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Examining the regulation of virulence factors in Francisella tularensis

Blake Wade Buchan
University of Iowa

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EXAMINING THE REGULATION OF VIRULENCE FACTORS IN
FRANCISELLA TULARENSIS

by

Blake Wade Buchan

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Microbiology
in the Graduate College of
The University of Iowa

December 2009

Thesis Supervisor:  Associate Professor Bradley D. Jones
ABSTRACT

*F. tularensis* is an intracellular pathogen, and is the causative agent of tularemia in humans. The ability of *F. tularensis* to parasitize host cells is largely dependent upon genes within a pathogenicity island (FPI), including those in the *iglABCD* operon. Specific mechanisms and gene products involved in regulation of the FPI are not well understood. I initiated the study of this regulatory system by creating an efficient Tn5-based mutagenesis system optimized for use in *F. tularensis*, and utilized this system to construct a *lacZ* reporter library. I identified genes differentially regulated in response to growth on two different media, including those in the *iglABCD* and *fslABCD* operons, and identified iron availability as a factor contributing to the differential regulation. One of these reporter strains, carrying a chromosomal *iglB-lacZ* fusion, was used as the basis for a secondary transposon mutagenesis to identify mutations that affect *iglABCD* expression. One such mutation is in FTL_1542 (*migR*), a hypothetical protein, and reduces expression of the *iglABCD* approximately 8-fold. The effect of this mutation on *igl* expression is likely through its effect on another known virulence regulator, *fevR*, as demonstrated by data from RT-PCR experiments. I compared the phenotypes of LVS *fevR* and *migR* mutant strains in primary macrophage and epithelial cell lines and in neutrophils. The mutation in *migR* effects growth and intracellular trafficking in macrophages but not epithelial cells, and reverses the ability of wild type *F. tularensis* to block the respiratory burst in neutrophils. When similar mutations were examined in the human virulent *F. tularensis* strain Schu S4, *migR* retained its regulatory role, but did not impair replication in macrophages. The *migR* mutation in Schu S4 did however have an attenuating effect when administered to mice intranasally. Comparison of LVS and Schu S4 in primary human airway epithelial cell infections revealed an inability of LVS to replicate within these cells, which is in contrast to the robust replication of LVS in cultured epithelial cell lines. Together, this work contributes to the understanding of
regulatory mechanisms governing virulence gene expression in *F. tularensis* and highlights differences between LVS and Schu S4 strains.

Abstract Approved: __________________________

Thesis Supervisor

____________________________

Title and Department

____________________________

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

Blake Wade Buchan

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Microbiology at the December 2009 graduation.

Thesis Committee:  
______________________________
Bradley D. Jones, Thesis Supervisor

______________________________
Alexander R. Horswill

______________________________
Lee-Ann H. Allen

______________________________
Linda L. McCarter

______________________________
W. Scott Moye-Rowley
To my wife Cindy for continued encouragement, support, and understanding, my mentor Brad for constant energy and optimism, and myself for hard work and dedication
Like a bat out of hell, I’ll be gone when the morning comes.

Meat Loaf
ABSTRACT

*F. tularensis* is an intracellular pathogen, and is the causative agent of tularemia in humans. The ability of *F. tularensis* to parasitize host cells is largely dependent upon genes within a pathogenicity island (FPI), including those in the *iglABCD* operon. Specific mechanisms and gene products involved in regulation of the FPI are not well understood. I initiated the study of this regulatory system by creating an efficient Tn5-based mutagenesis system optimized for use in *F. tularensis*, and utilized this system to construct a *lacZ* reporter library. I identified genes differentially regulated in response to growth on two different media, including those in the *iglABCD* and *fslABCD* operons, and identified iron availability as a factor contributing to the differential regulation. One of these reporter strains, carrying a chromosomal *iglB-lacZ* fusion, was used as the basis for a secondary transposon mutagenesis to identify mutations that affect *iglABCD* expression. One such mutation is in FTL_1542 (*migR*), a hypothetical protein, and reduces expression of the *iglABCD* approximately 8-fold. The effect of this mutation on *igl* expression is likely through its effect on another known virulence regulator, *fevR*, as demonstrated by data from RT-PCR experiments. I compared the phenotypes of LVS *fevR* and *migR* mutant strains in primary macrophage and epithelial cell lines and in neutrophils. The mutation in *migR* effects growth and intracellular trafficking in macrophages but not epithelial cells, and reverses the ability of wild type *F. tularensis* to block the respiratory burst in neutrophils. When similar mutations were examined in the human virulent *F. tularensis* strain Schu S4, *migR* retained its regulatory role, but did not impair replication in macrophages. The *migR* mutation in Schu S4 did however have an attenuating effect when administered to mice intranasally. Comparison of LVS and Schu S4 in primary human airway epithelial cell infections revealed an inability of LVS to replicate within these cells, which is in contrast to the robust replication of LVS in cultured epithelial cell lines. Together, this work contributes to the understanding of
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<th>Definition</th>
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<tr>
<td>ATI</td>
<td>alveolar type I epithelial cells</td>
</tr>
<tr>
<td>ATII</td>
<td>alveolar type II epithelial cells</td>
</tr>
<tr>
<td>CDM</td>
<td>Chamberlain’s defined medium</td>
</tr>
<tr>
<td>CR3</td>
<td>complement receptor 3</td>
</tr>
<tr>
<td>FPI</td>
<td>Francisella pathogenicity island</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>lethal dose 50, dose at which 50% of infected individuals die</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LVS</td>
<td>F. tularensis live vaccine strain</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDM</td>
<td>monocyte derived macrophage</td>
</tr>
<tr>
<td>MHC II</td>
<td>major histocompatibility complex II protein</td>
</tr>
<tr>
<td>MMH</td>
<td>modified Mueller Hinton</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte (neutrophil)</td>
</tr>
<tr>
<td>proSP-C</td>
<td>prosurfactant protein C</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>T4P</td>
<td>Type IV pili</td>
</tr>
<tr>
<td>T6SS</td>
<td>Type six secretion system</td>
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**Francisella tularensis**

*Francisella tularensis* is a non-motile gram-negative coccobacillus first isolated from diseased rodents in Tulare County, California in 1911 by McCoy and Chapin (115). A facultative intracellular pathogen, *F. tularensis* has fastidious growth requirements that include the addition of glucose, cysteine, and blood or blood components (e.g. IsoVitaleX) to culture medium to support growth (57). Since its initial isolation from a ground squirrel, *F. tularensis* has been isolated from over 250 host species including fish, birds, and small mammals, as well as arthropod vectors such as ticks and biting flies (25, 105, 132, 141). In susceptible hosts, *F. tularensis* can cause a wide range of symptoms depending on the route of infection. This can range from a localized cutaneous ulceration at the site of infection, to a lethal plague-like or pneumonic illness or a granulomatous disease of the liver and spleen (57, 135). There are four recognized subspecies of *F. tularensis*: *tularensis*, *holarctica*, *novicida* and *mediasiatica*. These subspecies differ in their global distribution and ability to cause the zoonotic disease tularemia in humans. Subspecies *tularensis*, known as type A, is found primarily in North America and is associated with the most severe cases of tularemia in humans. Strain Schu S4 is most commonly studied as a representative of this subspecies. Subspecies *holarctica*, or type B, is distributed throughout the Northern hemisphere and is also capable of causing disease in humans, though symptoms are milder than those caused by type A strains and the disease is rarely fatal. This subspecies is also the basis for a live vaccine strain (LVS) which affords protection against low dose cutaneous infection by type A strains, but provides little protection against aerosol exposure (30). This poor efficacy combined with the undefined status of attenuation has led the LVS to no longer be licensed for use in the United States. However, the LVS is still often used as a model organism to study...
*F. tularensis* pathogenesis because it mimics type A strains in in vitro cell culture infection models as well as mouse infection models. Subspecies *novicida* and *mediasiatica* are found in Australia and Asia, respectively, and are generally not considered to be human pathogens (57, 137). Still, *novicida* strains are commonly used as model organisms to study *F. tularensis* because they retain virulence in mouse models and are capable of survival and replication in human macrophages in vitro (74, 95, 193).

Despite the widespread distribution of human pathogenic type A and type B strains, there are very few reported fatalities as a result of *Francisella* infection. This is due, in part, to the infection route-dependent manifestation of the disease. Most natural exposures to *Francisella* are the result of direct contact with the blood of an infected animal or transmission from the bite of an infected arthropod vector into cutaneous tissue (180). In either case, the outcome of infection is most often a non-life threatening ulceration at the site of infection that may include swelling of local lymph nodes (174). If inhaled however, *F. tularensis* causes a rapidly progressing pneumonic form of tularemia characterized by fever, shortness of breath, and severe malaise (137). In the case of a type A infection, inhalation of as few as 10 viable organisms can cause abrupt onset of severe symptoms and can be 30-60% fatal if untreated (136, 180). Because of the extreme virulence of type A strains, the low ID$_{50}$ and LD$_{50}$, and the relative ease of dissemination by aerosol, *F. tularensis* has been developed as a biological weapon (53, 87).

The genome of *F. tularensis* consists of approximately 1,825 open reading frames (ORFs), of which 35% encode hypothetical proteins. Another 11% of the ~1,825 ORFs encode non-functional pseudogenes, many of which are constituents of various metabolic pathways (93). This inordinately high percentage of disrupted metabolic pathways is believed to be responsible for the fastidious nature of the bacterium and suggests a bacterium that is moving evolutionarily toward an obligate intracellular lifestyle. Of note, a region of DNA containing 17 ORFs is present in two identical copies on the
chromosome of subspecies *tularensis* and *holarctica* (93, 129, 130). Because this region of the chromosome has a lower G+C content (28%) than the rest of the chromosome (33%) and many of the genes in this region have been associated with the pathogenicity of this bacterium (10, 47, 104, 129, 130, 162, 164), it has been given the designation “*Francisella* Pathogenicity Island” (FPI). Specific genes and functions of the FPI will be discussed later; however, it is interesting to note that random mutagenesis screens have rarely identified genes on the FPI as a source of attenuation, suggesting that one intact copy of each gene is sufficient to maintain a virulent phenotype.

**Interactions with Host Cells**

*F. tularensis* has the ability to persist or replicate within phagocytes such as monocyte-derived macrophages (MDM) and neutrophils and other cell types including bronchial airway epithelial cells, and tissue culture cell lines such as HEP-2, A549, and J774A.1 (69, 95, 99, 113, 130, 149, 170, 175). While capable of infecting this wide variety of cells, the primary target of *F. tularensis* early in pulmonary infection appears to be the macrophage (134, 137, 184). Furthermore, the ability to survive and replicate within phagocytic cells seems to be central to the ability of *F. tularensis* to cause disease (57). Uptake by monocytes is mediated, at least in part, by the mannose receptor (MR), complement receptor 3 (CR3), and scavenger receptor A (SRA) (143, 170). Of these host cell receptors, CR3 appears to play a dominant role in bacterial uptake, since the use of heat-inactivated serum or complement factor C3 depleted serum results in dramatically reduces uptake of *F. tularensis* by macrophages (34, 35, 170). Physical uptake of the bacterium by macrophages has been reported to involve a novel “looping phagocytosis” mechanism (35) in which a pseudopod “reaches out” to envelop a single bacterium and enclose it in a loose phagosome, which then progresses to a more typical phagosome tightly encasing the pathogen. The specific bacterial ligands recognized by each receptor remain largely unknown, however periodate treatment to disrupt surface carbohydrates or the addition of soluble mannan both have a negative effect on bacterial
uptake by macrophages (34, 35, 170). Comparatively little is known about entry of \textit{F. tularensis} into non-phagocytic cells, however entry is blocked by cytochalasin D and nocodazole and does not depend on live bacteria, suggesting that uptake relies on a functional host cell cytoskeleton and is host cell dependent (42, 80, 99). Bacterial replication within cultured epithelial cells is robust, reaching up to 1000-fold multiplication and filling the entire cytosolic space within 24 hours of infection (23, 99, 140, 149).

Following phagocytosis by a macrophage, \textit{F. tularensis} resides in a phagosome which accumulates both early and late endosomal marker proteins such as early endosomal antigen-1 (EEA-1), CD63, LAMP-1 and -2, and rab5 (Fig. I.1) (29, 37, 69, 163). Following uptake, phagosomes containing latex beads or killed \textit{Francisella} progress to a fully mature phagolysosome which displays lysosomal markers and becomes relatively acidic. In contrast, phagosomes containing live \textit{Francisella} are stalled at the late endosomal stage, acquiring only late endosomal markers (6, 29). In a matter of 1-4 hours, the phagosomal membrane begins to degrade allowing bacteria access to the cytosol where they begin rapid replication (29, 69, 164). Although the subject of some debate, it appears that the transient acidification of the nascent phagosome is not required for bacterial escape (33, 36, 37). However, inhibition of phagosome acidification by blockade of vacuolar ATPase activity does result in delayed phagosomal escape and cytosolic replication. The significance of acidification to efficient bacterial escape remains unclear but does not appear to be the result of altered expression of FPI genes in response to lowered pH (33). Acidification of the phagosome may be required to maintain bacterial access to iron (62).

Once in the macrophage cytosol, \textit{F. tularensis} undergoes rapid replication with a doubling time estimated at as little as 60 min. for subspecies \textit{tularensis} strain SchuS4 (33). Within the cytosol \textit{F. tularensis} elicits activation of the inflammasome (66, 98), a set of cytosolic proteins that recognize bacterial components. Inflammasome activation
is dependent on *F. tularensis* gaining access to the cytosol, since mutants incapable of phagosomal escape do not trigger activation (91), however cytosolic replication is not necessary for inflammasome activation (171). When activated, the inflammasome proteins, along with an adapter protein (ACS), cleave pro-caspase-1 into its active form which in turn, triggers the production and secretion of the proinflammatory cytokines IL-1\(\beta\) and IL-18 (196). In contrast, mature caspase-3 is associated with the initiation of apoptosis. Apoptosis can function as a mechanism of host defense against intracellular pathogens, functionally eliminating their replicative niche. The exact mechanism of inflammasome activation by *F. tularensis* is unclear and is the subject of considerable research. Interestingly, one research group suggests that *F. tularensis* actively inhibits inflammasome activation and apoptosis to preserve its replicative niche (111). This hypothesis is supported by the characterization of two mutations that result in accelerated cytotoxicity and increased IL-1\(\beta\) secretion (193). These proinflammatory, hyper-cytotoxic events are ablated in caspase-1\(^{-/}\) host cells, suggesting that the characterized genes are involved in modulation of normal host cell function through the inflammasome. However, these studies involved murine macrophages and the non-human pathogenic subspecies *novicida*, so it is difficult to correlate the significance of these findings with human pathogenesis.

After 18-24 hrs of cytosolic replication in murine macrophages, approximately 62% of *F. tularensis* organisms appear to re-enter a double membrane-bound vacuole containing the marker protein LC3 (29, 83). Both the double membrane structure and the presence of LC3 are characteristic of autophagic vacuoles, which generally serve to engulf and degrade damaged host cell organelles and recycle nutrients during amino acid starvation (44). Autophagy can also play a role in defense against intracellular pathogens by enveloping cytosolic bacteria and delivering them to lysosomes, giving the host cell a second chance to clear the infection and aiding in MHC class II display of intracellular antigens (44, 77, 128). *Francisella* appears to inhibit the expression of autophagy-related
host genes during early cytosolic growth (43), temporarily inhibiting autophagy before later entering autophagosomes (29). Additionally, unlike *Listeria*, re-entry of *Francisella* into these autophagic vacuoles is dependent on continued bacterial protein synthesis (29, 154). This finding suggests that entry of *Francisella* into the autophagic pathway is a bacterial-active process, possibly requiring specific gene bacterial products. Combined, these data hint at the possibility that the bacterium may be modulating autophagy for its own benefit. The temporary inhibition of the autophagic process may prolong the life of the infected host cell, in turn providing *Francisella* with a longer-lived intracellular replicative niche.

In addition to macrophages, polymorphonuclear leukocytes (PMNs), or neutrophils, play a role in host defense against *F. tularensis*. Mice depleted of neutrophils are highly susceptible to killing by intradermal or intravenous *Francisella* infection, even at doses significantly less than the reported LD$_{50}$ (176). Like macrophages, neutrophils efficiently engulf opsonized *Francisella* and enclose the bacterium in a cytosolic vacuole (113). Once engulfed, foreign particles including bacteria, are subjected to toxic levels of oxidants such as hydrogen peroxide (H$_2$O$_2$), superoxide anion (O$_2^-$), hydroxyl radicals (OH), and hypochlorous acid (HOCl), which are generated by the multi-subunit enzyme NADPH oxidase (56). This event is often referred to as the oxidative burst. A key aspect of *F. tularensis* virulence is the ability to inhibit assembly of the NADPH oxidase complex on *Francisella* containing phagosomes, thereby preventing the production of these reactive oxygen species (113, 114). Furthermore, interference with NADPH oxidase assembly is not limited to the *Francisella*-containing vacuole, rather, it is a global effect throughout the infected neutrophil preventing NADPH oxidase assembly even at distal phagosomes that have engulfed other stimulatory particles (113). A recent screen to identify the genetic basis for the ability of *Francisella* to inhibit the respiratory burst isolated mutants defective in the synthesis of uracil (171). A respiratory burst inhibiting phenotype could be restored
by supplementation of the bacterial growth medium with uracil. It is not immediately clear how these mutations exert their effect on neutrophil function, but the genetic basis for NADPH oxidase inhibition remains an area of intense research. In addition to preventing the oxidative burst, the bacterium also escapes the cytosolic vacuole with kinetics similar to those seen in macrophages. However, unlike what is observed in macrophages, *F. tularensis* does not appear to undergo rapid replication within the neutrophil (113). Ironically, although neutrophils appear to be somewhat effective at containing *F. tularensis* and preventing replication, they are among the last cell type to respond in a pulmonary infection and recruitment may actually be suppressed by *Francisella* (81).

**Virulence Determinants**

*F. tularensis* does not appear to encode any canonical exotoxins, so pathogenicity is due in large part to its ability to invade and replicate in host cells, thereby causing tissue damage and organ failure (136). Indeed, the virulence of *F. tularensis* may stem from its ability to escape detection by the host immune system and avoid killing by innate defenses. Evasion of innate immune defenses is accomplished by the bacterium through the use of both active and passive mechanisms. Purified LPS from *F. tularensis* is ~1000-fold less immuno-stimulatory than that of *E. coli* (160), and is not recognized by Toll-Like receptor 4 (TLR4) (79, 182). This is a result of the unique lipid A within the *Francisella* lipopolysaccharide (LPS), which has a tetra-acylated structure rather than the hexa-acylated structure of the highly inflammatory lipid A species found in LPS of most enteric pathogens (142, 190). This unique lipid A initially allows *Francisella* to be undetected by the immune system. *Francisella* also produces a capsular material, which is believed to be involved in resistance to killing by serum components, most notably the complement system (177). In addition to not being easily recognized by the innate immune system, *Francisella* also actively interferes with cellular signaling and cytokine production. Following infection, the production of pro-inflammatory cytokines TNF-α
and IL-1β is diminished in host cells (181). This effect is dependent on viable bacteria, which implies a bacterial active process. Infection by Francisella also appears to dampen the inflammatory response to secondary stimuli such as E. coli LPS, which suggests a global interference with the pro-inflammatory response of macrophages (181, 182). While mechanisms are still unknown, modulation of the inflammatory response by F. tularensis is at least partially achieved through the blockade of phosphorylation of proteins in the TLR/mitogen activated protein kinase (MAPK) regulatory cascade (182).

The ability of F. tularensis to replicate within host cells is largely dependent on genes located within the Francisella Pathogenicity Island (FPI). The FPI is comprised of 17 genes that lie within 2 putative operons (Fig. I.2) (130). This island is present and highly conserved among F. tularensis subspecies. An interesting feature of the FPI is that it is present in 2 identical copies on the chromosome of the human pathogenic subspecies tularensis and holarctica, while non-human pathogenic subspecies novicida contains only one functional copy of the FPI (93, 157). Early random mutagenesis screens in subspecies novicida revealed that mutations in genes within the FPI were attenuating for growth in primary human macrophages and other phagocytic cell types (71, 74, 130). Some of the transposon insertions on the FPI were found to be in genes comprising a four gene operon, iglABCD. More thorough examination of this operon has directly implicated these genes in bacterial escape from the phagosome and/or the ability to replicate in the host cell cytosol (47, 91, 130, 161, 164). Recent targeted mutagenesis studies involving other genes within the FPI, including pdpA and pdpD, have shown similar attenuation phenotypes to those observed in iglABCD mutants (104, 167, 168). In general, this attenuated phenotype is characterized by the inability of FPI mutants to escape from, or disrupt development of, the nascent phagosome. This deficiency results in bacteria that are trapped in fully mature phagolysosomes, and as such are unable to reach the cytosol and undergo replication. This failure to escape the phagocytic or endocytic compartment as a result of mutations in FPI genes is likely the cause of
attenuation of these mutants in murine infections. Moreover, the function of the FPI genes appears to be central to the pathogenicity of *F. tularensis* since mutations in FPI genes also render the bacterium non-replicative within insect cell lines (151).

For the most part, FPI genes show little homology to any other known genes although it has been suggested that IglA and IglB form a complex involved in protein secretion (20, 47, 130). This is based on stabilizing interactions between IglA and IglB as well as limited homology and conserved gene arrangement between *iglAB* and *impBC*, a proposed secretion system in *Rhizobium leguminosarum*. More recently *iglAB* have been proposed to encode proteins similar to those of a newly described type 6 secretion system (T6SS) first characterized in *Vibrio cholerae* (20, 147). Additionally, altered expression of another FPI gene, *pdpD*, has been shown to affect the cellular localization of IglA, IglB, and IglC (104). Taken together, these data suggests that FPI genes may function together to encode both effector proteins as well as a secretion apparatus to export or directly deliver the effectors to host cells to enable survival of the bacteria.

While the FPI is the focus of much research regarding the genetic basis of *F. tularensis* pathogenesis, there are other virulence factors linked to genes outside the pathogenicity island. Among these are Type IV pili (T4P), acid phosphatases, and a secreted zinc metalloprotease. Long filamentous fibers similar in ultrastructure to T4P and having a polar localization pattern were first identified on the surface of LVS by Gil *et al.* (68). Genome sequence analysis of LVS and SchuS4 strains revealed a cluster of genes sharing both spatial arrangement and amino acid sequence homology to T4P gene clusters in *Pseudomonas aeruginosa* and *Neisseria meningitidis* (68, 93). The role of these genes in the pathogenicity of *F. tularensis* was initially evaluated by examination of a spontaneous mutant lacking 3 of the predicted T4P genes. It was found that this strain was unaffected for growth within macrophages, but was attenuated for the ability to disseminate from the intradermal site of infection and cause disease mice (26, 61). Data collected using site directed *pil* mutants confirm these phenotypes but also demonstrate a
mild reduction in attachment to host cells by the mutant strains (26). Studies using the non-human pathogenic subspecies *novicida* implicate the T4P genes in the secretion of at least 10 proteins, however no secreted proteins were detected in the culture supernatant of LVS (78). Furthermore, in contrast to LVS, mutation of the T4P genes or PepO, a T4P secretion dependent protein, actually increased the virulence of *F. novicida* in murine infections (78). PepO is homologous in sequence and catalytic activity to a zinc metalloprotease family of proteins found in both mammalian and bacterial species (78). It functions in mammalian systems to process a modulator of vasoconstriction to its active form. Hagar *et al.* hypothesize that deletion of pepO in *F. novicida* results in a more virulent phenotype because of reduced vasodilatation in the host, which leads to more rapid dissemination by *F. tularensis*. Interestingly, a functional pepO is only found in *novicida* subspecies (78).

Acid phosphatases produced by bacterial pathogens have been implicated in aiding intracellular survival through the inhibition of the respiratory burst in neutrophils (9, 158). An enzyme with limited sequence homology to other bacterial acid phosphatases and phospholipases C was purified from several different strain of *F. tularensis* and was found to have a broad substrate specificity which included AMP, ATP, and mannose-6-phosphate (60, 153). Added exogenously in vitro, this purified enzyme suppressed the respiratory burst of activated neutrophils (153). The role of AcpA in vivo is unclear. One group reported a transposon insertion mutation in *acpA* did not affect intramacrophage growth or virulence in mice (12), while another group deleted the entire gene and saw a mild attenuation in mice and a reduction in intramacrophage growth which was related to delayed phagosomal escape (123). Further examination of the *F. tularensis* genome sequence revealed four genes encoding putative acid phosphatases (93, 125), which when knocked out in conjunction in *F. novicida* have an additive effect on *F. tularensis* virulence (125), suggesting partially redundant function of
these genes with respect to pathogenicity. In contrast, mutation of these genes in a human virulent Type A strain appears to have no effect on virulence (32).

**Regulation of Virulence Factors**

*Francisella* is able to parasitize a wide range of organisms and cell types which suggests the ability to adapt to different niches through variable gene expression. Indeed, a comparison of protein profiles of *F. tularensis* after broth growth or intracellular growth revealed the appearance or increased abundance of several proteins in bacteria harvested from infected macrophages (70). More recently, global transcriptional profiling of *F. tularensis* at different times post infection in macrophages confirmed this result, revealing variable expression of whole sets of genes dependent on the stage of the infection (192). These observations support the notion that *F. tularensis* is able to sense and respond to changing environments during the course of infection, although few “signals” have been identified that can be directly linked to modulation of virulence gene expression. Indeed, the only identified environmental cue resulting in altered FPI expression is iron starvation which results in only a modest increase in expression of the *iglABCD* operon (24, 52).

At the initiation of this work only one regulator of virulence gene expression, MglA, had been identified. During a screen for acid phosphatase mutants, a strain was isolated that carried a spontaneous point mutation that resulted in reduced cleavage of the chromogenic phosphatase substrate 5-bromo-4-chloro-3-indolylphosphate (XP) (11). In addition to reduced phosphatase activity, this strain was also severely defective for growth within several types of macrophages. Complementation experiments identified the mutated gene and homology analysis revealed significant similarity to the stringent starvation regulatory protein (*sspA*) in *E. coli* (11). Because of the effect of the mutation on phosphatase activity and intracellular survival, and sequence similarity to an RNA polymerase associated regulatory protein in *E. coli*, the authors posited a regulatory role for MglA in virulence gene expression. This was later confirmed by real time RT-PCR
experiments demonstrating a loss of expression of several FPI genes in an *mglA* mutant (95) and again by microarray analysis implicating MglA in the regulation of up to 102 genes throughout the *Francisella* genome (22, 28). Not surprisingly, mutation of *mglA* has pleiotropic effects ranging from reduced in vitro growth rate, to an inability to replicate within host cells and a general sensitivity to cellular stresses (11, 19, 23, 28, 75, 95, 164). Unexpectedly, a second gene product with significant homology to *sspA* was identified during protein studies when it co-precipitated with both MglA and RNA polymerase (28). During the course of these studies it was also established that the alpha subunits of *F. tularensis* RNA polymerase are not identical, which is in contrast to other bacteria, and that these subunits are of different molecular weights. Together, these findings led to a model which involves the hetero-dimerization of MglA and SspA which then associate with the unique alpha C-terminal domains of RNA polymerase in *Francisella sp.* to elicit regulatory effects. This is supported by data demonstrating near 100% overlap of *mglA* and *sspA* regulons (28). The regulatory effects of *mglA/sspA* are not thought to be direct since neither protein contains a recognizable DNA binding domain.

A third regulatory protein, FevR, was among five genes identified from a random mutagenesis screen employing a plasmid-borne reporter of *pepO* (known to be part of the *mglA/sspA* regulon) expression (21). Each of the mutations resulted in reduced reporter activity. Microarray analysis comparing *fevR* and *mglA* strains revealed identical regulons (21), suggesting that FevR works in parallel with MglA/SspA to modulate gene expression. FevR shares some homology with *merR* family DNA binding regulators, but lacks the metal binding domain characteristic of MerR. Because of the DNA binding domain within FevR, it is attractive to speculate that FevR is the intermediary between *mglA/sspA* modulated expression and specific regulatory sequences on the chromosome, although, no protein-protein interactions have been demonstrated between FevR and
MglA or SspA, nor has FevR been demonstrated to bind to any specific DNA sequences (21).

Two-component regulatory systems consisting of a membrane-bound sensor kinase and a cytoplasmic response regulator are ubiquitous among bacterial species and provide a mechanism to sense and respond to different environments through altered gene regulation (59, 76, 110, 155). The Francisella genome encodes only two such systems, neither of which is paired (93). One of these unpaired response regulators is homologous to \textit{pmrA} in Salmonella enterica, where it is involved in the upregulation of genes for LPS modification in response to membrane perturbations, particularly due to cationic peptides (76). Inactivation of this gene in \textit{F. tularensis} did increase susceptibility to killing by antimicrobial peptides, and also ablated growth in macrophages (124). Microarray analysis of this mutant revealed a change in expression of 65 genes as compared to wild type bacteria grown under the same conditions. The most profoundly affected genes are located proximal to \textit{pmrA} itself, but the mutation also had a minor negative effect on \textit{fevR} and genes within the FPI (124) (Fig. I.3). Because of the relatively minor affect on FPI gene expression, it is difficult to determine if the attenuation observed in this mutant is due to reduced FPI expression, or the result of the affect of the \textit{pmrA} mutation on expression another gene in the PmrA regulon.

It is increasingly apparent that small non-coding RNAs (sRNA) are responsible for wide ranging affects on gene expression (191). The regulatory mechanism of these sRNA is based on paring with complementary sequences within mRNA messages (109). Sometimes sRNA activity is dependent upon interaction with a specific protein, which can aid in stabilization of the regulatory RNA or in targeting to an mRNA (73, 127). One such protein, Hfq, has been identified in many bacterial species, including \textit{F. tularensis}, and often is involved in the regulation of a wide range of genes (97, 148). Not surprisingly, mutation of \textit{hfq} in \textit{F. tularensis} resulted in pleiotropic effects including an increased sensitivity to elevated salt concentrations, detergents, and high temperatures.
In addition to these in vitro phenotypes, the *hfq* mutant was attenuated for growth in several types of macrophages and reduced virulence in mice (84, 118). Microarray and RT-PCR analysis of the mutant revealed an effect on the expression of 104 genes, among which are 10 genes within the FPI. Again, because of the large Hfq regulon, it is difficult to attribute specific phenotypes of the mutant to specific genes or to assess the impact of reduced FPI gene expression on virulence in this mutant.

**Thesis**

The overall aim of this thesis is to gain a better understanding of the genetic basis of gene regulation that enables *F. tularensis* to efficiently infect, and adapt to conditions within host cells. Herein I describe the development of a transposon-based mutagenesis system that enables the creation of mutant libraries, some of which carry reporters of gene expression. These libraries are initially used to identify differentially regulated genes and to examine the effect of iron availability on the expression of genes in two operons, *fslABCD* and *iglABCD*, associated with the virulence of *F. tularensis*. Following this characterization, I utilized one member of the mutant library carrying a *lacZ* reporter insertion in *iglB* as the basis for a second round of mutagenesis to identify a new regulator of virulence gene expression, MigR. Further, the role of this regulator in the pathogenesis of *F. tularensis* live vaccine strain is examined via phenotypic characterization using in vitro cell infection assays. Finally, these findings are applied to the fully virulent *F. tularensis* strain Schu S4, and comparisons are drawn between the two bacterial strains and corresponding mutations in primary human airway epithelial cells and murine infections. These studies contribute to a better overall understanding of gene regulation in *F. tularensis* pathogenesis and also highlight key differences between the live vaccine strain and fully virulent Schu S4 strain.
Figure I.1. Intracellular trafficking of *F. tularensis* in macrophages. Following phagocytosis, *F. tularensis* resides in a phagosome that sequentially acquires early and late endosomal markers such as Rab5, EEA1 and lamp-1. Maturation of the *Francisella* containing phagosome is stalled at the late endosomal stage and does not acquire lysosomal markers such as cathepsin D. Finally, *F. tularensis* escapes the phagosome to replicate within the host cell cytosol.
Figure I.2. The *Francisella* pathogenicity island. The *Francisella* pathogenicity island (FPI) is comprised of 17 genes composing two proposed operons. The region spans ~28 kb and is present in two identical copies on the *F. tularensis* chromosome. Genes within the island, including *iglABCD*, have been associated with the ability to replicate within macrophages.
Figure I.3. Model for regulation of FPI gene expression. MglA and SspA heterodimerize and associate with unique alpha subunits of RNA polymerase (RNAP). This interaction is necessary for positive activation of about 102 genes including those within the FPI, as well as others throughout the \textit{F. tularensis} chromosome. The regulatory activity of the MglA/SspA/RNAP complex is dependent on FevR, as a \textit{fevR} mutant demonstrates regulatory defects identical to \textit{mglA} or \textit{sspA} mutants with the exception that \textit{fevR} is not autoregulated. PmrA, annotated as an orphan response regulator, highly regulates genes proximal to itself on the chromosome but also has a modest effect (\~2-fold) on the expression of \textit{fevR} and FPI genes.
CHAPTER II
IDENTIFICATION OF DIFFERENTIALLY REGULATED FRANCISELLA TULARENSIS GENES BY USE OF A NEWLY DEVELOPED TN5-BASED TRANSPOSON DELIVERY SYSTEM

Introduction

The development of genetic techniques and tools to study Francisella pathogenesis has driven much of the progress that is being made in understanding the molecular pathogenesis of this organism. Early efforts to identify virulence factors of Francisella relied upon nonspecific mutagenesis techniques (17, 116, 159) or unstable transposon insertions (Tn10 or Tn1721) (7, 11, 12, 15, 39, 74, 94, 117). More recently, groups have used EZ::TN (Epicentre) transposon-transposase complexes to obtain stable Tn5 insertions in the chromosome of F. tularensis LVS (86, 178), F. tularensis novicida (65, 193) or F. tularensis Schu S4 (149). While the EZ::TN system is capable of creating random insertion mutants in Francisella sp., the insertion frequency is low, making it difficult to obtain a saturating library. Additionally, Maier et al. reported the development of a Himar1-based transposon system to create mutants in F. tularensis (108). Escherichia coli-Francisella shuttle vectors constructed for site-directed mutagenesis experiments (90, 103, 107, 131, 150) have been reported but work with variable success depending on the gene and F. tularensis subspecies being mutagenized. A single reporter of gene expression, chloramphenicol acetyltransferase, has been reported and utilized to screen a promoter fusion library (90), however the assay for reporter activity is cumbersome in the context of a large scale screen.

The development of microarray and proteomic technologies provides alternative approaches to traditional transposon promoter fusion constructions for the study of gene and protein expression in bacteria. One advantage of a microarray or proteomic approach is that expression of every bacterial gene or gene product can be compared under two, or
more, conditions. A comprehensive view of the bacterial genome or proteome is difficult to achieve with transposon promoter fusions since one must simultaneously examine thousands of individual mutant strains. However, a transposon-based reporter library has unique and important advantages over either of the more global approaches. For instance, insertion of a transposon reporter into a given gene creates a mutant strain, in addition to creating a promoter reporter strain. With the mutant isolate in hand, work to characterize the mutated gene can be initiated very quickly. Another significant advantage is the ability to re-mutagenize a reporter strain to identify regulatory elements that govern expression of the gene. This allows regulatory pathways to be uncovered and characterized, which significantly increases my understanding of bacterial gene expression and signal transduction.

In this chapter, I report the construction of a highly efficient *Francisella tularensis* mutagenesis system that employs the hyperactive Tn5 transposase. Expression of the transposase has been placed under the dual control of the *Francisella* groES promoter and the lac operator and LacI repressor to increase plasmid stability. The transposase, which resides outside of the Tn5 insertion sequences (mosaic ends), catalyzes insertion of the transposable element into the *Francisella* chromosome. Contained within the transposable element is a kanamycin resistance gene, which I engineered to be flanked by FLP recombination target (*FRT*) sequences for the creation of unmarked mutations, and the *pir*-dependent R6K origin of replication for recovery cloning and sequencing of the Tn5 insertion site. The transposon is delivered from a conditionally replicating (temperature-sensitive) *F. tularensis* plasmid (107) that, at high temperature, allows the selection of insertions into the *Francisella* chromosome due to the loss of replication of the temperature-sensitive plasmid. My results indicate that this transposon mutagenesis system produces single, random, stable insertions into the chromosomes of *F. tularensis* strains and is capable of creating a saturating Tn5 insertion library in a single experiment. In addition, derivatives of this system have been
engineered to allow the creation of chromosomal *lux*CDABE or *lacZ* as transcriptional reporters.

I have utilized this system to create and screen a transposon library of *F. tularensis* LVS for differential gene expression when grown on modified Mueller Hinton (MMH) or Chamberlains Defined Medium (CDM). Reports in the literature indicate that growth of *F. tularensis* on CDM results in increased capsule production as well as increased type IV pili expression (31, 68). In addition, it has been reported that growth on CDM causes a general increase in virulence of *Francisella* in a mouse model (31). Based upon the idea that CDM may upregulate virulence gene expression, I screened *F. tularensis* LVS transcriptional reporter libraries on MMH and CDM growth media. These screens have successfully identified established virulence genes as well as new genes that may play a role in the pathogenic lifestyle of *F. tularensis*. Some of the genes identified are involved in iron acquisition, suggesting that low iron availability is one of the signals sensed by *Francisella* on CDM agar that leads to upregulation of gene expression. Other groups have also reported iron availability as a signal resulting in differential expression of genes in *F. tularensis* (52, 96, 179). Classically, the ferric uptake regulator (Fur) functions as an iron-dependent transcriptional repressor by binding to DNA in the presence of ferrous iron (58). Sequences resembling Fur binding sites (Fur boxes) have been identified upstream of iron-regulated genes in *F. tularensis* (52, 179), although data linking Fur to regulation of these genes has not been presented. Based upon my observations, and data published by others, I examined the role of Fur in the transcriptional regulation of two *Francisella* gene clusters that respond to iron concentration. I present evidence that suggests that Fur may be involved in the regulation of only one of these two gene clusters.

In summary, in this chapter I describe the construction and use of a Tn5 transposon delivery system that is capable of creating random, stable insertions in *F. tularensis ssp*. I also present data demonstrating that chromosomal *lacZ* transcriptional
reporters can be used to identify differentially regulated genes and quantify gene expression in *F. tularensis*. Finally, I include preliminary data that Fur is a negative regulator of transcription of the *fslABCD* operon but not the *iglABCD* operon.

**Materials and Methods**

**Bacterial strains and growth conditions**

*F. tularensis* LVS (ATCC 29684) and *F. tularensis tularensis* Schu S4 were grown in Mueller Hinton Broth (Becton Dickinson, Sparks, MD) or on Mueller Hinton Agar (Acumedia, Lansing, MI) supplemented with 1% glucose (w/v), 0.025% ferric pyrophosphate, and 2% IsoVitaleX. Spectinomycin and kanamycin were added to the growth media to a final concentration of 25 µg/ml, when appropriate. Chamberlain’s Defined Medium (CDM) was prepared as described (27), or with 28 µM or 350 nM FeSO₄, as dictated by experiment. Plasmid pMKM219 is a derivative of the temperature-sensitive plasmid pFNLTP9 (107) in which the kanamycin resistance gene has been replaced with a spectinomycin resistance gene. The *F. tularensis* Fur expressing plasmid was constructed by amplifying the fur gene from *F. tularensis* LVS using oligonucleotide primers tailed such that the full length fur gene could be cloned downstream of the P<sub>groES</sub> promoter sequence in pBB103, a derivative of pKK202, to create pBB110. All cultures were grown at 30°C, 37°C, or 41°C as dictated by experiment. *F. tularensis* strain Schu S4 was handled within a BSL3 laboratory in accordance with all CDC/NIH regulatory and safety guidelines.

**Construction of Tn5 delivery vector**

The DNA fragment encoding the *Francisella groES* promoter, *lac* operator, and *lacI*<sup>q</sup> was first constructed in pCR2.1 by cloning PCR fragments that were amplified with primers (sequences will be supplied upon request) that introduced the desired restriction sites at the end of *lacI*<sup>q</sup> and the *groES-lac* operator fragment. This DNA fragment was removed from pCR2.1 by digestion with *BmgBI* and *NdeI* sites and ligated into the *BmgBI* and *NdeI* sites of pRL27 (92). These genetic elements were oriented in the
pRL27 vector such that the *lac* operator sequence was positioned immediately downstream of *groES* in the same orientation as the hyperactive transposase, while *lacI* was upstream of *groES* on the complementary strand (Fig. II.1). This plasmid intermediate was next modified by PCR amplifying and cloning the *Francisella omp26* promoter sequence upstream of the *aphA3* gene within the transposable element. This modification ensures expression of kanamycin resistance independent of the position of the chromosomal insertion. Finally, the *aphA3* gene was amplified from pRL27 using primers that included the *FRT* recognition sequence for FLP recombinase and the DNA fragment was used to replace the existing *aphA3* gene. These combined modifications resulted in the creation of pBDJ303, a stable, kanamycin-resistant transposon delivery vector suitable for use in *F. tularensis*. One feature worth mentioning in pBDJ303 is the presence of unique *EcoRI* and *KpnI* sites immediately inside the right side mosaic end of the transposable element. These sites allow the directional cloning of reporter genes that can be used to create transcriptional promoter fusions. For conditional (temperature-sensitive) maintenance in *F. tularensis*, pBDJ303 was joined to a pFNLT9-derived *E. coli-F. tularensis* shuttle vector (pMKM219) at unique *SpeI* sites present in each vector to create the final transposon delivery vector, pBB107.

**Cryotransformation**

Plasmid DNA was introduced into *F. tularensis* strains by a cryotransformation protocol (126). Briefly, 500 ng of DNA was added to ~10⁸ CFU *F. tularensis* LVS that were suspended in *Francisella* transformation buffer (0.2 M MgSO₄, 0.1 M Tris acetate, pH 7.5), frozen in liquid nitrogen, and then thawed. The transformed bacteria were grown in either modified Mueller-Hinton (MMH) broth or on MMH agar without selection at 30°C for 7 hours. Dilutions of the transformed bacteria were plated on MMH agar with 25 μg/ml spectinomycin at 30°C to select for *F. tularensis* containing the transposon delivery plasmid, pBB107.
Transposon selection protocol

Colonies obtained after ~3 days growth at 30°C on MMH agar containing 25 µg/ml spectinomycin were inoculated into 5 ml MMH broth with 25 µg/ml spectinomycin and were grown at 30°C with agitation to an OD$_{600}$ of ~0.1. Cultures of LVS were serially diluted and plated on MMH agar with no selection to quantitate the viable cells or on MMH agar with 25 µg/ml kanamycin at 41°C to select for Tn5 insertions into the *F. tularensis* chromosome with concomitant loss of the transposon delivery plasmid. Selection of *F. tularensis* Schu S4 transposition events was performed at 40°C, because the strain grew poorly at 41°C; the frequency of transposition was similar to those obtained with LVS at 41°C. *F. tularensis* Tn5 mutants were arrayed to 96-well cell culture plates in 100 µl MMH broth and were incubated at 37°C until turbid. Freezer stocks were made by adding 100 µl of 2X freezing medium (1.0 M sucrose, 20% glycerol).

Identification of transposon insertion sites

To identify the sites of Tn5 insertions, genomic DNA was isolated from individual colonies and digested with EcoRI (no reporter), PciI (*lux* reporter), or NdeI (lacZ reporter) to create a DNA fragment containing the oriR6K origin, the *aphA3* gene and flanking chromosomal sequence. The digested DNA was ligated, transformed into a *pir*+ *E. coli* strain and plated onto agar plates with kanamycin to select for transformants that carried the plasmid of interest. Plasmid DNA was isolated and sequenced using a primer with the sequence 5’CATGCAAGCTTCAGGGTTGAG 3’ that anneals to the 3’ end of the *aphA3* gene and produces sequence of the flanking chromosomal DNA. Sequence data was used to search the sequenced bacterial chromosomal database using NCBI BLAST to identify Tn5 insertion sites within the *F. tularensis* chromosome.

Screening *lacZ* and *luxCDABE* mutants for reporter activity

Tn5 mutants were recovered from freezer stocks and plated on MMH agar at 37°C using a 96-prong replicator (Boekel, Feasterville, PA). After ~24 hrs, reporter enzyme
activity was detected using a 60 min exposure time in a Fujifilm LAS-1000 luminescence imager (lux reporters), or were visualized by overlaying #1 Whatman filter paper pre-soaked with 20 mg/ml X-gal in dimethylformamide diluted 1:4 in water (lacZ reporters). Quantitation of lacZ activity was done according to the method of Miller (121). Duplicate cultures of tested strains were grown to mid log phase (OD$_{600}$ 0.5 – 0.6) or late log phase (OD$_{600}$ 0.9-1.1) and β-galactosidase assays were performed on triplicate samples of each culture.

Cloning and expression of FLP recombinase in F. tularensis

The gene encoding FLP recombinase was amplified from pFT-K plasmid template (145) DNA using upstream 5’AGCAGCGGTACCCAAGGGGTGTATGCCCACAATTGATATATTAT GTAAACACC 3’ and downstream 5’ATCGATCGGTGACTTATATGCGTCTATTT ATGTAGGATG 3’ oligonucleotide primers. At the 5’ end of the upstream primer, a Shine-Dalgarno sequence from aphA3 was included to ensure effective translation of the FLP gene in F. tularensis, as well as a KpnI site to facilitate cloning. At the 5’ end of the downstream primer a SalI site was included in the sequence of the primer. This PCR-amplified fragment was subcloned into pCR2.1 before being moved to an E. coli-Francisella shuttle vector containing the temperature-sensitive origin of replication. Expression of FLP is driven by the Francisella groES promoter. The shuttle vector containing FLP recombinase, pBB111, was introduced into Tn5 insertion mutants of Francisella by cryotransformation and transformants were selected on MMH agar containing 25 µg/ml spectinomycin. Spectinomycin-resistant colonies were passaged once on MMH agar containing spectinomycin, and isolated colonies were streaked to MMH agar with or without 25 µg/ml kanamycin to screen for FLP-mediated deletion of the aphA3 gene. Southern blot confirmation of the loss of the aphA3 gene was conducted using a DIG-labeled DNA probe generated using the oriR6K region of the transposon as template DNA.
Western blotting for IglC

Wild type *F. tularensis* LVS or LVS harboring the fur expression plasmid pBB110 was grown to mid-log phase of growth in CDM with either high (28 µM) or low (350 nM) FeSO₄. Cultures were normalized for cell number based on OD₆00 and an equal amount of bacterial cells were used for IglC quantification by Western blot. Polyclonal goat anti-IglC raised against IglC purified from *F. novicida* was used to probe IglC in the samples. Detection of IglC was achieved using horseradish peroxidase conjugated secondary antibody and SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Results

Construction of a Tn5 transposon delivery system in *Francisella*

My initial efforts to create Tn5 kanamycin-resistant transposon mutants in *Francisella* by electroporation or conjugation of the R6K pir-dependent plasmid pRL27 (92) into *F. tularensis* were unsuccessful. Factors that may have contributed to my inability to initially obtain mutants include poor transformation or conjugation efficiency, low expression of the transposase and/or low expression of the kanamycin resistance marker. In response to these failed experiments, I created a new *Francisella* Tn5 transposon delivery system that overcame these experimental concerns. First, I created plasmid pBDJ303 by optimizing several features of pRL27 for use in *Francisella* (Fig. II.1). The *Francisella groES* promoter was placed upstream of the hyperactive Tn5 transposase gene to drive expression of the gene in *Francisella* strains. In the process of making this modification, it became apparent that the plasmid was quite unstable, probably due to the high activity of the transposase. To alleviate plasmid instability, I placed the expression of the Tn5 transposase under the control of the lac operator and cloned the lacI* gene onto the plasmid so that, in *E. coli*, transposase expression is repressed by LacI* binding to the lac operator sequence between the groES promoter and
the transcriptional start site of the transposase. These modifications resolved the plasmid stability issues and also significantly reduced the likelihood of transposition occurring in *E. coli* during cloning. In addition, two other modifications were made to pBDJ303 before it was fused to a conditionally replicating *E. coli-Francisella* shuttle plasmid. First, I placed expression of the kanamycin resistance gene in the transposon under the control of the *Francisella omp26* promoter to ensure high level expression of kanamycin resistance from chromosomal insertions. Second, the kanamycin resistance gene was amplified by PCR with oligonucleotide primers that contain *FRT* sequences and recloned into the vector. This modification provides the option of deleting the kanamycin resistance gene from a *Francisella* Tn5 mutant, when desired. Finally, I fused pBDJ303 to pMKM219 (a derivative of pFNLT9) (107), an *E. coli-Francisella* shuttle vector containing a *Francisella* conditional origin of replication, to produce the Tn5 transposon delivery vector pBB107 (Fig. II.1).

**Transformation/transposition and rescue of Tn5 insertions**

*F. tularensis* LVS was transformed with the *E. coli-Francisella* Tn5 transposon shuttle plasmid pBB107 using a cryotransformation protocol (126, 131, 138). In preliminary experiments, no kanamycin resistant colonies were obtained by directly plating transformed *F. tularensis* LVS and selecting at 41°C indicating that the combined transformation and transposition frequencies were below a detectable threshold. Accordingly, the creation of transposon mutants was performed in two steps. First, transformants were selected at 30°C on plates containing spectinomycin which yielded ~100 spectinomycin-resistant transformants (frequency of 1 transformant per 10^6 recipient bacteria). The efficiency of transformation was not significantly altered by broth or plate outgrowth (data not shown). A single spectinomycin-resistant colony was inoculated into MMH broth containing 25 µg/ml spectinomycin and grown at 30°C to an OD_{600} ~0.1. To determine the frequency of transposition from the pBB107 plasmid, cultures were serially diluted and plated on MMH agar with or without 25 µg/ml
kanamycin at 41°C (40°C for Schu S4) to simultaneously cure the delivery plasmid and select for Tn5 insertion mutants. My results indicated that ~1 in a 1000 organisms containing pBB107 gave rise to a kanamycin-resistant Tn5 mutant (i.e. transposition frequency of $10^{-3}$) (Table II.2). Fifteen kanamycin-resistant LVS mutants were randomly selected for identification of Tn5 insertions sites. Of the fifteen Tn5 insertions that were recovered, each mapped to a unique location on the *F. tularensis* chromosome (data not shown), which is consistent with the findings of others (86, 149). This same frequency of transposition ($1-4 \times 10^{-3}$) has also been observed repeatedly in *F. tularensis* Schu S4, as part of the process of constructing various *F. tularensis* Schu S4 transposon libraries. Sequencing the transposon insertion site of individual mutants from these libraries revealed that the insertions are random. The Schu S4 libraries are the focus of work beyond the scope of the experiments in this chapter, however, they do serve as the basis for screens being conducted by other researchers in this and other laboratories. The frequency of transposition in *Francisella* is virtually identical to the transposition frequency observed in other bacterial species using the Tn5 hypertransposase (72, 92). A clear advantage of making Tn5 mutants with this method over the EZ::TN transposome system is that the number of mutants that can be created in a single selection is virtually unlimited.

**Creation of unmarked Tn5 mutations in *F. tularensis* using FLP recombinase**

To improve the utility of the Tn5 transposon delivery system, the 18 base-pair *FRT* sequence was added to each end of the *aphA3* gene present within the Tn5 transposable element by amplification of the *aphA3* gene with *FRT*-tailed oligonucleotide primers. Following mutagenesis with the transposon containing the *aphA3* gene flanked by *FRT* sites, I sought to remove the antibiotic marker by expressing FLP recombinase. A conditionally replicating (temperature-sensitive) *E. coli*-*F. tularensis* shuttle plasmid (pBB111) that expresses FLP recombinase from the *F. tularensis groES* promoter was
cryotransformed into a *F. tularensis* LVS Tn5 mutant strain and transformants were selected on solid agar with spectinomycin. Single transformants were purified and isolated colonies were patched to MMH agar with and without kanamycin. Twenty of twenty *F. tularensis* colony re-streaks grew on plates with no antibiotics but failed to grow on plates with kanamycin, indicating that FLP recombinase was extremely efficient at deleting the kanamycin resistance gene present in the Tn5 mutant. Additionally, Southern blotting was performed on a selected mutant and a ~1 kb deletion was detected, compared to the parent strain, which corresponds to the loss of the *aphA3* gene (Fig.II.2).

**Screening for regulated promoter activity using luxCDABE and lacZ reporters**

Separate libraries have been constructed with derivatives of pBB107 that create *luxCDABE* promoter fusions or *lacZ* promoter fusions with genes on the *F. tularensis* chromosome. Following selection of Tn5lux or Tn5lacZ mutants, individual mutant isolates were arrayed into the wells of a 96-well master plate and then replica plated onto large MMH or CDM agar plates. The objective of these screens was to identify genes that were differentially regulated by growth on the different media.

The activity of *lux* reporters in randomly generated *F. tularensis* strains was analyzed using a Fujifilm LAS-1000 luminescence imager. My experimental results revealed that detection of *lux* activity had several technical concerns. First, activity of bacterial luciferase, encoded by the *Vibrio harveyi luxCDABE* operon, was extremely low at 37°C compared with the activity of the luciferase complex at the optimal temperature of 25°C (55). This concern was magnified by the relatively low sensitivity of the photoimager which was unable to detect the luminescence of strains grown at 37°C. These detection issues could be partially overcome by first incubating the *F. tularensis lux* strains at 25°C for 4 hours, followed by a relatively long exposure time (1 hour) in the photoimager. However, even with this relatively elaborate detection method, less than 1% of *F. tularensis* strains carrying Tn5lux transposon insertion produced detectable luminescence.
luciferase activity. Despite these difficulties, I identified three strains carrying luciferase reporters that were upregulated when grown on CDM agar (Fig. II.3). Sequence analysis of the transposon insertion sites in these strains revealed that the Tn5 insertions were in genes encoding a 16S rRNA (FTL_R0003), fslD (FTL_1835), and iglC (FTL_0113/1159). Quantitation of the luciferase reporter activity levels in these strains, using a luminometer, revealed significant upregulation of each of these genes when grown in CDM, although the highest level of luciferase activity was near the lower limit of detection of the luminometer.

*F. tularensis* mutants, generated using the *lacZ* reporter, did not grow when plated directly onto differential media containing X-gal; however, the *lacZ* expression of individual isolates could be detected by first growing the strains in the absence of X-gal and then exposing the colony to a filter soaked with X-gal. Following a short incubation period (15-30 min.) at 37°C, *lacZ*+ strains were readily detectable by the characteristic blue precipitate observed when X-gal is cleaved. When β-galactosidase filter assays were performed with randomly selected colonies, ~30% of the strains expressed β-galactosidase to various degrees. Of ~1500 individual mutants screened in this manner, 24 were identified as carrying *lacZ* reporters in genes that were differentially expressed on the two media (Fig.II.4). When quantitative β-galactosidase assays were performed after growth in MMH or CDM broth to quantify gene expression, results for several strains did not match the plate-grown *lacZ* expression phenotypes. However, if gene expression was compared after growing in MMH versus CDM with only 350 nM FeSO₄ (instead of 7 µM FeSO₄), the expression profiles of broth-grown strains mirrored that of their plate-grown counterparts (Table II.3). This finding led us to conclude that iron starvation was responsible for the observed increase in expression of at least some of the reporter strains, which was detectable on plates due to local depletion of iron around the colonies.
The differential growth condition screen identified three of the four genes in the *fsl* operon to be among the most highly upregulated when grown on CDM agar or in CDM broth. It is likely that strains carrying reporters in these genes were identified on CDM plates because local iron levels in the iron-limiting CDM agar were depleted, resulting in induction of these iron-regulated genes. This mechanism would be consistent with low reporter activity in liquid media with the same iron concentration because effective iron concentrations would remain higher in liquid, due to mixing. When CDM broth was used with 350 nM iron, I saw induction of genes in the *fsl* operon, as well as other genes identified by the plate screen. Two other strains identified by the screen to be highly regulated were those containing lacZ fusions in *iglB* (FTL_0112/1158), as well as another hypothetical protein (FTL_0122/1168) located on the *Francisella* Pathogenicity Island (FPI). Because of these results, I believe that low iron concentration is likely one factor that contributes to the increased pathogenicity reported for *Francisella* grown on CDM agar.

**Expression of Fur in *fsl* and *igl* reporter strains grown in low and high iron media**

Since low iron growth conditions resulted in the induction of genes in the *fsl* operon as well as both *iglB* and *iglC*, I examined the role of Fur in the regulation of these genes. The *fur* gene was PCR amplified from the *F. tularensis* chromosome, cloned into the *E. coli-Francisella* shuttle plasmid, pBB110, and introduced into the *fslC-lacZ* and *iglB-lacZ* reporter strains. Since I wanted to use the *iglB-lacZ* reporter as an indicator of gene expression of the entire *igl* gene cluster, I first demonstrated that the four genes are likely transcribed as a single mRNA using RT-PCR (Fig.II.5). *F. tularensis* strains with lacZ reporters in *fslC* or *iglB* carrying pBB110 for expression of Fur, or without the expression vector as a control, were grown in CDM containing 28 µM (high iron condition) or 350 nM (restricted iron condition) FeSO₄. β-galactosidase assays were conducted on the strains after growth to mid log and late log growth phase.
When the \textit{fslB-lacZ} reporter strain was grown in CDM containing high iron, the reporter produced ~15 Miller units of activity regardless of growth phase. In contrast, the same strain showed a ~5-fold increase in expression in mid log phase and a ~10-fold increase during late log phase when grown under iron limiting conditions (Fig.II.6). This result is consistent with an increase in gene expression as a result of iron depletion in the growth medium. When the \textit{fslB-lacZ} reporter strain harboring the \textit{F. tularensis} Fur expression plasmid was examined, it exhibited little \textit{lacZ} expression (<5 Miller units) when grown in high iron media and a ~10-fold reduction in activity as compared to the parent strain when grown in iron restricted media (Fig.II.6). The observed repression of \textit{fslB} by overexpression of Fur was not surprising, given the strong consensus Fur box that overlaps the predicted \textit{fsl} promoter region. Similar experiments in \textit{V. vulnificus} demonstrated that overexpression of Fur in bacteria grown in iron replete or deplete media can have a repressing effect on Fur-regulated genes (101).

When similar experiments were conducted using the \textit{iglB} reporter strain, I also observed iron-dependent induction of the gene. Regardless of growth phase, the parent strain produced ~200 Miller units of activity when grown in CDM broth containing high iron. When grown in iron limiting media I observed a ~1.5 fold induction in mid log phase and a ~3-fold induction in late log phase. These data are similar to that obtained from the \textit{fslC} reporter, in that as iron is depleted from the growth medium induction of \textit{iglB} is increased. Unexpectedly, when I expressed the \textit{F. tularensis} Fur protein in this strain I observed a modest increase in activity from the reporter in both iron rich and iron limiting media (Fig.II.6). To confirm this result, I conducted a Western blot for IglC in LVS strains grown under normal or iron limiting conditions, either containing or lacking the Fur overexpression plasmid. Again, it was apparent that more IglC was present when LVS is grown under iron limiting conditions, however, the overexpression of Fur had no effect on IglC abundance regardless of iron concentration in the medium (Fig.II.7). These results provide surprising preliminary evidence that Fur acts as a repressor for the
fsl operon, but not the igl operon. These data allow the possibility that Fur regulates the
igl operon by a mechanism different from that of fslABCD, or not at all. I have explored
this potentially interesting regulatory mechanism and the role of Fur in the regulation of
iglABCD in more detail in the following chapter.

Discussion

My effort to create an efficient Tn5 transposon delivery system has relied upon
the observations and work of other research groups. Use of Tn10 and Tn1721 for
mutagenesis have both been found to produce unstable insertions (24) and the
commercially available Tn5-based in vitro system used to create mutant libraries in
different Francisella strains (65, 86, 149, 178, 193) has a reported efficiency of
transposition of ~1 mutant per 10^8 CFU in the transformation mix (86). My own efforts
to use the in vitro transposition system yielded small numbers of mutants per reaction that
quickly consumed resources and made it difficult to obtain enough mutants for a
saturating library. Combined, this information led me to create a more efficient system
for creating transposon mutants in Francisella strains.

Here, I have described the construction of a Tn5 mutagenesis system that has been
optimized for use in Francisella tularensis. This approach takes advantage of the
hyperactive Tn5 transposase which increases transposition ~1000-fold compared to wild
type Tn5 (92). Transcription of the transposase gene and the kanamycin resistance gene
has been placed under the control of Francisella promoters to achieve sufficient
expression in Francisella strains for activity and detection. In addition, expression of the
transposase gene has been placed under the control of the lac operator and LacI repressor
to stabilize the transposon delivery plasmid. I have also increased the utility of the
system by flanking the kanamycin resistance gene with FRT sequences to allow the
creation of unmarked mutations. A key aspect of my system is the use of a temperature-
sensitive F. tularensis plasmid origin of replication that was described by Maier et al.
(107) as the delivery platform for the Tn5 transposon. The use of this plasmid overcomes
the problem of low plasmid transformation frequencies into *F. tularensis* strains because a single transformant, recovered at 30°C, can be grown to provide sufficient numbers of bacteria to obtain virtually limitless numbers of transposon mutants. Furthermore, the temperature-sensitive replicon provides a strong selection against maintenance of the plasmid, allowing mutants to be recovered with ease at 41°C. My experimental results have validated the usefulness of this approach.

In addition to creating the *Francisella* Tn5 transposon mutagenesis system, I have also made two derivatives of Tn5 that create promoter fusions with *luxCDABE* or *lacZ* when inserted into the *F. tularensis* chromosome. The experimental data indicate that both reporters can be used to detect promoter activity in *F. tularensis*, although *lacZ* cleavage of the X-gal substrate is much more sensitive and able to produce more consistent results than light production from the *luxCDABE* gene fusions. I have used strains carrying randomly inserted Tn5lacZ reporters to identify genes differentially regulated when *F. tularensis* is grown on MMH as compared to CDM agar. Results from the qualitative plate screen were corroborated using β-galactosidase assays performed on broth grown bacteria and it was determined that iron depletion was responsible for upregulation of several genes. Among the genes found to be most highly regulated were genes in the *fsl* operon and *igl* operons. As previously described, genes within the *iglABCD* operon are critical to the pathogenicity of *F. tularensis* and mutation of these genes results in a bacterium that is unable to escape the maturing phagosome and is incapable of causing disease (71, 74, 130). Genes within the *fslABCD* operon share homology with bacterial siderophore systems have been demonstrated to be involved in iron acquisition (88, 179). Mutation of these genes results in a reduced ability to grow in iron restricted media, but has little effect on the ability to replicate within cultures macrophages (52, 179).

The Fur protein is associated with regulation of genes that respond to iron concentration in the growth medium. Ferrous iron binds to Fur as a co-repressor, causing
an allosteric change in the protein that results in Fur binding to conserved nucleotide sequences which often overlap the promoter region of Fur-regulated genes (40, 48). When iron becomes limiting, Fur adopts a non-DNA binding conformation and repression is relieved at these promoters. Fur-regulated genes are often involved in iron acquisition, but specific virulence factors in several pathogens have also been shown to be directly or indirectly regulated by Fur (102, 146). Given the strong consensus Fur box upstream of the *fsl* operon, it was not surprising that overexpression of *F. tularensis*-Fur resulted in super-repression of transcription of the *fsl* operon, under iron replete growth. Likewise, overexpression of Fur during iron restricted growth also resulted in significant repression of the *fslB-lacZ* reporter. These data strongly implicate the *F. tularensis* Fur protein as a repressor of the *fsl* operon in the presence of iron.

I, and others, have also found that genes in the *igl* operon, which are essential for intracellular survival and fundamental to the virulence of this pathogen, are upregulated when *F. tularensis* is grown in iron-restricted medium (52, 96, 179). Deng et al. (52) proposed that a Fur box resides upstream of *iglC* that shares 11 of 19 nucleotides with the consensus Fur box. However, it is difficult to reconcile how a functional Fur box upstream of *iglC* could control iron regulation of other genes in the *igl* operon, specifically upstream genes *iglA* and *iglB*. To determine if Fur from *F. tularensis* plays a role in the regulation of *iglABCD* similar to that which I have observed for *fslABCD*, I expressed the Fur protein in an *F. tularensis iglB-lacZ* reporter strain. Surprisingly, overexpression of Fur in this strain did not result in decreased expression of *iglB* reporter activity. In fact, overexpression of Fur resulted in a minor increase in *iglB-lacZ* activity. To rule out activity of Fur on the proposed Fur binding site upstream of the *iglC* gene, I conducted a Western blot for IglC under normal and iron restricted growth conditions and in the presence or absence of the Fur overexpression plasmid. Again, I observed induction of IglC in iron deplete media. Overexpression of Fur appeared to have no effect on IglC abundance in either growth condition. While it is unclear from these
experiments how Fur expression induces an increase in iglB transcription, I believe that these data clearly indicate that Fur is not acting as an iron-dependent repressor of the igl operon.

Two models are proposed to explain these data. First, F. tularensis Fur could positively regulate the expression of iglB in the absence of iron either directly, through productive contacts with RNA polymerase or by bending the DNA to favor transcription, or indirectly through repression of a transcriptional activator elsewhere on the chromosome. A mechanism of direct activation by Fur would involve the binding of Fur upstream of the iglA promoter in the absence of iron. This model fits my data and would explain the lack of an obvious Fur box upstream of iglA. Since iron binding to Fur causes an allosteric change, the DNA binding site for Fur not bound by iron would be expected to be different than the canonical Fur-Fe consensus binding site (50). The second model holds that overexpression of Fur simply allows Fur to act as an intracellular chelator of iron, which would trigger the activation of a second iron sensitive system that then regulates the expression of the igl operon. This model also fits my data, although the annotated LVS genome lacks an obvious alternative iron regulator.

Genetic approaches have been, and continue to be, invaluable in identifying and characterizing a wide range of bacterial characteristics including mechanisms of pathogenesis. In particular, transposon mutagenesis and the creation of chromosomal reporters of transcriptional activity are valuable techniques to identify bacterial virulence genes and study their regulation. Tn5-based transposons are well characterized and widely used because of their high frequency of transposition, functionality in many Gram-negative bacterial species, low sequence specificity for insertion, and stability when inserted into the host genome (46). The Tn5 transposon delivery system described in this report supplies an additional tool in work aimed at identifying and characterizing virulence factors, and their regulation, in F. tularensis strains. My data indicate that this transposon mutagenesis system produces virtually limitless numbers of single, random,
stable insertions in the chromosomes of *F. tularensis* strains. Modifications to the transposon provide additional features that are actively being used by our research group to explore and characterize *F. tularensis* pathogenesis. In addition, I have been able to demonstrate that reporters delivered by this transposon can be used to identify virulence genes (i.e. *igl* genes) and to study the regulation of various *Francisella* genes (iron regulation). The future work in this laboratory could focus on utilizing these new genetic tools to identify virulence genes and regulatory pathways that have been, until recently, inaccessible to characterization.
Table II.1. Bacterial strains and plasmids used in this chapter

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>F. tularensis</em> LVS</td>
<td>Live Vaccine Strain</td>
<td>K. L. Ekins</td>
</tr>
<tr>
<td><em>F. tularensis Schu S4</em></td>
<td><em>F. tularensis tularensis</em> Schu S4 “type A” strain</td>
<td>BEI Resources</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRL27</td>
<td>Suicide plasmid carrying hyperactive transposase and Tn5 transposable element</td>
<td>(92)</td>
</tr>
<tr>
<td>pBDJ303</td>
<td>pRL27 modified for use in <em>F. tularensis ssp.</em></td>
<td>This study</td>
</tr>
<tr>
<td>pMKM219</td>
<td><em>E. coli – F. tularensis</em> shuttle vector with temperature-sensitive <em>F. tularensis</em> origin of replication</td>
<td>This study, (107)</td>
</tr>
<tr>
<td>pBB107</td>
<td>Fusion of pBDJ303 and pMKM219, final Tn5 delivery plasmid for mutagenesis of <em>F. tularensis ssp.</em></td>
<td>This study</td>
</tr>
<tr>
<td>pBB108</td>
<td><em>E. coli – F. tularensis</em> shuttle vector containing cloned FLP gene downstream of <em>F. tularensis</em> P&lt;sub&gt;groES&lt;/sub&gt; promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pBB110</td>
<td><em>E. coli – F. tularensis</em> shuttle vector containing cloned LVS <em>fur</em> gene downstream of <em>F. tularensis</em> P&lt;sub&gt;groES&lt;/sub&gt; promoter</td>
<td>This study</td>
</tr>
</tbody>
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Table II.2. Transposition frequency of Tn5 in *F. tularensis*

<table>
<thead>
<tr>
<th>Trial</th>
<th>CFU plated(^a)</th>
<th>No. of Tn5 insertions(^b)</th>
<th>Frequency of transposition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.45 X 10(^7)</td>
<td>1.94 X 10(^5)</td>
<td>2.29 X 10(^{-3})</td>
</tr>
<tr>
<td>2</td>
<td>3.51 X 10(^8)</td>
<td>4.55 X 10(^5)</td>
<td>1.29 X 10(^{-3})</td>
</tr>
<tr>
<td>3</td>
<td>2.85 X 10(^8)</td>
<td>2.11 X 10(^3)</td>
<td>7.40 X 10(^{-4})</td>
</tr>
</tbody>
</table>

\(^a\)Total number of CFU determined by plating on MMH agar with no added antibiotics at 41°C

\(^b\)Total number of Tn5 insertions determined by plating on MMH agar with 25µg/ml kanamycin at 41°C
Table II.3. *F. tularensis* LVS genes upregulated by growth on Chamberlain’s Defined Media.

<table>
<thead>
<tr>
<th>Gene</th>
<th>MMH</th>
<th>CDM</th>
<th>Fold increase</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTL_1834</td>
<td>24</td>
<td>237</td>
<td>9.9</td>
<td><em>fslC</em></td>
</tr>
<tr>
<td>FTL_1835</td>
<td>13</td>
<td>101</td>
<td>7.8</td>
<td><em>fslD</em></td>
</tr>
<tr>
<td>FTL_0122</td>
<td>21</td>
<td>99</td>
<td>4.7</td>
<td>Hypothetical in FPI</td>
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<tr>
<td>FTL_1832</td>
<td>51</td>
<td>215</td>
<td>4.2</td>
<td><em>fslA</em></td>
</tr>
<tr>
<td>FTL_0112</td>
<td>211</td>
<td>665</td>
<td>3.1</td>
<td><em>iglB</em></td>
</tr>
<tr>
<td>FTL_1576</td>
<td>7</td>
<td>21</td>
<td>3.0</td>
<td>DNA mismatch repair</td>
</tr>
<tr>
<td>FTL_1576</td>
<td>15</td>
<td>38</td>
<td>2.6</td>
<td>DNA mismatch repair</td>
</tr>
<tr>
<td>FTL_1863</td>
<td>9</td>
<td>20</td>
<td>2.2</td>
<td>Glutamate decarboxylase</td>
</tr>
<tr>
<td>FTL_1352</td>
<td>7</td>
<td>14</td>
<td>2.0</td>
<td><em>tatD</em></td>
</tr>
<tr>
<td>FTL_1492</td>
<td>22</td>
<td>38</td>
<td>1.7</td>
<td>Fructokinase</td>
</tr>
<tr>
<td>FTL_0515</td>
<td>38</td>
<td>57</td>
<td>1.5</td>
<td>ABC Transporter</td>
</tr>
</tbody>
</table>

*a*Gene interrupted by the Tn5 transposon.

*b*Numbers of Miller units are averages of at least two experiments.

*c*Annotation according to gene homology with *F. tularensis* strain Schu S4.

FPI, *Francisella* pathogenicity island
Figure II.1. Construction of plasmids carrying a modified mini-Tn5 transposon. The plasmid pBDJ303 was derived from pRL27. Plasmid pRL27 carries a hyperactive Tn5 transposase outside of the mosaic ends, which define the transposed element. Within the mosaic ends are the R6K plasmid origin and the kanamycin resistance gene, *aphA3*. This plasmid was modified by cloning a DNA fragment encoding *lacI*\(^q\), the *Francisella* groES promoter and the *lac* operator upstream of the hyperactive transposase gene, *tnp*. In addition, the *Francisella* omp26 promoter was cloned upstream of the *aphA3* gene which was modified by flanking with *FRT* sequences. The original features of plasmid pRL27 are shown in black and the modifications are gray. Plasmid pMKM219 (features are shown in white) was digested with *SpeI* and ligated to *SpeI*-cut pBDJ303 to form the *E. coli-Francisella* temperature-sensitive Tn5 delivery plasmid, pBB107. Plasmid pBB107 confers kanamycin and spectinomycin resistance and is 12.4 kb in size.
Figure II.2. FLP recombinase, expressed from a temperature-sensitive shuttle vector, deletes the kanamycin resistance gene flanked by FRT sequences. Panel A. Southern blot analysis of EcoRI-digested chromosomal DNA from a Tn5 insertion mutant before (lane 1) and after (lane 2) FLP-mediated deletion of the kanamycin-resistance gene. The ~1.0 kb loss of size in the hybridizing band is the expected deletion size. The probe used for this experiment hybridizes to the R6Kori DNA, contained in the 0.8 kb region of the chromosome as depicted in the figure. Panel B. Depiction of the Tn5 transposon inserted into the *F. tularensis* LVS chromosome. Features in grey represent the *F. tularensis* chromosome while features in white represent Tn5 elements. The two black boxes represent the FRT recognition sites for FLP recombinase.
Figure II.3. *F. tularensis* LVS strains containing the *luxCDABE* reporter created by Tn5 mutagenesis. Strains containing transcriptional *lux* fusions in 1, *isflu* (control); 2, *fslD*; 3, FTL_R0003; or 4, *iglC* were streaked to either MMH or CDM agar and were photographed in light field (top panels) or using a photoimager (bottom panels).
Figure II.4. *F. tularensis* LVS strains containing the *lacZ* reporter created by Tn5 mutagenesis. Random mutants containing the *lacZ* reporter were arrayed to 96-well plates and were replica plated to MMH (A) or CDM (B) agar. Following ~24 hours of growth, plates were overlaid with filter paper pre-soaked in X-gal substrate. After ~ 20 minutes, a characteristic blue precipitate was observed in strains expressing *lacZ*. Several strains contained insertions in genes apparently causing auxotrophy for growth on CDM. Mutants were identified that demonstrated an increase in *lacZ* activity when grown on CDM agar (box).
Figure II.5. Genes in the *iglABCD* cluster are operonic. **A**: DNA or RNA was isolated from WT *F. tularensis* LVS and RT-PCR was conducted using primer sets that amplified DNA spanning intragenic regions between (A) *iglA*-*iglB*, 300 bp, (B) *iglB*-*iglC*, 350 bp, or (C) *iglC*-*iglD*, 400 bp. Lanes: 1, 4, 7 used DNA template; 2, 5, 8 used RNA template without addition of RT; 3, 6, 9 used RNA template with the addition of RT. **B**: Schematic drawing of the *iglABCD* gene cluster and location of primers used to amplify intragenic regions of DNA.
Figure II.6. Overexpression of *F. tularensis* LVS *fur* in chromosomal *lacZ* reporter strains.  

**A:** *F. tularensis* LVS carrying a *lacZ* reporter in *fslC* alone (parent strain, grey bars), or harboring the *fur* expression plasmid pBB110 (white bars) was grown to mid-log phase in CDM broth with either 28 µM (high), or 350 nM (low) FeSO$_4$.  

**B:** *F. tularensis* LVS carrying a *lacZ* reporter in *iglB* alone (parent strain, grey bars), or harboring the *fur* expression plasmid pBB110 (white bars) was grown to mid-log phase in CDM broth with either 28 µM (high), or 350 nM (low) FeSO$_4$. Miller units are the average of six independent samples. Error bars indicate +/- one standard deviation.
Figure II.7. Overexpression of *F. tularensis* LVS *fur* effect on IglC. Wild type *F. tularensis* LVS or LVS harboring the *fur* expression plasmid pBB110 was grown to mid-log phase in CDM broth with either 28 µM (high), or 350 nM (low) FeSO$_4$. Samples were normalized for cell number and a Western blot was conducted. Growth in iron restricted media increases the amount of IglC present in the cells, however, overexpression of Fur has no effect IglC protein level at either FeSO$_4$ concentration.
CHAPTER III
IDENTIFICATION OF MIGR, A REGULATORY ELEMENT OF THE
FRANCISELLA TULARENSIS LIVE VACCINE STRAIN IGLABCD VIRULENCE
OPERON REQUIRED FOR NORMAL REPLICATION AND TRAFFICKING IN
MACROPHAGES

Introduction

Previous studies have shown that *F. tularensis* has the ability to persist or
replicate within phagocytes and other cell types including human monocyte-derived
macrophages (MDM), human neutrophils, bronchial airway epithelial cells, and other
tissue culture cell lines such as HEp-2, and J774A.1 (69, 95, 99, 130, 149, 170, 175).
Replication within host cells is dependent on genes located within the *Francisella*
Pathogenicity Island (FPI) but intracellular survival and growth is likely to involve
additional genes as well. Most notably, genes comprising the *iglABCD* operon have been
directly implicated in escape from the phagosome and/or the ability to replicate in the
host cell cytosol (47, 91, 130, 161, 164). Genome-wide screens have also identified
genesis outside the FPI that are involved in other aspects of virulence, such as
dissemination, in animal models of tularemia (149, 178, 193).

While some genetic screens have identified genes critical to the intracellular life
cycle of *F. tularensis*, little has been done to examine the regulation of these genes or the
environmental stimuli leading to their differential expression. The first gene identified to
encode a regulator of virulence gene expression was *mglA* (11, 22, 95). Homologous to
the stringent starvation protein in *E. coli*, MglA positively regulates genes in the FPI
including those in the *iglABCD* operon (95). More recent work has shown that the
regulatory activity of MglA on the *igl* operon to also requires SspA, a second
transcriptional activator capable of associating with RNA polymerase (28).
Heterodimerization of MglA and SspA facilitates their interaction with the unique alpha
subunits of *F. tularensis* RNA polymerase, which are required for expression of FPI genes as well as numerous other genes throughout the chromosome (22, 28). A third regulatory gene, *fevR*, is among those non-FPI genes positively regulated by MglA/SspA and is reported to control the expression of the same set of genes as MglA/SspA (21). Finally, disruption of an orphan response regulator, *pmrA*, which is predicted to contain a DNA binding domain, also has been found to have a negative effect on transcription of many genes including the *igl* operon (124) (Fig.I.3). The regulatory activity of *pmrA* is apparently not exerted by altering *mglA* or *sspA* expression; nevertheless, the *pmrA* regulon does overlap with the genes regulated by *mglA/sspA*, specifically genes residing on the FPI. The exact mechanism of regulation by these factors is unknown, as are the environmental and/or host signals leading to this regulation.

Generally, the ability to sense and rapidly respond to environmental signals through modification of gene expression is vital to the ability of a bacterium to adapt to and survive within different conditions, including those found in various host cell environments. Studies of gene expression in *F. tularensis* have shown an upregulation of FPI genes when bacteria are grown intracellularly as compared with growth in broth (70). An increase in capsule production and surface pili have also been demonstrated when *F. tularensis* is grown in Chamberlain’s defined medium (CDM) as compared to rich growth medium (31, 68). While the specific signal or signals leading to these changes are unknown, iron availability has emerged as an environmental signal that influences expression of numerous *Francisella* genes. Among these are the genes in the *fslABCD* and *iglABCD* operons, which are involved in iron acquisition and intracellular growth, respectively (24, 52, 179). Previous studies have shown a role for the ferric uptake regulator protein (Fur) in the iron-dependent regulation of *fsl* but not *igl* transcription (24).

I initiated the work in this chapter by considering iron as an environmental signal leading to regulation of the *iglABCD* operon and more closely examining the role of Fur.
in this regulation. I conducted a genetic screen for new regulators of the *igl* operon and identified a new gene, *migR* (FTL_1542) that is involved in its regulation. Here, the effect of *migR* mutation on transcriptional regulation of the *iglABCD* operon has been partially characterized, as well as effects of this mutation on the interactions of *F. tularensis* with host cells. Specifically, I have examined whether this mutation alters intracellular growth in human phagocytes and epithelial cell lines. Additionally, I examined the specific effects of this mutation on phagosome maturation in macrophages and inhibition of the neutrophil oxidative burst.

**Materials and Methods**

**Bacterial strains, plasmid construction, growth conditions and antibiotics**

*F. tularensis* LVS (ATCC 29684), *F. novicida* U112 and *F. novicida* fur::TnKn (65) were grown in Modified Mueller Hinton (MMH) Broth (Becton Dickinson, Sparks, MD) or on Mueller Hinton Agar (Acumedia, Lansing, MI) supplemented with 1% glucose (w/v), 0.025% ferric pyrophosphate, and 2% IsoVitaleX. Spectinomycin (25 µg/ml for LVS, 100 µg/ml for *F. novicida*), kanamycin (25 µg/ml) and hygromycin (200 µg/ml) were added to the bacterial growth media when appropriate. CDM was prepared as described (27), or with 28 µM, 350 nM, or no added FeSO₄, as dictated by experimental parameters. Iron replete growth conditions were achieved by overnight growth of bacterial cultures in CDM containing 28 µM FeSO₄, followed by dilution into the same media before performing Miller assays for β-galactosidase quantitation (121). Iron depletion was achieved by growing bacterial cultures in CDM containing 7 µM FeSO₄, followed by a 1:1000 dilution into CDM containing 350 nM FeSO₄ (LVS), or by direct colony inoculation into CDM containing no added FeSO₄ (*F. novicida*) before performing β-galactosidase assays. LVS strains containing chromosomal lacZ reporters in *iglB* and *fslC* were described in previous work (24).
For plasmid-borne study of iron-responsive DNA elements, PCR was preformed on LVS chromosomal template to amplify ~300 nucleotide segments of DNA upstream of fslA, iglA, and iglC. Oligonucleotide primers were all designed with 5’ NcoI, BamHI “tails” and 3’ KpnI “tails” to facilitate cloning into the NcoI, KpnI restriction sites of pTrc99A (5). Successful cloning of these DNA elements was followed by the amplification of a promoterless lacZ gene from pA23 (188) template with oligonucleotide primers containing a 5’ KpnI “tail” and a 3’ SalI “tail”. The lacZ amplicon was then cloned into the KpnI, SalI restriction sites of the pTrc99A plasmids containing the previously described DNA fragments to create reporters of transcription for each of the three genes. Finally, the entire transcriptional reporter cassettes were removed from the pTrc99A backbone by BamHI, SalI digest, and were cloned into the same sites in the Francisella-E. coli shuttle plasmid pBB103 (sequences for oligonucleotide primers available upon request). As a control, the promoterless lacZ reporter alone was cloned into the same BamHI, SalI restriction sites in pBB103. These plasmids were designated pBB119, pBB125, pBB133, and pBB134 (Table III.1) and were introduced to F. novicida strains by rubidium chloride cryotransformation as described elsewhere (27).

Complementation plasmids pBB114 and pBB135 (Table III.1) were created by amplifying the entire FTL_1542 (migR) or FTL_0449 (fevR)gene using LVS chromosomal DNA as template and oligonucleotide primers containing 5’ KpnI and 3’ SalI “tails” (sequences for oligonucleotide primers available upon request). These amplicons were cloned downstream of the F. tularensis groES promoter in pTrc99A, and the entire expression cassette was transferred to pBB103 using BamHI, SalI restriction sites present on both plasmids.

**Mutagenesis, screening and identification of regulators of iglB transcription**

The LVS strain containing a chromosomal lacZ reporter of iglB transcription was transformed with Tn5 delivery plasmid pBB109 (Table III.1). Colonies obtained after ~3
days growth at 30ºC on MMH agar with 25 µg/ml spectinomycin were inoculated into 5 ml of MMH broth with 25 µg/ml spectinomycin and were grown at 30ºC with agitation to an OD$_{600}$ of ~0.1. Cultures were then serially diluted and plated on MMH agar with 200 µg/ml hygromycin at 41ºC to select for isolates with Tn5 insertions into the *F. tularensis* chromosome and simultaneous loss of the temperature sensitive transposon delivery plasmid. Resulting hygromycin-resistant colonies were arrayed to 96-well cell culture plates in 100 µl of MMH broth and were incubated at 37ºC until turbid. Freezer stocks were made by adding 100 µl of 2X freezing medium (1.0 M sucrose, 20% glycerol). To identify strains with reduced iglB expression, Tn5 mutants were recovered from freezer stocks and plated on MMH or CDM agar at 37ºC using a 96-prong replicator (Boekel, Feasterville, PA). After ~24 hrs, reporter enzyme activity was visualized by overlaying bacterial growth with #1 Whatman filter paper pre-soaked with 20 mg/ml X-gal in dimethylformamide diluted 1:4 in water. Quantitation of β-galactosidase activity was accomplished by conducting β-galactosidase assays in triplicate on duplicate cultures of bacteria grown to mid-log phase in MMH broth. IgIC was detected by Western blotting using a goat anti-IgIC antibody raised against IgIC purified from *F. novicida* generously provided by Dr. Karl Klose (University of Texas, San Antonio).

To identify the sites of Tn5 insertions affecting iglB expression, genomic DNA was isolated from individual colonies and digested with KpnI to create a DNA fragment containing the oriR6K origin, the hyg gene and flanking chromosomal sequence. The digested DNA was ligated, transformed into pir$^+$ *E. coli* and plated onto agar plates with 200 µg/ml hygromycin to select for transformants that carried the plasmid of interest. Plasmid DNA was isolated and sequenced using a primer with the sequence 5’GTGACAGGGGCCCTTTTATC 3’ that anneals to the 3’ end of the hyg gene and produces sequence of the flanking chromosomal DNA. Sequence data was used to search the sequenced bacterial chromosome database using NCBI BLAST to identify Tn5 insertion sites within the *F. tularensis* chromosome.
Creation of site-directed mutants using
intron-directed mutagenesis

Site-directed insertion mutants were created using a modified TargeTron (Sigma-Aldrich, St. Louis, MO) mutagenesis system (156). Briefly, the coding sequence of each gene of interest was entered into the Sigma TargeTron primer design site to determine appropriate oligonucleotides for retargeting the intron. Importantly, an XhoI restriction site was substituted for the HindIII site when designing the IBS primer. Retargeted PCR products were generated using Intron PCR Template (Sigma-Aldrich, TA0100) according to the recommendations of the manufacturer. The resulting fragment was introduced into the delivery vector pKEK1140 and cloning was verified by BglII digestion. LVS transformed with the retargeted plasmid was grown at 30°C on MMH agar with 25 µg/ml kanamycin. Individual colonies were purified once by growth at the permissive temperature and resulting colonies were screened by PCR to identify mutants before passaging at 37°C to cure the plasmid.

Real time RT-PCR for quantification of

RNA was isolated from wild-type LVS, and the site-directed FTL_1542 (migR) mutant cultures grown to mid log phase in MMH broth using a MasterPure complete DNA and RNA isolation kit (Epicentre, Madison, WI). High quality cDNA was generated using Superscript III reverse transcriptase and random primers (Invitrogen, Carlsbad, CA) according to the recommendations of the manufacturer. cDNA was quantified using Power Syber green PCR master mix (Applied Biosystems, Foster City, CA). Transcript levels for each gene were determined by comparing to a standard curve generated with each corresponding primer set using dilutions of genomic DNA template. Relative transcript abundance was determined by normalizing message in the mutant and wild type strains to that of the tul4 gene.
Neutrophil and macrophage isolation

Heparinized venous blood was obtained from healthy adult volunteers in accordance with a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa. Neutrophils (polymorphonuclear leukocytes, PMN) were isolated using dextran sedimentation and density gradient separation on Ficoll-Hypaque followed by hypotonic lysis of erythrocytes (51). PMN (~98% purity) were resuspended in HBSS without divalent cations, counted, and then diluted into appropriate media as indicated. Mononuclear cells were isolated by centrifugation on Ficoll-Hypaque, washed twice in RPMI-1640 (Cambrex), resuspended in RPMI + 20% autologous serum at a concentration of 2 x 10^6/ml, and differentiated into monocyte-derived macrophages (MDM) by incubation in Teflon jars for 5–7 days at 37°C (166, 170).

Intracellular growth assays

Wild-type or mutant LVS strains were used to infect MDM (MOI ~20:1), A549 cells, or HEp-2 cells (MOI ~ 100:1) in 24-well tissue culture plates. Approximately 10^5 MDM were seeded to individual wells in RPMI with 10% autologous serum and allowed to adhere overnight. Wells were washed and cells were re-suspended in RPMI with 2.5% autologous serum. Bacteria grown to mid-log phase in MMH broth were quantified by absorbance at 600 nm and quantitation was confirmed by plate counting. To optimize phagocytosis, bacteria were opsonized by incubation in 50% fresh autologous serum for 30 min at 37°C as described previously (113, 170). The appropriate numbers of bacteria were added to each well and infection was synchronized by centrifugation at 600 x g, 12°C for 4 min (170, 172). Initial infection efficiency was quantified after 1 hr co-incubation at 37°C. MDM monolayers were washed extensively with PBS to remove uningested bacteria and then processed immediately or incubated for another 23 hr at 37°C in fresh medium. Host cell lysis was achieved by addition of 1% saponin to each well and serial dilutions were plated on MMH agar to enumerate live organisms.
Similarly, 2x10^5 HEP-2 cells or A549 cells were seeded to individual wells in MEM with 10% FBS and were allowed to adhere overnight. Bacteria were added and infection was synchronized as described above. After 4 hr incubation at 37°C, gentamicin (10 µg/ml) was added for 1 hr to eliminate extracellular bacteria. Host cells were washed to remove gentamicin, lysed using 1% final concentration saponin, and the bacteria enumerated as described above to quantify bacterial uptake. For 24 hour time points, wells were replenished with gentamicin-free growth medium and incubated an additional 19 hrs before lysis and enumeration to quantify intracellular growth.

Confocal analysis of *F. tularensis* phagosomes

Previously established methods were used to assess phagosome composition in macrophages (113, 170, 172). In brief, MDM attached to chamber slides (LabTek) were infected with opsonized *F. tularensis* at MOI 20:1. Phagocytosis was synchronized as described above, and after 1 hr at 37°C, monolayers were washed extensively to remove uningested bacteria. After a total of 1-24 h at 37°C, MDM were fixed in 10% formalin, permeabilized with cold methanol:acetone, blocked, and then double-stained to detect bacteria and lamp-1 or cathepsin D. Bacteria were detected using rabbit anti-*F. tularensis* antiserum (BD Biosciences) or mouse anti-*F. tularensis* lipopolysaccharide T-14 (Novus Biologicals). Mouse anti-human lamp-1 hybridoma supernatants (clone H4A3) were from the Developmental Studies Hybridoma Bank of the University of Iowa. Rabbit anti-cathepsin D polyclonal antibodies were from Upstate Biotechnology, Inc. Fluorescein isothiocyanate- and rhodamine-conjugated F(ab')2 secondary antibodies were from Jackson ImmunoResearch Laboratories. Samples were viewed using an LSM-510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY). For each experiment, phagosomes in 50-100 infected cells were scored in duplicate or triplicate samples for each marker and time point.
Neutrophil infection and measurement of respiratory burst

Bacteria were grown on MMH agar for 48 h at 37°C in 5% CO₂ and harvested into HBSS containing divalent cations. Washed bacteria were opsonized with 50% fresh autologous serum for 30 min at 37°C as described above, washed again with HBSS lacking divalent cations, and quantified by measurement of absorbance at 600 nm. Generation of reactive oxygen species (ROS) by neutrophils was assessed using luminol-enhanced chemiluminescence (CL) assays as described previously (3, 45). Briefly, neutrophils were incubated at 5 x 10⁶/mL in RPMI-1640 (without phenol red) supplemented with 250 μM luminol and 4% human serum albumin for 10 min at room temperature. 200 μl aliquots were dispensed in triplicate into 96-well microtiter dishes (Perkin Elmer white/opaque OptiPlate 96) and infected at 37°C with the indicated strains of bacteria at MOI 50:1 unless otherwise specified. CL was recorded at 30 sec. intervals for 1 hr using a BMG Laboratories Novostar luminometer (BMG LabTech Inc., Durham, NC).

Results

Iron-dependent, Fur-independent regulation of the iglABCD operon

To examine the role of the iron-dependent regulator Fur on expression of the iglABCD operon, I grew strains containing either an iglB-lacZ or fslC-lacZ chromosomal reporter, which were identified using my transposon delivery system (24), in CDM containing high (28 μM) or low (350 nM) FeSO₄. β-galactosidase assays were conducted on strains grown to mid- or late-log growth phase before examining reporter expression. When grown in high iron conditions, the expression of each reporter was unchanged from the mid-log to the late-log phase of growth. When grown in iron-limiting conditions, there was an increase in expression of each reporter, a phenomenon that was exacerbated as the cultures progressed from mid-log to late-log growth phase. This trend is presumably observed because iron was being actively depleted in late-log phase by bacterial growth and utilization (Fig.III.1). Because this growth phase-dependent
increase in expression was observed in the low but not high iron-containing medium, I believe the induction is likely due to decreased iron availability and not the result of other growth phase effects. To assess the role of Fur in the regulation of these genes, I overexpressed Fur in *trans* in each strain and repeated the assay. The data in Figure III.1 clearly show repression of the *fslC-lacZ* reporter regardless of iron availability and growth phase, whereas expression of *iglB* was unaffected.

**Identification of an iron-responsive DNA segment upstream of *iglABCD***

Since the *iglB* reporter appeared to be responding to iron in a Fur-independent manner, I wanted to identify the region of DNA that was capable of eliciting the iron-dependent regulation. To this end, I amplified DNA upstream of *iglA* (containing no recognizable Fur binding site), *iglC* (containing a weak putative Fur-binding site) (179), and *fslA* (containing a near consensus Fur-binding site) and cloned each fragment into a *Francisella-E. coli* shuttle vector in front of a promoterless *lacZ* reporter gene (Fig.III.2B). Wild type *F. tularensis* or an isogenic strain containing an insertion mutation in *fur* were transformed with each of three different reporter plasmids or a promoterless *lacZ* control and grown in high or low iron-containing medium. The *lacZ* control reporter exhibited the same low-level expression irrespective of iron availability in both genetic backgrounds. The reporter of *fslA* activity demonstrated a ~33-fold induction in response to growth in iron-limiting medium. When assayed in the *fur* mutant background, the *fslA* reporter was further induced (~73-fold), and lost its responsiveness to media iron concentration (Fig.III.2A). The *iglA* reporter also responded positively to growth in iron-limiting medium, albeit to a lesser extent (~2-fold) than the *fslA* reporter. Expression of the *iglA* reporter in the Fur mutant background was increased approximately threefold over the level measured in iron-replete growth conditions (Fig.III.2A). The reporter of *iglC* activity, containing a weak putative consensus Fur-binding site, was unaffected by either iron availability or genetic background.
Identification of \textit{iglABCD} operon regulators

Four gene products that appear to control expression of the \textit{iglABCD} operon have been described, MglA, SspA, FevR and PmrA (28, 95, 124). None of these regulators have a known role in iron-mediated regulation. To identify additional genes encoding proteins affecting expression of this operon, I mutagenized an \textit{F. tularensis} strain containing a chromosomal \textit{lacZ} reporter of \textit{iglB} activity (24). Using a transposon delivery system described in chapter I and elsewhere (24), I generated and screened a library of 2,500 mutants for changes in \textit{iglB} expression. Three unique mutants were isolated, each of which exhibited significantly reduced $\beta$-galactosidase activity of the \textit{iglB-lacZ} reporter when streaked to MMH agar (Fig.III.3).

Chromosomal transposon insertion sites were cloned and sequenced to identify the location of each insertion. One of the transposon insertions resided within the \textit{lacZ} coding sequence, causing the complete loss of $\beta$-galactosidase activity. Since \textit{F. tularensis} LVS has two copies of the FPI, I performed a Western blot for IglC to examine the effect of these transposon insertions on expression of the \textit{iglABCD} operon not containing the \textit{lacZ} reporter. Results from the Western blot indicate that indeed each secondary transposon mutation affects both copies of the \textit{iglABCD} operon (Fig. III.4A). As expected, the transposon insertion in the \textit{lacZ} reporter itself caused no obvious change in IglC levels and this mutant was not pursued further (Fig.III.4A). A second transposon insertion mapped to nucleotide 163 of FTL_0347, a $\sim$0.6 kb gene that encodes a hypothetical protein with predicted transmembrane domains. This mutant displayed a 7-fold reduction in \textit{iglB-lacZ} expression and concomitant reduction in cell-associated IglC accumulation observed by Western blot (Fig.III.4A, III.4C). Unfortunately, additional studies including an inability to complement and the lack of a regulatory phenotype in a site-directed FTL_0347 mutant indicated that the transposon insertion was not responsible for the phenotype of this mutant, and it was not pursued further. The final transposon insertion mapped to nucleotide 211 of FTL_1542 (Fig.III.4D), a 2.1 kb gene
present in all *F. tularensis* subspecies sequenced to date. This gene was also recently identified as a regulator of *pepO*, a virulence-associated gene unique to *F. novicida* (21). A transposon insertion in FTL_1542 resulted in a fivefold reduction in *iglB-lacZ* expression, which was similar to the reduction in cell-associated IgIIC accumulation observed by Western blot (Fig.III.4A, III.4C). These findings suggest that an insertion mutation in FTL_1542 affects expression of the *iglABCD* operons on both pathogenicity islands.

**In vitro growth and complementation and creation of site-directed mutants**

The FTL_1542 (*migR*) mutant was transformed with a complementation plasmid (pBB114) containing the full length FTL_1542 gene, whose expression was driven by the *F. tularensis* *groES* promoter. The complementing clone was initially identified by the restoration of blue color when exposed to X-gal. β-galactosidase assays were conducted to confirm and quantify the extent to which complementation restored *iglB* transcription (data not shown). Consistent with the X-gal plate screen, expression of the *iglB-lacZ* reporter in the FTL_1542 mutant was restored by a functional copy of the FTL_1542 gene provided in trans; however, the level of *iglB-lacZ* activity only reached 50% that of the parent strain (data not shown). The maximal doubling time of the FTL_1542 mutant, using my growth conditions, was ~72% that of the parent *iglB-lacZ* strain. The effect of this mutation on maximal in vitro growth rate is similar to that of a *fevR* mutant (data not shown), and those reported for *mglA* and *sspA* mutants (28). Complementation of the FTL_1542 mutant restored the growth rate to 93% that of the parent (data not shown). The apparent lack of complete complementation in the FTL_1542 strain in each of these assays may be the result of inappropriate protein stoichiometry or some other unforeseen effect of overexpression of this protein from the complementation plasmid.

To further demonstrate that the transposon insertion in FTL_1542 was responsible for the reduced expression of *iglB* and *iglC*, I utilized a modified intron-
directed mutagenesis system to create a site-directed mutant in *F. tularensis* (156). Creation of this mutant allowed us to assess the mutant phenotype in the absence of the compounding *iglB* mutation present in the original mutant strain. Wild type and mutant strains created using the intron-directed mutagenesis system were transformed with pBB125, which carries a plasmid-borne *iglA-lacZ* reporter, and β-galactosidase assays were conducted. The *iglA-lacZ* reporter exhibited a 5-fold reduction in activity in the site-directed FTL_1542 (*migR*) mutant as compared to the wild-type strain. This finding is in agreement with my chromosomal reporter data for a mutation in this gene.

**Effect of *migR* mutation on the expression of known regulators of virulence genes**

Mutations in *mglA, sspA, fevR*, and *pmrA* have all been shown to affect the expression of genes within the FPI, including the *iglABCD* operon (11, 21, 22, 95, 124). The precise mechanism of action of these regulators is unknown, but the available data suggest that additional gene products may play a significant role in activating expression of genes within these overlapping regulons (28, 124). Therefore, I examined the effect of the *migR* mutation on transcript levels of each of these four known virulence gene regulators using real time quantitative RT-PCR (qRT-PCR). I also examined the expression of *iglC* in the *migR* mutant using qRT-PCR to confirm my results from *lacZ* reporter and Western blot assays. The qRT-PCR analysis confirmed the reduction in expression of *iglC*, which is consistent with my previous observations in Fig. III.4B and III.4C. Specifically, the data indicate that the FTL_1542 (*migR*) mutant strain contains 8.5-fold less *iglC* transcript that the wild-type strain (p<0.001, two-tailed Student’s t-Test) (Fig.III.5). The relative amount of transcript of *mglA* and *pmrA* was not significantly different in the mutant vs. wild-type strain (p>0.05, two-tailed Student’s t-Test). There was a slight but significant (p=0.034, two-tailed Student’s t-Test) difference in *sspA* transcript, which was reduced 1.4-fold in the FTL_1542 mutant strain. Most interesting was the 15-fold reduction in *fevR* transcript in the *migR* mutant strain.
(p<0.001, two-tailed Student’s t-Test) (Fig.III.5). Since FevR is essential for expression of the \textit{iglABCD} operon (21), it is likely the reduction in \textit{igl} expression observed in the FTL_1542 (\textit{migR}) mutant is due to a reduction in \textit{fevR} expression in this strain.

**Intracellular survival and growth in HEP-2 cells.**

A549 cells and MDM

As mutations in \textit{migR} reduced expression of the \textit{iglABCD} operon, I examined the effect of these mutations on intracellular survival and growth in both epithelial cells and macrophages. Additionally, since the regulatory effect of the \textit{migR} mutation on \textit{iglABCD} seemed to be through \textit{fevR}, I created a \textit{fevR} mutant using the intron-directed mutagenesis system as a means for comparison. Human monocyte-derived macrophages (MDM) were infected with opsonized wild-type LVS, the original \textit{migR} transposon mutant, or the site directed \textit{migR} or \textit{fevR} mutant strains and their \textit{trans}-complemented counterparts. Infection efficiency was similar for all six strains after 1 hr of co-incubation with MDM. To quantify intracellular replication, host cells were lysed 24 hr post-infection and viable bacteria were enumerated. Wild-type LVS multiplied approximately 50- to 70-fold over the 24 hr time course of the experiment, similar to the rate reported by us and others in primary macrophages or macrophage cell lines (70, 106, 139). The \textit{migR} and \textit{fevR} mutants were greatly impaired for growth in MDM, reaching a maximum of 5-fold and 2-fold replication over the course of the experiment (p<0.001, two-tailed Student’s t-Test), respectively (Fig.III.6A). This was an expected result since \textit{igl} expression is greatly reduced in the \textit{migR} strain (Fig.III.4B, III.5). There was no significant difference between the original FTL_1542 Tn5 insertion mutant and the site-directed \textit{migR} mutant (p=0.951). Complementation of the \textit{migR} mutant significantly restored the intracellular growth (p<0.001, two-tailed Student’s t-Test), albeit to a level lower than that of the parent strain (Fig.III.6A). These data are consistent with the incomplete complementation observed in \textit{iglB}-\textit{lacZ} expression and in vitro growth experiments.
Comparable results were also obtained using the original transposon insertion mutant strain and its isogenic trans-complemented strain (data not shown).

To assess the effect of migR and fevR mutations on bacterial growth in epithelial cell lines, a similar set of experiments was carried out using human cervical epithelial (HEp-2) and human airway epithelial (A549) tissue culture cells. Uptake of each of the five strains by HEp-2 and A549 cells was comparable after a 4 hr. incubation period. Thereafter, wild-type LVS underwent rapid replication, multiplying about 500-fold in A549 cells (Fig.III.6B and III.6C) and nearly 1,000-fold in HEp-2 cells (data not shown) by 24 hr. post infection. Interestingly, while the fevR mutant achieved only minimal growth in these cell lines (Fig.III.6B), the kinetics of growth for the migR mutant was indistinguishable from that of the parent strain over the course of the experiment (Fig.III.6C). This was an unexpected result given the dramatic reduction in igl expression in this strain and the reduced ability of the mutant to grow in primary human macrophages. Thus, I report the identification of a mutant defective for growth in MDM that replicates normally in HEp-2 and A549 epithelial cells.

**Intramacrophage trafficking of migR and fevR mutants**

After uptake by MDM, *F. tularensis* prevents phagosome-lysosome fusion and resides in a compartment that accumulates late endosome membrane glycoproteins prior to phagosome egress and replication in the cytosol (34, 37, 163, 164). In contrast, mutant bacteria that lack functional mglA or an intact FPI are defective for phagosome escape and reside in mature phagolysosomes (19, 163, 164). Since the data in Figure III.6A indicated that intracellular growth of the migR and fevR mutants are impaired, confocal microscopy was used to assess the intracellular fate of these mutants. These experiments, performed by my collaborator Dr. Lee-Ann Allen, were used to determine whether either strain exhibited an aberrant trafficking phenotype as the cause for attenuated intracellular replication. Infected MDM were analyzed by Dr. Allen 1 hr or 18-22 hr after initiation of infection, and in each case cells containing wild-type LVS, mutant organisms or their
trans complemented counterparts were directly compared. Representative images are shown in Figures III.7A-C, and pooled data from three independent experiments are summarized in Figures III.7D-E.

The findings by Dr. Allen indicate that early in infection (1 hr after uptake) the vast majority of the migR and fevR mutants, as well as the trans complemented strains migRc and fevRc, resided in phagosomes that accumulated the late endosome membrane glycoprotein lamp-1 but not the lysosomal marker cathepsin D, and in this manner resembled wild-type LVS (Figs.III.7A-D). No significant differences in initial infection efficiency were detected, and ~75% of the macrophages in each monolayer contained intracellular bacteria (not illustrated). However, as infection progressed, it became apparent that the migR and fevR mutants had distinct fates in MDM that differed both from one another and from wild-type LVS. Thus, by 5 hr after uptake, ~50% of wild-type LVS had breached the phagosome membrane (data not shown); and after 18-22 hr at 37°C, robust growth of wild-type bacteria in the MDM cytosol was readily apparent (Fig.III.7A), and these organisms did not colocalize with lamp-1 or cathepsin D (Fig.III.7A and III.7E). Analysis of MDM infected 19-22 hrs with the migR or fevR mutants demonstrated that the vast majority of both strains remained trapped inside phagosomes and did not reach the cytosol (Fig.III.7E). Compartments containing the migR mutant resembled mature phagolysosomes as indicated by their accumulation of lamp-1 and cathepsin D (Fig.III.7B and III.7E). Similar data were obtained for the original Tn5 insertion mutant in FTL_1542 (Dr. Allen, data not shown). In contrast, the fevR mutant appeared trapped in a more immature compartment that lacked cathepsin D despite sustained accumulation of lamp-1 (Fig.III.7C and III.7E). Robust replication of both complemented strains (migRc and fevRc) in the MDM cytosol 18-22 hr after uptake (Figs.III.7B-C) confirms a role for both migR and fevR in manipulation of macrophage membrane trafficking by F. tularensis strain LVS.
Mutations in \textit{migR} and \textit{fevR} affect the ability of \textit{F. tularensis} to block NADPH oxidase activity in neutrophils

An important aspect of \textit{F. tularensis} virulence is its ability to prevent neutrophil activation (2, 113). To assess whether the \textit{migR} or \textit{fevR} mutants were compromised in their ability to block NADPH oxidase activity, I performed luminol-enhanced CL assays to measure production of oxidants during infection of PMN. Concordant with other published data (4, 113), infection of PMN with wild-type LVS did not trigger a respiratory burst (Fig.III.8). In marked contrast, both the strain carrying the Tn5 insertion in FTL_1542 and the site-directed \textit{migR} mutant stimulated similar NADPH oxidase activation in PMN as judged by the luminol CL assay. Not surprisingly, the \textit{fevR} mutant was also unable to prevent oxidant production (Fig.III.8). For both \textit{migR} and \textit{fevR} mutant strains, PMN NADPH oxidase inhibition was restored by complementation with the wild-type gene in \textit{trans}. Collectively, these data demonstrate that \textit{migR} and \textit{fevR} play a role in disruption of neutrophil function, likely via effects on the expression of genes in overlapping regulons.

\textbf{Discussion}

The facultative intracellular pathogen \textit{F. tularensis} is capable of subverting the early innate immune response and replicating within macrophages and epithelial cells to cause significant morbidity and mortality in humans. Previous studies have demonstrated that genes within the \textit{iglABCD} operon are induced in response to growth in iron limiting conditions (24, 52, 96, 122, 179), however, the underlying mechanism remained obscure. One research group has identified sequences upstream of the \textit{iglC} gene with similarity to the consensus Fur binding site (52), although a Fur binding site in the middle of an operon would be an unusual regulatory arrangement. I have examined the effect of iron concentration and overexpression of the Fur protein in strains containing chromosomal reporters of either \textit{iglB} or \textit{fslC} transcription. The \textit{fslC} gene is part of an operon that is
both regulated by iron and carries a strong consensus Fur binding site upstream of *fslA*, the first gene of the operon (52, 179). Chromosomal reporters of *iglB* and *fslC* were induced when grown under iron-limiting conditions, although induction of the *iglB* reporter was mild. As expected, the chromosomal reporter of *fslC* activity was repressed by overexpression of Fur in both iron replete and iron depleted growth media. In contrast, overexpression of Fur had no significant effect on the expression of *iglB* regardless of the iron concentration in the media.

To identify the region of DNA containing iron-responsive regulatory sequences, I cloned DNA fragments upstream of *iglA*, *iglC*, and *fslA* into a *Francisella*-*E. coli* shuttle plasmid containing a *lacZ* reporter gene. Wild-type or *fur* mutant *Francisella* strains were transformed with each reporter plasmid. The *fslA* DNA fragment contains a well conserved Fur box DNA sequence, and reporter activity is increased ~33-fold in the wild-type strain when grown in iron-limiting medium. As expected, this reporter plasmid also produced much more *lacZ* activity (~73-fold) in the *fur* mutant than the wild-type background, regardless of iron concentration in the growth medium. These data provide compelling support for the notion that the *fslA* operon is regulated by Fur in response to iron in a conventional manner. A reporter containing DNA upstream of the *iglA* gene, likely to include promoter and regulatory sequences, underwent a mild ~2-3-fold induction in the wild type background, but was also induced slightly in the *fur* mutant genetic background when grown in iron-limiting medium. These data are consistent with results from my *fur* overexpression studies and suggest, at best, a modest role for Fur in the regulation of the *iglABCD* operon. The *lacZ* reporter plasmid containing DNA sequences upstream of the *iglC* gene with a weak putative Fur box showed no responsiveness to changes in iron concentration, or to the presence or absence of Fur. I conclude that iron-responsive DNA sequences are present upstream of *iglA*, but not *iglC*. Together these data suggest a minor role for iron and the Fur protein in the regulation of
iglABCD, and that the regulatory effects are exerted through DNA sequence upstream of the operon.

Since my initial data demonstrated that Fur did not have a dominant regulatory role on the igl operon, I mutagenized an F. tularensis LVS strain carrying a chromosomal reporter of iglB transcription in an attempt to identify new regulators of this operon. A mutant library screen identified one mutant of interest with a transposon insertion in the F. tularensis LVS gene FTL_1542 (migR) that resulted in reduced expression of genes in the igl operon as indicated by use of transcriptional reporters as well as Western blotting and qRT-PCR. migR was also identified in a F. novicida screen for regulators of pepO (21); and it is noteworthy that the pepO gene is thought to be nonfunctional in tularensis and holarctica subspecies (78). Based on limited homology migR was annotated as caiC in the Schu S4 chromosome in GenBank, a designation that has since been removed. Because of its regulatory effects on fevR and iglABCD, and its intracellular growth phenotype in macrophages, I have given the FTL_1542 ORF the designation of migR (macrophage intracellular growth regulator).

Transposon and site directed mutagenesis of migR resulted in decreased expression of igl genes and a reduced ability of mutant bacteria to grow in primary MDM. Gene orientation and the short intragenic regions between predicted genes suggest that migR could be the first gene in an operon that includes FTL_1541 (mraW, S-adenosyl-methyltransferase), FTL_1539 (hypothetical protein), and FTL_1538 (ftsI, penicillin binding protein) (Fig. III.4D). This gene arrangement is shared among F. tularensis subspecies. mraW and ftsI are also found in a similar arrangement in several enteric bacteria, however, there is not a homolog of migR proximal to these genes in those chromosomes. migR is annotated as a hypothetical protein that contains two conserved domains, luxE and an acyl carrier protein synthetase domain, each of which contain AMP binding sites. Proteins sharing this functional domain are commonly involved in fatty acid modifications, such as the activation of fatty acids by addition of
CoA as they enter the cytoplasm to both sequester the fatty acid as well as initiate metabolism of the energy-rich substrate (89). Although it is possible that migR is operonic with one or more of the downstream genes, the ability to complement the various phenotypes of this mutant with just the FTT_1542 ORF alone in several assays is strong genetic evidence that the mutation is not polar, or that downstream genes are not strongly associated with the regulatory function of migR. Furthermore, the regulatory effect exerted by migR is specific, and not due to a general downregulation of gene expression in *F. tularensis* since other genes examined by qRT-PCR are unaffected by this mutation. While it is not immediately clear how this gene product is exerting a regulatory affect on *fevR*, and thus *iglABCD*, proteins sharing domain homology regulate gene expression in *E. coli* through modification of fatty acids, enabling them to interact with DNA-binding regulatory proteins (18, 54). Further discussion of the possible mechanisms of MigR activity is found in chapter V.

To determine if the regulatory effect of the *migR* mutation was exerted through any of the previously identified regulators of the *iglABCD* operon, I used quantitative real time RT-PCR to compare the level of *mglA*, *sspA*, *pmrA*, or *fevR* transcription in the *migR* mutant with the parent strain. As expected, based on the reporter and protein blot assays, I found that the *iglC* transcript was reduced in the *migR* mutant. Interestingly, the levels of *fevR* transcript were significantly reduced in this strain, and to a much lesser extent the *sspA* transcript was also reduced. The reduction in *fevR* was dramatic in the mutant strain (15-fold, p<0.001), while the reduction in *sspA* transcript was 1.4-fold (p=0.043) and may not be biologically significant. This led us to conclude that the regulatory effect of *migR* on the *igl* operon is indirect, likely via downregulation of FevR (Fig.III.9). Previous studies have also shown that *fevR* expression requires MglA, SspA, and, to a lesser extent, PmrA (21, 124). Expression of *fevR* in an *mglA* mutant is reduced 5- to 10-fold (21, 22), while expression of *fevR* in a *pmrA* mutant is reduced only 2.4-fold compared to the wild-type strain (124). These observations suggest that FevR is a central regulator of
FPI gene expression with several other gene products, in turn, modulating expression of *fevR*. It is attractive to speculate that different stimuli encountered by *F. tularensis* alter *fevR* expression through different signaling cascades, although no specific data is available to support this hypothesis, at this time.

Wild-type *F. tularensis* is capable of rapid, robust growth in both epithelial cells and macrophages (37, 99). Mutations in genes in the *iglABCD* operon nearly ablate replication in macrophages in vitro, and markedly attenuate virulence in a mouse model of infection (71, 193). Given the finding that the *migR* mutation described here resulted in the net reduction of *igl* expression, I expected that the mutant strain would be impaired for intracellular replication within host cells. Upon infection of primary human macrophages, I found the *migR* mutant to be capable of only modest replication beyond the initial infection numbers. This was in sharp contrast to wild-type LVS, which multiplied up to 70-fold over the same 24 hour time period. As indicated by measurement of colony forming units, the defect in intra-MDM growth was partially ameliorated by complementation with the full-length wild-type *migR* (FTL_1542) (Fig.III.7A); and confocal analysis revealed that 18 hr after uptake MDM infected with wild-type LVS contained ~50% more bacteria than cells infected with the *migRc* strain (Fig. III.7A-B, and L. Allen unpublished data). Nevertheless, by 30-48 hr post-infection, both the *migRc* strain and wild-type LVS led to destruction of the MDM monolayer (L. Allen, unpublished data). Thus, although the *migRc* strain grew more slowly than wild-type bacteria in MDM, my data strongly suggest that mutation of *migR* accounts in large part for the reduced *igl* gene expression and the intracellular growth defects I describe.

Similar infection and intracellular growth experiments were also carried out in HEp-2 and A549 epithelial cell lines. These cell lines were more permissive than MDM for the intracellular replication of wild-type LVS, which increased 500- to 1,000-fold over the 24 hour experiment. Surprisingly, the *migR* mutant grew normally in both of these cell types while the *fevR* mutant did not. These data indicate that *migR* is required
for intracellular survival and replication in MDM but not for growth in cultured epithelial cells. In contrast, FevR is required for growth in all cell types tested. The distinct phenotypes of the mutant strains in epithelial cell lines could be due to at least one of the following mechanisms. First, reduced iglABCD expression in the migR mutant, as opposed to a complete loss of iglABCD expression in the fevR mutant, could account for the different intracellular growth phenotypes of the strains in MDM and A549 cells. This explanation would require that a low level of iglABCD expression in the migR mutant would be sufficient to allow endosomal escape and replication in the less hostile environment of epithelial cells, while not being sufficient to avert the phagosomal maturation process of macrophages. Alternatively, the different growth phenotypes of migR and fevR in epithelial cells could be the result of MigR responding to different signals that are present in one cell type but not another. The contrasting phenotypes of fevR and migR mutants, along with mutants in igl genes, will enable future studies to examine role of the igl operon in the fate of F. tularensis in epithelial cells.

Neutrophils provide an essential first line of defense against invading microbes and a key component of their killing arsenal is the NADPH oxidase. This enzyme catalyzes the conversion of molecular oxygen into superoxide anions, which are then converted into other reactive oxygen species (ROS) including H₂O₂ and HOCl (8). McCaffrey and Allen have shown previously that F. tularensis strain LVS evades killing by neutrophils via its ability to inhibit NADPH oxidase assembly and activation at the phagosome membrane (4, 113). Virulence factors that prevent neutrophil activation during tularemia are not well defined. In collaboration with Drs. McCaffrey and Allen, I now show that while wild-type bacteria prevent ROS production, phagocytosis of the migR or fevR mutant strains triggers a moderate respiratory burst that is abrogated by expression of a full-length copy of FTL_1542 (migR) or FTL_0449 (fevR) in trans. These data suggest a role for migR in disruption of neutrophil NADPH oxidase activity. Indeed, migR was also identified by direct screening of an LVS Tn5 mutant library (24,
171) for mutants that no longer prevent neutrophil activation (L. Allen, unpublished data). Thus, I favor a model in which migR regulates genes in addition to those in the iglABCD operon, perhaps including acpA, which encodes an acid phosphatase that can inhibit porcine NADPH oxidase activity in vitro (153).

The current model for lethal infection by *F. tularensis* involves inhalation of as few as 1-10 viable bacteria, which are engulfed by alveolar macrophages (144, 194). *F. tularensis* rapidly replicates in this niche, and infected macrophages likely mediate dissemination to the liver and spleen (63). Accordingly, research efforts and infection models have focused on understanding the interactions between *F. tularensis* and MDM, elicited murine peritoneal macrophages and murine bone marrow-derived macrophages as well as human and murine macrophage-like cell lines. Since the endotoxin of *F. tularensis* is non-stimulatory, it is generally believed that pathogenesis is mediated by the ability of the bacteria to replicate intracellularly, the principal target of growth being macrophages (137, 184). In general, mutants defective for growth in macrophages are also attenuated for in vivo virulence. However, a few mutants have been reported that grow normally inside macrophages yet exhibit reduced virulence in mouse infection models (26, 61, 104, 193). In this study, I report the identification of mutations that differentially affect PMN activation and intra-macrophage growth that are dispensable for replication in A549 and HEp-2 cells. My collaborator Dr. Allen shows for the first time that mutants lacking a functional migR (FTL_1542) reside in compartments with features of mature phagolysosomes in MDM, and in this manner resemble mutants that lack functional mglA or iglC (19, 163, 164). At the same time, the fact that this mutant grew normally in A549 and HEp-2 cells suggests that replication of *F. tularensis* in epithelial cells may represent an important, yet understudied aspect of tularemia. In this same vein, recent work has demonstrated that uracil auxotrophic mutants of LVS are killed by MDM and neutrophils, yet replicate normally in macrophage- and epithelial-like cell lines (171). What accounts for these cell-type specific virulence defects is unknown and merits
further investigation. In this regard it is also noteworthy that the \( fevR \) and \( migR \) mutants, while both defective in phagosome escape and intracellular growth, appeared to reside in distinct compartments in MDM since phagosomes containing the \( migR \) mutant accumulated the lysosomal marker cathepsin D whereas phagosomes containing the \( fevR \) mutant did not. Although these data suggest that the \( migR \) and \( fevR \) mutants differentially affect macrophage membrane trafficking, additional studies are needed to ascertain whether \( fevR \) mutants exhibit specific defects in phagosome escape while retaining at least partial capacity to induce phagosome maturation arrest.

In summary, I conclude that reduced iron availability and Fur play modest roles in regulation of the \( iglABCD \) operon. Perhaps this represents a “fine tuning” of expression of these genes necessary in a specific survival niche. Importantly, I have identified a gene in \( F. \) tularensis LVS that regulates the expression of \( iglABCD \), likely through the reduced expression of \( fevR \), and may also influence expression of other genes throughout the \( F. \) tularensis chromosome. Mutations in \( migR \) result in reduced expression of \( fevR \) and genes in the \( igl \) operon and also negatively affect growth in primary human macrophages but not epithelial cells. Moreover, the effects of the \( fevR \) and \( migR \) mutants on neutrophil NADPH oxidase activity and macrophage membrane trafficking are novel, and provide avenues for further study.
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<th>Strain or plasmid</th>
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<td><em>F. novicida</em> carrying an insertion mutation in <em>fur</em></td>
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<td>pBB135</td>
<td>Complementation plasmid carrying full length <em>fevR</em> gene driven by native promoter</td>
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Figure III.1. Examination of the effect of iron concentration and Fur overexpression on \( fslC \) and \( iglB \) chromosomal reporters. Duplicate cultures of each reporter strain were grown in CDM with high (28 \( \mu \)M) or low (350 nM) concentration FeSO\(_4\). \( \beta \)-galactosidase assays were conducted at the mid- or late-log phases growth. The \( fslC \) reporter is induced ~ 10-fold in late log-phase at low Fe concentration (\( p < 0.001 \)). This induction was repressed by the overexpression of Fur in \textit{trans}, which returned \( fslC\text{-lac}Z \) expression to the same level seen when grown in iron replete broth (\( p = 0.103 \)). The \( iglB \) reporter is induced ~ 2-fold in late log-phase growth at low Fe concentration (\( p < 0.001 \)). Expression of Fur in \textit{trans} had no significant effect on \( iglB \) reporter expression (\( p = 0.112 \)). Data is the average of at least two independent experiments performed in triplicate +/- one standard deviation.
Figure III.2. Determination of iron-responsive DNA sequence. A: Wild-type (WT) or fur (fur) mutant strains of *F. novicida* were transformed with plasmid-borne reporters of *fslA, iglA*, or *iglC* and were grown under iron replete (+), or iron deplete (-) conditions to measure transcriptional activity. Miller assays were carried out on at least two replicate cultures and were assayed in triplicate. The promoterless *lacZ* control reporter is unaffected by iron or genetic background. The *fslA* reporter is upregulated by growth in low iron media (p<0.001) and is further induced in the fur mutant background (p<0.001). Expression of the *iglA* reporter is modestly induced by both growth in iron-deplete media (p<0.001), or when in the *fur* mutant strain (p<0.001). Activity of the *iglC* reporter remains unchanged under tested iron availabilities and in either genetic background. B: Schematic representation of DNA amplified and assayed for reporter activity. Data are the average of at least six independent experiments. Error bars represent +/- one standard deviation.
Figure III.3. Screen to identify secondary mutations that affect expression of iglB. A *F. tularensis* LVS strain carrying a chromosomal *lacZ* reporter of *iglB* expression was re-mutagenized to identify mutations affecting *iglB* expression. Individual mutants were arrayed to a 96-well plate and were replica plated onto CDM agar (panel A). Replica plates were overlaid with filter paper pre-soaked in an X-gal solution and incubated 15 min. prior to visualization (panel B). Three mutants which displayed reduced *iglB-lacZ* activity were re-streaked and overlaid with filter paper pre-soaked in an X-gal solution to confirm the phenotype. The parent strain and well designations of each mutant are indicated (panels C and D).
Figure III.4. Identification of genes affecting \textit{iglB} transcription. A: Western blot of cell lysates isolated from each indicated strain using anti-IglC antibody. B: Coomassie stain of SDS-PAGE gel run on cell lysate from each indicated strain, loading control. C: \(\beta\)-galactosidase assay and ORF number of each identified putative \textit{iglB} regulator mutant. Miller assay results demonstrate a \(~5-7\)-fold reduction in \textit{iglB} transcription in FTL_1542 and FTL_0347 mutant strains (p<0.001). The IglC Western blot confirms that the mutations affect the expression of \(iglC\) as well as \(iglB\), and suggest that the mutations affect both chromosomal copies of the FPI. D: Schematic representation of the transposon insertion (black) at nucleotide 211 and the site directed insertion (white) at nucleotide 1458 of FTL_1542 and surrounding genes. FTL_1542 is upstream of genes encoding MraW, a hypothetical protein, and FtsI. The intragenic regions separating these genes are 6, -3, and -7 nucleotides, respectively. A gene encoding a 30S ribosomal protein is 106 nucleotides downstream of \textit{ftsI}. 
Figure III.5. Effect of *migR* mutation on expression of virulence regulators. Quantitative real time RT-PCR was conducted on mRNA template obtained from wild-type LVS (♦) or the site-directed *migR* mutant (□). Transcript of each gene was normalized to transcript of *tul4*, and the wild-type transcript for each gene was set to 1.0. The amount of *mglA* and *pmrA* transcripts were unaffected by the mutation in *migR* (p>0.1). The *migR* mutation resulted in a modest 1.4-fold decrease in *sspA* transcript (p=0.034). The *iglC* and *fevR* transcript levels were reduced in the *migR* mutant strain by 8.5- and 15-fold, respectively (p<0.001).
Figure III.6. Intracellular growth of migR and fevR mutant strains. A: Wild-type LVS (LVS), the original Tn5 migR mutant (Tn), site directed migR (migR) and fevR (fevR) mutants, and their corresponding complemented strains migRC and fevRC were used to infect MDM (MOI 20:1) cells in vitro, and intracellular growth was quantified as described in Materials and Methods. There was no significant difference in growth between the original Tn5 migR mutant and the site-directed migR mutant (p=0.951). Complementation of the migR strain with the full length FTL_1542 gene in trans restored growth in MDM (p<0.001). B-C: Wild-type LVS, site directed migR and fevR mutants, and their corresponding complemented strains migRC and fevRC were used to infect A549 (MOI 100:1) cells in vitro. Uptake of each strain was quantified after 1 hour for MDM or 4 hrs for A549 cells. Intracellular growth for each cell type was determined 24 hrs after infection. Data was normalized by dividing the 24 hrs time point by the 1 hr or 4 hrs time point. Both migR and fevR mutant strains were impaired for growth in MDM, while only the fevR mutant was defective for growth defect in A549 cells. Representative data from one of three experiments performed in triplicate is presented.
Figure III.7 Composition of *migR* and *fevR* mutant phagosomes in MDM.
Figure III.7., continued. Composition of migR and fevR mutant phagosomes in MDM. (A-C) Representative confocal sections of MDM infected for 1 h or 19-22 h (overnight) at 37°C with LVS (A), the migR mutant or its trans complemented strain migRc (B), or the fevR mutant and its trans complemented strain fevRc (C). In each case, samples were stained to detect bacteria and lamp-1 or cathepsin D, as indicated. Arrows indicate positive phagosomes. (D-E) Percentage of bacteria inside MDM that were infected for 1h (D) or overnight (E) that were inside lamp-1 or cathepsin-D-positive phagosomes. Data are the average ± SEM from three independent experiments performed in triplicate.
Figure III.8. The migR and fevR mutants activate human neutrophils. Neutrophils were left untreated (UN) or were infected with LVS, the original Tn5 FTL_1542 insertion mutant (Tn), the migR mutant (migR), its trans complemented strain migRc, the fevR mutant (fevR), or its trans complemented strain (fevRc) at 37°C, and reactive oxygen species production was measured at 30 sec intervals for 1 hr using the luminol assay. Data indicate luminol CL in counts per second (cps) and are the average ± SEM (grey bars) of triplicate samples from one representative experiment.
Figure III.9. Model for the role of MigR in regulation of \textit{iglABCD}. MglA and SspA form a heterodimer that is required for \textit{fevR} and \textit{iglABCD} expression. MigR does not affect the expression of \textit{mglA}, \textit{sspA}, or \textit{pmrA}. However, \textit{fevR} expression is reduced 15-fold in a \textit{migR} mutant. This reduction in \textit{fevR} results in an eightfold reduction in expression of the \textit{iglABCD} operon.
CHAPTER IV
ASSESSMENT OF THE EFFECT OF MIGR, AND FEVR MUTATIONS IN THE FULLY VIRULENT FRANCISELLA TULARENSIS STRAIN SCHU S4 IN MURINE AND PRIMARY HUMAN CELL INFECTIONS.

Introduction

Much of what is known about the genetics and pathogenicity of \textit{F. tularensis} has been learned through studies using non-human pathogenic model strains such as the live vaccine strain (LVS) or subspecies \textit{novicida} (21, 69, 167, 182). Further, the pathogenesis of these strains or the effect of specific mutations in these strains is often evaluated in model in vitro systems using either human or murine cell lines or primary murine phagocytic cells (42, 47, 64, 124). Because of the high level of genetic similarity between these model strains and the fully virulent subspecies \textit{tularensis} strain Schu S4, results from such experimentation have provided valuable data without the worry of accidental laboratory exposure to a potentially lethal pathogen. The use of non-human pathogenic model strains also generally leads to more rapid results as work is not encumbered by the inherently slower pace of work in a biosafety level 3 (BSL3) environment. Indeed, many findings using these model organisms have translated well when examined in Schu S4. These include the attenuation of Schu S4 \textit{iglC} and \textit{purMCD} mutants in mouse and macrophage infections (140, 186). However, the dramatic difference in virulence between LVS, subspecies \textit{novicida} and subspecies \textit{tularensis} suggests important differences between the strains. Recent reports have begun to highlight differences in pathogenesis between wild type strains of the different subspecies (81). Additionally, mutations in \textit{katG}, \textit{hfq}, \textit{tolC}, and \textit{chiA} have been reported to have different effects on the virulence of Schu S4 and LVS in murine infections (84, 100, 119). Taken together, these data suggest that while the use of model organisms and cell culture systems has value, findings do not always translate well across subspecies lines. Results
using such model systems should be carefully evaluated and confirmed using a fully virulent type A strain, such as Schu S4, and primary host cells to ensure the relevance of the obtained data.

The bulk of research to identify specific genes or evaluate specific mutations thought to be central to the virulence of *F. tularensis* has been conducted using primary or cultured murine and human phagocytic cells (33, 95, 130, 167). This is due to the central role that macrophages are believed to play in the pathogenesis of *F. tularensis* (134, 184). Far less work has been done to examine the role of non-professional phagocytes such as bronchial or airway epithelial cells to infection and pathogenesis. The distal airway is composed of a mosaic of different cell types including alveolar type I (ATI) and type II (ATII) epithelial cells, interstitial fibroblasts and alveolar macrophages (82), all of which have the potential to interact with and become targets of *F. tularensis* in the course of respiratory infection. ATI cells account for only ~10% of alveolar cells, but because of their flat morphology account for up to 95% of the alveolar airway surface area (41). These cells are the primary sites of gas and liquid exchange in the lung and can be identified by characteristic surface proteins such as aquaporin 5 (AQP-5) and T1-α (82, 195). In contrast, ATII cells account for ~15% of alveolar cells, but only cover ~5% of the alveolus (41). Type II cells serve the primary function of surfactant production in the lung, which aids in reducing surface tension in the lung, allowing air to enter the alveolar sacs (82). As such, they can be identified by non-secreted forms of surfactant proteins such as proSP-C. In addition to surfactant production, ATII cells also contribute to innate immunity through the production and secretion of opsonins and regulatory cytokines which may play an important role in the early activation of alveolar macrophages (85, 165, 197). Interestingly, type II cells also express on their surfaces MHC II (169), although it is not known whether these cells play a significant role in antigen presentation.
Reports on the ability of LVS to grow within immortalized epithelial cell lines such as TC-1, HEp-2, and the ATII-like cell line A549 have been published (23, 42, 99). Additionally, Kawula et. al. have demonstrated some preference of LVS for growth in ATII cells in the mouse lung, although LVS was also observed in other alveolar cell types as well (80). A small scale screen for Schu S4 mutants defective for growth in the human cervical carcinoma cell line Hep-G2 revealed 18 mutations that affected growth to some extent in these cells. Most of these mutants were auxotrophs or contained mutations in genes involved in nucleotide metabolism, protein modification, or were annotated as putative transporters (149). Only one study has been conducted using both Schu S4 and primary airway epithelial cells, and the focus of that study was the induction of cytokine secretion by the host cells in response to *F. tularensis* or conditioned media (67).

Therefore, it is still unclear if airway epithelial cells play important roles as host cells or as sites of replication during *F. tularensis* type A infections. Since respiratory exposure to *F. tularensis* is associated with the most severe form of tularemia, I wanted to examine interactions between airway epithelial cells and *F. tularensis*. To begin to examine a more relevant system and determine if current model systems using LVS and cultured epithelial cells lines are sufficient to study the pathogenesis of *F. tularensis*, I chose to characterize the infection of primary human small airway epithelial cells (SAECs) with the fully virulent type A *F. tularensis* strain Schu S4. SAECs are a mixed population of ATI and ATII cells obtained from the human alveolus. They are capable of polarizing and forming tight junctions and will better serve to replicate conditions encountered in the human lung.

In this chapter, I describe work in which I constructed *migR* and *fevR* mutants in subspecies *tularensis* (type A) strain Schu S4 and assessed the consequence of each mutation on expression of *iglABCD*, growth in primary human phagocytic and epithelial cells, and the effect of these mutations in murine infection. Specifically, I compared the ability of each mutant strain to grow in human monocyte derived macrophages (MDM)
and primary human small airway epithelial cells (SAECs). In addition, I compared the growth pattern of wild type LVS and Schu S4 in SAECs both by viable cell count and using confocal microscopy to shed light on differences in infection between bacterial strain as well as host cell. I also assessed the effect of migR and fevR mutations on LD$_{50}$ in mice and examined the dissemination pattern of each strain as compared to wild type Schu S4. The goal of these experiments was to confirm the regulatory and phenotypic effects of each mutation in LVS that were presented in Chapter 3 and to establish any differences between subspecies in these assays. Finally, I wanted to examine the validity of the use of wild type LVS in the airway epithelial cells line A549 as a quality model system for Schu S4 infection of the human airway.

**Materials and Methods**

**Bacterial strains, growth conditions, and plasmids**

*F. tularensis* LVS (ATCC 29684) and *F. tularensis tularensis* Schu S4 were grown in Mueller Hinton Broth (Becton Dickinson, Sparks, MD) or on Mueller Hinton Agar (Acumedia, Lansing, MI) supplemented with 1% glucose (w/v), 0.025% ferric pyrophosphate, and 2% IsoVitaleX. Spectinomycin and kanamycin were added to the growth media to a final concentration of 25 µg/ml, when appropriate. Ampicillin was added to a final concentration of 100 µg/ml for plating of organ homogenates. Plasmids containing a promoterless lacZ reporter gene (pBB119), an iglA-lacZ fusion (pBB125), and the full length migR (pBB114) or fevR (pBB135) genes driven by the groES promoter were transformed into various *F. tularensis* strains where indicated, and have been previously described (Table III.1).

**Quantitation of gene expression using the lacZ reporter**

Quantitation of lacZ activity was done according to the method of Miller (121). Duplicate cultures of tested strains were grown to mid log phase (OD$_{600}$ 0.4 – 0.7) in MMH broth, spun down and washed once in Z buffer, and β-galactosidase assays were performed on triplicate samples of each culture.
Creation of site-directed mutants using
intron-directed mutagenesis

Site-directed insertion mutants were created using a modified TargeTron (Sigma-Aldrich, St. Louis, MO) mutagenesis system (156). Briefly, the coding sequence of each gene of interest was entered into the Sigma TargeTron primer design site to determine appropriate oligonucleotides for retargeting the intron. Importantly, an XhoI restriction site was substituted for the HindIII site when designing the IBS primer. Retargeted PCR products were generated using Intron PCR Template (Sigma-Aldrich, TA0100) according to the recommendations of the manufacturer. The resulting fragment was introduced into the delivery vector pKEK1140 and cloning was verified by BglII digestion. LVS transformed with the retargeted plasmid was grown at 30°C on MMH agar with 25 µg/ml kanamycin. Individual colonies were purified once by growth at the permissive temperature and resulting colonies were screened by PCR to identify mutants before passaging at 37°C to cure the plasmid.

Intracellular growth assays

Wild-type or mutant LVS strains were used to infect MDM (MOI ~20:1), A549 cells, or SAECs (MOI ~ 100:1) in 24-well tissue culture plates. Approximately 10^5 MDM were seeded to individual wells in RPMI with 10% autologous serum and allowed to adhere overnight. Wells were washed and cells were re-suspended in RPMI with 2.5% autologous serum. Bacteria grown to mid-log phase in MMH broth were quantified by absorbance at 600 nm and quantitation was confirmed by plate counting. To optimize phagocytosis, bacteria were opsonized by incubation in 50% fresh autologous serum for 30 min at 37°C as described previously (113, 170). The appropriate numbers of bacteria were added to each well and infection was synchronized by centrifugation at 600 x g, 12°C for 4 min (170, 172). Initial infection efficiency was quantified after 1 hr co-incubation at 37°C. MDM monolayers were washed extensively with PBS to remove uningested bacteria and then processed immediately or incubated for another 23 hr at
37°C in fresh medium. Host cell lysis was achieved by addition of 1% saponin to each well and serial dilutions were plated on MMH agar to enumerate live organisms. Similarly, 2x10⁵ A549 or SAEC host cells were seeded to individual wells in MEM with 10% FBS, or in small airway cell growth medium (SAGM) (Lonza, Bassel, Switzerland). A549 cells were allowed to adhere overnight. SAECs were allowed to grow 4-6 days until reaching confluence. Bacteria were added and infection was synchronized as described above. After 4 hr incubation at 37°C, gentamicin at 15 µg/ml (LVS) or 30 µg/ml (Schu S4) was added for 90 min. to eliminate extracellular bacteria. Host cells were washed to remove gentamicin and lysed by the addition of 1% final concentration saponin. Additionally, SAECs were also mechanically disrupted using a cell scraper. Contents of the wells then were diluted and enumerated as described above to quantify bacterial uptake after 4 hours. For 24 hour time points, wells were replenished with gentamicin-free growth medium and incubated an additional 19 hrs before lysis and enumeration to quantify intracellular growth.

Confocal microscopy of *F. tularensis* infected host cells

Preparation and staining of infected host cells was conducted based on protocols established by Allen, L.A. (1). In brief, A549 or SAEC host cells attached to collagen coated glass slides were infected with *F. tularensis* at MOI 100:1. Bacterial uptake was synchronized as described above, and after 4 hr at 37°C, monolayers were washed extensively and were gent treated as described above to remove uningested bacteria. After a total of 4 or 24 h at 37°C, A549 infected with LVS were fixed in 4% paraformaldehyde for 20 min., permeabilized for 20 min. with 0.1% triton X-100 and 0.1 % BSA in PBS. Schu S4 infected A549 cells were subjected to a second 20 min. incubation in 4% paraformaldehyde to kill intracellular bacteria. Host cells were lysed with 1% saponin and plated to ensure sterilization of the sample. SAEC host cells were prepared in essentially the same way, but were permeabilized for 45 min. Samples were then blocked and double- or triple-stained to detect *F. tularensis*, lamp-1, and actin.
Bacteria were detected using rabbit anti-\textit{F. tularensis} antiserum (BD Biosciences) or expressed GFP. Mouse anti-human lamp-1 hybridoma supernatants (clone H4A3) were from the Developmental Studies Hybridoma Bank of the University of Iowa. Alexa conjugated anti-rabbit or anti-mouse secondary antibodies were obtained from the central microscopy research facility at the University of Iowa. For experiments using fluorescently labeled dextran, Alexa647 dextran (Invitrogen, Carlsbad, CA) was added to the cell culture wells at the time of infection at a concentration of 125 \( \mu \text{g/ml} \). Samples were fixed and permeabilized according to methods used by Tsang and Swanson (185). Samples were viewed using either a Bio-Rad Radiance multiphoton/confocal (Bio-Rad Laboratories, Hurcules, CA) or an LSM-510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY).

\textbf{Infection of mice and determination of organ burden}

Female Balb/c mice were inoculated intranasally with \textit{F. tularensis} strains in a total of 20 \( \mu \text{l} \) PBS by pipetting 10 \( \mu \text{l} \) of bacterial dilution in each nostril. Mice were anesthetized by administering 300 \( \mu \text{l} \) of avertin (tribromoethanol, 12.5 mg/ml) intraperitoneally 15 min. before infection. Following infection mice were housed with corn cob bedding in the University of Iowa BSL3 animal care facility and were monitored daily. To determine organ burden, mice were sacrificed by \textit{CO}_2 asphyxiation at indicated times and organs were removed, weighed, and homogenized in 2 ml of 1\% saponin in PBS using closed tissue grinders (Fisher, Pittsburgh, PA). Serial dilutions of the organ homogenates were plated on MMH agar with 100 \( \mu \text{g/ml} \) ampicillin to reduce contaminating flora.

\textbf{Results}

\textit{Creation of migR and fevR mutants and the effect on \textit{igl} gene expression}

Insertional inactivation mutants were constructed using a modified intron-directed mutagenesis system as described previously (23, 156), and in Materials and Methods.
The insertions lie between nucleotide 957/958 of FTT0694 (migR, total gene length 2100 nucleotides) and 223/224 of FTT0383 (fevR, total gene length 336 nucleotides). PCR using oligonucleotide primers flanking the insertion site as well as specific to intron sequence were used in conjunction to confirm the insertion sites (Fig.IV.1). To confirm the regulatory effect of each mutation on igl gene expression each mutant strain was transformed with a plasmid bearing either a promoterless lacZ reporter (pBB119) or an iglA-lacZ promoter fusion (pBB125). β-galactosidase assays were conducted on each Schu S4 mutant and were compared to results from wild type Schu S4 and corresponding LVS strains (Fig.IV.2). The lacZ control reporter ranged from ~1000 to ~1400 Miller units of activity regardless of strain or genetic background. In LVS, there was a 3.8-fold reduction in iglA-lacZ reporter activity in the migR mutant and a 5-fold reduction in the fevR mutant strain. As expected, the reduction in iglA-lacZ reporter activity was similar (4-fold) in the Schu S4 migR mutant. Unexpectedly, however, the baseline of iglA-lacZ reporter activity in the Schu S4 strain was approximately 3-fold higher than in LVS. Thus, despite the 4-fold reduction in iglA-lacZ reporter activity in the Schu S4 migR mutant strain, it retains 65% of the iglA-lacZ reporter activity as compared with the wild type LVS. Additionally, the larger 25.8-fold reduction in iglA-lacZ activity in the Schu S4 fevR mutant could be attributable to the higher baseline expression of iglA in Schu S4 in conjunction with a minimum amount of “leaky” lacZ expression.

Intracellular growth of Schu S4 and isogenic migR and fevR mutants in MDM

Wild type Schu S4, migR, fevR, complemented migR or complemented fevR strains were used to infect primary human monocyte derived macrophages (MDM). The wild type Schu S4 strain was capable of approximately 90-fold intracellular multiplication over a 23 hour outgrowth period (Fig.IV.3). This is virtually identical to the intracellular growth of LVS in MDM reported in Chapter 3 and elsewhere (23, 171). Interestingly, the migR mutation had no detectable effect on the ability of Schu S4 to
replicate within MDM. This is in contrast to the ~20-fold reduction in intramacrophage growth observed in the LVS migR strain (23). The fevR mutation in Schu S4 leaves the bacterium nearly incapable of growth in MDM (< 2-fold) and trans complementation restores growth to that of the parent strain. These results match data obtained from the LVS fevR mutant in similar experiments (23).

Intracellular growth of LVS, Schu S4, and migR and fevR mutants in SAECs

To examine the possible role of airway epithelial cells in the pathogenesis of F. tularensis, I infected primary alveolar epithelial cells with the attenuated LVS strain, the highly virulent Schu S4 strain, and isogenic migR and fevR mutants. The epithelial cells utilized are primary small airway epithelial cells (SAECs) (Lonza, Bassel, Switzerland), which are a heterogeneous population of ATI and ATII cells obtained from the human alveolus. Data from these infections indicate a consistent, low uptake of F. tularensis, generally between 0.01% and 0.1% of the input bacteria after 4 hours co-incubation regardless of strain or mutation (data not shown). This is similar to results obtained using cultured epithelial cells lines (23, 42, 80, 99). Surprisingly, the parent LVS strain was incapable of intracellular growth beyond initial uptake numbers (Fig.IV.4). This is in contrast to the ~1000-fold replication of LVS within A549, HEp-2, and other cultured cell lines (23, 42, 99). Schu S4 was capable of replication within SAECs, reaching ~15-fold increase over the initial uptake number. The inactivation of migR in Schu S4 had a mild deleterious effect on intracellular growth but did not rise to the level of statistical significance (p=0.075, two-tailed Student’s t-Test). Inactivation of fevR in Schu S4 significantly affected intracellular replication, leaving the mutant essentially non-replicative in SAECs (Fig.IV.4). Of note, intracellular growth of wild type Schu S4 and the migR mutant strain within SAEC cells varied from experiment to experiment, ranging from as low as 8- to as high as 40-fold replication over the course of an experiment. However, replication was most commonly between 10- and 20-fold for wild type Schu
S4, and the migR mutant was never statistically different from the parent strain regardless of the amount of replication achieved. Neither LVS nor the Schu S4 fevR mutant achieved more than 2-fold growth in any experiment. I believe this inconsistency is a result of the use of primary cells and the inherent differences between donors, passage, and variable host cell type composition of each individual experiment.

Since wild type Schu S4 appeared capable of modest replication in SAECs when compared to the ~1000-fold replication observed by LVS in A549 cells, I wanted to determine if this growth pattern was the result of a less permissive phenotype of primary epithelial cells or a general characteristic of the Schu S4 strain. To this end, I infected A549 cells with either wild type Schu S4 or LVS and assessed the intracellular replication of each strain. Results indicate 30-50-fold replication of Schu S4 in this cell line as compared to ~1000-fold multiplication of LVS (Fig.IV.4). This was a surprising finding given the superior virulence of the Schu S4 strain. Together, these data indicate that studies of human airway epithelial cell interactions with F. tularensis using the LVS and A549 model system may not be an ideal model to study the in vivo interactions of pulmonary tularemia.

Wild type strains Schu S4 and LVS display different intracellular growth patterns in cultured human cell lines and primary human cells

To better assess the dramatically different intracellular growth capabilities of wild type Schu S4 and LVS in primary cells and immortalized epithelial cells I observed each bacterial strain in each cell type using microscopy. LVS organisms were evident within A549 cells at 4 hours post infection. A typical sample had host cells containing a single bacterium, lacking lamp-1 co-localization. Less than 1% of host cells were infected (Fig.IV.5, IV.6). By 24 hours post infection, LVS replicated to fill the entire host cell cytosol (Fig.IV.7, IV.8) which correlates with the high intracellular growth numbers reported earlier. Despite the significant amount of bacterial growth in infected A549
cells, there is no visual indication of direct cell to cell spread at 24 hours post infection as adjacent host cells are free of bacteria. When observed in SAECs at 4 hours post infection, LVS is seen as single, lamp-1 negative bacteria present in less than 1% of the host cell population (Fig.IV.9). At 24 hours post infection, LVS still appears as single cells, ranging in number from one to few bacteria per host cell (Fig.IV.10, IV.11). This is consistent with the lack of replication observed in SAECs in the intracellular growth assays. The bacteria also remain lamp-1 negative at 24 hours post infection (Fig.IV.10, IV.11). The failure of LVS to co-localize with lamp-1 in A549 cells at 4 and 24 hours post infection was an expected result based on previous work by Craven et. al. in which LVS is maximally co-localized with lamp-1 at 2 hours post infection using the airway epithelium cell line TC-1. By 4 hours, and certainly 24 hours post infection, LVS has escaped the endosomal compartment and is freely replicating in the A549 host cell cytosol. It may be expected that the failure of LVS to replicate in SAECs is due to an inability to escape the endosome. While this can not be ruled out based on these results, it is clear that the bacteria are not confined to a typical late endosomal compartment.

Similar to the LVS infection of A549 cells and SAECs, Schu S4 was present as single bacteria at 4 hours post infection in less than 1% of SAECs (data not shown). Again, there appeared to be no co-localization with lamp-1 at this time point. At 24 hours post infection, bacterial replication was clearly visible in the SAECs. Interestingly, growth of Schu S4 appeared to occur predominately in a perinuclear location in the SAECs and occupied nearly the same cytosolic space as lamp-1 positive endosomes (Fig.IV.12, IV.13), although careful inspection revealed no specific co-localization with lamp-1. This is in contrast to the LVS infection of A549 cells, whose growth pattern filled the entire cytosolic space and seemed to exclude lamp-1 positive endosomes all together. Finally, I examined Schu S4 infection of A549 cells. As with the other three infection scenarios less than 1% of host cells were infected, reflecting an overall poor ability of F. tularensis to enter epithelial cells. In contrast to the other three infections
however, the intracellular growth pattern was unique. At both 4 and 24 hours post infection Schu S4 organisms were observed in circular formations. At 4 hours post infection there was generally only one such formation per infected host cell (Fig.IV.14). The bacteria within these circles appeared to occupy similar space as lamp-1, but upon careful inspection it was observed that these circles are composed of alternating Schu S4 staining and lamp-1 positive staining material. By 24 hours post infection, Schu S4 infected cells contained numerous circular formations that stained positive using a *F. tularensis* antiserum (Fig.IV.15, IV.16). It was also clear that by 24 hours post infection that bacteria in these structures excluded lamp-1 staining. *F. tularensis* has been shown to express a capsular material (31, 159) which may be shed during the course of infection. As the anti-*F. tularensis* antiserum being used in these experiments recognizes primarily LPS antigens but may also react with capsular antigens, it is possible that the circular formations being observed are actually the result of material shed from the bacterium within a vacuolar compartment. To explore this possibility, I repeated the infections using *F. tularensis* strains expressing GFP from a plasmid. Microscopic examination revealed the same circular patterns of bacteria within A549 cells (Fig.IV.17), suggesting that whole bacteria are being observed and not bacterial components.

The failure of lamp-1 to colocalize with Schu S4 growing in A549 cells indicates that these bacteria are not contained within a typical late-endosome type compartment, although it does not rule out that the bacteria might still be membrane bound within the host cell. To examine the intracellular growth pattern more closely and to begin to better characterize the circular pattern phenomenon, I performed co-uptake experiments in which A549 cells were infected with Schu S4 strains expressing GFP in the presence of high molecular weight dextran conjugated to a fluorophore. I reasoned that if the dextran was taken up along with *F. tularensis* in an endosome, the bacteria may co-localize with the dextran if further development of the endosomal vesicle was arrested by *F. tularensis*. At 24 hours post infection bacteria were again seen in circular clusters and they did not
co-localized with the fluorescent dextran (Fig.IV.18). While this result does not conclusively indicate the cellular localization of the bacteria it can be speculated that the bacteria were cytosolic, had re-entered a compartment after initial uptake, or were contained within a compartment that had undergone several fusion events, effectively diluting out the dextran signal. It is also possible that uptake of *F. tularensis* excluded uptake of the dextran during endocytosis.

**Mutation of migR or fevR in Schu S4**

*F. tularensis* has a very low LD$_{50}$ through intranasal or intraperitoneal routes of infection mice. Mutations in FPI genes or their regulators have been shown to dramatically increase the LD$_{50}$ of *F. tularensis* strains (21, 22, 38) so I assessed the ability of migR and fevR mutants to cause disease in mice following intranasal infection. Groups of 5 female Balb/c mice were inoculated intranasally with 10-fold dilutions of wild type Schu S4, the migR or fevR mutant strain in PBS. Dilutions of each inoculum were plated to enumerate the CFU delivered. Mice were monitored daily and the number of mice that succumbed to infection on each day was recorded. Results showed that 80% of mice infected with 87 CFU of wild type Schu S4 died 6 days post infection and 100% of mice were dead by day 11. In contrast, only 40% of mice infected with 250 CFU of the migR mutant strain succumbed to infection. Mice infected with the fevR mutant strain remained healthy for the entire 14 day course of the experiment, even at the highest dose, which approached 1000 CFU (Fig.IV.19). Using the Reed and Muench method for calculating for LD$_{50}$ (152), these results indicate that the LD$_{50}$ for wild type Schu S4 and the migR mutant were 42 CFU and 631 CFU, respectively. The 42 CFU LD$_{50}$ dose for wild type *F. tularensis* is similar to the reported literature value of ~10 CFU, and equates to a 15-fold increase in LD$_{50}$ for the migR mutant in my experiment. For comparison, mglA or iglC mutants in subspecies *novicida* are reported to have an LD$_{50}$ greater than 100,000 times that of the parent strain (95). The comparatively small increase in LD$_{50}$
seen in my experiment likely reflects the retention of the ability to replicate in macrophages observed in the Schu S4 migR mutant, despite a reduction in igl gene expression. It is worth mentioning that the experiment was conducted twice with similar attenuation results, although the absolute CFU corresponding to the LD$_{50}$ dose of each strain was 10-fold higher. I believe the increased LD$_{50}$ in this experiment was due to poor delivery of bacteria to the lungs of the mice, which was related to the type of bedding used in the experiment. Standard absorptive bedding material disseminates a fine dust that likely dries out the nasal passages of mice, reducing the spread of the intranasal inoculation to the lungs.

**Mutation of migR in Schu S4**

does not affect the dissemination pattern in mice

Following infection via intranasal, intraperitoneal, or intravenous routes, *F. tularensis* rapidly disseminates to the lung, liver and spleen where it is capable of robust replication (63). Based on results from the LD$_{50}$ studies presented above, I wanted to assess the effect of migR and fevR mutations on the ability of Schu S4 to disseminate, replicate, and/or persist within the lung, liver and spleen of mice following intranasal infection. Mice were infected with ~500 CFU of wild type or mutant strains and were sacrificed at 24, 60, or 96 hours, or 10 days post infection. Organs from the infected mice were homogenized and plated to determine bacterial burden. The *F. tularensis* chromosome carries the *blaB* gene which encodes a beta-lactamase (103, 156), so to reduce contamination from normal flora, ampicillin was added to the bacteriological growth medium used for viable cell counts. Unexpectedly, the fevR mutant strain was incapable of growth on media containing ampicillin while both wild type and migR mutant strains grew in the presence of the antibiotic. Both wild type Schu S4 and the migR mutant strains were detectable in the lung by 24 hours post infection, reaching up to 4.8 X 10$^4$ CFU/g organ, however neither strain was detectable in the liver or spleen at this time point (Fig.IV.20). By 60 hours post infection bacteria were present in the lungs of
two of three wild type infected and one of three migR infected mice, and bacterial replication was also apparent in the lung as viable cell count increased 10- to 100-fold from the 24 hour time point. Wild type Schu S4 was also present in the liver of two of three mice and the spleen of one of three mice ranging from $4.6 \times 10^4$ to $1.2 \times 10^5$ CFU/g organ. The migR strain was also present at similar CFU/g organ in the liver and spleen at 60 hours post infection, but was only detectable in one of three mice sacrificed. By 96 hours post infection bacterial replication was evident for both wild type and migR mutant strains in all three organs. Bacterial burden ranged from $3.1 \times 10^6$ to $1.9 \times 10^8$ in the lung, $5.4 \times 10^5$ to $6.5 \times 10^8$ in the liver, and $7.3 \times 10^6$ to $5.6 \times 10^9$ in the spleen and appeared to be similar for wild type and mutant strains (Fig.IV.20).

Discussion

The work in this chapter was initiated by creating migR and fevR mutants in the highly human pathogenic type A F. tularensis strain Schu S4. My goal was a comparison of the regulatory and phenotypic characteristics of these mutants with the corresponding mutants in the non-human pathogenic F. tularensis live vaccine strain (LVS) that I have described in a previous chapter. I also wanted to further the characterization of Schu S4 and these mutants in primary cell and murine infections to better establish the role of each gene in the pathogenesis of F. tularensis in vivo. Mutations in migR (FTT0694) and fevR (FTT0383) in the Schu S4 strain were successfully created and confirmed by PCR. My effort to characterize each mutant began with an examination of the effect of each mutation on igl gene expression using a plasmid borne iglA-lacZ reporter. Previous work has established that this reporter contains nucleotide sequence and binding sites required for differential regulation of the iglABCD operon. The mutations in Schu S4 had a negative effect on iglA expression, reducing expression ~4-fold in the migR mutant and reducing expression to a minimum in the fevR mutant. These reductions in iglA-lacZ activity are very similar to those observed in LVS migR and fevR mutant strains carrying the same plasmid-borne reporters. These data confirm the regulatory roles of MigR and
FevR on *iglABCD* expression in Schu S4 and suggests a similar regulatory network in both strains. Interesting, however, was the difference in baseline expression of *iglA* in the two wild type strains. Wild type Schu S4 exhibited approximately 3-fold higher expression of *iglA* than its live vaccine (LVS) counterpart. To my knowledge this is the first direct comparison of *igl* expression level between LVS and Schu S4 and illustrates a fundamental difference between the two strains. It is possible that this difference in virulence gene expression contributes to the difference in virulence between the two strains. Point mutations affecting promoter strength of virulence genes is consistent with the lack of major genetic differences between the pathogenic and live vaccine strains. Indeed, this hypothesis has been suggested in the context of *pdpD*, another virulence gene which lies directly upstream of *iglABCD* and whose promoter may have residual activity on the operon. The *pdpD* ORF contains substantial pleiomorphisms between the different *F. tularensis* subspecies and it has been suggested that these differences may affect the supplementary contribution of the *pdpD* promoter to *igl* expression (130).

The effect of *migR* and *fevR* mutations in Schu S4 was assessed by intracellular survival and growth experiments using human monocyte derived macrophages (MDM) as the host cell. Schu S4 was capable of nearly 100-fold replication in MDM over a 23 hour infection. This is nearly indistinguishable from the intracellular growth of LVS in MDM over the same period of time. I find this interesting given the dramatic difference in virulence between the two strains. It is possible that this apparent similarity between the strains is strictly an in vitro phenomenon resulting from the lack of some in vivo factor that inhibits LVS growth in MDM *in situ*. It is also a possibility that the attenuating mutation present in LVS does not affect its ability to survive and replicate within MDM *in vivo*. The latter possibility would also suggest that despite the large research focus on interactions of *F. tularensis* and macrophages, more subtle interactions between this organism and other host cell types may play vital roles in the virulence strategy for this pathogen.
The effect of the \textit{migR} mutation on intracellular growth of Schu S4 in MDM was negligible. This was a somewhat surprising result given the 20-fold reduction in intracellular growth of the LVS \textit{migR} mutant in MDM, especially since the \textit{migR} mutation in Schu S4 resulted in the same 4-fold reduction in \textit{iglA} expression that was seen in LVS. Again, the explanation of these data may relate to the difference in basal \textit{igl} expression between Schu S4 and LVS. While the \textit{migR} mutation results in a 4-fold reduction in \textit{iglA} expression in both Schu S4 and LVS, because of the higher basal \textit{iglA} expression in Schu S4, the \textit{migR} mutant still retains 65\% of the \textit{igl} expression that is observed in the wild type LVS. The \textit{fevR} mutation in Schu S4 rendered the strain essentially non-replicative in MDM, which was consistent with data obtained using LVS as the parent strain. The \textit{fevR} mutation caused a more severe reduction in \textit{iglA} expression, which even in the Schu S4 strain reduced \textit{iglA} expression to a minimal level. Together, these data support a hypothesis that a minimum threshold level of \textit{iglABCD} is needed to efficiently overcome host defenses, which in this case refers to the modulation of and escape from the macrophage phagosome. Additionally, this “threshold hypothesis” could help to explain the ability of LVS \textit{migR} but not \textit{fevR} mutants to replicate within cultures epithelial cell lines presented in Chapter 3.

Examination of LVS, Schu S4 and isogenic \textit{migR} and \textit{fevR} mutants in primary small airway epithelial cell (SAEC) infections yielded intriguing results. Unlike MDM infections, LVS was consistently unable to replicate within SAECs while wild type Schu S4 reached up to 40-fold replication over the 24 hour course of the experiment. This represents another fundamental difference between the pathogenic and non-pathogenic wild type strains and could potentially be important in the context of in vivo pulmonary infection. The \textit{migR} mutation had no significant effect on the ability of the Schu S4 strain to replicate within SAECs, which matched results using the LVS \textit{migR} mutant in A549 cells. Also similar to data obtained from LVS/A549 experiments, the Schu S4 \textit{fevR} mutant was unable to replicate within the SAECs beyond the initial uptake numbers. It
should be noted that there was significant variation in the absolute amount of intracellular growth of wild type Schu S4 and the migR mutant in SAECs from experiment to experiment. This ranged from ~8-fold up to 40-fold replication over the 24 hour course of infection; however, most commonly replication was in a 10- to 20-fold range. The primary nature of the cells, as well as donor and passage differences likely contributed to this variation. Additionally, I was unable to discern the relative proportion of different host cell types such as ATI and ATII within the population. This may be important since Hall et. al. have suggested a preference of *F. tularensis* for infecting ATII cells (80). Further, there are reports that primary ATII cells may differentiate into ATI upon in vitro passage (82). Combined, these factors may have contributed to the variation I have observed in these infections and should be taken into account when considering further work. Importantly, regardless of the absolute amount of replication, intracellular growth of Schu S4 and migR was statistically indistinguishable while LVS and fevR never surpassed 2-fold replication in any experiment. The consistent inability of fevR mutants in either LVS or Schu S4 to replicate within any primary or cultured cell line, phagocytic or epithelial, supports a model where FevR is a central regulator of virulence gene expression indispensable to the pathogenicity of the bacterium. In contrast, while the regulatory mechanism of MigR appears to be similar in Schu S4 and LVS, mutation of migR appears to be deleterious only in the context of LVS infection of phagocytic cells. The phenotypic differences caused by migR mutation in each strain hints at a more complex regulatory scheme in Schu S4 in which MigR plays a more peripheral role as compared with FevR. It is also possible that Schu S4 encodes functional gene products with overlapping or redundant function to that of MigR which may be absent in LVS and can sufficiently mask phenotypic effects of the mutation.

To follow up on observed differences in intracellular replication between wild type Schu S4 and LVS strains in A549 cells and primary SAECs, I performed confocal microscopy on infections of both host cell types using either Schu S4 or LVS. Consistent
among the four different infection scenarios was a low infection frequency. Less than 1% of host cells contained bacteria, and there was no indication of direct cell to cell spread in any of the infections, as host cells adjacent to infected host cells did not contain any bacteria. Intracellular growth patterns of the bacteria, however, were unique in each of the four infection scenarios. Growth of LVS within A549 cells 24 hours post infection was massive. Bacteria appeared to fill every available space within the cytosolic region of the cell leaving only the nuclear space free of bacterial growth. Visually, this was consistent with the ~1000-fold replication of LVS in A549 cells determined by viable cell count. This growth pattern was in stark contrast to LVS infection of SAECs in which bacteria appear as single cells which have perhaps undergone a single round of replication. The role of alveolar or airway epithelial cells in pneumonic tularemia is largely unstudied and it has yet to be determined whether the ability of *F. tularensis* to replicate within these cells is critical to the pathogenesis of the bacterium. However, the glaring contrast of LVS infection of A549 cells (a commonly used airway epithelial cell line) compared to infection of primary small airway epithelial cells highlights a shortcoming of the use of model systems to predict in vivo interactions. This is especially pertinent if these interactions are critical to the pathogenesis of *F. tularensis* type A strains. It is impossible from these preliminary studies to discern if the inability LVS to grow within primary human epithelial cells is related to its attenuation in humans, but it is an interesting conjecture given the similar phenotypes of LVS and Schu S4 in MDM. Further examination of other non-human pathogenic *F. tularensis* strains such as subspecies *novicida* in SAEC infection may help to address this question.

Another intriguing result obtained from epithelial cell infection experiments was that wild type Schu S4 only replicated ~15-fold within SAECs over 24 hours post infection. While this intracellular growth is significant compared to the lack of replication of LVS in these host cells, it is also far less than the ~1000-fold replication observed using the LVS infection of A549 cells model. Schu S4 replication in SAECs is
also less than the ~100-fold replication of LVS and Schu S4 in MDM. Microscopically, Schu S4 appeared to be replicating moderately but efficiently within SAECs, but was restricted primarily to the perinuclear space. This corresponds with the same region of the host cell that is highly enriched for late endosomes as indicated by staining for the lamp-1 marker. Close examination reveals little, if any, colocalization of Schu S4 and lamp-1, indicating that the bacteria are not contained within typical late endosomes and are likely to be cytosolic. It is not clear whether this perinuclear localization pattern is the result of some type of active cellular trafficking or simply the result of a physical barrier. It is possible that the bacteria localize to the perinuclear space based solely on the more spacious three dimensional character of the host cell around the nucleus, however, bacteria are present at more peripheral locations directly following infection. Experiments using other endosomal markers or real time microscopy may be better able to determine if bacteria are actively trafficked to the perinuclear space before replication, or if only those bacteria that infect a host cell proximal to the nucleus are able to replicate due to physical space restrictions of the peripheral host cell.

Overall, intracellular growth of Schu S4 in SAECs was modest compared to growth within MDM. To determine if this is a characteristic of Schu S4 within all epithelial cells or if it was the result of primary epithelial cells being generally less permissive for growth than cultured cell lines, I infected A549 cells with wild type Schu S4. Results show a 30-50-fold replication of Schu S4 over the course of the A549 infection. This result was unexpected since the avirulent live vaccine strain (LVS) replicates up to 1000-fold in these same cells. Upon microscopic examination, Schu S4 was found to be located in circular groupings within infected host cells. These structures were present in numbers ranging from 4 or 5 up to 15 per infected cell and often appeared in clusters. These formations were not restricted to a specific region of the host cell and were not lamp-1 positive, again indicating that the bacteria are not contained within a typical late endosome. However, due to the circular pattern, it is tempting to speculate
that the bacteria are contained within some type of membrane bound compartment rather than residing free in the cytosol.

The trafficking of phagosomal and endosomal compartments during infection has been studied using high molecular weight dextrans conjugated to fluorophores, which are taken up with bacteria during the normal endocytic or pinocytic process (16, 133). This allows the study of endosomal trafficking and fusion by following the localization of dextrans from initial uptake through the endpoint of maturation of the vesicle. Infection of A549 cells with Schu S4 in the presence of fluorescently labeled dextrans revealed good co-localization of lamp-1 and dextran, as expected, but failed to show any co-localization of dextran with Schu S4 in these unusual circular structures. I do not believe that this result rules out the possibility that the bacteria residing within these circular formations are contained within a compartment. It is possible that after initial uptake, Schu S4 escapes the endosome and later re-enters a different membrane bound compartment or organelle. It is also possible that the initial endosome undergoes heterotypic fusion with other cellular vesicles which results in the dilution of the dextrans and a loss of the ability to detect the fluorescent signal microscopically. I also explored the possibility that the circular structures might be bacterial fragments composed of capsular material or LPS components released by Schu S4 by repeating the infections using bacteria expressing GFP. In these infections the circular formations were still clearly visible, providing additional support that the bacteria themselves were the main constituent of the circular structures.

It is difficult to determine from these data exactly what is occurring within A549 cells during the course of infection by Schu S4. The use of different endosomal markers and the examination of infection at earlier time points (prior to 4 hours post infection) may help to better define the mechanism responsible for this unique phenotype. It is also difficult to determine if there is a bacterial genetic component, either gain or loss of function in LVS or Schu S4, which results in this growth phenotype. Assuming that there
are gene products responsible for the phenotype, a mutant hunt or a Schu S4 genomic expression library expressed in LVS might allow identification of genes involved in the phenotype. Likewise, similar experiments using Schu S4 should be able to relieve this phenotype and allow Schu S4 to grow freely in the cytosol of A549 cells. From these experiments it is clear that Schu S4 behaves very differently from LVS in the context of A549 infection. It is also apparent that Schu S4 itself behaves very differently in A549 and SAEC infection experiments. Both of these observations again highlight inherent concerns about using model cell lines or bacterial strains to study the pathogenesis of bacteria in vitro or in vivo.

Finally, I conducted in vivo mouse infections to determine the contribution of each mutation, migR and fevR, in Schu S4 to the LD$_{50}$ and the ability of the strains to disseminate from the initial site of infection to distal organs. Although the migR mutation in Schu S4 did not have a significant effect on intracellular growth of the mutant in in vitro infections, it did have an effect on igl gene expression. This negative effect on virulence gene expression may have a more pronounced effect on host infection kinetics than in vitro parameters. For instance, there may be other genes within the migR regulon that are dispensable for survival and growth within host cells but may play a role in serum resistance, attachment or dissemination of the bacterium. The LD$_{50}$ for wild type Schu S4 and each of the mutants was determined by administering 10-fold serial dilutions of broth grown bacteria in PBS intranasally. Mice were monitored daily for signs of illness or death. All mice that were infected with ~90 CFU of wild type Schu S4 succumbed to infection, with three of five mice dying on day 5 and the remaining two mice surviving until day 8 and 11 post infection. Only one of the five mice infected with ~9 CFU succumbed to infection. The migR mutation did affect LD$_{50}$, as only two of five mice succumbed to infection with ~250 CFU (one on day 6 and one on day 7 post infection), while the remaining three mice survived the entire 14 day course of the experiment. A dose of 10-fold higher CFU however was lethal in all infected mice. All
mice infected with the highest dose of the *fevR* mutant, which approached 1000 CFU, remained healthy throughout the experiment. This was not surprising since the *fevR* mutant has shown at least three to five log$_{10}$ attenuation in murine infections in previous studies (21). Based on this data, I used the Reed and Muench (152) method for estimating fifty percent endpoints. When applied to my data, this calculation estimates that the LD$_{50}$ of wild type Schu S4 to be 42 CFU and the LD$_{50}$ of the *migR* strain to be 631 CFU. The LD$_{50}$ of Schu S4 calculated from my data is similar to other reports which estimate the LD$_{50}$ of Schu S4 in mice via aerosol inoculation at approximately 10 CFU (173, 187). The calculated LD$_{50}$ of wild type Schu S4 and *migR* from my experiments represents a 15-fold increase in the LD$_{50}$ for the *migR* strain. Similar attenuation was also seen in a second independent experiment where mice were housed on traditional bedding, although the absolute LD$_{50}$ of each strain was approximately 10-fold higher, likely due to poor delivery of bacteria to the lung (data not shown). I believe this represents a real attenuating effect of the *migR* mutation in the context of murine infections, although it is less severe than mutations in other regulators of virulence gene expression such as FevR and MglA.

Based on the increased LD$_{50}$ observed for *migR* and *fevR* mutants in mice, I examined the ability of each strain to grow within the lung after infection as well as disseminate and grow within the liver and spleen of mice. Four groups of three mice for each bacterial strain were infected intranasally, this time with ~500 CFU of each strain. One group of three mice for each strain was sacrificed at 24, 60, or 96 hours, or 10 days post infection. Lung liver and spleen were removed, homogenized, and serial dilutions were plated to enumerate bacteria within each organ. The rich nature of *Francisella* growth medium and incubation at 5% CO$_2$ was found to allow the growth of fastidious normal flora of the upper airway. To overcome this complication as well as reduce other contaminants introduced into samples during the removal of the organs I supplemented the plating agar with ampicillin. This has no effect on the growth of *F. tularensis* which
in resistant to ampicillin due to the presence of a gene encoding a beta-lactamase. Wild
type and migR mutant strains were already replicating to detectable numbers in the lung
by 24 hours post infection. By 60 hours post infection replication of each strain in the
lung had risen approximately 100-fold. Replication continued but was not as dramatic
from 60 to 96 hours, reaching a maximum of $1.8 \times 10^8$ CFU per gram lung. At this point
both wild type and migR mutant infected mice were clearly sick and appeared moribund.
Neither wild type nor mutant strain appeared in the liver or spleen until 60 hours post
infection, at which time both strains had reached similar bacterial loads in each organ.
Bacterial replication by each strain continued rapidly in both organs, reaching a
maximum of $6.5 \times 10^8$ per gram liver and $5.6 \times 10^9$ per gram spleen. Interestingly, at 60
hours post infection only one of three mice infected with the migR mutant had detectable
bacteria in the liver and spleen while two of three wild type infected mice carried a
detectable bacterial load. Because of the small sample size it is difficult to determine if
this is significant, but it may suggest slightly slower dissemination by the migR mutant.
Overall, these results indicate little, if any, role of migR in the dissemination of bacteria
to distal organs following intranasal infection. It is worth noting that all of the wild type
infected mice died before the 10 day organ counts could be conducted while two of three
migR infected mice survived. These mice were sacrificed but no bacteria were detectable
in the lung liver or spleen. Together, these data support the observed attenuation of the
migR mutant but do not directly implicate any mechanism responsible for the attenuation.
Based on these data one could speculate a role of MigR in the establishment of infection,
perhaps through the regulation of pili or other surface adhesins, however more
experiments would have to be conducted to reach specific conclusions.

One potentially useful observation from these studies is that the fevR mutant was
sensitive to ampicillin. While this effectively prevented study of its dissemination
pattern, it also suggests that FevR is directly or indirectly involved in the regulation of
blaB, the gene encoding resistance to beta lactams in F. tularensis. This phenotype could
be the basis for a relatively simple and high throughput screen to identify upstream regulators of \textit{fevR} expression and/or other regulatory proteins within the \textit{fevR} regulon that may directly effect \textit{blaB} (and perhaps other virulence associated gene) expression.

In summary, I present the first report of mutations in two regulators of virulence gene expression in the human pathogenic type A \textit{F. tularensis} strain Schu S4. I have confirmed the regulatory effect of each mutation on \textit{iglA} expression and demonstrated a negative effect of a \textit{fevR} mutation on bacterial replication in both MDM and primary airway epithelial cells. In contrast to work with a \textit{migR} mutant in LVS, there was no apparent intracellular growth defect in a Schu S4 strain containing a \textit{migR} mutation. This is suggestive of a more complex regulatory scheme in Schu S4 that may involve additional gene products that are absent or non-functional in LVS. This difference may also be the result of an overall higher basal expression of the \textit{iglABCD} operon in the highly pathogenic Schu S4 strain. I have highlighted differences in both intracellular replication capabilities as well as intracellular growth patterns of Schu S4 and LVS in primary (SAEC) and cultured airway epithelial cells (A549). Data obtained from these experiments points out potential disparities between common model systems used to study the pathogenesis of \textit{F. tularensis} and conditions more similar to in vivo interactions of Schu S4 in the human airway. Last, I have demonstrated a mild attenuation attributable to a \textit{migR} mutation in Schu S4 in the context of intranasal infection of mice which is not due to a reduced ability to disseminate to distal organs. In total, the findings presented in this chapter begin to transition results obtained using the live vaccine strain to a more relevant bacterial strain and infection systems. I have also established baseline observations regarding the interaction of Schu S4 with primary airway epithelial cells that can be utilized to design further experiments to better define the role of these cells in the pathogenesis of \textit{F. tularensis}.
1. Add in IglC and iron Western
2. Fur box sequence like in deng paper but make my own for iglA, IglC, fslA
3. Proof of Targetron mutagenesis,
4. Perhaps the igl operon figure with different tnlacZ and tn-lux hits rolled into chapter 1 or 2, both fsl and igl operons from ppt
5. Restreak plates of iglB regulator screen where it says data not shown,
6. Work in migR domain map and re-work that results/discussion section to update,
7. In vitro MMH growth curves for LVS and S4 mutants and comps?

Figure IV.1. Construction of Schu S4 insertional inactivation mutants. A modified intron-directed mutagenesis system was used to create insertional inactivation mutations in FTT0694 (migR) and FTT0383 (fevR) in the Schu S4 strain. The insertions were located after nucleotide 957 of the migR gene and 224 of the fevR gene (dark triangles). Insertions were confirmed using PCR and primer sets either flanking the insertion site (1, 3) or with one primer within the inserted DNA (1, 2). The inserted intron DNA is 950 nucleotides in length. PCR product based on chromosomal DNA template from wild type (WT) or migR or fevR mutant strains was run on agarose gel.
Figure IV.2. Effect of mutations in migR or fevR on iglA expression in LVS and Schu S4. Wild type LVS, Schu S4, and isogenic migR and fevR mutants of each strain were transformed with plasmids carrying either a promoterless lacZ gene or an iglA-lacZ transcriptional fusion cassette. β-galactosidase assays were conducted on each reporter strain grown to mid-log growth phase in modified Muller Hinton broth. The promoterless lacZ reporter exhibited similar expression regardless of parental strain or genetic background. Expression of the iglA-lacZ reporter was 3-fold higher in the wild type Schu S4 strain as compared to the wild type LVS strain. Expression of the iglA-lacZ reporter in the migR mutant background was 4-fold lower in the migR mutant background in both Schu S4 and LVS. Expression of the iglA-lacZ reporter in the fevR mutant background was 25-fold lower than in the Schu S4 parent strain, reaching a baseline similar to that of the LVS fevR mutant strain.
Figure IV. 3. Replication of Schu S4 or isogenic \textit{migR} or \textit{fevR} mutants in MDM. Wild type (Schu S4), mutant (MigR, FevR), or complemented mutant (MigRc, FevRc) strains were used to infect MDM at an MOI of 20. MDM were washed and lysed 1 or 24 hours post infection and bacteria were enumerated. Results are expressed as a ratio of viable bacteria at 24 hours divided by viable bacteria at 1 hour post infection. Only the \textit{fevR} mutant strain was impaired for growth in MDM to a significant level.
Figure IV. 4. Replication of Schu S4, isogenic migR or fevR mutants, or wild type LVS in SAECs. Wild type (Schu S4), isogenic mutants (migR, fevR), or wild type LVS (LVS) strains were used to infect SAECs at an MOI of 100. SAECs were washed and lysed 4 or 24 hours post infection and bacteria were enumerated. Results are expressed as a ratio of viable bacteria at 24 hours divided by viable bacteria at 4 hour post infection. Wild type LVS and the Schu S4 fevR mutant were unable to replicate beyond initial uptake numbers detected at 4 hours post infection. Replication of the Schu S4 migR mutant was not statistically different (p>0.05) from that of the wild type Schu S4 strain in SAECs.
Figure IV.5. LVS infection of A549 cells 4 hours post infection. A single bacterium is present within A549 host cell and does not co-localize with the late endosomal marker lamp-1. Panel A: LVS only, green. Panel B: lamp-1 only, pseudocolored red. Panel C: merge of panel A and B. Panel D includes staining for actin to define host cell boundaries, LVS is shown in green, lamp-1 is shown in blue and actin is shown in red.
**Figure IV.6.** LVS infection of A549 cells 4 hours post infection. A single bacterium is present within A549 host cell and does not co-localize with the late endosomal marker lamp-1. Panel A: LVS only, green. Panel B: lamp-1 only, pseudocolored red. Panel C: merge of panel A and B. Panel D includes staining for actin to define host cell boundaries, LVS is shown in green, lamp-1 is shown in blue and actin is shown in red.
Figure IV.7. LVS infection of A549 cells 24 hours post infection. LVS is present within A549 host cell and has replicated to fill the entire host cell cytosol, excluding lamp-1 positive endosomes. Only the nuclear space remains free of bacteria. Adjacent host cells do not contain bacteria, indicating a lack of direct cell to cell spread. Panel A: LVS only, green. Panel B: lamp-1 only, pseudocolored red. Panel C: merge of panel A and B. Panel D includes staining for actin to define host cell boundaries, LVS is shown in green, and actin is shown in red.
Figure IV.6. LVS infection of A549 cells 24 hours post infection. LVS expressing GFP is present within A549 host cell and has replicated to fill the entire host cell cytosol, excluding lamp-1 positive endosomes. Only the nuclear space remains free of bacteria. Adjacent host cells do not contain bacteria, indicating a lack of direct cell to cell spread. Panel A: LVS only, green. Panel B: lamp-1 only, pseudocolored red. Panel C: merge of panel A and B. Panel D includes staining for actin to define host cell boundaries, LVS is shown in green and actin is shown in red.
Figure IV.9. LVS infection of SAEC cells 4 hours post infection. A single bacterium is present within the SAEC host cell and does not co-localize with the late endosomal marker lamp-1. Panel A: LVS only, green. Panel B: lamp-1 only, pseudocolored red. Panel C: merge of panel A and B. Panel D includes staining for actin to define host cell boundaries, LVS is shown in green, lamp-1 is shown in blue and actin is shown in red.
Figure IV.10. LVS infection of SAEC cells 24 hours post infection. A single bacterium is evident within the SAEC host cell 24 hours after infection. This reflects the inability of LVS to replicate within primary airway epithelial cells in vitro. Failure of LVS to co-localize with the late endosomal marker lamp-1 suggests that LVS may be free in the host cell cytosol. Panel A: LVS only, green. Panel B: lamp-1 only, pseudocolored red. Panel C: merge of panel A and B. Panel D includes staining for actin to define host cell boundaries, LVS is shown in green, lamp-1 is shown in blue and actin is shown in red.
Figure IV.11. LVS infection of SAEC cells 24 hours post infection. A single bacterium is evident within the SAEC host cell 24 hours after infection. This reflects the inability of LVS to replicate within primary airway epithelial cells \textit{in vitro}. Failure of LVS to colocalize with the late endosomal marker lamp-1 suggests that LVS may be free in the host cell cytosol. Panel A: LVS only, green. Panel B: lamp-1 only, pseudocolored red. Panel C: merge of panel A and B. Panel D includes staining for actin to define host cell boundaries, LVS is shown in green, lamp-1 is shown in blue and actin is shown in red.
Figure IV.12. Schu S4 infection of SAEC cells 24 hours post infection. Schu S4 is capable of replication within primary airway epithelial cells \textit{in vitro}. Growth of the bacterium appears to be localized to the same region of the cytosol as lamp-1 positive endosomes, however, Schu S4 does not directly co-localize with these endosomes. Failure of Schu S4 to co-localize with the late endosomal marker lamp-1 suggests that LVS may be free in the host cell cytosol. Panel A: LVS only, green. Panel B: lamp-1 only, pseudocolored red. Panel C: merge of panel A and B. Panel D includes staining for actin to define host cell boundaries, LVS is shown in green, lamp-1 is pseudocolored red and actin is pseudocolored blue.
Figure IV.13. Schu S4 infection of SAEC cells 24 hours post infection. Schu S4 is capable of replication within primary airway epithelial cells *in vitro*. Growth of the bacterium appears to be localized to the same region of the cytosol as lamp-1 positive endosomes, however, Schu S4 does not directly co-localize with these endosomes. Failure of Schu S4 to co-localize with the late endosomal marker lamp-1 suggests that LVS may be free in the host cell cytosol. Panel A: LVS only, green. Panel B: lamp-1 only, pseudocolored red. Panel C: merge of panel A and B. Panel D includes staining for actin to define host cell boundaries, LVS is shown in green, lamp-1 is shown in blue and actin is shown in red.
Figure IV.14. Schu S4 infection of A549 cells 4 hours post infection. Schu S4 appears as a circular cluster composed of a few bacteria 4 hours post infection. While maintaining a spherical pattern, these bacteria do not co-localize with lamp-1. Panel A: LVS only, green. Panel B: lamp-1 only, pseudocolored red. Panel C: merge of panel A and B. Panel D includes staining for actin to define host cell boundaries, LVS is shown in green, lamp-1 is shown in blue and actin is shown in red.
Figure IV.15. Schu S4 infection of A549 cells 24 hours post infection. Schu S4 appears in circular clusters composed of a few bacteria 24 hours post infection. This reflects the ability to replicate within A549 cells while maintaining a unique organizational pattern. Bacteria within these structures do not co-localize with lamp-1. Panel A: LVS only, green. Panel B: lamp-1 only, pseudocolored red. Panel C: merge of panel A and B. Panel D includes staining for actin to define host cell boundaries, LVS is shown in green, lamp-1 is shown in blue and actin is shown in red.
Figure IV.16. Schu S4 infection of A549 cells 24 hours post infection. Schu S4 appears in circular clusters composed of a few bacteria 24 hours post infection. This reflects the ability to replicate within A549 cells while maintaining a unique organizational pattern. Bacteria within these structures do not co-localize with lamp-1. Panel A: LVS only, green. Panel B: lamp-1 only, pseudocolored red. Panel C: merge of panel A and B. Panel D includes staining for actin to define host cell boundaries, LVS is shown in green, lamp-1 is shown in blue and actin is shown in red.
Figure IV.17. Schu S4 expressing GFP within A549 cells 24 hours post infection. Schu S4 appears in circular clusters composed of a few bacteria 24 hours post infection. The use of GFP expressing bacteria for infection experiments strengthens the notion that the circular pattern of growth is a result of bacterial organization rather than shed material that is recognized by the anti-Francisella antiserum used for fluorescence labeling of the bacteria. Bacteria within these structures do not co-localize with lamp-1. Panels A-D are four representative images. LVS is shown in green, lamp-1 is shown in blue and actin is shown in red.
Figure IV.18. Schu S4 expressing GFP within A549 cells 24 hours post infection does not co-localize with dextran. A549 cells were infected with Schu S4 in the presence of fluorescently labeled alexa647 dextran. At 24 hours post infection there is no co-localization of Schu S4 with the dextrans, suggesting that the bacteria were not trafficked in the same manner as the inert dextran substrate. Still, Schu S4 appears in circular clusters composed of several bacteria 24 hours post infection. Panels A and C show dextran alone (pseudocolored red), panels B and D show dextran (pseudocolored red) and Schu S4 (green).
Figure IV.19. Effect of migR and fevR mutations in Schu S4 on LD50 in mice. Groups of five mice were infected intranasally with 10-fold dilutions of wild type (A) or mutant (B and C) Schu S4. All mice infected with 87 CFU of wild type Schu S4 died, while 40% of mice infected with 250 CFU of the migR mutant succumbed to infection. Mice infected at the highest dose of the fevR strain all remained healthy throughout the course of the experiment.
Figure IV.20. Effect of \textit{migR} and \textit{fevR} mutations in Schu S4 on dissemination in mice. Mice were infected intranasally with ~ 500 CFU of wild type or \textit{migR} mutant Schu S4. Mice were sacrificed at 24, 60, or 96 hours or 10 days post infection. Lung (A), liver (B), and spleen (C) were homogenized, diluted, and plated to enumerate bacterial burden in the organs. The \textit{migR} mutant was not impaired in its ability to disseminate to distal organs following intranasal infection.
Initial classification and characterization of an organism such as \textit{F. tularensis} often begins with basic observations of the bacterium including morphological analysis to identify surface structures and simple biochemical assays to determine its metabolic capabilities. In the context of a pathogen, basic interactions with surrogate model host cell lines are also established and often include the ability of the pathogen to adhere to, invade, and replicate within these cells. These early characterizations rely primarily on observational experiments and are capable of establishing a solid base of knowledge from which to design further experiments. An obstacle to the advanced study, particularly genetic, of novel organisms is the lack of tools with which to manipulate the organism. Such was the case when I initiated my study of virulence gene regulation in \textit{F. tularensis}. Limitations included the availability of only a single plasmid origin of replication for use in \textit{Francisella ssp.}, the lack of a robust system to efficiently make large numbers of mutants, and the absence of any described reporters of gene expression. Through the development of a reliable transposon mutagenesis system and the evaluation of two different reporters of gene expression I was able to not only create limitless random mutant strains, but also constructed libraries to monitor gene expression under different environmental conditions. These libraries have been used by me and others to identify genes involved in capsule biogenesis, triggering the inflammatory response in macrophages, and subverting the respiratory burst in neutrophils. In particular, I was able to identify genes in the \textit{fslABCD} and \textit{iglABCD} operons as being upregulated when grown on Chamberlain’s defined medium (CDM) and determine that a major contributor to this induction of expression was iron restricted growth. A more comprehensive study of these two operons, both associated with the virulence of \textit{F. tularensis}, revealed the \textit{fslABCD} operon to be regulated by the ferric uptake regulator protein (FUR) in a canonical
manner. Surprisingly, although expression of the $iglABCD$ operon was also upregulated by growth in iron limiting conditions, it was independent of the FUR protein. Experiments utilizing plasmid-borne reporters containing different fragments of DNA adjacent to or within the $iglABCD$ operon identified an iron-responsive region of DNA upstream of the $iglA$ gene that likely also contains the promoter region for this operon. Further study of the iron-dependant regulation of this operon will be necessary both to identify the specific protein or mechanism of regulation and the specific nucleotides within the regulatory sequence governing $iglABCD$ expression. One possibility is that iron-dependant regulation of this operon relies on a gene product other than FUR such as $ryhB$ or $spoT$. RyhB is a small non-coding RNA that is involved in the regulation of several different operons in $E. coli$ and $V. cholerae$ which range in function from iron utilization to biofilm formation (112, 120). While it is unlikely that the $iglABCD$ mRNA is a direct target of RyhB, an activator or repressor of the operon could lie within the RyhB regulon. SpoT, along with RelA is involved in synthesis and degradation of the nucleotide (p)ppGpp. It is reported that iron limitation can increase SpoT dependent (p)ppGpp synthesis, and that (p)ppGpp is involved in the regulation of genes involved in iron uptake as well as others (189). In either case, further mutagenesis screens could potentially identify this factor.

The $iglABCD$ operon plays a critical role in the intracellular survival of $F. tularensis$; however, at the outset of this work the regulation of this operon was not well defined. The regulatory model included only MglA, a non-DNA binding activator of $iglABCD$ expression. To address this incomplete model of regulation, I utilized an LVS mutant strain containing a $lacZ$ reporter of $iglB$ expression as the basis for a second round of mutagenesis to identify mutations that resulted in the loss of reporter activity. The screen identified a mutation in a hypothetical protein (FTL_1542) which shares domains of homology with AMP binding fatty acid CoA-ligases and acyl carrier proteins. I confirmed the regulatory effect of this mutation, which I named $migR$, on $iglABCD$
expression using several techniques including protein blots and real time RT-PCR. Based on real time RT-PCR results I was able to incorporate MigR into a regulatory scheme as a positive regulator of FevR, a protein which contains a DNA binding domain and is essential for the expression of iglABCD. While this data strongly suggests that the migR effect on iglABCD expression is through the reduction of a more central and direct regulator, FevR, it does not explain the mechanism of regulation. Because of the lack of any predicted DNA binding domain within MigR, and the role of proteins with homologous domains in fatty acid modification, it seems likely that the regulatory effect of MigR on fevR is through some sort of signal modification. A regulatory mechanism involving the addition of ACoA to fatty acids, which then interact with a regulatory protein is present in the FadD/FadR regulatory system (18, 54, 89). Similarly, MigR could be involved in modification of fatty acids or sensing fatty acid starvation. Either effect could lead to the upregulation of other systems that may have an effect on fevR and thus iglABCD expression. Indeed, a recently published study has demonstrated interaction between the SpoT protein and an acyl carrier protein in E. coli. This binding dictates the (p)ppGpp synthesis or hydrolysis activity of SpoT (13, 14). Another recent publication has examined the phenotype of a relA mutant in F. tularensis. This mutant strain loses the ability to produce (p)ppGpp under amino acid starvation and is attenuated for growth in macrophages (49).

Based on these data, I propose a regulatory model in which MigR or a product or MigR activity (such as a modified fatty acid) interacts with SpoT to induce the synthesis of (p)ppGpp. The (p)ppGpp may then contribute to expression fevR, and thus iglABCD, and perhaps other genes critical to the pathogenesis of F. tularensis (Fig.V.1). The involvement of (p)ppGpp in the expression of virulence genes is not novel, as (p)ppGpp has been reported to be a positive regulator of SPI2 gene expression in S. enterica (183). In a migR mutant the SpoT interaction partner would be lacking, and (p)ppGpp would not be synthesized, thus fevR (and iglABCD) expression would not be induced. This model is
consistent with the recently published reports mentioned above as well as with data obtained from my own experiments with the *migR* mutant and its putative annotated function. In particular, it could explain the partial loss of *iglABCD* expression in a *migR* mutant, as well as the different growth phenotypes of LVS *migR* in MDM and epithelial cell lines. Since both SpoT and RelA are capable of synthesizing (p)ppGpp, the *migR* mediated interference with the SpoT pathway would still allow RelA rescue of the phenotype under specific environmental conditions, namely amino acid starvation. This hypothesis could be tested by comparing the levels of (p)ppGpp in wild type versus *migR* mutant strains. Further, *relA* and *spoT* mutants as well as double knockouts should have dramatically reduced levels of (p)ppGpp, and if the model is correct, a concomitant reduction in *fevR* and thus *iglABCD* expression.

Phenotypic characterization of LVS *fevR* and *migR* mutants revealed an inability of either mutant to replicate substantially within MDM and diminished ability to inhibit the respiratory burst of neutrophils. Interestingly, the *fevR* mutant was also incapable of replication within two different epithelial cell lines while the *migR* mutant was capable of replication similar to that of wild type LVS. To further examine this phenomenon, as well as the general characteristics and consequences of mutations in *migR* and *fevR*, I constructed site directed *migR* and *fevR* mutant strains in the human pathogenic strain Schu S4. The effect of the *migR* and *fevR* mutations on *iglA* expression was similar to those observed in LVS, although the baseline expression of *iglA* was significantly higher in the Schu S4 strain. Interestingly, the *migR* mutation did not result in attenuated growth in MDM. This may be the result of higher basal expression of *iglABCD* in Schu S4 which could minimize the phenotypic effects of a *migR* mutation. It could also be the result of a more complex regulatory scheme in Schu S4, possibly involving redundancy of gene function which could obscure the effects of a *migR* mutation. Sequence comparison of *migR* to other genes within the *F. tularensis* chromosome reveals significant similarity to genes annotated as *fadD1* and *fadD2*. These proteins are
involved in the addition of acetyl CoA to fatty acids as they enter the cell (54). Comparison of fadD1 and fadD2 between LVS and Schu S4 reveals few nucleotide substitutions, however, it possible that one of these mutations renders the gene non-functional in LVS. If these two genes can serve a redundant function to MigR, it is possible that a point mutation inactivating the gene in LVS accounts for the more dramatic phenotype of a migR mutant in LVS. Mutation of fadD1, fadD2 and migR alone and in combination in each strain could be performed to further examine this possibility.

In addition to macrophages, F. tularensis almost certainly comes into contact with airway and alveolar epithelial cells upon inhalation. I examined the interaction of wild type LVS with the airway epithelial cell line A549, as well as the effect migR and fevR mutations had on this interaction. Results indicated that fevR was essential for growth within A549 cells, while migR was not required for replication within these cells. Having created migR and fevR mutants in the Schu S4 strain, I had intended to examine the effect of these mutations on Schu S4 growth in both A549 cells and primary human small airway epithelial cells (SAECs). However, initial experiments to examine wild type LVS and Schu S4 interactions with each host cell type revealed remarkable differences between these interactions. The uptake of either bacterial strain was similar in either host cell, however bacterial replication was vastly different depending on the bacterial strain and host cell. LVS replicated nearly 1000-fold in A549, but was incapable of replication in SAECs. Schu S4 replicated only 30- to 50-fold in A549, and 15-fold in SAECs. In light of these results, I chose to focus on the characterization of each of the four infection scenarios (LVS/A549, LVS/SAEC, Schu S4/A549, Schu S4/SAEC) in the absence of any mutation.

The first noteworthy observation was the dramatic difference replication of LVS within A549 versus SAEC host cells. This suggests a fundamental difference between the cultured A549 cells, often used as a model, and primary SAECs. The foundation of this difference which has a significant impact on susceptibility to parasitization by LVS
could be the result of active or passive differences between LVS or host cells. The composition of the host cell cytosol may differ in the availability of certain essential nutrients critical for the growth of LVS. For example, previous work has shown that an LVS mutant incapable of pyrimadine biosynthesis can replicate within the cytosol of cultured epithelial cells, but not MDM (171). This is consistent with the observation that LVS does not appear to co-localize with lamp-1, suggesting that it is free in the cytosol but unable to replicate. In this case it may be possible to restore intracellular growth by the addition of a given nutrient, for example uracil, to the cell culture medium. Alternatively, the failure of LVS to replicate within SAECs could be the result of an active defense mechanism present or functional in primary epithelial cells that has been lost from a cultured cell line. Examination of this possibility would require a much more thorough inspection and comparison of host cell mechanisms and markers associated with endocytosis of *F. tularensis* between the two types of host cells.

Another interesting observation, and key difference between LVS and Schu S4, is the ability of Schu S4 to replicate within SAECs. This is striking not only because of the difference of replication within SAECs, ~15-fold for Schu S4 and less than 2-fold LVS, but also the implications of the result. Whereas LVS and Schu S4 have indistinguishable intracellular growth phenotypes in MDM infections in vitro, this observation highlights a potentially important difference between the avirulent LVS strain and the highly virulent Schu S4 strain. While difficult to prove, it is likely that *F. tularensis* comes into contact with epithelial cells in the distal airway at a greater frequency that with the resident alveolar macrophages. This would be increasingly important at low dose exposure where only 10 organisms enter the airway. Therefore, a model could be proposed in which airway epithelial cells serve as an important initial site of replication for *F. tularensis* preceding interaction with phagocytic cells. In this model, the inability of LVS to replicate within primary airway epithelial cells would aid in explaining its inability to cause disease, especially at low dose. To examine this hypothesis I would test the ability
of different strains and subspecies of *F. tularensis* to replicate within primary human airway epithelial cells and determine if there is a corollary between intracellular replication and the ability of the strain to cause disease in humans.

Provided the ability to replicate in epithelial cells is central to the pathogenesis of *F. tularensis*, a more complete understanding of the interaction between Schu S4 and SAECs would be valuable. While the Schu S4 strain does replicate within SAECs, the growth and localization pattern of the bacteria is unique and different from what I see in LVS infection of A549 cells. Bacterial growth of Schu S4 seems to be restricted to the perinuclear region of the host cell, occupying an almost identical space as lamp-1 positive endosomes. The ability of Schu S4 to replicate and the lack of direct co-localization of bacteria with lamp-1 suggest that the bacteria are cytosolic, however, it is possible that they are in a membrane bound compartment that is free of the lamp-1 marker. It would be worth observing infected host cells at later time points to determine whether the bacteria remain primarily within the lamp-1 enriched perinuclear region or if they replicate beyond the region to eventually fill the entire cytosol similar to what is observed in LVS infection of A549 cells. Likewise, observation at early time points using an array of endosomal markers or even live imaging would allow you to examine trafficking of the bacteria and determine if bacteria taken up at the periphery of the host cell are shuttled toward the nucleus or simply fail to replicate due to special restraints.

Finally, the interaction of Schu S4 with A549 cells produced perhaps the most surprising result. Not only was its 30- to 50-fold replication significantly less than the nearly 1000-fold replication of LVS within A549 cells, but the intracellular growth pattern was unique. In contrast to the cytosol filling growth of LVS, Schu S4 was observed to be replicating in circular formations. These formations were lamp-1 negative, did not stain for actin, and did not co-localize with fluorescently labeled dextran when observed in co-uptake experiments. These data may indicate cytosolic localization of the bacteria; however, it is difficult to explain the circular arrangement of the bacteria.
without the restraint of a host cell compartment. Further, it is not easy to assess the relevance of this phenomenon to pathogenesis or in the context of human infection, especially in light of the apparent differences between A549 and primary cells discussed above. Again, this could be attributable to differences between the bacterial strains or host cells. If it is the result of differences in the genetic repertoire of the LVS and Schu S4, or simply differences in expression of genes, then knockout or overexpression libraries may be of use to identify the underlying mechanism causing this growth pattern. However, at this point, I would find it hard to justify intense study of this phenomenon.

In summary, I have developed a mutagenesis and reporter system that has facilitated the study of gene regulation in *F. tularensis* and has enabled the identification of a new regulator of virulence gene expression. This regulator, *migR*, is involved in the positive regulation of *fevR*, a central regulator of pathogenicity island genes central to intracellular growth. MigR likely represents one of several convergent regulatory systems that result in virulence gene expression since it is necessary in some infections and dispensable in others. I have also created and characterized the effect of *migR* and *fevR* mutations in the highly virulent *F. tularensis* strain Schu S4 and have identified differences between the effects of the *migR* mutation between LVS and Schu S4 that suggest a more complex regulatory scheme in the human pathogenic strain. I have also begun to establish differences between wild type LVS and Schu S4 strains in their interaction with cultured and primary airway epithelial cells which may potentially contribute to the difference in virulence between the two strains. Together, this work has contributed tools for the study of *F. tularensis* and insight into the regulation of virulence factors of this pathogen. It has also highlighted differences between virulent and avirulent strains that can serve as a strong basis for future work.
Figure V.1. Proposed model for MigR-dependent regulation of \textit{iglABCD}. MigR homology to fatty acid (FA) modification enzymes suggests a role in the modification or transfer of fatty acids within the bacterial cell. A possible donor or acceptor of these fatty acids is the acyl carrier protein (ACP), a soluble protein that acts as a scaffold for fatty acid construction in the cell. SpoT is capable of association with ACP, and depending on the fatty acid state of the cell can either synthesize (under fatty acid starvation) or degrade (under fatty acid rich growth) the signaling molecule (p)ppGpp. Previous reports have demonstrated that \textit{fevR} expression to be dependent upon (p)ppGpp. Deletion of \textit{migR} may interrupt the native processing of fatty acids in the cell, disrupting the ability of \textit{F. tularensis} to perceive fatty acid starvation. This in turn could lead to the SpoT dependent degradation of (p)ppGpp, and a reduction in \textit{fevR}, and thus \textit{iglABCD} expression.
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