IDENTIFYING HOST AND BACTERIAL FACTORS REQUIRED FOR FRANCISELLA TULARENSIS INTRACELLULAR GROWTH

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ABSTRACT

Jason Brunton: Identifying host and bacterial factors required for *Francisella tularensis* intracellular growth
(Under the direction of Thomas Kawula)

*Francisella tularensis* is a small Gram negative coccobacillus that is the causative agent of the disease tularemia. A hallmark of *F. tularensis* pathogenesis is its extraordinary capacity to rapidly grow to high densities within the cytosol of host cells. To extensively replicate in the hostile environment of the host, the bacterium must expertly adapt to the cytosolic environment, evade destruction by immune defenses and acquire significant nutrients. However, few bacterial virulence factors have been identified and the mechanisms by which *F. tularensis* obtains nutrients and adapts to the cytosolic environment are poorly understood.

Here we performed a large scale transposon mutagenesis screen to identify novel bacterial factors required for intracellular growth. From the screen we identified a gene of unknown function, *FTT_0924*, which we demonstrate is required for intracellular growth and virulence. We show *FTT_0924* is required for resisting osmotic stress during bacterial replication indicating *FTT_0924* is required for maintaining cell wall integrity. Together these data suggest *F. tularensis* requires *FTT_0924* for strict control of cell wall dynamics to adapt to the cytosolic environment.

To replicate to high densities within host cells, *F. tularensis* must efficiently acquire significant nutrient sources, yet the major sources of host derived carbon, as
well as the strategies employed by the bacterium to acquire host carbon are unknown. Here we took a genetic approach to identify bacterial carbon metabolic pathways and define the major sources of host derived carbon that fuel bacterial replication. We show gluconeogenesis is essential for intracellular and in vivo growth and suggest glycerol 3-phosphate and amino acids are the primary carbon sources acquired by F. tularensis from host cells. We then investigated from where essential host derived nutrients are derived and identify host cell autophagy is required to provide F. tularensis with nutrients for bacterial replication. Specifically, we found F. tularensis infection induces flux through an ATG5-independent autophagy pathway to provide amino acids and bulk carbon to the bacterium. Overall, these studies provide significant steps in understanding how F. tularensis, and potentially other intracellular pathogens, adapt to the cytosolic environment and acquire essential nutrients for bacterial proliferation.
ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Tom Kawula, for all of the guidance and support throughout my graduate career. His mentorship has provided me with independence to develop as a scientist and solve problems, but also enough guidance to successfully complete the research studies described within this dissertation. His door was always open if I had questions and he always provided useful advice to fix whatever problem I had. Lastly, I appreciate the freedom he granted me to pursue projects outside the knowledge and expertise of the lab.

I would also like to thank everyone that helped me both design and perform these studies. My committee has provided me with significant direction, especially Tony Richardson and Nat Moorman, with whom I’ve had countless conversations about metabolism and metabolic signaling. Current and former members of the Kawula lab including Shaun, Sharon, Cheryl, Brittany, Eric and Todd have been instrumental in helping me design experiments and share workload of experiments in the BSL3. In particular, I would like to thank Shaun for working with me throughout the entire autophagy project which lead to a shared primary authorship on the publication and Sharon for help in experimental design, animal experiments, making sure the lab runs smoothly and all things BSL3 related.

Finally, I would like to thank everyone who made my graduate experience outside the lab great. There are too many people to mention but they include Kawula lab members, Microbiology department members, other BBSP students, non-scientists
and many, many others. Whether it was having drinks at a local dive bar, running marathons, camping on questionable islands, power hour-ing for football games, boating, collecting too many glasses at pint night, hosting house parties or doing just about anything, there were always several people willing to have fun.
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<table>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>3MA</td>
<td>3 methyladenine</td>
</tr>
<tr>
<td>AF</td>
<td>Alexa Fluor</td>
</tr>
<tr>
<td>AIM2</td>
<td>Absent in melanoma 2</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ATG</td>
<td>Autophagy-related gene</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Baf</td>
<td>Bafilomycin</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain-heart infusion</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow derived macrophage</td>
</tr>
<tr>
<td>Bref A</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BSL</td>
<td>Biosafety level</td>
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<td>C02</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CD</td>
<td>Cell Determinant</td>
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<tr>
<td>CDM</td>
<td>Chamberlain’s defined media</td>
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<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
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<tr>
<td>CQ</td>
<td>Chloroquine</td>
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<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DMEM</td>
<td>Dubelco’s modified eagle medium</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EEA-1</td>
<td>Early Endosomal antigen 1</td>
</tr>
<tr>
<td>EIEC</td>
<td>Enteroinvasive <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FCV</td>
<td>Francisella containing vacuole</td>
</tr>
<tr>
<td>FPI</td>
<td>Francisella pathogenicity island</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose 6-phosphate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human cytomegalovirus</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>hMDM</td>
<td>Human monocyte derived macrophages</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interlukin</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
</tr>
<tr>
<td>KD</td>
<td>Knock down</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosomal associate membrane protein</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule associate protein light chain 3B</td>
</tr>
<tr>
<td>LD_{100}</td>
<td>Lethal Dose 100%</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>LUX</td>
<td>Harboring a luminescence reporter plasmid</td>
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<tr>
<td>LVS</td>
<td>Live vaccine strain</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod-like receptor</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin binding protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PhoA</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<tr>
<td>RLU</td>
<td>Relative light units</td>
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<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
<td>RVFV</td>
<td>Rift Valley fever virus</td>
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<tr>
<td>SDS PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type 3 secretion system</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
</tr>
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CHAPTER 1: INTRODUCTION

Francisella tularensis History

*Francisella tularensis* is a small (roughly 0.5μm in diameter) Gram negative coccobacillus that is the causative agent of the disease tularemia. *F. tularensis* was discovered in 1911 in Tulare County California as a disease outbreak in ground squirrels that was first thought to be plague (119). Shortly after its initial isolation, interest in the bacterium’s potential as a bioweapon quickly grew due to the organism’s highly pathogenic and infectious nature. Leading up to World War II the United States, Japan and the former Soviet Union had developed and stockpiled weaponized *F. tularensis* (52). The United States finally destroyed the stocks of weaponized bacteria in 1973, whereas the former Soviet Union maintained its biological weapons development into the 1990’s (3, 52, 144, 163). Today, *F. tularensis* is still recognized as a high risk biological weapon and is defined as a Tier 1 Select Agent for bioterrorism due to its high infectivity and pathogenicity, as well as its ease of aerosolization and accessibility.

The organism’s highly infectious nature not only attracted research and development as a biological weapon but also detracted from basic tularemia research as *F. tularensis* became one of the most common laboratory acquired infections. Before major precautionary steps in biocontainment were established to prevent laboratory acquired infections, it was stated that very few long term researchers of *F.*
*tularensis* avoided contracting tularemia (143). This high potential for researchers to contract tularemia has no doubt impeded our understanding of *F. tularensis* biology and pathogenesis. Since then there has been major advancements in biocontainment and laboratory infections have dramatically decreased. Currently, virulent *F. tularensis* strains are handled under Biosafety Level 3 laboratory conditions to prevent laboratory acquired infections.

**F. tularensis Phylogeny, Ecology and Disease**

*F. tularensis* was first named *Bacterium tularense* upon its discovery (85,119). Since then the bacterium has been categorized under the genuses *Yersinia*, *Pasteurella* and finally to its own new genus *Francisella*, named after Edward Francis who performed much of the initial bacterial characterization after its discovery (142). Besides the parasitic *tularensis* species, other species have been categorized under the *Francisella* genus including species identified as fish pathogens, tick endosymbionts and soil dwelling organisms demonstrating that *Francisella* species inhabit several different niches (93). Although there is a significant diversity of *Francisella* species, the majority of *Francisella* research is focused on the mammalian pathogenic *tularensis* species and its virulence mechanisms.

*Francisella*’s closest relations are the fish pathogen *Piscirickettsia* and many deep ocean dwelling, nonpathogenic bacteria such as *Beggiatoa*, suggesting *Francisella* may have evolved from an ocean dwelling organism. *Francisella* and its ocean dwelling relatives are distantly related to commonly studied Gram negative organisms, thus a large portion of the *Francisella* genome encodes genes of unknown function. There have been many studies investigating *F. tularensis* genes of unknown
function including studies described in this thesis. These studies have identified uncharacterized Francisella genes encode several novel classes of proteins involved in a variety of bacterial processes as well as the extreme virulence of F. tularensis species.

**Francisella Species and Model Strains**

Due to the recent advancement of high throughput sequencing methods there has been a marked increase in the number of sequenced species and strains within the Francisella genus. The major species include *tularensis, novicida, philomiragia, noatunensis* and *piscida*. The *tularensis* species is the most widely studied and contains three subspecies: *tularensis, holarctica* and *mediasiatica* which are all highly virulent in humans and other mammals. The *tularensis* subspecies is generally considered the most virulent and *mediasiatica* the least virulent. The *novicida* species (previously annotated as a subspecies of *F. tularensis*) is not a primary human pathogen and only causes disease in immunocompromised individuals (13, 39, 58, 79, 86). The *philomiragia, noatunensis* and *piscida* species are generally avirulent in humans but are highly infectious fish pathogens that have a severe economic impact on fish farms in areas such as Scandenavia, Japan and Chile (14, 90, 141).

Although an increasingly larger diversity of Francisella strains are being discovered, there are primarily three strains used as models to study tularemia. The first is *F. tularensis* subsp. *tularensis* Schu S4, which was isolated in 1941 from a patient in Ohio (105). Schu S4 is one of the most virulent strains of *F. tularensis* and is designated a Tier 1 Select Agent. Consequently, the number of researchers using this strain is limited due to the necessity of a BSL3 facility to maintain the organism.
The second is *F. tularensis* subsps. *holarctica* Live Vaccine Strain (LVS), an attenuated *holarctica* strain that can be handled at BSL2 or BSL2+ conditions. LVS was artificially attenuated by researchers in the former Soviet Union in the 1950’s by successively passaging the strain in media and through mice to reduce, yet maintain virulence (52). One caveat of researching the LVS strain is that the specific mutations acquired from successive passaging and the specific effects of these mutations on the biology and virulence of the strain have not yet been fully characterized. Regardless of its artificial attenuation, LVS has long been a useful tool to study both *Francisella* biology and pathogenesis.

Lastly is *F. novicida* Utah 112 (U112), which was isolated from a water sample in Utah in 1955 (104). *F. novicida* is commonly used as a model system for *F. tularensis* as it is highly virulent in mice and is genetically manipulable (64,109). Although U112 causes a similar disease in mice as Schu S4, there are significant differences in the host response to *F. novicida* compared to Schu S4 in mice, as well as differences in the bacterial genetic requirements for infection (95,130). Therefore, when using the U112 strain to investigate bacterial pathogenesis applicable to human disease, analogous studies should be conducted using a human pathogenic strain, such as Schu S4, to verify both species employ similar virulence mechanisms. Since *F. tularensis* strains are most relevant to human disease, the experiments described in this document use LVS for a genetic screen in Chapter 2 and Schu S4 for the remainder of the described experiments.
**F. tularensis Host Range, Reservoirs and Transmission**

*F. tularensis* has one of the largest host ranges of any known pathogen. The current and growing tally exceeds 500 susceptible organisms and includes amoebae, fruit flies, mollusks, crayfish, fish, frogs, reptiles, birds, mice, rabbits, sheep and humans (1, 5, 6, 44, 128, 129, 138, 181). How *Francisella* can be transmitted to these organisms is not well understood but could occur through a variety of arthropod vectors including several species of biting flies, ticks and mosquitoes or from another unknown reservoir (57, 97). Direct transmission between susceptible hosts does not easily occur and arthropod vectors are not thought to be the sole mechanism for *F. tularensis* transmission suggesting there is likely an environmental reservoir from which susceptible species can also become infected. It is also likely that the reservoirs for *Francisella* species vary depending on the species, subspecies or geographic location (93).

**Clinical Manifestations of Tularemia**

Humans can acquire tularemia through a variety of routes resulting in varying disease progressions. Symptoms of tularemia generally include flu-like symptoms of fever, chills, cough, diarrhea and joint pain and also often include painful swelling of the draining lymph nodes (161, 162). *F. tularensis* can disseminate to distal organs including the liver, spleen and lungs from any site of infection resulting in further complications (143).

The most common form of tularemia is the ulceroglandular form which usually presents with a characteristic ulcer at the site of infection on the skin (179). Ulceroglandular tularemia is contracted by handling infected animals, contaminated
water or through arthropod vectors and it is unclear whether broken skin is required for infection. Contact of contaminated material with mucus membranes often results in infection. Contact of \textit{F. tularensis} with the eye can lead to ocular tularemia resulting in conjunctivitis, while ingestion of contaminated material leads to oropharyngeal tularemia resulting in ulcers and inflammation of the mouth and lips (stomatitis), throat (pharyngitis) and gastrointestinal infections (92, 186). Although cutaneous and mucosal infections can be debilitating, they are not often fatal. However, pneumonic tularemia is the most severe form of the disease and has high mortality rates if untreated.

Pneumonic tularemia results from dissemination to the lungs or via direct inhalation of bacteria causing pneumonia (78). Direct inhalation can occur by inhaling particles from disturbed hay, soil or infected animal carcasses and is best exemplified by a set of well documented cases where groundskeepers in Martha’s Vineyard acquired pneumonic tularemia (61). Finally, if the site of infection is unknown it is often designated as typhoidal tularemia.

The infectious dose of tularemia is extremely low for humans. Ethically questionable human studies conducted in the 1960’s where healthy “volunteers” from the Ohio State penitentiary system were inoculated with \textit{F. tularensis} via inhalation or intradermal injection (161, 162). Patients were then monitored and treated upon onset of symptoms. It was found that as few as 25 bacteria via inhalation or 10 bacteria administered through intradermal inoculation can cause disease in humans (161, 162). Pneumonic cases can be severe as 30-60% of untreated pneumonic tularemia cases are fatal, while fatalities from ulceroglandular tularemia are rare (52). Although \textit{F. tularensis} is highly virulent in humans, infections of small mammals including mice and
lagomorphs are even more severe as the LD$_{100}$ of *F. tularensis* Schu S4 for these species is a single bacterium.

**Bacterial Life Cycle and Virulence Factors**

*F. tularensis* is a facultative intracellular pathogen capable of infecting a variety of cell types including macrophages, dendritic cells, alveolar epithelial cells, hepatocytes, fibroblasts, neutrophils and even erythrocytes (74, 75, 81, 117, 151, 168). In eukaryotic cells *F. tularensis* grows to such high densities that more than 60% of the host cytosolic volume is bacterial mass (unpublished data). Further, *F. tularensis* rapidly grows over 1000-fold in eukaryotic cells within 24 hours and grows faster inside host cells than in defined media. This striking capacity for intracellular replication in host cells is essential to cause disease and is a major focus of *F. tularensis* research including studies in this thesis. Although *F. tularensis* infects and extensively replicates in a variety of eukaryotic cells the majority of researchers use macrophages as an infection model. Studies described in this document focus on infection of macrophages but also use epithelial cells or fibroblasts in specific experiments. Interestingly, we have identified very few differences among infection of different cell types in the described experiments suggesting *F. tularensis* may co-opt many ubiquitous host cell processes for intracellular proliferation.

**Intracellular Life Cycle**

*F. tularensis* is taken up by macrophages via a noncanonical mechanism termed looping phagocytosis where one eukaryotic phagocytic protrusion wraps around the bacterium creating a large spacious vacuole (41). This vacuole containing *F. tularensis*...
is then internalized and the bacterial-containing phagosome acquires the early endosomal marker EEA-1 then subsequently the late endosomal markers LAMP1, LAMP2 and CD63 (42, 45). Next, upon vacuolar acidification, *F. tularensis* breaks open the phagosome and escapes to the host cytosol in as early as 30 minutes post infection (37). The bacterium then replicates to high levels in the cytosol and ultimately leads to eukaryotic cell death via apoptosis presumably releasing the *F. tularensis* to infect new host cells (101, 146, 166, 184).

**F. tularensis Virulence Factors**

During an animal infection the majority of the *F. tularensis* life cycle is intracellular; however, there are some reports suggesting *F. tularensis* may have an extracellular phase (62). Regardless, intracellular growth is required for virulence in an animal. Transposon mutagenesis screens were developed and used to identify bacterial genes required for intracellular growth and virulence in murine infection models and these genetic screens have revealed surprisingly few factors specifically required for virulence (2, 26, 65, 91, 99, 102, 112, 113, 148, 151, 165, 171, 176, 183). *F. tularensis* lacks classical virulence factors including toxins and Type III, Type IV, Type V, Type VI secretion systems and no known protein effectors have been identified (105). This lack of classical virulence factors suggests *F. tularensis* may encode novel virulence mechanisms. Interestingly, some of the most attenuated mutants are those involved in metabolism and general cellular processes indicating adaptation to the intracellular environment is a particularly important aspect of *F. tularensis* virulence.
**Francisella Pathogenicity Island:** One major virulence locus is the Francisella Pathogenicity Island (FPI) and there are two copies of the FPI in *F. tularensis*, while only one copy exists in *F. novicida* and the fish pathogenic species (105, 133, 167). The FPI encodes a novel secretion system that some argue is a Type VI-like system; however, homology of the FPI to Type VI is poor and essential components are missing (20). Loci within the FPI are required for phagosomal escape and virulence in mice including *iglABCDEFGHIJ, pdpABCD, dotU, vgrG* and *anmK* while only *pdpE* is dispensable (8, 18, 19, 22, 49, 50, 110, 111, 133, 159, 160). Localization studies have shown that specific FPI components localize to the cytoplasm, inner membrane, periplasm or outer membrane but it is unknown how these components fit together to create the putative secretion apparatus (19, 50, 77). Our knowledge on the function of FPI gene products is sparse and many more studies will need to be performed before we understand how this system is assembled and what it may be secreting.

**Regulation of the FPI and Virulence Factors:** Regulation of FPI genes has been shown to be affected by several *F. tularensis* transcription factors. One of the first described virulence factors for *F. tularensis* was *mglA*, which encodes a transcription factor that regulates expression of over 100 genes including the FPI genes (22, 83, 106, 160). MglA forms a heterodimer with another transcriptional regulator that affects FPI gene expression, SspA, to bind RNA polymerase and affect transcription (32). Besides *mglA* and *sspA*, other transcriptional regulators including *fevR*, involved in ppGpp alarmone signaling, and *migR* affect transcription of FPI genes (21, 25, 31). Since
several overlapping regulatory networks control FPI gene expression, strict control FPI gene expression may be essential for FPI function and *F. tularensis* pathogenesis.

Many other factors including expression of bacterial chaperones and small molecules have also been shown to affect FPI gene transcription. Mutations in several genes affect FPI gene expression and virulence including disruptions of *hfq*, encoding the heat shock protein (121), *pmrA*, a response regulator (127) and genes involved in biotin biosynthesis (134). Further, the small molecules spermine and iron have also been shown to affect FPI expression (28, 51). How the effects of these genes and small molecules impact FPI gene regulation either through the previously described transcription factors or via independent mechanisms remains unknown. The observation that several seemingly unrelated signals affect FPI gene expression indicates that a complex, multisignal network controls FPI expression and many other factors to fine tune *F. tularensis* gene expression and virulence.

**Iron Acquisition:** As with all pathogens, iron acquisition is essential for *F. tularensis* virulence. Because *F. tularensis* does not encode a *tonB* homolog, an essential component that provides the energy for importing iron into the cell, it is likely that the bacterium encodes novel genes and mechanisms for iron acquisitions that have yet to be identified. *F. tularensis* encodes both canonical iron acquisition mechanisms including siderophore synthesis and novel mechanisms including *FTT_0918* that have been shown to be required for virulence. Interestingly, siderophore production is required for full virulence of the *F. novicida* U112 strain and LVS but not Schu S4, identifying a potential mechanism for differences in *in vivo* iron acquisition and virulence
between strains (149, 154, 172, 183). *F. tularensis* also contains the canonical ferrous iron transporter *feoB*, which has been implicated in LVS virulence (177). A gene of unknown function, *FTT_0918*, has been shown to be required for both siderophore mediated and free ferrous iron uptake (107, 154). *FTT_0918* encodes an outer membrane β-barrel protein, and an *FTT_0918* deletion mutant is strongly attenuated in a mouse, demonstrating the necessity of this novel iron acquisition protein product in *F. tularensis* virulence (180).

**Metabolic and Biosynthetic Pathways:** A strikingly large percentage of genes required for intracellular growth and virulence are involved in metabolic and biosynthetic pathways. Mutants incapable of *de novo* purine synthesis are some of the most attenuated of any *F. tularensis* mutants and are nearly avirulent in animals indicating that specific metabolic and biosynthetic machinery are particularly important for *F. tularensis* virulence (147). Other biosynthetic pathways required for virulence identified by mutant analysis are pyrimidine, biotin and multiple amino acid biosynthetic pathways as well as genes involved in glycerol and acetate metabolism (23, 80, 89, 120, 134, 148, 151, 155). Unlike mutants of purine biosynthesis, mutants of these metabolic and biosynthetic pathways are only moderately attenuated suggesting *F. tularensis* may partially circumvent *de novo* synthesis of specific molecules by direct import from the host.

Acquisition of amino acids has previously been shown to be required for *F. tularensis* intracellular growth as several amino acid and oligopeptide transporters are strongly attenuated for intracellular growth and virulence (4, 67, 116). This mutant data
aligns with the data demonstrating that \textit{F. tularensis} is auxotrophic for 13 amino acids indicating amino acid import from host cells is essential (30). Elucidating the metabolic requirements for \textit{F. tularensis} virulence is critical as both the nutrient availability of the host cytosol and the major nutrient sources available intracellular pathogens are poorly understood.

\textbf{Genes of Unknown Function:} A large portion of the \textit{F. tularensis} genome encodes proteins of unknown function and several of these genes have been identified to be required for virulence including \textit{fipB}, \textit{ripA}, \textit{dipA}, \textit{FTT\_1676}, \textit{FTT\_0918}, among others (35, 63, 152, 180, 182). Investigation of these gene products has helped elucidate the function of these \textit{Francisella} virulence associated genes. FipB is suggested to have oxidoreductase activity to help fold periplasmic proteins, \textit{FTT\_0918} is involved in iron acquisition and RipA is suggested to regulate Lipid A biosynthesis (123, 152, 154). Further identification of genes of unknown function that are required for virulence will potentially reveal elusive \textit{F. tularensis} virulence factors and aid in our understanding of the required physiological adaptations \textit{F. tularensis} undergoes to efficiently invade and replicate in the host cytosol.

Our understanding of \textit{F. tularensis} virulence is still lacking as no classical virulence factors have been identified and the function of the FPI proteins are poorly understood. Thus far, many of the most strongly attenuated \textit{F. tularensis} mutants identified have defects in metabolite biosynthesis, general cellular processes and genes annotated as having no known function. Therefore, we elected to perform a large scale transposon mutagenesis screen to identify novel \textit{F. tularensis} genes required for growth within macrophages and epithelial cells. Chapter 2 outlines the screen and the genes
identified as required for intracellular growth. The chapter also describes *FTT_0924*, a gene identified in the screen not previously identified as required for virulence. *FTT_0924* is a *Francisella* specific gene with no known predicted function and we suggest the role of this gene in modulating peptidoglycan dynamics, a process not previously implicated in *F. tularensis* virulence.

**Host-Francisella Interactions**

Bacterial and host factors required for uptake by host cells, phagosomal escape and cytosolic replication have been identified, but the mechanisms of how these and other unknown factors allow *F. tularensis* to co-opt host processes resulting in a successful infection are poorly understood. Many *F. tularensis* studies involve bacterial effects on the host immune system and *F. tularensis* immune evasion strategies are briefly described here. Host-*F. tularensis* interactions, other than those with the immune system, are essential for pathogenesis and the host-bacterial interactions described in this thesis involve *Francisella*-autophagy interactions and nutrient acquisition of intracellular *F. tularensis*, specifically focusing on bacterial assimilation of host derived carbon sources. Knowledge of bacterial interactions with host autophagy and the nutrient sources acquired by intracellular pathogens is lacking for nearly all intracellular pathogens and information learned from these studies will have a broad impact in aiding the general understanding of bacterial pathogenesis.

**Francisella Interactions with the Host Immune System**

In an animal infection there is a significant delay between infection and the onset of a host inflammatory response (114). This delay occurs because *F. tularensis*
employs several mechanisms to evade immune recognition and killing. Bacterial interactions with the host immune system include preventing immune detection, active suppression of the inflammatory response and resisting killing by host antimicrobials. Collectively, these immune evasion strategies allow F. tularensis to successfully establish infection and are a major topic of investigation in F. tularensis pathogenesis.

**Evasion of Immune Recognition:** F. tularensis evades detection by many pattern recognition receptors (PRRs) thereby avoiding activation of the host inflammatory response. Upon uptake by a host cell the first set of PRRs F. tularensis encounters are Toll-like receptors (TLRs), which are localized to the host cell surface or endosomal compartments. Most Gram negative bacteria stimulate a host response through binding of TLR4, as TLR4 detects lipopolysaccharide (LPS). F. tularensis does not stimulate TLR4 and evades detection by producing an abnormal lipid A structure (73). F. tularensis produces lipid A that lacks a 4’ phosphate on the glucosamine sugar and is tetra-acylated, compared to the hexa-acylated lipid A of many Gram negative organisms, preventing TLR4 recognition (140). Further, the acyl chains of F. tularensis lipid A are 16-18 carbons, whereas most Gram negative bacteria have shorter acyl chains of 12-14 carbons, which may further alter the properties of F. tularensis lipid A (123). Although F. tularensis avoids TLR4 activation during cellular entry, F. tularensis does not enter host cells completely unrecognized as F. tularensis infection activates TLR2, a sensor of bacterial lipoproteins (153).

Once in the cytosol, F. tularensis must evade recognition by several PRRs including Nod-like receptors (NLRs) that sense several ligands including Type III
Secretion System (T3SS) components, flagellin and damage from pore forming toxins (98, 122). *F. tularensis* does not encode T3SS, toxins or flagellin, thus preventing recognition by these NLRs. *F. tularensis* is also not recognized by the peptidoglycan sensing NLRs Nod1 and Nod2 nor the cytosolic double stranded DNA sensor AIM2 (184). *F. tularensis* may be well adapted to the cytosolic environment to prevent bacterial lysis that would release bacterial components, including DNA and peptidoglycan, into the host cell to be recognized by immunologic sensors. *F. novicida* however, is likely less well adapted to the cytosolic environment as DNA released by *F. novicida* lysis is recognized by AIM2 resulting in inflammasome activation and IL-1β secretion (115).

**Suppression of the Inflammatory Response:** *F. tularensis* infection suppresses the ability of macrophages, dendritic cells (DCs) and neutrophils to respond to immunologic stimuli (117, 174). Macrophages and DCs infected with *F. tularensis* do not induce a strong cytokine response to the TLR4 agonist *Escherichia coli* LPS and infected neutrophils do not produce a respiratory burst (118, 174) when stimulated with the Protein Kinase C activator phorbol methyl ester (118). Interestingly, crude lipid extracts from Schu S4 suppress the macrophage response to TLR4 agonists and the authors suggest a direct role of *F. tularensis* LPS in immunosuppression. Further, *F. tularensis* infection downregulates expression of several TLRs suggesting *F. tularensis* employs multiple mechanisms of suppressing the host inflammatory response (27, 84).

Infection of macrophages reduces production of the proinflammatory cytokines TNFα, IL-6, IL12, IL-1β and Type 1 interferon and induces secretion of the
antiinflammatory molecules TGF-β, IL-10 and Prostaglandin E₂ (9, 16, 17, 46, 87, 174, 187). This phenomenon along with reduced MHC-II expression may result in reduced T cell stimulation and IFNγ production that would inhibit bacterial growth (158, 187). Possible causative mechanisms for the reduced proinflammatory response may be that signaling through NF-κB, mitogen-activated protein kinase (MAPK) and Stat-1 are inhibited during infection (130, 158, 175). The mechanisms of inhibition of host signaling pathways remain unknown; however, bacterial mutants defective for inhibiting proinflammatory signaling have begun to elucidate these host-pathogen interactions. A ripA mutant that escapes the phagosome but fails to replicate in the cytosol had increased MAPK signaling resulting in proinflammatory cytokine secretion (82). Although the function of ripA in blocking MAPK signaling is likely indirect, this observation suggests the inhibition of this proinflammatory signaling may be an active F. tularensis process.

**Resistance to Host Antimicrobials:** Reactive oxygen species (ROS) are produced by host cells that can damage or kill pathogens by causing significant oxidative stress and F. tularensis employs several overlapping mechanisms to prevent damage from ROS. Acid phosphatases secreted by the bacterium dephosphorylate the p47phox component of the NADPH oxidase complex to prevent its formation and therefore ROS production (126). A second unknown bacterial mechanism prevents assembled NADPH oxidase complexes from producing ROS (118). A third F. tularensis mechanism to further prevent ROS damage, is to express the ROS detoxifying enzymes katG (catalase) and sodB (superoxide dismutase) (7, 108). It has been shown that acid
phosphatases are required for *F. novicida* virulence but acid phosphatase deficient mutants of the Schu S4 strain remain fully virulent indicating the reliance on bacterial mechanisms to prevent ROS damage may vary depending on the species of *Francisella* (34, 127). It is possible that the overlapping mechanisms in Schu S4 compensate for lacking any one process to prevent ROS damage. It is also likely that there are overlapping or redundant bacterial factors for many host-*Francisella* interactions and this redundancy may contribute to the difficulty in identification of responsible bacterial factors required for immunosuppression and virulence.

Although *F. tularensis* is an intracellular pathogen, the bacterium has developed strategies to evade killing by antimicrobials that it would often encounter in the extracellular space suggesting *F. tularensis* may have a more expansive extracellular phase than previously predicted. *F. tularensis* resists killing from complement by cleaving C3b, possibly by binding to Factor H, which recruits the C3b cleaving enzyme Factor I (37, 40). *Francisella* species have also been shown to be resistant to defensins, which are cationic antimicrobial peptides that can kill Gram negative bacteria by disrupting the negatively charged bacterial membrane. *F. novicida* is resistant to defensins at concentrations higher than what is observed *in vivo*, yet the mechanism of resistance is still poorly understood (76).

**Francisella Interactions with Host Autophagy**

Autophagy is a eukaryotic specific process first discovered in yeast over 40 years ago (53). Autophagy functions as a bulk recycling process to break down superfluous or damaged proteins and organelles into monomeric building blocks (amino acids, nucleotides, etc) to be reused by the cell. Autophagosomes are double membrane
vesicles, where the autophagic membrane is often derived from the endoplasmic reticulum, but can also be derived from the golgi, plasma membrane or mitochondria (124). These vesicles engulf the material to be degraded and fuse with lysosomes to break down their cargo (103). Cellular components to be degraded are tagged by ubiquitin and adapter molecules including p62, NBR1 and NDP52 traffic the cargo to autophagosomes (96, 178, 185, 190). Autophagic processes have been identified that can degrade specific cellular components such as ribosomes (ribophagy), mitochondria (mitophagy), lipid droplets (lipophagy), peroxisomes (pexophagy) and protein aggregates (aggrephagy). Further, the autophagic pathway can function as an immune mechanism to destroy invading pathogens. Intracellular pathogens are tagged with ubiquitin, engulfed and degraded in autophagosomes in a process termed xenophagy (156).

**The autophagy pathway:** Flux through the autophagic pathway is constantly occurring, yet autophagic flux can be increased or suppressed via multiple signals. Two major regulators of the autophagic pathway are AMP-activated protein kinase (AMPK), which positively regulates autophagy, and mammalian target of rapamycin (mTOR), which negatively regulates autophagy (94). Both AMPK and mTOR directly modulate autophagic flux via phosphorylation of Ulk1, an important component in the first protein complex involved in initiating autophagosome formation (94).

A series of protein complexes facilitate formation of an autophagosome from membrane budding to autophagosome formation and fusion with lysosomes. Autophagic initiation and membrane budding is mediated by two complexes acting as
kinases. The first complex includes Ulk1, Fip200, ATG101 and ATG13 which recruits the second complex that includes the Class III phosphatidylinositol-3-kinase VPS34, Beclin-1, and ATG14. The VPS34/Beclin1/ATG14 complex then recruits the final two protein complexes which are ubiquitin ligase-like systems that promote elongation and closure of the autophagosome. The first conjugates ATG12 to ATG5, while the second cleaves and lipidates LC3-I to LC3-II (124). Many of the individual autophagy components are not essential, but ATG5 is essential for canonical autophagy and LC3 is a commonly used marker for autophagy. ATG5 and LC3 independent autophagy pathways have been described but the exact functions of each of these pathways are unknown (135).

**General Bacterial-Autophagy Interactions:** Since cytosolic bacteria are tagged with ubiquitin, trafficked to autophagosomes and degraded, pathogens must evade killing by xenophagy to successfully replicate in the host cytosol. The interactions between host autophagy and intracellular pathogens have been observed for some time but are still poorly understood (157). Thus, further defining autophagy-pathogen interactions for any intracellular pathogen will significantly aid the understanding of the role of autophagy in bacterial pathogenesis.

Xenophagy can limit the replication of intracellular pathogens including *Mycobacterium tuberculosis* (71), *Streptococcus pyogenes* and *Salmonella enterica* Typhimurium (71, 132, 185). For *S. pyogenes* and S. Typhimurium, autophagy is required to degrade bacteria that escape from vacuoles from growing in the host cytosol. To combat killing by xenophagy, some intracellular pathogens have developed
specific mechanisms to actively avoid autophagic destruction. Cytosolic pathogens including *Shigella flexneri* and *Listeria monocytogenes* use genes involved in actin motility to prevent xenophagic destruction (139, 189). Besides genes involved in actin-based motility, other xenophagy evasion effectors are beginning to be identified. The *Legionella pneumophila* effector RavZ cleaves LC3 inhibiting host autophagy, while some *L. monocytogenes* bacteria transiently enter autophagosomes and require listeriolysin O and lipases to re-escape into the cytosol (15, 38, 173). More xenophagy evasion mechanisms likely exist, but the exact bacterial factors have yet to be identified.

While many pathogens avoid entering the autophagic pathway, some pathogens potentially benefit from entering autophagic vacuoles. *L. pneumophila* vacuoles acquire autophagic markers and may benefit from entering the autophagic pathway by providing an environment conducive for bacterial proliferation (55, 88, 170). *Anaplasma phagocytophilum* resides in an early autophagosome and the data suggests the bacterium consume autophagy derived nutrients to aid bacterial proliferation (136, 137). Although *L. pneumophila*, *A. phagocytophilum* and potentially other pathogens benefit from the autophagic pathway, the mechanisms by which the pathogens benefit have yet to be defined. Nonetheless, these studies indicate that complex host-pathogen interactions occur between host autophagy and intracellular pathogens which may be detrimental or beneficial to the pathogen depending on the organism or specific interaction.

**Francisella-Autophagy Interactions:** Interactions between *F. tularensis* and the host autophagic pathway have been described, yet the specific mechanisms involved
and effects of these interactions are unknown. Mutants deficient for intracellular replication and mutants lacking O-antigen are engulfed and degraded by autophagosomes but wild type Schu S4 evades autophagic destruction (29, 36). *F. tularensis* does not use actin based motility nor encodes any described xenophagy evasion mechanism suggesting *F. tularensis* encodes a novel mechanism of xenophagy evasion. Although *F. tularensis* replicates within the cytosol, there are reports that some *F. tularensis* are found in autophagolysosomes late during infection (53). These reports demonstrate multiple different interactions with the host autophagic pathway but whether autophagy is beneficial or detrimental to the pathogen is unclear. Experiments in Chapter 3 elucidate *F. tularensis*-autophagy interactions and determine the effects of these interactions on intracellular replication.

**F. tularensis Nutrient Acquisition from Host Cells**

Since *F. tularensis* replicates extensively in the cytosol of host cells, the bacterium must be capable of importing all essential nutrients for bacterial proliferation from this compartment. However, the low levels of simple available nutrients in the cytosol of a eukaryotic cell cannot support the high degree of bacterial replication that occurs in infected cells. There are large pools of nutrients in macromolecular complexes including proteins, lipid droplets, glycogen, ribosomes as well as extracellular nutrients that can be imported from the blood, yet these nutrients are not directly accessible to the bacterium. Therefore, *F. tularensis* must tap into host nutrient pools to acquire bulk carbon and other essential nutrients for bacterial replication. To acquire these nutrients *F. tularensis* must employ specific mechanisms to make these
inaccessible nutrients available, yet the specific nutrients acquired from the host and the strategies of bacterial manipulation of host metabolic processes are not known.

**Pathogen manipulation of host metabolism:** To acquire large pools of inaccessible host nutrients intracellular pathogens can directly degrade host macromolecules, co-opt host processes to degrade host constituents or induce host import of extracellular nutrients. Many intracellular pathogens likely manipulate the host to acquire nutrients although few specific examples of these processes have been described. Therefore, defining nutrient acquisition strategies for intracellular pathogens will have a significant impact in narrowing this large knowledge gap.

One common problem intracellular pathogens face, especially those residing in a vacuole, is trafficking nutrients to the specific bacterial-containing compartment and pathogens have developed diverse mechanisms to shuttle nutrients to their respective compartments. *Chlamydia* species create bacterial structures to traffic nutrients into the *Chlamydia* containing vacuole by producing projections, structurally similar to flagellin, to pierce the parasitophorous vacuole to acquire nutrients (188). Other pathogens co-opt host processes to acquire nutrients as *S. Typhimurium* may redirect vesicular transport to bring host nutrients to the Salmonella containing vacuole (54, 100). *L. pneumophila* employs an elegant nutrient acquisition mechanism that both degrades host macromolecular complexes and traffics nutrients to its vacuole by recruiting proteasome complexes to the *Legionella* containing vacuoles which degrade host proteins, thus providing the bacterium with amino acids (150). Many intracellular pathogens likely manipulate the host to obtain nutrients yet few bacterial mechanisms
are known suggesting there may be several novel mechanisms of pathogen nutrient acquisition that have yet to be identified. Chapter 3 elucidates mechanisms of how the cytosolic pathogen *F. tularensis* induces ATG5-independent autophagy to acquire nutrients derived from host macromolecules.

Another mechanism employed by intracellular pathogens is to modulate host metabolic signaling to provide nutrients to intracellular pathogens. Several viral, bacterial and eukaryotic parasites employ mechanisms to modulate activity of the metabolic regulators AMPK and mTOR. Besides generating nutrients, mTOR and AMPK modulation can suppress the host inflammatory response, as well as promote viral protein translation and lipid biogenesis for viral envelopes. Specific examples of pathogen manipulation of host AMPK and mTOR and the effects of these host-pathogen interactions are reviewed in Appendix 1. The impact of host metabolic signaling, including AMPK and mTOR, on *F. tularensis* infection is currently unknown and warrants investigation.

**Methods of Investigating Carbon Metabolism of Intracellular Pathogens:**

The nutritional capacities of intracellular pathogens have been extensively studied when growing in media, yet the knowledge of the required nutrients sources, including major carbon sources, for any intracellular bacterium growing in host cells or *in vivo* is either fragmentary or completely unknown. There are three main approaches for studying carbon metabolism of intracellular pathogens in a host cell: measuring transcription of carbon metabolic pathways, virulence analysis of mutants in specific carbon metabolic pathways and $^{13}$C metabolic flux analysis. Each approach has
benefits and limitations and no single analysis can completely define which carbon sources are essential for pathogens to import \textit{in vivo}.

To begin to describe intracellular carbon utilization by pathogens, microarray and RNAseq analyses are commonly used (24, 66). By comparing transcription from bacteria growing in defined media to bacteria growing in cultured eukaryotic cells, metabolic pathways specifically used by pathogens in host cells can be identified. The drawback of this analysis is that changes in transcription do not always directly correlate to changes in metabolic flux. A metabolic pathway used for growth in both defined media and host cells is difficult to identify from transcriptional analysis as bacterial pathogens may not significantly alter transcription of the metabolic genes between these two conditions. \textit{Chlamydia} species, for example, do not alter transcription of carbon metabolic genes when exposed to different carbon sources making transcriptional studies nearly useless in defining the carbon metabolism of these bacteria (83). Nonetheless, transcriptional analyses are excellent places to start for generating testable hypotheses to identify the potential carbon sources of intracellular bacteria.

Recently, studies providing $^{13}$C labeled glucose, glycerol and amino acids to cultured cell lines infected with various intracellular pathogens and measuring $^{13}$C incorporation into bacteria have begun to elucidate carbon sources used by intracellular pathogens. The $^{13}$C labeled substrate can be taken up by the bacteria in the host cell, converted into amino acids and incorporated into protein. Bacteria are then separated from host cells, bacterial protein is purified and then the purified protein is analyzed by mass spectrometry or NMR to quantify $^{13}$C incorporation. $^{13}$C incorporation into amino
acids of bacterially synthesized protein will determine if the specific carbon source provided at the beginning of the assay is used as an anabolic carbon source by the bacteria inside host cells. These studies have limitations in that they describe which carbon sources can be used by the bacterium, but cannot define which carbon sources are essential for bacterial growth or virulence. Also, many of these studies are performed in transformed cells, which are known to have altered carbon metabolism (56). Regardless, these assays are direct measures of nutrient uptake inside host cells and they have provided valuable information defining possible carbon sources for intracellular pathogens.

There are likely multiple sources of carbon available to pathogens and many bacteria often have redundant transporters and alternative pathways for importing and metabolizing the same nutrient. This redundancy makes mutant analysis difficult, especially for intracellular pathogens with relatively large genomes such as S. Typhimurium (10). However, successful mutant analyses are powerful as they define the specific carbon metabolic pathways essential for growth in host cells and virulence in animals.

To block a single carbon metabolic pathway multiple genes must often be deleted. This multiple knockout strain still may not yield an mutant attenuated for virulence as multiple carbon metabolic pathways may be used for intracellular and in vivo bacterial growth. Nonetheless, some information has been gleaned from analyzing mutants of specific carbon metabolic pathways even in pathogens encoding significant redundancy in metabolic pathways (47). By combining mutant analysis, to define the carbon metabolic pathways required for intracellular growth and virulence, with $^{13}$C
labeled substrate analysis, to directly demonstrate the uptake of the essential carbon sources, strong conclusions can be drawn for defining the in vivo carbon metabolism of intracellular pathogens.

**Identified Carbon Sources for Intracellular Pathogens:** The carbon metabolism of *M. tuberculosis* may be the best characterized of any intracellular pathogen. *M. tuberculosis* has been shown to import host cholesterol and fatty acids generated from host lipid droplets (48, 145). This observation coupled with reports that *M. tuberculosis* mutants of isocitrate lyase, required for the glyoxylate shunt of the tricarboxylic acid (TCA) cycle and therefore anabolism of acetyl-CoA, indicate that acetate generated from fatty acids and cholesterol is an essential source of anabolic carbon for *M. tuberculosis* (131). However, $^{13}$C metabolic flux analysis suggests *M. tuberculosis* may also acquire carbon from CO$_2$ and unidentified C$_3$ carbon substrates indicating *M. tuberculosis* consumes several carbon sources from host cells (12).

Numerous studies have identified essential nutrients required for *Chlamydia* intracellular growth, yet very little is known about which carbon sources are consumed by the bacterium. *Chlamydia* species have been shown to import several essential nutrients including amino acids, several lipids, NAD$^+$ and even ATP from host cells (43, 164, 169). Although these studies have provided a wealth of information on the variety nutrients *Chlamydia* species acquire from the host, these studies still do not define the major carbon sources for the pathogen. One possible carbon source may be fatty acids as lipid droplets are imported into the parasitophorous vacuole but no studies thus far
have directly investigated and identified major sources of carbon for Chlamydia species (43).

$^{13}$C studies have identified several potential carbon sources for L. monocytogenes, enteroinvasive E. coli (EIEC), M. tuberculosis, S. Typhimurium and L. pneumophila, and reveal that the carbon utilization by these pathogens varies greatly (59, 68, 69). Carbon uptake by intracellular pathogens can even vary by strain as some EIEC strains primarily use C$_3$ substrates while others use glucose in cultured epithelial cells (69). $^{13}$C labeling studies have also identified that pathogens acquire specific sources of host carbon as L. monocytogenes utilizes glycerol and glucose 6-phosphate extensively as anabolic carbon substrates in macrophages but not glucose, pyruvate or amino acids (60, 70). Although different pathogens use different carbon sources, one commonality is that no pathogen thus far has been shown to use amino acids as a major source of anabolic carbon.

**Identifying Carbon Sources for F. tularensis**: There are several potential carbon sources for intracellular F. tularensis, yet no studies have attempted to identify which carbon sources support F. tularensis intracellular growth. Genes required for the glyoxylate shunt of the TCA cycle are missing from the F. tularensis genome suggesting the bacterium cannot use host fatty acids as an anabolic carbon source, but fatty acids could function as an energy source by feeding the TCA cycle (105). The bacterium does encode the necessary pathways to use several carbon sources from the host cell cytosol including hexoses, nucleotides, pentoses, glycerol, pyruvate, lactate, amino acids and intermediates of the TCA cycle and glycolysis. The ability of F. tularensis to
assimilate many of these carbon sources in defined media has been described; however, the availability and use of these carbon sources in host cells or in a mouse infection model have yet to be investigated (72). The experiments described in Chapter 4 and Appendix 2 define the essential sources of carbon that \textit{F. tularensis} imports in host cells and the carbon sources \textit{F. tularensis} requires for growth in a murine pulmonary infection model.
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CHAPTER 2: IDENTIFYING FRANCISELLA TULARENSIS GENES REQUIRED FOR GROWTH IN HOST CELLS

Overview

Francisella tularensis is a highly virulent Gram negative intracellular pathogen capable of infecting a vast diversity of hosts, ranging from amoebae to humans. A hallmark of F. tularensis virulence is its ability to quickly grow to high densities within a diverse set of host cells including, but not limited to macrophages and epithelial cells. We developed a luminescence reporter system to facilitate a large scale transposon mutagenesis screen to identify genes required for growth in macrophage and epithelial cell lines. We screened 7500 individual mutants of which 269 exhibited reduced intracellular growth. Transposon insertions in the 269 growth defective strains mapped to 68 different genes. FTT_0924, a gene of unknown function but highly conserved among Francisella species, was identified in this screen to be defective for intracellular growth within both macrophage and epithelial cell lines. FTT_0924 was required for full Schu S4 virulence in a murine pulmonary infection model. The ΔFTT_0924 mutant bacterial membrane is permeable resulting in strongly reduced viability when grown in a...
hypotonic solution. The permeability and reduced viability was rescued when the mutant was grown in a hypertonic solution indicating that FTT_0924 is required for resisting osmotic stress. The FTT_0924 mutant was also significantly more sensitive to β-lactam antibiotics than Schu S4. Taken together, the data strongly suggest that FTT_0924 is required for maintaining peptidoglycan integrity and virulence.

**Introduction**

*Francisella tularensis* is a Gram negative facultative intracellular pathogen capable of infecting over 250 hosts, ranging from amoebae to humans (1). *F. tularensis* subspecies *tularensis* is highly virulent in mammals and as few as 25 organisms can cause a potentially fatal infection in humans (2). The bacterium infects a variety of cell types including, but not limited to, macrophages, dendritic cells, neutrophils, epithelial cells, fibroblasts and hepatocytes (3-7). *F. tularensis* replicates to high levels within these cell types, but the specific mechanisms *F. tularensis* uses to invade and replicate within host cells remain poorly understood.

It is essential for *F. tularensis* to replicate within host cells to successfully establish an infection and cause disease. Intracellular replication requires phagosomal escape followed by adaptation to and replication in the host cytosol. Upon internalization by a host cell the bacterium degrades the phagosome within 30 min and enters the cytosol (8). Escape from the phagosome is an essential step in the *Francisella* lifecycle and mutations in the *Francisella* Pathogenicity Island which encodes an alternative secretion system, fail to escape the phagosome (9-11). Other factors have been identified that are required for efficient *F. tularensis* vacuolar escape; however the specific mechanisms necessary for vacuolar escape have yet to be
determined (12-14). Once in the cytosol bacteria replicate to high levels before the host cell undergoes apoptotic cell death (15-18). Mutants that escape the phagosome but fail to replicate within the cytosol have been identified including mutants with genetic disruptions in purine biosynthesis, dipA or ripA (12,19,20). Francisella mutants defective for phagosomal escape or cytosolic replication exhibit reduced virulence in murine infection models indicating both processes are essential for F. tularensis virulence.

A large portion of the Francisella genome encodes proteins of unknown function. Several of these genes of unknown function are conserved across Francisella species and are required for intracellular growth and virulence including dipA, ripA, FTT_1676 and others (12,20,21). To better understand Francisella pathogenesis it is important to identify and elucidate the mechanisms by which these genes affect bacterial intracellular growth and virulence. Here we perform a large scale transposon mutagenesis screen to identify novel virulence factors required for intracellular growth. Because F. tularensis infects a diverse set of host cell types and likely requires different mechanisms for entry and replication within distinct cell types, we screened our library of mutants within both macrophage-like (J774) and alveolar epithelial cell lines (TC-1). To validate the efficacy of the screen, we identified and further investigated FTL_1286, a previously unidentified gene, to be required for virulence. FTL_1286 is a gene of unknown function that is conserved among Francisella species. To identify mechanisms required for F. tularensis pathogenesis we wanted to further elucidate the function of FTL_1286 and its homolog in the highly virulent Schu S4 strain, FTT_0924, in intracellular growth and virulence.
Materials and Methods

Bacterial Strains

*F. tularensis* subspecies *holarctica* LVS was obtained from the Center for Disease Control in Atlanta, GA and *F. tularensis* subspecies *tularensis* Schu S4 was obtained from BEI Resources. Each *F. tularensis* strain was cultured using chocolate agar plates supplemented with 1% IsoVitaleX (chocolate agar), Brain Heart Infusion broth supplemented with 1% IsoVitaleX (BHI), or Chamberlains Defined Media (CDM) (22). *Escherichia coli* DH10B was used for cloning and cultured using Luria-Bertani (LB) broth or LB agar. For selection, kanamycin was used at 50µg/ml for *E. coli* strains and 10µg/ml for *F. tularensis* or 200µg/ml of hygromycin for *E. coli* and *F. tularensis*. All cultures were grown at 37°C.

Cell Culture

J774A.1 (ATCC TIB-67) cells are a murine macrophage-like cell line and were maintained in 75cm² tissue culture flasks containing DMEM with 4.5g/L glucose supplemented with 10% fetal bovine serum, 2mM L-glutamine and 1mM sodium pyruvate. TC-1 (ATCC CRL-2785) are a murine alveolar epithelial cell line and were maintained in 75cm² tissue culture flasks containing RPMI 1640 supplemented with 10% fetal bovine serum, 2mM L-glutamine, 10mM HEPES, 1.5g/L sodium bicarbonate.
and 0.1mM nonessential amino acids. All tissue culture lines were maintained at 37°C and 5% CO₂.

Plasmid Vectors and Allelic Exchange

The luminescent reporter plasmid (pJB1) was created by cloning the *Photorhabdus luminescens luxCDABE* operon from pXB173 (23) into the low copy shuttle plasmid pMP831 (24). In-frame, markerless deletions in *F. tularensis* LVS and *F. tularensis* Schu S4 were constructed using pEDL50, a modified version of the suicide vector pMP812 containing an origin of transfer (25). Suicide vectors were mated into *F. tularensis* LVS or *F. tularensis* Schu S4 using *E. coli* S17-1λpir and primary recombinants were selected with 200µg/mL polymyxin B and 10µg/mL kanamycin. Primary recombinants were grown overnight without selection in BHI then plated on chocolate agar containing 10% sucrose. Complementing vectors were constructed by cloning into pJB3, which was derived from the plasmid pMP831 (24) respectively each constitutively expressing *luxCDABE*. GFP and PhoA fusion constructs were constructed by cloning the fusions into pEDL17 to allow controlled expression by anhydrotetracycline addition (26).

Mapping Transposon Insertion Sites

Colonies of identified transposon insertion mutants were suspended in water and boiled for 10 minutes to release the genomic DNA. The lysates were centrifuged to remove the bacterial debris and the remaining supernatant was used as template DNA
for amplification. Regions surrounding the transposon were amplified using a primer specific to the transposon and random primers containing a tagged sequence (GGACACGCCTCGACTAGTGG(N_{10})AA). The amplified products from the PCR reaction were used as a template in a subsequent reaction using a primer complementary to the tag (GGACACGCCTCGACTAGTGG) and a primer specific to the transposon. The following products were sequenced (Genewiz) by using a primer to amplify outward from the transposon.

**Transposon Mutagenesis Screen**

A EZ::TN <kan-2> transposome complex (Epicentre) containing a *F. tularensis* codon optimized kanamycin cassette (Blue Heron) was electroporated into *F. tularensis* LVS harboring pJB1. Transposon mutants were selected on chocolate agar plates containing 10μg/mL kanamycin and 200μg/mL hygromycin. Individual transposon mutants were grown in BHI broth, 10μg/mL kanamycin and 200μg mL⁻¹ hygromycin overnight in 96 well black wall/clear bottom plates (Corning). The following day, overnight cultures were screened for growth at OD_{600} and luminescence using an Infinite M200 Series plate reader (TECAN). Mutants that failed to grow or luminesce were removed from future experiments. Overnight cultures were diluted 1:50 into 200μL of either J774 media or TC-1 media. 50μL of the dilution was added to either 10^6 J774 cells or 10^6 TC-1 cells in 96 well black wall/clear bottom tissue culture treated plates, which yielded an average MOI of 100. At 4 hours post infection for J774 cells and 6 hours post infection for TC-1 cells, the bacteria were removed and 200μL of their respective tissue culture media containing 25μg/mL of gentamicin was added to each
infected well. Luminescence was read at 4, 6 and 24 hours using the TECAN plate reader in order to determine intracellular growth.

**Gentamycin Protection Assays**

*F. tularensis* LVS strains cultured for 3 days on chocolate agar containing 1% IsoVitaleX were used to make overnight cultures in CDM. The following day, the cultures were diluted to a Klett 100 (10^9 CFU/mL) in PBS. This suspension was diluted 1:10 into either J774 media or TC-1 media and 1mL is added to 10^6 J774 or TC-1 cells respectively resulting in a multiplicity of infection (MOI) of 100. At 2 hours post infection for J774 cells and 3 hours post infection for TC-1 cells, the bacteria were removed and 1mL of media containing 25µg/mL of gentamicin was added to the infected wells. At 2 hours post gentamicin treatment the media was removed, washed once with PBS, cells were scraped up, vortexed hard for one minute and dilution plated to enumerate intracellular bacteria.

**Mouse Infections**

Groups of 4 of 6-8 week old C57BL/6 mice (Jackson Labs) were infected with approximately 100 CFU of *F. tularensis* Schu S4, ΔFTT_0924 or ΔFTT_0924 p0924 intranasally. Mice were sacrificed at 2 hours, 1 day, 3 days, 4 days or 7 days post infection to determine organ burdens in the lung, liver and spleen. Organs were homogenized using a Biojector (Bioject) and homogenates were serially diluted and
plated onto chocolate agar. Individual colonies were counted after incubation for 4 days at 37°C to quantify organ burdens.

**Subcellular Fractionations**

50mL of cultures grown overnight in CDM were lysed via mechanical lysis by 0.1mM silicon beads in a bead beater and crude membrane fractions were pelleted from whole cell lysates by centrifugation at 100,000×g for 2 hours. Supernatant was removed for the soluble fraction. The membrane pellet was resuspended in 150mM NaCl, 10mM Tris, 0.5% Sarkosyl pH 7.5 and agitated overnight. The solubilized membrane fraction was then pelleted at 100,000×g for 1.5 hours. The soluble inner membrane fraction was removed, and the Sarkosyl insoluble fraction was washed then resuspended in 150mM NaCl, 10mM Tris pH 6.5. All fractions were standardized to equal protein using a BCA assay (Pierce), run on a 4-20% polyacrylamide gradient gel (BioRad) and transferred to nitrocellulose membrane. The membranes were probed with antibodies recognizing HA peptide (Sigma), RipA (27), IgIc (BEI Resources) and Tul4 (BEI Resources) for primary probes and antibodies conjugated to fluorophores as secondary probes (LI-COR Biosciences). Blots were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences).

**GFP and PhoA Fusion Localization Assay**
Overnight cultures were grown in Chamberlains Defined Media broth containing 100ng/mL anhydrotetracycline. GFP fluorescence assays were performed by pelleting 1.5mL of an overnight culture and resuspending samples in 50mM Tris-HCl pH 8.0, 200mM NaCl and 15mM EDTA. 200µL of resuspended pellet was transferred to a black walled 96 well dish (Nunc) and the plate was read using a TECAN plate reader with an excitation filter of 485nm and emission filter of 512nm. PhoA activity assays were performed by pelleting and resuspending overnight cultures in 1M Tris-HCl pH 8.0, 1mM ZnCl$_2$, 0.01% Sodium Dodecyl Sulfate and 5% chloroform. The resuspended cultures were permeabilized for 5 minutes at 37°C. 100µl of permeabilized culture was transferred to a 96-well dish and 20µl of 0.4% p-nitrophenyl phosphate in 1M Tris-HCl pH 8.0 was added and was incubated at 37°C and OD$_{420}$ was measured every 5 minutes using the TECAN Infinite 200 series plate reader for 16 hours. The time taken to reach an arbitrary OD$_{420}$ was recorded. PhoA activity was calculated as $(OD_{420} \times 1000)/(\text{minutes} \times OD_{600} \times \text{mL culture volume})$.

**Growth Curves**

Overnight cultures of *F. tularensis* grown in CDM were diluted to OD$_{600}$ of 0.05 and seeded into 200µl of CDM or BHI per well in a 96 well plate (Corning). Cultures were incubated in an Infinite 200M Pro series TECAN plate reader (TECAN) at 37°C with orbital shaking. OD$_{600}$ was measured every 15 minutes for 48 hours. A final concentration of 2.5µM propidium iodide was used to measure membrane permeability and fluorescence was measured using an 535nm excitation and 617nm emission. For sucrose viability rescues, overnight cultures were diluted to OD$_{600}$ of 0.05 in 10mL of
CDM with or without sucrose. Aliquots were removed at specific time intervals to measure optical density.

Intracellular growth was measured by infecting $10^5$ J774 or TC-1 cells with $10^7$ F. tularensis in a 96 well black wall/clear bottom plate for 2 or 4 hours respectively. Extracellular bacteria were then removed from cells and fresh media containing 25µg/ml gentamicin was added and the plate was placed in the TECAN plate reader maintaining 37°C and 5% CO$_2$. Luminescence was measured every 15 minutes for 48 hours.

**Disc Diffusions**

Disc diffusions were performed on modified Mueller-Hinton Agar supplemented with 1% Tryptone, 0.5% NaCl, 0.05% L-cysteine freebase, 1% glucose and 0.00025% Fe pyrophosphate. F. tularensis was resuspended in PBS (to an OD$_{600}$ of 1) and a lawn of bacteria was swabbed onto each plate. Paper discs impregnated with specific antibiotics or compounds (Becton Dickinson) were placed on the agar. Plates were incubated at 37°C for two days before the diameters of inhibition zones were measured.

**Fluorescence Microscopy**

J774 cells were plated onto 8 well chamber slides (Nunc) at $3 \times 10^4$ cells per well and allowed to replicate overnight. F. tularensis Schu S4-GFP was used to infect the J774 cells at an MOI of 100 for 2 hours, the bacteria were removed and fresh media containing 25µg/mL gentamicin was added to the cells. At 24 hours post infection, the media was removed, washed once with PBS and 4% paraformaldehyde was added for 30 minutes to fix the cells. The slide was then washed in 50mM ammonium chloride to
remove residual fixative. To stain the eukaryotic plasma membrane, the slide was incubated in 10µg/mL wheat germ agglutinin conjugated to AF647 for 10 minutes then washed with PBS. DAPI containing mounting medium (Vector Shield) was then added to stain the nucleus.

Transmission Electron Microscopy

Wild type *F. tularensis* Schu S4 and ΔFTT_0924 were grown overnight in CDM containing 300mM sucrose to maintain structural integrity of the mutant strain. Bacteria were then reseeded into fresh CDM containing 300mM sucrose at an OD_{600} of 0.1 and allowed to grow an additional 6 hours. Bacteria were then pelleted and resuspended in fixative buffer 2% glutaraldehyde, 2% paraformaldehyde, 300mM sucrose, 150mM sodium phosphate pH7.4 for at 4°C overnight. Samples were then fixed in 0.15M sodium phosphate and 1% osmium tetroxide. Fixed samples were dehydrated through a series of treatments: 30%, 50%, 75%, 90% then 100% ethanol and propylene oxide and embedded in Spurr’s low viscosity epoxy resin (Polysciences). 70-80nm sections were cut with a diamond knife and mounted on 200 mesh formvar/carbon-coated copper grids and stained with 4% aqueous uranyl acetate and Reynolds’ lead citrate. Samples were observed using a LEO EM910 transmission electron microscope operating at 80kV (Carl Zeiss Microscopy, LLC) and digital images were acquired using a Gatan Orius SC1000 CCD Digital Camera with Digital Micrograph 3.11.0 (Gatan, Inc).

Results
**F. tularensis luminescence reporter functions as a surrogate measurement for intracellular growth**

*F. tularensis* LVS expressing pJB1, a luminescence reporter plasmid constitutively expressing *Photorhabdus luminescens* operon *luxCDABE*, was used to determine if luminescence could act as a reporter for intracellular growth within host cells. J774A.1 macrophages (J774) were infected with either LVS, LVS harboring pJB1 (LVS LUX) or ΔripA, a mutant that does not grow inside macrophages, harboring pJB1 (ΔripA LUX) (20). Luminescence was measured at 4 and 24 hours post inoculation; after each reading the intracellular bacteria were enumerated by dilution plating. From 4 to 24 hours post inoculation both LVS and LVS LUX had increased intracellular numbers of roughly 100-fold indicating that pJB1 did not significantly affect intracellular growth (Figure 1). There was roughly a 100-fold increase in luminescence between 4 and 24 hours post inoculation in LVS with pJB1 indicating that increased luminescence was directly proportional to the increase in intracellular bacterial burdens. There was no increase in luminescence in the LVS vector only control between 4 and 24 hours indicating there were minimal background levels for this reporter system. Also, there was no significant change in luminescence or intracellular burden between 4 and 24 hours for ΔripA LUX further indicating luminescence can be used as an accurate measurement for intracellular growth.

**Transposon mutagenesis screen identifies growth-deficient mutants in J774A.1 macrophage or TC-1 epithelial cells using a luminescence reporter system**

LVS LUX was transformed with an EZ::TN <kan-2> transposome complex (Epicentre) containing a kanamycin resistance cassette that was codon optimized for *F.*
*tularensis* (Blue Heron). 7500 individual mutants were picked and propagated in BHI broth overnight. Mutants that failed to grow or luminesce were discounted from further experiments. J774 macrophages and TC-1 epithelial cells propagated in a 96 well format were infected with each individual mutant. Initial luminescence was measured at 4 (J774) or 6 (TC-1) hours and at 24 hours to determine intracellular growth. 356 mutants from the primary screening exhibited an intracellular growth defect in either or both cell types. These 356 mutants were re-evaluated in triplicate for intracellular growth in both J774 and TC-1 cells using the same luminescence reporter system. 269 mutants from the primary screen repeated a growth defect. The transposon insertion sites of all 269 mutants were identified by amplifying the region surrounding the transposon insertion by semi-degenerate PCR, followed by DNA sequence analysis (GeneWiz), and comparison to the LVS whole genome sequence (Table S2). The 269 transposon insertions mapped to 74 genes and 3 pseudogenes. To further eliminate false positives, representative mutants from genes with only 1 transposon mutant were tested in standard gentamicin protection assays identifying 68 genes and 2 pseudogenes as being required for intracellular growth (Figure 2). To the best of our knowledge, 14 of the 68 genes were previously unidentified as required for virulence or intracellular growth in any strain of *Francisella*.

**The FTL_1286 and FTT_0924 loci are required for intracellular growth**

One transposon mutant mapped to FTL_1286, a gene of unknown function that has not been identified as a virulence factor for any *Francisella* species. The transposon mutant exhibited no growth within J774 macrophage cells but had observable growth within the TC-1 epithelial cells (Table S2). To determine if the
FTL_1286 locus was required for intracellular growth a markerless, in-frame deletion was constructed of the FTL_1286 locus and growth in macrophages was determined. In J774 cells, the transposon mutant disrupting the FTL_1286 locus and ΔFTL_1286 expressing pJB3 (shuttle vector expressing P. luminescens luxCDABE) had no observable intracellular growth as determined by the luminescence reporter system (Figure 3A). However, growth of the ΔFTL_1286 p1286 complemented strain grew to similar levels as wild type LVS in macrophages indicating that FTL_1286 is required for intracellular replication of LVS (Figure 3A). Since there is 100% amino acid sequence identity between FTL_1286 and the FTT_0924 homolog in the highly virulent Schu S4 strain, we hypothesized that FTT_0924 was also required for intracellular growth of Schu S4. Deletion of the homologous FTT_0924 locus in the Schu S4 strain resulted in reduced intracellular growth in J774 cells that was complemented by expressing FTT_0924 in trans (Figure 3B) in the mutant strain. Interestingly, the LVS transposon mutant in FTL_1286 exhibited some growth within the TC-1 epithelial cells; however, the ΔFTL_1286 strain of LVS and the ΔFTT_0924 strain of Schu S4 exhibited no observable growth within epithelial cells indicating the transposon did not fully abolish the function of FTL_1286 or the transposon mutant acquired a mutation partially compensating for the genetic disruption (Figure 3D and data not shown).

To verify that FTT_0924 was required for intracellular growth and did not simply affect the bacterial luminescence reporter we performed gentamicin protection assays in J774 and TC-1 cells and intracellular burdens were quantified by dilution plating (Figure 3C and 3D). In both J774 and TC-1 cells, the ΔFTT_0924 strain did not exhibit intracellular growth and intracellular growth could be complemented to near wild type
levels by expressing \textit{FTT\_0924} \textit{in trans}. Fluorescence microscopy analysis of GFP-expressing strains was also performed to visualize intracellular growth of \textit{ΔFTT\_0924} (Figures 3E and 3F).

\textbf{\textit{FTT\_0924} is required for virulence in mice}

Since \textit{FTT\_0924} is required for \textit{F. tularensis} Schu S4 intracellular growth, we wanted to determine if \textit{FTT\_0924} was required for Schu S4 virulence in a mouse pulmonary infection model. C57BL/6 mice were inoculated intranasally with 100 CFU of wild type Schu S4, \textit{ΔFTT\_0924} or \textit{ΔFTT\_0924} p0924. At 2 hours and 1, 3, 4 and 7 days post inoculation bacterial burdens were enumerated from the lungs, livers and spleens of the infected mice. 2 hours post infection, lungs of mice were harvested and dilution plated. Equal numbers of bacteria were recovered from the lungs of mice infected with each bacterial strain indicating that the mutant and wild type organisms accessed the lung at equivalent efficiencies (data not shown). At day 1, mice infected with \textit{ΔFTT\_0924} had significantly reduced burdens in the lungs compared to the wild type and complemented strain. No bacteria were detected in livers or spleens of animals infected with any strain at day 1. By day 3, mice infected with \textit{ΔFTT\_0924} had significantly reduced burdens in the lungs and spleens compared to the wild type and complemented strains. The \textit{ΔFTT\_0924} strain did not robustly infect the livers of the animals, whereas there was significant bacterial burdens observed in both the wild type and complemented strains (Figure 4). Interestingly, there was no change in bacterial burden between day 3, day 4 and day 7 in mice infected with \textit{ΔFTT\_0924} in any organ.
tested, indicating that the \( \Delta FTT_0924 \) mutant had reached a burden where bacterial growth and killing occurred at equal rates, while significant growth was observed in the mice infected with the wild type and complemented strains (Figure 4). No data was taken for animals infected with wild type Schu S4 or the complemented strain at day 7 because animals infected with these strains became moribund at day 4 that required the animals to be euthanized. Overall, these data indicate that \( FTT_0924 \) was required for full virulence of \( F. \) tularensis Schu S4 in a pulmonary murine infection model.

**\( FTT_0924 \) is required for resisting osmotic stress during growth in liquid culture**

To characterize the \( FTT_0924 \) mutant, we monitored general growth characteristics of \( \Delta FTT_0924 \) in liquid culture. Schu S4 and \( \Delta FTT_0924 \) were grown in Chamberlains defined media (CDM) for 36 hours and the OD\(_{600}\) was measured every 6 hours. After 36 hours of growth, Schu S4 reached an OD\(_{600}\) of 2.75, while \( \Delta FTT_0924 \) reached an OD\(_{600}\) of 1.05 indicating a defect for growth in liquid culture (Figure 5A). To determine if there was any difference in the number of viable bacteria at each point, wild type Schu S4 and \( \Delta FTT_0924 \) were enumerated by dilution plating. Strikingly, there was 100-1000 fold fewer viable bacteria per OD\(_{600}\) in cultures with \( \Delta FTT_0924 \) (Figure 5B). We next aimed to determine whether the viability defect of the \( \Delta FTT_0924 \) mutant strain occurred in other conditions including growth on solid agar or in PBS suspension. This viability defect was not observed after bacterial growth on chocolate agar plates, resuspended in PBS to an OD\(_{600}\) of 1 and dilution plated indicating the mutant strain specifically did not tolerate growth in liquid culture (Figure 5C). Further, this viability defect in liquid culture was only observed with actively replicating bacteria as
suspension and shaking in PBS for 24 hours at 37°C did not reduce viable CFU recovered of either Schu S4 or ΔFTT_0924 (Figure 5D).

One possible reason for reduced viability in liquid culture is that ΔFTT_0924 is sensitive to osmotic stress from a hypotonic solution. To test this hypothesis we increased the osmolarity of the media to prevent the influx of water into the bacterial cytoplasm resulting in bacterial swelling and eventual lysis by adding the nonionic osmolite sucrose to the media. Wild type Schu S4 and ΔFTT_0924 were inoculated at and OD_{600} of 0.05 in Chamberlain's defined media supplemented with either 0mM, 100mM or 300mM sucrose and grown for 24 hours at 37°C. Both OD_{600} and viable bacteria were measured after 24 hours of growth to determine if growth in media with high osmolarity prevented the viability defect of the ΔFTT_0924 mutant after growth in liquid culture. In media containing sucrose, ΔFTT_0924 reached a terminal OD_{600} equal to that of the wild type indicating a rescue of the ΔFTT_0924 growth defect determined by OD_{600}. Likewise the number of viable mutant bacteria per OD_{600} was restored to near wild type levels in the presence of sucrose (Figure 5E and 5F) suggesting that the ΔFTT_0924 peptidoglycan integrity was compromised resulting in membrane permeability and an increased susceptibility to osmotic stress.

**ΔFTT_0924 membrane is permeable under hypotonic conditions**

Since ΔFTT_0924 viability was rescued with the addition of sucrose, we wanted to determine whether the membranes of the mutant bacteria were permeable specifically during growth under hypotonic conditions. Wild type and the ΔFTT_0924 strains were grown in CDM containing propidium iodide (PI), which is membrane impermeable and will only enter the bacterial cell and incorporate into the chromosomal
DNA if the integrity of the bacterial membrane is compromised. Once PI binds DNA it becomes fluorescent and therefore an increase in PI fluorescence indicates the bacterial membranes are permeable. Wild type and ΔFTT_0924 strains were grown in CDM, CDM with sucrose and CDM with gentamicin, a bacteriolytic antibiotic providing a positive control for PI incorporation. Optical density (Figure 6A) and PI fluorescence (Figure 6B) were measured simultaneously every 30 minutes for 24 hours. Wild type bacteria grown in CDM or CDM with added sucrose did not have any appreciable increase in PI fluorescence while bacteria grown in CDM plus gentamicin (inoculated at a higher OD<sub>600</sub> of 0.3) exhibited significant PI fluorescence (Figure 6B). In contrast, the ΔFTT_0924 strain had a significant increase in the PI fluorescence within the first 4 hours of growth in CDM, which did not occur in CDM with added sucrose (Figure 6B) indicating the ΔFTT_0924 strain’s bacterial membrane was permeable only when grown in hypotonic solution. Further, this increase in membrane permeability (Figure 6B) in CDM correlated with the decrease in viability within 6 hours of growth in CDM (Figure 5B) indicating the cellular defect was observable immediately upon initiation of bacterial replication.

The observed membrane permeability of ΔFTT_0924 suggests that FTT_0924 may be involved in maintaining the overall structure of F. tularensis. Bacteria growing in CDM with sucrose were fixed and transmission electron microscopy was performed to determine if the bacteria had any observable gross morphological differences compared to wild type bacteria. No obvious observable physiological differences in cell size, shape, membrane structure or other defects were visible between the wild type and
ΔFTT_0924 strains (Figure S1). This suggests that membrane permeability in hypotonic solution is not due to an altered bacterial structure of ΔFTT_0924.

**FTT_0924 localizes to the inner membrane facing the periplasm**

Determining FTT_0924 localization could provide insight into how the protein contributes to osmotic stress resistance. Membrane fractions were prepared from ΔFTT_0924 expressing FTT_0924 with a C-terminal HA tag (FTT_0924-HA). Soluble, inner membrane and outer membrane fractions were probed with antibodies recognizing HA, IglC, RipA or Tul4 to determine protein localization. FTT_0924 localized to the inner membrane fraction and fractionated with the same pattern as RipA, a known inner membrane protein (Figure 7A) (20).

FTT_0924 localized to the inner membrane; since there is only one predicted transmembrane domain (TMHMM) at the extreme N-terminus, the majority of the protein must be located within the cytoplasm or periplasm. To discern between these two possibilities, we adapted a GFP/PhoA fusion system to function in Francisella species (27). Constructs expressing translational fusions of GFP or PhoA to the C-terminus of FTT_0924 were constructed and expressed in *F. tularensis* Schu S4. Since GFP fluoresces only when expressed in the cytoplasm and PhoA is only active in the periplasm, cytoplasmic or periplasmic localization can be determined based on fluorescence/activity ratios expressed by the fusion construct strains. GFP and PhoA activities were measured for each fusion and the ratio of GFP and PhoA activities were used to determine localization. BioF, involved in biotin biosynthesis, and BlaB, β-lactamase, were used as cytoplasmic and periplasmic controls, respectively. Fusion of the *FTT_0924-GFP* expressing strain exhibited near background levels of fluorescence
and the FTT_0924-PhoA expressing strain lysate exhibited PhoA activity indicated periplasmic location of the expressed fusion proteins (Figure 7B). Together, these data indicate that FTT_0924 was localized in the inner membrane and facing the periplasmic space where FTT_0924 may be directly involved in preventing bacterial lysis from osmotic stress during bacteria replication.

**ΔFTT_0924 is sensitive to β-lactam antibiotics**

Because FTT_0924 is localized in the periplasm and is sensitive to osmotic stress, we hypothesize that FTT_0924 is involved in regulating peptidoglycan remodeling and therefore that the ΔFTT_0924 mutant would be sensitive to specific stresses affecting the cell wall and peptidoglycan. We therefore tested the sensitivity of the ΔFTT_0924 mutant to specific antibiotics and other compounds via disc diffusion assays. Suspensions of wild type Schu S4, ΔFTT_0924, or ΔacrB, a Francisella mutant with a defect in a drug efflux system, were swabbed onto modified Mueller-Hinton agar plates and paper discs containing each compound were placed on each plate. Diameters of zones of inhibition were measured after two days of incubation at 37°C. The ΔacrB displayed increased sensitivity to several hydrophobic drugs as previously described, whereas ΔFTT_0924 displayed a different pattern of antibiotic sensitivities indicating the sensitivities of ΔFTT_0924 do not affect the function of the known drug efflux mechanisms (Table 2) (28). Interestingly, ΔFTT_0924 displayed significantly increased sensitivity to β-lactam antibiotics as compared to wild type. It should be noted that ΔFTT_0924 was also sensitive to macrolide antibiotics and
rifampicin, which may be a result of either damaged peptidoglycan or that FTT_0924 may function in other cellular processes. Overall, these data further demonstrate that FTT_0924 is required for maintaining peptidoglycan integrity and dynamics.

Discussion

Luminescence is a commonly used reporter in prokaryotic systems and was recently first used in F. tularensis (23). Here we use bacterial luminescence as a reporter for growth within macrophages where bacterial burden increased proportionally with luminescence. Using this reporter system, we screened 7500 transposon mutants for growth in macrophage and epithelial cell lines. From these mutants 269 transposon insertions had a growth defect in one or both cell types, which mapped to 74 specific genes. The list of 74 genes was further narrowed to 68 genes by targeted phenotypic screening of the transposon mutants. The F. tularensis LVS genome contains about 1800 genes and testing 7500 transposon insertion mutants should identify an average of 4 transposon insertions mutants per gene if there was an unbiased distribution of transposon insertions. The 269 transposon mutants that impacted intracellular growth mapped to 68 genes, roughly 4 fold fewer genes. All 8 genes in the purine biosynthesis pathway (that are predicted as non-redundant and not essential) and 5 of 6 genes required for biotin biosynthesis were identified in this screen suggesting that the library of 7500 mutants contained minimal bias of insertion locations with a high level of genomic coverage (29).

The majority of mutants identified in the screen had similar growth defects in both J774 and TC-1 cell lines (disruptions in 50 of 68 genes identified). The majority of
genes involved in purine biosynthesis and transporters did not replicate to any detectable level in either cell type reiterating the known dependence on purine biosynthesis and dependence on host nutrients for intracellular growth and virulence (7,19,30,31). Mutants that grew to intermediate levels in both cell types included those involved in essential bacterial processes, carbon metabolism and DNA replication and repair. It is possible that the defect in intracellular replication of many of these mutants could be contributed to an overall fitness defect and are not specifically required within a host cell. Interestingly, the screen identified two predicted pseudogenes as required for intracellular growth, both predicted to be disrupted genes encoding transporters. This finding suggests these and potentially other predicted pseudogenes are not inactive and may encode functional proteins or untranslated elements required for virulence or other cellular processes.

Mutants with growth defects in macrophages but not TC-1 epithelial cells included insertions in genes encoding proteins involved in LPS and biotin biosynthesis. Mutants in the LPS biosynthesis pathway likely did not show observable luminescence in the J774 cells due to the hypercytotoxic phenotype previously observed (32). Interestingly, these mutants grew to wild type levels in the TC-1 epithelial cells indicating that functional LPS is not essential for intracellular growth in all cell types and that the host recognition receptors or signaling pathways that results in hypercytotoxicity may be myeloid-specific. Mutants with defects in biotin biosynthesis grew in TC-1 but not J774 cells because the tissue culture media for TC-1 cells was supplemented with biotin, whereas the media for J774 cells were not. Growth in J774 cells of the biotin biosynthetic mutants was restored by supplementing the media with biotin (data not
shown). The limited number of mutants that grew within macrophages but not epithelial cells had defects in synthesis or uptake of aromatic amino acids. It is possible that the reduced growth of these mutants in TC-1 cells was due to lower concentrations of phenylalanine, tyrosine and tryptophan in TC-1 media compared to J774 media. The growth defects observed in the purine, biotin and aromatic amino acid biosynthetic pathways illustrate that several essential nutrients are limiting in the cytosolic compartment.

Here we identify *FTT_0924* as a factor required for osmotic stress resistance, intracellular growth within macrophages and epithelial cells, and virulence in a pulmonary murine infection model. *FTT_0924* is highly conserved among all *Francisella* species and encodes a 132 amino acid protein of unknown function but contains no sequence similarity to proteins outside the *Francisella* genus. We found that *FTT_0924* is required for maintaining resistance to osmotic stress in liquid culture during replication and that deletion of the *FTT_0924* locus results in membrane permeability in a hypotonic solution. In solution, the OD_{600} of the *FTT_0924* mutant was maintained but viability was severely reduced suggesting that the mutant bacteria are leaky but full bacterial lysis does not occur. This viability defect can be rescued when increasing the osmolarity of the solution indicating the viability defect and membrane permeability only occurs in hypotonic solution. Interestingly, the total solute concentration of our defined media is 456mM and the isotonic solute concentration of a J774 cell is roughly 290mM (33). The virulence defect and sensitivity to hypotonic solutions of the *FTT_0924* mutant suggests that the intracellular compartment is hypotonic for *F. tularensis* and thus requires peptidoglycan to resist turgor pressure.
In Schu S4, FTT_0924 localizes with the inner membrane and faces the periplasm placing it in the optimal location to directly modulate peptidoglycan dynamics. The ΔFTT_0924 mutant is also hypersensitive to β-lactam antibiotics further suggesting the role of FTT_0924 in peptidoglycan stability. Transmission electron microscopy analysis revealed no gross morphological differences in the bacterial shape, size or membrane structure in the ΔFTT_0924 strain compared to wild type Schu S4 indicating the mutant bacteria maintained a structure similar to the wild type. Together, these data demonstrate that FTT_0924 has a role in maintaining integrity of the peptidoglycan but is not required for maintaining gross bacterial structure.

To survive and replicate bacterial pathogens must adapt to and resist stresses within different host environments. Identifying the specific stresses pathogens must overcome within host cells will help define the mechanisms required for intracellular growth and virulence and identify potential targets for therapeutics. Here we performed a large scale genetic screen to identify genes required for F. tularensis intracellular growth. We identified several genes, not previously recognized as required for intracellular growth and virulence, involved in metabolite biosynthesis and uptake indicating the host cell cytoplasm is limiting in several essential nutrients. We also identified FTT_0924, a gene of unknown function, and determined FTT_0924 is involved in modulating peptidoglycan dynamics. By further investigating the role of FTT_0924 in peptidoglycan dynamics we can understand how F. tularensis modulates its peptidoglycan and elucidate how optimal peptidoglycan dynamics is required for intracellular growth and virulence. Identifying new genes required for F. tularensis intracellular growth, such as specific amino acid biosynthetic genes and FTT_0924,
and defining the role of these genes in intracellular replication will help define the specific mechanisms of how \textit{F. tularensis} infects and replicates within host cells and causes disease.

**Figures**

![Graph](image)

\textbf{Figure 2.1: Luminescence as a Reporter for Intracellular Growth.} Intracellular replication of LVS, LVS LUX and LVS \textit{ΔripA} LUX in J774 macrophage cells was measured via luminescence (right axis) and dilution plating for CFU (left axis) each at 4 and 24 hours post infection. Each sample was read for luminescence then the infected
macrophages were lysed and the intracellular bacteria were enumerated by dilution plating. Graphs represent three independent experiments performed in triplicate.

Figure 2.2: Screening Procedure to Identify Genes Required for Intracellular Growth.

Each mutant identified in the primary screen with reduced growth in J774 or TC-1 cells was rescreened in triplicate in J774 and TC-1 cells. Transposon insertions were mapped in rescreened mutants with growth defects in either or both cell lines. If
only one transposon mutant mapped to a specific gene, the mutant was rescreened in a standard gentamicin protection assay.

Figure 2.3: Impact of FTL_1286 and FTT_0924 on Intracellular Growth.

Intracellular growth of A) LVS LUX, Tn:FTL_1286, ΔFTL_1286 LUX, ΔFTL_1286 p1286 and B) Schu S4, ΔFTT_0924 LUX and ΔFTT_0924 p0924 measured via luminescence every 30 minutes. C) J774 or D) TC-1 cells infected with Schu S4, ΔFTT_0924 or
ΔFTT_0924 p0924 measured at 4 and 24 hours by dilution plating. Representative images (n=30) of J774 cells at 24 hours post infection with E) Schu S4 GFP or F) ΔFTT_0924 GFP. Scale bars represent 10µm and red indicates wheat germ agglutinin staining, blue indicates DAPI and Green indicates GFP bacteria. Three independent experiments were performed for each panel.
Figure 2.4: Effect of *FTT_0924* on Schu S4 Growth and Dissemination in a Mouse Model of Infection. C57BL/6 mice were infected intranasally with 100 CFU of Schu S4 (WT), Δ*FTT_0924*, or Δ*FTT_0924* p0924. At 2 hours and days 1, 3, 4, and 7 post infection, organs were harvested, homogenized, serially diluted and plated on chocolate agar to determine bacterial burdens. Horizontal dashed line indicates the limit of detection of the assay. ** p<.01 and *** p<.0001 as determined by the Mann Whitney U test.
Figure 2.5: Bacterial Viability of ΔFTT_0924 During *in vitro* Growth. Schu S4 and ΔFTT_0924 were grown in CDM and A) OD$_{600}$ and B) viable bacteria per OD$_{600}$ were measured. C) Bacterial viability was measured after 24 hours growth on chocolate agar, resuspended in PBS and dilution plated. D) Schu S4 and ΔFTT_0924 viability was measured from inoculating PBS at an OD$_{600}$ of 1, allowed to shake for 24 hours and dilution plated. E) OD$_{600}$ and F) viable bacteria per OD$_{600}$ were quantified at 24 hours post inoculation from Schu S4 and ΔFTT_0924 grown in CDM with varying amounts of sucrose. Each graph is representative of at least 3 independent experiments. * p<.05, **p<.01 and n.s.= not significant as determined by Student’s T test.
Figure 2.6: Membrane Permeability of Schu S4 and ΔFTT_0924 in Liquid Culture.

Schu S4 and ΔFTT_0924 were grown in CDM, CDM plus 300mM sucrose or CDM plus 50μg/mL gentamicin where each media also contained 2.5μM propidium iodide. A) OD₆₀₀ of Schu S4 and ΔFTT_0924 was measured every 30 minutes over 24 hours. B) Fluorescence from propidium iodide incorporation into bacterial DNA of Schu S4 and ΔFTT_0924 was measured every 30 minutes over 24 hours. Each graph is representative of at least 3 independent experiments.
Figure 2.7: Localization of FTT_0924. A) Inner membrane (IM), outer membrane (OM), soluble (sol) and whole cell lysate (WCL) were prepared via Sarkosyl extraction from Schu S4 and ΔFTT_0924 p0924-HA (FTT_0924-HA). Proteins that reside in the cytosol (IglC), inner membrane (RipA) and outer membrane (Tul4) served as fraction purity controls. Lanes were loaded with equal protein as determined by BCA. B) Schu S4 expressing BioF, BlaB or FTT_0924 as translational fusions to PhoA or GFP were tested for GFP or PhoA activity and presented as the ratio of the two activities. Data presented represent at least three independent experiments.
Figure 2.8: (S1) Morphology of Schu S4 and ΔFTT_0924 by TEM. ΔFTT_0924 were grown to mid-log phase in CDM with 300mM sucrose, pelleted and fixed for analysis by TEM. Scale bars represent 200nm.
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### Table 2.2: List of Transposon Insertions and Phenotypes

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<td>AroG</td>
<td>Aromatic AA Synthesis</td>
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<td>N N 5</td>
<td>*MFS</td>
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<td>FTL_1930</td>
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WT indicates growth equal to WT control, I indicates Intermediate growth, and N indicates no observable growth.

* indicates not previously identified as required for virulence in *Francisella*.
Table 2.3: Numbers of Genes Identified by Mutant Phenotype

<table>
<thead>
<tr>
<th>Growth Phenotype</th>
<th>Total Genes</th>
<th>Pseudogenes</th>
<th>Bacterial Processes</th>
</tr>
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<tbody>
<tr>
<td>No Growth</td>
<td>20</td>
<td>1</td>
<td>Purine Biosynthesis; Transporters</td>
</tr>
<tr>
<td>Intermediate Growth</td>
<td>30</td>
<td>1</td>
<td>Essential processes; Carbon metabolism</td>
</tr>
<tr>
<td>Growth In Macrophages Only</td>
<td>5</td>
<td>0</td>
<td>Aromatic AA biosynthesis and transport</td>
</tr>
<tr>
<td>Growth In Epithelial Cells Only</td>
<td>13</td>
<td>0</td>
<td>Biotin biosynthesis; LPS biosynthesis</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>2</td>
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### Table 2.4: Sensitivity of ΔFTT_0924 to specific compounds

<table>
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<tr>
<th>Antibiotic</th>
<th>Class</th>
<th>Schu S4</th>
<th>Δ0924</th>
<th>ΔacrB</th>
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<tr>
<td>Nalidixic Acid</td>
<td>Quinolone</td>
<td>32.7 ± 0.6</td>
<td>31 ± 1</td>
<td>33.7 ± 0.7</td>
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<td>Ciprofloxacin</td>
<td>Fluoroquinolone</td>
<td>36.7 ± 0.7</td>
<td>37.7 ± 0.7</td>
<td>38.7 ± 0.7</td>
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<td>Erythromycin</td>
<td>Macrolide</td>
<td>19 ± 0</td>
<td>20.3 ± 1.5</td>
<td>34.3 ± 1.2</td>
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<td>Tetracycline</td>
<td>Tetracycline/polyketide</td>
<td>21 ± 0</td>
<td>21.3 ± 0.6</td>
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<tr>
<td>Ethidium Bromide</td>
<td>Intercalator</td>
<td>15.7 ± 0.6</td>
<td>12.3 ± 0.6</td>
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<td>SDS</td>
<td>Detergent</td>
<td>14 ± 0</td>
<td>17 ± 0</td>
<td>29.3 ± 0.6</td>
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<td>Ceftriaxone</td>
<td>β-Lactam</td>
<td>6 ± 0</td>
<td>24.3 ± 2.3</td>
<td>6 ± 0</td>
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<tr>
<td>Amoxicillin + Clavulonic Acid</td>
<td>β-Lactam</td>
<td>6 ± 0</td>
<td>17.3 ± 2.5</td>
<td>6 ± 0</td>
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<tr>
<td>Kanamycin</td>
<td>Aminoglycoside</td>
<td>21.3 ± 0.6</td>
<td>26 ± 0</td>
<td>23 ± 0</td>
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<tr>
<td>Gentamicin</td>
<td>Aminoglycoside</td>
<td>26 ± 0</td>
<td>31.3 ± 0.6</td>
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<tr>
<td>Rifampicin</td>
<td>Rifamycin</td>
<td>22.3 ± 0.6</td>
<td>35.3 ± 0.6</td>
<td>26 ± 0</td>
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Distances are measured in mm ± s.d.
REFERENCES


CHAPTER 3: FRANCISELLA TULARENSIS HARVESTS NUTRIENTS DERIVED VIA ATG5-INDEPENDENT AUTOPHAGY TO SUPPORT INTRACELLULAR GROWTH\textsuperscript{1}

**Overview**

*Francisella tularensis* is a highly virulent intracellular pathogen that invades and replicates within numerous host cell types including macrophages, hepatocytes and pneumocytes. By 24 hours post invasion, *F. tularensis* replicates up to 1000-fold in the cytoplasm of infected cells. To achieve such rapid intracellular proliferation, *F. tularensis* must scavenge large quantities of essential carbon and energy sources from the host cell while evading anti-microbial immune responses. We found that macroautophagy, a eukaryotic cell process that primarily degrades host cell proteins and organelles as well as intracellular pathogens, was induced in *F. tularensis* infected cells. *F. tularensis* not only survived macroautophagy, but optimal intracellular bacterial growth was found to require macroautophagy. Intracellular growth upon macroautophagy inhibition was rescued by supplying excess nonessential amino acids or pyruvate, demonstrating that autophagy derived nutrients provide carbon and energy sources that support *F. tularensis* proliferation. Furthermore, *F. tularensis* did not


\textsuperscript{2}Indicates co-primary authorship
require canonical, ATG5-dependent autophagy pathway induction but instead induced an ATG5-independent autophagy pathway. ATG5-independent autophagy induction caused the degradation of cellular constituents resulting in the release of nutrients that the bacteria harvested to support bacterial replication. Canonical macroautophagy limits the growth of several different bacterial species. However, our data demonstrate that ATG5-independent macroautophagy may be beneficial to some cytoplasmic bacteria by supplying nutrients to support bacterial growth.

**Author Summary**

*Francisella tularensis* is a highly virulent bacterial pathogen that infects hundreds of different animal species including humans. During infection, *F. tularensis* bacteria invade and rapidly multiply inside host cells. Within the host cell environment, basic nutrients that bacteria require for growth are in limited supply, and the majority of nutrients are tied up in complex molecules that are not readily available in forms that can be used by bacteria. In this study we asked and answered a very simple question; how does *F. tularensis* harvest sufficient carbon and energy sources from the host cell to support rapid intracellular growth? We found that *F. tularensis* induces a host recycling pathway in infected cells. Thus the host cell degrades nonessential proteins and releases amino acids. *F. tularensis* harvests the host-derived amino acids to generate energy and build its own more complex molecules. When we inhibited the host recycling pathway, growth of the intracellular bacteria was limited. Therefore, manipulation of host cell metabolism may be a means by which we can control the growth of intracellular bacterial pathogens during infection.
**Introduction**

When intracellular bacterial pathogens invade host cells, the bacteria must scavenge energy sources and anabolic substrates from the nutrient-limited intracellular environment. Most of the potential nutrient sources inside a host cell are stored within complex structures such as lipid droplets, glycogen and proteins, which are not immediately available to intracellular pathogens. To obtain nutrients for proliferation, intracellular bacteria must degrade these complex structures into their constituents (fatty acids, carbohydrates and amino acids respectively) or increase nutrient import. The strategies that bacteria use to acquire nutrients could potentially have widespread effects on the host cell. For example, pathogens that import amino acids from the host cell cytoplasm may starve the cell. Host cell amino acid starvation leads to mammalian target of rapamycin (mTOR) inhibition, thereby inhibiting mRNA transcription and other critical cellular homeostatic processes [1]. Thus, nutrient acquisition is an important step in the pathogenesis of intracellular bacteria and is critical to understand how a pathogen interacts with the host.

Autophagy is a highly conserved eukaryotic cell process that can be initiated by a variety of factors such as amino acid starvation, energy depletion, mTOR inhibition and immune signaling[2], [3]. Autophagy is a process by which multi-membranous vesicles called autophagosomes surround and degrade cellular constituents (during starvation) or cytoplasmic bacteria (during infection through a related innate immune response termed xenophagy [4]. The autophagosomes fuse with lysosomes to become autolysosomes, which then degrade the engulfed material. During starvation, autophagy can degrade nonessential proteins, thereby releasing free amino acids that are recycled
into new proteins. Current studies of the interactions between host autophagy and intracellular bacterial pathogens are primarily focused on xenophagy [5]–[7]. However, a few intracellular pathogens are known to benefit from autophagy [8]–[10]. Autophagosome formation is induced during infection with *Anaplasma phagocytophilum* and the autophagy derived nutrients are harvested and used by *A. phagocytophilum* to enhance intracellular replication [9]. Likewise, dengue virus uses autophagic byproducts to acquire lipids for viral replication [10]. Pathogens such as *Listeria monocytogenes* express active mechanisms that prevent bacterial degradation via xenophagy, yet autophagy still occurs in the infected cell and has the potential to provide nutrient sources for the bacteria [11]. These and other recent studies highlight the potential role of autophagy in providing nutrients or other benefits for intracellular pathogens.

*Francisella tularensis* is a facultative intracellular bacterium that infects over 200 different species (from amoeba to humans) [12]. The highly virulent *F. tularensis* subsp. *tularensis* Schu S4 strain has an infectious dose of fewer than 25 bacteria and a mortality rate of 30–60% in untreated pneumonic infections [13], [14]. *F. tularensis* infects a diverse range of cell types including macrophages, which are a key replicative niche for *F. tularensis* in humans and other susceptible mammals. *F. tularensis* also invades and replicates within several other cell types including epithelial cells and endothelial cells [12], [15]. *F. tularensis* enters the host cell through phagocytosis and proceeds to escape the phagosome and replicate in the host cell cytoplasm. By 24 hours post inoculation, *F. tularensis* replicates up to 1000-fold inside host cells. This rapid intracellular replication plays a major role in *F.*
*tularensis* pathogenesis but the mechanisms by which this organism acquires nutrients are not well characterized. Therefore, we sought to determine how these nutrients become available to support efficient *F. tularensis* intracellular replication.

In primary murine macrophages, *F. tularensis* induces the formation of a multiemembranous, autophagosome-like structure termed the Francisella containing vacuole (FCV) through an autophagy related process [16]. FCV formation occurs between 20 and 36 hours post inoculation, after the majority of *F. tularensis* replication has taken place. Blocking FCV formation late during infection does not increase *F. tularensis* proliferation, suggesting that FCV formation does not play a role in controlling intracellular *F. tularensis* replication [16]. However, the formation of FCVs hints that autophagy may be induced during *F. tularensis* infection. Additionally, replication deficient and chloramphenicol treated *F. tularensis* bacteria, but not wild type *F. tularensis* bacteria, are degraded via canonical autophagy [17]. This observation implies that *F. tularensis* avoids xenophagy. Lastly, treating *F. tularensis* infected macrophages 2 hours post inoculation with chloroquine or autophagy-inhibiting levels of ammonium chloride impairs *F. tularensis* intracellular replication [18]–[20]. Although chloroquine and ammonium chloride inhibit acidification of cellular compartments and have broad effects on the host cell, these data raise the intriguing possibility that autophagy may contribute to *F. tularensis* intracellular replication.

Taken together these observations suggest that intracellular *F. tularensis* avoids xenophagy yet induces autophagy or an autophagy-like process that contributes to *F.*
*tularensis* proliferation. We therefore examined the potential role of autophagy in aiding *F. tularensis* intracellular growth.

**Results**

*Host cell constituents are sufficient to support *F. tularensis* intracellular proliferation*

*F. tularensis* replicates efficiently and rapidly in host cells. Indeed, transmission electron microscopy analysis showed that *F. tularensis* consumed over half of the area of the cell cytoplasm of infected mouse embryonic fibroblasts (MEFs) by 16 hours post inoculation (Figure S1). *F. tularensis* cannot make all of the nutrients it needs *de novo* and must interact with the host to acquire certain metabolites to support rapid proliferation. In particular, *F. tularensis* is auxotrophic for 13 amino acids, some of which mammalian cells also do not synthesize. Thus, for sustained proliferation within infected cells, the bacteria must either take up amino acids imported by the host cell or degrade host proteins and reuse the resulting amino acids. To distinguish between these possibilities, we determined if decreasing the availability of free amino acids limited *F. tularensis* intracellular growth. We replaced the media on infected MEFs with media lacking amino acids at 3 hours post inoculation. *F. tularensis* replicated to similar numbers with or without amino acids present in the tissue culture media (Figure 1A). This result demonstrates that *F. tularensis* can acquire the amino acids it needs to sustain growth directly from the host cell. Since the majority of host amino acids are typically sequestered in proteins inside the cell, protein degradation likely occurs to provide sufficient amino acids to support *F. tularensis* intracellular growth. Additionally,
amino acid depletion results in starvation induced autophagy [21]. Starvation induced autophagy will degrade proteins to produce amino acids. Thus, *F. tularensis* may take advantage of host cell autophagy to acquire free amino acids.

**Autophagy supplies energy and anabolic substrates that support *F. tularensis* growth in fibroblasts**

To determine if autophagy had any impact on *F. tularensis* intracellular growth we measured bacterial replication inside cells treated with several different autophagy inhibitors. MEFs were treated with 3-methyladenine (3MA), which inhibits autophagosome formation, thereby blocking autophagy. *F. tularensis* replication inside 3MA treated MEFs was significantly reduced (Figure 1B), suggesting that intracellular *F. tularensis* benefit from host cell autophagy. Since autophagy is both a starvation response and a process by which damaged organelles and non-essential proteins are degraded we considered the possibility that *F. tularensis* may scavenge and utilize amino acids released by this process. We therefore wanted to determine if exogenous amino acid supplementation would rescue *F. tularensis* growth in MEFs that have impaired autophagy function. Indeed, *F. tularensis* intracellular growth in the presence of 3MA was restored by the addition of excess amino acids to the culture media (Figure 1B). These results, which were corroborated using confocal fluorescence microscopy of cells infected with GFP-expressing *F. tularensis* Schu S4 (Figure 1C) indicate that autophagy provides a source of nutrients that support *F. tularensis* replication.

To determine if degradative autophagy was responsible for optimal bacterial growth, we quantified *F. tularensis* intracellular growth in the presence of Bafilomycin
A(1) (Baf) or chloroquine (CQ), each of which inhibits autophagy by blocking functional autolysosome formation. We tested the effect of these drugs on *F. tularensis* replication kinetics by infecting MEFs with *F. tularensis* containing a bioluminescence reporter plasmid (Schu S4-LUX) [22] and measuring luminescence every 30 minutes to determine the bacterial growth kinetics. The limit of detection for this assay was approximately 50 relative light units (RLUs) or approximately $10^5$ bacteria in a 96 well format (data not shown). We verified this technique by treating *F. tularensis* infected cells with 3MA or 3MA supplemented with amino acids and observed similar results to the standard intracellular proliferation assays (Figure S2A, S2B). Additionally, CQ significantly reduced *F. tularensis* growth and amino acid supplementation rescued bacterial growth in CQ treated cells (Figure S2C, S2D). Similar to 3MA and CQ, treating MEFs with Baf also significantly reduced *F. tularensis* intracellular growth and growth was rescued with amino acid supplementation (Figure S2E, S2F). None of the inhibitors affected *F. tularensis* growth in broth culture (Figure S3B). Although 3MA, CQ, and Baf were each cytotoxic to MEFs, viability was comparable between treatments with and without amino acid supplementation (Figure S3A). Thus, the observed rescue was not due to increased eukaryotic cell viability upon amino acid supplementation.

Since all chemical inhibitors have the potential to confer off-target or non-specific effects on host cell processes we wanted to confirm the inhibitor results using genetic approaches. Beclin-1 is required for autophagosome formation in most autophagy pathways [23]. We therefore reasoned that depletion of Beclin-1 should limit bacterial replication if autophagy is required for *F. tularensis* growth. We created two Beclin-1 knock down MEF cell lines, Beclin-1 KD-1 and KD-2 that expressed 63.8% (+/-14.4%)
and 59.2% (+/−12.9%) of the scrambled shRNA control Beclin-1 mRNA, respectively (Figure S4). Despite the relatively modest reduction of Beclin-1 mRNA, F. tularensis replication was significantly reduced in the knockdown cell lines compared to the scrambled control (Figure 1D); supporting the conclusion that autophagy may have a pro-bacterial role in F. tularensis infected cells. Interestingly, the infection frequency of the knock down cell lines was approximately 2-fold higher than the scrambled control (data not shown) suggesting that Beclin-1 activity may modestly impair F. tularensis infection of host cells.

**Autophagy supports F. tularensis replication in primary human monocyte derived macrophages**

During the course of infection F. tularensis invade and replicate within many different cell lineages and types. Intracellular growth properties of F. tularensis vary depending on host cell type. For example, F. tularensis infects monocytes at a significantly higher frequency than epithelial cells or fibroblasts. On the other hand, F. tularensis intracellular growth is more prolonged, and achieves nearly 10-fold higher peak numbers in MEFs as compared to monocytes (data not shown). Growth within monocytes is a property that is fundamental to F. tularensis virulence. F. tularensis is also a human pathogen; we therefore wanted to determine the relevance of autophagy in supporting F. tularensis growth within human macrophages. Inhibition of autophagy with 3MA significantly decreased F. tularensis growth in hMDMs, and growth was rescued in 3MA treated hMDMs by supplementing the media with excess amino acids (Figure 1E). Therefore, autophagy provides amino acids that support F.
F. tularensis intracellular growth in primary human monocytes, a property that is crucial to F. tularensis pathogenesis.

**F. tularensis infection increases autophagic flux**

We compared the rate of degradation of long-lived proteins in uninfected and infected cells to determine if F. tularensis infection impacted autophagic flux. Since we were attempting to quantify a specific infected host cell response we performed this analysis in the J774A.1 monocyte cell line (J774) where the F. tularensis infection frequency is much greater than the infection frequency in MEFs (data not shown). We first labeled cellular proteins by incubating J774 cells in media containing $^{35}$S methionine and cysteine for 18 hours and chased for 2 hours to remove any remaining labeled free amino acids. The labeled cells were inoculated with F. tularensis and incubated for 16 hours. Following infection, infected cells had a 49.5%+/−7.9% (Average +/− SEM) decrease of $^{35}$S label in the TCA insoluble fraction of the cytoplasm (which will primarily contain proteins) compared to uninfected J774 cells (Figure 2A). Thus, infected cells had increased turnover of long lived proteins than uninfected cells. This result is consistent with autophagy induction in F. tularensis infected J774 cells. The decrease of total $^{35}$S label in both host and bacterial proteins in infected cells may indicate that the transfer of amino acids from the host to the bacteria is inefficient or that the majority of amino acids are used by F. tularensis for energy rather than protein synthesis. Uninfected and infected J774 cells had similar levels of cytotoxicity at 16 hours post inoculation, indicating that the loss of label in infected compared to uninfected cells was not due to cell lysis (Figure S3D).
Autophagy derived amino acids are transferred from host proteins to F. tularensis

To confirm that *F. tularensis* imports amino acids derived from host proteins, we monitored transfer of radiolabelled amino acids from host proteins into bacterial proteins. MEFs were first metabolically labeled with $^{35}$S-labeled methionine and cysteine for 18 hours to fully label all host proteins. Then the radiolabel was removed and the cells were incubated in unlabeled media for two hours prior to infection with *F. tularensis* to remove $^{35}$S that was not incorporated into protein. At 16 hours post infection (18 hours after the radiolabel was removed) we lysed the MEFs and purified *F. tularensis* by mixing cell lysate from either uninfected or infected cells with magnetic beads linked to an anti- *F. tularensis* lipopolysaccharide (LPS) antibody. We then determined if *F. tularensis* proteins contained radiolabeled amino acids by examining the trichloroacetic acid (TCA) insoluble fraction of purified *F. tularensis*. There was a significant increase of radiolabel in the TCA insoluble, *F. tularensis* bead purified fraction from infected MEFs as compared to uninfected control samples (Figure 2B). Indeed, 6.22%+/−4.15% (average +/- SEM, n = 5 samples) of the TCA insoluble radiolabel present prior to infection transferred to the bacteria during the 16 hour infection. To control for possible direct transfer of labeled amino acids that were not incorporated into host proteins we analyzed infected MEFs that were treated with cycloheximide during $^{35}$S labeling prior to infection. There were negligible amounts of radiolabel present in the bead purified fraction of cycloheximide treated cells (Figure 2B). *F. tularensis* survived and replicated within cycloheximide pre-treated cells and *F. tularensis* was present in the bacterial purified fraction (data not shown). Thus, host cell lysis due to the cycloheximide treatment was not solely responsible for the lack of
radiolabel in the bacterial fraction. $^{35}$S radiolabel was primarily incorporated into host proteins, rather than as free $^{35}$S labeled amino acids. Taken together, these data demonstrate that *F. tularensis* synthesized proteins using amino acids derived from host cell proteins.

Treating the radiolabeled cells with either Baf or 3-MA resulted in significantly decreased incorporation of the radiolabel by *F. tularensis* (Figure 2C). Since *F. tularensis* proliferation is reduced in 3MA and Baf treated MEFs, several fold fewer bacteria were present in the bacteria purified fraction of the treated MEFs (data not shown). Nevertheless, the median $^{35}$S counts per bacteria were significantly lower in the 3MA or Baf treated samples compared to untreated samples (untreated: 0.016 CPM/bacteria, 3MA: 0.000 CPM/bacteria, Baf: 0.000 CPM/bacteria [n = 3 experiments done in duplicate]). Therefore, transfer of radiolabeled amino acids to bacterial proteins was reduced by both 3MA and Baf treatment, indicating that under normal culture conditions, amino acids derived by the degradation of host cell proteins via autophagy were used by *F. tularensis*.

**F. tularensis uses autophagy by-products primarily for energy**

*F. tularensis* is capable of using amino acids as an energy source when simple carbohydrates such as glucose are not available (Figure 3A). Thus, autophagy derived amino acids could conceivably be used by intracellular *F. tularensis* for either the synthesis of new proteins or to provide energy for other bacterial processes. Although we found that *F. tularensis* uses host-derived amino acids for protein synthesis (Figure 2B), the proportion of amino acids used for protein synthesis as opposed to energy is
unknown. To determine if *F. tularensis* uses autophagy-derived amino acids primarily as anabolic precursors or as an energy source, we supplemented autophagy inhibited, *F. tularensis* infected MEFs with either serine or the metabolite pyruvate. Annotation of the *F. tularensis* genome indicates that *F. tularensis* encodes the protein L-serine dehydratase, which degrades serine directly into pyruvate. The addition of either pyruvate or serine alone rescued *F. tularensis* intracellular growth in Baf treated cells (Figure 3B). Fibroblasts cannot convert serine or pyruvate into all of 13 of the amino acids required to fulfill *F. tularensis* auxotrophies. Thus, host autophagy-derived nutrients are used by *F. tularensis* primarily as a source of energy. Although *F. tularensis* can incorporate autophagy derived amino acids into bacterial proteins (Figure 2B), these data indicate that energy, rather than amino acids for protein synthesis, was the limiting factor for *F. tularensis* proliferation in autophagy-deficient cells cultured in tissue culture media.

**ATG5 is not required for autophagy in Francisella infected cells**

Canonical autophagy is typically induced by the inhibition of mammalian target of rapamycin (mTOR). Thus, monitoring mTOR activity through downstream substrates such as S6 kinase is likely to correlate well with canonical autophagy induction. To determine if *F. tularensis* infection activates the autophagy signaling cascade, we assessed mTOR activity in infected J774 cells by measuring phosphorylation of the mTOR substrate S6 ribosomal protein. The ratio of phospho-S6 ribosomal protein to unphosphorylated S6 ribosomal protein decreased progressively over the course of infection, which is consistent with mTOR inhibition and thus autophagy induction (Figure 4A, 4B) [24]. However, loss of phospho-S6 ribosomal protein was not evident before 8
hours post inoculation suggesting that mTOR inhibition occurred after some bacterial replication had already taken place.

In the canonical autophagy pathway the protein ATG5 is essential for autophagosome formation. Thus, we would predict that ATG5 expression would be required for autophagic degradation of host proteins to amino acids that support *F. tularensis* intracellular growth. However, it was recently shown that *F. tularensis* replicates efficiently within ATG5−/− macrophages [17]. We also found that *F. tularensis* replication was not impaired in ATG5−/− MEFs (Figure 5A). In fact, there was a slight but statistically significant increase in bacterial replication in ATG5−/− MEFs compared to wild type MEFs (Figure 5A). Therefore, ATG5 is not required for efficient *F. tularensis* intracellular proliferation. Treatment of ATG5−/− MEFs with 3MA resulted in decreased bacterial proliferation and bacterial growth was rescued by supplementing treated cells with amino acids (Figure 5B). Taken together, these data suggest that *F. tularensis* intracellular growth is supported by nutrients generated by an ATG5-independent autophagy pathway.

Unlike canonical autophagy, ATG5-independent autophagy generates autophagosomes from the trans-Golgi apparatus [25]. Brefeldin A (Bref A) inhibits ATG5-independent autophagosome formation but does not affect canonical autophagosome formation [24]. To determine if ATG5-independent autophagy provides metabolites for *F. tularensis* in macrophages, we measured *F. tularensis* replication in J774 cells in the presence and absence of Bref A. Cells were infected with Schu S4-LUX and growth was monitored by measuring luminescence every 30 minutes. We
found that *F. tularensis* replication was significantly reduced in Bref A-treated J774 cells (Figure 5C, 5D), and growth was significantly rescued in Bref A treated cells by the addition of amino acids (Figure 5C, 5D). Bref A cytotoxicity was comparable regardless of amino acid supplementation, indicating that the increase in bacterial replication was not due to decreased eukaryotic cell cytotoxicity in amino acid treated cells (Figure S3C). The ability of amino acids to rescue bacterial replication in Bref A-treated cultures indicates that Bref A affects *F. tularensis* nutrient availability. This result is consistent with the conclusion that ATG5-independent autophagy provides nutrients that support *F. tularensis* growth in macrophages as well as in MEFs.

We wanted to determine the extent to which autophagosomes are formed during *F. tularensis* infection, and the spatial relationship between the bacteria and autolysosomes in ATG5−/− cells. Analysis of transmission electron microscopy (TEM) micrographs revealed that autophagic vacuoles constituted a greater percentage of the cytoplasm in *F. tularensis* infected as compared to uninfected ATG5−/− MEFs (Figure 6A–D) confirming that autophagy is induced in ATG5−/− MEFs.

Since morphological analysis of autophagic structures by TEM is inexact, we used fluorescence confocal microscopy as a secondary means to identify acidified autophagic vacuoles in infected MEFs. We stained and quantified the number of LysoTracker Red positive acidic vacuoles in infected and uninfected ATG5−/− MEFs. There were significantly more acidic vacuoles in the infected ATG5−/− MEFs as compared to uninfected ATG5−/− MEFs (Figure 6E). LysoTracker Red can also stain other acidic vacuoles including lysosomes and phagosomes. However, the increased
number of acidic vacuoles found in infected wild type and ATG5−/− MEFs as compared to uninfected and 3MA treated infected control cells strongly argues that the increase in acidic vacuoles correlate with an increase in autophagic vacuoles. Combined with the morphological analysis of the infected-cell vacuoles by TEM this data demonstrates that *F. tularensis* induced ATG5-independent autophagy in infected cells.

**Neither canonical autophagy nor xenophagy are induced during *F. tularensis* intracellular replication**

The slight but statistically significant increase in *F. tularensis* growth observed in ATG5−/− MEFs suggested that canonical autophagy may be induced in infected cells and exert some control over bacterial growth. It is also possible that in addition to destroying the bacteria, canonical autophagy could serve as a redundant mechanism for nutrient acquisition. To determine if canonical autophagy was induced in addition to ATG5-independent autophagy during infection with *F. tularensis*, we analyzed infected MEFs that were transiently transfected with a GFP-LC3 plasmid for an increase in GFP-LC3 puncta. LC3 puncta formation is stimulated by canonical autophagy; however, ATG5-independent autophagy does not induce LC3 puncta formation [24],[26]. LC3 puncta levels were unchanged in infected compared to uninfected MEFs at 16 hours post inoculation, whereas both the amino acid starvation and Torin1 controls conferred an increase in LC3 puncta (Figure 7A, B). Thus, it appears that canonical autophagy remained at basal levels in *F. tularensis* infected cells during late stages of infection.

To determine if induction of canonical autophagy would either increase bacterial clearance or generate additional nutrients that support bacterial replication, we
artificially induced autophagy throughout infection with the mTOR inhibitor Torin1. Torin1 treatment throughout infection had no impact on *F. tularensis* intracellular survival or growth in MEFs (Figure 7C). Thus, *F. tularensis* evades destruction by canonical autophagy and increased canonical autophagy did not benefit *F. tularensis* intracellular replication.

*F. tularensis* induces ATG5-independent autophagy while canonical autophagy remains at basal levels during infection. Little is known about the functional differences between canonical and ATG5-independent autophagy. However, xenophagy is known to occur via canonical autophagy whereas xenophagy via ATG5-independent autophagy has not been addressed. In canonical autophagy, cytosolic pathogens including chloramphenicol treated *F. tularensis* are targeted for xenophagy when bound to p62/SQSTM1 and polyubiquitin [17], [27]–[29]. We therefore investigated the role of polyubiquitin and p62/SQSTM1 in ATG5-independent autophagy induction in *F. tularensis* infected cells.

There was a significant decrease in the number of polyubiquitin puncta in the cytoplasm of infected wild type and ATG5−/− MEFs as compared to uninfected MEFs (Figure 8A). If polyubiquitin was degraded upon ATG5-independent autophagy induction, we would expect a corresponding increase in co-localization between polyubiquitin and acidic vacuoles in infected cells. However, the number of acidic vacuoles co-localizing with polyubiquitin in uninfected cells (15.2%+/−2.2%) and infected cells (20.0%+/−3.5%) was not significantly different (n>25 cells, mean +/−
SEM) (Figure 8B). These data indicate that the decrease in polyubiquitin aggregates in infected cells was independent of autophagy.

In addition, there were similar numbers of p62/SQSTM1 puncta in infected MEFs compared to uninfected MEFs (Figure 8C, S5C–S5E). Interestingly, although there were similar total numbers of p62/SQSTM1 puncta, there was increased co-localization of p62/SQSTM1 with acidic vacuoles in infected wild type MEFs. However, there was no difference in p62/SQSTM1 co-localization between uninfected and infected ATG5−/− MEFs (Figure 8D). The increased co-localization of p62/SQSTM1 with acidic vacuoles may indicate that some basal level of xenophagy is occurring in an ATG5-dependent manner, which is consistent with the increase in bacterial replication that we observed in ATG5−/− MEFs. Taken together, these data indicate that F. tularensis induced ATG5-independent autophagy is not associated with polyubiquitin, LC3B, or p62/SQSTM1.

**F. tularensis is adjacent to autophagic vacuoles**

A recent study demonstrated that *Salmonella enterica* associates with ubiquitinated aggregates that are degraded by autophagy [30]. Although these aggregates likely target *S. enterica* for degradation rather than supplying nutrients, these data suggest that mechanisms exist which target autophagosomes to bacteria or vice versa. We hypothesized that *F. tularensis* may recruit autophagic vacuoles, resulting in bacteria localizing in close proximity to autophagosomes to facilitate bacterial nutrient acquisition. Indeed, *F. tularensis* was frequently found within 250 nm of autophagic vacuoles in both ATG5−/− MEFs and J774 cells as determined by TEM
Indeed, 25.8+/−4.0% (average +/− SEM) of the autophagic vacuoles in ATG5−/− MEFs were also within 250 nm of a bacterium.

We confirmed the TEM results using confocal microscopy. Since ATG5-independent autophagy does not appear to require ubiquitination or any known target marker, we were limited to examining the relationship between bacteria and acidified vacuoles. Infected cells were stained with LysoTracker Red and Z-stacks from infected cells were analyzed by confocal microscopy. 28.0%+/−3.7% of bacteria in wild type MEFs and 35.1%+/−5.1% of bacteria in ATG5−/− MEFs were within 250 nm of an acidic vacuole (Average +/− SEM, n>10 cells) (Figure S6 C–H). At least 1 bacterium was within 250 nm of an acidic vacuole in every cell. The number of bacteria within 250 nm of an acidic vacuole was significantly lower in 3MA treated MEFs compared to the untreated MEFs (p = .01) (Figure S3 H). These data suggest that F. tularensis may recruit or traffic to autophagic vacuoles. Further investigation may reveal that not only autophagy induction, but also proximity to an autophagic vacuole contributes to F. tularensis nutrient acquisition.

Discussion

Intracellular pathogens have evolved to thrive within the hostile nutrient-limited host cell environment. Successful pathogens disarm or avoid innate and adaptive immune responses while simultaneously extracting carbon and energy sources to support their proliferation. Autophagy is a highly conserved degradation process that serves a multitude of functions including cell development, stress response and resistance to cytoplasmic pathogens. Herein we investigated the interaction between F.
tularensis and the host cell autophagy response. Our results demonstrate that ATG5-independent autophagy is triggered in F. tularensis infected cells and that intracellular bacterial replication was enhanced by this process. Furthermore, F. tularensis can replicate in cells when there are no amino acids present in the media, indicating that F. tularensis obtains all of the amino acids necessary to fulfill its 13 amino acid auxotrophies from the host cell through processes such as autophagy. F. tularensis acquires amino acids, and possibly other nutrients, via autophagy. These nutrients are then used for both energy and protein synthesis, although decreased bacterial replication in ATG5-independent autophagy deficient cells is primarily due to a lack of available energy. Autophagy derived nutrients are necessary for optimal F. tularensis replication, but F. tularensis still replicated in cells with decreased ATG5-independent autophagy. This indicates that F. tularensis uses other nutrient acquisition strategies in conjunction with ATG5-independent autophagy to supply nutrients for rapid and efficient proliferation.

Rapid bacterial proliferation requires readily available and abundant carbon and energy sources, commodities that are typically limited in the eukaryotic cell environment. Intracellular pathogens must acquire all required nutrients from the host cell, but the strategies that these pathogens employ to accomplish this task are only beginning to be characterized and vary widely between pathogens [9], [10], [31]–[33]. For example, Legionella pneumophila uses the byproducts of host proteosomal degradation rather than autophagy to obtain amino acids for energy [31]. Dengue virus growth is supported by autophagy mediated release of lipids while autophagosome formation increases nutrient availability for Anaplasma phagocytophilum [9],[10]. It is
likely that other intracellular pathogens that successfully avoid autophagic destruction benefit from the nutrients that are released by this process. Thus, autophagy subversion through various means may be a more common strategy for pathogens to acquire nutrients from the host than previously thought.

The conclusion that autophagy derived amino acids were sufficient to rescue intracellular growth was supported by the fact that the absence of amino acids in tissue culture media did not appreciably affect *F. tularensis* intracellular replication. Thus, host cell amino acid import was not required to support bacterial growth. This result would seem to contradict the recent observation that knocking down expression of the amino acid transporter SLC1A5 decreases *F. tularensis* LVS growth approximately 2-fold [32]. LVS is an attenuated *F. tularensis* vaccine strain that, like fully virulent *F. tularensis*, grows within macrophages and other cell types, but is significantly less virulent than *F. tularensis* and other wild type *F. tularensis* strains in humans and animal models of infection. Unlike *F. tularensis* Schu S4, we found that LVS intracellular growth was significantly impaired in ATG5−/− MEFs and growth in these cells was restored by supplying excess amino acids, implying that LVS harvests nutrients via ATG5-dependent autophagy or another ATG5-dependent mechanism (data not shown). It is therefore likely that LVS is less reliant on ATG5-independent autophagy to support efficient intracellular growth. It is also possible that SLC1A5 contributes to the export of free amino acids out of autolysosomes thereby making autophagy derived amino acids available to the cytoplasmic bacteria. Amino acid transporters export amino acids from autolysosomes to the cytosol in *Saccharomyces cerevisiae*, and a similar system likely exists in mammalian cells [34]. This latter possibility highlights the fact that currently
little is known about how free amino acids derived from autophagic degradation of host proteins are transported within eukaryotic cells.

Canonical autophagy destroys several different pathogens, including replication deficient and chloramphenicol treated F. tularensis [17]. The slight increase in bacterial replication in ATG5−/− MEFs compared to wild type MEFs supports the notion that canonical autophagy can degrade wild type bacteria in MEFs, although this may be cell type specific as there is no difference in F. tularensis replication between wild type and ATG5−/− bone marrow derived macrophages [17]. Also, induction of autophagy by starvation or Torin1 treatment did not reduce bacterial replication. Surprisingly, although we observed mTOR inhibition in J774 cells and autophagy induction in ATG5−/− MEFs, our results suggest that canonical autophagy is either at or close to basal levels 16 hours post inoculation. Our results suggest that F. tularensis suppresses canonical autophagy downstream of mTOR or that mTOR is inhibited in ATG5-independent autophagy and other signals help determine which autophagy pathway is induced.

In contrast to xenophagy via canonical autophagy, ATG5-independent autophagy is involved in the lifecycle of two other intracellular bacterial pathogens. Mycobacterium marinum and Brucella abortus are each sequestered into an autophagosome-like structure via an ATG5-independent pathway as part of their intracellular lifecycles [8], [35]. It is unclear why M. marinum is sequestered, but bacterial sequestration by autophagy appears to be part of the B. abortus intracellular lifecycle and may benefit the bacteria by increasing cell to cell spread rather than providing nutrients [8], [35]. Both of these interactions with ATG5-independent autophagy are
different from that of *F. tularensis*. What remains to be determined is if this difference is due to bacterial manipulation, if there are multiple ATG5-independent autophagy pathways, or if there are different functions for the same ATG5-independent autophagy pathway. Unfortunately, there is little information about how the various autophagy pathways are functionally different. We found that ATG5-independent autophagy, unlike canonical autophagy, does not appear to use two proteins associated with xenophagy during infection. Further characterization of how xenophagy and ATG5-independent autophagy are associated may reveal why certain pathogens induce ATG5-independent autophagy.

Little is known about how ATG5-independent autophagy is induced or the role that it plays in a healthy eukaryotic cell, let alone during pathogenesis. However, there appears to be distinct benefits for certain pathogens to induce ATG5-independent autophagy over the canonical autophagy pathway. Determining how this pathway is induced in *F. tularensis* infected cells may give us insight as to how different autophagy pathways are initiated and how these pathways differentially impact intracellular pathogen survival and growth.

**Materials and Methods**

**Bacteria and plasmids**

*Francisella tularensis* subsp. *tularensis* Schu S4 was obtained from Biodefense and Emerging Infections Research Resources Repository. For inoculation of eukaryotic cells Schu S4, Schu DSred, Schu S4-GFP [15] and Schu S4 – LUX (plasmid from [22])
were each grown initially on chocolate agar supplemented with 1% isovitalex then overnight in Chamberlain's defined broth media (CDM).

**Cell culture**

J774A.1 macrophage-like cells (J774) cells were maintained in 4.5 g/L glucose Dulbecco's minimal essential media (DMEM) with 10% FBS and supplemented with L-glutamine and sodium pyruvate. Mouse embryonic fibroblasts (MEFs) were maintained in 4.5 g/L glucose DMEM with 10% FBS. For treatment of MEFs without amino acids, DMEM with 4.5 g/L glucose was made following the ATCC DMEM protocol without adding amino acids and supplemented with 10% dialyzed FBS.

Human monocyte derived macrophages (hMDMs) were obtained by isolating peripheral blood mononuclear cells (PBMCs) from blood via ficoll gradient centrifugation. PBMCs were cultured for 2 hours in RPMI with 10% FBS and then washed to remove non-adherent cells. The adherent cells were cultured for 2 weeks in RPMI containing 10% FBS and 3 ng/ml GM-CSF (Biolegend). The media was replaced every 2 days. Experiments were performed using PBMCs isolated from peripheral blood from 2 healthy volunteers who gave informed, written consent following a protocol approved by the Institutional Review Board for human volunteers at University of North Carolina at Chapel Hill. Peripheral blood was obtained specifically for these experiments.

Stable Beclin-1 knockdown (TRCN0000087289 or TRCN0000087291) and scramble cell lines were generated by transducing MEFs with lentivirus encoding each shRNA. Cells were propagated in media containing 1 µg/ml puromycin for 2 weeks prior
to the first experiment to select for transduced cells. Concurrent with the first experiment and last intracellular bacterial proliferation assay in the knockdown cell lines, mRNA was harvested from the transduced cells, subjected to reverse transcription, and was analyzed by quantitative RT-PCR to determine the amount of Beclin-1 mRNA present in each sample. The results were normalized to a GAPDH control. Primer sequences in are in Table S1.

**Drug treatments**

3-methyladenine (10 mM) (Sigma), bafilomycin A(1) (200 nM) (Sigma), and chloroquine (160 µM) (Sigma) were each added with 25 µg/ml of gentamicin to the MEFs 3 hours post bacterial inoculation. Brefeldin A (17 µM) (Sigma) was added to J774 cells 3 hours post inoculation. Torin1 (250 nM) (Tocris Biosciences) was added overnight prior to inoculation and maintained throughout the infection. The excess amino acid mixture (12 mM L-amino acids containing aspartic acid, arginine, cysteine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tyrosine, and valine), L-serine (15 mM) or pyruvate (18 mM) were added at the same time as the inhibitors. All media was brought to a pH of 7.5.

Inhibitor cytotoxicity in MEFs was determined using a Live/Dead Fixable Green Dead Cell Stain kit (Invitrogen) following the manufacturer's instructions. Drugs were placed on cells for the same duration they would be on cells during infection (21 hours for Baf and CQ, 29 hours for 3MA). Percent cytotoxicity by flow cytometry was determined by gating. Cytotoxicity of *F. tularensis* in J774 cells 16 hours post inoculation was determined by testing the amount of lactate dehydrogenase (LDH) in
the supernatant with a CytoTox-Glo cytotoxicity kit (Promega) following the manufacturer's instructions. Percent cytotoxicity was determined based on media and digitonin treated controls. Brefeldin A cytotoxicity was determined 21 hours post treatment using an In vitro Toxicology Assay Kit (Sigma) to measure LDH release from J774 cells.

**Intracellular growth assays**

MEFs were plated at $2 \times 10^5$ cells per well in 24 well tissue culture treated plates and grown overnight. MEFs were inoculated with a multiplicity of infection (MOI) of 100 with wild type Schu S4. The media was removed 3 hours post inoculation and replaced with media containing 25 µg/ml of gentamicin to inhibit the growth of any remaining extracellular bacteria. MEFs were lysed by vortexing for 1 minute and the lysates were serially diluted and plated on chocolate agar to calculate the number of intracellular bacterial cells at the indicated times.

hMDM cells were inoculated with an MOI of 100 wild type Schu S4 in RPMI containing 10% FBS. At 2 hours post inoculation, the media was replaced with media containing 10 µg/ml of gentamicin. At 4 hours post inoculation, the media was replaced with media that did not contain gentamicin. Intracellular bacteria were quantified as described previously.

Bacterial intracellular growth kinetics was calculated by measuring luminescence of Schu S4 – LUX infected MEFs or J774 cells. MEFs and J774 cells were plated at $5 \times 10^4$ cells per well in 96 well black wall clear bottom polystyrene plates (Corning) the night before infection. Each well was inoculated at an MOI of 100 with Schu S4- LUX
and treated with gentamicin and inhibitors as described above. Luminescence was measured every 30 minutes using an Infinite M200 Pro plate reader (Tecan) maintaining constant 37°C temperature and 5% carbon dioxide.

All intracellular growth assays were performed in triplicate for each independent experiment. All of the inhibitors were added 3 hours post inoculation to reduce the impact of the inhibitors on \textit{F. tularensis} phagosomal escape.

**Growth curves**

Bacterial growth curves of broth cultures were generated by measuring the optical density at 600 nm (OD$_{600}$) every 15 minutes using an Infinite M200 Pro plate reader (Tecan) maintaining constant temperature (37°C). To test toxicity of each drug on Schu S4, the bacteria were grown in CDM overnight, and then diluted to an OD$_{600}$ of 0.05 in CDM containing the indicated inhibitors. CDM glucose substitution media were made without added glucose and 30 mM of the defined amino acid or carbon source. 50 mM MES buffer was added to all CDM media in the glucose substitution experiments.

**Fluorescence microscopy**

For confocal fluorescent microscopy images depicting the number of bacteria in drug treated cells, MEFs were plated at 1×10$^4$ cells per well in an 8 well chamber slide (Nunc) and grown overnight. MEFs were inoculated at a MOI of 100 with Schu S4-GFP or Schu S4- DSred and treated with 25 µg/ml of gentamicin as described above. At the indicated time post inoculation, the MEFs were washed and fixed with 4% paraformaldehyde for 15 minutes and then washed again in PBS. To stain the plasma membrane, 10 µg/ml of AF647 conjugated wheat germ agglutinin (Invitrogen) was
added to the fixed cells for 5 minutes and then washed away. DAPI containing mounting media (Vector Shield) was added to the slides to identify the nucleus.

Infection frequency was determined by fixing GFP infected MEFs 5 or 6 hours post inoculation and comparing the number of cells containing green puncta to the total number of cells completely within the field of view.

To quantify LC3B puncta, GFP-LC3 MEFs were generated by transfecting MEFs attached to an 8 well chamber slide (Nunc) with an eGFP-LC3 plasmid (Addgene plasmid 21073) [26]. 18 hours after transfection, the media was replaced with fresh media for one hour. After one hour, the cells were either infected with Schu-DSred or placed in fresh media. 3 hours post inoculation, the media in all wells was replaced with media containing 25 µg/ml gentamicin. 14 hours post inoculation, Torin1 or media lacking amino acids was added to the appropriate wells. The cells were fixed as above and stained with a mouse anti-GFP antibody (1:250 dilution, Millipore) followed by an AF488 anti-mouse secondary antibody (Invitrogen) as previously described.

To quantify acidic vacuoles and determine co-localization with polyubiquitin and p62, MEFs were initially prepared as described above but were incubated for 2 hours in the presence of 150 ng/ml of LysoTracker red (Invitrogen) beginning at 14 hours post inoculation. The cells were washed and MEF media was added for an additional 10 minutes at 16 hours post inoculation. The cells were fixed in 4% paraformaldehyde and treated with 10 mM ammonium chloride following fixation. The MEFs were incubated with a polyubiquitin antibody (1:1000 dilution, Enzo Life Sciences) or a p62/SQSTM1 primary antibody (1:250 dilution, Abnova) followed by an AF647 conjugated anti-mouse
secondary antibody (Invitrogen). DAPI containing mounting media (Vector Shield) was added to the slides to identify the nucleus. Images were acquired using a Zeiss 700 confocal laser scanning microscope (Carl Zeiss SMT, Inc.). Image acquisition, contrast adjustments, and cropping were all performed using Zen 2011 (Carl Zeiss SMT, Inc.).

Acidic vacuoles, p62, and polyubiquitin puncta were quantified by setting thresholds using ImageJ [36]. Only polyubiquitin puncta outside of the nucleus were counted. Co-localization of p62 or ubiquitin puncta with acidic vacuoles was determined by manual counting overlap. Any acidic vacuole or bacteria that overlapped any portion of the puncta was considered to co-localize.

To determine the distance between acidic vacuoles and \textit{F. tularensis}, Z-stacks of LysoTracker red stained cells were taken using a Flow View 500 confocal laser scanning microscope (Olympus America). The distance between the bacteria and the acidic vacuoles was determined using ImageJ [36]and Corsen [37], following the protocols described in Jourdren et al. Additional protocol information and ImageJ plugins were available at http://transcriptome.ens.fr/corsen. The distance between objects was measured from the surface of the bacteria to the closest surface of the nearest acidic vacuole. To decrease the impact of noise, acidic vacuoles and bacteria with a volume of less than 0.05 µm (as determined by the Corsen program) were not included in the analysis.

\textbf{Radiolabel experiments}

To monitor transfer of amino acids from the host cell to \textit{F. tularensis}, \(4 \times 10^5\) MEFs were incubated in cysteine and methionine free DMEM containing 10% dialyzed FBS.
and 0.125 mC of $^{35}$S radiolabelled cysteine and methionine (EasyTag Express $^{35}$S, Perkin-Elmer) for 18 hours. 10 µg/ml of cycloheximide was added with the radiolabel in the indicated sample. The MEFs were then washed once and then incubated with DMEM containing 10% FBS for 2 hours. DMEM contains in excess of 100,000 times more cysteine and methionine than the initial radiolabel. The MEFs were then inoculated with $F. tularensis$ Schu S4 at an MOI of 100 for 3 hours in fresh media. At 3 hours post inoculation, the media was replaced with media containing 25 µg/ml of gentamicin and either Baf or 3MA, as indicated, and supplemented with either a 12 mM amino acid mixture or 18 mM serine. The cells were washed in PBS, scraped from the plate, and lysed by vortexing the in PBS 16 hours post inoculation. The cell lysates were mixed with streptavidin coated magnetic beads (Solulink) that were pre-bound to biotinylated anti-$F. tularensis$ lipopolysaccharide antibody (US biological). The anti-$F. tularensis$ LPS antibody was biotinylated using a Biotin-xx protein labeling kit following the manufacturer's instructions (Invitrogen). The bead lysate mixture was incubated at room temperature for 20 minutes and then washed three times on a magnet. After the final wash, an equal volume of beads was added to 20% trichloroacetic acid (TCA) to make a final concentration of 10% TCA. The TCA mixture was mixed with an equal volume of 5% BSA and spun to pellet the TCA insoluble fraction. The TCA soluble fraction was removed and the TCA insoluble fraction was resuspended in PBS, added to scintillation fluid, and the number of counts was measured. An aliquot of the sample after the final wash was plated on chocolate agar to determine the number of bacteria present. The percent of radiolabel that was incorporated into $F. tularensis$ was
calculated by dividing the radiolabel counts from samples taken immediately prior to infection by the difference between the infected and uninfected samples.

To evaluate host protein degradation, J774 cells were radiolabeled for 24 hours, chased with non-radioactive media, inoculated and treated with gentamicin as described above. At 16 hours post inoculation, the cells were washed in PBS and lysed in RIPA buffer. The lysate was spun immediately to pellet the insoluble fraction. The soluble fraction was harvested and added to an equal volume of 20% TCA. The TCA insoluble fraction was then prepared and quantified as above.

**Electron microscopy**

Uninfected and Schu S4 infected J774 cells or ATG5\(^{-/-}\) MEFs were maintained on small plastic tissue culture dishes. 25 µg/ml of gentamicin was added 2 hours post inoculation for J774 cells and 3 hours post inoculation for MEFs. 16 hours post inoculation the cells were fixed for 1 hour at room temperature in 2% paraformaldehyde, 0.5% glutaraldehyde in 0.15 M sodium phosphate buffer at pH 7.4. The cells were then rinsed in buffer and post-fixed with 0.5% osmium tetroxide/0.15 M sodium phosphate buffer, pH 7.4, for 10 minutes.

TEM samples for J774 cells were prepared similarly, although the cells were post-fixed for 1 hour in 1% osmium tetroxide in 0.15 M sodium phosphate buffer at pH 7.4 and then stained en bloc with 2% aqueous uranyl acetate for 20 minutes.

Both fixed samples were dehydrated in ethanol (30%, 50%, 75%, 100%, 5 minutes each step) and infiltrated and embedded in L.R. White Resin (Electron Microscopy Sciences). The dehydrated samples were sectioned en face (parallel to the
substrate) at 70 nm, mounted on 200 mesh nickel grids, and post-stained with 4% uranyl acetate followed by Reynolds' lead citrate. Samples were observed with a LEO EM910 transmission electron microscope operating at 80 kV (Carl Zeiss SMT, Inc.) and digital images were acquired using a Gatan Orius SC1000 CCD Digital Camera with Digital Micrograph 3.11.0 (Gatan).

**Western blot analysis**

For the phospho- S6 ribosomal protein western blots, J774 cells were inoculated with Schu S4 at an MOI of 100 and treated with 25 µg/ml gentamicin 2 hours post inoculation. The uninfected sample had media replaced and media containing gentamicin added at the same times as infected samples. The uninfected samples were harvested 24 hours post inoculation. At the indicated times, cells were lysed by adding water containing phosphatase (Roche) and protease inhibitor cocktails (Pierce) and vortexing. The lysates were filtered through two 0.22 µm filters, separated on an SDS-PAGE gel under reducing conditions and then transferred to a nitrocellulose membrane. The membranes were probed with rabbit anti- S6 ribosomal protein or rabbit anti-phospho S6 ribosomal protein (Ser 235/236). All primary antibodies were obtained from Cell Signaling Technologies. Membranes were then probed with a horse radish peroxidase conjugated goat anti-Rabbit IgG (KPL) and bands were detected using an ECL Western Blotting Detection Kit (GE Life Sciences). Densitometry analysis was performed using ImageJ and comparing the amount of phosphor S6 ribosomal protein to the total amount of S6 ribosomal protein at the same time point [36]. The densities were then normalized to the uninfected sample.
Data analysis

Fold change was determined by subtracting each sample from the average of 3 samples taken at 5 hours post inoculation and a Mann-Whitney test was used to determine significance. The rest of the bacterial proliferation assays were pooled across experiments, log_{10} transformed, and then analyzed by a two-tailed Student's t-test were used to measure statistical significance. Significance for bacterial kinetic experiments was performed by pooling the maximum luminescence of each replicate for each experiment and performing a Mann-Whitney test. All luminescence and bacterial proliferation experiments were performed in triplicate in each experiment unless otherwise stated. Statistical significance for the distance measurement between \( F.\) \textit{tularensis} and acidic vacuoles was performed using a two tailed Student's t-test on the pooled distance measurements across all 3 experiments for each sample. Significance for radiolabel incorporation into \( F.\) \textit{tularensis} was determined by a Mann-Whitney test.

Morphology analysis was performed on the transmission electron micrographs by outlining the whole cell, nucleus, and each bacteria or autophagic vacuole in ImageJ to determine the area of each [36]. Morphology was determined with the aid of the following references [38]–[40]. Any rips in the slice were excluded from this analysis. Each micrograph depicted the nucleus and all infected cells had at least one bacteria present in the slice. The area of cytoplasm was determined by subtracting the area of the nucleus and bacteria from the area of the whole cell. At least 20 cells of each sample were examined and significance was determined by a two tailed Student's t-test.
Figure 3.1: Autophagy Derived Nutrients Enhance *F. tularensis* Intracellular Growth. (A) Number of intracellular *F. tularensis* 5 and 24 hours post-inoculation of MEFs cultured in DMEM with or without amino acids (mean +/- SD, 4 independent experiments). (B) Number of intracellular *F. tularensis* 5 and 32 hours post-inoculation of untreated and 3MA treated MEFs with or without amino acid supplementation (AA) (mean +/- SD, 3 independent experiments). (C) Representative confocal microscopy images of infected MEFs 32 hours post inoculation that were untreated, 3MA treated or each treatment with amino acid supplementation. Each scale bar represents 10 µm. GFP- Schu S4 bacteria are depicted in green, DAPI (nucleus) in blue, and wheat germ agglutinin (WGA) (plasma membrane) in red. (D) Intracellular bacterial proliferation from 5 to 24 hours post-inoculation of MEFs transduced with a scrambled control or one of two different shRNA's to Beclin-1 (mean +/- SEM, 5 independent experiments). (E) The
number of intracellular *F. tularensis* 4 and 24 hours post inoculation of untreated or 3MA treated hMDMs with or without amino acid supplementation (AA) (mean +/- SD, 4 independent experiments). (* p<0.05, **p<0.01).
Figure 3.2: Autophagic Flux and Transfer of Amino Acids in *F. tularensis* Infected Cells. (A) $S^{35}$ counts in the TCA insoluble fraction of uninfected or infected J774 cells 16 hours post inoculation (mean +/− SEM, 3 independent experiments). (B) $S^{35}$ counts in the bead purified *F. tularensis* fraction that was TCA insoluble from either uninfected MEFs or *F. tularensis* infected MEFs exposed to the indicated treatments (mean +/− SEM, 6 independent experiments) (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).
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other carbon source (each point represents an average of triplicate wells, 3 independent experiments). (B) Number of intracellular *F. tularensis* 5 and 24 hours post-inoculation of untreated or Baf treated MEFs. MEFs were supplemented with a 12 mM amino acid mixture, 15 mM serine, or 18 mM pyruvate (mean +/- SD, 3 independent experiments). (C) Representative confocal microscopy images of infected MEFs 24 hours post inoculation that were untreated, Baf treated or each condition plus amino acid supplementation. Each scale bar represents 10 μm. GFP- Schu S4 bacteria are depicted in green, nuclei (DAPI) in blue, and wheat germ agglutinin (WGA) (plasma membrane) in red. (***p<0.001).
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amino acid supplementation (each point represents an average of triplicate wells) as measured by luminescence (3 independent experiments). (D) Maximum luminescence values expressed in relative light units (RLUs) from kinetic growth assays for Schu S4 LUX infected J774 cells untreated and treated with brefeldin A (4 independent experiments). (* p<0.05, ** p<.01, *** p<0.001).
Figure 3.6: *F. tularensis* Induces ATG5-Independent Autophagy in Infected Cells.

Representative transmission electron micrographs of (A) uninfected and (B) *F. tularensis* infected ATG5<sup>−/−</sup> MEFs. (C) Higher magnification of representative infected MEF. *F. tularensis* is depicted with open faced arrows (>) and autophagosomes with solid arrows (▸). All scale bars represent 0.5 um. (D) The percentage of cytoplasm that is autophagic in ATG5<sup>−/−</sup> MEFs in uninfected and infected cells (• represents 1 cell, n≥20 per sample). (E) The number of acidic vacuoles per cell in wild type and ATG5<sup>−/−</sup> MEFs. MEFs were uninfected, infected, or infected and treated with 3MA (mean +/- SEM, n>30 cells per sample from 6 independent experiments). (* p<0.05, **p<0.01, ***p<0.001).
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Figure 3.8: p62/SQSTM1 and Polyubiquitin Puncta in *F. tularensis* Infected Cells. 

(A) The number of polyubiquitin puncta in the cytoplasm of uninfected and *F. tularensis* infected wild type and ATG5\(^{-/-}\) cells 16 hours post inoculation (* represents 1 cell, n\(\geq\)25 per sample, 3 independent experiments). (B) The number of acidic vacuoles that co-localized with a polyubiquitin puncta per cell in uninfected and *F. tularensis* infected wild type MEFs 16 hours post inoculation (mean +/- SEM, n>25 cells
per sample, 3 independent experiments). (C) The numbers of p62/SQSTM1 puncta per cell in uninfected and F. tularensis infected wild type and ATG5−/− cells 16 hours post inoculation (• represents 1 cell, n≥35 per sample, 3 independent experiments). (D) The numbers of p62 positive acidic vacuoles in wild type or ATG5−/− MEFs that were untreated, infected, or infected and treated with 3MA where infected samples were enumerated 16 hours post inoculation (mean +/− SEM, n>30 cells per sample, 3 independent experiments). (ns p>0.05, * p<0.05, **p<0.01, ***p<0.001).
Figure 3.9: (S1) *F. tularensis* Replicates to High Densities in the Host Cell

**Cytoplasm.** Representative transmission electron micrographs depicting (A) uninfected or (B) infected MEFs at 16 hours post inoculation. The scale bars represent 5 µm.
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Figure 3.12: (S4) Beclin-1 shRNA depletes Beclin-1 mRNA in MEFs. qRT-PCR quantification of Beclin-1 mRNA in MEFs transduced with a lentivirus encoding a Beclin-1 or scramble shRNA. KD-1 and KD-2 are independently derived lines transduced with different Beclin-1 shRNA's. Results were normalized to GAPDH and are expressed as percent of the scramble control.
Figure 3.13: (S5) *F. tularensis* Infection Decreases Polyubiquitin Puncta but Increases the Number of p62\(^+\) Acidic Vacuoles. Representative fluorescence confocal microscopy images of (A) uninfected and (B) infected wild type MEFs depicting polyubiquitin. Representative fluorescence confocal microscopy images of (C) uninfected, (D) infected, or (E) infected 3MA treated wild type MEFs stained for p62/SQSTM1. Scale bars represent 10 µm at the low magnification and 2 µm for the higher magnification inset. Nuclei (DAPI) is depicted in blue, GFP-Schu is depicted in green, acidic vacuoles are depicted in red, and polyubiquitin or p62/SQSTM1 are depicted in white.
Figure 3.14: (S6) *F. tularensis* Localizes Adjacent to Autolysosomes.

Representative transmission electron (TEM) microscopy images of Schu S4 (open faced arrows [>] adjacent to an autophagosome (solid arrows [•]) in (A) J774 cells or (B) ATG5⁻/⁻ MEFs 16 hours post inoculation. The scale bar for the TEM micrograph represents 200 nm. Representative compiled Z-stack images showing the distance (yellow line) between Schu S4 (green) and acidic vacuoles (red) in (C) wild type untreated, (E) ATG5⁻/⁻ untreated or (G) wild type 3MA treated MEFs 16 hours post inoculation. Scale bars for the 3D images represent 10 µm. The distance between Schu S4 and the closest acidic vacuole in (D) untreated wild type (n = 342 bacteria), (F) ATG5⁻/⁻ (n = 401 bacteria) or (H) 3MA treated wild type (n = 194 bacteria) MEFs. The distribution histograms are pooled from 3 independent experiments.
Table 3.1: Quantitative RT-PCR primer sequences. Primer sequences for assaying the amount of Beclin-1 or GAPDH mRNA in lentiviral transduced MEFs by qRT-PCR.

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<th>Primer</th>
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<td>Beclin-1 Forward</td>
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<tr>
<td>Beclin-1 Reverse</td>
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<td>GAPDH Forward</td>
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<tr>
<td>GAPDH Reverse</td>
<td>ATGCCAGTGAGCTCCGTCAG</td>
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REFERENCES


33. McArdle J, Moorman NJ, Munger J (2012) HCMV targets the metabolic stress response through activation of AMPK whose activity is important for viral replication. PLoS Pathog 8 ((1)) e1002502.


CHAPTER 4: DEFINING THE HOST-DERIVED CARBON SOURCES AND METABOLIC PATHWAYS REQUIRED FOR *FRANCISELLA TULARENSIS* IN VIVO GROWTH

**Overview**

Carbon metabolism is a fundamental process essential for growth of all living things and the encoded metabolic capacities of organisms, including bacterial pathogens, to assimilate specific carbon sources are generally well understood. However, within a host, the specific carbon metabolic pathways and host derived carbon sources acquired by pathogens essential for replication are poorly understood. *Francisella tularensis*, a Gram negative bacterial pathogen, quickly replicates to high densities in the cytosol of host cells indicating the bacterium efficiently acquires significant host derived nutrients, including sources of carbon, to fuel rapid proliferation. Therefore, we aimed to identify essential bacterial carbon metabolic pathways and major host derived carbon sources required for *F. tularensis* replication *in vivo*. We found that the gluconeogenic genes *glpX* and *pckA* were essential for *in vivo* bacterial replication. Furthermore, *glpA* and *gdhA* were essential for growth in bone marrow derived macrophages demonstrating that *F. tularensis* requires *glpA* and *gdhA* for acquiring gluconeogenic carbon essential to intracellular growth.
Introduction

In order to proliferate in a host, pathogens must both survive host immune defenses and obtain all essential nutrients for replication. A vast number of studies have focused on how pathogens subvert the host immune system, while surprisingly few studies focus on how pathogens acquire the large quantities of essential nutrients for replication from the nutrient restricted host spaces. Pathogens can acquire essential nutrients by direct import from the host, salvaged from a similar imported host molecule or generated de novo using host derived sources of carbon, nitrogen, sulfur, iron, etc. A significant portion of precursor metabolites must be synthesized de novo to create essential simple molecules and macromolecules, thus pathogens must import large quantities of host substrates to be used for bulk carbon, nitrogen, sulfur, metals, etc. to fuel proliferation.

To acquire nutrients in the limited host space, pathogens employ targeted strategies to obtain nutrients from specific host sources. Although the mechanisms by which pathogens obtain specific nutrients, such as iron, are well understood, the strategies employed by pathogens to obtain most nutrients are poorly studied. Many studies have demonstrated that pathogens use siderophores, hemoglobin binding molecules, transferrin receptors and high affinity transporters to obtain iron from host sources including free iron, iron storage molecules, metalloenzymes and hemoglobin (2, 19, 24, 28). Conversely, the host derived sources and acquisition mechanisms employed by pathogens to obtain host carbon remain poorly understood. This knowledge gap is particularly surprising as carbon metabolism is a fundamental process, yet the carbon metabolism of nearly all pathogens in vivo is poorly understood.
In order to replicate, a bacterium must acquire far more carbon than any other nutrient because carbon functions as the main source of energy and is also required for synthesis of all metabolic precursors and macromolecules. Since pathogens, especially intracellular pathogens, often do not encounter large quantities of freely available carbon sources in the nutrient restricted spaces of the host, many bacteria employ active mechanisms to acquire host carbon. *Mycobacterium tuberculosis* and *Chlamydia tracomatis* secrete lipases that degrade lipid droplets then import the resulting fatty acids for sources of carbon (6, 8, 25). *Salmonella enterica* Typhimurium secretes effector molecules that alter host vesicular trafficking and redirect nutrients, which likely include sources of carbon, to the Salmonella containing vacuole (9, 17). The observation that many pathogens employ active mechanisms to obtain carbon indicates that carbon acquisition by pathogens requires complex host-pathogen interactions and these interactions are only beginning to be elucidated.

Defining the carbon sources for intracellular pathogens is challenging as many pathogens import multiple different host molecules for carbon sources. For example, the intracellular pathogen *M. tuberculosis* consumes several sources of host derived carbon including lipids, cholesterol, glycerol, CO$_2$ and other unidentified metabolites (1, 8, 23, 25). Additionally, bacteria often employ redundant and compensatory mechanisms for importing carbon metabolites as pathogens likely acquire several carbon sources, express transporters with overlapping substrate specificities, use multiple metabolic pathways, and express redundant metabolic enzymes to acquire and metabolize carbon *in vivo*. Nonetheless, defining the specific carbon sources of pathogens in a host will elucidate an understudied aspect of pathogenesis.
Some pathogens are capable of quickly growing to extremely high densities within a host and must robustly and efficiently acquire significant amounts of carbon to fuel proliferation. One bacterium capable of rapid and extensive proliferation in vivo is *Francisella tularensis*. *F. tularensis* is a small Gram negative coccobacillus that is the causative agent of the disease tularemia. *F. tularensis* is a facultative intracellular pathogen that infects both phagocytic and nonphagocytic host cells including macrophages, dendritic cells, epithelial cells, neutrophils, hepatocytes, as well as many other cell types (15, 16, 22, 26). Within each of these cell types, *F. tularensis* quickly grows to high densities and it is commonly reported that the organism can replicate 1000-fold within 24 hours in a single host cell (11, 29). Upon internalization by a host cell the bacterium rapidly escapes the phagosome and accesses the cytosol (5). It is within the cytosolic compartment that *F. tularensis* extensively replicates and from which the bacterium must acquire large quantities of nutrients for proliferation.

We have previously shown that *F. tularensis* induces host autophagy, which produces nutrients used by the bacterium as a carbon or energy source and that this nutrient production from host autophagy is required for intracellular growth (29). However, the specific nutrient sources assimilated by *F. tularensis* that fuel rapid bacterial growth in the host cytosol remain unknown. We think that *F. tularensis* represents an excellent model for studying the in vivo carbon metabolism of bacterial pathogens for several reasons. First, the pathogen has adapted its metabolism to efficiently acquire and assimilate significant quantities of host derived carbon to fuel its exceptional capacity to quickly replicate to high intracellular densities. Second, the host cytosol does not contain sufficient nutrients for bacterial replication as the bacterium
modulates host processes, such as autophagy, to amass significant carbon for growth. Lastly, \textit{F. tularensis} likely encodes a small and decaying genome to transition into an obligate intracellular lifecycle (4). Thus, \textit{F. tularensis} likely contains a relatively simple set of carbon metabolic pathways focused for growth in the intracellular space. To define the host derived nutrient sources that allow the extremely high growth of \textit{F. tularensis} and help elucidate this surprisingly large gap in knowledge of bacterial pathogenesis, we aimed to define the essential carbon metabolic pathways and carbon sources required for \textit{F. tularensis} growth \textit{in vivo}.

\textbf{Materials and Methods}

\textbf{Bacterial Strains}

\textit{Francisella tularensis} subspecies \textit{tularensis} strain Schu S4 was obtained from BEI resources. \textit{F. tularensis} was maintained on solid chocolate agar medium supplemented with 1\% IsoVitaleX (chocolate agar), solid modified Mueller-Hinton agar supplemented with 1\% Tryptone, 0.5\% NaCl, 0.05\% L-cysteine freebase, 1\% glucose and 0.00025\% Fe pyrophosphate (MMH agar), liquid Brain Heart Infusion (BHI) media supplemented with 1\% IsoVitaleX, Chamberlains Defined Media (CDM) or modified CDM as described (3). For selection, 10\(\mu\)g/mL kanamycin, 10\% sucrose or 200\(\mu\)g/mL hygromycin B were added to chocolate agar. All cloning was performed in \textit{Escherichia coli} DH5\(\alpha\) and \textit{E. coli} S17-1\(\lambda\)pir was used to mate suicide vectors into \textit{F. tularensis}. \textit{E. coli} strains were maintained on Luria Bertani medium containing 50\(\mu\)g/mL kanamycin or 200\(\mu\)g/mL hygromycin B.
**Eukaryotic Cell Culture**

J774A.1 cells (ATCC TIB-67), a mouse macrophage-like cell line, were maintained in Dubelco’s Modified Eagle Medium (DMEM) (Gibco) with 4.5g/L glucose supplemented with 2mM L-glutamine, 1mM sodium pyruvate and 10% fetal bovine serum. Bone marrow derived macrophages (BMDMs) were prepared from C57BL6 mice (Jackson Labs). Femurs were isolated, flushed and recovered cells were plated in Petri dishes for 7 days in L929 cell conditioned DMEM containing 10% fetal bovine serum. For glucose free conditions DMEM was prepared using the recipe as described from (Gibco) without glucose or sodium pyruvate. The glucose free DMEM was supplemented with 10% dialyzed serum and used for infections with both J774s and BMDMs.

**Bacterial Genetics**

Markerless, in-frame deletions were created for all genetic deletions in *F. tularensis* except *glpA*. All suicide vectors were created using pEDL50, a modified version of pMP812 that contains an origin of transfer for mating into *Francisella* (21). All suicide vectors were mated into *F. tularensis* Schu S4 via *E. coli* S17λpir by mixing the bacteria and plating on LB agar overnight. The mixture was then plated on Chocolate agar or MMH agar with 10μg/mL kanamycin and 200μg/mL polymyxin B to select for primary integrants. These primary integrants were then grown overnight without selection to allow recombination and plated on chocolate agar containing 10% sucrose to enrich for *sacB* deletion recombinants. Verification of the deletion was determined by PCR and sequencing (Genewiz). The *glpA* gene was disrupted using the Targetron
system modified for use in *Francisella* (27). The suicide vector was created as described using primers assigned by the Targetron Primer design program (Sigma). The vector was transformed into the Schu S4 and the mutant was isolated as described (27).

Complementation plasmids were created by cloning genes with their native promoter into pJB3. Genes lacking an obvious native promoter were cloned into pJB2 which is a version of pJB3 that contains a *pblaB* promoter driving expression of the target gene (Chapter 2). Inducible, HA tagged expression constructs were cloned into pEDL17 and expressed as described (20). Expression and complementing vectors were transformed into *F. tularensis*. For transformations, *F. tularensis* was grown overnight in liquid BHI, washed three times with 0.5M sucrose and electroporated in a 1mm gap cuvette at 2kV, 25μF and 200Ω. The transformants were recovered for 3 hours shaking in BHI at 37°C then plated on chocolate agar or MMH agar with appropriate selection.

**Growth Curves**

Overnight cultures were resuspended and diluted to OD\(_{600}\) 0.05 and grown in 200μL of CDM or modified CDM a 96 well dish. All CDM contained 50mM MES at pH 6.2 to supplement the buffering capacity of the media to account for ammonia production from amino acid catabolism in experiments where modified CDM contained amino acids as a main carbon source. Each major carbon sources was added to a final concentration of 0.4%. Bacteria were grown at 37°C with orbital shaking in a TECAN M200 Pro (TECAN) and OD\(_{600}\) were read every 15 minutes for 48 hours.
**Macrophage Infections**

J774 macrophages or BMDMs were plated in a 96 well white walled/white bottom tissue culture treated plate at 100,000 cells/well or 75,000 cells/well respectively. *F. tularensis* harboring a luminescence reporter plasmid (Chapter 2) was used to infect macrophages at a multiplicity of infection 100 in 50μL/well for two hours. The inoculation media was removed and replaced with 200μL of media containing 25μg/mL (J774s) or 10μg/ml (BMDM) gentamycin for the remainder of the experiment. The plate was incubated at 37°C and 5% CO$_2$ in a TECAN M200 Pro and luminescence was measured every 15 minutes for 48 hours to measure intracellular growth.

**Mouse Infections**

Groups of 6-8 week old female C57BL6 mice (Jackson Labs) were inoculated intranasally with approximately 100 CFU of *F. tularensis*. Infected and control mice were housed in a recirculating air Techniplast system (Techniplast) within a BSL-3 facility. At 3 days post infection mice were sacrificed and the lungs, livers and spleens were harvested and homogenized using a Biojector (Bioject). The homogenates were serially diluted and plated onto chocolate agar or MMH agar to quantify organ burdens.

**Results**

**A Targeted Mutagenesis of *F. tularensis* Carbon Metabolic Pathways Identified**

**Gluconeogenesis is Essential for Intracellular Growth**

To define the intracellular carbon metabolism of *F. tularensis*, we focused on identifying the *F. tularensis* carbon metabolic pathways required for intracellular growth.
We first aimed to identify a *F. tularensis* mutant deficient for a major carbon metabolic pathway that was significantly attenuated for intracellular growth (Figure 1 Box A). The first metabolic genes tested to be required for virulence were *pfkA* (1), which is required for glycolysis, and *glpX* (2) which is required for gluconeogenesis. The GlpX (2) protein performs the opposite reaction as PfkA (1). Deletion of *pfkA* (1) would prevent *F. tularensis* from converting glucose or glucose 6-phosphate (G6P) imported from the host for energy and anabolic precursors while deletion of *glpX* (2) would prevent *F. tularensis* from converting several carbon sources into G6P, an essential precursor metabolite for the pentose phosphate pathway and *de novo* synthesis of lipopolysaccharide, peptidoglycan, pentoses and nucleotides. We hypothesized that if glucose represented a major carbon source for *F. tularensis* then *pfkA* (1) would be required for intracellular growth while *glpX* (2) would be essential if glucose was unavailable to the bacterium and therefore required gluconeogenesis to produce significant levels of G6P.

To confirm the predicted functions of *pfkA* (1) and *glpX* (2) for glycolysis and gluconeogenesis respectively, markerless, in-frame deletions were created for *pfkA* (1) and *glpX* (2) and grown in defined media containing either glycolytic or gluconeogenic carbon sources (Figure 2ABC). As predicted, a Δ*pfkA* (1) mutant did not grow using glucose, a glycolytic carbon source, but did grow on the gluconeogenic carbon source glutamate to levels equal to that of wild type Schu S4 (Figure 2B). Conversely, a Δ*glpX* (2) mutant grew using glycolytic, but not gluconeogenic, carbon substrates (Figure 2C). The growth defects of each mutant were fully restored to wild type levels of growth when the mutations were complemented *in trans* (Figure 2BC).
**F. tularensis requires glpX (2) to Acquire Host Derived Carbon in Macrohages**

We then wanted to determine the requirement of pfkA (1) and glpX (2) in intracellular growth. To measure intracellular growth we used F. tularensis strains expressing a luminescence reporter previously described (Chapter 2) and measured luminescence every 15 minutes over 48 hours. Increases in intracellular growth were directly proportional to increases in reporter luminescence (Chapter 2). BMDM were infected with wild type Schu S4, the ΔpfkA (1) mutant or the ΔglpX (2) mutant, with each harboring a plasmid expressing the luminescence reporter. After 24 hours growth, the wild type and the ΔpfkA (1) mutant grew to similar levels within BMDM while no growth was observed for the ΔglpX (2) mutant (Figure 2D). Furthermore, growth of the ΔglpX (2) mutant was restored when complemented in trans with a plasmid expressing glpX (2) (Figure 2D). These data indicate that F. tularensis glycolysis is dispensable for F. tularensis intracellular growth, while gluconeogenesis is essential. Additionally, these data suggest host glucose does not function as a major carbon source for F. tularensis and the bacteria exclusively use gluconeogenic carbon sources for replication in host cells.

We hypothesize that a glpX (2) mutant strain cannot replicate in host cells because the mutant cannot produce sufficient G6P from carbon sources acquired from the host cell. Therefore, supplementation of glucose or G6P to the glpX (2) mutant in host cells should rescue growth of the mutant strain. J774A.1 (J774) cells are a transformed macrophage cell line constitutively expressing c-Myc, which undergo increased aerobic glycolytic metabolism and must import large quantities of glucose (7). We infected J774 cells with wild type Schu S4 a ΔpfkA (1) mutant or a ΔglpX (2) mutant
and supplied the infected cells with either high glucose DMEM, which contains 4.5 g/L glucose, or glucose free DMEM and measured bacterial growth over 24 hours (Figure 2EF). Wild type Schu S4 and the ΔpfkA (1) mutant strain exhibited significant growth in J774s with or without glucose supplementation. The ΔglpX (2) mutant strain did not replicate in J774s supplied with glucose free DMEM as expected, but intracellular growth was rescued in the presence of high glucose DMEM. These data indicate that the ΔglpX (2) mutant does not acquire adequate carbon to replicate in BMDMs and J774s supplied glucose free DMEM and suggest that the ΔglpX (2) mutant assimilates a glycolytic carbon source that is only available in J774 cells supplied with high glucose DMEM.

The rescue of the ΔglpX (2) mutant does not occur in BMDMs as all BMDM infections were performed in DMEM containing 4.5g/L glucose. This observation suggests that the reduced glucose import and glycolytic flux exhibited by BMDMs is insufficient to permit intracellular F. tularensis from acquiring significant glucose for intracellular growth. This observation correlates with the data that pfkA (1), and therefore glycolysis, are dispensable for intracellular growth if glucose is not available to intracellular F. tularensis in primary cells. Future experiments to directly test whether intracellular F. tularensis acquires glucose in J774 cells and BMDMs are described in Appendix 2.

**glpX (2) and not pfkA (1) is required for in vivo F. tularensis growth**

We next tested whether F. tularensis similarly relied on glpX (2) and not pfkA (1) for replication in host tissue using a murine pulmonary infection model. Groups of C57BL/6 female mice were infected with approximately 100 CFU of wild type, ΔpfkA (1)
or ΔglpX (2) Schu S4 strains. 3 days post infection the lungs, livers and spleens were harvested and dilution plated to determine organ burdens (Figure 3). Strikingly, organ burdens of the ΔpfkA (1) strain were similar to that of the wild type strain in all organs. However, the number of CFU recovered from the lungs of mice infected with the ΔglpX (2) strain was similar to the number of bacteria in the original inoculum and bacteria were below the limit of detection in the livers and spleens of these mice. These data align with the observed in vitro data confirming that glpX (2), and therefore gluconeogenesis, was essential for in vivo growth, while pfkA (1) and glycolysis was dispensable in vivo.

**glpA (3), ppdK (4) and pckA (5) Are Not Individually Required for Assimilation of the Major Carbon Sources Required for F. tularensis in vivo Growth**

Since a ΔglpX (2) strain cannot use a large number of gluconeogenic carbon sources, including glycerol, pentoses, nucleotides, amino acids, lactate, pyruvate and TCA cycle intermediates, we next generated mutant strains unable to use fewer specific anabolic carbon sources. A combination of disrupting three independent genes, glpA (3), ppdk (4), pckA (5), would likely result in preventing *F. tularensis* from converting carbon substrates into G6P similar to a ΔglpX (2) strain (Figure 1 Box 2). glpA (3) is predicted to be required for *F. tularensis* growth using glycerol or glycerol 3-phosphate as major carbon sources. ppdK (3) is predicted to be required for *F. tularensis* to convert lactate, some amino acids and the glycolytic intermediate pyruvate to G6P. pckA (4) and ppdK (3) are involved in gluconeogenesis and can independently be
involved in converting TCA cycle intermediates, and therefore carbon sources that feed into the TCA cycle, eventually into G6P and other essential precursor metabolites.

Growth characteristics of each mutant were analyzed in defined media with specific metabolites to confirm the metabolic function of each gene. A ΔglpA (3) strain did not grow with glycerol 3-phosphate as the major carbon source, while wild type Schu S4 could assimilate glycerol 3-phosphate for replication (Figure 4A). A ΔpckA (5) strain did not exhibit any growth defect on the gluconeogenic carbon source glutamate, while a ΔppdK (4) strain did not grow with glutamate as the major carbon source indicating ppdK (4), and not pckA (5), is primarily used for gluconeogenesis in defined media (Figure 4BC). The growth defects of each mutant were restored to wild type levels when the corresponding genes were complemented in trans (Figure 4ABC).

We then tested ΔglpA (3), ΔppdK (4), and ΔpckA (5) mutants individually for intracellular growth in BMDMs. Interestingly, only glpA (3) was found to be required for intracellular growth in BMDMs (Figure 5A) and growth was restored upon expression of the glpA (3) gene in trans (Figure 5A). These data suggest that significant host carbon requires flux through glpA (3) to support F. tularensis intracellular growth. Interestingly, a ΔppdKΔpckA (4,5) strain replicated within BMDMs suggesting that these gluconeogenic pathways are not required for F. tularensis intracellular growth (data not shown).

We next tested these same mutants for replication in J774 cells with and without glucose (Figure 5B). A ΔglpA (3) mutant replicated in J774s with and without supplemented glucose to intermediate levels indicating that the ΔglpA (3) mutant
replicates in J774 cells and not BMDMs, but addition of glucose cannot rescue full growth of the \( \Delta glpA \) (3) mutant similar to the \( \Delta glpX \) (2) mutant strain. Interestingly, a \( \Delta ppdK \) (4) mutant exhibited significantly reduced intracellular growth in J774s supplied with glucose free DMEM, but replication was restored upon glucose supplementation similar to the \( \Delta glpX \) (2) mutant. These data suggest that \( ppdK \) (4) may contribute to assimilation of host derived carbon sources in J774 cells. Since \( ppdK \) (4) and \( glpA \) (3) are required for replication under different conditions in BMDMs or J774s, these data indicate that \emph{F. tularensis} requires different carbon metabolic pathways for intracellular growth in these systems and suggests that the bacterium may acquire different primary host derived carbon sources in each cell type. Future proposed experiments described in Appendix 2 aim to directly test whether \( glpA \) (3) is required for assimilation of specific carbon sources by intracellular \emph{F. tularensis}.

\textbf{\emph{F. tularensis} pckA (5), but not ppdK (4) is Required for in vivo Growth}

We then tested \( \Delta ppdK \) (4) and \( \Delta pckA \) (5) mutants and a \( \Delta ppdK\Delta pckA \) (4,5) double mutant for \emph{in vivo} replication (Figure 6). Interestingly, the \( \Delta ppdK \) (4) strain grew to similar burdens as the wild type strain in all organs tested, while the \( \Delta pckA \) (5) mutant was minorly attenuated. Further, the \( \Delta pckA \) (5) mutant was similarly attenuated as the \( \Delta ppdK\Delta pckA \) (4,5) double mutant strain indicating that function of \( ppdK \) (4) cannot compensate for lack of \( pckA \) (5) function \emph{in vivo}. This data suggests \( pckA \) (5) may be the primary gluconoeogenic pathway used \emph{in vivo} whereas \( ppdK \) (4) was shown to be used primarily in defined media and J774 cells (Figure 4C, 5B and 6). Since the \( pckA \) (5) single mutant did not display a severe virulence defect similar to the \( \Delta glpX \) (2) strain, multiple carbon sources are likely used by \emph{F. tularensis}. These data suggest that the
lack of gluconeogenic flux through \textit{pckA} (5) can be compensated for by assimilation of carbon though another metabolic pathway, possibly \textit{glpA} (3). The requirement of \textit{glpA} (3) has not yet been tested for \textit{in vivo} growth; however, we predict that a Δ\textit{glpA} (3) mutant will display an \textit{in vivo} growth phenotype similar to a Δ\textit{pckA} (5) mutant as we hypothesize that \textit{F. tularensis} assimilates host derived carbon through both metabolic pathways. Future experiments to further investigate \textit{F. tularensis} intracellular and \textit{in vivo} carbon acquisition through Δ\textit{pckA} (5) and Δ\textit{glpA} (3) are described in Appendix 2.

**Essential Host Derived Carbon Sources Enter the TCA Cycle through \textit{gdhA} (6)**

Since \textit{pckA} (5) is required for \textit{F. tularensis} virulence, we hypothesize that the bacterium requires \textit{pckA} (5) for anabolism of carbon sources acquired from the host. Potential carbon sources \textit{F. tularensis} could consume in host cells that require \textit{pckA} (5) for conversion into G6P (and other precursor metabolites) are amino acids that feed into the TCA cycle or TCA cycle intermediates. To discern between these possibilities, we tested the requirement of \textit{gdhA} (6) for \textit{F. tularensis} intracellular growth (Figure 1 Box C). \textit{gdhA} (6) is predicted to be required for interconversion of glutamate and the TCA cycle intermediate 2-oxoglutarate. \textit{F. tularensis} is predicted to require this enzyme to shuttle several amino acids into the TCA cycle including glutamate, glutamine, proline, arginine and potentially aspartate and asparagine. Therefore, if \textit{F. tularensis} imports amino acids that feed into the TCA cycle and does not directly assimilate TCA cycle intermediates, then a Δ\textit{gdhA} (6) mutant would be required for assimilating host carbon \textit{in vivo} similar to a Δ\textit{pckA} (5) mutant.

To validate the function of \textit{gdhA} (6), we tested the requirement of \textit{gdhA} (6) for growth on glucose, glycerol and glutamate as a major carbon source in defined media.
We found that \textit{gdhA} (6) was required for growth on the gluconeogenic carbon substrate glutamate, but not glucose or glycerol, and that the growth defect was restored when the \textit{gdhA} (6) gene was expressed \textit{in trans} (Figure 7A). These data indicate that \textit{gdhA} (6) is not essential for glutamate synthesis, but is required for growth on glutamate as major carbon source.

Next, a \textit{ΔgdhA} (6) mutant was tested for intracellular growth in BMDMs and we found that \textit{ΔgdhA} (6) is indeed required for \textit{F. tularensis} intracellular growth (Figure 7B) and that growth of the \textit{ΔgdhA} (6) mutant was restored upon expression of \textit{gdhA} (6) \textit{in trans}. We next infected J774 macrophages with a \textit{ΔgdhA} (6) mutant and provided DMEM supplemented with or without glucose to determine the requirement of this gene for \textit{F. tularensis} utilization of host derived carbon sources (Figure 7C). Growth of \textit{ΔgdhA} (6) mutant was rescued to upon supplementation of high glucose DMEM in J774 cells suggesting glucose supplementation provided significant carbon to intracellular \textit{ΔgdhA} (6) mutant bacteria for replication similar to a \textit{ΔglpX} (2) and a \textit{ΔppdK} (4) mutant. Together, these data suggest \textit{ΔgdhA} (6) is required for \textit{F. tularensis} utilization of host derived carbon sources in macrophages. This requirement for \textit{ΔgdhA} (6) in intracellular growth suggest that \textit{F. tularensis} acquires amino acids that feed into the TCA cycle and does not directly acquire TCA cycle intermediates as a major carbon source in host cells. The requirement for \textit{gdhA} (6) \textit{in vivo} has not yet been tested, but we predict that a \textit{ΔghdA} (6) mutant will exhibit a minor growth defect similar to a \textit{ΔpckA} (5) mutant and these future experiments are described in Appendix 2. Further proposed experiments described in Appendix 2 aim to directly test the requirement of \textit{gdhA} (6) for assimilation of specific carbon sources in host cells.
Discussion

To elucidate the carbon metabolism of \textit{F. tularensis} growing in cultured cells and animals, we performed a targeted mutagenesis to identify major carbon metabolic pathways that are essential for \textit{F. tularensis} growth in macrophages. We found that \textit{glpX} (2), required for gluconeogenesis, was essential for bacterial growth \textit{in vivo} and in cultured macrophage cells and that \textit{pfkA} (1), required for glycolysis, was dispensable. Since a \textit{ΔglpX} (2) strain is predicted to be incapable of converting several carbon sources to G6P, we generated mutants that inhibited the anabolism of fewer carbon substrates. We found that \textit{ΔglpX} (2), \textit{ΔglpA} (3) and \textit{ΔgdhA} (6) mutant strains did not replicate in BMDMs but could grow in transformed cells when supplied with DMEM containing 4.5g/L glucose. These data suggest that the major host derived carbon sources enter central carbon metabolism via \textit{glpA} (3) and \textit{gdhA} (6) and the gluconeoegensis gene \textit{glpX} (2) is required to convert these carbon sources into essential \textit{F. tularensis} metabolites.

The requirements for converting carbon sources that enter central carbon metabolism through \textit{gdhA} (6), or other metabolic pathways, via gluconeogenesis differ based on the infection model. \textit{pckA} (5) is required in a murine infection model, while \textit{ppdK} (4) is required in transformed macrophages indicating differences in the bacterial metabolic requirements between these systems. Since transformed macrophages undergo altered metabolism compared to primary cells, it is likely that the intracellular carbon sources available to \textit{F. tularensis} are also altered in these cells. For example, transformed cells produce significant amounts of lactate from increased aerobic glycolysis and it is possible \textit{F. tularensis} assimilates lactate in J774 cells. \textit{ppdK} (4) is
required for *F. tularensis* assimilation of lactate as an anabolic carbon source and may explain the requirement of *ppdK* (4) specifically within this cell line.

Based on the annotated genomic sequence of *F. tularensis* subspecies *tularensis* Schu S4 (18), a Δ*glpA* (3) mutant strain cannot use glycerol or glycerol 3-phosphate as an anabolic carbon source. Interestingly, complementation of the Δ*glpA* (3) mutant strain rescued growth on glycerol 3-phosphate, but not glycerol, thus indicating that an independent polar mutation also blocked growth on glycerol (data not shown). However, our (partially) complemented strain replicated within BMDMs, suggesting that *F. tularensis* acquires glycerol 3-phosphate, and not glycerol in host cells.

A Δ*gdhA* (6) mutant cannot convert glutamine, glutamate, arginine, proline, and possibly aspartate and asparagine into TCA cycle intermediates to be used as anabolic carbon sources or energy generation. The predicted requirement for *gdhA* (6) in converting aspartate and asparagine into TCA cycle intermediates is indirect. The only predicted mechanism for aspartate and asparagine to enter the TCA cycle is through a transaminase that converts aspartate to the TCA intermediate oxaloacetate but simultaneously removes a TCA cycle intermediate by converting a 2-oxoglutarate into glutamate, thus adding and removing a C₄ carbon molecule from the TCA cycle. Therefore, carbon flux through the TCA cycle requires *gdhA* (6) to remove the amino group of glutamate for conversion into 2-oxoglutarate when growing on aspartate and asparagine as major carbon sources. *F. tularensis* can use each of these amino acids as a major carbon source in defined media (data not shown) and it is likely that *F. tularensis* imports several, or possibly all, of these carbon sources from the host for anabolic carbon.
*F. tularensis* requires significant quantities of carbon to replicate to high densities in host cells and how the bacterium manipulates host carbon metabolism, metabolic signaling and cellular processes to produce the levels of amino acids and glycerol 3-phosphate required for extensive intracellular growth is not fully elucidated. We have previously shown host autophagy produces amino acids and sources of bulk carbon for *F. tularensis*. Our data suggests that amino acids and possibly glycerol 3-phosphate are major nutrients assimilated by intracellular *F. tularensis* produced from host autophagy (29). However, autophagy does not contribute all nutrients for *F. tularensis* growth and further studies are needed to elucidate other carbon acquisition mechanisms employed by the bacterium to specifically acquire glycerol 3-phosphate and amino acids (29).

*F. tularensis* is capable of infecting over 250 different host species and can infect a variety of cells within hosts. Therefore, the pathogen must metabolically adapt to these diverse environments in order to replicate. Our data indicates that the carbon sources and metabolic pathways required for replication may vary depending on the host infected, inoculation route and host cell types infected. An interesting possibility is that *ppdK* (4) could be required for assimilating carbon in other *in vivo* systems where the major carbon sources available are similar to those in our cultured macrophage system but not our *in vivo* pulmonary infection model. Based on the annotated *F. tularensis* Schu S4 genome sequence, the use of serine, glycine and threonine, pyruvate and lactate all require *ppdK* (4) to be converted into G6P and other metabolites (18). *F. tularensis* has been shown to assimilate several of these carbon sources *in vitro*, which could function as sources of carbon in other *in vivo* systems (14).
By exploiting the metabolic differences between a primary and transformed macrophage cell line we found that *F. tularensis* likely assimilates glucose or G6P in the transformed J774 cells, but not in primary macrophages or our *in vivo* system. Our observation differs from observations with other intracellular bacterial pathogens including *Listeria monocytogenes, Legionella pneumophila* and enteroinvasive *Escherichia coli* species which have been shown to import glucose or glucose 6-phosphate in host cells (10, 12, 13). One possible reason for the observed differences is that many studies are performed in transformed cell lines, and not primary cell lines, where these pathogens may assimilate glucose specifically within transformed cell lines, similar to *F. tularensis*. Another possible explanation is that other studies are performed in non-macrophage cell lines which may naturally have significant intracellular glucose concentrations. Lastly, other intracellular pathogens may induce host cell glucose uptake thus providing significant glucose for assimilation by the pathogen.

The targeted mutagenesis of *F. tularensis* metabolic pathways allowed us to identify multiple metabolic pathways required for assimilation of host derived carbon sources. Glucose supplementation of J774 cells infected with these mutants rescued intracellular growth indicating the added glucose provided an anabolic carbon source to these mutants allowing bacterial growth. Together, these analyses revealed that gluconeogenesis is essential for acquisition of host derived carbon sources and *in vivo* replication and we propose that glycerol 3-phosphate and amino acids are primary carbon sources acquired by *F. tularensis* in host cells. Overall, these studies begin to unravel the complex nutrient acquisition of *F. tularensis* in host cells and *in vivo*. 

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**Figure 4.1: Francisella tularensis Central Carbon Metabolism.** Each numbered enzyme (red) is required for flux of specific carbon sources (orange) in central carbon metabolism. Green arrows indicate reactions that are specific for gluconeogenesis.
**Box A** contains two enzymes required for major carbon metabolic pathways: PfkA (1) is required for glycolysis and GlpX (2) is required for gluconeogenesis. When glucose is not an available carbon source, *F. tularensis* requires GlpX (2) for conversion of gluconeogenic carbon sources into glucose 6-phosphate (G6P), an essential precursor for synthesis of several molecules. **Box B** contains enzymes that are required to contribute potential sources of gluconeogenic carbon. **Box C** contains TCA cycle intermediates and one enzyme GdhA (6) that is required for conversion of specific amino acids into TCA cycle intermediates.
Figure 4.2: Growth of $\Delta pfkA$ (1) and $\Delta glpX$ (2) in Defined Media and Host Cells.

Growth of **A**) wild type Schu S4, **B**) the $\Delta pfkA$ (1) mutant, the $\Delta pfkA$ (1) complemented strain, **C**) the $\Delta glpX$ (2) mutant and the $\Delta glpX$ (2) complemented strain in CDM containing a specific major carbon source. CDM denotes defined media lacking a major carbon source. **D**) Growth of wild type Schu S4, the $\Delta pfkA$ (1) and the $\Delta glpX$ (2) mutants in bone marrow derived macrophages in high glucose DMEM. Growth of **E**) the $\Delta pfkA$ (1) mutant, **F**) the $\Delta glpX$ (2) mutant and the $\Delta glpX$ (2) complemented strain in J774 cells supplied with either high glucose or glucose free DMEM. All growth curves are representative of at least three independent experiments.
Figure 4.3: *glpX* (2), and not *pfkA* (1), is Essential for *in vivo* Replication. 6 week old female C57BL/6 mice were infected intranasally with about 100 CFU of wild type Schu S4, the Δ*pfkA* (1) or Δ*glpX* (2) mutant strains. Three days post infection lungs, livers and spleens were harvested, homogenized, serially diluted and plated on chocolate agar to determine organ burdens. Data is pooled from three independent experiments. ***p<.001 was determined by Students T test.
Figure 4.4: Growth of ΔglpA (3), ΔppdK (4) and ΔpckA (5) mutants in defined media. A) Growth of wild type Schu S4, the ΔglpA (3) mutant and the ΔglpA (3) complemented strain were grown in CDM containing no major carbon source, glucose or glycerol 3-phosphate. Growth of B) the ΔppdK (4) mutant, the ΔppdK complemented strain and C) the ΔpckA (5) mutant in defined media containing no major carbon source,
glucose or glutamate. All panels indicate the OD$_{600}$ after 36 hours growth shaking at 37°C. CDM denotes defined media lacking a major carbon source. At least three independent experiments were performed for each panel.
**Figure 4.5: Growth of ΔglpA (3), ΔppdK (4) and ΔpckA (5) in Macrophages.**

A) Growth of wild type Schu S4, the ΔglpA (3) mutant and the ΔglpA (3) complemented strain in bone marrow derived macrophages supplied with high glucose DMEM.  

B) Growth of wild type Schu S4, the ΔglpA (3) mutant and the ΔglpA (3) complemented strain in J774 cells supplied with either high glucose or glucose free DMEM.  

C) Growth of wild type Schu S4, the ΔppdK (4) mutant and the ΔppdK (4) complemented strain in bone marrow derived macrophages supplied with high glucose DMEM.  

D) Growth of wild type Schu S4 and the ΔpckA (5) mutant or  

E) wild type Schu S4, the ΔppdK (4) mutant and the ΔppdK (4) complemented strain in J774 cells supplied with our without glucose. Each panel is a representative of at least 3 independent experiments.
Figure 4.6: *pckA* (5), and not *ppdK* (4), is Required for *in vivo* Growth. 6 week old female C57BL/6 mice were intranasally inoculated with about 100 CFU of wild type Schu S4, the Δ*ppdK* (4), Δ*pckA* (5) or Δ*ppdKΔpckA* (4,5) mutants. 3 days post infection the lungs, livers and spleens were harvested, homogenized, serially diluted and plated on chocolate agar to determine organ burdens. *p<0.05, **p<0.01 and ***p<0.001 as determined by Students T test. Data is pooled from three independent experiments.
Figure 4.7: Growth of ΔgdhA (6) in defined media and macrophages. Growth of wild type Schu S4, the ΔgdhA (6) mutant, and the ΔgdhA (6) complemented strain in A) defined media supplied with either no major carbon source, glucose, glycerol or glutamate, B) bone marrow derived macrophages supplied with high glucose DMEM
and C) J774 macrophages supplied with either high glucose or glucose free DMEM. Each panel is representative of at least 3 independent experiments.
REFERENCES


Chapter 5: SUMMARY AND DISCUSSION

Introduction

A hallmark of *F. tularensis* pathogenesis is its extreme virulence in mammalian hosts and the majority of *F. tularensis* research, including studies described in this thesis, focus on understanding the biology and specific host-bacterial interactions that contribute to disease. One striking feature of *F. tularensis* pathogenesis is its ability to infect and quickly replicate within diverse host cells to incredibly high densities. Studies described in this thesis elucidate specific bacterial genetics, metabolism, physiology and interactions with host processes required for replication to high burdens within an infected host cell.

The impact of these studies not only defines *F. tularensis* virulence mechanisms, but also broadly impacts the understanding of bacterial pathogenesis by demonstrating that pathogens require targeted mechanisms to manipulate host processes for acquisition of specific host derived nutrients essential for replication. Specifically, we define the requirement of host autophagy to produce nutrients essential for bacterial replication and suggest that glycerol 3-phosphate and amino acids function as the major carbon sources for intracellular *F. tularensis*. Together these studies describe that complex interactions between the pathogen and host metabolism, cellular processes
and host signaling are required for assimilation of host derived nutrients essential for pathogen proliferation.

**Characterization of Genes of Unknown Function will Identify New *F. tularensis* Biology Required for Pathogenesis**

The development of genetic tools for *F. tularensis* has facilitated the identification of several bacterial factors essential for pathogen virulence and intracellular growth. Chapter 2 describes a bacterial luminescence system that we adapted from Bina et al. to function as reporter for intracellular growth (3). This reporter allowed us to efficiently quantify replication of over 7500 transposon mutants in multiple eukaryotic cell lines. A striking observation from our mutagenesis screen and previously published screens is that *F. tularensis* encodes few virulence specific factors. However, a large portion of annotated *F. tularensis* genes have no known function and many uncharacterized genes have been shown to be required for intracellular growth and virulence. Further investigation has identified roles of these virulence-associated genes in a variety of biological processes including iron acquisition, disulfide bond formation, and Lipid A biosynthesis, among others (18, 23, 24). Defining the function of these uncharacterized genes has both elucidated the requirements for *F. tularensis* pathogenesis as well as defined uncharacterized biological processes used by *F. tularensis* and potentially other organisms.

From the luminescence reporter screen described in Chapter 2, we identified **FTT_0924**, a gene of unknown function to be required for intracellular growth that had not previously been implicated in *F. tularensis* virulence. **FTT_0924** encodes a small, 132 amino acid protein that is highly conserved among *Francisella* species, but has no
homology to known proteins outside of *Francisella*. Further characterization in this thesis (Chapter 2) has identified a requirement for this gene in *F. tularensis* resistance to osmotic stress during bacterial replication, but the exact function of *FTT_0924* remains unknown. We found that the FTT_0924 protein localizes to the inner membrane facing the periplasm, suggesting that FTT_0924 may play a direct role in cell wall stabilization or regulating periplasmic factors required for cell wall integrity (Chapter 2).

*Further Elucidating the Function of FTT_0924 will Help Define how *F. tularensis* Regulates Peptidoglycan Dynamics*

FTT_0924 contains no known conserved domains but is predicted to contain a single alpha helix (besides the predicted signal sequence) with one face of the helix consisting completely of 5 lysine residues. This highly charged helical face suggests to us that FTT_0924 may interact with another protein to perform its function. Therefore, to identify possible protein interactors of FTT_0924 we identified proteins that co-immunoprecipitated with FTT_0924 via mass spectrometry. AcrB, a periplasmic protein which functions as a membrane spanning protein required for Type I secretion was shown to co-immunoprecipitate with FTT_0924 (unpublished data). However, possible interactions between FTT_0924 and AcrB do not likely contribute to the observed deficiencies of a ΔFTT_0924 mutant as a ΔacrB mutant does not exhibit a viability defect when growing in liquid culture or an intracellular growth defect in macrophages (unpublished data, 22). Nonetheless, we hypothesize that specific lysines in the predicted alpha helix of FTT_0924 are required for protein function and
that FTT_0924 interactions with other proteins are required for *F. tularensis* osmotic stress resistance.

Since the sensitivity of a \(\Delta FTT\_0924\) mutant to osmotic stress strongly suggests FTT_0924 somehow affects peptidoglycan structure, we wanted to determine if \(FTT\_0924\) affected expression of penicillin binding proteins (PBPs), proteins that dynamically alter peptidoglycan remodeling. We mixed lysates of wild type Schu S4 and a \(\Delta FTT\_0924\) mutant with bocillin-FL, an ampicillin analog conjugated to a fluorescent dye that binds PBPs, then separated proteins via SDS-PAGE and imaged for fluorescent protein bands. Analysis of whole cell lysates identified a prominent band in the wild type Schu S4 strain that was missing in the \(\Delta FTT\_0924\) mutant strain (unpublished data). The predicted size of the identified bocillin-binding protein band does not match any known *F. tularensis* PBP suggesting a novel PBP, a PBP cleavage product or an off-target bocillin-FL binding protein is affected by the lack of FTT_0924. Studies aimed at identifying the bocillin-FL binding protein missing in the \(\Delta FTT\_0924\) strain and determining whether this protein contributes to the observed defects in the \(\Delta FTT\_0924\) mutant may help define the function of FTT_0924 in peptidoglycan dynamics.

*F. tularensis* effectively adapts to the intracellular environment to efficiently replicate and evade cellular defenses

Interestingly, many genes identified to be most important for *F. tularensis* replication include genes involved in biosynthetic pathways, such as purine synthesis, and bacterial physiological processes such as \(dsbB\), required for facilitating disulfide
bond formation in the bacterial periplasm (23). The importance of these metabolic and physiologic processes in virulence indicates *F. tularensis* adaptation to the host environment may be particularly critical for *F. tularensis* virulence. Although *F. tularensis* actively suppresses the host immune response, the bacterium has also adapted its physiology to evade immune activation (11, 16, 17, 25, 28). *F. tularensis* Lipid A has a noncanonical structure preventing TLR4 activation while *F. tularensis* lipopolysaccharide is required for evading destruction by host xenophagy (4, 5, 21).

Within the host cytosol, virulent strains of *F. tularensis*, but not *F. novicida*, avoid inflammasome activation through Nod-like receptors (NLRs) that sense bacterial DNA (15, 30). This suggests that virulent *F. tularensis* strains regulate their physiology by preventing significant lysis and release of stimulatory molecules in the host cytosol, thus avoiding inflammasome activation.

Chapter 2 identifies a bacterial gene, *FTT_0924*, required for maintaining cell wall and membrane integrity, while replicating in low osmolarity environments, which likely includes the host cell cytosol. Multiplex cytokine array analysis was performed, analyzing supernatants of bone marrow derived macrophages infected with several *F. tularensis* mutants including a Δ*FTT_0924* mutant and a Δ*ripA* mutant. The cytokine profiles of 21 different proinflammatory cytokines were identical between supernatants from BMDMs infected with a Δ*FTT_0924* mutant and a Δ*ripA* mutant, but significantly higher than supernatants from BMDMs infected with wild type Schu S4 (unpublished data). Further validation of proinflammatory cytokine secretion was confirmed via ELISA by detecting increased secretion of IL-1β by Δ*FTT_0924* infected BMDMs compared to wild type Schu S4 infected BMDMs (unpublished data). These data are consistent with
the idea that infection with these mutants results in a general proinflammatory response, and that these genes are not required for suppression of a specific proinflammatory pathway. Together these data suggest that *FTT_0924* is required for maintaining bacterial structural integrity essential for replication and viability within a host, but also to prevent bacterial lysis and release of pathogen associated molecular patterns that would trigger a proinflammatory immune response. These findings further the idea that the ability of *F. tularensis* to expertly adapt to the host cytosol is essential for intracellular proliferation, immune evasion and virulence, and that regulating cell wall integrity during replication, partially via *FTT_0924*, is required for *F. tularensis* adaptation to the cytosolic environment.

Obligate intracellular organisms such as *Wolbachia*, *Chlamydia* and *Mycoplasma* species produce little to no cell wall and are sensitive to osmotic stress indicating peptidoglycan is not essential to resist osmotic stress in host cells for these bacteria. Recent studies have suggested cell wall associated factors are required for cell division for intracellular *Chlamydia* and *Wolbachia* species but are not thought to provide resistance to osmotic stress (19, 29). Since *FTT_0924* is required for resisting turgor pressure, thus implying *FTT_0924* is required for cell wall function, it is somewhat surprising that *F. tularensis* requires *FTT_0924* for maintaining membrane integrity in host cells given other organisms do not require a cell wall to resist osmotic stress in the intracellular space. It is possible that *Francisella* has acquired *FTT_0924* to resist osmotic stress during intracellular replication since *Francisella* oddly experiences turgor pressure in the host cell cytosol.
*F. tularensis* co-opts Host cell Processes for Intracellular Replication

Previous studies have identified that autophagy can be detrimental to *F. tularensis* as mutants unable to produce full length LPS or capsule are degraded by the host autophagic pathway within the cytosol of host cells (3, 4). However, Chapter 3 outlines experiments describing that an ATG5-independent autophagy pathway benefits *F. tularensis* by producing amino acids and other nutrients required for intracellular growth. Our study reveals an interesting situation where host cell autophagy can function detrimentally or beneficially for *F. tularensis* depending on the circumstance. In order to both benefit from interactions with autophagy and prevent xenophagic killing, *F. tularensis* must simply evade degradation by host autophagy, as opposed to actively suppressing host autophagy pathways. *F. tularensis* evasion of xenophagic killing still allows autophagic machinery to degrade host cell components to produce nutrients for bacterial growth, whereas active suppression of autophagy would likely prevent nutrient production from autophagic breakdown of host constituents. Supporting this idea we found that there is an increase in autophagosome formation in infected host cells suggesting *F. tularensis* induces autophagy during infection, thus increasing nutrient production while simultaneously avoiding recognition and killing by xenophagy (Chapter 3).

The Signals Resulting in Autophagy Induction during *F. tularensis* Replication are Unknown

Although *F. tularensis* infection activates host ATG5-independent autophagy, the signals that trigger the ATG5-independent autophagic pathway and whether autophagy
activation is actively induced by a *F. tularensis* effector molecule or simply a result of bacterial infection is unknown. Active manipulation of autophagy suggests that *F. tularensis* produces a specific effector molecule that either interacts directly with host autophagic machinery or upstream factors resulting in autophagy activation. Indirect activation of autophagy by *F. tularensis* infection would imply that autophagy results from the host sensing several possible effects of *F. tularensis* infection. Possible indirect autophagy activation mechanisms include direct sensing of *F. tularensis* bacteria from cytosolic immunologic sensors, energy and nutrient depletion from bacterial consumption of host nutrients resulting in a starvation response, host sensing of ammonia production from *F. tularensis* amino acid catabolism or potentially many other signals.

We demonstrate that *F. tularensis* infected cells have increased autophagic flux during late stages of infection (16 hours post infection). However, it is unknown when and how autophagy must be induced to produce significant nutrients for *F. tularensis* replication. A possible mechanism for activating autophagy late during infection may be through mTOR and/or AMPK signaling. We found that AMPK is activated and mTOR activity is suppressed at roughly 12 hours post infection (unpublished data), which should result in activating Ulk1 and inducing autophagy (12). It is also possible that autophagy is initiated earlier than 12 hours post infection and may be initiated as early as initial binding and phagocytosis of *F. tularensis*. It has been demonstrated that pathogen binding to TLR4 results in a signaling cascade that eventually induces autophagy (7, 26). Although *F. tularensis* does not stimulate TLR4, *F. tularensis* does stimulate TLR2, which may also result in autophagy initiation. However, it is unlikely
that TLR signaling accounts for the increased autophagic flux occurring late during infection as extracellular bacteria (the TLR agonist) were removed 2 hours post infection in experiments measuring autophagy induction in host cells (Chapter 3). Further investigation into when these, or other signals, activate autophagy will help define the mechanisms by which *F. tularensis* manipulates host autophagy to provide nutrients for intracellular growth.

**Multiple Autophagic Pathways may Function in *F. tularensis* Nutrient Production**

Further investigation into the cellular biology of the autophagic process has identified several different autophagic pathways which result in degrading specific host constituents. Similarly, a single autophagic pathway does not produce sufficient nutrients for intracellular growth of all *F. tularensis* strains, as we have shown the pathways of autophagy required for intracellular growth vary in a *F. tularensis* strain specific manner. The live vaccine strain (LVS) requires ATG5-dependent autophagy to produce nutrients for optimal growth (unpublished data), while Schu S4 requires an ATG5-independent pathway for nutrient production (Chapter 3). However, it is possible that both strains acquire nutrients from both pathways, but each strain relies more heavily on separate pathways. For example, inhibiting nutrient production from ATG5-dependent pathways in Schu S4 infected cells could be compensated for by increasing nutrient production from an ATG5-independent pathway (but not the inverse). Thus, only ATG5-independent pathways are required for Schu S4 growth, but both pathways can produce nutrients for the bacterium.

The observation that LVS and Schu S4 rely on different sets of autophagic pathways for intracellular growth suggests each strain requires host autophagy to
produce different amounts or different types of nutrients for growth. Even more broadly, it is possible that other intracellular pathogens utilize autophagy for nutrient acquisition and the specific autophagic pathways induced may reflect the specific nutrients produced by autophagy and consumed by the pathogen. Therefore, investigating the requirement of autophagy for virulence of other intracellular pathogens may identify other autophagic pathways that produce nutrients which aid bacterial growth. Further investigation may also identify benefits of host autophagy for pathogens in addition to nutrient acquisition.

**Acquisition of Host Carbon by Intracellular Pathogens Requires Active Processes**

The cytosolic space is becoming more appreciated as a nutrient limiting environment for intracellular pathogens as more examples of modulation of host metabolic processes by pathogens are being discovered. This trending phenomenon indicates that pathogens do not simply import all free essential nutrients for replication, but must employ targeted mechanisms to acquire specific host derived nutrients. The concept that the host cytosol is a nutrient limiting environment is not new, yet very few nutrients are recognized as unavailable to pathogens. For example, it is well understood that iron is a limiting nutrient in host cells and pathogens actively acquire host derived iron from specific sources. Iron acquisition molecules such as siderophores and heme binding proteins are used by pathogens to acquire bound host iron from metalloenzymes, iron storage molecules and hemoglobin that would otherwise be unavailable to the pathogen (14, 20, 27). In contrast, host derived sources of other essential nutrients, such as carbon, are not well understood and neither are the strategies that pathogens use to acquire host carbon.
We have uncovered a novel mechanism by which an intracellular pathogen subverts a host process (autophagy) to acquire source of bulk carbon and amino acids essential for *F. tularensis* growth. Although, to our knowledge, this is the first study to demonstrate that autophagy provides a pathogen with a source of carbon, other intracellular pathogens have been shown to acquire host derived carbon by manipulating other host processes. Relatively few, but diverse, carbon acquisition mechanisms have been identified from other intracellular pathogens. Two examples include *Mycobacterium tuberculosis*, which secretes lipases to degrade host lipid droplets and imports the freed fatty acids and *Salmonella enterica* serovar Typhimurium, which alters vesicular trafficking to redirect nutrients to the Salmonella containing vesicle (6, 13). Considering that all intracellular bacteria must acquire host derived carbon for replication, it is likely that many more mechanisms of host nutrient acquisition have yet to be discovered.

All organisms require more carbon than any other nutrient and since *F. tularensis* rapidly replicates to extremely high densities, the bacterium must efficiently obtain vast quantities of carbon in the host cytosol to proliferate. Although autophagy contributes significant sources of nutrients to *Francisella* species, it is likely the bacterium employs other mechanisms for co-opting host processes to acquire carbon for fueling bacterial growth. Investigating how *F. tularensis* infection affects other potential sources of host carbon, including degradation of host macromolecules, increasing host cell nutrient import and altering flux through host metabolic pathways may reveal more mechanisms by which *Francisella* species manipulate the host to acquire sources of carbon.
Carbon Sources Acquired by Intracellular Pathogens are Diverse, yet Specific

Studies described in Chapter 3 illustrate how F. tularensis assimilates autophagy derived amino acids, which are directly incorporated into protein, and major sources of bulk carbon. However, whether amino acids or other nutrients provide the essential sources of carbon for F. tularensis proliferation was not determined. Therefore, we aimed to further identify the carbon sources acquired by F. tularensis in host cells (Chapter 4). These studies represent the first studies aimed to identify the host derived carbon sources of F. tularensis and will significantly aid the understanding of F. tularensis pathogenesis.

The major sources of carbon for all intracellular pathogens are poorly understood and this lack of knowledge is, at least partially, due to the technical difficulties in answering these questions. Distinguishing the metabolic flux of host carbon metabolism from flux through the comparatively smaller bacterium complicates metabolic analysis. Also, intracellular bacteria likely acquire multiple carbon sources from the host and inhibiting flux through one carbon metabolic pathway may be compensated for by increasing flux through another metabolic pathway, thus further increasing the complexity of the system. Nonetheless, information on host derived carbon sources has been gleaned from studies of intracellular pathogens. Analysis of M. tuberculosis mutants identified fatty acids and cholesterol are major nutrients sources for the bacterium, while $^{13}$C metabolite assays suggests M. tuberculosis fixes CO$_2$ and acquires an unidentified C$_3$ carbon source in macrophages (2, 6). Metabolic labeling studies in enteropathogenic Escherichia coli, Listeria monocytogenes, Legionella pneumophila and other intracellular pathogens have identified glucose, glucose 6-
phosphate, glycerol, glycerol 3-phosphate, and lactate, among other sources of carbon can be assimilated by different pathogens (8-10). Interestingly, each pathogen does not use all of these carbon sources, but is capable of using only a few specific sources. Together, these and other studies demonstrate that the sources of carbon acquired by pathogens are extremely diverse, yet each pathogen focuses on acquisition of specific sources of carbon.

Another observation which supports the idea that pathogens focus on acquisition of specific carbon sources, is that pathogens that occupy the same niche do not acquire the same host carbon sources. For example, uropathogenic *E. coli* and *Proteus mirabilis* both replicate in the urinary tract but *E. coli* acquires glycolytic carbon sources while *P. mirabilis* acquires gluconeogenic carbon sources (1). These data indicate that both gluconeogenic and glycolytic carbon sources can be acquired from the same niche, but each pathogen only focuses on acquisition of one carbon source or the other. Therefore, each pathogen likely employs carbon acquisition strategies to acquire glycolytic or gluconeogenic carbon sources specifically. More broadly, this idea suggests that pathogen manipulation of host processes for carbon acquisition is targeted to specific host processes for production of the desired nutrients.

*Defining the Carbon Metabolism of F. tularensis*

Due to the diversity of carbon sources suspected to be acquired by intracellular pathogens and that pathogens within the same niche acquire different carbon sources, we took a broad, but targeted mutagenesis approach to identify the specific carbon molecules assimilated by *F. tularensis* within host cells. By identifying specific carbon metabolic pathways required for intracellular growth and virulence, we could then
predict the specific molecules acquired by *F. tularensis* in host cells. We first probed major metabolic pathways, glycolysis and gluconeogenesis, for their requirement for intracellular growth and virulence in a pneumonic murine infection model. From these analyses we found glycolysis to be completely dispensable but gluconeogenesis to be essential for intracellular growth and virulence (Chapter 4).

Since a mutant defective for the gluconeogenic enzyme fructose 1,6-bisphosphatase (*glpX* FTT_1631) prevents anabolism of several potential carbon sources to create essential precursor metabolites and feed the pentose phosphate pathway, we tested mutants unable to assimilate specific gluconeogenic carbon sources for intracellular growth and virulence. We identified glutamate dehydrogenase (*gdhA*, FTT_0380) and glycerol 3-phosphate dehydrogenase (*glpA* FTT_0132) as required for replication within macrophages. *glpX*, *gdhA* and *glpA* are all likely required for assimilation of host carbon as intracellular growth of a mutant defective for each gene can be rescued upon addition of excess glucose in transformed cell lines.

A Δ*glpA* mutant cannot convert glycerol or glycerol 3-phosphate into the glycolytic intermediate dihydroxyacetone phosphate for use in anabolic or catabolic metabolism. We could complement the growth of the Δ*glpA* mutant on glycerol 3-phosphate, but not glycerol, in defined media, thus indicating a polar mutation independently resulted in preventing glycerol assimilation. Interestingly, the (partially) complemented strain still rescued growth of the mutant in macrophages, suggesting glycerol 3-phosphate, and not glycerol, is a major carbon source used by *F. tularensis* in host cells.
A \textit{gdhA} mutant is unable to convert glutamate, glutamine, arginine or proline into the TCA cycle intermediate 2-oxoglutarate; however, assimilation of aspartate and asparagine may also require \textit{gdhA}. The only annotated mechanism of converting aspartate to oxaloacetate by \textit{F. tularensis} Schu S4 requires a transaminase which also converts a 2-oxoglutarate into glutamate resulting in the addition and simultaneous removal of one C$_4$ molecule from the TCA cycle. Thus, GdhA would be required to convert the produced glutamate back into 2-oxoglutarate for net positive carbon flux into the TCA cycle and central carbon metabolism to be used for anabolism. Overall, we think that glycerol 3-phosphate, along with several amino acids including glutamate, glutamine, proline, arginine, aspartate and asparagine provide the major carbon sources for \textit{F. tularensis} Schu S4 in host cells.

\textbf{F. tularensis may Require Different Carbon Metabolic Pathways in Different Cellular Environments}

We also identified the gluconeogenic gene phosphoenolpyruvate carboxykinase (\textit{pckA FTT_0449}) to be required for full virulence \textit{in vivo}. PckA converts the TCA cycle intermediate oxaloacetate into phosphoenolpyruvate (PEP) to allow TCA cycle intermediates to be converted into metabolic precursors such as G6P. Therefore, creating G6P from the amino acids glutamate, glutamine, proline and arginine requires GdhA to convert the amino acids into TCA cycle intermediates and also PckA to convert the TCA cycle intermediates into PEP to eventually create G6P. \textit{F. tularensis} encodes an alternative gluconeogenic pathway that bypasses PckA by using maelic enzyme (\textit{maeA FTT_0917}) and pyruvate phosphate dikinase (\textit{ppdK FTT_0250}) to convert TCA
cycle intermediates into PEP. However, \textit{ppdK} was shown to be dispensable for \textit{in vivo} growth indicating \textit{F. tularensis} primarily uses \textit{pckA} for gluconeogenic flux of TCA cycle intermediates \textit{in vivo}.

Interestingly, \textit{ppdK}, but not \textit{pckA}, was found to be required for replication within the transformed macrophage cell line J774A.1. Since cultured macrophages, especially immortalized macrophage lines, likely use different metabolic pathways than host cells \textit{in vivo}, the carbon sources available to \textit{F. tularensis} within these systems may also differ. Therefore, \textit{F. tularensis} likely requires different metabolic pathways to grow in these different systems. \textit{ppdK} is required for \textit{F. tularensis} to assimilate lactate, pyruvate and specific amino acids as an anabolic carbon source, thus it is possible that \textit{F. tularensis} acquires host produced lactate, or other nutrients requiring \textit{ppdK} for assimilation, in transformed macrophages which may not be available in significant quantities to \textit{F. tularensis} in our \textit{in vivo} pneumonic infection model.

Since \textit{F. tularensis} is capable of efficiently growing in a myriad of host cell types and hosts, it is likely that the bacterium acquires different sources of host carbon and uses different carbon metabolic pathways under different conditions. The requirement of different gluconeogenic pathways for replication of \textit{F. tularensis} in transformed and primary macrophages suggests that \textit{F. tularensis} may acquire different sources of carbon in different host cell types and different hosts. We have identified the carbon metabolic pathways required for replication in macrophages and in a murine pulmonary infection model. However, it would be interesting to determine if \textit{F. tularensis} acquires different carbon sources in other cell types such as epithelial cells. Differences observed in the requirements for infection of these or other cell types may be reflected
by the requirements for infection through different inoculation routes, such as an intradermal route compared to our model of an inhalational infection route.

**Future Directions: Knowing the Specific Carbon Sources Assimilated by Intracellular Pathogens Allows for Targeted Inquiry to Define How Nutrients are Acquired by Pathogens in the Limiting Host Environment**

The next step, after defining which carbon sources are acquired by the bacterium, is to determine from where the identified carbon sources acquired by the bacterium are derived. Since *F. tularensis* replicates in host cells to levels where greater than 60% of the host cytosolic volume is bacterial mass (our unpublished data), it is unlikely that there are sufficient preexisting free cytosolic nutrients to support this extensive bacterial proliferation. Knowing which nutrients are consumed by *F. tularensis* will allow for targeted studies to determine how specific nutrients are produced in the host cell and define novel mechanisms of bacterial subversion of host processes to acquire nutrients.

As an example, we think that glycerol 3-phosphate is assimilated by intracellular *F. tularensis*. Therefore, *F. tularensis* infection likely results in production of significant free glycerol 3-phosphate in the host cytosol. It is possible that the ATG5-independent autophagy pathway, which aids in intracellular growth, may degrade lipid droplets or membranes producing glycerol 3-phosphate, but this hypothesis remains untested. Other mechanisms by which *F. tularensis* infection could increase cytosolic glycerol 3-phosphate levels could be by directly increasing host glycerol import, inducing glycerol
3-phosphate synthesis from other host carbon sources or inducing host breakdown of complex molecules to produce glycerol 3-phosphate independent of autophagy.

It is possible that much of the carbon assimilated by *F. tularensis* in the host cytosol derives from nutrients imported by the host. It has been shown that increased glucose uptake from infected host cells contributes energy and nutrients to several intracellular pathogens and may indirectly contribute to *F. tularensis* nutrient acquisition (Appendix 1). Imported host glucose, or other nutrients, could be converted to glycerol 3-phosphate or amino acids which are then assimilated by *F. tularensis*. In conjunction with increasing host carbon uptake, infection by *F. tularensis* may also alter flux of host carbon to increase production of the particular sources of carbon acquired by the bacterium. Finally, via mechanisms independent of autophagy, *F. tularensis* may induce host cell processes to degrade complex host molecules into simple nutrients to be acquired by pathogens. Determining which, if any, of these proposed mechanisms provide nutrients for *F. tularensis* will significantly add to the understanding of bacterial pathogenesis by identifying new strategies bacterial pathogens use to acquire nutrients in the limiting environment of the host.

A mechanism by which intracellular pathogens could induce host cell processes to increase the availability of simple nutrients is to directly target host metabolic signaling. Many viral, bacterial and eukaryotic pathogens manipulate host metabolic signaling and the effects of pathogens on the major metabolic regulators AMPK and mTOR are reviewed in Appendix 1. We have determined that in host cells infected with *F. tularensis*, AMPK becomes activated while mTOR activity is suppressed. We found that AMPK activity is required for full *F. tularensis* intracellular growth as AMPK null host
cells permit significantly reduced *F. tularensis* proliferation (unpublished results). However, we do not know how AMPK activity is required for *F. tularensis* replication, whether by affecting host metabolism or other host processes. Further investigation into the effects of AMPK on *F. tularensis* growth as well as the contribution of other host metabolic signaling to *F. tularensis* infection will elucidate mechanisms of *Francisella* virulence by potentially linking the assimilation of host nutrients by *Francisella* to effects of *F. tularensis* infection on host metabolism and metabolic processes.

Overall, we have identified specific host derived nutrients *F. tularensis* acquires in host cells and a mechanism by which *F. tularensis* acquires host derived nutrients. By further defining these processes and investigating the effects of *F. tularensis* infection on host carbon metabolism, metabolic signaling and other host metabolic processes will begin to untangle the extremely complex web of host-bacterial interactions resulting in nutrient production for bacterial growth. Not only will these studies elucidate *F. tularensis* pathogenesis, but will broadly aid the understanding of bacterial pathogenesis as the interactions between intracellular pathogens and host metabolism are underappreciated.
REFERENCES


APPENDIX 1: FEEDING UNINVITED GUESTS: MTOR AND AMPK SET THE TABLE FOR INTRACELLULAR PATHOGENS

Introduction

Most pathogenesis studies focus on pathogen virulence attributes that mediate host colonization, toxicity, or immune evasion. Some studies focus on how pathogens employ active mechanisms to acquire essential nutrients such as iron and vitamins from the host by producing siderophores or avidins. In order to prevent pathogen nutrient acquisition, host cells employ a process called nutritional immunity to sequester these nutrients, particularly iron, from invading pathogens [1]. However, relatively little attention has been paid to understanding the mechanisms by which pathogens parasitize energy and catabolic substrates from the host even though several host and pathogen metabolic genes, including those in central carbon metabolism, are regularly identified as required for growth in the host [2], [3]. This issue is particularly important for intracellular pathogens that must compete with the host cell for energy and nutrient sources.

How and where do intracellular pathogens obtain sufficient amounts of energy and nutrients to support their replication? Pathogens may either parasitize existing energy stores or manipulate the host cell to create usable energy and anabolic precursor metabolites. Several recent studies have identified the host AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) kinases as two

1This chapter previously appeared as an article in the Journal of PLoS pathogens. The original citation is as follows: Brunton J., et al. "Feeding Uninvited Guests: mTOR and AMPK Set the Table for Intracellular Pathogens." PLoS pathogens 9(10): e1003552.
important regulators of cellular metabolism whose activities are often altered during infection. However, the AMPK/mTOR pathway also regulates autophagy, which can destroy cytosolic pathogens. While the evasion of autophagy by pathogens is well appreciated, recent work suggests that both the AMPK/mTOR pathway and autophagy itself can provide intracellular metabolites that support intracellular pathogen replication.

**AMPK and mTOR Regulate Energy Homeostasis**

During times of limited nutrient availability, intracellular ATP levels fall, with a corresponding increase in AMP levels. Within eukaryotic cells the increased AMP:ATP ratio induces AMPK activity, which in turn initiates a series of signaling events that stimulate energy and nutrient acquisition [4]. For example, activated AMPK stimulates glycolytic flux, increases glucose uptake, and induces fatty acid oxidation (Figure 1). Together these events allow the cell to use its existing metabolic stores and also acquire new sources of energy. At the same time, activated AMPK limits energy consuming processes. Activated AMPK conserves energy by globally reducing protein synthesis, which perhaps is the most energy-intensive process in eukaryotic cells. AMPK limits protein synthesis by antagonizing the mTOR kinase, and mTOR kinase activity is necessary for formation of the eIF4F complex, which is critical for translation initiation. In addition, mTOR and AMPK inversely regulate the recycling of existing intracellular metabolites through their effects on autophagy. Active AMPK stimulates autophagic breakdown of macromolecular complexes in the cell, thus producing energy and nutrients. In contrast, active mTOR suppresses autophagy to promote cell growth and proliferation. In a simplified view, when energy is low AMPK is active and mTOR is inhibited. This stimulates energy-producing processes and inhibits energy consumption.
thereby providing sufficient energy to support cell viability. Although AMPK and mTOR have additional roles outside of cellular metabolism, here we focus on the effects of AMPK and mTOR on cellular metabolism during infection by intracellular pathogens.

**Manipulation of Both AMPK and mTOR by Intracellular Pathogens**

In order to achieve optimal levels of proliferation, many pathogens must manipulate activity of AMPK and mTOR. Interestingly, several viral pathogens have evolved strategies that allow for the induction of both AMPK and mTOR activity. For example, infection with human cytomegalovirus (HCMV) increases both AMPK and mTOR activity [5]. To acquire sufficient energy for viral growth, HCMV infection increases glycolytic flux in an AMPK-dependent manner [2], [6]. However, HCMV must strictly regulate AMPK activity during infection, as treatment of infected cells with chemicals that strongly activate or inhibit AMPK can limit viral replication [6], [7]. Interestingly, HCMV replication also requires fatty acid synthesis, which should be inhibited when AMPK is activated. Yet fatty acid synthesis is maintained during HCMV infection through a mechanism that requires mTOR activation [8]. How does HCMV allow for the activation of both AMPK and mTOR? The answer lies in part in the activity of the HCMV UL38 protein (pUL38). pUL38 binds and inhibits the TSC1/2 complex, which is necessary for antagonism of mTOR by activated AMPK [9]. HCMV thus uncouples AMPK/mTOR signaling resulting in increased energy production and lipid synthesis, both of which contribute to virus replication.

Simian virus 40 (SV40) infection also stimulates both AMPK and mTOR activity. SV40 small T antigen is both necessary and sufficient for AMPK activation [10], [11].
This function of small T antigen may provide critical nutrients needed for viral replication. mTOR activity is induced early in infection but inhibited as infection progresses. The mechanism driving the early induction of mTOR activity is unknown, but may be the result of Akt activation by the SV40 T antigens. However, the inhibition of mTOR activity during the late stage of infection is due to the effects of the SV40 small T antigen [11]. While activated AMPK would seemingly reduce SV40 protein synthesis, the expression of SV40 late proteins is driven by an internal ribosome entry site (IRES) that allows for efficient late mRNA translation when mTOR is inhibited [12]. It is likely that other pathogens employ active mechanisms to balance AMPK and mTOR signaling to allow for both catabolic and anabolic processes essential for pathogen replication, similar to HCMV and SV40.

**Inhibiting AMPK or Inducing mTOR Can Provide Essential Substrates for Pathogen Replication**

Enveloped viruses require host lipids to generate the virion membrane. Activated mTOR stimulates fatty acid and lipid synthesis, and therefore could prove beneficial for virus assembly. In fact, host lipid metabolism is essential for the hepatitis C virus (HCV) life cycle and is highly regulated during infection [13], [14]. HCV infection limits AMPK activity and chemical induction of AMPK suppresses viral replication and inhibits fatty acid synthesis in HCV-infected cells [15]. Consistent with AMPK suppression, mTOR activity is increased during HCV infection through increased Akt signaling and decreased TSC1/2 expression [16]. However, this raises the question of how HCV acquires significant energy sources for viral replication in an AMPK-inhibited, mTOR-activated metabolic state? The answer may be the temporal regulation of host signaling.
and nutrient usage. Glucose import is required for viral replication and glycolytic flux is induced early during HCV infection [14], [17]. The products of glycolysis are likely diverted to fatty acid synthesis, as TCA flux and oxidative phosphorylation are reduced in HCV-infected cells [14], [18]. Later during infection, glucose uptake is reduced, while β-oxidation and amino acid catabolism are increased [14]. It is therefore possible that HCV temporally regulates AMPK and mTOR activity to achieve significant viral protein translation and lipid production, yet still obtain sufficient energy to support virus replication. Some bacterial pathogens may benefit from inhibiting AMPK and activating mTOR by inducing lipid synthesis, as *Mycobacterium tuberculosis* and *Chlamydia trachomatis* utilize fatty acids derived from lipid droplets [19], [20]. However, it is unknown how these bacteria affect host metabolic signaling to acquire nutrients.

AMPK activation also inhibits the replication of several arboviruses, including Rift Valley fever virus (RVFV) [21]. RVFV replication can be rescued in the presence of activated AMPK by providing cells with excess palmitate [21]. This suggests that AMPK inhibition is required to provide lipids essential for viral replication. The HIV-1 Tat protein inhibits the host SIRT1 protein resulting in AMPK inhibition [22]. Interestingly, AMPK induction inhibits lytic HIV replication, but is involved in reactivation of latent HIV genomes suggesting that AMPK activity may have different roles in acute and persistent infection [23].

**AMPK Activation May Benefit Replication of Diverse Pathogens**

It takes a lot of energy to make hundreds, thousands, or potentially millions of new parasites, bacteria, or viruses. It seems logical that intracellular pathogens that
undergo significant intracellular growth would activate AMPK due to the energetic demands placed on the infected cell. Activation of AMPK could provide several benefits for intracellular pathogens. The increased glucose uptake, glycolysis, and fatty acid breakdown would increase available intracellular energy and nutrient pools needed for pathogen replication. For example, *Leishmania donovani* amastigotes (the parasitic form that grows inside macrophages) preferentially generate energy through fatty acid oxidation and amino acid catabolism [24], suggesting *L. donovani* acquires fatty acids and amino acids from the infected host cell. Consistent with this finding, transcriptomic analysis of macrophages infected with the related parasite *Leishmania major* suggests that infected cells increase glucose transport, glycolysis, and starch degradation [25]. While it is currently unknown how *Leishmania* alters host metabolic processes, a reasonable hypothesis is that intracellular *Leishmania* activates AMPK to benefit parasite replication. Activated AMPK could stimulate increased glucose utilization and autophagy, thus creating elevated levels of anabolic precursor pools for parasite growth. Parasite replication requires the *Leishmania* protein GP63, which cleaves and inactivates mTOR to reduce type I interferon production, thus AMPK activation could further benefit parasite replication by inhibiting mTOR [26]. Viral pathogens may also benefit from AMPK activation. Measles virus requires β-oxidation for replication [27], but it is unknown if the virus manipulates AMPK for energy generation. It would be interesting to determine if these intracellular pathogens and others induce AMPK to generate energy and nutrients for growth.
Autophagy Provides Intracellular Pathogens with Nutrients

Autophagy is an essential cellular process that recycles cellular constituents from macromolecular complexes under conditions of nutrient stress. As discussed above, autophagy is positively regulated by AMPK and negatively regulated by mTOR. However, autophagy also functions as a host defense mechanism that destroys intracellular pathogens through a process termed xenophagy. While generally viewed as detrimental for intracellular pathogens, some bacteria and viruses use autophagosomes as a replicative niche [28]. Whether these pathogens benefit or simply tolerate residing in autophagosomes remains unclear. However, it may be that replicating in a site where free nutrients are accumulating provides pathogens with a competitive edge for the acquisition of nutrients. This concept is supported by recent evidence that intracellular pathogens may use autophagy to acquire energy and nutrients for growth. Dengue virus–induced autophagy degrades lipid droplets. This increases free fatty acids levels in the cell and stimulates β-oxidation, which is required for efficient dengue virus replication [29]. Similarly, we have found that *Francisella tularensis* growth is impaired in autophagy-deficient host cells. Bacterial growth was restored in autophagy-deficient cells by supplying the infected cells with excess pyruvate or amino acids. Since *F. tularensis* replicates within the cytosol of host cells, our results suggest that intracellular *F. tularensis* uses autophagy to increase cytosolic nutrient pools that support bacterial growth [30]. Interestingly, *F. tularensis* avoids engulfment by classical autophagosomes [31] and instead induces an alternative form of autophagy that is required for bacterial replication [30]. It is attractive to speculate
that other intracellular pathogens manipulate autophagy to avoid xenophagic destruction, while simultaneously benefiting from autophagy-derived nutrients.

**Conclusion**

AMPK and mTOR are critical regulators of host cell metabolism making them logical targets for manipulation by invading pathogens. The energetic burden of the host cell to create hundreds or more pathogens should deplete cellular ATP levels, thus activating AMPK. AMPK induction stimulates host processes to produce energy and nutrients that the pathogen could then steal from the host. This idea suggests AMPK activation may be a common theme among infection by successful intracellular pathogens. On the other hand, mTOR signaling stimulates protein and lipid synthesis, which could be beneficial for many viral pathogens; whereas mTOR modulation is likely less important for free-living bacteria pathogens and parasites that supply their own biosynthetic and translation machinery. Identifying what nutrient sources are required for intracellular growth and how host metabolic signaling is manipulated by infection is being investigated in viral pathogenesis, yet remains poorly understood in bacterial and parasitic pathogenesis.

Manipulating host metabolism is an attractive approach to controlling infection as targeting the host rather than the pathogen should considerably reduce the ability of pathogens to develop drug resistance. Several drugs already in clinical use target the AMPK or mTOR kinases to treat diseases such as cancer and diabetes. The studies described above suggest that these drugs may have additional uses in treating infections with intracellular pathogens. As our understanding of pathogen manipulation
of host metabolism grows, it may also be possible to develop inhibitors of specific host metabolic pathways hijacked by intracellular pathogens. Identifying the essential nutrients required for intracellular pathogen proliferation and the host pathways manipulated to acquire these nutrients will be a significant step in understanding the requirements for viral, bacterial, and parasitic pathogenesis and identifying new targets for novel therapeutics
Figures

Figure A1.1: Infection by Diverse Pathogens Impacts AMPK and mTOR Signaling.
Several intracellular pathogens manipulate the AMPK/mTOR pathway during infection through either directly targeting AMPK or mTOR or by targeting the upstream or downstream pathways. Depicted here are specific points of manipulation in the
mTOR/AMPK pathway by human cytomegalovirus (HCMV), hepatitis C virus (HCV), Rift Valley fever virus (RVFV), simian virus 40 (SV40), *Leishmania*, and *Francisella* species. The table summarizes the resulting effects on the activities of mTOR and AMPK from infection by the specific pathogen.
REFERENCES


The studies described in this section are proposed experiments to both supplement the data generated in Chapter 4, as well as to more comprehensively describe the carbon sources acquired by intracellular *F. tularensis*. The proposed experiments generally fall into two categories. The first category includes determining the requirement of specific *F. tularensis* carbon metabolic pathways in an *in vivo* pneumonic murine infection model to identify all major carbon metabolic pathways. The second involves quantifying uptake of $^{13}$C labeled substrates by intracellular *F. tularensis* to directly measure uptake and assimilation of specific carbon molecules in host cells. Overall we believe successful completion of these studies will significantly add to the understanding of *F. tularensis* metabolic requirements for infection and identify the major host derived carbon sources used by *F. tularensis*.

**Elucidating the Essential *F. tularensis* Carbon Metabolic Pathways *in vivo* Will Comprehensively Identify the Major Host Derived Carbon Sources**

Experiments described in Chapter 4 identify gluconeogenesis, specifically *glpX* (2) and to a lesser extent *pckA* (5), are required for *in vivo* growth. The data also suggest that *glpA* (3) and *gdhA* (6) are required to assimilate host derived carbon in primary cells. We hypothesize that specific carbon sources shuttled into central carbon metabolism through *gdhA* (6) also requires *pckA* (5) for conversion into specific precursor molecules including glucose 6-phosphate *in vivo*. Additionally, we hypothesize that *glpA* (3) and *gdhA* (6) are required for *in vivo* growth and that a mutant
defective for each carbon metabolic pathway is only partially attenuated, similar to a
*pckA* (5) mutant. We also hypothesize that a Δ*glpAΔgdhA* (3,6) double mutant or a
Δ*glpAΔpckA* (3,5) double mutant will be significantly more attenuated compared to
mutants defective for only one pathway. If a Δ*glpAΔgdhA* (3,6) double mutant and a
Δ*glpAΔpckA* (3,5) double mutant is similarly attenuated to a Δ*glpX* (2) mutant, these
data would indicate that all major gluconeogenic carbon sources enter through *glpA* (3)
and *gdhA* (6). Therefore, we propose to test the *in vivo* growth of Δ*glpA* (3), Δ*gdhA* (6),
Δ*glpAΔpckA* (3,5) and Δ*glpAΔgdhA* (3,6) mutant strains in our mouse pulmonary
infection model.

If a Δ*glpAΔgdhA* (3,6) mutant is found to not be significantly attenuated similar to
a Δ*glpX* (2) mutant, the data would indicate that *F. tularensis* acquires other major
carbon sources *in vivo* besides sources that enter central carbon metabolism through
*glpA* (3) and *gdhA* (6). Two major possible groups of carbon sources include TCA cycle
intermediates and carbon sources requiring *ppdK* (4) for assimilation. If TCA cycle
intermediates or other sources of carbon entering the TCA cycle function as major
sources of carbon for intracellular *F. tularensis*, then a Δ*glpAΔpckA* (3,5) mutant will be
significantly more attenuated than a Δ*glpAΔgdhA* (3,6) double mutant. Carbon sources
requiring *ppdK* (4) for assimilation include lactate, pyruvate, serine, glycine and
threonine and could potentially be used by intracellular *F. tularensis*. If these carbon
sources are major sources host derived carbon for *F. tularensis* then disrupting *ppdK* (4)
in a Δ*glpAΔpckA* (3,5) or a Δ*glpAΔgdhA* (3,6) mutant background will further attenuate
*F. tularensis in vivo* growth.
Direct Measurement of $^{13}$C Labeled Metabolic Substrate by Intracellular *F. tularensis*

Data demonstrating *glpA* (3) and *gdhA* (6) are required for *F. tularensis* growth in host cells and that addition of excess glucose to J774 cells infected with Δ*glpA* (3) or Δ*gdhA* (6) rescues bacterial growth, provides strong evidence that these genes are required for assimilation of host carbon. However, these studies indirectly demonstrate acquisition of host carbon by *F. tularensis*. To directly test uptake of specific carbon sources by intracellular *F. tularensis* and the requirement of *glpA* (3) and *gdhA* (6) to assimilate these carbon sources, $^{13}$C labeled carbon sources will be provided to *F. tularensis* macrophages and incorporation of $^{13}$C into bacterial protein will be quantified. Figure 1 diagrams the intracellular labeling process.

Wild type Schu S4, a Δ*glpA* (3) mutant or a Δ*gdhA* (6) mutant strain expressing an arbitrary protein, FTT_1703 (involved in the Francisella Pathogenicity Island), with an HA tag can be created and used to infect BMDMs. The *F. tularensis* infected cells are then provided $^{13}$C labeled glycerol or glutamine and induced with anhydrotetracycline to produce FTT_1703-HA. After several hours of incubation, the infected cells and bacteria are lysed and FTT_1703-HA protein is column purified via anti-HA magnetic beads (Miltenyi). The eluted protein will be separated via SDS-PAGE and the band containing FTT_1703-HA will be excised and analyzed by mass spectrometry to quantify $^{13}$C incorporation into FTT_1703-HA.

We hypothesize that incorporation of $^{13}$C labeled substrates into wild type Schu S4 produced FTT_1703 indicates Schu S4 can assimilate the specific substrate in host cells. Additionally, significantly reduced incorporation of $^{13}$C glutamine into FTT_1703-HA produced by the Δ*gdhA* (6) strains indicates *gdhA* (6) is required for glutamine
assimilation in host cells. Analogous experiments would be conducted using $^{13}$C labeled glycerol instead of glutamine and infecting macrophages with the $\Delta glpA$ (3) mutant instead of the $\Delta gdhA$ (6) mutant. Further, incorporation of $^{13}$C labeled glucose into wild type $F. tularensis$ can be determined in J774 cells and BMDMs to determine if glucose is significantly assimilated in host cells. If these hypothesizes are correct we would expect data to be similar to that described in Figure 2. Overall, these studies will directly test the ability of $F. tularensis$ to acquire specific carbon sources in host cells and determine the requirement of $glpA$ (3) and $gdhA$ (6) to assimilate these molecules into bacterial protein.
Figure A2.1: Metabolic Labeling of Intracellular *Francisella tularensis*. Bone marrow derived macrophages will be infected with wild type Schu S4 or specific metabolic mutants and fed $^{13}$C labeled substrate. The labeled carbon source will be taken up by the host cell and subsequently by *F. tularensis*. If wild type Schu S4 is capable of assimilating significant levels of the labeled substrate as a major carbon source, the bacterium will convert the substrate into amino acids and incorporate the $^{13}$C labeled amino acids into FTT_1703-HA. If the mutant strain is unable to convert the $^{13}$C labeled carbon source into amino acids, there will be significantly less incorporation into FTT_1703-HA compared to the wild type strain.
Figure A2.2: Predicted Outcomes of $^{13}$C Metabolic Labeling Experiments.

Incorporation of $^{13}$C into FTT_1703-HA expressed from wild type Schu S4 or specific metabolic mutants grown in bone marrow derived macrophages or J774s. Labeled A) glycerol or B) glutamine will be supplied to infected bone marrow derived macrophages for several hours. C) Labeled glucose will be added to infected bone marrow derived macrophages or J774 cells for several hours. After incubation with labeled substrate, all host cells and bacteria will be lysed in 1% NP40 and FTT_1703 will be purified from total lystate via anti-HA magnetic bead. Eluted protein will be separated by SDS PAGE, the protein band containing FTT_1703-HA will be excised and $^{13}$C incorporation will be quantified by mass spectrometry.