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Biotin limitation in the phagosome and *Francisella* virulence

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Biotin limitation in the phagosome and *Francisella* virulence

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B.A., Agnes Scott College, 2007

Advisor: David Weiss, Ph.D.

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Abstract

Biotin limitation in the phagosome and *Francisella* virulence

By Brooke Ann Napier

Cytosolic bacterial pathogens require extensive metabolic adaptations within the host to replicate intracellularly and cause disease. In phagocytic cells such as macrophages, these pathogens must respond rapidly to nutrient-limitation within the harsh environment of the phagosome. Many cytosolic pathogens escape the phagosome quickly (15-60 min) and thereby subvert this host defense, reaching the cytosol where they can replicate. While a great deal of research has focused on strategies used by bacteria to resist antimicrobial phagosomal defenses and transiently pass through this compartment, the metabolic requirements of bacteria within the phagosome are largely uncharacterized. We previously identified a novel *Francisella* protein, FTN_0818, as an essential factor for intracellular replication and involved in virulence *in vivo*; however, it was unclear how this new virulence determinant affects bacterial pathogenesis. I demonstrate in this work that FTN_0818, now named BioJ, is involved in biotin biosynthesis and required for rapid escape from the *Francisella*-containing phagosome (FCP), as well as replication *in vitro* and *in vivo.* These results demonstrate that biotin is critical for promoting rapid escape during the short time that the bacteria are in the phagosome, implying biotin may be a limiting factor during infection. Furthermore, functional anaylsis found BioJ works as a carboxyl-esterase that, as a monomer, catalyzes the hydrolysis of Me-pimeloyl-ACP into pimeloyl-ACP, and is necessary for the second step of biotin biosynthesis. Phylogenetic and structural analyses identified BioJ as an evolutionarily distinct *Francisella*-specific biotin gatekeeper enzyme. Additionally, bioinformatic analysis of BioJ revealed a conserved carboxylesterase catalytic triad, Ser-Asp-His. We found that all three residues within the catalytic triad are required for BioJ function and subsequently *Francisella* replication in macrophages and survival in mice. Thus, I have demonstrated that biotin is required for phagosomal escape of *Francisella* and we have characterized BioJ as a novel *Francisella*-specific biotin gatekeeper enzyme. This is the first demonstration of a bacterial metabolite required for phagosomal escape of an

intracellular pathogen, providing new insight into the link between bacterial metabolism and virulence, likely serving as a paradigm for other cytosolic pathogens.

Biotin limitation in the phagosome and *Francisella* virulence

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Chapter 1

Introduction and Background

History and background of *Francisella*

Francisella tularensis was first identified as the causative agent of a fatal plague-like disease in a population of ground squirrels in Tulare County, California in 1911 (1). Originally called *Bacterium tularense*, it was later renamed *Francisella tularensis* in honor of Dr. Edward Francis who spent his career extensively studying and characterizing the transmission and growth of this bacterium (2) Although it causes disease in squirrels, rabbits, and numerous other mammals, none have been identified as a reservoir, which instead is thought to be fresh water or amoeba living therein. As there is no person-to-person spread, *Francisella* is primarily spread to humans via arthropod vectors or zoonotic transmission, though it can also be transmitted by inhalation of aerosolized bacteria or ingestion of contaminated food or water (3). Inhalation of *F. tularensis* causes the most severe infections, and only 10 bacteria can lead to a potentially fatal disease. This high infectivity, along with its ease of aerosolization and genetic manipulation, led Japan, the Soviet Union, and the United States to weaponize *Francisella* during WWII (2).

Francisella species are endemic only in the northern hemisphere. *F. tularensis* subsp. *tularensis* is the most virulent etiologic agent of tularemia in humans and is the primary disease-causing *Francisella* species in North America. *F. tularensis* subsp. *holarctica* is responsible for the majority of reported cases of tularemia in Europe and Asia. The current vaccine is an attenuated live vaccine strain (LVS) derived from virulent *F. holarctica* by serial passage. LVS causes a very mild infection in humans but can cause a lethal infection in mice and is therefore commonly used as a model to study *Francisella*

pathogenesis. The closely related *F. novicida* species rarely causes disease in humans, though some cases have been documented (4, 5). However, *F. novicida* is highly virulent in mice, has over 98% identity to *F. tularensis* at the DNA level (6), shares many of the same virulence genes (7), and is also used as a model system to study *Francisella* virulence. Finally, *F. mediasiatica* is a species of intermediate virulence in humans and is found in Central Asia, while *F. philomiragia* and *F. noatunensis* can cause infections in aquatic organisms including wild and farmed fish (8). Throughout this chapter "*Francisella*" will refer to general characteristics shared by numerous species and subspecies, and will otherwise refer to specific species and subspecies by name.

Tularemia

Francisella is the etiological agent of the disease tularemia, also known as rabbit fever. Tularemia is characterized by a 3-5 day incubation period (2) during which the bacteria replicate almost "silently" in macrophages and other types of host cells. The eventual release of bacteria from these cells coincides with the presentation of flu-like symptoms. There are several manifestations of tularemia, each dependent on the route of acquisition (9). The most common form of tularemia is ulceroglandular disease, which can result from insect bites, or by contact with infected animal tissues following mechanical damage to the skin. A cutaneous ulcer develops at the site of infection and bacteria drain to lymph nodes, subsequently causing a systemic infection. Less common forms of the disease include pneumonic, oculoglandular, and oropharyngeal tularemia. Streptomycin

or doxycycline is indicated for treatment. Tularemia may be fatal, however, survivors gain robust immunity found to last for up to 30 years (10).

Francisella intracellular life cycle

Though *Francisella* shows an extracellular phase during bacteremia in mice, *Francisella*'s primary replicative niche is the cytosol of phagocytic and non-phagocytic cells including macrophages, dendritic cells, polymorphonuclear neutrophils, hepatocytes, endothelial, and type II alveolar lung epithelial (11-16). The life cycle of *Francisella* within non-phagocytic cells is still fragmented, however the life cycle within macrophages, an important cell type during *Francisella* infection, has been under extensive investigation in the last decade.

After initial engulfment by macrophages, *Francisella* is taken up into the nutrient-limiting phagosome that contains an array of toxic antimicrobials aimed at degrading the bacteria (Fig 1). After a very brief (30 min – 1 h) period within the phagosome, *Francisella* escapes through unknown mechanisms to replicate within the permissive cytosolic compartment (Fig 1). Once in the cytosol, *Francisella* must combat detection from pathogen recognition receptors (PRRs) such as AIM2, which recognizes bacterial DNA and induces inflammatory mediated cell death (17). After successful stealth replication in the cytosol, *Francisella* then spreads via undefined mechanisms to neighboring cells.

Francisella and the phagosome

Mechanisms of Entry and Fate of Intracellular *Francisella*

As mentioned above, macrophages are one of the sentinels of infection and one of the first cell types to encounter *Francisella* (16). Upon initial contact with macrophages, *Francisella* binds to host receptors and is taken up via spacious, asymmetrical protrusions called pseudopod loops (18). Ultra-structural analyses have shown that pseudopod loops are formed during uptake of unopsonized and opsonized *Francisella*, and are present in multiple cell types (18) (Napier and Weiss, unpublished data). This novel phenomenon differs from other described bacterial uptake mechanisms including coiling phagocytosis seen at the surface of phagocytes during *Legionella pneumophila* infection and conventional phagocytosis observed during infection with multiple bacterial and viral species (19-22).

The efficiency of *Francisella* uptake by macrophages depends in large part on whether the bacteria are opsonized, since serum- or antibody-opsonized bacteria are taken up by macrophages at 10-fold higher levels than unopsonized bacteria (23). While this increased efficiency of uptake helps the bacteria evade extracellular defenses, it comes at a cost since the intracellular fate of opsonized and unopsonized bacteria are different (Fig 2). Opsonized bacteria escape the phagosome with delayed kinetics and replicate modestly in the host cell cytosol. In contrast, unopsonized *Francisella* escape the phagosome rapidly and replicate robustly in the cytosol. Therefore, the route of uptake has a profound impact on the outcome of infection, and as discussed below, *Francisella*

uses several strategies to promote entry mechanisms that favor its survival and replication.

Uptake of unopsonized *Francisella* depends largely on the mannose receptor (MR). When the MR is blocked with antibody or mannan, uptake of unopsonized *Francisella* decreases by more than 50% (24, 25). Unopsonized *Francisella* taken up by the MR escape the phagosome rapidly and replicate to high numbers. In addition, the MR is known to induce relatively weak inflammatory responses compared to other phagocytic receptors (26). All of these outcomes favor the bacteria, and therefore, MR-mediated uptake makes host cells more permissive for *Francisella* replication, similar to what is observed during uptake of *Mycobacterium tuberculosis* (Fig 2) (27).

In contrast, serum-opsonized bacteria are bound by complement, leading to uptake mediated in large part by complement receptor 3 (CR3). Ablation of CR3 ($CR3^{-/-}$ macrophages) results in at least a 50% decrease in uptake of serum-opsonized LVS (24). The scavenger A receptor (SRA), although traditionally known to play a role in uptake of unopsonized bacteria, contributes to the uptake of serum-opsonized *Francisella* since $SRA^{-/-}$ macrophages exhibit a 20% reduction in the uptake of serum-opsonized LVS (28). Macrophage cell surface-exposed nucleolin has also been implicated in uptake of serum-opsonized *Francisella* by binding the bacterial membrane protein EF-Tu (29). Finally, MR-mediated uptake also plays a role in uptake of serum-opsonized *Francisella* (roughly 30% decreased uptake when blocked) (23)**.** As mentioned above, this multi-receptor

uptake pathway predominated by CR3 results in delayed phagosomal escape and replication and is therefore non-optimal for *Francisella*.

Opsonization with antibody, specifically IgG, leads to FcγR-mediated uptake of *Francisella*. FcγR$^{-/-}$ macrophages exhibit a 90% reduction in uptake of IgG-opsonized *Francisella*, although they have no defect in the uptake of unopsonized or serum-opsonized bacteria (23). FcγR-mediated uptake leads to increased activation of the NADPH oxidase, a phagosomal enzyme complex that produces toxic reactive oxygen species (ROS) and is discussed in greater detail below. This activation results in a more inhospitable environment for the bacteria in the phagosome, acting to delay phagosomal escape and severely limit subsequent bacterial replication (Fig 2). The efficacy of this host defense is revealed when ROS production is abrogated (using gp91$^{phox-/-}$ macrophages), since IgG-opsonized *Francisella* are rescued and can replicate to high levels (23).

To limit uptake by the FcγR pathway and maximize intracellular replication levels, *Francisella* binds plasmin to degrade soluble antibody at the bacterial surface (30). *Francisella* also subverts the CR3-mediated pathway using LPS O-antigen and capsule that inhibit binding of complement factors. It should be noted that when complement binding does occur, the bacteria alter the complement pathway to prevent MAC formation and lysis (17). However, the alternate complement fragments generated actually promote phagocytosis via CR3. While uptake via this pathway is not optimal, it does still support bacterial replication and is a much better outcome for the bacteria than

complement-mediate lysis. The most optimal entry pathway, however, is mediated via the MR. Therefore, *Francisella* uses the aforementioned subversion mechanisms to promote uptake by the more favorable MR-mediated pathway, facilitating the highest levels of intracellular replication.

Phagosomal Acidification

After uptake by the macrophage, *Francisella* resides within the *Francisella*-containing phagosome (FCP) and subsequently escapes into the cytosol where it can replicate (Fig 1) (31)]. The brief time spent in the FCP is a dynamic step in infection during which *Francisella* must actively evade host antimicrobial defenses including acidification of the FCP, reactive oxygen species, antimicrobial peptides, and nutrient limitation. Acidification of the phagosome is intended to lower the pH in this compartment, preventing bacteria from efficiently replicating. Many intracellular pathogens must subvert this host defense mechanism by either blocking it, escaping the phagosome, or by maintaining intracellular pH in spite of the lowered pH in the environment (31). It is interesting to note that acidification is actually required for the rapid phagosomal escape of the intracellular pathogen *Listeria monocytogenes* (32). Studies with *Francisella* have shown a brief acidification of the FCP that is dependent on the route of uptake, although there has been debate about whether this step is required for rapid escape and about its effect on the outcome of infection.

Uptake of unopsonized *Francisella* leads to transient acidification of the FCP and rapid escape (33, 34). Santic *et al.* reported acidification of the phagosome 15 to 30 minutes post-infection (pi) of human monocyte-derived macrophages (MDM) with unopsonized *F. novicida*, followed by escape 30 minutes to 1 hour pi (33). Interestingly, they showed that inhibition of the proton vATPase pump by bafilomycin A (BFA) does not block escape, but results in delayed escape (6 to 12 h). These results suggest that acidification is required for rapid escape from the FCP (33). Additionally, Chong *et al.* observed brief acidification of the FCP in macrophages infected with unopsonized *F. tularensis* (34). Similarly, BFA did not inhibit phagosomal escape or cytosolic replication, but slowed infection kinetics (34). Together these data show that acidification of the FCP ensures rapid escape and robust replication of unopsonized *Francisella*, however, it is not required for these processes.

Concurrently, it has been reported that uptake of serum-opsonized *Francisella* results in acidification of a modest 20% to 30% of phagosomes containing serum-opsonized LVS, and that it is not required for escape or replication (35). These authors found that after uptake with serum-opsonized *Francisella*, the maturing FCP does not acquire high levels of the acid hydrolase cathepsin D or the endosomal-lysosomal markers CD63, LAMP1 and LAMP2, which are cellular markers of phagosomal maturation and acidification (18, 35). In contrast to the previously mentioned work using unopsonized bacteria, BFA did not significantly delay infection kinetics in these experiments using serum-opsonized bacteria (35). These data demonstrate that acidification of the FCP is not required for

escape and replication of unopsonized or serum-opsonized *Francisella*, but is required for rapid escape of unopsonized *Francisella*.

The fact that *Francisella* does not exhibit a significant survival or replication defect in the presence or absence of acidification demonstrates that this pathogen has devised ways to efficiently resist this host defense. This resistance is mediated in part by physically escaping the FCP where acidification takes place (discussed in the *Francisella* Escape from the Phagosome section). It is also likely that *Francisella* has evolved mechanisms to maintain intracellular pH while in the acidified environment of the FCP.

Inhibition of Reactive Oxygen Species (ROS)

In addition to subverting the potentially toxic effect of acidification of the FCP, *Francisella* must contend with reactive oxygen species (ROS) produced in this compartment. ROS are produced by the NADPH oxidase, a membrane-bound multi-component enzyme system that converts molecular oxygen into toxic superoxide anions (36). In a resting phagocyte, NADPH oxidases are unassembled with the $gp91^{phox}$ and $p22^{phox}$ (also called flavocytochrome b_{558}) components localizing to the plasma membrane, and $p47^{phox}$, $p40^{phox}$, $p67^{phox}$ and Rac2 in the cytosol (14, 36). Upon phagocytosis of a microbe, the cytosolic subunits traffic to the phagosome and assemble with the membrane subunits to create the active NADPH oxidase that then produces ROS. Similar to numerous extracellular and intracellular bacterial species including

Helicobacter pylori and *Salmonella* spp., multiple *Francisella* species block NADPH oxidase assembly in neutrophils and macrophages (14, 37-40).

Francisella species use several approaches to inhibit ROS including blocking initial assembly of NADPH oxidase components at the phagosomal membrane, blocking ROS production in complexes that have assembled, and detoxifying ROS that are generated. *F. novicida* requires four putative acid phosphatases (AcpA, AcpB, AcpC, and Hap) for inhibition of NADPH oxidase assembly (41, 42) . AcpA co-localizes with the cytosolic NADPH oxidase component $p47^{phox}$ during infection, and purified AcpA dephosphorylates $p47^{phox}$ and $p40^{phox}$ (41). Without phosphorylated membrane-bound components, the cytosolic NADPH oxidase components are not recruited and assembly cannot occur (43). These data suggest that AcpA interacts directly with NADPH oxidase components to block complex assembly.

While AcpA plays a role in limiting the oxidative burst, its relative contribution to virulence is unclear. Two studies found that AcpA is required for replication of *F. novicida* in human macrophage-like cells, indicating that blocking the NADPH oxidase is an important factor in facilitating replication (41, 44). In contrast, another study using *F. novicida* determined that AcpA did not play a role in replication in murine macrophages (45). In *F. tularensis*, deletion of *acpA* or even *acpA, acpB*, and *acpC* together, did not influence virulence in murine macrophages or human monocytes (46, 47)]. These conflicting data indicate that AcpA is not required for *Francisella* replication in all

conditions tested. Its requirement may depend on the species or host cells used and specific infection conditions.

In addition to blocking assembly, *F. tularensis* can also inhibit the generation of ROS when NADPH oxidase assembly is induced by exogenous stimuli (47). In spite of the formation of this complex, *F. tularensis* can inhibit the production of ROS. These data show that *F. tularensis* can block NADPH oxidase-dependent ROS production post-assembly (47), although the mechanism by which this occurs has not yet been elucidated.

Although *Francisella* uses the mechanisms described above to significantly suppress activation of the NADPH oxidase, low levels of ROS are produced in the phagosome during infection (40). Like many pathogens, *Francisella* can directly detoxify ROS using proteins including catalase (48-50) and superoxide dismutases (50-52) whose specific mechanisms of action are reviewed extensively elsewhere (53). In addition to these well-known mechanisms of ROS resistance, we identified a previously uncharacterized protein, FTN_1133, that is required for virulence and resistance to organic hydroperoxides (53). FTN_1133 has sequence similarity to Ohr proteins involved in resistance to organic hydroperoxides created during the interaction of ROS with lipids of the bacterial cell membrane (53). The FTN_1133 mutant was attenuated for replication, although this could be restored in $gp91^{phox-/-}$ macrophages and mice (53). These data together indicate that *Francisella* has numerous overlapping mechanisms with which to subvert the NADPH oxidase and ROS, facilitating pathogenesis.

Francisella Escape from the Phagosome

Phagosomal escape is the last step in *Francisella*'s subversion of the phagocytic pathway, allowing it to escape the toxic phagosome and reach the cytosol where it can replicate. The timing of escape has been a topic of debate, and as mentioned previously is largely dependent on the route of *Francisella* uptake by host cells. Unopsonized *Francisella* escape the phagosome rapidly (within 1 h) whereas opsonized *Francisella* exhibit delayed escape (2-4 h) (Napier and Weiss, unpublished observation) (54).

The exact mechanism of escape is not yet known, however, the proteins encoded in the *Francisella* pathogenicity island (FPI) are absolutely required. The FPI encodes a putative type VI secretion system (T6SS) that is essential for *Francisella* replication and pathogenesis (55). The requirement of a specialized secretion system for phagosomal escape would be in line many intracellular bacteria including *Shigella flexneri* (T3SS), *Listeria monocytogenes* (Sec pathway), and *Burkholderia* spp. (T3SS) (56-60). The FPI proteins IglA and IglB share high homology with proteins encoded in T6SS clusters in multiple bacterial species, are required for phagosomal escape, and may form the putative outer tube of the T6SS "needle" (61, 62). IglC, also required for phagosomal escape, has been proposed to form the inner tube of the T6SS (61). Recently, two conserved components of the T6SS cluster, VgrG and DotU, were shown to be required for phagosomal escape and subsequent replication in murine macrophages (63). VgrG was shown to form multimers, consistent with its suggested role as a trimeric membrane-

puncturing device in T6SSs, and DotU was shown to stabilize PdpB/IcmF, another core component of the T6SSs (64).

Currently, there are conflicting reports about the occurrence of FPI-dependent substrate secretion. In both *F. novicida* and LVS it was shown that IglI and VgrG are secreted (55, 64); however the data in LVS was conflicting regarding the requirement for other FPI proteins since the findings in *F. novicida* suggested that only secretion of IglI was FPI-dependent, whereas the findings in LVS conclude that IglI as well as VgrG secretion was FPI-dependent (64). Additionally, it was identified that 6 more FPI encoded proteins (IglE, IglC, PdpE, PdpA, IglJ, and IglF) were secreted, and in all cases the core components DotU, VgrG, IglC, and IglG were required (64). Interestingly, all secreted proteins, with the exception of VgrG, are *Francisella*-specific proteins, displaying the unique nature of T6SS of *Francisella*. Combined, these findings suggest an important role for T6SS proteins encoded within the FPI for *Francisella* escape and replication in the cytosol; however, there may be varying T6SS substrates among different *Francisella* species and fundamental differences in the *Francisella* T6SS mechanism compared to other bacterial T6SSs (64). In addition to the T6SS proteins, numerous proteins that are not encoded in the FPI have also been implicated in phagosome escape and are reviewed elsewhere (34, 44).

To facilitate phagosomal escape, *Francisella* must subvert the action of host factors that have evolved to slow or block this process in order to control infection. Activation of the PI3K/Akt pathway by bacteria leads to production of pro-inflammatory cytokines,

increased ROS production, and retention of bacteria in the phagosome (65-67). In accordance, activation of the PI3K/Akt pathway during *Francisella* infection blocks phagosomal escape and cytosolic replication (67). Interestingly, *Francisella* infection of macrophages activates the SH2 domain-containing inositol phosphatase (SHIP) by an unknown mechanism that is dependent on live bacteria (68). SHIP antagonizes activation of the PI3K/Akt pathway, leading to rapid escape from the phagosome and robust cytosolic replication (68). Additionally, the PI3K/Akt pathway can be activated by cell surface receptors including FcγR (69) and TLRs (70). Therefore, *Francisella* mechanisms to evade these receptors (reviewed in (17)) may also function to block activation of the PI3K/Akt pathway and ensure rapid phagosomal escape.

Thesis overview

As summarized, nearly all mechanisms of *Francisella* evasion from phagosomal antimicrobial properties have been extensively studied, however *Francisella* evasion of the nutrient-limiting properties within the phagosome remains unclear. Though direct analysis of nutrient availability within the FCP is not yet determined, composition of nutrients within this compartment can begin to be defined through studies of the infection kinetics of nutrient-deficient mutants. Recent *in vitro* genetic screens have identified multiple metabolic pathways required for wild-type *Francisella* FCP escape; however, verification of requirement and mechanism of requirement for these metabolites have yet to be explored. The implication of various metabolic pathways in FCP escape is not

surprising due to the numerous antimicrobial evasion mechanisms required to efficiently evade the FCP to replicate within the permissive cytosol.

In this dissertation, we have identified biotin (vitamin H) as a metabolite required for *Francisella* rapid escape from the FCP. In the absence of a novel biotin enzyme (BioJ), we find that *Francisella* cannot express FPI encoded genes to wild-type levels, thus delaying escape from the FCP, inhibiting replication within the host cytosol and survival within mice (Chapter 2). From these results, we next characterized the molecular contribution of BioJ in *Francisella* biotin biosynthesis. In Chapter 3, we find BioJ is a carboxyl-esterase required for *de novo* synthesis of biotin and is working as the previously missing, *Francisella*-specific biotin gatekeeper enzyme.

Since nutrient limitation in the FCP as not been fully characterized, it has been difficult to study what other nutritional limitations affect *Francisella* virulence within this environment; however, directed studies, such as those described in this dissertation, have implicated the *de novo* production of biotin (vitamin H) within the phagosome as an important virulence determinant contributing to the knowledge of cytosolic intracellular pathogens and providing a possible new target for antimicrobial therapeutics.

Figures

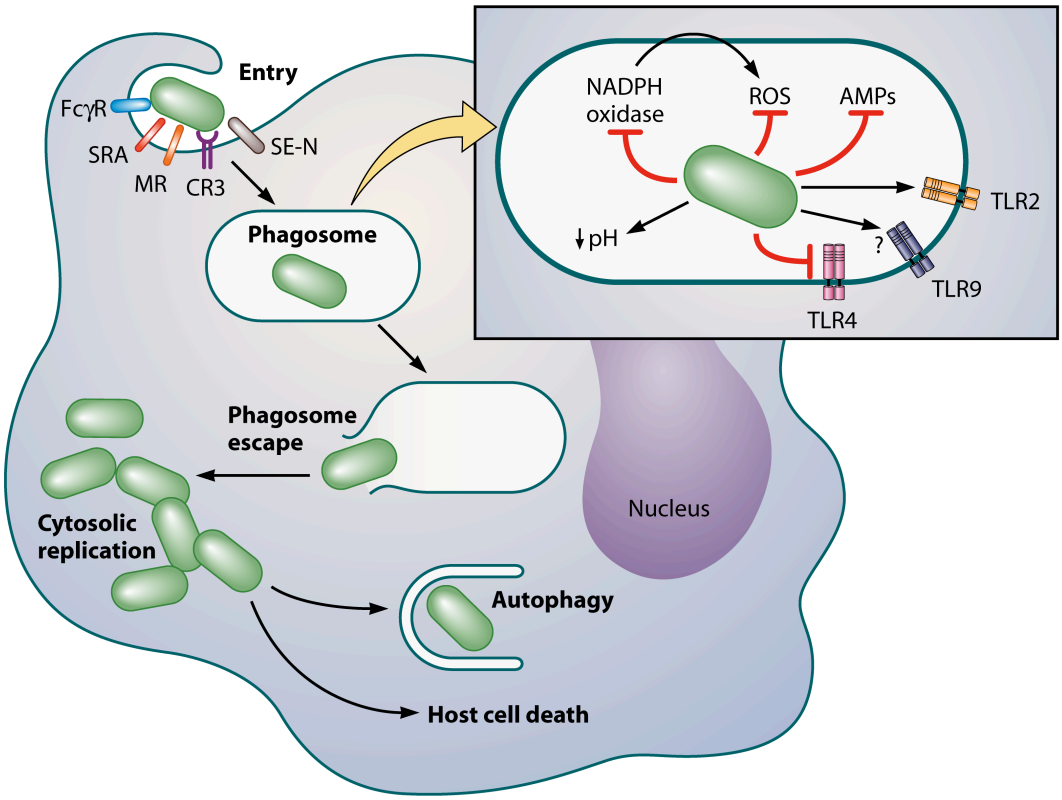

Figure 1. Stages of *Francisella* pathogenesis in the macrophage. *Francisella* can be detected by multiple macrophage receptors and is engulfed by a unique pseudopod loop mechanism. It then traffics to an early phagosome called the *Francisella*-containing phagosome (FCP). *Francisella* uses multiple 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 and may induce TLR9 signaling. *Francisella* then escapes the FCP to replicate within the cytosol. Subsequently, *Francisella* associates with autophagosomes although the outcome of this interaction is unknown. *Francisella* can also induce host cell death.

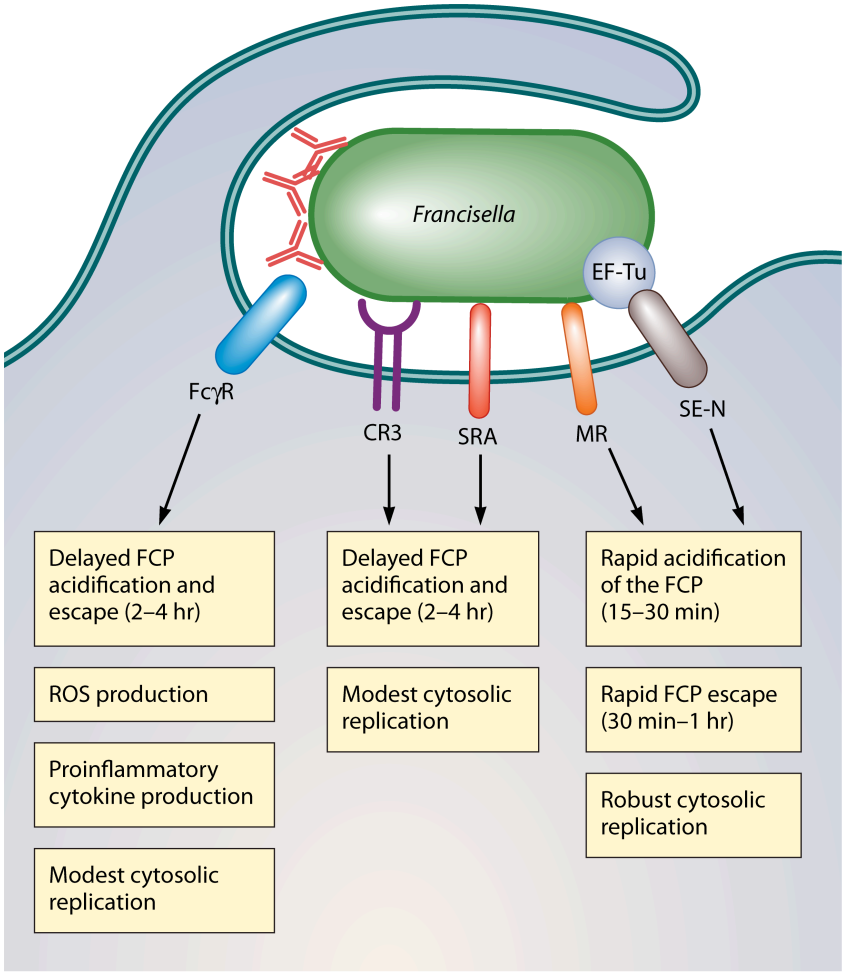

Figure 2. The intracellular fate of *Francisella* after uptake by different macrophage receptors. Antibody-opsonized *Francisella* are taken up via the FcγR, leading to increased ROS production and induction of proinflammatory cytokines, delayed FCP acidification and bacterial escape (2-4 h) from the phagosome, and only moderate levels of cytosolic replication. Uptake of serum (complement)-opsonized *Francisella* is mainly mediated by CR3 (complement receptor 3) and SRAs (scavenger A receptors), which lead to slowed FCP acidification and phagosomal escape (2-4 h) and results in modest cytosolic replication. Lastly, uptake of unopsonized *Francisella* is mediated by the MR (mannose receptor) and SE-N (surface-exposed nucleolin), leading to rapid acidification (15-30 min) and escape from the FCP (30 min-1h) and robust cytosolic replication.

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Chapter 2

A link between intraphagosomal biotin and rapid phagosomal escape in *Francisella*

A link between intraphagosomal biotin and rapid phagosomal escape in *Francisella*

By

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Abstract

Cytosolic bacterial pathogens require extensive metabolic adaptations within the host in order to replicate intracellularly and cause disease. In phagocytic cells such as macrophages, these pathogens must respond rapidly to nutrient limitation within the harsh environment of the phagosome. Many cytosolic pathogens escape the phagosome quickly (15-60 min) and thereby subvert this host defense, reaching the cytosol where they can replicate. While a great deal of research has focused on strategies used by bacteria to resist antimicrobial phagosomal defenses and transiently pass through this compartment, the metabolic requirements of bacteria in the phagosome are largely uncharacterized. We previously identified a novel *Francisella* protein, FTN_0818, as being essential for intracellular replication and involved in virulence *in vivo*. We now show that FTN_0818 is involved in biotin biosynthesis and required for rapid escape from the *Francisella*-containing phagosome (FCP). Addition of biotin complemented the phagosomal escape defect of the *FTN_0818* mutant, demonstrating that biotin is critical for promoting rapid escape during the short time that the bacteria are in the phagosome. Biotin also rescued the attenuation of the *FTN_0818* mutant during infection *in vitro* and *in vivo*, highlighting the importance of this process. The key role of biotin in phagosomal escape implies biotin may be a limiting factor during infection. This is the first demonstration of a bacterial metabolite that is required for phagosomal escape of an intracellular pathogen, providing new insight into the link between bacterial metabolism and virulence, likely serving as a paradigm for other cytosolic pathogens.

Introduction

Subversion of the hostile phagosomal environment is required for the survival of intracellular bacteria. While bacterial strategies to resist antimicrobial phagosomal defenses have been studied in great detail (1, 2), the ways in which bacteria counter phagosomal nutrient limitation are largely unknown. This is especially true for cytosolic pathogens that are often in the phagosome for a very limited time (15-60 min), before escaping this compartment to reach their replicative niche in the cytoplasm. During this brief and dynamic time, it is unclear if cytosolic pathogens require sequestration of nutrients or synthesis of *de novo* metabolites to promote their virulence strategies and escape the toxic phagosome.

Francisella tularensis is a cytosolic intracellular Gram-negative bacterial pathogen that uses a multitude of mechanisms to evade phagosomal host defenses (3). This pathogen is highly virulent and causes the potentially fatal disease tularemia. *Francisella novicida* U112 and *Francisella holarctica* LVS are less virulent yet highly related strains that are often used as models to study *F. tularensis*. Like other cytosolic bacterial pathogens, after initial contact with the host macrophage, *Francisella* spp. are taken up into a phagosome and rapidly escape (30-60 min) this compartment to reach and replicate within the cytosol (3-5). The mechanism by which *Francisella* escapes the *Francisella*-containing phagosome (FCP) is unknown, however, this process requires expression of the *Francisella* pathogenicity island (FPI), a cluster of 17 genes encoding a putative type VI secretion system (T6SS) (6-8).

We previously identified FTN_0818, a hypothetical protein with no known function, as one of the most critical genes for *F. novicida* replication in mouse macrophages (9). We also identified FTN_0818 as being required for infection of mice using an unbiased genome-wide, *in vivo* negative selection screen (10), a finding later supported by another group as well (11). FTN_0818 was also identified in an intracellular replication screen in arthropod-derived cells (12). Here we characterize FTN_0818 and highlight a novel adaptation of *Francisella* to the FCP by linking intraphagosomal metabolic requirements with rapid escape from this compartment.

Our studies demonstrate that FTN_0818 is required for growth in nutrient-limiting environments and, by use of a phenotypic microarray, we identified the enzymatic co-factor biotin as being able to fully complement the growth defect of the *FTN_0818* mutant. The addition of exogenous biotin alleviated the requirement of FTN_0818 for rapid FCP escape, intracellular replication and pathogenesis in mice. Our data suggest that biotin may be a limiting factor that, when absent, restricts cytosolic pathogens to the phagosome, blocking their escape and preventing them from reaching their replicative niche in the cytoplasm. This is the first time bacterial metabolism within the phagosome has been shown to be vital for rapid phagosomal escape and likely serves as a paradigm for other cytosolic bacterial pathogens.

Results

FTN_0818 is required for rapid escape from the FCP and intracellular replication

The screens that identified FTN_0818 as being required for *Francisella* virulence used transposon insertion mutants that can have defects in genes other than the one targeted. We therefore wanted to validate the identification of *FTN_0818* and constructed a clean deletion mutant in *F. novicida* (*ΔFTN_0818*). We infected macrophages and found that at 7.5 h post-infection (pi), wild-type (WT) bacteria replicated almost 10-fold whereas *ΔFTN_0818* was unable to replicate (Fig. 1). To ensure that this phenotype was due solely to deletion of *FTN_0818* and not an unknown second-site mutation, we complemented the deletion strain with a WT copy of *FTN_0818*. The complemented strain replicated to levels similar to the WT (Fig. 1). These data confirm that *FTN_0818* is indeed required for *F. novicida* replication in macrophage

Several steps are required for *Francisella* replication in macrophages including passage through the highly nutrient-limiting FCP (13), and we set out to determine at which step Δ*FTN_0818* was defective. To test whether Δ*FTN_0818* had a deficiency in entry, we infected macrophages and determined the levels of intracellular colony forming units (cfu) at 30 min pi, before any bacterial replication occurs. WT, Δ*FTN_0818* and the complemented strain were present at similar levels (Fig. 2A), demonstrating that FTN_0818 is not required for initial uptake of *F. novicida* by macrophages.

Escape from the FCP is essential for *Francisella* to evade this non-permissive environment to successfully replicate in the cytosol (4), and this process requires the expression of *Francisella* pathogenicity island (FPI) genes. We therefore measured the expression of the FPI gene *iglA* during macrophage infection with either the WT or Δ*FTN_0818* strain. At 30 min pi, *iglA* expression in the Δ*FTN_0818* mutant was significantly lower than that in the WT strain, although its expression increased by 4 h pi (Fig. 2B). The kinetics of FCP escape correlated with this *iglA* expression defect. At 30 min pi, both WT and *ΔFTN_0818* were almost exclusively (>95%) within phagosomes (Fig. 2C-D, G). At 3 h pi, WT had largely escaped as >95% of the bacteria were cytosolic (Fig. 2E, G), while *ΔFTN_0818* was still almost completely retained within the FCP (Fig. 2F, G). However, *ΔFTN_0818* escaped the FCP at 6 h pi after *iglA* expression increased in this strain (Fig. 2G). These results indicate that FTN_0818 is required for WT expression of an FPI gene early in infection and subsequent rapid escape from the FCP, correlating with the severe growth defect of the Δ*FTN_0818* mutant during macrophage infection.

FTN_0818 plays a role in biotin metabolism

Since FTN_0818 is required for regulation of *iglA* in the nutrient-limiting FCP, a process critical for escape from this compartment (Fig. 2B), and recent literature has emphasized the importance of *Francisella*'s metabolic state for virulence (14), we hypothesized that FTN_0818 may play a role in the acquisition of nutrients or production of metabolites. To determine if FTN_0818 might be involved in these processes, we compared the

growth of *ΔFTN_0818* in rich (Tryptic soy broth, TSB) and defined minimal medium (Chamberlain's medium, CHB) (15). We found that *ΔFTN_0818* replicated to WT levels in TSB (Fig. 3A), however, it exhibited a severe growth defect in CHB in comparison to the WT and complemented strains (Fig. 3B). These data demonstrate that FTN_0818 is specifically required for growth in a nutrient-limiting environment (13), suggesting that it may contribute to the acquisition and/or biosynthesis of nutrients that are required for growth in these conditions.

In order to determine if a specific metabolite could complement the growth defect of Δ*FTN_0818* in minimal media, we used a Biolog Phenotypic Microarray. As expected, the WT strain grew well in minimal medium [modified CHB (Table 2)] while the *FTN_0818* mutant did not (Fig. 4A). Only biotin was able to complement growth of the *FTN_0818* mutant (Fig. 4A). We further validated these results, showing that biotin complemented *ΔFTN_0818* growth in CHB (Fig. 4B). These data suggest that the *FTN_0818* mutant has insufficient levels of biotin and that FTN_0818 is involved in the acquisition or synthesis of biotin in *F. novicida*.

Biotin is required for numerous metabolic pathways and is covalently attached (biotinylation) to proteins to facilitate their activity. Therefore, one method for quantifying biotin levels in bacteria is to measure the level of biotinylated proteins. Using immunoprecipitation with anti-biotin covered beads, we quantified the total concentration of biotinylated proteins and detected much lower levels in the *FTN_0818* mutant as

compared to WT (Fig. 4C). Furthermore, exogenous addition of biotin to CHB restored the levels of biotinylated proteins in *ΔFTN_0818* to those of the WT (Fig. 4C). Therefore, these data further suggest that the *FTN_0818* mutant has a biotin deficiency.

Biotin alleviates the requirement of FTN_0818 for phagosomal escape and replication in macrophages

We next tested whether exogenous biotin could also rescue the intracellular defects of the *ΔFTN_0818* mutant. At 30 min pi, exogenous biotin complemented *iglA* expression in the Δ*FTN_0818* mutant (Fig. 5A). We employed immunofluorescence microscopy to determine if FCP escape kinetics correlated with the rescue of *iglA* expression in the presence of biotin. We observed that Δ*FTN_0818* had a phagosomal escape defect (Fig. 5B-G, K), similar to our previous results using electron microscopy (Fig. 2F-G). At 30 min pi, biotin-supplemented *ΔFTN_0818* localized to the FCP (Fig. 5K). However, at 2 h pi, this strain was within the cytosol (Fig. 5H-K), similar to WT. These data clearly demonstrate that biotin is required for the rapid escape of *Francisella* from the FCP.

Since biotin rescued *iglA* gene expression and subsequent escape of the *FTN_0818* mutant, and escape is required for intracellular replication, we tested whether biotin could also rescue replication. During macrophage infection, the WT strain replicated nearly 30-fold while *ΔFTN_0818* exhibited a severe replication defect (Fig. 5L), in agreement with our previous data (Fig. 1). However, when biotin was added to the macrophages at the

time of infection, the *ΔFTN_0818* replication defect was significantly complemented (Fig. 5L). We further tested whether pre-treatment with biotin prior to infection would rescue the intracellular growth defect of the *FTN_0818* mutant, or if biotin had to be present during the infection. *ΔFTN_0818* grown in CHB supplemented with biotin overnight, but without exogenous biotin during infection, was unable to replicate in macrophages (Fig. 6). This demonstrates that biotin must be present at the time of infection to facilitate replication. These data demonstrate that biotin is required to promote escape when the bacteria are present within the FCP.

FTN_0818 is required for FCP escape in multiple *Francisella* species

In order to determine whether the role of FTN_0818 was conserved in other *Francisella* species, we first generated a deletion mutant lacking the *FTN_0818* ortholog, *FTT_0941* (99% amino acid identity), in the human pathogenic *Francisella tularensis* strain SchuS4. Similar to our findings with *F. novicida*, the *FTT_0941* mutant in *F. tularensis* had a defect in escape from the FCP (Fig. 7). However, when biotin was added to the media, the *FTT_0941* mutant escaped with WT kinetics (Fig. 7). We also generated and tested a mutant in the Live Vaccine Strain (LVS), a derivative of highly pathogenic *F. holarctica*. We found that the *FTN_0818* ortholog, *FTL_1266* (99% amino acid identity), was also required for LVS escape from the phagosome, as well as growth in minimal media, and that these phenotypes were complemented by biotin (Fig. 8A-D). Furthermore, *FTL_1266* was also required for replication in macrophages (Fig. 8E), in agreement with

the role of *FTN_0818* in *F. novicida*. Together, these data highlight the conserved role of FTN_0818 in multiple *Francisella* species.

FTN_0818 is necessary for pathogenesis in mice, and this requirement is alleviated by biotin

We and others identified *FTN_0818* as being required for *Francisella* virulence in mice using *in vivo* screens (10, 11). To validate these findings, we performed competition experiments in which a 1:1 mix of the WT and *ΔFTN_0818* or the complemented strain was used to infect mice. Forty-eight hours pi, *ΔFTN_0818* levels were 1 to 2 logs lower in spleens compared to WT (Fig. 9A). In contrast, the complemented strain colonized the spleen of mice similarly to WT bacteria (Fig. 9A). We also infected mice with the WT or *ΔFTN_0818* strain separately and determined that *ΔFTN_0818* was attenuated 100-fold in the spleen (Fig. 9B) and almost 10-fold in the skin (Fig. 9C), as compared to WT. In agreement, the *FTN_0818* ortholog, *FTL_1266*, was required to reach WT LVS levels in spleens 48 h pi (Fig. 8F). Together, these results demonstrate the requirement of FTN_0818 for *Francisella* virulence *in vivo*.

To determine if exogenous biotin could rescue the attenuation of the *FTN_0818* mutant during *in vivo* infection, as we observed during macrophage infection, we added biotin to the inoculum. *ΔFTN_0818* without biotin was attenuated nearly 10-fold compared to WT in the skin at the site of infection, whereas when biotin was added, *ΔFTN_0818* was

present at WT levels (Fig. 9D). Furthermore, addition of biotin resulted in rescue to levels similar as genetic complementation, as observed with the complemented strain (Fig. 9D). These results confirm that FTN_0818 is required for virulence in mice and that biotin can alleviate this requirement. Taken together, we have characterized a novel metabolic protein that links the requirement for biotin in the phagosome with rapid phagosomal escape and virulence *in vivo*.

Discussion

Evasion of the harsh phagosomal environment is imperative for the survival of intracellular bacterial pathogens. We have characterized a novel metabolic protein, FTN_0818, revealing a unique link between metabolism and rapid escape from the FCP during *F. novicida* infection of macrophages. Exogenous biotin overrode the requirement of FTN_0818 for rapid phagosomal escape, replication in macrophages, and *in vivo* pathogenesis. Pre-treatment with biotin prior to infection of macrophages was unable to complement the mutant strain. However, when biotin was added at 6 h (after the mutant escaped the phagosome), or when the mutant was microinjected with biotin into the host cytosol (bypassing the phagosome), the mutant's replication defect was rescued (Fig. 10A, B). This suggests that *Francisella* requires biotin in the FCP to promote rapid escape and in the cytosol for intracellular replication. These data contribute to current literature highlighting the link between *Francisella* metabolism and virulence (3). Specifically, it has been shown that utilization of glutathione as a cysteine source is required for intracellular replication (14). Similarly, utilization of uracil has been shown to be required for inhibition of the neutrophil respiratory burst (16). It will be interesting to delineate the full metabolic requirements of *Francisella* within host cells, and specifically determine how these control phagosomal escape and other virulence traits.

In support of our current data, biotin biosynthetic genes have been identified as being important for *Francisella* replication *in vitro* and *in vivo* (9, 12, 17). In addition, Wehrly *et al.* previously published a transcriptional profile of *F. tularensis* within the

macrophage and identified *bioB*, a gene required for biotin biosynthesis, as being up-regulated (18). Additionally, Asare *et al.* published a screen for mutants with defects in phagosomal escape and identified *birA*, a biotin associated gene (12). Taken together, these data provide additional evidence that biotin, and biotin associated genes, play important roles during intracellular infection by *Francisella*.

Bioinformatic analysis revealed that FTN_0818 shares high sequence similarity with the carboxyl esterase family of proteins, similarly to lipases and serine proteases, this family of proteins harbors a conserved catalytic triad: a serine, glutamic acid or aspartic acid, and histidine (19). Our analysis hypothesized serine residue S151 of FTN_0818 to be the required catalytic serine within this triad (data not shown). Interestingly, we showed that when the putative catalytic serine (S151) in FTN_0818 was mutated to an alanine residue, *Francisella* could no longer grow in minimal media (this phenotype was rescued by exogenous biotin) (Fig 11A). This was not attributable to a decrease in the level of expression of the point mutant compared with WT FTN_0818 (Fig 11B). Futhermore, disruption of this catalytic residue led to retention of *Francisella* within the FCP (which could be rescued by the addition of biotin) (Fig 11C), and inhibition of replication in macrophages and during *in vivo* infection (Fig 11D and E). These data provide evidence that the FTN_0818 is a putative member of the carboxyl-esterase family of proteins and harbors a catalytic serine (S151), required for FTN_0818 activity.

Taken together, the work presented here strongly suggests that biotin availability may be a limiting factor for *Francisella* spp., and likely other bacterial pathogens, during infection. Biotin has been reported as being required for *Mycobacterium tuberculosis* virulence in mice and *Vibrio cholera* colonization of the mouse intestine, both through unknown mechanisms (20-22). In addition, several antimicrobials target the biotin pathway by causing the degradation of biotin or biotin precursors including amiclenomycin, actithiazic acid and the biotin analogue α-dehydrobiotin (23-25), further demonstrating the importance of biotin during infection as well as the therapeutic utility of limiting biotin availability to pathogens.

Our data show that biotin is required in the phagosome to promote rapid escape, suggesting that biotin is limiting in this compartment. Iron is also limiting in the phagosome and numerous host factors such as transferrin play a critical role in the control of infection by depleting phagosomal iron. Similarly, the host innate immune system has been shown to target biotin. Chicken embryo fibroblasts and yolk-sac macrophages induce the production of avidin, which binds and sequesters biotin, in response to *E. coli* infection, treatment with lipopolysacchride (LPS), or interleukin-6 (26, 27). These data suggest that sequestration of biotin may be a form of nutritional immunity by the host innate immune system and support the idea that biotin might be a critical and limited commodity during infection. Sequestration of biotin could restrict cytosolic pathogens to the phagosome, blocking their escape and preventing them from reaching their replicative niche in the cytoplasm. Understanding more about how specific bacterial metabolites are generated and how the host attempts to sequester these compounds will provide new

insight into host-pathogen interactions and may reveal new targets for the development of novel antimicrobials to inhibit bacteria at an early step in pathogenesis and combat infection.

Materials and Methods

Bacterial strains

WT *F. novicida* strain U112 was grown in TSB/0.2% cysteine or Chamberlain's Medium (CHB) (Teknova, Hollister, CA) (15), while LVS cultures were grown in modified Mueller-Hinton (MH) broth described previously (9). *F. tularensis* (SchuS4) was cultured in modified Mueller Hinton broth (MMH broth supplemented with 10 g/L tryptone, 0.1% glucose, 0.025% ferrous pyrophosphate, 0.1% L-cysteine, and 2.5% calf serum) or in Brain Heart Infusion (BHI) broth (BHI supplemented with 50ug/mL hemin, 1.4% agar (w/v), and 1% (v/v) IsoVitalex; BBL, Cockeysville, MD).

Mutagenesis and complementation

To generate the kanamycin (kan)-marked *FTN_0818* deletion mutant, the regions of the chromosome 5' and 3' to *FTN_0818* were amplified by PCR, using primers found in *SI Table 1*. A kan-resistance cassette flanked by Flp-FRT recombinase sites was introduced between these flanking regions using overlapping PCR. The sewn PCR construct was gel purified (Qiagen, Valencia, CA) and chemically transformed into competent U112 as previously described (9).

In order to create the *FTN_0818* clean deletion mutant, plasmid pLG72 encoding the flippase gene was transformed into the kan-marked *FTN_0818* mutant and clones in which the kan cassette had been deleted were isolated as previously described (28). To complement the deletion, constructs were made using overlapping PCR by amplification of the 5' and 3' regions, the wild-type gene, and a kan-cassette for selection and this

construct was transformed into the *FTN_0818* clean deletion mutant. All strains were verified by PCR and sequencing (Eurofins EWG Operon, Huntsville, AL).

The serine to alanine *FTN0818* point mutant (*FTN0818*-S151A) was constructed by overlapping PCR using a primer encoding a single amino acid change and using a chloramphenicol cassette for selection. This construct was transformed into the *FTN0818* kan-resistant deletion mutant and selected for on chloramphenicol plates chloramphenicol (3 μg/ml).

To generate a mutant lacking the *FTN_0818* ortholog, *FTL_1266*, in the *Francisella holarctica* Live Vaccine Strain (LVS), we used a group II intron (as previously described) (29). We constructed primers targeting *FTL_1266* using the TargeTron Gene Knockout System (Sigma-Aldrich) and the PCR product was cloned into the *Francisella* targeting vector, pKEK1140, a generous gift from Dr. Karl Klose (UT San Antonio). LVS was then transformed with the targeting vector and *FTN_1266* deletion clones (Δ*FTL_1266*) were isolated. All cloning primers are listed in *SI Table 1*.

Wild-type *F. tularensis* (SchuS4) was obtained from the Centers for Disease Control and Prevention (CDC, Atlanta, GA). SchuS4 was cultured in modified Mueller Hinton broth (MMH broth supplemented with 10 g/L tryptone, 0.1% glucose, 0.025% ferrous pyrophosphate, 0.1% L-cysteine, and 2.5% calf serum) or in Brain Heart Infusion (BHI) broth (BHI supplemented with 50ug/mL hemin, 1.4% agar (w/v), and 1% (v/v) IsoVitalex; BBL, Cockeysville, MD). Counter selection for SchuS4-pΔ*FTT_0941* co-

integrants was performed on cysteine heart agar (CHA) containing 5% sucrose, plus kanamycin at 10 ug/mL when necessary.

The Δ*FTT_0941* construct was created by PCR overlap extension using primers listed in Table S1. The PCR product was then ligated into plasmid pXB186 , using SacI and BamHI sites, and p*DFTT_0941* was introduced by electroporation into electrocompetent SchuS4. Electrocompetent cells were prepared using an overnight MMH broth culture of SchuS4 to inoculate 50 mL of fresh MMH broth. The culture was then incubated, shaking at 37˚C until reaching an OD_{600} of 0.3, transferred to a 50 mL conical tube, pelleted at 4˚C, resuspended in 4 mL of prechilled 0.5 M sucrose, divided into 2x 2 mL screw-capped vials and centrifuged at 4˚C. The cells were then washed (x3) with 2 mL of prechilled 0.5 M sucrose. Next, the washed pellet was resuspended into 70 uL 0.5 M sucrose, placed on ice, and 70 uL of the purified pFTT_0941 was added. The mixture was transferred to a prechilled 0.1 cm electroporation cuvette and electroporated (1.5 kV, 25 uF, and 200 ohms). Immediately after, 1 mL of MMH broth was added, transferred to a 50 mL conical tube, and the cells were incubated, shaking at 37˚C for 2 hr. Cells were collected by centrifugation, resuspended in 500 uL of MMH broth and spread onto BHI agar plates containing 10 ug/mL kanamycin. After 3 days of incubation at 37˚C, colonies were patched onto fresh BHI agar plates, grown overnight, and used to inoculate 1 mL of MMH broth without antibiotics. The resulting broth culture was incubated with shaking at 37˚C until early log phase and spread onto CHA-5% sucrose plates. Sucrose-resistant colonies (4-5 days later) were then patched onto fresh CHA sucrose plates and the plates incubated at 37˚C. The resulting colonies were then replica-plated onto BHI agar plates in

the presence and absence of kanamycin. The resulting clones were re-streaked onto BHI agar and a single colony was selected, cultured, and screened by PCR for the *FTT_0941* deletion.

Infection stocks of *F. tularensis* SchuS4 were prepared by pelleting bacterial cells from mid-log phase cultures via centrifugation, resuspending cells in fresh MMH with 20% glycerol, and cryogenically storing 100-200 uL aliquots at -80˚C. Infection stocks were enumerated by dilution plating several times over a span of 3 months to ensure stability of the cryogenically preserved bacteria. To initiate infections, the frozen stocks were thawed and diluted to the desired concentration in sterile PBS. All primers used in this study are listed in Table 1.

Macrophage Preparation and Infection

Bone marrow-derived macrophages (mBMDMs) were prepared as described previously (17). Briefly, bone marrow was collected from the femurs of mice. Bone marrow cells were plated into sterile petri dishes and incubated in DMEM supplemented with 10% heat-inactivated FBS and 10% macrophage colony-stimulating factor (M-CSF)-conditioned medium (collected from M-CSF-producing NIH3T3 cells). Bone marrow cells were incubated at 37°C with 5% CO_2 and harvested after 6 days. All mBMDMs were incubated before and during infection in 24-well plates at 37°C with 5% CO_2.

For infection, mBMDMs were seeded at 5 x 10^5/well and incubated overnight at 37°C with 5% CO_2. mBMDMs were infected at a multiplicity of infection (MOI) of 10:1 (*F.*

novicida and *F. tularensis*) or 100:1 (LVS) and colony forming units (cfu) were quantified at specified timepoints (9). Macrophage experiments were analyzed by using the Student's unpaired *t* test. *,$p<0.05$, **, $p<0.001$, ***, $p<0.0001$.

Growth Curves

Bacteria were subcultured to an OD_{600} of 0.03 in TSB/0.2% cysteine or Chamberlain's Medium (CHB) (Teknova, Hollister, CA) (15) for *F. novicida*, while LVS cultures were grown in modified Mueller-Hinton (MH) broth described previously (9). Subcultures were read hourly using a SynergyMX BioTek plate reader (Applied Biosystems, Foster City, CA). Biotin (0.25 μM) (Merck KGaA, Darmstadt, Germany) or pimelate (3 μg/mL) (Sigma-Aldrich, St. Louis, MO) was added when appropriate.

Transmission Electron Microscopy (TEM)

Infected mBMDMs were fixed with 1 ml of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight at 4°C. Cells were then post-fixed in 1% buffered osmium tetroxide, dehydrated, and embedded in Eponate 12 resin (Ted Pella Inc., Redding, CA). Ultrathin sections were cut on a Leica UC6rt ultramicrotome (Leica Microsystems, Bannockburn, IL) at 70-80 nm and counter-stained with 4% aqueous uranyl acetate and 2% lead citrate. Sections were examined using a Hitachi H-7500 transmission electron microscope (Hitachi High Technologies of America, Inc., Pleasanton, CA) equipped with a Gatan BioScan CCD camera. Multiple fields were examined for a total of 100 bacteria/timepoint. The criterion for being considered within the phagosome was

visualization of a phagosomal membrane that was at least 90% intact surrounding a bacterium.

RNA collection and qRT-PCR

RNA was collected during macrophages infections as previously described (9). qRT-PCR was performed with the Power SYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems, Foster City, CA) and primers (Table S1) using the StepOnePlus Real-time PCR System (Applied Biosystems).

Nutrient Supplementation Phenotypic Microarray

Metabolic profiling was performed according to modified Biolog guidelines (http://www.biolog.com). An 85% transmittance cell culture was made by using a damp cotton swab to transfer colonies from TSA/0.1% cysteine into 15 mL modified CHB (Table 2), and measuring in a turbidometer. Inoculating media was prepared by mixing 50mL of 1.2x mCHB (pH5.5), with 360 µL dye (Biolog Dye Mix A, cat#74221), and 360 µL of the 85% transmittance cells. Sterile water was added for a final volume of 60mL. Phenotypic Microarray plate 5 (PM5, Biolog cat #12141) was inoculated with 100 µL of the inoculating media per well. The plate was incubated in a Biolog Omnilog Phenotypic Microarray incubator for 48h at 37°C.

Immunoprecipitation

Bacteria were subcultured to an OD_{600} of 0.03 in TSB/0.2% cysteine or CHB and grown for 4 h shaking at 37°C, pelleted and resuspended in PBS. Bacteria were then freeze-

thawed 3 times, centrifuged to discard unlysed bacteria, and the whole cell lysate (WCL) was applied to Protein G sepharose beads (Invitrogen, Frederick, MD) conjugated to anti-biotin antibody (Invitrogen). Eluted proteins were then quantified using the BCA Protein Assay (Thermo Fisher Scientific, Rockford, IL).

Immunofluorescence Microscopy

For immuno-fluorescence, mBMDMs were infected at an MOI of 100:1 and fixed at specified time points (30). Staining, imaging, and quantification of phagosomal escape was performed as previously described (30). Statistics indicate average percent escape for 3 independent experiments.

Microinjection

Microinjection of J774A.1 cells. 2 x 10^5 cells of the murine macrophage-like cell line J744 were cultured overnight in glass bottom Petri dishes (MatTek Corporation, Ashland, MA) in DMEM supplemented with 10 % FBS. Before the injection, the medium was replaced by fresh DMEM containing 10 % FBS, 5 μg/ml of gentamicin and 1 μg/ml of Cytochalasin D and the cells were allowed to recover for 90 minutes. Plate-grown GFP-expressing bacteria were resuspended in saline at a density of 1 X 10^9 cfu/ml and mixed with rhodamine dextran (25 mM solution in Tris-HCl pH 7.5, Sigma Aldrich). Injections were carried out with standardized Femtotips II (Eppendorf, Hamburg, Germany) with an injection pressure of 60 hPa. Injected cells were washed with DMEM containing gentamicin and kept in DMEM with 10 % FBS and 5 μg/ml of gentamicin with or without 25 μM biotin at 37°C and 5 % CO2. Pictures were taken after 2, 14, and 24 h

with a live cell microscope (Nikon Eclipse Ti-E equipped with an Andor iXon + EMCCD camera). For each injection experiment, on average 150 cells per strain were injected whereby 2 – 10 bacteria and the injection marker rhodamine dextran were delivered into the cell cytosol. The number of bacteria per cell was determined by microscopic counting at 24 h after injection. Twenty-four hour time points were possible because J774A.1 cells do not undergo inflammasome-dependent cell death, therefore are still viable at 24 h post injection.

To assess the statistical significances of the microinjection data, the number of bacteria/cell was determined by microscopic counting and categorized. The total number of infected cells in each category was the basis for the statistical comparisons that were performed using the non-parametric Wilcoxon rank-sum test.

Mouse infections

For mouse infections, female C57BL/6 mice (6-8 wk) (Jackson Laboratory, Bar Harbor, ME) were housed under specific pathogen-free housing at Emory University. Experimental studies were performed in accordance with the Institutional Animal Care and Use Committee guidelines. Competitive index [CI = (mut output/WT output)/(mut input/WT input] and infections with single strains were carried out as described previously (9). Statistical analysis for CI experiments described previously (10).

Acknowledgements

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Tables

Table 1. Primers used in this study.

FTN_0818-Kan insertion mutant		
	FTN0818 f1	aaccattagtcagtattgac
	FTN0818 r1	accactaaatagtacacaac
	FTN0818 arm1-sKAN fwd	ggagtcatatatcaaaccaaatctctttgggttgtcact
	FTN0818 arm1-sKAN rev	ggagtcatatatcaaaccaaatctctttgggttgtcact
	FTN0818 sKAN-arm2 fwd	cagaattggttaattggttgttccttattaaatcttgtgtac
	FTN0818 sKAN-arm2 rev	gtacacaagatttaataaggaacaaccaattaaccaattctg
	FTN0818 check	aaacatcagaggtattggag
	FTN0818 checkR	attgatatcggagctgaatc
FTN_0818 complement		
	FTN0818 f1	
	FTN0818 r1	
	818Comp arm1 F	agaattggttaattggttgtatattggtttgatatatgact
	818Comp arm1 R	agtcatatatcaaaccaatatacaaccaattaaccaattct
	818Comp sKan F	attgagtttataaacaaaagataaatctctttgggttgtcact
	818Comp sKan R	agtgacaacccaaagagatttatcttttgtttataaactcaat
FTN_0818 clean deletion		
	FTN0818 f1	
	FTN0818 r1	
	arm1-FRTsKAN fwd	ggagtcatatatcaaaccaagaggtcgacggtatcgataa
	arm1-FRTsKAN rev	ttatcgataccgtcgacctcttggtttgatatatgactcc
	FRTsKAN-arm2 fwd	tatcgatcctgcagctatgcttccttattaaatcttgtgtac
	FRTsKAN-arm2 rev	gtacacaagatttaataaggaagcatagctgcaggatcgata
	FTN0818 check	
	FTN0818 checkR	
LVS FTL_1266 disruption		
	FTL1266 r1	attgatatcggagctgaatc
	FTL1266 f1	gcaccaccaaatccagtacc
	FTL_1266 Check F1	atttctcaaatagagtcagc
	IBS-1	aaaaaagcttataattatccttataggtccgataagtgcgcccagataggtg
	EBS1d-1	cagattgtacaaatgtggtgataacagataagtccgataaattaacttacct ttctttgt
	EBS2-1	tgaacgcaagtttctaatttcggttacctatcgatagaggaaagtgtct
qRT-PCR		
FTN_0818	*FTN_0818* real-time F	acagcaaggaacttatgttga
	FTN_0818 real-time R	ccaaggatgcccatgaaaccat
uvrD qRT-PCR	*uvrD* real-time F	gggatgtcgccttttgattttc
	uvrD real-time R	ctcttttgtcccttgtgcttgc
iglA qRT-PCR	*iglA* real-time F	cgccaacaactaggactctg
	iglA real-time R	tcccaaattcaaggttgatg
***F. tularensis* SchuS4 FTT_0941 deletion**		
	FTT_0941 F2	agggatccatatgtgagtcttggttgctcaggag
	FTT_0941 R1-SacI	gcattattagaccctaaaatagctttgaataaggtgattgag
	FTT_0941-F1-BamHI	aggagctcaggtattggagcagttatcgcagcag
	FTT_0941-R2	aagctattttagggtctaataatgcttctaatgatggattgtatgg

Table 2. Modified Chamberlain's medium

	CHB	mCHB
	g/L	g/L
KH_2PO_4	1	4.096
H_2HPO_4	1	1.707
NaCl	10	10
$MgSO_4$*7H2O	0.135	0.135
$FeSO_4$*7H2O	0.002	0.002
Ca-Panthothenate	0.002	0.002
Thiamine-HCl	0.004	0.004
Spermine-PO_4	0.04	0.04
Ammonium Sulfate		2
Glucose	4	2
Pyruvate		2
His	0.2	0.155
ARG	0.4	0.174
MET	0.4	0.149
CYS	1.2	1.158
LYS	0.4	0.183
PRO	2	0.115
LEU	0.4	0.131
ILE	0.4	0.131
VAL	0.4	0.117
THR	2	0.119
TYR	0.4	0.181
ASP	0.4	0.133
GLU		0.147
PHE		0.165
GLN		0.146
ASN		0.132
ALA		0.089
GLY		0.075
TRP		0.204
SER	0.4	0.105
pH	6.2-6.4	5.5

Figures

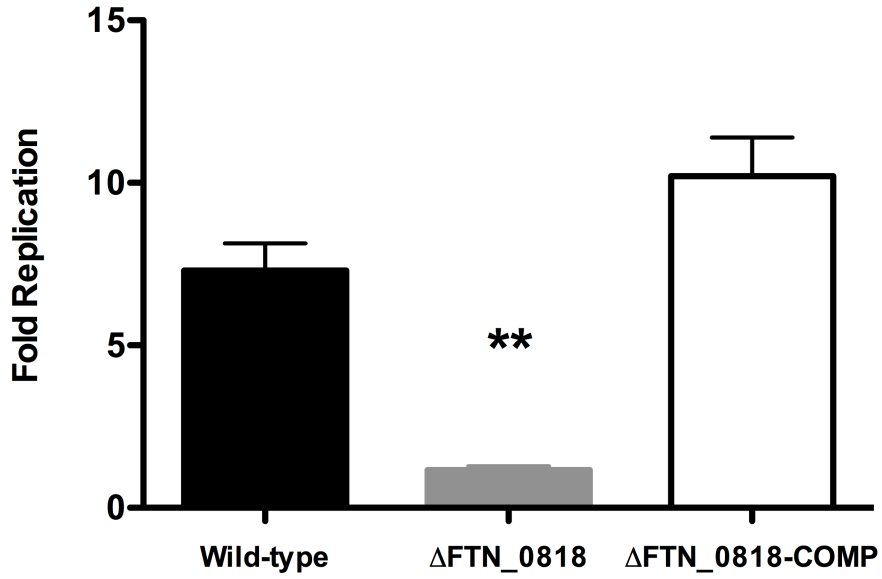


Figure 1. FTN_0818 is required for replication in macrophages. Macrophages were infected with WT, Δ*FTN_0818*, and Δ*FTN_0818* complemented (FTN_0818-COMP) *F. novicida* strains. Colony-forming units were quantified at 30 min and 7.5 h post infection (pi), and fold replication was calculated. ***P*<0.001.

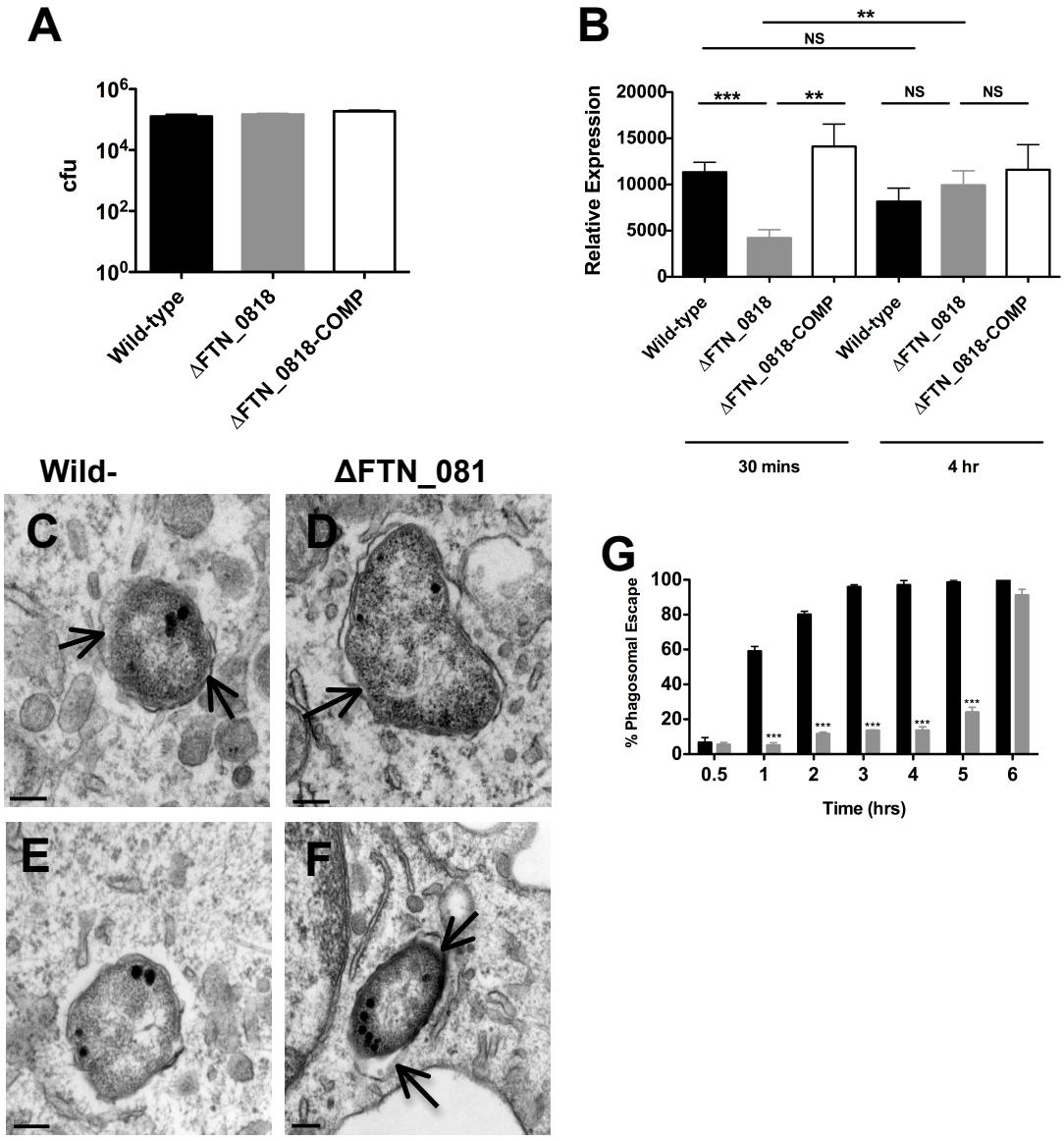


Figure 2. FTN_0818 is required for rapid phagosomal escape. (A and B) Macrophages were infected with the indicated strains, and colony-forming units were quantified at 30 min pi (A) or qRT-PCR was used to measure the expression of *iglA* and normalized to the expression of *uvrD* at 30 min and 4 h pi (B). (C-F) Transmission electron microscopy of infected macrophages at (C and D) 30 min pi and (E and F) 3 h pi (arrows, intact FCP). (G) Phagosomal escape of WT (black) and Δ*FTN_0818* (gray) was quantified 30 min to 6 h pi. One hundred bacteria per condition were viewed and the percentage of phagosomal

escape was determined for three independent experiments. *P <0.05; **P <0.001; ***P <0.0001.

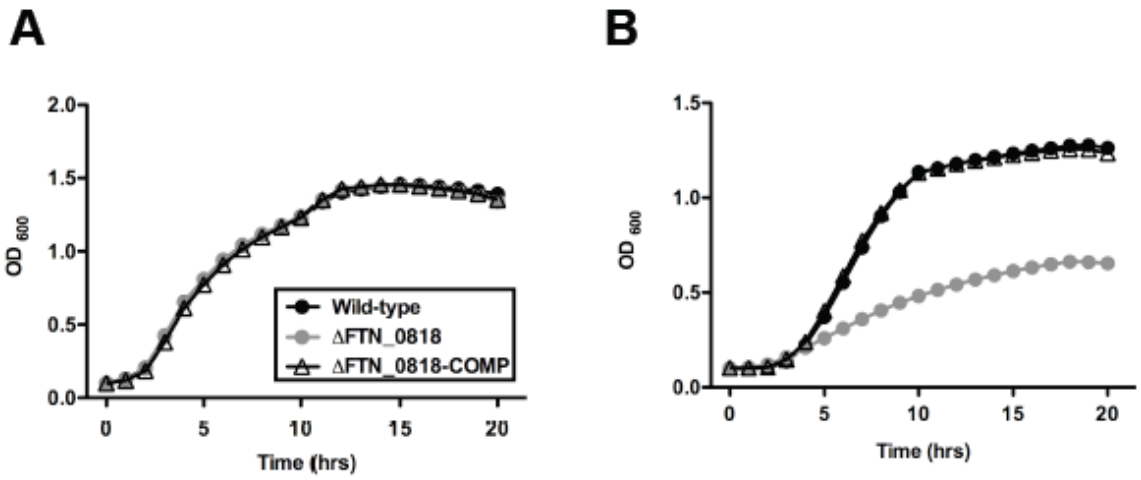
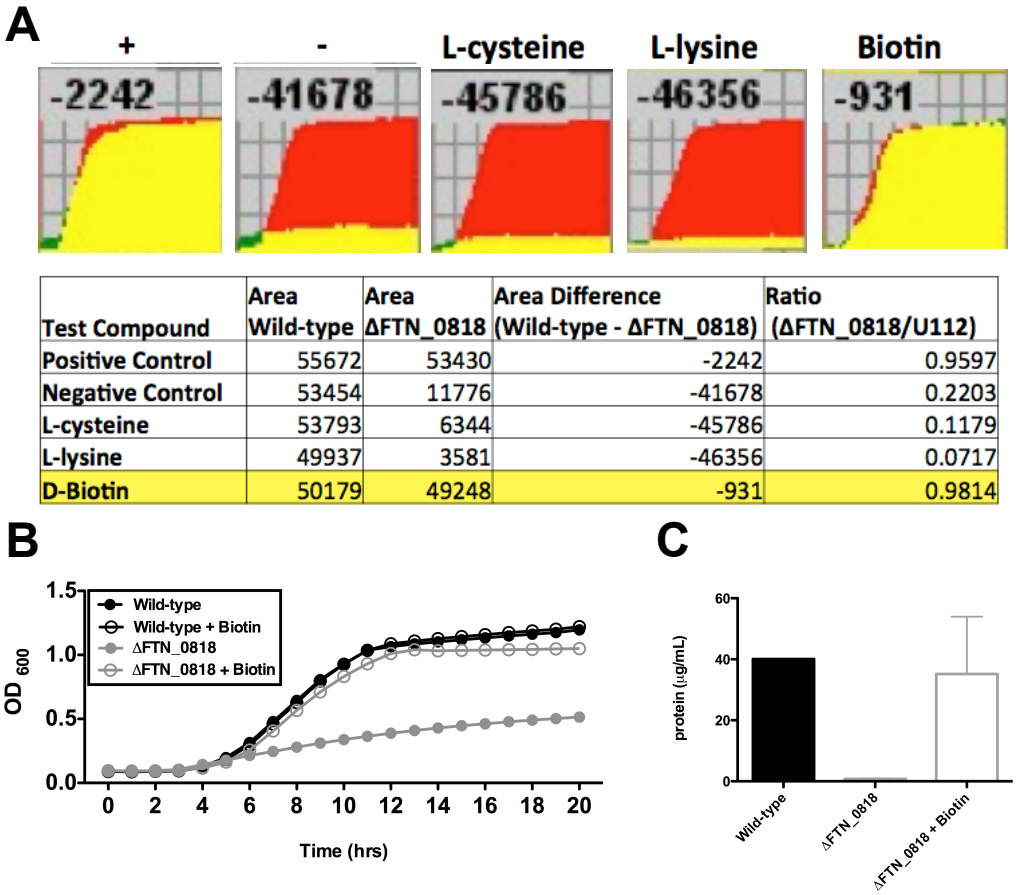


Figure 3. FTN_0818 is required for replication in minimal medium. *F. novicida* strains were grown overnight in TSB and subcultured into (A) TSB or (B) CHB and the OD_{600} was measured every hour (black circles = WT; grey circles =*ΔFTN_0818*; clear triangles = *ΔFTN_0818-COMP*).



Test Compound	Area Wild-type	Area ΔFTN_0818	Area Difference (Wild-type - ΔFTN_0818)	Ratio (ΔFTN_0818/U112)
Positive Control	55672	53430	-2242	0.9597
Negative Control	53454	11776	-41678	0.2203
L-cysteine	53793	6344	-45786	0.1179
L-lysine	49937	3581	-46356	0.0717
D-Biotin	50179	49248	-931	0.9814

Figure 4. Biotin can complement *ΔFTN_0818* growth in minimal media. (A) WT or *ΔFTN_0818* *F. novicida* strains were subcultured into modified Chamberlain's medium, added to Biolog Phenotypic Microarray plate 5 (PM5) and incubated for 48 h at 37°C (wild-type growth = red, *ΔFTN_0818* growth = green, overlapping growth = yellow). Areas under the curve and ratio of wild-type/*ΔFTN_0818* growth are shown for the Biolog positive and negative controls, L-cysteine, L-lysine, and D-biotin. Growth to at least 80% of wild-type levels was used as a cutoff for full complementation. (B) WT and *ΔFTN_0818* were grown in CHB with or without pimelate and the OD_{600} was measured every hour. (C) The concentration of biotinylated proteins in whole cell lysates of all strains was quantified after immunoprecipitation with anti-biotin antibodies

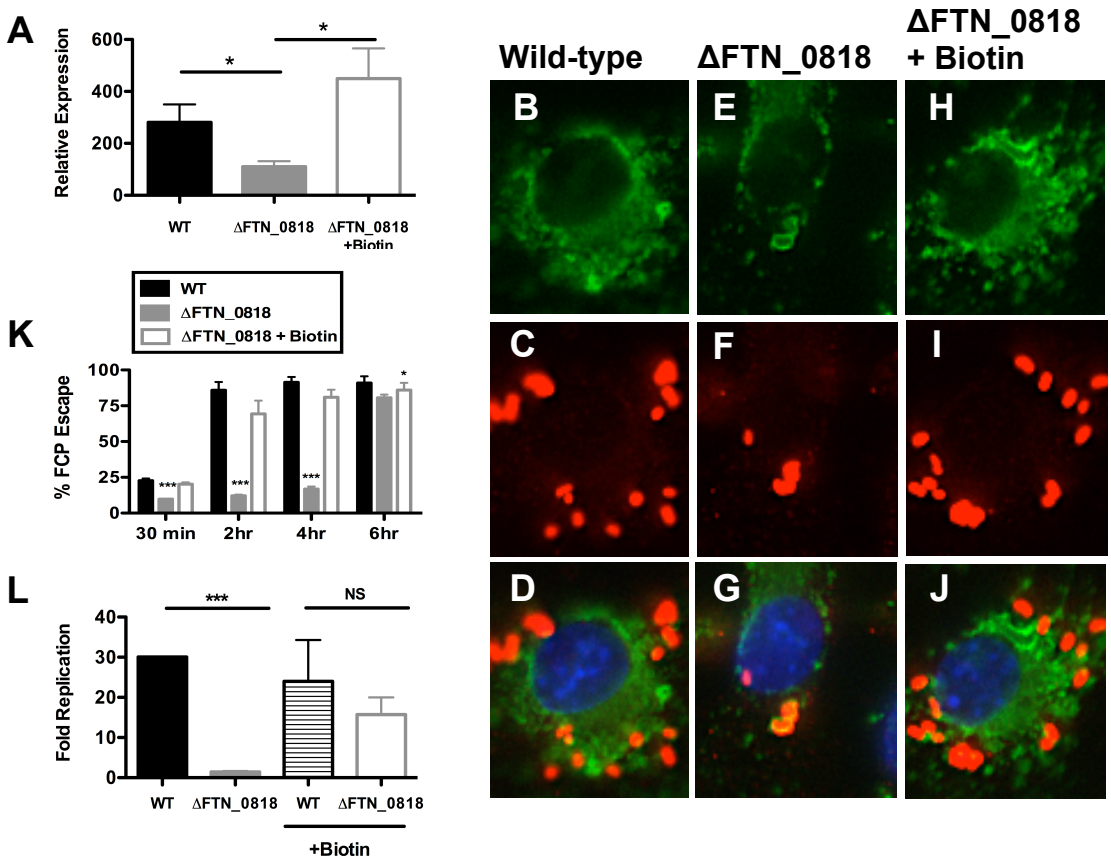


Figure 5. Biotin rescues rapid phagosomal escape and the *ΔFTN_0818* replication defect in macrophages**.** Macrophages were infected and (A) qRT-PCR was used to measure the expression of *iglA* and normalized to the expression of *uvrD* at 30 min pi, and (B) immunofluorescence microscopy was used to determine escape kinetics of WT (B-D), *ΔFTN_0818* (E-G), and *ΔFTN_0818* supplemented with biotin (H-J) 2 h pi FITC stained LAMP-1 (green), anti-*Francisella* (red), and DAPI (blue). (K) Two hundred bacteria were counted per sample and co-localization with LAMP-1 was used as a marker for phagosomal localization. (L) Macrophages were infected with WT or *ΔFTN_0818* strains in media with or without biotin. CFUs were quantified 30 min and 6 h pi, and fold replication was calculated.

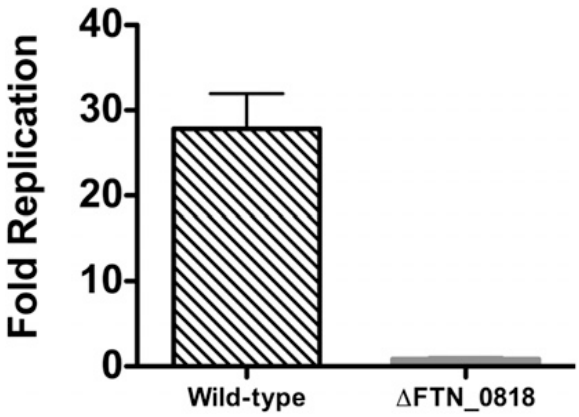


Figure 6. FTN_0818 growth defect in macrophages is not rescued by pretreatment with biotin. WT and Δ*FTN_0818 F. novicida* strains were grown overnight in CHB supplemented with biotin and used to infect macrophages in the absence of biotin at a 10:1 (bacteria: macrophage) ratio. Intracellular colony-forming units were enumerated at 30 min and 5 h pi, and fold replication was calculated.

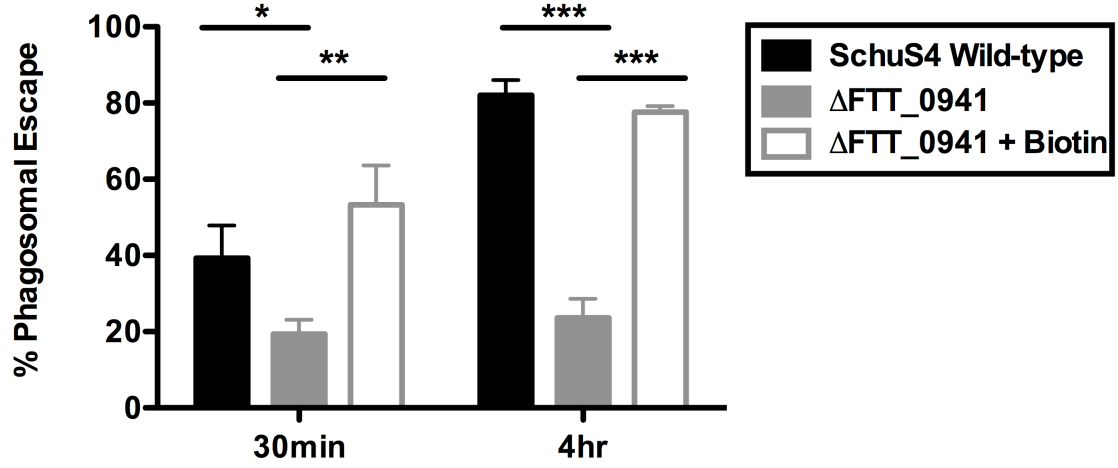


Figure 7. *FTT_0941*, the *FTN_0818* ortholog in *F. tularensis* SchuS4, is required for rapid FCP escape. Quantification of FCP escape at 30 min and 4 h pi in macrophages infected with wild-type *F. tularensis* SchuS4, Δ*FTT_0941*, or Δ*FTT_0941* supplemented with biotin (t=0), by immunofluorescence microscopy. Two hundred bacteria were counted per sample and co-localization of DAPI with LAMP-1 was used as a marker for phagosomal localization. **P*<0.05; ***P*<0.001; ****P*<0.0001.

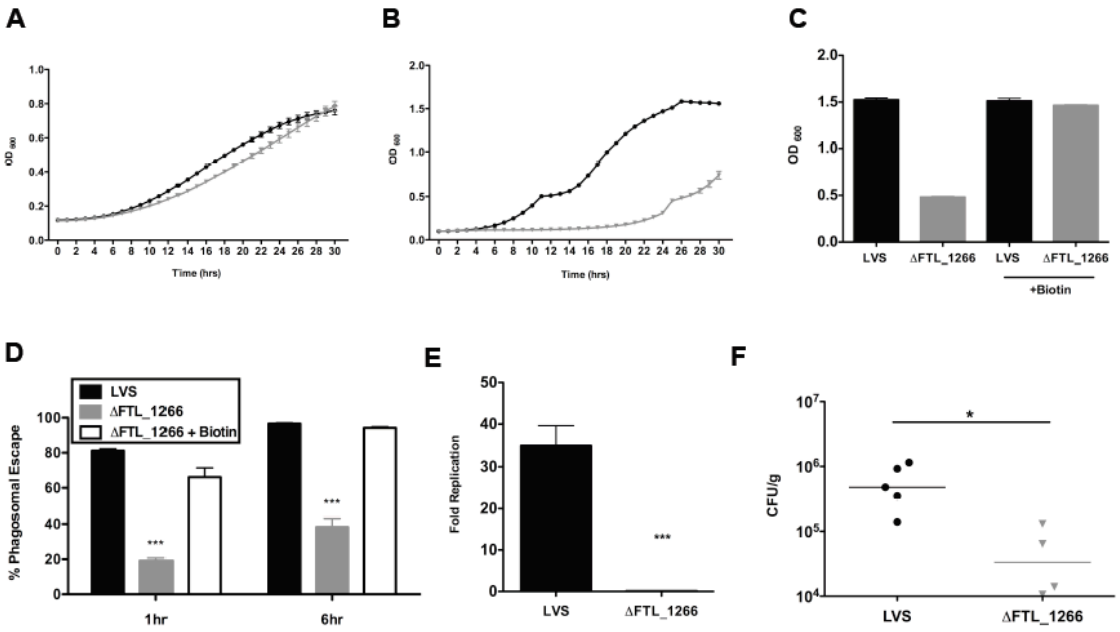


Figure 8. *FTL_1266*, the LVS *FTN_0818* ortholog, is required for escape from the FCP and *in vitro* and *in vivo* replication. Wild-type (black circles) and *ΔFTL_1266* (grey triangles) LVS strains were grown in (A) TSB or (B) CHB for 30 h and the OD_{600} was measured every hour. (C) Wild-type and *ΔFTL_1266* LVS strains were grown for 30 h in CHB supplemented with and without biotin. (D) FCP escape was quantified at 1 h and 6 h pi in macrophages infected with wild-type LVS, *ΔFTL_1266*, or *ΔFTL_1266* supplemented with biotin (t=0), using immunofluorescence microscopy. Two hundred bacteria were counted per sample and co-localization of DAPI with LAMP-1 was used as a marker for phagosomal localization. (E) Macrophages were infected with wild-type and *ΔFTL_1266* LVS strains, cfus were enumerated at 30 min and 24 h pi, and fold replication calculated. (F) Mice were infected subcutaneously with 10^6 CFU of wild-type or the *ΔFTL_1266* LVS strains. At 48 h pi, spleens were harvested and cfu/g was quantified. . **P*<0.05; ****P*<0.0001.

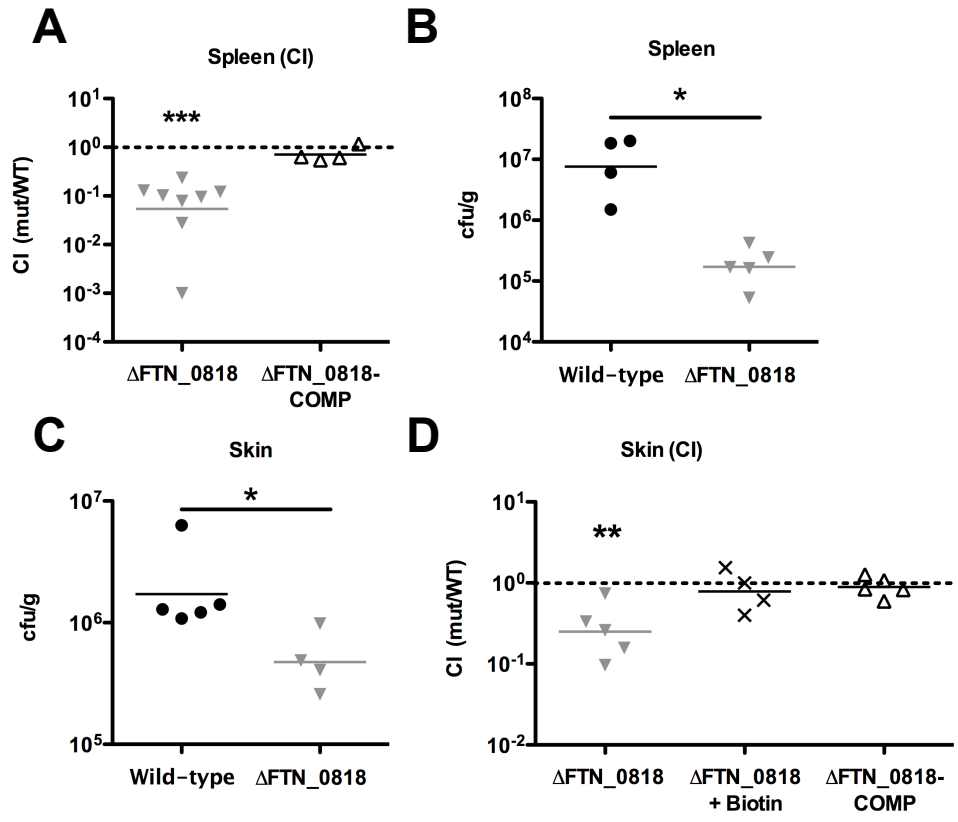


Figure 9. Biotin rescues the Δ*FTN_0818* virulence defect *in vivo.* (A) Mice were infected subcutaneously with a 1:1 mix of WT with the *ΔFTN_0818* or the *ΔFTN_0818* complemented strain (ΔFTN_0818-COMP). At 48 h pi, spleens were harvested to quantify bacterial levels, and the competitive index was calculated. (B, C) Mice were subcutaneously infected with 10^6 cfu of WT or *ΔFTN_0818*. At 48 h pi, the spleen (B) and skin at the site of infection (C) were harvested and bacterial levels quantified. (D) A competition assay was performed with WT and *ΔFTN_0818* in the absence of biotin, or WT and the *ΔFTN_0818* complemented strain in the presence of biotin. At 24 h pi, the skin at the site of infection was harvested to quantify bacterial levels, and the competitive index was calculated. . *P<0.05; **P<0.001; ***P<0.0001.

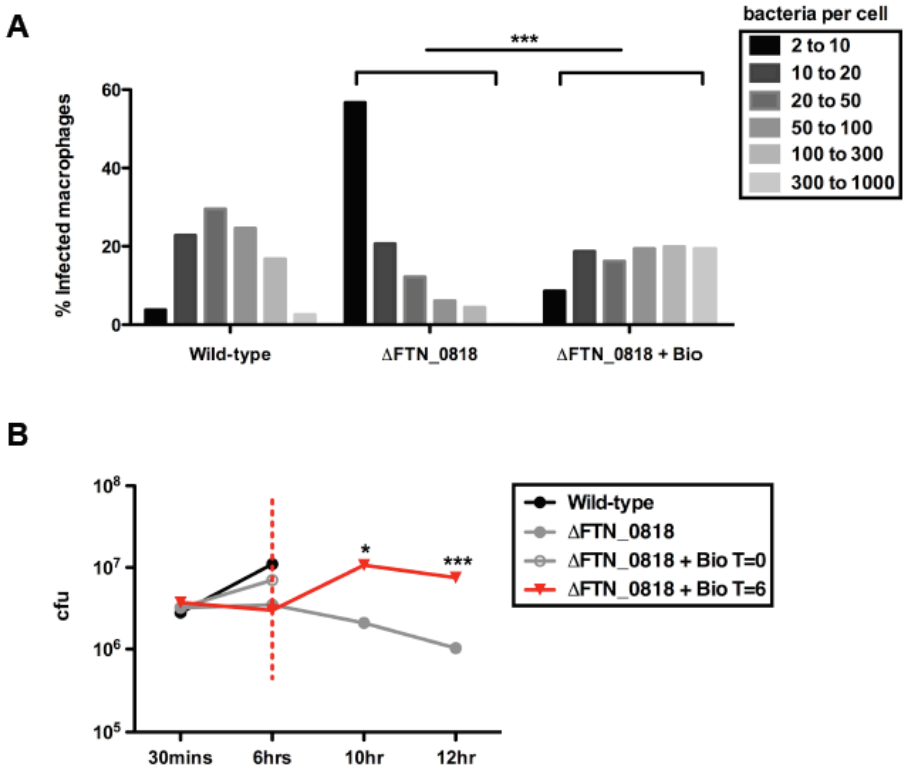


Figure 10. FTN_0818 is required for *F. novicida* replication in the cytosol. (A) J774 cells were injected with GFP-expressing U112 or the Δ*FTN_818* mutant and infection followed for 24 h. Pictures were taken at 24 h with live-cell imaging microscope. The number of bacteria/cell was determined by microscopic counting and categorized as indicated. The total number of infected cells for each different strain was the basis for the statistical comparisons. The non-parametric Wilcoxon rank-sum test was used to determine Δ*FTN_0818* without biotin compared to Δ*FTN_0818* with biotin was significantly different (p value < 2.2^{-16}). (B) Macrophages were infected with WT, Δ*FTN_0818*, and Δ*FTN_0818* supplemented with biotin at time zero (Bio T=0) or at 6 h (Bio T=6; indicated by the red dashed line). Intracellular cfus were quantified for all strains/conditions at 30 min and 6 h pi Additional timepoints were taken at 10 h and 12 h pi for Δ*FTN_0818* and Δ*FTN_0818* + Bio T=6. . *P<0.05; ***P<0.0001.

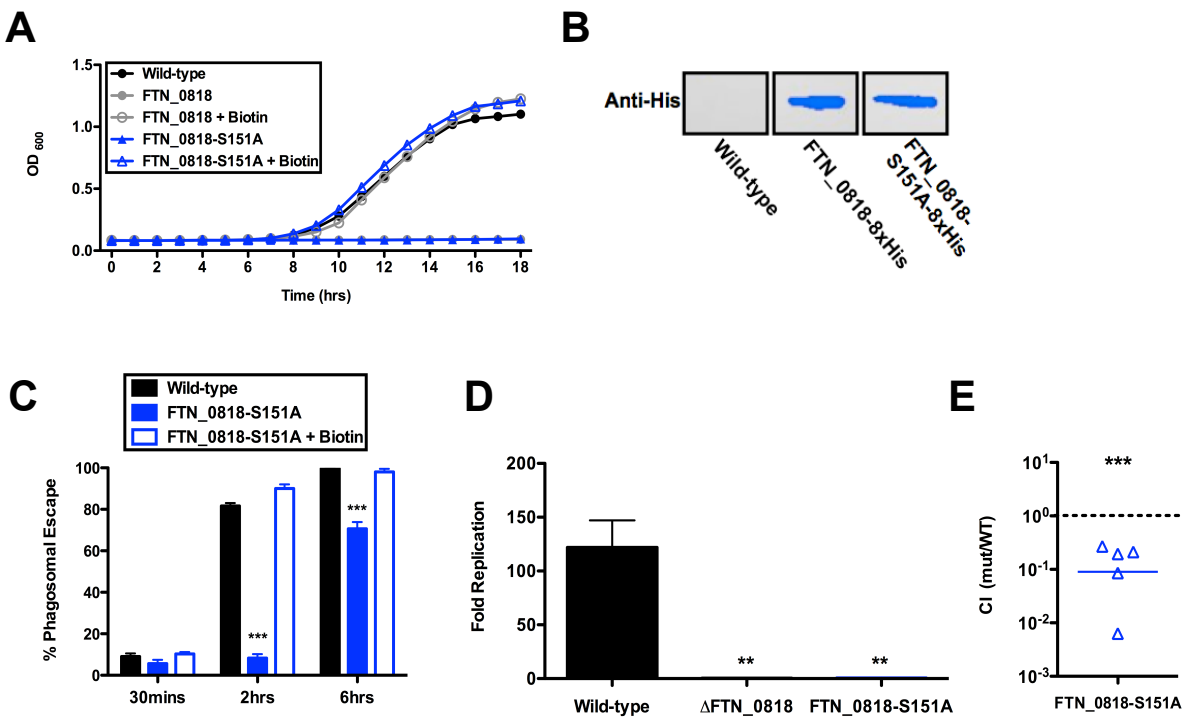


Figure 11. Putative catalytic serine residue (S151) is required for FTN_0818 function. (A) All strains were grown in CHB medium for 18 h, and the OD600 was measured every hour. (B) WT, His-tagged *FTN_0818* (FTN_0818-8xHis), and His-tagged *FTN_0818-S151A* (FTN_0818-S151A-8xHis) were grown in TSB and whole cell lysates (WCL) were resolved by SDS-PAGE and visualized with anti-His antibody. (C) FCP escape was quantified at 30 min, 2 h, and 6 h pi in macrophages infected with WT, *FTN_0818-S151A,* or *FTN_0818-S151A* supplemented with biotin. Two hundred bacteria were counted per sample, and colocalization of *F. novicida* with LAMP-1 was used as a marker for phagosomal localization. (D) Macrophages were infected with the indicated strains, colony-forming units were quantified 6 h pi, and fold replication was calculated. (E) Mice were infected s.c. with a 1:1 mixture of WT with the *FTN_0818-S151A* strain. At 48 h pi, spleens were harvested to quantify bacterial levels, and the CI was calculated. ***P*<0.001; ****P*<0.0001.

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Chapter 3

BioJ is a *Francisella*-specific carboxyl-esterase required for pimelate generation during biotin biosynthesis and virulence

Note: Chapter 3 is adapted from a manuscript currently under preparation in collaboration with Youjun Feng from the John E. Cronan laboratory at University of Illinois Champagne Urbana.

Abstract

Recently we identified the enzyme FTN_0818 as a protein that links biotin biosynthesis and virulence in *Francisella*. However, these studies did not elucidate the detailed molecular function of this novel virulence determinant. Here we reveal that FTN_0818, now named BioJ, is the missing gatekeeper enzyme for the first step of biotin biosynthesis in *Francisella*. Biochemical and enzymatic assays demonstrate that BioJ, as a monomer, acts as an enzyme catalyzing the hydrolysis of Me-pimeloyl-ACP into pimeloyl-ACP, the required precursor for the second step of biotin synthesis. Phylogenetic and structural analyses confirmed that BioJ is distinct from the previously characterized biotin gatekeeper enzymes; therefore, BioJ represents a novel *Francisella*-specific biotin gatekeeper protein. Furthermore, alignment of BioJ revealed conservation of the known carboxyl-esterase catalytic triad, Ser-Asp-His. We show alanine substitution of each residue within the catalytic residue inhibits BioJ activity and growth in nutrient-limiting medium. However, the addition of biotin or pimelate to the growth medium alleviates the requirement of BioJ function. Finally, we show that all three catalytic residues are required for *Francisella* replication in macrophages in the absence of exogenous biotin and for survival in mice. Our findings represent the first detailed molecular evidence for bridging the enzymatic activity of a biotin gatekeeper protein to bacterial virulence.

Introduction

We recently identified FTN_0818 as the novel link between biotin biosynthesis and virulence in *Francisella*. In this previous study we showed that FTN_0818 is required for biotin biosynthesis, up-regulation of virulence proteins in the phagosome, rapid escape from the phagosome, macrophage replication, and survival in mice (1). This previous study linked biotin limitation in the phagosome with *Francisella* virulence, however we did not identify the functionality or molecular characteristics of FTN_0818.

Biotin is an essential enzyme cofactor required for all three domains of life, and is required for the virulence of multiple bacterial pathogens including *Francisella* spp., *Mycobacterium tuberculosis*, and *Vibrio cholerae*; however, our knowledge of its biosynthesis remains fragmented (1-5). Currently it is known that the synthesis of biotin consists of two major steps: the first step, which is still unclear in most bacterial species, is dedicated to the acquisition of a pimelate moiety which is required to generate a valeryl side chain (Fig 1), and the well characterized second step which involves the synthesis of two fused heterocyclic rings on the aforementioned valeryl side chain (2, 6).

Recently, in the first described complete biotin biosynthetic pathway, Lin *et al.* determined in *E. coli* that modification of the fatty acid synthesis pathway allows for the synthesis of the required pimelate moiety by two enzymes, BioC and BioH (2). BioC is required for the methylation of the malonyl acid-ACP (acyl carrier protein) (Fig 1); this methyl group disguises the biotin synthetic intermediates such that they are accepted as

substrates by the fatty acid synthetic pathway (Fig 1) (2, 6). The fatty acid synthetic pathway sends the methylated malonyl acid-ACP through two cycles of elongation resulting in a methylated pimeloyl-ACP (Fig 1) (2). The methyl ester of this product is then cleaved by methyl ester carboxyl-esterase BioH to generate pimeloyl-ACP (Fig 1). Demethylation of the pimeloyl-ACP is required so that it can interact with BioF to generate the first intermediate of the biotin bicyclic ring assembly during the second step of biotin synthesis (2).

Interestingly, multiple groups have noted that the methyl ester carboxyl-esterase BioH, or biotin gatekeeper enzyme, has been replaced with BioG, BioK, or BioZ within the biotin synthetic pathways of different bacteria, suggesting diverse evolution of this protein (7, 8). Interestingly, there is currently no known annotated biotin gatekeeper enzyme in any *Francisella* species, which may be due to the diversity of the biotin gatekeeper proteins throughout different bacterial species.

In our current study we find that our previously identified *Francisella* virulence determinant, FTN_0818, works upstream of the pimelate moiety in biotin biosynthesis as a *Francisella*-specific biotin gatekeeper enzyme. We have thus renamed this protein BioJ. Functional analysis identified BioJ as a carboxyl-esterase that works as a monomer to hydrolyze Me-pimeloyl-ACP into pimeloyl-ACP in order for the second step of biotin biosynthesis to occur. Additional phylogenetic and structural analyses of the known gatekeeper enzymes along with BioJ provides evidence that we have identified a novel,

Francisella-specific methyl ester carboxyl-esterase. Furthermore, BioJ shares homology with known carboxyl-esterases, specifically the catalytic triad (Ser-Asp-His), shown to be required for enzymatic activity of these enzymes (9-11). We demonstrate that BioJ requires all three catalytic residues for hydrolysis of the pimeloyl-ACP methyl ester and growth in minimal medium. Finally, we show all three catalytic residues are required for *Francisella* replication in macrophages and survival in mice. These findings are the first to provide direct biochemical and molecular evidence linking the enzymatic activity of the biotin gatekeeper enzyme to virulence of a bacterial pathogen.

Results

BioJ is required upstream of pimelate generation in biotin biosynthesis

Our previous report on *bioJ* (*FTN_0818*) determined *bioJ* is required for biotin biosynthesis in *Francisella*; therefore, without the addition of biotin, Δ*bioJ* cannot replicate to WT levels in nutrient limiting medium [Chamberlain's medium (CHB)] (12). In order to determine the molecular contribution of *bioJ* during biotin biosynthesis, we next needed to elucidate where in the pathway *bioJ* is required: the first step of biotin synthesis, which involves the acquisition of pimelate, or the second step of biotin synthesis, which involves the addition of the bicyclic ring to the pimelate backbone.

In order to determine if BioJ is required for the first step of biotin biosynthesis, we tested whether pimelate could complement the growth defect of Δ*bioJ* in CHB. Interestingly, when pimelate was added to CHB, it rescued the Δ*bioJ* growth defect to wild-type (WT) growth levels, with a minor delay (Fig 2A). These data place BioJ upstream of pimelate generation during the first step of biotin biosynthesis.

As previously mentioned, there are two required enzymes in the first step of biotin synthesis, BioC and BioH. In *Francisella*, the biotin metabolism loci annotated within all *Francisella* species are present in the bioBFCD/A operon, which encodes BioC; however, the bioH gene is missing (Fig 2B), suggesting functional replacement by an unknown enzyme. We therefore hypothesized that BioJ is acting as a genetically distinct,

but functionally similar, biotin gatekeeper enzyme, upstream of pimelate generation in the biotin biosynthesis pathway.

BioJ catalyzes the hydrolysis of Me-pimeloyl-ACP into pimeloyl-ACP

In order to determine if BioJ was functionally similar to the known biotin gatekeeper enzymes (BioH, BioG, BioK, and BioZ) we first over-expressed recombinant BioJ from *Francisella philomiragia*, Fphi_1796, using the prokaryotic expression system in *E. coli* and purified it to homogeneity using Ni^{2+}-chelate chromatography. The purified BioJ was analyzed using size exclusion chromatography, which revealed that our protein of interest was eluted in a monomeric form, which is not unexpected considering both BioH and BioG function as monomers (data not shown) (10).

Using a modified enzymatic reaction system originally established by Lin *et al.* to identify the function of BioH in multiple bacterial species, we examined if BioJ demethylates Me-pimeloyl-ACP, the physiological BioH substrate, to pimeloyl-ACP (Fig 3A). In this assay, the carboxyl-esterase activity of BioJ can be assessed using a gel electrophoretic mobility shift assay that ensures conformational integrity of the protein. The Me-pimeloyl-ACP substrate migrates faster than the pimeloyl-ACP product, allowing for definitive resolution between substrate and product (Fig 3A) (2).

When we added purified BioJ to Me-pimeloyl-ACP, we found hydrolysis of the methyl ester bond of Me-pimeloyl-ACP was observed within as little as 2 min in the reaction with 3.6 pmol BioJ, and within 1 h the hydrolysis reaction was fully completed (Fig 3B). Subsequent MALDI mass spectrometry of the reaction mixtures with or without BioJ revealed that the molecular weight of the product pimeloyl-ACP is around 14-15.7 Da less than those of the substrate Me-pimeloyl-ACP, as seen in previous studies with this method (Fig 3C), validating the migratory shift of the product was due to the loss of the methyl moiety (2). These data conclude BioJ is working as the biotin gatekeeper enzyme by converting Me-pimeloyl-ACP into pimeloyl-ACP during the first step of biotin biosynthesis.

BioJ is an evolutionarily and structurally distinct biotin gatekeeper protein

As mentioned previously, four distinct biotin gatekeeper enzymes (BioH, BioG, BioK, and BioZ) have been found to hydrolyze the methyl ester of Me-pimeloyl-ACP during the first step of biotin biosynthesis in various bacterial species. Our studies indicate BioJ is performing the same functional requirement during biotin biosynthesis as the previously described enzymes, thus identifying BioJ as a new biotin gatekeeper enzyme. Interestingly, the four previously described enzymes recognize the same physiological substrate (Me-pimeloyl-ACP), however, their sequence similarity is low (2). Therefore, we attempted to unveil the potential phylogenetic relationships of these enzymes in relation to BioJ. BioH, BioG, BioK, and BioZ sequences were sampled from the Pfam database, along with all 6 *Francisella* BioJ sequences (13). Interestingly, all of the

Francisella BioJ proteins grouped to form a distinct sub-clade when compared to known biotin gatekeeper enzymes (Fig 4). Additionally, based on the known structure of BioH, the *E. coli* biotin gatekeeper enzyme, and the modeling of BioG, BioK, BioZ, and BioJ, we found that all five groups were structurally distinct, consistent with the diversity observed in the phylogenetic evolutions (Fig 4) (2, 7). Together these data show that *Francisella* BioJ proteins form a unique sub-clade and possess distinct structural models; therefore, we propose that the BioJ is an evolutionarily distinct, species-specific carboxyl-esterase that acts as the biotin gatekeeper enzyme by demethylating Me-pimeloyl-ACP into pimeloyl-ACP in the first step of biotin biosynthesis in *Francisella*.

The carboxyl-esterase catalytic triad, Ser-Asp-His, is required for BioJ activity

To further chacterize BioJ as a novel biotin gatekeeper enzyme we used bioinformatic and comparative structural analysis of the hypothetical BioJ structure with structurally defined *E. coli* BioH to identify possible important residues. It was previously described that BioH in *E. coli* and *Salmonella enterica* features a conserved catalytic Ser-Asp-His triad, consisting of a S82 serine residue, D207 aspartic acid residue, and a H235 histidine residue (Fig 5A) (2, 11, 14). Each residue in the catalytic triad is required for BioH functionality in both *E. coli* and *S. enterica* (10, 11, 14). Our previous report on BioJ (FTN_0818) provided preliminary data that this protein was a member of the carboxyl-esterase family of proteins that harbors the required catalytic serine residue (1). In our current study, comparative analyses of the modeled BioJ structure with the known BioH structure revealed similar results: though sequence similarity with BioH is low, BioJ

harbored the entire catalytic triad consisting of the Ser-Asp-His, and nearly identical 3D configuration of the catalytic triad with BioH (Fig 5B,C). Subsequently, the predicted BioJ catalytic triad consists of previously reported serine (S151), and the additional aspartic acid (D248) and histidine (H278) residues (Fig 5A).

In order to determine if the conserved Ser-Asp-His catalytic triad is required for BioJ activity we created three distinct alanine point mutants: Serine mutant S151A, aspartic acid mutant D248A, and histidine mutant H278A. We next prepared and purified all three mutants, as well as the WT BioJ. Using our modified enzymatic reaction system described above, we found in contrast to the WT BioJ protein, all three point mutants were no longer able to hydrolyze the methyl ester on Me-pimeloyl-ACP (Fig 5D-G). These data clearly demonstrate that each residue in the proposed catalytic triad is necessary for enzymatic function of BioJ.

To assess the physiological relevance of the inactivity of BioJ by alanine substitutions in each of the three catalytic residues, we quantified growth in nutrient-limiting CHB. Expectedly, the inactivation of BioJ by disruption of either the S151, D248, or H278 residues resulted in a severe growth defect as compared to WT *Francisella* (Fig 6A-B). Similarly to what was seen with the complete deletion of BioJ in our previous study, the addition of biotin was able to complement the growth defect of all 3 catalytic residue point mutants (Fig 6A). Additionally, as we have shown previously with the BioJ deletion mutant above (Fig 2A), exogenous pimelate was also able to complement the

growth defect of all 3 catalytic residue point mutants (Fig 6B). Taken together, these results definitively illustrate the physiological importance of each catalytic residue in the Ser-Asp-His triad of BioJ, indicating *Francisella*-specific BioJ is functionally similar to known biotin gatekeeper proteins.

BioJ catalytic residues are required for replication in macrophages and survival in mice

In our previous study of *bioJ* (*FTN_0818*) we found that deletion of this gene from the genome inhibited WT replication in macrophages and survival in mice (1). Since loss of any of the three catalytic residues is required for BioJ function and growth in minimal medium, we hypothesized that S151A, D248A, and H278A would exhibit a severe replication defect in macrophages compared to wild type. We infected murine bone marrow-derived macrophages (mBMDM) with WT *Francisella*, *ΔbioJ*, and all three BioJ catalytic mutants (S151A, D248A, and H278A) and found that 6 h post-infection (pi), WT bacteria replicated almost 10-fold, whereas the *ΔbioJ* and all three catalytic triad mutants replicated from ~2-3 fold (Fig 7). However, when biotin was added to the mBMDMs at the time of infection, the *ΔbioJ,* S151A, D248A, and H278A replication defects were significantly complemented 6 h pi (Fig 7). These data illustrate the importance of biotin availability during infection, and specifically the functional importance of BioJ in the production of biotin during intracellular replication.

Finally, we wanted to identify if the BioJ catalytic triad was required for *Francisella* survival in mice. We performed competition experiments in which a 1:1 mixture of the WT and *ΔbioJ,* S151A, D248A, or H278A was used to infect mice. Forty-eight hours pi, *ΔbioJ* levels were ~1-2 logs lower in the spleen and livers compared to WT *Francisella* (Fig 8A). Similarly to the deletion mutant, all three catalytic triad mutants (S151A, D248A, and H278A) displayed a 1-2 log defect of survival in the mouse spleen and liver (Fig 8B-D). These results demonstrate the requirement of BioJ to be fully functional during *Francisella* infection *in vivo*. Taken together, we have characterized BioJ as a novel *Francisella*-specific biotin gatekeeper enzyme that is functionally required for bacterial replication in nutrient limiting media and macrophages and systemic survival in mice.

Discussion

Biotin availability has been shown to be important in multiple bacterial pathogens during infection including *Francisella* spp., *Mycobacterium tuberculosis*, and *Vibrio cholerae* (1-5). Our previous publication identified a novel virulence determinant in *Francisella*, BioJ (FTN_0818), as required for biotin production in nutrient-limiting environments and WT infection kinetics *in vitro* and *in vivo* (1). However, this study did not elucidate the molecular function of BioJ and how this contributes to biotin biosynthesis and virulence in *Francisella*. Here we identify BioJ as the missing biotin gatekeeper enzyme required for complete biotin biosynthesis in *Francisella*.

Recently, it has been shown that there is remarkable diversity among characterized biotin gatekeeper enzymes within different bacterial pathogens (7). Interestingly, there was no known biotin gatekeeper enzyme within the *Francisella* genome (Fig 2B). Here we have described BioJ as the *Francisella*-specific biotin gatekeeper enzyme that hydrolyzes the methyl ester of Me-pimeloyl-ACP in order to produce pimeloyl-ACP (Fig 3-4), which is required for the commencement of the second step of biotin biosynthesis (Fig 1).

Furthermore, phylogenetic and structural analyses show that BioJ has the conserved carboxyl-esterase catalytic triad (Ser-Asp-His), found in all biotin gatekeeper enzymes characterized (6, 7, 10). All three of these residues are required for functional activity of BioJ and subsequently growth in nutrient-limiting medium, and this functional requirement is alleviated by the addition of biotin or pimelate (Fig 6). Additionally, these

three catalytic residues are required for replication in macrophages and survival in mice (Fig 7 and 8), which reiterates the importance of biotin availability within the bacterium during infection. Together, we have characterized a new *Francisella*-specific biotin gatekeeper enzyme and shown the first biochemical and molecular link between the functionality of a biotin gatekeeper enzyme and bacterial virulence.

Given that biotin plays crucial roles in metabolism throughout the three domains of life, it is imperative to understand its contribution to bacterial pathogenicity. Interestingly we have found like *E. coli*, there is only one protein covalently bound to biotin within the entirety of *Francisella* (AccB) (data not shown). Therefore, the requirement of biotin within the host cell is not dependent on the covalent attachment of biotin to known virulence proteins. We hypothesize biotin is working as a nutritional virulence determinant that is required for providing energy to *Francisella* during infection. Since biotin is a known costly metabolic pathway (15, 16), *Francisella* most likely does not rely on this metabolic cofactor in nutrient-rich environments [as we have shown WT *Francisella* does not have a growth defect in nutrient rich medium (Fig 2A)].

Additionally, since biotin is acting as a nutritional virulence factor most likely providing energy to the invading bacterial pathogen, there is a possibility that lipoic acid, the other fatty acid-derived vitamin, could be playing a role in bacterial virulence. The intracellular pathogen *Listeria monocytogenes* relies on the host-derived lipoic acid for its intracellular growth and virulence (17, 18). Intriguingly, *Francisella* has evolved two mechanisms for

obtaining lipoic acids 1) *de novo* synthesis and 2) scavenging. Since *Francisella* lacks the gene required for biotin scavenging (*bioY*), it will be interesting to understand the differences between the requirement of *de novo* biotin within the host cell and *de novo* or scavenging requirements of lipoic acids within the host cell during infection (19).

Recently it was found that the bacterial pathogen *Chlamydia trachomatis* has the capability of localizing the mammalian sodium multivitamin transporter (SMVT), which transports lipoic acids, biotin, and pantothenic acid into cells, to *Chlamydia*-containing vacuoles within 20-24 h pi (15). Our previous study on *Francisella* showed biotin as a requirement within the *Francisella*-containing phagosome (1); however, since *Francisella* escapes the FCP within 30-60 min pi, we hypothesize that in contrast to *C. trachomatis*, *Francisella* will depend on *de novo* synthesis of lipoic acids within the FCP. However, it is currently unknown whether *Francisella* recruits host nutrient transport systems to the FCP, so it will be interesting to determine if mammalian transporters, such as SMVT, can be recruited within 30-60 min to the FCP.

Our data here has provided functional and molecular insight into how biotin production is affecting *Francisella* virulence, specifically providing insight into BioJ and the recognition of its physiological substrates. Since we currently lack the capabilities to define all of the metabolic requirements of *Francisella* within the FCP or the cysotol, with directed studies looking at specific nutrient availability (like biotin or lipoic acids) we will continue to shed light on the relationship between nutritional availability in the

host and virulence. These efforts will contribute to identification of potential metabolic targets in development of small molecule inhibitors and therapeutics to treat intracellular bacterial infections. Alternatively, future investigation may lead to biotin gatekeeper enzymes or biotinylated AccB as potentially new targets for engineered antibodies used for treatment against lethal infection by intracellular pathogens.

Materials and Methods

Bacterial strains and mutagenesis

All *F. novicida* strains were grown in TSB/0.2% cysteine or Chamberlain's Medium (CHB) (Teknova, Hollister, CA) (12). Generation of the kanamycin (kan)-marked *FTN_0818* deletion mutant, *FTN_0818* clean deletion mutant, and serine to alanine *FTN_0818* serine point mutant (FTN_0818-S151A) were reported previously (1).

The aspartic acid to alanine and histidine to alanine point mutants (FTN0818-D248A and FTN0818-H278A, respectively) were constructed by overlapping PCR using primers encoding a single amino acid change and using a kan-cassette for selection. This construct was transformed into the FTN0818 clean deletion mutant and selected for on kanamycin plates (30μg/ml). All strains mentioned above were verified by PCR and sequencing (Eurofins EWG Operon, Huntsville, AL).

All the *E. coli* strains (Table 1) were routinely maintained at 37^{o}C using the liquid LB medium (Luria-Bertani medium containing 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl per liter) or LB agar plate.

Site-directed mutagenesis in *F. philomiragia* was performed as described by Zheng *et al*. (20). The PCR reaction system (25 μl) included the following components: 2.5 μl of 10x *pfx* buffer (Invitrogen), 0.5 μl of 50 mM $MgSO_4$ (Invitrogen), 0.5 μl of 40 mM dNTP mix

(10 mM each), 1.0 µl of forward/reverse primers (*Fphi*(S151A)-F plus *Fphi*(S151A)-R, 10 pmol/µl) (Table S2), 1.0 µl of pBAD322-Fphi (or pET28-Fphi) as template (10 ng/µl), 0.5 µl of Platinum pfx (2.5 U/µl, Invitrogen), and 17.0 µl of distilled sterilized H_2O. The reaction was done using the program consisting of a denaturing cycle at 95°C for 5 min; 20 cycles comprised of 95°C for 50 s, 55°C for 50 s, and 68°C for 6.5 min and a final step of 8 min at 68°C. To remove the residual template plasmid, the gel purified PCR products were digested for overnight with DpnI (20 U/µl, NEB) at 37 °C. Subsequently, the processed PCR products were transformed into chemically competent cells of Topo 10 (Invitrogen) and the inserts of purified plasmids were confirmed by direct DNA sequencing. The two mutant plasmids pBAD322-Fphi (S151A) and pET28-Fphi (S151A) were transformed into STL24 (MG1655, Δ*bioH*) (10, 11) and BL21 (DE3), respectively.

Growth Curves

Bacteria were subcultured to an OD_{600} of 0.03 in TSB/0.2% cysteine or Chamberlain's Medium (CHB) (Teknova, Hollister, CA) (12) for *F. novicida*, while LVS cultures were grown in modified Mueller-Hinton (MH) broth described previously (21). Subcultures were read hourly using a SynergyMX BioTek plate reader (Applied Biosystems, Foster City, CA). Biotin (0.25 µM) (Merck KGaA, Darmstadt, Germany) or pimelate (200 µg/mL) (Sigma-Aldrich, St. Louis, MO) was added when appropriate.

Protein expression and purification

The N-terminal 6X histidine tagged BioJ protein (wild type version plus three versions of mutants) was over-expressed using BL21/ pET28-*Fphi* prokaryotic expression system (Table S1). 0.3 mM IPTG was added into 500 ml of bacterial cultures with an $OD_{600\ nm}$ of 1.0 and kept at 30°C for 6 h (22-24). The bacterial cells were pelleted by centrifugation (4200 g, 20 min), washed three times with ice-cold PBS buffer (101.4 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 12% glycerol, pH7.4), and resuspended in PBS buffer containing 25 mM imidazole. Following lysis in a French pressure cell and removal of bacterial debris by centrifugation (16,000xg for 60 min), the clarified supernatant was loaded onto a nickel chelate column (Qiagen) for no less than 2 h. After washing the column with 10 column volumes of PBS buffer contatining 50mM imidazole, the BioJ protein (in wild type or mutant version) was eluted with 150 mM imidazole, dialyzed against PBS buffer and then concentrated by ultra-filtration (10 kDa cut-off, Amicon Ultra) (23). The protein purity was visualized by gradient SDS-PAGE (4-20%), and further confirmed by liquid chromatography quadruple time-of-flight (qTOF) mass spectrometry of tryptic peptides as described previously (22).

Enzymatic assays

The *in vitro* enzymatic activity of BioJ and its derivatives was determined using the protocol established for the *E. coli* BioH with appropriate modifications (7, 11, 25). The enzymatic reaction was reconstituted in the 50 mM HEPES buffer (pH 7.0) containing 5% glycerol. In each reaction with the total volume of 10 ul, the enzyme BioJ (~14 μg/ml, 3.6 μM) was mixed with 150 μM of its physiological substrate pimeloyl-ACP

methyl ester (or a shorter or longer homologue) and incubated at 37°C for 1 h. The generated products of these reactions were loaded into 20% PAGE gel containing 2.5 M urea and then run at 130V for 2.5 h to acquire the satisfied separation.

MALDI TOF/TOF mass spectrometry

The mixture of BioJ-catalyzing reaction (30 μl in total) was subjected to dialysis in buffer of 20 mM ammonium acetate (2L) overnight at 4 °C (26). After ammonium acetate was evaporated under a stream of nitrogen (24), the dried protein samples then were dissolved in methanol and mixed with 50 mg/mL 2,5-dihydroxybenzoic acid (DHB) matrix (Sigma, St. Louis, MO) in 50% acetonitrile and 0.1% trifluoroacetic acid in a 1:10 ratio and deposited on a standard stainless steel target. The mass spectra were collected in positive ion mode on an UltrafleXtreme MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a frequency tripled Nd:YAG solid state laser using the FlexControl 1.4 software package (Bruker Daltonics). Following external calibration, 2000 spectra were acquired at 500 Hz using a randomized raster, summed, and saved for analysis. Data processing was done using the FlexAnalysis 3.4 software package (Bruker Daltonics). Spectra were smoothed and a baseline correction was applied using the built-in features of the software package (26).

Bioinformatics

The BioJ homologues were aligned using the program of ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and resulting output was expressed by the help of the ESPript 2.2 server (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) (26). The protein sequence of *Francisella* BioJ (or other related proteins BioG, BioK, BioH and BioZ) was submitted to the CPHmodels 3.0 Server (http://www.cbs.dtu.dk/services/CPHmodels), giving their PDB files of the modeled structures (26). The modeled tertiary structures then were analyzed using either Swiss_PDBViewer 4.0.1 software from the Swiss Institute of Bioinformatics (http://spdbv.vital-it.ch) or the UCSF Chimera program (http://www.cgl.ucsf.edu/chimera). Clustal Phylogeny (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny) was applied to generate the phylogenetic tree on the basis of the multiple alignment of protein sequence by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo).

Macrophage Preparation and Infection

Bone marrow-derived macrophages (mBMDMs) were prepared as described previously (21). Briefly, bone marrow was collected from the femurs of mice. Bone marrow cells were plated into sterile petri dishes and incubated in DMEM supplemented with 10% heat-inactivated FBS and 10% macrophage colony-stimulating factor (M-CSF)-conditioned medium (collected from M-CSF-producing NIH3T3 cells). Bone marrow cells were incubated at 37°C with 5% CO_2 and harvested after 6 days. All mBMDMs were incubated before and during infection in 24-well plates at 37°C with 5% CO_2.

For infection, mBMDMs were seeded at 5 x 10^5/well and incubated overnight at 37°C with 5% CO_2. mBMDMs were infected at a multiplicity of infection (MOI) of 10:1 (21).

Mouse infections

For mouse infections, female C57BL/6 mice (6-8 wk) (Jackson Laboratory, Bar Harbor, ME) were housed under specific pathogen-free housing at Emory University. Experimental studies were performed in accordance with the Institutional Animal Care and Use Committee guidelines. Competitive index [CI = (mut output/WT output)/(mut input/WT input], as described previously (1, 21). Statistical analysis for CI experiments described previously (27).

Acknowledgements

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Tables

Table 1: Bacterial strains and plasmids used in this study. Contributed by Youjun Feng

Bacteria or plasmids	Relevant characteristics	Origins
Bacterial strains		
Topo10	F^-, ΔlacX74, a cloning host for recombinant plasmids	Invitrogen, (23, 24)
BL21(DE3)	The engineered E. coli host for protein expression	Lab stock, (23, 24)
STL24	MG1655, ΔbioH	(10)
STL25	MG1655, ΔbioC ΔbioH	(10)
FYJ390	Topo10 carrying pBAD322-Fphi	This work
FYJ391	STL24 (MG1655, ΔbioH) carrying pBAD322	This work
FYJ392	STL24 (MG1655, ΔbioH) carrying pBAD322-Fphi	This work
FYJ393	Topo10 carrying pET28-Fphi	This work
FYJ394	BL21(DE3) carrying pET28-Fphi	This work
FYJ396	Topo10 carrying pBAD322-Fphi(S151A)	This work
FYJ397	Topo10 carrying pET28-Fphi(S151A)	This work
FYJ398	STL24 (MG1655, ΔbioH) carrying pBAD322-Fphi(S151A)	This work
FYJ399	BL21(DE3) carrying pET28-Fphi(S151A)	This work
FYJ400	STL25 (MG1655, ΔbioC ΔbioH) carrying pBAD322-Fphi	This work
FYJ401	Topo10 carrying pBAD322-Fphi (D248A)	This work
FYJ402	Topo10 carrying pBAD322-Fphi (H278A)	This work
FYJ403	Topo10 carrying pET28-Fphi (D248A)	This work
FYJ404	Topo10 carrying pET28-Fphi (H278A)	This work
FYJ405	STL24 (MG1655, ΔbioH) carrying pBAD322-Fphi(D248A)	This work
FYJ406	STL24 (MG1655, ΔbioH) carrying pBAD322-Fphi(H278A)	This work

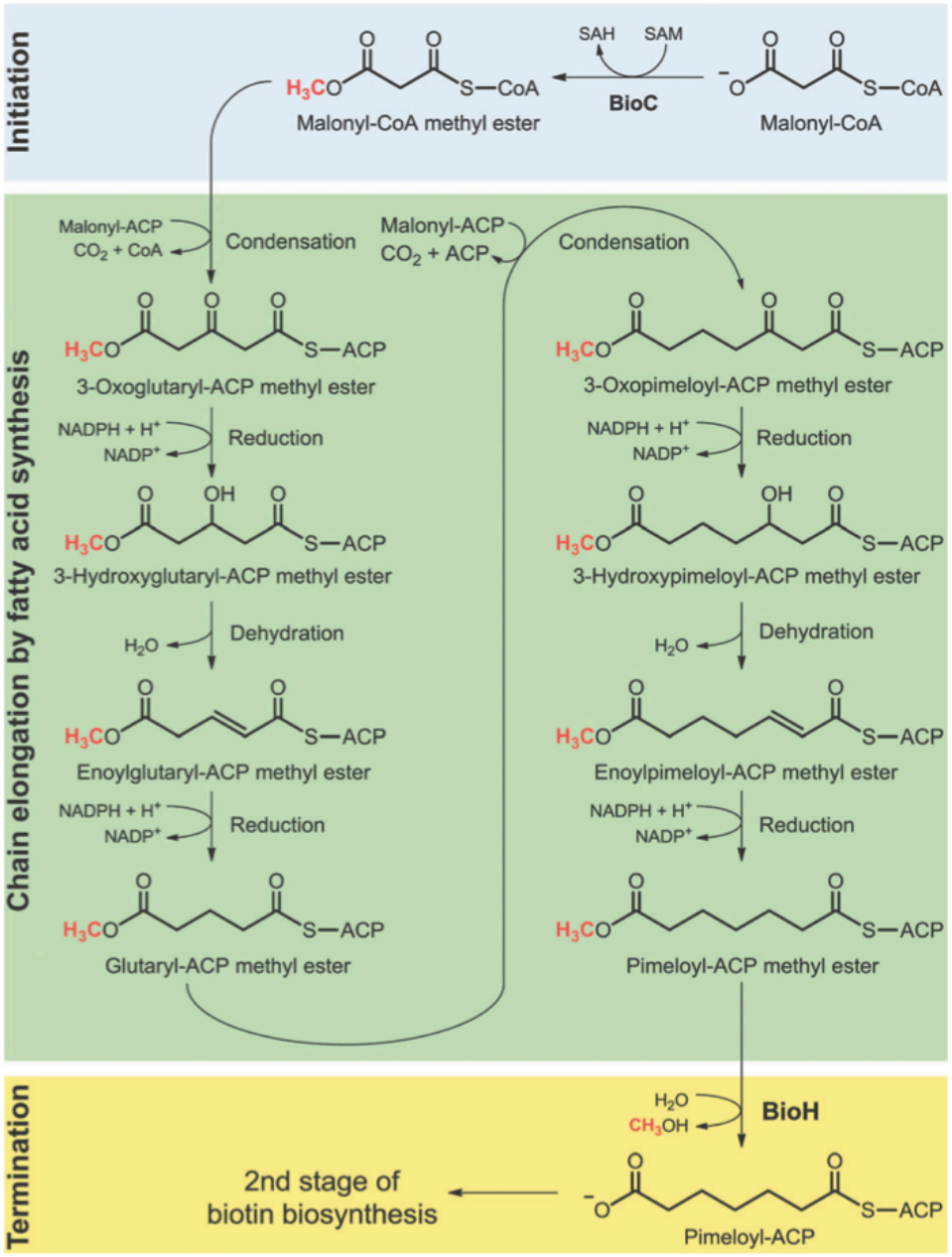
FYJ407	BL21 (DE3) carrying pBAD322-Fphi(D248A)	This work
FYJ408	BL21 (DE3) carrying pBAD322-Fphi(H278A)	This work
Plasmids		
pET28(a)	Commercial T7-driven expression vector, Km^R	Novagen
pBAD322	An arabinose-inducible expression vector, Amp^R	(28)
pBAD322-Fphi	pBAD322 encoding F. philomiragia Fphi_1796, Amp^R	This work
pBAD322-Fphi(S151A)	pBAD322 encoding a S151A mutant version of F. philomiragia Fphi_1796, Amp^R	This work
pBAD322-Fphi(D248A)	pBAD322 encoding a D248A mutant version of F. philomiragia Fphi_1796, Amp^R	This work
pBAD322-Fphi(S278A)	pBAD322 encoding a H278A mutant version of F. philomiragia Fphi_1796, Amp^R	This work
pET28-Fphi	pET28(a) encoding F. philomiragia Fphi_1796, Km^R	This work
pET28-Fphi(S151A)	pET28(a) encoding a S151A mutant version of F. philomiragia Fphi_1796, Km^R	This work
pET28-Fphi(D248A)	pET28 encoding a D248A mutant version of F. philomiragia Fphi_1796, Km^R	This work
pET28-Fphi(S278A)	pET28 encoding a H278A mutant version of F. philomiragia Fphi_1796, Km^R	This work

Table 2: Primers used in this study. Contributed by Youjun Feng.

Primers	**Sequences**
Fphi-F1 (XmaI)	5'-ACTTGA CCCGGG ATG CCA TAC CAT CCA GCA TT-3'
Fphi-R1 (SphI) for pBAD322	5'-AGTGCT GCATGC TTA TCT TTT ATT TAT AAA CTC AAT T-3'
Fphi-F2 (BamHI)	5'-CG GGATCC ATG CCA TAC CAT CCA GCA TT-3'
Fphi-R2 (XhoI)	5`-CCG CTCGAG TTA TCT TTT ATT TAT AAA CTC AAT T-3'
For pET28(a)	
Fphi(S151A)-F	5'-TC TTT GTA ATG GGC GAT GCT GCT GGT GGA AAT CTT GT-3' AGT
Fphi(S151A)-R	5'-AC AAG ATT TCC ACC AGC AGC ATC GCC CAT TAC AAA GA-3'
Fphi(D248A)-F	5'-TT GTA GCG GCT ACT CAT GCT ATC CTT ATA GAT GGG AT-3' GAT
Fphi(D248A)-R	5'-AT CCC ATC TAT AAG GAT AGC ATG AGT AGC CGC TAC AA-3'
Fphi(H278A)-F	5'-AT GAT GAT GAA ATG TAT GCT GGT TTT ATT GGA GGA CT-3' CAT
Fphi(H278A)-R	5'-AG TCC TCC AAT AAA ACC AGC ATA CAT TTC ATC ATC AT-3'

*The underlined sequences in italics are restriction sites.

Figures



Lin and Cronan, 2011

Figure 1. The first step of biotin biosynthesis in *E. coli*. Published by Lin and Cronan in 2011, this figure is the BioC-BioH pathway for the synthesis of pimelate in *E. coli*. This pathway comprises of three main steps: 1) The initiation step is catalyzed by BioC *O*-methyltransferase which transfers a methyl group from an amino donor (*S*-adenosyl-ι-

methionine, SAM) to the ω-carboxyl group of malonyl-CoA, to give malonyl-CoA methyl ester, a primer dedicated to biotin biosynthesis, 2) next, the chain elongation cycle of fatty acid synthesis will occur twice, 3) finally, chain elongation termination occurs when BioH cleaves the methyl ester moiety to produce pimeloyl-ACP, a substrate for the second stage of biotin biosynthesis.

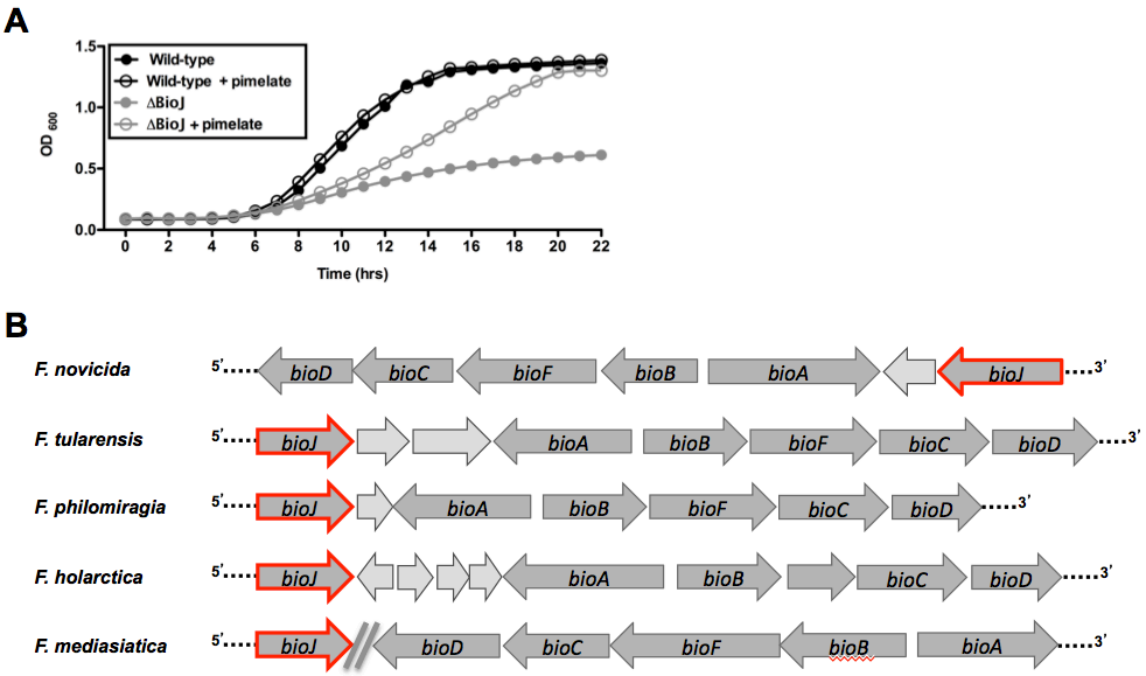
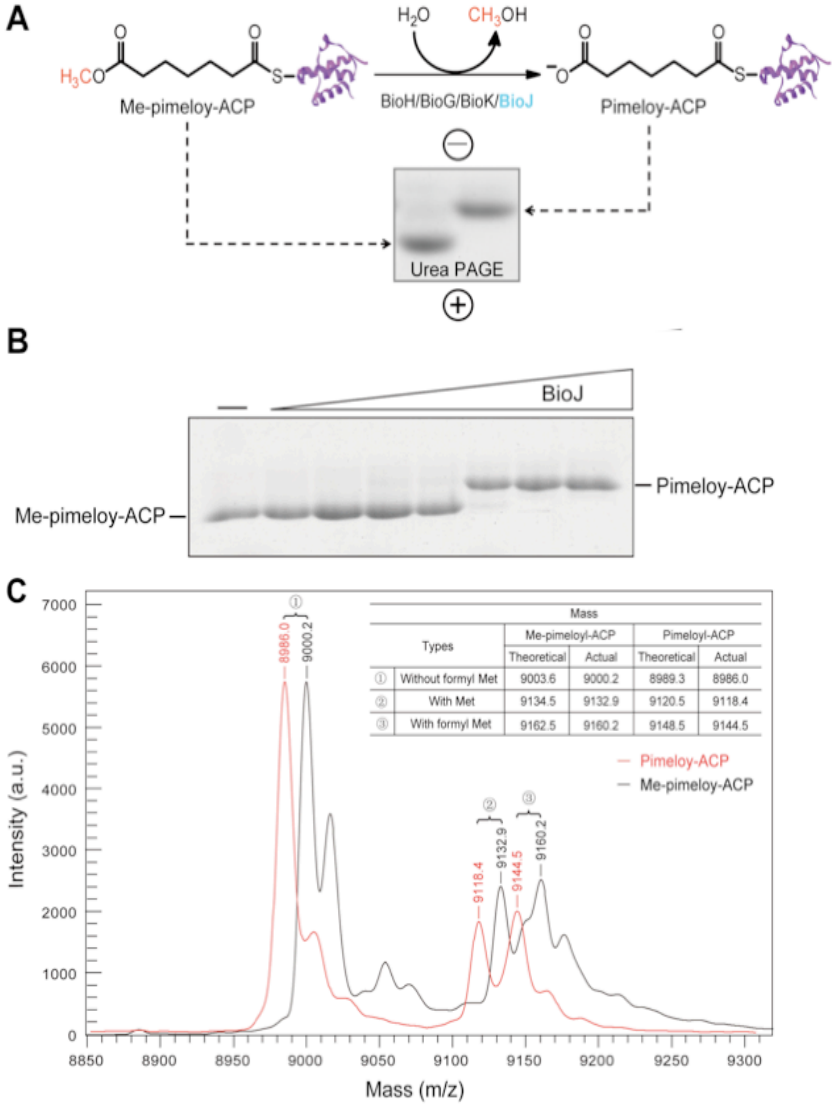


Figure 2. *bioJ* is required for pimelate acquisition and is conserved throughout *Francisella* species. (A) WT *Francisella* and *ΔbioJ* were grown in Chamberlain's medium (CHB) with or without pimelate and the OD_{600} was measured every hour (12). (B) A schematic representation of the annotated biotin operon in all human virulent *Francisella* species and *F. philomiragia*. *bioJ* annotations were based on 100% sequence identity to *FTN_0818, Francisella novicida bioJ. bioJ* genes identified as follows; *FTT_0941c, Francisella tularensis bioJ; Fphi_1796, Francisella philomiragia bioJ; FTL_1266, Francisella holarctica LVS bioJ; FTM_1009, Francisella mediasiatica bioJ.*



Youjun Feng, *et al.*

Figure 3. Integrated evidence that *Francisella* BioJ is a functional carboxyl-esterase. (A) Schematic diagram of the enzymatic reaction catalyzed by the biotin gatekeeper enzymes, including BioJ in blue. (B) Enzymatic assays for hydrolysis of pimeloyl-ACP methyl ester by *Francisella* BioJ into pimeloyl-ACP. (C) Maldi-MS identification of products

produced in BioJ-catalyzed hydrolysis reaction. Abbreviations: Met, methionine; Me, methyl ester. Contributed by Youjun Feng.

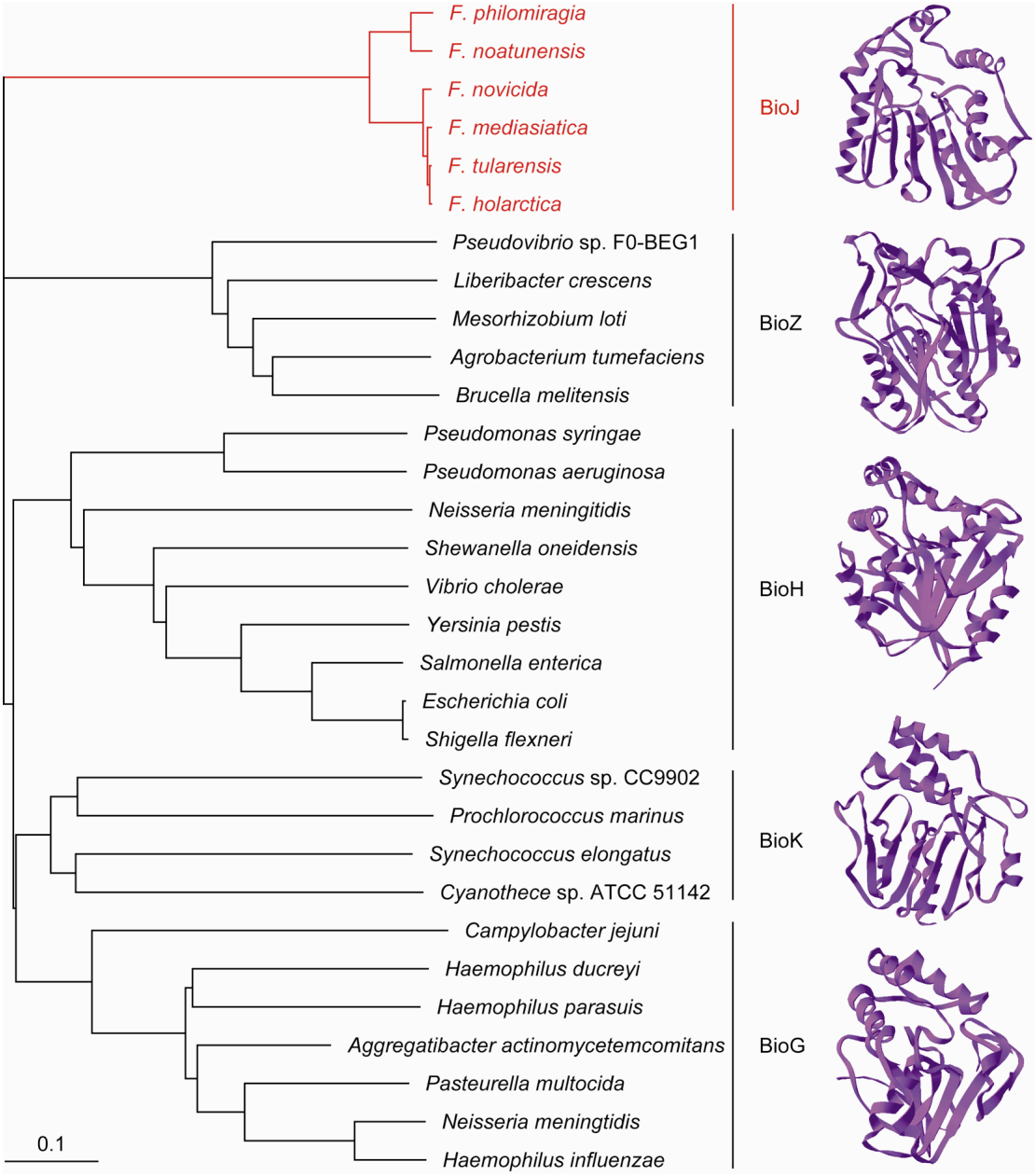
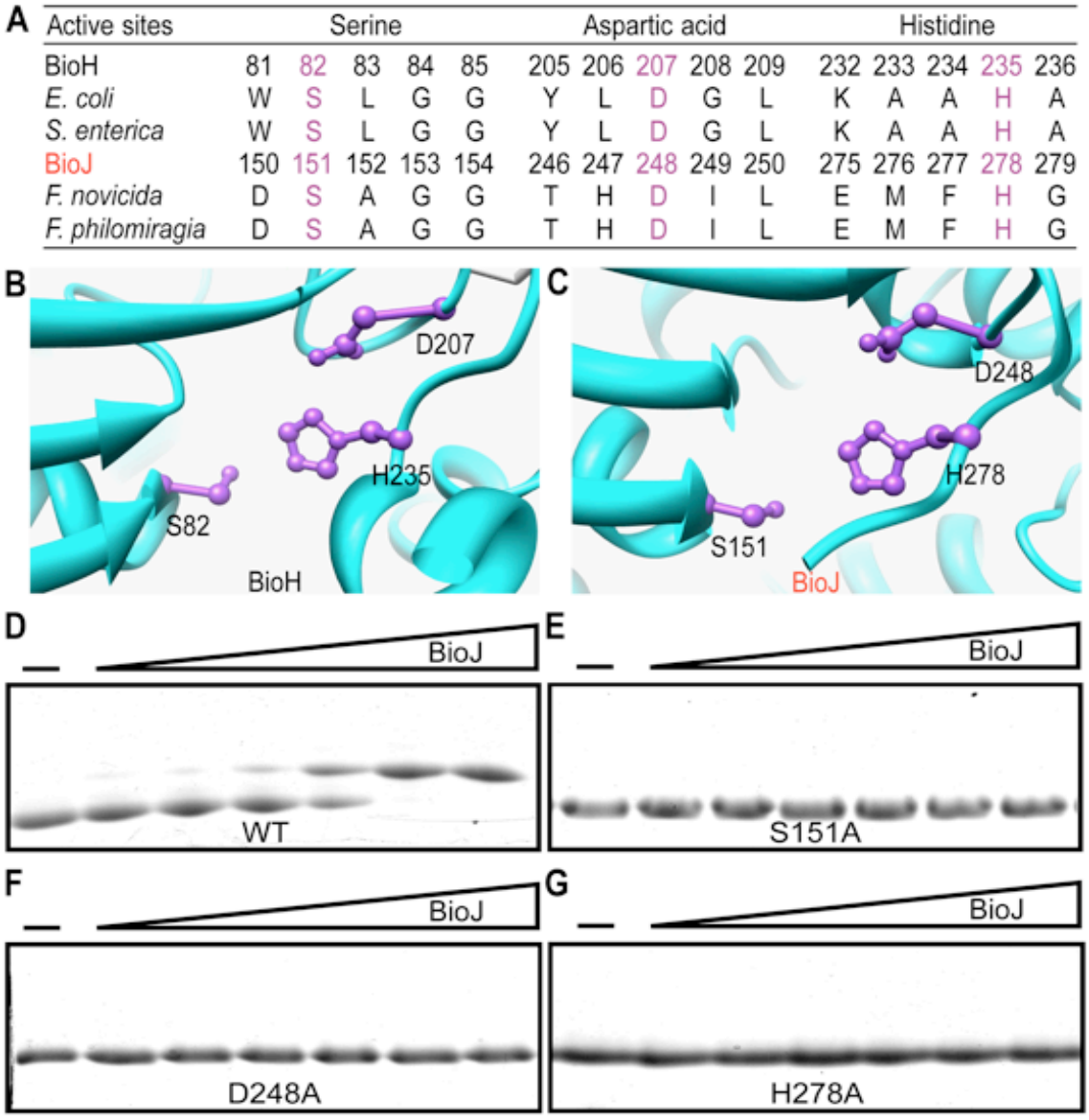


Figure 4. Phylogenetic and structural analyses of the gatekeeper enzymes required for bacterial biotin synthesis. Sequences were sampled from the Pfam database of all annotated biotin gatekeeper enzymes. The structure of each subclade member (in ribbon)

is shown on the right hand. All the BioJ homologues are restricted to *Francisella* species and highlighted in red. Contributed by Youjun Feng.



Youjun Feng, *et*

Figure 5. Identification of a catalytic Ser-Asp-His triad motif required for BioJ function. (A) Comparison of the putative active sites in BioJ with known residues of BioH from *E. coli* and *S. enterica*. (B) Structural visualization of the known triad motif of *E. coli* BioH, and (C) the predicted triad for *Francisella* BioJ. The relevant three catalytic residues are shown in purple. Enzymatic assays for BioJ (D) and the single point mutant S151A (E), D248A (F) and H278A (G) using the conformationally-sensitive electrophoretic mobility

shift assays. Minus denotes no addition of BioJ (or mutant protein), whereas the triangle on the right hand represents protein level in series of dilution (0.034, 0.068, 0.17, 0.34, 0.7, 1.7, 3.4 pmol). The enzymatic reaction (10 μl in total) that was conducted contains 150 μM of pimeloyl-ACP substrate. The reaction mixture was separated using 20% PAGE with 2.5 M urea. Contributed by Youjun Feng.

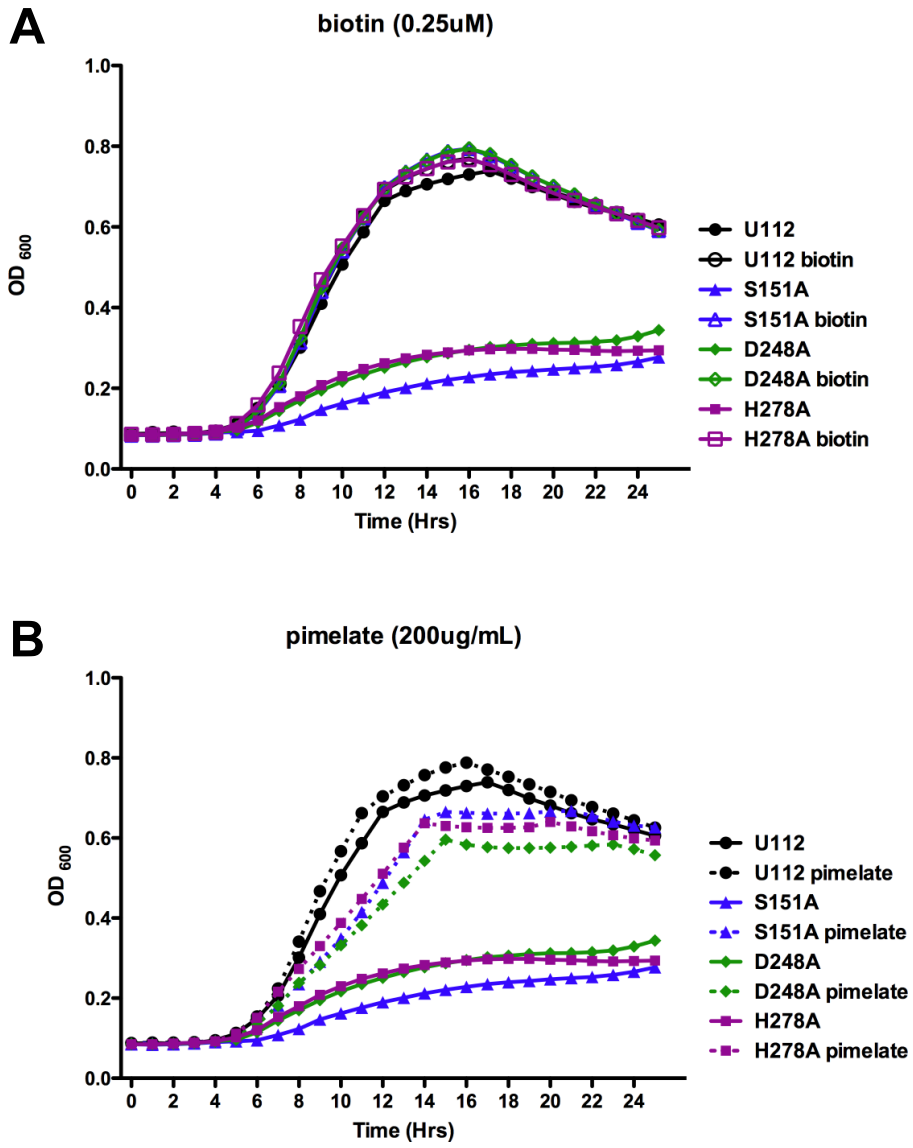


Figure 6. The catalytic triad of BioJ is required for growth in minimal media, and can be alleviated by the addition of biotin or pimelate. WT *Francisella* and all three BioJ catalytic point mutants (S151A, D248A, and H278A) were grown in Chamberlain's medium (CHB) with or without (A) biotin or (B) pimelate and the OD_{600} was measured every hour.

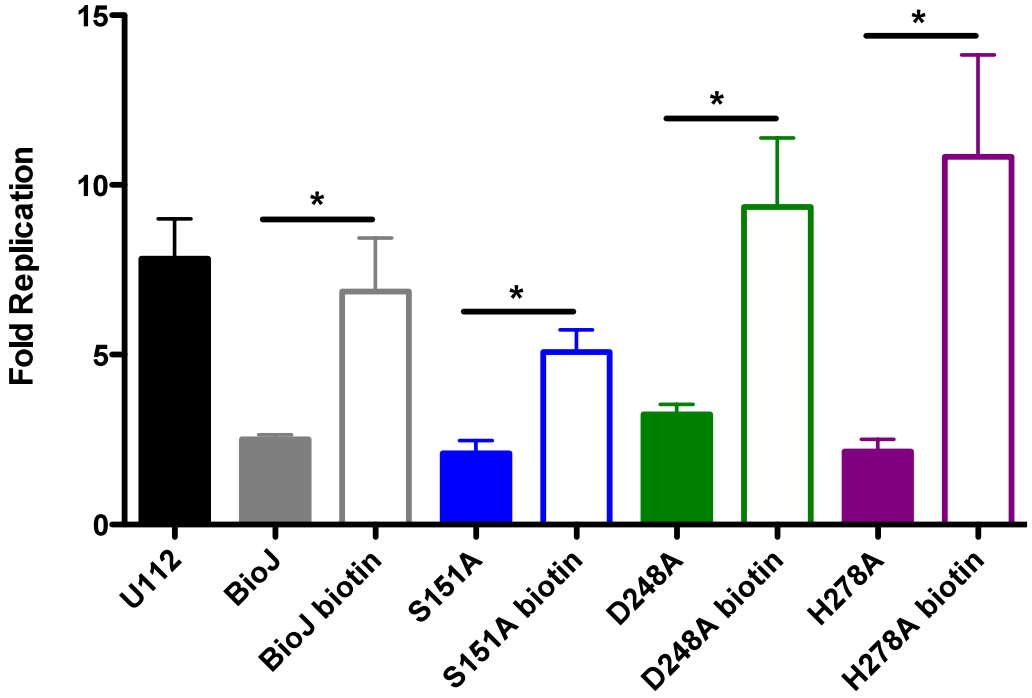


Figure 7. The catalytic triad of BioJ is required for replication in macrophages, without exogenous biotin. Macrophages were infected with WT, *ΔbioJ*, *ΔbioJ-S151A* (S151A), *ΔbioJ-D248A* (D248A), and *ΔbioJ-H278A* (H278A) *F. novicida* strains, with (empty bars) or without (filled bars) 25 μM of exogenous biotin. Colony-forming units were quantified at 30 min and 6 h post-infection (pi), and fold replication was calculated. *$P<0.0509$.

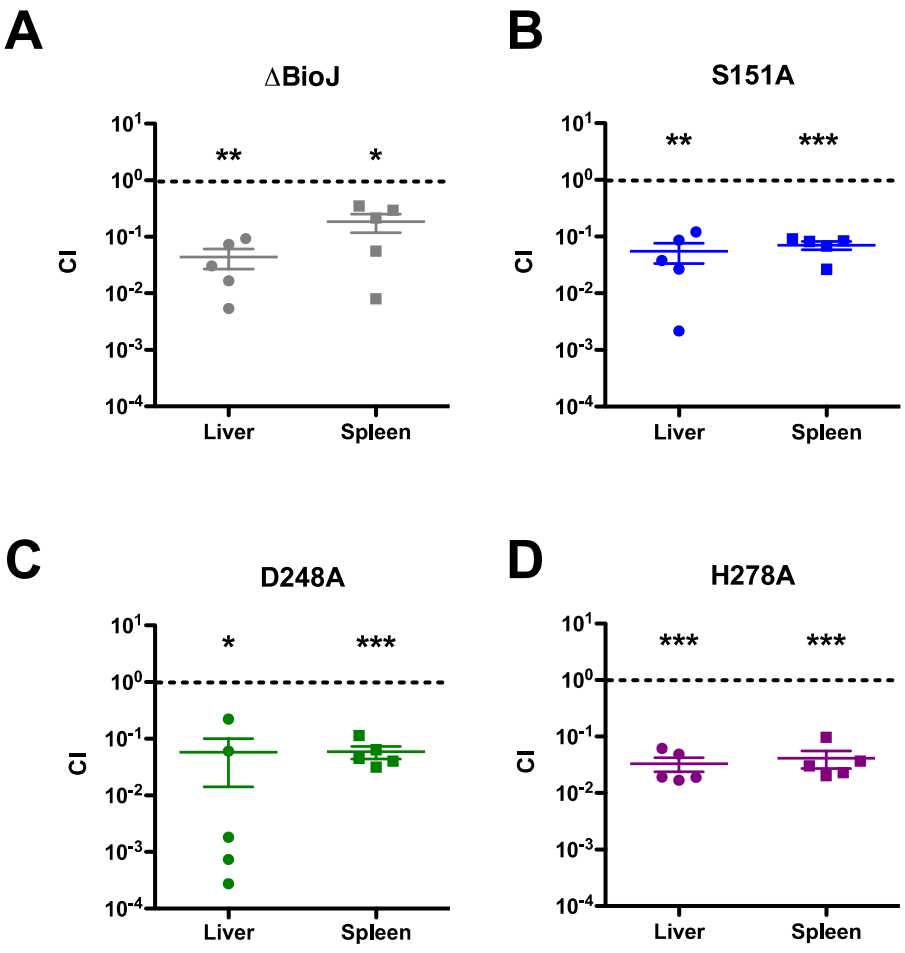


Figure 8. The catalytic triad of BioJ is required for survival in mice. Mice were infected sub-cutaneously with a 1:1 mixture of WT *Francisella* with *ΔbioJ*, S151A, D248A, or H278A. At 48 h pi, spleens and livers were harvested to quantify bacterial levels, and the competitive index (CI) was calculated. Statistical analysis was described previously (27). **P*<0.05, ***P*<0.001, ****P*<0.0001.

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Chapter 4

Discussion and conclusion

Francisella is a cytosolic intracellular Gram-negative bacterial pathogen that uses a multitude of mechanisms to evade phagosomal host defenses. This pathogen is the causative agent of the potentially fatal disease tularemia. Despite recognition as a Category A Select Bioterrorism Agent by the Centers for Disease Control (CDC), there is not a Food and Drug Administration (FDA) approved vaccine against *Francisella*.

As a cytosolic pathogen, after *Francisella* is taken up by the host macrophage it resides within the highly toxic phagosomal compartment for nearly 1 h. After this brief, but dynamic, period within the phagosome, *Francisella* escapes the *Francisella*-containing phagosome (FCP) to replicate within the permissive cytosolic compartment. The introduction of this dissertation reviewed all of the antimicrobial host defenses *Francisella* must evade while it resides within the FCP, however it is unknown how *Francisella* combats the nutrient limitation within this compartment in order to escape.

The nutrient composition within the FCP is not yet defined; in fact no known microbe-containing vacuole has been entirely nutritionally defined, due to the difficulty in detection methods. It will be a very daunting and tedious task to isolate intact microbe-containing vacuoles during intracellular infection and to then define their nutritional composition. However, with directed studies looking at specific nutrient-deficient mutants and their infection kinetics, we can start to define these microbe-containing compartments and the effect of this nutrient limitation on bacterial pathogenesis.

This dissertation focuses on a *Francisella* biotin biosynthesis mutant that we characterized to define the link between biotin availability within the FCP and *Francisella* virulence. Biotin (vitamin H) is an enzymatic co-factor and is essential for all three domains of life (1). Specifically, biotin is a co-factor for carboxylase enzymes involved in synthesis of fatty acids, isoleucine, valine, and gluconeogenesis (1, 2). Interestingly, biotin has been reported to be necessary for *Mycobacterium tuberculosis* survival within mice and *Vibrio cholerae* colonization of intestinal epithelial cells within mice (3-5). Despite the overt link between bacterial pathogenesis and biotin availability during infection, this relationship has not been defined.

In Chapter 2, FTN_0818, now referred to as BioJ, was identified as a novel virulence factor required for *Francisella* biotin production and rapid escape from the phagosome. We show that within 30 minutes post-infection of macrophages (when *Francisella* is located within the FCP) the *Francisella*-pathogenicity island (FPI) encoded transcripts required for phagosomal escape could not be expressed to wild-type levels in the absence of BioJ. Subsequently, we showed that without BioJ, *Francisella* could no longer rapidly escape the phagosome, replicate within macrophages, and survive in mice. Interestingly, we found that BioJ is also required for growth in a defined minimal medium, which like the phagosomal compartment is a nutrient-limiting environment. We found that these two phenotypes were intertwined, in that BioJ is involved in biotin biosynthesis and when exogenous biotin is present during growth minimal medium, replication in macrophages, and infection of mice, BioJ is no longer required.

Considering biotin synthesis is a costly metabolic pathway (6, 7) it makes sense that BioJ is not required in nutrient rich environments, however we show it becomes a necessity in nutrient-deplete circumstances. There is only one detectable covalently biotinylated protein (AccB) within *Francisella*, which acts as the biotinylated subunit of acetyl-coenzyme A carboxylase (ACC), an enzyme complex required for fatty acid synthesis (16). Therefore, we hypothesize that biotin may be influencing *Francisella* pathogenesis either 1) by acting as a cofactor for fatty acid synthesis, which may be required within the phagosome, 2) by covalently or transiently biotinylating an undetected protein involved in virulence, or 2) biotin utilization in nutrient-limiting environments is essential for a stress signaling cascade necessary to up-regulate FPI genes. It has been seen that when the bacterial pathogen *Clostridium difficile* is grown in biotin-limiting environments there is a significant increase in two major pathogenic factors, toxin A and toxin B production (8). It will be interesting to define a stress response in *Francisella*, possibly regulated by the utilization of specific metabolites, like biotin.

Important to note, though we saw BioJ is necessary for escape from the phagosome and replication in macrophages, we only saw a 1-2 log defect in survival in mice. Mammalian cells do not synthesize biotin, within the eukaryotes only a few plants and fungi can synthesize biotin *de novo* (9). Generally the intestinal bacteria within mammals produce in excess of the mammalian body's daily requirements (10). Additionally, biotin can be found throughout the mammalian diet. Therefore, the commensal organisms and the diets of the mice infected could be unintentionally partially complementing the requirement of biotin for *Francisella* replication without BioJ. In order to avoid this complementation

we would have to clear the mice of their commensals by treatment with a cocktail of antibiotics, which will inherently make them immunocompromised (11), inadvertently affecting the outcome of *Francisella* infection. Therefore, we have not pursued that line of experimentation in order to find a more drastic phenotype in the BioJ mutant.

Together, the work in Chapter 2 has defined biotin as a novel *Francisella* virulence determinant that is required for wild-type infection kinetics within macrophages and mice (12). However, this study did not define the molecular role of BioJ as an enzyme required for *Francisella* biotin biosynthesis. In Chapter 3, we defined the enzymatic role of BioJ within the biotin biosynthetic pathway and reveal the importance of the functionality of BioJ during infection. We defined BioJ as the *Francisella*-specific missing biotin gatekeeper enzyme that is required for pimelate acquisition during the first step of biotin synthesis. Enzymatic assays show that BioJ hydrolyzes the methyl ester of Me-pimeloyl-ACP in order to produce pimeloyl-ACP, which is required to carry out the second step of biotin synthesis (2). We found BioJ harbors the carboxyl-esterase catalytic triad, Ser-Asp-His (S151, D248, and H278), and all three of these residues are necessary for enzymatic activity of BioJ, as well as *Francisella* growth in minimal medium, replication within macrophages, and survival in mice. With these data we were able to functionally characterize BioJ and provide the first molecular and biochemical characterization of the link between biotin availability and bacterial virulence.

Some intracellular bacterial pathogens, including *Chlamydia* spp, have evolved a multifaceted approach to ensuring biotin availability within the host cell (7). One mechanism involves BioY, the biotin transport protein required for bacteria to take up environmental biotin (7, 13, 14). Interestingly, *Francisella,* along with previously mentioned *M. tuberculosis,* do not encode an annotated BioY; therefore, it is most likely that these intracellular pathogens are not using this route to ensure biotin availability during infection. However, in our studies when an overabundance of exogenous biotin is available either in broth or during infection, *ΔbioJ* can be complemented, suggesting that there is a mechanism in which *Francisella* can take up biotin from the environment without BioY. These results suggest that there is a yet undefined *Francisella* biotin transporter, or high concentrations of biotin can override the requirement for a biotin-specific transporter by utilizing a different undefined transporter.

Additionally, the second defined mechanism *Chlamydia* spp. use to ensure biotin availability is sequestration of host-cell transporters to the *Chlamydia*-containing vacuoles (7). Specifically, within 20-24 h post-infection, *Chlamydia*-containing vacuoles (or inclusion membranes) contain the mammalian sodium multivitamin transporters (SMVTs), which transport biotin, lipoic acid, and pantothenic acids into cells (7). We hypothesize that *Francisella* may be sequestering host-derived transporters to the FCP. Although *Francisella* only spends 30 min to 1 h within the FCP, it is known that *M. tuberculosis* can manipulate phagosomal trafficking within 4 minutes post-infection, providing evidence that manipulation of the host vacuolar membrane can occur quickly during infection (15). Considering *M. tuberculosis* can manipulate the trafficking of the

phagosome into a lysosome within 4 minutes, we suggest it is possible that *Francisella* can modify host-cell transporters in the FCP membrane during its short stay. Accordingly, we would hypothesize that *M. tuberculosis* may also be modifying the abundance of host-cell transporters in the *Mycobacteria*-containing vacuole. This is a fascinating and unchartered area of research for nearly all intracellular bacterial pathogens, and is a worthy area of interest.

Since we currently lack the capabilities to define the entire nutritional composition of microbe-containing phagosomes, directed studies looking at specific nutrient availability (like biotin) will continue to provide insight on the relationship between nutritional availability in this compartment and virulence of intracellular bacterial pathogens. Identification of metabolic requirements within these compartments will provide new targets for the design of novel therapeutics or microbial inhibitors. By defining microbial metabolic requirements we not only learn more about the intracellular life of these bacterial pathogens, but we can also use these metabolites as targets to stop progression of infection by intracellular bacterial pathogens before they start replicating and cause disease.

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