

Characterization of the *Francisella* virulence factor RipA

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## ABSTRACT

James Robertson Fuller: Characterization of the *Francisella* virulence factor RipA  
(Under the direction of Thomas Kawula)

*Francisella tularensis* is a highly virulent facultative intracellular pathogen; however, the mechanisms by which *Francisella* virulence factors mediate this virulence are not well defined. We identified the *Francisella tularensis* LVS FTL\_1914 locus in a transposon mutagenesis screen for genes required for intraepithelial replication. Creation and characterization of a *F. tularensis* LVS  $\Delta ripA$  strain revealed that the locus was necessary for intracellular replication and we termed the gene *ripA* (Required for Intracellular Propagation, Factor A). We have studied the *Francisella* virulence factor RipA in an attempt to define its importance in pathogenesis. RipA is required for *Francisella* virulence in a mouse model of tularemia. A *Francisella*  $\Delta ripA$  mutant fails to replicate after intracellular colonization. The mutant invades at the same rate as wild type, escapes the phagosome, then fails to replicate in the cytoplasm. RipA localizes to the cytoplasmic membrane of *Francisella* and interacts with the transcriptional regulator IclR. How this interaction impacts virulence phenotypes and IclR transcriptional regulation are currently unknown. RipA is repressed by the *Francisella* virulence regulator MglA and is suppressed during intracellular growth after replication in the cytosol. MglA is upregulated after cell invasion. Most other virulence factors regulated by MglA are positively impacted by MglA suggesting that other factors act

together with MglA to negatively influence gene expression. We screened a transposon library to identify loci that can influence *ripA* gene expression as identified using a *lacZ* reporter system. HU form B and NusA were identified as possible regulators that influence *ripA* expression. RipA may be part of a programmed gene response of *Francisella* during different stages in its intracellular lifecycle. MglA acting with other regulators may act to positively and negatively impact gene expression during different phases of intracellular growth and in the much more complex setting of *in vivo* pathogenesis.

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## List of Abbreviations and Symbols

$\Delta$ , gene deletion

$\Phi$ , gene fusion

AMCA, aminomethylcoumarin acetate

ANOVA, analysis of variance

ATII, alveolar epithelial Type II cells

BSA, bovine serum albumin

CDC, Centers for Disease Control and Prevention

CFU, colony forming units

CPRG, chlorophenol red- $\beta$ -D-galactopyranoside

DAPI, 4',6-diamidino-2-phenylindole

EDTA, ethylenediaminetetraacetic acid

EEA1, early endosome antigen 1

ELISA, enzyme-linked immunosorbent assay

FACS, fluorescence-activated cell sorting

FBS, fetal bovine serum

FCV, *Francisella* containing vacuole

GFP, green fluorescent protein

HCCA,  $\alpha$ -cyano-4-hydroxycinnamic acid

HPLC, high performance liquid chromatography

HRP, horse radish peroxidase

IACUC, Institutional Animal Care and Use Committee

ID-MS, isotope dilution-mass spectrometry

IPTG, isopropyl  $\beta$ -D-1-thiogalactopyranoside

LAMP-1, lysosome associated membrane protein 1

LD<sub>50</sub>, 50% lethal dose

LPS, lipopolysaccharide

LVS, live vaccine strain

MA, microagglutination

MALDI, matrix assisted laser desorption ionization

MCS, multiple cloning site

MOI, multiplicity of infection

MS/MS, tandem mass spectrometer

MU, miller units

NF- $\kappa$ B, nuclear factor kappa B

ONPG, *ortho*-Nitrophenyl- $\beta$ -galactoside

PAGE, polyacrylamide gel electrophoresis

PCR, polymerase chain reaction

PBS, phosphate buffered saline

PFA, paraformaldehyde

PI, post inoculation

RBS, ribosome binding site

RT-PCR, reverse transcriptase polymerase chain reaction

SDS, sodium dodecyl sulfate

SISCAPA, stable isotope standards with capture by anti-peptide antibodies

SOE, splice overlap extension

TC, tetracysteine

TEM, transmission electron microscopy

TIGR CMR, The Institute for Genomic Research Comprehensive Microbial Resource

TLR, toll like receptors

TOF, time of flight

X-gal, 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside

## Chapter 1

### INTRODUCTION

*Francisella tularensis* is a small, gram negative, pleomorphic coccobacillus that is the etiologic agent of the disease tularemia. Tularemia had mortality rates as high as 40% in the pre-antibiotic era (Stuart and Pullen 1945) and even with modern antibiotic therapy, tularemia is lethal in 2% of reported cases (Dennis, Inglesby et al. 2001). This highly successful pathogen was developed as a biological weapon by both the United States and the Soviet Union during the cold war (Dennis, Inglesby et al. 2001) and recent concerns that *Francisella tularensis* could be used as a agent of bioterrorism have renewed interest in understanding *F. tularensis* pathogenesis (Oyston, Sjostedt et al. 2004). As few as 1 to 10 bacteria can initiate a lethal disease by the inhalational route of transmission (Saslaw, Eigelsbach et al. 1961; Saslaw, Eigelsbach et al. 1961). This makes *F. tularensis* one of the most infectious bacteria known. The mechanisms that make *F. tularensis* so infectious are unclear. Insight into the strategies that make *F. tularensis* such a virulent pathogen may carry over into the pathogenesis of other virulent bacteria. This would enable the coherent design of detection, prevention, and therapeutic protocols.

In this era of worldwide travel, the potential for the emergence of virulent pandemics is staggering. Historically pandemics used to traverse the globe over decades and years, now air travel has potentiated pandemics that can spread much more rapidly

(Colizza, Barrat et al. 2007). Public health agencies are on the watch for known threats such as influenza and SARS; however, emerging pathogens may represent the biggest threat. The rapidity with which emerging diseases may spread will limit the amount of time available to research their pathogenesis, properties, and to develop countermeasures. To be prepared for emerging highly virulent pathogens we need to understand the mechanisms that make other pathogens, including *F. tularensis*, virulent. The strategies used by highly virulent pathogens to evade, subvert, and resist the immune system will likely be conserved by other virulent organisms including emerging pathogens because of one fact, they work. What has worked once will likely evolve again.

**History.** In 1910, George McCoy first recognized a plague-like disease in ground squirrels that infected a wide array of animal species in Tulare county, California (McCoy 1910). He surmised that this disease could infect humans, and indeed in 1914 Wherry and Lamb reported two cases of human “Deer-fly fever” in Ohio (Wherry and Lamb 1914). McCoy and Chapin first cultured the bacteria that caused “Deer-fly fever” on egg yolks and named it *Bacterium tularense* (McCoy and Chapin 1912). Edward Francis studied *Bacterium tularense* for many years and as a consequence contracted Deer-fly fever on four different occasions (Francis 1936). *Bacterium tularense* was later renamed *Francisella tularensis* in his honor. “Deer-fly fever” and other diseases caused by *F. tularensis* were named tularemia on the recommendation of Edward Francis. Tularemia was derived from the words Tulare and bacteremia based upon the location of the initial work and the clinical manifestations of the disease.

**Clinical manifestations.** Tularemia is clinically categorized into ulceroglandular and typhoidal forms based on the involvement of the skin, mucous membranes, and the presentation of lymphadenopathy (Nigrovic and Wingerter 2008). Approximately 75% of tularemia cases can be categorized as ulceroglandular tularemia based upon the development of a maculopapular lesion and the development of lymphadenopathy. Typhoidal tularemia presents without skin involvement or lymphadenopathy. Typhoidal tularemia patients have a high fever and hepatosplenomegaly. Pneumonia develops in 30% of ulceroglandular and 80% of typhoidal tularemia cases. Tularemia in humans is a rare disease in most countries; however, occasional epidemic outbreaks of tularemia have helped to maintain clinical interest in *F. tularensis* detection and treatment. More recently, interest in *F. tularensis* has been sparked because of its classification as a Category A Select agent by the United States based upon the potential for its use as an agent in bioterrorism (Dennis, Inglesby et al. 2001).

**Epidemiology.** Extensive epidemiological data has been gathered about tularemia, but the details of the *F. tularensis* lifecycle are still unclear. *F. tularensis* is endemic in the Northern hemisphere, but is a rare disease in most countries (Johansson, Farlow et al. 2004). The frequency of the disease does not correlate with the persistence of *F. tularensis* in the environment (Sjostedt 2007). Tularemia occurs mainly in localized epidemic outbreaks (Berdal, Mehl et al. 1996; Feldman, Enscoe et al. 2001; Eliasson, Lindback et al. 2002; Reintjes, Dedushaj et al. 2002; Hofstetter, Eckert et al. 2006; Wik 2006). Based on current surveys, the most likely reservoirs of *F. tularensis* are rodents and lagomorphs (Centers for Disease Control and Prevention (CDC) 2005), although *F. tularensis* has been found to be infectious in all animals that have been tested, from amoeba to humans (Lavender, Stagg et

al. 1956; Abd, Johansson et al. 2003). Transmission is most commonly linked to insect vectors such as ticks (Centers for Disease Control and Prevention (CDC) 2005) and mosquitoes (Eliasson, Lindback et al. 2002; Eliasson and Back 2007). Other routes of transmission include physical contact with infected animal tissues (Centers for Disease Control and Prevention (CDC) 2005), contaminated water (Reintjes, Dedushaj et al. 2002; Celebi, Baruonu et al. 2006), and inhalation of aerosolized organisms (Feldman, Ensore et al. 2001). Aerosol transmission of *F. tularensis* results in pneumonic tularemia. Pneumonic tularemia is a case of typhoidal tularemia known to be acquired by inhalation. Pneumonic tularemia is the most aggressive and lethal form of the disease (Stuart and Pullen 1945; Matyas, Nieder et al. 2007; Nigrovic and Wingerter 2008). Outbreaks of pneumonic tularemia are rare but are a significant public health threat when they occur (Feldman, Ensore et al. 2001; Matyas, Nieder et al. 2007).

*F. tularensis* has been divided into the following subspecies and the virulent forms into two subtypes: *Francisella tularensis* (Type I *F. tularensis*), *Francisella holarctica* (Type II *F. tularensis*), *Francisella novicida*, and *Francisella mediasiatica* (Keim, Johansson et al. 2007). Type I and Type II *F. tularensis* are the only clinically relevant subspecies (Sjostedt 2007) that cause disease in humans. Type II *F. tularensis* has a wider geographic distribution being found throughout the Northern hemisphere and usually results in a milder, self limiting disease (Johansson, Farlow et al. 2004). Type II *F. tularensis* was used by the Soviet Union to produce a Live Vaccine Strain (LVS) (Tigertt 1962). This strain is attenuated in humans, but retains virulence in mice. LVS is used a model system for virulent *F. tularensis* infection (Rick Lyons and Wu 2007). Type I *F. tularensis* is typically localized to North America and results in aggressive, virulent, and highly infectious cases of

tularemia (Centers for Disease Control and Prevention (CDC) 2002; Farlow, Wagner et al. 2005). The routes of transmission and initial disease presentation are similar in the two forms with differences being noted in the severity of the disease and associated mortality rate (Eliasson, Broman et al. 2006). *F. tularensis* is most infectious by the inhalational route of transmission. Less than 10 bacteria can result in an aggressive, lethal case of pneumonic tularemia (McCrum 1961; Conlan, Chen et al. 2003; Sjostedt 2003). As a comparison, pneumonic transmission of plague and anthrax spores have 50% lethal dose as low as 16 bacteria (Heckly and Blank 1980) and 1000 bacteria (Lyons, Lovchik et al. 2004) respectively.

**Pathogenesis.** *F. tularensis* pathogenesis is complex and poorly understood. *F. tularensis* is a facultative intracellular pathogen that replicates in macrophages (Thorpe and Marcus 1964; Thorpe and Marcus 1965; Nutter and Myrvik 1966). Intracellular replication has so far correlated well with virulence in animal models of tularemia, but this property does not fully explain the remarkable infectivity and virulence of the bacterium. Other pathogenic bacteria, such as *Mycobacterium tuberculosis* (Pieters 2008) and *Legionella pneumophila* (Cianciotto 2001), replicate in macrophages yet are less virulent in an acute infection. *F. tularensis* is taken up by phagocytosis into macrophages (Clemens, Lee et al. 2005; Balagopal, MacFarlane et al. 2006; Clemens and Horwitz 2007), blocks phagosome maturation, and quickly escapes to the cytosol where it replicates to high density (Clemens, Lee et al. 2004; Santic, Molmeret et al. 2005). Recent advances have demonstrated that *F. tularensis* reenters autophagosomal vacuoles in a process that is dependent on *F. tularensis* replication (Checroun, Wehrly et al. 2006). The vacuoles are double membrane bound and are positive

for the autophagosomal marker LC3, and the late endosomal marker LAMP-1. However, the mechanisms by which *F. tularensis* disseminates from one host cell to another are not understood.

Intracellular replication clearly is not the only mechanism of *F. tularensis* pathogenesis. The host immune system is modulated during both infection in a mouse model of tularemia and during intracellular replication *in vitro*. *F. tularensis* actively inhibits the ability of macrophages to produce pro-inflammatory cytokines when stimulated post invasion (Telepnev, Golovliov et al. 2003; Telepnev, Golovliov et al. 2005; Cole, Santiago et al. 2008). *F. tularensis* also stimulates the secretion of prostaglandin E2 which promotes a Th2 response and blocks T cell proliferation (Woolard, Wilson et al. 2007). However, the molecular mechanisms of *F. tularensis* immune system modulation have not been described.

***Francisella* virulence determinants.** Work on identifying and characterizing *F. tularensis* virulence factors has revealed that a set of genes on a putative pathogenicity island and their regulator, MglA, are essential for intracellular replication (Baron and Nano 1998; Lauriano, Barker et al. 2004). A number of *F. tularensis* strains with insertional mutations in pathogenicity island genes are attenuated in animal models of tularemia and deficient for intra-macrophage replication. The *F. tularensis* pathogenicity island is an approximately 30 kb chromosomal genomic island that is present in one copy in *F. novicida* and duplicated in Type I and Type II *F. tularensis*. The pathogenicity island has been linked to virulence by transposon mutagenesis of *F. novicida*. Transposon mutants in the *F. novicida* pathogenicity island do not replicate after macrophage invasion and fail to escape the phagosome in mutants where intracellular trafficking has been studied (Nano, Zhang et al. 2004). No

transposon insertions in the pathogenicity islands of the Type I and Type II *F. tularensis* have been linked to virulence, presumably because of the island duplication in these strains. The island contains from 16 to 19 open reading frames that do not have significant similarity to any proteins of known function. Gene regulation of many of the pathogenicity island genes has been linked to the transcriptional regulator MglA (Lauriano, Barker et al. 2004).

MglA is a positive transcriptional regulator that is homologous to stringent starvation protein A or SspA (Baron and Nano 1998). Both *sspA* and *mglA* are present in *F. tularensis* species and have been found to be necessary for intracellular replication and virulence in mouse models of tularemia (Lauriano, Barker et al. 2004). MglA and SspA cooperatively interact with RNA polymerase. Microarray analysis of gene expression in  $\Delta mglA$  and  $\Delta sspA$  strains demonstrate that the *mglA* and *sspA* regulons almost totally overlap (Charity, Costante-Hamm et al. 2007). MglA and SspA act as positive regulators of gene expression in the *F. tularensis* pathogenicity island (Lauriano, Barker et al. 2004). Many MglA regulated genes play roles in stress responses and some are required for intracellular replication, including the genes of the *F. tularensis* pathogenicity island (Guina, Radulovic et al. 2007). The four genes of the *iglABCD* operon were among the first loci identified as being required for intracellular replication (Golovliov, Ericsson et al. 1997; Gray, Cowley et al. 2002). The *iglABCD* operon is located in the *F. tularensis* pathogenicity island (Titball and Petrosino 2007). This operon is MglA regulated and is upregulated after macrophage invasion by *F. tularensis* (Baron and Nano 1999; Kovarova, Halada et al. 2002; de Bruin, Ludu et al. 2007). *F. tularensis* *iglA* and *iglC* mutants fail to escape the phagosome and do not replicate in the intracellular niche (Lindgren, Golovliov et al. 2004; de Bruin, Ludu et al. 2007). Although, some of the predicted proteins in the *F. tularensis* pathogenicity island

show distant similarity to some of the loci of described Type VI secretion systems (Nano and Schmerk 2007), the mechanisms by which IglC and IglA act remain a mystery. These predictions of a Type VI secretion system in *F. tularensis* have not been validated and no secreted proteins in Type I or Type II *F. tularensis* have been identified to date.

Seven secreted proteins have been identified in *F. novicida*. The secreted proteins were identified as the proteinase (PepO), two chitinases (ChiA and ChiB), a chitin binding protein (CbpA), beta glucosidase (BglX), and two proteins of unknown function. PepO, ChiA, and BglX were found to be regulated by MglA (Hager, Bolton et al. 2006; Forsberg and Guina 2007). The secretion of these factors in *F. novicida* was found to be dependent on a functional *pilA* gene in culture (Forsberg and Guina 2007). Mutation of the *pilA* gene abrogated secretion and resulted in increased virulence in a mouse model of tularemia. Type I and Type II *F. tularensis* have sequence differences or mutations that result in a *pilA* pseudogene. Protein secretion has not been demonstrated in those strains; however, pili have been observed in electron micrographs (Forsberg and Guina 2007). The role of Type IV pili in *F. tularensis* secretion or attachment is not well characterized.

**Pneumonic Type I *Francisella tularensis* infections are the most virulent cases of tularemia.** What makes Type I strains more virulent than other *F. tularensis* species is unknown even though the genome of a *F. tularensis* Schu S4, a Type I strain, has been sequenced (Larsson, Oyston et al. 2005; Rohmer, Fong et al. 2007). The pathogenicity island is common to all sequenced *Francisella* species (Titball and Petrosino 2007). Another gap in the *F. tularensis* knowledge base is why aerosol transmission is more infectious and results in more aggressive infections. Mortality in tularemia is generally surmised to be due

to multiple organ failure with large bacterial burdens and not specifically linked to infection of the lungs (Conlan, Chen et al. 2003). So why does pneumonic tularemia have increased mortality rates?

*F. tularensis* can infect and replicate in alveolar macrophages (Nutter and Myrvik 1966), which is generally considered to be an important factor in the pathogenesis of *F. tularensis*. However, the ultrastructure of the alveolus consists of much more than alveolar macrophages. The alveolar epithelium consists of Type I and Type II epithelial cells which produce surfactant and enable gas exchange. The alveolar epithelium must be breached or bypassed to enable the dissemination seen during the course of a pneumonic *F. tularensis* infection. These alveolar epithelial cells have largely been ignored as potential sites of infection and replication, even though they comprise the majority of alveolar surface area. We hypothesize that *F. tularensis* comes in to contact with and infects alveolar epithelial cells and that a subset of *F. tularensis* genes are necessary for intraepithelial replication. In this work we have characterized a gene we have termed *ripA*. The *ripA* gene encodes a protein required for intracellular replication and virulence in a mouse model of tularemia.

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## Chapter 2

### ***Francisella tularensis* replicates within alveolar type II epithelial cells *in vitro* and *in vivo* following inhalation**

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#### ABSTRACT

*Francisella tularensis* replicates in macrophages and dendritic cells, but interactions with other cell types have not been well described. *F. tularensis* LVS invaded and replicated within alveolar epithelial cell lines. Following intranasal inoculation of C57BL/6 mice, *Francisella* localized to the alveolus and replicated within alveolar type II epithelial cells.

#### INTRODUCTION

*Francisella tularensis*, the etiological agent of tularemia, is a zoonotic pathogen with a broad host range spanning from protists to humans. The organism is transmitted to humans through handling of contaminated material, insect bites, or inhalation (1, 10). It has been determined that contact with 25 or fewer bacteria is sufficient to cause disease (16, 17). Following contact, the bacteria disseminate to liver, spleen and lungs regardless of initial transmission route (7, 10). The low infectious dose, myriad of transmission routes, broad host range, and severity of disease caused by *F. tularensis* led a number of nations to develop and

stockpile this organism as a biological weapon (9), yet little is known about the basis of its pathogenesis or virulence. It has been reported that macrophages and potentially dendritic cells serve as the primary host cells for *Francisella* growth in humans and other animals (6, 18). This conclusion is supported by the observation that *F. tularensis* and other closely related *Francisella* species survive and replicate in human and rodent macrophages (2, 5), and that many of the identified attenuating mutations impair intra-macrophage growth (3, 11, 12, 14, 15).

Clearly intra-macrophage survival is an important *Francisella* virulence property. However, apart from one report of hepatocyte association in infected liver (8), the potential contribution of other host cell interactions to *F. tularensis* virulence and pathogenesis remains untested. Given the severity of inhalation-acquired tularemia, we sought to address this possibility by using *in vitro* and *in vivo* models to examine whether *Francisella* invades and replicates within epithelial cells of the lung.

## RESULTS

### ***Francisella tularensis* LVS invades and replicates within lung epithelial cells *in vitro*.**

To determine if *Francisella* could invade and replicate within lung epithelial cells, *in vitro* invasion and replication assays were performed using a human alveolar type II (ATII) epithelial-derived cell line A549 (ATCC), a mouse ATII-derived cell line MLE-12 (ATCC), and a mouse lung epithelial-derived cell line Tc-1 (ATCC). For comparison, the mouse macrophage-like cell line, J774A.1 (ATCC) was also used. Intracellular bacteria were enumerated using a gentamicin protection assay. The cell lines were grown to confluency in 24-well plates and inoculated with *F. tularensis* LVS at a multiplicity of infection (MOI) of

100:1. Four hours following inoculation, the cells were washed with PBS and media containing gentamicin (25 µg/mL) was added to kill extracellular bacteria.

Intracellular organisms were recovered from all cell lines 6 and 24 hours post-inoculation (Figure 2.1A). The number of intracellular organisms recovered 6 hours post-inoculation as well as the percentage of infected cells varied among the lung epithelial cell lines with the fewest intracellular *Francisella* recovered from the human A549 cell line, A549. The percentage of infected epithelial cells ranged from 0.2% infected A549 cells infected to 17% infected Tc-1 cells (Figure 2.1A). The number of intracellular bacteria recovered from each lung epithelial cell line at 6 hours was less than the recovery from J774A.1 cells as was the percentage of infected cells, indicating that entry into epithelial cells may be a less efficient process than uptake by macrophages.

The number of intracellular bacteria increased by an average of 100 fold during the following 18 hours in each cell line representing an intracellular doubling time of roughly 3 hours (Figure 2.1A). Intracellular localization and proliferation was confirmed using fluorescence microscopy and digital image deconvolution (Figure 2.1B-E). From 6 to 24 hours post-inoculation, the relative increase of intracellular bacteria within lung epithelial cells was similar to that observed within J774A.1 cells, indicating that *F. tularensis* LVS replicated to the same degree within lung epithelial cells as in macrophages.

There are many reports of *Francisella* replicating within macrophages *in vitro* and here we report *Francisella's* ability to replicate within cultured lung epithelial cells. Whereas some work has been done to examine *Francisella* localization in the liver (8), little is known about the localization of inhaled *Francisella* in the lung. Bosio *et al* recently reported that *Francisella* was associated primarily with dendritic cells isolated from

bronchioalveolar lavage fluid of mice one hour following inhalation (6). However, the localization of bacteria in lung tissue throughout the course of infection has not been addressed.

**Following inhalation, *Francisella* localizes to the alveolus.**

To determine the localization of *Francisella* in the airway following inhalation, anesthetized 6-8 week old female C57BL/6 mice were inoculated intranasally with  $10^5$  CFU of *F. tularensis* LVS expressing GFP suspended in 50  $\mu$ l of PBS (All mouse work was performed under IACUC-approved protocol.). Immunofluorescence analysis was performed on formalin fixed and paraffin embedded tissue sections obtained from nasal turbinates, trachea, and lungs harvested 1, 3 and 7 days post-inoculation. No bacteria were observed in turbinates or trachea. In the lung, few bacteria associated with the apical surface of bronchial epithelial cells while the majority localized with alveolar cells (Figure 2.2). Fluorescence imaging of sequential vertical planes revealed that most infected alveolar cells contained multiple bacteria throughout the cytoplasm, indicative of intracellular replication (data not shown). Bacterial replication within ciliated or non-ciliated (Clara) bronchial epithelial cells was not observed. The absolute number of bacteria (Figure 2.2D) and the number of infected alveolar cells increased by day 3 (Figure 2.2B). By day 7, *F. tularensis* within the lung was widespread but remained exclusively in the alveolus (Figure 2.2C). Throughout the course of infection, the amount of extracellular space observed in the network of alveolar cells decreased leading to the alveolar cells appearing more condensed (Figure 2.2).

**Inhaled *Francisella* co-localizes to and replicates within ATII cells *in vivo*.**

Given that *Francisella* invaded and replicated within ATII-derived cell lines *in vitro*, we probed infected lung tissue sections with antibodies to non-secreted surfactant protein

precursors proSP-B and proSB-C (Chemicon) to determine if *Francisella* localized to and replicated within ATII cells *in vivo* following inhalation. proSP-B is produced by ATII cells and non-ciliated bronchiolar Clara cells (13) whereas proSP-C is produced by ATII cells exclusively (4). Three days following inoculation, the majority of alveolar cells associated with *F. tularensis* also bound antibodies to non-secreted surfactant protein precursors, proSP-B and proSB-C (Figure 2.3A-D). Some cells associated with bacteria were not positive for proSP-B or proSP-C indicating that *Francisella* interacts with other cell types in the lung following inhalation.

In addition to the co-localization with proSP proteins, microscopy of sequential vertical planes confirmed that bacteria were inside infected ATII cells (data not shown). Rabbit antibody specific for  $\beta$ -tubulin (Abcam) did not co-localize with *F. tularensis* (Figure 2.3E) indicating that the co-localization of *Francisella* with rabbit proSP antibodies was not due to bacteria non-specifically binding rabbit antibodies, nor did the proSP antibodies cross-react with cultured *Francisella* (data not shown). These collective data demonstrate that *F. tularensis* replicates within ATII cells following inhalation, and the proportion of ATII cells harboring bacteria increases during the course of pneumonic tularemia.

Bacteria were also observed in probed sections that did not co-localize with proSP-B and proSP-C (Figure 2.3) indicating that ATII cells are not the only alveolar cell type harboring replicating *Francisella*. Macrophages and dendritic cells have been reported as potential host cells for replicating *Francisella* (2, 5, 6). Due to difficulties in staining for surface macrophage and dendritic cell markers in embedded lung sections, we utilized dispase digestion of infected mouse lungs to stain for surface markers and also to assess our results from the embedded tissue staining.

Three days following inoculation with LVS expressing GFP, lungs were infused and incubated with the neutral protease, dispase (BD Biosciences). Digested tissue was washed in PBS and subsequently filtered through 40  $\mu\text{m}$  and 20  $\mu\text{m}$  mesh. Staining with fluorescently-labeled antibody specific for the surface markers F4/80 (eBioscience) and CD11c (eBioscience) was performed prior to fixation with 4% paraformaldehyde. Staining for the intracellular markers proSP-B and proSP-C occurred following fixation and required the use of Cytoperm (BD Biosciences) to permeabilize the cells. *F. tularensis* LVS was observed within cells expressing F4/80, CD11c, proSP-B, and proSP-C (Figure 2.4) indicating that following inhalation, bacteria survive and replicate within macrophages, dendritic cells, and ATII cells.

Here we report that in addition to replicating within macrophages and dendritic cells, *F. tularensis* also invades and replicates within alveolar type II epithelial cells, indicating that interaction with these cells following inhalation may be an important component of pneumonic tularemia. Future work will analyze the proportion of various cell types that are infected in the lung as well as attempt to identify the contribution of ATII cell invasion and replication to the disease progression of pneumonic tularemia.

### **ACKNOWLEDGEMENTS**

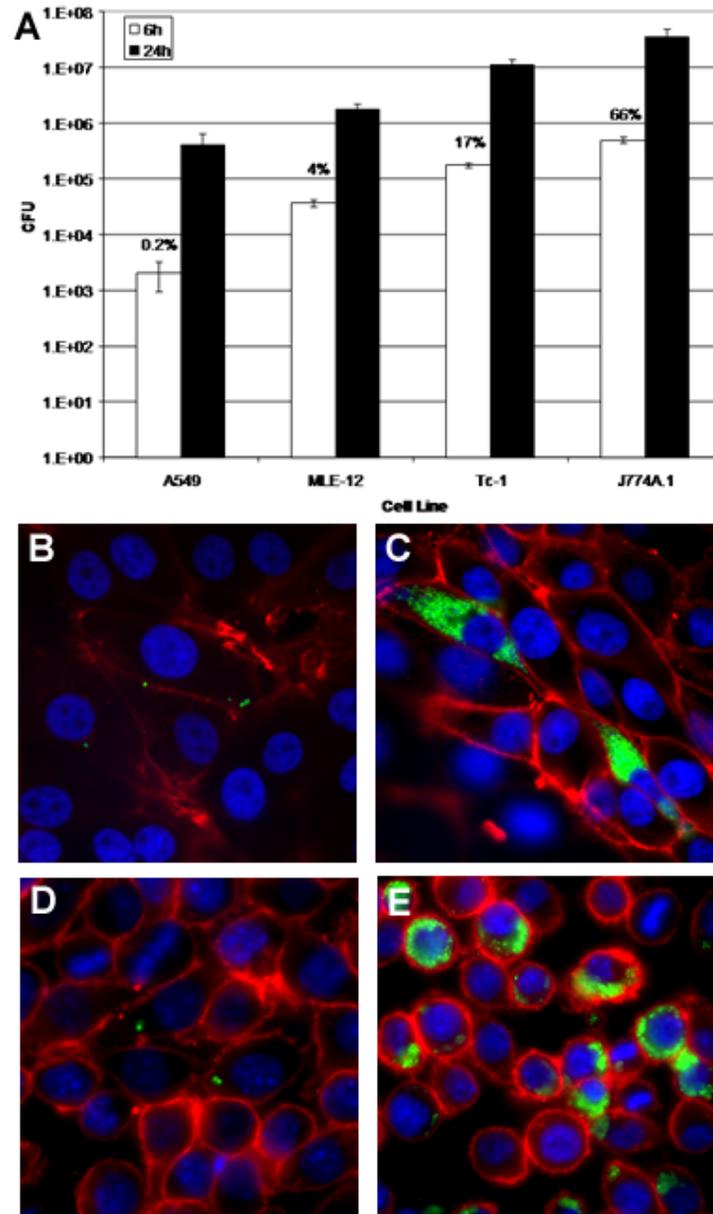
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### **ATTRIBUTIONS**

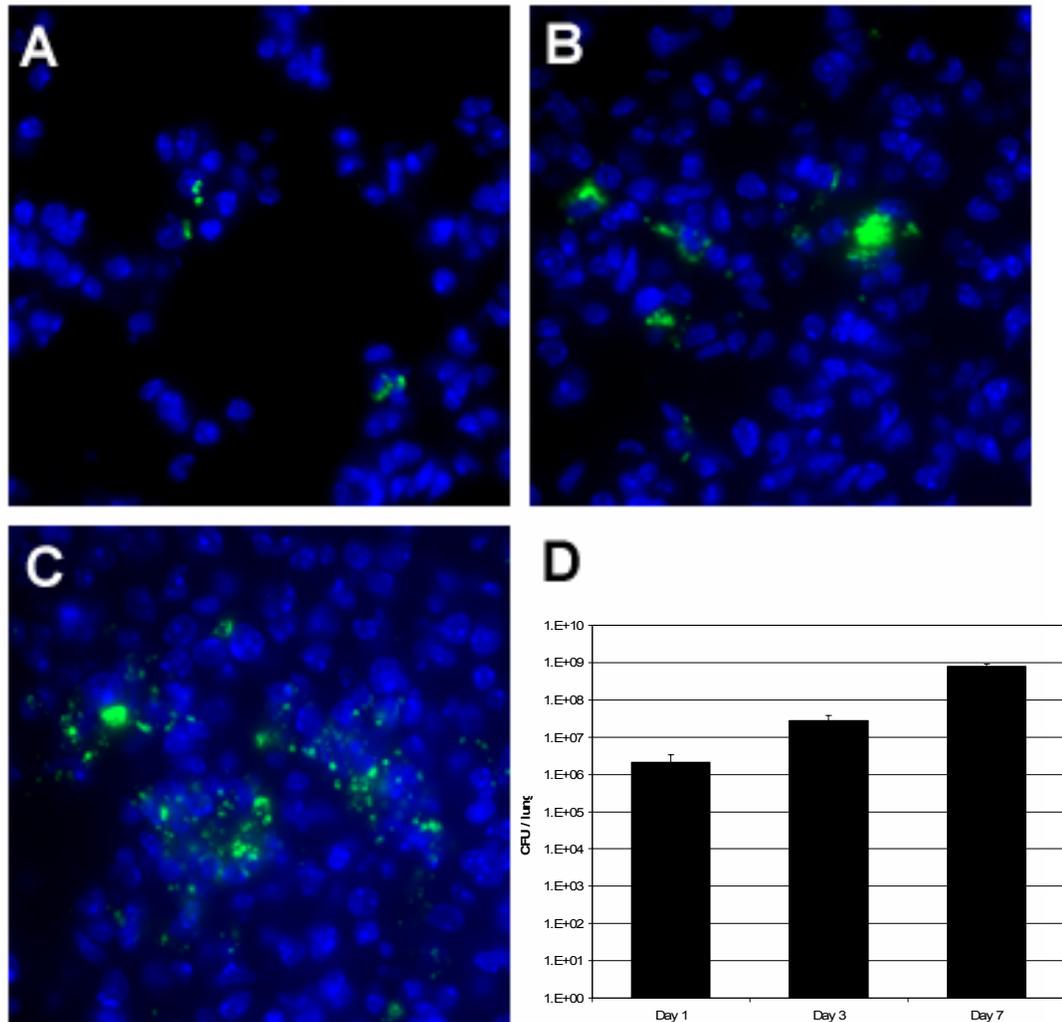
Josh Hall performed all experiments presented in this chapter with the following exceptions. Robin Craven and James Fuller assisted with mouse infections, lung collection, and organ burden experiments. Robin Craven acquired J774 fluorescence microscopy images. This work was published, Copyright © American Society for Microbiology, *Infection and Immunity*, Volume 75, pages 1034-1039, 2006. Permission has been granted to reprint this material.

## FIGURES

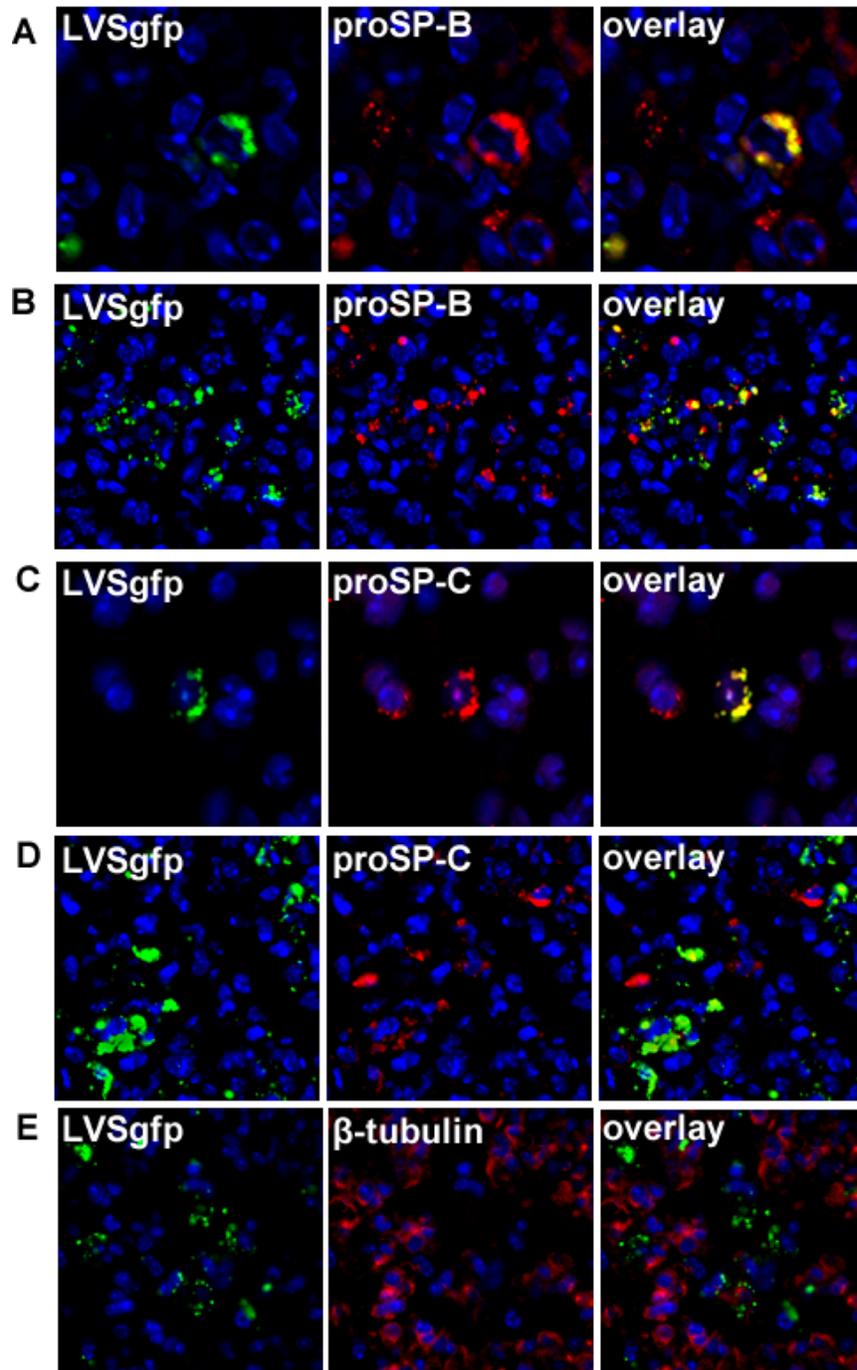


**Figure 2.1. *F. tularensis* LVS invades and replicates within ATII cell lines *in vitro*.** (A) Intracellular bacteria recovered from A549, MLE-12, Tc-1, and J774A.1 cells 6 and 24 hours post inoculation. Percentages above bars represent percentage of infected cells. (B) Fluorescence imaging of A549s inoculated with GFP-expressing LVS 6 and (C) 24 hours following inoculation. Cell borders were visualized by rhodamine-phalloidin (Molecular Probes) staining (red) and nuclei visualized with DAPI (blue). (D) Fluorescence imaging of J774A.1 cells inoculated with GFP-expressing LVS (green) 6 and (E) 24 hours post-inoculation. Cell borders were visualized using biotinylated lectin from *Lens culinaris* and streptavidin-conjugated Alexa Fluor 647 (Molecular Probes) (red) and nuclei were stained

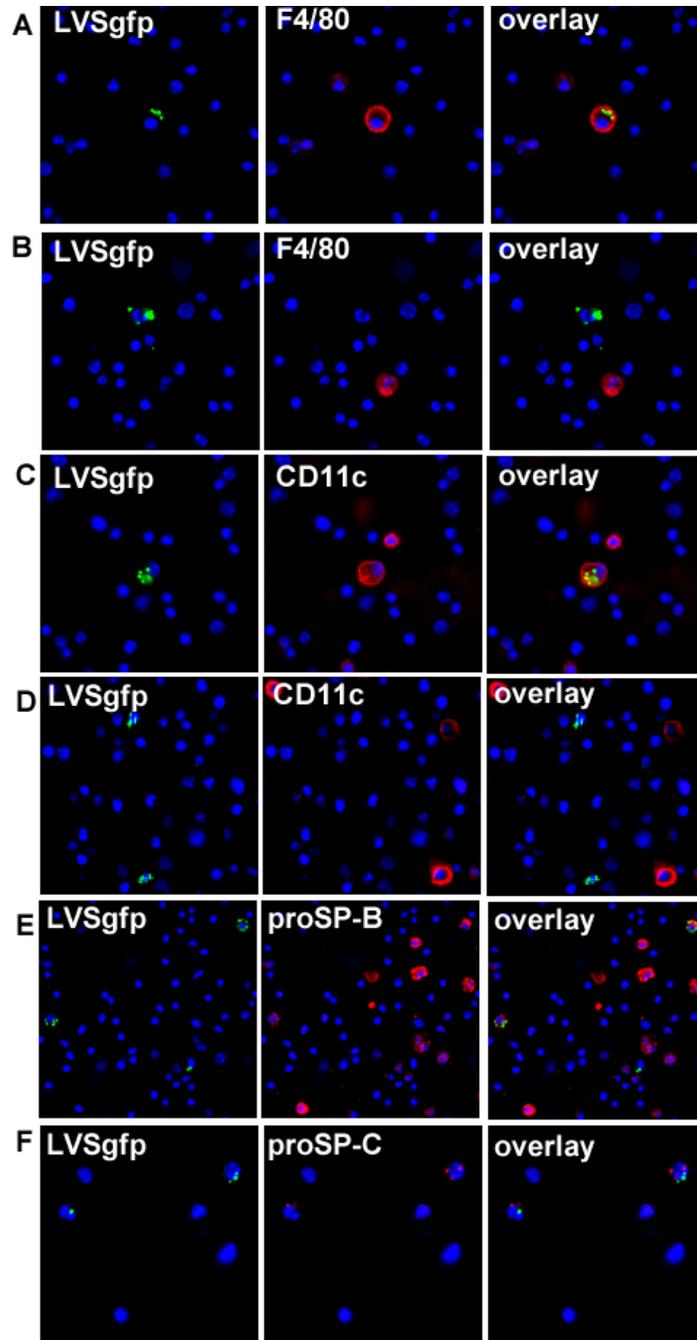
with DAPI (blue). Intracellular replication experiments were carried out in triplicate with error bars representing standard deviations of the means.



**Figure 2.2. Following inhalation *F. tularensis* localizes to the alveolus.** Mice were intranasally inoculated with  $10^5$  CFU of *F. tularensis* LVS expressing GFP. One, three, and seven days post-inoculation, lungs were harvested and prepared for immunofluorescence analysis. Bacterial localization was determined by probing lung sections with a fluorescently-labeled antibody to GFP (green). Nuclei were stained with DAPI (blue) to visualize lung cells. Representative images of the alveolus of infected mice (A) 1, (B) 3, and (C) 7 days post-inoculation. (D) Bacterial recovery from lungs 1, 3, and 7 days following intranasal inoculation with  $10^5$  CFU LVS. Each bar represents mean recovery from 3 mice with error bars representing standard deviations of the means.



**Figure 2.3. Following inhalation, *F. tularensis* LVS expressing GFP co-localized with proSP-B and proSP-C; proteins produced by ATII epithelial cells.** Bacterial localization was determined with a fluorescently-labeled antibody to GFP (green). Nuclei were stained with DAPI (blue). Sections were probed with fluorescently-labeled antibody to (A-B) proSP-B (red), (C-D) proSP-C (red) to identify ATII cells and (E)  $\beta$ -tubulin (red). Representative images are from lung sections 3 days post-inoculation.



**Figure 2.4. *F. tularensis* LVS expressing GFP co-localized with cells expressing the macrophage marker F4/80, the dendritic cell marker CD11c, and the ATI cell markers proSP-B and proSP-C.** Nuclei were stained with DAPI (blue). Mouse lung cells were probed with fluorescently-labeled antibody to (A-B) F4/80 (red), (C-D) CD11c (red), and (E) proSP-B (red). Representative images are from lung cells 3 days post-inoculation.

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## Chapter 3

### *Francisella tularensis* Invasion of Lung Epithelial Cells

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#### ABSTRACT

*Francisella tularensis*, a gram-negative facultative intracellular bacterial pathogen, causes disseminating infections in humans and other mammalian hosts. Macrophages and other monocytes have long been considered the primary site of *F. tularensis* replication in infected animals. However, recently it was reported that *F. tularensis* also invades and replicates within alveolar epithelial cells following inhalation in a mouse model of tularemia. TC-1 cells, a mouse lung epithelial cell line, were used to study the process of *F. tularensis* invasion and intracellular trafficking within nonphagocytic cells. Live and paraformaldehyde fixed *F. tularensis* live vaccine strain (LVS) associated with, and were internalized by, TC-1 cells at a similar frequency and with indistinguishable differences in kinetics. Inhibitors of microfilament and microtubule activity resulted in significantly decreased *F. tularensis* invasion, as did inhibitors of PI3 kinase and tyrosine kinase activity. Collectively these results suggest that *F. tularensis* epithelial cell invasion is mediated by a preformed ligand on the bacterial surface and driven entirely by host cell processes. Once internalized, *F.*

*tularensis* containing endosomes associated with EEA1 followed by LAMP-1 with peak co-association frequencies occurring at 30 and 120 minutes post-inoculation, respectively. By 2 hours post-inoculation 70.0% ( $\pm 5.5\%$ ) of intracellular bacteria were accessible to antibody delivered to the cytoplasm indicating vacuolar breakdown and escape into the cytoplasm.

## INTRODUCTION

*Francisella tularensis*, the causative agent of the disease tularemia, infects a wide range of animal hosts. Humans can be infected by a variety of routes, including physical contact with infected animals, insect bites, ingestion of contaminated food or water, and inhalation of organisms (13, 52). Disease severity is affected by both the route of inoculation and bacterial subtype (13, 48, 52). *F. tularensis* strains are subdivided into two groups, A and B; the more severe form of tularemia in humans is caused by type A strains (13, 48, 52). The type A strain *F. tularensis* subspecies *tularensis* is found almost exclusively in North America, whereas the type B strain *F. tularensis* subspecies *holarctica* is found throughout Europe, as well as in North America (35). There is a 5% to 15% mortality rate associated with untreated human tularemia caused by type A strains, however that rate reaches 30% to 60% for untreated pneumonic and typhoid forms of the disease (11). The live vaccine strain (LVS) is an attenuated type B strain that causes a tularemia-like disease in mice, and is used as a model organism to study *F. tularensis* pathogenesis. There has been heightened interest in the study of this organism in recent years due to its history of weaponization and potential for use as an agent of biological warfare. *F. tularensis* is a category A select agent on the

CDC's bioterrorism agent list, which includes organisms with the potential to cause high numbers of casualties if disseminated in an aerosol form (26).

Much of the *Francisella* pathogenesis research has focused on survival and replication of *F. tularensis* in macrophages and dendritic cells, and many of the genes identified to date that are required for full virulence contribute to survival or replication in the macrophage (1, 6, 17, 20, 27, 30, 32, 37, 42). We recently demonstrated that *F. tularensis* also invades and replicates in alveolar type II (ATII) epithelial cells of infected mice (21). ATII cells have a number of biological functions, including the production, secretion and recycling of surfactant, proliferation to produce additional type II cells as well as transdifferentiation into type I cells, maintenance of alveolar fluid balance, and production of antimicrobial and anti-inflammatory substances(29). Due to the close proximity between the blood and alveolar epithelial cells required for gas exchange and fluid balance, these cells are ideally located to provide a portal through which bacteria could disseminate to distal organs.

*F. tularensis* attaches to and invades nonphagocytic cells, including the ATII cell lines A549 and MLE 12 and the lung epithelial cell line TC-1 (21, 28). Melillo et al. also reported that *E. coli* expressing the *F. tularensis* surface protein FsaP bind to A549 cells (31), identifying a surface protein that may play a role in ATII cell association.

A primary function of monocytes is to engulf bacterial cells and other foreign particles (24, 44). However, invasion of epithelial and other nonphagocytic host tissue cells requires bacterially mediated exploitation of host cell functions to gain entry (10, 15, 16). Alveolar epithelial cells provide a site where *F. tularensis* can replicate in the infected host (21), and as such it is important to understand how bacterial interaction with these cells may

differ from interaction with macrophages. Herein we describe our efforts to understand how *F. tularensis* invades lung epithelial cells.

## MATERIALS AND METHODS

### Bacterial strains

*Francisella tularensis* LVS was obtained from the Centers for Disease Control and Prevention, Atlanta, Ga. LVSgfp was constructed using the pKK214GFP plasmid (a gift of Mats Forsman). *F. tularensis* LVS and LVSgfp were propagated on chocolate agar supplemented with 1% IsoVitaleX (Becton-Dickinson). *Listeria monocytogenes* EGD1/2a, *Salmonella enterica* serovar Typhimurium, *Yersinia pseudotuberculosis*, and *Campylobacter jejuni* strain 81-176 were gifts from Paul Orndorff, Craig Altier, Ralph Isberg, and Deborah Threadgill, respectively. All bacterial strains were grown on LB agar, with the exception of *C. jejuni*, which was grown on Mueller-Hinton agar with 5% CO<sub>2</sub>, and *F. tularensis* LVS, which was grown on chocolate agar as described above. All bacterial strains were grown at 37°C. *Salmonella enterica* was grown overnight in LB broth under static conditions for invasion assays.

### Cell culture

TC-1 (ATCC CRL-2785) is a tumor cell line derived from primary lung epithelial cells of C57BL/6 mice. The cells were immortalized with HPV-16 E6 and E7 and transformed with the c-H-*ras* oncogene. These cells were grown in RPMI 1640

supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids and 10% FBS. Cell cultures were maintained at 37°C and 5% CO<sub>2</sub>.

### **Attachment, Invasion and Vacuolar Escape Assay**

To evaluate the percentage of TC-1 cells with associated bacteria synchronized infections were carried out by adding *F. tularensis* LVS expressing GFP (LVSgfp) at a multiplicity of infection (MOI) of 100 to TC-1 cells chilled to 4°C. Bacterial suspensions were made in tissue culture media from bacteria grown as described above. Bacterial concentration of suspensions was determined using a Klett meter. Dilutions of suspensions were plated to verify MOI. Plates were centrifuged at 300 x g for 5 minutes at 4°C, and then rapidly warmed by placing in a 37°C water bath for 2 minutes before transferring to a 37°C, 5% CO<sub>2</sub> incubator. For experiments using killed LVS, organisms were treated with 4% paraformaldehyde (PFA) for 10 minutes. There was no growth from a PFA treated bacterial aliquot that was plated on chocolate agar, demonstrating that PFA treatment killed all organisms. At 10 minutes post-inoculation samples were washed with PBS to remove unattached bacteria and pre-warmed media was added to monolayers. Cells were collected by trypsinization at 10, 20, 30 and 60 minutes post-inoculation and samples were processed at 4°C. Cells to be analyzed for bacteria association were fixed in 4% PFA for 10 minutes, and analyzed by flow cytometry.

Parallel wells were analyzed for intracellular bacteria by collecting samples as described above. Extracellular bacteria were labeled using anti-*F. tularensis* LPS antibody

(USBiological) conjugated to Pacific Blue (Molecular Probes) 1:1000 for 30 minutes at 4°C. Cells were then lysed with water and centrifuged at 300 x g to removed eukaryotic cell debris. Supernatant containing bacteria was centrifuged at 16,000 x g, fixed with 4% PFA, and analyzed by flow cytometry to differentiate intracellular (GFP only) from extracellular (GFP and Pacific Blue) bacteria.

To evaluate bacterial escape into the cytoplasm, synchronized inoculations of TC-1 cells and staining of extracellular bacteria was done as described above. Cells were collected by trypsinization at 10, 20, 30, 60 and 120 minutes post-inoculation. TC-1 cells were treated with 50 µg/ml digitonin in KHM buffer (110 mM potassium acetate/20 mM Hepes/2 mM MgCl<sub>2</sub>, pH 7.3) for 1 minute, washed in KHM buffer, and incubated with anti-*F. tularensis* LPS antibody conjugated to Alexa Fluor 647 (Molecular Probes) 1:1000 for 30 minutes at 4°C. Cells were then washed in KHM buffer, lysed with water and centrifuged at 300 x g to removed cellular debris. Supernatants were centrifuged at 16,000 x g and bacterial pellets were fixed in 4% PFA and analyzed by flow cytometry to differentiate extracellular (GFP, Pacific Blue and Alexa Fluor 647), cytoplasmic (GFP and Alexa Fluor 647) and vacuolar (GFP only) bacteria.

Flow cytometry of whole cells and bacteria was performed using a CyAn ADP flow cytometer (Dako Cytomation). Data was analyzed using Summit Software (Dako), with gating for single events for whole cell samples and GFP positive single events for bacterial samples.

## **Inhibitor Assays**

Inhibitor assays were carried out with cytochalasin D, colchicine, wortmannin and genistein. Cytochalasin D, an actin polymerization inhibitor, was used at 0.5 and 1.0  $\mu\text{M}$  concentrations. Colchicine, a microtubule polymerization inhibitor, was used at 0.1 and 0.25  $\mu\text{M}$  concentrations. Wortmannin, an inhibitor of PI3 kinase activity, was used at 100 and 200 nM concentrations. Genistein, an inhibitor of tyrosine kinase activity, was used at 50 and 100  $\mu\text{M}$  concentrations. Monolayers were preincubated for one hour with escalating concentrations of inhibitor, and then bacteria suspended in media containing inhibitor were added at an MOI of 100 for LVS, or 25 to 50 for other bacteria. Samples without inhibitor included bacteria suspended in tissue culture media alone, or media containing the carrier in which the inhibitor was reconstituted where appropriate. After four hours, cells were washed with PBS to remove inhibitor. Extracellular bacteria were killed by adding media containing 25  $\mu\text{g}/\text{ml}$  of gentamicin and incubated 2 hours before monolayers were washed with PBS and scraped from the plate using sterile applicator sticks. Serial dilutions were plated on chocolate agar to quantify colony forming units (CFU) of intracellular bacteria. Inhibitors did not affect LVS viability as demonstrated by incubating bacteria in tissue culture media containing inhibitor for four hours, followed by plating onto chocolate agar to quantify CFU. Inhibitors did not affect eukaryotic cell viability at the concentrations used as determined by Trypan Blue exclusion. The effect of inhibitors on bacterial attachment to cells was determined as described above for invasion, except that gentamicin was not added, allowing bacteria attached to cells but not internalized to be included in the CFU recovered. Inhibitors had no effect on LVS attachment to TC-1 cells. Results are expressed as the percentage of LVS or control organism that survived gentamicin treatment, relative to the sample without inhibitor inoculated with the same organism. Invasion without inhibitor is defined as 100%

invasion. Data presented are the results of assays done in triplicate, and are representative of multiple repetitions of each experiment. Data were analyzed for statistical significance by paired two-tailed *t* test, and considered significantly different from the untreated control when  $P < 0.01$ .

### **Fluorescence microscopy**

Cells were grown on poly-L-lysine coated coverslips in 24-well cell culture plates to ~90% confluency. LVS<sub>gfp</sub> was added at an MOI of 100 to pre-chilled TC-1 plates, and centrifuged at 300 x g for three minutes to synchronize infection as described above. At 10, 20, 30, 60, 120 and 180 minutes post-inoculation monolayers were washed with PBS to remove unattached bacteria and fixed using 4% PFA. Samples were blocked with PBS/Fc block/5% donkey serum, incubated with anti-*F. tularensis* LPS diluted 1:1000 for 30 minutes at 4°C followed by donkey anti-mouse AMCA (7-amino-4-methylcoumarin-3-acetic acid, Jackson ImmunoResearch) diluted 1:100, 30 minutes, 4°C to label extracellular bacteria. For early endosome antigen 1 (EEA1) or lysosome associated membrane protein 1 (LAMP-1) staining samples were blocked using PBS/0.1% saponin/5% serum of host species of secondary antibody followed by goat polyclonal anti-EEA1 (N-19; Santa Cruz Biotechnologies) diluted 1:200 in blocking solution, or rat monoclonal anti-mouse LAMP-1 (1D4B, developed by J. T. August, obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242) diluted 1:200 in blocking solution. Secondary antibodies used were donkey anti-goat Cy5 (Jackson ImmunoResearch) or goat anti-rat Alexa Fluor 647 (Molecular Probes), diluted 1:500 in PBS/0.1% saponin.

Samples were examined using a Zeiss Axioplan 2 epifluorescence microscope and SlideBook digital deconvolution software (Intelligent Imaging Innovations). 100 intracellular bacteria were counted for each time point and condition. Images of sequential vertical planes were acquired to determine the location of bacteria within EEA1 or LAMP-1 containing vacuoles. Data presented are the results of three independent experiments.

### **Electron microscopy**

Cell monolayers grown on polystyrene plates were rinsed with PBS or serum-free medium and fixed in 3% glutaraldehyde/0.15M sodium phosphate, pH 7.4. Following three rinses with sodium phosphate buffer, the monolayers were post-fixed for 1 hour in 1% osmium tetroxide/1.25% potassium ferrocyanide/0.15M sodium phosphate buffer, rinsed in deionized water, dehydrated using increasing concentrations of ethanol (30%, 50%, 75%, 100%, 100%, 10 minutes each) and embedded in Polybed 812 epoxy resin (Polysciences, Inc., Warrington, PA). The embedded samples were sectioned parallel and perpendicular to the substrate at 70nm using a diamond knife. Ultrathin sections were collected on 200 mesh copper grids and stained with 4% aqueous uranyl acetate for 15 minutes, followed by Reynolds' lead citrate for 7 minutes. Sections were observed using a LEO EM910 transmission electron microscope at 80kV (LEO Electron Microscopy, Thornwood, NY) and photographed using a Gatan Bioscan Digital Camera (Gatan, Inc., Pleasanton, CA).

## **RESULTS**

### **Kinetics of *F. tularensis* epithelial cell attachment and invasion**

*Francisella tularensis* LVS invades and replicates within TC-1, MLE 12 and A549 lung epithelial cell lines (21). While the intracellular replication rates within these lines are indistinguishable, TC-1 cells support the highest initial invasion frequency (21). In addition, TC-1 cells are a mouse cell line, and LVS infection causes a tularemia-like disease in mice similar to that seen with virulent strains in humans. We therefore used TC-1 cells to examine the initial stages of *F. tularensis* invasion.

To determine kinetics of lung cell attachment and invasion by *F. tularensis* we synchronized infection of TC-1 cells at an MOI of 100 with LVSgfp. At 10, 20, 30 and 60 minutes post-inoculation, cells were washed to remove nonadherent bacteria and collected to quantify TC-1 cells associated with GFP expressing bacteria by flow cytometry (Figure 3.1A). At these same time points, in parallel samples, extracellular bacteria were stained using a Pacific Blue labeled antibody to *F. tularensis* LPS and TC-1 cells were lysed to recover cell-associated bacteria. GFP positive bacteria recovered from cells were analyzed by flow cytometry to determine intracellular (GFP alone) versus extracellular (GFP and Pacific Blue) localization (Figure 3.1B). At 10 minutes 1.7% ( $\pm 0.4\%$ ) of TC-1 cells had associated bacteria, of which 1.4% ( $\pm 0.1\%$ ) of TC-1 cell-associated bacteria were intracellular. At 20 minutes post-inoculation 1.1% ( $\pm 0.2\%$ ) of TC-1 cells had cell-associated bacteria, of which 7.0% ( $\pm 0.6\%$ ) were internal. At 30 minutes post-inoculation 1.0% ( $\pm 0.2\%$ ) of TC-1 cells had cell-associated bacteria, of which 21.3% ( $\pm 6.3\%$ ) were internal. At 60 minutes post-inoculation 1.4% ( $\pm 0.1\%$ ) of TC-1 cells had cell-associated bacteria, of which 23.6% ( $\pm 5.8\%$ ) were intracellular.

To determine if LVS entry into lung epithelial cells required viable bacteria, we repeated the above experiments using killed LVSgfp. At 10 minutes 0.9% ( $\pm 0.2\%$ ) of TC-1

cells had dead bacteria associated with them, of which 1.2% ( $\pm 0.4\%$ ) of TC-1 cell-associated bacteria were intracellular. At 20 minutes post-inoculation 1.1% ( $\pm 0.7\%$ ) of TC-1 cells had cell-associated dead bacteria, of which 5.7% ( $\pm 0.6\%$ ) were internal. At 30 minutes post-inoculation 0.6% ( $\pm 0.2\%$ ) of TC-1 cells had cell-associated dead bacteria, of which 17.3% ( $\pm 3.5\%$ ) were internal. At 60 minutes post-inoculation 0.4% ( $\pm 0.1\%$ ) of cells had dead bacteria associated with them, of which 25.7% ( $\pm 1.6\%$ ) were intracellular. These results indicated that both live and dead LVS attached to and invaded lung epithelial cells. Further, invasion frequency and kinetics were not significantly different between live and dead bacteria ( $P < 0.01$ ), except cell association at 60 minutes (Figure 3.1). The ability of nonviable *F. tularensis* to invade lung epithelial cells suggested that a preformed ligand on the bacterial surface may interact with the host cell.

### **Effects of cytoskeleton and signaling pathway inhibitors on *F. tularensis* invasion of epithelial cells.**

Inhibitors of eukaryotic cell function were used to determine the contribution of host cell signaling and cytoskeleton rearrangement to LVS invasion of lung epithelial cells. Bacterial entry into host cells generally requires rearrangement of cytoskeletal structures; either microfilaments alone, as is the case with *Salmonella*, *Shigella*, *Listeria* and *Yersinia* spp. (12), or both microfilaments and microtubules, as is the case with *Neisseria gonorrhoeae* and *Campylobacter jejuni* (4, 43).

To determine the contribution of actin polymerization to LVS invasion we incubated TC-1 lung epithelial cells with cytochalasin D, an actin polymerization inhibitor that disrupts microfilaments, and measured bacterial internalization by gentamicin protection assay.

Treatment of TC-1 cells with 0.5 and 1.0  $\mu\text{M}$  cytochalasin D decreased LVS invasion more than 99% ( $\pm 0.1\%$ ) (Figure 3.2A), whereas *Salmonella enterica* invasion, which is known to be actin dependent (36), decreased by 84.6% ( $\pm 2.3\%$ ) and 88.1% ( $\pm 2.5\%$ ) at the same inhibitor concentrations, respectively. TC-1 cell invasion by *Campylobacter jejuni* strain 81-176, a strain which does not require actin for invasion of intestinal epithelial cells (34), was not decreased by actin inhibition.

Microtubules are responsible for the cytoplasmic organization of eukaryotic cells, control of organelle transport, and are a primary component of cilia and flagella (55). Microtubule polymerization has been shown to contribute to the epithelial cell invasion of *Neisseria gonorrhoeae* and *Campylobacter jejuni* (4, 43). To determine the contribution of microtubules to LVS invasion we treated lung epithelial cells with colchicine, which binds tubulin and inhibits microtubule polymerization, and determined invasion by gentamicin protection assay. Treatment of TC-1 cells with colchicine decreased LVS invasion by 55.2% ( $\pm 18.3\%$ ) and 94.2% ( $\pm 2.4\%$ ) at 0.1 and 0.25  $\mu\text{M}$  concentrations, respectively (Figure 3.2B). TC-1 cell invasion by *Campylobacter jejuni* strain 81-176, a strain for which invasion is blocked by microtubule depolymerization in intestinal epithelial cells (34), was decreased significantly at the higher colchicine concentration. *Salmonella enterica* invasion, which is not considered microtubule dependent (4), did not demonstrate a statistically significant decrease in invasion at these concentrations.

Signaling pathways are frequently manipulated by bacteria to cause the cytoskeletal rearrangement necessary to gain entry into nonphagocytic cells (10, 15, 16). PI3 kinase and tyrosine kinase signaling are exploited by other pathogens for invasion (25, 45). These

pathways were examined for their contribution to *Francisella tularensis* entry into lung epithelial cells.

PI3 kinases phosphorylate inositol phospholipids, forming lipid products that are in turn involved in cellular functions such as cell growth, actin rearrangement and vesicular trafficking (51). To determine the importance of PI3 kinase signaling to *F. tularensis* invasion, lung epithelial cells were treated with wortmannin, an inhibitor of PI3 kinase activity, and invasion was assessed by gentamicin protection assay. Wortmannin decreased LVS invasion of TC-1 cells by 69.4% ( $\pm 4.6\%$ ) and 84.1% ( $\pm 1.8\%$ ) when cells were exposed to 100 and 200 nM concentrations, respectively (Figure 3.3A). Invasion by *Listeria monocytogenes*, which is PI3 kinase dependent (25), was significantly decreased. *Salmonella enterica* invasion, which is not PI3 kinase dependent (49), was not significantly decreased at these concentrations in TC-1 cells.

Cells monitor and respond to their external environment via receptors that lead to intracellular signaling events (53). Activation of receptor tyrosine kinases can lead to receptor internalization, as well as initiation of a cascade of downstream signaling events (53). Some organisms, such as *Yersinia pseudotuberculosis*, exploit signaling through host cell receptors to gain entry into cells, and this entry can be blocked by tyrosine kinase inhibitors (45). To determine the contribution of tyrosine kinase signaling to LVS invasion, the inhibitor genistein was added to lung epithelial cells and the number of intracellular organisms measured by gentamicin protection assay. Genistein decreased LVS invasion of TC-1 cells by 68.1% ( $\pm 1.1\%$ ) and 77.1% ( $\pm 1.1\%$ ) at 50 and 100  $\mu\text{M}$  concentrations, respectively (Figure 3.3B). Invasion by *Yersinia pseudotuberculosis* was significantly

reduced at these concentrations, while *Salmonella enterica* invasion, which is tyrosine kinase independent (45), was not significantly decreased in TC-1 cells.

Collectively, these results indicate that actin and microtubule rearrangement both contribute to the entry of *F. tularensis* into lung epithelial cells. PI3 kinase and tyrosine kinase, proteins which regulate cytoskeletal rearrangement, both impact *F. tularensis* invasion of lung epithelial cells.

### **LVS traffics along the endocytic pathway in lung epithelial cells**

To characterize the trafficking of *F. tularensis* LVS along the endocytic pathway in lung epithelial cells, we synchronized the addition of LVSgfp to TC-1 cells and evaluated these cells microscopically for the presence of bacteria inside vacuoles containing the early endosomal marker EEA1 or the late endosomal/lysosomal marker LAMP-1 (Figure 3.4A and 4.4B). LVS association with EEA1 containing phagosomes increased from 1.4% ( $\pm 2.4\%$ ) at 10 minutes to 40.4% ( $\pm 5.3\%$ ) at 30 minutes, before decreasing (Figure 3.4C). LVS association with LAMP-1 containing vacuoles peaked two hours post-inoculation at 49.3% ( $\pm 3.5\%$ ) (Figure 3.4C). These results indicate that LVS traffics along the endocytic pathway in lung epithelial cells, associating first with early endosomes before progressing to late endosomes/lysosomes.

### **LVS escapes the phagosome and replicates in the cytoplasm of lung epithelial cells**

Once inside host cells invasive bacteria either replicate within the endosome or escape the vacuole and replicate in the cytoplasm. To determine the intracellular location of bacteria, TC-1 cells were inoculated with LVS at an MOI of 100 and examined at one and 24 hours by transmission electron microscopy. At 1 hour post-inoculation LVS were TC-1 cells in a membrane-bound vacuole (Figure 3.5A). The membrane was easily visualized, and in some cases was beginning to degrade (Figure 3.5A). By 24 hours all observed intracellular bacteria were no longer contained within a visible membrane, and appeared to be free in the cytoplasm (Figure 3.5B), though they were surrounded by an electron lucent zone that has been noted by other researchers (8, 19).

To determine the kinetics of LVS escape from the endocytic pathway into the cytoplasm of lung epithelial cells, we differentially labeled extracellular, cytoplasmic and vacuolar LVS<sub>gfp</sub> and analyzed recovered bacteria by flow cytometry (modified from Checroun et al.). We first labeled bacteria that were extracellular and attached to TC-1 cells using Pacific Blue conjugated anti-*F. tularensis* LPS. We then permeabilized the cytoplasmic membrane of TC-1 cells using digitonin, allowing Alexa Fluor 647 conjugated anti-*F. tularensis* LPS to access cytoplasmic bacteria, but not those enclosed in intact vacuoles, which are digitonin impermeable. TC-1 cells were lysed and cell-associated bacteria recovered and analyzed by flow cytometry, gating on GFP to differentiate bacteria from residual TC-1 debris. The resulting three populations of bacteria were analyzed: extracellular (Pacific Blue, Alexa Fluor 647 and GFP), cytoplasmic (Alexa Fluor 647 and GFP), and vacuolar (GFP only) (Figure 3.6A and 3.6B). At 10 minutes post-inoculation 19.3% ( $\pm 8.1\%$ ) of intracellular bacteria were accessible to antibody delivered to the cytoplasm, and therefore considered cytoplasmic (Figure 3.6C). This number increased to 70.0% ( $\pm 5.5\%$ ) at 2 hours

post-inoculation, indicating that the majority of intracellular bacteria were no longer contained within intact endocytic vacuoles. It should be noted that once the vacuolar membrane begins to degrade bacteria are accessible to antibody. As a result, bacteria that were associated with LAMP-1 staining vacuoles at 2 hours post-inoculation (Figure 3.4C) would be identified as cytoplasmic by this assay if the vacuolar membrane was no longer intact. When TC-1 cells were treated with saponin, which permeabilizes both vacuolar and cytoplasmic membranes, greater than 99% of bacteria were accessible to antibody labeling (data not shown), demonstrating that a population of bacteria were protected from staining when only the cytoplasmic membrane was permeabilized. Microscopic examination of saponin treated TC-1 cells revealed that anti-*F. tularensis* antibody was able to access bacteria in EEA1 containing vacuoles, while antibody was excluded from these vacuoles in digitonin treated cells (data not shown). When TC-1 cells were inoculated with PFA killed bacteria >90% of organisms were vacuolar at 60 minutes post-inoculation, indicating that killed bacteria did not escape into the cytoplasm (data not shown). Thus, bacterial viability was required for escape from the epithelial cell endosome.

## DISCUSSION

The ability of many facultative intracellular bacterial pathogens to cause disease is dependent upon their ability to invade and replicate within various host cells (14, 22, 41). *F. tularensis* survival and replication within macrophages and dendritic cells has been well described (1, 5, 6, 8, 33). We previously demonstrated that *F. tularensis* LVS also localizes to and replicates within alveolar type II epithelial cells following inhalation of organisms in a

mouse model of infection (21), prompting us to investigate lung epithelial cell invasion by this organism.

Alveolar type II cells account for about 12% of the total cells, and 2% of the surface area, of the alveolar region in mice (50). Approximately 98% of the surface area, and 10% of the total cell number, is comprised of type I cells, which provide structure and are the site of gas exchange (50). Type II cells have a number of biological functions, including the production, secretion and recycling of surfactant, proliferation to produce additional type II cells as well as transdifferentiation into type I cells, maintenance of alveolar fluid balance, and production of antimicrobial and anti-inflammatory substances (29). Due to the close proximity between the blood and alveolar epithelial cells required for gas exchange and fluid balance, these cells are ideally located to provide a portal through which bacteria could disseminate to distal organs.

To characterize the interactions of *F. tularensis* with lung epithelial cells we investigated the initial interactions of LVS with the lung epithelial cell line TC-1. We determined that bacteria associated with and were internalized by lung epithelial cells within 10 minutes of inoculation, and that viable bacteria were not necessary for gaining entry into these cells. We previously found that the frequency with which *F. tularensis* invaded lung epithelial cell lines was low (21). The low invasion frequency may be due to the inherent inability of a transformed cell line grown in a non-polarized fashion to recapitulate the complex environment within the host lung. These cells may also intermittently express a relevant receptor or other process that facilitates *F. tularensis* invasion *in vivo*. A system using primary cells grown in a polarized manner may more realistically reproduce the environment in the lung (18). Given the quantity of cells required for studies, cell culture

lines provide a reasonable place to begin the investigation of the interaction of bacteria with epithelial cells, and have been described as a model for such interactions (28).

Pathogens have developed numerous means of exploiting host cell functions to gain entry into nonphagocytic cells (10, 15). These invasion strategies are typically classified as either a zipper mechanism, which is utilized by pathogens such as *Yersinia pseudotuberculosis* and *Listeria monocytogenes* or a trigger mechanism, which is utilized by organisms such as *Salmonella typhimurium* and *Shigella flexneri*. The zipper mechanism is characterized by bacterial surface proteins binding to host cell receptors leading to internalization. The trigger mechanism is characterized by the injection of bacterial effector proteins into the host cell via a type III secretion system resulting in bacterial engulfment via induced macropinocytosis. Entry by either of these mechanisms requires the manipulation of host cell cytoskeletal components and signaling pathways. A number of host cell receptors play a role in *F. tularensis* entry into macrophages, including Fc $\gamma$ , CR3, MR and SRA receptors (3, 38, 47). However, very little is known about how this organism is able to access cells that are not considered to be professional phagocytes.

To better understand the process of *F. tularensis* entry into lung epithelial cells we investigated cytoskeletal components and signaling pathways that are involved in epithelial invasion by other bacterial pathogens for their contribution to internalization of LVS by a lung epithelial cell line. Lindemann et al. previously demonstrated that actin and microtubules are necessary for *F. tularensis* invasion the human epithelial cell line HEp-2 (28). We determined that both actin and microtubules contribute to *F. tularensis* invasion of lung epithelial cells, as do PI3 kinases and tyrosine kinases, both of which can control signaling events leading to cytoskeletal rearrangement. We showed that *F. tularensis*

internalization by lung epithelial cells is dependent upon a variety of host cell mechanisms, and that interruption of any of these mechanisms interferes with bacterial invasion.

We were unable to identify the characteristic membrane ruffling that is seen with *Salmonella* invasion either by phalloidin staining or Field Emission Scanning Electron Microscopy (FESEM) under conditions where we were able to clearly identify membrane ruffling with *Salmonella* (data not shown). While this points toward *F. tularensis* not causing massive actin reorganization at the site of entry it is possible that membrane ruffling occurs but was not detected by us. *Listeria monocytogenes*, another organism that is able to invade epithelial cells, also requires PI3 kinase and tyrosine kinase function for invasion, in a cell type dependent manner (39, 40). This organism has two invasion proteins, InlA and InlB, which interact with different host receptors, leading to different signaling events. It is possible that *F. tularensis* also uses multiple receptors for entry into lung epithelial cells, and that interruption of various signaling pathways could decrease uptake via various receptors. Toll-like receptors (TLRs) may play a role in modulation of the immune response to *F. tularensis* (9, 23, 54), and functional TLRs are present on AIIII cells (2). However, TLRs have not been implicated among macrophage receptors for *F. tularensis* internalization identified to date, which include Fc $\gamma$ , CR3, MR and SRA receptors (3, 38, 47). Melillo et al. have reported that *E. coli* expressing the *F. tularensis* surface protein FsaP were able to bind A549 cells (31). We have also demonstrated that killed *F. tularensis* was taken up by lung epithelial cells. This information, taken together with the absence of genes predicted to encode a type III secretion system, point toward the presence of a preformed ligand receptor interaction as with the zipper mechanism of uptake, rather than injection into the host cell of

effector proteins as with organisms that gain entry via the trigger mechanism. Further studies are needed to identify receptors necessary for uptake of *F. tularensis* by nonphagocytic cells.

To investigate *F. tularensis* trafficking along the endocytic pathway in lung epithelial cells we examined association of LVS with EEA1 and LAMP-1 containing vacuoles, as well as escape into the cytoplasm. *F. tularensis* traffics along the endocytic pathway in macrophages before escaping to the cytoplasm where replication occurs. The timing of this escape seems to be dependent upon the *Francisella* species and host cell type tested. *F. tularensis* subsp.*novicida* begins to disrupt the phagosomal membrane of quiescent human macrophages at 4 hours and is free in the cytoplasm by 12 hours post-infection (46). LVS and clinical isolates of *F. tularensis* associate with EEA1 then LAMP-1 containing vacuoles in mouse bone marrow derived macrophages, human macrophages, and mouse and human macrophage-like cell lines before degrading the phagosomal membrane and beginning to escape into the cytoplasm between one and two hours post-inoculation (7, 8, 19). We determined that LVS was initially associated with EEA1 containing vacuoles, then LAMP-1 containing vacuoles, before being found free in the cytoplasm of lung epithelial cells, in a manner and kinetic consistent with that seen in macrophages.

*F. tularensis* LVS is found in ATII cells in the lungs of C57BL/6 mice 1, 3 and 7 days post-intranasal inoculation, replicates in these cells as disease progresses, and by day 7 organisms are widespread in the alveolar epithelium (21). These observations demonstrate that that ATII cell invasion and replication is a part of the *F. tularensis* disease process. Understanding how this bacterium gains access to these cells, and just as importantly, how

they replicate and establish a stronghold in the lung epithelium, is necessary to understanding the progression of respiratory tularemia, as well as potentially providing insight into methods that may be used to block bacterial uptake or replication, and thus prevent disease.

### **ACKNOWLEDGEMENTS**

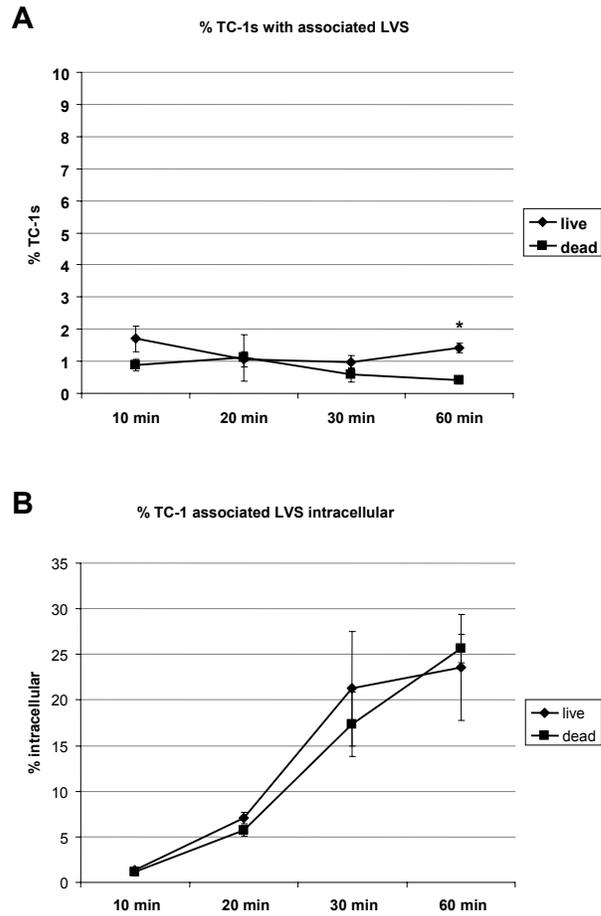
We gratefully acknowledge Victoria Madden of the Microscopy Services Laboratory and Larry Arnold of the Flow Cytometry Facility at UNC-CH for their expertise. We thank Richard Cheney and Jean Celli for helpful advice. We also thank Robert Fulcher and Todd Kijek for editing assistance and general advisement.

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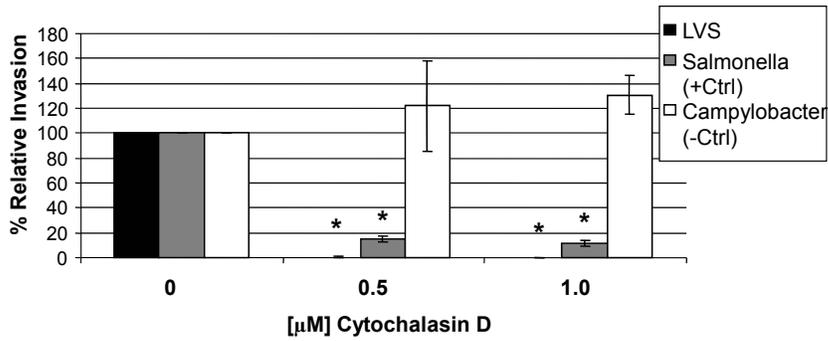
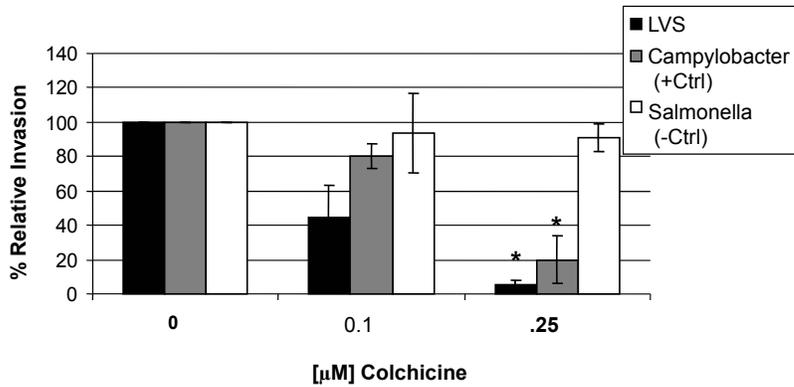
### **ATTRIBUTIONS**

All experiments described in this paper were conducted by Robin Craven, except for TEM, which was done by Joshua Hall. James Fuller and Joshua Hall provided assistance with Gentamicin protection assays. This work has been previously published, Copyright © American Society for Microbiology, *Infection and Immunity*, Volume 76, pages 2833-42, 2008. Permission has been granted to reprint this material.

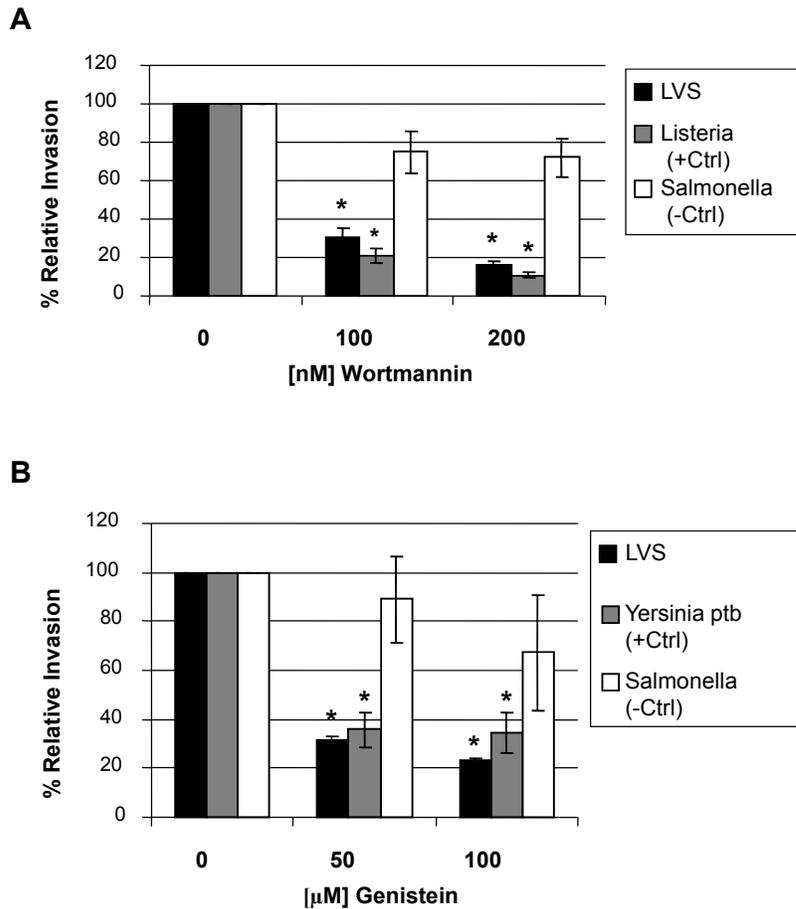
## FIGURES



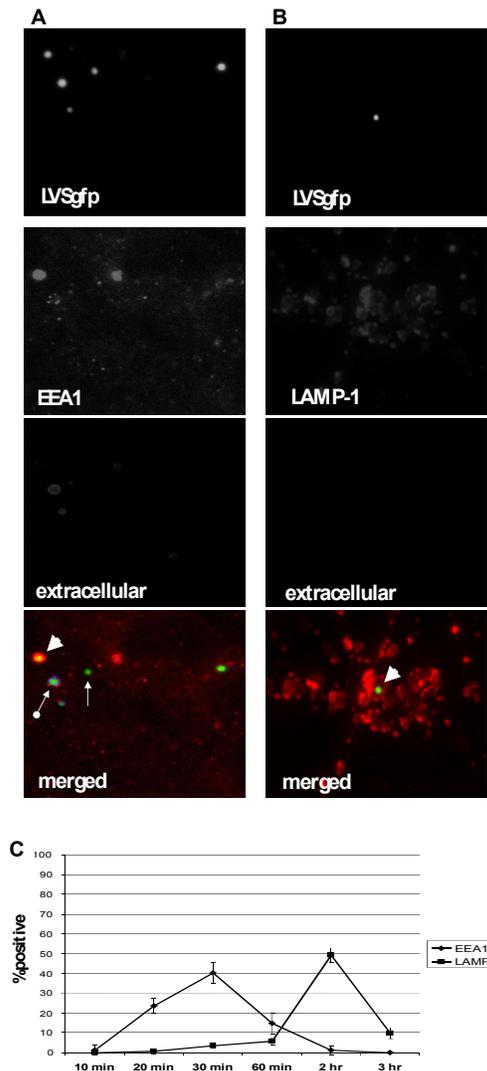
**Figure 3.1.** *F. tularensis* LVS association with and internalization by TC-1 lung epithelial cells. (A) Percent of TC-1 cells with associated live (◆) or PFA-fixed (■) LVS at designated times post-inoculation. \* Data are significantly different from untreated control,  $P < 0.01$  by paired two-tailed  $t$  test. (B) Percent of TC-1 cell-associated live (◆) or PFA-fixed (■) LVS that are intracellular at designated times post-inoculation.

**A****B**

**Figure 3.2.** The effect of actin and microtubule polymerization on *F. tularensis* LVS invasion of lung epithelial cells. TC-1 cells were treated with designated concentrations of (A) cytochalasin D or (B) colchicine. Results are expressed as the percentage of LVS, *Salmonella* or *Campylobacter* that survived gentamicin treatment, relative to the sample without inhibitor inoculated with the same organism (defined as 100% invasion). \* Data are significantly different from untreated control,  $P < 0.01$  by paired two-tailed  $t$  test.

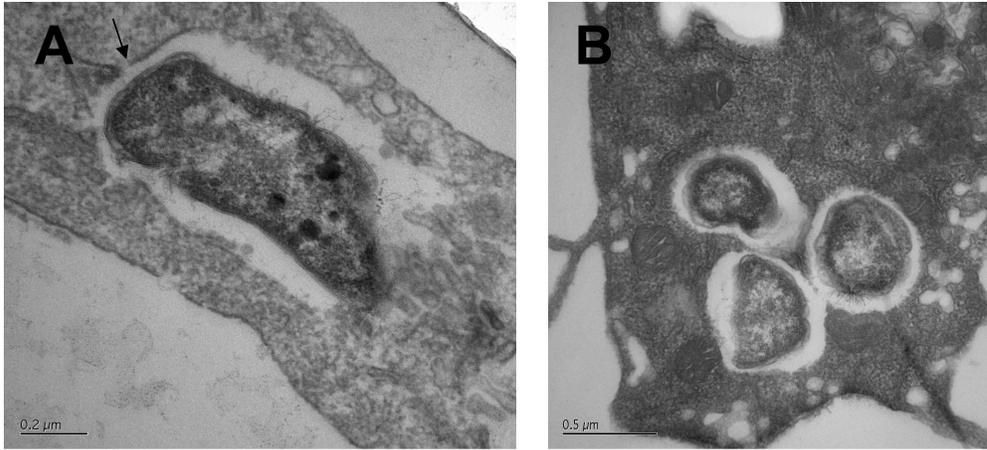


**Figure 3.3.** The effect of PI3 kinase and tyrosine kinase activity on LVS invasion of lung epithelial cells. (A) Wortmannin or (B) genistein were added to TC-1 cells at the indicated concentrations. Results are expressed as the percentage of LVS or control organism that survived gentamicin treatment, relative to the sample without inhibitor inoculated with the same organism (defined as 100% invasion). \* Data are significantly different from untreated control,  $P < 0.01$  by paired two-tailed  $t$  test

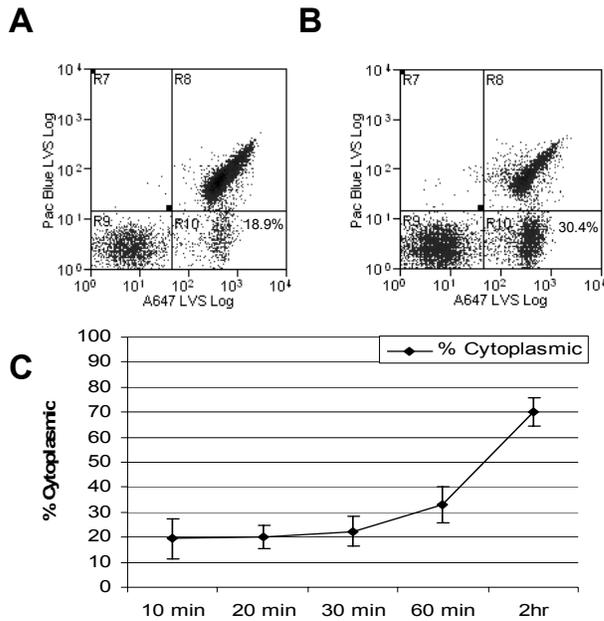


**Figure 3.4.** Representative fluorescence microscopy images demonstrating LVSgfp localization within EEA1 or LAMP-1-containing endosomes in TC-1 cells. Images depict (A) TC-1 cells stained for EEA1 30 minutes post-inoculation with LVSgfp or (B) 2 hours post-inoculation stained for LAMP-1. Single color images: LVSgfp images depict bacteria alone, EEA1 and LAMP-1 images depict staining for EEA1 or LAMP-1 only, and extracellular images depict staining of extracellular bacteria (no extracellular bacteria were present in B). The merged color images depict LVSgfp (green), vacuoles labeled with anti-EEA1 antibody (N19)(red) or anti-LAMP-1 (1D4B) (red), or extracellular LVS labeled with anti-*F. tularensis* LPS antibody conjugated to Pacific Blue (blue). Extracellular LVSgfp (arrow with round end), intracellular but not EEA1 associated LVSgfp (small arrow), and LVS associated with EEA1 containing vacuoles (large arrowhead). (C) Trafficking of LVSgfp in TC-1 cells. 100 intracellular bacteria were counted for each condition and scored for association with

EEA1 (◆) or LAMP-1 (▪) containing vacuoles. Three replicates were examined for each time point and condition.



**Figure 3.5.** Transmission electron micrographs of TC-1 infected with *F. tularensis* LVS. (A) One hour post-inoculation TC-1 cells showing LVS in a membrane bound vacuole that in some cases appeared to be degrading (arrow). (B) 24 hours post-inoculation LVS were free in the cytoplasm.



**Figure 3.6.** LVSgfp was analyzed by flow cytometry for escape from vacuoles into the cytoplasm of TC-1 cells. Extracellular bacteria were labeled with anti-*F. tularensis* LPS antibody conjugated to Pacific Blue (region R8). Cytoplasmic bacteria were identified by labeling with anti-*F. tularensis* LPS conjugated to Alexa Fluor 647 after digitonin permeabilization of the cytoplasmic membrane (region R10). Vacuolar bacteria were inaccessible to antibody, and therefore GFP positive only (region R9). Representative flow cytometry data of bacteria recovered (A) 10 minutes or (B) 60 minutes post-inoculation. The value shown in region R10 represents the percent of intracellular bacteria that are cytoplasmic. (C) Percent of intracellular bacteria present in the cytoplasm at designated times post-inoculation

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## Chapter 4

### **Use of transposon-transposase complexes to create stable insertion mutant strains of *Francisella tularensis* LVS**

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#### **ABSTRACT**

*Francisella tularensis* is a highly virulent zoonotic bacterial pathogen capable of infecting numerous different mammalian species, including humans. Elucidation of the pathogenic mechanisms of *F. tularensis* has been hampered by a lack of tools to genetically manipulate this organism. Herein we describe the use of transposome complexes to create insertion mutations in the chromosome of the *F. tularensis* live vaccine strain (LVS). A Tn5-derived transposon encoding kanamycin resistance and lacking a transposase gene was complexed with transposase enzyme and transformed directly into *F. tularensis* LVS by electroporation. An insertion frequency of  $2.6 \times 10^{-8} \pm 0.87 \times 10^{-8}$  per cell was consistently achieved using this method. There are 178 described Tn5 consensus target sites distributed throughout the *F. tularensis* genome. Twenty-two of 26 transposon insertions analyzed were within known or predicted open reading frames, but none of these insertions was associated with the Tn5 target site. Analysis of the insertions of sequentially passed strains indicated that the transposons were maintained stably at the initial insertion site after more than 270

generations. Therefore, transformation by electroporation of Tn5-based transposon-transposase complexes provided an efficient mechanism for generating random, stable chromosomal insertion mutations in *F. tularensis*.

## INTRODUCTION

*Francisella tularensis* is a gram-negative bacterial pathogen and is the etiologic agent of tularemia. The manifestations of tularemia depend on the initial route of inoculation, but all modes of contact can result in sepsis and disseminated disease, with organisms found in the liver, spleen, lymph nodes, kidney, and lungs (6, 20, 24, 25). Skin contact results in ulcer formation at the site of inoculation, where the organisms multiply and spread to the draining lymph nodes. Inhalation of *F. tularensis* leads to bronchial hemorrhaging, mediastinal lymphadenopathy (27), and pneumonia without a corresponding productive cough. Ingestion of the organisms can result in oropharyngeal tularemia, where patients typically develop exudative ulcerative pharyngitis and pharyngeal lymphadenopathy (1, 4).

*F. tularensis* strains are divided into two groups, A and B, which are distinguished by acid production from glycerol and by citrulline ureidase activity. The two groups of organisms exhibit similar pathogenesis; however, group A strains are considered to be highly virulent for humans and other animals, whereas group B strains typically cause milder disease (28). A live attenuated vaccine strain (LVS) derived from a group B *F. tularensis* strain has been developed and used to vaccinate laboratory workers (7). This vaccine provides significant protection against the highly virulent strains initiated by skin contact (21) and inhalation (13, 22). However, the basis for attenuation of this strain in humans is not known, and it has not been licensed for general public use in the United States.

*F. tularensis* can survive within macrophages, a feature that is thought to be important in the pathogenesis of this organism (5, 12, 17, 26). *F. tularensis* subsp. *novicida*, a related organism historically referred to as *Francisella novicida*, also survives within macrophages, but it is an animal pathogen that does not infect humans. Two different genetic loci, termed *mglAB* for macrophage growth locus (2) and *iglABCD* for intracellular growth locus (11), have been identified in *F. tularensis* subsp. *novicida* that contribute to its survival in macrophages. The function of the *igl* gene products in promoting this survival has not been determined. MglA of *F. tularensis* subsp. *novicida* has recently been shown to function as a positive regulator for at least seven different genes (16) that are normally induced in macrophages, including *iglC*. An *F. tularensis* subsp. *novicida* mutant lacking *mglA* is unable to survive in cultured macrophages and is attenuated in mice (16). Homologues of these genes are present in both group A and B *F. tularensis* strains, and they presumably serve similar functions in these organisms.

Apart from the described macrophage survival phenotype and the identification of a limited number of genetic loci that contribute to intracellular survival (11), very little is known about the molecular mechanisms that support *F. tularensis* pathogenesis and virulence. The lack of tools for the genetic manipulation of *F. tularensis* has made it difficult to identify and dissect the bacterial products and processes that contribute to this organism's extraordinary virulence and pathogenesis. Mechanisms of transformation and allelic exchange have only recently been described (3, 8, 15), and most of these procedures have been developed in *F. tularensis* subsp. *novicida*. Lauriano et al. (15) recently described the use of allelic exchange to generate targeted insertion mutations in *F. tularensis* LVS. They also reported that Tn10- and Tn1721-based transposon insertions were unstable in *F.*

*tularensis* and that bacterial gene-encoded transposase-complementing activity may function to promote the movement of these transposons. Herein we describe a procedure that uses a Tn5 derivative to create insertion mutations in *F. tularensis* LVS which, unlike Tn10 and Tn1721, were stably maintained at the initial insertion site.

## MATERIALS AND METHODS

*F. tularensis* LVS was obtained from the Centers for Disease Control and Prevention, Atlanta, Ga., and were propagated at 37°C on chocolate medium supplemented with 1% IsoVitalex (BBL). Multiple colonies from an overnight culture of LVS grown on chocolate agar were picked and swabbed onto fresh plates to achieve confluent growth, and they were then incubated for 16 h. Cells from one plate of freshly confluent LVS were suspended in 6 ml of wash buffer, consisting of 0.5 M sucrose and 10% glycerol, and then were centrifuged at 16,000 x g for 3 min. The pellet was suspended in wash buffer and centrifuged again for a total of four washes. The pellet resulting from the final centrifugation was suspended in wash buffer to a total volume of 100 µl.

A procedure initially described by Goryshin et al. (9) to create mutations in *Salmonella*, *Proteus*, and *Escherichia* species was used to produce similar transposon insertion mutations in *F. tularensis* LVS. One microliter of EZ::TN <kan-2> transposome complex (Epicentre) containing 0.1 pmol of transposon and 1 U of transposase was added to 100 µl of the washed cell suspension, consisting of 10<sup>9</sup> to 10<sup>10</sup> cells in 0.5 M sucrose and 10% glycerol. The contents were then mixed and transferred to a 0.1-cm-gap electroporation cuvette.

The EZ::TN <kan-2> transposome contains a derivative of Tn5 that lacks a transposase gene and has the transposase enzyme bound to the inverted repeat ends of the transposon. The transposase is stably associated with the transposon but is inactive in the absence of Mg<sup>2+</sup>. Magnesium ions present inside the bacterium activate the transposase following transformation, facilitating transposition into the *Francisella* chromosome. Thus, transposition is dependent simply upon activation of the enzyme and not the expression of a foreign transposase gene.

The transposome was introduced into *F. tularensis* LVS by electroporation using a Bio-Rad Gene Pulsar set at 2.5 kV, 25 µF, and 200 Ω. Immediately following electroporation the cells were suspended in 1 ml of brain heart infusion broth (BBL) supplemented with 50 µg of hemin/ml, incubated for 1 h at 37°C, and then plated on chocolate agar containing 10 µg of kanamycin/ml.

## RESULTS

We consistently achieved an insertion frequency of  $2.6 \times 10^{-8} \pm 0.87 \times 10^{-8}$  (Table 4.1), as determined by the number of antibiotic-resistant colonies divided by the total number of potential recipient organisms. The insertion frequency was not appreciably affected by the number of bacteria, nor was it improved by increasing the concentration of transposome complexes. Recovering the organisms with cold or prewarmed media following electroporation as described for other organisms (23) also did not alter the frequency with which we isolated antibiotic-resistant organisms.

Ideally a transposon will insert randomly throughout a genome in order to be a useful tool for creating insertion mutation libraries. The transposon insertion sites were mapped by directly sequencing the transposon-chromosome junctions by using oligonucleotide primers that hybridize within the transposon. Primers Kan-2 FP-1 (ACCTACAACAAAGCTCTCATCAACC) and JF119 (GGATCAGATCACGCATCTTC) hybridize 70 and 150 bp, respectively, from the end of the transposon adjacent to the 3' end of the Kan resistance gene, and primer KAN RP-1 (GCAATGTAACATCAGAGATTTTGAG) hybridizes 43 bp from the other end of the transposon. Individual kanamycin-resistant colonies were picked and restreaked on selective media. Chromosomal DNA was prepared from strains by using the MasterPure DNA purification kit according to the manufacturer's instructions (Epicentre).

The precise transposon-chromosome junction sites were determined in a total of 26 kanamycin-resistant colonies from each of three different transformations. One microgram of chromosomal DNA was mixed with 100 pM primer, and DNA sequence was generated by the University of North Carolina Genome Analysis Center. The transposon insertion site was determined by aligning the sequence to the *F. tularensis* LVS strain genome database produced by the Biology and Biotechnology Research Program Sequencing Group at Lawrence Livermore National Laboratory (<http://bbrp.llnl.gov/bbrp/html/microbe.html>).

Tn5, the transposon upon which EZ::TN is based, has a reported insertion bias for the sequence A-GNTYWRANC-T (where W is A or T, R is A or G, Y is C or T, and N is any base) (10). The *F. tularensis* 1.8-Mbp LVS genome has 178 of these insertion bias sequences, but none of the 26 insertions that we analyzed occurred within one of these sites. Of the 26

junction sites sequenced, 21 were within potential open reading frames and 1 was in the transcription termination sequence of an open reading frame, which is consistent with the observation that, apart from the Tn5 insertion bias site, this transposon has a propensity for inserting into actively transcribed DNA and regions of high superhelical density (18, 19). One insertion, B5, was in a gene encoding the RNA polymerase  $\beta$  subunit; this is an essential gene. The insertion is located 12 bases from the 3' end of the gene. *F. tularensis* LVS does not possess a second copy of this gene; thus, it is likely that the B5 mutant still produces a functional RNA polymerase  $\beta$  subunit. Ironically, one of the insertions (strain A10; Table 4.2 and Fig. 4.1) was within a gene reported to encode a *Francisella* transposase (14). The insertions did not appear to cluster within a specific chromosomal segment (Fig. 4.1).

Insertions in potential reading frames were examined further by translating the sequence and blasting the protein database to tentatively identify the products of interrupted genes. The majority, but not all, of these mutations occurred in genes that encoded proteins with significant homology to proteins with known functions or conserved hypothetical proteins (Table 2).

Lauriano et al. reported that insertion mutations created by derivatives of the Tn10 and Tn1721 transposons in the closely related organism *F. tularensis* subsp. *novicida* were unstable and subject to movement to other chromosomal locations, possibly through the activity of a host-encoded transposase (15). We picked five transposon insertion mutation strains representing insertions within and outside of potential reading frames, including the strain with the insertion in the reported *Francisella* transposase gene. These strains were passed daily for 10 days on kanamycin-containing media, and chromosomal DNA was

prepared from cells on passages 0, 5, and 10. Southern blots were performed by digesting 5  $\mu\text{g}$  of chromosomal DNA with EcoRI, which does not cleave the transposon. Digested DNA segments were separated by agarose gel electrophoresis, transferred to nylon, and probed with digoxigenin-labeled Tn5 probe. The Tn5 probe hybridized to identically sized fragments in each of the DNA samples prepared from different passages of the same insertion strain (Fig. 4.2). Given that a single colony is composed of at least  $10^8$  organisms, a single passage equates to at least 27 generations of growth. Thus, after 10 passages the analyzed insertions were stable for a minimum of 270 generations.

Electroporation of a Tn5-derived transposon-transposase enzyme complex into *F. tularensis* LVS resulted in the creation of random insertion mutant strains at a frequency that is sufficient to generate mutant libraries of this organism. These insertions are genetically stable and are apparently unaffected by the activities of any potential chromosomally encoded transposases. Thus, this scheme will facilitate the use of genetic approaches to study the mechanisms of *F. tularensis* physiology and pathogenesis.

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## ATTRIBUTIONS

Josh Hall and I each performed replicates of the transposon mutagenesis reactions used to calculate insertion frequencies. I performed experiments optimizing the transposition efficiency. Josh Hall isolated and characterized the 27 mutants described in the chapter. Julie Clark performed Southern blot analysis for 5 of the mutants under the direction of Robin Craven. This work was previously published, Copyright ©American Society for Microbiology, Applied and Environmental Microbiology, Volume 70, pages 6901-6904, 2004. Permission has been granted to reprint this material.

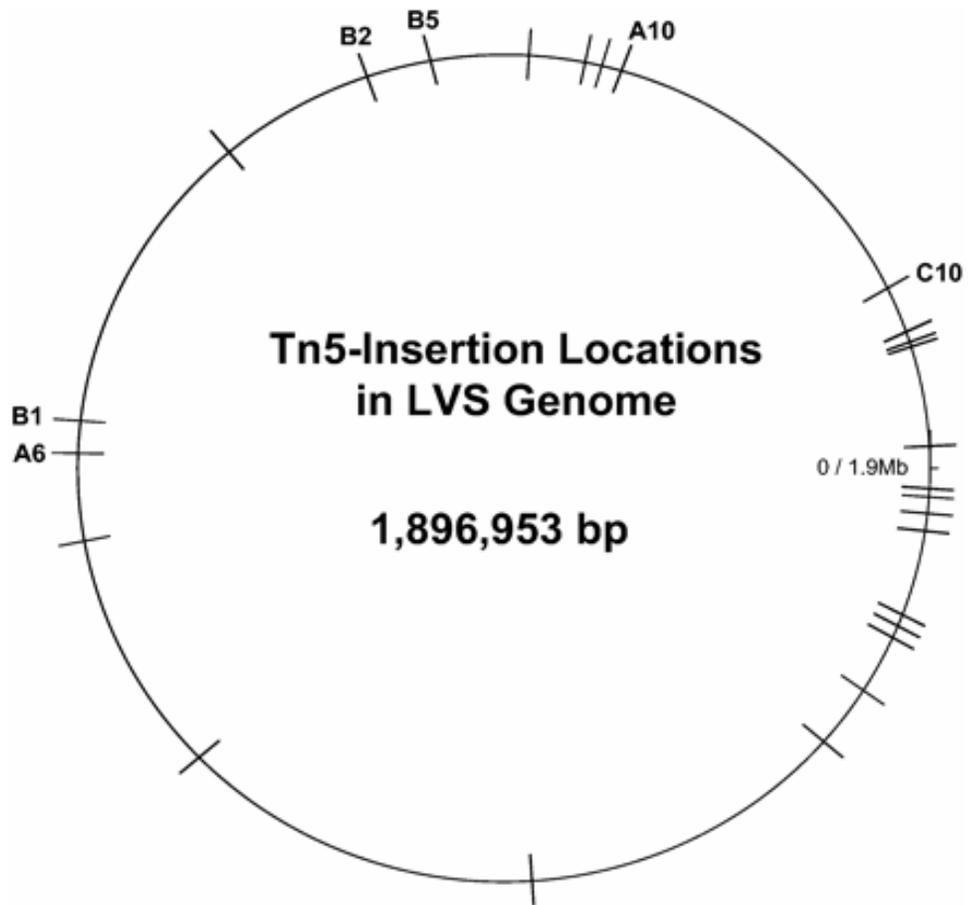
## TABLES AND FIGURES

**Table 4.1. Transposon insertion frequencies**

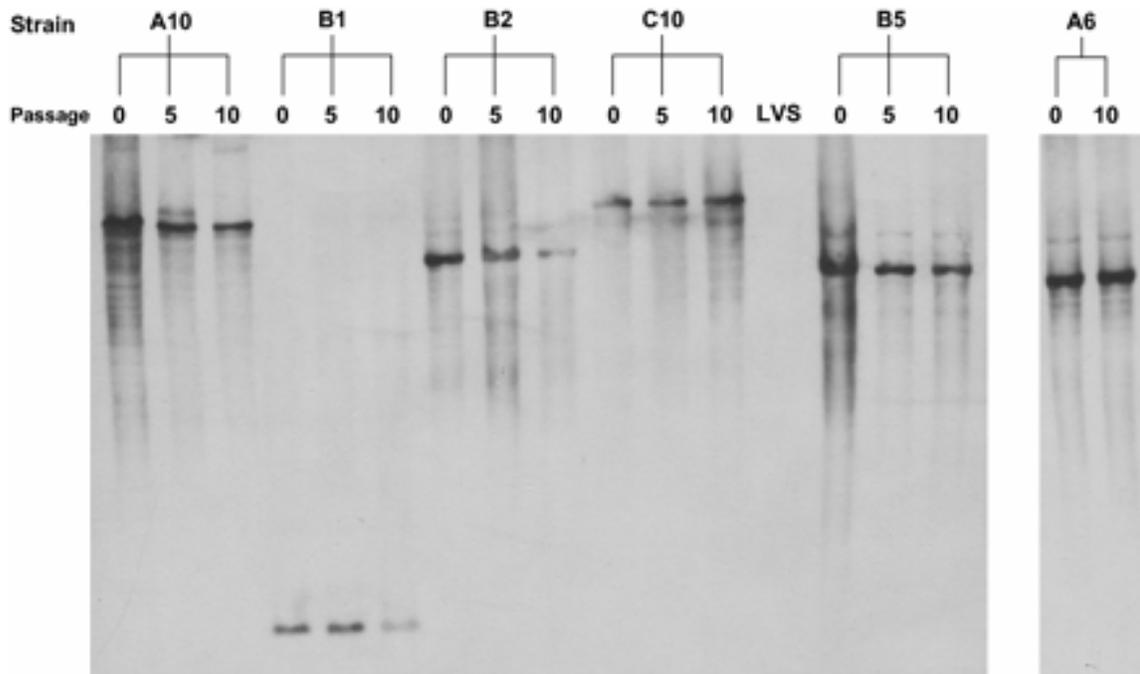
Input (CFU)	No. of mutants	Mutants/CFU
$2.8 \times 10^{10}$	344	$1.0 \times 10^{-8}$
$4.9 \times 10^9$	62	$1.2 \times 10^{-8}$
$1.1 \times 10^9$	50	$4.5 \times 10^{-8}$
$2.6 \times 10^9$	207	$7.0 \times 10^{-8}$
$5.7 \times 10^{10}$	1,097	$1.9 \times 10^{-8}$
$2.3 \times 10^{10}$	550	$1.0 \times 10^{-8}$
$1.4 \times 10^{10}$	210	$1.5 \times 10^{-8}$

**Table 4.2. Identified transposon insertion sites**

Strain designation	Predicted insertion
A1	Extragenic
A2	Deacyclase
A3	Guanosine polyphosphate pyrophosphohydrolases
A4	Hydrolase of HD superfamily
A5	Metal-dependent hydrolase
A6	Extragenic
A8	Hypothetical protein gi34496297
A9	Extragenic
A10	<i>Francisella</i> transposase (14)
B1	ATPase
B2	Extragenic
B4	Eflux pump protein
B5	RNA polymerase $\beta$ subunit
B6	Transcription terminator
B7	Predicted membrane protein of unknown function, pfam03956
B8	Phosphate acetyltransferase
B9	Adenylosuccinate synthase
B10	UsoAp of <i>Emericella nidulans</i>
C1	Pyruvate phosphate dikinase
C2	Methyltransferase
C3	Isocitrate dehydrogenase
C4	Chloride channel protein EriC
C6	Anthranilate synthase
C7	Tryptophan synthase alpha chain
C8	Multidrug resistance protein
C10	Integral membrane protein



**Figure 4.1. Graphic representation of the chromosomal positions of the 27 identified insertion sites.** The insertions assessed for stability and depicted in Fig. 4.2 are labeled.



**Figure 4.2. Southern blot of five insertion mutants (A6, A10, B1, B2, B5, and C10) and wild-type *F. tularensis* LVS probed with labeled Tn5.** Chromosomal DNA was prepared from mutants after 0, 5, and 10 passages, digested with EcoRI, and probed. All insertions analyzed in this manner retained the transposon at the initial insertion site.

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## Chapter 5

### **RipA, a Cytoplasmic Membrane Protein Conserved Among *Francisella* Species is Required for Intracellular Survival**

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#### **ABSTRACT**

*Francisella tularensis* is a highly virulent bacterial pathogen that invades and replicates within numerous host cell types including macrophages and epithelial cells. In an effort to better understand this process we screened a transposon insertion library of *F. tularensis* LVS for mutant strains that invaded but failed to replicate within alveolar epithelial cell lines. One such strain isolated from this screen contained an insertion in the gene *FTL\_1914*, which is conserved among all sequenced *Francisella* species, yet lacks significant homology to any gene with known function. A deletion strain lacking *FTL\_1914* was constructed. This strain did not replicate in either epithelial or macrophage – like cells, and intracellular replication was restored by the wild type allele *in trans*. Based on the deletion mutant phenotype *FTL\_1914* was termed *ripA* (Required for Intracellular Proliferation, Factor A). Following uptake by J774.A1 cells, *F. tularensis* LVS  $\Delta ripA$  co-localized with LAMP-1 then escaped the phagosome at the same rate and frequency as wild type infected cells. Electron micrographs of *F. tularensis* LVS  $\Delta ripA$  demonstrated reentry of the mutant bacteria into double membrane vacuoles characteristic of autophagosomes in a

process that was not dependent on replication. *F. tularensis* LVS  $\Delta ripA$  was significantly impaired in its ability to persist in the lung and in its capacity to disseminate and colonize the liver and spleen in a mouse model of pulmonary tularemia. The RipA protein was expressed during growth in laboratory media, and localized to the cytoplasmic membrane. Thus, RipA is a cytoplasmic membrane protein conserved among *Francisella* species that is required for intracellular replication within the host cell cytoplasm, as well as disease progression, dissemination and virulence.

## INTRODUCTION

*Francisella tularensis* is a highly virulent zoonotic pathogen that is the etiologic agent of the disease tularemia. *Francisella* species have been isolated from over 250 animal species including mice, rabbits, and squirrels (5). Transmission to humans from infected animals occurs through arthropod bites (41), physical contact with infected animal tissues (10), consumption of contaminated water (9, 50), or inhalation of aerosolized organisms (19). Transmission by inhalation results in the most aggressive form of tularemia where as few as 10 colony forming units (CFU) can lead to a disease (55) that can rapidly progress (62) and result in mortality rates as high as 60% if untreated (17). Individual cases of tularemia occur throughout the Northern hemisphere (35) with clusters of outbreaks occurring in Scandinavia (63), Europe (28, 33), and the American Midwest (56). Reported cases of tularemia peaked in the United States during the 1930's and 1940's, but have since declined with only 1368 cases between 1990 and 2000 (11).

*F. tularensis* strains infectious for humans are subdivided into two groups, Type A (*Francisella tularensis tularensis*) and Type B (*Francisella tularensis holarctica*). Type A

is characterized by high virulence for humans and geographic localization to North America; although recently Type A was isolated in Europe as well (29). Type B is distributed throughout the Northern hemisphere and infections with this strain are associated lower mortality than Type A. A Type B *F. tularensis* strain was used to create an attenuated live vaccine strain (LVS) for use in the Soviet Union (59); however, this vaccine is not universally protective nor is it licensed for use in the United States. *F. tularensis* LVS is attenuated in humans, but remains pathogenic for mice and other rodents, making it an excellent model for laboratory studies on *Francisella* pathogenesis (21).

*F. tularensis* is a facultative intracellular pathogen. The ability to replicate in various cell types such as alveolar macrophages (3, 45, 48, 58), dendritic cells (8), and lung alveolar Type II epithelial cells (22, 31) are all considered to be important in the pathogenesis of *Francisella*. Virulence factors such as *mglA* (4), *iglA* (16), *iglC* (38, 39, 54), *tolC* (24), *clpB* (42), Type IV pili (20, 23, 30), and a recently identified 58 kDa protein (60) have all been characterized using macrophage and mouse models of infection. Yet the actual function of many of these proteins remains to be determined. We screened a transposon insertion library to identify additional genes that contribute to the intracellular growth of *F. tularensis*. Herein we describe one such gene that is required for intracellular growth and virulence in a mouse model of tularemia.

## MATERIALS AND METHODS

**Bacterial strains and Cell culture.** *Escherichia coli* TOP10 and M15 strains were used for cloning and expression of 6HIS fusion proteins, respectively. *E. coli* cultures were propagated in Luria's broth supplemented with ampicillin at 100µg/ml or kanamycin at

20µg/ml as necessary for maintaining antibiotic selection. *Francisella tularensis* Live Vaccine Strain (LVS) was obtained from the Centers for Disease Control and Prevention, Atlanta, Ga. *F. tularensis* LVS was propagated on chocolate agar (25g/l BHI, 50µg/ml hemoglobin, 15g/l agarose) supplemented with 1% IsoVitalex (Becton-Dickson), complete BHI broth (35g/l BHI, 50µg/ml hemin, 1% IsoVitalex), or Chamberlains Defined Media (12). All bacterial strains cultured on chocolate agar were grown at 37°C. Broth cultures were incubated in a shaking water bath at 37 °C.

TC-1 (ATCC CRL-2785) is a tumor cell line derived from mouse primary lung epithelial cells that was cultured in RPMI 1640 supplemented with 2mM L-glutamine, 1.5g/liter sodium bicarbonate, 10mM HEPES, 1.0mM sodium pyruvate, 0.1mM nonessential amino acids, and 10% fetal bovine serum. J774A.1 (ATCC TIB-67) is a reticulum cell sarcoma mouse macrophage-like cell line that was cultured in D-MEM with 4.5g/l glucose, 2mM L-glutamine, and 10% fetal bovine serum. Cell lines were cultured at 37 °C and 5% CO<sub>2</sub> atmosphere.

**Plasmids and Molecular Techniques.** Cloning of *F. tularensis* LVS DNA was conducted by PCR amplification of genomic DNA using Pfu turbo DNA polymerase (Stratagene) and cloned into pCR Blunt-II TOPO vector (Invitrogen) using the manufacturer's protocols. Plasmids for complementation experiments were created by ligating cloned regions of the *F. tularensis* LVS genome into pKK MCS, a pKK214 GFP (1) derivative where a *gfp tet<sup>R</sup>* fragment was removed and replaced with a fragment containing a multiple cloning site (MCS) and a *kan<sup>R</sup>* allele. Kanamycin selection was maintained at 20 µg/ml for *E. coli* TOP10, and 10 µg/ml for *F. tularensis* LVS. The kanamycin resistance gene was a *F.*

*tularensis* codon optimized version of *aphA1* synthesized by Blue Heron Biotechnology that was expressed from a modified *F. tularensis groEL* promoter (18).

**Gentamicin Protection Assay.** To determine the rate of intracellular invasion and replication, *F. tularensis* LVS strains were cultured to mid-exponential phase in Chamberlains Defined Media and then added to J774A.1 or TC-1 monolayers at a multiplicity of infection (MOI) of 100 in 200 $\mu$ l prewarmed tissue culture media. Gentamicin protection assays were then conducted as described previously (31). Assays were done in triplicate and statistical significance was determined using unpaired t tests with unequal variance on the log transforms of recovered CFU to compare mutant strains to the wild type.

**Mutagenesis and Allelic Exchange.** A *ripA* deletion construct was made by Splice Overlap Extension (SOE) PCR (34) using primers designed to delete the *ripA* locus and while maintaining 1kb flanking regions. All *ripA* sequence between the start and stop codons was deleted while leaving the start and stop codons intact. The construct was sequenced to confirm the deletion was in frame and to ensure the integrity of the flanking DNA sequence. A *BamHI-NotI* fragment containing the deleted allele was ligated into pMP590 *kan<sup>R</sup>sacB* for allelic exchange (40). Kanamycin (10 $\mu$ g/ml) was used to select for plasmid integration and 10% sucrose to counterselect for resolution as described by LoVullo et al. (40).

**Invasion and Vacuolar Escape Assay.** Intracellular bacteria accessible to cytoplasmically delivered antibodies were enumerated by the methods described previously (15). *F. tularensis* LVS  $\Delta$ *ripA* accessibility to the cytoplasm was compared to wild type at 20

minutes, 60 minutes, and 180 minutes post invasion in J774A.1 cells in triplicate assays per time point. Bacteria accessible to the cytoplasm were described as cytoplasmic and bacteria inaccessible to the cytoplasm as vacuolar.

**Fluorescence microscopy.** J774A.1 cells were cultured in 8 well chamber slides (Nunc). *F. tularensis* LVS pKK214 gfp and *F. tularensis* LVS  $\Delta ripA$  pKK214 gfp were prepared for inoculation as done for Gentamicin protection assays. Invasion assay synchronization, epifluorescent microscopy, and LAMP1 co-localization quantitation was done as in Craven et. el. (15) at the described times post inoculation in J774A.1 mouse macrophage-like cells. Three independent assays were conducted for quantitation with multiple captures at each time point examined. LVS GFP positive objects at 24 hours contained multiple bacteria and were scored as a group. Three independent counts of over 100 GFP positive objects were scored as positive or negative for LAMP-1 co-localization. The mean percent co-localization  $\pm$  the standard deviations were recorded and mutant values compared to wild type with an unpaired two-tailed t test.

**Electron microscopy.** Cell monolayers grown on polystyrene plates were rinsed with PBS and fixed in 3% glutaraldehyde/0.15M sodium phosphate, pH 7.4. Following three rinses with sodium phosphate buffer, the monolayers were post-fixed for 1 hour in 1% osmium tetroxide/1.25% potassium ferrocyanide/0.15M sodium phosphate buffer, rinsed in deionized water, dehydrated using increasing concentrations of ethanol (30%, 50%, 75%, 100%, 100%, 10 minutes each) and embedded in Polybed 812 epoxy resin (Polysciences, Inc., Warrington, PA). The embedded samples were sectioned parallel and perpendicular to the substrate at

70nm using a diamond knife. Ultrathin sections were collected on 200 mesh copper grids and stained with 4% aqueous uranyl acetate for 15 minutes, followed by Reynolds' lead citrate for 7 minutes. Sections were observed using a LEO EM910 transmission electron microscope at 80kV (LEO Electron Microscopy, Thornwood, NY) and photographed using a Gatan Bioscan Digital Camera (Gatan, Inc., Pleasanton, CA).

**Mouse Model of Pulmonary Infection.** *Francisella* strains were prepared for intranasal inoculation by culturing in Chamberlains Defined Media to mid-exponential phase prior to suspension in PBS. Anesthetized 6- to 8-week-old female C57BL/6 mice were inoculated intranasally with  $10^5$  CFU of *F. tularensis* LVS suspended in 50  $\mu$ l of PBS. Inoculations were conducted in groups of four mice per strain for each time point. Organ burdens were evaluated by homogenization and serial dilution before plating on Chocolate agar to determine recovered CFU per organ. Statistical analysis was conducted on log transforms of organ burdens using independent unpaired t-tests to compare mutant strains to wild type.

**Membrane Fractionation.** *F. tularensis* LVS was cultured to mid-exponential phase in Chamberlains Defined Media, pelleted by centrifugation, and resuspended in lysis buffer (10mM Tris pH 7.5, 150mM NaCl). Lysozyme was added to 0.1mg/ml and the bacterial suspension incubated on ice for 30 minutes. A 1:1 cell suspension with 0.1mm silica beads was beaten for 20 minutes in a Disruptor Genie (Scientific Industries). Cell lysates were collected after the beads settled and the beads were washed 3 times with lysis buffer. Cell lysates were clarified by centrifugation at 12,000 x g for 4 minutes. Crude membrane fractions were collected by ultracentrifugation at 100,000 x g for 90 minutes, and the

supernatants were saved as the cytosolic fractions. Crude membrane fractions were washed one time by re-suspension in lysis buffer and ultracentrifugation. Cytoplasmic membrane fractions were solubilized with lysis buffer and extracted with 10% sarkosyl (Sigma) added to a 0.2% final concentration. Outer membrane fractions were then pelleted by ultracentrifugation at 100,000 x g for 60 minutes (16, 44).

**Recombinant RipA and anti-RipA Antiserum Production.** The *F. tularensis* LVS *ripA* gene was cloned and sequenced to confirm the integrity of the DNA sequence. The *ripA* allele was ligated into a pQE30 (Qiagen) vector to construct an N terminal 6x His tag fusion. The *ripA::6His* allele was induced using 0.1mM IPTG for 4 hours and cells were harvested by centrifugation. The cell pellet was resuspended (40 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1M NaCl) and lysozyme treated at 1 mg/ml for 30 minutes before sonication. The lysate was clarified by centrifugation. The crude membrane fraction was pelleted by ultracentrifugation at 115,000 x g for 60 minutes. RipA::6His was purified from the crude membrane fraction using a Qiagen native purification protocol with buffers containing 1% Triton X-100 and 10mM  $\beta$ -mercaptoethanol. The purified RipA::6His was analyzed for purity by SDS-PAGE and Coomassie staining. The purified protein was methanol chloroform precipitated (61) and resuspended in PBS. Purified RipA::6His was sent to Proteintech Group, Inc for the production of Rabbit antiserum by the standard Proteintech long protocol. Preimmune bleeds were collected from each rabbit used in the protocol. Antiserum was screened for reactivity and specificity by Western blotting of the purified protein.

**SDS-PAGE and Western Analysis.** SDS-PAGE was conducted by the methods of Laemmli et al (36) utilizing a denaturing discontinuous 12.5% acrylamide gel. Total protein loaded in each sample was equivalent as determined by a BCA assay (Pierce). Proteins were transferred to nitrocellulose and blocked in 5% BSA blocking buffer (0.05% Tween 20, PBS) overnight. Antibody incubations were conducted in 0.5% BSA Wash buffer (0.05% Tween 20, PBS). Membranes were washed in 0.05% Tween 20 PBS. Primary incubations and secondary antibody incubations were for 60 minutes. Rabbit anti-RipA serum was used at 1:1000, Chicken anti-AtpB IgY at 1:5000 (AgriSera), Mouse anti-GFP IgG (Abcam) at 1:1000, Goat anti-rabbit IgG-HRP at 1:10,000 (Sigma), and Goat anti-Chicken IgG HRP at 1:10,000 (Sigma). Development was conducted using Pierce SuperSignal Pico substrate as the manufacturer's protocols and exposed to autoradiography film.

## RESULTS

**Identification of RipA.** To identify genes involved in *Francisella* intracellular growth, we created and screened a transposon mutant library of *F. tularensis* (LVS) for strains that entered but did not replicate within TC-1 cells, which are a transformed lung epithelial cell line. We identified one mutant strain that invaded epithelial cells at the same frequency as *F. tularensis* LVS, but did not replicate following invasion (Fig. 5.1A). At 6 hours post inoculation there was no significant difference between the number of CFU recovered from mutant or wild type infected-TC-1 cells. However, by 24 hours post inoculation there were significantly fewer ( $P < 0.001$ ) mutant organisms ( $1.9 \times 10^5$  CFU) than wild type LVS ( $1.1 \times 10^7$  CFU) recovered from the Tn5 mutant and wild type LVS -infected cells, respectively.

To compare the intracellular growth of the Tn5 mutant strain and wild type LVS in epithelial and macrophage – like cell lines, identical assays were performed using the J774A.1 mouse macrophage-like cell line (Fig. 5.1B). As in the TC-1 epithelial cell line, the mutant and wild type strains entered J774A.1 cells at the same frequency. At 24 hours post inoculation,  $8.2 \times 10^7$  versus  $8.7 \times 10^6$  CFU ( $P < 0.01$ ) were recovered from LVS and Tn5 mutant infected cells, respectively. Thus, in contrast to the no growth phenotype observed in TC-1 cells, the Tn5 mutant replicated within J774A.1 cells, but to a lesser extent than wild type LVS. Similar results were obtained in other lung epithelial cell lines (MLE-12, A549) and in bone marrow derived mouse macrophages (data not shown). The monocyte and epithelial cell lines are propagated in modestly different media formulations, but swapping the media had no impact on the cell type specific growth defect of the mutant (data not shown).

Analysis of the DNA sequence flanking the Tn5 insertion showed that the interrupted allele was *FTL\_1914* (Fig. 5.2A). This allele was termed *ripA* (required for intracellular proliferation, factor A). The *ripA* locus is predicted to encode a 178 amino acid membrane protein conserved in all sequenced *Francisella* strains. There is only one copy of the locus in each sequenced genome. pBLAST (25) was used to identify similar non-*F. tularensis* proteins ( $E < 1$ ). These were hypothetical membrane proteins of unknown function in *Streptomyces coelicolor* A3(2) ( $E = 1 \times 10^{-10}$ ), *Beggiatoa* sp. PS ( $E = 2 \times 10^{-10}$ ), *Moritella* sp. PE36 ( $E = 1 \times 10^{-9}$ ), *Sulfitobacter* sp. NAS-14.1 ( $E = 0.006$ ), *Clostridium perfringens* C strain JGS1495 ( $E = 0.044$ ), a predicted membrane protein linked to a retron element in an *Escherichia coli* clinical isolate ( $E = 0.36$ ), and a nitric oxide reductase (NorW) in *Aeromonas salmonicida* ( $E = 0.60$ ). NorW plays a role in nitric oxide detoxification through

interactions with NorR and NorV; however, there are no NorR or NorV homologs encoded in the *Francisella* genome and the amino acid similarity is to a NorW fragment not part of the functional oxidoreductase. Thus, at this time protein homology analyses did not reveal any obvious clues to RipA function

**Creation and initial characterization of *ripA* deletion mutant.** Intracellular growth of the LVS *Tn5::ripA* mutant was not restored by *in trans* complementation with wild type *ripA*. Thus, to circumvent potential complications caused by the transposon insertion a, *F. tularensis* LVS  $\Delta ripA$  strain lacking all but the start and stop codons was created for use in subsequent phenotypic assays. The deletion allele was generated by splice overlap extension (SOE) PCR (Fig. 5.2B), cloned into the allelic exchange vector pMP590 (40) and introduced into LVS as described (27, 40). Following sucrose selection to isolate resolved plasmid integrants, strains lacking wild type *ripA* and possessing the *ripA* deletion locus were identified by PCR. Integrity of the  $\Delta ripA$  locus and flanking DNA was confirmed by sequencing. Growth of the mutant strain in Chamberlains Defined Media was not significantly different than that of wild type LVS. After 24 hours of growth in Chamberlains Defined Media, the mutant and wild type strains reached equivalent maximum densities (data not shown).

***Francisella* intracellular growth requires *ripA*.** Mutant and wild type strains were subjected to gentamicin protection assays conducted in J774A.1 (Fig. 5.3B) and TC-1 cell lines (Fig. 5.3A) to determine the contribution of *ripA* on intracellular growth. Invasion frequencies of the deletion mutant were not significantly different from wild type (P

values > 0.4) in either cell line. However, in contrast to wild type, the number of recovered intracellular mutant CFU decreased after 24 hours in both cell types indicating that the *ΔripA* mutant had an intracellular survival defect. Mean recovery for LVS and LVS *ΔripA* was  $1.23 \times 10^7$  versus  $2.60 \times 10^2$  CFU ( $P < 0.001$ ), respectively, at 24 hours in TC-1 lung epithelial cells. Mean recovery for LVS and LVS *ΔripA* was  $1.04 \times 10^8$  versus  $1.07 \times 10^5$  CFU ( $P < 0.001$ ), respectively, at 24 hours in J774A.1 cells. The intracellular growth defect of LVS *ΔripA* was not due to increased sensitivity to gentamicin (data not shown). Intracellular replication of LVS *ΔripA* was restored by *in trans* complementation with *ripA* demonstrating a direct cause and effect relationship between *ripA* expression and intracellular growth.

***F. tularensis ΔripA* is unaffected in its ability to escape the phagosome.** Following uptake by host cells, wild type *F. tularensis* escape the phagosome and replicate within the cytoplasm (13, 26). With the notable exception of *F. novicida ΔiglD* (53), *F. tularensis* LVS *ΔpurMCD* (47), and *F. tularensis* LVS *clpB* (42), most intracellular growth defective mutants strains of *Francisella* fail to escape the phagosome (39, 54). To determine the contribution of *ripA* to this property, we compared the phagosome escape frequencies and rates of wild type and *ΔripA* in J774A.1 cells. Briefly, monolayers were inoculated with GFP-expressing strains at an MOI of 100. Invasion was synchronized as described (13). Infected cells were treated with digitonin to selectively permeabilize the cytoplasmic, but not phagosomal, membrane 20, 60, and 180 minutes post invasion and probed with Alexa 647 conjugated anti-*F. tularensis* antibody. Extracellular and cytoplasmic bacteria were antibody accessible, but not bacteria contained by an intact phagosomal membrane. Antibody bound and unbound organisms were quantified via FACS, and the contribution of extracellular

bacteria was accounted for in corresponding samples not treated with digitonin. At each time point, the percentage of antibody accessible mutant and wild type bacteria were statistically identical (Fig. 5.4A). Thus, the  $\Delta ripA$  strain accessed the cytoplasm at the same rate as wild type *F. tularensis* LVS, demonstrating that *ripA* is not required for phagosome escape and that failure to escape the phagosome does not account for the intracellular growth defect conferred by the  $\Delta ripA$  allele.

***Francisella ripA* mutant colocalizes with LAMP-1.** *Francisella* containing phagosomes associate with early and late maturation antigens EEA-1 and LAMP-1 before membrane integrity is compromised and the bacteria are released to the cytoplasm. There is evidence to suggest that early phagosome maturation steps are required for *Francisella* survival and subsequent intracellular replication (17,62). We compared mutant and wild type colocalization with LAMP-1 in infected cells to determine if the lack of *ripA* affected phagosome trafficking. J774A.1 cells were inoculated with GFP-expressing wild type and  $\Delta ripA$  strains as described above, permeabilized and probed with Alexa 647 conjugated LAMP-1 antibody. Co-localization of LAMP-1 with bacterial cells was evaluated by epifluorescence. Consistent with what has been reported by other groups (13,27), at 20 and 180 minutes post inoculation 50% and 22% respectively, of intracellular LVS co-localized with LAMP-1 (Fig. 5.4B). The number of wild type organisms co-localizing with LAMP-1 reached a minimum of 1.5 % at 12 hrs post inoculation, which was followed by an increase to 26% at 24 hrs (Fig. 5.4B). This reacquisition of LAMP-1 co-localization has been described previously as the replication dependent formation of *Francisella* containing vacuoles (13). The co-localization of LVS  $\Delta ripA$  with LAMP-1 was not statistically different

from wild type at any point tested ( $P > 0.05$ , unpaired two tailed t test) save for 24 hrs post inoculation where 40% of the mutant organisms co-localized with LAMP-1. It should be noted that LVS *ΔripA* infected cells contained only 1 – 3 bacterial per cell, whereas wild type infected cells had what appeared to be hundreds of bacteria 24 hours post inoculation. Thus, the concentration of wild type bacteria in these cells may impact the LAMP-1 co-localization calculations. More important than the difference in proportion of mutant and wild type organisms that co-localized with LAMP-1 in 24 hr infected cells is the observation that LAMP-1 colocalization with LVS *ΔripA* increased between 12 and 24 hours. This process of re-association with LAMP-1 positive vacuoles was previously thought to require bacterial replication. These results demonstrate that replication *per se* is not required for this process, and that failure to re-associate with LAMP-1 positive vacuoles does not account for the intracellular growth deficiency of *ΔripA*.

***Francisella ripA* mutant reenters a membrane bound vacuole after escape to the cytoplasm.** *Francisella* escapes from the phagosome quickly after cell invasion before replicating in the cytosol. *Francisella* then reenters a vacuole with autosomal characteristics in a replication dependent process (13) We compared mutant and wild type subcellular localization by electron microscopy to determine if the mutant bacteria were associated with *Francisella* containing vacuoles (FCV). J774A.1 cells were inoculated with wild type and *ΔripA* strains as described above.

Mutant and wild type bacteria were found in membrane bound vacuoles at 15 minutes post inoculation and were free in the cytoplasm at 3 hours post inoculation (Fig. 5.5). No difference in the subcellular localization was evident when the wild type and mutant bacteria were compared. By 24 hours post inoculation, evidence of bacteria in membrane bound

vacuoles was present. Wild type bacteria were present at high density in most cells and could be located in either the cytosol or membrane bound vacuoles. Mutant bacteria were present in small clusters of 1 to 3 bacteria that could be found in either the cytosol or membrane bound vacuoles. Some bacteria containing vacuoles demonstrated the double membranes characteristic of autophagosomes (Fig. 5.5). The reentry of *F. tularensis* LVS  $\Delta ripA$  into *Francisella* containing vacuoles did not appear to be dependent on replication, differentiating it from the wild type phenotype.

**RipA is Required for Virulence in a Mouse Model of Infection.** We used a mouse model of pulmonary tularemia to assess the role of *ripA* in *F. tularensis* virulence and pathogenesis. Anesthetized C57BL/6 mice were inoculated intranasally with  $10^5$  CFU of either LVS pKK MCS (wild type + vector), LVS  $\Delta ripA$  pKK MCS (mutant + vector), or LVS  $\Delta ripA$  pKK MCS *ripA* (mutant + plasmid complement). Bacterial burdens were measured by serial dilution, and plating of homogenized organs. Lung burdens were determined two hours post inoculation to monitor the delivery of each strain to the lung. Lung, liver, and spleen organ burdens (Fig. 5.6) were determined 1, 3, 7, and 14 days post inoculation.

The CFU recovered from the lungs at 2 hours post inoculation,  $2.48 \times 10^4$ ,  $1.18 \times 10^4$ , and  $1.12 \times 10^4$  for LVS, LVS  $\Delta ripA$ , and LVS  $\Delta ripA$  pJRF146, respectively, were not statistically different ( $P > 0.2$ , independent two tailed t-test with unequal variance), thus equivalent numbers of each strains were delivered to the lung. By day 1 the organ burdens of LVS  $\Delta ripA$  infected animals were significantly lower than those of wild type and complemented mutant strains ( $P < 0.001$ ). Within the first 24 hours post inoculation, the number of LVS  $\Delta ripA$  organisms in the lung decreased, whereas the number of both wild type and complemented strains increased by at least one order of magnitude over the number

of organisms initially delivered. In contrast to the wild type and complemented mutant strains, LVS *ΔripA* was not detected in the liver or spleen before day seven. At day seven, all LVS *ΔripA*-infected mice had detectable liver burdens but only 1 LVS *ΔripA* –infected mouse had a detectable spleen burden (> 30 CFU). In mice infected with LVS *ΔripA* or LVS *ΔripA* pJRF146 *ripA* organ burdens were reduced at day 14 relative to day 7 (P<0.01). Mice infected with wild type LVS were euthanized at day 7 due to significant morbidity. Collectively, these data demonstrate the importance of *ripA* in establishing a *Francisella* infection.

**Characterization and localization of the RipA Protein.** Subcellular fractions of LVS, LVS *ΔripA*, and LVS *ΔripA* pJRF146 *ripA* were analyzed by Western blotting using anti-RipA antiserum (Fig. 5.7A). RipA migrated at a relative molecular weight of 17 kDa. RipA protein was not detected in LVS *ΔripA* lysates. Complementation with the native *ripA* allele in a multicopy plasmid resulted in increased RipA expression relative to the wild type strain.

When the translated amino acid sequence was analyzed with TopPred (14), RipA was predicted to be localized to the cytoplasmic membrane with three transmembrane domains and the amino terminus in the cytoplasm. Western analysis of cytoplasmic, cytoplasmic membrane, and outer membrane enriched protein fractions (16, 44) prepared from GFP-expressing LVS revealed that RipA localized to the cytoplasmic membrane (Fig. 5.7B). RipA was differentiated from proteins that reacted non-specifically with the anti-serum by probing corresponding samples with preimmune serum. Antibodies recognizing the F<sub>0</sub>F<sub>1</sub> ATP synthase β subunit (AtpB) (37) and GFP were used as markers for cytoplasmic membrane and cytoplasmic components, respectively. No GFP was detected in either

membrane fraction. Low levels of AtpB and RipA were detected in the outer membrane fraction, indicating that the presence of low level contamination with cytoplasmic membrane proteins. However, the relative band intensities demonstrate that the majority of RipA was present in the cytoplasmic membrane fraction of *F. tularensis*.

## DISCUSSION

The highly virulent nature of *F. tularensis* was recognized even before it was isolated and identified in the 1920's as the etiologic agent of a plague like disease in rodents. It is evident that the ability to survive and replicate within diverse host cell types is fundamental to *F. tularensis* pathogenesis. While the processes of host cell entry, phagosome escape, and intracellular replication have been described, the mechanisms used by *F. tularensis* to achieve these have not. The recent creation and improvement of tools and procedures for the genetic manipulation of *F. tularensis* has led to the identification of a number of genes that are required for intracellular growth and/or virulence in animal models of tularemia.

We screened a transposon insertion mutant library of *F. tularensis* LVS to identify previously unstudied genes required for proliferation within host cells. One such mutant strain contained a transposon insertion in FTL\_1914 (*ripA*), which is conserved among *Francisella* species. The transposon insertion mutant possessed an unusual phenotype in that it failed to replicate within cell lines of epithelial origin, yet retained the ability to replicate within monocyte cell lines and bone marrow derived macrophages. We created a *F. novicida* U112 Tn5::*ripA* mutant strain by replacing the wild type gene with the LVS Tn5::*ripA* allele via homologous recombination, and this strain had the same intracellular growth phenotype as LVS Tn5::*ripA* (data not shown). However, the *F. tularensis* LVS  $\Delta$ *ripA* deletion mutant

strain failed to replicate in any cell type tested. We cannot account for the differences in intracellular growth conferred by the transposon insertion and deletion mutant alleles. Only the deletion mutant phenotype was complemented by wild type *ripA* *in trans* suggesting that the transposon insertion may have exerted *cis*-acting or polar effects, or possibly created selective pressure for secondary unlinked mutations that compensated for the growth defect in macrophages.

Even though *ripA* has homology to a limited number of genes in other unrelated bacteria, this information does not provide significant insight into the *ripA* function. Thus, we thoroughly characterized the expression and subcellular localization of RipA, as well as the  $\Delta ripA$  mutant phenotypes to provide a basis for discerning the mechanism(s) by which this protein contributes to intracellular growth.

Intracellular pathogens must overcome the host innate immune response to successfully colonize the intracellular niche. The primary host defense is centered on the antimicrobial properties of the phagosome. Most successful intracellular pathogens either escape the phagosome or divert phagosome maturation to their own ends. *Francisella* quickly escapes the phagosome into the cytoplasmic environment where it replicates (13, 26, 39). Since the recovery of LVS  $\Delta ripA$  from infected epithelial cells or macrophages decreased dramatically after the initial invasion, we wanted to assess if the decreased survival was due to altered intracellular trafficking of LVS  $\Delta ripA$ . LVS  $\Delta ripA$  became accessible to the cytoplasm at the same rate as wild type LVS. The mutant also co-localized with the late endosomal/lysosomal marker LAMP-1 with the same frequency as wild type. Thus, the intracellular survival defect was not due to failure to escape from and subsequent killing in the phagolysosome.

Autophagy is another important means by which host cells clear pathogens from the host cytoplasm (43, 51). Most cytoplasmic pathogens studied to date either evade or hijack autophagic mechanisms. For example, *Shigella flexneri* and *Listeria monocytogenes* evade autophagy in a process requiring *de novo* protein synthesis (46, 51). While *Legionella pneumophila*, *Salmonella enterica*, and *Coxiella burnetti* exploit the autophagic machinery (6, 7, 32, 52, 57). *Francisella* is different from other intracellular pathogens that have been examined because evasion of autophagy is not dependent on *de novo* protein synthesis (13). *Francisella* reenters autophagosome like, LAMP-1 positive, vacuoles after replication in the cytoplasm. LVS  $\Delta ripA$  also localizes with LAMP-1 after escape to the cytoplasm. Electron micrographs showed *F. tularensis* LVS  $\Delta ripA$  present in vacuoles with double membranes characteristic of autophagosomes by 24 hours post inoculation. Reentry of the mutant bacteria into *Francisella* containing vacuoles was not dependent on replication differentiating the phenomenon from the wild type process. We hypothesize that autophagy of intracellular *F. tularensis* LVS  $\Delta ripA$  occurs in the cytoplasm although this may or may not have a direct bacteriocidal effect on the bacteria. Further work needs to be done to determine if LVS  $\Delta ripA$  requires *de novo* protein synthesis to stimulate compartmentalization during the cytoplasmic phase of its lifecycle and what effect compartmentalization of LVS  $\Delta ripA$  has on intracellular bacterial survival.

The survival defect of LVS  $\Delta ripA$  in the cytoplasm is likely due to increased sensitivity to host anti-microbial factors, its inability to acquire a necessary nutrient, or failure to adapt to the cytoplasmic environment. Nutritional deprivation or auxotrophy would have a bacteriostatic effect on the bacteria (2). For example, *F. tularensis*  $\Delta purF$ , a purine auxotroph, fails to replicate in host cell cytoplasm, but persists for an extended period

before declining gradually (49). This is not the phenotype demonstrated by *F. tularensis*  $\Delta ripA$ , which declines dramatically between 6 hours and 24 hours in the intracellular niche. Though not conclusive, this rapid loss of intracellular viability coupled with the fact that the  $\Delta ripA$  strain replicates at the same rate and extent as wild type LVS in chemically defined minimal Chamberlains media suggests that *F. tularensis*  $\Delta ripA$  is not an auxotroph. We are currently working to determine if the  $\Delta ripA$  strain is affected in its ability respond and adapt to the host cell cytoplasmic environment and are identifying RipA interacting proteins with the aim of inferring its function based on the properties of interacting proteins.

#### **ACKNOWLEDGEMENTS**

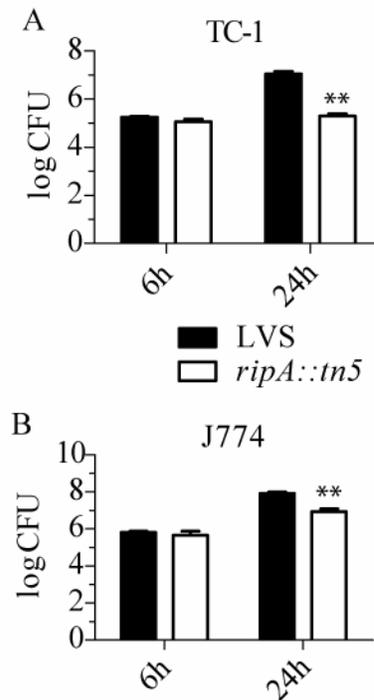
We gratefully acknowledge Victoria Madden of the Microscopy Services Laboratory and Larry Arnold of the Flow Cytometry Facility at UNC-CH for his expertise.

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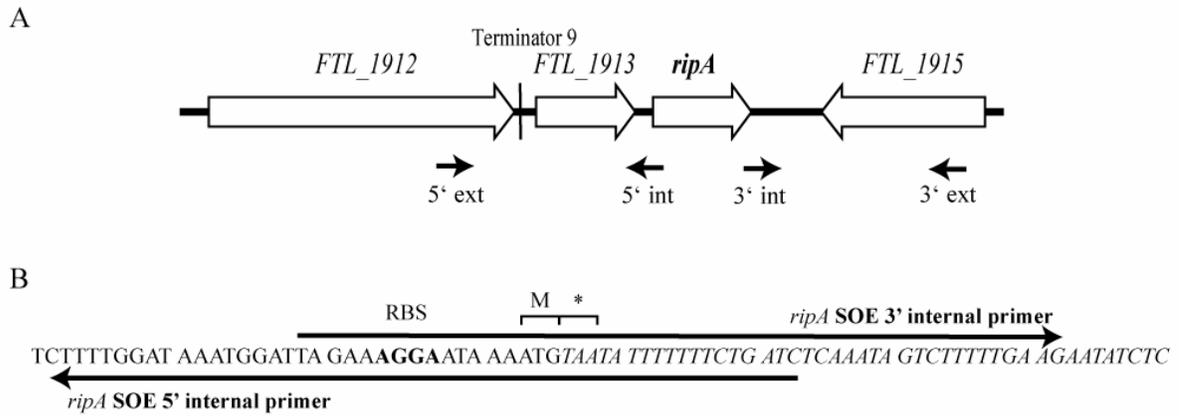
#### **ATTRIBUTIONS**

I performed all work in this chapter with the following exceptions. Josh Hall isolated and characterized the original transposon mutant and assisted in mouse experiments. Robin Craven performed flow cytometric analyses for cytoplasmic accessibility. Todd Kijek and Sharon Taft-Benz assisted in animal experiments. This work has been previously published,

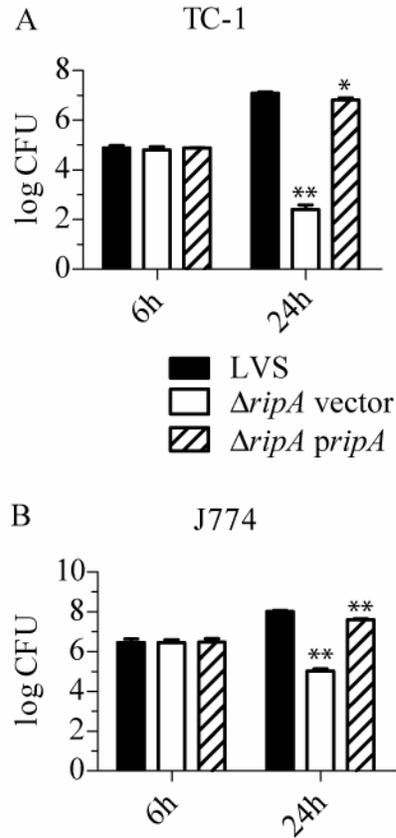
### FIGURES



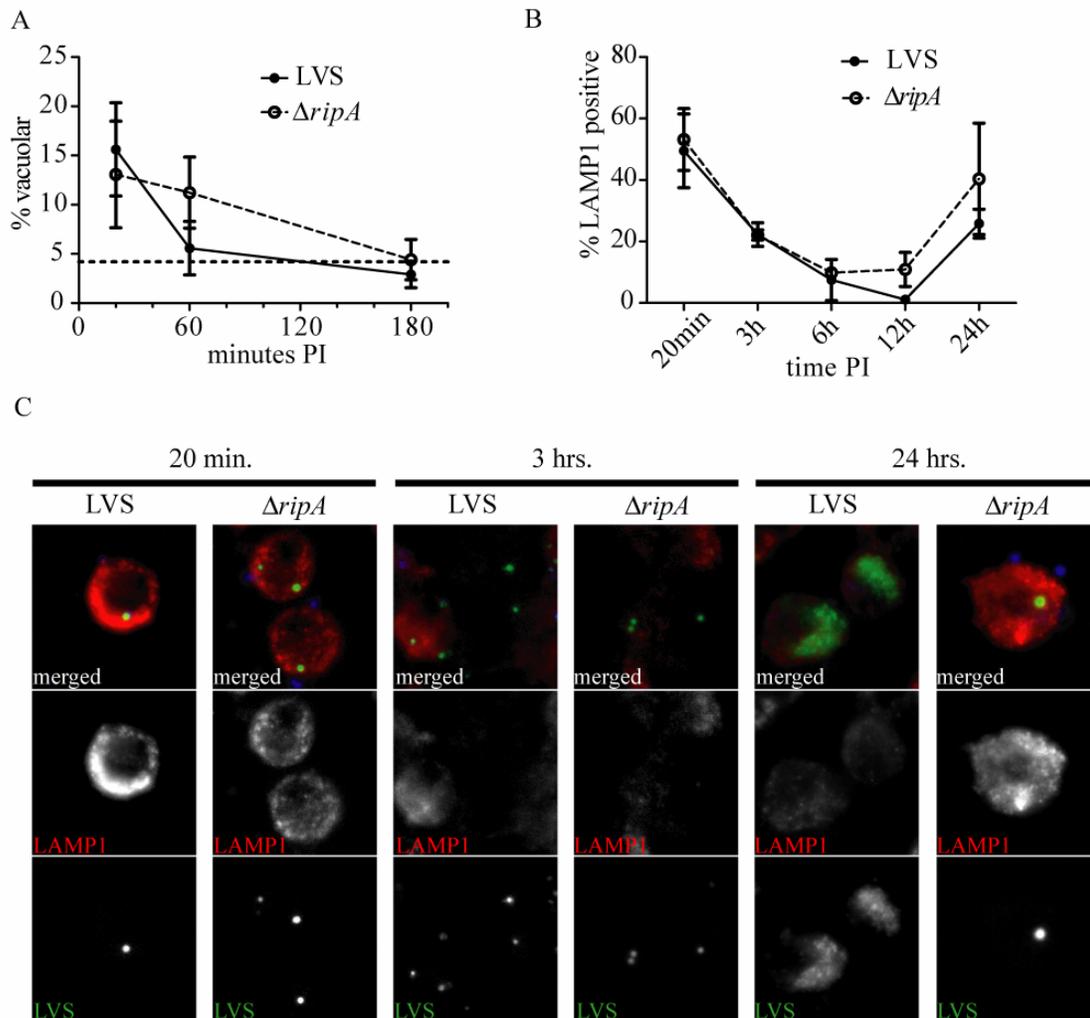
**Fig. 5.1.** Gentamicin protection assays with LVS *ripA::tn5*. (A) TC-1 lung epithelial cells or (B) J774A.1 mouse macrophage-like cells were infected with either LVS or LVS *ripA::tn5* at an MOI of 100. Mean CFU (N=3) at 6 hours and 24 hours post inoculation with error bars  $\pm$  one standard deviation. Student's t-tests were conducted comparing LVS to LVS *ripA::tn5* at each time point (P<0.01 demarcated by \*\*).



**Fig. 5.2.** (A) Graphical representation of the genomic organization of the LVS *ripA* locus. Loci in close proximity to *ripA* (FTL\_1914) on the chromosome are FTL\_1912 (30S ribosomal protein S1), FTL\_1913 (Sua5/YciO/YrdC family protein), and FTL 1915 (Acetyltransferase). Primers utilized for SOE PCR are marked by arrows. (B) DNA sequence of the *ripA* deletion marked with the forward and reverse overlapping internal primers used in the SOE PCR. The predicted ribosome binding site (RBS) and the remaining *ripA* codons (M and \*) are marked.

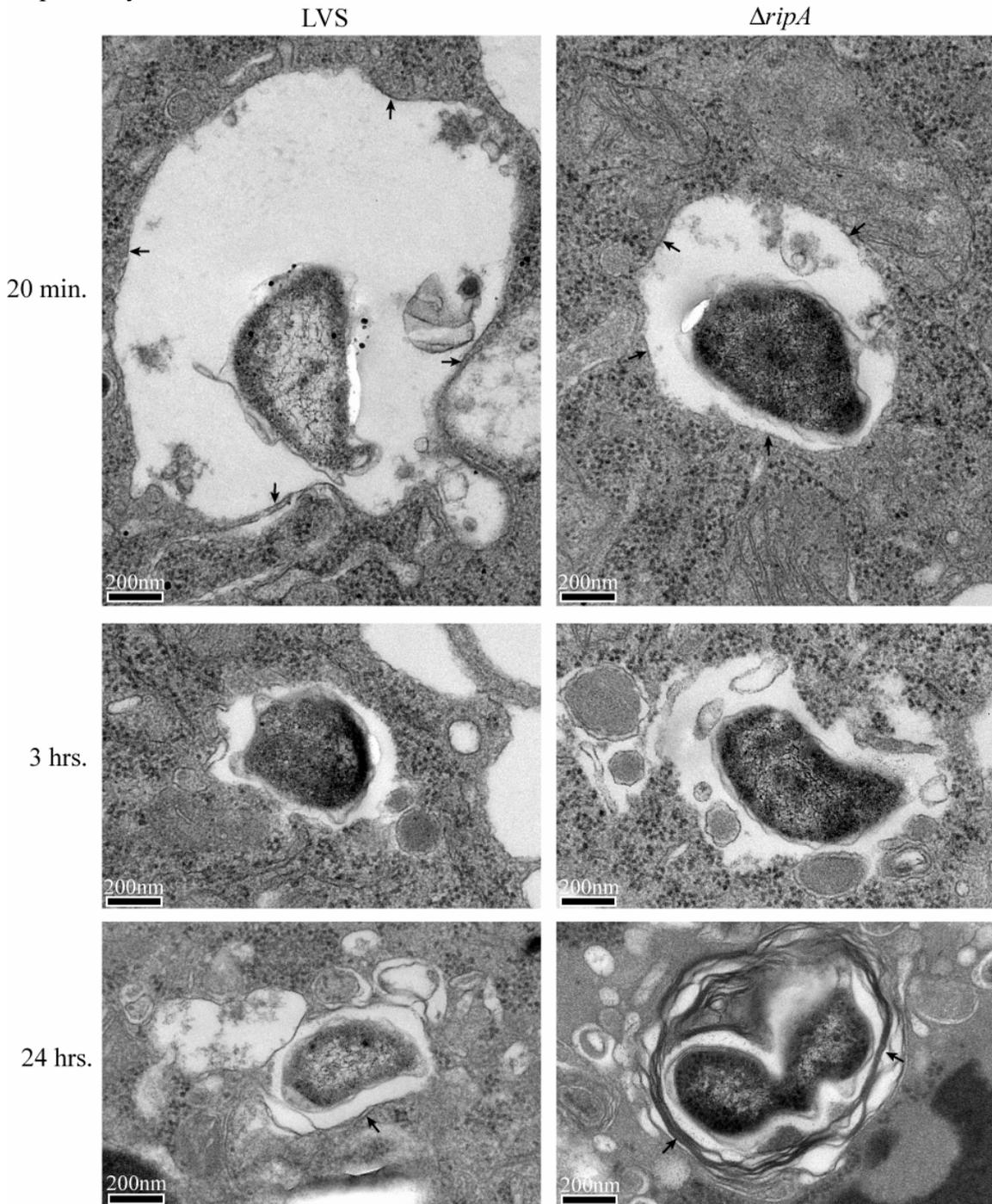


**Fig. 5.3.** Gentamicin protection assays with  $\Delta ripA$ . (A) TC-1 lung epithelial cells or (B) J774A.1 mouse macrophage-like cells were infected at an MOI of 100. Mean CFU (N=3) at 6 hours and 24 hours post inoculation with error bars  $\pm$  one standard deviation. Student's t-tests were conducted comparing strains to wild type LVS at each time point ( $P < 0.05$  demarcated by \* and  $P < 0.01$  demarcated by \*\*).



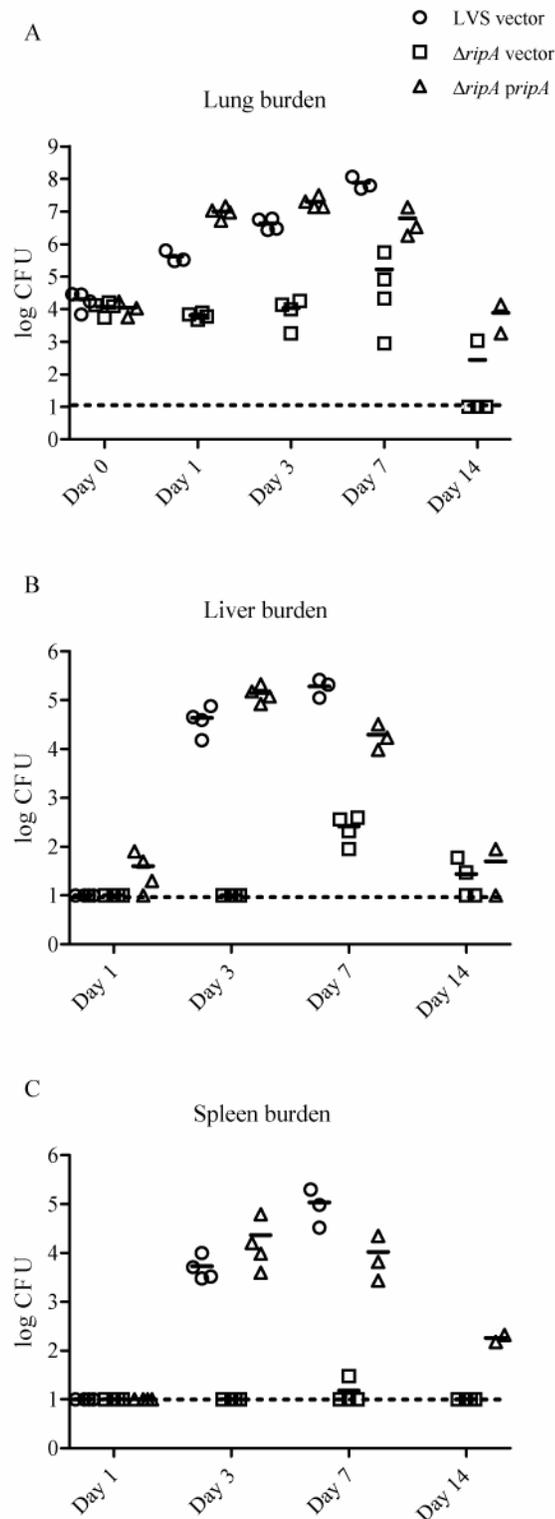
**Fig. 5.4.** Intracellular trafficking of LVS  $\Delta ripA$ . (A) Cytoplasmic accessibility of LVS gfp and LVS  $\Delta ripA$  gfp in J774A.1 macrophage-like cells at 20 minutes, 1 hr, and 3 hrs post inoculation (PI). Vacuolar bacteria were defined as bacteria inaccessible to the cytoplasmically delivered  $\alpha$ -*Francisella* LPS antibodies. Mean % vacuolar (N=3) values plotted and error bars are representative of  $\pm 1$  standard deviation. Percent accessibility with saponin permeabilization marked with a broken line. (B) Co-localization of LVS gfp and LVS  $\Delta ripA$  gfp with LAMP-1 in J774A.1 macrophage-like cells. Percent LAMP-1 co-localization was determined from 3 independent experiments and recorded as mean % LAMP-1 positive with error bars representative of  $\pm 1$  standard deviation. At 24 hours PI, LAMP-1 co-localization done on a per cells basis. Individual bacteria were not distinguishable in wild type images. (C) Representative epifluorescent images of LVS gfp and LVS  $\Delta ripA$  gfp at 20 minutes, 3 hrs, and 24 hrs PI in J774A.1 macrophage-like cells. The 20 minute and 3 hour images demonstrate a similar staining pattern for LVS gfp and LVS  $\Delta ripA$  gfp. The 24 hours image demonstrates that LVS  $\Delta ripA$  gfp may be visualized as individual bacteria co-localized with LAMP-1 at this time point. Extracellular bacteria were stained with AMCA and colorized blue. GFP captures demarked by LVS and colorized green in merged images. LAMP-1 stained with Alexa 647 and colorized red. Individual

channels for GFP and Alexa 647 captures marked LVS and LAMP-1 respectively.



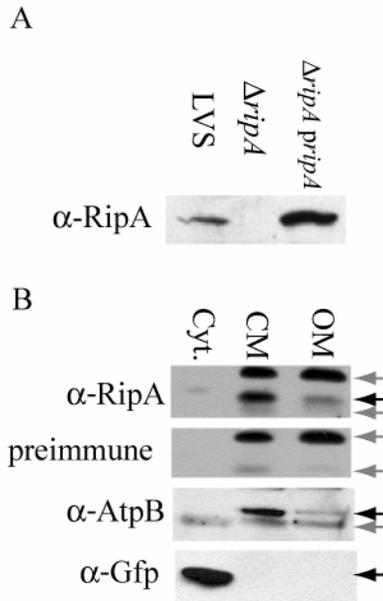
**Fig. 5.5.** *Francisella ripA* reenters membrane bound vacuoles after phagosomal escape. J774A.1s were infected with LVS and LVS  $\Delta ripA$ . Samples were processed for TEM at 20 min, 3 hrs, and 24 hrs post inoculation. TEM micrographs demonstrating individual bacteria enclosed by membranes at 20 minutes and 24 hours post inoculation (indicated by arrows). Arrows on 24 hrs post inoculation micrographs demonstrate double membrane regions

characteristic of autophagosomes. The 3h post inoculation micrographs demonstrate *F. tularensis* not enclosed by membranes. (Scale bars: 200 nm)



**Fig. 5.6.** Bacterial organ burdens from infected mice (N=4) were quantified as CFU per organ and graphed as individual data points. Mean organ burdens are demarcated by horizontal lines. Organ burdens in mice inoculated with LVS vector only ( $\circ$ ), LVS  $\Delta ripA$

vector only ( $\square$ ), and LVS  $\Delta ripA$  pJRF146 *ripA* ( $\Delta$ ) strains. Lung (A), liver (B), and spleen (C) burdens were determined at 2 hours (lung only), 1 day, 3 days, 7 days, and 14 days (LVS  $\Delta ripA$  vector only and LVS  $\Delta ripA$  pJRF146 *ripA*) post inoculation. The limit of detection is marked as a broken line at  $\sim 10$  CFU. Burdens that were below the limit of detection are plotted at 10 CFU.



**Fig. 5.7.** (A) Western blot analysis of cytoplasmic membrane enriched fractions of LVS, LVS  $\Delta ripA$ , and LVS  $\Delta ripA$  pJRF146 *ripA*. Rabbit anti-RipA (1:1000) antiserum was used as the primary antibody. (B) Subcellular localization of RipA. Western analysis of cytosolic (Cyt.), cytoplasmic membrane (CM), and outer membrane (OM) enriched fractions of LVS pKK214 *gfp*. Non specific bands were determined with preimmune serum from the mouse used to generate the mouse anti-RipA serum. AtpB was used as a marker for the cytoplasmic membrane and GFP for the cytoplasm. Specific bands (RipA, AtpB, and Gfp) are marked with black arrows. Non-specific bands are marked with gray arrows.

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## Chapter 6

### ***Francisella ripA* expression is suppressed during intracellular growth and by MglA.**

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#### ABSTRACT

*Francisella tularensis* is a highly virulent, facultative, intracellular pathogen that is the etiologic agent of the zoonotic disease tularemia. We recently identified a cytoplasmic membrane protein, RipA, which is conserved among *Francisella* species and is required for intracellular growth. Characterization of *ripA* expression by Northern analysis of total RNA determined that the *ripA* allele was predominantly expressed as a monocistronic message and not as part of an operon. Multi- and single copy transcriptional and translational *lacZ* fusions were constructed to facilitate *ripA* expression analyses. Site directed mutagenesis of the predicted *ripA* promoter and ribosome binding sites significantly reduced  $\Phi(\textit{ripA}'\textit{-lacZ})$  expression. LacZ activity produced by the *ripA* fusions accurately reflected changes in both *ripA* transcript and protein levels. The  $\Phi(\textit{ripA}'\textit{-lacZ})$  strains were used to quantify *ripA* expression in response to different environmental conditions and during different stages of intracellular growth. The expression of *ripA* was as much as two fold higher in laboratory cultures grown at pH 7.5 as compared to pH 5.5. Further, *ripA* expression levels associated with different stages of intracellular growth mirrored that of the pathogenicity island gene *iglA*. However, in contrast to *iglA*, the *ripA* gene is moderately repressed by the

transcriptional regulators MglA and SspA. The expression of *ripA* was two-fold or more higher in  $\Delta mglA$  or  $\Delta sspA$  mutant backgrounds whereas *iglA* expression was significantly lower in these two strains. Expression of *ripA* and *iglA* were returned to near wild type levels by complementation in the respective mutants with the native *mglA* and *sspA* alleles *in trans*. The activation and suppression of genes required for intracellular growth by *mglA* suggests a temporally compartmentalized expression profile of *Francisella* virulence factors related to the expression levels of *mglA*.

## INTRODUCTION

*Francisella tularensis* is the etiologic agent of the highly virulent zoonotic disease tularemia. Routes of transmission include arthropod bites (Markowitz *et al.*, 1985), physical contact with infected animal tissues (Centers for Disease Control and Prevention (CDC), 2005), contaminated water (Celebi *et al.*, 2006; Reintjes *et al.*, 2002), and inhalation of aerosolized organisms (Feldman *et al.*, 2001). Pulmonary tularemia is the most lethal form of the disease with a mortality rate as high as 60% (Dennis *et al.*, 2001). This lethal disease rapidly progresses after colonization of the lungs by as few as 10 colony forming units or CFU (Saslaw *et al.*, 1961; White *et al.*, 1964).

*F. tularensis* is a facultative intracellular pathogen that escapes the phagosome and replicates in the cytoplasm (Checroun *et al.*, 2006; Clemens *et al.*, 2004; Golovliov *et al.*, 2003). *Francisella* mutations that result in the loss of intracellular replication have been linked to either a lack of replication in the cytoplasm (Fuller *et al.*, 2008; Meibom *et al.*, 2008; Mohapatra *et al.*, 2007; Pechous *et al.*, 2006) or a failure to escape the phagosome (Lindgren *et al.*, 2004; Santic *et al.*, 2005). Regulation of *Francisella* virulence factors

required for phagosomal escape and intracellular replication have been in many cases linked to the transcriptional regulator MglA (Brotcke *et al.*, 2006; Charity *et al.*, 2007; Lauriano *et al.*, 2004; Santic *et al.*, 2005). Snapshots of *Francisella* intracellular protein expression have been taken using proteomics studies (Golovliov *et al.*, 1997; Twine *et al.*, 2006), but studies on how the expression of *Francisella* virulence factors change during the course of intracellular infection are limited. Existing studies include measurement of intracellular IgLA by Western blotting (de Bruin *et al.*, 2007) and *mglA* expression through the use of a *mglA-cat* reporter (Baron & Nano, 1999). Recent advances in the understanding of subcellular localization of *Francisella* during intracellular growth (Checroun *et al.*, 2006; Clemens *et al.*, 2004; Lindgren *et al.*, 2004) have enhanced the value of examining how intracellular gene expression changes during different phases of intracellular growth and subcellular localization.

The *ripA* locus is dispensable for growth in chemically defined media, yet is essential for intracellular replication (Fuller *et al.*, 2008). Many loci necessary for intracellular replication are positively regulated by MglA (Charity *et al.*, 2007; Lauriano *et al.*, 2004); however, data on whether MglA regulates *ripA* expression is contradictory. Microarray data on MglA regulated loci show *ripA* expression unchanged (Charity *et al.*, 2007), while proteomics data demonstrate RipA to be repressed by MglA (Guina *et al.*, 2007). This repression is seemingly in conflict with the upregulation of MglA after cell invasion and the necessity of *ripA* for intracellular replication. To clarify this conflict, we used *lacZ* as a reporter gene to study *F. tularensis* LVS *ripA* extracellular and intracellular gene expression. Gene expression of bacteria in intracellular niches has been studied using *lacZ* reporter fusions in *Listeria monocytogenes* (Moors *et al.*, 1999) and *Salmonella typhimurium* (Chen *et*

*al.*, 1996). In *Francisella*, *lacZ* has been used successfully as a reporter gene introduced by transposon mutagenesis and evaluated during extracellular growth (Buchan *et al.*, 2008). This is the first use of a *lacZ* reporter system in *Francisella* to evaluate intracellular gene expression.

## METHODS

**Bacterial strains and Cell culture.** *F. tularensis* LVS (Table 6.1) was propagated on chocolate agar (25 g BHI l<sup>-1</sup>, 50 µg hemoglobin ml<sup>-1</sup>, 1.5 g agarose l<sup>-1</sup>) supplemented with 1% (v/v) IsoVitaleX (Becton-Dickson), BHI broth (35 g BHI l<sup>-1</sup>, 1% (v/v) IsoVitalex), or Chamberlains defined media (Chamberlain, 1965) . All bacterial strains cultured on chocolate agar were grown at 37 °C. Broth cultures were incubated in a shaking water bath at 37 °C. J774A.1 (ATCC TIB-67) reticulum cell sarcoma mouse macrophage-like cells were cultured in DMEM plus 4 mM L-glutamine, 4500 mg glucose l<sup>-1</sup>, 1 mM sodium pyruvate, 1500 mg sodium bicarbonate l<sup>-1</sup>, and 10% (v/v) FBS at 37 °C and 5% CO<sub>2</sub> atmosphere.

**Reverse Transcriptase PCR.** Total RNA was isolated from mid exponential phase cultures using a mirVana RNA isolation kit (Ambion). DNA was removed by incubation with RQ1 DNase (Promega) for 1 hour at 37 °C. First strand cDNA was generated using SuperScript III Reverse transcriptase (Invitrogen) and random primers. cDNA was quantified using a ND-1000 spectrophotometer (Nanodrop). PCR analysis of *ripA* and *tul4* expression was accomplished using 20 ng cDNA per 50 µl PCR reaction. As a control for DNA contamination, a Reverse transcriptase reaction was conducted without the Reverse

transcriptase enzyme. Ten percent of each reaction was analyzed by agarose gel electrophoresis, ethidium bromide staining, and densitometry using BioRad Quantity One software. Trace intensity (Int mm) of *ripA* was normalized to the mean *tul4* expression (Charity *et al.*, 2007). Mean normalized expression and standard deviation were calculated on four independent samples. Significance was determined using an unpaired two tailed t test with unequal variance.

**Agarose Formaldehyde Electrophoresis and Northern Analysis.** Total RNA was harvested from mid exponential phase *F. tularensis* LVS grown in Chamberlains defined media using RNeasy columns (Qiagen), concentrated using ethanol/sodium acetate precipitation and quantified with a ND-1000 spectrophotometer (Nanodrop). RNA was separated using agarose-formaldehyde (2% agarose, 2.2 M Formaldehyde) electrophoresis followed by capillary transfer to nitrocellulose as described (Rueger *et al.*, 1997). Additional lanes of the membrane containing duplicate samples were stained with methylene blue to assess rRNA bands for degradation and equality of loading. Digoxigenin labeled RNA probes were generated using a Northern Starter Kit (Roche). Probe generation, hybridization, washing, and detection were performed using the manufacturer's (Roche) protocols.

**Plasmids and Molecular techniques.** Specific *F. tularensis* LVS DNA fragments were produced by PCR amplification of genomic DNA using Pfu turbo DNA polymerase (Stratagene). *Francisella* translational *lacZ* fusions were generated by ligating a *KpnI/EcoRV* fragment containing a native RBS and translational start site into *KpnI/SmaI* digested pBSK

*lacZ cat* (Table 6.1). *Francisella* transcriptional *lacZ* fusions were generated by ligating a *NotI/PacI* fragment to *NotI/PacI* digested pBSK *lacZ aphAI* (Table 6.1) containing the *lacZ* RBS and translational start site. The pBSK *lacZ* fusion clones that lack a *Francisella* origin of replication were used as co-integration vectors to generate single copy expression strains of *Francisella*. This allowed the subsequent use of *Francisella* shuttle vectors in these strains. The *cat* and *aphAI* alleles were expressed by a *Francisella* promoter derived from the *groEL* gene. *Francisella* multicopy *lacZ* vectors were created by transferring the fusion constructs to the *Francisella* shuttle vector pKK MCS (Table 6.1). All *Francisella* transformations were performed as described (LoVullo *et al.*, 2006).

**Intracellular  $\beta$ -galactosidase Assay.** To determine the activity of *Francisella* promoter *lacZ* fusions in the intracellular environment, intracellular invasion and replication assays were conducted by adding *F. tularensis* LVS strains cultured to mid exponential phase in BHI to J774A.1 monolayers at a multiplicity of infection (MOI) of 100 in 200  $\mu$ l tissue culture media. Assays were synchronized as described (Checroun *et al.*, 2006; Craven *et al.*, 2008). At 15 minutes post inoculation, monolayers were washed 3 times with pre-warmed tissue culture media to remove extracellular bacteria. At 1, 6, and 24 hours post inoculation samples were washed with PBS and scraped into 200  $\mu$ l PBS. CFU present in each sample were determined by serial dilutions and plating on Chocolate agar. 100  $\mu$ l of each sample was lysed in 2x lysis buffer (1% NP40, 0.5 M Tris pH 7.4, 5 mM EDTA) and assayed for  $\beta$ -galactosidase activity using the substrate Chlorophenol red- $\beta$ -D-galactopyranoside (CPRG). Twenty  $\mu$ l of each sample was mixed with 130  $\mu$ l of CPRG buffer (2 mM CPRG, 25 mM MOPS pH 7.5, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 50 mM  $\beta$ -mercaptoethanol) and incubated at

37 °C until visible color developed. Enzymatic activity was stopped by adding 80 µl of 0.5 M Sodium carbonate and measured by absorbance at OD<sub>580</sub>. Background β-galactosidase activity was determined at each time point using duplicate samples of J774A.1 cells infected with wild type *F. tularensis* LVS. Mean background activity was subtracted from each sample before calculating relative activity. Relative β-galactosidase activity was calculated by normalizing OD<sub>580</sub> readings with time of development, dilution of sample, and CFU recovered per sample. Data are presented as activity per 10<sup>10</sup> bacteria which results in an activity range similar to Miller units. All assays were performed using four wells of infected cells from a 24 well tissue culture plate per time point. Inoculum activities were determined using the same techniques before addition to cell culture in replicates of four.

$$\text{Relative activity} = 10^{10} * \text{OD}_{580} * (\text{Dilution} * \Delta T * \text{CFU})^{-1}$$

Statistical analysis was as applied to the mean activity and standard deviation of four independent samples. Significance was calculated using an unpaired two tailed t test assuming unequal variance. P values of less than 0.05 were considered significant.

**Mutagenesis and Allelic Exchange.** A  $\Phi(\text{ripA}'\text{-TC})$  fusion was made by Splice Overlap Extension (SOE) PCR (Horton *et al.*, 1989) using primers designed to insert the tetracycline (TC) tag sequence with a glycine linker between the last *ripA* codon and the stop codon (Fig. 6.3b). Deletion constructs were made by SOE PCR designed to keep the start and stop codons of *mgla* (fusion of first four and last two codons) and *sspA* (fusion of first four and last four codons) in frame with 0.8 kb of flanking sequence. The constructs were cloned into pMP590 (Table 6.1) and sequenced to confirm the integrity of the flanking DNA sequence. Allelic exchange was achieved by transformation, selection for plasmid co-integrates,

counter selection on sucrose containing media and screening via PCR for replacement of the wild type allele with the deletion mutant allele as described (LoVullo *et al.*, 2006). Each mutation was confirmed by DNA sequencing. Site directed mutagenesis was performed using the Stratagene QuickChange XL kit and the manufacturers protocols. DNA sequencing was used to confirm all *ripA* promoter mutations.

**Extracellular  $\beta$ -galactosidase Assay.** Overnight cultures of *lacZ* reporter strains were diluted 1:10 in Chamberlains defined media and cultured until mid exponential phase (0.2-0.8 OD<sub>600</sub>).  $\beta$ -galactosidase activity was measured as OD<sub>420</sub> using the substrate ONPG (Sigma) as described elsewhere (Miller, 1972). Relative promoter activity was normalized using OD<sub>600</sub> of culture, time of development, and cell to buffer ratio (CBR).

$$\text{Miller Units} = 1000 * \text{OD}_{420} * (\Delta T * \text{CBR} * \text{OD}_{600})^{-1}$$

Statistical analysis was performed to determine the mean Miller units and standard deviation of replicates of three independent samples and significance calculated using an unpaired two tailed t test with unequal variance.

**SDS-PAGE and FIAsH™ labeling.** Proteins were separated by SDS-PAGE. Total protein loaded in each sample was equivalent as determined by a BCA assay (Pierce). FIAsH™ labeling was accomplished using the manufacturer's protocols (Invitrogen). In gel fluorescence of the arsenical fluorescein and total protein stain was conducted on a Typhoon 9200 laser scanner (488 nm laser/ 520 nm BP 40 filter and 633 nm laser/ 670 nm BP 30 filter). Densitometry was conducted using ImageQuant XL software and sample comparisons made using the same gel and scan. Mean intensity and standard deviation of

four independent samples was calculated and significance determined using an unpaired two tailed t test with unequal variance.

## RESULTS

**Analysis of the *ripA* transcript.** The *Francisella tularensis* LVS *ripA* gene is required for intracellular replication and virulence in a mouse model of tularemia (Fuller *et al.*, 2008). The *ripA* gene is preceded by two other genes, FTL\_1912 and FTL\_1913, that are predicted to be transcribed in the same orientation, and thus the *ripA* locus may be a part of a three gene operon. A Rho independent terminator is predicted to exist between FTL\_1912 and FTL\_1913, but not between FTL\_1913 and *ripA*. Results from an analysis of this locus with the operon prediction tool FGENESB ([www.softberry.com](http://www.softberry.com)) were inconclusive. Thus, we analyzed *F. tularensis* LVS *ripA* transcripts by RT PCR and Northern blot.

One set of primers was designed to amplify coding regions from each of the 3 genes, and another set was designed to amplify RNA that bridged adjacent genes (Fig. 6.1a). 20 ng of synthesized first strand cDNA was subjected to 25 or 30 cycles of amplification to synthesize intra-genic and potential gene bridging products, respectively. There was no detectible product following amplification with primers bridging FTL\_1912 and FTL\_1913 (Fig. 6.1b), suggesting that the predicted Rho independent terminator is functional. A faint amplicon was present in reactions using FTL\_1913 – *ripA* bridging primers (Fig. 6.1b). However, the band intensity was significantly lower than that of the *ripA* intra-genic amplicon and was detectable only after the additional cycles of amplification. This result suggests that FTL\_1913 transcription terminates, albeit less efficiently than FTL\_1912, and that *ripA* expression was initiated by a promoter independent of FTL\_1913 expression.

Northern analysis of total RNA harvested from mid exponential phase *F. tularensis* LVS and *F. tularensis* LVS *ripA*::Tn5 (Table 6.1) was used to evaluate the size of *ripA* containing transcripts. The *ripA* coding sequence is 537 nucleotides, and an approximately 600 nucleotide RNA fragment hybridized to an anti-sense *ripA* probe confirming that the *ripA* gene was transcribed (Fig. 6.1c). No *ripA* message was detected in the *F. tularensis* LVS *ripA*::Tn5 RNA samples.

**Construction and characterization of *Francisella*  $\Phi(ripA'$ -*lacZ*) transcriptional and translational fusions.** Transcriptional  $\Phi(ripA'$ -*lacZ*)<sub>2</sub> and translational  $\Phi(ripA'$ -*lacZ*)<sub>1</sub> fusions were constructed to facilitate studies on *ripA* expression (Table 6.1). The sequence 5' of *ripA* was analyzed using BPROM ([www.softberry.com](http://www.softberry.com)) to identify potential promoters.  $\Phi(ripA'$ -*lacZ*)<sub>1</sub> was constructed by amplifying and cloning the *ripA* ATG start codon and 1 kB of preceding sequence in frame with the *Escherichia coli lacZ* gene (AAA24053) in a pBSK *lacZ cat* vector.  $\Phi(ripA'$ -*lacZ*)<sub>2</sub> lacks the *ripA* ATG and preceding 6 bp of untranslated sequence but includes the *E. coli lacZ* initiation codon and ribosome binding site in a pBSK *lacZ aphA1* vector (Fig. 6.2a). Each construct was sequenced to ensure that no mutations were introduced. The  $\Phi(ripA'$ -*lacZ*)<sub>1</sub> *cat* and  $\Phi(ripA'$ -*lacZ*)<sub>2</sub> *aphA1* regions were cloned into the *Francisella* shuttle vector pKK MCS to produce multicopy reporter vectors.

The pBSK  $\Phi(ripA'$ -*lacZ*) clones (Table 6.1), which cannot replicate in *Francisella*, were used to create single copy reporters by co-integration into the chromosome. Use of integrated reporters facilitated the use of *Francisella* shuttle vectors in analyses. Mid exponential phase cultures grown in Chamberlains defined media were assayed for  $\beta$ -galactosidase activity (Miller, 1972). Activity was recorded as mean Miller units  $\pm$  standard

deviation of three replicates. Background  $\beta$ -galactosidase activity in *Francisella tularensis* LVS was less than 1 Miller unit (Data not shown).

The LVS  $\Phi(ripA'-lacZ)1$  and LVS pKK  $\Phi(ripA'-lacZ)1$  reporter strains expressed  $17.4 \pm 0.43$  and  $163.6 \pm 3.91$  Miller units, respectively, whereas the LVS  $\Phi(ripA'-lacZ)2$  and LVS pKK  $\Phi(ripA'-lacZ)2$  reporter strains expressed  $2823 \pm 75.9$  and  $7887 \pm 43.0$  Miller units, respectively (Fig. 6.2b). To validate that *lacZ* expression accurately reflected *ripA* promoter activity we measured the impact of mutations in the predicted -10 promoter region, the RBS, and a frameshift mutation in pKK  $\Phi(ripA'-lacZ)1$  (Fig. 6.2a). In the -10 box mutant the TAGCATTAT sequence was replaced by TAGCTTAC (pKK  $\Phi(ripA'-lacZ)1a$ ). The predicted RBS AGGA was mutated to ACCA (pKK  $\Phi(ripA'-lacZ)1b$ ). The frameshift mutation consisted of a one base deletion in the fourth codon in pKK  $\Phi(ripA'-lacZ)1c$ .  $\beta$ -galactosidase assays were performed on strains containing these constructs in multi-copy. The *ripA* promoter mutations decreased  $\beta$ -galactosidase activity to 67.4% (-10 box), 12.7% (RBS), and 0.4% (frameshift) of the parent reporter (Fig. 6.2c). Thus, the predicted *ripA* promoter and *ripA* RBS are responsible for *lacZ* expression in the reporter strains.

**Quantification of RipA protein.** Attempts to quantify RipA protein concentrations in *Francisella* were complicated by the fact that antisera that we generated produced high background in Western blots and ELISA (Fuller *et al.*, 2008). We therefore generated an expression construct  $\Phi(ripA'-TC)$  fusing a tetracysteine (TC) tag to the C-terminal end of RipA to facilitate the use of FLAsH™ (Invitrogen) reagents to directly measure RipA protein concentrations.

SOE PCR was used to fuse a TC tag with a glycine linker to the C terminus of RipA (GGCCPGCCGGG) while maintaining native *Francisella* flanking DNA sequence (Fig. 6.3b). The TC tag was codon optimized using *F. tularensis* codon usage tables (<http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=119857>).  $\Phi(ripA'-TC)$  was cloned into pMP590 for allelic exchange and pKK MCS for multi-copy expression in *Francisella*.

Whole cell lysates prepared from mid exponential phase bacteria growing in Chamberlains defined media were suspended in FLAsH™ loading buffer containing biarsenical fluorescein and subjected to SDS-PAGE. The RipA-TC fusion protein was detected and quantified by relative mean fluorescence with wild type *F. tularensis* LVS lacking any TC fusion protein serving as a control to determine background fluorescence. To determine the limit of detection of LVS  $\Phi(ripA'-TC)$  and LVS pKK  $\Phi(ripA'-TC)$  (Table 6.1), labeled whole cell lysates (6000 ng to 60 ng total protein) were separated using SDS-PAGE and in - gel fluorescence measured. Three nonspecific biarsenical fluorescein binding proteins were detected between 22 kDa and 30 kDa in size in wild type *F. tularensis* LVS; however, these were easily distinguishable from RipA-TC which migrated at approximately 18 kDa (Fig. 6.3c). RipA-TC expressed from multi-copy plasmid was detectable in 60ng of whole cell lysates while single copy was detected in 600 ng (Fig. 6.3c). The concentration of RipA-TC (multi-copy) was approximately 6.5 fold greater than RipA-TC (single copy). Thus, the use of the RipA-TC fusion in conjunction with biarsenical labeling provided a sensitive and reproducible method to detect and quantify RipA in *Francisella*.

**Expression of *ripA* is pH sensitive.** In separate studies designed to determine the function of RipA we found that  $\Delta ripA$  mutants were sensitive to increasing pH (data not shown). We therefore used  $\Phi(ripA'-lacZ)$  strains to examine the effects of pH on *ripA* expression.  $\beta$ -galactosidase levels from mid exponential phase *F. tularensis* LVS  $\Phi(ripA'-lacZ)$  reporter strains grown in Chamberlains defined media at pH 5.5 and pH 7.5 were measured. The LVS pKK  $\Phi(ripA'-lacZ)1$  reporter strain expressed  $125 \pm 3$  and  $223 \pm 2$  Miller units at pH 5.5 and 7.5, respectively (Fig. 6.4a) representing a 1.8 fold difference (  $P < 0.001$ ). The LVS  $\Phi(ripA'-lacZ)2$  reporter strain expressed  $2618 \pm 121$  and  $3419 \pm 71$  Miller units at pH 5.5 and 7.5, respectively (Fig. 6.4b) representing a 1.3 fold difference ( $P = 0.0016$ ).

RT-PCR and FIASHTM labeling of RipA-TC were used as complementary assays to evaluate the pH effects on *ripA* transcription and protein concentration revealed by the  $\Phi(ripA'-lacZ)$  expression results. RT-PCR of *ripA* was conducted in replicates of four independent cultures and normalized using *tul4* expression (Lauriano *et al.*, 2004). Primers internal to *ripA* and *tul4* were designed with matched melting temperatures and amplicon sizes. Total RNA was collected from *F. tularensis* LVS to compare *ripA* transcript levels at mid exponential growth in Chamberlains Defined media at pH 5.5 and pH 7.5. cDNA was generated from the RNA samples using random primers in a reverse transcriptase reaction. Samples lacking reverse transcriptase negative were used to monitor DNA contamination. Quantitation of *ripA* expression was achieved by densitometry of gene-specific products isolated by agarose electrophoresis. Mean normalized expression of *ripA*  $\pm$  standard deviation at pH 5.5 was  $1.527 \pm 0.1656$  and  $2.448 \pm 0.2934$  at pH 7.5 (Fig. 6.4c) representing a 1.6 fold expression differential ( $P = 0.0033$ ). This result was consistent with the 1.3 fold difference in expression exhibited by the  $\Phi(ripA'-lacZ)2$  transcriptional fusion strain.

The concentration of RipA protein present at pH 5.5 and pH 7.5 were measured by FLAsH™ labeling RipA-TC in LVS  $\Phi(ripA'-TC)$  whole cell lysates (Table 6.1). Six  $\mu$ g of total protein was incubated with TC specific FLAsH™ reagents, separated by SDS-PAGE and subjected to in-gel fluorescence. Mean intensity of RipA-TC  $\pm$  standard deviation of four independent samples at pH 5.5 was  $1.083 \times 10^7 \pm 6.340 \times 10^5$  arbitrary units as compared to  $1.551 \times 10^7 \pm 8.734 \times 10^5$  arbitrary units at pH 7.5 (Fig. 6.4d), representing a 1.43 fold change in expression (P = 0.00031) as compared to the 1.8 fold difference expressed by the  $\Phi(ripA'-lacZ)1$  translational fusion.

***F. tularensis* LVS *ripA* expression is suppressed during intracellular growth.** In a previous study we found that an *F. tularensis* LVS  $\Delta ripA$  mutant entered host cells and escaped from the phagosome, but failed to replicate upon release to the cytoplasm (Fuller *et al.*, 2008). Since *ripA* expression is required for late, but not early stages of host cell interaction, we hypothesized that *ripA* may be differentially regulated during intracellular growth. To test this hypothesis, we measured  $\beta$ -galactosidase produced by *F. tularensis*  $\Phi(ripA'-lacZ)2$  at different stages of intracellular growth in J774A.1 macrophage-like cells. Expression of *iglA*, a gene located on the pathogenicity island, is induced upon entry into host cells (de Bruin *et al.*, 2007). We therefore constructed a strain expressing an  $\Phi(iglA'-lacZ)$  transcriptional fusion (Table 6.1) for use as a control in these experiments. Host cell entry was synchronized by centrifugation of reporter strains onto chilled J774A.1 monolayers as described (Craven *et al.*, 2008) and LacZ activity was measured in the inocula, and at 1, 6, and 24 hours post inoculation using a modified  $\beta$ -galactosidase assay based on the rate and amount of chlorophenol red- $\beta$ -D-galactopyranoside (CPRG) conversion per CFU.

The mean activity ( $\pm$  standard deviation) of  $\beta$ -galactosidase produced by LVS  $\Phi(\textit{ripA}'\textit{-lacZ})_2$  at 1, 6, and 24 hours was 155.9 ( $\pm$  12.96), 193.5 ( $\pm$  23.99), and 80.6 ( $\pm$  17.83) units, respectively (Fig. 6.5a). The activity of the inoculum was 199.7 ( $\pm$  13.32) units. Each data point was significantly different from all others ( $P < 0.05$ ). The most significant change in intracellular *ripA* expression was at 24 hours post inoculation as compared to the inoculum. By this time,  $\Phi(\textit{ripA}'\textit{-lacZ})_2$  expression was approximately two fold lower than the expression at all previous time points.

The mean ( $\pm$  standard deviation)  $\beta$ -galactosidase activity of LVS  $\Phi(\textit{iglA}'\textit{-lacZ})$  at 1, 6, and 24 hours was 97.8 ( $\pm$  9.59), 199.4 ( $\pm$  26.24), and 112.0 ( $\pm$  24.21) (Fig. 6.5b). The activity of the inoculum was 135.0 ( $\pm$  9.59). The 6 hours activity was significantly higher than both the 1 hour and 24 hours time points ( $P < 0.01$ ). This result is in accordance with the published data on intracellular IglA expression (de Bruin *et al.*, 2007). Both  $\Phi(\textit{ripA}'\textit{-lacZ})_2$  and  $\Phi(\textit{iglA}'\textit{-lacZ})$  were marginally, but significantly ( $P < 0.05$ ), down at 1 hour post inoculation as compared to the inoculums.

### **The transcriptional regulators *mglA* and *sspA* decrease $\Phi(\textit{ripA}'\textit{-lacZ})_2$ expression.**

MglA and SspA are transcriptional regulators that associate with DNA and RNA-polymerase and modulate the expression of a number of stress response and virulence associated genes in *F. tularensis* (Brotcke *et al.*, 2006; Charity *et al.*, 2007; Guina *et al.*, 2007; Lauriano *et al.*, 2004). In a recent study comparing protein expression profiles of wild type and *mglA* mutant strains, the concentration of IglA was decreased and RipA increased in an *mglA* mutant compared to wild type (Guina *et al.*, 2007). We investigated further the relationship between

the regulators MglA and SspA, and RipA expression using the  $\Phi(ripA'-lacZ)_2$  and  $\Phi(iglA'-lacZ)$  transcriptional fusions in  $\Delta mglA$  and  $\Delta sspA$  mutants (Table 6.1).

$\beta$ -galactosidase assays were conducted on mid exponential phase reporter strains grown in Chamberlains defined media. The mean expression of *ripA* was nearly two-fold higher in the  $\Delta mglA$  ( $4091 \pm 75$ ) and  $\Delta sspA$  ( $4602 \pm 52$ ) strains as compared to wild type ( $2549 \pm 128$ ) (Fig. 6.5a). Wild type levels of expression were restored by the wild type alleles in the complemented mutant strains (Fig. 6.5a). The differences of *ripA* expression in the mutant backgrounds were all significantly different from wild type ( $P < 0.01$ ).

The *mglA* and *sspA* deletions had the opposite effect on *iglA* expression. The mean expression ( $\pm$  standard deviation) of LVS  $\Phi(iglA'-lacZ)$  was 2757 ( $\pm 98$ ) Miller units, but only 80 ( $\pm 2.2$ ) and 67 ( $\pm 0.9$ ) Miller units in  $\Delta mglA$  and  $\Delta sspA$  strains, respectively (Fig 6.6b). The differences of *iglA* expression in the mutant backgrounds were all significantly different from wild type ( $P < 0.01$ ), and near wild type levels of expression were restored by complementation with *mglA* and *sspA* *in trans* (Fig. 6.6b). Together, these results confirm that *mglA* and *sspA* expression positively influence *iglA* expression, and conversely demonstrate that these two regulators negatively influence *ripA* expression.

## DISCUSSION

As a facultative intracellular pathogen *F. tularensis* is able to survive and replicate within several different types of eukaryotic cells as well as in a number of extracellular environments (Abd *et al.*, 2003; Bosio & Dow, 2005; Craven *et al.*, 2008; Forestal *et al.*, 2007; Hall *et al.*, 2006; Kobayasi, 1960; Thorpe & Marcus, 1964). Organisms similarly capable of adapting to multiple environments such as *Salmonella typhimurium* (Chen *et al.*,

1996), *Legionella pneumophila* (Bruggemann *et al.*, 2006), and *Listeria monocytogenes* (Chatterjee *et al.*, 2006; Moors *et al.*, 1999) exhibit differential gene expression in response to entering or exiting host cells and even as they transition between intra-vacuolar and cytoplasmic niches. These studies have helped identify bacterial products that function specifically in adaptation to, and manipulation of the host cell environment.

Studies on intracellular gene expression by *Francisella* species revealed a number of genes including *iglC* (Golovliov *et al.*, 1997), *iglA* (de Bruin *et al.*, 2007) and *mglA* (Baron & Nano, 1999), that are induced upon entry and growth in macrophages. IglC protein concentrations increase between 6 hours and 24 hours post host cell invasion (Golovliov *et al.*, 1997). Similarly IglA protein concentrations increase between 8 hours and 12 hours post invasion as measured by Western blot (de Bruin *et al.*, 2007), and in this study we found that *iglA* expression was induced during intracellular growth, but not immediately after host cell invasion.

MglA is a transcriptional regulator (Baron & Nano, 1998; Charity *et al.*, 2007). Transcription of the *mglA* gene is induced in macrophages as early as 1 to 4 hours post invasion (Baron & Nano, 1999). Results from microarray and proteomic (Brotcke *et al.*, 2006; Charity *et al.*, 2007; Guina *et al.*, 2007; Lauriano *et al.*, 2004) analyses demonstrate that IglC and IglA expression are regulated by MglA. After entry into the host cell, *Francisella* is transiently associated with a membrane bound phagosome, but within 2 hours post invasion greater than 90% of internal bacteria are free in the cytoplasm (Checroun *et al.*, 2006; Craven *et al.*, 2008; Golovliov *et al.*, 2003). Coupled with the kinetics of gene expression changes, there is a strong correlation between MglA regulated genes and adaptation to the cytoplasmic environment.

The *ripA* gene was not identified as being regulated by MglA or SspA in transcriptional profiling studies of *mglA* and *sspA* mutant strains by microarray (Charity *et al.*, 2007). However, in complementary proteomic studies RipA (FTN\_0157) is present in two fold higher amounts in an *mglA* mutant strain as compared to wild type *F. novicida* in mid exponential phase bacteria cultured in rich media (Guina *et al.*, 2007). This result suggested that MglA has a repressive effect, directly or indirectly, on RipA expression. We tested this hypothesis using  $\Phi(\textit{ripA}'\textit{-lacZ})$  reporters and found that *ripA* expression was increased significantly in both  $\Delta\textit{mglA}$  and  $\Delta\textit{sspA}$  mutants. Thus, results using the  $\Phi(\textit{ripA}'\textit{-lacZ})$  reporters correlated with proteomics analysis of MglA regulation and not with published microarray – based results. This may be due at least in part to the relative magnitude of the MglA and SspA effects on *ripA* expression.

We found that *F. tularensis ripA* and *iglA* gene expression changes during the first 24 hours of growth in the intramacrophage environment progressing from vacuolar, to early cytoplasmic, and then late cytoplasmic stages of infection. MglA, which is involved in the regulation of a number of genes that function in intracellular growth, had opposing effects on *iglA* and *ripA* expression exerting positive and negative effects, respectively. On the surface this appears to present a paradox with respect to *ripA* expression patterns and the requirement of *ripA* for intracellular growth and survival. However, it is important to note that under no condition or circumstance tested was *ripA* completely repressed. Genes required for *Francisella* virulence may be either promoted or suppressed by MglA. This suggests that the intracellular regulation of MglA causes a temporal compartmentalization of gene expression with different patterns of up or down regulation during the different phases of the *Francisella* intracellular lifecycle. Further work on the transcriptomes and proteomes during these

phases of the *F. tularensis* intracellular lifestyle may give insight into the programmed response of gene expression that makes *F. tularensis* such a successful pathogen in the host intracellular environment.

### **ACKNOWLEDGEMENTS**

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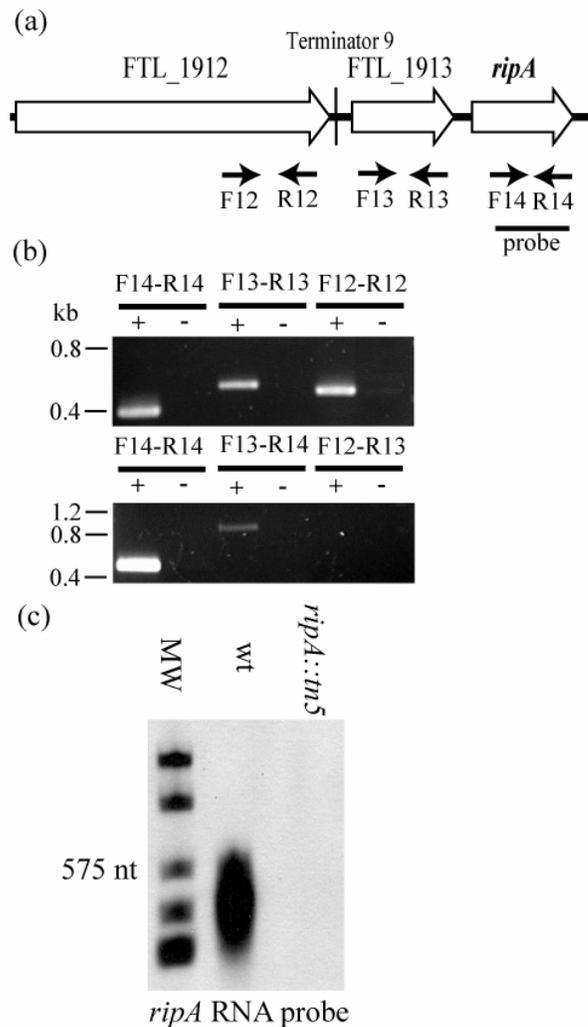
### **ATTRIBUTIONS**

I performed all experiments in this chapter with the following exceptions. Sharon Taft-Benz and Todd Kijek provided assistance with the  $\beta$ -galactosidase assays.

## TABLES AND FIGURES

**Table 6.1. Bacterial strains and plasmids.**

Strains or Plasmid	Description	Source or Reference
<b>Bacteria</b>		
<i>Francisella tularensis</i>		
LVS	<i>F. tularensis</i> live vaccine strain	CDC, Atlanta, GA
LVS <i>ripA</i> ::Tn5	Tn5 <i>ripA</i> transposon mutant	(Fuller <i>et al.</i> , 2008)
LVS $\Phi$ ( <i>ripA</i> '-lacZ)1	BSK $\Phi$ ( <i>ripA</i> '-lacZ)1 <i>cat</i> cointegrate	This work
LVS $\Phi$ ( <i>ripA</i> '-lacZ)2	BSK $\Phi$ ( <i>ripA</i> '-lacZ)2 <i>aphA1</i> cointegrate	This work
LVS $\Phi$ ( <i>iglA</i> '-lacZ)	BSK $\Phi$ ( <i>iglA</i> '-lacZ)1 <i>aphA1</i> cointegrate	This work
LVS $\Phi$ ( <i>ripA</i> '-TC)	Replacement of <i>ripA</i> with $\Phi$ ( <i>ripA</i> '-TC)	This work
LVS $\Delta$ <i>mglA</i>	Inframe deletion of <i>mglA</i>	This work
LVS $\Delta$ <i>mglA</i> $\Phi$ ( <i>ripA</i> '-lacZ)2	BSK $\Phi$ ( <i>ripA</i> '-lacZ)2 <i>aphA1</i> cointegrate	This work
LVS $\Delta$ <i>mglA</i> $\Phi$ ( <i>iglA</i> '-lacZ)	BSK $\Phi$ ( <i>iglA</i> '-lacZ)1 <i>aphA1</i> cointegrate	This work
LVS $\Delta$ <i>sspA</i>	Inframe deletion of <i>sspA</i>	This work
LVS $\Delta$ <i>sspA</i> $\Phi$ ( <i>ripA</i> '-lacZ)2	BSK $\Phi$ ( <i>ripA</i> '-lacZ)2 <i>aphA1</i> cointegrate	This work
LVS $\Delta$ <i>sspA</i> $\Phi$ ( <i>iglA</i> '-lacZ)	BSK $\Phi$ ( <i>iglA</i> '-lacZ)1 <i>aphA1</i> cointegrate	This work
LVS $\Delta$ <i>mglA</i> $\Delta$ <i>sspA</i>	Inframe deletion of <i>sspA</i> and <i>mglA</i>	This work
LVS $\Delta$ <i>mglA</i> $\Delta$ <i>sspA</i> $\Phi$ ( <i>ripA</i> '-lacZ)2	BSK $\Phi$ ( <i>ripA</i> '-lacZ)2 <i>aphA1</i> cointegrate	This work
LVS $\Delta$ <i>mglA</i> $\Delta$ <i>sspA</i> $\Phi$ ( <i>iglA</i> '-lacZ)	BSK $\Phi$ ( <i>iglA</i> '-lacZ)1 <i>aphA1</i> cointegrate	This work
<b>Plasmids</b>		
pBSK <i>bla lacZ</i>	pBluescript cloning vector	Stratagene
pBSK <i>lacZ aphA1 bla</i>	Amp <sup>R</sup> , <i>Francisella</i> Km <sup>R</sup> , <i>lacZ</i> reporter	This work
pBSK <i>lacZ cat bla</i>	Amp <sup>R</sup> , <i>Francisella</i> Cm <sup>R</sup> , <i>lacZ</i> reporter	This work
pBSK $\Phi$ ( <i>ripA</i> '-lacZ)2 <i>aphA1 bla</i>	<i>Francisella</i> Km <sup>R</sup> , Transcriptional fusion	This work
pBSK $\Phi$ ( <i>ripA</i> '-lacZ)1 <i>cat</i>	<i>Francisella</i> Cm <sup>R</sup> , Translational fusion	This work
pBSK $\Phi$ ( <i>iglA</i> '-lacZ)2 <i>aphA1</i>	<i>Francisella</i> Km <sup>R</sup> , Transcriptional fusion	This work
pMP590	Km <sup>R</sup> , <i>sacB Francisella</i> suicide vector	(LoVullo <i>et al.</i> , 2006)
pMP590 $\Delta$ <i>mglA</i>	<i>mglA</i> allelic exchange vector	This work
pMP590 $\Delta$ <i>sspA</i>	<i>sspA</i> allelic exchange vector	This work
pMP590 $\Phi$ ( <i>ripA</i> '-TC)	$\Phi$ ( <i>ripA</i> '-TC) allelic exchange vector	This work
pMP633	Hyg <sup>R</sup> , <i>E. coli</i> - <i>F. tularensis</i> shuttle vector	(LoVullo <i>et al.</i> , 2006)
pMP633 <i>mglA</i> <sup>+</sup>	<i>mglA</i> <sup>+</sup> allele with native promoter	This work
pMP633 <i>sspA</i> <sup>+</sup>	<i>sspA</i> <sup>+</sup> allele with native promoter	This work
pKK MCS	Km <sup>R</sup> , <i>E. coli</i> - <i>F. tularensis</i> shuttle vector	(Fuller <i>et al.</i> , 2008)
pKK MCS $\Phi$ ( <i>ripA</i> '-lacZ)1	Km <sup>R</sup> , translational fusion of <i>ripA</i> and <i>lacZ</i>	This work
pKK MCS $\Phi$ ( <i>ripA</i> '-lacZ)1a	Km <sup>R</sup> , $\Phi$ ( <i>ripA</i> '-lacZ)1, -10 mutation	This work
pKK MCS $\Phi$ ( <i>ripA</i> '-lacZ)1b	Km <sup>R</sup> , $\Phi$ ( <i>ripA</i> '-lacZ)1, RBS mutation	This work
pKK MCS $\Phi$ ( <i>ripA</i> '-lacZ)1c	Km <sup>R</sup> , $\Phi$ ( <i>ripA</i> '-lacZ)1, <i>lacZ</i> frameshift	This work
pKK MCS $\Phi$ ( <i>ripA</i> '-lacZ)2	Km <sup>R</sup> , transcriptional fusion	This work
pKK MCS $\Phi$ ( <i>ripA</i> '-TC)	$\Phi$ ( <i>ripA</i> '-TC), CT tetracycline tag fusion	This work



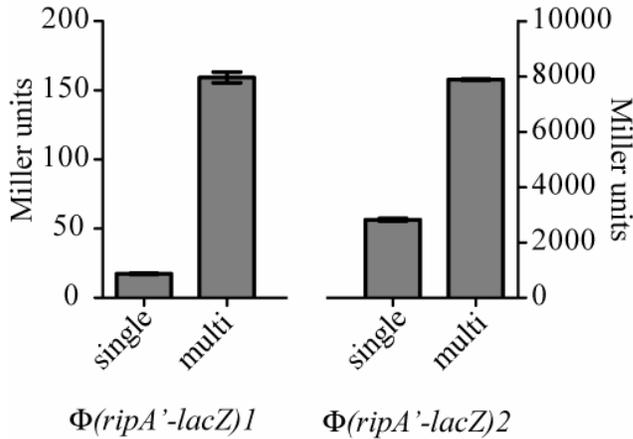
**Figure 6.1.** (a) Graphical representation of the *ripA* region. Primers utilized for RT-PCR are marked with arrows while the region complementary to the RNA probe used in the Northern analysis is demarcated by a solid line. (b) RT-PCR analysis of the expression of genes FTL\_1912 (F14-R14), FTL\_1913 (F13-R13), and *ripA* (F14-R14) shown in the upper image. Analysis for transcripts bridging FTL\_1912 to FTL\_1913 (F12-R13) and FTL\_1913 to *ripA* (F13-R14) shown in lower image. PCR of cDNA demarcated by a (+) and reverse transcriptase negative reactions to assess DNA contamination marked as (-). (c) Northern analysis to determine the transcript size of *ripA* containing RNA. Roche digoxigenin labeled RNA ladder is present in the left most lane followed by total RNA from *F. tularensis* LVS (wt) and *F. tularensis* LVS *ripA*:: Tn5. This analysis used a *ripA* complementary digoxigenin labeled RNA probe demonstrating the presence of monocistronic *ripA* transcript in LVS and the absence of the transcript in *F. tularensis* LVS *ripA*::Tn5.

(a)

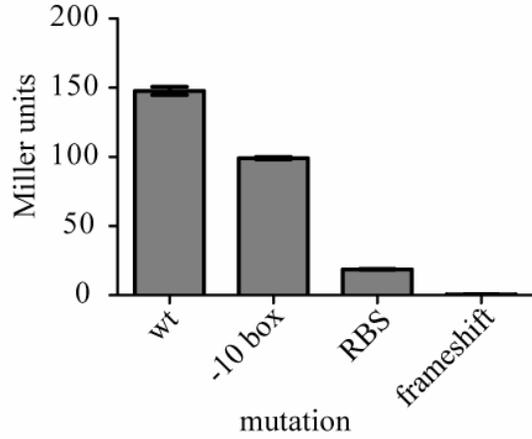
```
translational fusion
Φ(ripA'-lacZ)1  ATGTAATAGCATTATCAGTGTA AATTC TTTGGATAAATGGATTAGAAAGGAATAAA  M D G D P
-10 mutant     ATGTAATAGCCTTACAGTGTA AATTC TTTGGATAAATGGATTAGAAAGGAATAAA  ATG GAt ggg gat ccc
RBS mutant     ATGTAATAGCATTATCAGTGTA AATTC TTTGGATAAATGGATTAGAAAGGAATAAA  ATG GAt ggg gat ccc
frameshift     ATGTAATAGCATTATCAGTGTA AATTC TTTGGATAAATGGATTAGAAAGGAATAAA  ATG GAt ggg g-t ccc

transcriptional fusion
Φ(ripA'-lacZ)2  ATGTAATAGCATTATCAGTGTA AATTC TTTGGATAAATGGATTAGAAAGGttaattaaaggaggaaacagct atg  M
```

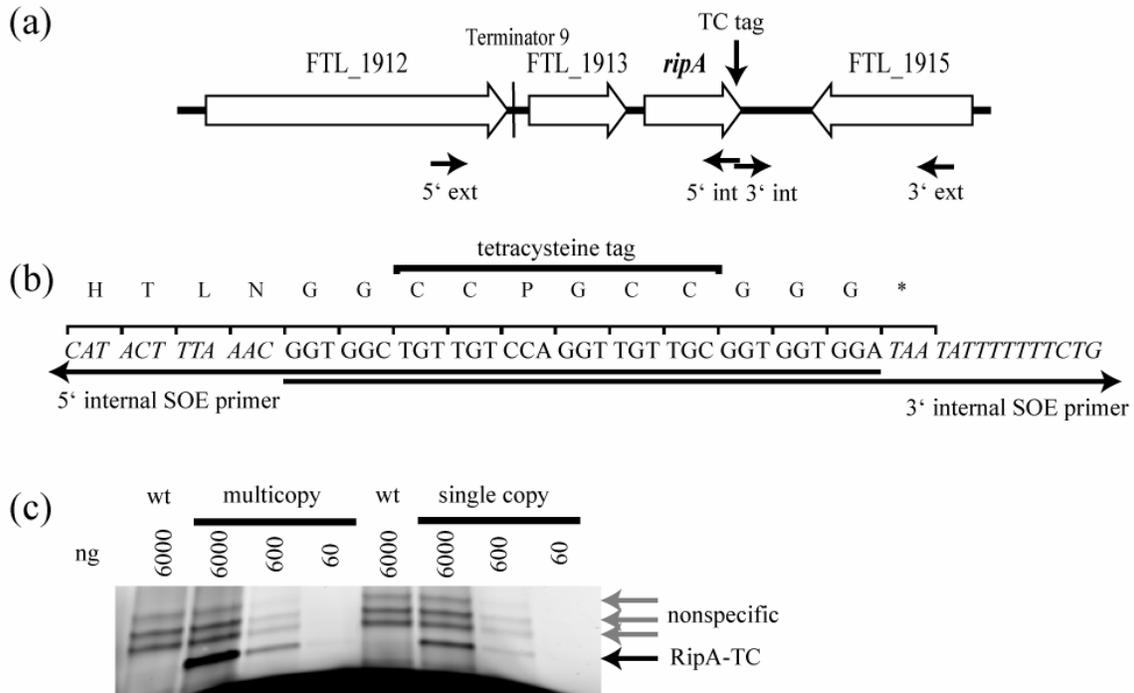
(b)



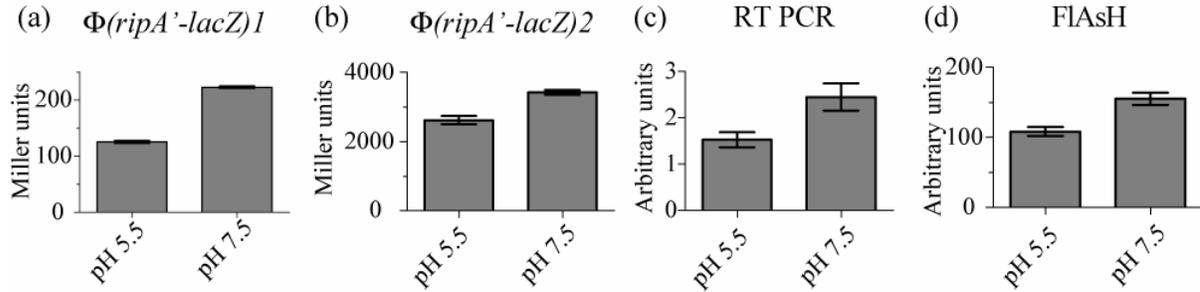
(c)



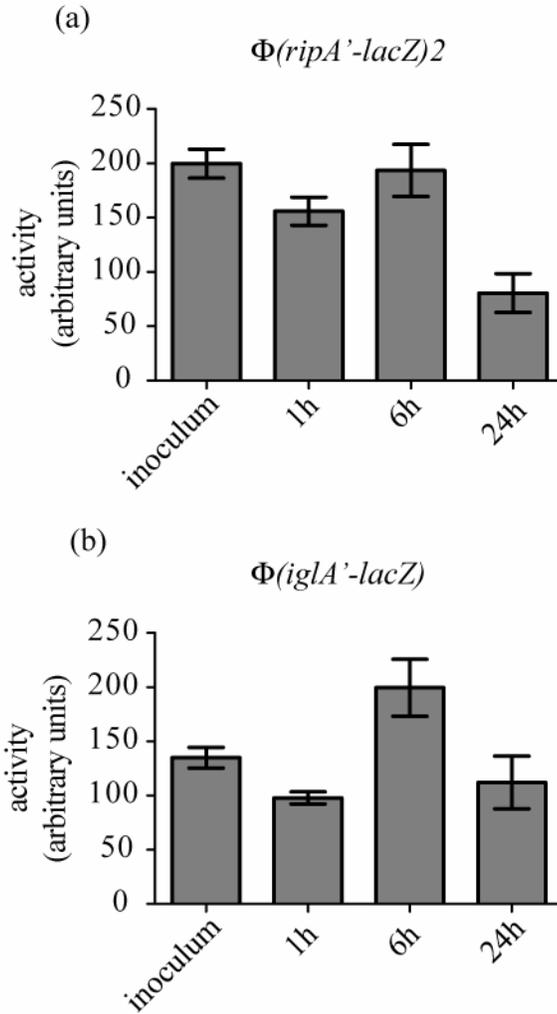
**Figure 6.2.** (a) Multiple sequence alignment of translational and transcriptional  $\Phi(ripA'-lacZ)$  fusions. Predicted -10 and RBS sequences are boxed with introduced mutations in each highlighted. (b)  $\beta$ -galactosidase activity of single and multi-copy translational and transcriptional *F. tularensis* LVS  $\Phi(ripA'-lacZ)$  reporter strains displayed as mean Miller units. Error bars represent standard deviation of three independent samples. (c)  $\beta$ -galactosidase activity of *F. tularensis* LVS multi-copy translational  $\Phi(ripA'-lacZ)1$  promoter mutations displayed as mean Miller units. Error bars represent standard deviation of three independent samples.



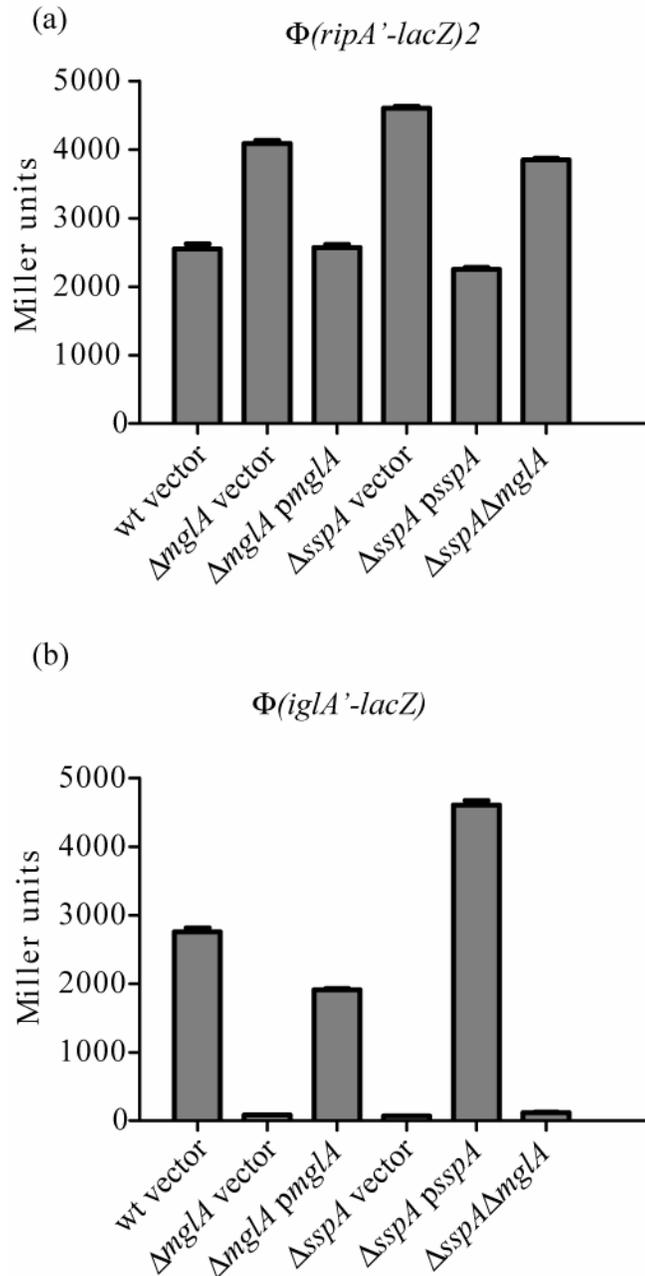
**Figure 6.3.** (a) Graphical depiction of *F. tularensis* LVS *ripA* locus showing the location of SOE PCR primers used to insert the C terminal TC tag. (b) Nucleotide and amino acid sequence of the C terminal TC tag showing the overlapping sequence of the SOE PCR primers. (c) In gel fluorescence of RipA-TC (black arrow) from dilution series of *F. tularensis* LVS (multi-copy) pKK  $\Phi(ripA'-TC)$  and *F. tularensis* LVS (single copy)  $\Phi(ripA'-TC)$  using 6000 ng to 60 ng total protein of whole cell lysates. *F. tularensis* LVS lysates (wt) used as a non TC tagged control displaying three non specific bands (gray arrows) at a higher molecular weight than RipA-TC.



**Figure 6.4.** Effect of pH on *F. tularensis* LVS *ripA* expression. All experiments were performed using mid exponential phase bacteria cultured in Chamberlains defined media at pH 5.5 or pH 7.5. Data are presented as mean values with error bars representing one SD. (a)  $\beta$ -galactosidase activity of *F. tularensis* LVS pKK  $\Phi(ripA'-lacZ)1$  at pH 5.5 and pH 7.5. Difference in expression levels were significant ( $P < 0.01$ ). (b)  $\beta$ -galactosidase activity of *F. tularensis* LVS  $\Phi(ripA'-lacZ)2$  at pH 5.5 and pH 7.5. Difference in expression levels were significant ( $P < 0.01$ ). (c) *F. tularensis* LVS *ripA* RNA concentrations displayed as *tul4* normalized mean trace (Int mm) on four independent RT-PCR reactions using purified total RNA samples of mid exponential *F. tularensis* LVS cultured at pH 5.5 and pH 7.5. Difference in expression levels were significant ( $P < 0.01$ ). (d) RipA-TC concentration in whole cell lysates of mid exponential phase *F. tularensis* LVS  $\Phi(ripA'-TC)$  cultured at pH 5.5 and pH 7.5. Concentrations were measured using densitometry of the specific in-gel fluorescence of FIAsh™ labeled RipA-TC. Four independent samples were used to calculate mean expression. Difference in expression was significant ( $P < 0.01$ ).



**Figure 6.5.** Intracellular expression of LVS  $\Phi(ripA'-lacZ)2$  (a) and LVS  $\Phi(iglA'-lacZ)$  (b) in J774A.1 mouse macrophage like cells infected at an MOI of 100. All assays were performed on four independent samples and reported as mean activity  $\pm$  standard deviation. Activities were calculated from four samples taken before application of the inoculums. Mean  $\beta$ -galactosidase activity is normalized by time of development and CFU per well minus the activity from the control samples. All differences in expression were significant ( $P < 0.05$ ) with the exception of comparisons between  $\Phi(ripA'-lacZ)2$  inoculum to 6h, and  $\Phi(iglA'-lacZ)$  1h to 24h.



**Figure 6.6.** MglA and SspA effects on *ripA* and *iglA* expression. Mid exponential phase cultures of the indicated transcriptional *lacZ* reporter strains cultured in Chamberlains defined media were assayed for  $\beta$ -galactosidase activity in replicates of three and reported as mean Miller units  $\pm$  standard deviation. (a) *F. tularensis* LVS  $\Phi(ripA'-lacZ)2$  expression in wild type (wt),  $\Delta mglA$ ,  $\Delta sspA$ , and  $\Delta mglA\Delta sspA$  backgrounds. *In trans* complementation (*pmglA* and *psspA*) was accomplished using wild type alleles and native promoters cloned into pMP633. *F. tularensis* LVS pMP633 was used as the vector only control (vector). (b) *F. tularensis* LVS  $\Phi(iglA'-lacZ)$  expression in wild type (wt),  $\Delta mglA$ ,  $\Delta sspA$ , and  $\Delta mglA\Delta sspA$  backgrounds.

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## Chapter 7

### Transposon screen for regulators of *ripA* expression

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#### ABSTRACT

Expression of the *Francisella* virulence factor RipA changes through the course of intracellular growth. We screened a transposon mutant library to identify potential regulators of *ripA*. Insertions in FTL\_0439 and FTL\_0073 which are predicted to encode two putative envelope proteins of unknown function resulted in a 3.95 fold increase and 0.63 fold decrease in *ripA* expression respectively. Insertions in two known regulatory loci, *hupB* and *nusA*, resulted in 0.68 and 0.17 fold decreases in *ripA* expression respectively. HU form B is encoded by *hupB* and is a DNA binding protein, whereas NusA regulates termination and anti-termination by Rho. The *nusA::Tn5* mutant was not complemented by the wild type allele. However, expression of *nusA* in the wild type background resulted in decreased *ripA* expression. We concluded that the change in *ripA* expression was due to polar effects on the downstream *infB* locus and possible autoregulation by NusA on *nusA-infB* expression.  $\Delta hupB$  and  $\Delta FTL\_0073$  mutants were constructed and analyzed for their effects on *ripA* expression. The  $\Delta hupB$  mutation resulted in a 0.40 fold decrease in *ripA* expression that was

partially restored by the wild type allele *in trans*. The  $\Delta$ F<sub>FTL\_0073</sub> mutation resulted in a 2.45 fold increase in *ripA* expression that was restored by the wild type allele *in trans*.

## INTRODUCTION

Research on the regulation of virulence factors in *Francisella tularensis* has focused almost exclusively on the transcriptional factors MglA and SspA (Barker & Klose, 2007; Baron & Nano, 1998; Charity *et al.*, 2007; Guina *et al.*, 2007; Lauriano *et al.*, 2004). MglA and SspA bind cooperatively to RNA polymerase to positively regulate the transcription of many *F. tularensis* genes required for intracellular replication (Charity *et al.*, 2007). MglA is necessary for intracellular replication and is induced after cell invasion (Baron & Nano, 1999). RipA is a cytoplasmic membrane protein in *F. tularensis* that is required for intracellular replication and virulence in a mouse model of tularemia (Fuller *et al.*, 2008a). RipA expression is repressed after cell invasion (Chapter 6). This seeming paradox of intracellular repression of a protein required for intracellular replication is mitigated by the fact that RipA expression is only lowered, not completely absent at this stage of infection (Chapter 6). A remaining question is how MglA, described as a positive regulator of transcription, acts to repress gene expression. We hypothesize that MglA acts indirectly through other loci to negatively impact *ripA* expression. To test this hypothesis, we constructed and screened a transposon mutant library for mutations that effected *ripA* expression.

## MATERIALS AND METHODS

**Bacterial strains and Cell culture.** *Escherichia coli* TOP10 cells were used for propagating clones. *E. coli* cultures were grown in Luria's broth at 37 °C with shaking. *Francisella tularensis* Live Vaccine Strain (LVS) was obtained from the Centers for Disease Control and Prevention, Atlanta, Ga. *F. tularensis* LVS was propagated on chocolate agar (25g/l BHI, 50µg/ml hemoglobin, 15g/l agar) supplemented with 1% (v/v) IsoVitaleX (Becton-Dickson), complete BHI broth (35g/l BHI, 50µg/ml hemin, 1% (v/v) IsoVitallex), or Chamberlains Defined Media (Chamberlain, 1965). All bacterial strains cultured on chocolate agar were grown at 37°C. Broth cultures were incubated in a shaking water bath at 37 °C.

**Plasmids and Molecular Techniques.** Cloning of *F. tularensis* LVS DNA was accomplished as described previously (Fuller *et al.*, 2008a). Sequence integrity was confirmed by DNA sequencing and comparison to the *F. tularensis* LVS genome (NC\_007880). The *Francisella* shuttle vector pKK MCS (Fuller *et al.*, 2008a) was used for *trans* complementation experiments. Kanamycin selection was maintained at 20 µg/ml for *E. coli* TOP10, and 10 µg/ml for *F. tularensis* LVS.

**Transposon mutagenesis and colony selection.** The *Francisella* Tn5 kan<sup>R</sup> linear transposon was removed from the pMOD-2 backbone (Epicentre) by restriction digest with PvuII. The transposon was purified by agarose gel electrophoresis in the absence of ethidium bromide, since ethidium bromide reduces the efficiency of later transposition reactions. Linear transposon was mixed with transposase by the manufacturer's protocols to form DNA-protein transposome complexes (Epicentre). The transposome was introduced into *F. tularensis* LVS  $\Phi(ripA'-lacZ)1$  (Fuller *et al.*, 2008b) by electrotransformation using a Bio-

Rad Gene Pulsar set at 2.0 kV, 25  $\mu$ F, and 200  $\Omega$ . Immediately following electrotransformation the cells were suspended in 1 ml of BHI broth, incubated for 4 h at 37°C, and then plated on chocolate agar containing 10  $\mu$ g of kanamycin/ml and 50  $\mu$ g/ml X-gal. Colonies were chosen for screening based on altered blue/white phenotype from the parent reporter strain.

**Galactosidase assays.** The activities of reporter strains were analyzed by quantitative  $\beta$ -galactosidase assay as described (Fuller *et al.*, 2008b; Miller, 1972). Reporter strains were cultured overnight in Chamberlains defined media and diluted 1:10 before growth to mid exponential phase (OD<sub>600</sub> 0.2 to 0.8) (Fuller *et al.*, 2008b). Activity was calculated as follows:

$$\text{Miller Units} = 1000 * \text{OD}_{420} * \Delta T^{-1} * \text{CBR}^{-1} * \text{OD}_{600}^{-1}$$

Statistical analysis was calculated as mean Miller units and standard deviation in replicates of three independent samples and significance determined using an unpaired two tailed t test with unequal variance.

**Transposon insertion location.** Transposon insertion sites were determined by two-stage semi-degenerate PCR and DNA sequencing (Jacobs *et al.*, 2003) and compared to the *F. tularensis* LVS genome to determine insertion location and orientation.

**Statistical analysis.** The predicted number of *F. tularensis* LVS reading frames containing a transposon insertion was calculated using a neutral-base pair model (Jacobs *et al.*, 2003). In this model, every base-pair is assumed to be equally likely to contain a transposon insertion.

The probability of an open reading frame containing an insertion ( $p_j$ ) is based upon the open reading frame size and chromosome size (1,895,994 bp). Coverage was calculated based upon the number of transposon insertions ( $n = 16,000$ ) and the number of annotated reading frames ( $k = 1,967$ ) with the addition of an extra open reading frame consisting of non-coding DNA. For a neutral-base pair model the expected number of open reading frames hit is.

$$k - \sum_{j=1}^k (1 - p_j)^n$$

**Mutagenesis and Allelic Exchange.** Deletion constructs were made by splice overlap extension (SOE) PCR as described (Fuller *et al.*, 2008a; Horton *et al.*, 1989) using primers designed to delete the *hupB* and FTL\_0073 loci. The deletion constructs were ligated into pMP590 to make suicide vectors for allelic exchange. Allelic exchange was conducted as described (LoVullo *et al.*, 2006). Native loci with predicted promoters were cloned into pKK MCS for *in trans* complementation experiments (Fuller *et al.*, 2008a).

## RESULTS AND DISCUSSION

**Construction of an improved *Francisella* transposon.** Transposon mutagenesis of *Francisella tularensis* is a viable means to create a library of mutants that can be screened for informative phenotypes (Kawula *et al.*, 2004). Mutagenesis of *F. tularensis* with existing transposons resulted in insufficient coverage of the genome and insertion bias. However, Gallagher *et al.* improved upon this by using a *Francisella* promoter to drive an antibiotic resistance gene on a transposon. Using this transposon they isolated mutations in 84% of the predicted open reading frames of *F. novicida* (Gallagher *et al.*, 2007). To construct an

improved Tn5 based transposon for use in *Francisella* we synthesized a *Francisella* codon optimized *aphA1* allele expressed by the *Francisella groEL* promoter and cloned it into the Epicentre pMOD-2 <MCS> EZ-Tn5™ transposon construction vector. The resulting transposon was purified as described and mixed with purified transposase enzyme creating a transosome. The transposon complexed with transposase is inactive in solution with limiting magnesium. Following electroporation into *Francisella* the increased intracellular magnesium concentration activates the transposase enzyme (Kawula *et al.*, 2004) initiating insertion of the transposon into the chromosome. The highest efficiency of transformation was achieved using mid-exponential phase *F. tularensis* cultured in BHI broth. Greater than 10<sup>4</sup> kanamycin resistant mutants were generated with each electrotransformation with no insertional bias (data not shown).

**Screen for *ripA* regulators.** Investigation of *Francisella* gene regulation has focused on the positive transcriptional regulator MglA (Charity *et al.*, 2007). MglA has a repressive role with a limited number of genes including *ripA* (Guina *et al.*, 2007). Based on the hypothesis that other genes operate in conjunction with MglA to negatively impact *ripA* expression we screened for genes that when interrupted by Tn5 insertion impacted *ripA* expression. We used  $\beta$ -galactosidase activity of a *F. tularensis* LVS  $\Phi(ripA'-lacZ)1$  reporter strain to analyze *ripA* expression. This strain was chosen because of the low basal level of  $\beta$ -galactosidase activity expressed, because *F. tularensis* is sensitive to high concentrations of the X-gal cleavage product (Baron & Nano, 1999). This strain grew with no apparent difference from wild type on Chocolate agar plates containing 50  $\mu\text{g ml}^{-1}$  X-gal. Strains with native  $\Phi(ripA'-lacZ)1$  expression (~20 Miller units) formed light blue colonies. Changes in  $\Phi(ripA'-lacZ)1$

expression were identified by alterations in colony color when grown in Chocolate agar containing X-gal.

The  $\Phi(ripA'-lacZ)$  strain was transformed with transposome and plated on Chocolate agar containing kanamycin to select for insertions and X-gal to screen for  $\Phi(ripA'-lacZ)$  expression variations.  $1.6 \times 10^4$  transposon mutants from two independent electrotransformations were screened on X-gal Chocolate agar for altered  $\Phi(ripA'-lacZ)$  expression based on color different from the parent reporter strain. Control electrotransformations with no transposome mix resulted in a spontaneous kanamycin resistance at a frequency of less than 1 in  $10^{11}$  (no resistant colonies were detected in five independent electrotransformations). Using a neutral-base pair model (Jacobs *et al.*, 2003), 1917 of the annotated 1967 loci in the genome should be covered in the library; although insertions in only non-essential genes will be recovered in the screen. 137 mutants exhibited altered  $\Phi(ripA'-lacZ)$  expression. Each strain was subjected to quantitative  $\beta$ -galactosidase assay using mid exponential phase bacteria in Chamberlains defined media and compared to the parent reporter strain. Twenty strains failed to grow in Chamberlains defined media. Chamberlains defined media is a minimal media and these mutants were most likely auxotrophs. Mutants displaying activity greater than three standard deviations from the mean wild type  $\Phi(ripA'-lacZ)$  activity (99% confidence interval) were identified by DNA sequencing (Jacobs *et al.*, 2003).

Of these 43 mutants, 21 had transposon insertions in the *lacZ* gene and 22 had insertions in 14 unique loci (Table 7.1). Twelve exhibited decreased and 10 increased  $\Phi(ripA'-lacZ)$  activity relative to wild type. Multiple transposon insertions were isolated in three loci, FTL\_0439 (induced, seven insertions), FTL\_0073 (repressed, two insertions), and

FTL\_1790 (induced, two insertions). FTL\_0439 and FTL\_0073 are designated as envelope proteins of unknown function, while FTL\_1790 is described as a transport and binding protein of unknown specificity by the TIGR Comprehensive Microbial Resource (<http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi>). Two possible known regulatory proteins were identified in the screen that repressed  $\Phi(ripA'-lacZ)$  expression, FTL\_0895 (*hupB*) and FTL\_1810 (*nusA*). FTL\_0895 encodes HU form B which is a chromosome associated DNA binding protein that can impact global gene regulation (Balandina *et al.*, 2001), stationary phase survival (Claret & Rouviere-Yaniv, 1997), DNA repair (Kow *et al.*, 2007), and DNA replication in *E. coli* (Jaffe *et al.*, 1997). HU form B is currently the only annotated histone-like protein in the *Francisella* genome. FTL\_1810 encodes a homolog to NusA. NusA regulates gene expression in *E. coli* via termination and anti-termination activity of Rho (Greenblatt & Li, 1981; Shankar *et al.*, 2007).

Seven independent transposon insertions in FTL\_0439 resulted in increased  $\Phi(ripA'-lacZ)$  expression (Figure 7.1). FTL\_0439 is a hypothetical envelope protein of unknown function. FTL\_0439 is a fusion of the 5' end of FTT0918 with the 3' terminus of FTT0919 created by the deletion of the intervening sequence in *F. tularensis* LVS (Figure 7.1). FTT0918 and FTT0919 are important in the virulence of *F. tularensis* Schu4 in a mouse model of tularemia (Twine *et al.*, 2005). Further characterization of FTL\_0439 was not pursued in this study due to the mutation of the native allele in the LVS strain. Further work in the virulent *F. tularensis* Schu4 strain is needed to clarify the roles of FTT0918 and FTT0919 in *ripA* expression. FTL\_1810 and FTL\_0895 were chosen for further investigation because of a possible role in transcriptional regulation and FTL\_0073 because of its identification in other published screens for *Francisella* virulence factors.

### **Insertional inactivation and NusA overexpression repress $\Phi(\text{ripA}'\text{-lacZ})$ activity.**

FTL\_1810 *nusA* was investigated in depth because of its possible role as a transcriptional regulator and the strongly repressed expression of  $\Phi(\text{ripA}'\text{-lacZ})$  in the *nusA::Tn5* strain. The *nusA* gene is essential in *E. coli* (Nakamura & Uchida, 1983) and was determined to be essential in *Francisella* by analysis of a saturating transposon mutagenesis screen in *F. novicida* (Gallagher *et al.*, 2007). The *nusA::Tn5* mutant isolated in this study had an insertion in the 3' end of the gene. NusA is predicted to be 490aa in length. The transposon insertion results in a truncation of NusA(441-490). In *E. coli*, the C terminal domain (431-490), of NusA plays a role in protein-protein interactions as well as RNA binding to create a functional anti-termination complex with Rho (Eisenmann *et al.*, 2005). While this might not be the case in *Francisella*, the high degree of similarity between NusA in *E. coli* and *F. tularensis* makes this a valid hypothesis. To assess the role of *nusA* in  $\Phi(\text{ripA}'\text{-lacZ})$  expression, the wild type allele was cloned and placed under control of the *groEL* promoter in the *F. tularensis* shuttle vector pKK214 (*pnusA*). Expression of  $\Phi(\text{ripA}'\text{-lacZ})$  was assessed in the *nusA*<sup>+</sup>, *nusA::Tn5*, *nusA::Tn5 pnusA*, and *nusA*<sup>+</sup> *pnusA* backgrounds by assay for  $\beta$ -galactosidase activity in mid exponential phase bacteria cultured in Chamberlains defined media (Figure 7.2). The wild type allele did not restore expression of  $\Phi(\text{ripA}'\text{-lacZ})$  to wild type levels in the *nusA::Tn5* background, and in fact, repressed expression of  $\Phi(\text{ripA}'\text{-lacZ})$  in a *nusA*<sup>+</sup> background. The failure to complement the mutation may have been due to polar effects on the downstream FTL\_1809 *infB* locus; and in fact, *nusA* and *infB* constitute an operon in *E. coli* (Cenatiempo *et al.*, 1987). Translation initiation factor 2 (IF-2) is encoded by *infB*. IF-2 plays a role in the regulation of translation initiation in *E. coli*

(Cole *et al.*, 1987). However, this did not explain the repression we observed in the wild type background. NusA has been found to be autoregulatory in *E. coli* and overexpression resulted in the downregulation of the *nusA-infB* operon (Cenatiempo *et al.*, 1987). This could result in lowered expression of *infB* in both the *nusA::Tn5* strain and the *pnusA* strain. The *infB* locus was determined to be essential in *F. novicida* and no transposon insertions have currently been described in this locus (Gallagher *et al.*, 2007).

***F. tularensis*  $\Delta$ hupB and  $\Delta$ FTL\_0073 impacts *ripA* expression.** Transposon insertions into FTL\_0073 and FTL\_0895 (*hupB*) both resulted in decreased expression of  $\Phi$ (*ripA'*-*lacZ*). Deletion strains were created via allelic exchange to further assess the role of these loci in *ripA* expression. Slice overlap extension PCR was used to create inframe deletion constructs of FTL\_0073 (first two codons fused to the last six codons) and FTL\_0895 (first four codons to the last four codons). The integrity of flanking DNA in deletion constructs was confirmed by DNA sequencing. Constructs were cloned into the *F. tularensis* suicide vector pMP590 and allelic exchange achieved as described (Fuller *et al.*, 2008a; LoVullo *et al.*, 2006). pBSK  $\Phi$ (*ripA'*-*lacZ*)1 *cat* (Chapter 6) was integrated into *F. tularensis* LVS  $\Delta$ FTL0073 and  $\Delta$ hupB as a single copy reporter to compare expression to the wild type reporter strain. The native FTL\_0073 and *hupB* loci were cloned with their predicted native promoters and subcloned into the pKK MCS *Francisella* shuttle vector for *trans* complementation experiments (Fuller *et al.*, 2008a).

$\Phi$ (*ripA'*-*lacZ*)1 activity was measured in wild type *F. tularensis* LVS, *F. tularensis* LVS  $\Delta$ hupB or  $\Delta$ FTL\_0073, and *F. tularensis* LVS  $\Delta$ hupB pKK *hupB*<sup>+</sup> or *F. tularensis* LVS  $\Delta$ FTL\_0073 pKK FTL\_0073<sup>+</sup> strains.  $\Phi$ (*ripA'*-*lacZ*)1 activity was as follows for the

analysis of the  $\Delta hupB$ : wild type, 26.7 ( $\pm$  0.04) Miller units;  $\Delta hupB$ , 10.7 ( $\pm$  0.6) Miller units; and  $\Delta hupB$  pKK  $hupB^+$ , 17.2 ( $\pm$  1.3) Miller units.  $\Phi(ripA'-lacZ)l$  activity was as follows for the analysis of the  $\Delta FTL\_0073$ : wild type, 32.0 ( $\pm$  5.2) Miller units;  $\Delta FTL\_0073$ , 78.4 ( $\pm$  2.9) Miller units; and  $\Delta FTL\_0073$  pKK  $FTL\_0073^+$ , 25.6 ( $\pm$  1.1) Miller units (Figure 7.3). All results are significantly different from each other by ANOVA analysis ( $P < 0.05$ ). Thus,  $\Delta hupB$  had a negative influence on  $\Phi(ripA'-lacZ)l$  expression and  $\Delta FTL\_0073$  had a positive influence on  $ripA$  expression. The wild type alleles *in trans* resulted in partial restoration of wild type expression. The change in expression of  $\Phi(ripA'-lacZ)l$  in the  $\Delta FTL\_0073$  strain and the  $FTL\_0073::Tn5$  strain were the opposite. Polar effects and the expression of a truncated protein in the transposon insertion could result in the observed differences. We concluded that the results obtained with the  $\Delta FTL\_0073$  strain were accurate because the changes in  $ripA$  expression were restored to wild type levels by the native allele. Although the differences observed in the transposon mutant may lead to future avenues of investigation.

The  $FTL\_0073$  locus was identified as important in lung pathogenesis in a Signature tagged mutagenesis screen for *Francisella* virulence factors (Su *et al.*, 2007).  $FTL\_0073$  encodes a potential envelope protein of unknown function. Since its function is unknown, the route by which it impacts  $ripA$  expression is not easily definable. Mutations in envelope proteins may lead to varied effects in permeability, transport, membrane potential, or membrane composition. Characterization of *F. tularensis* LVS  $\Delta FTL\_0073$  may shed light on the function of  $FTL\_0073$  in the envelope and hence how it impacts  $ripA$  expression.

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### **ATTRIBUTIONS**

Screening of the transposon library was done in conjunction with Todd Kijek. NusA complementation experiments and FTL\_0073 allelic exchange were performed by Todd Kijek. All other experiments were conducted by myself.

## TABLES AND FIGURES

**Table 7.1. *F. tularensis* LVS Tn5 mutants that impact *ripA* expression.** *F. tularensis* LVS loci identified as impacting  $\Phi(ripA'-lacZ)$  expression based upon transposon mutagenesis. Expression falling outside a 99% confidence interval of the wild type  $\Phi(ripA'-lacZ)$  expression was used as a criteria from selection. All differences in expression listed were significant ( $P < 0.01$ ) using an unpaired student's t test from that parent reporter strain. Fold change is calculated as the ratio of mutant expression to native expression ( $MU_{Tn5}/MU_{wt}$ ). Positive or negative MglA regulation is based upon proteomics and microarray data in *F. novicida mglA*.

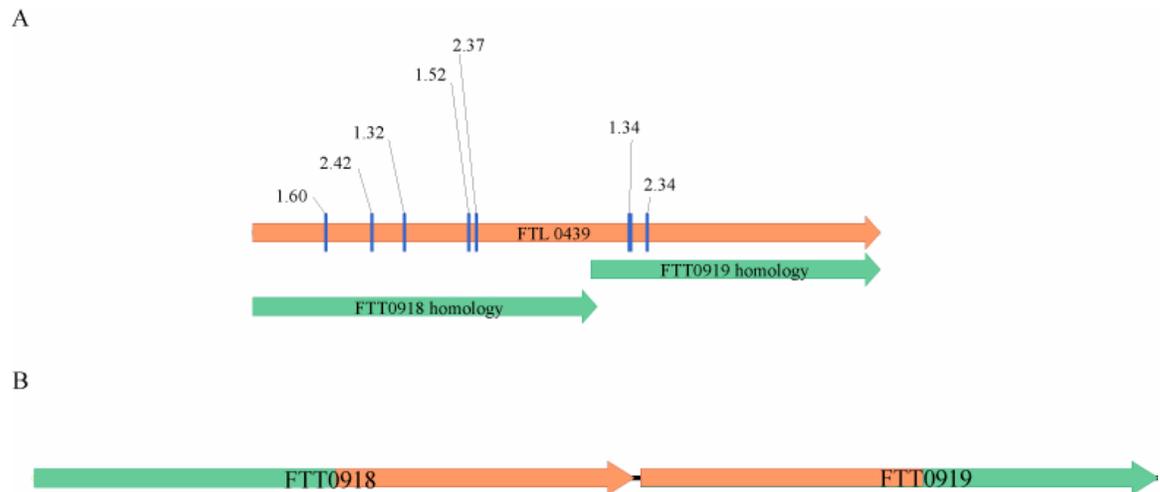
<i>locus</i>	<i>Putative identification</i>	<i>fold change</i>	<i>TIGR Cellular Role Category</i>	<i>MglA regulated</i>
FTL_1810	<i>nusA</i>	0.17	Transcription	yes <sup>a</sup>
FTL_1059	hypothetical protein	0.34	Hypothetical proteins	no <sup>a</sup>
tRNA-Val2	tRNA-Val2	0.43	tRNA	no <sup>a</sup>
FTL_0329	CDP-alcohol phosphatidyltransferase	0.53	Fatty acid and phospholipid metabolism	yes <sup>a</sup>
FTL_1666	DNA Pol I	0.57	DNA metabolism	yes <sup>a</sup>
FTL_0073 <sup>d</sup>	hypothetical protein	0.63	Cell envelope	no <sup>a</sup>
tRNA-Arg1	tRNA-Arg1	0.64	tRNA	no <sup>a</sup>
FTL_0895	HU Form B ( <i>hupB</i> )	0.68	DNA metabolism	yes <sup>a</sup>
FTL_0690	Acyl-CoA synthetase (long-chain-fatty-acid--CoA ligase)	0.69	Fatty acid and phospholipid metabolism	no <sup>a</sup>
FTL_0699	ribosomal large subunit pseudouridine synthase D	0.70	Protein synthesis	yes <sup>a</sup>
FTL_1306	hypothetical protein	0.82	Hypothetical proteins	yes <sup>a</sup>
FTL_0597	NAD dependent epimerase ( <i>wcaG</i> )	1.37	Energy metabolism	yes <sup>a</sup>
FTL_1790 <sup>d</sup>	MFS transporter ( <i>ampG</i> )	1.78	Transport and binding proteins	yes <sup>b</sup>
FTL_0439 <sup>c</sup>	hypothetical protein	3.95	Cell envelope	no <sup>a</sup>

<sup>a</sup> (Guina *et al.*, 2007)

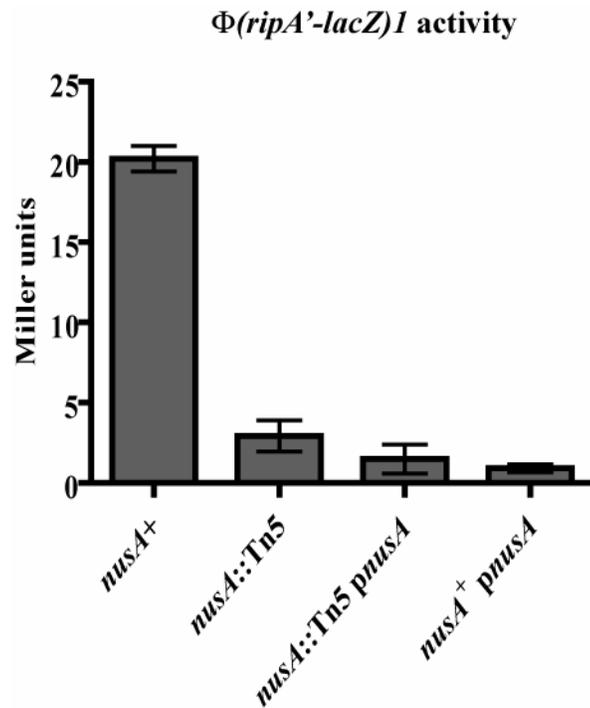
<sup>b</sup> (Charity *et al.*, 2007)

<sup>c</sup> seven Tn5 insertions

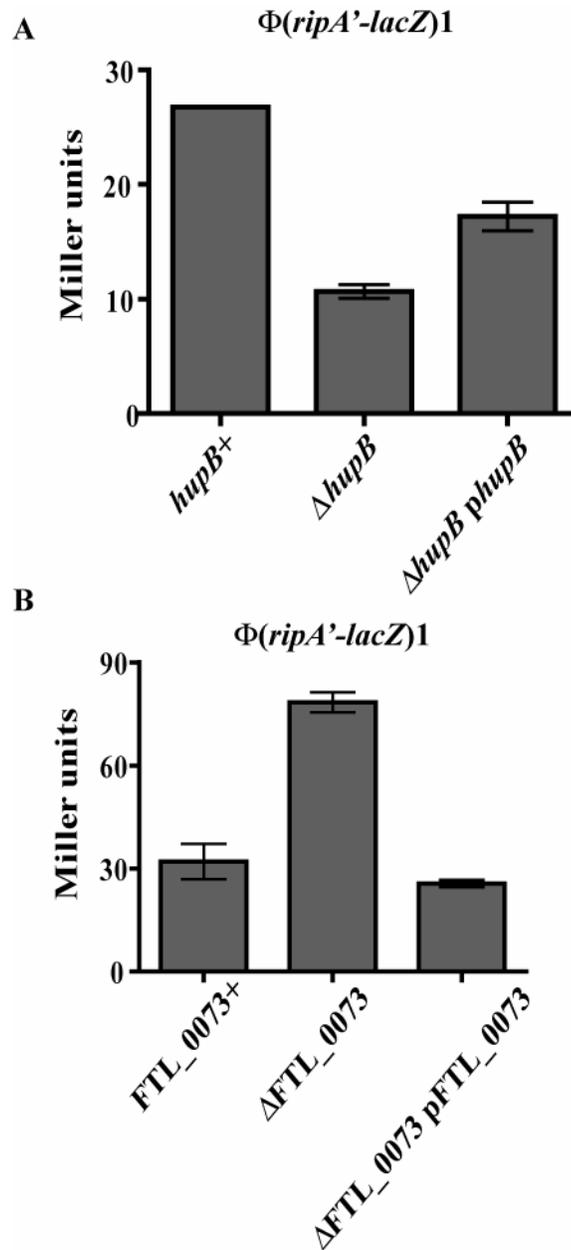
<sup>d</sup> two Tn5 insertions



**Figure 7.1. Organization of FTL\_0439, FTT0918, and FTT0919.** (A) Graphical representation of the Tn5 insertions in *F. tularensis* LVS FTL\_0439. Numerical designation of each insertion designated above the locus. FTL\_0439 is a gene fusion of the 5' of FTT0918 and the 3' of FTT0919. (B) The organization of FTT0918 and FTT0919 in *F. tularensis* Schu4. The regions of FTL\_0439 homologous to FTT0918 and FTT0919 are demarcated in green.



**Figure 7.2. Impact of *nusA* on  $\Phi(\text{ripA}'\text{-lacZ})1$  activity.** Activity of the *F. tularensis* LVS  $\Phi(\text{ripA}'\text{-lacZ})1$  reporter in wild type (*nusA*<sup>+</sup>), *nusA::Tn5*, *nusA::Tn5 pnusA*<sup>+</sup>, and *nusA*<sup>+</sup> *pnusA*<sup>+</sup> backgrounds. *pnusA*<sup>+</sup> is the wild type *nusA* allele expressed from the *Francisella groEL* promoter in the *Francisella* shuttle vector pKK214. Reporter activity is significantly repressed relative to wild type expression in all cases (P < 0.001).



**Figure 7.3. Impact of  $\Delta hupB$  and  $\Delta FTL\_0073$  on  $\Phi(ripA' lacZ)1$  activity.** (A) Activity of the *F. tularensis* LVS  $\Phi(ripA' lacZ)1$  reporter in wild type (*hupB*<sup>+</sup>),  $\Delta hupB$ ,  $\Delta hupB$  *phupB* backgrounds. *phupB* is the native *hupB* allele and promoter in the *Francisella* shuttle vector pKK MCS. Reporter expression is significantly ( $P < 0.01$ ) reduced by  $\Delta hupB$  and partially restored to wild type expression by the wild type allele *in trans*. (B) Activity of the *F. tularensis* LVS  $\Phi(ripA' lacZ)1$  reporter in wild type (FTL\_0073<sup>+</sup>),  $\Delta FTL\_0073$ ,  $\Delta FTL\_0073$  pFTL\_0073 backgrounds. pFTL\_0073 is the native FTL\_0073 allele and promoter in the *Francisella* shuttle vector pKK MCS. Reporter expression is significantly ( $P < 0.001$ ) increased by  $\Delta FTL\_0073$  and restored to wild type expression by the wild type allele *in trans*.

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## Chapter 8

### *Francisella tularensis* RipA protein-protein interactions

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#### ABSTRACT

RipA is a cytoplasmic membrane protein required for virulence in *Francisella tularensis*, but its function is not currently known. We used co-immunoprecipitation to identify proteins that interact with RipA to gain insight on the functions of this protein. RipA co-precipitated with the transcriptional regulator IclR (FTL\_1364) and the glycosyltransferases FTL\_0265 and FTL\_0604. IclR localized to the cytoplasm and the cytoplasmic membrane fraction in wild type and  $\Delta ripA$  strains suggesting that IclR-RipA protein interactions do not impact IclR localization. FTL\_0265 localized to the cytoplasmic membrane fractions of both wild type and  $\Delta ripA$  strains. The localization of RipA, IclR, and FTL\_0265 to the cytoplasmic membrane strengthens the hypothesis that they interact.

#### INTRODUCTION

RipA is a *Francisella tularensis* virulence factor required for intracellular replication and for virulence in a mouse model of tularemia (Fuller *et al.*, 2008). There are a small group of conserved hypothetical membrane proteins in diverse bacterial species with similarity to RipA. None of these proteins have a known function. Assessment of the impact

of RipA on phenotypes related to virulence such as intracellular replication and trafficking have so far been descriptive and do not directly address the function of RipA. Most proteins do not function in isolation; rather they interact with other proteins to fulfill their function. Identifying proteins that interact with RipA that have a known function may shed light on the function of this protein.

## MATERIALS AND METHODS

**Bacterial strains and Cell culture.** *Escherichia coli* TOP10 cells were used for cloning. *E. coli* cultures were grown in Luria's broth at 37 °C with shaking. *Francisella tularensis* Live Vaccine Strain (LVS) was obtained from the Centers for Disease Control and Prevention, Atlanta, Ga. *F. tularensis* LVS was propagated on chocolate agar (25g/l BHI, 50µg/ml hemoglobin, 15g/l agarose) supplemented with 1% (v/v) IsoVitaleX (Becton-Dickson), complete BHI broth (35g/l BHI, 50µg/ml hemin, 1% (v/v) IsoVitalax), or Chamberlains Defined Media (Chamberlain, 1965). All bacterial strains cultured on chocolate agar were grown at 37°C. Broth cultures were incubated in a shaking water bath at 37 °C.

**Fusion of C terminal epitopes.** Gene fusions to C terminal epitope tags were constructed by splice overlap extension PCR (Chapter 6). Overlapping primers were designed that fused either HA (Chen *et al.*, 1993; Kolodziej & Young, 1991) or V5 (Dunn *et al.*, 1999; Grabar & Cain, 2004) epitope tags to the C terminus of the coding sequence. Glycine residues were added as a linking sequence between the tag and the C terminus (Figure 8.1). Sequence integrity was confirmed by DNA sequencing. The 5' flanking sequence of  $\Phi(ripA'-HA)$ ,  $\Phi(FTL_{1364}'-V5)$ , and  $\Phi(FTL_{0265}'-V5)$  contained the predicted promoter sequences

allowing constructs to be cloned into either the *Francisella* shuttle vector pKK MCS (Fuller *et al.*, 2008) for expression or pMP590 for allelic exchange. Allelic exchange was done as described (LoVullo *et al.*, 2006).

**Co-immunoprecipitation assays.** RipA-HA was immunoprecipitated from mid-exponential phase *F. tularensis* LVS whole cell lysates. Mid exponential phase broth cultures grown in BHI were pelleted by centrifugation. Bacterial pellets were lysed in non-denaturing lysis buffer (1% (v/v) NP40, 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM MgCl<sub>2</sub>) for 1 hour on ice. Lysates were clarified in a microcentrifuge at 16,000 xg for 15 minutes. Antibody agarose, anti-HA agarose or anti-V5 agarose (Sigma), was washed once in lysis buffer and clarified lysates added to agarose beads. Immunoprecipitations were incubated overnight with shaking at 4 °C. Beads were washed three times in either low salt wash buffer (1% (v/v) NP40, 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM MgCl<sub>2</sub>) or high salt wash buffer (1% (v/v) NP40, 20 mM Tris pH 7.5, 300 mM NaCl, 1 mM MgCl<sub>2</sub>) followed by two washes in microcentrifuge spin columns with the same buffer. Low pH glycine elution (0.2% (v/v) NP40, 0.1 M Glycine pH 2.5) using two volumes equal to the amount of antibody agarose used was done and pooled. The pH of the eluate was neutralized with 1 M Tris pH 9.5 and eluates separated using SDS-PAGE followed by either Coomassie staining or Western blot. Coomassie stained specific co-precipitating proteins were identified using MALDI MS/MS at the UNC-Duke Michael Hooker Proteomics Center.

**SDS-PAGE and Western analysis.** Western blot analysis of immunoprecipitated proteins was as described (Fuller *et al.*, 2008). Non-specific binding to the membrane was blocked

with 7% (w/v) non-fat milk (0.05% (v/v) Tween 20, Dulbecco's phosphate buffered saline (PBS)). Antibody dilutions (1:10,000 mouse monoclonal anti-HA clone HA-7 or 1:1000 mouse monoclonal anti-V5 clone V5-10, Sigma) were in 0.5% (w/v) BSA (0.05% (v/v) Tween 20, PBS).

**Membrane fractionation.** Membranes were fractionated as described previously by ultracentrifugation and Sarkosyl extraction (Fuller *et al.*, 2008). Mid exponential phase bacteria were lysed by bead beating for 20 minutes. The lysates were clarified at max speed in a microcentrifuge for 5 minutes and crude membranes pelleted by ultracentrifugation for 1 hour at 100,000 x g. The cytoplasmic membrane fraction was enriched for by extraction with 0.2% sarkosyl and the outer membrane fraction re-pelleted by ultracentrifugation.

**Construction of chromosomal mutant alleles.** Allelic exchange protocols were as described (LoVullo *et al.*, 2006). pMP590 suicide vectors containing mutant versions of the targeted alleles were integrated using Kanamycin selection (10 µg/ml) and resolved by counterselection with 10% sucrose on chocolate agar plates. PCR with primers specific to either the HA tag (5'-CACCAGCATAATCTGGAACATCATAAGGATAGCC-3') or V5 tag (5'-AAACCAATACCAAACCCACTACTAGGACTAGATTCTAC-3') were used to form an amplicon with chromosomal specific primers to screen for retention of the fusion allele. Expression was confirmed by Western blot with HA or V5 specific antibodies.

## RESULTS AND DISCUSSION

**MS/MS Identification of RipA co-precipitating proteins.** *F. tularensis* RipA is homologous to a limited number of conserved hypothetical membrane proteins of unknown function in diverse bacterial species. Identifying protein-protein interactions may help elucidate unknown protein functions (Hishigaki *et al.*, 2001). Co-immunoprecipitation with specific antibodies is a well documented method to accomplish this (Elion, 2007). Rabbit anti-RipA antiserum to recombinant RipA bound numerous *Francisella* proteins and thus was of limited use for immunoprecipitations (Fuller *et al.*, 2008). Therefore, we constructed C terminal HA epitope tagged RipA fusion protein so that monoclonal antibodies to HA could be used in the immunoprecipitation assays.

The amino acid sequence of the HA epitope was reverse translated to a codon optimized sequence (Figure 8.1) based on the *Francisella* codon usage tables. This sequence was inserted 5' to the native *ripA* stop codon by splice overlap PCR. The native DNA sequence 5' and 3' to the HA tag was included in the construct to allow incorporation in the *Francisella* chromosome via allelic exchange as described (Fuller *et al.*, 2008; LoVullo *et al.*, 2006). Cointegrates were selected by growth on kanamycin and plasmid resolution identified by sucrose counterselection. Retention of the fusion allele was determined by PCR amplification using a primer specific the epitope tag sequence and a primer specific to the native chromosomal sequence. Validation of the fusion protein expression was accomplished by Western blot using monoclonal mouse anti-HA antibodies.

Mid exponential phase *F. tularensis* LVS  $\Phi(ripA'$ -HA) were lysed with non-denaturing NP40 lysis buffer and immunoprecipitated with anti-HA agarose. Samples were separated by SDS-PAGE and stained by Coomassie blue to identify specific co-precipitating proteins relative to the control precipitation with RipA-HA negative whole cell lysate. Co-

precipitating proteins were identified by MALDI MS/MS and Mascot database search by the UNC-Duke Michael Hooker Proteomics Center. Three proteins were identified that were specific and did not precipitate in the negative controls. These proteins were identified as FTL\_1364, FTL\_0265, and FTL\_0604. Two of these proteins (FTL\_1364 and FTL\_0265) retained interaction with RipA-HA after higher stringency high salt washes. FTL\_1364 was identified as a homolog to the IclR superfamily of transcriptional regulators.

**RipA and IclR coprecipitate.** To evaluate interactions between RipA and FTL\_0265 or IclR, C terminal V5 epitope tagged constructs of FTL\_0265 and IclR (Figure 8.1) were constructed utilizing SOE PCR. Fusion proteins were introduced into *F. tularensis* via allelic exchange as described above and expression of V5 fusion proteins was confirmed by Western blot with anti-V5 monoclonal antibodies (clone V5-10, Sigma).

RipA-HA and IclR-V5 were immunoprecipitated using anti-HA (HA-7) or anti-V5 agarose (V5-10) after non-denaturing lysis of mid exponential phase broth cultures using NP40 low salt lysis buffer. Immunoprecipitates were analyzed by Western blot using monoclonal anti-HA or anti-V5 antibodies (Figure 8.2). Both RipA-HA and IclR-V5 coprecipitated the opposing partner (Figure 8.2). RipA-HA and IclR-V5 were not pulled down by nonspecific antibody agarose validating the interaction (Figure 8.2).

**IclR and FTL\_0265 localize to the Cytoplasmic membrane.** The possibility that IclR, a putative transcriptional regulator, interacted with RipA, an integral cytoplasmic membrane protein raises the question of where IclR is localized in *F. tularensis*. Bioinformatics predictions of the subcellular localization of IclR based on the predicted amino acid sequence

were inconclusive. To investigate this question experimentally, we analyzed subcellular fractions for the presence of IclR-V5 by Western blot. Cytosolic, cytoplasmic membrane, and outer membrane fractions were isolated as described previously (Fuller *et al.*, 2008). The resulting cytosolic, cytoplasmic membrane and outer membrane fractions were normalized to an equivalent number of bacteria and analyzed by Western blot using anti-V5 antibodies. IclR-V5 was present in the cytoplasmic and the cytoplasmic membrane fractions in similar amounts (Figure 8.3). RipA was used as a marker for the purity of the cytoplasmic membrane fraction. Previous studies have demonstrated that RipA is localized to the cytoplasmic membrane fraction (Fuller *et al.*, 2008)(Figure 8.3).

To see if RipA impacted the localization of IclR or FTL\_0265, membrane fractions were analyzed for IclR-V5 and FTL\_0265-V5 localization. Crude membrane fractions were isolated from the cytosolic fractions by ultracentrifugation. Samples were loaded with equivalent amounts of total protein and analyzed by Western blot with anti-V5 antibodies. No difference in IclR-V5 localization or FTL\_0265-V5 was noted between the *ripA*<sup>+</sup> to  $\Delta$ *ripA* backgrounds (Figure 8.4). Based on these results we conclude that RipA and IclR or FTL\_0265 interact in the cytoplasmic membrane in a fashion that does not impact the subcellular localization of IclR or FTL\_0265. This interaction may impact IclR transcriptional regulation and future work in this area will explore the possibility.

#### **ACKNOWLEDGEMENTS**

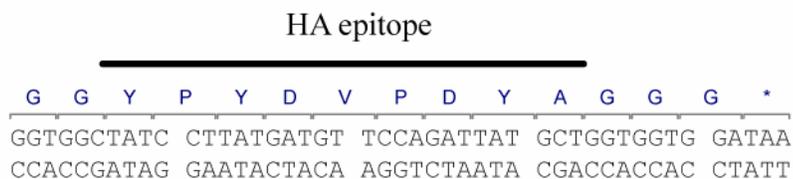
This work was supported by a Southeast Regional Center of Excellence in Biodefense and Emerging Infections grant (NIH/NIAID U54-AI057157) and by the National Institutes of Health (R21-AI053399).

## **ATTRIBUTIONS**

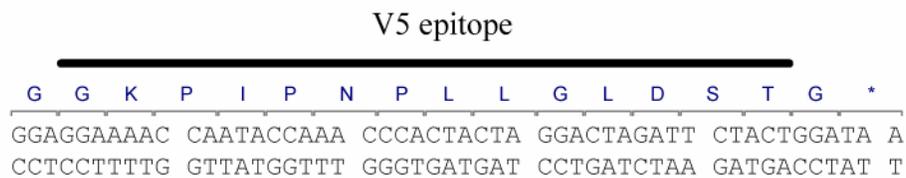
All experiments performed in this chapter were by myself.

## FIGURES

A



B



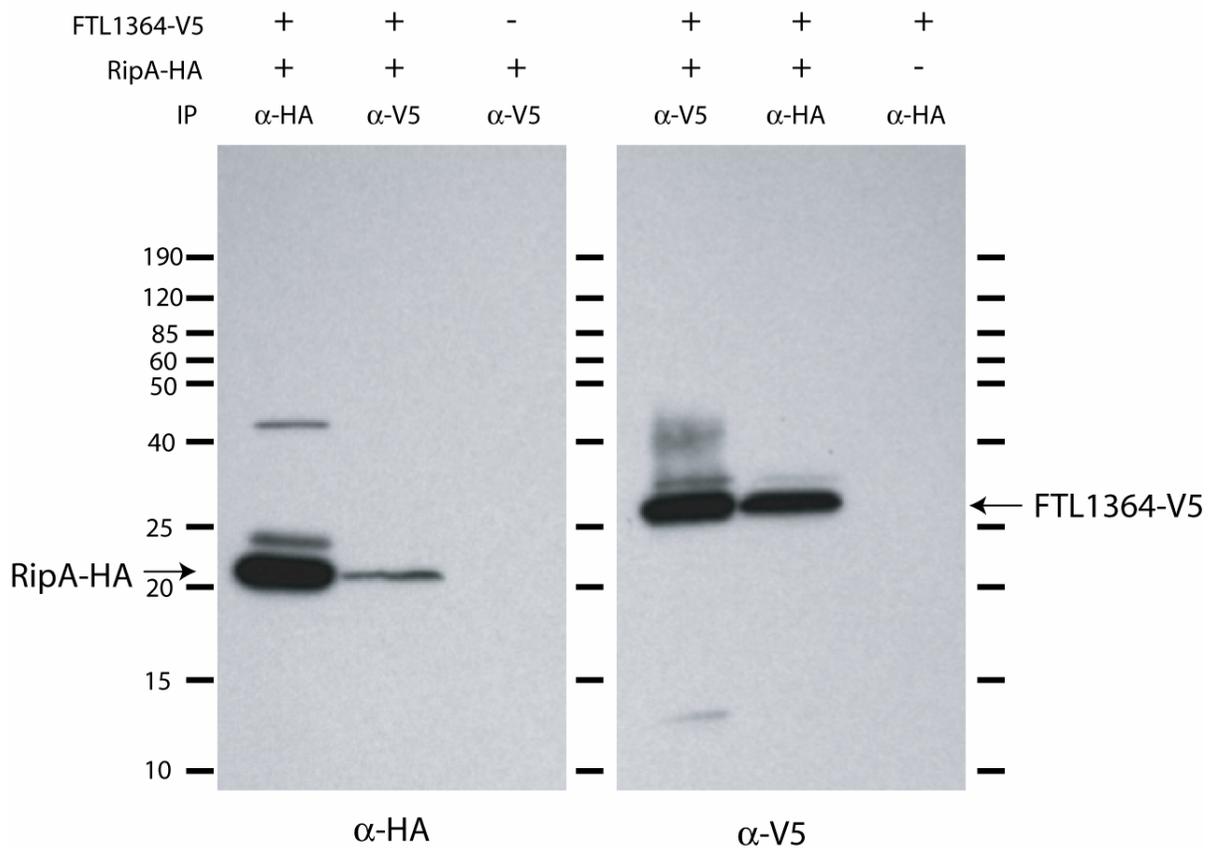
C



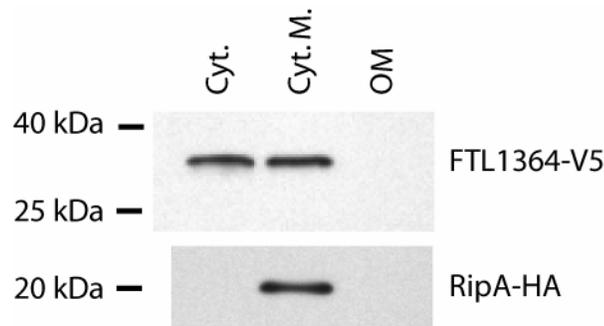
D



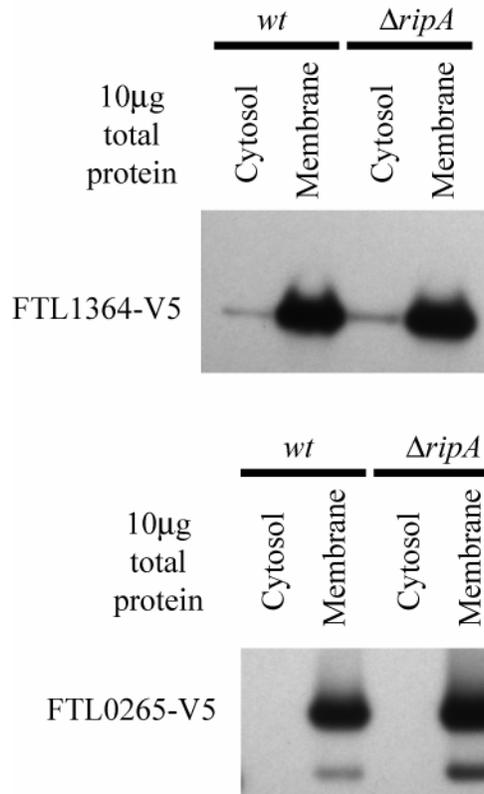
**Figure 8.1.** HA (A) and V5 (B) epitope tag sequences inserted by splice overlap extension PCR between last coding sequence and stop codons in designated loci. Glycine linkers have been added to the C and N terminus of epitopes. (C) Cartoon of RipA with the transmembrane domains (TM), HA epitope (HA), the C terminus (CT), and N terminus (NT) demarcated. (D) Cartoon of IclR with the Helix-Turn-Helix (HTH) DNA binding domain, IclR substrate binding domain (IclR), V5 epitope (V5), the C terminus (CT), and N terminus (NT) demarcated.



**Figure 8.2. RipA-HA and IclR-V5 co-precipitate.** Western blot of immuno-precipitated proteins. Presence of C terminal V5 and HA fusions RipA-HA and FTL\_1364-V5 (IclR-V5) designated with “+” and absence as “-“. Antibody-agarose used in immuno-precipitation listed under IP ( $\alpha$ -HA or  $\alpha$ -V5). Antibody used as primary in Western blot listed at the bottom of the image. Immuno-precipitation of RipA-HA co-precipitates FTL\_1364-V5 (center lane, right panel). Immuno-precipitation of FTL\_1364-V5 co-precipitates RipA-HA (center lane, left panel). Positive control for precipitation of the targeted fusion protein in the left lane of each panel (probe Western with the same antibody used in the precipitation). Antibody agarose does not non-specifically precipitate FTL\_1364-V5 or RipA-HA (note absence of bands in right lane of each panel).



**Figure 8.3. Subcellular localization of IclR.** FTL\_1364 (IclR) localizes to the cytosolic (Cyt.) and cytoplasmic membrane (Cyt. M.) subcellular fractions of *F. tularensis* LVS  $\Phi(ripA'-HA)$   $\Phi(FTL\_1364'-V5)$  and does not localize to the outer membrane fraction (OM). Western blot of subcellular fractions utilizing anti-HA and anti-V5 antibodies specific for RipA-HA and FTL\_1364-V5 fusion proteins. Samples normalized to load equivalent numbers of CFU per lane. Crude membrane fraction isolated by ultracentrifugation and cytoplasmic membrane fraction enriched by Sarkosyl extraction.



**Figure 8.4. Impact of RipA of IclR subcellular localization.** FTL\_1364 (IclR) and FTL\_0265 are predicted to interact with RipA based upon immunoprecipitation. However, the subcellular localization of FTL\_1364 and FTL\_0265 were not impacted in  $\Delta ripA$  background. FTL\_1364 localized in both the cytosolic and crude membrane fractions. FTL\_0265 localized in the crude membrane fraction only. Western blots were conducted using anti-V5 antibodies specific to FTL\_1364-V5 and FTL\_0265-V5 fusion proteins. Gene fusions are C terminal and have replaced the wild type chromosomal allele. Samples are normalized to be equivalent in total protein loaded (10  $\mu$ g total protein). Crude membrane fractions (Membrane) were isolated by ultracentrifugation and cytosolic fractions (Cytosol).

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## Chapter 9

### Conclusions

*F. tularensis* was recognized as a highly virulent pathogen in the early 1900's. Outbreaks of "Deer-fly fever" in Tulare County California after the San Francisco earthquake in 1906 led to the isolation of *F. tularensis* in 1912. Early on, *F. tularensis* was described as a fastidious intracellular pathogen and intracellular replication by this pathogen was noted to be important for *F. tularensis* virulence. While intracellular replication has been well described, the processes and mechanisms that form the basis for the pathogenesis and virulence remain largely unknown. A lack of genetic tools for *F. tularensis* is a major limitation. We have developed genetic tools for use in *F. tularensis* and used them to identify and characterize the virulence factor RipA. RipA is required for virulence and intracellular replication.

**Replication in the cytosol requires RipA.** Intracellular pathogens evade some host immune responses by colonization of the intracellular niche; however, host immunity also includes intracellular mechanisms. Low pH, antimicrobial peptides, and nutrient limitation synergistically make the phagosomal environment very hostile for most bacteria. Intracellular bacteria must resist, alter, or escape the phagosomal environment to survive. *Francisella* escapes from the phagosome early after invasion and replicates in the cytosol before re-entering a vacuole with autophagosomal characteristics late in its intracellular life

cycle (Checroun *et al.*, 2006; Golovliov *et al.*, 2003; Lindgren *et al.*, 2004). *F. tularensis* LVS  $\Delta ripA$  escapes from the phagosome, but fails to replicate in the cytosol. Electron micrographs of infected macrophages demonstrated that *F. tularensis* LVS  $\Delta ripA$  was free in the cytoplasm at similar time points post invasion as wild type. However, unlike wild type, the mutant  $\Delta ripA$  bacteria in the cytosol did not increase in density by 24 hours post inoculation. Some of the other *Francisella* mutations that result in failure to replicate in the cytoplasm are auxotrophs (Pechous *et al.*, 2006; Qin & Mann, 2006). However, *F. tularensis* LVS  $\Delta ripA$  replicates in minimal media with the same doubling time and to the same maximum density as wild type. This leads to the conclusion that failure of  $\Delta ripA$  strains to replicate was not due to nutrient limitation.

*F. tularensis* re-enters *Francisella* containing vacuoles in a process that is dependent upon replication (Checroun *et al.*, 2006). *F. tularensis* LVS  $\Delta ripA$  re-enters vacuoles in a manner different from wild type. Individual *F. tularensis* LVS  $\Delta ripA$  are found in autophagosomal like vacuoles at 24 hours post inoculation. The lack of replication of the mutant contrasts with the described requirement for replication before re-entry into vacuoles for the wild type bacteria (Checroun *et al.*, 2006). This suggests that either re-entry is mediated in a different fashion or that the triggering of the processes is different in the  $\Delta ripA$  mutant. Another interpretation is that reentry is dependent of protein synthesis and not replication. Checroun *et. al.* used chloramphenicol to block vacuole re-entry (Checroun *et al.*, 2006). Future work blocking bacterial replication with a DNA synthesis inhibitor instead of a protein synthesis inhibitor may be able to differentiate these hypotheses.

Autophagy is a process that can be triggered by the inflammatory response. *Francisella* modulates the host inflammatory response although this has not been linked to

re-entry into a vacuole. We hypothesize that modulation of the inflammatory response is different in the *ΔripA* mutant leading to increased autophagy of the bacteria. Further work on inflammation and autophagy with the *ΔripA* mutant would help clarify this issue.

**RipA is modulated during intracellular growth and by the *Francisella* regulator of virulence MglA.** Virulence factor regulation is a significant area of research in pathogenesis. Many genes that are necessary for growth in the intracellular niche, but not under laboratory conditions are regulated by facultative intracellular pathogens. In *Salmonella*, for example, SifA differentially regulates virulence factors and is upregulated under intracellular conditions (Buchmeier *et al.*, 1997; Spory *et al.*, 2002). *Legionella* presents programmed transcriptional responses that change between the replicative and transmissive phases of its life cycle (Bruggemann *et al.*, 2006). The *Francisella* transcriptional regulator MglA positively regulates some genes required for virulence (Baron & Nano, 1998; Charity *et al.*, 2007; de Bruin *et al.*, 2007) and itself is upregulated in the intracellular environment (Baron & Nano, 1999). Shotgun proteomics studies in a *F. novicida mglA* background revealed that RipA is repressed by MglA; however, microarray analysis of *ΔmglA* strains did not reveal any differences in *ripA* transcription. We clarified these contradictory findings through the use of  $\Phi(ripA'-lacZ)$  reporters to analyze *ripA* expression in the intracellular niche and in a *ΔmglA* background (Chapter 6).

$\Phi(ripA'-lacZ)$  reporter expression was increased in a *ΔmglA* background. Expression was returned to wild type levels by the introduction of the wild type *mglA* allele *in trans*. MglA exerts a negative influence on RipA expression. Since MglA predominantly has a

positive effect on gene regulation it logically follows that other genes work in conjunction with MglA to exert a negative effect on gene expression.

We identified NusA as a possible regulator of RipA expression in a transposon screen. Overexpression of NusA resulted in suppression of  $\Phi(ripA'-lacZ)$  expression, and NusA expression was greater than 20 fold changed in the MglA shotgun proteomics analysis. Although these findings relating RipA expression to NusA and MglA are significant, they do not thoroughly explain the mechanisms of RipA regulation. NusA is also repressed, not induced by MglA. We were not able to complement the *nusA::Tn5* phenotype with the wild type allele. Likely, other genes either downstream of *nusA* in the *nusA* operon or in other transcripts play a significant role in repression of gene expression by MglA. Since *mglA* was found to be increased after cell invasion, we measured *ripA* expression in the intracellular niche as well. Early (1 hour PI), early cytoplasmic (6 hours PI), and late cytoplasmic (24 hours PI) stages were evaluated. RipA was suppressed at the late cytoplasmic time point, possibly through an intermediary such as NusA that would act concert with MglA. Analysis of *iglA*, a gene induced by MglA, gene expression showed an induction during early cytoplasmic growth that was reduced by late cytoplasmic growth. We hypothesize that this programmed gene expression in the intracellular niche serves a role in adaptation to the cellular niche and maintenance of the intracellular lifecycle. Further work on the genes up-regulated during each stage of the *Francisella* lifecycle may give clarity to the genes necessary for adaptation and successful propagation of *F. tularensis*.

**RipA interacts with the transcriptional regulator IclR.** Phenotype analysis showed that RipA is necessary for intracellular replication and virulence in a mouse model of tularemia,

but insight into what function RipA plays in *Francisella* is currently elusive. RipA protein-protein interactions were analyzed to try to clarify this issue. We found that RipA coprecipitates with IclR (FTL\_1364). IclR is a common transcriptional regulator in gram negative bacterial species. It is characterized by the IclR domain superfamily which consists of a helix-turn-helix DNA binding domain and a substrate binding domain in characterized IclR superfamily members. IclR regulates diverse systems and binds to a wide variety of small molecule effectors in the bacteria where it has been studied. Based on the hypothesis that RipA may modulate IclR transcriptional regulation through sequestration to the bacteria membrane, we assessed the subcellular localization of IclR. RipA did not appear to impact IclR localization; however, an interesting finding was that IclR itself localizes to the cytoplasmic membrane. Thus IclR may form a membrane complex with RipA or possibly as transient association with RipA. Such as transient association could function to modify IclR or act as a cofactor in IclR transcriptional regulation. How this impacts IclR mediated transcriptional regulation and what genes are regulated by IclR are currently under investigation.

In summary, RipA is a factor necessary for virulence in a mouse model of tularemia that modifies *F. tularensis* replication and trafficking after escape from the phagosome. RipA may interact with the transcriptional regulator IclR. How this interaction may impact IclR gene regulation and virulence phenotypes remains to be determined. The *Francisella* regulator of virulence MglA represses RipA expression. However, the biologic significance of the changes in RipA expression is unknown. What is clear is that MglA regulated genes show patterns of induction and repression during the intracellular lifecycle. Clarification of programmed gene response during different phases the intracellular lifecycle would give

insight into the importance of different loci during these stages and the intracellular environment during that stage. Also, research on the modulation of inflammatory cytokines by wild type and  $\Delta ripA$  *Francisella* strains may help clarify the mechanisms underlying relocalization into *Francisella* containing vacuoles. Taken together these future studies will lead to a cohesive story on the place of RipA in *Francisella* pathogenesis.

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## Appendix A

### **An immunoaffinity tandem mass spectrometry (iMALDI) assay for detection of *Francisella tularensis***

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#### **ABSTRACT**

*Francisella tularensis* (*F. tularensis*) has been designated by the CDC as 1 of the 10 organisms most likely to be engineered for bioterrorism. Symptoms of tularemia in humans are nonspecific, thus making the disease difficult to diagnose. If not quickly diagnosed and treated, the disease has a high mortality rate – thus methods for early and specific diagnosis are of critical importance.

This immunoaffinity MALDI MS/MS (iMALDI) assay provides unambiguous detection of *F. tularensis* peptides at attomole levels from peptide solutions, and at low CFU levels from bacteria. The addition of stable-labeled versions of the peptide as internal standards allows absolute quantitation of *F. tularensis* peptides with a linear dynamic range spanning two orders of magnitude. The ability of mass spectrometry to obtain amino acid sequence data on affinity-captured peptides provides absolute specificity and avoids “false positives” from the non-specific binding. The *F. tularensis* iMALDI assay has been applied to different samples, such as nasal swabs.

This novel quantitative diagnostic *F. tularensis* iMALDI assay allows the safe, sensitive, and specific detection of *F. tularensis*. The assay can be easily adapted to other target peptides and therefore has broad application potential in clinical diagnosis of other pathogens and diseases.

## INTRODUCTION

Tularemia is caused by *Francisella tularensis* (*F. tularensis*), a small, non-motile, aerobic Gram-negative cocco-bacillus. Although normally causing a relatively rare tick-borne disease in humans, *F. tularensis* has the potential to be engineered for bioterrorism. The bacteria can penetrate unbroken skin, can survive and multiply within macrophages, and can then spread to various organs through the blood [1]. Ten bacteria injected subcutaneously [2], 10–50 on contact with unbroken skin [3], 10–50 given by aerosol [4,5], or  $10^2$ – $10^8$  bacteria by ingestion [3] are sufficient to cause infection. Humans who have direct physical contact with infected animals or insects, or have inhaled aerosolized bacteria, have a good chance of becoming infected. Untreated, the mortality rate can be 30% [6].

Initial non-specific flu-like symptoms usually appear 3 days after exposure [7], and general laboratory tests (CRP, LDH, alkaline phosphatase, leukocytes, *etc.*) are insufficient for diagnosis. Because early antibiotic therapy (with streptomycin or gentamicin) can greatly reduce the lethality rate [8], immediate diagnosis of an infection with *F. tularensis* is critical. Because of the virulence of *F. tularensis*, the severity of the disease, the rapidity of progression from initial onset to seriousness or death, and the high mortality rate, several agencies [9] have been concerned about the weaponization of *F. tularensis*, especially the use of aerosolized bacteria by terrorists as an airborne pathogen [10–12]. *F. tularensis* has been designated as one of the organisms most likely to be engineered for bioterrorism [13], and one of the six “category A bioterrorism organisms” [1,11,14,15].

Bacteriological methods can be used for detecting *F. tularensis* [16], but culturing the

organism is difficult [2,4,10,17], time-consuming (sometimes taking several days), and is potentially hazardous to laboratory personnel [18]. Moreover, several studies have shown [19] that the sensitivities and specificities of these methods are low (note: “specificity” means that there are no “false positives” – *i.e.*, the test gives a positive result only for the target organism).

Since culturing *F. tularensis* is difficult, serological tests such as the bacterial microagglutination (MA) test, have been used to diagnose tularemia, but this takes 1 week for measurable levels of antibodies to develop, and an additional week for sufficient antibody levels for a reliable test [10,20–23]. Antibodies against *F. tularensis* may cross-react with other organisms such as *Brucella*, *Proteus OX19*, and *Yersinia spp.* [10], which reduces the specificity and could lead to false positives. More recently, another type of serological test, an enzyme-linked immunosorbent assay (ELISA), achieved a detection limit of  $10^3$  bacteria  $\text{mL}^{-1}$  in PBS and  $10^4$  bacteria  $\text{mL}^{-1}$  in human serum [24]. However, when samples are contaminated with other microorganisms[25], accurate diagnosis with ELISA or culturing methods is very difficult due to their low specificities.

PCR is a molecular method for detection of *F. tularensis* that usually takes several hours per reaction. A handheld PCR assay could detect *F. tularensis* in 3 h, and achieved the analytical sensitivity of 100 bacteria  $\text{mL}^{-1}$  PBS or  $10^3$ – $10^4$  bacteria  $\text{mL}^{-1}$  serum [26]. However, PCR may give false positives from contamination with other DNA, which lowers its specificity. Moreover, simultaneous assaying for multiple species or virulence factors by PCR is difficult [27].

In this paper, we describe a peptide-based immunoaffinity MALDI mass spectrometry (iMALDI) assay for detection of *F. tularensis*, which circumvents many of the problems

associated with traditional detection methods. It is based on capture of the *F. tularensis* IglC aa49–61 peptide. This *F. tularensis* peptide assay is capable of fast, safe, sensitive, and specific detection of *F. tularensis* in PBS solution. It can be used for absolute quantitation of target peptides and, therefore, for absolute quantitation of their parent proteins. We also demonstrate the applicability of iMALDI to the detection of *F. tularensis* in clinical samples, such as human plasma and nasal swabs.

## EXPERIMENTAL

In iMALDI (Fig. A.1) [28,29], anti-peptide antibodies are first produced and immobilized on affinity beads. Next, the proteome of interest is proteolytically digested. Isotopically-labeled epitope-containing peptides, called “heavy” peptides, are added into the digest as internal standards for quantitation. The digest, containing both the labeled “heavy” peptides and the unlabeled native (“light”) peptides, is incubated with the antibody beads and both types of peptides are adsorbed. After immunoadsorption, the antibody beads are arranged in a microarray/spot format on the MALDI-target plate. MALDI matrix solution is then added, which enables the elution of the affinity-bound peptides from the immobilized antibodies permitting MALDI analysis of the peptides. The relative abundances of the molecular ion signals corresponding to the original “light” and “heavy” peptides are used for quantification. Absolute specificity can be achieved by mass spectrometric sequencing of the epitope-containing peptide, using MALDI–MS/MS.

### **2.1. Target protein**

The 23 kDa protein, IglC, from *F. tularensis* bacteria is encoded by *iglC*, whose sequence has no significant homology to any other gene present in the GenBank database [29]. Live

Vaccine Strain (LVS) bacteria, at a concentration of  $2.25 \times 10^9$  CFU (colony forming units)  $\text{mL}^{-1}$  in 70% ethanol, were used. The total protein concentration of the bacteria sample was  $700 \text{ ng mL}^{-1}$  as determined with an ELX800 Universal Microplate Reader.

## **2.2. Nasal swab samples**

Mice were inoculated intranasally with  $1 \times 10^5$  LVS. Two days post inoculation, a small, moist, alginate swab was used to rub across the nostrils of the mice. The swab was then swirled in a tube containing 100  $\mu\text{L}$  PBS. Next, 100  $\mu\text{L}$  of 95% ethanol was added to the sample to ensure inactivation.

## **2.3. Tryptic digestion**

Digestion of human plasma (UNC blood bank) was carried out in 25 mM ammonium bicarbonate (Sigma) at  $37^\circ\text{C}$  overnight. A  $\sim 1:10$  enzyme:substrate ratio of trypsin (sequencing-grade modified trypsin, Promega) to protein was used. The digested human plasma was spiked with *F. tularensis* peptides or a bacterial digest to mimic clinical samples. Before digestion, the nasal swab solution was diluted with ammonium bicarbonate until the final concentration of ethanol was 38%, which is compatible with tryptic digestion.

## **2.4. Antibody production and immobilization of antibodies on beads**

Four *F. tularensis* IglC tryptic peptides, which are absolutely unique to, and thus diagnostic of *F. tularensis*, were selected for their high sensitivity in the MALDI-MS mode (Fig. A.2 and Table A.1). These four peptides were synthesized by Sigma-Genosys. Antibodies were raised against each peptide, and tested by ELISA to determine the detection sensitivi-

ties/efficiencies. The peptide that showed the highest binding efficiency was selected for use in the *Francisella* peptide assay, and the corresponding antibody was purified by Sigma-Genosys.

The anti-peptide antibody was then immobilized on CNBractivated sepharose beads (Amersham Pharmacia) according to the manufacturer's instructions [30]. Briefly, by reacting with the primary amine groups on the antibody, the sepharose beads covalently linked to the antibody. Excess binding sites on the beads were blocked by incubation with an amine-containing buffer, (in this case, Tris). The process was completed by a series of washes at alternating pH.

### ***2.5. Immunoadsorption protocol***

An aliquot of settled antibody-bead slurry (1–5  $\mu\text{L}$ ) was added to a compact reaction column (USB) and washed 4–5 times with 400  $\mu\text{L}$  of  $0.1 \times \text{PBS}$ . A bacterial digest or synthetic *F. tularensis* Ig1C peptide [CNIVAIEGGEDVTK, calculated MW = 1446.71 (note: the N-terminal cysteine, C, is not part of the native sequence, but was added to facilitate antibody production)] was dissolved in water/buffer and diluted to a series of concentrations, *e.g.* 25  $\text{ng } \mu\text{L}^{-1}$ , 5  $\text{ng } \mu\text{L}^{-1}$ , 0.5  $\text{ng } \mu\text{L}^{-1}$ , *etc.* A 20  $\mu\text{L}$  aliquot of each solution was incubated separately with a small amount (1–5  $\mu\text{L}$ ) of antibody beads to immuno-adsorb the epitope-containing peptide. Peptide solutions or a bacterial digest spiked into a plasma digest mixture was used to mimic clinical samples. Three negative control experiments were performed by incubating (a) unconjugated agarose beads with PBS; (b) unconjugated agarose beads with synthetic peptide or bacteria digest sample; and (c) antibody beads with PBS, respectively.

After incubation for 2–4 h at room temperature with end-to-end rotation on a “Labquake”

rotator (Lab Industries), the beads were washed six times with 400  $\mu$ L of freshly made 50 mM ammonium bicarbonate. When high levels of nonspecific binding were expected in complex samples such as plasma, the beads were washed three times with 400 mM of NaCl (Sigma) followed by three times with 50 mM ammonium bicarbonate. Then the beads were re-suspended in a small volume of 50 mM ammonium bicarbonate (1–5  $\mu$ L) and an aliquot of the beads (0.5  $\mu$ L) was spotted directly onto the MALDI target as described in Section 3.

### ***2.6. Isotopic labeling for absolute quantitation***

The *F. tularensis* IglC aa49–61 peptide <sup>49</sup>NIVAIEGGEDVTK<sup>61</sup> (calculated MW = 1343.69) containing an isotopically-labeled valine at position 59 (underlined) (the “heavy” peptide) was synthesized at the UNC Peptide Synthesis Facility, using a <sup>13</sup>C-labeled fmoc valine purchased from Isotec/Sigma–Aldrich according to the fmoc approach described in details elsewhere [31]. The increase in mass from the unlabeled (“light”) *F. tularensis* IglC aa49–61 peptide was 6 Da.

### ***2.7. Absolute quantitation***

The “heavy” peptide, dissolved in water (HPLC grade, Pierce) was used as an internal standard. Heavy peptide was spiked into a *F. tularensis* bacterial digest in various amounts, immuno-adsorbed on anti-*F. tularensis* IglC peptide antibody beads, and analyzed by MALDI–MS of the beads on the MALDI target.

### ***2.8. Mass spectrometric analysis***

MALDI–MS experiments were performed on Bruker Dal-tonics’ (Billerica, MA) Reflex III and Ultraflex MALDI-TOFs, using Bruker’s Anchor-chip MALDI-target plates (400 or 600

$\mu\text{m}/384$  spot format). MS/MS analyses were carried out on an Applied Biosystems Voyager 4700 (Framingham, MA) MALDI-TOF/TOF. The matrix used for all experiments was re-crystallized (from hot methanol)  $\alpha$ -cyano-4-hydroxycinnamic acid (HCCA) (Sigma). A saturated solution of HCCA in 50:49.9:0.1 acetonitrile (Caledon Laboratories):water (HPLC grade, Pierce):trifluoroacetic acid (Pierce) was used. A small volume (1–5  $\mu\text{L}$ ) of ammonium bicarbonate (ABC) solution (50 mM) was added for easy placement of the beads on the MALDI target. Following placement of the antibody beads on the target, 0.3–0.5  $\mu\text{L}$  of HCCA matrix was added, and the spot was allowed to dry at room temperature.

## RESULTS AND DISCUSSION

The iMALDI technique belongs to a general category of techniques which couples affinity capture with direct mass spectrometric analysis of target proteins (Fig. A.1) [32–48]. In iMALDI, anti-*peptide* antibodies are immobilized on sepharose beads for affinity capture, rather than on the surface of a plate, thus eliminating the need for special surface chemistry. Additional advantages of using affinity beads are less-stringent sample storage requirements after antibody immobilization, fewer complications resulting from denaturation of the capture antibody in solution, and the ability to incubate several types of beads with small volumes of tryptic-digested biological fluid instead of “soaking” the entire target. Because peptides are more stable and less susceptible to denaturation, the iMALDI should be more reproducible from sample to sample than the techniques based on anti-protein antibodies, and sample storage requirement should not be as stringent.

Unlike conventional affinity capture/elution/MALDI techniques [33,34], the “stable isotope standards with capture by anti-peptide antibodies” (SISCAPA) approach [35], or on-

target elution followed by removal of affinity beads as was done by Schriemer and Li [49], in iMALDI the affinity beads are placed directly on the MALDI target and analyzed without prior elution of the bound analytes [32–48], which greatly reduces the sample loss and therefore increases the detection sensitivity. In iMALDI, after immunoadsorption, the antibody beads are arranged in a microarray/spot format on the MALDI target plate. Addition of MALDI matrix solution releases the affinity-bound peptides from the immobilized antibodies permitting MALDI analysis of the peptides. Multiple samples can be analyzed simultaneously by mass spectrometry as an array of different anti-peptide antibody beads on a MALDI target. Furthermore, since complete inactivation of the bacteria can be achieved by enzymatically digesting the lysate at the time of sample collection, the iMALDI technique carries low risk to laboratory personnel.

### ***3.1. Sensitivity***

#### *3.1.1. Detection of *F. tularensis* bacteria in PBS solution*

By direct MALDI–MS analysis of the beads, the detection limit for the synthetic *F. tularensis* IglC peptide (cysteine was added for antibody production) was determined to be in the low attomole range (14 amol) in buffer (HPLC-grade water or PBS). Without enrichment by iMALDI, in MALDI–MS analysis, the detection sensitivity of the peptide in buffer was in the low femtomole range (17 fmol) (Fig. A.3). In the enrichment process, the peptides are bound to antibody beads, which greatly reduce losses due to adsorption by tubes and tips. Moreover, when spotted on the target plate, even after elution by the matrix solution, the peptides do not spread out all over the spot area. Instead, they tend to aggregate at “hot spots”, and when the laser is focused at these “hot spots”, greatly increased detection sensitivities can be obtained.

The *F. tularensis* iMALDI assay, based on the anti-*F. tularensis* IglC aa49–61 antibody, can also detect the target *F. tularensis* peptide from a bacterial digest. The target *F. tularensis* peptide was determined in a bacterial digest equivalent to 80 bacteria. The bacteria were digested and incubated with a small aliquot (1–5  $\mu\text{L}$ ) of anti-aa49–61 NIVAIEGGEDVTK antibody beads. One tenth of the antibody beads were spotted on the MALDI target plate and analyzed directly. Although there were signals from some unknown impurities or non-specifically bound peptides from, for example, the bacterial digest, the target peptide was still detectable by its mass. The singly charged, epitope-containing tryptic peptide (aa49–61 NIVAIEGGEDVTK) from the IglC protein was observed at  $m/z = 1344.7$  (Fig. A.4). Therefore, the detection limit for bacteria in PBS solution is determined to be 8 CFU bacteria on target by using 50% of the antibody beads incubated with 50  $\mu\text{L}$  of a bacterial solution in PBS, at a concentration of  $320 \text{ CFU mL}^{-1}$ , which is as low as the most sensitive detection systems yet developed [50,51]. It should be noted that 8 CFU does not represent the lowest possible detection limit in real applications, but is an estimate of the lowest possible detection limit of our method, as the tryptic digestion of the protein equivalent of 8 CFU might be difficult.

### 3.1.2. Detection of *F. tularensis* bacteria in blood

The following detection limits were determined for the synthetic *F. tularensis* IglC and *F. tularensis* bacteria spiked into blood samples (plasma): 69 amol of the *F. tularensis* IglC synthetic peptide and 800 CFU of bacteria (Fig. A.5) on target by using 50% of the antibody beads incubated with 125  $\mu\text{L}$  of spiked plasma, at a concentration of  $1 \text{ fmol mL}^{-1}$  IglC aa49–61 peptide and  $1280 \text{ CFU mL}^{-1}$  bacteria, respectively. Although this method is not as sensitive for the detection of *F. tularensis* bacteria in blood as it is for the detection of

bacteria in PBS solution, the detection limit is still comparable to most ELISA methods [24,52]. However, our technology has the advantage of greater safety because we are detecting bacterial *peptides* after the bacteria have been inactivated by lysis and proteolysis. In addition, our technology has higher specificity, since tandem mass spectrometry is a molecular approach with sequencing capabilities to provide absolute identification of the target molecule.

Due to the high virulence of bioterrorism pathogens, high sensitivity is one of the most important requirements in order to avoid “false negative” results. The sensitivity in plasma (800 CFU) is lower than in PBS solution (*ca.* 10 CFU bacteria), maybe due to incomplete digestion and to high levels of nonspecific binding of other plasma proteins which can suppress the ion signal from the target peptide. Optimized sample preparation protocols may alleviate this problem. In particular, a major advantage of this MS/MS-based technique is that the proteins which are the source of these non-specifically bound peptides can be identified from the MS/MS spectra of their peptides, and depletion protocols can be specifically designed to remove these proteins prior to digestion and affinity capture of the target peptides [53]. Improved sample preparation protocols and improvements in mass spectrometric detection technology, should lead to routine detection sensitivities of ~ 10–100 amol of peptide.

### *3.1.3. Detection of *F. tularensis* bacteria in nasal swab samples*

The current technique is also usable for nasal swab analyses. Nasal swabs are widely used in clinical tests, and are a simple and useful method for collecting a wide range of respiratory viruses. The collection of a nasal swab is easy and painless, and it can easily be performed in remote locations [54]. In the mouse nasal swab samples collected 2 days after inoculation, a

very low level of target peptide ( $m/z = 1344.7$ ) was detected (Fig. A.6a). The absence of the signal for this peptide in the uninfected mouse samples supports these results (Fig. A.6b). MS/MS was not performed on the ion at  $m/z = 1344.7$  because the ion abundance was below the instrument threshold for MS/MS acquisition. No bacteria were observed in a culture of the nasal swab solution from the infected animal (which later developed tularemia), thus demonstrating that iMALDI could detect *F. tularensis* at levels too low for successful culturing of the bacteria.

### **3.2. Specificity**

By “specificity” of the assay, we mean that the target peptide is unique to the organism (as determined by a BLAST search of the peptide sequence). Thus, the detection of this peptide, at the appropriate MW and with the correct sequence is a positive indication of the presence of *F. tularensis*. The iMALDI can provide this specificity by combining two molecular characteristics of the epitope-containing peptides: (i) the molecular weight, typically measured by MALDI–MS within an error of  $\leq 100$  ppm and (ii) the amino acid sequence, determined by performing MS/MS on the same sample.

We have demonstrated that this iMALDI technology is able to accurately determine the molecular weight of an immunoaffinity-enriched *F. tularensis* peptide from a proteolytic digest of *F. tularensis* bacteria in PBS solution and blood. The molecular weight determination was accurate to within 60 ppm. Sequence information on the affinity-bound peptide was also obtained by MALDI–MS/MS (Fig. A.7a). MS/MS analysis produces high-abundance sequence-specific ions, resulting in an almost complete amino acid sequence of the peptide. These sequence-specific ions allow confident assignment of the target peptide (Fig. A.7b). Using the existing NCBI nr database (2006.02.16), searching with only MS data,

we retrieved 74,528 hits out of more than three million entries; in contrast, searching with combined MS and MS/MS data, resulted in only one hit. This demonstrates the specificity of this iMALDI technique for detecting *F. tularensis*. Here, we used polyclonal antibodies because of their ready availability. Polyclonal antibodies often contain more than a single epitope within a particular stretch of amino acids, and therefore, are able to capture even modified peptides since they are likely to contain an unmodified stretch of amino acids. This makes this detection method tolerant of small sequence variations. On the other hand, the use of polyclonal antibodies might lead to cross-reactivity with peptides from other species. This could decrease specificity and/or the detection limit of our method. This issue could be resolved by using a several monoclonal antibodies, or perhaps even both types of antibodies, for the final clinical version of this technique.

### **3.3. Quantitation**

Mass spectrometry is well suited for peptide/protein quantitation. MS can perform *relative* quantitation using reference peptides from two different samples that have been differentially labeled with stable isotopes, or *absolute* quantitation if a known amount of a stable-isotope labeled peptide has been added as an internal standard.

To determine the absolute concentration of a given peptide in a sample, and, therefore, the absolute concentration of the original protein, as is done in isotope dilution-mass spectrometry (ID-MS) [55], synthetic stable-isotopically labeled “heavy” peptide is used as an internal standard. These heavy peptides are identical in amino acid sequence to the native epitope containing “light” peptides from the protein in the sample, but are higher in mass. They are added to the proteolytically digested protein sample prior to incubation with the immobilized anti-peptide antibody. Following incubation, MALDI-MS analysis of the antibody beads

shows doublets of ion signals from the “light” and “heavy” peptides. The relative abundances of the molecular ion signals corresponding to the “light” and “heavy” peptides are used to quantify the amount of the “light” peptide and therefore its parent protein in the original sample.

For quantifying *F. tularensis* IglC protein, the *F. tularensis* IglC aa49–61 peptide <sup>49</sup>NIVAIEGGEDVTK<sup>61</sup> was synthesized with an isotopically labeled valine at position 59 (underscored). This “heavy” peptide (H) is identical in amino acid sequence to the native epitope-containing “light” peptide (L), but is 6 Da higher in mass ( $m/z = 1350.7$ ) due to six <sup>13</sup>C’s in place of 6 <sup>12</sup>C’s. In these experiments, the heavy peptide was added in various amounts (100 fmol–5 pmol) to a proteolytic digestion of *F. tularensis* bacteria in PBS solution, and was immuno-adsorbed using the anti-*F. tularensis* IglC aa49–61 antibody immobilized on affinity beads (Fig. A.8a–d). Analysis of an aliquot of these beads spotted directly on the MALDI target reveals monoisotopic protonated molecular ions from the two peptides, separated by 6 Da. (Fig. A.8e) shows a linear correlation ( $R^2 = 0.9988$ ) between the ratio of the two peptides (H/L) and the amount of H spiked in as internal standard over near two orders of magnitude (from 100 fmol to 5 pmol). The nearly perfect linear correlation demonstrates the stability of iMALDI for the absolute quantitation of *F. tularensis* peptides using an internal standard.

Compared to the traditional culturing method and microagglutination test [2,4,10,17,20–23], iMALDI is more rapid, sensitive and specific. The peptide sequencing capability of tandem mass spectrometry in iMALDI provides higher molecular specificity than ELISA. The sensitivity of iMALDI is comparable to the most sensitive PCR method [26], but may not be as rapid when the digestion and incubation time is considered. However, the digestion

time of iMALDI can be greatly reduced by using immobilized trypsin [56], and the incubation time can be reduced by using an antibody with high affinity. Moreover, compared to other methods, iMALDI has higher safety, better absolute quantitation capabilities, and improved high-throughput analysis capability which significantly reduce the cost per sample. One major disadvantage of iMALDI is that its overall quality highly depends on the affinity and specificity of the antibody. However, this problem should be able to be solved by using different strategies of antibody optimization, or alternative affinity-capture materials such as aptamers [57,58].

## CONCLUSIONS

We have shown that the combination of two mass spectrometric approaches (MALDI–MS and MALDI–MS/MS), inherent in our iMALDI approach, can unambiguously identify affinity-bound peptides, and therefore, permits specific detection of *F. tularensis* bacteria. The *F. tularensis* iMALDI can detect *F. tularensis* bacteria in PBS solution at a sensitivity of better than 10 CFU of bacteria. Since both the molecular weight and the sequence of the bacterial peptide is determined at high accuracy, the high sensitivity of this technique means a low rate of “false negatives”, and the high specificity means a low rate of “false positives”. In addition, the iMALDI *F. tularensis* peptide assay is adequate for accurate and absolute quantitation of the target protein concentration. With the addition of robotic liquid handling systems, it would be an even safer technique, as well as being capable of high-throughput analyses. This would make iMALDI an extremely valuable tool for the early detection of *F. tularensis* and other bioterrorist agents.

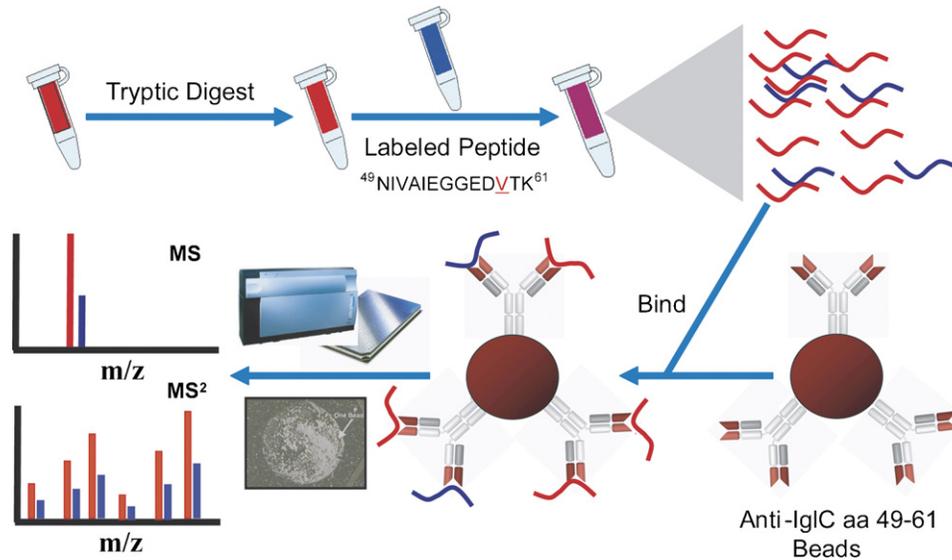
## ACKNOWLEDGEMENTS

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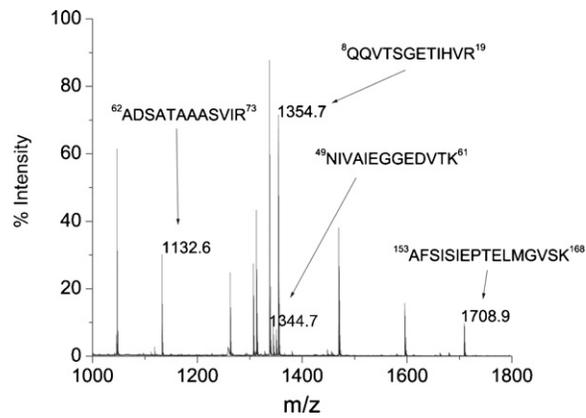
## ATTRIBUTIONS

I performed the molecular biology, protein expression, and protein purification work on the *Francisella tularensis* proteins. Collection of *Francisella tularensis* samples was done in the Kawula lab. MALDI MS/MS analysis was performed in the UNC-Duke Michael Hooker Proteomics Center. All other work was performed by Jian Jiang. This work was previously published, Copyright © Elsevier, Analytica Chimica Acta, Volume 605, pages 70-79,2007. Permission has been granted to reprint this material.

## TABLES AND FIGURES



**Fig. A.1** – Analytical scheme of the iMALDI assay. Epitope-containing peptides,  $^{49}\text{NIVAIEGGEDVTK}^{61}$ , synthesized using isotopically labeled amino acid V, are added to the proteolytic digest of *F. tularensis* bacteria and are subsequently incubated with the anti-aa49–61 (*F. tularensis* IgC) antibody beads to immuno-adsorb both isotopically labeled and unlabeled peptides. After immunoabsorption of the differentially labeled peptides, the antibody beads are arranged in a microarray/spot format on the MALDI-target plate. MALDI matrix solution added to the affinity-bound peptides elutes the peptides from the immobilized antibodies, permitting MALDI analysis of the peptides. The relative abundances of the molecular ion signals corresponding to “heavy” (isotopically labeled) and “light” (unlabeled) peptides are used to quantify the amount of this protein in the original sample. Absolute specificity can be achieved by mass spectrometric sequencing of the epitope-containing peptide, using MALDI–MS/MS.

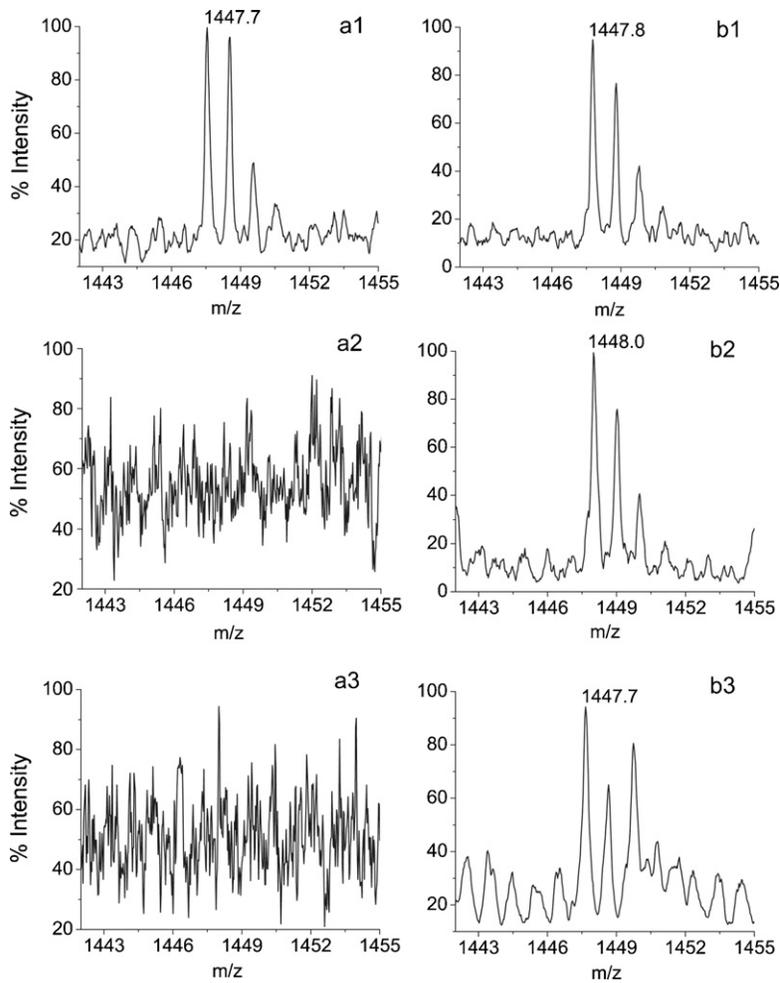


**Fig. A.2** – Selection of *F. tularensis* IglC peptides for raising antibodies to be used for the *F. tularensis* iMALDI assay. MALDI–MS of proteolytic *F. tularensis* IglC peptides obtained by in-solution digestion of IglC with trypsin. Four “true” (C-terminal cleavage of K or R residues) tryptic peptides of IglC were selected for antibody production based on the high sensitivity in the MALDI–MS.

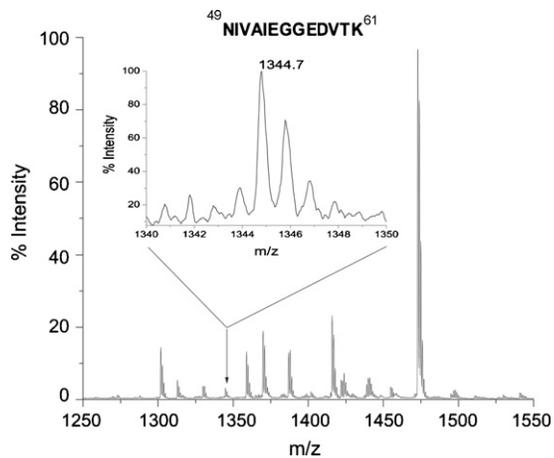
**Table A.1** - Affinity determination of the four anti-(*F. tularensis* IglC) peptide antibodies against their epiptope peptide by ELISA (the cysteine residue marked with asterisk has been added to the native sequence because of ease of conjugation with the carrier protein)

<i>F. tularensis</i> IglC peptides used for antibody production	Anti-( <i>F. tularensis</i> IglC) peptide Antibodies	ELISA response (titer)
<sup>153</sup> AFSISIESPTELMGVS <sup>167</sup>	[( <i>F. tularensis</i> IglC) aa153-167]	1/50,000
<sup>8</sup> QQVTSGETHIHVR <sup>29</sup>	[( <i>F. tularensis</i> IglC) aa8-19]	1/3,000
<sup>48</sup> C*NIVAIEGGEDVTK <sup>61</sup>	[( <i>F. tularensis</i> IglC) aa48-61]	1/500,000
<sup>62</sup> ADSATAAASVIR <sup>73</sup>	[( <i>F. tularensis</i> IglC) aa62-73]	1/10,000

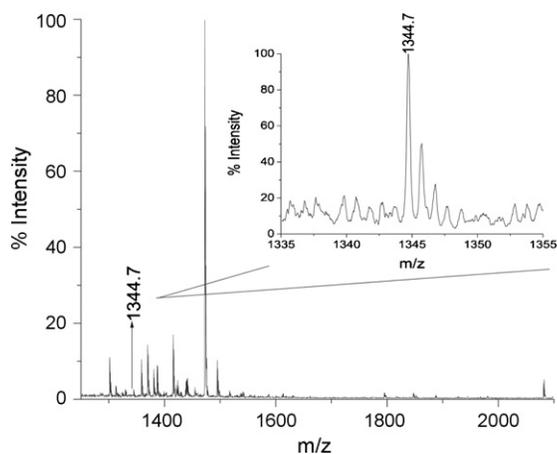
The cysteine residue marked with asterisk has been added to the native sequence because of ease of conjugation with the carrier protein.



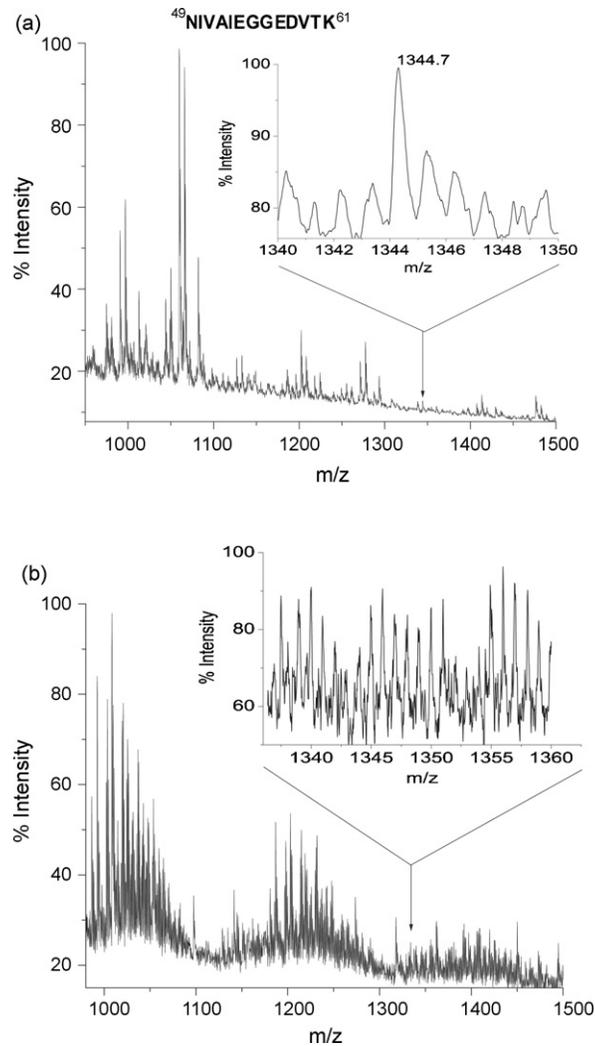
**Fig. A.3** – Detection sensitivity of synthetic *F. tularensis* IgIC peptides in solution and using the *F. tularensis* iMALDI assay. MALDI–MS spectrum of synthetic *F. tularensis* IgIC peptide CNIVAIEGGEDVTK (a) in solution: (a-1) 100 fmol; (a-2) 1 fmol; (a-3) 10 amol. (b) Affinity-bound to anti-aa48-61 (*F. tularensis* IgIC) antibody beads: (b-1) 138 fmol; (b-2) 1.38 fmol; (b-3) 13.8 amol.



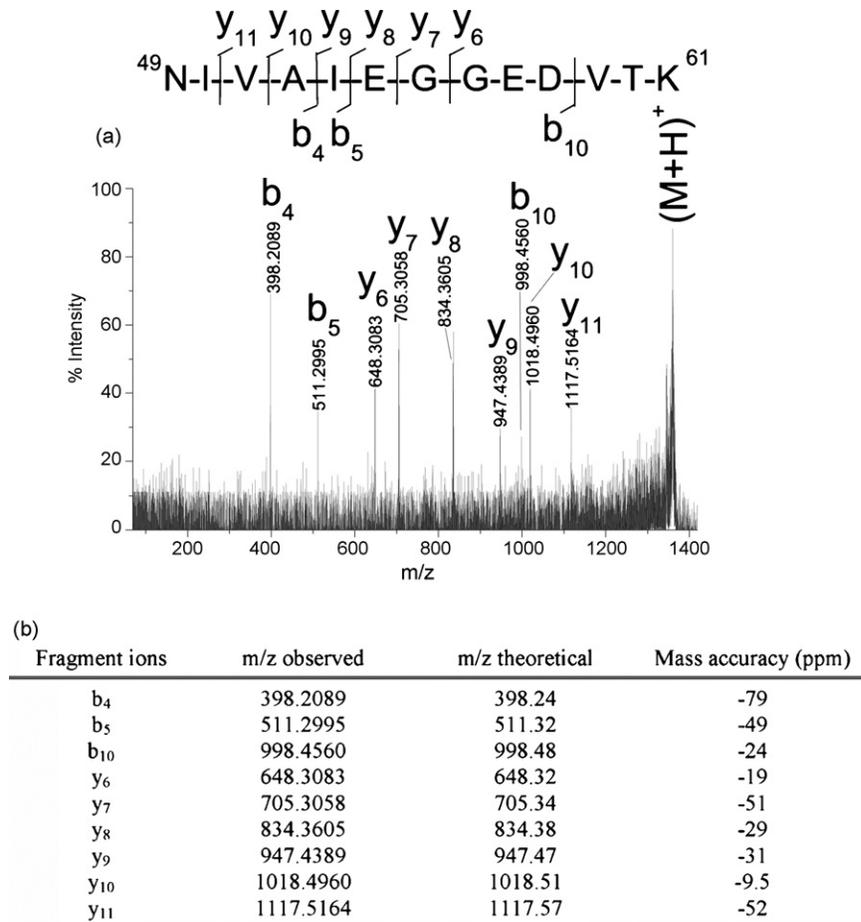
**Fig. A.4** – Detection of *F. tularensis* bacteria in PBS solution using the *F. tularensis* iMALDI assay. MALDI-MS spectrum of peptides obtained after proteolysis of *F. tularensis* bacteria in PBS solution, affinity-bound to anti-aa49–61 (*F. tularensis* IglC) antibody beads. The singly charged epitope-containing tryptic peptide (aa49–61 *F. tularensis* IglC protein) is observed at  $m/z = 1344.7$  (see inset). The spectrum represents the amount of IglC peptide equivalent to 8 CFU for *F. tularensis* bacteria.



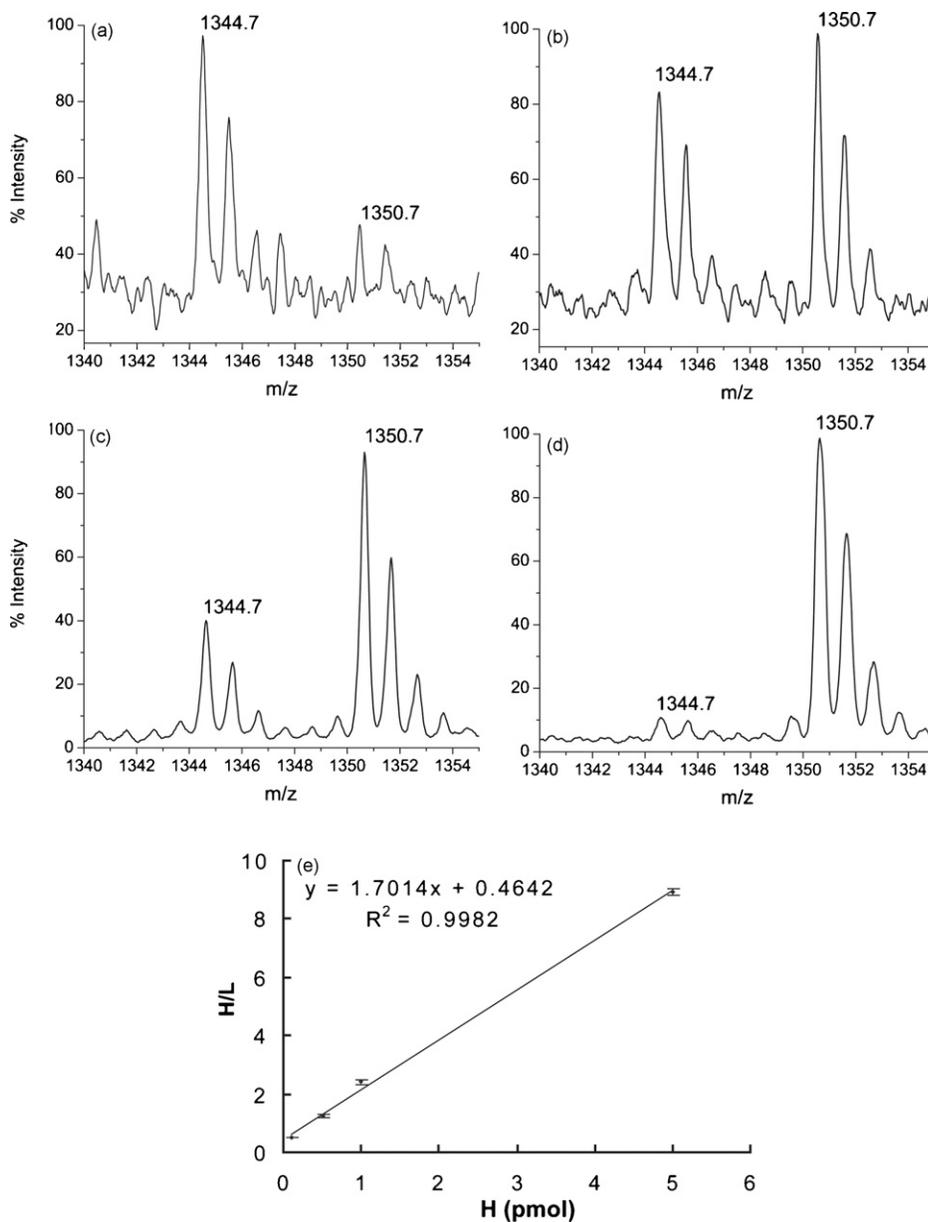
**Fig. A.5** – Detection of *F. tularensis* bacteria in plasma samples using the *F. tularensis* iMALDI assay. MALDI-MS spectrum of peptides obtained from proteolyzed *F. tularensis* bacteria spiked in plasma digest, affinity-bound to anti-aa49–61 (*F. tularensis* IglC) antibody beads. The singly charged epitope-containing tryptic peptide (aa49–61 *F. tularensis* IglC protein) is observed at  $m/z = 1344.7$  (see inset). The spectrum represents the amount of IglC peptide equivalent to 800 CFU for *F. tularensis* bacteria.



**Fig. A.6** – Detection of *F. tularensis* bacteria in nasal swab samples using the *F. tularensis* iMALDI assay. (a) MALDI–MS spectrum of peptides affinity-bound to anti-aa49–61 (*F. tularensis* Ig1C) antibody beads, obtained after proteolysis of mouse nasal swab extracts collected 2 days after the mice were inoculated with *F. tularensis*. The singly charged, epitope-containing, tryptic peptide, NIVAIEGGEDVTK, corresponding to aa49–61 of the *F. tularensis* Ig1C protein, was observed at  $m/z = 1344.7$  (see inset). (b) MALDI–MS spectrum of anti-aa49–61 (*F. tularensis* Ig1C) antibody beads obtained after proteolysis of nasal swab extracts collected from uninfected mice on the same day.



**Fig. A.7** – Highly specific detection of *F. tularensis* bacteria in PBS solution by mass spectrometric sequencing of the immunoaffinity-enriched *F. tularensis* IglC aa49–61 peptide, using the *F. tularensis* iMALDI assay. (a) MALDI–MS/MS spectrum of the peptide at  $m/z = 1344.7$  affinity-bound to anti-aa49–61(*F. tularensis* IglC) antibody beads, obtained after proteolysis of *F. tularensis* bacteria in PBS solution. All of the abundant ions can be assigned to sequence-specific y- and b-ions of the *F. tularensis* IglC peptide aa49–61, resulting in unambiguous identification of the immunoaffinity-enriched peptide. (b) This table shows the mass accuracies of the fragment ions, demonstrating that the assignment of these ion signals is correct. This therefore demonstrates that, using our *F. tularensis* iMALDI assay, *F. tularensis* bacteria can be detected with high specificity and correspondingly low “false positive” rates.



**Fig. A.8** – Quantitation of *F. tularensis* bacteria using the *F. tularensis* iMALDI assay. Absolute quantitation of the IgIc peptide NIVAIEGGEDVTK (aa48–61) (L, light peptide,  $m/z = 1344.7$ ) in a bacterial sample. *F. tularensis* bacteria were digested and incubated with different amounts of heavy peptides (H,  $m/z = 1350.7$ ) as internal standards: (a) 0.1 pmol, (b) 0.5 pmol, (c) 1 pmol, (d) 5 pmol, (e) Plot of the observed ratios of monoisotopic abundances of H and L in the MALDI–MS spectra (a–d) versus the absolute amount of H added. Note, only a 10% aliquot has been used for the analysis.

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