processing chain A, pseudogene
FTT0834	*aroQ*	chorismate mutase
FTT0838	*tolR*	tolR protein
FTT0842		Peptidoglycan-associated lipoprotein
FTT0857c		conserved hypothetical protein
FTT0876c	*aroC*	chorismate synthase
FTT0881c		amino acid permease
FTT0886		DNA repair protein recN
FTT0889c*		Type IV pili fiber building block protein
FTT0890c		Type IV pili fiber building block protein
FTT0892*	*folD*	methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase putative bifunctional protein
FTT0896	*purE*	phosphoribosylaminoimidazole carboxylase, catalyic subunit
FTT0899c*	*prlC*	Oligopeptidase A
FTT0921		
FTT0930c		acetoacetate decarboxylase, fragment
FTT0939c	*add*	adenosine deaminase
FTT0954c		hypothetical protein
FTT0955c	*gor*	Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase component
FTT0956c		hypothetical membrane protein
FTT0957c		conserved hypothetical protein
FTT0977c*		conserved hypothetical protein
FTT0992		conserved hypothetical membrane protein
FTT1020c		amino acid permease
FTT1025c		hypothetical protein
FTT1026c		hypothetical protein
FTT1039	*dacB*	D-alanyl-D-alanine carboxypeptidase (Penicillin binding protein) family protein
FTT1040		conserved hypothetical lipoprotein
FTT1041		conserved hypothetical protein
FTT1050c	*cysN*	sulfate adenylate transferase subunit 1
FTT1060c	*rplI*	50S ribosomal protein L9
FTT1061c	*rpsR*	30S ribosomal protein S18
FTT1066c		hypothetical protein
FTT1068c		hypothetical protein
FTT1069c		hypothetical protein
FTT1085		conserved hypothetical protein
FTT1098c		UvrD/REP helicase family protein, pseudogene
FTT1103*		conserved hypothetical lipoprotein
FTT1104	*bcr1*	major facilitator superfamily (MFS) transport protein, pseudogene

Table III.1. Continued

Schu S4 gene designation	Gene name	Annotation (Schu S4)
FTT1110		hypothetical protein
FTT1112c	*rpoH*	RNA polymerase sigma-32 factor
FTT1113c		hypothetical protein
FTT1130c	*cphA*	cyanophycin synthetase
FTT1188		hypothetical membrane protein
FTT1205	*gidA*	glucose inhibited division protein A
FTT1206		hypothetical lipoprotein
FTT1230*	*serA*	D-3-phosphoglycerate dehydrogenase
FTT1233c	*yjdL*	Proton-dependent oligopeptide transport (POT) family protein
FTT1234*		choloylglycine hydrolase family protein
FTT1235	*IpcC*	glycosyltransferase group 1 family protein
FTT1236*		hypothetical protein
FTT1237*		glycosyltransferase family 8 protein
FTT1252		conserved hypothetical protein
FTT1254	*fadD1*	Acyl-CoA synthetase (long-chain-fatty-acid—CoA ligase)
FTT1257*	*emrA1*	HlyD family secretion protein
FTT1261c		flavodoxin family protein, pseudogene
FTT1299	*hitA*	histidine triad (HIT) family protein
FTT1304c	*murB*	UDP-N-acetylenolpyruvoylglucosamine reductase
FTT1334c*		hypothetical protein
FTT1341		membrane protein
FTT1342		conserved hypothetical membrane protein
FTT1366c	*pyk*	pyruvate kinase
FTT1368c	*gapA*	Glyceraldehyde-3-phosphate dehydrogenase
FTT1369c*	*tktA*	Transketolase
FTT1374		malonyl coA-acyl carrier protein transacylase
FTT1376	*acpP*	acyl carrier protein
FTT1383*	*sun*	Sun protein
FTT1390*	*panC*	Pantoate-beta-alanine ligase
FTT1406c		hypothetical membrane protein
FTT1413		aspartate/tyrosine/aromatic aminotransferase
FTT1428c		acetyltransferase
FTT1440c		major facilitator superfamily (MFS) transport protein, pseudogene
FTT1441		conserved hypothetical protein
FTT1448c	*manC*	Mannose-1-phosphate guanyltransferase
FTT1451c	*wbtL*	Glucose-1-phosphate thymidylyltransferase
FTT1456c*	*wbtH*	asparagine synthase
FTT1458c	*wzy*	Membrane protein/O-antigen protein
FTT1460c*	*wbtE*	UDP-glucose/GDP-mannose dehydrogenase
FTT1462c*	*wbtC*	UDP-glucose 4-epimerase
FTT1463c	*wbtB*	galactosyltransferase
FTT1469c	*nadA*	quinolinate synthetase A
FTT1470*	*gmk*	guanylate kinase
FTT1471c*	*deaD*	Cold-shock DEAD-box protein A
FTT1472c*	*ppiC*	Peptidyl-prolyl cis-trans isomerase
FTT1483c*	*lpd*	dihydrolipoamide dehydrogenase
FTT1484c	*aceF*	pyruvate dehydrogenase, E2 component

FTT1485c	*aceE*	pyruvate dehydrogenase, E1 component
FTT1489*		hypothetical protein
FTT1495c		hypothetical membrane protein
FTT1522c		conserved hypothetical protein
FTT1554c	*truB*	tRNA pseudouridine synthetase B
FTT1555c	*rnc*	Ribonuclease III
FTT1557c		Two-component response regulator
FTT1570c	*fabZ*	(3R)-hydroxymyristoyl-(acyl-carrier protein) dehydratase
FTT1571c	*lpxD*	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase
FTT1586c*		hypothetical membrane protein
FTT1611*		hypothetical protein
FTT1612		hypothetical protein
FTT1614c		hypothetical protein
FTT1628c		metal ion transporter
FTT1641c	*hdR2*	HdR protein, fragment
FTT1642c	*hdR3*	HdR protein, fragment
FTT1688*		aromatic amino acid transporter of the HAAAP family
FTT1749	*secB1*	preprotein translocase, subunit B, chaperone protein
FTT1753	*tdcD*	propionate kinase
FTT1796c		conserved hypothetical protein
FTT1803c*	*trpR*	trp operon repressor

Note: Asterisk denotes that one or more previously published high-throughput studies have also implicated this gene as potentially important for virulence.

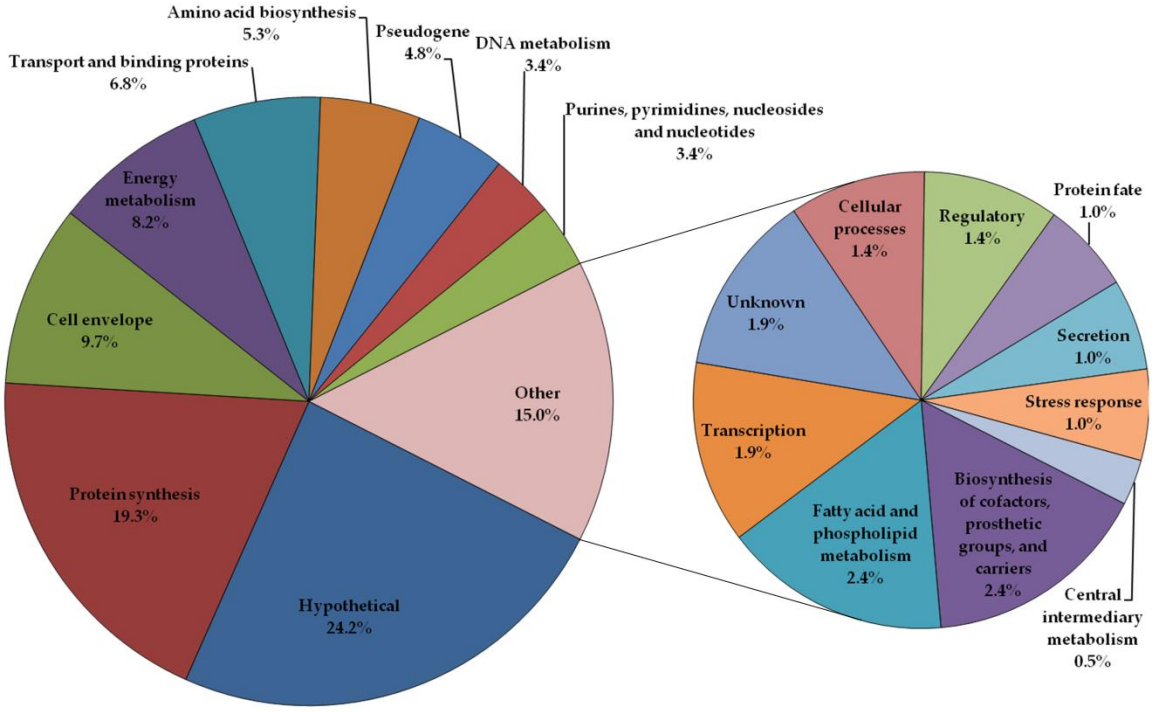

Figure III.1. Categorization of Schu S4 genes negatively selected in human primary macrophages. Categories are as defined by Larsson, *et al.*, 2005. *Nature Genetics*, **37**:153-159, with the addition of categories for stress response and secretion.

CHAPTER IV

CHARACTERIZATION OF A *FRANCISELLA TULARENSIS* LOCUS REQUIRED FOR SURFACE POLYSACCHARIDE BIOSYNTHESIS AND PREVENTION OF EARLY MACROPHAGE DEATH

Introduction

While classified among the γ-proteobacteria, *Francisella,* the only genus of family *Francisellaceae*, has no pathogenic near neighbors phylogenetically. Based upon 16S rRNA sequencing, *Francisella* has more in common with the tick endosymbiont *Wolbachia* than with bacteria known to have similar intracellular lifestyles in humans, such as *Coxiella* and *Legionella* (134). Furthermore, there are marked differences in the modes of virulence between these nearest-neighbor intracellular pathogens and *Francisella*, not least of which is the fact that *Francisella* escapes its phagosome and replicates within the cytosol while *Coxiella* and *Legionella* grow intravacuolarly (75). Many genes which have been implicated in virulence do not have an annotation based upon significant homology to known proteins of other organisms and are annotated simply as hypothetical proteins (156), and it is further likely that the phylogenetic distance has resulted in some genes receiving misleading annotations. These factors have made *in silico* annotation of *Francisella* genes and prediction of protein function difficult, especially with respect to genes involved in prosecuting *Francisella's* intracellular lifestyle. Attempting comparison to other bacteria that escape the phagosome and grow within the cytosol is also of little help, as *Francisella*'s virulence strategy is increasingly perceived to be dissimilar from other intracytosolic bacteria such as *Listeria, Shigella*, *Burkholderia*, and *Rickettsia.* Because *Francisella* lacks virulence factors common to intracytosolic bacteria, such as a type III secretion system, known pore-forming toxins, and actin-based

motility (197), it is likely that many of the genes required for *Francisella*'s successful parasitism of host cells will be without homology to those of other intracellular bacteria and therefore remain unannotated.

The lack of annotation poses an additional problem when attempting to select genes implicated in virulence from high-throughput screening for further study, as little or no indication of the role or importance of a gene in virulence can be ascertained from sequence analysis. One method to reduce uncertainty in the discovery of interesting genes is to select genes that have been implicated by other high-throughput screens under different conditions and possibly in varying strains. Another is to utilize both weak homology to other genes of interest and localization in the chromosome near other genes of known function or importance to select genes for further study. One of the limitations of the TraSH system is that it does not result in easily obtainable mutants to further examine for virulence phenotypes; consequently, I used both strategies to select genes to mutagenize for further study. Of particular note in the TraSH results were several contiguous genes that were negatively selected in MDMs (*FTT1235, FTT1236,* and *FTT1237* as well as the neighboring gene *FTT1238*) that I hypothesized may constitute a locus required for intramacrophage growth. In *F. novicida*, homologs of *FTT1236*, *FTT1237,* and *FTT1238* were found to be selected against in mouse spleen after intraperitoneal infection (238). In Schu S4, mutants in *FTT1236* are known to be attenuated for growth in human hepatocyte-like cell line HepG2 and also in mouse macrophage-like cell line J774A.1 (191). Furthermore, a mutant in the LVS homolog of *FTT1238* (*FTL0706*) was detected in a high-throughput screen for mutants with reduced cytopathogenicity to J774A.1 cells (145).

I also hypothesized that these genes may be important for synthesis of extracellular polysaccharides. *FTT1235* bears homology to LPS core

mannosyltransferase *lpcC* from *Brucella suis* and appears, by protein family analysis (using the hmmsearch algorithm from HMMER 3.0, www.hmmer.janelia.org, against all Pfam-A alignments as of May 17, 2010) to contain a conserved group 1 glycosyltransferase domain (amino acids 151 to 327) with an E-value of 6e-41, while *FTT1237* encodes a conserved group 8 glycosyltransferase domain (amino acids 5 to 264) at an E-value of 1.9e-41. *FTT1236* and *FTT1238* exhibit no significant conserved domains (E-value < .001). While its function cannot be predicted based upon the current analysis, *FTT1238* is thought to encode a membrane protein that may contain ten transmembrane alpha-helices utilizing the TMpred algorithm (http://www.ch.embnet.org/software/TMPRED_form.html). Furthermore, *FTT1238* is known to be required for O-antigen synthesis in Schu S4 (145). Lipopolysaccharide (LPS) O-antigens (O-Ags) are required for virulence in many pathogenic bacteria. These polysaccharides, composed of repeating glycan units, are attached to a core polysaccharide affixed to lipid A through 3-deoxy-D-manno-octulosonic acid (Kdo) (100) and have diverse functions in bacterial pathogenesis: they function in part to prevent deposition of complement proteins on the bacterial surface and prevent complement-mediated lysis when exposed to serum and also to mask bacterial antigens from pattern-recognition receptors (PRRs) like toll-like receptors and specific antibodies (52). These polysaccharides are usually highly antigenic and, due in part to their exposure to the immune system, the bacterial polysaccharides such as O-Ag and capsule are highly diverse molecules, even within a species – *Escherichia coli* alone is known to express 186 O-serotypes and 80 capsular serotypes (101). The *F. tularensis* O-antigen shares two internal carbohydrate residues with *F. novicida* (α-D-GalNAcAN- α-D-GalNAcAN) but expresses different outer residues (β-D-Qui4NFm and β-D-Qui4NAc), and is therefore structurally and antigenically

distinct from that of *F. novicida* (100, 153). While our understanding of the genetics of O-Ag biosynthesis in *F. tularensis* is incomplete, it is known that mutants in the *wbt* operon of *F. tularensis* LVS and Schu S4 do not express an O-Ag (47, 139, 145, 198, 216, 230). These strains bind to C3b more avidly and are sensitive to bile salts and to complement-mediated lysis in the presence of serum when compared to wild type strains. These mutants have also been found to be defective for intracellular growth in the mouse macrophage-like cell line J774A.1 and also are reduced for virulence and dissemination in mice. Of note, an LVS mutant in *FTL0706*, designated *FTT1238* in *F. tularensis* Schu S4, lacks an O-Ag and exhibits decreased replication within and cytotoxicity to J774A.1 cells. A Schu S4 mutant in *FTT1238* has been shown to lack an O-Ag but its virulence properties have not yet been described (145).

Francisella has long been thought to have an extracellular structure that resembles a capsular polysaccharide (113). In many pathogenic bacteria, capsular polysaccharides function similarly to O-Ag but have also been observed to inhibit phagocytosis of the encapsulated organism and also to mediate adherence to various cell types (169). First observed by Hood in 1976, the nature of this capsule has remained elusive. Acridine orange treatment was used to produce an undefined LVS mutant that appears to lack an extracellular polysaccharide structure (204). This strain was designated to be Cap^- (alternately termed "rough" in more recent work). This mutant exhibits increased C3b deposition and serum sensitivity (47, 204, 221). Furthermore, expression of the capsular polysaccharide was observed to be increased by passage on Chamberlain's defined medium, resulting in increased virulence in mice (42). Recently, others in our group have discovered a capsular polysaccharide and have generated a novel monoclonal antibody that recognizes this structure as distinct from LPS O-Ag, although O-Ag and capsule appear to share a common

repeating subunit. This capsule has been found to have distinct immunological properties compared to purified LPS, and appears to have protective potential when delivered to mice as a vaccine (7).

Based upon my TraSH results, others' studies suggesting negative selection for mutants in this locus of the *Francisella* chromosome, as well as my interest in surface polysaccharide synthesis, I selected *FTT1235*, *FTT1236*, and *FTT1237* as targets to generate site-directed mutants using group II intron-mediated mutagenesis to determine whether they were attenuated for intracellular growth in the MDM model system. I also selected *FTT1238*, based upon its proximity to the locus of interest, previous characterization of its LPS O-antigen phenotype, and negative selection in previous iterations of the TraSH screen through MDMs with slightly different experimental conditions (data not shown). In this chapter, I describe the mutagenesis and characterization of these genes, which I determined to be required for synthesis of the LPS O-antigen and capsular polysaccharide. I further demonstrate that these genes are required for serum resistance, intracellular growth within macrophages, and that mutants in these genes exhibit a novel defect in the *F. tularensis* lifecycle, being fully capable of escaping the phagosome and growing intracellularly but inducing premature cell death in infected macrophages and destroying their intracellular replicative niche.

Materials and Methods

Bacterial strains and growth conditions

All *Francisella* strains studied in this work are isogenic with *F. tularensis* ssp. *tularensis* Schu S4. Bacteria were routinely cultured on modified Mueller-Hinton plates (Acumedia, Lansing, MI) or in modified Mueller-Hinton broth supplemented with 150 mM NaCl (MMH-Na). Where necessary to maintain plasmids, 25 μg/ml spectinomycin (Spec) or 25 μg/ml kanamycin (Kan) were

added to the media. *F. tularensis* wild type Schu S4 and mutant strains expressing green fluorescent protein (GFP) were generated by cryotransforming them with pBB103-sGFP, a kind gift of Justin Schwartz. *Escherichia coli* DH12S and DH5α λ pir were utilized in plasmid construction and were routinely cultured on Lennox agar and broth supplemented with 50μg Spec or 50μg Kan as appropriate. All work with Schu S4 was performed within the Carver College of Medicine Biosafety Level 3 Core Facility and all experimental protocols were reviewed for safety by the BSL-3 Oversight Committee of the University of Iowa Carver College of Medicine. Recombinant DNA work with Schu S4 was approved by the Institutional Biosafety Committee.

Macrophage isolation and culture

Heparinized venous blood was drawn from healthy adult volunteers using a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa, and all subjects provided informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated as described previously (213, 214). Briefly, PBMCs were isolated from venous blood by centrifugation on Ficoll-Hypaque (GE Healthcare, Piscataway, NJ), washed twice in HEPES-buffered RPMI 1640 with L-glutamine (RPMI; Cambrex, Walkersville, MD), seeded into Teflon jars at 2 x 10^6 cells/ml and allowed to differentiate into monocyte-derived macrophages (MDMs) for 5-7 days in RPMI 1640 + 20% autologous serum at 37°C.

Group II intron-mediated site-directed mutagenesis

Site-directed mutants of genes indicated by negative selection to be putatively important for growth within human MDMs were generated using a modified TargetTron (TA0100; Sigma-Aldrich, St. Louis, MO) mutagenesis system as previously described (201). Briefly, primers were designed through the TargetTron algorithm to modify the insertion site of the intron into two sites

per targeted gene. Utilizing the TargetTron kit, a retargeted fragment was generated by PCR and cloned into the XhoI/BsrGI sites of the pKEK1140 temperature-sensitive *Francisella-E. coli* shuttle vector and screened for LacZ activity. Clones were confirmed by BglII digest, cryotransformed into Schu S4, and plated at 30°C on MMH + Kan. Transformants were restreaked on MMH + Kan at 30°C and many colonies from the second or third streak were streaked twice on nonselective media at 37°C, a restrictive temperature. Ten colonies were then patched to MMH +/- Kan to determine whether the plasmid had completely cured; genomic DNA from cured clones was then purified using a DNeasy Blood and Tissue Kit (QIAgen) and subjected to PCR for confirmation of insertions. Generally, one in four colonies screened for one or both of the retargeted introns bore the desired insertion (data not shown).

In vitro growth assay

To determine whether mutants in the genes chosen were negatively selected due to their inability to grow at an equivalent rate to wild type Schu S4 in MMH-N broth, wild type and mutant strains were grown to saturation shaking at 37°C and diluted into fresh MMH-N broth to $OD_{600} = 0.1$. Broth cultures were shaken at 200 rpm at 37°C and the optical density was determined at intervals. Doubling time (T) was calculated according to the formula $N = N_o e^{kt}$ where $T = (ln\ 2)/k$ and growth indices were computed by dividing maximal mutant doubling times by maximal wild type Schu S4 growth rates.

Serum sensitivity assay

Bacteria were grown on MMH plates (with 25 μg spectinomycin for complemented mutants) for 2 days at 37°C + 5% CO_2, resuspended and washed in Hanks' balanced salt solution (HBSS; Gibco Invitrogen, Carlsbad, CA) with calcium and magnesium, and quantitated by OD_{600}. Bacteria were then diluted to 1×10^7 CFU/ml in 50% HBSS with calcium and magnesium and 50% normal

human serum and incubated shaking at 37°C for 90 min. Before and after incubation, bacteria were serially diluted in PBS, plated on MMH plates, grown for 2 days at 37°C + 5% CO_2, and enumerated to determine the concentration of viable cells both before and after treatment.

Isolation and immunoblotting of capsule and LPS

Isolation of *Francisella* capsule-like material and LPS was performed as described recently (7) with minor modifications. Bacteria grown as a lawn on MMH plates (with 25 μg spectinomycin for complemented mutants) were collected by scraping and resuspended in a solution of 6 mM Tris (Research Products International Corp. (RPI), Mt. Prospect, IL), 10 mM EDTA (RPI), and 2% sodium dodecyl sulfate (w/v) (Amresco, Solon, OH), pH 6.8, treated with proteinase K (New England Biolabs, Ipswitch, MA) and heat-treated at 65°C to sterilize. Lysates were ethanol precipitated three times and treated with micrococcal nuclease (New England Biolabs) to digest chromosomal DNA. Samples were then phenol extracted, ethanol precipitated three more times, and separated into capsule and LPS fractions by centrifugation in the presence of Triton X-114 (Sigma-Aldrich). Fractions were lyophilized and 5 μg of each was combined with NuPAGE (Invitrogen) Sample Reducing Agent and Sample Buffer, boiled for 10 minutes, and electrophoresed on NuPAGE Novex 4-12% Bis-Tris gels in NuPAGE MES SDS Running Buffer. Samples were transferred to nitrocellulose and probed with primary antibodies 11B7 to detect capsule, a kind gift of Dr. Michael Apicella, The University of Iowa, and FB11 (QED Bioscience, San Diego, CA) to detect LPS. Blots were detected with goat anti-mouse IgG (H+L) conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) and visualized by chemiluminescence with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL).

Intramacrophage growth and cytotoxicity assay

To quantify the extent of replication, MDMs were plated in 24-well dishes (Costar) at a density of 1 x 10^5 cells/well and allowed to adhere overnight at 37°C. Monolayers were washed with PBS twice and infected in triplicate with wild type Schu S4, mutant, or complemented mutant *F. tularensis* in RPMI + 2.5% heat-inactivated (56°C for 30 min) normal human serum (hiNH). After 1 h at 37°C, monolayers were washed twice with PBS to remove unassociated bacteria and fresh RPMI + 2.5% hiNH was added. Wells were lysed in 1% saponin at intervals. Lysates were diluted in PBS and viable bacteria were enumerated by plating on MMH as above. For microscopic analysis, MDMs were seeded onto 8-well Nunc chamberslides (Thermo Fisher Scientific, Rochester, NY) at a density of 40,000 cells per chamber, allowed to adhere overnight, and washed and infected as described above. For quantitation of MDM lysis by lactate dehydrogenase (LDH) release, macrophages were plated and infected as described above, except that 50 µl of culture supernatant was collected at intervals from triplicate wells and LDH in the supernatant was quantitated using the CytoTox-Fluor Cytotoxicity Assay (Promega, Madison, WI) according to the manufacturer's recommendations and read on an FLUOStar Optima microplate reader (BMG LabTech, Offenburg, Germany) using plate mode settings and averaging two readings per time point. Percent cytotoxicity was calculated by comparing an average of triplicate wells at each time point to an average of at least six uninfected positive control wells lysed in 0.9% Triton X-100.

Confocal microscopy

Macrophages infected with *F. tularensis* were processed for microscopic analysis as previously described (Allen, 1996) with minor modifications. Cells were fixed in 10% formalin, permeablized by the addition of -20°C acetone and methanol (1:1), and blocked at 4°C for five days in PBS + 0.5 g/L sodium azide +

10% bovine serum albumin (BSA) while lysed control slides were examined for sterility to be removed from the BSL-3 facility. Where possible (wild type Schu S4 and mutants) GFP-expressing bacteria were utilized. The GFP-encoding plasmid could not be introduced into complemented mutants due to plasmid incompatibility between the complementation vectors and pBB103-sGFP. Consequently, complemented mutants were detected using anti-*Francisella* antiserum (Becton, Dickinson, and Co., Franklin Lakes, NJ) together with an Alexa Fluor 488-conjugated goat anti-rabbit IgG $F(ab')_2$ secondary antibody (Invitrogen). In all cases, lysosome-associated membrane protein 1 (lamp-1) was detected using an anti-human lamp-1 mAb (H4A3) from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City) and an Alexa Fluor 568-conjugated goat anti-mouse IgG $F(ab')_2$ secondary antibody (Invitrogen). Images were obtained using a Zeiss LSM-510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY).

Results

FTT1236 and *FTT1237* constitute an operon required, along with *FTT1238*, for LPS O-antigen and capsule production

I successfully generated intron insertions between nt 202-203 of *FTT1236*, nt 409-410 of *FTT1237*, and nt 93-94 and nt 480-481 of *FTT1238* (in all assays performed, the *FTT1238* intron insertion in nt 480-481 performs identically to the one between nt 93-94, thus, in all further experimentation, data is reported for the upstream mutant only). I was not able to generate a stable mutant in *FTT1235*, as sequential passage of bacteria transformed with the intron delivery plasmid at 30°C generated increasingly smaller colonies until viable cells could not subsequently be passaged, suggesting that a mutation in *FTT1235* may be lethal under the conditions we utilized to generate our mutants (data not

shown). In order to ensure that the genes I had selected were not identified by our screen because of an inability to grow as rapidly as wild type Schu S4 in the culture conditions utilized in our initial screening, I obtained growth curves of the *FTT1236*, *FTT1237*, and *FTT1238* mutants as well as wild-type Schu S4 in broth culture and determined their maximal rate of growth. None of the mutants exhibited an *in vitro* growth rate significantly different from wild type (data not shown).

Because the infection of MDMs involved opsonization of the input bacteria in 50% normal human serum for uptake by macrophages, I hypothesized that the mutants may have been negatively selected based upon sensitivity to serum, as wild type Schu S4 is serum-resistant. Consequently, I exposed wild type Schu S4 and mutants in *FTT1236*, *FTT1237* and *FTT1238* to 50% human serum and performed a viable cell count. All of the mutants were shown to be serum sensitive, consistent with disruption of extracellular polysaccharide biosynthesis (Fig. 1). A mutant in *FTT1236* appears to be significantly more sensitive to serum than mutants in either *FTT1237* or *FTT1238*, though all these strains are significantly killed by exposure to serum as compared with wild type Schu S4.

Due to the orientation of *FTT1236* and *FTT1237* and because merely 8 bp separate their coding sequences, I hypothesized that the genes composed an operon. In order to determine with certainty that the insertions into *FTT1236*, *FTT1237* and *FTT1238* were responsible for the mutants' sensitivity to serum, and to functionally test this hypothesis, I cloned fragments encoding both *FTT1236* and *FTT1237*, *FTT1237*alone, and *FTT1238* alone into plasmid pBB103, forming plasmids pSL129, pSL130, and pSL149 respectively. I also deleted the *FTT1237* gene from plasmid pSL129 by restriction digest with SalI and religation, creating a plasmid with just a functional *FTT1236* gene, pSL129Δ37. I then

cryotransformed mutants with the appropriate complementation vectors and assayed their sensitivity to serum. Serum resistance was significantly restored by the addition of pSL129 to the *FTT1236* mutant, pSL130 to the *FTT1237* mutant, and pSL149 to the *FTT1238* mutant, indicating that these mutations were responsible for the serum sensitive phenotype observed. Interestingly, complementation of the *FTT1236* mutant could not be achieved by adding back either pSL129Δ37 or pSL130, suggesting that transcription of both *FTT1236* and *FTT1237* are disrupted by a polar mutation in *FTT1236*. The inability of the pSL129Δ37 plasmid to complement a mutant in *FTT1237* confirms that *FTT1237* expression has been ablated by the pSL129Δ37 deletion (Figure IV.1).

While increased serum sensitivity was suggestive of disruption in extracellular polysaccharide biosynthesis, I further endeavored to determine what physical effects the disruption of *FTT1236*, *FTT1237*, and *FTT1238* had upon LPS O-Ag and the newly described capsule. I isolated capsule from wild type Schu S4, mutants in *FTT1236*, *FTT1237*, and *FTT1238* and their complements and, in collaboration with Jason Hunt and Jed Rasmussen, performed immunoblots with antibodies that recognize the O-antigen (FB11) and the capsular material (11B7) (7). Mutants in *FTT1236*, *FTT1237*, and *FTT1238* lacked O-Ag, as determined by reactivity to FB11, while complementation of *FTT1236* with pSL129, *FTT1237* with pSL130, and *FTT1238* with pSL149 restored O-Ag reactivity (Figure IV.2). Complementation of *FTT1236* with pSL129Δ37 or pSL130 or *FTT1237* with pSL129Δ37 failed to restore O-Ag, correlating with the inability of these plasmids to restore serum resistance to these mutants. Interestingly, while mutants in *FTT1236* or *FTT1238* are capsule-deficient or lack 11B7-recognized epitopes of capsule, a mutant in *FTT1237* retains 11B7-reactive material of significantly higher molecular weight as compared with wild type Schu S4. Complementation of *FTT1237* with pSL130 restored the molecular

weight of the capsular material to that of wild type. Complementation of *FTT1238* with pSL149 restored reactivity to 11B7, while complementation of *FTT1236* with pSL129 restored only small amounts of reactivity in comparison, possibly due to a stoichiometric effect of expression from a multicopy plasmid. Together, the serum sensitivity and the LPS and capsule immunoblot data suggest that *FTT1236* and *FTT1237* compose an operon required, along with *FTT1238*, for the biosynthesis of O-Ag and CLM in *F. tularensis* Schu S4.

Mutants in *FTT1236*, *FTT1237*, and *FTT1238* exhibit increased uptake by macrophages

Having determined that mutants in *FTT1236*, *FTT1237*, and *FTT1238* were likely selected against in my initial MDM assay, at least in part, due to their sensitivity to serum, I further examined the mutants to determine whether they exhibited a virulence defect in MDMs in a complement-free infection model. To that end, I infected MDMs in medium supplemented with heat-inactivated normal human serum with Schu S4 and mutants in *FTT1236*, *FTT1237*, and *FTT1238*, lysing samples at 8 h intervals for 24 hpi, and performed a viable cell count. Routinely, association with MDMs at the 1 h time point was three-to-five-fold higher for the mutants as compared with wild type bacteria; the increase in uptake appeared to be more dramatic for the *FTT1236* and *FTT1238* mutants as compared with mutants in *FTT1237*. After uptake, mutants in *FTT1236*, *FTT1237* and *FTT1238* grew at similar rates to wild type Schu S4 over the first 16 h of infection. At 16 hpi, however, viable cell counts of mutant bacteria ceased to increase while Schu S4 continued to grow within MDMs over the entire 48 h course of the experiment. Mutant bacterial growth reached a plateau at approximately 10-fold growth over 16 h, while Schu S4 growth increased 50-fold growth over that time period and reached 300-500 fold growth over the 48 h course of the infection (Figure IV.3).

One possible explanation for the increased uptake and reduced growth exhibited by the *FTT1236*, *FTT1237*, and *FTT1238* mutants was that their altered surface properties enhanced their adherence to the tissue culture wells or diminished the macrophages' ability to internalize them as seen, for example, in *bvrR/S* mutants of *Brucella abortus* (220), and thus a majority of the bacteria were growing extracellularly throughout the course of infection. To test this hypothesis, I exposed Schu S4 and mutant bacteria to extracellular gentamicin after a 4 h infection. Addition of gentamicin did not significantly impact bacteria in coculture with MDMs either immediately after treatment or over a 24 h time course, while bacteria exposed to gentamicin in wells without cells were killed (Figure IV.4). These data suggested that the bacteria were internalized by macrophages and the observed growth was intracellular.

Mutants in *FTT1236*, *FTT1237*, and *FTT1238* escape the phagosome and demonstrate reduced intracytosolic growth

A second possible explanation for the observed elevated uptake of mutant bacteria yet reduction in intramacrophage growth compared with wild type Schu S4 was that a significant fraction of the mutant bacteria was unable to escape the phagosome and/or arrest the maturation of these phagosomes and ended up either trapped or destroyed within lysosomes. Furthermore, it was possible that mutant bacteria were trapped within autophagosome-like vesicles at late time points as observed by Checroun and coworkers (37), although our group has never observed autophagy in human MDMs infected with Schu S4 under our experimental conditions. I, in collaboration with Lee-Ann Allen, tested this hypothesis by performing a microscopic analysis of MDMs infected with Schu S4 and mutant bacteria over a 32 h time course to determine if mutant bacteria were preferentially enclosed within lamp-1 positive vesicles as compared to wild type.

At 1 hpi, we could find no significant evidence of colocalization of either wild type or mutant bacteria with lamp-1, suggesting that both wild type and mutant bacteria had successfully escaped the phagosome (see Figure IV.5). Quantitation of macrophages infected with Schu S4 revealed 22% of the MDMs were infected with 1.23 +/- 0.57 bacteria per MDM, while 94% and 95% of macrophages were infected with mutants in *FTT1236* and *FTT1238* with 6.02 +/- 4.09 and 6.75 +/- 4.72 bacteria each, respectively. These data correlate well with the uptake observed quantitatively in the viable cell count.

By 16 hpi, macrophages infected with mutant bacteria appeared significantly less healthy compared with those infected with wild type bacteria although the apparent average bacterial burden in Schu S4-infected cells was considerably higher. MDMs infected with mutants in *FTT1236* (Figure IV.8) or *FTT1238* (Figure IV.7) exhibited increased rounding, blebbing, and nuclear condensation as compared with macrophages infected with wild type Schu S4 (Figure IV.6). In contrast with wild type, which generally grew diffusely throughout the cytosol of infected macrophages, mutant bacteria tended to cluster and appeared to have a predominantly perinuclear localization. Neither wild type nor mutant bacteria appeared to have any significant colocalization with lamp-1 at 16, 24 or 32 hpi, suggesting that autophagy was not induced. Complementation of mutants in *trans* with the corresponding gene restored near wild type growth patterns and macrophage morphology (Figure IV.8,9).

In order to gain some quantitative sense of the degree to which the mutants were altering macrophage morphology and viability, I performed microscopic counts of MDMs infected with Schu S4, mutants, and their complements. At 24 hpi, ~90% of Schu S4-infected MDMs appeared generally healthy, as indicated by their flat, spread morphology and large nuclei, although approximately 10% appeared rounded with condensed nuclei. In contrast, by 24

hpi with mutants in *FTT1236* and *FTT1238*, ~95% of MDMs appeared to have morphologies consistent with diminished viability and death, as indicated by cell rounding, surface blebbing, and/or nuclear condensation. In a subset of MDMs infected with the mutants, these morphological changes were apparent as early as 16 hpi. The phenotype of the *FTT1237* mutant appeared to be somewhat less severe than the other two, with only ~65% of infected macrophages appearing rounded at 24 hpi. Macrophages infected with complemented *FTT1236c* and *FTT1237c* strains appeared generally similar to Schu S4, with ~60% of infected cells displaying an adherent and flat phenotype at 24 hpi (see Figure IV.6-9). Taken together, these data suggest that Schu S4-infected MDMs eventually die by a similar mechanism as mutant-infected cells, yet with slower kinetics, and that complementation of the mutants in *trans* restores *F. tularensis*' ability to grow intracellularly without inducing premature cell death. Additionally, cell death observed in mutant-infected macrophages appeared to be dependent upon bacterial load, as cells exhibiting few bacteria in the cytosol appear to retain an adherent and flat phenotype (see Figure IV.10). Furthermore, bystander macrophages were not impacted by rampant growth of either Schu S4 or mutant bacteria within neighboring MDMs and retained an adherent and flat morphology (Figure IV.6, IV.8). At 32 hpi, few cells infected with mutant bacteria remained and observation of cell debris was common, while ~75% of cells infected with Schu S4 still appeared relatively healthy despite showing extensive intracytosolic bacterial replication (Figure IV.6). Interestingly, over time lamp-1 signal in infected MDMs became more clustered in the cytoplasm and/or disappeared in parallel with diminished macrophage viability (i.e. Figure IV.7, Figure IV.8, 32 h). After cell death and disruption, mutant bacteria appeared both in conjunction with cell debris and freely extracellular (Figure

IV.8). In contrast, extracellular bacteria were seldom observed in chambers infected with Schu S4, even at 32 hpi, as the majority of MDMs remained intact.

Taken together, these data suggest that mutants in *FTT1236*, *FTT1237*, and *FTT1238* are significantly attenuated for intramacrophage growth as compared with Schu S4 independently of their sensitivity to serum. In addition, these data suggest that one possible explanation for the decreased intracellular growth observed in the mutant strains is that macrophages infected with these mutants die prematurely and therefore limit the mutants' ability to parasitize infected MDMs to the same extent as wild type Schu S4.

Infection with mutants in *FTT1236*, *FTT1237*, and *FTT1238* triggers early cell death in human macrophages

In order to quantify the magnitude of cytopathogenicity and kinetics of cell death in macrophages infected with mutants in *FTT1236*, *FTT1237* and *FTT1238* compared to wild type Schu S4, we assayed culture supernatants from infected MDMs for the cytosolic enzyme lactate dehydrogenase (LDH) as a marker of loss of membrane integrity. In keeping with the microscopic data, all of the mutants assayed caused a significant release of LDH from infected macrophages, visible as early as 8 hpi and increasing over the course of the experiment (Figure IV.11). MDMs infected with mutants in *FTT1236* exhibited the most severe phenotype, resulting in the largest and most rapid release of LDH, while mutants in *FTT1237* and *FTT1238* presented a similar, slower release of LDH than *FTT1237* and *FTT1238*. Infection of MDMs with Schu S4 elicited much less cytopathogenicity over the first 24 hpi, only becoming significantly different from mock-infected MDMs at the 24 h time point. Similarly, restoration of the interrupted genes and the corresponding extracellular polysaccharides dramatically reduced the rate at which infected MDMs release LDH, suggesting

that *FTT1236*, *FTT1237*, and *FTT1238* are required for the avoidance of premature cell death and, therefore, for intramacrophage growth.

Discussion

In this chapter, I report the characterization of a locus (*FTT1235*, *FTT1236*, *FTT1237*, and *FTT1238*) of the Schu S4 chromosome that appears to be important for intramacrophage growth and was identified by high-throughput screening of my mutant library by transposon site hybridization. Three of these genes (*FTT1236*, *FTT1237*, and *FTT1238*) had been previously implicated in *Francisella* virulence by studies that utilized virulent (191) or attenuated (145, 238) *Francisella* strains, but no characterization of the mechanism of intracellular attenuation had previously been described. Finally, of particular interest to our group, sequence analysis suggests that two of the four genes (*FTT1235* and *FTT1237*) contained putative glycosyltransferase domains and might have a potential role in carbohydrate biosynthesis. Furthermore, a mutant in neighboring *FTT1238* of Schu S4 had previously been determined to express no O-antigen. In order to characterize the phenotypes of mutants in these genes, I constructed site-directed mutants in *FTT1236*, *FTT1237*, and *FTT1238* utilizing group II intron-mediated mutagenesis adapted for *Francisella* (201). I determined that these mutants exhibit defects in both O-antigen and capsule biosynthesis and are sensitive to complement-mediated lysis as compared with wild type Schu S4. Complementation studies showed that *FTT1236* and *FTT1237* compose an operon required for surface polysaccharide synthesis and that *FTT1238* is required for the synthesis of the *Francisella* O-antigen capsular polysaccharide that has recently been described by our group (7) in addition to its involvement in LPS O-antigen biosynthesis. I further determined that mutants in these genes exhibit increased uptake by human monocyte-derived macrophages in the absence of complement as compared with wild type Schu S4 and are fully able to

escape the phagosome and grow within the cytosol, as evidenced by their lack of colocalization with lamp-1 at 1 hpi. However, these mutants exhibit truncated intramacrophage growth, observed both as a reduced number of bacteria per MDM as well as a reduction in the total number of viable bacteria per well and significant and rapid cytotoxicity to MDMs as compared with wild type Schu S4. These mutant phenotypes were restored to wild type upon complementation with the appropriate genes in *trans*. The observed reduction in intramacrophage growth did not appear to be mediated by autophagy, as we observed no significant colocalization of either Schu S4 or mutant bacteria with lamp-1 at late time points in infection. Taken together, these data suggest that Schu S4 mutants in *FTT1236*, *FTT1237*, and *FTT1238* are attenuated in the MDM model due to their inability to express O-antigen, capsule, or both, which in turn results in their inability to prevent rapid macrophage death. This induced death appears morphologically similar, if not identical, to that caused, at later time points, by wild type Schu S4 and appears to be dependent upon bacterial burden, suggesting that macrophages infected with mutants in *FTT1236*, *FTT1237*, and *FTT1238* may die by the same mechanism as those infected with wild type Schu S4, but more rapidly and at lower bacterial loads.

Furthermore, these mutants in a virulent strain of *Francisella* exhibit a novel defect in human macrophage pathogenesis, as they possess the ability to escape from the phagosome and grow significantly within the macrophage cytosol but then rapidly induce cytotoxicity in infected macrophages. In contrast, most mutants in the *Francisella* pathogenicity island and its regulators are trapped within phagosomes that mature into lysosomes. I hypothesize that the reason for the observed deficiency in intramacrophage growth of these mutants is the premature destruction of their intracellular replicative niche.

The mechanism by which cells infected with *Francisella* die *in vivo* is a subject of considerable debate, and both apoptotic and pyroptotic cell death have been observed with varying *Francisella* strains, cell types, and conditions. Apoptosis is an anti-inflammatory form of programmed cell death in which either intrinsic or extrinsic stimuli induce activation of caspases, centrally caspase-3. Cells undergoing apoptosis exhbit rounding, shrinkage, nuclear condensation, DNA fragmentation and membrane blebbing into apoptotic bodies, and are eventually phagocytosed by macrophages (130). In contrast, pyroptosis is a proinflammatory form of programmed cell death in which recognition of pathogen-associated molecular patterns (PAMPs) induces activation of caspase-1 and the assembly of the inflammasome, a multimeric structure that serves to process proinflammatory cytokines for secretion. Pyroptotic cell death is characterized morphologically by cell swelling, membrane rupture, and vesiculation, as well as the nuclear condensation and DNA fragmentation observed in apoptosis (74).

Recent evidence has emerged that Schu S4 triggers widespread caspase-3-mediated apoptosis in the lungs, livers and spleens of infected mice, while LVS and *F. novicida* U112 induce caspase-1 activation and pyropotosis (241). The mechanism of induced cell death appears to correlate histopathologically with containment of LVS and U112 within microgranulomas, while Schu S4 causes extensive cell death (178). Interestingly, Weiss and coworkers identified two mutants in *F. novicida* (homologs of *FTT0584* and *FTT0748*) which caused greatly increased caspase-1- and ASC-mediated cytotoxicity in BMDMs as well as decreased mouse virulence but no significant diminution of intramacrophage growth, suggesting that their hypercytotoxicity does not negatively impact their replicative niche (238). In addition, an LVS mutant in *tolC* was recently shown to exhibit a defect in intracellular growth within murine bone marrow-derived

macrophages (BMDM) (91), decreased virulence in the mouse model (91, 188) and increased cytotoxicity in BMDM (188). The intracellular localization of the *tolC* mutant and the precise mechanism for this increased cell death is not yet understood, but it is thought to involve caspase-3, but not caspase-1 (188). The intramacrophage growth and cytotoxicity phenotypes of a Schu S4 *tolC* mutant remain unknown, but recent mouse survival experiments suggest that its contribution to virulence in this strain is modest (122). Taken together, these data suggest that different *Francisella* strains and species induce cell death by different mechanisms, which significantly complicates our understanding of the molecular basis for and overall significance of mutants expressing a hypercytotoxic phenotype. Consequently, to develop a clear understanding of relevant hypercytotoxicity phenotypes for primary host cells, including macrophages, it will be important to study mutants in human-virulent strains of *Francisella tularensis*.

Specifically, the contribution of O-antigen and capsular polysaccharide to the inhibition of premature cell death in virulent *F. tularensis* infection must be further elucidated. To date, the large majority of studies involving the interactions of *Francisella* mutants with diminished or ablated expression of O-antigen with host cells have been undertaken in avirulent *Francisella* strains. While images suggestive of a capsule were published as early as 1976 by Hood (113) and this structure was increasingly expressed in serial passage on Chamberlain's defined medium (42), the polysaccharide has only recently been isolated and characterized (7). Consequently, these earlier studies did not describe differential O-antigen and capsular phenotypes and it is likely that the mutations described abrogate capsule biosynthesis in addition to O-antigen biosynthesis. Early studies of an undefined rough mutant generated by acridine orange treatment of LVS exhibited increased complement deposition (204, 221) as

well as serum sensitivity, and decreased virulence in mice (204). Analysis of spontaneously-arising "grey" variants of LVS, which display an altered (47, 57) or missing O-antigen (108) suggests that they, too, exhibit increased complement deposition (47), serum sensitivity (47, 108), reduced intracellular growth in J774A.1 mouse macrophage-like cell line and diminished virulence in mice (108). Mutational studies of O-antigen biosynthesis have been predominantly focused upon the *wbt* locus, likely because its large size and the probable polarity of mutations generated upstream in the operon onto downstream genes makes it a high-frequency target of transposon mutagenesis. A likely mutant in *wbtA* is among the first *F. novicida* transposon mutants found to exhibit defects in O-antigen biosynthesis (56) and both a polar (47, 145, 198) and a nonpolar (216) insertion within *wbtA* of LVS exhibited diminished O-antigen, sensitivity to complement mediated lysis (47), lack of growth within J774A.1 cells (145, 198), and reduced virulence and dissemination in the mouse model (198, 216). A spontaneous LVS mutant in the *wbtI* gene (139), as well as insertion mutants in *wbtC* (145) and *wbtM* (47, 145) exhibit similar phenotypes. Interestingly, an insertion mutant in the *wbtDEF* genes of Schu S4 and *F. novicida* ablates O-antigen biosynthesis in both strains but only prevents intracellular growth in Schu S4, suggesting strain-specific differences in the requirement for intact LPS for intracellular growth and may point to divergent bacterial mechanisms of intracellular parasitism (230). Taken together, these data suggest that the *wbt* operon is important to biosynthesis of LPS, which is in turn important to intracellular growth and pathogenicity to mice. As expected, several genes within the *wbt* operon were implicated in our negative selection through human macrophages. However, prior to our study, only one gene outside the *wbt* locus, *FTT1238*, had been proven to be important for O-antigen biosynthesis in LVS and Schu S4 (145). An LVS insertion mutant in this gene exhibited reduced growth in

J774A.1 cells and virulence in mice (145). Interestingly, however, Maier and coworkers selected the *FTT1238* mutant for study due to its reduced cytopathogenicity for J774A.1 cells (145). Qin and Mann also determined that a Schu S4 mutant in *FTT1236* is incapable of growth in J774A.1 cells, but did not determine whether these mutants induced cell death in J774A.1 (191). In contrast to the results of Maier, *et al*, I observed that Schu S4 mutants in *FTT1238* exhibit increased cytotoxicity in human primary macrophages. Maier and coworkers did not characterize the intracellular growth of an *FTT1238* mutant in Schu S4 in J774A.1 cells, nor have we, thus the explanation for the divergence in cytotoxicity may be a result of strain variation (Schu S4 vs. LVS) or the specific point of transposon insertion within the *FTT1238* gene. As the mechanisms involved in regulation of cell death processes must, by definition, be altered to generate an immortalized cell line, it is also possible that the *FTT1238* mutant may be incapable of inducing cell death in the J774A.1 cell line but able to induce death in primary human macrophages, which again highlights the importance of studying mutants of human virulent strains in primary host cells.

In summary, in this chapter I presented mutants in hypothetical genes in a virulent strain of *Francisella* that are incapable of biosynthesizing either LPS O-antigen or capsular polysaccharide. These mutants exhibit a previously undescribed defect in *Francisella* virulence, being able to be internalized by primary human macrophages, escape the phagosome, and replicate within the cytosol but exhibit limited growth due to the premature death of their intramacrophage replicative niche. Further study of these genes will help elucidate both the biosynthetic pathway for O-Ag and capsule production and also help characterize the mechanism by which rough mutants trigger cell death, as is discussed in detail in Chapter V.

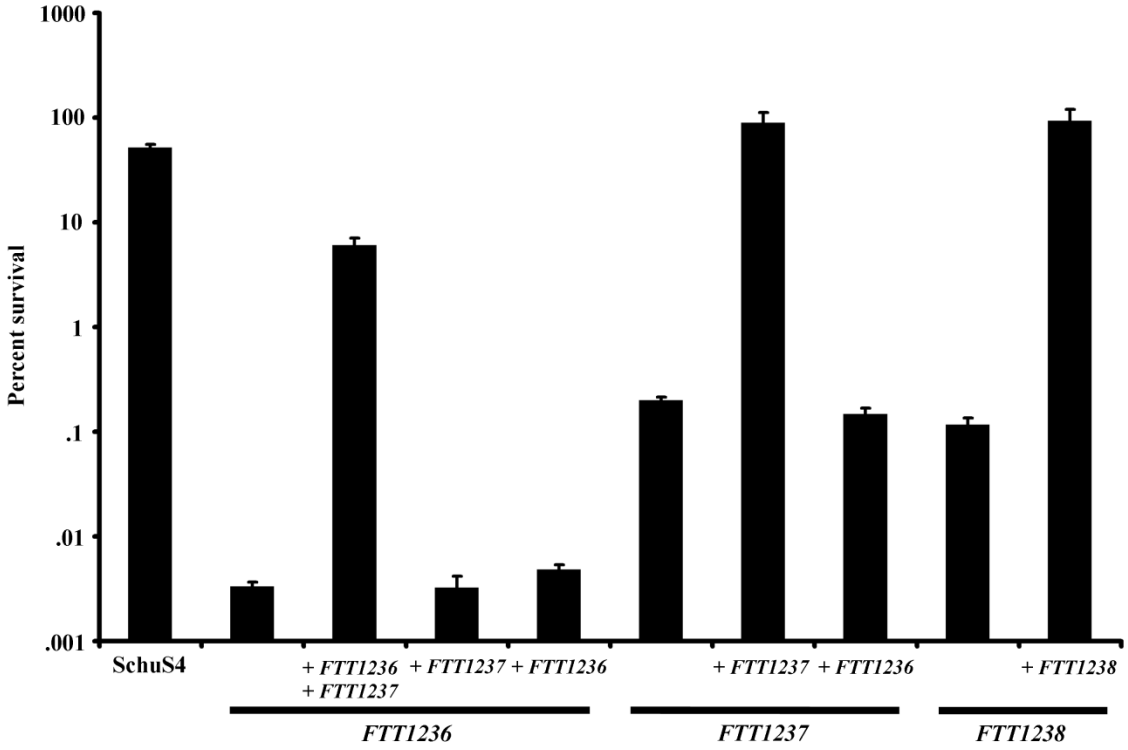

Figure IV.1. Serum sensitivity of *F. tularensis* Schu S4, mutants in *FTT1236*, *FTT1237*, and *FTT1238*, and complemented strains. Viable cell counts were performed by growing bacteria on MMH plates, resuspending them in HBSS with calcium and magnesium, and shaking them in 50% normal human serum for 1.5h at 37º C. Pre- and post-exposure samples were diluted in PBS and plated on MMH to calculate percent survival. Error bars represent one standard deviation. Data shown are representative of four total experiments.

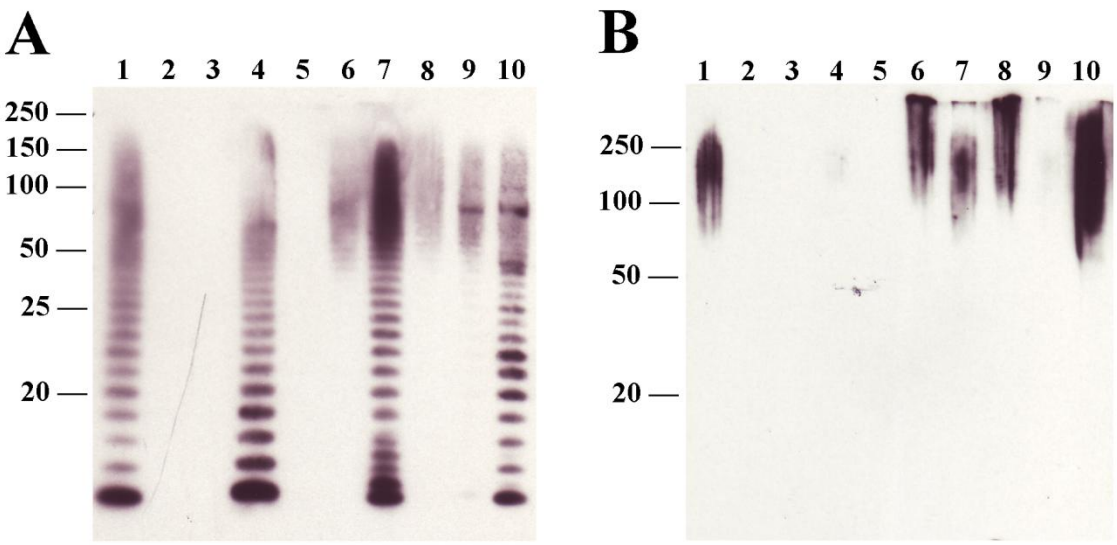

Figure IV.2. Immunoblots of purified lipopolysaccharide and capsule from *F. tularensis* Schu S4, mutants in *FTT1236*, *FTT1237*, and *FTT1238*, and complemented strains. Bacteria were grown on MMH plates for 2 days, lysed by exposure to detergent at 65º C, and LPS and capsule were purified as described previously (7). A. Purified LPS was deteced with the FB11 monoclonal antibody, which recognizes the *Francisella* LPS O-antigen. B. Purified capsule was detected with the recently-described 11B7 antibody, which binds preferentially to the *Francisella* capsular polysaccharide. In both panels, the lane order is as follows: Schu S4 (1), *FTT1236* (2), *FTT1236* + *FTT1237* (3), *FTT1236* + *FTT1236* + *FTT1237* (4), *FTT1236* + *FTT1236* (5), *FTT1237* (6), *FTT1237* + *FTT1237* (7), *FTT1237* + *FTT1236* (8), *FTT1238* (9), and *FTT1238* + *FTT1238* (10). Complementation of *FTT1236* with *FTT1236* and *FTT1237*, *FTT1237* with *FTT1237*, and *FTT1238* with *FTT1238* restored reactivity to FB11, and complementation of *FTT1237* with *FTT1237*, and *FTT1238* with *FTT1238* restored reactivity to 11B7.

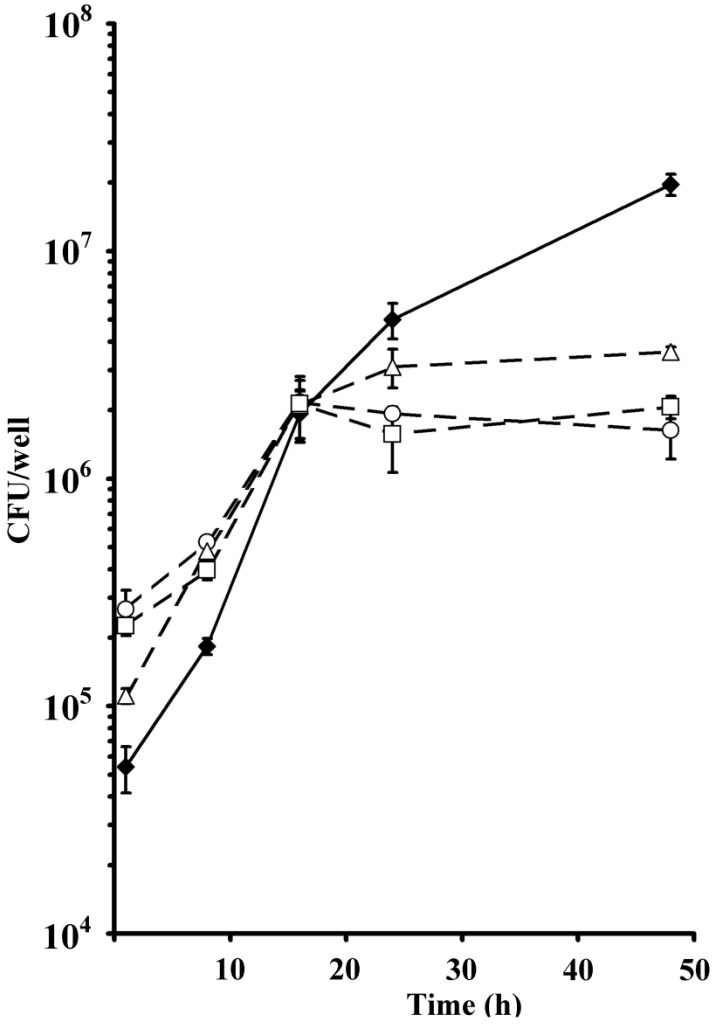

Figure IV.3. Intracellular growth of Schu S4 and mutants in *FTT1236*, *FTT1237*, and *FTT1238* within human primary macrophages. Macrophages were infected for 1 h with wild type Schu S4 or mutant bacteria, washed to remove extracellular organisms, and then lysed with 1% saponin at 1, 8, 16, 24, and 48 hpi to enumerate viable bacteria. Mutant bacteria (dashed line, *FTT1236*, squares; *FTT1237,* triangles; and *FTT1238*, circles) did not grow significantly after 16 hpi, while wild type Schu S4 (solid line, diamonds) continued growing throughout the course of the experiment. Error bars denote one standard deviation. Data are from one experiment representative of three.

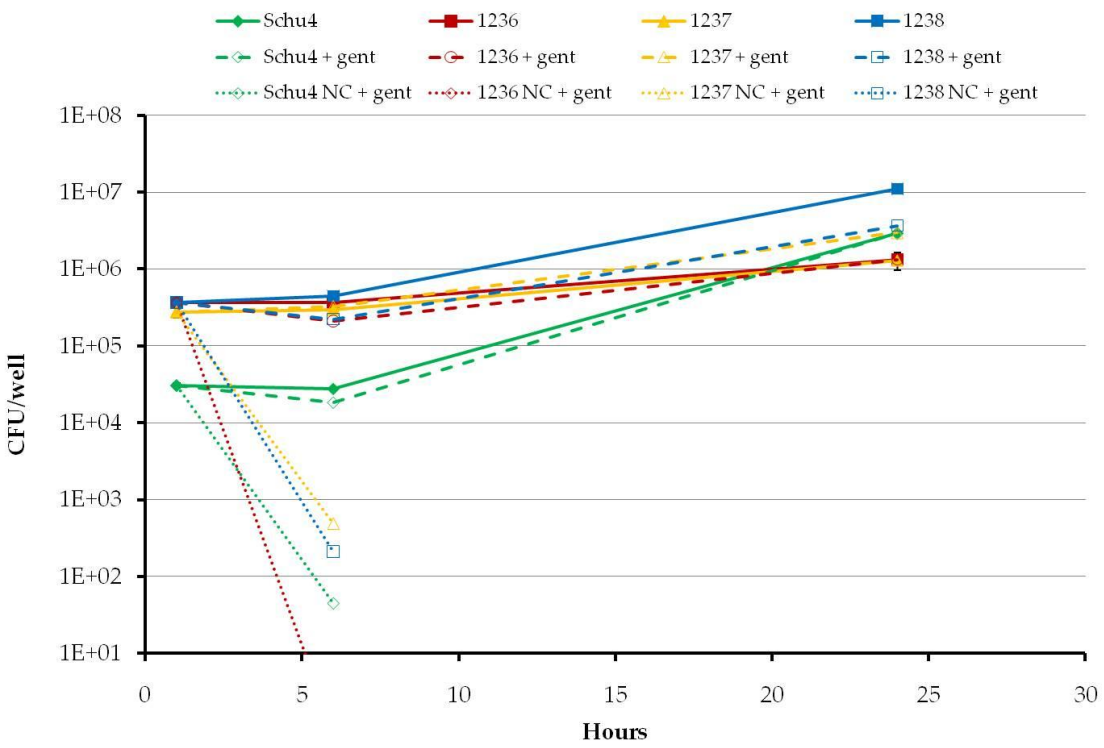

Figure IV.4. Gentamicin protection of wild-type Schu S4, mutants, and complements within macrophages. MDMs were infected at an MOI ~100, allowed to phagocytose bacteria for 4 h, and then washed in triplicate. Media containing 25 μg/ml was added for 1 h. Bacteria were enumerated at 1, 5 and 24 hpi by viable cell count.

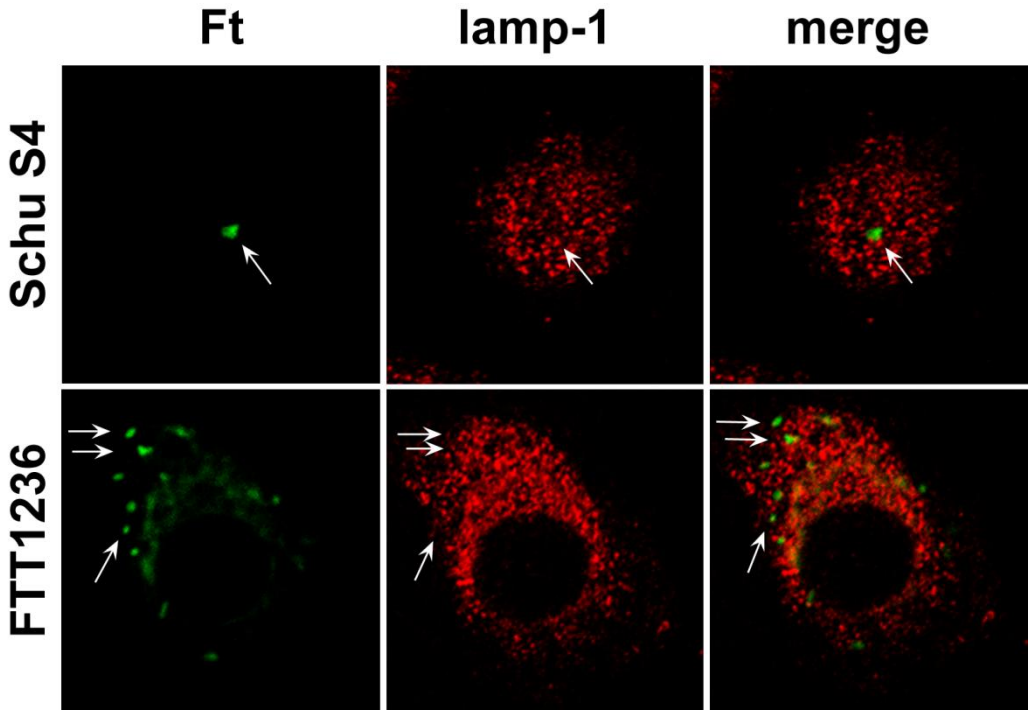

Figure IV.5. Infection efficiency of wild type Schu S4 and a mutant in *FTT1236*. MDM were infected with unopsonized Schu S4 or *FTT1236* at MOI of 100:1 for 1 h at 37°C and then processed for confocal microscopy. Images show bacteria in green and lamp-1 in red. *Arrows*, indicate phagosomes.

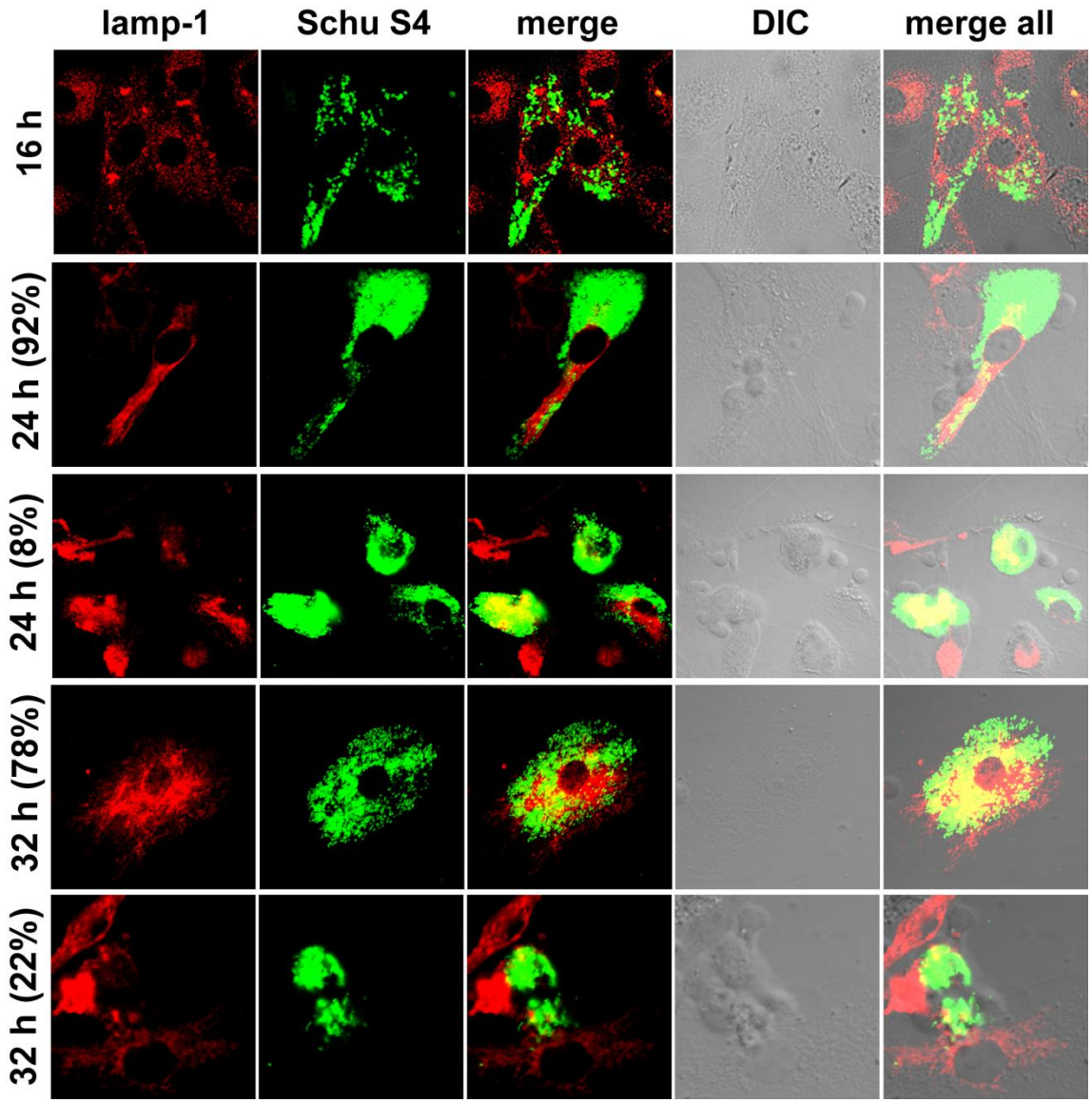

Figure IV.6. Robust growth of Schu S4 in MDM. MDM infected with unopsonized Schu S4 were processed for confocal microscopy at 16, 24 or 32 h post infection. Confocal sections show lamp-1 in red and bacteria in green. Differential interference contrast (DIC) images show MDM morphology. *Arrowheads* in lower 24 h and 32 h panels indicate rounded cells that contain large numbers of bacteria

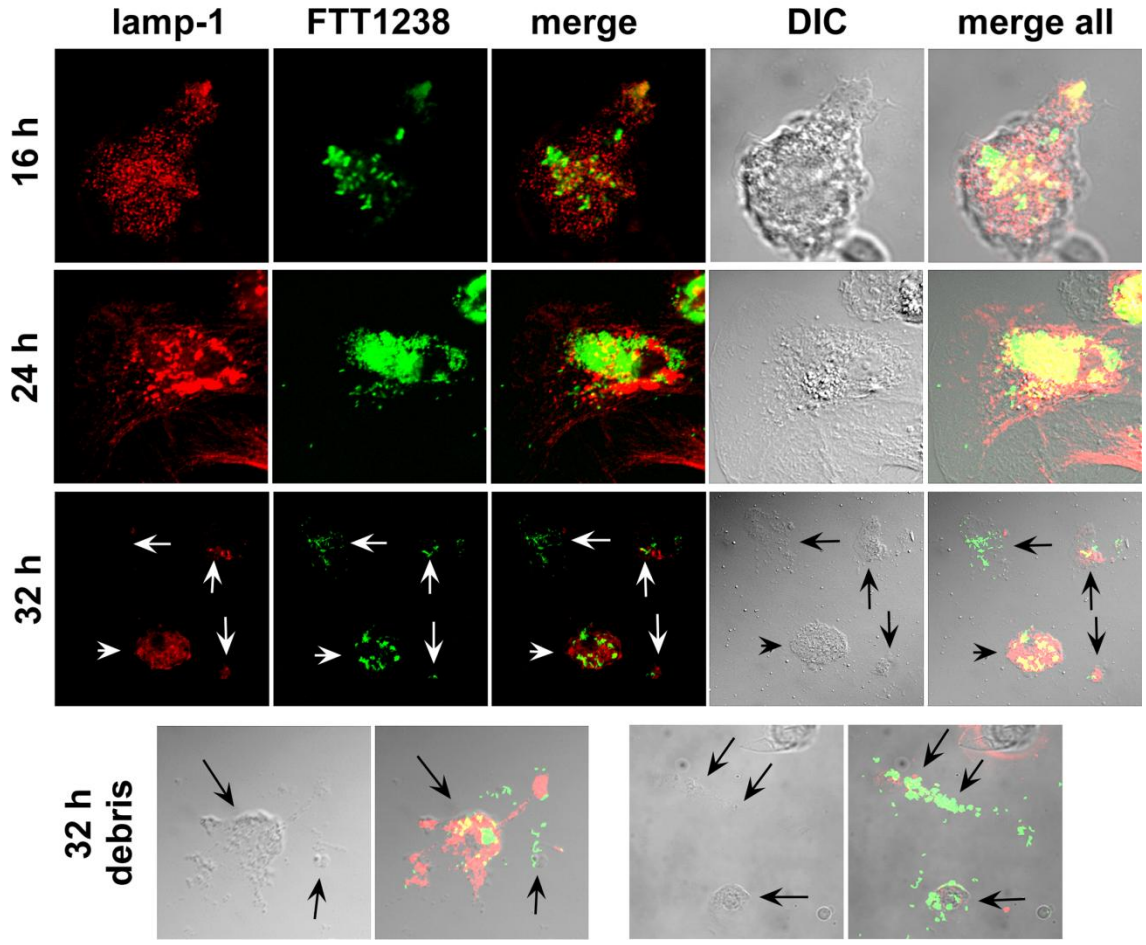

Figure IV.7. Accelerated death of MDM infected with *FTT1238*. MDM were infected with unopsonized *FTT1238* for 16, 24 or 32 h and then processed for confocal microscopy. Images show bacteria in green and lamp-1 in red. MDM morphology and integrity was assessed using DIC optics. *Arrowhead* in 32 h panels indicates an infected MDM with rounded morphology whereas *arrows* indicate bacteria associated with MDM debris.

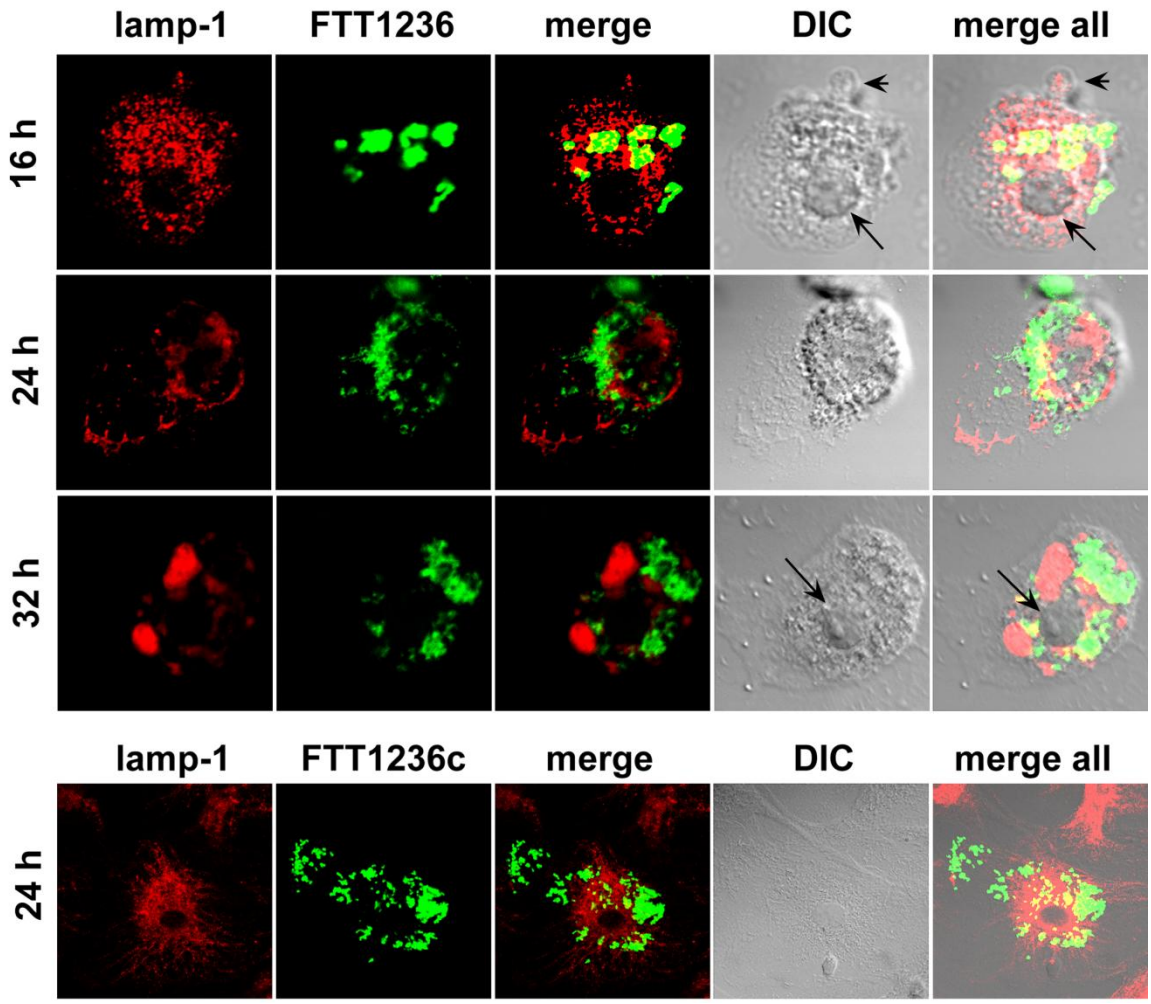

Figure IV.8. Intracellular growth within and morphology of MDM infected with *FTT1236* and complemented mutant strain. MDM were infected with unopsonized *FTT1236* for 16, 24 or 32 h, or the complemented strain *FTT1236c* for 24 h at 37°C and then processed for confocal microscopy. Images show bacteria in green and lamp-1 in red. MDM morphology is shown using DIC optics. *Arrows* and *arrowheads* indicate condensed MDM nuclei and plasma membrane blebs, respectively.

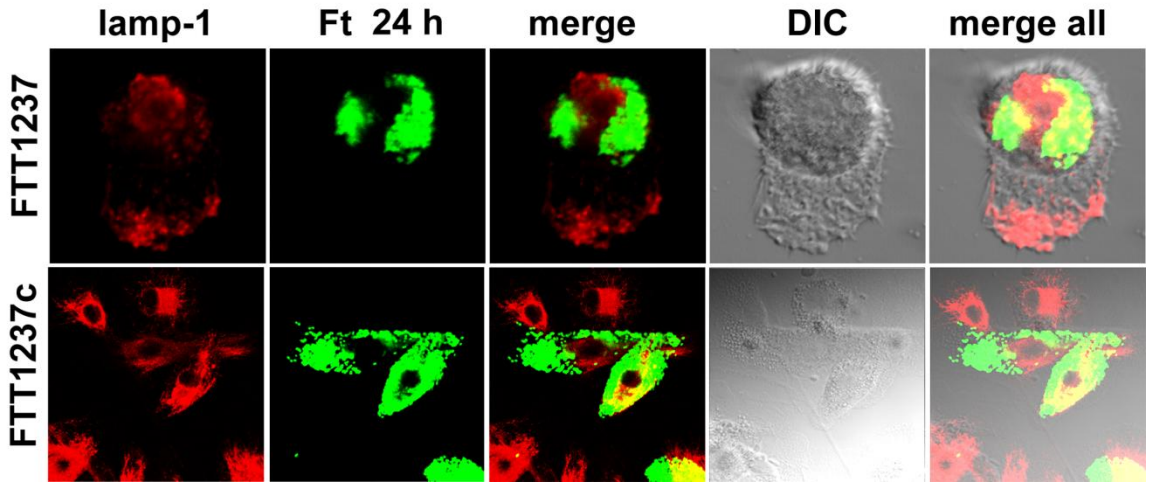

Figure IV.9. Complementation of *FTT1237* favors bacterial growth and MDM viability. Confocal images of MDM infected for 24 h with *FTT1237* (top panels) or *FTT1237c* (bottom panels) show bacteria in green and lamp-1 in red. MDM morphology is shown using DIC optics. Note that MDM infected with *FTT1237c* retain a normal, spread morphology despite extensive bacterial replication, whereas MDM infected with *FTT1237* do not.

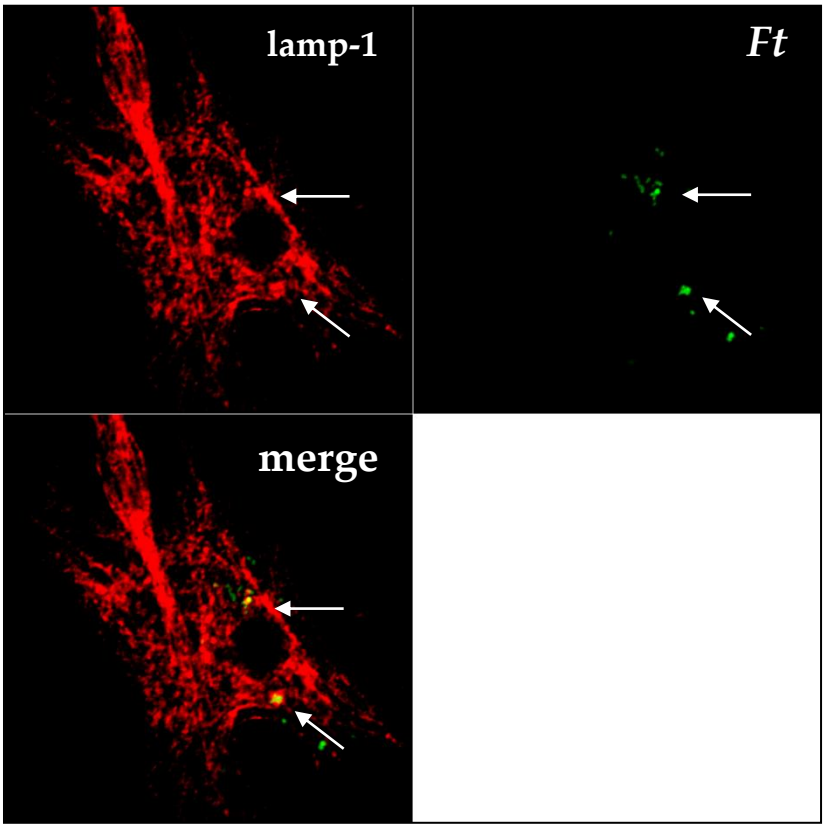

Figure IV.10. Cell fate in *FTT1236*-infected macrophages is dependent upon bacterial burden and/or phagosome escape. MDMs were infected with *FTT1236* for 24 hpi, at which point the cells were fixed and processed for confocal microscopy. Images show bacteria in green and lamp-1 in red. *Arrows*, indicate bacteria colocalizing with lamp-1, a possible indicator of entrapment within the phagosome.

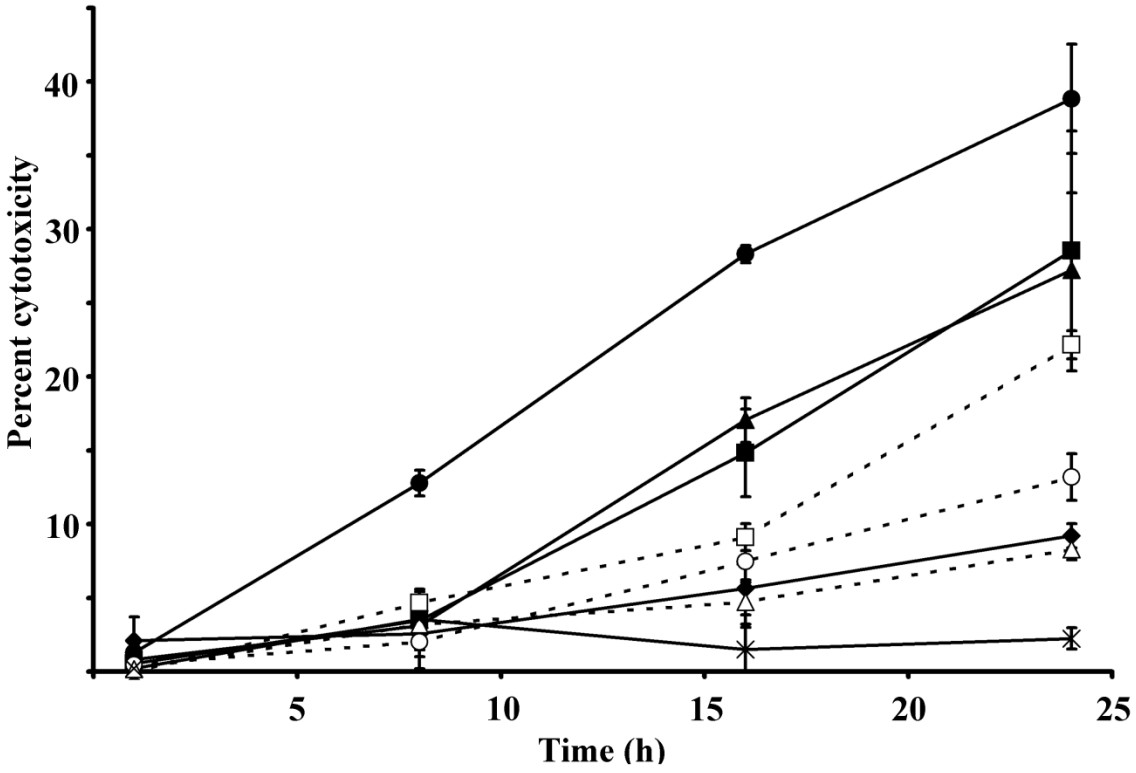

Figure IV.11. Lactate dehyrdogenase release from human primary macrophages infected with Schu S4, mutants in *FTT1236, FTT1237,* and *FTT1238* and their complements. Supernatant was collected from wells containing infected MDMs and assayed for lactate dehydrogenase (LDH) activity at 1, 8, 16, and 24 hpi. Percent cytotoxicity was computed by dividing LDH activity from experimental wells by uninfected wells lysed with 0.9% Triton X-100, representing total LDH in the well. Schu S4 (solid line, diamonds) induced little LDH release, only becoming significantly different from uninfected (solid line, crosses) at 24 hpi. In contrast, all of the mutant strains (solid lines and symbols, *FTT1236*, squares; *FTT1237*, triangles; and *FTT1238*, circles) induced significantly earlier and greater LDH release, while complemented mutants (dashed lines, open symbols – *FTT1236*, squares; *FTT1237*, triangles; and *FTT1238*, circles) exhibited substantially less cytotoxicity. Error bars denote 95% confidence interval (2 standard deviations). Data are from one experiment representative of four.

CHAPTER V

CONCLUSIONS AND PROPOSED FUTURE DIRECTIONS

An initial part of the characterization of any relationship between host and pathogen is the generation of a reproducible and robust system in which to characterize the interactions between the two organisms. At the outset of this work, no such system existed for the study of *Francisella tularensis*' potential interactions with human epithelial cells. Early observations of *F. tularensis* growth in coculture with nonphagocytic cells existed, even including some published descriptions and images of microscopic analyses which suggested intracellular localization, but these studies had been performed nearly a half-century ago and under conditions that are now hard or impossible to replicate (64, 219). In the interim, the entire *Francisella* world had changed; *Francisella* had grown from a little-known bacterial agent of disease in rodents and, occasionally, humans and an agent of biowarfare, though its abandonment as a bioweapon in 1970 (at least in the United States), eventually becoming a rare zoonosis worldwide and a fearful specter of potential bioterrorist threat yet to come. More recently, cryptic evidence of *Francisella*'s intracellular interaction with cells of epithelial (53) and endothelial (77) origin began to resurface. Very little had remained the same in the intervening time; the research community had changed, becoming less military and more academic, the strains studied had shifted from the predominant study of virulent strains to avirulent ones such as LVS and *F. novicida*, and even widely accepted culture conditions for the bacterium, to say nothing of the advent of genetic systems, had changed as general microbiological research improved. Consequently, when I began my investigations into the interactions of *F. tularensis* with epithelial cells, especially

those of airway origin, there was no recent model system available for studying these interactions nor was it possible to recapitulate the old systems.

As the preponderance of surface area of the airway, and even the alveolus, is covered by epithelial cells, I hypothesized that previously ignored interactions with cells of epithelial origin may contribute to *F. tularensis*' extreme infectivity by the aerosol route. Through the generation of an *in vitro* system to investigate these interactions, I was able to show that *Francisella* is able to productively infect epithelial cell lines in culture. Significantly, I observed specific adherence of LVS to these epithelial cell lines but a rather inefficient invasion of these cells. Once inside, however, LVS was capable of rampant multiplication within epithelial cell lines in culture, completely occupying the cytosolic volume of the cell. These data, in conjunction with more recent evidence that has observed both extensive bacterial growth in airway epithelial cells after intranasal infections of mice (58, 114) as well as the retained mouse virulence in mutants incapable of growth within primary mouse macrophages *in vitro* (114), strongly suggests that invasion and growth within epithelial cells is an important virulence determinant of the organism. The inefficiency of uptake in my current *in vitro* system suggests that it fails to mimic an unknown central aspect of the *in vivo* infection of epithelial cells; this may be the result of the physiological state of the bacteria as utilized for this system, a defect in the cell lines employed (such lack of expression of a receptor), or both. My optimization of bacterial culture conditions, including prior intracellular growth in J774A.1 cells, failed to yield increased invasion of the epithelial cells. While I had hoped to utilize my system to detect genes important for growth in epithelial cells globally within the *Francisella* genome by transposon site hybridization, my inability to improve the efficiency of bacterial uptake led me to convert to a phagocytic model for high-throughput screening. Regardless, this system presents a useful means to

characterize mutants that are suspected of being incapable of adherence to or growth within epithelial cells. At the same time, a model system in which epithelial cells can be utilized for high-throughput analysis of the TraSH-capable library should be earnestly sought, as current evidence suggests my original hypothesis may be correct and the ability to parasitize nonphagocytes, such as epithelial cells and hepatocytes is important to the overall virulence of *F. tularensis*, at least by the inhalational route (114).

In selecting a phagocytic model system, I chose to screen my near-saturating Schu S4 library through primary human macrophages to generate results that were most significant to human tularemia. Overall, this screen implicated 207 genes as important for intracellular survival and growth within primary macrophages. A plurality of the genes implicated was not annotated, owing principally to *Francisella*'s genetic distance from any well-studied near neighbors and unique mechanism of intracellular pathogenesis. Further, the screen implicated genes in protein synthesis, metabolism, and transport, as well as regulation, secretion, and stress response, among others. Several of the implicated genes were annotated to be involved in cell envelope biosynthesis, suggesting that the generation of the bacterial surface was important to pathogenesis in a primary macrophage model, which is in agreement with previous reports that had identified mutants in O-antigen biosynthesis as exhibiting no or reduced growth within macrophages and macrophage-like cell lines for unknown reasons (56, 108, 139, 145, 198, 230). Further, I found that several unannotated genes encoding hypothetical proteins detected by my screen, *FTT1235-FTT1238*, reside contiguously within the Schu S4 chromosome. Two of the four genes were postulated to encode putative glycosyltransferases, and a mutant in one gene of the locus, *FTT1238*, expressed no O-antigen (145). Additionally, *FTT1236*, *FTT1237*, and *FTT1238* had been implicated as important

for virulence in various models and strains of *Francisella* (145, 191, 238). I hypothesized that these genes, together, composed a locus required for surface polysaccharide biosynthesis, and, therefore were attenuated for intramacrophage growth.

There are several possible reasons that a gene could be negatively selected by this screen, some interesting and some relatively uninteresting. I was interested in mutants that are serum sensitive or are unable to survive or grow inside of macrophages. Potentially, however, it is possible that mutants might be negatively selected due to relatively uninteresting phenomena, such as conditional auxotroph or those exhibiting a reduced growth rate in broth culture or tissue culture media. I was successfully able to construct site-directed mutants in *FTT1236*, *FTT1237*, and *FTT1238* utilizing retargeted group II introns; however, a mutant in *FTT1235* was unobtainable under the conditions employed. Mutants in this locus exhibited broth growth rates indistinguishable from those of wild type Schu S4, but were determined to be serum sensitive. Further, mutants in *FTT1236, FTT1237*, and *FTT1238* exhibited no reactivity against antibodies raised against the O-antigen or the recently described capsular polysaccharide. Complementation of these mutants generally restored both serum resistance and reactivity to antibodies directed against O-antigen and capsule, and complementation of a mutant in *FTT1236* required restoration of both *FTT1236* and *FTT1237* in *trans*, functionally determining that the mutation in *FTT1236* was polar and that these two genes composed a novel operon required for the synthesis of both O-antigen and capsule.

As the TraSH screening protocol had employed opsonization in normal human serum, it was likely that mutants in this locus had been negatively selected due to their serum sensitivity. I further endeavored to determine whether mutants in these genes had a defect in intramacrophage growth in the

absence of active complement; surprisingly, they exhibited increased uptake by MDMs but reduced growth, reaching a plateau at approximately 16 hpi. In contrast, wild type Schu S4 was capable of continuous growth over the entire 48 h assay. Attempts to microscopically determine the mechanism of attenuation, in concert with the quantitation of cytotoxicity by the release of lactate dehydrogenase (LDH), determined that the mutants were fully capable of phagosomal escape and cytosolic growth but appeared to induce premature cell death in infected MDMs as compared with wild type Schu S4. Macrophages infected with mutants in this locus demonstrated signs of cell death, such as rounding, shrinkage, nuclear condensation, and membrane blebbing, much more rapidly than did those infected with wild type Schu S4. Complementation of the mutants in large part restored their ability to prevent premature cell death, both morphologically and as determined by release of LDH.

It is unknown which cell death pathway, or combination of pathways, are induced in macrophages by mutants lacking O-antigen or capsular polysaccharide. While morphology alone is insufficient to determine which pathway is engaged, our observations of the changes induced in infected macrophages suggest death by apoptosis. In the event of the induction of autophagy, the generation of large, lamp-1-positive, double membranous vesicles should be observed, as are in mouse macrophages infected with Schu S4 24 hpi (37), which we do not see. Further, in both oncotic and pyroptotic cell death, the dying cell swells and membrane integrity is lost; in contrast, we observe cell shrinkage, which is more typical of apoptotic cell death. Our observation of nuclear condensation and membrane perturbation and blebbing could be indicative of either apoptotic or pyroptotic cell death. As widespread caspase-3 activation has been observed in the livers and spleens of mice infected with a type A strain of *F. tularensis* (178, 241), which is indicative of apoptotic cell

death, and cell death in macrophages in mutants in *FTT1236*, *FTT1237*, and *FTT1238* appears indistinguishable morphologically though more rapid kinetically from the eventual cellular fate induced in Schu S4-infected macrophages consistent with apoptosis, it seems reasonable to hypothesize that the cells infected with either Schu S4 or mutants lacking LPS O-antigen or capsular polysaccharide die apoptotically.

Further work must be performed, however, in order to convincingly show that the cell death observed is indeed apoptotic in nature. In early stages of apoptosis, plasma membrane asymmetry is lost and phosphatidylserine is exposed on the surface of dying cells (115). This membrane asymmetry can be determined microscopically by treatment with a fluorescent annexin V conjugate, which specifically binds phosphatidylserine but is not permeable through intact membranes (224). Permeability of the plasma membrane, as would be expected in pyroptotic and necrotic cell death, can then be determined by treatment with the membrane impermeant nucleic acid-binding fluorophore propidium iodide (PI). Macrophages that are dying apoptotically will generally appear annexin V-positive but PI-negative. Additionally, TdT-mediated dUTP-biotin nick end-labeling can be employed to detect DNA fragmentation (164), indicative of either apoptosis or pyroptosis (130). Finally, should apoptosis or pyroptosis be implicated as the probable mechanism of death, activation of executioner caspase-3 and proinflammatory caspase-1, which in both cases results in cleavage of a procaspase into a large and small subunit, should be assayed by immunological detection methods such as immunoblotting and immunofluorescence. Cells dying apoptotically will characteristically exhibit caspase-3 cleavage, while caspase-1 activation should be apparent in pyroptosis. Further, inhibition of cell death utilizing inhibitors to specific caspases has been

successfully employed to determine which are involved in cell death induced by rough *Brucella* mutants (38).

Inhibition of apoptosis is becoming an increasingly recognized strategy in intracellular lifestyle of several pathogenic bacteria. Furthermore, the inability of mutants to prevent apoptosis is correlated with reduction of virulence in bacteria such as *Brucella melitensis* (38), *Mycobacterium tuberculosis* (126), and several others (69). It is generally thought that induction of apoptosis limits the replication of these intracellular bacteria due to loss of its replicative niche. Induction of apoptosis can be required for either successful parasitic or symbiotic relationships with a host; interestingly, *Francisella*'s near neighbor and arthropod endosymbiont *Wolbachia* is capable of prevention of apoptosis in human neutrophils via *Wohlbachia* surface protein (Wsp) (16) and is required endosymbiotically for maturation of oocytes in the parasitoid wasp *Asobara tabida* (177). In many cases, the bacterial determinants of inhibition of apoptosis remain poorly understood, however, LPS has been implicated as having a role in prevention of apoptosis via activation of NF-κB through TLR4-mediated signaling (176). As TLR4 primarily recognizes the lipid A moiety of LPS, and not the O-antigen, it is unknown how bacteria expressing a noncanonical lipid A structure like *Brucella* and *Francisella* inhibit apoptosis, although rough mutants in both are attenuated for intramacrophage growth (116).

Mounting evidence suggests that expression of surface polysaccharide plays a critical role in the ability of other intracellular bacterial pathogens to successfully prevent macrophage activation and premature cell death, especially in bacteria such as *Brucella* and *Coxiella* that establish longer-term infection of macrophages. It is conceivable that the surface polysaccharides in these bacteria, as well as in *F. tularensis* as described in this work, functionally accomplishes this in one of two ways (or both). First, the polysaccharide may actively prevent cell

death by mediating interactions with the host cell, such as suppressing pro-apoptotic signals or directly stimulating anti-apoptotic signaling. Second, the surface carbohydrates may be functioning as a shield to prevent the recognition of the intracellular bacterium by cellular sensors within macrophages such as TLRs and NLRs, which may function as an intracellular tripwire for invading pathogens, or alter the mechanism by which these bacteria are taken up by the macrophage or trafficked within it. Virulent *Brucella* and *Coxiella* appear to utilize both strategies to prevent macrophage apoptosis, though neither is thought to express a separate capsular polysaccharide in addition to their O-antigen. In rough strains of both bacteria (180, 218), as well as in my mutants in *FTT1236, FTT1237*, and *FTT1238*, mutants without surface polysaccharides are taken up more efficiently by macrophages. *B. melitensis* rough mutants induce cell death in human MDMs and are incapable of growth in these cells (72). Induction of cell death appears to be mediated in *B. melitensis*-infected RAW264.7 murine macrophage-like cells by caspase-2, has hallmarks of both apoptosis and necrosis, and macrophages infected with *B. melitensis* are more resistant to exogenous stimulators of apoptosis than uninfected cells (38). *B. abortus* rough mutants induce apparent oncosis in J774A.1 cells, human macrophage-like cell line THP-1, and primary bovine macrophages, but, interestingly, not in Vero cells, BHK cells, or primary bovine epithelia, suggesting that the induction of apoptosis observed is specific to professional phagocytes (180). Induction of cell death by *B. abortus* rough mutants is dependent upon viable bacteria and requires a functional type IV secretion system (181, 182), suggesting that the bacteria play an active role in prevention of cell death. In contrast, *Coxiella* phase II mutants, which lack an O-polysaccharide, do not induce apoptosis in infected macrophages but do activate human dendritic cells (DCs) (218). This activation is not suppressed by coinfection of smooth and rough strains (218), which

suggests that the O-antigen is functioning passively as a shield to hide bacterial components that would otherwise stimulate the DCs. *Coxiella* phase II mutants are taken up more efficiently than smooth phase I strains by a different mechanism (31). One significant difference between *Brucella* and *Coxiella* is that both of these create an intracellular replicative niche within vacuoles while *Francisella* escapes to the cytosol. Schu S4 mutants in *FTT1236, FTT1237*, and *FTT1238* are able to escape from the phagosome and replicate, early on, in a cytosolic environment at rates identical to wild type Schu S4, suggesting either that a potentially divergent initial route of uptake of the mutants induces macrophage death or that one or both of the surface polysaccharides of *F. tularensis* either actively prevent macrophage apoptosis or mask *Francisella* components from recognition by the death-inducing recognition by the macrophage. Spontaneous *F. tularensis* variants either lacking or expressing a significantly truncated O-antigen are thought to occur *in vitro* at a frequency of approximately 10^{-3} to 10^{-4} per generation (174). Interestingly, rough variants of *Brucella* (215) and *Coxiella* (104) are known to spontaneously occur, leading some to speculate that spontaneous rough variants may be part of a virulence strategy employed by these intracellular pathogens to facilitate cell-to-cell spread (181). It is possible that the phase variation observed in *Francisella* as "blue" versus "gray" (100) may indeed be a virulence factor in itself and potentially form part of a larger strategy employed by intracellular bacteria to balance prevention of apoptosis as required for preservation of the intracellular replicative niche with the necessity of eventual egress from a cell that can support no further growth.

Beyond characterization of the type of cell death induced, further experimentation will be required to determine the mechanism by which mutants in *F. tularensis* lacking O-antigen or capsular polysaccharide induce premature cell death in infected macrophages. Initially, it may be productive to determine

whether active bacterial protein synthesis and growth is required, possibly by incubating MDMs with formalin-killed *FTT1236* mutant bacteria or treating intracellular mutant bacteria with chloramphenicol at various time points after infection to stop protein synthesis. From the macrophage point of view, it may be advantageous to generate specific double mutants within the context of the *FTT1236* mutant to determine whether cytosolic localization and growth is required for induction of premature cell death. A double mutant in *FTT1236/iglI* or *FTT1236/iglJ* will result in a mutant incapable of escape from the phagosome (data not shown) in concert with an inability to generate O-antigen and capsule, while a double mutant in *FTT1236/pyrB* should result in a mutant capable of escape from the phagosome (214) but incapable of growth within the cytosol of primary macrophages (114, 191). Additionally, it would be instructive to determine whether the *FTT1236* mutant exhibits an adherence or invasion phenotype in infections of epithelial cells, as induction of apoptosis by *Brucella* rough mutants is macrophage-specific (180). Finally, random remutagenization of the *FTT1236* mutant may uncover other *F. tularensis* genes required for modulation of cell death induction and its kinetics.

In sum, this study has contributed to the overall understanding of the virulence genetics of *F. tularensis* by generating new tools to study interactions of *Francisella* with host cells and by employing those tools to detect and characterize genes required for pathogenicity within cells. Legacies of this work include both an *in vitro* model system to study adherence, invasion, and growth within nonphagocytic cells which have been further employed by myself and others to study interactions with epithelial cells (214) as well as a TraSH-capable, ~15,000 member library in virulent strain Schu S4, which can further screened through other model systems to improve our understanding of genes required for virulence in disparate hosts, such as the tick. Additionally, in this work I have

identified 207 genes that may be important for human tularemia and described a poorly characterized locus required for O-antigen, capsule biosynthesis, and prevention of premature macrophage death in primary human cells. Mutants in this locus may be useful in the future to elucidate both *Francisella* mechanisms for intracellular parasitism in virulent strains as well as host responses to *F. tularensis* and, potentially, important to the study of the intracellular lifestyle of pathogenic bacteria broadly.

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