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Investigating *Francisella* intracellular survival mechanisms

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Abstract

Investigating *Francisella* intracellular survival mechanisms

Anna C. Llewellyn

Despite being designated a Category A potential biothreat by the Centers for Disease Control and Prevention, there is relatively little known about the molecular genetics of *Francisella* species pathogenesis. Recent *in vivo* genetic screens have identified hundreds of *Francisella* genes predicted to be involved in virulence, though in what way many of these genes may contribute to disease is unknown. Therefore, I began my thesis work with an intracellular replication screen to identify which virulence factors are specifically required for intracellular replication, an important component of *Francisella* infection. From the results of this screen, two novel proteins were chosen for further study: FTN_1133 and NaxD. Characterization of FTN_1133 revealed the role of this novel Ohr-like organic hydroperoxide resistance protein in withstanding reactive oxygen species both in macrophages and in mice. This is the first report of an Ohr-like protein involved in virulence. Next, NaxD, a member of the previously functionally uncharacterized YdjC superfamily of proteins, was shown to function as a deacetylase required for a lipid A modification important for pathogenesis, intramacrophage proliferation, and resistance to polymyxin B. Importantly, the role of NaxD was conserved in the human pathogenic *F. tularensis* as well as the mammalian pathogenic *Bordetella bronchiseptica*. Further studies indicate that this lipid A modification is required for resistance to specific intracellular host CAMPs and evasion of innate immune recognition. Taken together, the work presented in this dissertation elucidated the role of *Francisella* proteins in intramacrophage proliferation and resistance to host innate antimicrobial defenses. These studies have contributed not only to the understanding of *Francisella*'s genetic requirements for intracellular proliferation, but have revealed the role of Ohr-like proteins in pathogenesis and elucidated the function and role in virulence of members of the previously uncharacterized YdjC superfamily of proteins. Given that both Ohr and YdjC proteins are encoded by many virulent bacteria, this work has implications for our understanding of the pathogenesis of both intracellular and extracellular organisms and may represent attractive targets for drug and vaccine development.

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Chapter 1: Introduction

History

Francisella species are small Gram-negative coccobacilli and are the causative agents of the zoonotic disease tularemia, which is primarily endemic to the northern hemisphere (Sjostedt, 2007). Tularemia was first described by McCoy and Chapin in 1911 after an outbreak of plague-like illness in Tulare County, California (for which the bacteria would in part later be named) (McCoy & Chapin, 1911). *Francisella tularensis* was then isolated as the etiologic agent a year later (McCoy, 1912). In 1914, the initial documented human cases were reported by Wherry and Lamb after treating patients from Ohio who had both been in contact with wild hares (Wherry, 1914).

After these initial discoveries, Dr. Edward Francis became the leading researcher involved in characterization of the transmission, clinical manifestations, and microbiology of this pathogen (Keim *et al.*, 2007). Indeed, it was Francis who originally coined the term "tularemia" and also who first identified the role of arthropods in transmission of the disease (Keim *et al.*, 2007). Incidence of tularemia was believed to be isolated to the United States until 1925 when Ohara noticed the similarities of a hare associated human outbreak in Japan and sent bacterial cultures to Francis who confirmed them to be *Francisella* (Keim et al., 2007). Shortly thereafter, *Francisella* was also identified as the etiologic source of outbreaks in the former Soviet Union, Europe, and Canada (Petersen & Schriefer, 2005). After a series of taxonomic reassignments from the original genus *Bacterium* to *Pasteurella* and then *Brucella*, in 1947 the bacterium was

found to be unique enough to belong to its own genus, which was named *Francisella* in honor of the decades of research by Dr. Francis (Nigrovic & Wingerter, 2008).

Taxonomy

Based on 16S rRNA analysis, *Francisella* is a deeply branching member of the modern taxonomic γ-subclass of proteobacteria (Larsson *et al.*, 2005). The closest related human pathogens are *Coxiella burnetii* (Q fever) and *Legionella pneumophila* (Legionnaire's disease) (Oyston *et al.*, 2004, Larsson *et al.*, 2005). *Francisella*, the only genus of the family *Francisellaceae*, includes four recognized species (Jones *et al.*, 2012). The human pathogenic species, *F. tularensis*, includes three subspecies, *F. tularensis* subspecies *tularensis* (*F. tularensis*), *F. tularensis* subspecies *holarctica* (*F. holarctica*), and *F. tularensis* subspecies *mediasiatica* (*F. mediasiatica*). While not considered to be a human pathogenic species, *F. novicida* has been reported to cause disease in rare instances, typically in immunocompromised individuals (Keim *et al.*, 2007). Finally, *F. philomiragia* and *F. noatunensis* are the most distantly related of the genus and are pathogens of wild and aquarium fish (Jones *et al.*, 2012). The majority of human tularemia cases are caused by *F. tularensis* and *F. holarctica*, with *F. tularensis* causing the most severe disease. Cases of infection with *F. mediasiatica* are considered to be less common than *F. tularensis* and *F. holarctica* while *F. novicida* is rarely associated with human disease, though it can act as an opportunistic pathogen in immunocompromised humans (Keim *et al.*, 2007). Importantly, *F. tularensis* and *F. novicida* retain approximately 98% nucleotide identity (Rohmer *et al.*, 2007). This is interesting as hundreds of ORFs present in *F. novicida*, thought to be most closely related to the

ancestral *Francisella* species, are annotated as pseudogenes in the virulent subpecies *F. tularensis* and *F. holarctica* (Rohmer *et al.*, 2007). It has been suggested that this genetic decay indicates recent adaptation to a new niche (Rohmer *et al.*, 2007).

The primary strains used in laboratory research include *F. tularensis*, *F. novicida*, and an attenuated live vaccine strain (LVS; see below) derived from *F. holarctica* (Table 1). Experiments involving *F. tularensis* require the use of BSL-3 Select Agent laboratories. Consequently, researchers often use *F. novicida* and LVS when conducting pathogenesis research. Both of these strains are considered strong model organisms since they cause a tularemia-like disease in mice, have 98-99% nucleotide identity with *F. tularensis*, are genetically tractable, and are BSL-2 pathogens (Pechous *et al.*, 2009)(Table 1).

Tularemia

Tularemia, also known as rabbit fever, deerfly fever, and meat-cutter's disease, is transmitted to humans through a variety of mechanisms including arthropod bites, aerosol inhalation, handling infected animals, and ingestion of contaminated food or water (Petersen & Schriefer, 2005). However, there is no person-to-person spread of tularemia (Nigrovic & Wingerter, 2008)). Onset of symptoms usually begins 3-5 days after exposure, with a range of 1-14 days (Nigrovic & Wingerter, 2008). Disease progression is determined by the route of infection and can result in two main types of tularemia: ulceroglandular and pneumonic (Nigrovic & Wingerter, 2008).

Bites from arthropods such as ticks, deer flies, and mosquitos result in ulceroglandular tularemia, the most common form of the disease (Nigrovic & Wingerter, 2008). Disease presentation typically begin with nonspecific flu-like symptoms and an ulcer at the site of the insect bite followed by swelling of the draining lymph nodes after which it can spread systemically, possibly leading to organ failure and death (Nigrovic & Wingerter, 2008)).

Pneumonic tularemia is the most fatal form of the disease and manifests as fever and a dry cough (Nigrovic & Wingerter, 2008). When aerosolized, *F. tularensis* can cause a potentially lethal disease with an infectious dose as low as 10 bacteria and result in a mortality rate as high as 30-60% if left untreated (Nigrovic & Wingerter, 2008, Oyston, 2008). However, with antibiotic treatment the overall fatality rate is about 2% (Nigrovic & Wingerter, 2008). The current recommended treatments for both ulceroglandular and pneumonic tularemia include fluoroquinolones (such as ciprofloxacin) and tetracyclines (such as doxycycline) (http://www.bt.cdc.gov/agent/tularemia/faq.asp).

Epidemiology

Francisella species are endemic across the northern hemisphere (approximately 30°-70° north latitude) on multiple continents and in diverse ecologies (Nigrovic & Wingerter, 2008, Petersen *et al.*, 2009). However, the preponderance of reported cases of disease occurred in the United States, Europe, Russia, and Japan (Keim *et al.*, 2007). In fact, tularemia has been reported in every US state except Hawaii (Nigrovic & Wingerter, 2008). Of the human pathogenic subpecies, *F. holarctica* is found throughout the Northern Hemisphere, *F. tularensis* has only been identified in North America, and

reports of *F. mediasiatica* are limited to Central Asia (Petersen *et al.*, 2009). *F. holarctica* is thought to primarily be associated with aquatic environments while *F. tularensis* has been shown to be both aquatic and terrestrial (Petersen *et al.*, 2009).

While *F. tularensis* has become an increasing concern due to potential use as a bioterrorism agent (see "Weaponization" below), the actual incidence of reported cases of tularemia has decreased since the 1950s (Petersen *et al.*, 2009). For example, in the United States in 1939, 2,291 cases of tularemia were reported, the highest incidence on record, whereas only 1,368 cases from 44 states were reported for the entire decade of 1990-2000 (CDC MMWR 2002). In order of largest incidence, the majority of these cases were reported in Missouri, Arkansas, Oklahoma, and Martha's Vineyard, Massachusetts (Nigrovic & Wingerter, 2008, Petersen *et al.*, 2009).

A zoonotic pathogen, *Francisella* can infect an unusually wide range of amplifying host animals, though lagomorphs and rodents are thought to be the primary reservoirs for *F. tularensis* and *F. holarctica*, respectively (Petersen *et al.*, 2009). Mosquitos have been shown to be important transmitting arthropod vectors for *F. holarctica* in Europe while ticks and deer flies are the primary vectors for *F. tularensis* (Nigrovic & Wingerter, 2008). Landscapers, farmers, veterinarians, hunters, and meat handlers all have increased risk for tularemia acquisition (Nigrovic & Wingerter, 2008).

Vaccine

In the 1930s, scientists at the Gamaleya Institute in Moscow developed an attenuated strain of *F. holarctica* that was used to vaccinate over 60 million soldiers during WWII after 10,000 soldiers from the former Soviet Union were disabled by presumed tularemia infections (Cross *et al.*, 2007). An ampoule of this strain was brought to the United States in 1956 and developed into what is now referred to as live vaccine strain (LVS) (Cross *et al.*, 2007). In the1960s, the federal Food and Drug Administration (FDA) granted LVS investigatory drug status in order to vaccinate at-risk military and laboratory workers (Conlan, 2005). The incidence of laboratory-acquired pulmonary tularemia dropped dramatically, though ulceroglandular incidents did not decrease (Nigrovic & Wingerter, 2008). LVS never attained full vaccine status due to insufficient characterization of the basis of its attenuation and how it elicits protection, reports of reversion to virulence, and incomplete vaccine efficacy (Conlan, 2005). The development of characterized attenuated strains with increased coverage will contribute greatly not only to protection from disease, but also from the destruction of a potential bioterrorism attack.

Weaponization

In response to the rising threat of terrorism and bioterrorism, in 1999 the Centers for Disease Control and Prevention (CDC) declared *F. tularensis* a category A potential bioweapon, alongside pathogens such as *Bacillus anthracis*, *Yersinia pestis*, variola major, and viral hemorrhagic fevers (Sjostedt, 2007). *F. tularensis* was placed in this category due to its low infectious dose, high untreated mortality rate, ease of aerosolization and dissemination, genetic tractability, large potential cost in response to

an attack, and history of weaponization (Sjostedt, 2007). To date, *F. tularensis* remains one of the least characterized category A biothreats.

In the 1930s-1960s, Japan, USA, and the former Soviet Union all actively tested and stockpiled weaponized *F. tularensis* with the USA and the former Soviet Union developing drug and vaccine resistant strains (Sjostedt, 2007, Nigrovic & Wingerter, 2008). During WWII, the US biodefense medical research program, Operation Whitecoat, conducted experiments with weaponized tularemia on volunteers from the Seventh-day Adventist Church who could not engage in combat on religious grounds but who wanted to contribute to the war effort (Cross *et al.*, 2007). In the 1950s, studies supported by the US Department of Defense that tested antibiotic treatment regimens and the protective efficacy of vaccines against *F. tularensis* challenge were performed by researchers from the University of Maryland and Ohio State University on human volunteers, many of whom were prisoners (Cross *et al.*, 2007). Japan is known to have performed experiments on civilian and military prisoners of war (men, women, and children) using various chemical and biological agents, including *F. tularensis* (Sjostedt, 2007). In addition, there is anecdotal evidence that outbreaks of tularemia among German and Soviet soldiers on the eastern front during WWII resulted from intentional release (Dennis *et al.*, 2001) (Nigrovic & Wingerter, 2008). In 1969, President Richard Nixon officially dissolved the US offensive biological warfare program and then in 1973, stockpiles of weaponized *F. tularensis* were destroyed (Dennis *et al.*, 2001). It has also been reported that Russia maintained its stockpile and weaponization program into the 1990s (Sjostedt, 2007).

Intracellular life cycle

Francisella's primary replicative niche is thought to be the cytosolic compartment of both phagocytic and non-phagocytic cells such as macrophages, neutrophils, hepatocytes, alveolar epithelial cells, and fibroblasts (Fujita *et al.*, 1993, Qin & Mann, 2006, Hall *et al.*, 2007, Schulert *et al.*, 2009). Uptake has been shown to be mediated by opsonized and unopsonized pathways, both of which result in phagocytosis involving a distinctive pseudopod loop formation (Figure 1) (Clemens *et al.*, 2005). Multiple receptors have been identified to be involved in *Francisella* cell entry, the most important of which include FcγR for opsonized bacteria and the mannose receptor (MR) for unopsonized bacteria (Jones *et al.*, 2012).

After entry into the phagosomal compartment and before escape into the cytosol, *Francisella* has been shown to transiently encounter various antimicrobial defenses including acidification, reactive oxygen species (ROS), and cationic antimicrobial peptides (CAMPs) (Figure 1) (Jones *et al.*, 2012). Though a lowered pH is generally detrimental to bacteria, brief phagosomal acidification has been shown to be required for rapid escape of *Francisella* into the cytosol (Jones *et al.*, 2012). Similar to many bacteria, *Francisella* resists oxidative stress with the help of multiple proteins important for detoxification and suppression of ROS (discussed in detail below).

CAMPs have been shown to be required for control and clearance of bacterial infections and it is known that they are attracted to and bind the negatively charged lipid A of

Gram-negative pathogens (Koprivnjak & Peschel, 2011). However, the presence or extent of bactericidal activity due to membrane disruption is still under debate (Koprivnjak & Peschel, 2011). While in the phagosome, *Francisella* must resist the action of host CAMPs such as cathelicidins (LL-37, CRAMP) and even lysozyme, which is known to have an enzyme-independent CAMP domain (Koprivnjak & Peschel, 2011). *Francisella* subverts the action of these and other CAMPs via both lipid A modifications (discussed in detail below) and efflux systems (Jones *et al.*, 2012). The AcrAB/TolC efflux system has been shown to be important for *Francisella* resistance to multiple antibiotics and it is thought that host CAMPs could also be exported by this complex (Jones *et al.*, 2012). In addition, *Francisella* encodes other putative efflux proteins belonging to an uncharacterized transport system which may complement AcrAB/TolC pump functions (Jones *et al.*, 2012).

Francisella escapes the phagosome within 1-4 hours, depending on route of entry (Jones, 2012). Though the mechanisms of escape are still being determined, it is known that the *Francisella* Pathogenicity Island (FPI), which encodes a putative type VI secretion system (T6SS), is required (Jones *et al.*, 2012). Apart from structural T6SS proteins, the FPI also encodes virulence factors that have been shown to be secreted in a T6SS-dependent manner, such as IglI and VgrG, though their functions have not been defined (Jones *et al.*, 2012).

Upon release into the cytosolic compartment of the host cell, *Francisella* is able to replicate quickly to high numbers (Jones *et al.*, 2012). In order to accomplish this without triggering induction of host defenses, this pathogen must evade multiple pattern recognition receptors (PRRs) during its intracellular replication cycle. Indeed, *Francisella* is known to evade nearly all Toll-like receptors (TLRs), which can recognize bacterial components at the cell surface or within phagosomes (Figure 1) (Jones *et al.*, 2012). *Francisella* even subverts recognition by TLR4, a principal sensor of Gram-negative pathogens (discussed in detail below). In fact, TLR2, which recognizes bacterial lipoproteins and possibly peptidoglycan, is the only TLR that has been shown to be involved in the host response to *Francisella* infection, though the bacteria is also able to dampen this response (Jones *et al.*, 2012). Once in the cytosol, bacteria can be recognized by PRRs such as Nod-like receptors, though a role for these proteins during *Francisella* infection has not been established (Jones *et al.*, 2012). However, *F. novicida* does trigger the cytosolic PRR AIM2, which recognizes bacterial DNA and induces inflammatory mediated cell death (Jones, 2012). Interestingly, dead or dying *Francisella* have recently been shown to trigger autophagy, which may help diminish the amount of bacterial DNA in the cytosol (Figure 1) (Chong *et al.*, 2012). Whether or not this pathogen is capable of cell to cell spread is still being elucidated, though it is thought that the bacteria generally escape from host cells upon induction of either pyroptotic or apoptotic programmed cell death (Jones *et al.*, 2012).

Francisella and oxidative stress

Within macrophages, ROS are primarily produced by the NADPH oxidase, a multi-protein complex that assembles in the phagosomal membrane (Nauseef, 2004). The NADPH oxidase components, including $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p40^{phox}$, $p67^{phox}$, and Rac2 function together to convert molecular oxygen into superoxide species (Nauseef, 2004). During infection, *Francisella* has been shown to suppress both the assembly of the NADPH oxidase complex as well as the function of assembled complexes. AcpA, AcpB, AcpC, and Hap are all *Francisella* acid phosphatases that have been shown to inhibit complex assembly, however the requirement of these proteins for intracellular replication is still under debate (Jones *et al.*, 2012). In addition, *F. tularensis* has been shown to block ROS production by NADPH oxidase complexes assembled in response to exogenous stimuli through an undetermined mechanism (McCaffrey *et al.*, 2010).

Francisella also encodes multiple proteins important for the detoxification of ROS including the superoxide dismutase proteins SodB and SodC. These proteins, which convert superoxide radicals into less toxic hydrogen peroxide, have been shown to be essential for *in vivo* survival of this pathogen (Bakshi *et al.*, 2006, Melillo *et al.*, 2009). In addition, the catalase KatG, which decomposes hydrogen peroxide into water and oxygen, is also required for virulence in mice (Lindgren *et al.*, 2007). Also, MoxR has been shown to be involved in *Francisella* resistance to multiple stresses including oxidative stress and is important *in vivo* (Dieppedale *et al.*, 2011).

In addition, *Francisella* encodes proteins involved in detoxification of the byproducts of ROS, in particular organic hydroperoxides produced during lipid peroxidation. Chapter 2 of this work identifies the *Francisella* protein FTN_1133 as a novel *Francisella* oxidative stress resistance protein, specific for stress induced by organic hydroperoxides, and is required for virulence and replication in macrophages. According to the NCBI database, *Francisella* also encodes a predicted AhpC homolog. This protein has been shown to be important for resistance to both inorganic and organic hydroperoxides in other bacterial pathogens such as *P. aeruginosa*, *Brucella abortus*, and *Salmonella typhimurium* (Ochsner *et al.*, 2000, Parsonage *et al.*, 2008, Steele *et al.*, 2010). Consistent with a role in virulence, an *ahpC* transposon mutant showed a marked increase in time to death in a low dose challenge in mice (Kadzhaev *et al.*, 2009).

Francisella Lipid A

Expression of a modified lipid A is another mechanism by which *Francisella* dampens the host response. While the host pattern recognition receptor TLR4 is considered a primary sensor of Gram-negative bacteria, *Francisella* LPS does not efficiently activate TLR4 when compared to LPS from *E. coli* and other Gram-negative pathogens (Raetz *et al.*, 2009). Many Gram-negative bacteria that elicit robust TLR4 signaling synthesize a hexaacylated lipid A portion of LPS with acyl chains of 12-14 carbons and phosphate groups at the 1 and 4' positions (Figure 2) (Raetz *et al.*, 2009). However, *Francisella* modifies or removes these structures. For example, *Francisella* lipid A acyl chains are two to six carbons longer than those in *E. coli* LPS (Raetz *et al.*, 2009). In addition, *Francisella* lipid A is tetraacylated as it lacks the canonical 3' double acyl chain, and

both the 1 and 4' phosphate groups are absent (Figure 2) (Raetz *et al.*, 2009). These modifications are critical for virulence as Wang *et al*. have shown that an *F. novicida* mutant lacking the *lpxF* gene and producing a pentaacylated lipid A containing the 4' phosphate group was rapidly cleared in a mouse infection model (Wang *et al.*, 2007). This mutant exhibited hypersensitivity to the cationic antimicrobial peptide polymyxin B, elicited an increased TLR4-independent local cytokine response and increased neutrophil recruitment *in vivo*.

Unlike other Gram-negative bacteria, between 70% and 90% of total *Francisella* lipid A exists as "free lipid A" that does not contain core polysaccharides or O-antigen (Figure 2)(Wang *et al.*, 2006, Zhao & Raetz, 2010). Similar to the complete LPS, free lipid A is tetraacylated with elongated acyl chains and lacks the 4' phosphate group. However, instead of being removed as it is in complete LPS, the phosphate at the 1 position is present and modified with a galactosamine (Wang *et al.*, 2006, Zhao & Raetz, 2010). This modification to free lipid A alone is critical for pathogenesis since an *flmK* mutant that does not add the galactosamine to free lipid A but has wild-type complete LPS, is highly attenuated *in vivo* (Kanistanon *et al.*, 2008). Chapter 3 of this manuscript elucidates the role of a novel protein that is essential for the addition of galactosamine to *Francisella* free lipid A and is required for virulence and intracellular replication. Chapter 4 explores the role of this modification in evasion of intracellular host antimicrobial peptides and innate signaling recognition.

Thesis overview

Overall, there is still much to be learned about *Francisella* pathogenesis, in particular the genetic requirements for the different phases of its intracellular lifecycle. Recent *in vivo* genetic screens have identified hundreds of *Francisella* genes predicted to be involved in virulence, though how many of these genes may contribute to disease is unknown (screen refs). For my thesis project, I assembled a transposon mutant library of genes previously identified to be required *in vivo* and performed an intracellular replication screen to determine which of these virulence factors were specifically required for *Francisella*'s intracellular lifecycle (Chapter 2). From the results of this screen, we then characterized two proteins that were required for intracellular proliferation: FTN_1133, a protein important for detoxifying the byproducts of oxidative stress (Chapter 2), and NaxD, a protein required for a lipid A modification essential for virulence (Chapter 3) and the evasion of host antimicrobial peptides (Chapter 4). Further studies into the roles of these proteins and others identified in my screen have the potential to contribute to knowledge of virulence mechanisms of not only *Francisella* species, but many homolog-encoding intracellular pathogens.

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Chapter 1 Tables

Laboratory Strain	LD_{50} CFU (Mice)	LD_{50} CFU (Humans)	% Nucleotide identity w/ SchuS4	Biosafety level
F. tularensis (SchuS4)	< 10	< 10	-	3
Live vaccine strain (LVS)	< 10	ND	99.3%	2
F. novicida (U112)	< 10	$> 10^3$	97.7%	2

Table 1. Laboratory strains of *Francisella.* Adapted from Pechous, 2009, MMB

Chapter 1 Figures

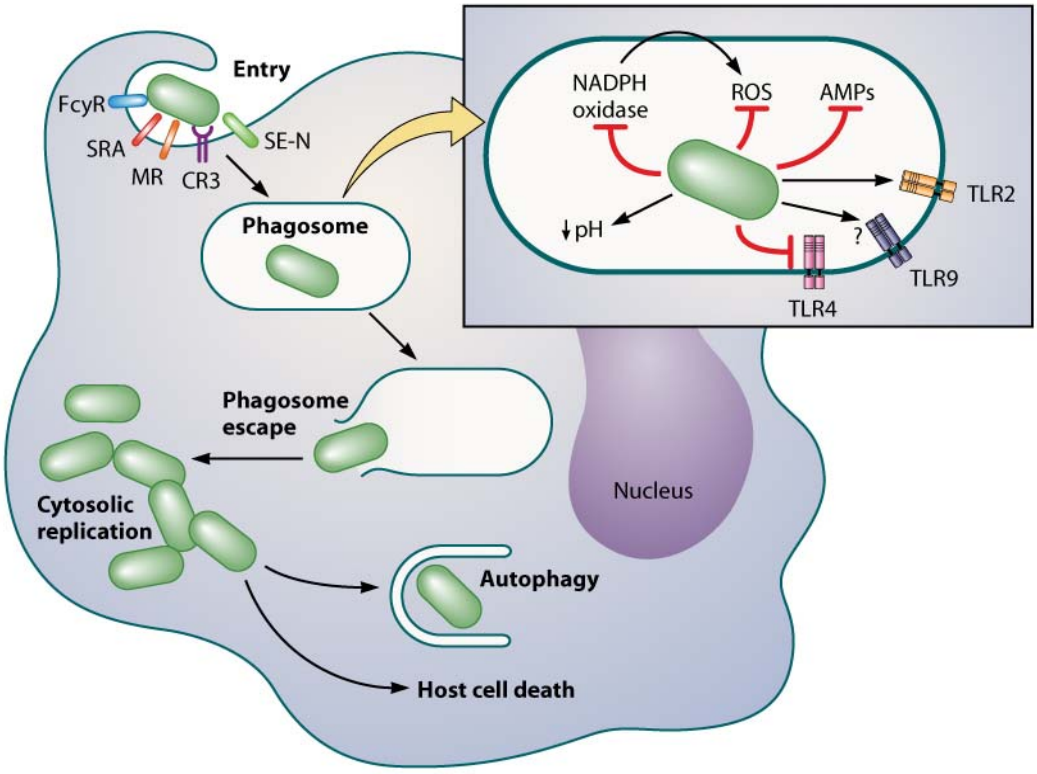

Figure 1. Stages of *Francisella* pathogenesis in the macrophage. *Francisella* can be detected by multiple macrophage receptors and is engulfed by a unique pseudopod loop mechanism and traffics to an early phagosome. *Francisella* uses multiple mechanisms to evade host defenses in this harsh environment (inset). *Francisella* blocks the NADPH oxidase and also detoxifies reactive oxygen species (ROS). It can also resist the action of antimicrobial peptides (AMPs). *Francisella* does not signal through TLR4 but does activate TLR2. *Francisella* then escapes the phagosome to replicate within the cytosol. Nonviable *Francisella* bacteria are cleared via autophagy. *Francisella* can also induce pyroptotic and apoptic host cell death. (From Jones, 2012).

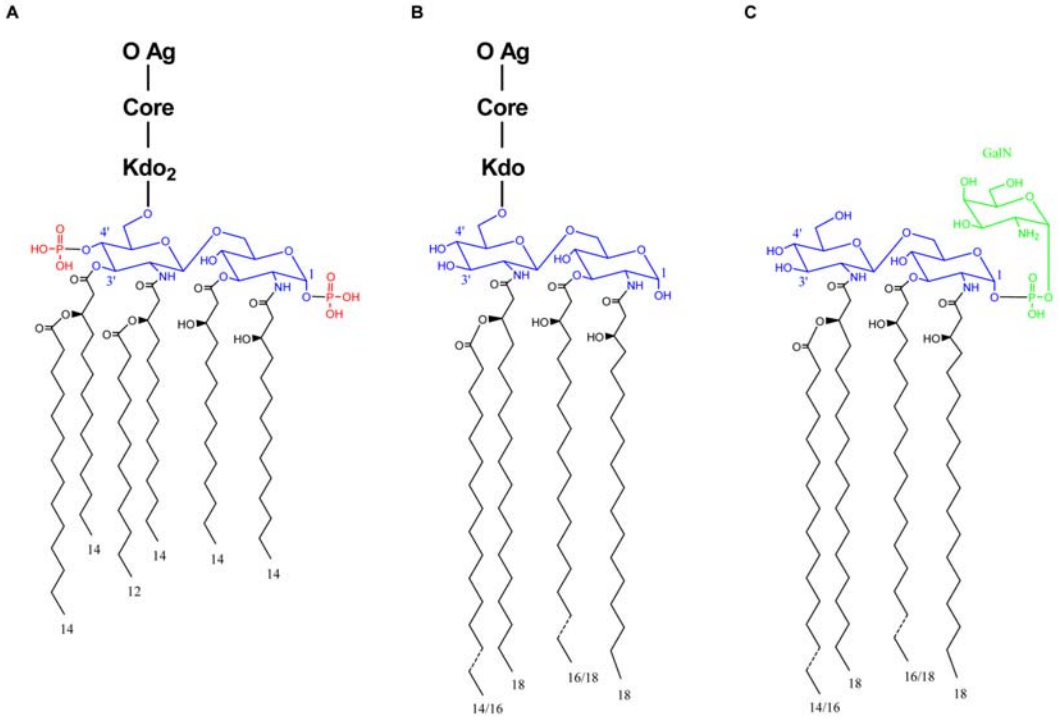

Figure 2. *E. coli* and *Francisella* LPS and lipid A structures. Structures of (A) *E. coli* LPS, (B) complete LPS from *Francisella* species, and (C) "free" lipid A of *Francisella* species are compared. (A, B) O-antigen (O Ag), core sugars (Core), and the specific core sugar Kdo (Kdo) are indicated. For all structures, lipid A backbone disaccharides are highlighted in blue and acyl chains are represented in black with numbers denoting length. *E. coli* lipid A 4'and 1 position phosphate groups (missing from the lipid A of complete *Francisella* LPS) are highlighted in red. Unlike the lipid A component of complete *Francisella* LPS, *Francisella* free lipid A includes a phosphate modified with galactosamine at the 1 position (highlighted in green). (From Chapter 3).

Chapter 2: Macrophage Replication Screen Identifies a Novel *Francisella* Hydroperoxide Resistance Protein Involved in Virulence.

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Chapter 2 Abstract

Francisella tularensis is a Gram-negative facultative intracellular pathogen and the causative agent of tularemia. Recently, genome-wide screens have identified *Francisella* genes required for virulence in mice. However, the mechanisms by which most of the corresponding proteins contribute to pathogenesis are still largely unknown. To further elucidate the roles of these virulence determinants in *Francisella* pathogenesis, we tested whether each gene was required for replication of the model pathogen *F. novicida* within macrophages, an important virulence trait. Fifty-three of the 224 genes tested were involved in intracellular replication, including many of those within the *Francisella* pathogenicity island (FPI), validating our results. Interestingly, over one third of the genes identified are annotated as hypothetical, indicating that *F. novicida* likely utilizes novel virulence factors for intracellular replication. To further characterize these virulence determinants, we selected two hypothetical genes to study in more detail. As predicted by our screen, deletion mutants of *FTN_0096* and *FTN_1133* were attenuated for replication in macrophages. The mutants displayed differing levels of attenuation *in vivo*, with the *FTN_1133* mutant being the most attenuated. *FTN_1133* has sequence similarity to the organic hydroperoxide resistance protein Ohr, an enzyme involved in the bacterial response to oxidative stress. We show that FTN_1133 is required for *F. novicida* resistance to, and degradation of, organic hydroperoxides as well as resistance to the action of the NADPH oxidase both in macrophages and mice. Furthermore, we demonstrate that *F. holarctica* LVS, a strain derived from a highly virulent human pathogenic species of *Francisella*, also requires this protein for organic hydroperoxide resistance as well as replication in macrophages and mice. This study expands our

knowledge of *Francisella*'s largely uncharacterized intracellular lifecycle and demonstrates that FTN_1133 is an important novel mediator of oxidative stress resistance.

Chapter 2 Introduction

Francisella tularensis is a unique facultative intracellular pathogen that can cause a potentially lethal disease with an infectious dose as low as 10 bacteria [1]. A small Gram-negative coccobacillus, *F. tularensis* is the causative agent of tularemia, a vector- and water-borne zoonotic disease resulting in non-specific, flu-like symptoms that may culminate in pneumonic, glandular, and systemic infections [1]. When left untreated, pneumonic tularemia can result in a mortality rate as high as 60% [2]. *F. tularensis* subspecies are endemic across the Northern Hemisphere, with the majority of reported cases of disease in the United States, Europe, Russia, and Japan [3]. Due to its extreme infectivity, high morbidity and mortality rates, history of weaponization, and ease of aerosolization, dissemination, and genetic manipulation, *F. tularensis* is considered a category A potential bioweapon by the Centers for Disease Control and Prevention (CDC) [4-6].

The virulence mechanisms of *F. tularensis* subspecies and other *Francisella* species are still being characterized, including the role of the *Francisella* pathogenicity island (FPI) which is thought to encode a Type VI secretion system that facilitates the release of virulence proteins into host cells [7-13]. While *F. tularensis* and *F. holarctica* are responsible for the majority of disease burden in humans [5], many important virulence

determinants are conserved among other *Francisella* species, including *F. novicida*. In addition to the FPI, these conserved virulence determinants include the presence of a non-inflammatory lipopolysaccharide (LPS), protective capsule, siderophores, and proteins involved in resistance to oxidative stress [14-21]. *F. novicida*, which has 98% nucleotide identity with the human pathogenic species, causes disease mainly in immunocompromised individuals but has also been shown to cause disease in healthy individuals [22-24]. The live vaccine strain (LVS) is an attenuated strain of the highly pathogenic species *F. holarctica* that was originally developed as a vaccine and retains 99.92% identity to its parental species [25,26]. Though work involving *F. tularensis* and *F. holarctica* is restricted to Select Agent Biosafety Level 3 (BSL3) laboratories, both *F. novicida* and LVS are approved for use in BSL2 laboratories, are readily genetically manipulated, and cause tularemia-like disease in mice, making them both good laboratory models for studying *Francisella* pathogenesis [27,28].

Francisella's primary replicative niche is thought to be the cytosolic compartment of both phagocytic and non-phagocytic cells such as macrophages, neutrophils, hepatocytes, alveolar epithelial cells, and fibroblasts [29-32]. After being engulfed by phagocytic host cells, the bacteria are taken up into phagosomes where they are confronted with a myriad of antimicrobial defenses including degradative enzymes, acidic pH, and oxidative stress [17-21,33-36]. The reactive oxygen species (ROS) which cause oxidative stress can directly damage bacterial macromolecules such as proteins, DNA, and lipids. They can also react with these macromolecules to generate more ROS and toxic oxygen compounds, including the highly toxic organic hydroperoxides that result from the

destructive lipid peroxidation of cell membranes [37-40]. Within host cells, ROS are generated by multiple mechanisms including the NADPH oxidase, myeloperoxidase, lipoxygenases, and cellular respiration [41,42]. The NADPH oxidase, which produces superoxide radicals that lead to ROS formation, has been shown to be important for the host response to infection with *Francisella* species [43-46]. These bacteria employ numerous strategies to resist oxidative stress including limiting the activation of the NADPH oxidase [35,36,47] and using multiple enzyme systems to detoxify reactive oxygen compounds [18,32,48,49].

While a general outline of *Francisella*'s interaction with host cells is known, the specific mechanisms of cell entry, phagosomal escape, cytosolic replication, and some of the ways it evades immune defenses are still unknown. Genome-wide *in vivo* screens have identified genes required for the virulence of several *Francisella* species but do not shed light on how the majority of these genes contribute to pathogenesis [50-52]. As replication within host cells is a major part of *Francisella*'s infectious cycle, we set out to determine which of the genes that are known to be required for virulence *in vivo* are also required for replication in host macrophages.

We performed an intracellular replication screen using transposon mutants representing 224 genes that have previously been shown to be required for virulence *in vivo*. Fifty-three of the genes tested were required for replication in macrophages including many of the FPI genes, validating the screen. We also identified biotin biosynthetic genes and the *fsl*/*fig* siderophore biosynthetic genes [16,53-55] as well as numerous proteins of

unknown function as being required for replication in macrophages. We validated the intracellular and *in vivo* requirement of two of these novel genes, *FTN_1133* and *FTN_0096*. We then further investigated the importance of *FTN_1133*, which encodes a protein with sequence similarity to Ohr, a protein involved in oxidative stress resistance [56-64]. Accordingly, we find that FTN_1133 is required for resistance to, and degradation of, organic hydroperoxides. Furthermore, the replication defect of the *FTN_1133* mutant is rescued in macrophages lacking a functional NADPH oxidase and partially rescued in mice with the same defect. We further demonstrate that *F. holarctica* LVS also requires this protein for organic hydroperoxide resistance and replication in macrophages and mice. Taken together, these data highlight the critical role that novel virulence factors play in *Francisella* pathogenesis and contribute to the elucidation of the requirements for this pathogen's largely uncharacterized intracellular lifecycle.

Chapter 2 Results

Intracellular replication screen. To further characterize the role in virulence of genes known to be required in animal infection models, we screened a library of corresponding transposon mutants for replication in RAW264.7 macrophages. We screened a total of 451 transposon mutants representing 224 genes and identified 53 of these genes to be required for *F. novicida* replication in RAW264.7 macrophages (Table 1). The screen results were validated by the identification of genes that have previously been reported to be required for intracellular replication, including genes encoded in the *Francisella* Pathogenicity Island (FPI) [8,9,11,12,77-80]. To the best of our knowledge, this study is the first to report the requirement of *Francisella*'s biotin biosynthetic genes and the *fsl*/*fig*

siderophore biosynthetic genes for replication in mammalian cells [16,53-55]. A more in-depth review of the genes identified in this screen is included in the Discussion. Interestingly, a large proportion of the genes identified to be required for intracellular replication by this screen encode proteins of unknown function. Two such proteins were chosen for further study: FTN_0096 and FTN_1133. FTN_0096 was selected because of the severe intracellular replication defect of the *FTN_0096* transposon mutant (Table 1). FTN_1133 was chosen because, although it is annotated as a hypothetical protein, we found that it has sequence similarity to Ohr, a protein involved in resistance to organic hydroperoxides which can induce oxidative stress, resistance to which is a critical virulence mechanism of *Francisella* species [17-21,56-63].

Validation of Screen Results. Deletion mutants for *FTN_0096* and *FTN_1133* were generated using allelic replacement as previously described [70]. The mutants exhibited wild-type replication kinetics when grown in both tryptic soy broth (TSB) supplemented with cysteine (Fig. S1A) and Chamberlain's chemically defined minimal medium (Fig. S1B). To validate the phenotypes of the corresponding transposon mutants in our screen, the replication phenotype of each deletion mutant was determined in RAW264.7 macrophages. Twenty-four hours post-infection, the *FTN_0096* mutant was severely attenuated for replication compared to wild-type *F. novicida*, with a fold replication value similar to that of the replication-deficient control strain, GB2, which we will refer to as *mglA* (Fig. 1A). This strain harbors a point mutation in *mglA*, a gene known to be essential for intracellular replication [65]. The *FTN_1133* mutant displayed an approximate six-fold replication deficiency compared to wild-type (Fig. 1C). In order to

ensure that the observed phenotypes resulted from deletion of the targeted gene and not unintended secondary site mutations, we generated complemented strains of each mutant in which the deleted gene was replaced. All of the complemented strains displayed wild-type levels of replication (Fig. 1A, C). These data further validate our screen and demonstrate the requirement of *FTN_0096* and *FTN_1133* for *F. novicida* replication in RAW264.7 macrophages.

To verify that the replication defects of these *F. novicida* mutants were not specific to RAW264.7 cells, we next infected primary murine bone marrow-derived macrophages (BMM) and measured replication levels. Compared to the wild-type strain, the *FTN_0096* mutant again displayed a severe replication deficiency similar to the *mglA* mutant, and the *FTN_1133* mutant had an approximate three-fold replication defect (Fig. 1B, D). Replication was restored to the wild-type level in the complemented strains (Fig. 1B, D). We measured bacterial replication at 5.5 hrs post-infection, before any macrophage cell death occurred, to ensure that the attenuated phenotypes of the mutants were not a consequence of the cell death response. RAW264.7 macrophages are known to be deficient in ASC/caspase-1 inflammasome-mediated cell death [81], an inflammatory host cell death pathway known to be triggered by *F. novicida* infection [82], explaining why we could measure bacterial replication at later time points in these cells. Taken together, these results demonstrate that in both of the macrophage cell types tested, *FTN_0096* and *FTN_1133* play a role in replication and that the deletion mutants lacking these genes displayed intracellular replication deficiencies similar to those predicted by our screen.

Deletion mutants are attenuated *in vivo*. To test whether the macrophage replication defects correlated with *in vivo* attenuation levels, competition experiments were performed in mice. Briefly, mice were infected with a 1:1 ratio of wild-type *F. novicida* and each mutant strain. Forty-eight hours post-infection, mouse organs were harvested, homogenized, and plated for enumeration of wild-type and mutant CFU. The number of *FTN_0096* deletion mutant CFU in the spleen and liver was one log below that of wild-type (Fig. 2B, C), although no attenuation was observed in the skin (Fig. 2A). The *FTN_1133* mutant had a one log attenuation in the skin, roughly two log attenuation in the spleen, and nearly three log attenuation in the liver (Fig. 2). Both mutant phenotypes were restored to wild-type levels in the complemented strains (Fig. 2). Taken together, these results demonstrate that FTN_0096 and FTN_1133 are involved in *F. novicida* pathogenesis *in vivo*, though FTN_1133 appears to have a more significant role in virulence in mice.

***FTN_1133* is required for virulence in single infections.** Since the *FTN_1133* mutant was the most severely attenuated *in vivo*, we chose this gene for further characterization. To ensure that the attenuation of this mutant was not only observed when in competition with wild-type bacteria, we performed single infection experiments. We observed that the *FTN_1133* mutant was attenuated in each organ to a similar degree as in competition experiments (Fig. 3A-C), confirming that FTN_1133 is required for full virulence of *F. novicida*. We further verified this result by monitoring the survival of these mice. By day

10 post-infection, only 40% of mice infected with wild-type bacteria survived while 100% of those infected with the *FTN_1133* deletion mutant survived (Fig. 3D).

***FTN_1133* is expressed during infection of macrophages and mice.** Given its importance in *F. novicida* replication and survival in macrophages and mice, we tested whether *FTN_1133* was transcribed during infection. Levels of *FTN_1133* transcript from samples of wild-type *F. novicida*-infected BMM at 30 minutes, 2 hours, and 4 hours post-infection were determined using quantitative real-time RT-PCR (qRT-PCR) (Fig. 4A). The expression of *FTN_1133* was induced during macrophage infection, as indicated by a moderate but significant increase in expression between 30 minutes and 4 hours (Fig. 4A). We also observed a similar level of expression in livers of infected mice 4 hours after intraperitoneal infection (Fig. 4B). Taken together and consistent with its role in pathogenesis, these results show that *FTN_1133* is expressed during infection of both macrophages and mice.

FTN_1133 is involved in resistance to organic hydroperoxides. Bioinformatic analysis revealed that FTN_1133 has sequence similarity to an organic hydroperoxide resistance protein (Ohr) from *Bacillus megaterium*. Specifically, the C-terminal half of Ohr has significant similarity to residues 18-86 of FTN_1133 (Fig. S2). Based on these findings, we tested whether FTN_1133 is involved in resistance to organic hydroperoxides in *F. novicida*. We quantified bacterial sensitivity by measuring zones of inhibition upon exposure to organic hydroperoxides via the disk diffusion method. The *FTN_1133* mutant showed increased sensitivity to cumene and tert-butyl hydroperoxides compared to the

wild-type and complemented strains (Fig. 5A, B). Since Ohr is often required for resistance to organic hydroperoxides but not inorganic hydroperoxides [59,60,62,63,83], we grew the bacterial strains in the presence of inorganic hydrogen peroxide and found that the wild-type, mutant, and complemented strains showed equal levels of sensitivity, indicating that FTN_1133 is indeed not required for resistance to an inorganic hydroperoxide (Fig. 5C). Finally, to further demonstrate the specificity of FTN_1133 for organic hydroperoxides and rule out a general sensitivity to stresses, we found that the wild-type, mutant and complemented strains were equally susceptible to SDS, a membrane-damaging detergent (Fig. 5D). Taken together, these data demonstrate that FTN_1133, similar to most Ohr proteins, is required for resistance to organic but not inorganic hydroperoxides.

FTN_1133 is required for degradation of an organic hydroperoxide. To determine if FTN_1133 is important not only for resistance to organic hydroperoxides, but also detoxification of these chemicals, we added tert-butyl hydroperoxide to cultures of wild-type *F. novicida* or the *FTN_1133* deletion mutant and measured its concentration over time. Between 15 minutes and 30 minutes, the *FTN_1133* deletion mutant degraded approximately 50% less tert-butyl hydroperoxide than wild-type bacteria (Fig. 6). These data indicate that FTN_1133 is involved in degradation of an organic hydroperoxide.

FTN_1133 is required for resistance to the action of the NADPH oxidase. To test if the sensitivity of the *FTN_1133* mutant to oxidative stress was the cause of its replication defect in macrophages, we infected BMM from both wild-type and gp91$^{phox-/-}$ mice with

wild-type *F. novicida* and the *FTN_1133* deletion mutant. gp91 is a subunit of the NADPH oxidase and is required for the generation of reactive oxygen species and the oxidative stress induced by this enzyme. While the *FTN_1133* mutant was attenuated for replication in wild-type BMM (Fig. 7A, and similar to Fig. 1), it replicated to the same levels as wild-type *F. novicida* in the $gp91^{phox-/-}$ BMM (Fig. 7B). In order to determine whether a similar phenotype is observed *in vivo*, we infected wild-type and $gp91^{phox-/-}$ mice with either wild-type *F. novicida* or the *FTN_1133* deletion mutant. Indeed, the two log attenuation of the *FTN_1133* deletion mutant in wild-type mice was rescued by one log in $gp91^{phox-/-}$ mice (Fig. 7C). Together, these data indicate that FTN_1133 is required to resist the oxidative stress generated by the NADPH oxidase and makes an important contribution to *F. novicida*'s intracellular and *in vivo* pathogenesis.

The *FTN_1133* ortholog, *FTL_0803*, confers resistance to an organic hydroperoxide. In order to determine whether *FTN_1133* is also involved in organic hydroperoxide resistance in other *Francisella* species, we constructed an *F. holarctica* LVS deletion mutant lacking the *FTN_1133* ortholog, *FTL_0803*. FTN_1133 and FTL_0803 share 98% amino acid identity. To test the requirement of FTL_0803 for LVS resistance to organic hydroperoxides, we performed disk diffusion assays on wild-type LVS and the *FTL_0803* deletion mutant. As with FTN_1133, we found that FTL_0803 is required for wild-type resistance to the organic hydroperoxide tert-butyl hydroperoxide (Fig. 8A), but not H_2O_2 (Fig. 8B) or SDS (Fig. 8C).

FTL_0803 is required for *F. holarctica* LVS virulence and resistance to the action of the NADPH oxidase. Next we investigated the importance of FTL_0803 in LVS pathogenesis by determining the replication phenotype of the deletion mutant both in macrophages and mice. First, RAW264.7 macrophages were infected with wild-type LVS and the *FTL_0803* mutant, and bacterial levels were measured at 24 hours post-infection. Similar to the phenotype of the *FTN_1133* mutant, the *FTL_0803* mutant exhibited a five-fold intracellular replication defect (Fig. 9A). Mouse infection experiments revealed that the *FTL_0803* deletion mutant was present at one log lower levels than LVS in the organs tested (Fig. 9B-D). Finally, to determine if the replication deficiency of this mutant in macrophages is also rescued in the absence of a functional NADPH oxidase, we infected both wild-type BMM and gp91$^{phox-/-}$ BMM with either LVS or the *FTL_0803* deletion mutant. The five-fold replication deficiency of the mutant in wild-type BMM was largely rescued in gp91$^{phox-/-}$ BMM (Fig. 10). These data demonstrate that the importance of FTN_1133/FTL_0803 in resisting oxidative stress, promoting intracellular replication, and contributing to *in vivo* virulence is conserved in multiple *Francisella* species.

Chapter 2 Discussion

Recently, *in vivo* screens have identified many genes required for *Francisella* virulence, though the functions of these genes and an understanding of the stage of infection at which they contribute to virulence are largely unknown [50,51]. In an effort to further characterize these virulence determinants and to begin to answer questions that remain regarding *Francisella*'s intracellular lifecycle, we performed an intracellular replication

screen using transposon insertion mutants of genes that were identified in the two mammalian *in vivo* genome-wide screens that were published at the time of this study: one using *F. novicida* transposon mutants [50] and one using transposon mutants of the *Francisella* live vaccine strain (LVS) [51]. Recently, Kraemer et al. published another mammalian *in vivo* genome-wide screen, the results of which were published after our screen was performed and therefore genes specifically identified in this study were not included [52].

Of the 224 genes screened, 53 were shown to be required for replication in murine macrophages. The 171 genes that were not identified in this screen may be required for replication in other cell types known to be infected by *Francisella*, such as neutrophils, hepatocytes, alveolar epithelial cells, or fibroblasts [29-32,84,85]. Alternatively, these genes may be required for processes other than intracellular replication such as immune evasion or systemic dissemination. To the best of our knowledge, the intracellular replication data from mutants for 140 of the 224 genes that were represented in this screen have not been previously reported in mammalian cells. One gene, *feoB* (*FTN_0066*), was included in the screen but was excluded from the results because of a growth defect on cysteine-enriched tryptic soy agar (TSA), despite no apparent growth defect in cysteine-enriched tryptic soy broth (TSB) (data not shown).

The results of our screen were validated by the identification of genes that have previously been shown to be required for replication in macrophages, including most of the *Francisella* pathogenicity island (FPI) genes [8,9,11,12,77-80,86]. Of the 18 genes in

the *F. novicida* FPI, mutants representing 13 genes were identified as attenuated for replication in macrophages. Among the FPI genes not identified in this screen (*pdpA*, *pdpC*, *pdpD*, *pdpE*, and *anmK*), *pdpD* has previously been reported to be unnecessary for replication in mouse macrophages [87]. Both *pdpC* and *pdpD* were also shown to be unnecessary for replication in an arthropod cell line [88] and *pdpC*, *pdpD*, *pdpE*, and *anmK* were all reported to have no role in virulence in a live arthropod model [78]. While *pdpA* has been shown to be required for intracellular replication within mammalian cells [10,78,86], it was not identified in this screen. One of the three *pdpA* transposon mutants we tested displayed a deficiency in intracellular replication, but it was just below the cut-off value used in this screen (Table S1). This may be due to retention of some protein function in the *pdpA* mutant strains used in this study, a potential problem when using transposon mutants as compared to deletion mutants.

In addition to the identification of FPI genes, this screen was further validated by the identification of other genes that have previously been shown to be required for intracellular replication, including *pyrB* [29,32,78], *carA* [29,32,78], *carB* [29,32,78,89], *purM* [90] and *clpB* [12,78,91,92]. Genes that have previously been shown to play no role in intracellular replication and were also shown to be unnecessary for replication in our screen include *FTN_0757* and *FTN_0720*, verifying the selectivity of our screen [50]. Though an *htpG* deletion mutant has previously been reported to be attenuated for replication in BMM macrophages [50], and one of the two transposon mutants with insertions in this gene displayed an approximate two-fold replication attenuation in this screen, the level of attenuation was not sufficient for our cut-off.

Our screen led to numerous novel insights into *Francisella*'s genetic requirements for replication within host cells. To the best of our knowledge, this is the first study to implicate a requirement of biotin for *Francisella's* replication in mammalian cells since each of the biotin synthetic genes, *bioA*, *bioB*, *bioC*, *bioD*, and *bioF* (*FTN_0812-FTN_0816*) was identified. Biotin is critical for various metabolic pathways and biotin biosynthetic genes are required for *Mycobacterium tuberculosis* intracellular replication and pathogenesis [93-95]. *bioF* was also recently reported to be important for *Francisella* replication in an arthropod cell line [96]. In addition, we report here for the first time *Francisella*'s requirement for replication in mammalian cells of at least three of the six siderophore biosynthetic genes: *fslA*, *fslB*, and *fslC*, also known as *figA*, *figB*, and *figC* (*FTN_1682-FTN_1684*), confirming that this iron acquisition system is essential for efficient replication within mammalian cells. Pathogens generally encounter iron-limiting conditions within the host, and thus iron acquisition proteins are critical virulence factors in numerous pathogens [97,98]. *fslA* and *fslB* were also recently reported to be important for replication in an arthropod cell line [96]. The other three genes from this group (*FTN_1685-FTN_1687*) were not included in our screen.

Of the 53 genes identified to be required for replication in this screen, 19 are annotated as hypothetical, indicating that *F. novicida* encodes novel genes that are required both for virulence *in vivo* and intracellular replication. To study the role of some of these hypothetical proteins during infection as well as further validate the screen results, we chose two genes to study: *FTN_0096* and *FTN_1133*. FTN_0096 is a member of the DUF1275 superfamily of proteins, which has members in other human pathogens such as

Vibrio spp, *Acinetobacter* spp, *Neisseria* spp, and *Burkholderia* spp. Analysis with the Simple Modular Architecture Research Tool (SMART) identified 7 potential transmembrane domains as well as a putative signal peptide, indicating that FTN_0096 is likely a membrane protein (http://smart.embl-heidelberg.de/). Interestingly, bioinformatic analyses revealed that FTN_1133 was found only in *Francisella* species but had significant similarity to the C-terminal domain of an organic hydroperoxide resistance protein, Ohr, from *Bacillus megaterium*.

We show that both *FTN_0096* and *FTN_1133* are required for replication in macrophages and virulence in mice. FTN_0096 was absolutely required for replication in both RAW264.7 macrophages and BMM (Fig. 1A, B). In addition, *FTN_0096* was recently reported to be important for replication in an arthropod *in vivo* model as well as murine macrophage-like J774 cells [78]. The *FTN_0096* deletion mutant displayed only a moderate one log attenuation in the spleen and liver of mice, despite having a severe intracellular replication defect (Fig. 2). The disparity in this mutant's *in vivo* versus *in vitro* phenotypes could be due to an ability of bacteria lacking *FTN_0096* to replicate efficiently in other cells types, particularly non-immune cells that have fewer defenses against intracellular infection. Indeed, Horzempa et al. recently showed that a uracil synthesis mutant unable to replicate in macrophages displayed a virulent phenotype in mice attributable to that mutant's ability to replicate in non-phagocytic host cells [99]. Conversely, the *FTN_1133* mutant was moderately attenuated for intracellular replication (3-6 fold) in macrophages (Fig. 1), displayed a marked two to three log attenuation in the spleen and liver following mouse infections (Fig. 2, 3), and did not cause lethal infection

in mice (Fig. 3D). Furthermore, we showed that *FTN_1133* was expressed during infection of both macrophages and mice (Fig. 4).

Bioinformatic analyses revealed that FTN_1133, a 127 amino acid protein, has significant similarity to the C-terminal domain of an organic hydroperoxide resistance protein (Ohr) from *Bacillus megaterium* (Fig. S2). The C-terminus of Ohr proteins has been shown to be important for enzymatic function in bacteria such as *Xylella fastidiosa* and *Pseudomonas aeruginosa* [83,100-102]. Originally identified in *Xanthomonas campestris*, Ohr is a 139 amino acid protein which has homologs in several bacterial species including *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* [56,57,59,61]. Ohr is thought to be a hydroperoxide reductase that converts organic hydroperoxides into less toxic metabolites [101]. This protein contributes to resistance to reactive oxygen species (ROS)-induced damage by degrading the highly toxic organic hydroperoxides that are created during lipid peroxidation when oxygen radicals react with the unsaturated and polyunsaturated lipids of cell membranes [37-40,100].

Interestingly, Ohr proteins are usually only involved in resistance to organic hydroperoxides, such as tert-butyl hydroperoxide and cumene hydroperoxide, but not inorganic hydroperoxides, such as hydrogen peroxide [59,60,62,63,83]. Our disk diffusion analysis indicates that FTN_1133, like many Ohr proteins, is required for resistance to organic hydroperoxides but not hydrogen peroxide (Fig. 5). Also similar to Ohr, we demonstrate that FTN_1133 is required for degradation of tert-butyl hydroperoxide (Fig. 6) [59,60,75,76]. The *FTN_1133* deletion mutant displays a

moderate amount of degradation compared to the wild-type strain, which indicates that *Francisella* may encode another system involved in degradation of these chemicals. Potential candidates for this function include *Francisella*'s uncharacterized AhpC homologs. Most proteins involved in oxidative stress resistance specifically detoxify inorganic oxygen species, such as oxide radicals (superoxide dismutases) or hydrogen peroxide (catalase). However, AhpC has been shown to be important for resistance to both inorganic and organic hydroperoxides in other bacterial pathogens such as *P. aeruginosa*, *Brucella abortus*, and *Salmonella typhimurium* [103-105]. Consistent with a role in virulence, Kadzhaev et al. demonstrated that an *F. tularensis ahpC* transposon mutant showed a marked increase in time to death in a low dose challenge in mice [106].

While expression of *ohr* genes is often increased in response to organic hydroperoxides, several *ohr* genes have been identified that are not induced in this manner [57,62,64]. Quantitative real-time PCR analysis of *FTN_1133* expression revealed that while the gene is induced during infection (Fig. 4), this gene was not significantly induced in broth in our hands in response to organic hydroperoxides (data not shown).

To the best of our knowledge, *ohr* mutants have not previously been characterized in host cells or animal models, though *ohr* has been shown to be co-expressed with other virulence factors during *Actinobacillus pleuropneumoniae* infection of pigs [60]. Further suggestive of a role in pathogenesis, *ohr* is sometimes encoded on mobile genetic elements, such as a genomic island in *Actinobacillus pleuropneumoniae* [107] and a plasmid in pathogenic *Acinetobacter baumannii* [61]. Our *in vitro* screen and previous *in*

vivo screens [51,52] identified *FTN_1133* as important for virulence. We hypothesized that increased sensitivity of the *FTN_1133* mutant to oxidative stress may explain its intracellular and *in vivo* replication defect. Indeed, we observed restoration of wild-type levels of intracellular replication for the *FTN_1133* deletion mutant in BMM from mice lacking gp91$^{phox-/-}$ (Fig. 7B), an essential subunit of NADPH oxidase that is required for the generation of superoxide radicals by this enzyme complex. In addition, the *in vivo* attenuation of the *FTN_1133* mutant was significantly rescued in gp91$^{phox-/-}$ mice (Fig. 7C). Though the mutant was not fully complemented in these mice as it was in the gp91$^{phox-/-}$ BMM, this is not surprising since there are alternate ROS generating pathways that do not exist in macrophages but are present during *in vivo* infection. For example, myeloperoxidase is present in neutrophils but not macrophages and can initiate lipid peroxidation and organic hydroperoxide generation [41]. Furthermore, we demonstrate that the importance of FTN_1133 in resistance to oxidative stress is conserved in multiple *Francisella* species by showing the requirement of *FTL_0803*, the *F. holarctica* LVS *FTN_1133* ortholog, for resistance to organic hydroperoxides (Fig. 8) and wild-type replication both in macrophages and mice (Fig. 9). Finally, we show that the *in vitro* attenuation of the *FTL_0803* mutant was largely rescued in macrophages deficient in oxidative burst (Fig. 10). The molecular bases for the attenuation of the LVS strain have yet to be fully characterized and as such, there may be underlying deficiencies that explain the incomplete complementation of the *FTL_0803* mutant strain in gp91$^{phox-/-}$ BMM [26].

Our data demonstrating a role for FTN_1133 in resistance to oxidative stress and *Francisella* virulence are consistent with the fact that many oxidative stress resistance genes are transcribed during *Francisella* infection of macrophages [108] and many of these have been identified in screens as being required for replication in macrophages [78,89] and virulence in arthropods [20,78,96]. *Francisella* species have been shown to use an array of genes to suppress activation of the NADPH oxidase [32,35,36,45,109]. *Francisella* species also use numerous genes to detoxify reactive oxygen compounds and thereby resist oxidative stress. Specifically, the catalase KatG [48,49,51,89] and the superoxide dismutases SodB and SodC [18,49,110] have all been shown to be essential for survival of *Francisella* species *in vivo*. Also, a novel oxidative stress resistance gene, MoxR, was recently described in LVS [21]. The data presented here identify FTN_1133 as a novel *Francisella* oxidative stress resistance protein, specific for stress induced by organic hydroperoxides.

In this report, we demonstrate for the first time the importance of an Ohr-like protein in virulence during *in vitro* and *in vivo* infections as well as its specific role in resistance to oxidative stress both in macrophages and in mice. Furthermore, we show that the importance of this protein for pathogenesis is conserved in multiple *Francisella* species. Taken together, the results of this screen highlight the requirement of numerous *F. novicida* virulence determinants for intracellular replication. The critical importance of resisting oxidative stress suggests that Ohr-like proteins, including FTN_1133, may represent attractive drug targets [111]. In this way, continued characterization of FTN_1133 and other novel proteins and mechanisms used by *Francisella* could

contribute to the development of new therapeutics and vaccines against this potential bio-threat.

Chapter 2 Materials and Methods

Ethics Statement. All experimental procedures were approved by the Emory University Institutional Animal Care and Use Committee (protocol #069-2008Y).

Bacterial strains and growth conditions. Wild-type *F. novicida* strain U112, a previously described *mglA* point mutant, GB2 [65], and the *F. holarctica* Live Vaccine Strain (LVS) were generous gifts from Dr. Denise Monack (Stanford University, Stanford, CA). *F. novicida* overnight cultures were grown at 37°C on a rolling drum in tryptic soy broth (TSB; Difco/BD, Sparks, MD) supplemented with 0.02% L-cysteine (Sigma-Aldrich, St. Louis, MO) while LVS cultures were grown in modified Mueller-Hinton broth (mMHB) supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% glucose (Sigma-Aldrich), 2% Isovitalex (Difco/BD), and 0.025% ferric pyrophosphate as previously described [66]. Growth in minimal medium was determined using Chamberlain's chemically defined minimal medium, prepared as previously described [67]. For the replication screen, *F. novicida* was plated for enumeration on tryptic soy agar (TSA; Difco/BD) and supplemented with 0.01% L-cysteine. Bacteria from all other *F. novicida* experiments were plated on modified Mueller Hinton (mMH) (Difco/BD) plates supplemented with 0.025% ferric pyrophosphate (Sigma-Aldrich), 0.1% glucose, and 0.01% L-cysteine. LVS was plated on mMH supplemented additionally with 2% Isovitalex. When appropriate, kanamycin (Kan; Fisher Scientific, Fair Lawn, NJ) was added to media at a concentration of 30 μg/ml for *F. novicida* and 10 μg/ml for LVS.

Macrophages. RAW264.7 murine macrophages (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle medium (high glucose, L-glutamine; DMEM; Lonza, Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT). Bone marrow-derived macrophages (BMM) were isolated from either wild-type C57BL/6 or $gp91^{phox-/-}$ mice (Jackson Laboratories, Bar Harbor, ME) and cultured as described previously [68] in DMEM supplemented with 10% heat-inactivated FCS and 10% macrophage colony-stimulating factor (M-CSF)-conditioned medium (collected from M-CSF-producing L929 cells). Macrophages were incubated before and during infection at 37°C with 5% CO_2.

Intracellular replication screen and macrophage infections. The screen library was assembled by inoculation of individual transposon mutants from the *F. novicida* two-allele transposon library [69] into 96-well plates containing cysteine-supplemented TSB. These were grown overnight at 37°C, glycerol (Fisher Scientific) was added to 20% final volume, and the plates were stored at -80°C. RAW264.7 murine macrophages were seeded at 10^5 cells/well in 96-well tissue culture plates for the replication screen or 5 x 10^5cells/well in 24-well tissue culture plates for subsequent infections and incubated overnight. The medium was then removed and the macrophages were infected with overnight cultures of individual mutants from the screen library that had been diluted in DMEM/10% FCS to achieve a multiplicity of infection (MOI) of twenty bacteria per macrophage. The plates were centrifuged for 15 minutes at 931 x g at room temperature and then incubated for 30 minutes. Next, the macrophages were washed twice with DMEM and incubated for an additional 30 minutes in DMEM/10% FCS containing 100 μg/ml of gentamicin (TekNova, Hollister, CA). The macrophages were again washed

twice and DMEM/10% FCS with 10 µg/ml gentamicin was added. At 1 and 24 hours post-infection, the macrophages were washed twice and then lysed with 1% saponin (Alfa Aesar, Heysham, Lancs., UK) in phosphate buffered solution (PBS) without calcium and magnesium (Lonza, Walkersville, MD). Serial dilutions of the resulting macrophage lysates were plated onto cysteine-supplemented TSA in sterile 24-well plates. Finally, the colony forming units (CFU) for each transposon mutant were counted and the fold replication (CFU at 24 hr/ CFU at 1 hr) was calculated and compared to the fold replication of wild-type *F. novicida* U112. A similar infection procedure was followed for both wild-type and gp91$^{phox-/-}$ BMM infections with the following modifications: 3 x 10^5 BMM were plated per well in a 24-well plate, DMEM/10% FCS/10% M-CSF was used throughout and the final time point was 5.5 hours for *F. novicida* infections or 24 hours for LVS infections. The replication screen was performed twice and the data were averaged to determine the final results. All transposon mutants that replicated less than or equal to 30% of the wild-type value (mutant fold replication/wild-type fold replication ≤ 0.3) were considered attenuated for replication *in vitro.* Results for all transposon mutants tested are listed in Table S1.

Mutagenesis and complementation. To generate *F. novicida* deletion mutants, PCR was used to amplify flanking DNA regions upstream and downstream of the gene of interest. A Kan-resistance cassette was sewn in between these flanking regions using overlapping PCR reactions. The final linear PCR products were then gel purified and transformed into chemically competent wild-type U112 as previously described [70]. The primers used to create the Kan-resistant deletion mutants contained FRT sites flanking the Kan-resistance cassette, which allowed removal of the cassette using the plasmid pFFlp encoding the

Flp-recombinase as previously described [71]. Constructs for the complementation of each mutant were generated by overlapping PCR using PCR-amplified fragments of the wild-type gene of interest, upstream and downstream flanking regions, and a Kan-resistance cassette. These constructs were then transformed into the appropriate chemically competent deletion mutants. Verification of allelic replacement in mutant and complemented strains was performed using check primers in PCR reactions on purified genomic DNA from each strain. PCR products of the correct size were subsequently sequenced (MWG Operon, Huntsville, AL) for final verification of allelic replacement. For LVS mutagenesis, we employed both targeted gene disruption via group II introns as previously described [72] and allelic replacement. Briefly, in order to perform targeted gene disruption, primers for targeting the *FTL_0803* allele were generated using the TargeTron system (Sigma-Aldrich) and the resulting PCR product was cloned into the *Francisella* targeting vector, pKEK1140 (generously provided by Dr. Karl Klose, UTSA, San Antonio, TX) [72]. LVS was then transformed with the resulting vector and *FTL_0803* insertion mutants were selected for as previously described [72]. To generate LVS deletion mutants via allelic replacement, PCR was used to amplify flanking DNA regions upstream and downstream of the gene of interest which were then sewn together using overlapping PCR reactions. The final linear PCR products were then gel purified, digested with BamHI restriction enzyme (New England Biolabs, Ipswich, MA), dephosphorylated with Antarctic phosphatase (New England Biolabs), and ligated using T4 ligase (New England Biolabs) into the *Francisella* suicide vector pXB186 that encodes the *sacB* enzyme (James Bina, University of Tennessee, Memphis, TN). LVS was then transformed and mutants selected as previously described [73,74]. Briefly,

pXB186 ligations were transformed into LVS via electroporation and plated on chocolate agar with kanamycin (10 ug/ml). Next, kanamycin resistant colonies were plated on 10% sucrose, 1% hemoglobin cysteine heart agar (CHA) and surviving colonies were then patched onto kanamycin plates. Finally, genomic DNA from the kanamycin sensitive colonies was PCR-verified and sequenced to confirm the deletion. The *FTL_0803* targeted disruption mutant was used in the RAW264.7 LVS macrophage experiment and the *FTL_0803* clean deletion mutant was used in all other LVS experiments. Neither *FTN_1133* nor *FTL_0803* appear to be in an operon as the genes adjacent to both are transcribed in opposing directions. All primers used in this study are listed in Table S2.

Mouse experiments. Female C57BL/6 and gp91$^{phox-/-}$ mice (Jackson Laboratory, Bar Harbor, ME) between 7 and 10 weeks of age were kept under specific pathogen-free conditions in filter-top cages at Emory University and provided with sterile food and water *ad libitum*. Experimental studies were performed in accordance with the Emory University Institutional Animal Care and Use Committee guidelines. For competition experiments, mice were inoculated subcutaneously with a 1:1 ratio of kanamycin-resistant deletion mutant and kanamycin-sensitive wild-type *F. novicida* for a total of 2 x 10^5 CFU in 50 µl sterile PBS. For single infections, mice were infected with 2 x 10^5 CFU subcutaneously. After 48 (*F. novicida* infections) or 72 hours (LVS infections), mice were sacrificed and the spleen, liver, and skin at the site of infection were harvested, homogenized (Tissue Tearor, Cole-Parmer, Vernon Hills, Illinois), plated for CFU on MH plates (with and without kanamycin for competition experiments), and then incubated overnight at 37°C. For single infections, organs were weighed before homogenization and the resulting CFU were divided by the weight of each organ to

determine CFU/gram. For survival experiments, mice were infected as described for single strain infections and then observed for illness and sacrificed if they appeared moribund. For collection of RNA, mice were infected intraperitoneally with an infectious dose of 2 x 10^6 CFU, four hours after which the mice were sacrificed and the livers collected for RNA isolation. Competitive index (CI) values were determined using the formula: (CFU mutant output/CFU WT output)/(CFU mutant input/CFU WT input).

RNA isolation and quantitative real-time PCR. At various time points post-infection, BMM were lysed and homogenized in trizol reagent (MRC, Cincinnati, Ohio). Similarly, liver samples from intraperitoneally-infected mice (4 hours post-infection) were homogenized in trizol reagent. For both *in vitro* and *in vivo* samples, RNA was isolated using the RNeasy Mini kit (QIAGEN, Germantown, MD). Gene-specific primers (Table S2) were used to amplify *FTN_1133* transcripts using the Power Sybr Green One Step Kit (Applied Biosystems, Foster City, CA) on an Applied Biosystems StepOnePlus Real Time PCR System per the manufacturers' instructions. Expression of *FTN_1133* transcript was calculated relative to the expression of the DNA helicase *uvrD* (*FTN_1594*).

Susceptibility assays. Overnight cultures of *F. novicida* or LVS strains were diluted to an OD_{600} of 1.0 and 100 µl of each were spread on mMH agar plates. Six mm filter disks (Bel-Art Scienceware, Lake Charles, LA) were then added to the center of each plate and 3 µl of the appropriate dilution of chemical agent was spotted on the disks. The following concentrations of chemical agents were used: 250 mM (*F. novicida*) and 25 mM (LVS) tert-butyl hydroperoxide, 150 mM cumene hydroperoxide, 3% H_2O_2, and 200 mg/ml

sodium dodecyl sulfate (SDS). Plates were then grown overnight at 37°C and the zones of inhibition measured.

Organic hydroperoxide degradation assay. Degradation of tert-butyl hydroperoxide was measured using a xylenol orange colorimetric assay based on previously described methods [59,60,75,76]. Briefly, overnight cultures of *F. novicida* were subcultured to an OD_{600} of 0.01-0.03 and then incubated with shaking at 37° C. Once cultures reached mid-log phase (~OD_{600} 1.0), they were diluted to OD_{600} 0.5 and a 2 ml sample of each culture or TSB alone were added to a 24 well plate. Tert-butyl hydroperoxide was then added to each sample to a final concentration of 300 μM, after which the plate was incubated with shaking at room temperature for 30 minutes. 20 μl samples were taken every five minutes from each well and immediately added to 80 μl 25 mM H_2SO_4. Once all samples were collected, 100 μl reaction buffer [200 μM xylenol orange (Alfa Aesar), 200 μM ammonium ferrous sulfate (Ricca Chemical, Arlington, TX), and 25 mM H_2SO_4 (Fisher Scientific) prepared in 9:1 methanol to water solution] was added to each well and the OD_{540} measured. The concentration of tert-butyl hydroperoxide in each sample was calculated based on a standard curve.

Statistical analysis. All macrophage replication, susceptibility, and qRT-PCR data were analyzed for significance using the unpaired Student's *t* test. The CI values from the mouse experiments were analyzed with the one-sample Student's *t* test and compared to 1, with the exception of the CI values comparing replication in wild-type versus $gp91^{phox-/-}$ mice which were analyzed using the unpaired Student's *t* test. The single strain mouse infection data were analyzed for significance using the Mann-Whitney test.

Chapter 2 Acknowledgements

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We have, to the best of our knowledge, included all mammalian *in vitro* and *in vivo* references for the genes listed in Table 1. However, we apologize in advance for any omissions that may have occurred.

Chapter 2 References

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Chapter 2 Tables

F. novicida **locus**	***F. tularensis*** **locus**	**Gene name**	**Gene description**	**Mammalian *in vivo***	**Mammalian *in vitro***
FTN_0019	FTT1665	*pyrB*	aspartate carbamoyltransferase	[50]	[29,32,78,89]
FTN_0020	FTT1664	*carB*	Carbamoyl-phosphate synthase large	[50,112]	[29,32,78,89]
FTN_0021	FTT1663	*carA*	Carbamoyl-phosphate synthase small	[50]	[29,32,78]
FTN_0035	FTT1648c	*pyrF*	Orotidine 5'-phosphate decarboxylase	[50]	[78]
FTN_0036	FTT1647c	*pyrD*	diyroorotate dehydrogenase	[50]	[78]
FTN_0096	**FTT1689c**	**NA**	**conserved hypothetical membrane**	**[50]**	**[78]**
FTN_0109	FTT1676	NA	hypothetical protein	[51,108]	[78,89,108]
FTN_0177	FTT0203c	*purH*	bifunctional purine biosynthesis protein	[50,112]	[29]
FTN_0178	FTT0204	*purA*	adenylosuccinate synthetase	[112,113]	[29,113]
FTN_0419	FTT0893	*purM*	Phosphoribosylaminoimidazol (AIR)	[50,112]	
FTN_0422	FTT0896	*purE*	phosphoribosylaminoimidazole	[50]	[114]
FTN_0430	FTT0904	*lpnB*	conserved hypothetical lipoprotein	[50,52]	
FTN_0544	FTT0453c	NA	conserved hypothetical protein	[50]	
FTN_0546	FTT0455c	*flmK*	dolichyl-phosphate-mannose-protein	[50,115]	
FTN_0593	FTT0503c	*sucD*	succinyl-CoA synthetase, alpha subunit	[51]	[89,114]
FTN_0594	FTT0504c	*sucC*	succinyl-CoA synthetase subunit beta	[51,112]	[114]
FTN_0599	FTT0509c	NA	conserved hypothetical protein	[50]	
FTN_0812	FTT0934c	*bioD*	dethiobiotin synthetase	[51]	
FTN_0813	FTT0935c	*bioC*	biotin synthesis protein BioC	[51]	
FTN_0814	FTT0936c	*bioF*	8-amino-7-oxononanoate synthase	[50,51]	

FTN_0815	FTT0937c	*bioB*	biotin synthase	[50]	
FTN_0816	FTT0938	*bioA*	Adenosylmethionine-8-amino-7-	[50]	
FTN_0818	FTT0941c	NA	lipase/esterase	[50,51]	
FTN_0848	FTT0968c	NA	amino acid antiporter	[50,51]	[78]
FTN_1133	**FTT1152**	**NA**	**protein of unknown function**	**[51,52]**	
FTN_1240	FTT1221	NA	hypothetical protein	[51]	[78]
FTN_1254	FTT1236	NA	hypothetical protein	[50]	[29,114]
FTN_1310	FTT1700;	*pdpB/*	conserved hypothetical protein	[50,79,112]	[78,79]
FTN_1311	FTT1701;	*iglE*	conserved hypothetical protein	[50]	[78]
FTN_1312	FTT1702;	*vgrG*	conserved hypothetical protein	[9,50,52]	[9,78]
FTN_1313	FTT1703;	*iglF*	conserved hypothetical protein	[50]	[78]
FTN_1314	FTT1704;	*iglG*	conserved hypothetical protein	[50]	[78]
FTN_1315	FTT1705;	*iglH*	conserved hypothetical protein	[50]	[78]
FTN_1316	FTT1706;	*dotU*	conserved hypothetical protein	[50]	[78]
FTN_1317	FTT1707;	*iglI*	conserved hypothetical protein	[9,50]	[9,78]
FTN_1318	FTT1708;	*iglJ*	conserved hypothetical protein	[50,79]	[78,79]
FTN_1321	FTT1711c;	*iglD*	intracellular growth locus, subunit D	[50,52]	[12,78,80]
FTN_1322	FTT1712c;	*iglC*	intracellular growth locus, subunit C	[8,50,51,116]	[8,12,77,78,1
FTN_1323	FTT1713c;	*iglB*	intracellular growth locus, subunit B	[50,51,117]	[12,78,117]
FTN_1324	FTT1714c;	*iglA*	intracellular growth locus, subunit A	[50,51]	[11,12,78]
FTN_1421	FTT1456c	*wbtH*	asparagine synthase	[50]	[89,114]
FTN_1423	FTT1457c	*wbtG*	glycosyl transferase	[50]	
FTN_1427	FTT1461c	*wbtD*	galacturonosyl transferase	[50]	
FTN_1501	FTT1490	NA	Na+/H+ antiporter	[50]	[29,91]
FTN_1586	FTT0129	NA	major facilitator superfamily sugar		[29]

FTN_1608	FTT0107c	*dsbB*	disulfide bond formation protein	[29,50,91,112]	[29,78,91]
FTN_1682	FTT0029c	*figA/*	conserved siderophore protein	[50,51]	
FTN_1683	FTT0028c	*figB/*	conserved siderophore protein	[50]	
FTN_1684	FTT0027c	*figC/*	diaminopimelate decarboxylase	[50]	
FTN_1699	FTT1720c	*purL*	phosphoribosylformylglycinamidine	[50,106,112]	[91]
FTN_1700	FTT1721c	*purF*	amidophosphoribosyltransferase	[50,106,113]	[91,113]
FTN_1715	FTT1736c	*kdpD*	two component sensor protein kdpD	[50,118]	
FTN_1743	FTT1769c	*clpB*	ClpB protein	[50,51,92,119]	[12,78,91,92]

Table 1. List of genes required for replication in RAW264.7 macrophages.

Strains highlighted in **bold** were chosen for further characterization in this study.

Chapter 2 Figures

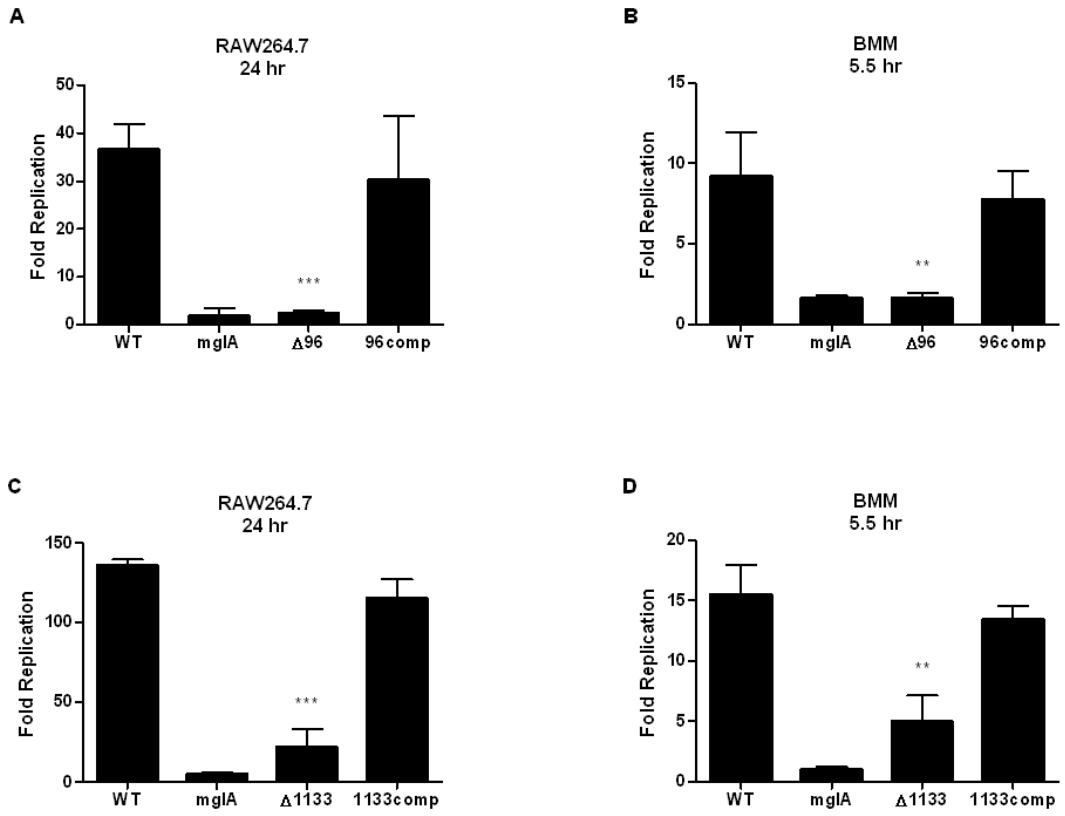

Figure 1. Validation of replication screen results. RAW264.7 macrophages (A and C) or primary murine bone marrow-derived macrophages (BMM) (B and D) were infected with a 20:1 MOI of the indicated bacterial strains. Twenty-four (A and C) or 5.5 (B and D) hours post-infection, fold replication was determined for the *FTN_0096* (A and B) and *FTN_1133* (C and D) deletion mutants (Δ96 and Δ1133) and their respective complemented strains. Wild-type *F. novicida* and an *mglA* point mutant strain (GB2) were used as positive and negative replication controls, respectively. For each strain, bars represent the average fold replication and error bars represent the standard deviation (n=3). Data shown is representative of at least three independent experiments. Asterisks indicate significance as compared to wild-type. (**) $p < 0.005$, (***) $p < 0.0005$.

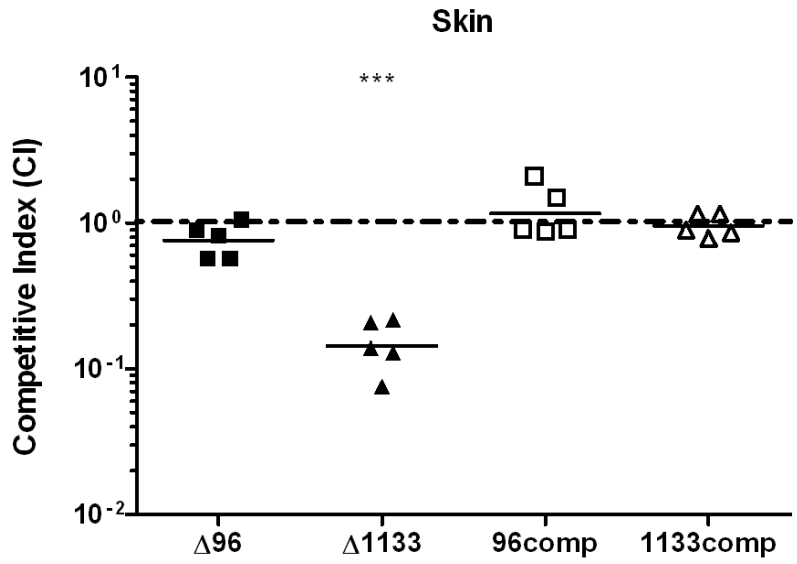

Figure 2. *FTN_0096* and *FTN_1133* deletion mutants are attenuated for virulence *in vivo*. Mice were subcutaneously infected with a 1:1 mixture of wild-type *F. novicida* and either Δ96 or Δ1133 (10^5 CFU each) or respective complemented strains. Forty-eight hours after infection, organs were harvested, CFU enumerated, and the competitive index (CI) calculated for the skin at the site of infection (A), spleen (B), and liver (C). CI = (CFU mutant output/CFU WT output)/(CFU mutant input/CFU WT input). Bars represent the geometric mean CI values from each group of mice (n=5). CI values below 1 (dashed line) indicate attenuation of the mutant strain. Data shown is representative of two independent experiments. Asterisks indicate significance as compared to a CI value of 1. (***) $p < 0.0005$.

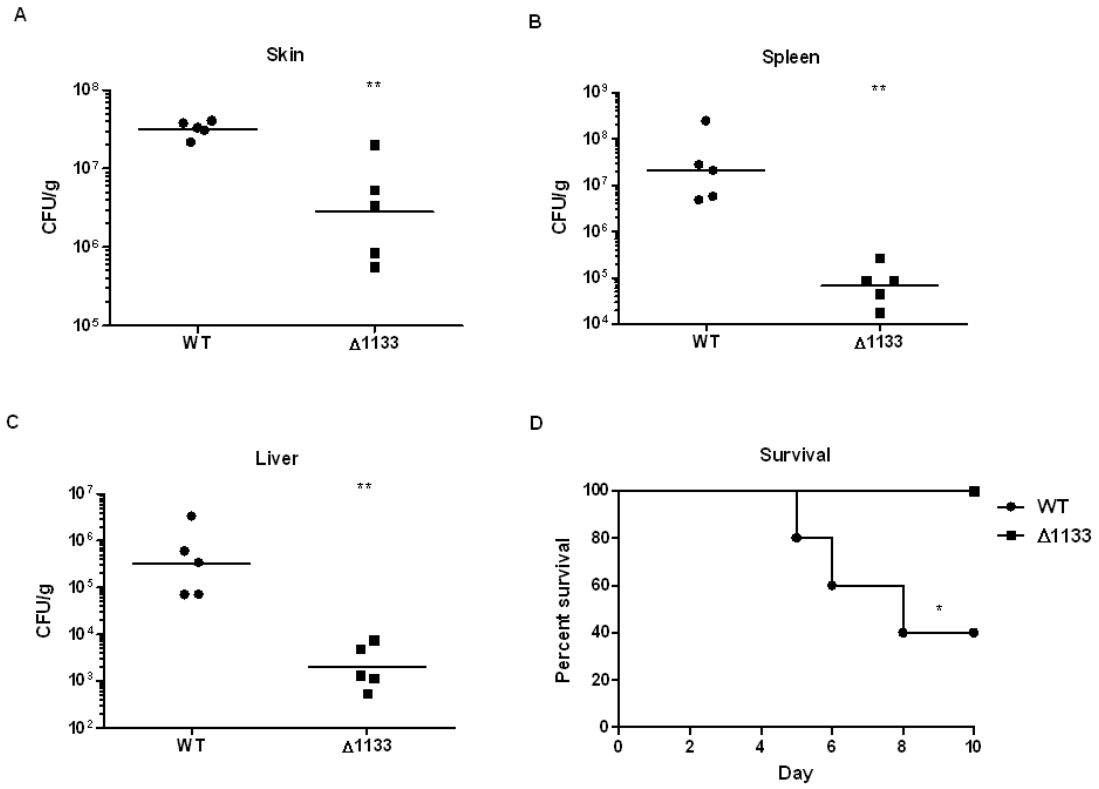

Figure 3. FTN_1133 is required for *F. novicida* pathogenesis in single infections. Mice were subcutaneously infected with $2x10^5$ CFU of either wild-type *F. novicida* (WT) or the *FTN_1133* mutant (Δ1133). Forty-eight hours after infection, organs were harvested and plated and CFU were enumerated 24 hours later for the skin at the site of infection (A), spleen (B), and liver (C). To test survival, mice were infected as described above and then sacrificed upon display of moribundity (D). Bars represent the geometric mean of each group of mice (n=5). Data shown is representative of two independent experiments. Asterisks indicate significance. (**) $p< 0.005$, (*) = $P < 0.05$.

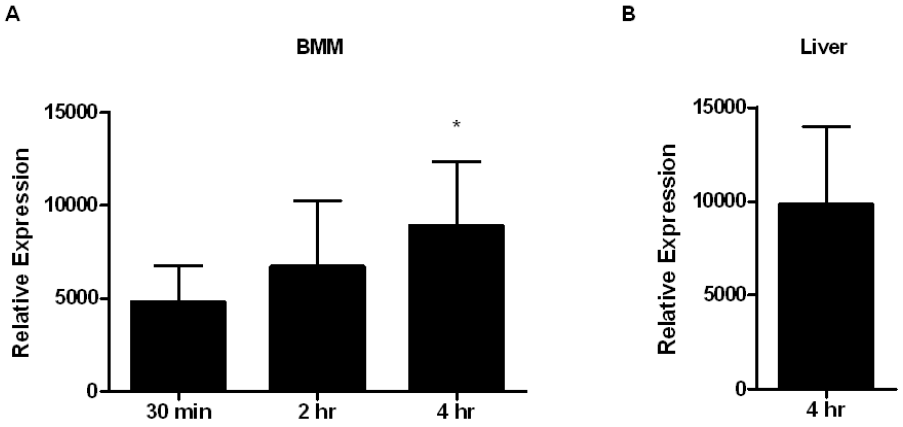

Figure 4. *FTN_1133* is expressed during infection of macrophages and mice.

Relative expression of *FTN_1133* was determined in wild-type *F. novicida*-infected BMM (A) at 30 minutes, 1 hour, and 4 hours post-infection with an MOI of 20:1 and (B) in the livers of intraperitoneally-infected mice at 4 hours. Relative expression of *FTN_1133* transcript was calculated by comparison with the expression levels of the DNA helicase *uvrD* (*FTN_1594*). Data shown is representative of two independent experiments. Bars represent the standard deviation from each set of samples (n=5). Asterisk indicates significance compared to 30 minutes. (*) $p < 0.05$.

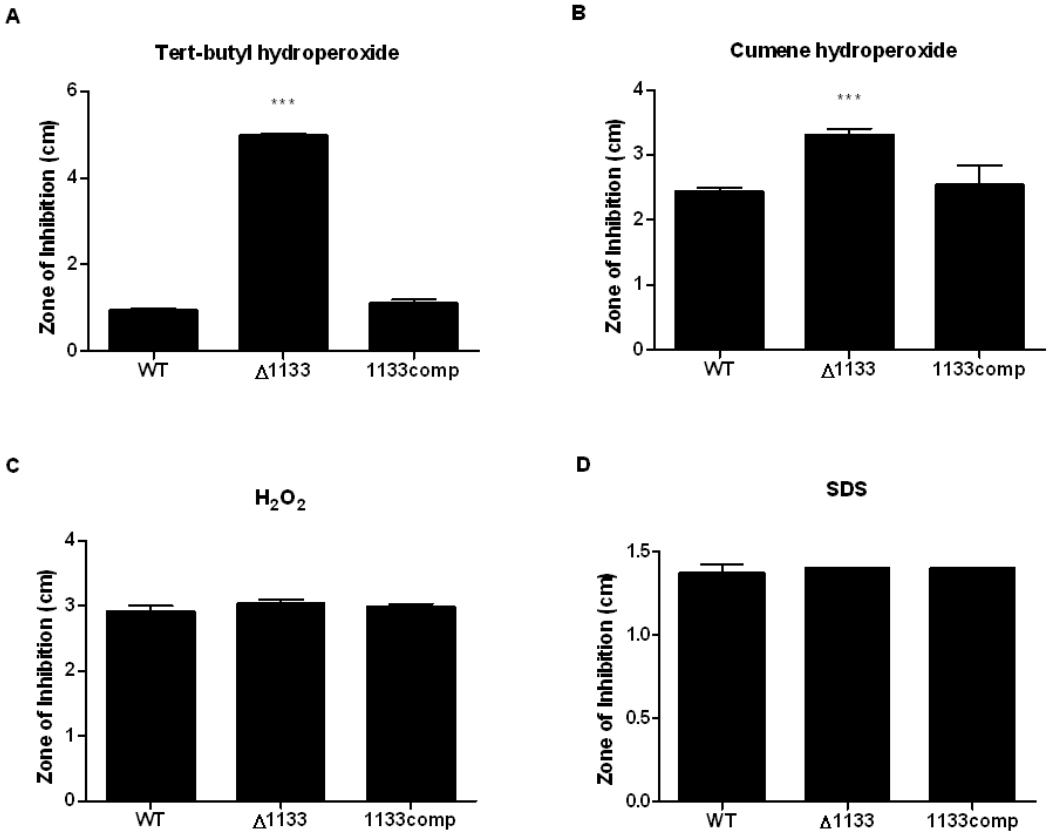

Figure 5. FTN_1133 confers resistance to organic hydroperoxides. Two hundred and fifty mM tert-butyl hydroperoxide (A), 150 mM cumene hydroperoxide (B), 3% hydrogen peroxide (C), and 200 mg/ml SDS (D) were spotted on filter disks placed on lawns of wild-type, *FTN_1133* mutant (Δ1133), and the complemented strain and the zones of inhibition for each were measured. In each graph, bars represent the mean and error bars represent the standard deviation (n=3). Data shown is representative of at least three independent experiments. Asterisks indicate significance as compared to wild-type. (***) $p<0.0005$.

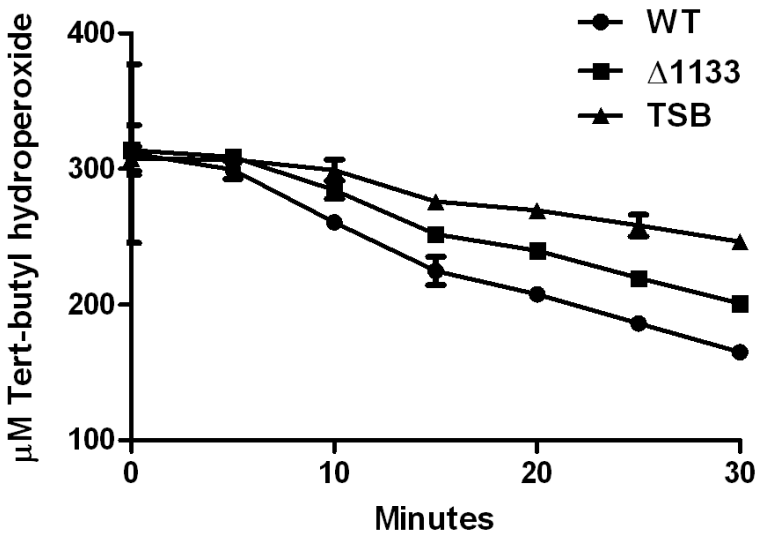

Figure 6. FTN_1133 is required for degradation of an organic hydroperoxide. Three hundred μM tert-butyl hydroperoxide was added to cultures of wild-type *F. novicida* (circles), the *FTN_1133* deletion mutant (Δ1133, squares), or TSB media alone (triangles) and its degradation was measured over time. Data points represent the mean and error bars represent the standard deviation (n=3). Data shown is representative of at least three independent experiments.

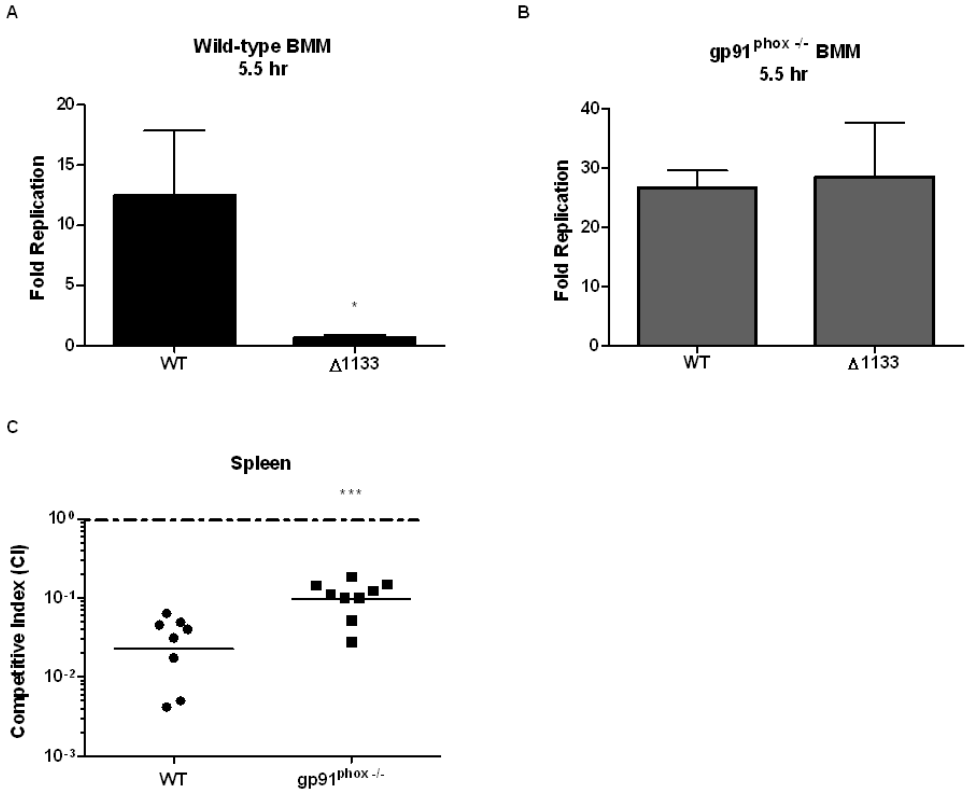

Figure 7. FTN_1133 is required for resistance to the action of the NADPH oxidase. Wild-type (A) or gp91$^{phox-/-}$ (B) bone marrow-derived macrophages were infected with a 20:1 MOI of the indicated bacterial strains. (A, B) Five and a half hours post-infection, fold replication (CFU at 5.5 hr/ CFU at 30 minutes) was determined for wild-type *F. novicida* and the *FTN_1133* deletion mutant (Δ1133). For each strain, bars represent the average fold replication and error bars represent the standard deviation (n=3). Data shown are representative of three independent experiments. (C) Wild-type and gp91$^{phox-/-}$ mice were subcutaneously infected with a 1:1 mixture of wild-type *F. novicida* and Δ1133 (10^5 CFU each). Forty-eight hours after infection, organs were harvested, CFU enumerated, and the competitive index (CI) calculated. Data shown include two

independent experiments. Asterisks indicate significance as compared to wild-type *F. novicida*. (*) $p < 0.05$, (***) $p < 0.0005$.

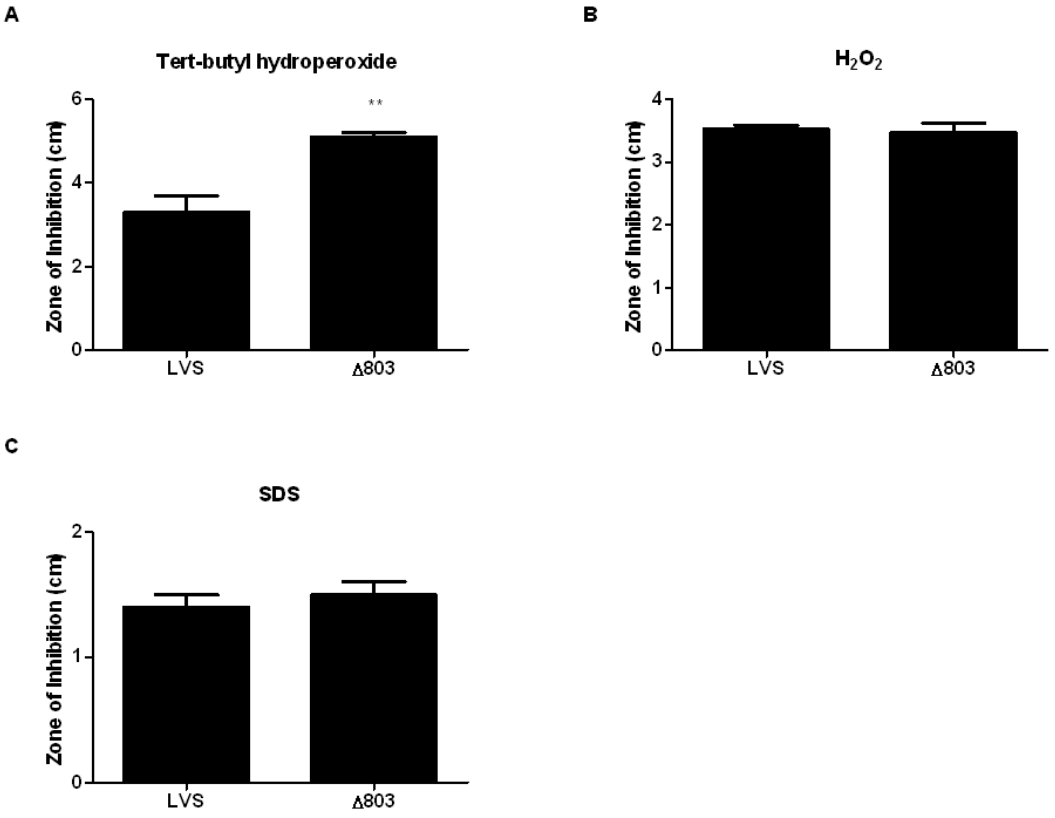

Figure 8. The *FTN_1133* ortholog, *FTL_0803*, confers resistance to tert-butyl hydroperoxide. Twenty-five mM tert-butyl hydroperoxide (A), 3% hydrogen peroxide (B), and 200 mg/ml SDS (C) were spotted on filter disks placed on lawns of wild-type LVS or the *FTL_0803* mutant (Δ803), incubated overnight, and then the zones of inhibition for each were measured. In each experiment, bars represent the mean and error bars represent the standard deviation (n=3). Data shown is representative of at least three independent experiments. Asterisks indicate significance as compared to wild-type. (**) $p<0.005$.

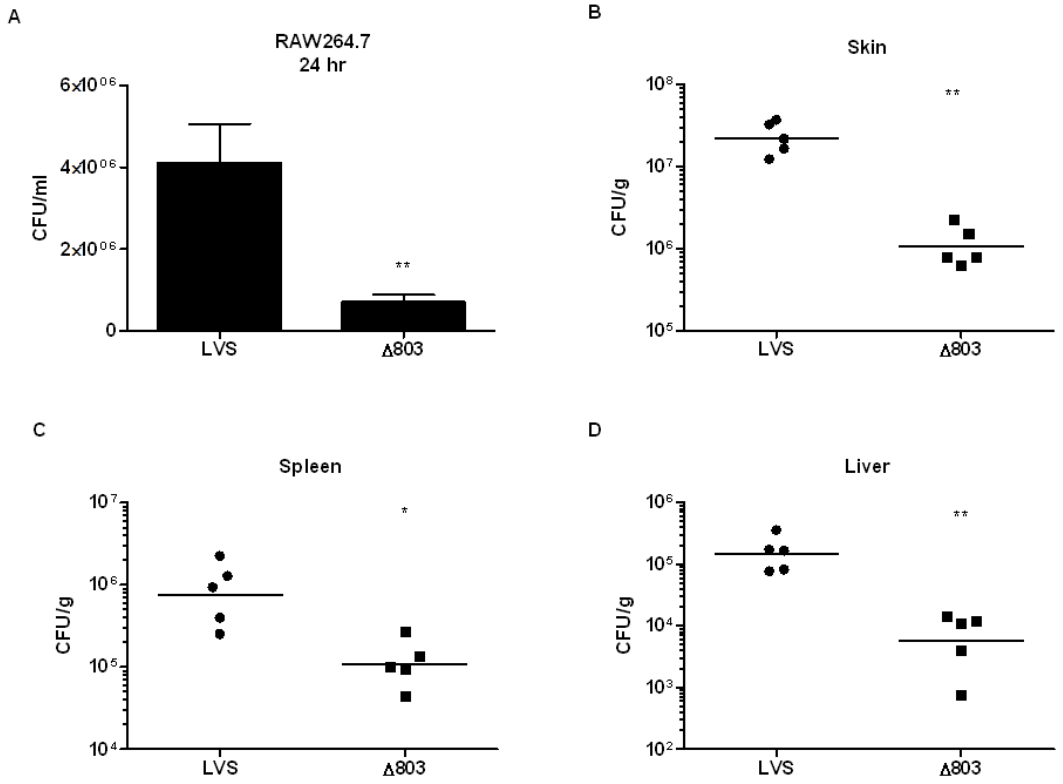

Figure 9. FTL_0803 is important for LVS replication in macrophages and mice. (A) RAW264.7 macrophages were infected with a 20:1 MOI of wild-type LVS and the *FTL_0803* mutant (Δ803). Twenty-four hours post-infection, intracellular CFUs were determined for both strains. Bars represent the mean and error bars represent the standard deviation (n=3). (B-D) Mice were subcutaneously infected with $2x10^5$ CFU of either wild-type *F. holarctica* LVS (WT) or the *FTL_0803* mutant (Δ803). Seventy-two hours after infection, organs were harvested, plated, and CFU were enumerated 48 hours later for the skin at the site of infection (B), spleen (C), and liver (D). Bars represent the geometric mean from each group of mice (n=5). Data shown is representative of two independent experiments. Asterisks indicate significance as compared to wild-type. (*) $p< 0.05$, (**) $p< 0.005$.

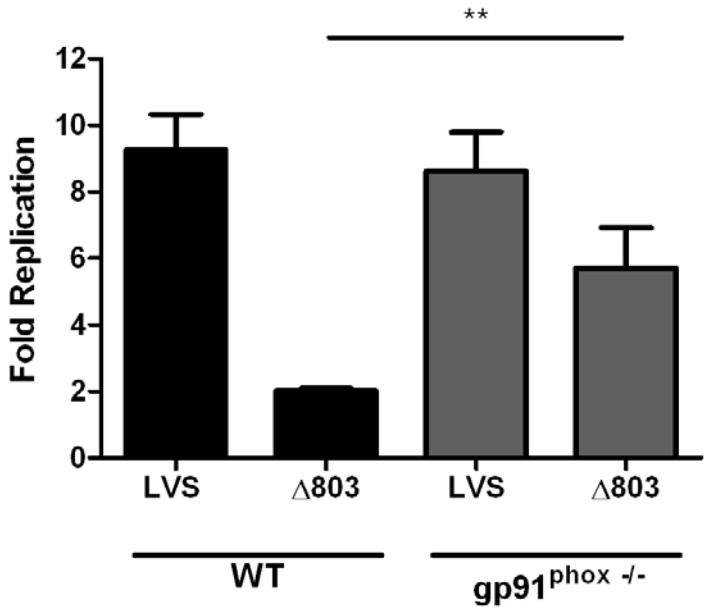

Figure 10. FTL_0803 contributes to resistance against the action of the NADPH oxidase. Bone marrow-derived macrophages (BMM) were infected with a 20:1 MOI of wild-type LVS or the *FTL_0803* mutant (Δ803). Twenty-four hours post-infection, fold replication was determined for both strains. Bars represent the mean and error bars represent the standard deviation (n=3). Data shown is representative of two independent experiments. Asterisks indicate significance as compared to wild-type. (**) $p < 0.005$.

Chapter 2 Supporting Tables

Table S1. Full list of transposon mutant replication phenotypes in RAW264.7 macrophages.

FTN locus	FTT locus	Gene name	Gene Description	AVG Mut/ WT	Strain Name	Plate Number	Well	Mammalian *in vivo* attenuation references	Mammalian *in vitro* attenuation references
FTN_0019	**FTT1665**	***pyrB***	**aspartate carbamoyltransferase**	**0.03**	**tnfn1_pw060510p01q109**	**NR-8063**	**A02**	**[1]**	**[2-5]**
FTN_0019	**FTT1665**	***pyrB***	**aspartate carbamoyltransferase**	**0.06**	**tnfn1_pw060323p03q119**	**NR-8037**	**C03**	**[1]**	**[2-5]**
FTN_0020	**FTT1664**	***carB***	**carbamoyl-phosphate synthase large chain**	**0.18**	**tnfn1_pw060328p01q118**	**NR-8043**	**B03**	**[1,6]**	**[2-4,7]**
FTN_0020	**FTT1664**	***carB***	**carbamoyl-phosphate synthase large chain**	**0.09**	**tnfn1_pw060323p08q120**	**NR-8042**	**D03**	**[1,6]**	**[2,3,5]**
FTN_0021	**FTT1663**	***carA***	**carbamoyl-phosphate synthase small chain**	**0.19**	**tnfn1_pw060510p02q160**	**NR-8064**	**D08**	**[1]**	**[3,5,8]**
FTN_0021	FTT1663	*carA*	carbamoyl-phosphate synthase small chain	0.50	tnfn1_pw060328p04q165	NR-8046	A09	[1]	[2,3,5]
FTN_0035	**FTT1648c**	***pyrF***	**orotidine-5'-phosphate decarboxylase**	**0.05**	**tnfn1_pw060510p01q129**	**NR-8063**	**E04**	**[1]**	**[5]**
FTN_0035	**FTT1648c**	***pyrF***	**orotidine-5'-phosphate decarboxylase**	**0.09**	**tnfn1_pw060323p04q176**	**NR-8038**	**D10**	**[1]**	**[5]**
FTN_0036	**FTT1647c**	***pyrD***	**dihydroorotate oxidase**	**0.22**	**tnfn1_pw060510p04q195**	**NR-8066**	**G12**	**[1]**	**[5]**
FTN_0036	**FTT1647c**	***pyrD***	**dihydroorotate oxidase**	**0.26**	**tnfn1_pw060323p03q144**	**NR-8037**	**D06**	**[1]**	**[5]**
FTN_0096	**FTT1689c**	**NA**	**hypothetical protein**	**0.00**	**tnfn1_pw060328p06q161**	**NR-8048**	**E08**	**[1]**	**[5]**
FTN_0096	FTT1689c	NA	hypothetical protein	0.65	tnfn1_pw060419p03q167	NR-8057	C09	[1]	[5]
FTN_0097	FTT1688	NA	hydroxy/aromatic amino acid permease (HAAAP) family protein	0.51	tnfn1_pw060328p06q162	NR-8048	F08	[1]	[8,9]
FTN_0097	FTT1688	NA	hydroxy/aromatic amino acid permease (HAAAP) family protein	1.43	tnfn1_pw060420p01q180	NR-8059	H10	[1]	[8,9]
FTN_0098	FTT1687c	*gidB*	glucose-inhibited cell division protein	0.65	tnfn1_pw060328p04q143	NR-8046	C06	[1]	

FTN_0098	FTT1687c	*gidB*	glucose-inhibited cell division protein	1.50	tnfn1_pw060419p01q136	NR-8055	D05	[1]	
FTN_0109	**FTT1676**	**NA**	**hypothetical protein**	**0.07**	**tnfn1_pw060328p06q147**	**NR-8048**	**G06**	**[10,11]**	**[4,5,11]**
FTN_0109	FTT1676	NA	hypothetical protein	1.28	tnfn1_pw060418p04q193	NR-8054	E12	[10,11]	[4,5,11]
FTN_0113	FTT1672	*ribC*	riboflavin synthase alpha chain	0.71	tnfn1_pw060420p02q191	NR-8060	C12	[1]	[5]
FTN_0113	FTT1672	*ribC*	riboflavin synthase alpha chain	2.13	tnfn1_pw060328p01q121	NR-8043	E03	[1]	[5]
FTN_0119	FTT1747	NA	conserved outer membrane protein of unknown function	0.56	tnfn1_pw060323p08q158	NR-8042	B08	[10]	
FTN_0119	FTT1747	NA	conserved outer membrane protein of unknown function	1.50	tnfn1_pw060419p03q120	NR-8057	D03	[10]	
FTN_0120	FTT1748	NA	rhodanese-related sulfurtransferase	1.00	tnfn1_pw060328p08q125	NR-8050	A04	[10]	
FTN_0120	FTT1748	NA	rhodanese-related sulfurtransferase	2.92	tnfn1_pw060418p03q181	NR-8053	A11	[10]	
FTN_0122	FTT1750	*recA*	recombinase A protein	0.50	tnfn1_pw060323p01q152	NR-8035	D07	[10]	
FTN_0122	FTT1750	*recA*	recombinase A protein	2.17	tnfn1_pw060510p04q169	NR-8066	E09	[10]	
FTN_0132	FTT0256c	NA	hypothetical protein	0.50	tnfn1_pw060323p02q111	NR-8036	C02	[1,12]	
FTN_0132	FTT0256c	NA	hypothetical protein	0.64	tnfn1_pw060510p01q123	NR-8063	G03	[1,12]	[4]
FTN_0177	**FTT0203c**	***purH***	**AICAR transformylase/IMP cyclohydrolase**	**0.07**	**tnfn1_pw060328p04q192**	**NR-8046**	**D12**	**[1,6]**	**[2]**
FTN_0177	**FTT0203c**	***purH***	**AICAR transformylase/IMP cyclohydrolase**	**0.07**	**tnfn1_pw060323p06q116**	**NR-8040**	**H02**	**[1,6]**	**[2]**
FTN_0177	FTT0203c	*purH*	AICAR transformylase/IMP cyclohydrolase	1.00	tnfn1_pw060510p03q162	NR-8065	F08	[1,6]	[2]
FTN_0178	**FTT0204**	***purA***	**adenylosuccinate synthetase**	**0.01**	**tnfn1_pw060323p03q133**	**NR-8037**	**A05**	**[6,13]**	**[2,13]**
FTN_0178	**FTT0204**	***purA***	**adenylosuccinate synthetase**	**0.04**	**tnfn1_pw060510p03q187**	**NR-8065**	**G11**	**[6,13]**	**[2,13]**
FTN_0183	FTT0209c	NA	manganese/Zinc/Iron chelate uptake transporter family protein	1.22	tnfn1_pw060419p01q165	NR-8058	C01	[10]	[9]
FTN_0183	FTT0209c	NA	manganese/Zinc/Iron chelate uptake transporter family protein	1.75	tnfn1_pw060419p01q165	NR-8055	A09	[10]	[9]
FTN_0196	FTT0282	*cyoB*	cytochrome bo terminal	0.75	tnfn1_pw060323p05q118	NR-8039	B03	[1]	

			oxidase subunit I						
FTN_0196	FTT0282	*cyoB*	cytochrome bo terminal oxidase subunit I	1.00	tnfn1_pw060418p02q121	NR-8052	E03	[1]	
FTN_0197	FTT0283	*cyoC*	cytochrome bo terminal oxidase subunit III	0.42	tnfn1_pw060419p02q183	NR-8056	C11	[1]	
FTN_0197	FTT0283	*cyoC*	cytochrome bo terminal oxidase subunit III	0.90	tnfn1_pw060328p08q152	NR-8050	D07	[1,10]	
FTN_0198	FTT0284	*cyoD*	cytochrome bo terminal oxidase subunit IV	0.85	tnfn1_pw060420p03q154	NR-8061	F07	[1]	
FTN_0198	FTT0284	*cyoD*	cytochrome bo terminal oxidase subunit IV	1.00	tnfn1_pw060328p04q194	NR-8046	F12	[1]	
FTN_0202	FTT0288c	*pdxY*	pyridoxal kinase	1.46	tnfn1_pw060418p04q144	NR-8054	D06	[1]	
FTN_0210	FTT0295	NA	hypothetical protein	0.57	tnfn1_pw060510p01q155	NR-8063	G07	[1]	
FTN_0210	FTT0295	NA	hypothetical protein	0.81	tnfn1_pw060323p08q153	NR-8042	E07	[1]	
FTN_0210	FTT0295	NA	hypothetical protein	0.81	tnfn1_pw060323p08q152	NR-8042	D07	[1]	
FTN_0211	FTT0296	*pcp*	pyrrolidone carboxylylate peptidase	0.50	tnfn1_pw060323p04q102	NR-8038	B01	[1]	[4]
FTN_0211	FTT0296	*pcp*	pyrrolidone carboxylylate peptidase	2.92	tnfn1_pw060418p03q177	NR-8053	E10	[1]	[4]
FTN_0265	FTT0351	*rplQ*	50S ribosomal protein L17	0.50	tnfn1_pw060323p03q185	NR-8037	E11	[1]	
FTN_0266	FTT0356	*htpG*	chaperone Hsp90, heat shock protein HtpG	0.52	tnfn1_pw060328p08q114	NR-8050	F02	[1,6]	[1]
FTN_0266	FTT0356	*htpG*	chaperone Hsp90, heat shock protein HtpG	0.62	tnfn1_pw060328p02q157	NR-8044	A08	[1,6]	[1]
FTN_0289	FTT1640c	*proQ*	activator of osmoprotectant transporter ProP	0.86	tnfn1_pw060419p04q137	NR-8058	E05	[1]	
FTN_0296	FTT1633c	*lysP*	lysine:H+ symporter	0.64	tnfn1_pw060328p04q193	NR-8046	E12	[1]	
FTN_0296	FTT1633c	*lysP*	lysine:H+ symporter	0.70	tnfn1_pw060419p04q126	NR-8058	B04	[1]	
FTN_0296	FTT1633c	*lysP*	lysine:H+ symporter	1.39	tnfn1_pw060419p04q133	NR-8058	A05	[1]	
FTN_0297	FTT1632c	NA	hypothetical protein	0.72	tnfn1_pw060420p04q134	NR-8062	B05	[1,12]	[4]
FTN_0297	FTT1632c	NA	hypothetical protein	0.81	tnfn1_pw060323p08q103	NR-8042	C01	[1,12]	
FTN_0298	FTT1631c	*gplX*	fructose 1,6-bisphosphatase II	1.00	tnfn1_pw060419p02q168	NR-8056	D09	[1,10,12]	[8]
FTN_0298	FTT1631c	*gplX*	fructose 1,6-bisphosphatase II	1.50	tnfn1_pw060328p05q168	NR-8047	D09	[1,10,12]	[8]

FTN_0325	FTT1611	NA	membrane protein of unknown function	0.40	tnfn1_pw060328p04q119	NR-8046	C03	[10]	[9]
FTN_0325	FTT1611	NA	membrane protein of unknown function	1.42	tnfn1_pw060418p03q169	NR-8053	E09	[10]	[9]
FTN_0330	FTT1606	*minD*	septum formation inhibitor-activating ATPase	0.58	tnfn1_pw060328p01q167	NR-8043	C09	[10]	[4,5]
FTN_0330	FTT1606	*minD*	septum formation inhibitor-activating ATPase	0.69	tnfn1_pw060328p01q104	NR-8043	D01	[10]	[5]
FTN_0331	FTT1605	*minC*	septum formation inhibitor	0.56	tnfn1_pw060323p08q146	NR-8042	F06	[10]	[5]
FTN_0331	FTT1605	*minC*	septum formation inhibitor	1.09	tnfn1_pw060420p02q170	NR-8060	F09	[10]	[5]
FTN_0337	FTT1600c	*fumA*	fumarate hydratase, class I	0.58	tnfn1_pw060328p03q144	NR-8045	D06	[6,10]	
FTN_0337	FTT1600c	*fumA*	fumarate hydratase, class I	1.11	tnfn1_pw060420p01q158	NR-8059	B08	[6,10]	
FTN_0358	FTT0843	NA	tRNA-methylthiotransferase MiaB protein	0.55	tnfn1_pw060328p06q121	NR-8048	E03	[10]	
FTN_0358	FTT0843	NA	tRNA-methylthiotransferase MiaB protein	0.69	tnfn1_pw060328p03q179	NR-8045	G10	[10]	[4]
FTN_0358	FTT0843	NA	tRNA-methylthiotransferase MiaB protein	1.75	tnfn1_pw060419p01q169	NR-8055	E09	[10]	[4]
FTN_0407	FTT0881c	NA	amino acid transporter (AAT) family protein	0.63	tnfn1_pw060323p07q130	NR-8041	F04	[10]	[9]
FTN_0407	FTT0881c	NA	amino acid transporter (AAT) family protein	1.75	tnfn1_pw060418p03q155	NR-8053	G07	[10]	[9]
FTN_0410	FTT0884c	NA	aspartate/tyrosine/aromatic aminotransferase	0.50	tnfn1_pw060323p01q142	NR-8035	B06	[10]	
FTN_0410	FTT0884c	NA	aspartate/tyrosine/aromatic aminotransferase	2.14	tnfn1_pw060420p01q150	NR-8059	B07	[10]	
FTN_0416	FTT0891	*lpxE*	lipid A 1-phosphatase	0.56	tnfn1_pw060328p01q185	NR-8043	E11	[1,14]	
FTN_0416	FTT0891	*lpxE*	lipid A 1-phosphatase	1.43	tnfn1_pw060418p04q182	NR-8054	B11	[1,14]	
FTN_0417	FTT0892	*folD*	methyleneTHF enzyme/ methenyltetrahydrofolate cyclohydrolase/ methylenetetrahydrofolate dehydrogenase	0.48	tnfn1_pw060328p05q131	NR-8047	G04	[1,12]	[9]
FTN_0419	**FTT0893**	***purM***	**phosphoribosylformylglycinamide cyclo-ligase**	**0.05**	**tnfn1_pw060323p08q193**	**NR-8042**	**E12**	**[1,6,15]**	

FTN_0419	FTT0893	*purM*	phosphoribosylformylglycinamide cyclo-ligase	0.35	tnfn1_pw060510p02q136	NR-8064	D05	[1,6,15]	
FTN_0421	FTT0895	*purN*	phosphoribosylglycinamide formyltransferase	1.08	tnfn1_pw060510p02q128	NR-8064	D04	[1]	
FTN_0422	**FTT0896**	***purE***	**N5-carboxyaminoimidazole ribonucleotide mutase**	**0.03**	**tnfn1_pw060323p08q194**	**NR-8042**	**F12**	**[1]**	**[9]**
FTN_0422	FTT0896	*purE*	N5-carboxyaminoimidazole ribonucleotide mutase	1.67	tnfn1_pw060510p04q172	NR-8066	H09	[1]	
FTN_0427	FTT0901	NA	lipoprotein of unknown function	1.67	tnfn1_pw060418p04q133	NR-8054	A05	[10]	
FTN_0429	FTT0903	NA	hypothetical protein	0.99	tnfn1_pw060328p06q195	NR-8048	G12	[1]	
FTN_0429	FTT0903	NA	hypothetical protein	1.42	tnfn1_pw060418p03q178	NR-8053	F10	[1]	
FTN_0430	**FTT0904**	**NA**	**hypothetical protein**	**0.30**	**tnfn1_pw060323p06q169**	**NR-8040**	**E09**	**[1,12]**	
FTN_0430	FTT0904	NA	hypothetical protein	1.00	tnfn1_pw060419p03q130	NR-8057	F04	[1,12]	
FTN_0431	FTT0905	NA	hypothetical membrane protein	0.71	tnfn1_pw060420p02q140	NR-8060	H05	[1]	
FTN_0431	FTT0905	NA	hypothetical membrane protein	2.13	tnfn1_pw060328p02q173	NR-8044	A10	[1]	
FTN_0434	FTT0908	*parB*	chromosome partition protein B	1.83	tnfn1_pw060510p03q196	NR-8065	H12	[10]	
FTN_0436	FTT0910	NA	lipolytic enzyme	0.38	tnfn1_pw060420p04q119	NR-8062	C03	[1,10]	
FTN_0436	FTT0910	NA	lipolytic enzyme	0.65	tnfn1_pw060323p06q132	NR-8040	H04	[1,10]	
FTN_0444	FTT0918	NA	membrane protein of unknown function	1.06	tnfn1_pw060420p03q175	NR-8061	C10	[10]	[4]
FTN_0444	FTT0918	NA	membrane protein of unknown function	1.50	tnfn1_pw060328p05q119	NR-8047	C03	[10]	[4]
FTN_0487	FTT0390c	NA	30S ribosomal protein S21	0.50	tnfn1_pw060323p04q101	NR-8038	A01	[10]	
FTN_0494	FTT0398c	NA	hypothetical membrane protein	0.50	tnfn1_pw060323p02q112	NR-8036	D02	[1]	
FTN_0494	FTT0398c	NA	hypothetical membrane protein	0.61	tnfn1_pw060510p01q167	NR-8063	C09	[1]	
FTN_0494	FTT0398c	NA	hypothetical membrane protein	1.50	tnfn1_pw060419p03q142	NR-8057	B06	[1]	
FTN_0495	FTT0399c	NA	BNR/Asp-box repeat protein	0.44	tnfn1_pw060328p06q124	NR-8048	H03	[1]	
FTN_0495	FTT0399c	NA	BNR/Asp-box repeat protein	0.89	tnfn1_pw060420p03q136	NR-8061	D05	[1]	

FTN_0504	FTT0406	NA	lysine decarboxylase	1.00	tnfn1_pw060323p05q182	NR-8039	B11	[1]	[4]
FTN_0504	FTT0406	NA	lysine decarboxylase	1.67	tnfn1_pw060510p04q152	NR-8066	D07	[1]	
FTN_0505	FTT0407	*gcvT*	glycine cleavage complex protein T	0.50	tnfn1_pw060323p03q128	NR-8037	D04	[1]	
FTN_0505	FTT0407	*gcvT*	glycine cleavage complex protein T	0.50	tnfn1_pw060323p07q143	NR-8041	C06	[1]	
FTN_0505	FTT0407	*gcvT*	glycine cleavage complex protein T	0.70	tnfn1_pw060328p03q121	NR-8045	E03	[1]	
FTN_0506	FTT0408	*gcvH*	glycine cleavage system H protein	1.17	tnfn1_pw060420p03q135	NR-8061	C05	[1]	
FTN_0507	FTT0409	*gcvP1*	glycine cleavage system P protein, subunit 1	0.53	tnfn1_pw060510p01q124	NR-8063	H03	[1]	[4]
FTN_0507	FTT0409	*gcvP1*	glycine cleavage system P protein, subunit 1	0.58	tnfn1_pw060323p06q129	NR-8040	E04	[1]	
FTN_0513	FTT0413c	*glgB*	1,4-alpha-glucan branching enzyme	0.50	tnfn1_pw060510p04q104	NR-8066	D01	[10]	[5]
FTN_0513	FTT0413c	*glgB*	1,4-alpha-glucan branching enzyme	1.60	tnfn1_pw060323p05q194	NR-8039	F12	[10]	[5]
FTN_0514	FTT0414	*pgm*	phosphoglucomutase	0.50	tnfn1_pw060510p02q132	NR-8064	H04	[1]	
FTN_0514	FTT0414	*pgm*	phosphoglucomutase	1.17	tnfn1_pw060328p03q191	NR-8045	C12	[1]	
FTN_0515	FTT0415	*glgC*	glucose-1-phosphate adenylyltransferase	1.00	tnfn1_pw060510p03q111	NR-8065	C02	[10]	
FTN_0515	FTT0415	*glgC*	glucose-1-phosphate adenylyltransferase	1.17	tnfn1_pw060328p04q138	NR-8046	F05	[10]	
FTN_0534	FTT0443	NA	hypothetical protein	1.33	tnfn1_pw060328p05q130	NR-8047	F04	[10]	[4]
FTN_0534	FTT0443	NA	hypothetical protein	1.50	tnfn1_pw060418p04q140	NR-8054	H05	[10]	
FTN_0535	FTT0444	NA	drug:H+ antiporter-1 (DHA1) family protein	0.67	tnfn1_pw060323p04q126	NR-8038	B04	[10,14]	
FTN_0535	FTT0444	NA	drug:H+ antiporter-1 (DHA1) family protein	1.44	tnfn1_pw060418p03q165	NR-8053	A09	[10,14]	
FTN_0544	**FTT0453c**	**NA**	**hypothetical protein**	**0.17**	**tnfn1_pw060418p02q107**	**NR-8052**	**G01**	**[1]**	
FTN_0544	FTT0453c	NA	hypothetical protein	0.65	tnfn1_pw060419p01q114	NR-8055	F02	[1]	
FTN_0544	FTT0453c	NA	hypothetical protein	0.94	tnfn1_pw060420p04q160	NR-8062	D08	[1]	
FTN_0545	FTT0454	NA	glycosyl transferase, group 2	0.34	tnfn1_pw060323p06q168	NR-8040	D09	[1]	[4]

FTN_0545	FTT0454	NA	glycosyl transferase, group 2	1.00	tnfn1_pw060419p01q187	NR-8055	G11	[1]	[4]
FTN_0546	**FTT0455c**	***flmK***	**dolichyl-phosphate-mannose-protein mannosyltransferase family protein**	**0.14**	**tnfn1_pw060420p02q141**	**NR-8060**	**A06**	**[1,16]**	
FTN_0546	**FTT0455c**	***flmK***	**dolichyl-phosphate-mannose-protein mannosyltransferase family protein**	**0.15**	**tnfn1_pw060328p08q168**	**NR-8050**	**D09**	**[1,16]**	
FTN_0554	FTT0463	NA	RNA methyltransferase, SpoU family	1.67	tnfn1_pw060510p04q133	NR-8066	A05	[10,12]	
FTN_0560	FTT0469	*ksgA*	dimethyladenosine transferase	1.00	tnfn1_pw060419p02q135	NR-8056	C05	[1]	
FTN_0560	FTT0469	*ksgA*	dimethyladenosine transferase	1.67	tnfn1_pw060418p03q144	NR-8053	D06	[1]	
FTN_0561	FTT0470	*apaH*	diadenosine tetraphosphatase	1.50	tnfn1_pw060419p02q156	NR-8056	H07	[1]	
FTN_0593	**FTT0503c**	***sucD***	**succinyl-CoA synthetase, alpha subunit**	**0.06**	**tnfn1_pw060328p02q141**	**NR-8044**	**A06**	**[10]**	**[9]**
FTN_0593	FTT0503c	*sucD*	succinyl-CoA synthetase, alpha subunit	1.00	tnfn1_pw060419p03q116	NR-8057	H02	[10]	[4,9]
FTN_0593	FTT0503c	*sucD*	succinyl-CoA synthetase, alpha subunit	1.19	tnfn1_pw060420p03q166	NR-8061	B09	[10]	[9]
FTN_0594	**FTT0504c**	***sucC***	**succinyl-CoA synthetase, beta chain**	**0.10**	**tnfn1_pw060323p06q147**	**NR-8040**	**G06**	**[6,10]**	**[9]**
FTN_0594	FTT0504c	*sucC*	succinyl-CoA synthetase, beta chain	1.50	tnfn1_pw060419p03q113	NR-8057	E02	[6,10]	[9]
FTN_0599	**FTT0509c**	**NA**	**hypothetical protein**	**0.25**	**tnfn1_pw060328p06q173**	**NR-8048**	**A10**	**[1]**	
FTN_0599	FTT0509c	NA	hypothetical protein	0.48	tnfn1_pw060418p02q187	NR-8052	G11	[1]	
FTN_0599	FTT0509c	NA	hypothetical protein	0.83	tnfn1_pw060510p04q147	NR-8066	G06	[1]	
FTN_0620	FTT0708	NA	major facilitator superfamily (MFS) transport protein	0.50	tnfn1_pw060323p01q145	NR-8035	E06	[10]	
FTN_0620	FTT0708	NA	major facilitator superfamily (MFS) transport protein	1.18	tnfn1_pw060419p04q167	NR-8058	C09	[10]	
FTN_0624	FTT0712c	NA	serine permease	0.63	tnfn1_pw060510p02q156	NR-8064	H07	[1]	[4,9]
FTN_0624	FTT0712c	NA	serine permease	1.00	tnfn1_pw060323p06q164	NR-8040	H08	[1]	[4,9]
FTN_0624	FTT0712c	NA	serine permease	2.50	tnfn1_pw060418p04q157	NR-8054	A08	[1]	[9]

FTN_0633	FTT0721c	*katG*	peroxidase/catalase	0.50	tnfn1_pw060418p02q128	NR-8052	D04	[10]	[4]
FTN_0633	FTT0721c	*katG*	peroxidase/catalase	0.90	tnfn1_pw060323p06q133	NR-8040	A05	[10]	
FTN_0643	FTT1334c	NA	hypothetical protein	0.81	tnfn1_pw060418p01q154	NR-8051	F07	[1]	[9]
FTN_0643	FTT1334c	NA	hypothetical protein	1.00	tnfn1_pw060323p08q109	NR-8042	A02	[1]	[9]
FTN_0643	FTT1334c	NA	hypothetical protein	1.75	tnfn1_pw060419p01q157	NR-8055	A08	[1]	[9]
FTN_0651	FTT1327	*cdd*	cytidine deaminase	1.00	tnfn1_pw060418p01q120	NR-8051	D03	[1]	
FTN_0651	FTT1327	*cdd*	cytidine deaminase	1.75	tnfn1_pw060419p01q168	NR-8055	D09	[1]	
FTN_0664	FTT1314	*fimT*	Type IV pili, pilus assembly protein	0.38	tnfn1_pw060328p03q183	NR-8045	C11	[10]	
FTN_0664	FTT1314	*fimT*	Type IV pili, pilus assembly protein	1.50	tnfn1_pw060419p03q141	NR-8057	A06	[10]	
FTN_0666	FTT1312	*uvrA*	excinuclease ABC, subunit A	0.70	tnfn1_pw060328p03q169	NR-8045	E09	[10,14]	
FTN_0666	FTT1312	*uvrA*	excinuclease ABC, subunit A	1.67	tnfn1_pw060510p04q168	NR-8066	D09	[10,14]	
FTN_0669	FTT0766	*deoD*	purine nucleoside phosphorylase	0.50	tnfn1_pw060510p03q113	NR-8065	E02	[1,10]	
FTN_0669	FTT0766	*deoD*	purine nucleoside phosphorylase	1.17	tnfn1_pw060323p04q172	NR-8038	H09	[1,10]	
FTN_0672	FTT0769	*secA*	preprotein translocase, subunit A (ATPase, RNA helicase)	0.53	tnfn1_pw060328p04q123	NR-8046	G03	[10]	[4,5]
FTN_0672	FTT0769	*secA*	preprotein translocase, subunit A (ATPase, RNA helicase)	0.62	tnfn1_pw060418p02q130	NR-8052	F04	[10]	[5]
FTN_0689	FTT1472	*ppiC*	parvulin-like peptidyl-prolyl isomerase domain	0.50	tnfn1_pw060323p01q118	NR-8035	B03	[10,12]	[9]
FTN_0689	FTT1472	*ppiC*	parvulin-like peptidyl-prolyl isomerase domain	0.88	tnfn1_pw060420p01q160	NR-8059	D08	[10,12]	[9]
FTN_0690	FTT1471	*deaD*	DEAD-box subfamily ATP-dependent helicase	0.81	tnfn1_pw060323p07q195	NR-8041	G12	[10,12]	[9]
FTN_0690	FTT1471	*deaD*	DEAD-box subfamily ATP-dependent helicase	2.38	tnfn1_pw060420p02q160	NR-8060	D08	[10,12]	[9]
FTN_0719	intergenic; FTT0747c/ FTT0748	NA	hypothetical protein	0.50	tnfn1_pw060323p05q171	NR-8039	G09	[1]	
FTN_0719	intergenic; FTT0747c/ FTT0748	NA	hypothetical protein	0.67	tnfn1_pw060328p05q133	NR-8047	A05	[1]	

FTN_0720	FTT0748	NA	transcriptional regulator, IclR family	1.22	tnfn1_pw060418p04q107	NR-8054	G01	[1,12]	
FTN_0728	FTT0756	NA	predicted Co/Zn/Cd cation transporter	1.04	tnfn1_pw060328p06q132	NR-8048	H04	[1]	[4]
FTN_0728	FTT0748	NA	predicted Co/Zn/Cd cation transporter	1.17	tnfn1_pw060328p05q123	NR-8047	G03	[1]	
FTN_0728	FTT0756	NA	predicted Co/Zn/Cd cation transporter	1.44	tnfn1_pw060418p03q149	NR-8053	A07	[1]	
FTN_0731	FTT0759	NA	hypothetical protein	0.50	tnfn1_pw060323p03q186	NR-8037	F11	[10]	
FTN_0731	FTT0759	NA	hypothetical protein	0.63	tnfn1_pw060328p02q104	NR-8044	D01	[10]	
FTN_0756	FTT0583	*fopA*	OmpA family protein	0.39	tnfn1_pw060323p04q187	NR-8038	G11	[6,10]	[9]
FTN_0756	FTT0583	*fopA*	OmpA family protein	0.49	tnfn1_pw060510p01q195	NR-8063	G12	[6,10]	[9]
FTN_0756	FTT0583	*fopA*	OmpA family protein	0.50	tnfn1_pw060420p03q180	NR-8061	H10	[6,10]	[9]
FTN_0757	FTT0584	NA	membrane protein of unknown function	0.75	tnfn1_pw060419p02q184	NR-8056	D11	[1]	
FTN_0757	FTT0584	NA	membrane protein of unknown function	0.82	tnfn1_pw060419p04q172	NR-8058	H09	[1]	
FTN_0771	FTT1103	NA	protein-disulfide isomerase	0.45	tnfn1_pw060419p04q194	NR-8058	F12	[10,17]	[2,8,9]
FTN_0772	FTT1102	NA	hypothetical protein	0.74	tnfn1_pw060419p02q111	NR-8056	C02	[10]	[5]
FTN_0806	FTT0928c	NA	glycosyl hydrolase family 3	0.50	tnfn1_pw060323p02q108	NR-8036	H01	[1]	
FTN_0806	FTT0928c	NA	glycosyl hydrolase family 3	1.00	tnfn1_pw060510p03q119	NR-8065	C03	[1]	
FTN_0812	**FTT0934c**	***bioD***	**dethiobiotin synthetase**	**0.05**	**tnfn1_pw060323p01q189**	**NR-8035**	**A12**	**[10]**	
FTN_0812	FTT0934c	*bioD*	dethiobiotin synthetase	0.44	tnfn1_pw060328p08q164	NR-8050	H08	[10]	
FTN_0812	FTT0934c	*bioD*	dethiobiotin synthetase	0.56	tnfn1_pw060418p01q175	NR-8051	C10	[10]	
FTN_0813	**FTT0935c**	***bioC***	**biotin synthesis protein BioC**	**0.05**	**tnfn1_pw060323p04q196**	**NR-8038**	**H12**	**[10]**	
FTN_0814	**FTT0936c**	***bioF***	**8-amino-7-oxononanoate synthase**	**0.28**	**tnfn1_pw060419p02q138**	**NR-8056**	**F05**	**[1,10]**	
FTN_0814	FTT0936c	*bioF*	8-amino-7-oxononanoate synthase	0.55	tnfn1_pw060323p07q185	NR-8041	E11	[1,10]	
FTN_0815	**FTT0937c**	***bioB***	**biotin synthase**	**0.05**	**tnfn1_pw060323p03q139**	**NR-8037**	**G05**	**[1]**	
FTN_0815	FTT0937c	*bioB*	biotin synthase	0.55	tnfn1_pw060419p02q174	NR-8056	B10	[1]	
FTN_0816	**FTT0938**	***bioA***	**adenosylmethionine-8-**	**0.12**	**tnfn1_pw060328p05q108**	**NR-8047**	**H01**	**[1]**	

			amino-7-oxononanoate aminotransferase						
FTN_0816	FTT0938	*bioA*	adenosylmethionine-8-amino-7-oxononanoate aminotransferase	0.58	tnfn1_pw060420p02q145	NR-8060	E06	[1]	
FTN_0817	FTT0940c	NA	hypothetical protein	0.88	tnfn1_pw060323p06q170	NR-8040	F09	[1]	
FTN_0817	FTT0940c	NA	hypothetical protein	1.00	tnfn1_pw060418p01q141	NR-8051	A06	[1]	
FTN_0818	**FTT0941c**	**NA**	**lipase/esterase**	**0.02**	**tnfn1_pw060323p02q160**	**NR-8036**	**D08**	**[1,10]**	
FTN_0818	**FTT0941c**	**NA**	**lipase/esterase**	**0.03**	**tnfn1_pw060323p02q159**	**NR-8036**	**C08**	**[1,10]**	
FTN_0818	**FTT0941c**	**NA**	**lipase/esterase**	**0.27**	**tnfn1_pw060328p04q175**	**NR-8046**	**C10**	**[1,10]**	
FTN_0818	FTT0941c	NA	lipase/esterase	0.36	tnfn1_pw060328p04q172	NR-8046	H09	[1,10]	
FTN_0821	FTT0944	NA	AMP-binding enzyme	0.39	tnfn1_pw060420p04q157	NR-8062	A08	[1]	
FTN_0821	FTT0944	NA	AMP-binding enzyme	5.00	tnfn1_pw060323p02q137	NR-8036	E05	[1]	
FTN_0822	FTT0945	NA	para-aminobenzoate synthase component I	0.57	tnfn1_pw060323p06q143	NR-8040	C06	[1]	[8]
FTN_0822	FTT0945	NA	para-aminobenzoate synthase component I	0.69	tnfn1_pw060420p04q108	NR-8062	H01	[1]	[4,8]
FTN_0823	FTT0946	*pabA*	para-aminobenzoate synthase component II	0.50	tnfn1_pw060323p01q148	NR-8035	H06	[1]	
FTN_0823	FTT0946	*pabA*	para-aminobenzoate synthase component II	1.50	tnfn1_pw060418p02q134	NR-8052	B05	[1]	
FTN_0842	FTT0963c	*aroG*	phospho-2-dehydro-3-deoxyheptonate aldolase	0.81	tnfn1_pw060510p04q131	NR-8066	G04	[10]	
FTN_0842	FTT0963c	*aroG*	phospho-2-dehydro-3-deoxyheptonate aldolase	1.50	tnfn1_pw060323p07q179	NR-8041	G10	[10]	
FTN_0848	**FTT0968c**	**NA**	**amino acid antiporter**	**0.02**	**tnfn1_pw060323p02q136**	**NR-8036**	**D05**	**[1,10]**	**[5]**
FTN_0848	FTT0968c	NA	amino acid antiporter	0.55	tnfn1_pw060418p04q151	NR-8054	C07	[1,10]	[5]
FTN_0855	FTT0975	NA	hypothetical protein	1.00	tnfn1_pw060420p04q176	NR-8062	D10	[10]	[4]
FTN_0855	FTT0975	NA	hypothetical protein	1.42	tnfn1_pw060328p05q125	NR-8047	A04	[10]	
FTN_0893	FTT1015	NA	hypothetical protein	0.50	tnfn1_pw060323p03q193	NR-8037	E12	[10]	
FTN_0893	FTT1015	NA	hypothetical protein	1.17	tnfn1_pw060420p03q160	NR-8061	D08	[10]	
FTN_0925	FTT1047c	NA	hypothetical protein	0.54	tnfn1_pw060328p03q147	NR-8045	G06	[1]	

FTN_0925	FTT1048c	NA	hypothetical protein	2.75	tnfn1_pw060419p04q188	NR-8058	H11	[1]	[4]
FTN_0945	FTT1056c	*rsuA*	16S rRNA pseudouridine synthase	0.78	tnfn1_pw060510p02q145	NR-8064	E06	[10]	
FTN_0945	FTT1056c	*rsuA*	16S rRNA pseudouridine synthase	1.17	tnfn1_pw060328p05q161	NR-8047	E08	[10]	
FTN_0998	FTT0685c	NA	potassium channel protein	0.67	tnfn1_pw060420p01q191	NR-8059	C12	[1]	[9]
FTN_0998	FTT0685c	NA	potassium channel protein	0.84	tnfn1_pw060323p05q184	NR-8039	D11	[1]	[9]
FTN_0999	FTT0684c	*udhA*	soluble pyridine nucleotide transhydrogenase	0.50	tnfn1_pw060323p01q131	NR-8035	G04	[10]	
FTN_0999	FTT0684c	*udhA*	soluble pyridine nucleotide transhydrogenase	1.31	tnfn1_pw060418p03q163	NR-8053	G08	[10]	
FTN_1007	FTT0675	*rplY*	50S ribosomal protein L25	0.50	tnfn1_pw060323p02q164	NR-8036	H08	[10]	
FTN_1016	FTT0667	NA	hypothetical protein	0.72	tnfn1_pw060328p01q165	NR-8043	A09	[1,12]	
FTN_1016	FTT0667	NA	hypothetical protein	1.50	tnfn1_pw060419p02q170	NR-8056	F09	[1,12]	
FTN_1029	FTT0654	*elbB*	DJ-1/PfpI family protein	0.50	tnfn1_pw060323p04q186	NR-8038	F11	[10,12]	
FTN_1029	FTT0654	*elbB*	DJ-1/PfpI family protein	1.50	tnfn1_pw060419p03q146	NR-8057	F06	[10,12]	
FTN_1038	FTT0645c	NA	hypothetical protein	0.70	tnfn1_pw060419p02q108	NR-8056	H01	[1]	
FTN_1048	FTT0633	*hflK*	HflK-HflC membrane protein complex, HflK	0.33	tnfn1_pw060420p01q192	NR-8059	D12	[10]	
FTN_1048	FTT0633	*hflK*	HflK-HflC membrane protein complex, HflK	0.68	tnfn1_pw060323p07q121	NR-8041	E03	[10]	
FTN_1050	FTT0631	*hflX*	protease, GTP-binding subunit	0.47	tnfn1_pw060419p04q125	NR-8058	A04	[10]	
FTN_1050	FTT0631	*hflX*	protease, GTP-binding subunit	0.67	tnfn1_pw060323p08q161	NR-8042	E08	[10]	
FTN_1055	FTT0626	*lon*	DNA-binding, ATP-dependent protease La	0.67	tnfn1_pw060418p01q153	NR-8051	E07	[10,12,14]	
FTN_1056	FTT0625	*clpX*	ATP-dependent Clp protease subunit X	0.65	tnfn1_pw060420p03q172	NR-8061	H09	[10]	
FTN_1057	FTT0624	*clpP*	ATP-dependent Clp protease subunit P	0.55	tnfn1_pw060328p02q113	NR-8044	E02	[10]	
FTN_1057	FTT0624	*clpP*	ATP-dependent Clp protease subunit P	1.00	tnfn1_pw060419p03q136	NR-8057	D05	[10]	
FTN_1058	FTT0623	*tig*	trigger factor (TF) protein (peptidyl-prolyl cis/trans isomerase)	0.40	tnfn1_pw060323p04q107	NR-8038	G01	[10,14]	[5]

FTN_1058	FTT0623	*tig*	trigger factor (TF) protein (peptidyl-prolyl cis/trans isomerase)	1.00	tnfn1_pw060418p03q174	NR-8053	B10	[10,14]	[5]
FTN_1058	FTT0623	*tig*	trigger factor (TF) protein (peptidyl-prolyl cis/trans isomerase)	1.21	tnfn1_pw060323p06q141	NR-8040	A06	[10,14]	[5]
FTN_1064	FTT0617c	NA	PhoH family protein, putative ATPase	1.17	tnfn1_pw060420p03q121	NR-8061	E03	[1]	[4]
FTN_1066	FTT0615c	NA	transporter-associated protein, HlyC/CorC family	0.44	tnfn1_pw060328p03q134	NR-8045	B05	[1]	
FTN_1066	FTT0615c	NA	transporter-associated protein, HlyC/CorC family	0.89	tnfn1_pw060420p04q123	NR-8062	G03	[1]	
FTN_1090	FTT0589	NA	membrane protein of unknown function	0.50	tnfn1_pw060323p02q135	NR-8036	C05	[1]	
FTN_1090	FTT0589	NA	membrane protein of unknown function	1.00	tnfn1_pw060418p02q191	NR-8052	C12	[1]	
FTN_1091	FTT0588	*aroA*	3-phosphoshikimate 1-carboxyvinyltransferase	0.42	tnfn1_pw060328p04q101	NR-8046	A01		[2,9]
FTN_1091	FTT0588	*aroA*	3-phosphoshikimate 1-carboxyvinyltransferase	0.72	tnfn1_pw060510p02q161	NR-8064	E08		[2,9]
FTN_1091	FTT0588	*aroA*	3-phosphoshikimate 1-carboxyvinyltransferase	0.77	tnfn1_pw060419p04q168	NR-8058	D09		[2,9]
FTN_1097	FTT1117c	NA	isochorismatase family protein	1.17	tnfn1_pw060418p02q101	NR-8052	A01	[10]	
FTN_1097	FTT1117c	NA	isochorismatase family protein	2.00	tnfn1_pw060418p04q122	NR-8054	F03	[10]	
FTN_1107	FTT1125	*metlQ*	methionine uptake transporter (MUT) family protein, membrane and periplasmic protein	0.71	tnfn1_pw060328p02q109	NR-8044	A02	[10,12]	[8]
FTN_1107	FTT1125	*metlQ*	methionine uptake transporter (MUT) family protein, membrane and periplasmic protein	1.08	tnfn1_pw060418p04q163	NR-8054	G08	[10,12]	[8]
FTN_1107	FTT1125	*metlQ*	methionine uptake transporter (MUT) family protein, membrane and periplasmic protein	1.67	tnfn1_pw060510p03q130	NR-8065	F04	[10,12]	[8]
FTN_1111	FTT1129c	NA	Mur ligase family protein	0.41	tnfn1_pw060323p06q130	NR-8040	F04	[1]	

FTN_1111	FTT1129c	NA	Mur ligase family protein	0.63	tnfn1_pw060418p04q102	NR-8054	B01	[1]	
FTN_1112	FTT1130c	*cphA*	cyanophycin synthetase	0.43	tnfn1_pw060420p03q118	NR-8061	B03	[1,14]	[9]
FTN_1112	FTT1130c	*cphA*	cyanophycin synthetase	1.04	tnfn1_pw060328p03q132	NR-8045	H04	[1,14]	[9]
FTN_1131	FTT1150c	*putA*	bifunctional proline dehydrogenase, pyrroline-5-carboxylate dehydrogenase	0.50	tnfn1_pw060323p04q179	NR-8038	G10	[1]	
FTN_1131	FTT1150c	*putA*	bifunctional proline dehydrogenase, pyrroline-5-carboxylate dehydrogenase	0.63	tnfn1_pw060328p02q175	NR-8044	C10	[1]	[4]
FTN_1133	**FTT1152**	**NA**	**hypothetical protein**	**0.30**	**tnfn1_pw060328p04q129**	**NR-8046**	**E04**	**[10,12]**	
FTN_1133	FTT1152	NA	hypothetical protein	0.37	tnfn1_pw060420p01q163	NR-8059	G08	[10,12]	
FTN_1146	FTT1165c	NA	aspartate aminotransferase	0.50	tnfn1_pw060323p02q150	NR-8036	B07	[6,10]	[8]
FTN_1146	FTT1165c	NA	aspartate aminotransferase	1.50	tnfn1_pw060419p03q174	NR-8057	B10	[6,10]	[8]
FTN_1157	FTT1179	NA	GTP binding translational elongation factor Tu and G family protein	0.67	tnfn1_pw060420p03q176	NR-8061	D10	[10,12,14]	
FTN_1157	FTT1179	NA	GTP binding translational elongation factor Tu and G family protein	0.89	tnfn1_pw060323p07q186	NR-8041	F11	[10,12,14]	
FTN_1159	FTT1181	*ggt*	gamma-glutamyltranspeptidase	1.00	tnfn1_pw060328p08q180	NR-8050	H10		[2,8]
FTN_1159	FTT1181	*ggt*	gamma-glutamyltranspeptidase	1.00	tnfn1_pw060418p01q146	NR-8051	F06		[2,8]
FTN_1199	FTT0807	NA	hypothetical protein	0.67	tnfn1_pw060323p04q135	NR-8038	C05	[10,12]	[8,9]
FTN_1199	FTT0807	NA	hypothetical protein	1.22	tnfn1_pw060418p04q179	NR-8054	G10	[10,12]	[8,9]
FTN_1200	FTT0806	*capC*	capsule biosynthesis protein CapC	0.59	tnfn1_pw060418p02q139	NR-8052	G05	[1,12]	[8]
FTN_1201	FTT0805	*capB*	capsule biosynthesis protein CapB	0.63	tnfn1_pw060328p02q103	NR-8044	C01	[1,10]	[8]
FTN_1201	FTT0805	*capB*	capsule biosynthesis protein CapB	2.92	tnfn1_pw060418p03q121	NR-8053	E03	[1,10]	[8]
FTN_1209	FTT0802	*cphB*	cyanophycinase	0.50	tnfn1_pw060323p04q177	NR-8038	E10	[10,12]	
FTN_1209	FTT0802	*cphB*	cyanophycinase	1.75	tnfn1_pw060419p01q138	NR-8055	F05	[10,12]	
FTN_1211	FTT0800	NA	haloacid dehalogenase-like	1.67	tnfn1_pw060323p01q185	NR-8035	E11	[1]	

			hydrolase						
FTN_1211	FTT0800	NA	haloacid dehalogenase-like hydrolase	2.50	tnfn1_pw060418p03q131	NR-8053	G04	[1]	
FTN_1212	FTT0799	NA	glycosyl transferase, group 1	0.67	tnfn1_pw060420p04q101	NR-8062	A01	[1]	
FTN_1212	FTT0799	NA	glycosyl transferase, group 1	0.78	tnfn1_pw060323p05q162	NR-8039	F08	[1]	
FTN_1213	FTT0798	NA	glycosyl transferase, family 2	0.50	tnfn1_pw060323p03q152	NR-8037	D07	[1]	
FTN_1213	FTT0798	NA	glycosyl transferase, family 2	1.17	tnfn1_pw060510p04q120	NR-8066	D03	[1]	
FTN_1214	FTT0797	NA	glycosyl transferase, family 2	0.83	tnfn1_pw060328p06q149	NR-8048	A07	[1]	
FTN_1214	FTT0797	NA	glycosyl transferase, family 2	0.90	tnfn1_pw060418p03q150	NR-8053	B07	[1]	
FTN_1214	FTT0797	NA	glycosyl transferase, family 2	0.92	tnfn1_pw060510p01q174	NR-8063	B10	[1]	
FTN_1217	FTT0793	NA	ATP-binding cassette (ABC) superfamily protein	2.50	tnfn1_pw060418p03q107	NR-8053	G01	[1]	[4,5,9]
FTN_1218	FTT0792	NA	glycosyl transferase, group 1	0.94	tnfn1_pw060420p04q161	NR-8062	E08	[1,12]	
FTN_1218	FTT0792	NA	glycosyl transferase, group 1	1.00	tnfn1_pw060323p07q127	NR-8041	C04	[1,12]	
FTN_1219	FTT0791	*galE*	UDP-glucose 4-epimerase	1.08	tnfn1_pw060328p08q135	NR-8050	C05	[1]	
FTN_1219	FTT0791	*galE*	UDP-glucose 4-epimerase	1.25	tnfn1_pw060510p02q175	NR-8064	C10	[1]	
FTN_1219	FTT0791	*galE*	UDP-glucose 4-epimerase	1.80	tnfn1_pw060510p02q177	NR-8064	E10	[1]	
FTN_1220	FTT0790	NA	sugar transferase involved in lipopolysaccharide synthesis	0.92	tnfn1_pw060420p03q139	NR-8061	G05	[1]	
FTN_1220	FTT0790	NA	sugar transferase involved in lipopolysaccharide synthesis	1.17	tnfn1_pw060323p06q191	NR-8040	C12	[1]	
FTN_1240	**FTT1221**	**NA**	**BolA family protein**	**0.24**	**tnfn1_pw060419p04q183**	**NR-8049**	**E10**	**[10]**	**[5]**
FTN_1242	FTT1223	NA	DedA family protein	0.71	tnfn1_pw060418p02q190	NR-8052	B12	[1]	
FTN_1242	FTT1223	NA	DedA family protein	0.90	tnfn1_pw060328p01q163	NR-8043	G08	[1]	
FTN_1252	FTT1234	NA	choloylglycine hydrolase family protein	0.50	tnfn1_pw060419p04q171	NR-8058	G09		[2,9]
FTN_1252	FTT1234	NA	choloylglycine hydrolase family protein	0.78	tnfn1_pw060323p04q169	NR-8038	E09		[2,9]
FTN_1254	**FTT1236**	**NA**	**hypothetical protein**	**0.20**	**tnfn1_pw060323p02q156**	**NR-8036**	**H07**	**[1]**	**[2,9]**
FTN_1254	**FTT1236**	**NA**	**hypothetical protein**	**0.30**	**tnfn1_pw060323p02q155**	**NR-8036**	**G07**	**[1]**	**[2,9]**
FTN_1254	FTT1236	NA	hypothetical protein	0.47	tnfn1_pw060510p04q135	NR-8066	C05	[1]	[2,9]

FTN_1254	FTT1236	NA	hypothetical protein	1.00	tnfn1_pw060510p03q135	NR-8065	C05	[1]	[2,9]
FTN_1255	FTT1237	NA	glycosyl transferase, family 8	1.00	tnfn1_pw060323p05q183	NR-8039	C11	[1]	[9]
FTN_1256	FTT1238c	NA	membrane protein of unknown function	0.75	tnfn1_pw060420p04q196	NR-8062	H12	[1]	
FTN_1257	FTT1239	NA	membrane protein of unknown function	0.50	tnfn1_pw060323p03q102	NR-8037	B01	[1]	[4]
FTN_1257	FTT1239	NA	membrane protein of unknown function	1.50	tnfn1_pw060419p03q150	NR-8057	B07	[1]	
FTN_1263	FTT1244c	*comL*	competence lipoprotein	0.76	tnfn1_pw060328p06q171	NR-8048	G09		[2,4,8]
FTN_1263	FTT1244c	*comL*	competence lipoprotein	1.66	tnfn1_pw060420p02q179	NR-8060	G10		[2,4,8]
FTN_1273	FTT1254	NA	long chain fatty acid CoA ligase	0.52	tnfn1_pw060510p04q149	NR-8066	A07	[1]	[9]
FTN_1273	FTT1254	NA	long chain fatty acid CoA ligase	0.75	tnfn1_pw060328p06q166	NR-8048	B09	[1]	[9]
FTN_1276	FTT1257	NA	membrane fusion protein	1.19	tnfn1_pw060420p03q109	NR-8061	A02	[1]	[9]
FTN_1276	FTT1257	NA	membrane fusion protein	1.42	tnfn1_pw060510p02q119	NR-8064	C03	[1]	[9]
FTN_1277	FTT1258	NA	outer membrane efflux protein	0.36	tnfn1_pw060510p01q136	NR-8063	D05	[1]	
FTN_1277	FTT1258	NA	outer membrane efflux protein	1.22	tnfn1_pw060418p04q138	NR-8054	F05	[1]	
FTN_1309	FTT1699; FTT1344	*pdpA*	hypothetical protein	0.38	tnfn1_pw060510p03q145	NR-8065	E06	[1,18-20]	[5,18,19,21]
FTN_1309	FTT1699; FTT1344	*pdpA*	hypothetical protein	0.67	tnfn1_pw060323p05q130	NR-8039	F04	[1,18,20]	[5,18,21]
FTN_1309	FTT1699; FTT1344	*pdpA*	hypothetical protein	1.00	tnfn1_pw060323p05q128	NR-8039	D04	[1,18,20]	[5,18,21]
FTN_1310	**FTT1700; FTT1345**	***pdpB; icmF***	**hypothetical protein**	**0.10**	**tnfn1_pw060323p05q179**	**NR-8039**	**G10**	**[1,6,20]**	**[5,20]**
FTN_1310	FTT1700; FTT1345	*pdpB; icmF*	hypothetical protein	0.44	tnfn1_pw060418p02q165	NR-8052	A09	[1,6,20]	[5,20]
FTN_1311	**FTT1701; FTT1346**	***iglE***	**hypothetical protein**	**0.24**	**tnfn1_pw060328p01q194**	**NR-8043**	**F12**	**[1]**	**[5]**
FTN_1312	**FTT1702; FTT1347**	***vgrG***	**hypothetical protein**	**0.25**	**tnfn1_pw060328p06q144**	**NR-8048**	**D06**	**[1,12,22]**	**[5,22]**
FTN_1312	FTT1702; FTT1347	*vgrG*	hypothetical protein	1.14	tnfn1_pw060419p03q132	NR-8057	H04	[1,12,22]	[5,22]
FTN_1313	**FTT1703;**	***iglF***	**hypothetical protein**	**0.28**	**tnfn1_pw060328p01q144**	**NR-8043**	**D06**	**[1]**	**[5]**

	FTT1348								
FTN_1313	FTT1703; FTT1348	*iglF*	hypothetical protein	0.60	tnfn1_pw060328p04q154	NR-8046	F07	[1]	[5]
FTN_1313	FTT1703; FTT1348	*iglF*	hypothetical protein	1.00	tnfn1_pw060419p03q114	NR-8057	F02	[1]	[5]
FTN_1314	**FTT1704; FTT1349**	***iglG***	**hypothetical protein**	**0.10**	**tnfn1_pw060323p03q179**	**NR-8037**	**G10**	**[1]**	**[5]**
FTN_1314	FTT1704; FTT1349	*iglG*	hypothetical protein	0.60	tnfn1_pw060418p04q141	NR-8054	A06	[1]	[5]
FTN_1315	**FTT1705; FTT1350**	***iglH***	**hypothetical protein**	**0.19**	**tnfn1_pw060328p06q163**	**NR-8048**	**G08**	**[1]**	**[5]**
FTN_1315	FTT1705; FTT1350	*iglH*	hypothetical protein	0.39	tnfn1_pw060420p04q133	NR-8062	A05	[1]	[5]
FTN_1316	**FTT1706; FTT1351**	***dotU***	**hypothetical protein**	**0.17**	**tnfn1_pw060323p06q162**	**NR-8040**	**F08**	**[1]**	**[5]**
FTN_1316	FTT1706; FTT1351	*dotU*	hypothetical protein	0.31	tnfn1_pw060419p01q125	NR-8055	A04	[1]	[5]
FTN_1317	**FTT1707; FTT1352**	***iglI***	**hypothetical protein**	**0.10**	**tnfn1_pw060323p02q142**	**NR-8036**	**B06**	**[1,22]**	**[5,22]**
FTN_1317	**FTT1707; FTT1352**	***iglI***	**hypothetical protein**	**0.30**	**tnfn1_pw060420p04q177**	**NR-8062**	**E10**	**[1,22]**	**[5,22]**
FTN_1318	**FTT1708; FTT1353**	***iglJ***	**hypothetical protein**	**0.12**	**tnfn1_pw060418p04q162**	**NR-8054**	**F08**	**[1,20]**	**[5]**
FTN_1318	**FTT1708; FTT1353**	***iglJ***	**hypothetical protein**	**0.16**	**tnfn1_pw060328p06q148**	**NR-8048**	**H06**	**[1,20]**	**[5]**
FTN_1319	FTT1709; FTT1354	*pdpC*	hypothetical protein	0.50	tnfn1_pw060323p01q171	NR-8035	G09	[1]	
FTN_1319	FTT1709; FTT1354	*pdpC*	hypothetical protein	1.33	tnfn1_pw060418p04q106	NR-8054	F01	[1]	
FTN_1320	FTT1710; FTT1355	*pdpE*	hypothetical protein	0.50	tnfn1_pw060323p03q140	NR-8037	H05	[1]	
FTN_1320	FTT1710; FTT1355	*pdpE*	hypothetical protein	0.88	tnfn1_pw060420p01q177	NR-8059	E10	[1]	
FTN_1321	**FTT1711c; FTT1356c**	***iglD***	**intracellular growth locus protein D**	**0.16**	**tnfn1_pw060510p01q110**	**NR-8063**	**B02**	**[1,12]**	**[5,23,24]**
FTN_1321	**FTT1711c; FTT1356c**	***iglD***	**intracellular growth locus protein D**	**0.28**	**tnfn1_pw060420p03q137**	**NR-8061**	**E05**	**[1,12]**	**[5,23,24]**

FTN_1322	**FTT1712c; FTT1357c**	***iglC***	**intracellular growth locus protein C**	**0.14**	**tnfn1_pw060418p02q116**	**NR-8052**	**H02**	**[1,10,19,25]**	**[5,19,23,25,26]**
FTN_1322	**FTT1712c; FTT1357c**	***iglC***	**intracellular growth locus protein C**	**0.24**	**tnfn1_pw060328p06q115**	**NR-8048**	**G02**	**[1,10,19,25]**	**[5,19,23,25,26]**
FTN_1323	**FTT1713c; FTT1358c**	***iglB***	**intracellular growth locus protein B**	**0.19**	**tnfn1_pw060328p02q115**	**NR-8044**	**G02**	**[1,10,27]**	**[5,23]**
FTN_1323	FTT1713c; FTT1358c	*iglB*	intracellular growth locus protein B	0.45	tnfn1_pw060419p01q141	NR-8055	A06	[1,10,27]	[5,23]
FTN_1324	**FTT1714c; FTT1359c**	***iglA***	**intracellular growth locus protein A**	**0.15**	**tnfn1_pw060323p05q159**	**NR-8039**	**C08**	**[1,10]**	**[5,23,28]**
FTN_1324	FTT1714c; FTT1359c	*iglA*	intracellular growth locus protein A	0.57	tnfn1_pw060420p03q125	NR-8061	A04	[1,10]	[5,23,28]
FTN_1325	FTT1715c; FTT1360c	*pdpD*	hypothetical protein	0.50	tnfn1_pw060323p02q114	NR-8036	F02	[1,18,29]	
FTN_1325	FTT1715c; FTT1360c	*pdpD*	hypothetical protein	0.89	tnfn1_pw060419p04q108	NR-8058	H01	[1,18,29]	
FTN_1326	FTT1716c; FTT1361c	*anmK*	anhydro-N-acetylmuramic acid kinase	1.00	tnfn1_pw060328p06q183	NR-8048	C11	[1]	
FTN_1326	FTT1716c; FTT1361c	*anmK*	anhydro-N-acetylmuramic acid kinase	1.17	tnfn1_pw060420p03q122	NR-8061	F03	[1]	
FTN_1357	FTT1394c	*recB*	ATP-dependent exoDNAse (exonuclease V) beta subunit	0.81	tnfn1_pw060323p04q156	NR-8038	H07	[1]	
FTN_1357	FTT1394c	*recB*	ATP-dependent exoDNAse (exonuclease V) beta subunit	1.00	tnfn1_pw060323p08q169	NR-8042	E09	[1]	
FTN_1357	FTT1394c	*recB*	ATP-dependent exoDNAse (exonuclease V) beta subunit	2.92	tnfn1_pw060418p03q158	NR-8053	B08	[1]	
FTN_1362	FTT1400c	NA	hypothetical protein	0.50	tnfn1_pw060323p04q171	NR-8038	G09	[10,12]	
FTN_1362	FTT1400c	NA	hypothetical protein	1.75	tnfn1_pw060419p01q161	NR-8055	E08	[10,12]	
FTN_1382	FTT1416	NA	hypothetical protein	1.75	tnfn1_pw060419p01q119	NR-8055	C03	[10]	
FTN_1410	FTT1441	*bfr*	bacterioferritin	1.17	tnfn1_pw060420p03q191	NR-8061	C12	[10]	[9]
FTN_1410	FTT1441	*bfr*	bacterioferritin	1.50	tnfn1_pw060328p05q190	NR-8047	B12	[10]	[9]
FTN_1412	FTT1442c	NA	DNA-directed RNA polymerase, alpha subunit/40 kD subunit	0.50	tnfn1_pw060323p04q184	NR-8038	D11	[10]	
FTN_1417	FTT1447c	*manB*	phosphomannomutase	0.70	tnfn1_pw060328p08q115	NR-8050	G02	[1]	[5]

FTN_1417	FTT1447c	*manB*	phosphomannomutase	1.67	tnfn1_pw060510p03q186	NR-8065	F11	[1]	[5]
FTN_1421	**FTT1456c**	***wbtH***	**glutamine amidotransferase/asparagine synthase**	**0.15**	**tnfn1_pw060323p06q119**	**NR-8040**	**C03**	**[1]**	**[9]**
FTN_1421	FTT1456c	*wbtH*	glutamine amidotransferase/asparagine synthase	0.56	tnfn1_pw060420p04q116	NR-8062	H02	[1]	[4,9]
FTN_1423	**FTT1457c**	***wbtG***	**glycosyl transferase, group 1**	**0.15**	**tnfn1_pw060323p03q189**	**NR-8037**	**A12**	**[1]**	
FTN_1423	FTT1457c	*wbtG*	glycosyl transferase, group 1	1.00	tnfn1_pw060418p01q194	NR-8051	F12	[1]	
FTN_1425	FTT1459c	*wbtF*	NAD dependent epimerase	0.33	tnfn1_pw060323p06q161	NR-8040	E08	[1,10]	
FTN_1425	FTT1459c	*wbtF*	NAD dependent epimerase	1.00	tnfn1_pw060510p04q137	NR-8066	E05	[1,10]	
FTN_1426	FTT1460c	*wbtE*	UDP-glucose/GDP-mannose dehydrogenase family protein	0.35	tnfn1_pw060328p03q164	NR-8045	H08	[1]	[9]
FTN_1426	FTT1460c	*wbtE*	UDP-glucose/GDP-mannose dehydrogenase family protein	1.07	tnfn1_pw060418p04q180	NR-8054	H10	[1]	[9]
FTN_1427	**FTT1461c**	***wbtD***	**glycosyl transferase, group 1**	**0.15**	**tnfn1_pw060323p01q134**	**NR-8035**	**B05**	**[1]**	
FTN_1427	FTT1461c	*wbtD*	glycosyl transferase, group 1	1.00	tnfn1_pw060510p03q107	NR-8065	G01	[1]	
FTN_1427	FTT1461c	*wbtD*	glycosyl transferase, group 1	1.17	tnfn1_pw060419p04q192	NR-8058	D12	[1]	
FTN_1431	FTT1464c	*wbtA*	dTDP-glucose 4,6-dehydratase	0.65	tnfn1_pw060323p06q123	NR-8040	G03	[1,10,30]	[8,30]
FTN_1431	FTT1464c	*wbtA*	dTDP-glucose 4,6-dehydratase	0.75	tnfn1_pw060419p03q166	NR-8057	B09	[1,10,30]	[8,30]
FTN_1433	FTT1525c	NA	hypothetical protein	1.00	tnfn1_pw060323p05q154	NR-8039	F07	[1,10]	
FTN_1433	FTT1525c	NA	hypothetical protein	1.11	tnfn1_pw060420p02q142	NR-8060	B06	[1,10]	
FTN_1438	FTT1530	NA	bifunctional protein: 3-hydroxacyl-CoA dehydrogenase/acyl-CoA-binding protein	0.50	tnfn1_pw060323p07q169	NR-8041	E09	[1,10]	
FTN_1438	FTT1530	NA	bifunctional protein: 3-hydroxacyl-CoA dehydrogenase/acyl-CoA-binding protein	1.28	tnfn1_pw060418p02q122	NR-8052	F03	[1,10]	
FTN_1470	FTT1562	*ispA*	geranyl diphosphate synthase/farnesyl diphosphate synthase	0.75	tnfn1_pw060419p04q138	NR-8058	F05	[1]	

FTN_1470	FTT1562	*ispA*	geranyl diphosphate synthase/farnesyl diphosphate synthase	0.89	tnfn1_pw060328p04q118	NR-8046	B03	[1]	
FTN_1471	FTT1563	*pcs*	(CDP-alcohol) phosphatidyltransferase	0.71	tnfn1_pw060323p06q184	NR-8040	D11	[1,12]	
FTN_1500	FTT1489	NA	hypothetical protein	0.89	tnfn1_pw060510p02q135	NR-8064	C05	[1]	[9]
FTN_1500	FTT1489	NA	hypothetical protein	1.00	tnfn1_pw060328p06q151	NR-8048	C07	[1]	[9]
FTN_1501	**FTT1490**	**NA**	**monovalent cation:proton antiporter-1**	**0.22**	**tnfn1_pw060328p03q177**	**NR-8045**	**E10**	**[1]**	**[2,8]**
FTN_1501	FTT1490	NA	monovalent cation:proton antiporter-1	1.00	tnfn1_pw060419p03q196	NR-8057	H12	[1]	[2,8]
FTN_1502	Intergenic (FTT1491c -1492c)	NA	hypothetical protein	0.50	tnfn1_pw060419p04q149	NR-8058	A07	[1]	
FTN_1502	Intergenic (FTT1491c -1492c)	NA	hypothetical protein	0.72	tnfn1_pw060323p04q133	NR-8038	A05	[1]	
FTN_1513	FTT1503	*xerC*	site-specific recombinase	0.75	tnfn1_pw060328p06q169	NR-8048	E09	[1]	
FTN_1513	FTT1503	*xerC*	site-specific recombinase	1.25	tnfn1_pw060510p03q193	NR-8065	E12	[1]	
FTN_1518	FTT1508c	*relA*	GDP pyrophosphokinase/GTP pyrophosphokinase	0.50	tnfn1_pw060323p06q110	NR-8040	B02	[1]	[4]
FTN_1518	FTT1508c	*relA*	GDP pyrophosphokinase/GTP pyrophosphokinase	0.50	tnfn1_pw060323p07q167	NR-8041	C09	[1]	[4]
FTN_1538	FTT1696	*groEL*	chaperonin GroEL (HSP60 family)	1.75	tnfn1_pw060419p01q178	NR-8055	F10	[1]	[4]
FTN_1538	FTT1696	*groEL*	chaperonin GroEL (HSP60 family)	1.50	tnfn1_pw060328p05q129	NR-8047	E04	[1]	[4]
FTN_1548	FTT0165c	NA	hypothetical protein	0.67	tnfn1_pw060420p03q194	NR-8061	F12	[1]	
FTN_1548	FTT0165c	NA	hypothetical protein	0.72	tnfn1_pw060328p01q101	NR-8043	A01	[1]	
FTN_1548	FTT0165c	NA	hypothetical protein	0.89	tnfn1_pw060323p06q150	NR-8040	B07	[1]	
FTN_1551	FTT0162	*ampD*	N-acetylmuramoyl-L-alanine amidase	1.36	tnfn1_pw060328p06q177	NR-8048	E10	[1]	
FTN_1551	FTT0162	*ampD*	N-acetylmuramoyl-L-alanine amidase	2.17	tnfn1_pw060510p04q129	NR-8066	E04	[1]	
FTN_1558	FTT0154	*xerD*	site-specific recombinase	0.65	tnfn1_pw060418p03q117	NR-8053	A03	[1]	

FTN_1558	FTT0154	*xerD*	site-specific recombinase	0.68	tnfn1_pw060328p01q175	NR-8043	C10	[1]	
FTN_1582	FTT0134	NA	hypothetical membrane protein	0.35	tnfn1_pw060420p02q102	NR-8060	B01	[1]	
FTN_1582	FTT0134	NA	hypothetical membrane protein	0.59	tnfn1_pw060323p08q185	NR-8042	E11	[1]	
FTN_1586	**FTT0129**	**NA**	**sugar transporter, MFS superfamily**	**0.10**	**tnfn1_pw060418p01q150**	**NR-8051**	**B07**		**[2]**
FTN_1586	**FTT0129**	**NA**	**sugar transporter, MFS superfamily**	**0.15**	**tnfn1_pw060328p01q110**	**NR-8043**	**B02**		**[2]**
FTN_1597	FTT0118	*prfC*	peptide chain release factor 3	0.71	tnfn1_pw060510p01q146	NR-8063	F06	[1,14]	[4]
FTN_1597	FTT0118	*prfC*	peptide chain release factor 3	0.81	tnfn1_pw060328p01q106	NR-8043	F01	[1,14]	
FTN_1602	FTT0113	*deoB*	phosphopentomutase	0.50	tnfn1_pw060323p04q137	NR-8038	E05	[1]	[9]
FTN_1602	FTT0113	*deoB*	phosphopentomutase	1.00	tnfn1_pw060418p01q174	NR-8051	B10	[1]	[9]
FTN_1607	FTT0108c	*cca*	tRNA nucleotidyl transferase	0.75	tnfn1_pw060328p04q195	NR-8046	G12	[1]	
FTN_1608	**FTT0107c**	***dsbB***	**disulfide bond formation protein**	**0.06**	**tnfn1_pw060510p02q157**	**NR-8064**	**A08**	**[1,2,6]**	**[2,5,8]**
FTN_1608	**FTT0107c**	***dsbB***	**disulfide bond formation protein**	**0.20**	**tnfn1_pw060323p05q173**	**NR-8039**	**A10**	**[1,2,6]**	**[2,5,8]**
FTN_1610	FTT0105c	NA	RND efflux transporter, AcrB/AcrD/AcrF family	0.50	tnfn1_pw060323p02q131	NR-8036	G04	[10]	
FTN_1610	FTT0105c	NA	RND efflux transporter, AcrB/AcrD/AcrF family	2.50	tnfn1_pw060418p04q118	NR-8054	B03	[10]	
FTN_1613	FTT0101	NA	peptidase, U61 family	0.65	tnfn1_pw060328p04q184	NR-8046	D11	[10]	
FTN_1613	FTT0101	NA	peptidase, U61 family	1.50	tnfn1_pw060419p03q180	NR-8057	H10	[10]	
FTN_1617	FTT0094c	NA	two-component regulator, sensor histidine kinase	0.66	tnfn1_pw060323p08q137	NR-8042	E05	[1]	
FTN_1617	FTT0094c	NA	two-component regulator, sensor histidine kinase	1.50	tnfn1_pw060418p04q101	NR-8054	A01	[1]	
FTN_1633	FTT0078	*apt*	adenine phosphoribosyltransferase	0.50	tnfn1_pw060323p01q166	NR-8035	B09	[1]	
FTN_1633	FTT0078	*apt*	adenine phosphoribosyltransferase	1.67	tnfn1_pw060510p02q107	NR-8064	G01	[1]	
FTN_1653	FTT0057	NA	hypothetical membrane protein	1.00	tnfn1_pw060328p05q181	NR-8047	A11	[1]	
FTN_1654	FTT0056c	NA	major facilitator superfamily	0.89	tnfn1_pw060419p04q155	NR-8058	G07	[1]	[2]

			(MFS) transport protein						
FTN_1654	FTT0056c	NA	major facilitator superfamily (MFS) transport protein	0.90	tnfn1_pw060328p08q189	NR-8050	A12	[1]	[2]
FTN_1655	FTT0055	*rluC*	ribosomal large subunit pseudouridine synthase C	0.50	tnfn1_pw060510p02q165	NR-8064	A09	[1]	[4]
FTN_1655	FTT0055	*rluC*	ribosomal large subunit pseudouridine synthase C	1.17	tnfn1_pw060323p04q160	NR-8038	D08	[1]	[4]
FTN_1656	FTT0054	NA	hypothetical protein	0.85	tnfn1_pw060420p02q127	NR-8060	C04	[1]	[4]
FTN_1656	FTT0054	NA	hypothetical protein	1.00	tnfn1_pw060328p08q182	NR-8050	B11	[1]	
FTN_1657	FTT0053	NA	major facilitator superfamily (MFS) transport protein	0.40	tnfn1_pw060418p02q115	NR-8052	G02	[1]	[8]
FTN_1657	FTT0053	NA	major facilitator superfamily (MFS) transport protein	0.64	tnfn1_pw060328p02q171	NR-8044	G09	[1]	[8]
FTN_1682	**FTT0029c**	***figA/ fslA***	**siderophore biosynthesis protein**	**0.15**	**tnfn1_pw060323p04q131**	**NR-8038**	**G04**	**[1,10]**	
FTN_1682	FTT0029c	*figA/ fslA*	siderophore biosynthesis protein	0.64	tnfn1_pw060419p04q180	NR-8058	H10	[1,10]	
FTN_1682	FTT0029c	*figA/ fslA*	siderophore biosynthesis protein	0.70	tnfn1_pw060328p05q150	NR-8047	B07	[1,10]	
FTN_1683	**FTT0028c**	***figB/ fslB***	**Conserved membrane protein**	**0.15**	**tnfn1_pw060323p03q163**	**NR-8037**	**G08**	**[1]**	
FTN_1683	**FTT0028c**	***figB/ fslB***	**Conserved membrane protein**	**0.18**	**tnfn1_pw060419p04q105**	**NR-8058**	**E01**	**[1]**	
FTN_1683	**FTT0028c**	***figB/ fslB***	**Conserved membrane protein**	**0.20**	**tnfn1_pw060418p02q193**	**NR-8052**	**E12**	**[1]**	
FTN_1683	**FTT0028c**	***figB/ fslB***	**Conserved membrane protein**	**0.20**	**tnfn1_pw060328p02q192**	**NR-8044**	**D12**	**[1]**	
FTN_1684	**FTT0027c**	***figC/ fslC***	**diaminopimelate decarboxylase**	**0.24**	**tnfn1_pw060510p02q193**	**NR-8064**	**E12**	**[1]**	
FTN_1684	FTT0027c	*figC/ fslC*	**diaminopimelate decarboxylase**	0.40	tnfn1_pw060328p03q166	NR-8045	B09	[1]	
FTN_1684	FTT0027c	*figC/ fslC*	**diaminopimelate decarboxylase**	1.00	tnfn1_pw060510p02q191	NR-8064	C12	[1]	
FTN_1699	**FTT1720c**	***purL***	**phosphoribosylformylglycin amide synthase**	**0.11**	**tnfn1_pw060510p03q121**	**NR-8065**	**E03**	**[1,6,14]**	**[2]**
FTN_1699	**FTT1720c**	***purL***	**phosphoribosylformylglycin amide synthase**	**0.12**	**tnfn1_pw060323p06q112**	**NR-8040**	**D02**	**[1,6,14]**	**[2]**

FTN_1700	**FTT1720c**	***purL***	**phosphoribosylformylglycinamide synthase**	**0.17**	**tnfn1_pw060419p01q190**	**NR-8055**	**B12**	**[1,13,14]**	**[2,13]**
FTN_1705	FTT1726	NA	peptidase, U32 family	0.83	tnfn1_pw060420p04q131	NR-8062	G04	[10]	
FTN_1705	FTT1726	NA	peptidase, U32 family	1.17	tnfn1_pw060328p05q105	NR-8047	E01	[10]	
FTN_1715	**FTT1736c**	***kdpD***	**two component regulator, sensor histidine kinase kdpD**	**0.11**	**tnfn1_pw060328p01q124**	**NR-8043**	**H03**	**[1,31]**	
FTN_1715	FTT1736c	*kdpD*	two component regulator, sensor histidine kinase kdpD	0.68	tnfn1_pw060510p03q184	NR-8065	D11	[1,31]	
FTN_1715	FTT1736c	*kdpD*	two component regulator, sensor histidine kinase kdpD	1.75	tnfn1_pw060419p01q150	NR-8055	B07	[1,31]	
FTN_1743	**FTT1769c**	***clpB***	**chaperone clpB**	**0.29**	**tnfn1_pw060420p01q140**	**NR-8059**	**H05**	**[1,10,32,33]**	**[5,8,23,32]**
FTN_1743	FTT1769c	*clpB*	chaperone clpB	1.07	tnfn1_pw060510p02q130	NR-8064	F04	[1,10,32]	[5,8,23,32]
FTN_1743	FTT1769c	*clpB*	chaperone clpB	1.17	tnfn1_pw060328p03q122	NR-8045	F03	[1,10,30,32]	[5,8,23,32]
FTN_1744	FTT1768c	*chiB*	chitinase	0.71	tnfn1_pw060328p03q184	NR-8045	D11	[1]	
FTN_1744	FTT1768c	*chiB*	chitinase	1.39	tnfn1_pw060419p04q131	NR-8058	G04	[1]	
FTN_1745	FTT1767c	*purT*	phosphoribosylglycinamide formyltransferase 2	0.78	tnfn1_pw060510p01q164	NR-8063	H08	[1]	
FTN_1745	FTT1767c	*purT*	phosphoribosylglycinamide formyltransferase 2	0.90	tnfn1_pw060328p08q169	NR-8050	E09	[1]	
FTN_1745	FTT1767c	*purT*	phosphoribosylglycinamide formyltransferase 2	1.00	tnfn1_pw060510p01q133	NR-8063	A05	[1]	
FTN_1750	FTT1762c	NA	acyltransferase	0.63	tnfn1_pw060510p02q173	NR-8064	A10		[2]
FTN_1750	FTT1762c	NA	acyltransferase	0.89	tnfn1_pw060328p02q134	NR-8044	B05		[2]
FTN_1750	FTT1762c	NA	acyltransferase	1.50	tnfn1_pw060418p03q116	NR-8053	H02		[2]
FTN_1750	FTT1762c	NA	acyltransferase	2.50	tnfn1_pw060419p03q128	NR-8057	D04		[2]
FTN_1762	FTT1782c	NA	(putative) drug resistance ATPase-1 (Drug RA1) family protein	1.00	tnfn1_pw060419p04q170	NR-8058	F09	[1,10,14]	
FTN_1762	FTT1782c	NA	(putative) drug resistance ATPase-1 (Drug RA1) family protein	1.07	tnfn1_pw060328p04q169	NR-8046	E09	[1,10,14]	

Table S1. Strains highlighted in **bold** were attenuated for intracellular replication. Each transposon mutant was screened 2-3 times and each time the fold replication of the mutant was compared to the fold replication of wild-type (fold Mut/ fold WT). AVG Mut/WT is the average of these ratios from all experiments for each mutant. Strain names, plate number, and well location are as annotated in the two-allele transposon mutant library from Gallagher, et al. [34].

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Table S2. Primers used in this study.

Primer name	Sequence
FTN_0096	
96 deletion check F1	accctctaatgtaatatcgc
96 Arm1 FWD	caattttccatggctgagta
96 Arm 1 REV	ttatcgataccgtcgacctctttttactctcctcttttagc
96 frt_sKAN_frt FWD	gctaaaagaggagagtaaaaaagaggtcgacggtatcgataa
96 frt_sKAN_frt REV	actctaaattaaaggctatgatgcatagctgcaggatcgata
96 Arm 2 FWD	tatcgatcctgcagctatgcatcatagcctttaatttagagt
96 Arm 2 REV	caattataataggcagctcc
96 deletion check R1	cagcgtttccaatactacta
96 comp Arm 1 REV	gagtgacaacccaaagagatttaaaggctatgatttttaactt
96 comp sKAN FWD	aagttaaaaatcatagcctttaaatctctttgggttgtcactc
96 comp sKAN REV	atttattaccaactctaaattaaaggctatgatacaaccaattaaccaattctg
96 comp Arm 2 FWD	cagaattggttaattggttgtatcatagcctttaatttagagttggtaataaat
96 comp check R2	gggaacttaagaattctagg
FTN_1133	
1133 deletion check F1	ctttctctaacttgtgcctt
1133 Arm1 FWD	ataggatattctcctgagtg
1133 Arm 1 REV	ttatcgataccgtcgacctctagattttaatccttataatatttt
1133 frt_sKAN_frt FWD	aaaatattataaggattaaaatctagaggtcgacggtatcgataa
1133 frt_sKAN_frt REV	tgagatatttaaacttattatttaagcatagctgcaggatcgata
1133 Arm 2 FWD	tatcgatcctgcagctatgcttaaataataagtttaaatatctca
1133 Arm 2 REV	acttttctcattaccttggc
1133 deletion check R1	gggctagagctattttgaat
1133 comp Arm 1 REV	gagtgacaacccaaagagatttagcttttattatcgatcaag
1133 comp sKAN FWD	cttgatcgataataaaagctaaatctctttgggttgtcactc
1133 comp sKAN REV	tgagatatttaaacttattatttaaacaaccaattaaccaattctg
1133 comp Arm 2 FWD	cagaattggttaattggttgtttaaataataagtttaaatatctca
1133 comp check R2	cagatgaatggtcaactctt
FTL_0803	
FTL_0803 192/3a-IBS	aaaactcgagataattatccttaacaatctgactgacttgtgcgcccagatagggt
FTL_0803 192/3a-EBS1d	cagattgtacaaatgtggtgataacagataagtctgacttagtaacttacctttctttgt
FTL_0803 192/3a-EBS2	tgaacgcaagtttctaatttcgattattgttcgatagaggaaagtgtct
FTL_0803 Arm 1 FWD	atatatatggatccgtcatactcgatcataaacg
FTL_0803 Arm 1 REV	gagatatttaaacttattatttaatagattttaatccttataatatttttttg
FTL_0803 Arm 2 FWD	caaaaaatattataaggattaaaatctattaaataataagtttaaatatctc
FTL_0803 Arm 2 REV	atatatatGGATCCgattaaagctcaagtcgttg

FTL_0803 Check F1	gatgcttttccataccaaca
FTL_0803 Check F2	gcaagatcttcatgcatataac
FTL_0803 Check R1	gaatatattgctggtatggc
RT Primers	
uvrD RT1	gggatgtcgccttttgattttc
uvrD RT2	ctcttttgtcccttgtgcttgc
FTN_1133 RT3	gatattcgttgtttagcttgc
FTN_1133 RT5	ggtcaactcttctcaatgag

Chapter 2 Supporting Figures

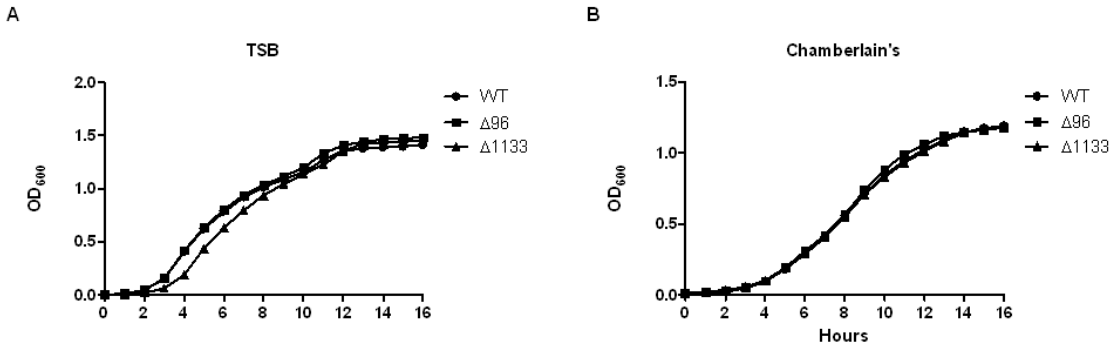

Figure S1. Selected deletion mutants of genes identified in the replication screen display wild-type growth in rich media and in defined minimal media. Bacterial growth at 37°C in (A) cysteine-enriched tryptic soy broth and (B) Chamberlain's minimal defined media is shown for wild-type *F. novicida* (circles), *FTN_0096* (Δ96, squares), and *FTN_1133* (Δ1133 triangles). Data shown is representative of at least three independent experiments.

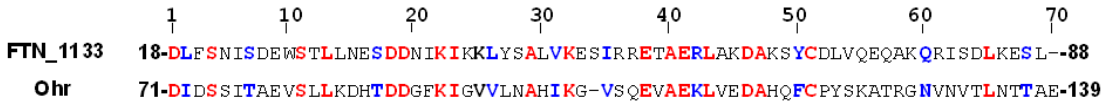

Figure S2. FTN_1133 has similarity to the organic hydroperoxide resistance protein Ohr. The sequences for *F. novicida* FTN_1133 (a.a. 18 - 86) and *Bacillus megaterium* Ohr (a.a. 71 - 139) were aligned using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical residues are highlighted in red and similar residues are highlighted in blue. The sequences have 28.6% identity and 42.9% similarity.

Chapter 3: NaxD is a deacetylase required for lipid A modification and *Francisella* pathogenesis.

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Chapter 3 Abstract

Modification of specific Gram-negative bacterial cell envelope components, such as capsule, O-antigen, and lipid A, are often essential for the successful establishment of infection. *Francisella* species express lipid A molecules with unique characteristics involved in circumventing host defenses, which significantly contribute to their virulence. In this study, we show that NaxD, a member of the highly conserved YdjC superfamily, is a deacetylase required for an important modification of the outer membrane component lipid A in *Francisella*. Mass spectrometry analysis revealed that NaxD is essential for the modification of a lipid A phosphate with galactosamine in *F. novicida,* a model organism for the study of highly virulent *F. tularensis*. Significantly, enzymatic assays confirmed that this protein is necessary for deacetylation of its substrate. In addition, NaxD was involved in resistance to the antimicrobial peptide polymyxin B and critical for replication in macrophages and *in vivo* virulence. Importantly, this protein is also required for lipid A modification in *F. tularensis* as well as *Bordetella bronchiseptica*. Since NaxD homologs are conserved among many Gram-negative pathogens, this work has broad implications for our understanding of host subversion mechanisms of other virulent bacteria.

Chapter 3 Introduction

Mammalian host defenses include multiple pathways for recognition of, and action against, Gram-negative bacterial cell wall components including capsule, O-antigen, and lipid A. Accordingly, many such pathogens have evolved modifications of these structural elements in order to evade host responses. The lipid A molecules of *Francisella*

species have multiple unique modifications, though the details of the pathways involved in generating these alterations are still being elucidated.

F. tularensis is a Gram-negative intracellular pathogen and the causative agent of tularemia. Due to its extreme infectivity, high morbidity and mortality rates, history of weaponization, and ease of aerosolization and dissemination, it is considered a category A select agent (potential bioweapon) by the Centers for Disease Control and Prevention (CDC) (Darling *et al.*, 2002). *F. novicida* is a less virulent species that rarely causes disease in humans but is frequently used as a laboratory model as it causes a tularemia-like disease in mice, is easily genetically manipulated, and is known to use many of the same virulence determinants as *F. tularensis* (Titball & Petrosino, 2007). These include the *Francisella* pathogenicity island (FPI), which is thought to encode a putative type VI secretion system, oxidative stress resistance proteins, siderophores, and outer membrane lipid A modifications that enable the bacteria to evade recognition and damage by host phagocytes (Bakshi *et al.*, 2006, Gunn & Ernst, 2007, Nano & Schmerk, 2007, Ramakrishnan *et al.*, 2008, Honn *et al.*, 2012).

Francisella LPS has a unique lipid A moiety that is distinct from canonical lipid A structures of other Gram-negative pathogens. For example, compared to the hexa-acylated lipid A expressed by *E. coli*, *Francisella* lipid A features only four acyl chains that are longer than those of *E. coli* by as many as six carbons (Raetz & Whitfield, 2002, Trent, 2004, Raetz *et al.*, 2009) (Fig. 1A, B). In addition, *Francisella* LPS lacks both the 1 and 4' position distal phosphates (Raetz et al., 2009). Also unique to *Francisella*

species, 70% of the total lipid A in the outer membrane exists in a "free" form that lacks the traditional Kdo, core, and O-antigen polysaccharides of complete LPS (Wang *et al.*, 2006, Zhao & Raetz, 2010) (Fig. 1C). Unlike the lipid A of complete LPS, free lipid A retains the 1 position phosphate that is further modified with a galactosamine residue.

As highly successful intracellular pathogens, *Francisella* species are able to utilize multiple phagocytic and nonphagocytic cell types for replication (Fujita *et al.*, 1993, Qin & Mann, 2006, Hall *et al.*, 2007, Hall *et al.*, 2008, Schulert *et al.*, 2009). Entry into host macrophages often occurs by a novel process involving the formation of unusually large and asymmetrical pseudopod loops (Clemens & Horwitz, 2007). One to three hours after uptake by phagocytes, *Francisella* species escape the phagosome before replicating within the host cytosol. However, many of the details of *Francisella*'s intracellular lifecycle are still unknown (Clemens & Horwitz, 2007).

Although much progress has been made in understanding *Francisella* virulence mechanisms, there are still many questions regarding how this pathogen is able to so effectively replicate within host cells and cause disease. To begin to answer these questions, we performed a genome-wide *in vivo* negative selection screen to identify genes required for pathogenesis (Weiss *et al.*, 2007). Next, we conducted an intracellular replication screen to determine which of those genes were important specifically for replication in macrophages (Llewellyn *et al.*, 2011). *FTN_0544* was identified in both of these screens. Though annotated as a hypothetical protein of unknown function in the NCBI database, FTN_0544 belongs to the YdjC superfamily of proteins. Interestingly,

proteins belonging to this family are encoded by multiple Gram-negative pathogens including *Bordetella bronchiseptica*, *Brucella abortus*, *Coxiella burnetii*, and *Legionella pneumophila*.

In this study, we show that FTN_0544 is a deacetylase involved in the galactosamine modification of *Francisella*'s unique free lipid A molecules. We have thus renamed this protein NaxD (*N*-acetylhexosamine deacetylase). Furthermore, we show that the action of NaxD is required for resistance to the cationic antimicrobial peptide polymyxin B, intracellular replication, and virulence *in vivo*. Importantly, we have shown that the role of this protein is conserved in human pathogenic *F. tularensis,* as well as *B. bronchiseptica*. Since NaxD is highly conserved in numerous Gram-negative pathogens, this work has broad implications for the elucidation of mechanisms of pathogenesis in other virulent bacteria.

Chapter 3 Results

NaxD is a member of the YdjC superfamily of proteins. Although *naxD* is annotated as encoding a hypothetical protein in the NCBI database, protein sequence analysis revealed that NaxD belongs to the YdjC superfamily. This family is highly conserved, with over 3,000 entries in the NCBI database. Homologs of NaxD are encoded by numerous pathogens including *Bordetella bronchiseptica*, *Brucella abortus*, *Legionella pneumophila*, and *Coxiella burnetii* (Fig. 2A). While a member of this family from *Bacillus stearothermophilus* had been putatively identified as a part of a cryptic cellobiose metabolism operon (Lai & Ingram, 1993), another member from *Thermus*

thermophilus, TTHB029, has been shown to have structural similarity to a deacetylase from *Streptococcus pneumoniae* (Imagawa *et al.*, 2008). Structural analysis revealed a putative active site containing three potential catalytic residues (Imagawa *et al.*, 2008). Importantly, these residues are conserved among YdjC superfamily proteins (Fig. 2B), suggesting that NaxD and other YdjC proteins may function as deacetylases.

NaxD is required for *F. novicida* replication in macrophages and virulence *in vivo*. We originally identified *naxD* as being required for virulence in an *in vivo* genome-wide negative selection screen (Weiss et al., 2007). In addition, we showed that this gene was required for intracellular proliferation in a macrophage replication screen (Llewellyn et al., 2011). Since both of these screens utilized transposon mutants, we wanted to ensure that the observed phenotypes resulted from disruption of *naxD* and not unintended secondary mutations. To do this, we generated an *F. novicida naxD* deletion mutant and a complemented strain. The *naxD* mutant exhibited wild-type growth kinetics in both rich and minimal media (Fig. S1). Macrophage replication experiments revealed that the *naxD* mutant was unable to replicate in either RAW264.7 macrophages or primary murine bone marrow-derived macrophages (BMM) (Fig. 3A, B). In fact, the level of attenuation of the *naxD* mutant was similar to that of a previously characterized strain lacking a functional copy of the gene encoding the virulence factor MglA, which is known to persist but not replicate in macrophages (Baron & Nano, 1998). In addition, the *naxD* complemented strain replicated to levels similar to wild-type. Given that *Francisella* must escape the phagosome in order to replicate, we used fluorescence microscopy to measure escape kinetics via colocalization of intracellular bacteria with the phagosomal marker LAMP-1

(Fig. S2). These experiments demonstrated that wild-type and *naxD* mutant *F. novicida* escaped the phagosomes of BMM with similar kinetics, indicating that the mutant's attenuation in macrophages is not due to a deficiency in phagosomal escape (Fig. S2). Overall, these results show that NaxD is required for intracellular proliferation but not for phagosomal escape.

While our *in vivo* negative selection screen identified *naxD* as being important for virulence, it did not provide quantitative data regarding the degree of attenuation of a *naxD* mutant. To determine this, we performed competition experiments. Briefly, mice were infected with a 1:1 ratio of wild-type *F. novicida* and either the *naxD* deletion mutant or complemented strain. Forty-eight hours post-infection, the *naxD* mutant displayed an approximate 2.5 log attenuation in the skin and a nearly 5 log attenuation in both the liver and spleen compared to wild-type (Fig. 3C). All mutant phenotypes were restored to wild-type levels in the complemented strain (Fig. 3C). To determine the consequence of the *naxD* mutant's virulence defect, we infected mice with either the wild-type or mutant strain and monitored survival. While mice infected with wild-type bacteria were moribund by 4 days after infection, the mutant did not kill mice up to 28 days post-infection (Fig. 3D). Taken together, these data show that *naxD* is required for both replication in host macrophages and virulence *in vivo*.

NaxD is involved in altering surface charge and resistance to polymyxin B. After validating the importance of NaxD in *F. novicida* infection of macrophages and mice, our next aim was to determine the role of this protein in pathogenesis. To further characterize

the phenotypes of the *naxD* mutant, we subjected both wild-type and the deletion mutant to different antimicrobials and compared the survival of each strain. While the wild-type was unaffected at the concentrations tested, the mutant displayed dose-dependent sensitivity to the cationic antimicrobial peptide polymyxin B (Fig. 4A), which acts on Gram-negative bacteria by binding to the negatively charged lipid A component of LPS (Morrison & Jacobs, 1976). Conversely, the mutant showed increased resistance to the anionic detergent SDS (Fig. 4B) and displayed wild-type levels of sensitivity to the nonionic detergent Triton X-100 (Fig. 4C). The altered response of the mutant to charged antimicrobials that act on the cell membrane suggested that NaxD might be involved in altering the net charge of the bacterial surface. To test this hypothesis, we measured the zeta electrokinetic potential of each strain, which gives an indirect reading of the bacterial surface charge. We determined that the mutant exhibited approximately a two-fold decrease in zeta potential compared to wild-type bacteria (Fig. 4D), indicating that NaxD is involved in increasing the charge of the bacterial surface. Taken together, the *naxD* mutant's decreased surface charge and increased sensitivity to cationic polymyxin B, which targets negatively charged lipid A, suggested that NaxD could be required for a modification to lipid A that alters its charge.

NaxD is required for lipid A modification with galactosamine. In order to determine if NaxD is involved in lipid A modification, we analyzed the lipid fractions of the wild-type and mutant strains using liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS). As mentioned previously, the majority of *Francisella* lipid A exists as free lipid A (Vinogradov *et al.*, 2002, Wang et al., 2006). ESI/MS analysis via

direct infusion of wild-type *F. novicida* free lipid A revealed an anticipated peak at *m*/*z* 1665.22 (Fig. 5A) (Phillips *et al.*, 2004, Wang et al., 2006), while the *naxD* mutant lipid A exhibited a peak at *m/z* 1504.15 (Fig. 5B). Interestingly, this shift corresponds to the molecular weight of galactosamine, and wild-type *Francisella* free lipid A is modified with a galactosamine at the 1 position phosphate (Phillips et al., 2004, Wang et al., 2006, Schilling *et al.*, 2007, Shaffer *et al.*, 2007, Kanistanon *et al.*, 2008, Kalhorn *et al.*, 2009, Song *et al.*, 2009, Wang *et al.*, 2009, Soni *et al.*, 2010, Beasley *et al.*, 2012). The absence of the galactosamine moiety on the free lipid A of the mutant would result in an exposed, negatively charged phosphate group, which correlates with the decreased surface charge of the mutant strain (Fig. 4D) (Phillips et al., 2004, Wang et al., 2006).

To determine why the *naxD* deletion mutant lacks galactosamine and where NaxD might act in the lipid A biosynthetic pathway, we measured the presence and quantities of precursor molecules required for the galactosamine modification. Galactosamine is added to *Francisella* free lipid A from undecaprenyl phosphate-galactosamine (undecaprenyl phosphate-GalN) (Song et al., 2009), a complex of the sugar with a lipid carrier molecule. Analysis of the wild-type lipid fraction revealed a singly charged peak corresponding to undecaprenyl phosphate-GalN at *m/z* 1006.76 (Fig. 5C). However, this glycolipid was not present in the mutant strain (Fig. 5D). Instead, the mutant exhibited a peak at *m/z* 1048.79, corresponding to undecaprenyl phosphate-*N*-acetylgalactosamine (undecaprenyl phosphate-GalNAc), the acetylated precursor of undecaprenyl phosphate-GalN (Fig. 5D, E). Conversely, this acetylated precursor was not detected in the wild-type lipid fraction (Fig. 5C). Taken together, these data show that NaxD is required for deacetylation of

undecaprenyl phosphate-GalNAc and that the absence of this deacetylation event prevents the galactosamine modification to *F. novicida* free lipid A.

NaxD is required for deacetylation of undecaprenyl phosphate-GalNAc. After MS analysis revealed NaxD was required for deacetylation of undecaprenyl phosphate-GalNAc, we set out to determine whether NaxD was directly responsible for this reaction. First, using a strain in which NaxD was labeled with an 8x histidine tag, we found that NaxD localizes to the *F. novicida* membrane fraction (Fig. S4). To determine if NaxD could deacetylate undecaprenyl phosphate-GalNAc in the membrane, we harvested crude membrane fractions from either wild-type or mutant strains and incubated them with synthetic undecaprenyl phosphate-GalNAc. The lipids were extracted from each reaction and analyzed using LC-ESI/MS. For both wild-type and mutant, the substrate peak (undecaprenyl phosphate-GalNAc, expected *m/z* 1048.74) was present at time zero (Fig. 6). After a 5 hour incubation, deacetylation of undecaprenyl phosphate-GalNAc was observed in the wild-type reaction, since a peak consistent with undecaprenyl phosphate-GalN was detected (Fig. 6A). In contrast, no product peak was observed in the mutant reaction (Fig. 6B). These results showed that NaxD in the membrane fraction was necessary for undecaprenyl phosphate-GalNAc deacetylation.

To determine if NaxD was responsible for this enzymatic activity, we overexpressed *naxD* in *E. coli*, which does not encode a NaxD homolog, does not synthesize undecaprenyl phosphate-GalNAc or undecaprenyl phosphate-GalN, and does not modify its lipid A with galactosamine. We demonstrated that NaxD localized to the *E. coli*

membrane fraction (Fig. S5), isolated membranes from strains that were either transformed with an empty vector control or the *naxD* expression plasmid, and assayed for enzymatic activity as described for *F. novicida* above. LC-ESI/MS analysis revealed that there was no deacetylated product (expected *m/z* 1006.73) detected for the reactions using a whole cell lysate from the *E. coli* empty vector control strain (Fig. 7A) or with soluble fraction from *E. coli* expressing *naxD* (Fig. 7B). However, the deacetylated product, undecaprenyl phosphate-GalN, was detected in assays that contained the membrane fraction from *E. coli* expressing NaxD (Fig. 7C). Together, these results using membrane fractions from *F. novicida* and *E. coli* respectively demonstrate that NaxD is necessary and suggest that it is sufficient for deacetylation of undecaprenyl phosphate-GalNAc.

The NaxD ortholog in *F. tularensis* is required for lipid A modification and virulence. The NaxD ortholog from human pathogenic *F. tularensis*, FTT_0453, retains 99% amino acid identity with *F. novicida* NaxD (Fig. 2A). In order to ascertain if NaxD function is conserved in *F. tularensis*, we generated a *FTT_0453* (*naxD*) deletion mutant in strain SchuS4. LC-ESI/MS analysis of wild-type *F. tularensis* free lipid A revealed the *m/z* 1665.21 peak that corresponds to *Francisella* lipid A modified with galactosamine (Fig. 8A), although this species was not detected in the mutant. Instead, the *naxD* mutant displayed a peak at *m/z* 1504.13 that corresponds to lipid A without galactosamine (Fig. 8B). This demonstrates the conserved role of NaxD in lipid A modification in highly virulent *F. tularensis*.

Next we tested the functional role of *F. tularensis* NaxD in polymyxin B resistance, intracellular replication, and *in vivo* survival. The *naxD* deletion mutant displayed an increased susceptibility to polymyxin B as compared to wild-type (Fig. 8C). Given that *F. tularensis* is a virulent human pathogen, we wanted to determine the importance of NaxD function during infection of human cells. Indeed, we observed a severe defect in replication of the *naxD* deletion mutant compared to wild-type *F. tularensis* 24 hours after infection of human THP-1 macrophage-like cells (Fig. 8D). In addition, similar to the *F. novicida naxD* mutant (Fig. 3B), the *F. tularensis naxD* deletion mutant was unable to proliferate in primary murine BMM (Fig. S3). Importantly, NaxD was also required for replication in mice, since 48 hours after subcutaneous infection, wild-type *F. tularensis* was recovered at a level 2.5 logs higher in the spleen of mice (Fig. 8E) and nearly 1.5 logs higher in the liver than the mutant strain (Fig. 8F). These data show that NaxD function is conserved in human pathogenic *F. tularensis*, in which it is required for the addition of galactosamine to free lipid A and is required for resistance to the cationic antimicrobial peptide polymyxin B, replication within human cells, and virulence in mice.

Conserved role of the NaxD homolog in *Bordetella bronchiseptica*. Given that the YdjC superfamily of proteins is conserved among many virulent bacteria, we wanted to determine if a NaxD homolog from a different pathogen shared a similar function in lipid A modification. To test this, we generated a *Bordetella bronchiseptica* deletion mutant lacking the gene encoding the NaxD homolog BB4267 (Fig. 2). *B. bronchiseptica* is a Gram-negative bacterium that colonizes mammalian respiratory tracts and is considered a

primary pathogen of domestic animals such as dogs, cats, rabbits, and pigs, but can also establish chronic infections in immunocompromised humans (Egberink *et al.*, 2009). Lipid A from this pathogen has two phosphate groups that are known to be modified with glucosamine, a stereoisomer of galactosamine (Tirsoaga *et al.*, 2007, Marr *et al.*, 2008, Basheer *et al.*, 2011). Though BB4267 is nearly 100 amino acids larger than NaxD, the majority of the protein is comprised of the YdjC superfamily domain, including the conserved putative active site residues, which suggests conserved function (Fig. 2B).

Indeed, LC-ESI/MS analysis of the wild-type lipid fractions revealed a doubly charged lipid A peak at *m/z* 1072.70 that corresponds to lipid A with glucosamine modifications at both the 1- and 4'-position phosphates (Fig. 9A). This peak that was absent in the mutant fractions, which instead displayed a doubly charged peak at *m/z* 911.64, corresponding to the lipid A molecule missing both glucosamine modifications (Fig. 9B). This data shows that the NaxD homolog BB4267, like NaxD in *Francisella* is required for the modification of lipid A phosphates with hexosamine sugars.

Similar to the addition of galactosamine to *Francisella* lipid A, the modification of *B. bronchiseptica* lipid A with glucosamine requires the deacetylation of undecaprenyl phosphate *N*-acetylglucosamine (undecaprenyl phosphate-GlcNAc) to form undecaprenyl phosphate-glucosamine (undecaprenyl phosphate-GlcN). LC-ESI/MS from the lipid fraction of the wild-type strain showed a singly charged peak at *m/z* 1006.85, corresponding to undecaprenyl phosphate-GlcN (Fig. 9C). In contrast, the *bb4267* mutant displayed a peak at *m/z* 1048.88, corresponding to undecaprenyl phosphate-GlcNAc (Fig.

9D), which was undetectable in the wild-type. This demonstrates that, similar to NaxD function in *Francisella*, BB4267 is required for a deacetylation reaction in *B. bronchiseptica*. To test the functional relevance of the lipid A modification with glucosamine, we measured the polymyxin B sensitivity of the wild-type and mutant strains and found that the *bb4267* mutant exhibited a dose-dependent increase in susceptibility to polymyxin B as compared to wild-type (Fig. 9E). These data confirm that the function of NaxD is conserved among multiple Gram-negative pathogens.

Chapter 3 Discussion

Using *in vivo* negative selection (Weiss et al., 2007) and intramacrophage replication (Llewellyn et al., 2011) screens, we have recently identified NaxD as an important virulence factor of *Francisella*. Here we have extended those findings by showing that this member of the YdjC protein superfamily is a deacetylase that is required for lipid A modifications that render bacteria more resistant to killing by the cationic antimicrobial peptide polymyxin B. Given our findings that the *B. bronchiseptica* NaxD homolog is also required for lipid A modification, this report suggests that NaxD/YdjC proteins are likely to have an important role in the pathogenesis of other virulent Gram-negative bacteria.

This work contributes to a greater understanding of the mechanisms by which *Francisella* is able to so effectively evade killing by antimicrobial peptides compared to other Gram-negative pathogens (Ishimoto et al., 2006, Mohapatra et al., 2007). For example, *Francisella* is nearly 1000x more resistant to polymyxin B than *E. coli* (Mohapatra et al.,

2007). Because polymyxin B is known to bind Gram-negative lipid A, it is interesting that the majority of the exposed surface of the *Francisella* outer membrane consists of free lipid A (Zhao & Raetz, 2010). To our knowledge, *Francisella* is the only Gram-negative pathogen shown to exhibit this sort of unique outer membrane composition. Mutants that lack the galactosamine modification on free lipid A have an exposed phosphate at the 1-position (Phillips et al., 2004, Bina *et al.*, 2006, Schilling et al., 2007, Shaffer et al., 2007, Kanistanon et al., 2008, Kalhorn et al., 2009, Song et al., 2009, Wang et al., 2009, Soni et al., 2010, Beasley et al., 2012), significantly altering the charge and likely the topography of the majority of the outer leaflet of the outer membrane. Interestingly, the small percentage of lipid A that is part of complete LPS lacks the 1-position phosphate and, therefore, the galactosamine modification as well (Zhao & Raetz, 2010). It is not clear why *Francisella* produces such a large amount of lipid A without O-antigen, given that O-antigen is critical for virulence (Sandstrom *et al.*, 1988, Sorokin *et al.*, 1996, Clay *et al.*, 2008). However, since the majority of *Francisella*'s outer membrane is composed of free lipid A, it is intuitive that the galactosamine modification to this moiety would be critical in resistance to host stresses, similar to the importance of modifications to complete LPS in other bacteria (Wang & Quinn, 2010). Indeed, given that this modification is important for resistance to polymyxin B, replication in macrophages, and during *in vivo* infection, it likely contributes to *Francisella* resistance to host cationic antimicrobial peptides such as cathelicidins, defensins, and ubiquicidin (Weiss et al., 2007, Kanistanon et al., 2008, Llewellyn et al., 2011). Future work will aim to elucidate whether there are advantages conferred by the novel cell surface component

free lipid A, e.g., whether free lipid A promotes enhanced resistance to host antimicrobials compared to full LPS.

The Gram-negative pathogen *B. bronchiseptica* has been shown to express lipid A species that have one or both phosphates modified with glucosamine, a stereoisomer of galactosamine (Tirsoaga et al., 2007, Marr et al., 2008, Basheer et al., 2011). Like galactosamine, addition of glucosamine neutralizes the negative charge of lipid A phosphates (Marr et al., 2008). In this study we show that the *B. bronchiseptica* NaxD homolog BB4267 is required for the glucosamine modification of lipid A phosphates and specifically is necessary for the deacetylation of undecaprenyl phosphate-*N*-acetylglucosamine. Similar to the *Francisella* galactosamine modification, we show that this glucosamine modification is important for resistance to the lipid A-binding cationic antimicrobial peptide polymyxin B. While no bacteria other than *Francisella* species have been reported to utilize free lipid A, our *B. bronchiseptica* data indicate that NaxD homologs could be involved in modifying the lipid A component of complete LPS of other Gram-negative pathogens.

This study has generated new insight into *Francisella* pathogenesis and lipid A biosynthesis as well as the function of YdjC superfamily proteins. Significantly, this study has broad implications for host-pathogen interactions of other highly virulent NaxD homolog-encoding Gram-negative bacteria, particularly intracellular pathogens such as *B. abortus*, *L. pneumophila*, and *C. burnetii*. Future studies on the role of this family of

proteins will likely further illuminate the virulence mechanisms of other NaxD homolog-encoding pathogenic bacteria.

Chapter 3 Materials and Methods

Bacterial strains and growth conditions. Wild-type *F. novicida* strain U112 and a previously described *mglA* point mutant, GB2 (Baron & Nano, 1998), were a generous gift from Dr. Denise Monack (Stanford University, Stanford, CA). These strains, the *naxD* deletion mutant, and the *naxD* complemented strain were grown at 37°C on a rolling drum in tryptic soy broth (TSB; Difco/BD, Sparks, MD) supplemented with 0.02% L-cysteine (Sigma-Aldrich, St. Louis, MO). *F. novicida* was plated for colony forming units on tryptic soy agar (TSA; Difco/BD) and supplemented with 0.01% L-cysteine, with the exception of bacteria from mouse experiments, which were plated on modified Mueller Hinton (mMH) agar plates (Difco/BD) supplemented with 0.025% ferric pyrophosphate (Sigma-Aldrich), 0.1% glucose (Sigma-Aldrich), and 0.01% L-cysteine. When appropriate, kanamycin (Fisher Scientific, Fair Lawn, NJ) was added to media at a concentration of 30 μg ml^{-1}. *F. tularensis* strains were grown in mMH broth or on Brain Heart Infusion (BHI) agar (BHI supplemented with 50 μg ml^{-1} hemin, 1.4% agar (w/v), and 1% (v/v) IsoVitalex (BBL, Cockeysville, MD). Counter selection for resolution of *F. tularensis FTT0453* deletion plasmid co-integrants was performed on cysteine heart agar containing 5% sucrose. Kanamycin was added to the plates when necessary at 10 μg ml^{-1} for *F. tularensis*. *B. bronchiseptica* wild-type strain RB50, the *bb4267* deletion mutant, and the *bb4267* complemented strain were grown under similar conditions as *F. novicida*, except using Stainer-Scholte broth supplemented with nicotinic

acid, glutathione, and ascorbic acid as previously described (Hulbert & Cotter, 2009), or Bordet-Gengou blood agar plates (Remel, Lenexa, KS). When appropriate, streptomycin (Fisher Scientific) and kanamycin were added at concentrations of 25 μg ml^{-1} and 50 μg ml^{-1}, respectively.

Mutagenesis and complementation. To generate the *naxD* deletion mutant in *F. novicida*, PCR was used to amplify flanking DNA regions upstream and downstream of the gene of interest. A kanamycin resistance cassette was sewn in between these flanking regions using overlapping PCR reactions. The final linear PCR product was then gel purified and transformed into chemically competent wild-type strain U112 as previously described (Anthony *et al.*, 1991). The primers used to create the kanamycin-resistant deletion mutant contained FRT sites flanking the kanamycin resistance cassette, which allowed a clean deletion of each mutant to be made using the plasmid pFFlp encoding the Flp-recombinase as previously described (Gallagher *et al.*, 2008). A construct for the complementation of the mutant was generated by overlapping PCR using PCR-amplified fragments of the wild-type gene of interest, upstream and downstream flanking regions, and a kanamycin resistance cassette. These constructs were then transformed into the chemically competent *naxD* clean deletion mutant. Verification of allelic replacement in the mutant and complemented strains was performed using check primers in PCR reactions on purified genomic DNA from each strain. PCR products of the correct size were subsequently sequenced (MWG Operon, Huntsville, AL) for final verification of allelic replacement. PCR constructs for *F. tularensis* mutagenesis were amplified as for *F. novicida* and then cloned into plasmid pXB186 containing a kanamycin resistance cassette and the *sacB* counter-selectable marker (making plasmid pΔFTT0453). To

generate the *FTT0453* deletion mutant, the deletion plasmid was introduced by electroporation into electrocompetent *F. tularensis* SchuS4. Electrocompetent cells were prepared on the day of the transformation as described in the Supplemental Experimental Procedures. The resulting clones were screened by PCR for the *FTT0453* deletion. To generate the *B. bronchiseptica bb4267* deletion mutant, linear PCR deletion constructs were amplified as described above for *F. novicida*. This *bb4267*-deleting fragment was cloned into the *Bordetella* allelic exchange plasmid pSS4245. The resulting plasmid was then transformed into wild-type *B. bronchiseptica* strain RB50, following procedures described previously (Inatsuka *et al.*, 2010). The loss of *bb4267* was confirmed by PCR and subsequent enzymatic digestions. To generate the complementation plasmid, the two external primers used to produce the *bb4267*-deleting fragment were employed and this complementing fragment was cloned into pUC18-Mini-TN7 plasmid that has a Tn7 integration sequence, which, along with a helper plasmid pTNS3, was mated into the deletion strain via tri-parental mating (Choi *et al.*, 2005) to generate the complemented strain. All primers and plasmids used in this study are listed in Table S1.

Antimicrobial assays. The antimicrobial peptide polymyxin B (USB, Cleveland, OH) was dissolved in peptide buffer (0.01% acetic acid, 0.2% BSA) and then serially diluted in the same buffer to desired concentrations. The detergents sodium-dodecyl-sulfate (SDS; Fisher Scientific) and Triton X-100 (Fisher Scientific) were serially diluted in 25% TSB. Overnight cultures of bacteria were diluted to 1 x 10^7 colony forming units (CFU) ml^{-1} in 25% TSB. Ninety microliters of diluted cultures were then added to 96 well plates containing 10 µl of the appropriate antimicrobial. Plates were incubated at 37°C on shaking platforms for 6 hours. Cultures were then serially diluted and plated to enumerate

CFU. *F. tularensis* antimicrobial susceptibility was determined by the gradient agar plate method as previously described (Szybalski & Bryson, 1952, Bina *et al.*, 2006, Bina *et al.*, 2008). Briefly, 35 ml of BHI-chocolate agar (without polymyxin B) was poured into a square Petri dish and allowed to solidify as a wedge by elevating one side of the plate. After the agar solidified, 35 ml of BHI-chocolate agar containing polymyxin B at 2 mg ml^{-1} was added to the leveled plate and allowed to solidify. Theses plates were inoculated with overnight mMH broth cultures of each respective strain and incubated at 37°C for two days when the length of growth along the polymyxin B gradient was recorded. The gradient agar plate tests were performed a minimum of three times and representative results are presented.

Zeta electrokinetic potential. Overnight cultures of bacteria were subcultured and grown to OD_{600} = 1.0. The bacteria were then pelleted (10,000 x *g*, 3 minutes) and resuspended at a 5X concentration in 20 mM potassium chloride. Twenty microliters of the concentrated bacteria were added to 3.2 ml of 20 mM potassium chloride in the zeta potential electrokinetic cuvette from Brookhaven Instruments Corporation (BIC, Holtsville, NY). The bacterial sizes and zeta electrokinetic potentials were measured using the 90Plus size and zeta potential analyzer (BIC). Data was analyzed using BIC Zeta Potential Analyzer Software Version 5.20, which takes into account the size of the bacteria when calculating the zeta potential.

Macrophages. RAW264.7 murine macrophages (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle medium (high glucose, L-glutamine; DMEM; Lonza, Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT). Bone marrow-derived macrophages (BMM) were isolated from

wild-type C57BL/6 mice and cultured as described previously (Schaible, 2002) in DMEM supplemented with 10% heat-inactivated FCS and 10% macrophage colony-stimulating factor (M-CSF)-conditioned medium (collected from M-CSF-producing L929 cells). THP-1 monocyte-like cells (ATCC) were cultured in RPMI (Lonza) with 10% heat-inactivated fetal calf serum (HyClone). Macrophages were incubated before and during infection at 37°C with 5% CO_2.

Macrophage infections. RAW264.7 macrophages were seeded in 24-well plates at 5 x 10^5 cells/well and incubated overnight. The following day, overnight cultures of the indicated strains were pelleted (10,000 x *g*, 3 minutes) and resuspended in DMEM/10% FCS. After removal of the overnight media, the macrophages were infected with bacteria at an MOI of 20:1 (bacteria to macrophage), centrifuged for 15 minutes at 900 x *g*, and then incubated for 30 minutes. Next, the macrophages were washed twice with warm DMEM and then incubated in DMEM/10% FCS with 10 μg ml^{-1} gentamicin. At 30 minutes and 24 hours post-infection, the macrophages were washed twice and then lysed with 1% saponin in phosphate buffered saline (PBS). Macrophage lysates were serially diluted and plated on mMH agar, the resulting CFU were enumerated, and the fold replication of each strain was determined. The same protocol as above was followed for the BMM infections with the following alterations: 3 x 10^5 BMM were plated per well, DMEM/10% FCS/10% M-CSF was used throughout, and the final time point was 6 hours instead of 24 hours. The difference in time point was due to the fact that *F. novicida* triggers inflammatory mediated cell death in BMM (Mariathasan *et al.*, 2005). Therefore, bacterial replication was measured at 6 hrs post-infection to minimize loss of bacterial counts as a consequence of the host cell death response. It is likely that we did

not observe this early cell death in RAW264.7 macrophages because this cell line is known to be deficient in ASC/caspase-1 inflammasome-mediated cell death (Pelegrin *et al.*, 2008). For *F. tularensis* experiments, THP-1 cells or BMM were seeded into 24-well tissue culture plates (3×10^5 cells/well) in a total volume of 1 ml of culture medium. THP-1 cells were treated with 200nM phorbol 12-myristate 13-acetate **(**PMA) immediately after cells were plated. The cells were infected 24 hours later with the indicated strains at an MOI of 50:1 bacteria to macrophage. Fifty micrograms per milliliter of gentamicin was added 2 hours later to kill any remaining extracellular bacteria. At 2 or 24 hours after infection, wells were washed twice with PBS, lysed, and then bacteria were enumerated by dilution plating in duplicate using an IUL Eddy Jet Spiral plater and a Flash and Go automated colony counter (Neutec Group, Farmingdale, NY).

Mice. For *F. novicida* experiments, female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) between 7 and 10 weeks of age were kept under specific pathogen-free conditions in filter-top cages at Emory University and provided with sterile food and water *ad libitum*. Experimental studies were performed in accordance with the Emory University Institutional Animal Care and Use Committee (IACUC) guidelines. For *F. tularensis* experiments, C57BL/6 mice were purchased from Charles River Laboratories. Mice were age-matched and used between 7 and 10 weeks of age. Mice were housed in sealed Allentown caging and HEPA-filtered cage racks with food and water *ad libitum*. All experimental protocols were reviewed and approved by the University of Tennessee Health Science Center IACUC.

Mouse experiments. For competition experiments, mice were inoculated subcutaneously with a 1:1 ratio of kanamycin-resistant deletion mutant and kanamycin-sensitive wild-type *F. novicida* for a total of 2 x 10^5 CFU in 50 µl sterile PBS. After 48 hours, the mice were sacrificed and the spleen, liver, and skin at the site of infection were harvested, homogenized, plated for CFU on MH plates with and without kanamycin, and then incubated overnight at 37°C. Competitive index (CI) values were determined using the formula: (CFU mutant output/CFU WT output)/(CFU mutant input/CFU WT input). For survival experiments, mice were infected subcutaneously with 2 x 10^5 CFU of either the deletion mutant or wild-type strain in 50 µl sterile PBS and then monitored for signs of illness and sacrificed if they appeared moribund.

For *F. tularensis* infection experiments, mice were challenged subcutaneously with 50 CFU in 100 µl sterile PBS. All procedures were performed under BSL3 containment according to standard operating procedures that have been fully vetted by the UTHSC Committee On Biocontainment and Restricted Entities (COBRE). Spleens, livers and lungs of challenged mice were homogenized with a disposable tissue homogenizer in 1 ml of sterile PBS and then 0.25 ml disruption buffer (2.5% saponin, 15% BSA, in PBS) was added with light vortexing. Appropriate dilutions of each sample were then plated in duplicate using an Eddy Jet spiral plater on mMH agar plates supplemented with 5% calf serum and incubated at 37°C for 48-72 hours. Colonies were counted using a Flash & Go automated colony counter.

Preparation of total lipids. Overnight cultures of *F. novicida* U112 wild-type or *naxD* mutant strains were subcultured to OD_{600} = 0.02 and grown at 37 °C in TSB supplemented with L-cysteine until the OD_{600} = 1.0. The cells were collected by

centrifugation (5,000 x *g*, 20 minutes) and washed with PBS. The cell pellets were resuspended in a single-phase Bligh-Dyer mixture (Bligh & Dyer, 1959) consisting of chloroform, methanol, and water (1:2:0.8, v/v), incubated at room temperature for 60 minutes, and centrifuged (10,000 x *g*, 20 minutes) to remove insoluble debris. The supernatant was converted to a two-phase Bligh-Dyer system by adding chloroform and water to generate a mixture consisting of chloroform, methanol, and water (2:2:1.8, v/v). The two phases of Bligh-Dyer system were separated under centrifugation and the lower phase was dried by rotary evaporation and under a stream of nitrogen. The total lipids were analyzed using thin layer chromatography (TLC) and liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS). The TLC plate was developed using the solvent chloroform, methanol, pyridine, acetic acid, and water (25:10:5:4:3, v/v). Lipids were detected by spraying 10% of sulfuric acid in ethanol and charring at 300°C.

Negative ion mode electrospray ionization (ESI) mass spectrometry (MS) and MS/MS analysis. All ESI/MS and MS/MS spectra were acquired on a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems, Foster City, CA) equipped with an ESI source. Lipid A samples were dissolved in chloroform and methanol (2:1, v/v) containing 1% piperidine and subjected to ESI/MS in the negative ion mode via direct infusion (Garrett & Yost, 2006, Guan *et al.*, 2007, Wang *et al.*, 2009). Nitrogen was used as the collision gas for MS/MS experiments (Garrett & Yost, 2006, Guan *et al.*, 2007, Wang *et al.*, 2009). Data acquisition and analysis were performed using the instrument's Analyst QS software.

Liquid Chromatography/Mass Spectrometry (LC/MS). LC/MS of lipids was performed using a Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps, and an SCL-10A system controller) coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (as above). LC was performed at a flow rate of 200 µl $minute^{-1}$ with a linear gradient as follows: 100% mobile phase A was held isocratically for 2 minutes and then linearly increased to 100% mobile phase B over 14 minutes and held at 100% B for 4 minutes. Mobile phase A consisted of methanol/acetonitrile/aqueous 1 mM ammonium acetate (60:20:20, v/v/v). Mobile phase B consisted of 100% ethanol containing 1 mM ammonium acetate. A Zorbax SB-C8 reversed-phase column (5 m, 2.1 x 50 mm) was obtained from Agilent (Palo Alto, CA). The postcolumn splitter diverted ~10% of the LC flow to the ESI source of the mass spectrometer.

Membrane fractionation. Fifty milliliters of *F. novicida* strains were harvested at OD_{600} = 1.0 by centrifugation for 20 minutes at 5,000 x *g* at 4°C. Cell pellets were washed with 50 mM K+HEPES, pH 7.5, resuspended in 5 ml of the same buffer, and passed through a French pressure cell at 18,000 p.s.i. Unbroken cells were then removed by centrifugation at 10,000 x *g* for 20 minutes at 4°C. Membrane fractions were pelleted from whole cell lysates by ultracentrifugation at 200,000 x *g* for 2 hours at 4°C. *F. novicida* fractionation and protein localization were verified using western blotting (see Supplemental Experimental Procedures). For *E. coli*, fractions were prepared similarly with the following exceptions: *E. coli* C41 (DE3) strains transformed with the empty vector or vector encoding *naxD* were grown in LB broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) with 100 µg ml^{-1} of ampicillin and were induced using 1 mM IPTG when cell

density reached OD_{600} = 0.8, then harvested when the OD_{600} = 2.0. NaxD protein expression was analyzed using 12% SDS-PAGE gel and Coomassie staining.

Undecaprenyl phosphate-GalNAc deacetylase assay. These assays measured the deacetylase activity of proteins from the *F. novicida* wild-type or *naxD* mutant membrane fractions, whole cell lysate of *E. coli* transformed with the empty vector, and the membrane and cytosolic fractions of *E. coli* transformed with vector encoding *naxD* (grown under inducing conditions). The 100 µl reaction mixture included 50 µg ml^{-1} protein from the bacterial fractions, 4.0 µM synthesized undecaprenyl phosphate-GalNAc (Song et al., 2009), 1 mM $MnCl_2$, 150 mM KCl, 1.0 mg ml^{-1} BSA, 0.1% Triton X-100, and 50 mM HEPES (pH 7.5) and was incubated at 30°C. A 20 µl sample was removed at 0 and 5 hours for *F. novicida* and 0 and 1 hour for *E. coli*. Samples were converted to a two-phase Bligh-Dyer system by the addition of chloroform and methanol. After centrifugation, the lower phase was dried under nitrogen and analyzed using LC-ESI/MS.

Statistical analysis. All macrophage replication, single infection, killing assay, and zeta potential data were analyzed for significance using the unpaired Student's *t* test. For zeta potential, values beyond three standard deviations of the mean were excluded as outliers. The CI values from the mouse competition experiments were analyzed with the one-sample Student's *t* test and compared to 1. The mouse survival infection data were analyzed for significance using the Gehan-Breslow-Wilcoxon Test.

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Chapter 3 Figures

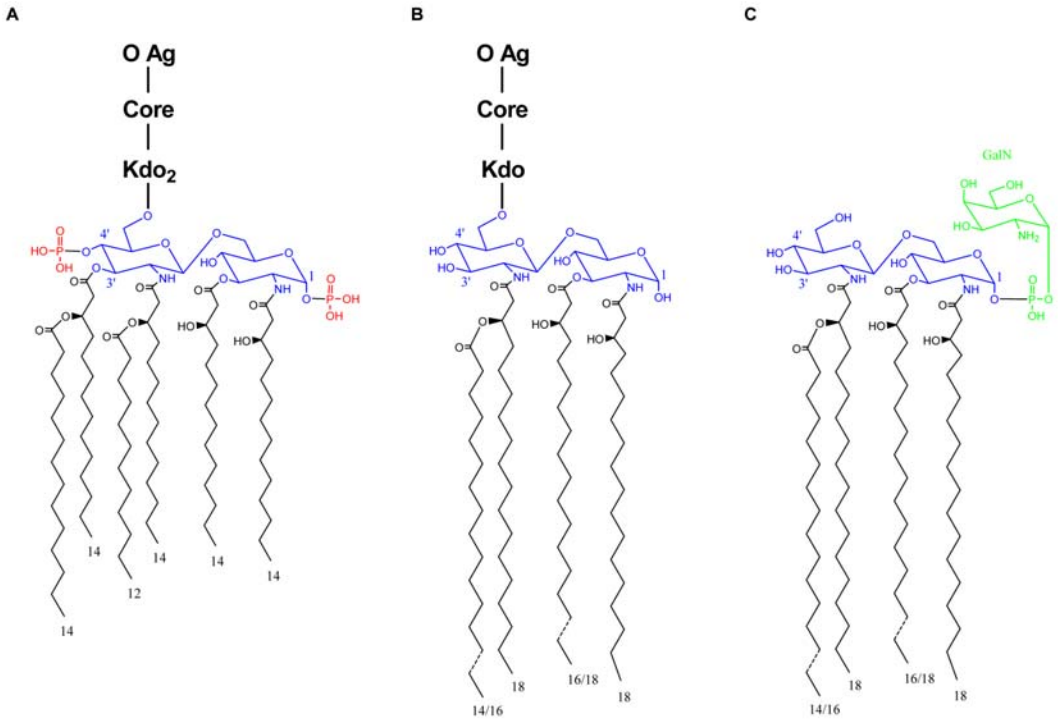

Figure 1. *E. coli* and *Francisella* LPS and lipid A structures. Structures of (A) *E. coli* LPS, (B) complete LPS from *Francisella* species, and (C) "free" lipid A of *Francisella* species are compared. (A, B) O-antigen (O Ag), core sugars (Core), and the specific core sugar Kdo (Kdo) are indicated. For all structures, lipid A backbone disaccharides are highlighted in blue and acyl chains are represented in black with numbers denoting length. *E. coli* lipid A 4'and 1 position phosphate groups (missing from the lipid A of complete *Francisella* LPS) are highlighted in red. Unlike the lipid A component of complete *Francisella* LPS, *Francisella* free lipid A includes a phosphate modified with galactosamine at the 1 position (highlighted in green).

A

NaxD and select YdjC Superfamily members

	Length	% Identity	% Similarity
F. novicida	**268**	-	-
F. tularensis	**268**	**98.5**	**98.9**
T. thermophilus	264	11.8	23.9
B. bronchiseptica	**367**	**18.3**	**30.5**
L. pneumophila	270	33.0	49.1
B. abortus	262	21.7	36.5
C. burnetii	273	34.4	47.9

B

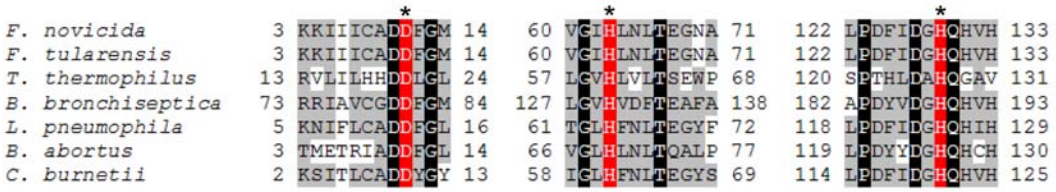

Figure 2. NaxD is a member of the YdjC superfamily. The amino acid sequences of *F. novicida* and *F. tularensis* NaxD (FTN_0544 and FTT_0453, respectively) were aligned with YdjC superfamily proteins from *Thermus thermophilus* (TTHB029), *Bordetella bronchiseptica* (BB4267), *Legionella pneumophila* (lp12_2472), *Brucella abortus* (BAbS19_II01260), and *Coxiella burnetii* (CBU_0580) using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). (A) The percent amino acid identity and similarity to *F. novicida* NaxD are shown. Proteins in bold and highlighted in red are described in this manuscript. (B) Amino acids surrounding putative active site residues are shown and numbers indicate their position in the sequence. Highlighting indicates conserved putative active site residues (red, asterisk), identical (black), and similar (grey) residues.

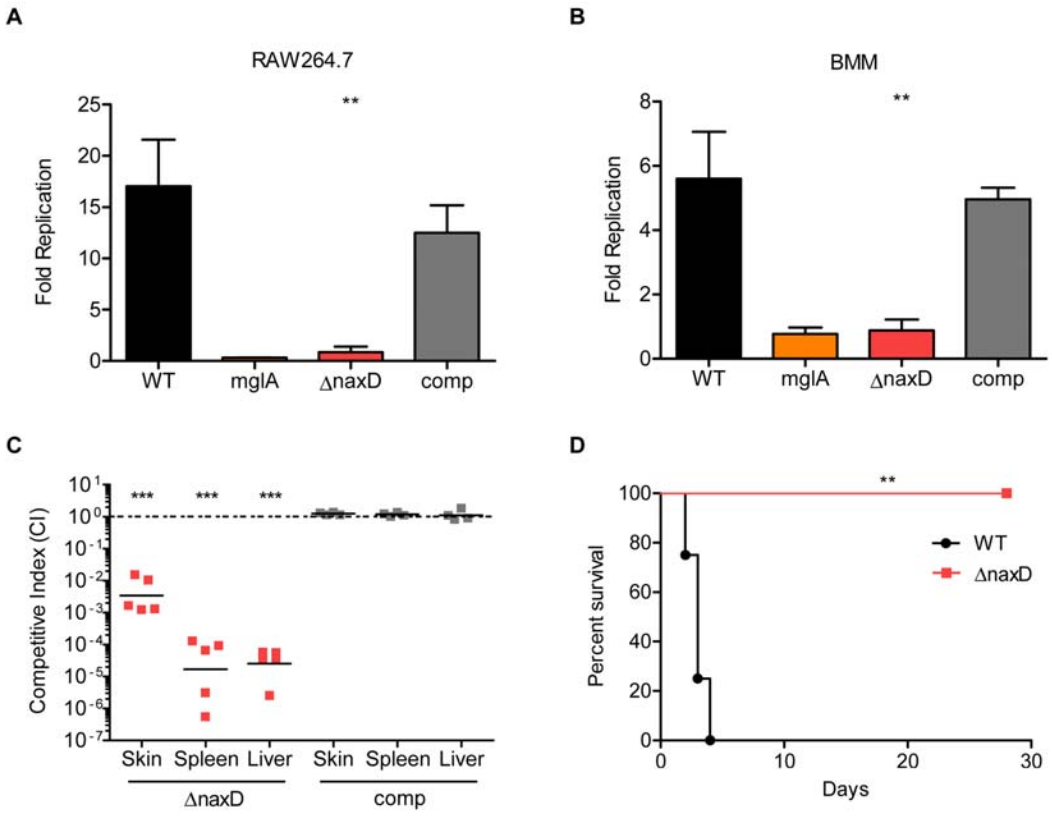

Figure 3. NaxD is required for replication in murine macrophages and mice. (A) RAW264.7 or (B) primary murine bone marrow-derived macrophages (BMM) were infected with a 20:1 MOI of wild-type *F. novicida* (WT), the *mglA* mutant (mglA), the *naxD* deletion strain (ΔnaxD), or the complemented strain (comp). CFU from lysates 30 minutes post-infection were compared to those from (A) 24 or (B) 6 hours post-infection to determine fold intracellular replication (n=3 biological replicates). (C) Mice were subcutaneously infected with a 1:1 mixture of 10^5 CFU each of wild-type and ΔnaxD (red) or wild-type and the complemented strain (grey). Forty-eight hours after infection, organs were harvested, CFU enumerated, and the competitive index (CI) calculated for the skin at the site of infection, spleen, and liver. CI = (CFU mutant output/CFU WT

output)/(CFU mutant input/CFU WT input). Bars represent the geometric mean CI values from each group of mice (n=5 mice). CI values below 1 (dashed line) indicate attenuation of the mutant strain. (D) Mice were subcutaneously infected with $2x10^7$ CFU of either wild-type or ΔnaxD and sacrificed if they appeared moribund (n=4 mice). In panels A and B, bars represent the average and error bars represent the standard deviation of three biological replicates from one experiment. Data shown in all panels are representative of at least three independent experiments. Asterisks indicate significance as compared to wild-type (A, B, D) or compared to 1 (C). (**) = $P < 0.005$, (***) = $P < 0.0005$.

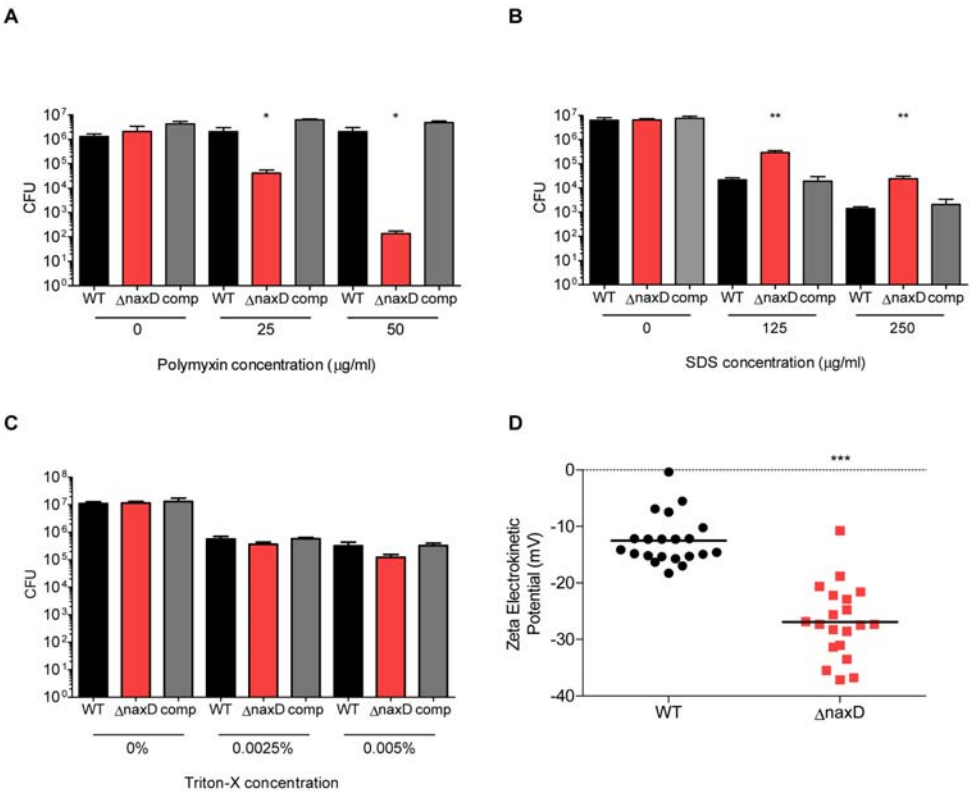

Figure 4. NaxD is involved in resistance to cationic antimicrobials and alteration of bacterial surface charge. Wild-type *F. novicida* (WT), the *naxD* deletion mutant (ΔnaxD) or the complemented strain (comp) were incubated with the indicated concentrations of (A) polymyxin B, (B) SDS, or (C) Triton X-100 for 6 hours. Cultures were then serially diluted and plated for CFU (n=3 biological replicates). (D) The zeta potential of wild-type and ΔnaxD was measured (n=10 technical replicates) and the results of three independent experiments were combined for statistical analysis. In panels A-C, bars represent the average and error bars represent the standard deviation of three biological replicates from one experiment. Data shown are representative of at least three independent experiments. Asterisks indicate significance as compared to wild-type. (*) = $P < 0.05$, (**) = $P < 0.005$, (***) = $P < 0.0005$.

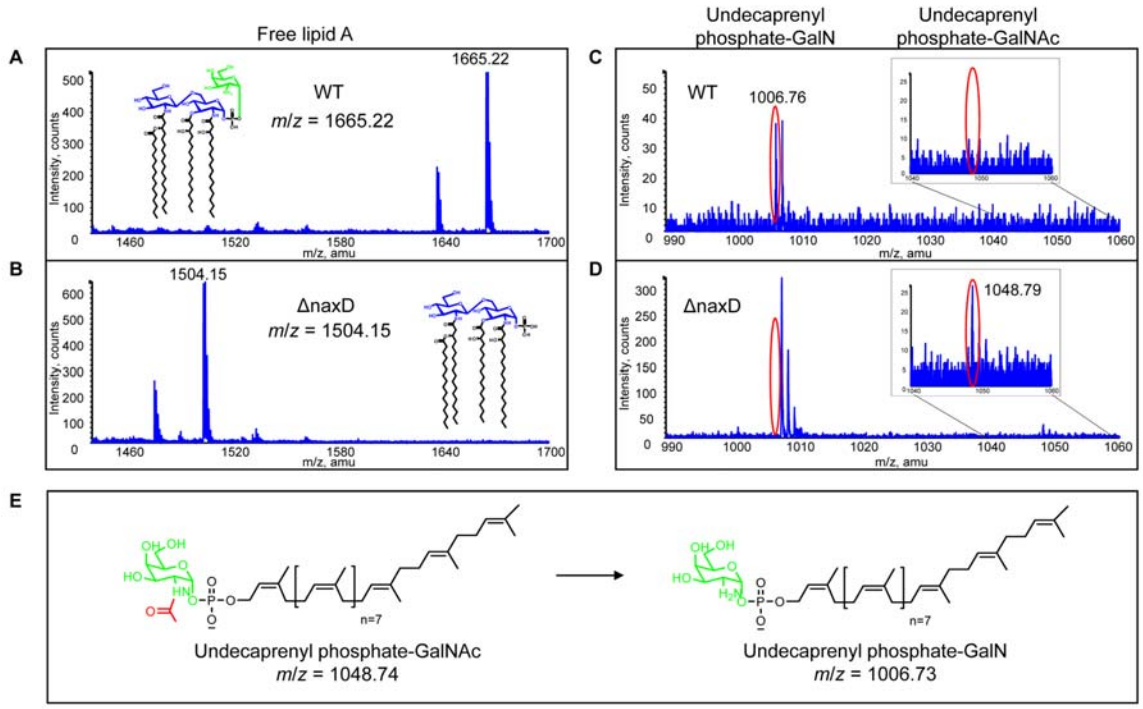

Figure 5. NaxD is required for the galactosamine modification of *F. novicida* free lipid A. Total lipids were extracted from (A, C) wild-type *F. novicida* (WT) and (B, D) *naxD* mutant (ΔnaxD) strains in mid-log phase and (A, B) free lipid A, (C, D) undecaprenyl phosphate-*N*-acetylgalactosamine (GalNAc), and undecaprenyl phosphate-galactosamine (GalN) were analyzed by ESI/MS in negative ion mode via direct infusion. (E) A schematic for the deacetylation of undecaprenyl phosphate-GalNAc (expected *m*/*z* = 1048.74) to undecaprenyl phosphate-GalN (expected *m*/*z* = 1006.73) is shown. (A, E) Galactosamine is highlighted in green and (E) the acetyl group is highlighted in red.

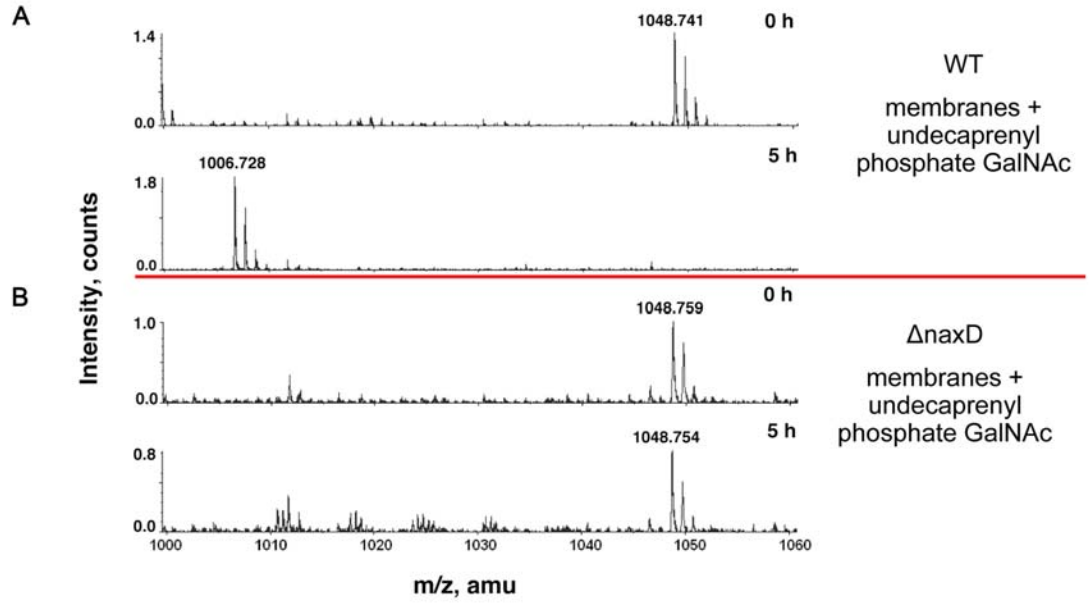

Figure 6. NaxD is necessary for deacetylation of undecaprenyl phosphate-*N*-acetylgalactosamine. Deacetylase activity assays using synthesized undecaprenyl phosphate-*N*-acetylgalactosamine and (A) 0.5 mg ml^{-1} *F. novicida* wild-type (WT) membrane fraction or (B) *F. novicida naxD* mutant (ΔnaxD) membrane fraction were incubated at 30°C for the indicated times and then analyzed using LC-ESI/MS in negative ion mode.

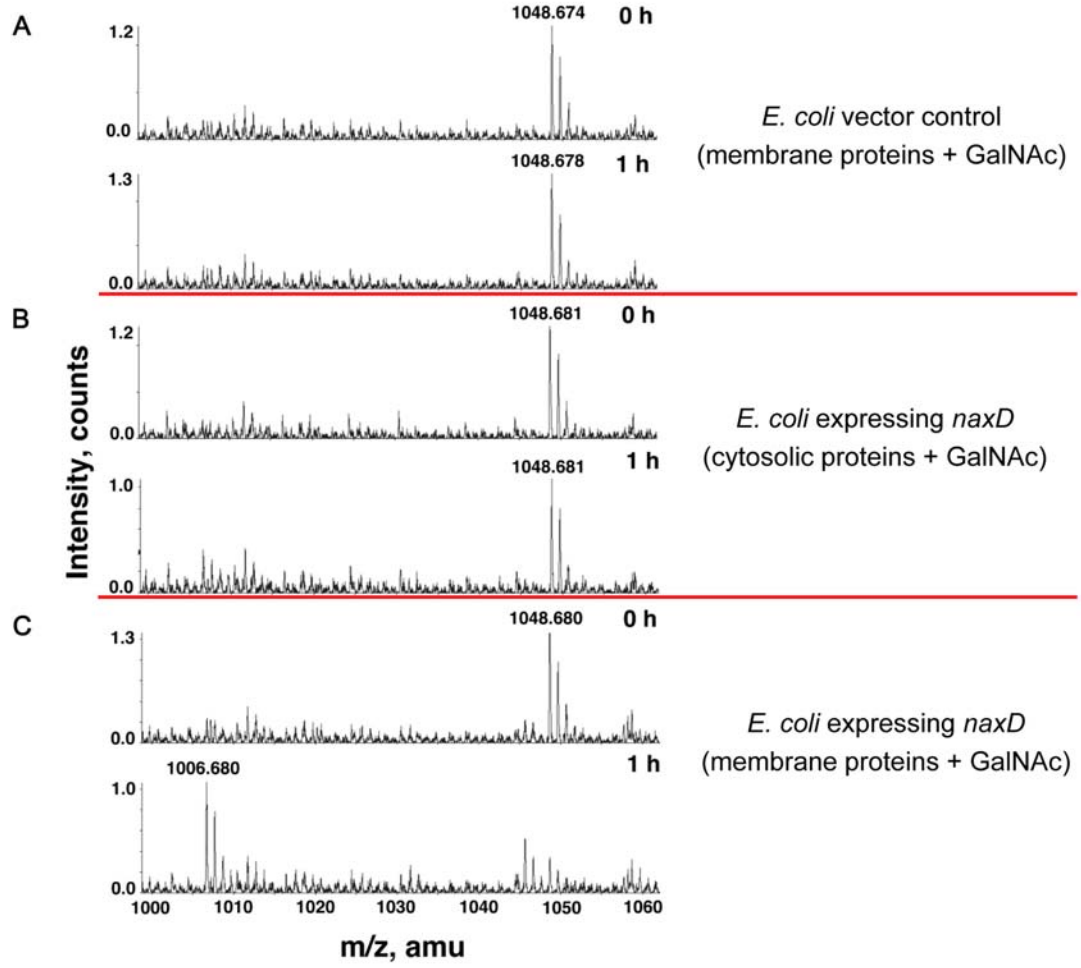

Figure 7. NaxD is required for deacetylation of undecaprenyl phosphate-*N*-acetylgalactosamine when exogenously expressed in *E. coli*. Deacetylase activity assays using synthesized undecaprenyl phosphate-*N*-acetylgalactosamine and (A) whole cell lysate from *E. coli* with an empty vector, (B) the soluble fraction from *E. coli* expressing *naxD*, or (C) the membrane fraction from *E. coli* expressing *naxD* were incubated at 30°C for the indicated times and then analyzed using LC-ESI/MS in negative ion mode.

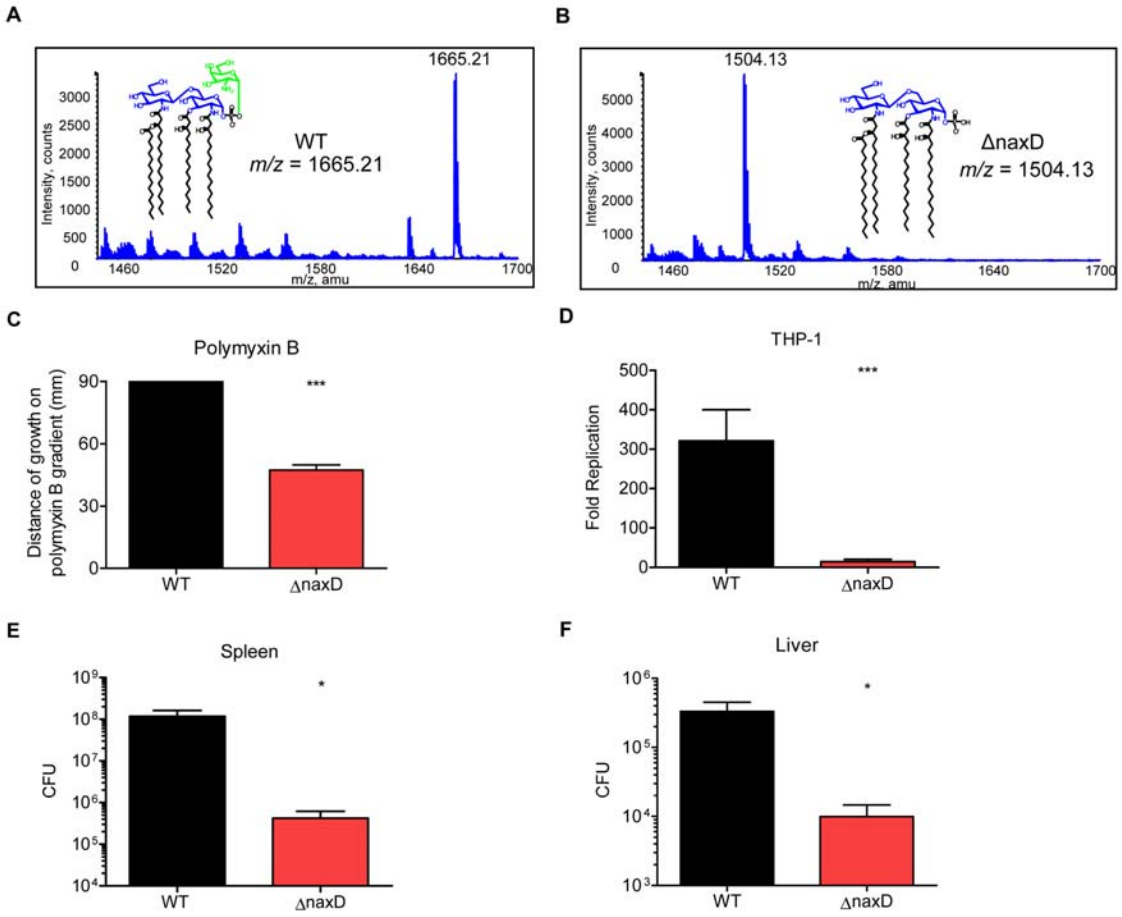

Figure 8. NaxD function is conserved in human pathogenic *F. tularensis*. Total lipids were extracted from (A) wild-type *F. tularensis* and (B) the ΔnaxD strain in mid-log phase and lipid A composition was analyzed by LC-ESI/MS. (A) Galactosamine is highlighted in green. (C) The distance of growth of wild-type or ΔnaxD along a 0-2 mg ml^{-1} gradient of polymyxin B was measured (n=3 biological replicates). (D) Human PMA-differentiated THP-1 macrophage-like cells were infected with either wild-type *F. tularensis* (WT) or the *naxD* deletion mutant (ΔnaxD) at a 50:1 MOI. CFU recovered from macrophages lysed 24 hours after infection were compared to CFU recovered at 2 hours post-infection to calculate fold replication (n=3 biological replicates). (E, F) Mice were subcutaneously infected with 50 CFU of either wild-type or ΔnaxD and 48 hours

after infection, organs were harvested and plated and CFU were enumerated for the (E) spleen and (F) liver (n=5 mice). In panels C and D, bars represent the average and error bars represent the standard deviation of three biological replicates from one experiment. Data shown in panels C-F are representative of at least three independent experiments. Asterisks indicate significance as compared to wild-type. (*) = $P < 0.05$, (***) = $P < 0.0005$.

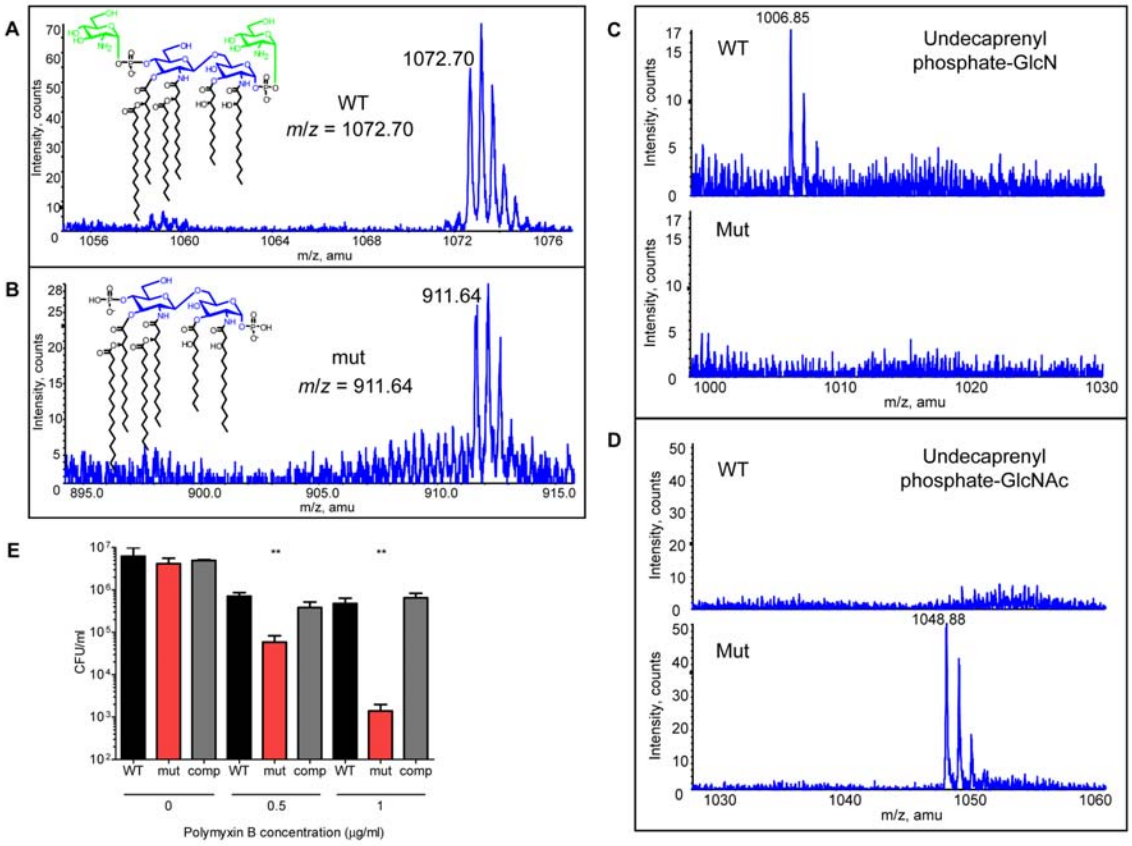

Figure 9. Conserved role of the *Bordetella bronchiseptica* NaxD homolog in lipid A modification. Total lipids were extracted from (A, C) wild-type (WT) and (B, D) *naxD* homolog mutant (mut) strains of *B. bronchiseptica* in mid-log phase and (A, B) lipid A, (C, D) undecaprenyl phosphate-*N*-acetylglucosamine (GlcNAc), and undecaprenyl phosphate-glucosamine (GlcN) analyzed by LC-ESI/MS. In panel A, glucosamine groups are highlighted in green. (E) WT, mut, or the complemented strain (comp) were incubated with the indicated concentrations of polymyxin B for 6 hours and CFU were enumerated (n=3 biological replicates). Bars represent the average and error bars represent the standard deviation of three biological replicates from one experiment. Data shown are representative of at least three independent experiments. Asterisks indicate significance as compared to wild-type. (**) = $P < 0.005$.

Chapter 3 Supporting Information Materials and Methods

***F. tularensis* transformation.** An overnight mMH broth culture of SchuS4 was used to inoculate 50 ml of mMH broth. The resulting culture was then incubated with shaking at 37°C until the culture reached OD_{600} = 0.3 before the culture was transferred to a 50 ml conical tube and the cells were collected by centrifugation at 4°C. The cell pellet was resuspended in 4 ml of 0.5 M sucrose that was pre-chilled to 4°C, equally distributed into two 2 ml screw-capped vials, before the cells were again collected by centrifugation at 4°C. The resulting cells were then washed three additional times with 2 ml of pre-chilled 0.5 M sucrose. Following the final wash, the cell pellet was resuspended to a total volume of 70 µl 0.5 M sucrose and placed on ice. One microgram of purified pΔFTT0453 was added to 70 µl of the electrocompetent SchuS4 cells and the mixture was transferred to a pre-chilled 0.1 cm electroporation cuvette (BTX, Holliston, MA). This mixture was then electroporated at 1.5 kV, 25 uF, and 200 ohms. Next, 1 ml of mMH broth was immediately added to the cuvette and then this cell suspension was transferred to a 50 ml conical tube. An additional 1 ml of mMH broth was added to the electroporated cell suspension and the cells were incubated with shaking at 37°C. After two hours incubation the cells were collected by centrifugation, resuspended in 500 µl of mMH broth and spread onto BHI agar plates containing 10 µg ml^{-1} kanamycin. Following three days incubation at 37°C, several kanamycin resistant colonies were then patched onto fresh BHI agar plates. Among the resulting clones, four colonies were selected and used to inoculate 1 ml of mMH broth without antibiotics. These broth cultures were incubated with shaking at 37°C until early log phase (OD_{600} = 0.4) and then aliquots of the culture were spread onto CHA-5% sucrose plates. When colonies appeared on the

sucrose plates (4-5 days later), 24 sucrose resistant colonies were then patched onto fresh CHA sucrose plates and the plates incubated at 37°C until colonies were visible. The resulting colonies were then replica-plated onto BHI agar plates with or without kanamycin. Kanamycin sensitive clones were subsequently assayed for the presence of the *naxD* deletion.

***F. novicida* growth curves.** *F. novicida* overnight cultures were grown at 37°C on a rolling drum in tryptic soy broth (TSB; Difco/BD, Sparks, MD) supplemented with 0.02% L-cysteine (Sigma-Aldrich, St. Louis, MO) and then subcultured in 96 well plates to OD_{600} = 0.03 in either TSB with 0.02% cysteine or Chamberlain's chemically defined minimal medium, prepared as previously described (Chamberlain, 1965). OD_{600} was measured hourly using a BioTek Synergy Mx (Winooski, VT) microplate reader.

Macrophage staining and immunofluorescence. To measure phagosomal escape, primary murine BMM were infected and stained as previously described (Weiss *et al.*, 2007, Jones *et al.*, 2011). Briefly, prechilled macrophages were infected at 4°C, rapidly warmed for 5 min in a 37°C water bath, and then incubated for 10 min at 37°C, 5% CO_2. At 30, 60, or 120 min after infection, cells were fixed with 4% paraformaldehyde and then permeabilized and stained with a rat monoclonal anti-LAMP-1 antibody (1D4B) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and chicken anti-*F. novicida* antibody (generous gift from Dr. Denise Monack, Stanford University, Stanford, CA) in PBS with 1% saponin and 3% BSA. Next, cells were respectively incubated with Alexa488 (anti-rat) and Alexa594 (anti-chicken) secondary antibodies. The coverslips were then mounted over SlowFade Gold antifade reagent

containing DAPI. Images were obtained using a Zeiss Axioscope Z.1 microscope equipped with a Zeiss Imager 2.1 camera, and images were taken at 63x magnification.

Western blot. For *F. novicida*, protein concentrations were normalized to colony forming units of initial cultures and amounts equaling 1 x 10^8 bacteria from each fraction were run on protein gels (Bio-Rad, Hercules, CA) along with a broad range protein standard (Bio-Rad) for 35 minutes at 200 volts using a Bio-Rad PowerPac HC. Next, the proteins were transferred onto nitrocellulose membranes (GE Osmonics, Trevose, PA) for 30 minutes at 100 volts. Western blots were then performed. Briefly, at room temperature, membranes were blocked 2 hours using 5% skim milk tris-buffered saline with 0.005% Tween-20 (TBST). Next, membranes were incubated for 2 hours with primary antibodies diluted in 5% skim milk TBST against His-tag (Abgent, San Diego, CA), FopA (generous gift from Dr. Michael Norgard, University of Texas Southwestern Medical Center, Dallas, TX)(Huntley *et al.*, 2010), or IglA (BEI Resources, Manassas, VA)(de Bruin *et al.*, 2007). The membranes were then incubated with secondary antibodies against IgG from mouse (Cell Signaling, Danvers, MA), rat (Cell Signaling), or rabbit (Cell Signaling). Finally, membranes were developed using SuperSignal (Bio-Rad) and imaged using a UVP BioSpectrum 600 (Upland, CA). UVP Vision Work CS Image Acquisition and Analysis Software 6.7.2 was used to merge white light and fluorescent images.

Chapter 3 Supporting Information References

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Chapter 3 Supporting Information Tables

Table S1. Primers used in this study.

Primer/Plasmid name	Sequence/Specifications
F. novicida FTN_0544	
FTN_0544 deletion check F1	atctgcaacctcaaatggta
FTN_0544 Arm 1 FWD	gtcctctgataaaacgacta
FTN_0544 Arm 1 REV	ttatcgataccgtcgacctcaacttttatttgctaattgaatta
FTN_0544 frt_sKAN_frt FWD	taattcaattagcaaataaaagttgaggtcgacggtatcgataa
FTN_0544 frt_sKAN_frt REV	ttataagaaagtaaaatagcagttatgcatagctgcaggatcga
FTN_0544 Arm 2 FWD	tcgatcctgcagctatgcataactgctattttactttcttataa
FTN_0544 Arm 2 REV	aacaaagtacgttggtatgg
FTN_0544 deletion check R1	gggatgtatcgcaatattgt
FTN_0544 comp Arm 1 REV	tgagtgacaacccaaagagatttatagtacaatattatgatctt
FTN_0544 comp sKAN FWD	aagatcataatattgtactataaatctctttgggttgtcactca
FTN_0544 comp sKAN REV	taagaaagtaaaatagcagttatacaaccaattaaccaattctg
FTN_0544 comp Arm 2 FWD	cagaattggttaattggttgtataactgctattttactttctta
FTN_0544 comp check R2	tggtcatcaacatgttcatc
F. tularensis FTT_0453	
pXB186	Kanamycin resistance cassette (*aph*), sucrose sensitivity cassette (*sacB)*
FTT0453c checkF1	aaaggtactaatgttggagc
FTT0453c checkF2	tatccaagttgcagaattgg
FTT0453c Arm 1 FWD	atatatatggatccttattattgggtggtgtagc
FTT0453c Arm 1 REV	aggtgataattcaattagcaaaataactgctattttactttct
FTT0453c Arm 2 FWD	agaaagtaaaatagcagttattttgctaattgaattatcacct
FTT0453c Arm 2 REV	atatatatggatccaaatgttgttgtcagtgttg
FTT0453c checkR1	tattattatcttcacgccag
FTT0453c checkR2	acagatatcaatctaagcgg
B. bronchiseptica BB4267	
pSS4245	Allelic exchange plasmid for *Bordetella* species. Tetracycline, streptomycin, ampicillin, bleomycin and kanamycin resistance cassettes
pXQ026	pSS4245 containing *bb4267* deletion fragment (used to generate strain RBXQ24)
pXQ042	pUC18-Mini-TN7 containing *bb4267* complement fragment [used to generate strain RBXQ27 (RBXQ24/pXQ042)]
4267F1	aagatgcctggccgacttc

4267R1	ctggcagccgtttatgagt
LK-4267F2	taaacggcttgccagtcgttcactggtcatttcag
4267R2	cctgcatacctatccgttcg
pUC18-Mini-TN7	ampicillin and kanamycin resistance cassettes
pTNS3	Helper plasmid for pUC18-Mini-TN7 vectors, ampicillin resistance cassette.
E. coli	
pET-21a	Empty expression vector plasmid
pET-21a-*FTN_0544*	pET-21a encoding *FTN_0544*

Chapter 3 Supporting Information Figures

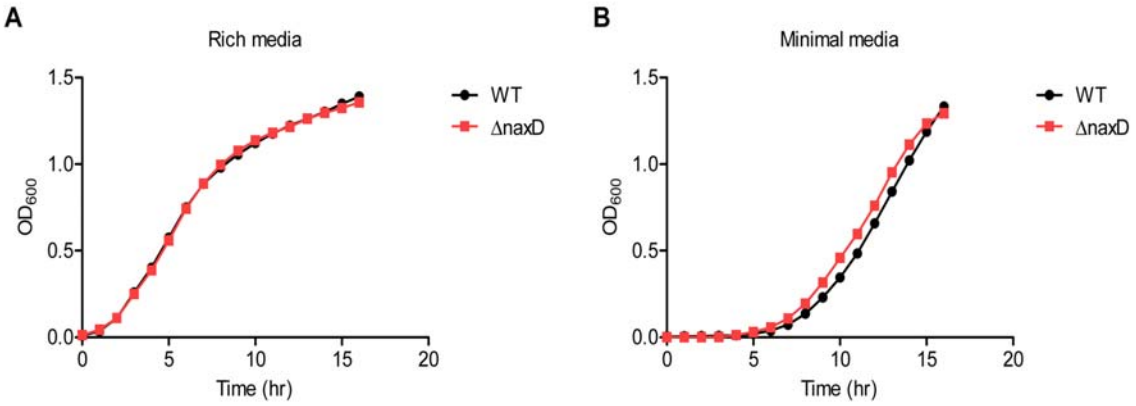

Figure S1. The *naxD* deletion mutant exhibits wild-type growth kinetics in both rich and minimal media. Wild-type (WT, black) and *naxD* deletion mutant strains (ΔnaxD, red) were grown in either (A) rich media (tryptic soy broth) or (B) minimal media (Chamberlain's broth) and the OD_{600} was measured hourly for 16 hours.

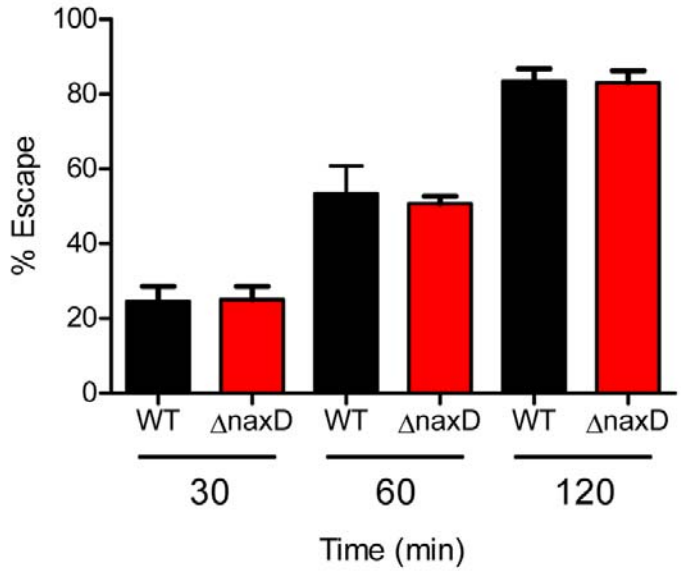

Figure S2. NaxD is not required for phagosomal escape. Flourescence microscopy imaging was used to quantify the escape of wild-type *F. novicida* (WT) and *naxD* deletion mutant (ΔnaxD) bacteria. Primary murine BMM were infected at 100:1 MOI and fixed with 4% paraformaldehyde at 30, 60, and 120 minutes post-infection. After sample staining and preparation, bacteria within phagosomes were identified by colocalization of LAMP-1 and *F. novicida*. The percentage of bacteria that escaped from the phagosome was determined by counting a minimum of 200 bacteria per strain from three different experiments. Bars represent the average and error bars represent the standard deviation.

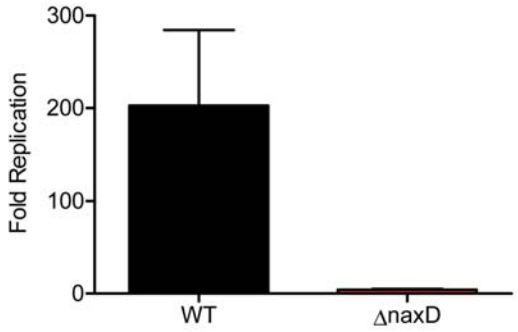

Figure S3. NaxD is required for *F. tularensis* proliferation within murine BMM. Primary murine bone marrow-derived macrophages (BMM) were infected with a 20:1 MOI of wild-type *F. tularensis* (WT) or the *naxD* deletion strain (ΔnaxD). CFU from lysates 30 minutes post-infection were compared to those from 24 hours post-infection to determine fold intracellular replication (n=3 biological replicates). Bars represent the average and error bars represent the standard deviation. Asterisks indicate significance as compared to wild-type. (*) = $P < 0.05$.

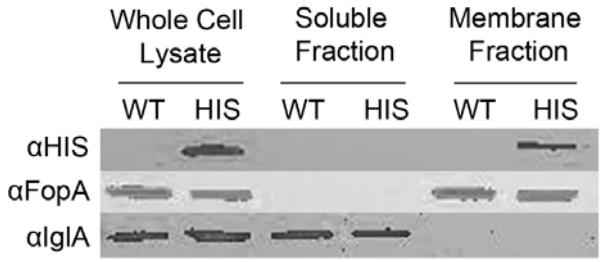

Figure S4. NaxD localizes to the membrane fraction. Western blot analysis of whole cell lysates, soluble fractions, and membrane fractions of wild-type *F. novicida* (WT) and a wild-type strain in which NaxD was tagged with an 8x His tag (HIS). αHis, αIglA (a cytosolic protein), or αFopA (a membrane protein) antibodies were used with the appropriate secondary antibodies.

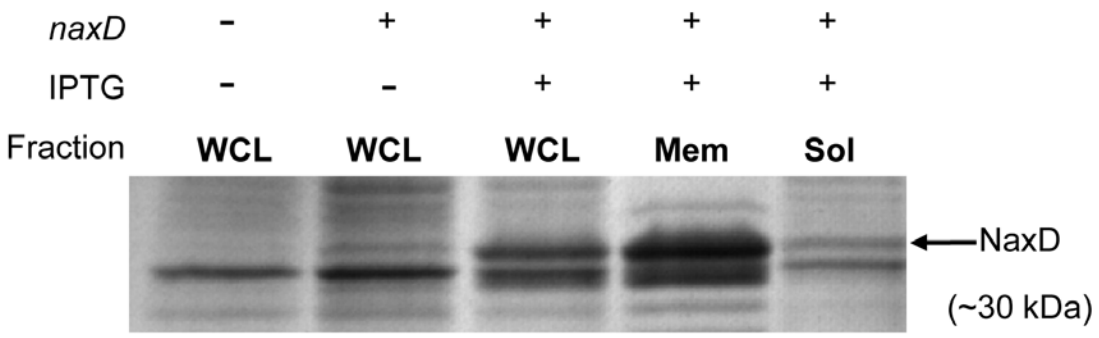

Figure S5. Exogenously expressed NaxD localizes to the membrane fraction in *E. coli*. Coomassie stain was used to analyze whole cell lysate (WCL), membrane (Mem) or soluble (Sol) protein fractions from *E. coli* transformed with either the vector control pET-21a or pET-21a encoding *naxD*, with or without IPTG induction.

Chapter 4: Future Directions

The work described in Chapter 3 revealed not only the function of NaxD and related YdjC proteins, but also suggested a role for this protein in resistance to host intracellular cationic antimicrobial peptides. Elucidating the biological role of this protein and the corresponding lipid A modification in the context of an infection is an important next step. Indeed the recent preliminary evidence described below illustrates that *Francisella*'s altered lipid A contributes to defense against antimicrobials and innate recognition during infection of macrophages.

To test the importance of *Francisella*'s free lipid A galactosamine modification in host antimicrobial resistance, the sensitivity of wild-type *F. novicida* versus the *naxD* deletion mutant to the human cathelicidin LL-37 (generous gift from William Shafer) was tested in broth culture. In the absence of modified free lipid A, *Francisella* displayed a dose-dependent increase in sensitivity to LL-37 (Figure 1A). In addition, this modification was also required for resistance to lysozyme (Figure 1B). Though this antimicrobial is typically thought of as important for degradation of Gram-positive bacteria, lysozyme has also been shown to have an enzyme-independent CAMP domain (Nash *et al.*, 2006). Further studies with heat-killed or enzymatic point mutant lysozyme will determine if the action of lysozyme against the *naxD* deletion mutant is enzyme-dependent, related to the CAMP domain, or both.

Next we wanted to determine if the difference in sensitivity of the wild-type and *naxD* deletion mutant to antimicrobials in broth culture was biologically relevant. Towards this

end, these bacterial strains were used to infect macrophages from wild-type mice and mice that were deficient for the production of CRAMP (the murine homolog of LL-37) or macrophage lysozyme (LysM). Importantly, the intramacrophage replication defect of the *naxD* deletion mutant was partially restored in macrophages lacking either CRAMP or lysozyme (Figure 1C). These results indicate that *Francisella*'s modified free lipid A shields the pathogen from the action of host antimicrobials during its intracellular life cycle.

We then hypothesized that the increased antimicrobial sensitivity of *F. novicida* lacking the free lipid A modification would result in the release of PAMPs and thus trigger host inflammatory signaling. Indeed, ELISA analysis of infected macrophage supernatants showed a dose-dependent increase in IL-6 production in response to infection with the *naxD* deletion mutant but not wild-type *F. novicida* (Figure 2A). Significantly, the same trend was observed in the blood of mice infected intraperitoneally with these strains (Figure 2B). These data indicated that the addition of galactosamine to *Francisella* free lipid A shields the bacteria not only from damage, but also indirectly from host recognition.

Given that the host CAMPs act against bacterial membranes, we hypothesized that bacterial lipoproteins (BLPs) were the most likely PAMP to be released upon exposure to these intramacrophage antimicrobials (Koprivnjak & Peschel, 2011). As BLPs are a primary agonist of TLR2 (Kawai & Akira, 2011), we performed ELISA analysis of supernatants of wild-type or TLR2-deficient macrophages infected with *F. novicida* wild-

type or *naxD* deletion mutant strains to see if this PRR plays a role in the observed inflammatory response. The results show that the IL-6 response to the *naxD* deletion mutant infection is completely TLR2-dependent, indicating that BLPs are in fact the PAMP being recognized in response to the action of intramacrophage antimicrobials in the absence of modified *Francisella* free lipid A (Figure 3A). In fact, IL-6 secretion is diminished in macrophages deficient in either CRAMP or lysozyme, which correlates with the increased ability of the *naxD* deletion mutant to replicate in these macrophages (Figure 3B, 1C). These preliminary data indicate that not only does this modification directly protect the pathogen against the damaging effects of CAMPs, but this protection serves to shield against host recognition and inflammatory signaling as well.

In order to determine if there is an additive or even synergistic effect in restoration of the *naxD* deletion mutant's virulence defects in the absence of more than one antimicrobial, it would be beneficial to breed CRAMP and LysM double knock-out mice. Then we could compare the requirement for *Francisella*'s free lipid A modification in intramacrophage and *in vivo* survival and evasion of innate immune recognition in the absence of one or both antimicrobials. Since there is a partial restoration of the *naxD* deletion mutant growth in macrophages lacking the individual antimicrobials, I would hypothesize macrophages lacking both CRAMP and lysozyme would be more permissive for mutant replication than BMM from single knockout mice.

Another interesting point to be addressed is that the numbers of viable intramacrophage *naxD* deletion mutant bacteria remain relatively stable from infection until initiation of

host cell death. We have shown that this mutant is able to escape from the phagosome (Chapter 3), but it would be informative to determine if these bacteria are surviving intracellularly but not replicating or if they are indeed replicating but getting killed. Therefore, we would like to use fluorescence microscopy to measure the number of live versus dead intracellular bacteria during infection of macrophages from wild-type or the CRAMP and LysM single and double knock-out mice. Collecting these data over a time course will provide meaningful information regarding how *Francisella*'s free lipid A modification and interactions with host antimicrobials affect it's ability to survive and proliferate intracellularly.

Though the importance of antimicrobials in controlling bacterial infections is accepted, the myriad intracellular and *in vivo* ramifications of the action of CAMPs against pathogens are not thoroughly defined. This and future work aims to elucidate the interplay between the antibacterial effects of CAMPs and downstream host signaling and control of infection. In addition to contributing to the understanding of *Francisella* pathogenesis, these studies would deepen the greater understanding of interactions of pathogens and host innate immune defenses and signaling.

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Chapter 4 Figures

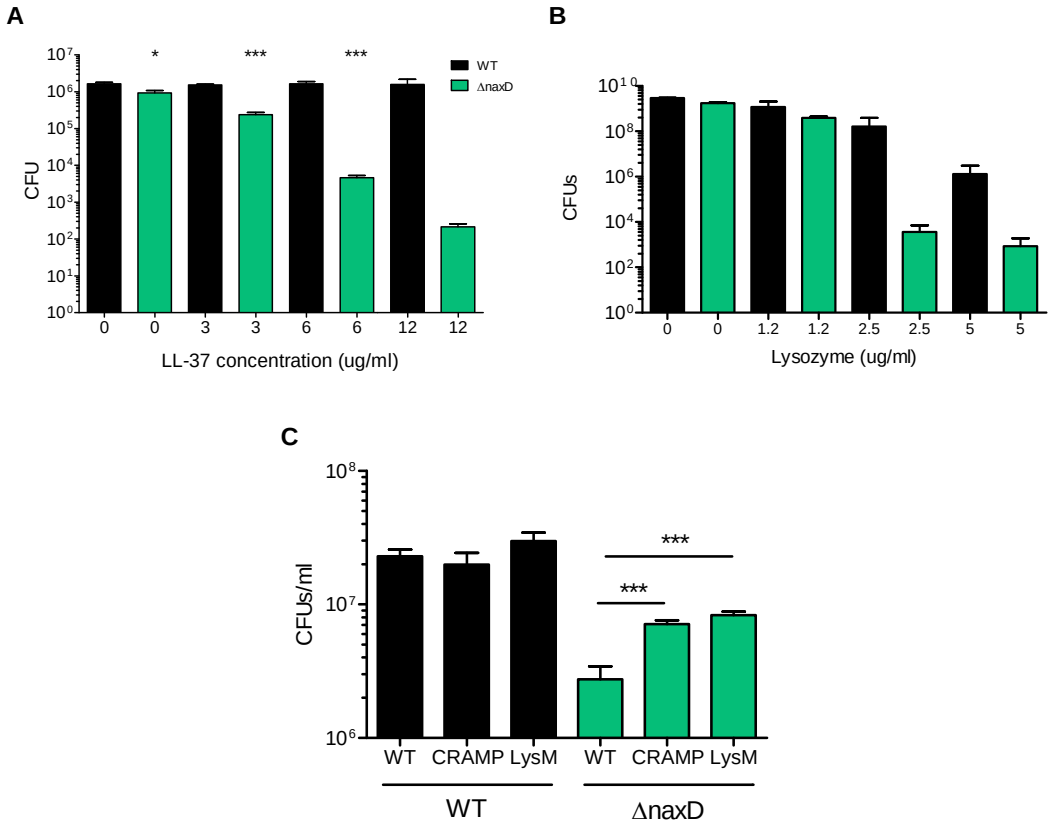

Figure 1. Free lipid A modification is important for resistance to host antimicrobials in broth and macrophages. Overnight bacterial cultures were subcultured to 1x10^7 bacteria/ml in 25% TSB broth diluted in water. Ninety microliters of either wild-type (WT) or *naxD* deletion mutant (ΔnaxD) *F. novicida* was then added to 10 µl of the desired antimicrobial and shaken at 37° C. After 6 hours, the cultures were then serially diluted and plated for colony forming units (CFUs) (A, B). Primary murine bone marrow macrophages (BMM) from wild-type (WT), CRAMP knock-out (CRAMP $^{-/-}$) and lysozyme M knock-out (LysM $^{-/-}$) mice were plated at 3.2 x 10^5/well in 24 well plates and infected the following day with WT or ΔnaxD at a 20:1 multiplicity of infection (MOI).

Six hours post-infection (PI), the macrophages were lysed with 1% saponin in PBS, serially diluted, and plated for CFUs (C).

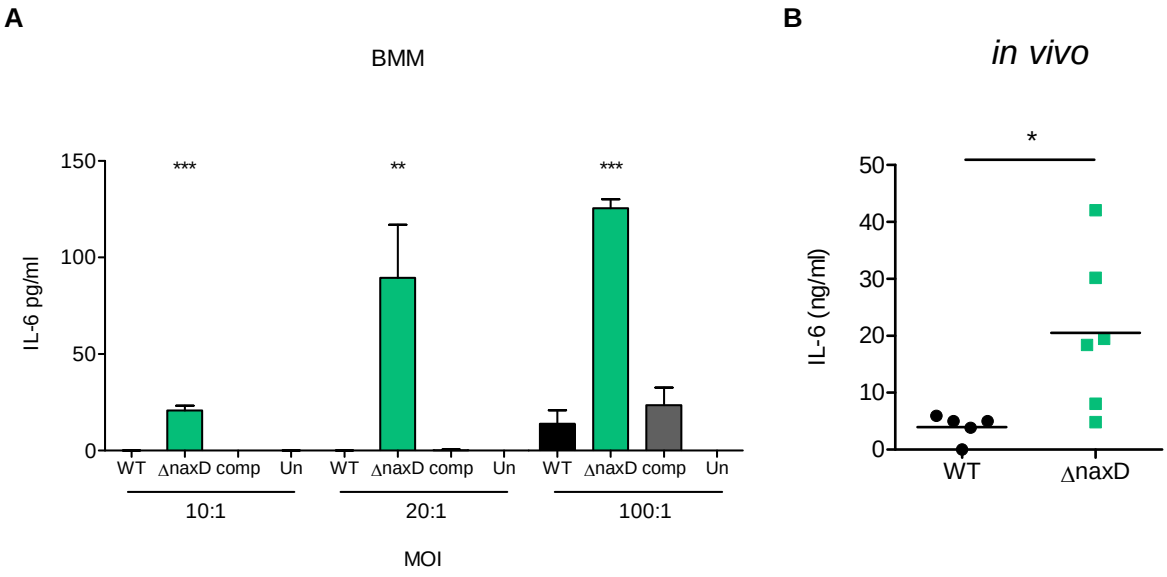

Figure 2. Free lipid A modification is important for evasion of the host inflammatory response in macrophages and *in vivo*. BMM were plated at 3.2 x 10^5/well in 24 well plates and infected the next day with wild-type (WT), *naxD* deletion mutant (ΔnaxD), or complemented (comp) *F. novicida* at a 10:1, 20:1, or 100:1 MOI. Six hours PI, the macrophage supernatant was collected and used to perform ELISA to quantify the presence of IL-6 (BD Biosciences, San Jose, California) (A). Mice were infected intraperitoneally with 1 x 10^5 CFU of either WT or ΔnaxD. Four hours PI, mice were sacrificed and the blood extracted and centrifuged (15,000 x g, 4° C, 15 min). Serum was then collected and analyzed for the presence of IL-6 via ELISA (Figure 2B).

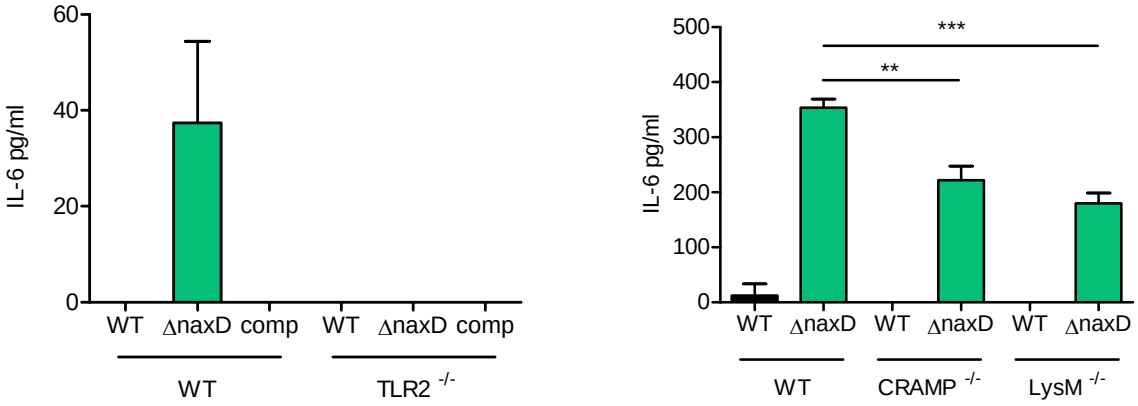

Figure 3. Evasion of host antimicrobials decreases TLR2-dependent inflammatory response. BMM from wild-type (WT) or TLR2 knock-out (TLR2 $^{-/-}$) mice were plated at 3.2 x 10^5/well in 24 well plates and infected the next day with wild-type (WT), *naxD* deletion mutant (ΔnaxD), or complemented (comp) *F. novicida* at a 20:1 MOI. Six hours PI, the macrophage supernatant was collected and used to perform ELISA to quantify the presence of IL-6 (Figure 3A). BMM from WT, CRAMP knock-out (CRAMP $^{-/-}$) and lysozyme M knock-out (LysM $^{-/-}$) mice were also infected with WT or ΔnaxD and IL-6 was quantified from supernatants collected 6 hours PI (Figure 3B).

Chapter 5: Discussion

Despite being designated a primary potential biothreat, there is relatively little known about how *Francisella* species cause disease in comparison to other bacterial pathogens. Recent *in vivo* screens have identified hundreds of genes required in *Francisella* pathogenesis (Su *et al.*, 2007, Weiss *et al.*, 2007). While providing valuable insight into the genetics of *Francisella* virulence, these screens did not indicate how these genes might contribute to disease. Therefore, to initiate my thesis work, I assembled a transposon mutant library representing genes identified in *in vivo* screens to determine which of the genes shown to be required for virulence were specifically required for replication in macrophages (Chapter 2). Fifty-three of the 224 genes screened were required for replication in murine macrophages. To the best of my knowledge, the intracellular replication data from mutants for 140 of the 224 genes that were represented in this screen had not been previously reported in mammalian cells, contributing valuable information to the field of *Francisella* pathogenesis.

The intracellular replication screen identified genes encoding multiple proteins with characterized and predicted functions including FPI proteins known to be required for phagosomal escape and metabolic genes required for cytosolic replication. In addition, the screen demonstrated for the first time that biotin is critical for *Francisella* intracellular replication, which my colleague Brooke Napier has now shown to be essential for expression of FPI genes and phagosomal escape (Napier *et al.*, 2012).

Interestingly, 19 of the 53 genes identified in the screen are annotated as encoding hypothetical proteins, suggesting that *Francisella* utilizes novel mechanisms for both *in vivo* virulence and intracellular replication. As our laboratory is particularly interested in investigating the role of hypothetical proteins in pathogenesis, the remainder of my thesis work focused on elucidating the function of two of these proteins: FTN_1133 and NaxD.

While the full-length protein does not have predicted homologs outside of *Francisella* species, bioinformatic analyses revealed that FTN_1133 has similarity to the C-terminal domain of an organic hydroperoxide resistance protein, Ohr, from *Bacillus megaterium*. Ohr homologs are encoded by multiple bacterial species including *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* (Mongkolsuk *et al.*, 1998, Fuangthong *et al.*, 2001, Ochsner *et al.*, 2001, Dorsey *et al.*, 2006). Like Ohr proteins, FTN_1133 is required for both resistance to, and degradation of, organic hydroperoxides (Chapter 2).

However, the significance of Ohr proteins has not before been shown in tissue culture or *in vivo* models (Lesniak *et al.*, 2002). Given that organic hydrogen peroxide is known to be a byproduct of lipid peroxidation, we hypothesized that FTN_1133 may contribute to *Francisella* resistance to ROS-induced damage incurred during transient interactions within the phagosome (Halliwell & Gutteridge, 1984, Storz *et al.*, 1990, Akaike *et al.*, 1992, Girotti, 1998, Oliveira *et al.*, 2006). Indeed, we found that intracellular growth of the *FTN_1133* deletion mutant was restored to wild-type in macrophages that lack a functional NADPH oxidase. In addition, *in vivo* virulence was only partially restored in

mice deficient for NADPH oxidase, indicating that FTN_1133 is likely required for resistance to ROS generated by other sources during in vivo infection, such as myeloperoxidases within neutrophils.

Furthermore, the role of FTN_1133 in resistance to oxidative stress is conserved in the *F. holarctica*-derived LVS FTN_1133 ortholog. Specifically, the LVS homolog was required for resistance to organic hydroperoxides and replication both in macrophages and mice. Finally, the inability of the LVS mutant to proliferate intracellularly was largely rescued in macrophages deficient in NADPH oxidase.

The intramacrophage replication screen also identified NaxD to be required for *Francisella* intracellular proliferation. Furthermore, the work described in Chapter 3 and Chapter 4 shows that NaxD, a member of the previously uncharacterized YdjC protein superfamily, is required for a lipid A modification that contributes to intramacrophage proliferation by providing resistance to host cationic antimicrobial peptides and evasion of host innate recognition and signaling.

Specifically, this work shows that NaxD deacetylates a precursor molecule for a substrate required to add galactosamine to *Francisella* free lipid A and alter the surface charge of the bacteria. Mass spectrometry analysis revealed that this protein is required for the addition of galactosamine to free lipid A and specifically for the deacetylation of a sugar carrier lipid which is essential for this lipid A modification. Significantly, enzymatic

assays showed that NaxD was required and likely sufficient for this deacetylation reaction.

The work in this thesis also illustrates that NaxD is required for virulence, intramacrophage survival, and resistance to the CAMP polymyxin B in both *F. novicida* and the human pathogenic *F. tularensis* (Chapter 3). In addition, the enzymatic function and role in CAMP resistance is conserved in the NaxD/YdjC homolog encoded by *B. bronchiseptica*, indicating a possible function in multiple other YdjC-encoding Gram-negative bacteria.

Finally, preliminary evidence indicates that NaxD is required for resistance to intramacrophage CRAMP and lysozyme (Chapter 4). In addition, increased sensitivity to these antimicrobials leads to increased TLR2-dependent inflammation in the host cells, likely due to outer membrane damage and release of bacterial lipoproteins. Further studies will deepen our understanding of the role of NaxD, lipid A modifications, and innate immune evasion in the success of *Francisella* and similar pathogens.

Taken together, the work described in this thesis has contributed not only to the understanding of *Francisella*'s genetic requirements for intracellular proliferation, but has revealed the role of Ohr-like proteins in pathogenesis and elucidated the function of members of previously uncharacterized YdjC superfamily of proteins in a lipid a modification important for virulence and innate immune evasion. Given that both Ohr and YdjC proteins are encoded by many virulent bacteria, this work has implications for

bacterial pathogenesis of both intracellular and extracellular organisms and may represent attractive targets for drug and vaccine development.

Taken together, the work presented in this thesis has addressed the role of *Francisella* proteins in resistance to host intracellularQ antimicrobial defenses. First, the intracellular replication screen identified virulence factors that were specifically required for proliferation inside of macrophages. Next, we elucidated the role of FTN_1133, a novel hydroperoxide resistance protein and demonstrated for the first time the requirement of an Ohr-like protein for resistance to host ROS. Finally, we characterized the function of NaxD, a member of the previously functionally uncharacterized YdjC superfamily and a deacetylase essential for a lipid A modification required for pathogenesis, intramacrophage proliferation, and resistance to CAMPs. Further studies will define the role of this lipid A modification in resistance to specific intracellular host CAMPs and evasion of innate immune recognition. Given that both Ohr and YdjC proteins are encoded by many virulent bacteria, this work has implications for bacterial pathogenesis of both intracellular and extracellular organisms and may represent attractive targets for drug and vaccine development.

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