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CRISPR-Cas Systems: New Players in Bacterial Gene Regulation, Innate Immune Evasion, Pathogenesis, and Beyond

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Abstract

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Timothy R. Sampson

CRISPR (clustered, regularly interspaced, short palindromic repeats) - Cas (CRISPR-associated) systems are a form of prokaryotic defense against invading foreign nucleic acids, particularly those derived from bacteriophages and plasmids. Such foreign nucleic acids are targeted and cleaved by CRISPR-Cas systems in an RNA-dependent, sequence-specific manner. Additionally, CRISPR-Cas systems are adaptive, providing protection against previously encountered foreign elements. Canonically, it has been thought that these restriction systems act solely in prokaryotic immunity against exogenous genetic elements. However, here, we reveal the very first demonstration of a unique role for CRISPR-Cas systems in the control of endogenous gene expression, a previously unappreciated form of prokaryotic gene regulation. We demonstrate that in the intracellular bacterial pathogen, *Francisella novicida*, the CRISPR-Cas endonuclease, Cas9, functions in association with two small RNAs to target and alter the stability of a particular endogenous transcript which encodes a bacterial lipoprotein (BLP). Since BLPs are recognized by the host innate immune receptor Toll-like Receptor 2 (TLR2), CRISPR-Cas-mediated repression of BLP expression dampens the activation of TLR2-dependent immune signaling. Furthermore, we demonstrate that control of BLP levels in *F. novicida* promotes resistance to antimicrobials and enhances the stability of the bacterial envelope, which additionally allows evasion of the host inflammasome complex. Dampening the activation of both TLR2 and the inflammasome by Cas9-mediate regulation ultimately promotes the successful survival of this pathogen in the mammalian host. Since ~45% of bacteria and ~83% of Archaea encode these machineries, this newly described regulatory function of CRISPR-Cas systems is likely to play a broad role in controlling the pathogenesis and physiology of diverse prokaryotes.

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"The most exciting phrase to hear in science, the one that heralds new discoveries, is not Eureka! (I found it!) but rather, 'hmm... that's funny...'"
- Isaac Asimov

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Chapter 1: Introduction

Part 1. CRISPR-Cas Systems: History and Discovery

CRISPR (clustered, regularly interspaced, short, palindromic repeats) - Cas (CRISPR-associated) systems are adaptive, sequence specific, nucleic acid restriction machineries found in many bacteria and Archaea (1). These systems provide prokaryotes with an effective defense against mobile genetic elements, in particular bacteriophages, plasmids, and transposons (2-5). Colloquially, CRISPR-Cas systems represent the "adaptive immune system" of prokaryotes, in contrast to restriction-modification systems, which represent the "innate immune system."

The defining feature of CRISPR-Cas systems is a chromosomal array consisting of short, repetitive, and sometimes palindromic nucleotide sequences (termed "repeats"), which are interspersed by short, unique, spacer sequences (termed "spacers"). Such genetic arrays of spacers and repeats were first identified over 25 years ago in 1987. Five, identical repeat sequences of 29 base pairs, with unique, 32 base pair, spacer sequences were found, directly downstream of the gene encoding the alkaline phosphatase isozyme convertase (*iap*) during sequencing of this gene in *E. coli* (6). At the time, the biological relevance was unknown. However, it was hypothesized that these repetitive sequences may form specific RNA hairpins that could mediate either termination of transcription, and/or increase the stability of the transcript (6).

Fifteen years later, these genomic loci were termed CRISPR (clustered, regularly interspaced, short, palindromic repeats) (7). Bioinformatic analysis subsequently revealed

that such CRISPR loci are widespread and found in many prokaryotic species (8). Additionally, directly adjacent to the CRISPR loci, there existed conserved groups of open reading frames, termed *cas* (CRISPR-associated) genes. Two *cas* genes, *cas1* and *cas2,* were found universally near all CRISPR loci. Others were found only in specific groups of bacterial or archeal species (1).

At the same time, the sequence databases were expanding. The unique spacer sequences were identified as having significant sequence identity to (or often, were completely identical to) numerous exogenous, extra-chromosomal elements, such as bacteriophages, plasmids, and transposons, with a small percentage also being identical to endogenous chromosomal sequences (9-11). Additionally, the most conserved *cas* genes were found to be similar to various endonucleases, helicases, integrases (8, 12). Together, this lead to the hypothesis that CRISPR-Cas systems were functioning together as a form of prokaryotic RNA interference against extra-chromosomal genetic elements.

This hypothesis was not confirmed until 2007, when Barrangou and colleagues clearly and elegantly demonstrated that CRISPR-Cas systems functions as adaptive restriction machineries against bacteriophage infection (2). Using *Streptococcus thermophilus* as a model system, this group demonstrated that the CRISPR locus, and the associated *cas* genes could mediate protection against bacteriophages with sequences identical to those found in the CRISPR locus. Additionally, this study successfully demonstrated that a percentage of bacterial cells which survived bacteriophage infection had acquired a unique spacer sequence, identical to the previously challenged bacteriophage. And

further, that this newly acquired spacer was necessary to mediate bacteriophage resistance.

Part 2. CRISPR-Cas Systems: Function and Types

Numerous prokaryotes harbor CRISPR loci, ~45% of bacteria and ~83% of Archea encode at least one CRISPR region (13). Interestingly, the number and type of associated *cas* genes adjacent to each CRISPR locus is not identical among all species (1, 8, 14). In fact, even among related strains, or within a single genome, CRISPR loci are found to have a varied assortment of *cas* genes. Computational sequence analysis has now allowed the clustering of these various CRISPR regions into three main types based on the encoded *cas* genes, other than the universal *cas1* and *cas2* (1, 8, 14).

Type I CRISPR-Cas systems are characterized by the presence of a six gene operon which forms the CRISPR-associated complex for antiviral defense (CASCADE), as well as a large endonuclease, Cas3. The *cas* genes within Type III CRISPR-Cas systems are similar in architecture to those in Type I, but share limited sequence similarity. These are characterized by a CASCADE-like group of genes (termed Csm or Csr) as well as a unique endonuclease, Cas6. Both of these systems are quite intricate, requiring eight or nine distinct genes to function. On the other hand, the Type II CRISPR-Cas system is significantly less complex, and requires only three or four genes to function completely. These systems are singly characterized by a very large endonuclease (~1,000-1,600 amino acids), termed Cas9 (1, 14-16).

While the Cas proteins involved in CRISPR-Cas function are distinct among the types of systems, their general mechanism of action is similar. Each act as a form of RNA-directed nucleic acid interference, guided by small RNAs which are formed from the CRISPR locus. First, the entire CRISPR repeat-spacer array is transcribed as a single, long transcript (the pre-crRNA array), and is subsequently processed into individual CRISPR RNAs (crRNAs) (Figure 1). Each crRNA contains an individual spacer sequence, as well as portions of the surrounding repeat sequence at both its the 5' and 3' ends (17-21). This maturation from the pre-crRNA array to the individual crRNAs requires of the action of distinct Cas proteins. This includes CASCADE in Type I systems, Cas6 in Type III systems, and Cas9 in Type II systems (1, 14).

Interestingly, Type II systems have a further requirement for an accessory RNA encoded adjacent to the CRISPR locus, termed the *trans*-activating crRNA (tracrRNA). This small RNA associates with the Cas9 endonuclease and subsequently hybridizes to the repeat sequences within the pre-crRNA array, forming a double-stranded RNA structures (15-17, 22). This structure is recognized by RNase III and subsequently cleaved into the mature crRNAs. which remain associated with Cas9 (17) (Figure 1). Subsequently, the spacer sequence of each mature crRNA hybridizes to complementary sequences in nucleic acid targets, ultimately triggering the cleavage of the target by the associated Cas proteins, Cas6 in Type I, Cas9 in Type II, and Csm/Cmr in Type III (2-5).

While the precise mechanism of targeting and cleavage for Type I and Type III systems is still unclear, the action of Cas9 on DNA targets is now well defined. After maturation

of the crRNAs, the dual RNA:Cas9 complex associates with double-stranded DNA (17, 22). A helicase domain within Cas9 acts to unwind double stranded DNA, and the protein travels along the DNA strand, pausing at short (3-7bp) recognition sequences, known as proto-spacer adjacent motifs (PAMs) (22-24). Upon pausing, if the crRNA is sufficiently identical to the sequence adjacent to the PAM, Cas9 mediates cleavage of both strands of the DNA using two endonuclease domains (22). The RuvC domain cleaves the non-complementary DNA sequence to the crRNA spacer, while its HNH domain cleaves the complementary strand (22). Following cleavage, the double stranded breaks catalyzed by Cas9 can cause the further degradation of the DNA target by cellular nucleases, and also limit the ability of transcription to occur from the targeted sequence. Overall, sequence-specific cleavage events catalyzed by crRNA targeting and Cas protein endonuclease activity, allow the successful restriction of invading exogenous genetic elements.

However, one of the most unique aspects of the CRISPR-Cas systems, which differentiates these from the canonical restriction-modification systems, is that CRISPR-Cas loci are uniquely adaptive (2, 25). In a currently incompletely defined process, it is thought that the universal Cas proteins, Cas1 and Cas2, recognize foreign nucleic acid that has entered the prokaryotic cell, and process it into a new spacer sequence(s) that is then directly integrated into the CRISPR locus (Figure 1) (25, 26). This allows the individual bacterial cell and its progeny to subsequently target the foreign nucleic acid if encountered again (2, 26, 27). Altogether, CRISPR-Cas systems provide prokaryotes with an unprecedented adaptive mechanism to prepare for, and mitigate, future threats from exogenous genetic elements.

While the majority of all prokaryotes encode a CRISPR-Cas system, and some species even encode different types, is interesting to note that the Type II systems, utilizing the Cas9 endonuclease, are found primarily within the genomes of pathogenic (including *Neisseria meningitidis*, *Campylobacter jejuni*, *Legionella pneumophila, Listeria monocytogenes* (1, 15, 16, 28) and commensal bacteria that interact with eukaryotic hosts. One such group of bacteria, is the genus of Gram-negative, intracellular pathogens, *Francisella*.

Part 3. *Francisella* species: Overview

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 (29). 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 (30). Although it causes disease in rodents, lagomorphs, and numerous other mammals, no animal has been conclusively identified as a reservoir. Instead, the reservoir may be amoeba living within fresh and brackish water. Additionally, there is no transmission between infected persons, so *F. tularensis* is primarily acquired by 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 (31). 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, have led to its history of weaponization (30).

Francisella species are endemic only in the northern hemisphere. *F. tularensis* is the most virulent etiologic agent of tularemia in humans and is the primary disease-causing *Francisella* species in North America. *Francisella 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 *Francisella novicida* species rarely causes disease in humans, though some cases have been documented (32, 33). However, *F. novicida* is highly virulent in mice, has over 98% identity to *F. tularensis* at the DNA level (34), shares many of the same virulence genes (35), and is also used as a model system to study *Francisella* virulence. However, there have been cases of *F. novicida* infection in both immune-compromised and immune-competent individuals. Finally, *Francisella mediasiatica* is a species of intermediate virulence in humans and is found in Central Asia, while *Francisella philomiragia* and *Francisella noatunensis* can cause infections in aquatic organisms including wild and farmed fish (36). Throughout this manuscript we will refer to "*Francisella*" when speaking about general characteristics shared by numerous species and subspecies, and will otherwise refer to specific species and subspecies by name.

F. tularensis and F. holarctica are the etiological agents of the disease tularemia, also known as rabbit fever. Tularemia is characterized by a 3-5 day incubation period (30) 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 (37). 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 (38).

Part 4. *Francisella* species: Intracellular Life Cycle

Upon infection, *Francisella* initially comes into contact with extracellular defenses such as complement, antibody, and cationic antimicrobial peptides (39-42). Binding of these components to bacteria directly or indirectly leads to lysis and killing. Therefore, *Francisella* uses multiple surface structures and outer membrane modifications (capsule, LPS O-antigen, modifications that increase surface charge, etc.) to resist these components and block killing. In addition, this prevents structural damage that would release proinflammatory bacterial components capable of initiating a strong immune

response. *Francisella* also enters host cells as an efficient way of evading such extracellular defenses.

After engulfment by phagocytic cells including macrophages, *Francisella* is taken up into phagosomes that contain an array of toxic antimicrobials aimed at degrading the bacteria (Figure 2). However, this pathogen has an equally diverse cache of defenses to counteract host antimicrobials. These once again not only prevent killing, but also the release of proinflammatory bacterial components that could be recognized by host innate immune receptors (including Toll-like Receptors) that stimulate inflammatory responses. Furthermore, similar to entering host cells to avoid extracellular antimicrobials, *Francisella* escapes the phagosome to avoid phagosomal antimicrobials and importantly, reach the cytosol where it can replicate (Figure 2). The cytosol is also, however, guarded by innate recognition and defense systems (including the inflammasome) with which the bacteria must contend.

Part 5. *Francisella* species: Evasion of Toll-like Receptor Signaling

Upon contact with host cells, extracellular and intracellular *Francisella* and other microbes encounter host pathogen recognition receptors (PRRs) that are capable of detecting conserved microbial components known as pathogen-associated molecular patterns (PAMPs) (43). These receptors can then trigger multiple pathways including phagocytosis and inflammatory signaling (43, 44). Toll-like receptors (TLRs) are important PRRs that can recognize PAMPs outside the host cell and in the endosome/phagosome (44, 45). TLR signaling is mediated by TIR domain-containing

adaptor proteins, including MyD88, TRIF, and TIRAP, that activate transcription factors such as NF-κB and IRF3 (44). These transcriptional regulators induce the expression of inflammatory cytokines and type I interferons, resulting in the activation of innate and adaptive immune cells (43, 44). Numerous TLRs recognize bacterial PAMPs including TLR2 which senses bacterial lipoproteins (BLP) and peptidoglycan (PGN)(46, 47), TLR4 which signals in response to lipopolysaccharide (LPS) from Gram-negative bacteria, TLR5 which recognizes flagellin, and TLR9 which senses bacterial CpG DNA (44).

A central component of *Francisella*'s success as a pathogen is its ability to avoid recognition and subvert the host inflammatory response, particularly in the early stages of infection. Indeed, *Francisella* can likely evade or suppress inflammatory signaling by all of the aforementioned bacteria-sensing TLRs. For example, *Francisella* does not encode flagellin, and therefore TLR5 is not activated in response to infection (48). Though mammalian host cells are capable of recognizing *Francisella* DNA through other receptors, TLR9 (present in the membranes of endosomes and phagosomes) is not important for the host response to *Francisella* infection *in vivo* (49). This suggests that *Francisella* may subvert TLR9 activation, possibly by limiting the release of its DNA in the phagosome through resistance to damaging antimicrobial agents and/or by directly modulating TLR9 signaling. In support of the idea that *Francisella* maintains strong structural integrity to prevent DNA release, Peng *et al*. have shown that some hyperinflammatory *F. novicida* mutants exhibit increased bacteriolysis and DNA release during *in vitro* infection (50).

Furthermore, while TLR4 is considered a primary sensor of Gram-negative bacteria, *Francisella* LPS does not efficiently activate TLR4 when compared to LPS from *E. coli* and other Gram-negative pathogens (51). Many Gram-negative bacteria that elicit robust TLR4 signaling synthesize a hexaacylated lipid A portion of LPS with acyl chains of 12-14 carbons and phosphate groups at the 1 and 4' positions (51). However, *Francisella* modifies or removes these important signaling structures. For example, *Francisella* lipid A acyl chains are two to six carbons longer than those in *E. coli* LPS (51). In addition, *Francisella* lipid A is tetraacylated as it lacks the canonical 3' double acyl chain, and both the 1 and 4' phosphate groups are absent (51, 52).

In part due to the absence of significant signaling from other TLRs, TLR2 is the primary TLR involved in the inflammatory response to *Francisella* infection (53). Known *Francisella* TLR2 agonists include the uncharacterized lipoproteins LpnA/Tul4 and FTT_1103 (54, 55). To our knowledge, TLR2 recognition of *Francisella* PGN has not been reported and the status of PGN as a TLR2 ligand is still under debate (46, 47, 56). Several reports have shown that TLR2 is essential for the early inflammatory response to *Francisella* infection in macrophages *in vitro*, as well as a critical component of the host response to *in vivo Francisella* infection as demonstrated by its requirement for control of pulmonary and intradermal infection (53, 57, 58).

However, while *Francisella* elicits TLR2-dependent signaling, it can also dampen this response. Specific *Francisella* genes have been identified that play a role in this suppression of the host inflammatory response. For example, a mutant in the FPI gene

iglC not only failed to escape the phagosome or replicate in macrophages, but was also unable to suppress TNFα production and other inflammatory responses (59-62). In addition, infection of macrophages with wild-type LVS blocked TLR2 and TLR4 activation in response to the addition of *E. coli* BLP and LPS, respectively, while the *iglC* mutant could not block this signaling (59). Further supporting the attenuation of TLR2 signaling by *Francisella*, infection with *F. tularensis* has been shown to reduce TLR2 expression (63). Mechanisms the bacteria use to resist damage by antimicrobials, and therefore the release of BLPs, are also an indirect way of evading TLR2 signaling. It has been reported that high molecular weight (HMW) carbohydrates from "host-adapted" LVS and *F. tularensis* capsules impede TLR2-dependent cytokine production in murine macrophages (*64*), possibly by shielding the bacteria from antimicrobials. Taken together, these data indicate that *Francisella* is capable of subverting TLR2 signaling, while the host uses this pathway as a mechanism of innate defense.

Part 6. *Francisella* species: Evasion of Inflammasome Activation

When *Francisella* reaches the cytosol, it has trafficked past TLRs and phagosomal defenses. It is nonetheless faced with a formidable challenge: replicate to high numbers without triggering an effective immune response. This is all the more challenging since the process of bacterial replication results in the release of PAMPs that can be recognized by cytosolic PRRs. Like the cell surface and the phagosome, the cytosol is equipped with numerous PRRs that recognize an array of bacterial products and elicit an immune response aimed at clearing the invaders. One large family of cytosolic PRRs is the Nod-like receptor (NLR) family whose 22 members respond to a diverse set of PAMPs

including PGN (Nod1 and Nod2), flagellin (NLRC4, NAIP5 and NAIP6) (*65*), components of bacterial type III secretion systems (NLRC4), as well as damage induced by pore-forming toxins (NLRP3)(66).

In particular, cytosolic DNA released during *Francisella* infection is recognized by the PRR absent in melanoma 2 (AIM2)(67, 68), whose expression is up-regulated by IFN-β (69). AIM2 is a member of the PYHIN (Pyrin and HIN-200) family of proteins that binds double-stranded DNA through a HIN-200 domain (70-72). AIM2 contributes to host defense by initiating the formation of a multiprotein complex called the inflammasome that is comprised of a PRR (from the NLR or PYHIN families), the scaffolding protein ASC, and the cysteine protease caspase-1 (73). Inflammasome activation causes infected cells to undergo an inflammatory form of programmed cell death called pyroptosis (74). This cell death may release bacteria into the extracellular environment where they can no longer replicate and can easily be taken up by cells such as neutrophils that are not permissive for replication (75). Additionally, pyroptosis is accompanied by the release of the pro-inflammatory cytokines IL-1β and IL-18 from dying cells, serving to recruit and activate other immune cells and further promote bacterial clearance. AIM2 inflammasome activation is essential for controlling *F. novicida* infection since mice lacking components of this defense system succumb to infection much more rapidly than their wild-type counterparts (72, 76). The NLRC4 and NLRP3 inflammasomes do not play an obvious role in combating *Francisella* during murine infection (76, 77). TLR2 signaling is necessary for not only the expression of IL-1β, but also accelerates the rate of inflammasome activation during *F. novicida* infection (78), while type I IFN is essential

for inflammasome activation (79). Therefore, dampening TLR2 and IFN signaling, two major host defense pathways that contribute to inflammasome activation,

Several *Francisella* genes have been implicated in modulating inflammasome activation (67, 80-82). However, Peng *et al*. recently showed that these genes were not necessarily actively modulating the inflammasome (50). Instead, the increased induction of macrophage death triggered by a panel of mutants lacking genes encoding membrane-associated proteins was due to increased bacteriolysis in the cytosol that allowed for the leakage of DNA and increased inflammasome activation. This study suggests that maintenance of membrane integrity is critical for *Francisella* to prevent the release of PAMPs and induction of the inflammasome.

Part 7. Thesis Overview

A number of genetic screens have been performed to identify those genes which *Francisella* species require for intracellular survival and virulence *in vivo*. However, while hundreds of genes have been identified for these pathogenic processes, their function is largely unknown. My thesis project began with the aim to identify how one particular gene identified from these screens, *FTN_0757,* contributed to the survival of *Francisella in vivo*. We found that this gene actively repressed the total content of BLP present in the *Francisella* envelope, dampening the activation of TLR2 *in vivo* (Chapter 2). Surprisingly, we found that *FTN_0757* encodes the CRISPR-Cas endonuclease, Cas9. We determined that *Francisella* Cas9 acts in conjunction with two small RNAs to directly modulate the stability of a BLP transcript, the first such description of CRISPR-

Cas components mediating gene regulation (Chapter 3). We next found that such regulation of total BLP content is a necessary attribute to allow *Francisella* to maintain its envelope integrity and subsequently resist the actions of antimicrobials and prevent release of PAMPs that are detected by the host during infection, allowing subversion of both TLR2 signaling and cytosolic inflammasome activation (Chapter 4). Interestingly, not all *Francisella* species contain an intact Cas9, or CRISPR-Cas locus, leading to interesting speculation regarding how CRISPR-Cas systems can shape genomic content (Chapter 5). Additionally, there is now increasing evidence that CRISPR-Cas systems may have important functions in other species as well (Chapter 6). Finally, there exists the strong potential to re-program the *Francisella* Cas9 to target any RNA of interest, which could provide a platform for a new form of RNA intereference in a number of experimental systems (Chapter 7). In total, this project has uncovered a new role for CRISPR-Cas systems in gene regulation that will have broad implications in understanding gene regulation, bacterial physiology, and virulence in the numerous species which encode these systems.

Chapter 1. References

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Figure 1.

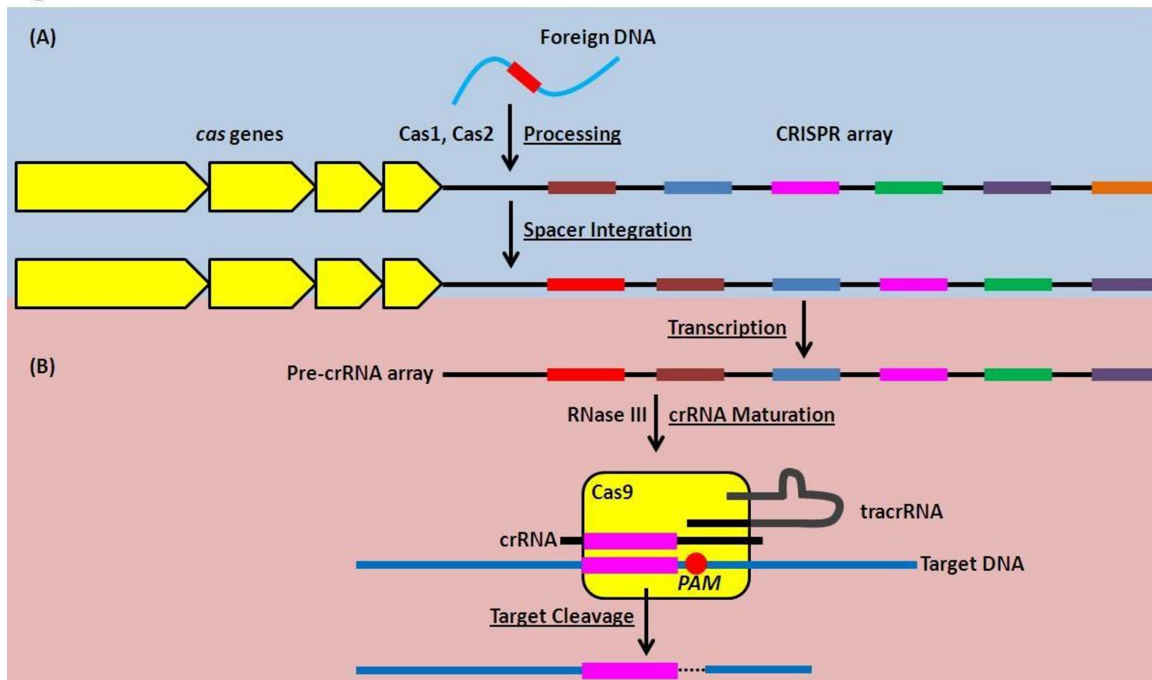

Figure 1. Function of the Type II CRISPR-Cas system in adaptive nucleic acid restriction. (A) Foreign DNA is recognized by Cas1 and Cas2 and is processed into a new spacer sequence (red) within the CRISPR array (Adaptation phase, blue). **(B)** To restrict foreign DNA, the CRISPR array is transcribed as a single transcript (pre-crRNA array) and matured into small targeting crRNAs in a process requiring RNase III and tracrRNA. The dsRNA complex of crRNA and tracrRNA is associated with Cas9 and the spacer sequence within the crRNA can hybridize to complementary DNA sequences. Cas9 then mediates cleavage of the targeted DNA downstream of the proto-spacer adjacent motif, or PAM, highlighted by the red circle (Effector phase, pink).

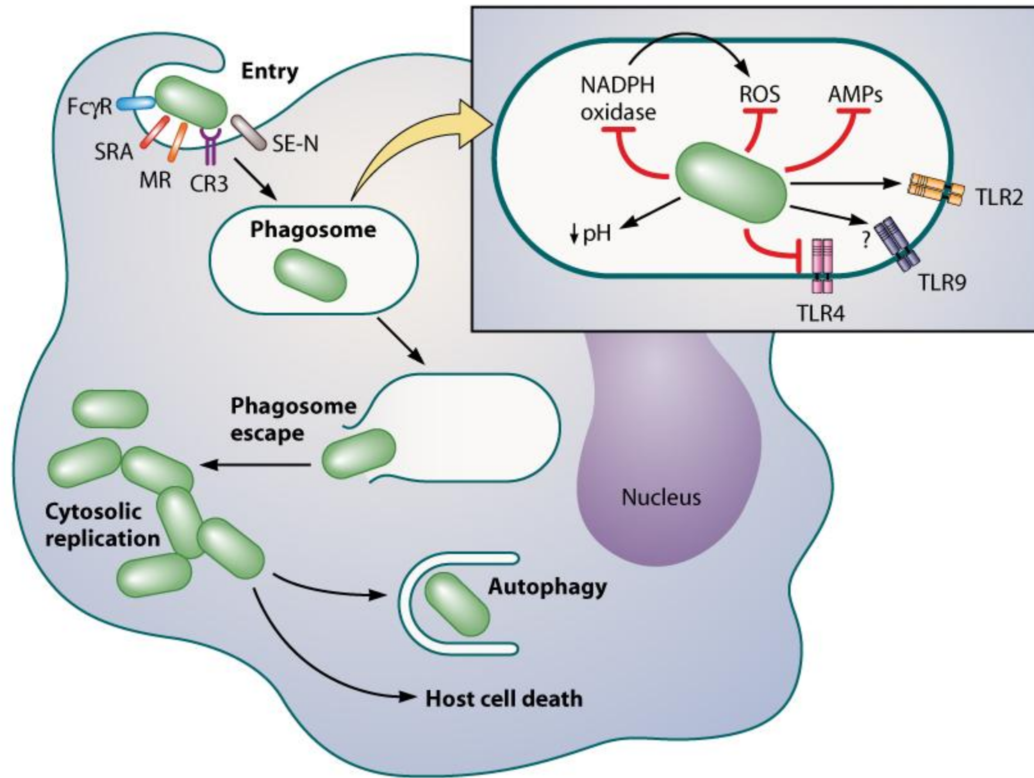

Figure 2. Stages of *Francisella* pathogenesis in the macrophage. *Francisella* can be detected by multiple macrophage receptors and is engulfed by a unique pseudopod loop mechanism. It then traffics to an early phagosome called the *Francisella*-containing phagosome (FCP). *Francisella* uses multiple 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.

Chapter 2: Repression of bacterial lipoprotein production by *Francisella novicida* facilitates evasion of innate immune recognition

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Chapter 2 Abstract

Innate recognition systems, including the Toll-like receptors (TLRs), play a critical role in activating host defenses and proinflammatory pathways in response to infection. Pathogens have developed strategies to subvert TLRs in order to survive and replicate within the host. The model intracellular pathogen, *Francisella novicida*, modulates host defenses to promote survival and replication in macrophages. TLR2, which recognizes bacterial lipoproteins (BLPs), is critical for activating host defenses and proinflammatory cytokine production in response to *Francisella* infection. Here we show that the *F. novicida* protein FTN_0757 acts to repress BLP production, dampening TLR2 activation. The Δ*FTN_0757* mutant strain induced robust TLR2-dependent cytokine production in macrophages compared to wild-type bacteria, and produced increased amounts of BLPs. The deletion of one BLP (FTN_1103) from Δ*FTN_0757* decreased the total BLP concentration to near wild-type levels and correlated with a decrease in the induction of TLR2 signaling. The overproduction of BLPs also contributed to the *in vivo* attenuation of the Δ*FTN_0757* mutant, which was significantly rescued when *FTN_1103* was deleted. Taken together, these data reveal a novel mechanism of immune evasion by the downregulation of BLP expression to subvert TLR2 activation, which is likely also used by other intracellular bacterial pathogens.

Chapter 2 Introduction

Early detection of microbial pathogens by pattern recognition receptors (PRRs) is an important component in the initiation of an effective immune response aimed at clearing infections (1). One group of PRRs, the Toll-like receptors (TLRs), are type I integral membrane proteins present on the surface of a diverse set of host cells, as well as in endosomes. TLRs are responsible for the recognition of a number of different microbial components, or pathogen-associated molecular patterns (PAMPs) (2). For example, lipid A from Gram-negative bacteria is recognized by TLR4, flagellin by TLR5, CpG DNA by TLR9, and bacterial lipoproteins (BLPs) by TLR2 (3-6). Upon recognition of their cognate PAMP, TLRs signal to activate transcription factors, including NF-κB, which lead to the production of proinflammatory cytokines, chemokines, and antimicrobial peptides (2). Production of these proteins stimulates an array of host defenses including the activation of macrophages and the recruitment of neutrophils, which help to fight infection (2, 7).

Pathogens have developed a variety of mechanisms to prevent TLR signaling. Some pathogens secrete effector proteins into host cells that block components of the TLR signaling pathways. For example, the enteropathogenic *Escherichia coli* effector NleE directly blocks NF-κB activation by preventing IκB degradation, and the *Brucella* spp. effector Btp1 binds the cytosolic TIR domain of TLR2 and TLR4, preventing the recruitment of downstream signaling proteins (8-10). Pathogens can also prevent TLR signaling by modifying PAMPs. For instance, alterations in the amino acid sequence of the flagellin monomer allow *Helicobacter* spp. to prevent recognition by TLR5 (11).

Specific modifications to the structure of lipid A, such as the addition of acyl chains (*Salmonella* spp.) or the removal of acyl chains (*Yersinia pestis*), facilitate evasion of TLR4 signaling (12, 13). Furthermore, recognition of pathogens by TLRs can be subverted by preventing the release of PAMPs. For example, the masking of flagella by a lipid membrane sheath is used by *Vibrio* spp. to prevent recognition of flagellin by TLR5 (14, 15).

The Gram-negative bacterium *Francisella novicida*, a model intracellular pathogen closely related to highly virulent *F. tularensis*, has evolved strategies to subvert host defense proteins including TLRs (16, 17). Similar to other *Francisella* spp., *F. novicida* can infect and replicate within host macrophages, which express numerous TLRs (18, 19). Extensive modification of its lipid A, including alterations in the length and number of acyl chains, results in a lack of signaling through TLR4 (20, 21). This is a critical component of *Francisella* pathogenesis since mutants that cannot modify lipid A are severely attenuated *in vivo* (20). Furthermore, *Francisella* does not encode flagellin and therefore does not activate TLR5 signaling (22, 23). The bacteria are, however, recognized by TLR2, which plays an important role in host defense as indicated by the increased susceptibility to infection of mice lacking TLR2 (22-25). Thus, molecular strategies used by *Francisella* spp. to subvert TLR2 signaling would likely promote pathogenesis.

The specific proteins used by *Francisella* spp. to suppress host defenses, and their mechanisms of action, are largely unknown. We and others previously used *in vivo*

genetic screens to identify critical *Francisella* virulence determinants (26-30). One gene that we identified, *FTN_0757* (also termed *FTT_0584* before the *F. novicida* genome was sequenced), is necessary for *F. novicida* virulence in mice and has been shown to be involved in the suppression of several pro-inflammatory cytokines (26, 30). However, its mechanism of action is unknown. Therefore, we set out to elucidate how *FTN_0757* contributes to the subversion of innate inflammatory responses and better define the breadth of its effect on the host response.

Here, we demonstrate that FTN_0757 action leads to the suppression of a large panel of NF-κB-dependent genes, as well as genes encoding other host defense proteins. We show that the increased production of cytokines and chemokines in response to infection by the *FTN_0757* mutant is due to hyperstimulation of TLR2. More specifically, we show that FTN_0757 functions to limit the expression and production of BLPs that induce proinflammatory mediators through TLR2. One BLP, FTN_1103, is highly overproduced in the *FTN_0757* mutant and accounts for the majority of the increased BLP content. Deletion of *FTN_1103* from the *FTN_0757* mutant significantly reduces the activation of TLR2 and rescues the virulence defect of the mutant *in vivo*. To our knowledge, this is the first demonstration that suppression of BLP content by an intracellular pathogen allows subversion of TLR2-dependent responses and promotes virulence. Furthermore, this work may provide insights into ways by which other pathogens escape recognition by TLR2.

Chapter 2 Results

***FTN_0757* Suppresses TLR2-dependent Proinflammatory Responses in Macrophages.** Previous studies by our laboratory and others showed that a Δ*FTN_0757* mutant of *F. novicida* induced increased macrophage secretion of several proinflammatory cytokines compared to wild-type bacteria (26, 30). However, the full scope of this hyperinflammatory response and the mechanism underlying this phenotype were unclear. As a first step towards defining the extent of this effect, we sought to measure the breadth of the inflammatory response induced by the Δ*FTN_0757* mutant during macrophage infection using microarray analysis. We harvested RNA from murine bone marrow-derived macrophages (BMDM) infected with wild-type *F. novicida* or the Δ*FTN_0757* mutant, as well as uninfected controls. Microarray analysis revealed that there was a broad and robust increase in the number and magnitude of macrophage genes expressed in response to infection by the Δ*FTN_0757* mutant compared to wild-type bacteria (Tables S1, S2 and Figure 1A [Tables available online at http://onlinelibrary.wiley.com/doi/10.1111/j.1462-5822.2012.01816.x/full]).

Furthermore, Ingenuity Pathway Analysis revealed that host defense pathways consisting of genes encoding PRRs that recognize bacteria and proteins important for proinflammatory cytokine signaling were induced to higher levels in Δ*FTN_0757*-infected macrophages than in macrophages infected with wild-type bacteria (Figure S1A). This analysis also identified 53 NF-κB-regulated genes as being induced in macrophages infected with the Δ*FTN_0757* mutant, compared to only 25 genes in wild-type-infected macrophages (Figures S1B, C).

To validate the microarray results, we first measured IL-6 production since it was the most differentially expressed gene in macrophages infected with the Δ*FTN_0757* mutant compared to those infected with wild-type bacteria (Figure 1A). In agreement with our microarray data, macrophages infected with the Δ*FTN_0757* mutant secreted significantly higher levels of IL-6 than wild-type-infected macrophages (Figure 1B). This response was dependent on TLR2 since TLR2$^{-/-}$ cells did not produce detectable levels of IL-6 (Figure 1B). In addition to increased cytokine production, macrophages infected with the Δ*FTN_0757* mutant secreted significantly higher amounts of the chemokines KC and MIP-1β compared to those infected with wild-type bacteria, further validating our microarray results (Figure1B). This response was also TLR2-dependent. To ensure that differences in cytokine and chemokine production were not due to differences in the ability of these strains to replicate within macrophages, we assessed the bacterial burden and found that both wild-type and Δ*FTN_0757* bacteria replicated with the same kinetics in wild-type and TLR2$^{-/-}$ macrophages (Figure S2A,B). Taken together, these findings validate our microarray results by demonstrating that TLR2 is required for the hyperinflammatory response elicited in macrophages infected with the Δ*FTN_0757* mutant.

***FTN_0757* Represses Production of Bacterial Lipoproteins.** Since we have shown that infection with Δ*FTN_0757* elicits a broad and robust increase in TLR2-dependent signaling compared to wild-type bacteria, we sought to explore the mechanism responsible for this phenotype. Bacteria can block TLR signaling through active

processes such as secretion of effectors, or dampen host signaling by modulating PAMPs in numerous ways that lessen the immunostimulatory capacity of the bacteria (8-13). To test whether the FTN_0757-dependent subversion of TLR2 signaling was an active process requiring live bacteria, we treated macrophages with heat-killed preparations of wild-type and Δ*FTN_0757* mutant bacteria. We used IL-6 as a marker for the proinflammatory response due to its robust induction during macrophage infection with Δ*FTN_0757* (Figures 1A, B). We found that heat-killed Δ*FTN_0757* induced a significant increase in IL-6 production compared to killed wild-type bacteria, and that this response was completely dependent on TLR2 (Figure 2A). This demonstrated that the suppression of cytokine production by FTN_0757 is not dependent on an active process, but is instead due to a difference in a heat-resistant component(s) of the bacteria. Since the immunostimulatory moiety of BLPs is heat-resistant, BLPs signal through TLR2, and *Francisella* is known to encode BLPs that activate TLR2 (22-25, 31, 32), we hypothesized that changes in BLPs were responsible for the hyperinflammatory phenotype of Δ*FTN_0757*.

BLPs are located in bacterial membranes, so we next isolated the total membrane protein fraction from the wild-type and Δ*FTN_0757* strains and tested them for their TLR2-stimulating activity. The membrane fraction from Δ*FTN_0757* induced increased IL-6 production compared to the wild-type fraction (Figure 2B). This response was TLR2-dependent, similar to what we observed with the heat-killed preparation (Figure 2A) and infection with live bacteria (Figure 1B). This is consistent with the hypothesis that differences in BLPs are responsible for the hyperinflammatory phenotype of

Δ*FTN_0757*. To further explore this possibility, we fractionated and quantified BLPs from the total membrane protein fraction of each strain. Strikingly, we found that the Δ*FTN_0757* strain contained roughly twice as much total BLP as wild-type bacteria (Figure 2C). This data provides a potential explanation for the increased induction of proinflammatory cytokines elicited by Δ*FTN_0757*, since higher levels of BLPs would likely lead to increased TLR2 activation. To rule out the possibility that BLPs from the Δ*FTN_0757* strain had increased activity on a molar basis compared to BLPs from the wild-type strain, we treated macrophages with equal concentrations of the BLP fraction from each strain. Both BLP fractions induced an equivalent amount of IL-6 production in a TLR2-dependent manner (Figure S3), demonstrating that the BLPs from each strain had the same intrinsic TLR2-stimulating activity. Taken together, these data suggest that the hyperinflammatory phenotype of Δ*FTN_0757* is due to its increased BLP content leading to more robust TLR2 activation, rather than differences in the ability of its BLPs to act as TLR2 ligands.

***FTN_0757* Represses the Expression of the Bacterial Lipoprotein FTN_1103.** The significant increase in BLP concentration in the Δ*FTN_0757* strain could be due to an increase in the levels of a small number of specific BLPs, or a more global increase in overall BLP production. In order to differentiate between these possibilities, we analyzed the respective protein composition of the BLP fraction from each strain via SDS-PAGE. While most protein bands were present at similar levels, a specific band of approximately 30 kD was highly enriched in the BLP fraction of the Δ*FTN_0757* strain compared to wild-type (Figure 3A). Utilizing an LC-MS/MS peptide mass fingerprinting approach, we

identified the band to be FTN_1103. Although annotated in the NCBI database as a hypothetical protein, FTN_1103 contains the typical amino acid motifs associated with BLPs, including a positively charged N-terminal region, a hydrophobic H-region, and a conserved lipobox motif (Leu-Gly-Ser) adjacent to the invariant cysteine at residue 29, which would serve as a lipidation site (Figure 3B). Also, consistent with its presence in the BLP fraction, FTN_1103 is predicted to be a BLP by the PRED-LIPO lipoprotein prediction server with a reliability score of 0.996 (33). To further show that the protein overproduced in Δ*FTN_0757* is indeed FTN_1103, we generated a Δ*FTN_0757*/Δ*FTN_1103* double deletion strain and analyzed its BLP content. The enriched protein that we previously identified as FTN_1103 was absent in the BLP fraction of the Δ*FTN_0757*/Δ*FTN_1103* mutant, confirming its identity as FTN_1103 (Figure 3A). Together, these data demonstrate that FTN_1103 is a BLP, which we showed is highly overproduced in Δ*FTN_0757*.

We next measured the proportion of the increased BLP pool in Δ*FTN_0757* that was due to the increase in production of FTN_1103. Deletion of *FTN_1103* in the Δ*FTN_0757* strain led to a large reduction in the total BLP concentration, almost to the level present in the wild-type strain (Figure 4A). Altogether these data demonstrate that FTN_0757 is required to repress expression of *FTN_1103*, and suggests that overproduction of the BLP, FTN_1103, may cause the increased TLR2-stimulating activity of the Δ*FTN_0757* strain.

To learn more about how FTN_0757 regulates FTN_1103, we tested whether this occurs at the transcriptional level by quantifying the expression level of *FTN_1103* mRNA isolated from broth-grown wild-type or Δ*FTN_0757* strains (Figure S4A).We observed a large increase in *FTN_1103* expression in the Δ*FTN_0757* strain compared to wild-type, confirming the observation that lack of FTN_0757 results in an increase in *FTN_1103* expression. Furthermore, to investigate whether overexpression occurred in the context of a macrophage infection, we analyzed RNA from wild-type- or Δ*FTN_0757*-infected macrophages and found that the Δ*FTN_0757* strain overexpressed *FTN_1103* compared to wild-type (Figure S4B). Additionally, in order to further prove that FTN_0757 is a regulator of *FTN_1103*, we generated a strain in which the *groE* promoter drives increased expression of *FTN_0757*, compared to its natural promoter (Figure S4C) (34). During growth in broth, this strain overexpressing *FTN_0757* exhibited a significant decrease in *FTN_1103* expression compared to wild-type (Figure S4D). This is notable, since this result implies that *FTN_1103* expression is controlled by the action of FTN_0757, rather than changes in *FTN_1103* expression being an indirect effect of the absence of *FTN_0757*. Furthermore, the effect of FTN_0757 on *FTN_1103* expression is specific since we did not observe a significant difference in gene expression of other predicted BLPs (such as *dsbA/ FTN_0771*)(32) or genes within the *Francisella* Pathogenicity Island (FPI) between the wild-type and Δ*FTN_0757* strains (data not shown). Altogether, these data suggest that FTN_0757 acts as a negative regulator of *FTN_1103*, and that FTN_1103 is the primary contributor to the increased BLP content in the Δ*FTN_0757* strain.

***FTN_0757* Represses *FTN_1103* to Evade TLR2 Activation in Macrophages.** To determine whether overproduction of FTN_1103 in Δ*FTN_0757* was the major basis for the increased TLR2 activation induced by this strain, we treated macrophages with either heat-killed preparations or total membrane protein fractions derived from wild-type, Δ*FTN_0757*, or Δ*FTN_0757*/Δ*FTN_1103*. Similar to our previous observation, macrophages stimulated with preparations from Δ*FTN_0757* elicited a significantly increased TLR2-dependent IL-6 response as compared to those treated with preparations from the wild-type strain (Figures 4B, C).

However, macrophages treated with equivalent fractions from the Δ*FTN_0757*/Δ*FTN_1103* mutant secreted significantly lower levels of IL-6 compared to those treated with Δ*FTN_0757* preparations (Figures 4B, C). Deletion of *FTN_1103* did not reduce the TLR2-stimulatory activity of Δ*FTN_0757* completely to wild-type levels. This is likely due to smaller increases in the production of other BLPs and correlates with the incomplete reduction of BLP levels in the Δ*FTN_0757*/Δ*FTN_1103* mutant (Figure 4A). These data demonstrate that the major cause of the increased TLR2-stimulating capacity of killed and membrane preparations of Δ*FTN_0757* is the overproduction of FTN_1103.

We next sought to determine whether FTN_1103 overexpression contributed to the increase in TLR2-dependent cytokine production elicited by Δ*FTN_0757* during infection of macrophages. First, however, we determined if *FTN_0757* and *FTN_1103* expression was altered during the course of macrophage infection. We found that *FTN_0757*

expression was induced early during infection (1 hr) (Figure 5A), when *Francisella* is located within host phagosomes and co-localizes with TLR2. This correlated with a decrease in expression of *FTN_1103* (Figure 5B). As the infection progressed past 2 hours, when *Francisella* escapes the phagosome and resides in the cytosol, *FTN_0757* expression decreased, correlating with a significant increase in *FTN_1103* expression. These data suggest that differential regulation of both *FTN_0757* and *FTN_1103* occurs during infection of host cells, and therefore, may contribute to subdued recognition by TLR2 by preventing *FTN_1103* overexpression during early phases of macrophage infection.

We next sought to determine the contribution of FTN_1103 to the increase in TLR2-dependent cytokines observed during Δ*FTN_0757* infection. We infected macrophages with the Δ*FTN_0757*/Δ*FTN_1103* mutant and notably, observed the amount of IL-6 induced by this strain was much less than that induced by macrophages infected with Δ*FTN_0757* (Figure 5C). This is consistent with our results for stimulation with heat-killed preparations and membrane protein fractions (Figures 4B, C). As a further control, we genetically restored *FTN_1103* into the Δ*FTN_0757*/Δ*FTN_1103* strain. Following infection of macrophages with this strain, we observed a restoration of the hyperinflammatory defect of the Δ*FTN_0757* strain (Figure S5). Collectively, these data show that the TLR2-dependent cytokine response induced by Δ*FTN_0757* is due to the over-production of FTN_1103, and suggest that *FTN_0757* alters *FTN_1103* expression during the course of infection to dampen recognition by TLR2.

***FTN_0757* Repression of BLP Expression is Critical for *F. novicida* Virulence *in vivo*.** The Δ*FTN_0757* mutant is severely attenuated in mice compared to wild-type bacteria (26). Since deletion of *FTN_1103* in the Δ*FTN_0757* mutant rescued the majority of its hyperinflammatory phenotype during macrophage infection, we tested whether it would also rescue its virulence attenuation *in vivo*. First, as a control, we tested whether *FTN_1103* expression was upregulated in the Δ*FTN_0757* mutant during *in vivo* infection. We measured the level of *FTN_1103* expression in the wild-type and Δ*FTN_0757* strains at 6 hours after subcutaneous infection of mice, an early timepoint when the loads of each strain were similar (data not shown). We found that the *FTN_1103* transcript was indeed present at higher levels in the Δ*FTN_0757* mutant compared to wild-type bacteria (Figure 6A). This demonstrates that the regulation of *FTN_1103* by FTN_0757 occurs during *in vivo* infection, similar to our findings with bacteria grown in rich media and during macrophage infection (Figures S4A, B).

To determine whether overexpression of FTN_1103 contributed to *in vivo* attenuation of the Δ*FTN_0757* mutant, we performed competition experiments. We infected mice subcutaneously with a 1:1 mixture of wild-type and either the Δ*FTN_0757* or Δ*FTN_0757*/Δ*FTN_1103* mutant and enumerated bacteria in the spleen at 48 hpi. The Δ*FTN_0757* mutant was >100,000-fold attenuated compared to wild-type bacteria (Figure 6B), in agreement with our previous work (26). In contrast, the Δ*FTN_0757*/Δ*FTN_1103* mutant was present at much higher levels than the Δ*FTN_0757* mutant, and was only ~100-fold attenuated compared to wild-type. This represents 1,000-fold complementation of the Δ*FTN_0757* mutant as a result of deleting *FTN_1103*. The

lack of complete complementation correlates with the BLP content, macrophage stimulation and infection experiments using the Δ*FTN_0757/*Δ*FTN_1103* strain, and might be attributed to an increase in the expression of other BLPs in the Δ*FTN_0757* mutant (Figures 4, 5). As a control, we tested the phenotype of the Δ*FTN_1103* mutant and found that it was present at levels close to those of the wild-type strain, indicating that *FTN_1103* alone does not play an important role in virulence under these infection conditions (Figure 6B). Taken together, these data indicate that increased FTN_1103 production in the Δ*FTN_0757* mutant significantly contributes to its attenuation *in vivo*.

Chapter 2 Discussion

During *in vivo* infection, macrophages are among the first cells that *Francisella* encounters after entering the host (35), and TLR2 expressed on the surface of these immune cells recognizes *Francisella* BLPs and induces a proinflammatory cytokine response aimed at clearing this pathogen. Our findings reveal a novel mechanism of TLR2 evasion: repression of BLP expression. We found that *F. novicida* FTN_0757 strongly represses the expression of FTN_1103, a BLP whose function is unknown but is not essential for bacterial replication *in vitro* or virulence *in vivo*. Furthermore, *F. novicida* temporally represses the expression of this BLP during macrophage infection. Based on these findings, we propose a model where *F. novicida* represses BLP expression during the first hour of macrophage infection (Figures 5A, B) when it could be recognized by TLR2 in the phagosome (36). Subsequently, *FTN_1103* expression is upregulated once this pathogen escapes the phagosome and enters the cytosol. A loss of membrane-associated proteins in *F. novicida* makes this bacterium more susceptible to

intracellular bacteriolysis (30). Therefore, the upregulation of BLP expression after phagosomal escape (37) could help to refortify and stabilize the outer membrane, preventing the leakage of PAMPs in the cytosol.

Since TLR2 is essential for controlling *Francisella* infection *in vivo* (24, 38), evading TLR2 activation may provide this bacterium with precious time to replicate inside the host without triggering an inflammatory response and reach high enough numbers to withstand attacks by the host immune system. The Δ*FTN_0757* mutant, which elicited robust production of a broad array of TLR2 and NF-κB-dependent inflammatory chemokines and cytokines in macrophages (Figures 1, S1), was severely attenuated *in vivo*. Macrophages infected with the mutant produced significantly higher levels of chemokines, such as CXCL1 and CXCL2, which promote neutrophil recruitment (39). Neutrophils are non-permissive for *Francisella* replication (7) and an influx of these cells *in vivo* could greatly reduce the bacterial burden. Furthermore, the Δ*FTN_0757* mutant elicited macrophages to produce a combination of TLR2-dependent cytokines that, if produced *in vivo*, can act to turn this replicative niche into inhospitable death chamber for *F. novcida*. For example, IL-12 was significantly upregulated in mutant-infected macrophages, and robust production of this cytokine *in vivo* can induce T cells and NK cells to produce IFN-γ (40), a potent inducer of macrophage antibacterial defenses including nitric oxide production. In turn, IFN-γ activates macrophages making them less permissive to *Francisella* replication (41). Taken together, these findings highlight the importance of this novel immune evasion mechanism because an inability of *F. novicida* to repress BLP production resulted in the induction of a robust TLR2-dependent

inflammatory cytokines response that activated innate immune cells to kill bacteria ultimately leading to the attenuation of the Δ*FTN_0757* mutant. In fact, this repression of BLPs by FTN_0757 to evade innate immune responses is somewhat reminiscent of the *Salmonella* virulence factor TviA represses flagellin expression when this pathogen invades the intestinal mucosa and can be recognized by TLR5, but not in the lumen where TLR5 is not present (42).

In addition to contributing to the evasion of innate defenses, suppression of BLP expression could also play an important role in evading adaptive immune responses. B cells provide early protection against *Francisella* infection (43), and antibodies generated in response to *Francisella* LPS provides long-term protection against challenge (44). These circulating antibodies could be contributing to protection by opsonizing disseminating bacteria in the blood during the extracelluar phase of *Francisella* infection (45). Interestingly, the serum of vaccinated individuals and patients with prior tularemia infections contained antibodies against highly immunogenic BLP LpnA/Tul4 (46); therefore, modulating the expression of BLPs during the extracelluar phase of infection could allow bacteria to escape antibody-mediated killing. The spirochete *Borrelia burgdorferi* downregulates the expression of one of its most highly immunogenic BLP, OspC, at the onset of the humoral immune response in order to avoid detection by antibodies and subsequent killing (47). *B. burgdorferi* strains that are unable to downregulate OspC expression *in vivo* are rapidly cleared from mice in an antibody-dependent manner (48). Our data suggest that another consequence of OspC downregulation may be the evasion of TLR2 activation. As TLR2 signaling can

contribute to antibody responses (49), this may represent a two-pronged approach to block this critical host defense: dampening the TLR2 activation signal as well as limiting the expression of a major antigen against which the antibody response is directed. By extension, the Δ*FTN_0757* mutant may induce a more potent antibody-mediated immune response than wild-type bacteria, and therefore downregulating FTN_1103 may also lead to evasion of antibody responses.

Our findings reveal a novel mechanism utilized by *F. novicida* to evade TLR2 activation. To our knowledge, this is the first demonstration of an intracellular bacterial pathogen that downregulates BLP expression to evade innate immune recognition. This may represent a new paradigm used by other intracellular pathogens to evade TLR2.

Chapter 2 Materials and Methods

Bacterial strains and growth conditions. *Francisella novicida* strain U112 was kindly provided by Dr. Denise Monack (Stanford University, Stanford, CA). All bacterial cultures were grown overnight at 37°C with aeration in tryptic soy broth (TSB) supplemented with 0.2% L-cysteine (BD Biosciences, Sparks, MD). When necessary, the media was supplemented with kanamycin (30 μg/ml) or tetracycline (20 μg/ml).

Bacterial mutagenesis. Mutant strains (Δ*FTN_0757* and Δ*FTN_1103*) were constructed by allelic replacement as described previously (50, 51) using primers in Table 1. To excise the Flippase Recognition Target (FRT)-flanked kanamycin resistance cassette and create unmarked strains, the kanamycin-resistant mutants were transformed with plasmid

pLG42 encoding the Flp-recombinase, performed as previously described (52). The Δ*FTN_0757*/Δ*FTN_1103* deletion strain was generated by transforming an unmarked Δ*FTN_0757* strain with genomic DNA from the marked Δ*FTN_1103* strain, and selecting for kanamycin resistance. *FTN_1103* was complemented in *cis* into the Δ*FTN_0757*/Δ*FTN_1103* deletion strain through allelic replacement, as described previously (26, 51).

Preparation of bacterial fractions. Overnight cultures of bacteria were subcultured 1:50 into 50ml of TSB with 0.2% cysteine and grown to an OD_{600} of 0.9 – 1.0. Cultures were centrifuged at 5,000 x *g* for 10 minutes to pellet the bacterial cells. For cell-free supernatants, the remaining supernatant was passed through a 0.22µm filter (Millipore, Billerica, MA), and stored at -20°C until use. For heat-killed bacteria, the bacterial pellet was resuspended in PBS (Lonza, Walkersville, MD), boiled at 100°C for 1 hour, and then stored at -20°C until use. For isolation of membrane fractions, resuspended cells were lysed via freeze-thawing for three cycles. The cell lysate was then centrifuged at 10,000 x *g* for 10 minutes to remove unlysed cells. The cleared supernatant was then centrifuged at 120,000 x *g* for 2 hours to pellet the total membrane fraction. Membrane pellets were resuspended in 1ml PBS and stored at -20°C until use. For enrichment of bacterial lipoproteins, pelleted membrane fractions were resuspended in 200µl PBS and 500µl *n*-butanol (Sigma-Aldrich, St. Louis, MO) and centrifuged at 27,000 x *g* for 90 minutes. The aqueous phase, containing an enrichment of bacterial lipoproteins, was collected and stored at -20°C until use (53). Protein fractions were normalized either by colony forming units (cfu) or by protein concentration, measured via the bicinchoninic acid assay

(Thermo Scientific, Waltham, MA), as indicated. Twenty micrograms or 10^8 cfu-equivalents of each fraction were separated via 12-20% SDS-PAGE (Bio-Rad, Hercules, CA) and stained with Coomassie Blue G-250 (Teknova, Hollister, CA).

Microarray analysis. All RNA samples were checked for purity using a ND-1000 spectrophotometer (NanoDrop Technologies) and for integrity by electrophoresis on a 2100 BioAnalyzer (Agilent Technologies). The samples were amplified using the Nugen WT Pico Kit (NuGEN Technology) and the target reactions were run with 25 ng of total RNA. The amplification products were processed through the EXON Module (NuGEN Technology), which creates sense-strand cDNA targets. The sense strand cDNA Targets were then fragmented and labeled using NuGEN's FL-Ovation™ cDNA Biotin Module V2 (NuGEN Technology). Labeled targets were hybridized to GeneChip® Mouse Gene 1.0ST arrays (Affymetrix, Inc.), following Standard Nugen Protocols for target hybridization to the Affymetrix Gene Arrays. The hybridizations were run for 16 hours, 45°C, 60 rpm in an Affymetrix Hybridization Oven 640. The Cartridge arrays were washed and stained using the Affyemtrix Fluidics Stations 450, following Affymetrix protocols. Scanning was performed on an Affymetrix GeneChip 3000 7G scanner, and Affymetrix GCOS software was used to perform image analysis and generate raw intensity data. Probe sets of all samples were normalized by RMA, which includes global background adjustment and quantile normalization. Using the gene annotation provided by Affymetrix, we discarded 11,537 probe sets that did not match to known genes. Student's t-test ($p < 0.02$) and a fold-change filter (mean fold-change > 25%) were used to identify genes differentially expressed in macrophages infected with Δ*FTN0757* strain

compared to those infected with wild-type strain for 4 hours. The expression levels of NF-κB-regulated genes were visualized using Ingenuity Pathway Analysis (Ingenuity Systems) software.

Protein identification by mass spectrometry. After staining with Coomassie Blue G-250, the band of interest was excised and subjected to in-gel digestion (12.5 µg/ml trypsin). Extracted peptides were loaded onto a C_{18} column (75µm inner diameter, 15cm long, ~300nl/min flow rate, 1.9µm resin) (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) and eluted using a 10-30% gradient (Buffer A: 0.1% formic acid, 1% ACN; Buffer B: 0.1% formic acid, 99.9% ACN). The eluted peptides were detected by Orbitrap (300-1600 *m/z*; 1,000,000 automatic gain control target; 500-ms maximum ion time; resolution, 30,000 full-width at half-maximum) followed by ten data-dependent MS/MS scans in the linear ion trap quadrupole (2 *m/z* isolation width, 35% collision energy, 5,000 automatic gain control target, 200-ms maximum ion time) on a hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The acquired tandem mass spectrometer (MS/MS) spectra were searched against and a decoy-concatenated *F. novicida* database (3,393 redundant protein targets) from the NCBI RefSeq protein database project (September 2011) using the Sorcerer-SEQUEST Algorithm version 3.11 r11 (Sage-N Research, San Jose, CA). Search results were filtered with a 1% FDR and summarized by in-house programs, as described by Gozal et al (54).

Macrophage experiments and infections. Murine bone marrow-derived macrophages (BMDM) were prepared from 6-8 week old wild-type and $TLR2^{-/-}$ C57BL/6 mice and

cultured as described (26). Macrophages were cultured in 96-well plates (5-8x10^4 cells/well) in high glucose Dulbecco's modified Eagle's medium (DMEM) (Lonza, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 10% L929-conditioned media (conditioned DMEM) containing M-CSF (macrophage colony stimulating factor) overnight. The media was removed and bacteria were added at a multiplicity of infection (MOI) of 20 or 100 bacteria per macrophage. Plates were centrifuged for 15 minutes at 335 x *g* at room temperature to promote uptake of bacteria. Macrophages were incubated for 30 minutes at 37°C and washed two times before adding warm conditioned DMEM. The concentrations of IL-6, KC, and MIP-1β in culture supernatants at the indicated timepoints after infection were quantified by ELISA (BD Biosciences, Sparks, MD). For treatment with bacterial components, cells were washed gently and media containing heat-killed bacteria, membrane fractions, or bacterial lipoprotein fractions at the given concentrations was added. Macrophages were stimulated for the indicated duration of time, before the cell-culture supernatant was collected.

RNA extraction and quantitative real-time PCR. Overnight cultures of the indicated bacteria were subcultured 1:50 into 50mL TSB with 0.2% L-cysteine and grown to an OD_{600} of 0.9 to 1.0. RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) and purified using the RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturers' instructions. Quantitative real-time RT-PCR (qRT-PCR) was performed using the Power Sybr Green RNA to CT 1-Step Kit (Applied Biosystems, Carlsbad, CA) and gene-specific primers (Table 1) using an Applied Biosystems StepOne

Cycler. Relative transcript levels were calculated by normalizing C_T values to DNA helicase II (*uvrD, FTN_1594*).

Mouse infections. For competition experiments, groups of five wild-type C57BL/6 mice were infected subcutaneously with a 1:1 ratio of wild-type and the indicated mutant strain of *F. novicida* (total of 1 x 10^5 cfu) in sterile PBS. At 48 hpi, the skin, spleen and liver of infected mice were harvested and homogenized in PBS. Serial dilutions were plated on Mueller-Hinton agar supplemented with 0.1% L-cysteine with or without kanamycin for enumeration of bacterial burden in each organ. The competitive index (CI) was calculated using the formula, CI = (mutant cfu in output/wild-type cfu in output)/(mutant cfu in input/wild-type cfu in input). All experimental procedures were approved by the Emory University Institutional Animal Care and Use Committee (protocol #069-2008Y).

Statistics. All experiments were analyzed using the unpaired Student's *t* test except Figure 6A, which was analyzed by a Mann-Whitney test. The CI values from competition experiments were analyzed using the one-sample Student's *t* test and CI values of the *ΔFTN_0757* and *ΔFTN_0757/ ΔFTN 1103* strains were both significantly different from 1 (***$p < 0.0001$).

Chapter 2 Acknowledgements

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Chapter 2 Figures

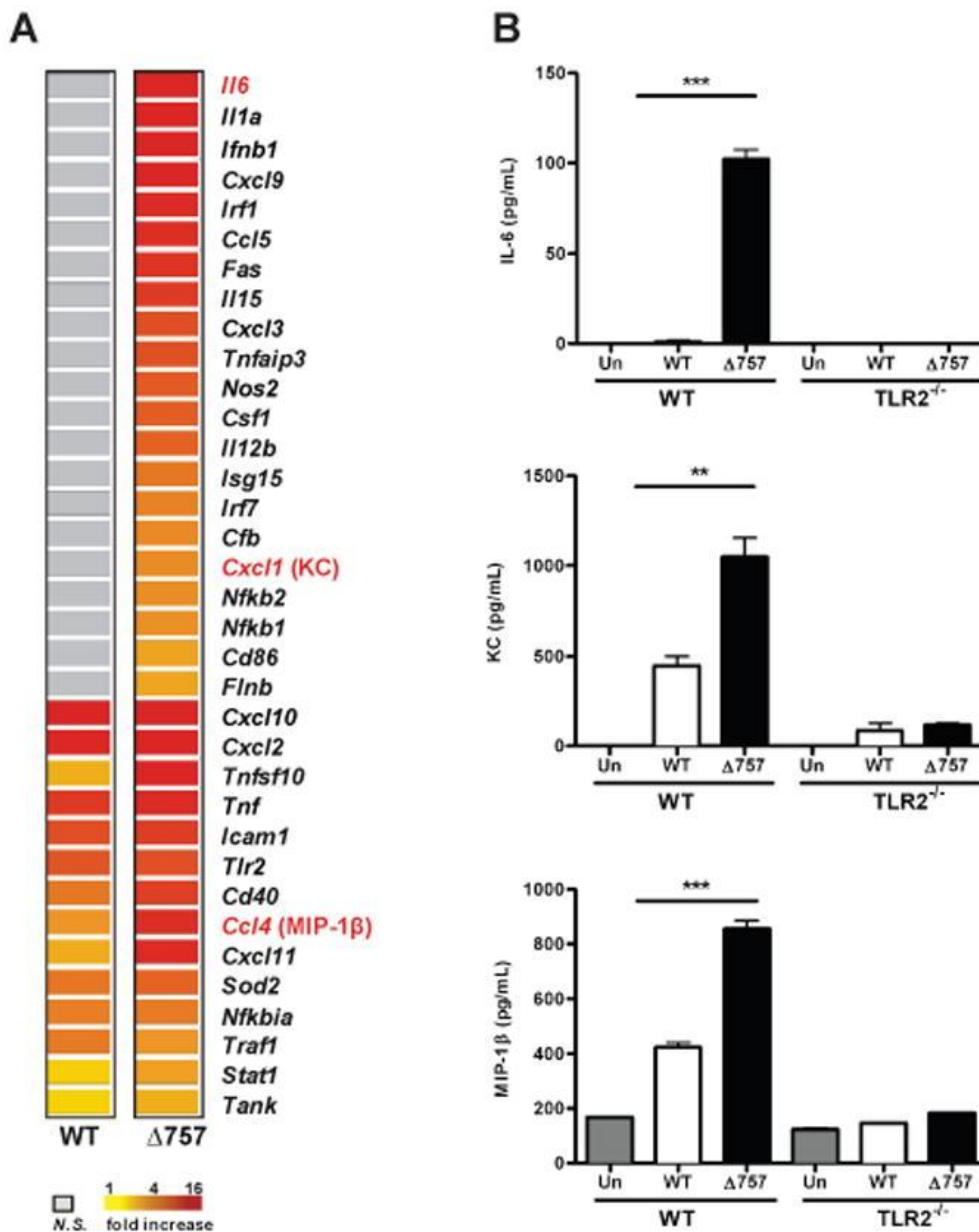


Figure 1. The Δ*FTN_0757* mutant induces robust TLR2-dependent macrophage activation. Wild-type and TLR2$^{-/-}$ bone marrow-derived macrophages (BMDM) were either left untreated (Un) or infected with wild-type (WT) or the Δ*FTN_0757* mutant (Δ757) of *F. novicida* for 4 hours at an MOI of 100:1. **(A)** A heat map of immune genes differentially expressed in infected

macrophages compared to uninfected macrophages. **(B)** The total amount of IL-6, KC and MIP-1β secreted into the culture supernatant by macrophages infected with the indicated strains 4 hours after infection with an MOI of 100:1 was measured by ELISA. Data are representative of three independent experiments. Error bars represent the standard deviation of triplicate samples. ** p = 0.0069; *** p < 0.0005.

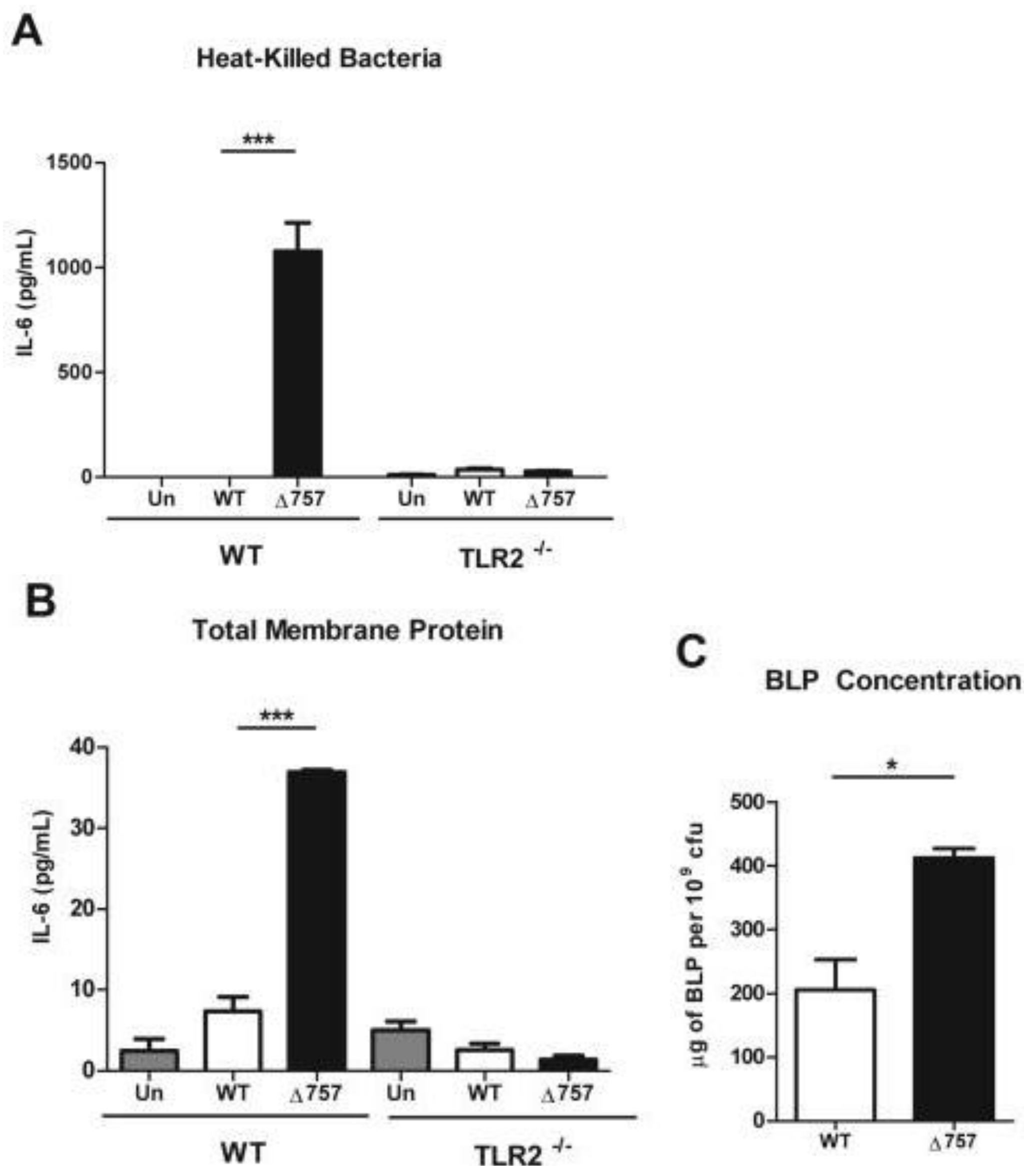


Figure 2. The Δ*FTN_0757* mutant has increased TLR2-stimulating activity and BLP levels. Wild-type or $TLR2^{-/-}$ macrophages were stimulated with **(A)** heat-killed wild-type or Δ*FTN_0757* (Δ757) at a ratio of 20:1 bacterial cell equivalents per macrophage or **(B)** total membrane protein

fractions derived from the indicated strains at a 1:1 ratio. At 4 hours, supernatants were collected and IL-6 concentrations were quantified by ELISA. **(C)** BLPs were extracted from the total membrane protein fraction and their concentrations measured via the BCA assay and normalized to bacterial cfu. Bars represent the mean and standard deviation. Data are representative of at least 2 independent experiments. * $p \leq 0.05$; *** $p < 0.0001$.

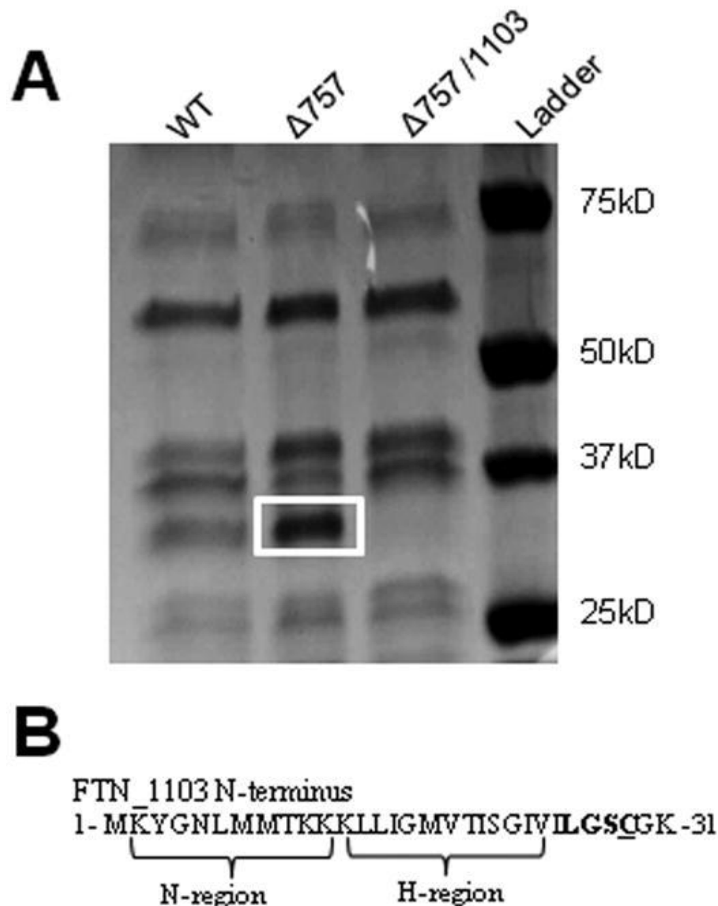


Figure 3. The Δ*FTN_0757* mutant produces increased amounts of the BLP FTN_1103. **(A)** 10^8 cfu equivalents of the BLP fraction from wild-type or the Δ*FTN_0757* mutant (Δ757) were separated by SDS-PAGE and stained with Coomassie G-250. The most enriched band in the Δ*FTN_0757* lane (white box) compared to the wild-type lane was subjected to LC-MS/MS analysis and identified as FTN_1103. Deletion of *FTN_1103* from the Δ*FTN_0757* mutant resulted in loss of the enriched band. **(B)** The N-terminus of FTN_1103 contains canonical BLP

motifs including a positively charged N-region, hydrophobic H-region, a lipobox motif (bold), and conserved cysteine (underline).

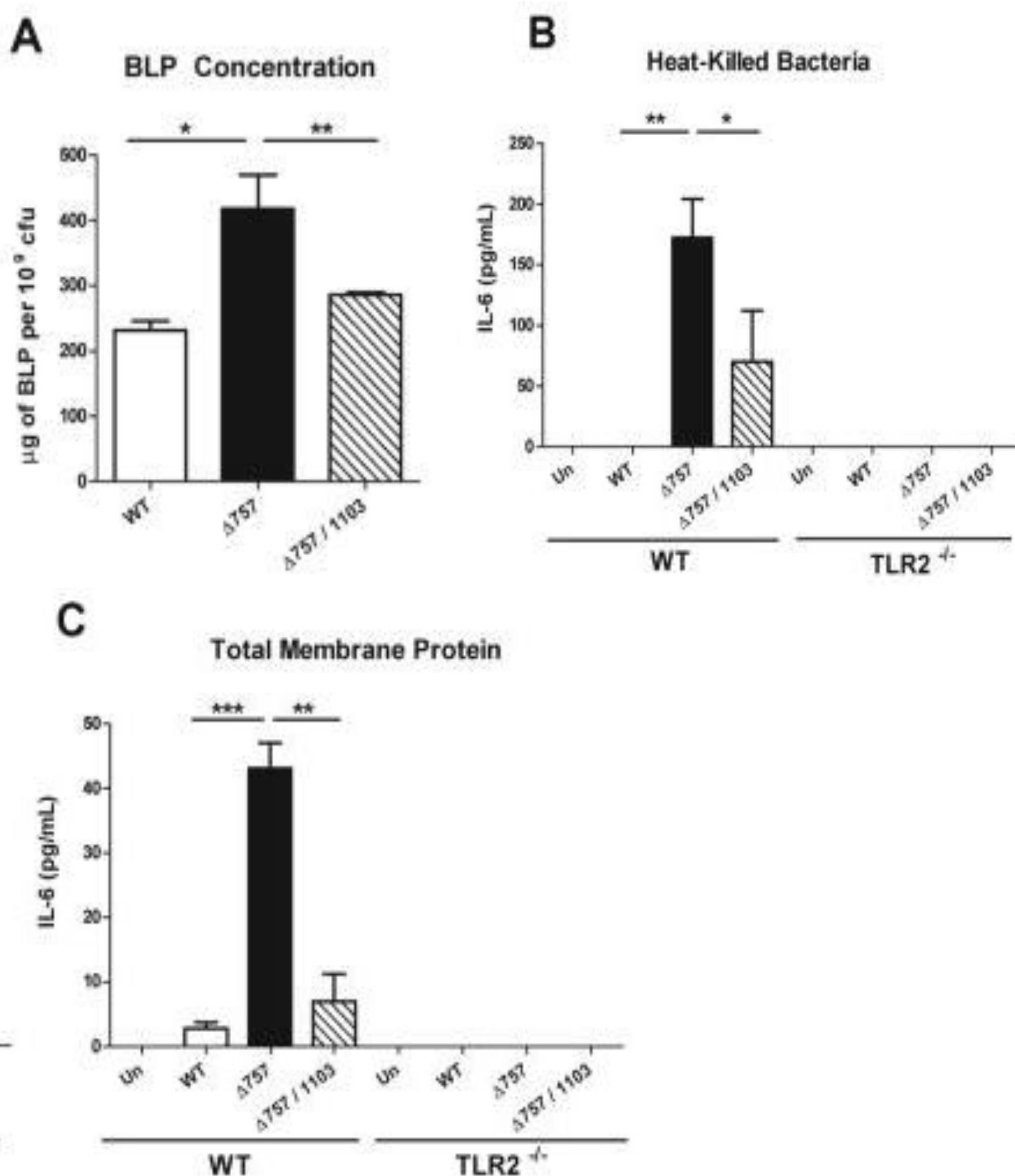


Figure 4. FTN_1103 is responsible for increased TLR2 signaling in the Δ*FTN_0757* mutant. **(A)** BLPs were extracted from the total membrane protein fraction of wild-type, the Δ*FTN_0757* mutant (Δ757), or the Δ*FTN_0757*/Δ*FTN_1103* (Δ757/1103) strains, and their concentrations

measured via the BCA assay and normalized to bacterial cfu. Wild-type or TLR2$^{-/-}$ macrophages were stimulated with **(B)** heat-killed bacteria at a ratio of 20:1 bacterial cell equivalents per macrophage, or **(C)** total membrane protein fractions at a 1:1 ratio for 4 hours. Supernatants were collected and IL-6 concentrations were quantified by ELISA. Bars represent the mean and standard deviation. Data are representative of at least 2 independent experiments. * $p \leq 0.05$; ** $p \leq 0.005$; *** $p < 0.0001$.

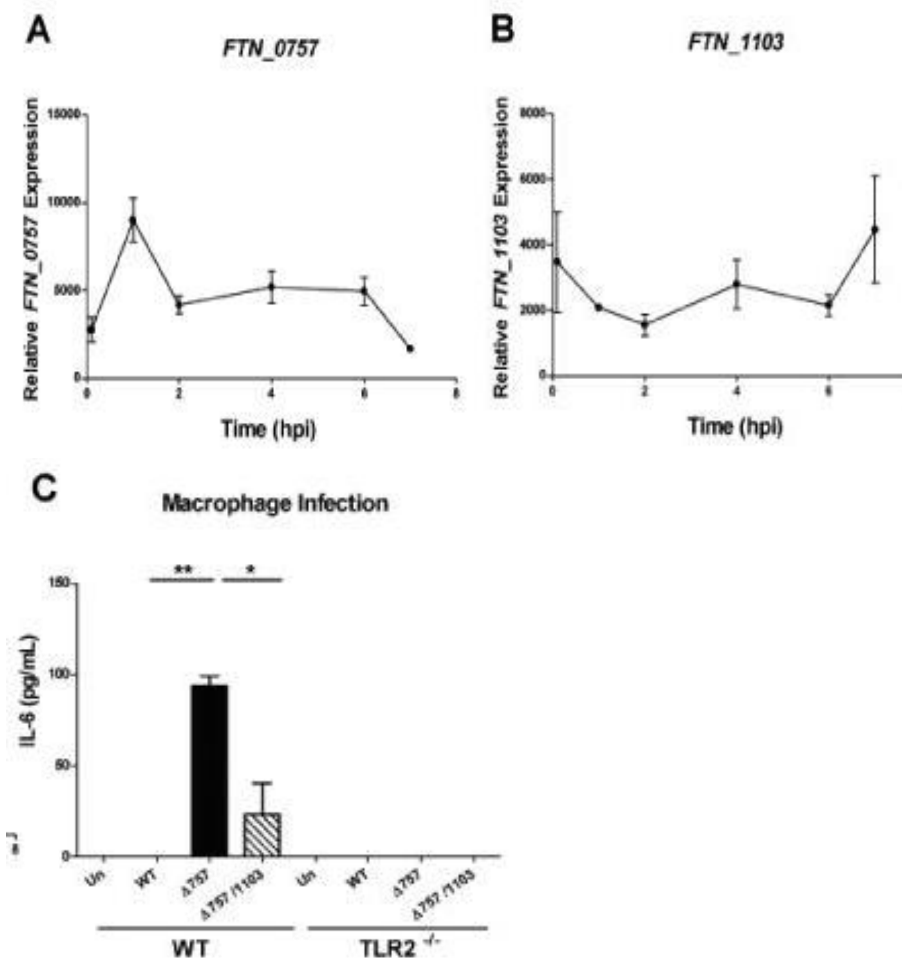


Figure 5. *FTN_0757* and *FTN_1103* expression are modulated during macrophage infection and correlate with evasion of TLR2 signaling. RNA was collected from wild-type *F. novicida* prior to infection of macrophages, or at 1, 2, 4, 6 and 7 hours after infection with an MOI of 20:1. Quantitative real-time PCR was performed for **(A)** *FTN_0757* or **(B)** *FTN_1103* and $\Delta\Delta C_T$ values

were normalized to those of the helicase, *uvrD* (*FTN_1594*). Points represent the mean and bars the standard deviation. **(C)** Wild-type or TLR2$^{-/-}$ macrophages were uninfected (Un) or infected with wild-type (WT), Δ*FTN_0757* (Δ757), or Δ*FTN_0757*/Δ*FTN_1103* (Δ757/1103) strains at an MOI of 20:1. Supernatants were collected at 4 hpi and IL-6 concentrations were quantified by ELISA. Bars represent the mean and standard deviation. Data are representative of at least 3 independent experiments. * $p \leq 0.05$; ** $p \leq 0.005$.

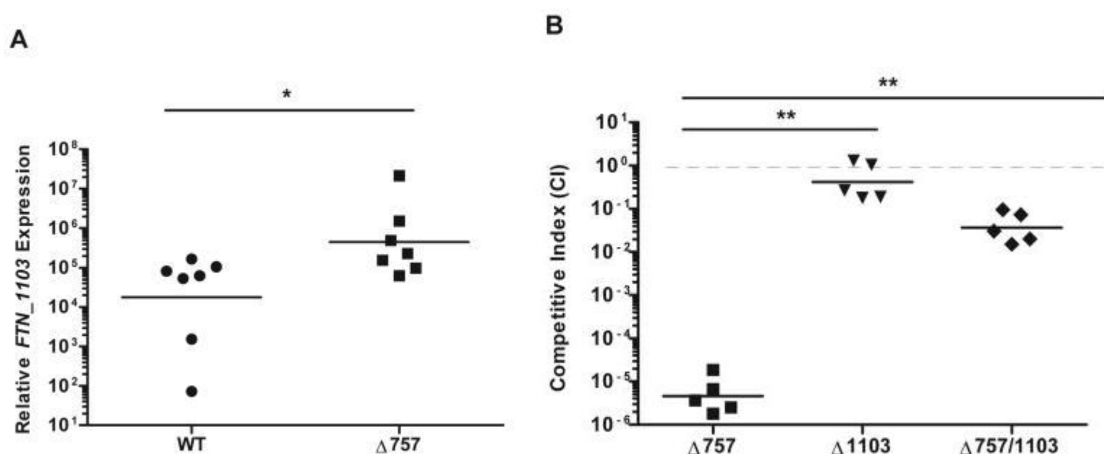
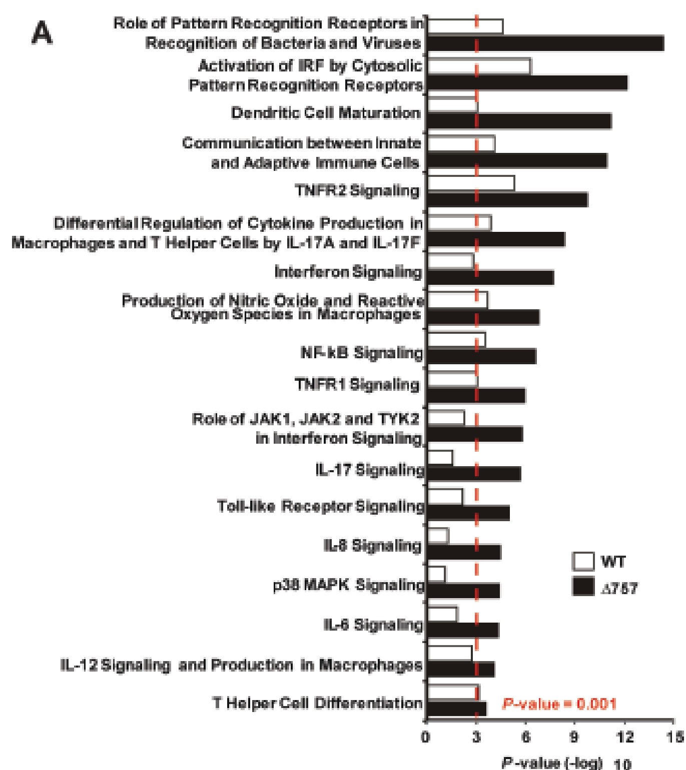
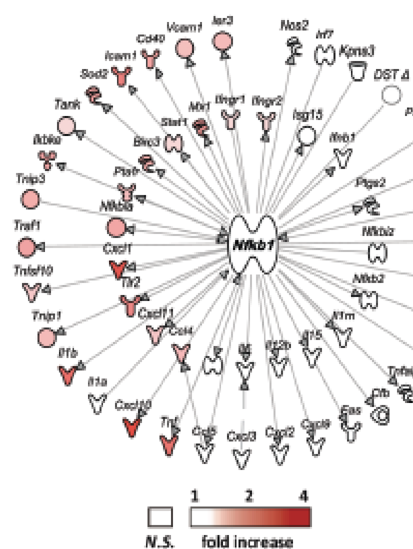
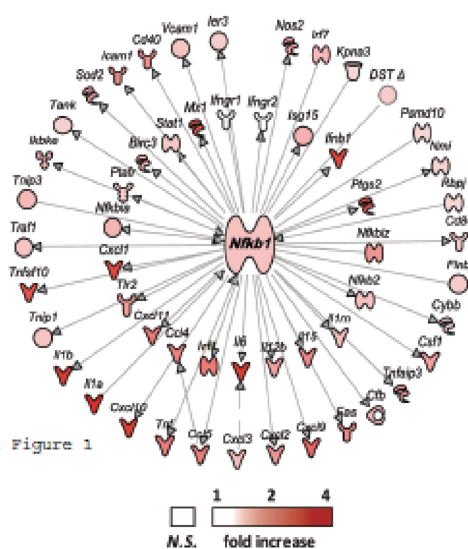


Figure 6. Deletion of *FTN_1103* significantly rescues the *in vivo* virulence defect of the Δ*FTN_0757* mutant. **(A)** Wild-type C57BL/6 mice were infected subcutaneously with 2×10^6 cfu of wild-type *F. novicida* or the Δ*FTN_0757* mutant. At 6 hpi, the skin was harvested and RNA was extracted. Quantitative real-time PCR was used to determine relative expression of *FTN_1103*, which was normalized to the constitutively expressed *uvrD*. **(B)** Mice were infected subcutaneously with a 1:1 mixture of wild-type *F. novicida* and either the Δ*FTN_0757* (Δ757), Δ*FTN_0757*/Δ*FTN_1103* (Δ757/1103), or Δ*FTN_1103* mutant (Δ1103). At 48 hpi, the spleen was harvested and the colony forming units (cfu) for each strain were enumerated after overnight growth. The competitive index (CI) = (mutant cfu_{output} / wild-type cfu_{output}) / (mutant cfu_{input} / wild-type cfu_{input}). Bars represent the geometric mean of CI values from each group of mice. Data shown are representative of three independent experiments. * $p \leq 0.05$; ** $p \leq 0.005$.

Chapter 2. Supplemental Materials

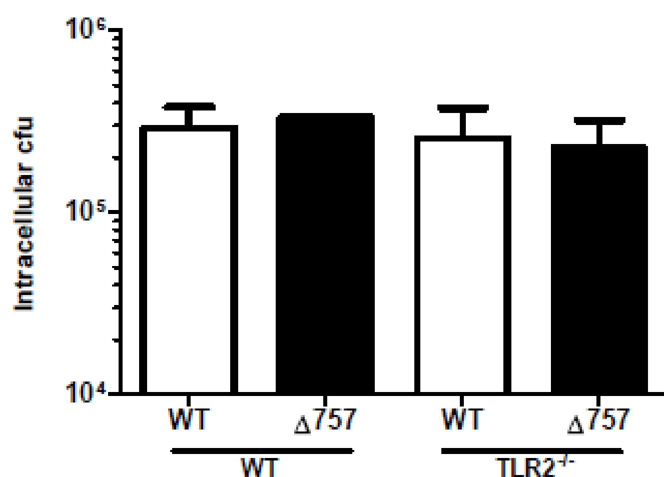




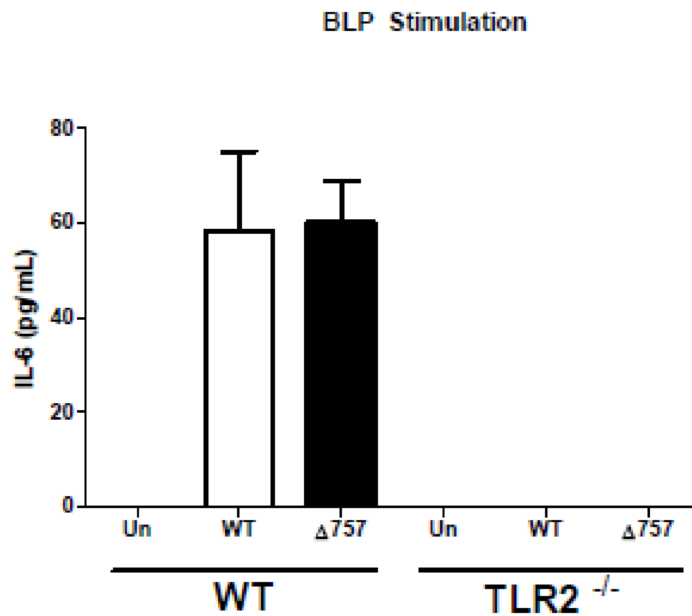


Supplemental Figure 1

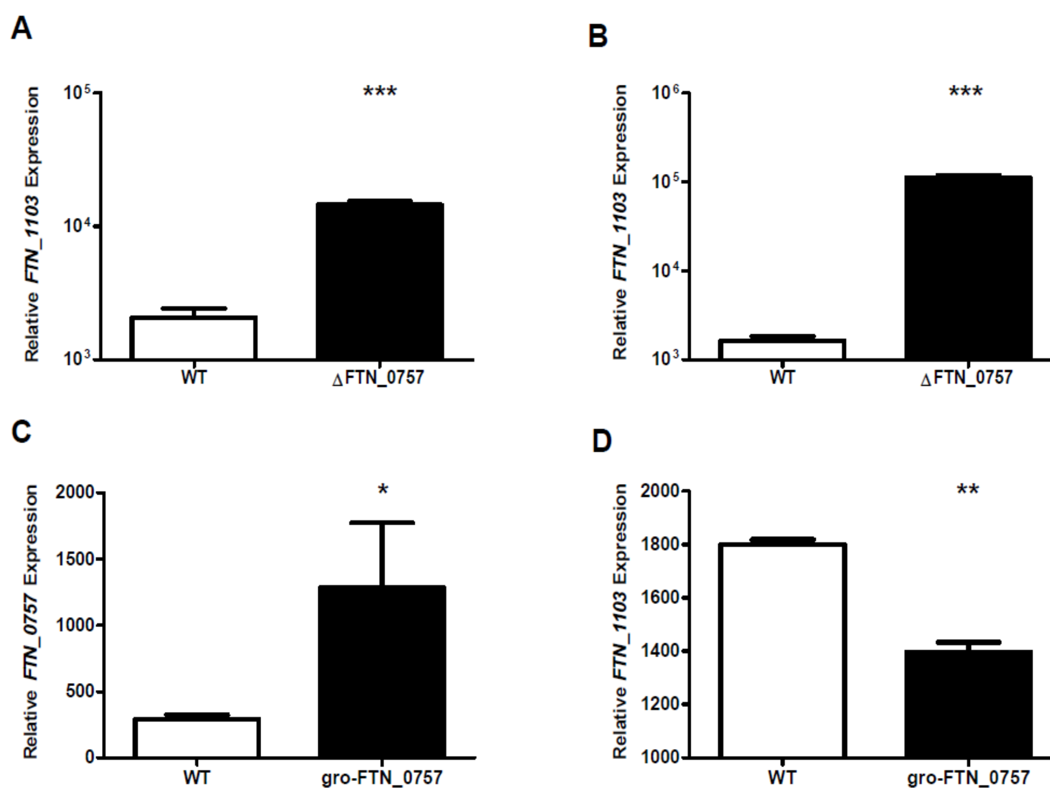
Supplementary Figure S1. Global transcriptional response to infection with the Δ*FTN_0757* mutant in macrophages. **(A)** Ingenuity Pathway Analysis was used to identify innate immune pathways whose genes were differentially expressed in macrophages infected with wild-type *F. novicida* (WT) or the Δ*FTN_0757* mutant (Δ757) at 4 hpi. The red dashed line represents a *P*-value (right-tailed Fisher exact test) cut-off of 0.001.**(B and C)** A network map of NF-κB-related genes differentially expressed in macrophages infected with **(B)** WT or **(C)** Δ757 at 4 hpi. Solid and dashed lines represent direct and indirect interactions reported for the genes respectively. The colours represent the mean fold change in gene expression at 4 hpi compared with control in two biological replicates.



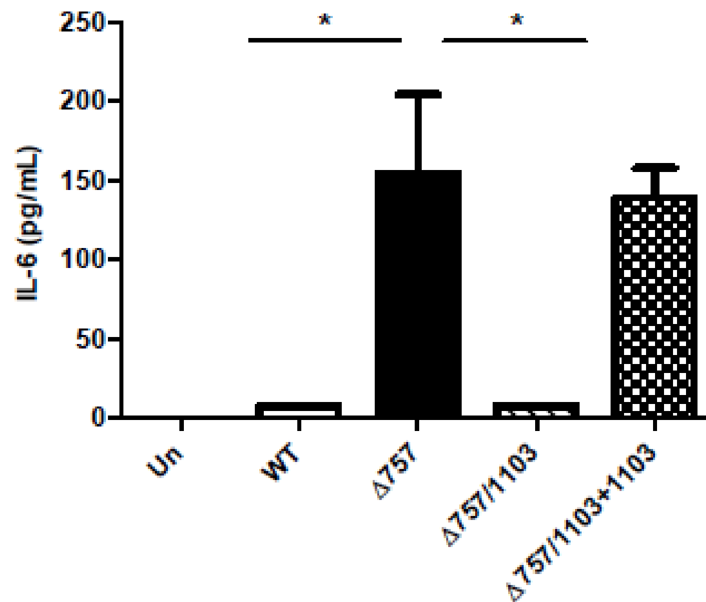
Supplementary Figure S2. Intracellular replication of Δ*FTN_0757* in macrophages. Wild-type or TLR2$^{-/-}$ macrophages were infected with wild-type (WT) or the Δ*FTN_0757* mutant (Δ757) bacteria at an MOI of **(A)** 20:1 or **(B)** 100:1. At 4 h post infection, macrophage lysates were plated, and colony-forming units (cfu) were counted. Data are representative of three independent experiments. Error bars represent the standard deviation of triplicate samples.



Supplementary Figure S3. BLP from wild-type and Δ*FTN_0757* strains activate TLR2 with equal potency. Wild-type or TLR2$^{-/-}$ macrophages were unstimulated (Un) or stimulated with 1 μg of total BLP preparations from wild-type (WT) or Δ*FTN_0757* (Δ757) strains. At 4 h post stimulation, supernatants were collected and IL-6 concentrations were quantified by elisa. Bars represent the mean and standard deviation.



Supplementary Figure S4. FTN_0757 represses expression of *FTN_1103*. RNA was harvested from wild type or Δ*FTN_0757* (Δ757) grown in **(A)** broth or **(B)** macrophages at an MOI of 20:1. Quantitative real-time PCR was performed for *FTN_1103* and $\Delta\Delta C_T$ values were normalized to those of the helicase, *uvrD* (*FTN_1594*). (C and D) RNA was harvested from wild type or a strain overexpressing *FTN_0757* (gro-Δ757) grown in broth culture, and qRT-PCR was performed for **(C)** *FTN_0757* and **(D)** *FTN_1103*. Bars represent the mean and standard deviation. $^*P \leq 0.05$; $^{**}P \leq 0.005$; $^{***}P < 0.0001$.



Supplementary Figure S5. Complementation of the Δ*FTN_0757*/Δ*FTN_1103* strain with *FTN_1103* restores the induction of inflammatory signaling. Macrophages were untreated (Un) or infected with wild-type (WT) *F. novicida*, Δ*FTN_0757* (Δ757), Δ*FTN_0757*/Δ*FTN_1103* (Δ757/1103) or the Δ*FTN_0757*/Δ*FTN_1103* mutant complemented with *FTN_1103* (Δ757/1103+1103), at an MOI of 20:1. At 4 hpi, the concentration of IL-6 secreted into the culture supernatant was measured by elisa. Data are representative of three independent experiments. Error bars represent the standard deviation of triplicate samples. *$P < 0.05$.

Chapter 3: A CRISPR-Cas system mediates bacterial innate immune evasion and virulence

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Chapter 3 Abstract

CRISPR/Cas (clustered regularly interspaced palindromic repeats/CRISPR-associated) systems are a bacterial defence against invading foreign nucleic acids derived from bacteriophages or exogenous plasmids (1-4). These systems use an array of small CRISPR RNAs (crRNAs) consisting of repetitive sequences flanking unique spacers to recognize their targets, and conserved Cas proteins to mediate target degradation (5-8). Recent studies have suggested that these systems may have broader functions in bacterial physiology, and it is unknown if they regulate expression of endogenous genes (9, 10). Here we demonstrate that the Cas protein Cas9 of *Francisella novicida* uses a unique, small, CRISPR/Cas-associated RNA (scaRNA) to repress an endogenous transcript encoding a bacterial lipoprotein. As bacterial lipoproteins trigger a proinflammatory innate immune response aimed at combating pathogens (11, 12), CRISPR/Cas-mediated repression of bacterial lipoprotein expression is critical for *F. novicida* to dampen this host response and promote virulence. Because Cas9 proteins are highly enriched in pathogenic and commensal bacteria, our work indicates that CRISPR/Cas-mediated gene regulation may broadly contribute to the regulation of endogenous bacterial genes, particularly during the interaction of such bacteria with eukaryotic hosts.

Chapter 3 Introduction

F. novicida is a model intracellular pathogen that evades host defences as it traffics through the phagosome of eukaryotic cells to replicate to high numbers within the cytosol. Specifically, it has developed mechanisms to prevent recognition by a variety of pattern recognition receptors (PRRs) that detect bacteria and localize to the surface and phagosomes of host phagocytic cells (13). One PRR, Toll-like receptor 2 (TLR2), recognizes bacterial lipoproteins (BLP) and is critical for defence against *F. novicida* (11-15). By dampening TLR2 activation, *F. novicida* reaches its replicative niche in the cytosol without inducing significant inflammatory signalling, promoting its pathogenesis (13).

We demonstrated that *F. novicida* gene *FTN_0757* is involved in the repression of a BLP, (FTN_1103), although its mechanism of action was unclear (16). Unexpectedly, bioinformatic analysis revealed that FTN_0757 has significant protein sequence similarity to the CRISPR/Cas system protein Cas9 (15–65% across conserved regions) (Supplementary Fig. 1 [Supplementary material available online at http://www.nature.com/nature/journal/v497/n7448/full/nature12048.html]), typically known to degrade foreign DNA (6, 7), and not currently known to regulate endogenous bacterial gene expression. Furthermore, *FTN_0757* is present in a complete type II CRISPR/Cas locus, similar to those in the genomes of pathogens and commensals such as *Streptococcus* spp., *Neisseria* spp., *Campylobacter* spp. and *Lactobacillus* spp. (Supplementary Fig. 2 and Supplementary Table 1). The locus contains Cas1, Cas2 and Cas4, all predicted to be involved in acquisition of new targeting crRNAs (1, 8, 17), as

well as a predicted trans-activating crRNA (tracrRNA), an accessory small RNA necessary for crRNA activity (18). It also contains a unique small RNA (19) not previously described in a CRISPR/Cas locus, distinct from the crRNAs and tracrRNA, which we term small CRISPR/Cas-associated RNA (scaRNA)(Fig. 1a).

Chapter 3 Results

Since *FTN_0757* (*cas9*) is in a CRISPR/Cas locus (Fig. 1a), we tested whether its ability to repress the bacterial lipoprotein *FTN_1103* required the canonical CRISPR/Cas system or if an alternative mechanism was involved. Deletion of *cas9*, but not other *cas* genes, led to 100-fold increased levels of *FTN_1103* transcript (Fig. 1b). Since Cas9 degrades DNA targeted by crRNAs, we tested whether the crRNA array or the tracrRNA were necessary for *FTN_1103* repression. Deletion of the crRNA array did not alter *FTN_1103* transcript levels (Fig. 1c); however, deletion of the tracrRNA resulted in increased *FTN_1103* transcript, similar to the *cas9* mutant (Fig. 1c). Additionally, deletion of the scaRNA resulted in increased *FTN_1103* transcript, indicating that it is also critical for *FTN_1103* repression. Complementation of the *cas9*, tracrRNA and scaRNA mutants restored *FTN_1103* expression to near wild-type levels, and levels of *FTN_1103* transcript in the mutants correlated with an increase in protein production (Supplementary Figs 3 and 4). Furthermore, a triple mutant lacking *cas9*, tracrRNA and scaRNA expressed similar levels of *FTN_1103* as the single mutants, providing genetic evidence that these components may act together within the same regulatory pathway.

CRISPR/Cas systems mediate degradation of their nucleic acid targets, so we tested whether Cas9, tracrRNA and scaRNA mediated repression of *FTN_1103* via degradation. Following treatment with rifampicin to prevent messenger RNA production, *FTN_1103* transcript was rapidly depleted in wild-type cells (Fig. 2a). In contrast, *FTN_1103* transcript was not degraded in *cas9*, tracrRNA or scaRNA mutants (Fig. 2a), indicating that each of these components is required for its degradation. Cas9 proteins contain four RuvC endonuclease domains (RuvC-I to RuvC-IV) and an HNH endonuclease domain (Fig. 2b and Supplementary Fig. 1) (17). RuvC-I and the HNH are necessary for degradation of target DNA (6, 7). We constructed point mutant strains lacking conserved residues17 in each endonuclease domain to determine if they were necessary for repression of *FTN_1103* (Fig. 2b). These strains maintained wild-type levels of *FTN_1103* (as well as *cas9*), indicating that none of these domains were required for this activity (Fig. 2c and Supplementary Fig. 5). Additionally, we found no role for known RNases in *FTN_1103* repression (Supplementary Fig. 6). Cas9 proteins also contain a previously uncharacterized, conserved, arginine-rich motif (ARM)(17) (Fig. 2b and Supplementary Fig. 1), motifs that are known to mediate protein–RNA interactions (20). A point mutation in the ARM (R59A) completely abrogated the ability of Cas9 to repress *FTN_1103* (Fig. 2c), implicating the potential importance of Cas9–RNA interactions.

We therefore analysed the sequences of the tracrRNA and scaRNA and predicted that the tracrRNA could hybridize to a degenerate repeat region in the scaRNA (Fig. 2d and Supplementary Fig. 7), similar to the interaction between the tracrRNA and the repeat region of a crRNA, which is necessary for targeting DNA18. We also predicted that a

region of the scaRNA could hybridize to a portion of the *FTN_1103* transcript encompassing the start codon and ribosomal binding site (Fig. 2d and Supplementary Fig. 7). To determine whether Cas9 and the RNAs associated, we immunoprecipitated Cas9 from a strain expressing a Flag-tagged version of this protein. tracrRNA, scaRNA and *FTN_1103* mRNA were significantly enriched in association with Cas9 (Fig. 2e-g). However, these associations were abrogated in the Cas9 ARM (R59A) mutant, suggesting this motif is necessary for Cas9 interaction with these RNAs. We then generated reverse complement mutations in the tracrRNA region (bases 13–17) predicted to interact with the scaRNA, as well as the scaRNA regions predicted to interact with the tracrRNA (bases 4–8) or *FTN_1103* mRNA (bases 48–54). Any of the three mutations resulted in the inability to repress *FTN_1103* (Fig. 2h), whereas a strain that expressed the altered but complementary versions of both the tracrRNA and scaRNA significantly restored *FTN_1103* repression (Supplementary Fig. 8a). Additionally, the mutations predicted to disrupt the interaction between scaRNA and tracrRNA significantly dampened the ability of either small RNA to associate with Cas9, which was immunoprecipitated with equal efficiency in all strains (Supplementary Figs 8b, c and 9). Thus, the sequence-specific association of Cas9, tracrRNA and scaRNA is necessary for the repression of *FTN_1103*.

Because Cas9, tracrRNA and scaRNA repress the expression of the FTN_1103 BLP, and BLPs are ligands for host TLR2, we tested if these CRISPR/Cas components were involved in evasion of TLR2 recognition. Membrane protein fractions of the tracrRNA and scaRNA mutants stimulated increased TLR2-dependent secretion of the

proinflammatory cytokine IL-6, similar to those from the *cas9* mutant as shown previously (16)(Fig. 3a). This response was rescued in double mutants lacking *FTN_1103*, indicating that overexpression of *FTN_1103* in these strains was largely responsible for the increased TLR2 signalling (Fig. 3a). Mutants lacking *cas9*, tracrRNA or the scaRNA also elicited enhanced TLR2-dependent IL-6 secretion during macrophage infection compared to wild-type *F. novicida*, which was dependent on *FTN_1103* (Fig. 3b). This is in contrast to mutants in other *cas* genes or the crRNA array, which did not alter TLR2 signalling (Supplementary Fig. 10). As a control, a mutant lacking only *FTN_1103* did not have observable differences in membrane protein content nor did it induce altered host signalling (Supplementary Fig. 11). Together these data indicate that CRISPR/Cas component mediated suppression of BLP facilitates evasion of TLR2.

To determine if repression of *FTN_1103* was an active evasion process, we analysed the temporal expression of CRISPR/Cas components during intracellular infection. We found that *FTN_1103* expression decreased when the bacteria were in the phagosome, as shown previously (16) (Fig. 3c), directly correlating with the approximately 100-fold induction of *cas9*, tracrRNA and scaRNA (Fig. 3d-f). In the absence of Cas9, tracrRNA or scaRNA, the temporal repression of *FTN_1103* was completely abrogated (Fig. 3c). These data indicate that *cas9*, tracrRNA and scaRNA are induced during intracellular infection, allowing temporal repression of *FTN_1103* when the bacteria are in the proximity of TLR2 in the phagosome, thus facilitating evasion of this innate immune pathway. Although *cas1*, *cas4* and the crRNA array are not required for *FTN_1103* repression, they were similarly expressed during infection (Supplementary Fig. 12).

However, their expression differed during *in vitro* growth (Supplementary Fig. 13), possibly indicating specific co-regulation of these CRISPR/Cas components during intracellular infection.

We tested the consequences of the inability to repress *FTN_1103* on fitness during murine infection. We performed competitive infections with wild-type *F. novicida*, and either the *cas9,* tracrRNA or scaRNA deletion mutants, and measured bacterial burden in the spleen 48 h post-infection. All three mutants were highly attenuated (1,000 to 10,000-fold) compared to wild-type (Fig. 4a), demonstrating that all three components are critical for *F. novicida* virulence. This attenuation was rescued by deletion of *FTN_1103* from the mutants. Notably, mutants lacking the crRNA array or other *cas* genes were not attenuated (Supplementary Fig. 14). The *cas9*, tracrRNA and scaRNA mutants were also highly attenuated when inoculated individually; they were non-lethal even at 100× the LD_{50} (the dose lethal to 50% of animals tested), whereas mice infected with wild-type or *cis*-complemented strains (which restored repression of *FTN_1103,* Supplementary Fig. 15) rapidly succumbed to disease (Fig. 4b). We conjectured that the mice surviving an initial infection might be protected against subsequent lethal challenge with *F. novicida*. Naive mice rapidly succumbed to a challenge, but mice immunized with *cas9,* tracrRNA or scaRNA mutants were completely protected (Fig. 4c), demonstrating that mutants lacking these CRISPR/Cas components can efficiently vaccinate mice. Given that CRISPR/Cas systems of other bacteria may also contribute to virulence, mutants of these genes may represent attractive vaccine strains for other pathogens.

Chapter 3 Discussion

Our results demonstrate that the Cas9 system has a non-canonical function in acting with a non-crRNA (scaRNA) to regulate gene expression via the degradation of an endogenous mRNA, leading to innate immune evasion and promoting virulence (Supplementary Fig. 16). This surprising observation shows that CRISPR/Cas components have been co-opted to perform functions distinct from foreign nucleic acid defence. Notably, they have also been implicated in DNA repair (10) and biofilm formation (9). Our work further indicates that predicted self-targeting crRNAs (21) may have natural roles in endogenous gene regulation, functioning with the Cas9 machinery.

Eighty-five of the 109 bacteria shown to encode Cas9 (by our and others work)(22) are known pathogens or commensals, making it interesting to speculate that the regulatory mechanism we describe may function in numerous other organisms that interact with eukaryotic cells (Supplementary Table 1). To further explore the breadth of this phenomenon, we generated a *cas9* deletion mutant in *Neisseria meningitidis* str. 92045 and assayed virulence traits. We observed a significant decrease in the ability of the *cas9* mutant to adhere to, invade and replicate in human epithelial cells, leading to an overall defect in survival (Supplementary Fig. 17), indicating that Cas9 plays an important role in *N. meningitidis* pathogenesis. Additionally, a recent study identified *Campylobacter jejuni* Cas9 as critical for interactions with host cells (23). Bioinformatic analysis predicted that *N. meningitidis*, *C. jejuni* and other pathogens may encode a scaRNA, which is critical for Cas9 targeting of endogenous mRNA (Supplementary Table 2). Together, these results clearly show that Cas9 controls virulence traits of several bacteria.

It is interesting to note, however, that the CRISPR/Cas locus in the highly virulent *Francisella tularensis* is probably non-functional, as it lacks the tracrRNA and contains an internal deletion within Cas9. *F. tularensis* is known to potently inhibit TLR signalling (13) and may therefore not limit, or use distinct mechanisms to limit, BLP expression. Although its role in different species may therefore vary, the enrichment of Cas9 within the genomes of pathogens and commensals and its demonstrated role in controlling virulence traits in *F. novicida*, *N. meningitidis* and *C. jejuni*, strongly indicate that it is involved in regulating the interaction of bacteria with eukaryotic hosts. Our data support a model whereby type II CRISPR/Cas systems can function in endogenous bacterial gene regulation, ultimately promoting both pathogenesis and commensalism.

Chapter 3 Materials and Methods

Bacterial Growth

Francisella novicida U112 was a gift from D. Monack, Stanford University. Cultures were grown overnight at 37 °C with aeration in tryptic soy broth supplemented with 0.2% L-cysteine (BD Biosciences) or on tryptic soy agar (BD Biosciences) (24). When necessary, media was supplemented with kanamycin (30 $\mu g\ ml^{-1}$) or tetracycline (20 $\mu g\ ml^{-1}$). Meningococcal strains were grown with 5% CO_2 at 37 °C on GC base (Difco) agar containing supplements of 0.4% glucose and 0.68 mM $Fe(NO_3)_3$, or GC broth with the same supplements and 0.043% $NaHCO_3$ (25). Brain heart infusion medium with 1.25% fetal bovine serum was used when kanamycin selection was required. *N. meningitidis* was transformed by the procedure of Janik *et al* (26). To

measure growth rate, overnight cultures of wild-type and the indicated *F. novicida* mutant strains were diluted to $D_{600\ \mathrm{nm}}$ of 0.03, incubated at 37 °C with aeration and $D_{600\ \mathrm{nm}}$ was measured hourly in a BioTek Synergy MX plate reader (BioTek) for 20 h.

Mutagenesis

Francisella deletion mutant and point mutant strains were constructed by allelic replacement as described previously(27) using primers listed in Supplementary Table 3. Double deletion strains were created using Flp-recombinase, as previously described (28), and transforming unmarked strains with the second targeting construct. scaRNA and tracrRNA were complemented *in trans* by ligation into the broad host range vector, pBAV1K-T5-GFP32 at the EcoRI and BamHI sites, and transformed into unmarked ΔscaRNA or ΔtracrRNA strains. The *N. meningitidis cas9* mutant was generated using a targeting construct generated by overlapping PCR25, using primers listed in Supplementary Table 3, that created a 2,615 base pair (bp) deletion in the *cas9* (3,246 bp) coding sequence. The final PCR product with the expected size was gel-purified and used directly for transformation of a meningococcal serogroup W135 strain Nm92045. Colonies were selected on brain heart infusion agar plates with 80 μg ml^{-1} of kanamycin. Removal of the *cas9* internal sequence was confirmed by PCR.

Membrane protein fractionation and SDS–PAGE analysis

Membrane protein fractions were prepared as previously described16. Membrane proteins were normalized by colony forming units (c.f.u.) to 10^8 c.f.u., separated via 12–20% SDS–PAGE (Bio-Rad) and stained with Coomassie blue G-250 (Teknova).

RNA isolation and qRT–PCR

RNA was isolated from bacterial cultures or macrophage infections at the given time points using TRI Reagent (Molecular Research Center) and purified using the RNeasy mini kit (Qiagen) and on-column DNase treatment (Qiagen) according to the manufacturers' instructions. Quantitative real-time PCR (qRT–PCR) was performed with 40 ng total RNA using the Power SYBR Green RNA to C_T 1-Step kit (Applied Biosystems) and gene-specific primers (Supplementary Table 3) using an Applied Biosystems StepOne cycler. Relative transcript levels were calculated by normalizing C_T values to DNA helicase II (*uvrD*, *FTN_1594*) and plotted as $2^{-\Delta\Delta C_T}$.

RNA degradation assay

RNA degradation assays were performed as previously described (29). Overnight cultures of bacteria were subcultured 1:10 into 10 ml of tryptic soy broth with 0.2% cysteine and grown to $D_{600\,nm}$ of ~0.4. Rifampicin (USB Corporation) was added to a final concentration of 500 μg ml^{-1}, cultures were incubated at 37 °C with aeration, and aliquots were taken at the indicated time points for RNA isolation.

Immunoprecipitation

Immunoprecipitation was performed on bacterial lysates using the Flag immunoprecipitation kit (Sigma) according to the manufacturer's instructions and the addition of 0.05% NP-40 during wash steps. Total RNA was isolated from the precipitate and qRT–PCR performed, normalizing to *uvrD*.

Macrophage Infections and Stimulations

Murine bone marrow-derived macrophages were prepared from 6 to 8-week-old wild-type and *Tlr2*$^{-/}$ C57BL/6 mice and cultured as described(30). Macrophages were seeded into 96-well plates (~5×10^4 cells per well) for cytokine analysis, or 24-well plates (~$3.2x10^5$ cells per well) for RNA isolation, in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and 10% L929-conditioned media (conditioned DMEM) containing macrophage colony stimulating factor (M-CSF) overnight. Bacteria were added at a multiplicity of infection (MOI) of 20:1 bacteria per macrophage and centrifuged for 15 min at 335 *g* at room temperature to facilitate bacterial uptake. Infected macrophages were incubated for 30 min at 37 °C and washed twice before adding warm conditioned DMEM26. The concentrations of IL-6 in culture supernatants at the indicated time points after infection were quantified by enzyme-linked immunosorbent assay (ELISA) (BD Biosciences). For stimulation with bacterial membrane protein fractions, cells were washed gently and media containing membrane fractions at a relative MOI of 20:1 were added. Macrophages were stimulated for the indicated duration of time, before the cell culture supernatant was collected and assayed for IL-6 by ELISA.

N. meningitidis intracellular survival assay

The A549 human lung adenocarcinoma cell line was cultured in DMEM supplemented with heat inactivated FBS (10%) at 37 °C and 5% CO_2. For the bacterial adherence and invasion assay, A549 cells were seeded at a density of 10^5 cells per ml in 24-well plates (Corning) two days before the experiment. To prepare the bacterial inoculum,

meningococcal strains were grown in GC broth to mid log phase, collected by centrifugation and resuspended in cell culture media. Bacterial cells were added to cell cultures at a MOI of 100, and serial dilutions of the inoculum were plated to determine the input c.f.u. After a 3-h infection, the monolayers were washed three times with sterile phosphate-buffered saline to remove free bacteria and the c.f.u. of attached bacteria determined. Separately infected cells were washed and then incubated in cell culture media containing 100 μg ml^{-1} of gentamicin for 1 h to kill extracellular bacteria. A549 cells were lysed by incubation with 1% saponin (Sigma) for 10 min to release intracellular bacteria at 4 h and 6 h post infection. Serial dilutions of lysates in PBS were plated on GC plates for c.f.u. counts of invasion efficiency. Each assay was conducted with 2–3 independently infected monolayers and repeated three times.

In vivo Experiments

Female C57BL/6 mice aged 7 to 10 weeks were kept under specific-pathogen free conditions in filter-top cages at Yerkes National Primate Center, and provided food and water *ad libitum.* All experimental procedures were approved by the Emory University Institutional Animal Care and Use Committee (protocol #YER-2000573-061314BN). For competitive infections, groups of five mice were infected subcutaneously with 1×10^5 c.f.u. of wild-type and the indicated mutant strain of *F. novicida* at a 1:1 ratio in sterile PBS. At 48 h post-infection, spleens were collected and homogenized in PBS. Appropriate dilutions were plated with or without kanamycin for enumeration of bacterial burden. The competitive index (CI) was calculated using the following formula: CI = (mutant c.f.u. output/wild-type c.f.u. output)/(mutant c.f.u. input/wild-type c.f.u.

input). For vaccination experiments, groups of five mice were infected subcutaneously with 1×10^5 c.f.u. of the indicated mutant strain of *F. novicida* in sterile PBS, or PBS alone. Twenty-eight days later, mice were challenged subcutaneously with 1×10^7 c.f.u. wild-type *F. novicida* in sterile PBS and euthanized when they appeared moribund.

Chapter 3 Acknowledgements

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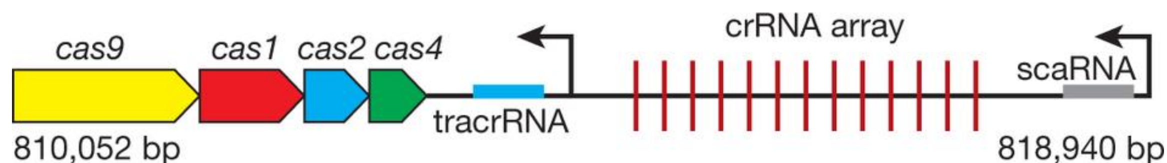
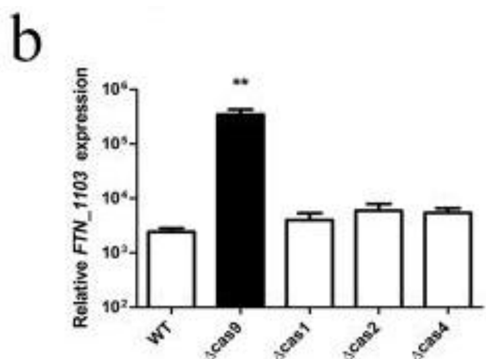
Chapter 3 Figures





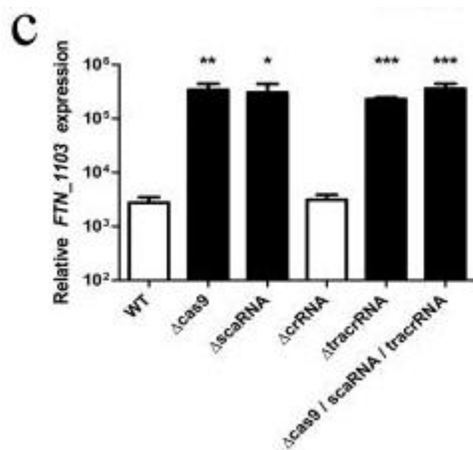


Figure 1. Cas9, tracrRNA, and scaRNA are necessary for *FTN_1103* repression. **(A)** Schematic of the *F. novicida* Type II CRISPR-CAS locus, containing *cas9*, *cas1*, *cas2*, and *cas4*, as well as the crRNA array (repeats indicated by vertical red lines), tracrRNA (blue), scaRNA (orange), and predicted promoters (black arrows). Relative expression of *FTN_1103* in **(B)** wild-type (WT), Δ*cas9*, Δ*cas1*, Δ*cas2*, and Δ*cas4* strains and **(C)** WT, Δ*cas9*, ΔscaRNA, ΔcrRNA, and ΔtracrRNA strains (n = 4, bars represent s.d.).

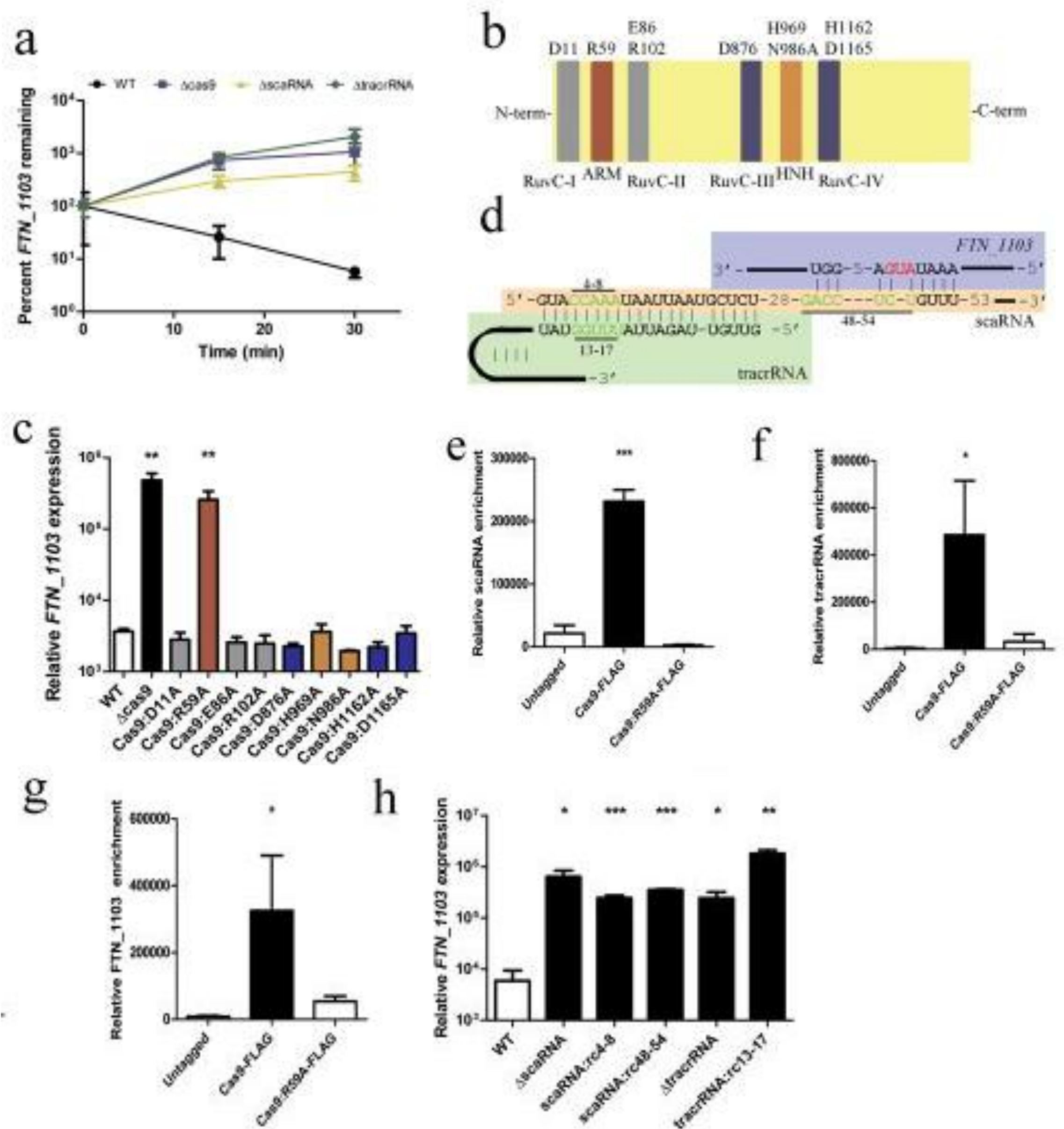


Figure 2. Cas9, tracrRNA, and scaRNA associate and mediate *FTN_1103* degradation. **(A)** *FTN_1103* stability in the indicated strains (n = 3). **(B)** Schematic of Cas9 indicating five endonuclease domains (RuvC-I - RuvC-IV, HNH) and the ARM. **(C)** Relative expression of *FTN_1103* in the indicated strains (n = 4). **(D)** Schematic of predicted hybridization between tracrRNA, scaRNA, and *FTN_1103*. Bars highlight mutated bases (green), red represents the *FTN_1103* start codon. **(E–G)** Immunoprecipitation from WT, Cas9-FLAG, or Cas9:R59A-

FLAG, and qRT-PCR for **(E)** scaRNA, **(F)** tracrRNA or **(G)** *FTN_1103* (n = 4). **(H)** Relative expression of *FTN_1103* in WT, ΔscaRNA, scaRNA:rc4-8 (reverse complement of bases 4–8), scaRNA:rc48–54, ΔtracrRNA, and tracrRNA:rc13-17 strains (n = 4, bars represent s.d.).

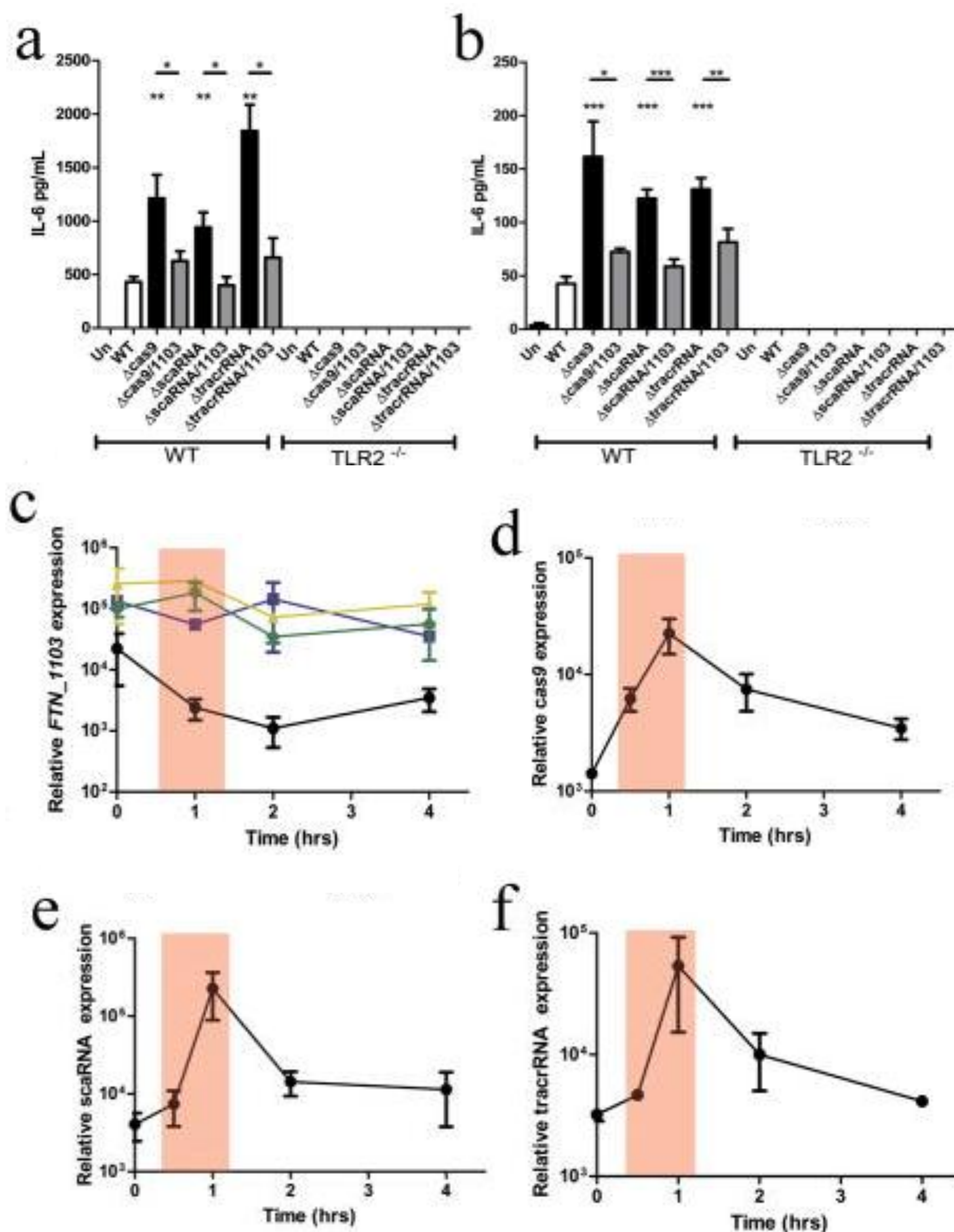


Figure 3. Cas9, tracrRNA, and scaRNA facilitate evasion of TLR2 signaling by temporal repression of *FTN_1103*. IL-6 secretion from wild-type (WT) and TLR2$^{-/-}$ bone marrow-derived macrophages (BMDM) **(A)** unstimulated (Un) or stimulated with membrane proteins

(relative MOI of 20:1 for 5 hours) from wild-type (WT), the indicated single mutants, or double deletion strains also lacking *FTN_1103* (n = 3), or **(B)** infected with the same strains at an MOI of 20:1 for 5 hours (n =6). Relative expression of **(C)** *FTN_1103*, **(D)** *cas9*, **(E)** scaRNA, and **(F)** tracrRNA during infection of BMDM with the indicated strains (n = 3, bars represent s.d.).

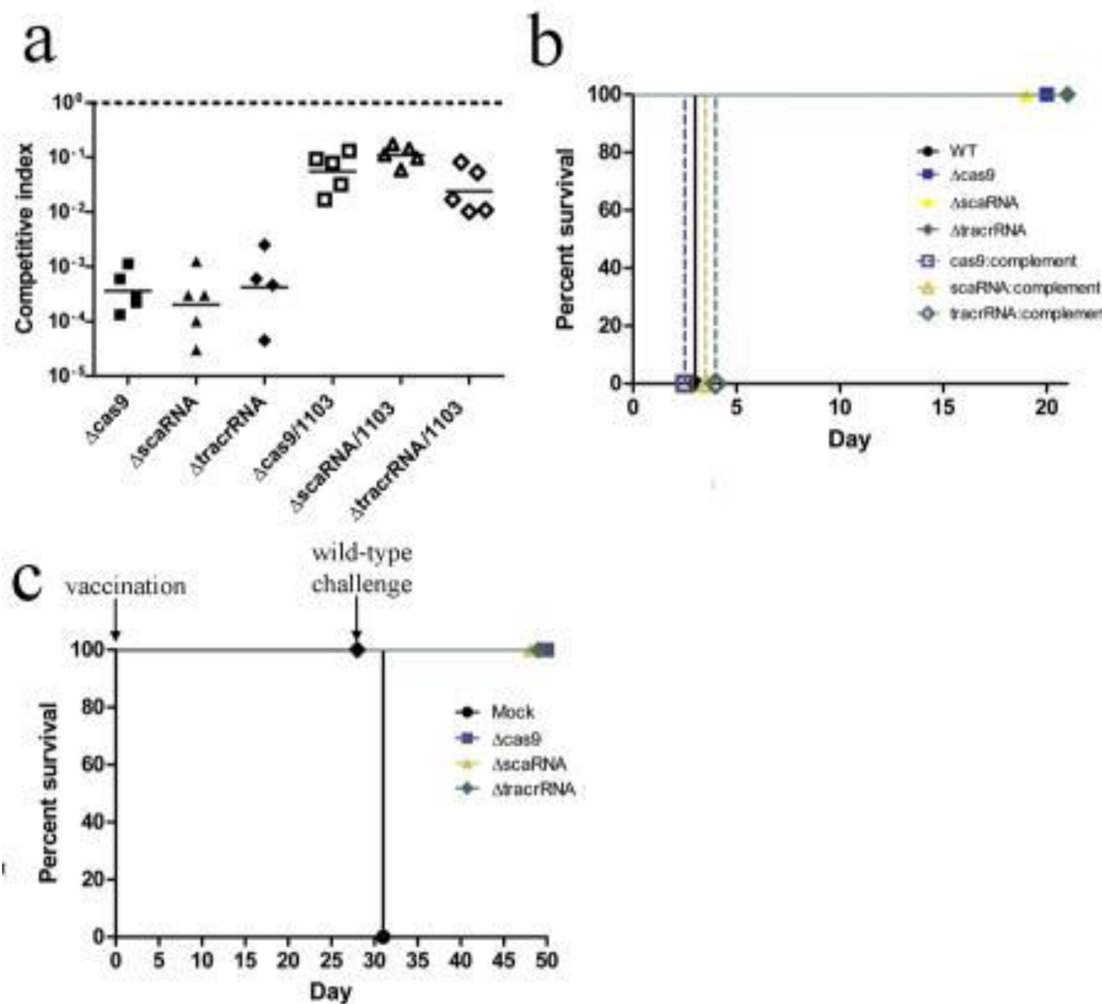


Figure 4. Cas9, tracrRNA, and scaRNA are necessary for virulence. **(A)** Competitive indices of wild-type and the indicated mutant or double mutant strains from murine spleens, 48 hours post-infection. Bars represent the geometric mean. **(B)** Mice were infected with 10^7 cfu of either wild-type (black circle), Δ*cas9* (blue square), ΔscaRNA (yellow triangle), ΔtracrRNA (green diamond), or the corresponding cis-complemented strains (open symbols), and survival monitored

over time. **(C)** Mice were vaccinated with 10^4 cfu of either Δ*cas9*, ΔscaRNA, or ΔtracrRNA strains, or PBS. Twenty-eight days later, mice were challenged with 10^7 cfu wild-type.

Chapter 3 Supplemental Materials

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Chapter 4. A CRISPR-Cas system enhances envelope integrity mediating antibiotic resistance and inflammasome evasion.

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Chapter 4 Abstract

CRISPR/Cas systems are RNA-directed bacterial defenses against invading foreign nucleic acids. Their fundamental roles in bacterial physiology remain unclear. Here, we identify a previously unappreciated role for the Cas protein, Cas9, in enhancement of envelope integrity and resistance to a membrane-targeting antibiotic in the bacterial pathogen *Francisella novicida*. This requires the recently described function of Cas9 as a repressor of a bacterial lipoprotein (BLP), a process also critical for evasion of host Toll-like Receptor 2 (TLR2) signaling during infection. We further find that Cas9-mediated BLP repression and enhancement of envelope integrity facilitates evasion of the host AIM2/ASC inflammasome. Highlighting the important role of Cas9 in evading multiple innate immune pathways, the virulence defect of the *cas9* mutant is rescued in ASC/TLR2-deficient mice. This work delineates the critical role of Cas9 as a regulatory factor controlling bacterial physiology, antibiotic resistance, and virulence, and serves as a paradigm for the numerous pathogens encoding this protein.

Chapter 4 Introduction

CRISPR/Cas (clustered, regularly interspaced, short palindromic repeats / CRISPR associated) systems are adaptive bacterial defenses against foreign nucleic acids, for example those derived from bacteriophages and plasmids (1). Foreign nucleic acids are targeted by direct hybridization of small CRISPR RNAs (crRNAs), which act in conjunction with conserved Cas proteins to mediate cleavage of the target. Interestingly, there is evidence that CRISPR/Cas components are upregulated during infection by bacteriophages or due to the misfolding of membrane proteins (2-4). Despite their induction at the transcriptional level, it is unknown if and how CRISPR/Cas systems might function to enhance the integrity of the bacterial envelope to combat membrane stress.

While strengthening the bacterial envelope would represent an alternative function for CRISPR/Cas systems, non-canonical roles have been postulated. For example, these systems have been implicated in DNA repair and biofilm formation, and most recently CRISPR/Cas components have been observed to function as virulence factors in bacterial pathogens (5-9). In this regard, we demonstrated a non-canonical role for components of a Type II CRISPR/Cas system [encoded predominantly in pathogens and commensals (10)] in the regulation of a membrane lipoprotein in the model intracellular pathogen, *Francisella novicida* (8). Through the action of the RNA-directed endonuclease Cas9 and two small RNAs, tracrRNA and scaRNA, the transcript for a bacterial lipoprotein (BLP; *FTN_1103*) is targeted and its stability altered, resulting in a decrease in protein production (Supplemental Figure 1). Since BLPs trigger activation of the host innate

immune receptor Toll-like Receptor 2 (TLR2), this Cas9-mediated regulation facilitates a dampening of the proinflammatory response and is essential for *F. novicida* to cause disease (8). To our knowledge, targeting of the *FTN_1103* transcript by the Cas9 regulatory axis in *F. novicida*, consisting of Cas9, tracrRNA, and scaRNA, is the only known example of CRISPR/Cas-mediated endogenous gene regulation. As such, the *F. novicida* CRISPR/Cas system represents an important model to understand how these common prokaryotic genetic elements can act as regulators to control bacterial physiology and function as virulence factors.

F. novicida is capable of causing disease in a number of mammalian species, including humans, due in part to its ability to invade and replicate to high levels in a variety of eukaryotic cells (11). In particular, one of its primary replicative niches within the mammalian host is the macrophage. Following phagocytosis, *F. novicida* escapes the macrophage phagosome and replicates to high titers in the cytosol. Throughout the infection cycle, the bacteria must resist, evade, or dampen innate immune responses (11). In addition to TLR2, which it encounters at both the plasma membrane and in the phagosome (12), *F. novicida* can be recognized in the cytosol by the AIM2/ASC inflammasome (13-15). This large, multimeric protein complex triggers activation of the cysteine protease caspase-1, which mediates the cleavage and secretion of pro-inflammatory cytokines and initiates an inflammatory host cell death. Cell death results in the loss of the intracellular replicative niche for *F. novicida* and is an important component of the host's defense against infection. Since both TLR2 and the AIM2/ASC inflammasome are important for host defense against *F. novicida* infection, dampening

the activation of these innate signaling pathways is critical for *F. novicida* pathogenesis (16-18).

During infection, *F. novicida* must also resist numerous antimicrobials present on mucosal surfaces and in phagosomes (11). In fact, compared to *E. coli* and many other Gram-negative species, it is extremely resistant to the effects of several antimicrobials, including antimicrobial peptides that disrupt bacterial membranes by interacting with lipid A in the outer membrane, causing lysis and death (19-21). The polymyxin class of antibiotics is often used as a surrogate for cationic antimicrobial peptides. Strikingly, the minimum inhibitory concentration of these antibiotic drugs against *F. novicida* is 40-80 fold more than that of *E. coli* (20, 21). We initially sought to identify *F. novicida* genes required for resistance to polymyxin and, surprisingly, identified the CRISPR/Cas gene *cas9*. We subsequently found that tracrRNA and scaRNA are also necessary for polymyxin resistance, and that this is dependent on their regulation of the FTN_1103 BLP. This provides intriguing evidence that CRISPR/Cas-mediated gene regulation can act as a resistance mechanism against a membrane targeting antimicrobial, and combat envelope stress.

The Cas9-dependent enhancement of envelope integrity occurs during *F. novicida* infection of host cells as well, where it facilitates evasion of the AIM2/ASC inflammasome. The importance of Cas9-mediated evasion of both the AIM2/ASC inflammasome and TLR2 in *F. novicida* virulence is highlighted by the demonstration that the *cas9* deletion mutant is nearly completely rescued for virulence in mice lacking

both ASC and TLR2. Thus, the work presented here shows that CRISPR/Cas systems are capable of enhancing the integrity of the bacterial envelope during intracellular infection, a previously unrecognized role in bacterial pathogenesis, ultimately facilitating the evasion of multiple spatially and temporally separated innate defense pathways. This reveals a new paradigm in CRISPR/Cas functionality that is likely relevant to the numerous bacterial pathogens encoding these systems.

Chapter 4 Results

Genetic screen for determinants of *F. novicida* resistance to polymyxin. *Francisella* species are extremely resistant to polymyxin in comparison to other bacteria. We therefore set out to identify genes necessary for *F. novicida* resistance to this antibiotic. We initiated a screen of 470 transposon mutants from a library (22) representing 229 genes that have previously been identified as being necessary for either virulence *in vivo* and/or intracellular replication in mammalian cells. Each transposon mutant was grown overnight in the presence of polymyxin B, and its subsequent growth compared to wild-type. Mutants that failed to reach at least 75% of wild-type growth were deemed to have mutations in genes important for mediating polymyxin resistance (Supplemental Tables 1 and 2).

The screen identified 120 genes as playing important roles in *F. novicida* resistance to polymyxin. The majority of these genes fall into the Unknown Function category by COG analysis, while the remainder are primarily in pathways necessary for the generation of envelope structures or within metabolic pathways that can act upstream of

envelope biogenesis and modification (Supplemental Figure 2). Resistance to polymyxin can be mediated by alterations to lipid A and O-antigen, components of lipopolysaccharide (LPS) in the bacterial outer membrane. Notably, we identified *FTN_0544*, *FTN_0545*, and *lpxE* which have previously been implicated in *Francisella* polymyxin resistance due to their roles in lipid A modification (23-25), providing validation for the results of the screen (Supplemental Table 1). We additionally identified *wbtD*, *wbtF*, and *wbtH*, which are part of the O-antigen biosynthetic machinery (Supplemental Table 1)(26). In order to further confirm the results of the screen, we generated deletion mutants in two genes encoding proteins of unknown function, *FTN_0109* and *FTN_1254*. Deletion of each of these genes resulted in a significant decrease in polymyxin resistance compared to wild-type, providing additional validation for the accuracy of the screen (Supplemental Figure 3).

Surprisingly, the screen also identified the CRISPR/Cas system RNA-directed endonuclease *cas9*. In order to confirm that *cas9* was indeed involved in resistance to polymyxin, we treated wild-type or a *cas9* deletion mutant with varying doses of this antibiotic. The *cas9* mutant was significantly hindered in its ability to grow at doses that had little effect on the growth of wild-type bacteria, confirming its identification in our screen (Figure 1a). Restoration of the *cas9* gene to the *cas9* deletion mutant successfully complemented polymyxin resistance to wild-type levels (Supplemental Figure 4). These data demonstrate the importance of a CRISPR/Cas system to enhanced resistance against a membrane-damaging antibiotic.

Cas9 regulatory axis promotes enhancement of envelope integrity. Since we previously demonstrated that *F. novicida* Cas9 interacts and functions with two small RNAs (tracrRNA and scaRNA) to regulate an endogenous transcript (*FTN_1103*) (Sampson et al., 2013), we analyzed whether mutants lacking these small RNAs had a diminished ability to resist the action of polymyxin as well. Dose-curve analysis revealed that tracrRNA and scaRNA deletion mutants exhibited near identical susceptibility phenotypes during growth in polymyxin compared to the *cas9* deletion strain (Supplemental Figures 5a, b). Together, these data demonstrate that *cas9* and the small RNA components of its regulatory axis are each necessary for resistance to polymyxin.

Cas9, tracrRNA, and scaRNA act to regulate the stability of the transcript for the FTN_1103 BLP. In their absence, *FTN_1103* mRNA and protein levels are drastically increased. We therefore analyzed whether the over-abundance of *FTN_1103* contributed to the increase in susceptibility to polymyxin observed in Cas9 regulatory axis mutants. Deletion of *FTN_1103* from the *cas9* mutant significantly restored its resistance to polymyxin (Figure 1a), and similar results were observed with tracrRNA or scaRNA deletion mutants (Supplemental Figures 5a, b). Furthermore, the susceptibility of the Cas9 regulatory axis mutants was observed during treatment with the non-ionic surfactant, Triton-X, but not hydrogen peroxide (Supplemental Figures 6 and 7). This suggests that this machinery mediates resistance to multiple, but not all, membrane stressors, via the regulation of *FTN_1103* expression.

Because polymyxin targets bacterial membranes, we sought to address more directly whether Cas9, tracrRNA, and scaRNA promoted resistance by enhancing the integrity of the bacterial envelope. We therefore analyzed the permeability of *cas9*, tracrRNA, and scaRNA deletion mutants by measuring their uptake of the nucleic acid staining dye, ethidium bromide, which fluoresces in the presence of DNA and RNA. The *cas9* deletion mutant demonstrated significantly increased fluorescence compared to wild-type, indicating that it is more permeable to ethidium bromide (Figure 1b). Similar increases in cellular permeability were also observed in both the tracrRNA and scaRNA deletion mutants (Supplemental Figure 5c). Furthermore, the increased permeability of all three strains could be restored to near wild-type levels by deletion of *FTN_1103* (Figure 1b and Supplemental Figure 5c). In order to ensure that the observed increase in permeability was not due to effects specific to ethidium bromide, we performed similar experiments with the nucleic acid staining dye propidium iodide and observed a near identical increase in fluorescence in the *cas9* mutant, that was dependent on *FTN_1103* (Supplemental Figure 8a). Importantly, similar levels of colony forming units were recovered from each strain during this experiment (Supplemental Figure 8b), indicating that while bacterial permeability was altered, viability was unaffected. Furthermore, the Cas9 regulatory axis mutants had no apparent defect in their ability to grow in either rich or defined minimal media (Supplemental Figures 9a, b), providing additional evidence that they are not inherently growth deficient in spite of their altered envelope permeability. Taken together, these data indicate that the CRISPR/Cas components, *cas9*, tracrRNA, and scaRNA directly enhance envelope integrity through their ability to regulate the production of a BLP, and thereby mediate antibiotic resistance.

Cas9 regulatory axis promotes enhanced bacterial integrity during intracellular infection. Since these data demonstrated a role for CRISPR/Cas components in enhancing envelope integrity during growth in broth culture, we examined whether they were necessary for a similar function during infection of macrophages, an important replicative niche for *F. novicida*. We first infected bone marrow-derived macrophages and quantified the intracellular levels of wild-type and mutant strains at four hours post infection, to determine if there were any differences between strains following phagosomal escape. Importantly, the Cas9 regulatory axis mutants or double deletion mutants lacking *FTN_1103* as well, replicated to wild-type levels (Figure 2a, and Supplemental Figure 10a). Next, we directly tested the permeability of cytosolic bacteria using propidium iodide (PI), measuring the co-localization of PI with each strain and quantifying 1,000 bacteria per sample. We observed that *cas9*, tracrRNA, and scaRNA deletion mutants displayed an almost 10-fold increase in PI staining (Figures 2b, c, and Supplemental Figures 10b, c). Additionally, permeability to propidium iodide was dependent on *FTN_1103*, further demonstrating the importance of repression of this membrane protein for the enhancement of envelope stability during infection (Figures 2b, c and Supplemental Figures 10b, c). These data provide evidence that CRISPR/Cas system components can control the enhancement of bacterial envelope integrity during an intracellular infection, which is mediated via the repression of a BLP.

Cas9, tracrRNA, and scaRNA are required for evasion of inflammasome activation. Because we observed an increase in the permeability of Cas9 regulatory axis mutants

during intracellular infection, we sought to determine if the lack of enhanced membrane stability might correlate with increased recognition of bacterial components by host cytosolic receptors that stimulate innate immune signaling. *Francisella* is recognized in the cytosol by the AIM2 inflammasome, which contains the adaptor protein ASC, and whose activation leads to an inflammatory host cell death. After infection of bone marrow-derived macrophages, mutants lacking *cas9*, tracrRNA, or scaRNA were significantly more cytotoxic than wild-type bacteria (Figure 3a and Supplemental Figure 11). Furthermore, the increase in cell death was completely dependent on ASC (Figure 3b and Supplemental Figure 11). Importantly, the increased cytotoxicity was due to the regulatory function of Cas9 and each of the small RNAs, since absence of FTN_1103 in the double deletion strains decreased cell death to near wild-type levels (Figure 3a and Supplemental Figure 11). In addition, increased cytotoxicity was only induced by mutants lacking *cas9*, tracrRNA, or scaRNA; deletion mutants of other components of the CRISPR/Cas system induced cell death at wild-type levels (Supplemental Figure 12). Together these data demonstrate that dysregulation of the FTN_1103 BLP is the main factor responsible for the increased activation of ASC-dependent cell death by Cas9 regulatory axis mutants.

Previous observations indicated that TLR2 signaling is capable of increasing the kinetics of inflammasome activation (27). Before testing the contribution of TLR2 signaling to inflammasome activation induced by Cas9 regulatory axis mutants, we first sought to confirm our previous findings (8) that FTN_1103 overexpression led to increased TLR2-dependent cytokine production. Indeed, the *cas9* mutant induced a robust increase in

TLR2-dependent secretion of IL-6, which was due to *FTN_1103* overexpression and was ASC-independent (Figure 3c). Since *FTN_1103* overexpression increased TLR2 activation, we next examined the contribution of TLR2 signaling to the increased cell death induced by Cas9 regulatory axis mutants. A significant portion of the increased cell death induced by *cas9*, tracrRNA, and scaRNA mutants was TLR2-dependent, yet these mutant strains nonetheless remained hypercytotoxic in TLR2-deficient cells as compared to wild-type bacteria (Figure 3b and Supplemental Figure 11). This suggested that the elevated cytotoxicity they induced was only partially due to the increase in TLR2 signaling resulting from overexpression of BLP.

Since these mutants also exhibited increased permeability compared to wild-type bacteria during intracellular infection, which was dependent on *FTN_1103*, we tested whether the increase in permeability during infection correlated with increased cytotoxicity. Indeed, the TLR2-independent cytotoxicity induced by the Cas9 regulatory axis mutants was decreased to wild-type levels in the absence of *FTN_1103* (Figure 3b and Supplemental Figure 11). Furthermore, only in macrophages deficient in both ASC and TLR2, was the induction of inflammatory cytokines and cell death by the *cas9* deletion mutant completely abrogated (Figures 3b, c). Taken together, this demonstrated that CRISPR/Cas-mediated repression of *FTN_1103* has two effects leading to evasion of two innate signaling pathways: 1) repression of a TLR2-stimulating ligand facilitates dampening of TLR2 signaling and both inflammasome activation and cytokine production (Figures 3b, c), and 2) enhancement of membrane integrity promotes evasion of TLR2-independent, ASC-dependent inflammasome activation (Figure 2b, c).

The *cas9* mutant is rescued for virulence in ASC/TLR2-deficient mice. *cas9* deletion mutants are severely attenuated and unable to cause lethal infection in mice (8). However, the cause of this attenuation *in vivo* is not clear. Since Cas9 is important for evasion of both the inflammasome and TLR2, we tested whether the *cas9* mutant was rescued for virulence in the absence of these innate inflammatory pathways. While the *cas9* mutant was undetectable in the spleen and liver of infected wild-type mice, strikingly, mice lacking both ASC and TLR2 were unable to restrict its replication (Figures 4a, b). The levels of the mutant increased at least 3 logs in the spleen and 2-3 logs in the liver (above the limit of detection) of infected ASC/TLR2-deficient mice, almost reaching the levels of wild-type bacteria observed in wild-type mice (Figures 4a, b). This robust increase in bacterial burden correlated with mortality, since the *cas9* deletion mutant did not cause a lethal infection in wild-type mice (Figure 4c), but >90% of infected ASC/TLR2-deficient mice succumbed to infection (Figure 4d). This astounding increase in virulence of the *cas9* mutant in ASC/TLR2-deficient mice highlights the essential role that Cas9 plays in facilitating the evasion of two distinct and critical host innate immune receptors, providing further evidence of the important roles that CRISPR/Cas systems can play in bacterial pathogenesis.

Chatper 4 Discussion

Through the use of a genetic screen, we identified 120 genes which contribute to *F. novicida* resistance to the membrane targeting antibiotic polymyxin. While the screen identified a number of genes known to be involved in the biogenesis or modification of

envelope structures, it also implicated numerous potential metabolic pathways in mediating polymyxin resistance. These pathways may be involved in creating necessary precursors for envelope structures, and/or increasing metabolic output, allowing sufficient energy to resist and repair damage induced by polymyxin. In either case, the results suggest an important interplay between the metabolic status of the bacterial cell and its ability to resist the action of polymyxin. Surprisingly, the screen also identified the CRISPR/Cas gene *cas9* as being required for polymyxin resistance. We subsequently revealed that regulation of the FTN_1103 BLP by Cas9 working in conjunction with tracrRNA and scaRNA is critical for enhancing the stability of the bacterial envelope, ultimately promoting resistance to polymyxin. Overall, to our knowledge, this is the first example of CRISPR/Cas components providing resistance to an antibiotic.

Previous studies have focused on the ability of CRISPR/Cas systems to limit antibiotic resistance by restricting the acquisition of mobile elements, which include those that carry antibiotic resistance cassettes. Studies in several bacteria revealed a correlation between increased antibiotic resistance and non-functional CRISPR/Cas systems (28-30). In fact, it has been demonstrated that acquisition of resistance traits can be restricted by CRISPR/Cas systems *in vivo* (31). However, the data presented here suggest that CRISPR/Cas systems with regulatory functions may provide bacteria with the capability of resisting certain antibiotics. Thus, loss of these systems in antibiotic resistant species may have currently unappreciated regulatory effects leading to altered bacterial physiology (i.e. envelope structure) and enhanced susceptibility to membrane-targeting antibiotics. Delineating the regulatory functions of CRISPR/Cas systems in diverse

bacteria in the future will be required to more broadly assess their putative roles as antibiotic resistance determinants.

Expression of CRISPR/Cas components can be induced in response to bacterial envelope stress. For instance, when a membrane-targeted GFP is expressed, causing aberrant envelope protein localization and dysfunctional envelope protein composition, the subsequent stress response triggers the induction of CRISPR/Cas system expression (2). Furthermore, these systems are induced in the presence of bacteriophage (3, 4), and we previously demonstrated an induction of CRISPR/Cas system components during infection of host cells (8). Together, these data strongly suggest that CRISPR/Cas systems are broadly activated by envelope stress. The data presented here show that subsequently, CRISPR/Cas activation can result in an enhancement of envelope integrity and resistance to membrane damaging agents. Therefore, it is tempting to speculate that the CRISPR/Cas response to envelope stress serves two purposes: the activation of the adaptive, foreign nucleic acid restriction system, and the regulation of membrane proteins to enhance the integrity of the bacterial envelope, representing a novel role in bacterial physiology and a paradigm shift in our understanding of these systems.

During infection, the ability of CRISPR/Cas systems to enhance envelope integrity has important ramifications for the virulence of *F. novicida*. We demonstrate here that Cas9 regulatory axis-mediated envelope enhancement is necessary to inhibit activation of the inflammasome and host cell death. This is broadly in agreement with the idea that mutant strains with membrane defects induce increased levels of inflammasome activation (16).

Because the AIM2/ASC inflammasome responds to DNA released from *Francisella*, it is likely that the increase in envelope stability prevents release of such ligands, thereby subverting inflammasome activation (13, 15, 16). Therefore, the regulation of BLP expression by the Cas9 regulatory axis not only prevents TLR2 activation, which occurs at the host cell surface and in the phagosome, but also the activation of cytosolic receptors after *Francisella* has escaped the phagosome and is replicating in the host cell cytosol. This provides the bacterial cell with a single system to dampen the activation of each of these compartmentally separated innate immune receptors. It is important to note that despite the decrease in membrane integrity in the *cas9*, tracrRNA, and scaRNA deletion mutants, these mutants maintain wild-type replication kinetics in both broth culture (Supplemental Figures 9a, b) and in macrophages (Figure 2a and Supplemental Figure 10a). This suggests that the increased BLP content, and subsequent increase in envelope permeability of these mutants, is not sufficient to grossly alter their ability to replicate, but rather inhibits them from being able to subvert the immune response induced by ASC and TLR2. It is only in the absence of both components, that the inflammatory response against the *cas9* mutant is completely abrogated during intracellular infection (Figure 3c). In fact, in mice lacking both of these receptors, the virulence of the *cas9* mutant is strikingly restored (Figure 4), inducing similar mortality as wild-type bacteria, demonstrating the importance of Cas9-mediated innate immune evasion in the ability of *F. novicida* to cause disease.

While *F. novicida* is the only known bacterial species with an established regulatory activity for Cas9, other species also utilize Cas9 as a virulence factor. *Neisseria*

meningitidis and *Campylobacter jejuni* each require Cas9 for invasion and replication in eukaryotic cells (8, 9). In addition, both of these species require Cas9 to attach to host cells, further suggestive of CRISPR/Cas system effects on the bacterial envelope (8, 9). Furthermore, the importance of Cas9 to *C. jejuni* virulence correlates with the presence of the CstII sialyltransferase (9). It is interesting to hypothesize that *C. jejuni* may utilize Cas9-dependent regulation to modulate the sialylation of its envelope, allowing it to not only efficiently attach to host cells, but also to evade detection and action by components of the innate immune system such as the complement system. Furthermore, the Type I CRISPR/Cas system in *Pseudomonas aeruginosa* (which does not encode *cas9*) is capable of modulating biofilm formation (6, 7). This is indicative of a broader CRISPR/Cas function in the modification and regulation of envelope structures that extends beyond only those organisms which encode Cas9.

Given that Cas9 is encoded in numerous pathogenic bacteria, including *Legionella pneumophila*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Haemophilus parainfluenzae* (8, 10), it is likely that Cas9-dependent regulation occurs in these and other species as well. Our data suggest that Cas9-containing Type II CRISPR/Cas systems, as well as other CRISPR/Cas subtypes, may broadly function as important regulators of envelope structure and integrity. This novel role for these systems would allow bacterial pathogens to respond to the stresses that occur during infection of host cells, ultimately facilitating evasion of innate immune defenses and virulence.

Chapter 4 Materials and Methods

Bacterial manipulations. *Francisella novicida* strain U112 and all derivatives used in this study were routinely grown at 37°C with aeration in tryptic soy broth (TSB) supplemented with 0.2% L-cysteine (BD Biosciences, Sparks, MD), or on TSA plates supplemented with 0.1% L-cysteine. Cas9 regulatory axis deletion mutants and complementation strains were described previously (8, 32). *FTN_1254* and *FTN_0109* mutants were constructed by allelic exchange as described previously (33, 34) using primers in Table S3.

Screen for polymyxin resistance determinants. Four hundred and seventy transposon mutants, representing 229 genes were obtained from the *Francisella* two-allele transposon mutant library (22, 35). Each transposon mutant was grown overnight in a well of a 96 well plate containing cation-adjusted Mueller Hinton broth (MH/C-A) with 0.2% L-cysteine (BD Biosciences). Subsequently, each mutant was diluted to an OD_{600} of 0.03 in MH/C-A containing 100μg/mL of polymyxin B (USB Corporation, Cleveland, OH). Following overnight growth at 37°C with aeration, the OD_{600} was measured and used to calculate the percent growth compared to wild-type bacteria. Strains that grew to an OD_{600} of less than 75% than that of wild-type were deemed to have increased sensitivity.

Polymyxin resistance assay. The indicated strains were grown overnight and subsequently diluted to an OD_{600} of 0.03 in MH/C-A with 0.2% L-cysteine containing the specified doses of polymyxin B. Following overnight growth at 37°C with aeration,

OD_{600} was measured and used to calculate the percent growth compared to the growth of the strain in media alone.

***In vitro* permeability.** The indicated strains were grown overnight and subsequently subcultured 1:50 in TSB and grown to an OD_{600} of ~0.8-0.9. Cells were washed twice in 50mM phosphate buffer and resuspended in 50mM phosphate buffer containing 30µg/mL ethidium bromide or 200µM propidium iodide. Fluorescence was measured immediately in a Biotek Synergy Mx plate reader (Winooski, VT) using an excitation of 250nm and emission of 605nm for ethidium bromide or excitation of 534nm and emission of 617nm for propidium iodide, correcting with samples lacking bacteria.

Macrophage culture and infection. Murine bone marrow-derived macrophages were prepared from wild-type C57BL/6 mice or the indicated knockout strains, and cultured as described previously (32). Macrophages were seeded overnight and infected with overnight cultures of the indicated bacterial strains at a multiplicity of infection (MOI) of 20:1, bacteria per macrophage. Plates were centrifuged for 15 minutes at 335 x *g* at room temperature to promote bacterial uptake. Infected macrophages were incubated for 30 minutes at 37°C and washed twice before adding DMEM containing 10µg/mL gentamicin.

Intracellular permeability. Wild-type murine bone marrow-derived macrophages were seeded onto glass cover slips and infected as above. At 4 hours post infection, macrophages were gently permeabilized for 15 minutes at room temperature with 0.1%

saponin / 3% BSA in PBS. Cells were first stained with 2.6µM propidium iodide (PI) and chicken-anti-*F. novicida* antibody (a kind gift from Dr. Denise Monack, Stanford University) for 12 minutes at 37°C. Following washing, cells were fixed with 4% paraformaldehyde and incubated with FITC-labeled anti-chicken antibody. Cover slips were mounted onto glass slides with SlowFade Gold reagent with DAPI (Life Technologies, Carlsbad, CA). Slides were imaged on a Zeiss Axioscope Z.1 microscope and a Zeiss Imager 2.1 camera. Images were analyzed with Volocity 5.5 software (Perkin Elmer, Waltham, MA). Colocalization was determined by no less than 50% overlap between PI and *Francisella* positive cells, and 1,000 cells were counted for each strain.

Cytotoxicity assays. Murine bone marrow-derived macrophages prepared from the indicated mice were infected with bacterial strains as described above. At 5.5 hours post infection, supernatants were collected and assayed for levels of LDH using the non-radioactive cytotoxicity assay kit (Promega, Madison, WI).

Murine infections. $ASC^{-/-}$ and $TLR2^{-/-}$ C57BL/6 mice were a generous gift from Bali Pulandren and were bred together to generate mice deficient in both ASC and TLR2. Mice were bred and kept under specific-pathogen free conditions, in filter-top cages at Yerkes National Primate Center, Emory University, and provided food and water *ad libitum*.For bacterial burden assays, female wild-type or ASC/TLR2-deficient mice (of 8 to 10 weeks of age) were infected subcutaneously with $2x10^5$ cfu of the indicated bacterial strains in sterile PBS. At 48 hours post infection, liver and spleen were harvested, weighed, homogenized in PBS, and serial dilutions plated to enumerate colony

forming units. For survival experiments, mice were infected with 10^8 cfu subcutaneously and monitored for signs of illness. Mice were euthanized when they appeared moribund. All experimental procedures were approved by the Emory University Institutional Animal Care and Use Committee (Protocol #069-2008Y).

Statistics. Two-tailed, Student's *t* tests were performed to analyze pairs of data as indicated, excluding the experiments in Figure 4A, which were analyzed with the Mann-Whitney test.

Chapter 4 Acknowledgements

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Chapter 4 Figures

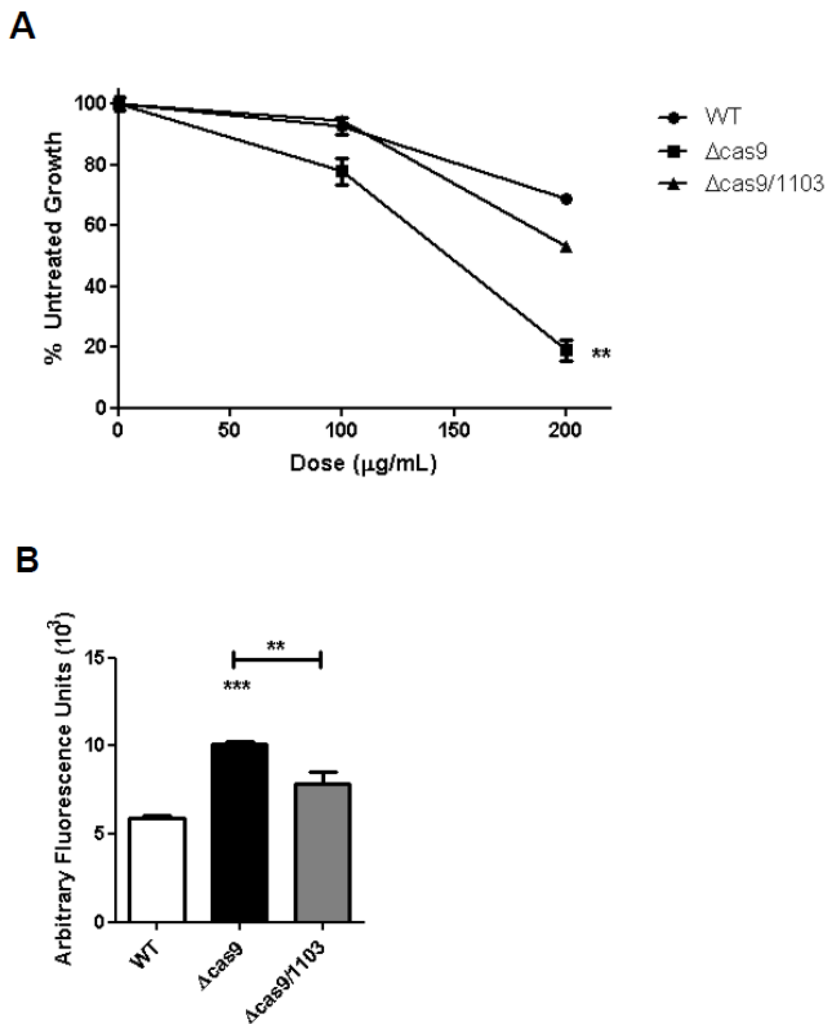


Figure 1. The Cas9 regulatory axis is necessary for polymyxin resistance. (A) Wild-type (WT), *cas9* or *cas9/1103* deletion mutants were grown overnight in broth culture containing the indicated concentration of polymyxin B. Percent growth compared to untreated cultures is plotted (n=3). **(B)** WT, *cas9*, or *cas9/1103* deletion mutants were grown to mid-log phase, washed, and stained with ethidium bromide and fluorescence measured (n=3). **; $p \leq 0.005$, ***; $p \leq 0.001$.

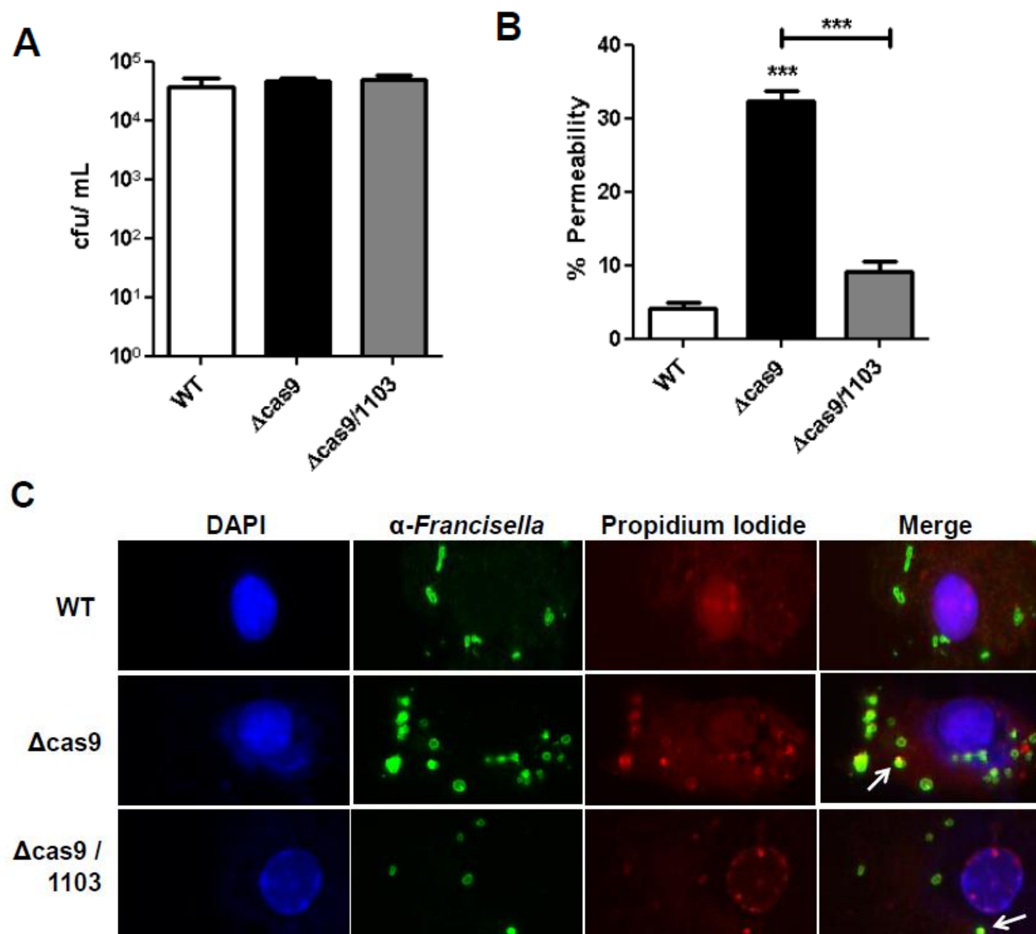


Figure 2. Cas9 is necessary for enhanced envelope integrity during intracellular infection. (A) Bone-marrow derived macrophages were infected with wild-type (WT), *cas9*, or *cas9/1103* deletion mutants at a multiplicity of infection (MOI) of 20:1 (bacteria per macrophage). At 4 hours post infection, macrophages were lysed, and plated to enumerate colony forming units. **(B)** Macrophages were infected as above, and at 4 hours post infection, macrophages were permeabilized with saponin and stained for anti-*Francisella* antibody (green), and propidium iodide (nucleic acids; red). Co-localization was quantified as no less than 50% PI overlap with *Francisella*, and 1,000 bacteria were counted for each strain. **(C)** Representative fluorescence micrographs of WT, *cas9*, or *cas9/1103* deletion mutants. DAPI (DNA; blue), anti-*Francisella*

antibody (green), and propidium iodide (nucleic acids; red). Arrows indicate representative PI and anti-*Francisella* co-localization. ***; $p \leq 0.001$.

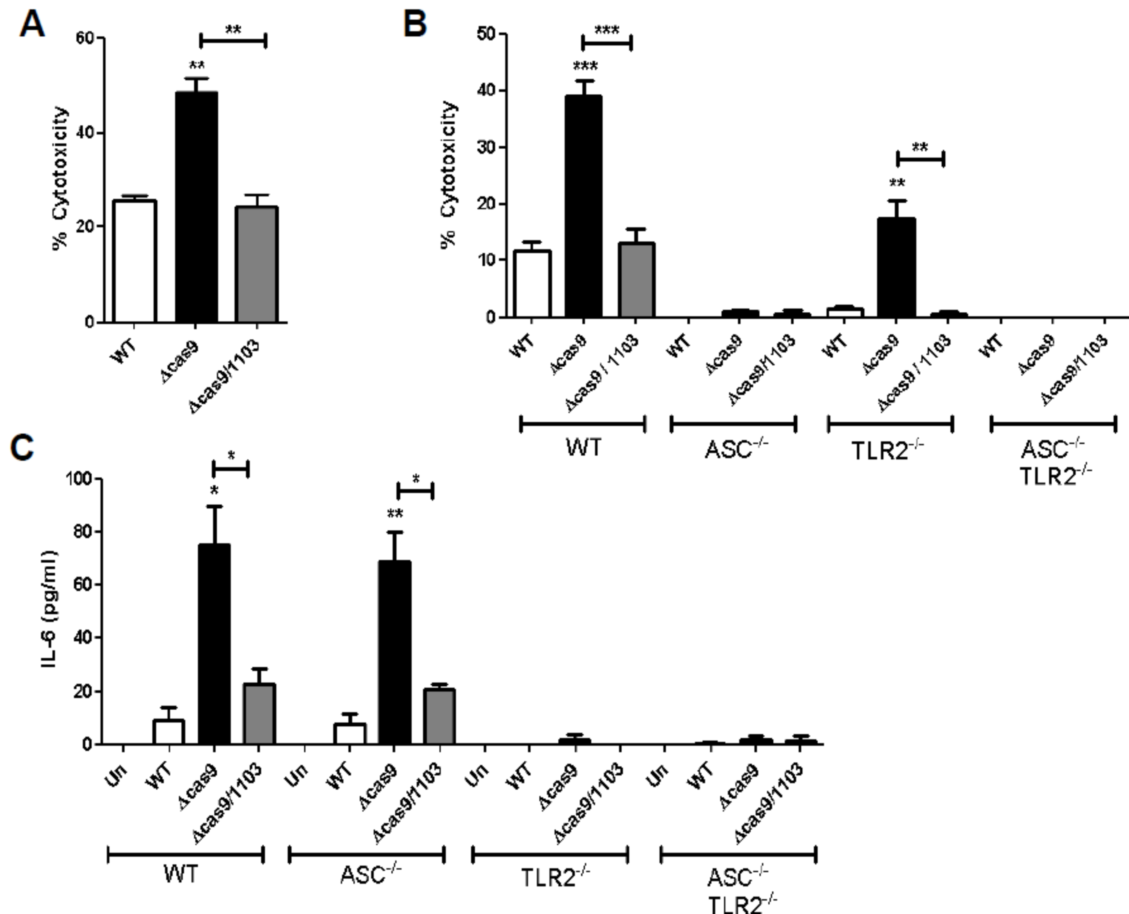


Figure 3. Cas9 promotes evasion of inflammasome activation and TLR2 signaling. **(A)** Wild-type (WT) bone marrow-derived macrophages were infected with WT, *cas9*, or *cas9/1103* deletion mutants at a multiplicity of infection (MOI) of 20:1 (bacteria per macrophage). At 5.5 hours post infection, cells were assayed for cytotoxicity using the LDH release assay. **(B, C)** WT, $ASC^{-/-}$, $TLR2^{-/-}$, and $ASC^{-/-}$/$TLR2^{-/-}$ double knockout macrophages were infected identically as in **(A)** and cytotoxicity measured **(B)** at 5.5 hours post infection or **(C)** IL-6 release was measured by ELISA at 4 hours post infection (n=3). *; $p \leq 0.05$, **; $p \leq 0.005$, ***; $p \leq 0.001$.

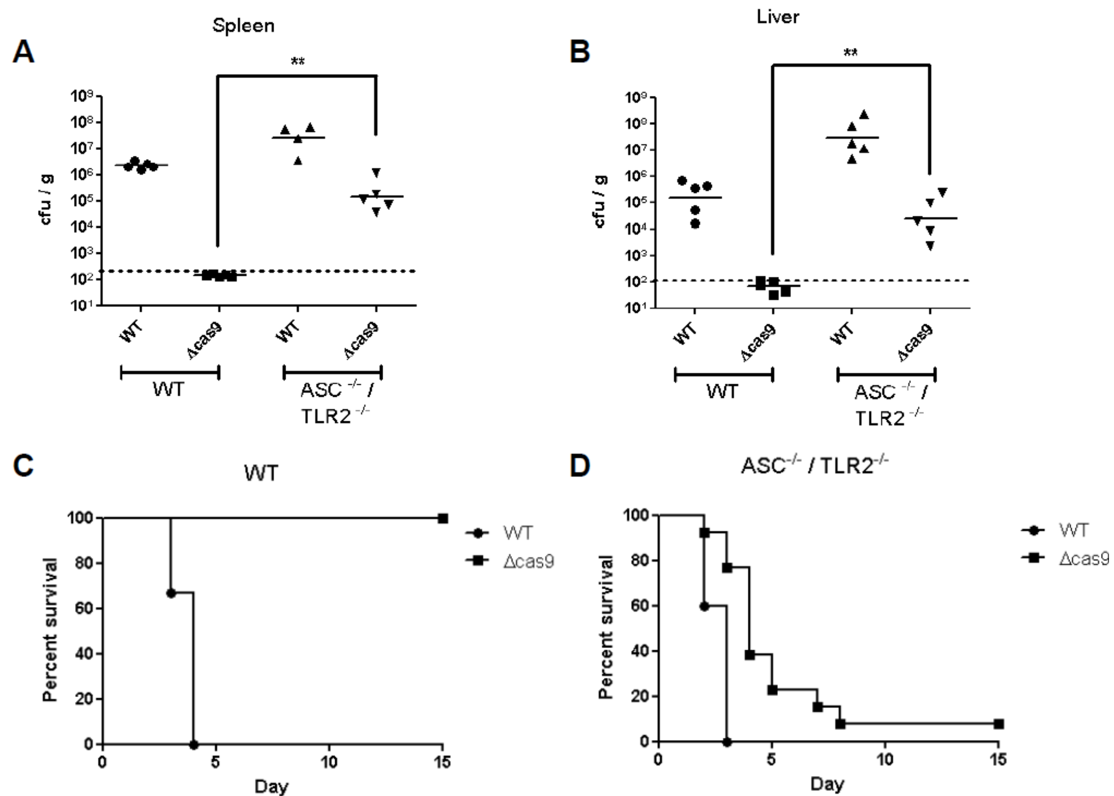
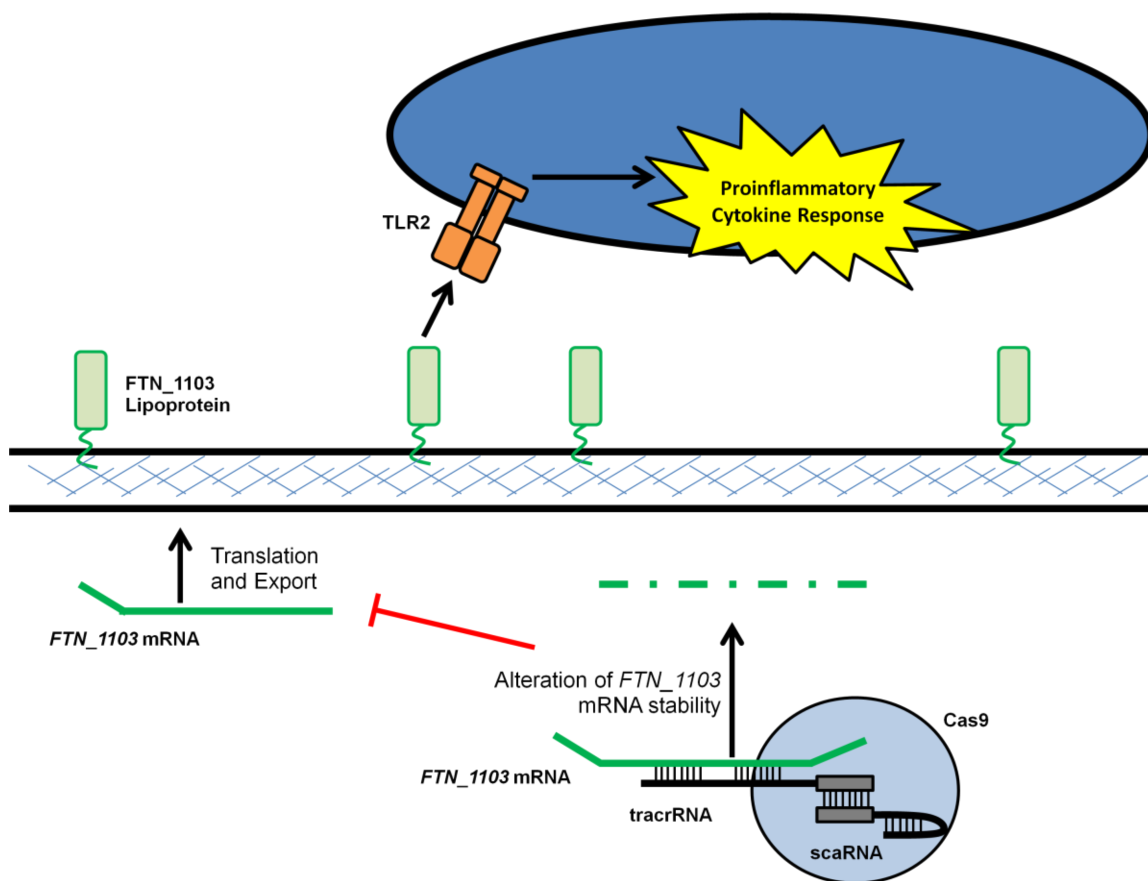
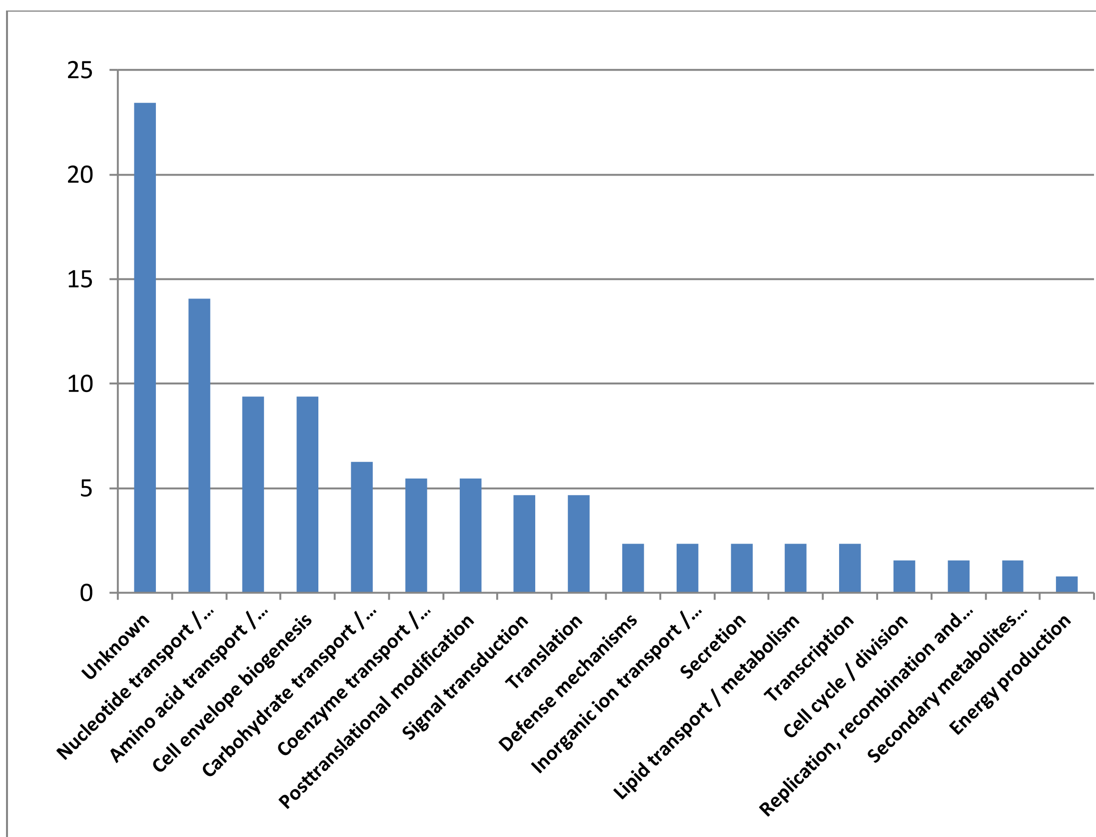


Figure 4. A *cas9* deletion mutant is rescued for virulence in mice lacking both ASC and TLR2. (A, B) Wild-type (WT) or ASC/TLR2-deficient mice were inoculated subcutaneously with 10^5 cfu of WT or the *cas9* deletion strain. Forty-eight hours post infection, the **(A)** spleen and **(B)** liver were harvested and plated to quantify bacterial levels (n=5). **(C, D)** Groups of 15 **(C)** WT or **(D)** ASC/TLR2-deficient mice were inoculated subcutaneously with 10^8 cfu of WT or *cas9* deletion strains. Mice were monitored for survival over 15 days. **; $p \leq 0.005$.

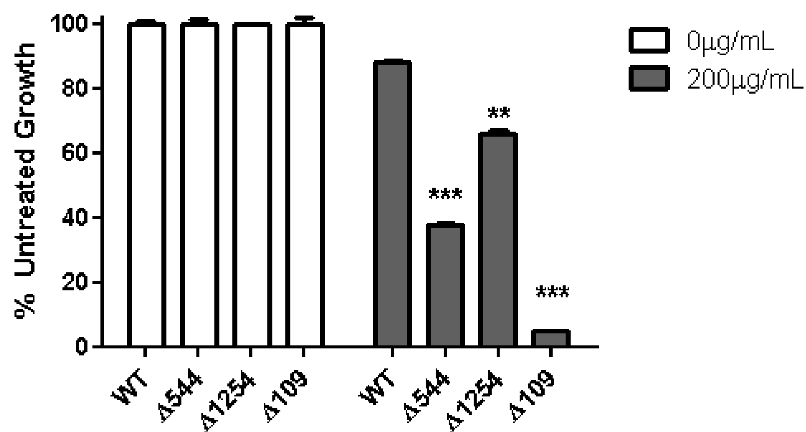
Chapter 4 Supplemental Figures



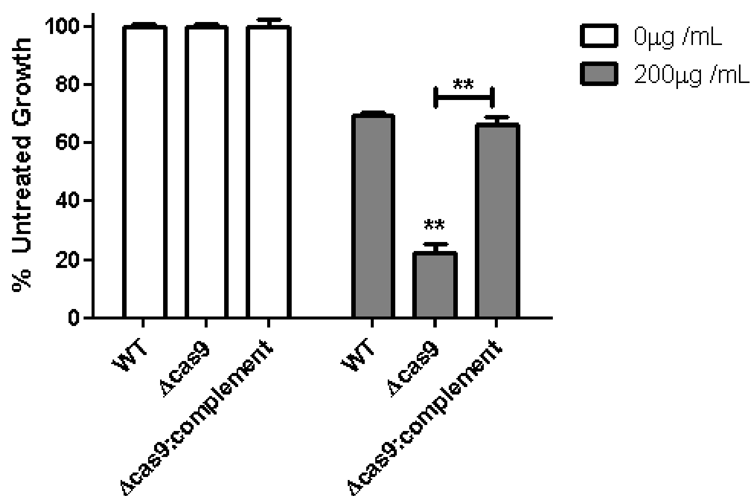
Supplemental Figure 1. Model of the Cas9/dual RNA complex mediating *FTN_1103* repression. Cas9 associates with two small RNAs, tracrRNA and scaRNA. This complex is then targeted to the *FTN_1103* transcript, encoding a bacterial lipoprotein (BLP), and ultimately mediates the repression of BLP production by altering the stability of its mRNA. Since BLP can be recognized by TLR2, leading to a proinflammatory innate immune response, the ability of Cas9 to act as a regulatory element against this transcript is critical for *Francisella* evasion of the innate immune response.



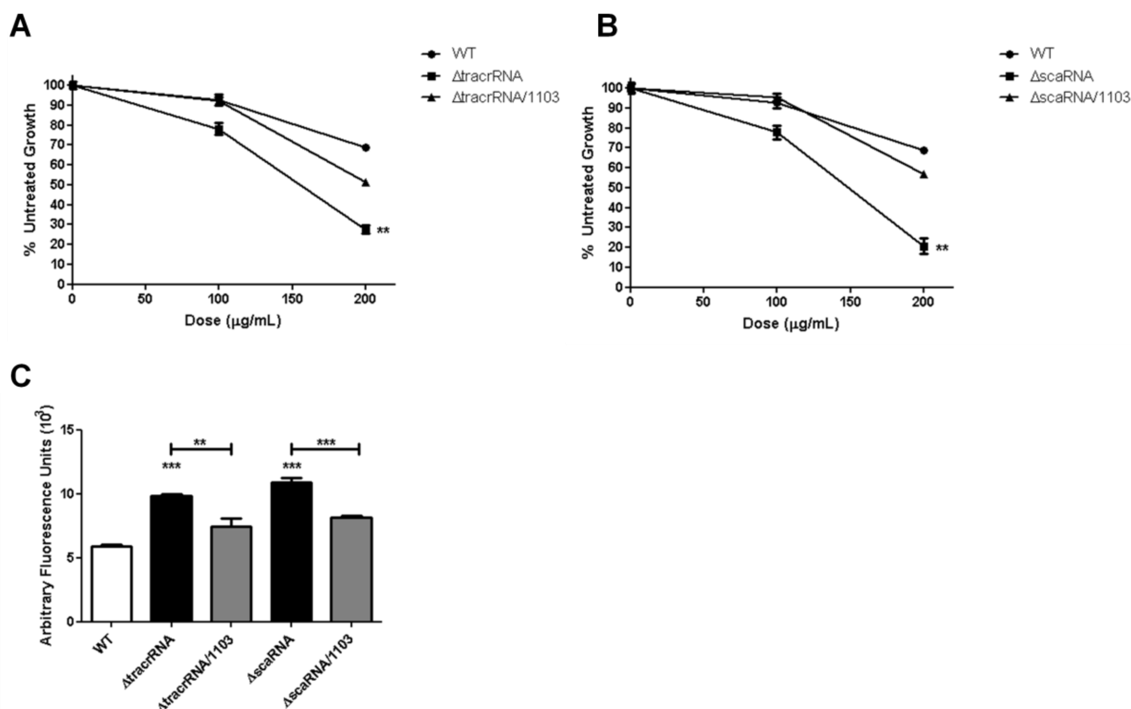
Supplemental Figure 2. COG categories of genes identified as being involved in polymyxin B resistance. COG categories were assigned to each locus identified within the screen as defined by the *Francisella novicida* U112 genome database through NCBI (Accession #: NC_008601.1) . Quantities of each COG category were plotted as percent of all categories identified within the screen.



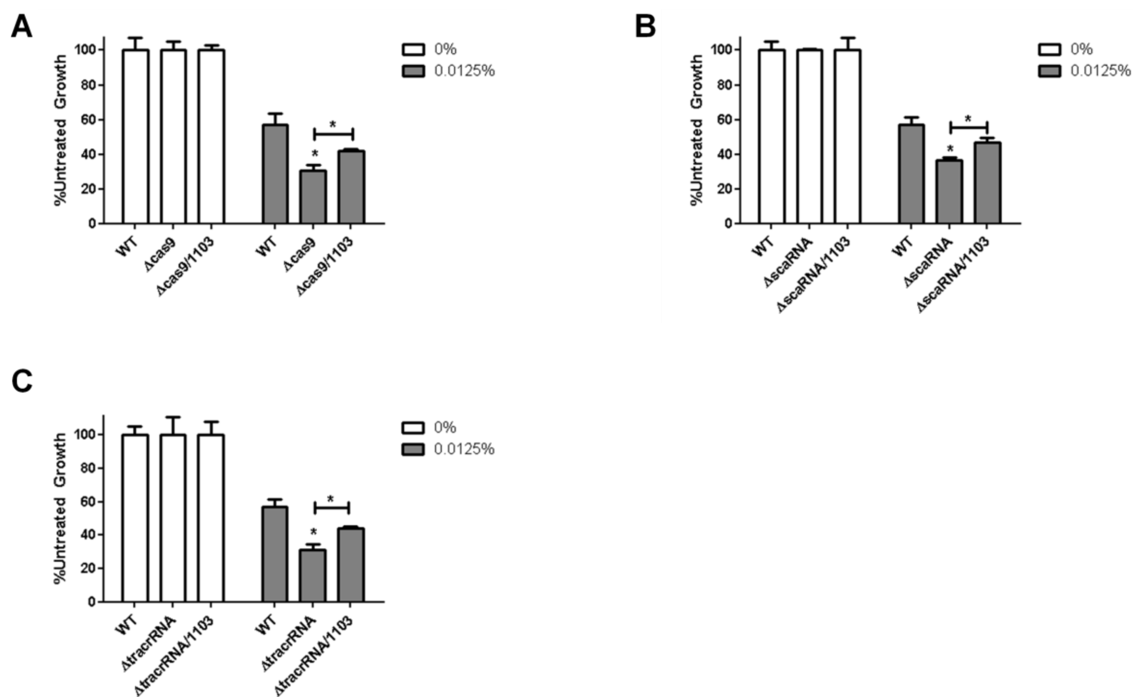
Supplemental Figure 3. *FTN_1254* and *FTN_0109* contribute to *F. novicida* polymyxin resistance. Wild-type (WT), *FTN_0544*, *FTN_1254,* or *FTN_0109* deletion mutants were grown overnight in TSB with or without polymyxin B (200 ug/mL). Percent growth compared to untreated cultures is plotted (n=3). **; $p \leq 0.005$, ***; $p \leq 0.001$.



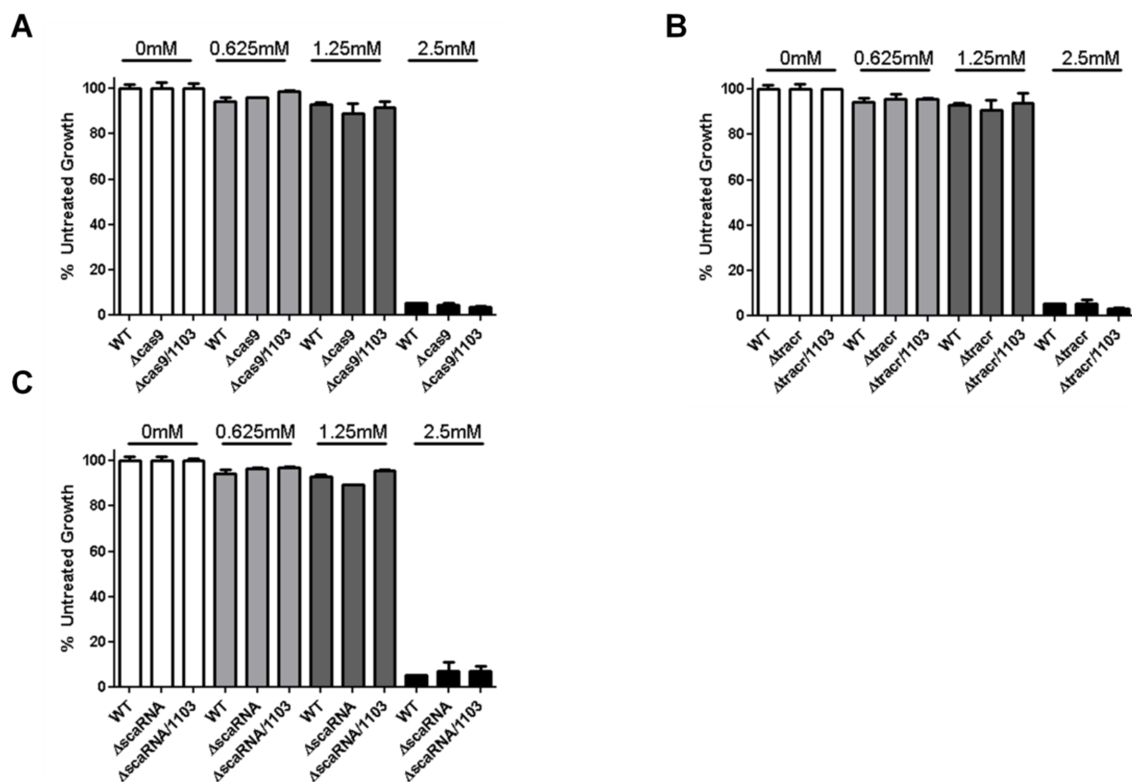
Supplemental Figure 4. Complementation of the *cas9* deletion mutant restores polymyxin resistance. Wild-type (WT), *cas9* deletion mutant, or a *cas9*:complement strain were grown overnight with or without polymyxin B (200 ug/mL). Percent growth compared to untreated cultures is plotted (n=3). **; $p \leq 0.005$.



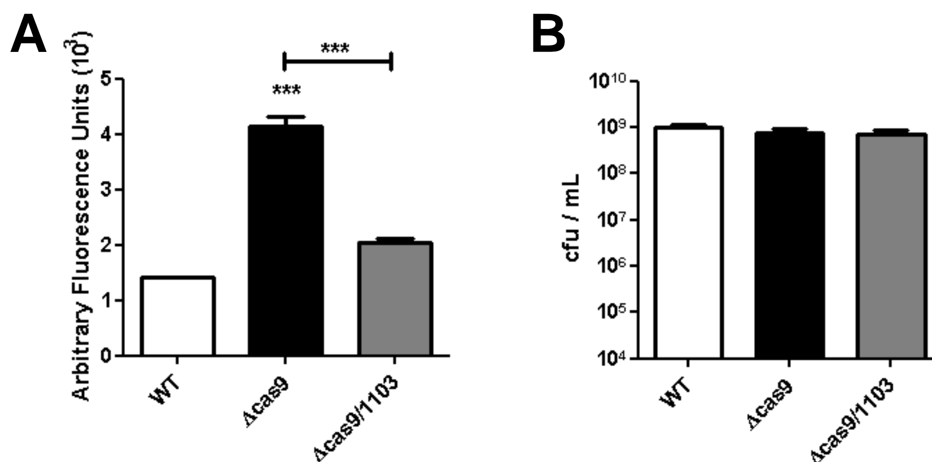
Supplemental Figure 5. ***FTN_1103*** **regulation by tracrRNA and scaRNA is necessary for polymyxin resistance. (A)** Wild-type (WT), tracrRNA or tracrRNA*/1103* deletion mutants, or **(B)** WT, scaRNA or scaRNA*/1103* deletion mutants, were grown overnight in TSB containing the indicated concentrations of polymyxin B. Percent growth compared to untreated cultures is plotted (n=3). **(C)** WT, tracrRNA, tracrRNA/*1103*, scaRNA, or scaRNA/*1103* deletion mutants were grown to mid-log phase, washed, and stained with ethidium bromide. Fluorescence was measured at excitation 250nm and emission 605nm (n=3). Data presented was generated during the same experiment as Figures 1a, b, utilizing the same controls, and plotted separately for clarity. **; $p \leq 0.005$, ***; $p \leq 0.001$.



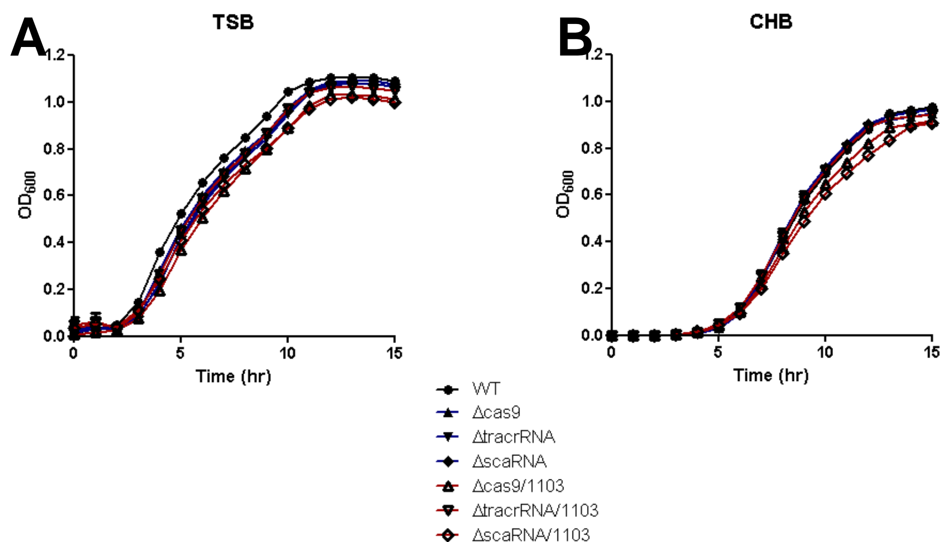
Supplemental Figure 6. Cas9 regulatory axis provides resistance to Triton X. **(A)** Wild-type (WT), *cas9* and *cas9/1103* deletion mutants, **(B)** tracrRNA and tracrRNA/*1103* deletion mutants, or **(C)** scaRNA and scaRNA/*1103* deletion mutants were grown overnight in TSB, in the presence or absence of the non-ionic detergent Triton X (0.0125%). Percent growth compared to untreated cultures is plotted (n=3). Data presented was generated during the same experiment, utilizing the same controls, and plotted separately for clarity. *; $p \leq 0.05$.



Supplemental Figure 7. Cas9 regulatory axis is not required for resistance to hydrogen peroxide. **(A)** Wild-type (WT), *cas9* and *cas9/1103* deletion mutants, **(B)** WT, tracrRNA and tracrRNA/*1103* deletion mutants, or **(C)** WT, scaRNA and scaRNA/*1103* deletion mutants were grown overnight in TSB containing the indicated concentrations of hydrogen peroxide. Percent growth compared to untreated cultures is plotted (n=3). Data presented was generated during the same experiment, utilizing the same controls, and plotted separately for clarity. $p > 0.05$.

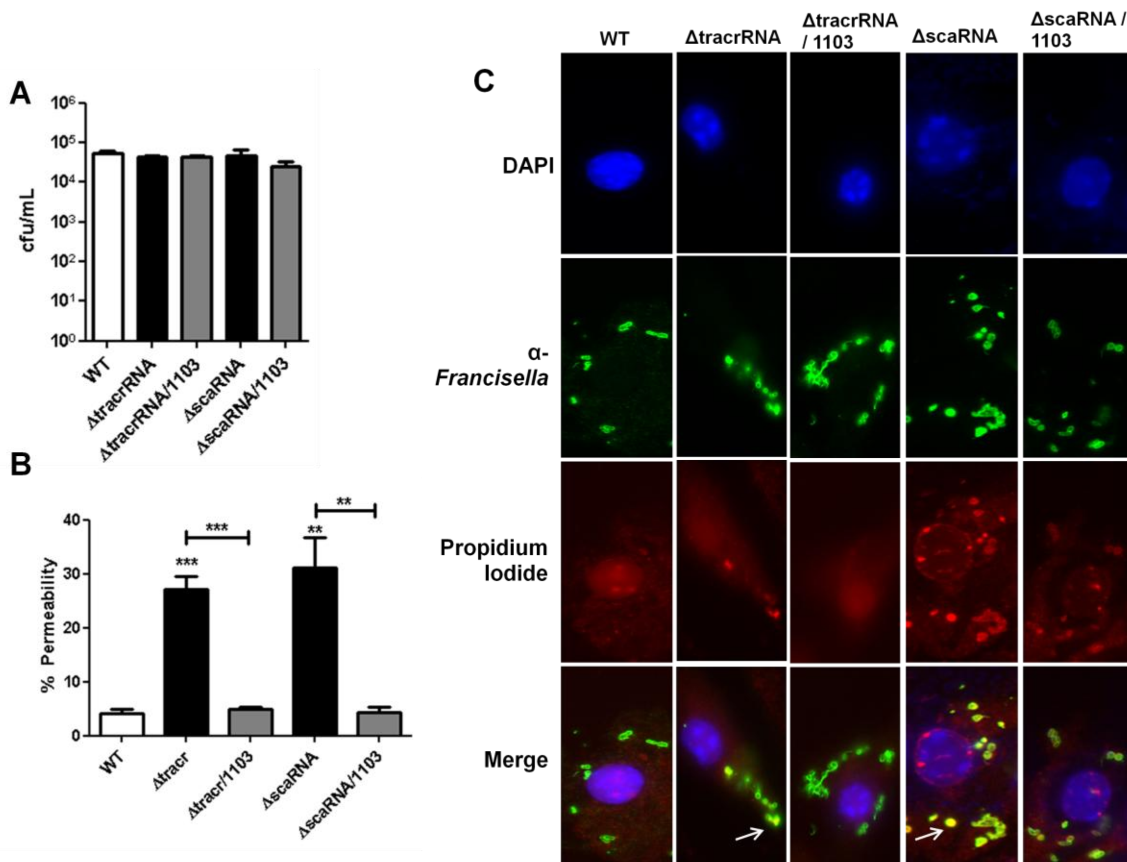


Supplemental Figure 8. *cas9* mutant exhibits increased permeability to propidium iodide during growth in broth. Wild-type (WT), *cas9*, or *cas9/1103* deletion mutants were grown to mid-log phase in TSB (n=3) and **(A)** washed and stained with propidium iodide (fluorescence was measured at excitation 534nm and emission 617nm), or **(B)** plated to enumerate colony forming units. ***; $p \leq 0.001$.



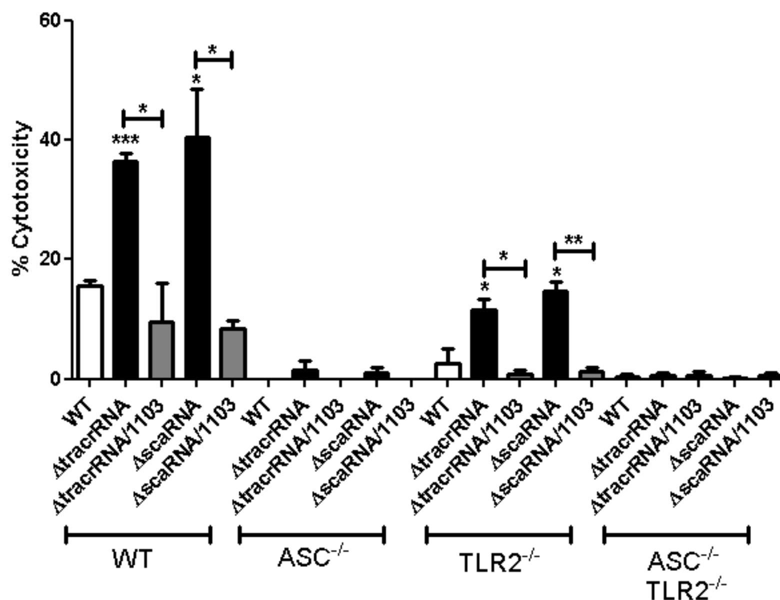
Supplemental Figure 9. Cas9 regulatory axis mutants exhibit wild-type growth kinetics in rich or synthetic media. Wild-type (WT), *cas9*, tracrRNA, scaRNA, *cas9/1103*, tracrRNA/*1103*

and scaRNA/*1103* deletion mutants were grown in **(A)** TSB or **(B)** Chamberlain's defined media (CHB) for 15 hours, and OD_{600} was measured every hour (n=3).

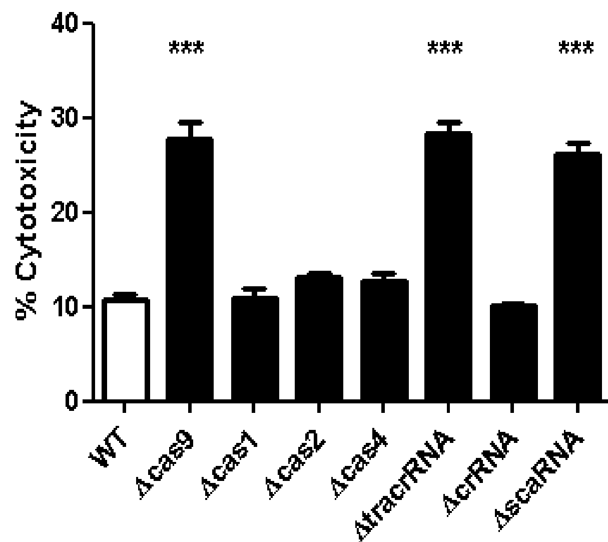


Supplemental Figure 10. *FTN_1103* regulation by tracrRNA and scaRNA is necessary for enhanced envelope integrity during intracellular infection. (A) Bone marrow-derived macrophages were infected with wild-type (WT), tracrRNA, tracrRNA/*1103*, scaRNA, or scaRNA*/1103* deletion mutants at a multiplicity of infection (MOI) of 20:1 (bacteria per macrophage). At 4 hours post infection, macrophages were permeabilized with saponin and lysates were plated to enumerate intracellular bacterial levels (n=3). **(B)** Macrophages were infected as above, and at 4 hours post infection, macrophages were permeabilized with saponin and stained with anti-*Francisella* antibody (green), and propidium iodide (nucleic acids; red). Co-localization was quantified as no less than 50% PI overlap with *Francisella*,and 1,000 bacteria were counted for each strain. **(C)** Representative fluorescence micrographs of WT, tracrRNA,

tracrRNA/*1103*, scaRNA, or scaRNA/*1103* deletion mutants. DAPI (DNA; blue), anti-*Francisella* antibody (green), and propidium iodide (nucleic acids; red). Arrows indicate representative PI and anti-*Francisella* co-localization. **; $p \leq 0.005$, ***; $p \leq 0.001$.



Supplemental Figure 11. *FTN_1103* regulation by tracrRNA and scaRNA promotes evasion of inflammasome. Wild-type (WT), TLR2$^{-/-}$, ASC$^{-/-}$ and ASC$^{-/-}$/TLR2$^{-/-}$ double knockout macrophages were infected with wild-type (WT), tracrRNA, scaRNA, tracrRNA/*1103* or scaRNA/*1103* deletion mutants at a multiplicity of infection (MOI) of 20:1. At 5.5 hours post infection, cells were assayed for cytotoxicity through LDH release (n=3). *; $p \leq 0.05$, **; $p \leq 0.005$, ***; $p \leq 0.001$.



Supplemental Figure 12. Other CRISPR/Cas components are not required for evasion of the inflammasome. Wild-type bone marrow-derived macrophages were infected with wild-type (WT), *cas9*, *cas1*, *cas2*, *cas4*, tracrRNA, crRNA, or scaRNA deletion mutants at a multiplicity of infection (MOI) of 20:1. At 5.5 hours post infection, cells were assayed for cytotoxicity through LDH release (n=3). ***; $p \leq 0.001$.

Chapter 5: Degeneration of a CRISPR-Cas system and its regulatory target during the evolution of a pathogen.

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Chapter 5 Abstract

CRISPR/Cas systems are bacterial RNA-guided endonuclease machineries that target foreign nucleic acids. Recently, we demonstrated that the Cas protein Cas9 controls gene expression and virulence in *Francisella novicida* by altering the stability of the mRNA for an immunostimulatory bacterial lipoprotein (BLP). Genomic analyses, however, revealed that *Francisella* species with increased virulence harbor degenerated CRISPR/Cas systems. We hypothesize that CRISPR/Cas degeneration removed a barrier against genome alterations, which resulted in enhanced virulence. Importantly, the BLP locus was also lost; likely a necessary adaptation in the absence of Cas9-mediated repression. CRISPR/Cas systems likely play regulatory roles in numerous bacteria, and these data suggest additional genomic changes may be required to maintain fitness after CRISPR/Cas loss in such bacteria, having important evolutionary implications.

Chapter 5 Introduction

CRISPR/Cas (clustered, regularly interspaced, short palindromic repeats / CRISPR-associated) systems are well-described RNA-guided endonuclease complexes that act to target and degrade foreign nucleic acids, such as those derived from bacteriophages (1). They consist of genomic or plasmid-encoded arrays of repetitive sequences that are interspaced by unique "spacer" sequences. These arrays are encoded adjacent to groups of conserved Cas genes, which distinguish three primary CRISPR/Cas subtypes (1). Following transcription of the CRISPR array, the transcript is processed into individual CRISPR RNAs (crRNAs) each containing partial repeat sequences and one unique spacer (2). These crRNAs form complexes with Cas proteins, hybridize to complementary nucleic acid targets, and the associated Cas genes catalyze the degradation of the target. Additionally, CRISPR arrays are adaptive. The Cas proteins Cas1 and Cas2 act to integrate new spacer sequences derived from invading foreign nucleic acids into the CRISPR array, allowing CRISPR systems to adapt and target these sequences in the future (3, 4). Due to their specificity and adaptivity, CRISPR/Cas systems are well established to play an important role in mediating defense against invading bacteriophages. These systems can also prevent transformation by plasmids as well as chromosomal DNA, clearly demonstrating that they represent broad barriers to horizontal gene transfer (HGT) (5, 6).

The Gram-negative intracellular pathogen, *Francisella novicida*, encodes a Type-II CRISPR/Cas system, which is characterized by the presence of the Cas9 endonuclease (1, 7). We recently established the importance of this system in the pathogenesis of *F.*

novicida (8). Like other *Francisella* species, *F. novicida* is capable of infecting and replicating within the cytosol of a variety of host cells, including phagocytic cells of the innate immune system (9). Upon phagocytosis by macrophages, *Francisella* spp. evade or block numerous phagosomal host defenses, before rapidly escaping this compartment to reach the host cell cytosol where they replicate to high titers (Reviewed by Jones et al. (10)). During this process, the bacteria can be detected by the host innate immune protein Toll-like Receptor 2 (TLR2), which recognizes bacterial lipoproteins (BLP) and is present at both the plasma membrane and in the phagosome. TLR2 plays a critical role in recognizing *Francisella* and mounting a proinflammatory response (Reviewed by Jones et al. (10)). Therefore, in order to reach its replicative niche in the cytosol without inducing a significant inflammatory response, *Francisella* dampens recognition by, and activation of, TLR2. We have demonstrated that components of the *F. novicida* Type II CRISPR/Cas system are capable of targeting and repressing the expression of an endogenous transcript (*FTN_1103*) encoding a TLR2-activating BLP.(8) Specifically, Cas9 forms a complex with the tracrRNA and a novel small RNA, termed small, CRISPR/Cas-associated RNA (scaRNA). Together, these components allow tracrRNA to interact with and target the *FTN_1103* transcript for degradation (8). Using this system, *F. novicida* is able to rapidly decrease the abundance of the *FTN_1103* transcript specifically when the bacteria are in the phagosome and in the presence of TLR2. Repression of this BLP via Cas9-dependent degradation allows *F. novicida* to dampen activation of TLR2 (8). Since mutants lacking components of the Cas9 regulatory complex are severely attenuated, the innate immune evasion mediated by this system is absolutely critical for *F. novicida* pathogenesis (8).

In addition to the role of components of the Type II CRISPR/Cas system in *F. novicida* pathogenesis, this system is predicted to be functional in the canonical role of targeting foreign nucleic acid (7). The Type II CRISPR/Cas locus in *F. novicida* genomes encodes full-length forms of all the necessary components for the adaptation (*cas1*, *cas2*, *cas4*) and effector phases (*cas9*, crRNA, tracrRNA – also required for crRNA processing and interaction of the crRNA with Cas9) of targeting foreign DNA, as compared to functional Type II systems in *Streptococcus* spp and other bacteria. Further suggesting that the Type II system is active in the targeting of foreign nucleic acid, most *F. novicida* genomes encode spacers identical to sequences in a predicted prophage present in the genome of a single known isolate of *F. novicida* (7). Interestingly, this isolate does not encode such spacers, potentially explaining why it harbors this prophage (7). In addition, *F. novicida* genomes encode a second CRISPR/Cas locus that most closely resembles a Type II locus in its architecture, but rather than Cas9, it encodes a novel Cas protein with no homology to known proteins (7). In contrast, we and others observe that the CRISPR/Cas systems present in the more virulent *F. holarctica*, *F. mediasiatica*, and *F. tularensis* species, have degenerated and lack critical components for CRISPR/Cas functionality (Figure 1a) (7).

Chapter 5 Results and Discussion

Analysis of the genome of highly virulent *F. tularensis* (strain SchuS4) provides strong evidence for the degeneration of its CRISPR/Cas systems compared to *F. novicida* (strain U112). Specifically, there are disruptions within all four *cas* genes. While *F. tularensis*

encodes the full-length DNA sequence for *cas1,* it contains a single base deletion (thymine 556 [815,478]) resulting in a -1 frame-shift mutation, leading to truncation of the protein by 125 amino acids (Figure 1d). Similarly, this truncation of the *cas1* gene is also present in *F. holarctica* (strain LVS) and *F. mediasiatica* (strain FSC147). The *cas2* gene of *F. tularensis* contains a single base insertion (adenine 83 [816,119 – 816,120]) resulting in a +1 frame-shift mutation, a premature stop codon downstream, and a Cas2 protein only 31 amino acids in length, compared to 98 in *F. novicida* (Figure 1e), whereas the *cas2* open reading frames in *F. holarctica* and *F. mediasiatica* appear to be full length in comparison to *F. novicida* (Figure 1a). Since *cas1* is likely non-functional in *F. tularensis* and other virulent *Francisella* species, these species would lack the ability to integrate new spacer sequences and therefore to adapt to new target sequences (3, 4). *F. tularensis cas4* has an internal deletion of 12 bases (567 – 578 [816,853 – 816,864]) resulting in a loss of 4 amino acids (Figure 1f). However, the predicted protein is in-frame, and it is therefore unclear if it retains function. A similar in-frame mutation is present in *F. holarctica,* while *F. mediasiatica* contains an early stop codon, resulting in truncation of this protein. The *cas9* open reading frame is the most divergent between these species. While *F. novicida* and *F. tularensis* have a single open reading frame corresponding to a *cas9* protein predicted to be produced, *F. holarctica* and *F. mediasiatica* contain a *cas9* sequence that has been degenerated into four or three truncated open reading frames, respectively, with the majority of these predicted to be pseudogenes (Figure 1a). On the other hand, detailed analysis of the *F. tularensis cas9* has revealed some striking differences. *F. tularensis cas9* has a large internal deletion of 1,572 bases (corresponding to bases 2992 through 4563 of the *F. novicida cas9* [813,044

– 814,617], and 524 amino acids)(Figure 1c). This deletion includes the predicted RuvC-IV endonuclease domain (8, 11), as well as a portion of the predicted HNH endonuclease domain, necessary for Cas9 cleavage of DNA targets (12). However, the deletion does not disrupt the conserved HNH catalytic residues. It is striking that the *cas9* sequence deleted from *F. tularensis* is flanked by the sequence GATAATAAAAA as a direct repeat in *F. novicida* (Figure 1c). In *F. tularensis*, there is only a single copy of this flanking sequence, highly suggestive of an intramolecular recombination event, which would have led to the excision of the 1,572 nucleotides present in *F. novicida*.

Furthermore, there is a large span of amino acids (681 aa through 784 aa in *F. novicida* Cas9) that are highly dissimilar between the two species (Figure 1b). Flanking this region of dissimilarity is a small, 4bp, inverted repeat (TATC – GATA) that may be an indication of the occurrence of an illegitimate recombination event or the product of double strand break repair. Small, inverted repeats may also be scars of transposition events, however we find no evidence of an inserted transposon within this sequence.

Not only are Cas proteins disrupted in the *F. tularensis* SchuS4 genome, but the content of CRISPR/Cas system RNAs is also altered. *F. tularensis* SchuS4 contains a transposable element (*Is-Ftu2*) inserted at the site within the *F. novicida* genome that encodes the crRNA array and the scaRNA, resulting in the deletion of these CRISPR/Cas components. The tracrRNA is still present in the *F. tularensis* genome, but in the absence of the crRNA array, it is unclear if, or how, this RNA would function to target foreign

nucleic acid. Similarly, in the absence of scaRNA, which is critical for the ability of *F. novicida* to repress production of FTN_1103(8), it is unlikely that the remaining components in the *F. tularensis* system could function equally in its regulation. Since this regulatory pathway is essential for evasion of TLR2 and virulence in *F. novicida*, its inactivation in *F. tularensis* would potentially be highly detrimental to the pathogen's ability to survive in mammalian hosts (8). This raises two important questions: what evolutionary pressures would select against a functional CRISPR/Cas locus in *F. tularensis*, and were there coincident changes that occurred in order to prevent the induction of the host TLR2 response?

Recent work has very clearly demonstrated that CRISPR/Cas systems represent a strong barrier to HGT. This restriction is extremely broad, as these systems prevent not only infection by bacteriophages (as well as their integration and the subsequent potential for lysogenic conversion), but also acquisition of plasmids, and both conjugative and free linear DNA. (5, 6, 13, 14) Therefore, acquisition of new genetic information from many sources is significantly inhibited by CRISPR/Cas systems. Additionally, since plasmids and bacteriophages can be carriers of transposons (15), CRISPR/Cas systems also present a blockade to prevent uptake of these and other mobile elements. Furthermore, CRISPR/Cas systems have been shown to prevent the induction of prophages (13). Therefore, it is interesting to speculate that this type of action may also inhibit the excision of other mobile elements, and may therefore prevent other mechanistically similar recombination events within the chromosome, such as gene duplication or phase variation through inversion or gene conversion (14). Thus, in the absence of a functional

CRISPR/Cas system, as observed in the highly virulent *Francisella* spp., genomes would be more likely to undergo these numerous types of genetic alterations.

Sequence analysis of highly virulent *Francisella* species reveals that they underwent a number of genomic changes compared to *F. novicida*, during their hypothesized patho-adaptation to mammalian hosts (16-18). *F. holarctica* and *F. tularensis* species contain forty-one genes not present in *F. novicida*, that are predicted to play important roles during infection of mammals (18). While the function of the majority of these unique genes has not been determined, six are predicted to play roles in the biosynthesis of O-antigen, a critical surface structure necessary for pathogenesis (18). Additionally, *F. tularensis* strains have nine genes unique to their genomes (18). Eight of these are located in a predicted remnant of a prophage or other mobile element flanked by transposons, and while its function is unknown, it has been postulated to be an *F. tularensis*-specific pathogenicity island (18). Furthermore, the *F. tularensis* genome has no less than twenty genetic duplication events (16-18). Notably, this includes duplication of the *Francisella* Pathogenicity Island (FPI), an event observed in all highly virulent species of *Francisella*(16-18). The FPI encodes a Type VI secretion system that is absolutely essential for intracellular replication and virulence of *Francisella* spp. in mammals (19). The FPI is flanked by transposable elements that likely facilitated its duplication by non-reciprocal recombination (17). Duplication of the region may result in an increased gene dosage and/or altered pattern of expression, enhancing the virulence of highly pathogenic strains. Additionally, *F. tularensis* genomes as a whole have gained a number of transposable elements (79 in *F. tularensis* SchuS4, compared to 26 in *F. novicida* U112)

which may have facilitated the aforementioned genetic duplications and acquisitions, as well as large-scale transposon-mediated inversions (16-18). Together, these global genetic changes are generally thought to have been essential for the increased virulence of *F. tularensis.*

Because CRISPR/Cas systems are capable of inhibiting the acquisition of new genetic information, we hypothesize that the loss of functional CRISPR/Cas systems facilitated those widespread genomic changes that occurred in highly virulent *Francisella* species. However, since *F. novicida* absolutely requires the Cas9 regulatory system to repress an immunostimulatory BLP (*FTN_1103*)(8), the loss of CRISPR/Cas systems in highly virulent species would have likely come at the cost of a decreased ability to dampen BLP levels and thus recognition by the host innate immune receptor TLR2. This is paradoxical in light of the many studies that have clearly demonstrated that *F. tularensis* is much less inflammatory, and in some cases even anti-inflammatory, compared to the less virulent *F. novicida* (10). Therefore, additional changes likely occurred in highly virulent *Francisella* species to prevent the activation of TLR2 and host innate immune defenses, even in the absence of the Cas9-encoding CRISPR/Cas locus.

DNA sequence analysis demonstrates that significant degeneration of the *FTN_1103* region occurred in *F. tularensis* (Figure 2). The region encompassing *FTN_1103* degenerated completely in *F. tularensis* (as well as in *F. holarctica* and *F. mediasiatica*), lacking any nucleotide sequence directly corresponding to the gene. Furthermore, there is no *FTN_1103* ortholog elsewhere within the *F. tularensis* genome. *FTN_1102* is also

absent, and the *FTN_1104* ortholog (*FTT1122c*) is truncated by 78 bases (Figure 2). There is evidence of a transposon insertion occurring within this region of the more virulent strains (Figure 2), as each contains an *ISFtu6* sequence (now predicted to be a non-functional pseudogene). This insertion may have facilitated the loss of the ~2kbp region that contains *FTN_1101, FTN_1102,* and *FTN_1103* in the *F. novicida* genome. The *FTN_1103* region within *F. novicida* is flanked by *ygiH*, a gene predicted to be involved in glycerolipid metabolism and *tgt,* a predicted queuine-tRNA ribosyltransferase. Both of these genes remain highly conserved between *F. novicida* and the more virulent *Francisella* genomes, providing clear boundaries to the genetic changes which occurred. These data clearly delineate the widespread loss of *FTN_1103* among virulent *Francisella* species, as well as the degeneration of the surrounding genomic region.

While its physiological function is unknown, *FTN_1103* is dispensable for *F. novicida* virulence since mutants lacking this gene are not significantly attenuated in a mouse model of infection (8, 20). Thus, loss of this gene does not have a significant adverse effect on the fitness of the organism. Taken together, we hypothesize that virulent *Francisella* species lost the *FTN_1103* coding sequence (as well as some of the surrounding genetic region) previously to, or concurrently with, the degeneration of the CRISPR/Cas locus. This coincident change would have allowed *F. tularensis* to undergo significant genome alterations (in the absence of CRISPR/Cas–mediated HGT restriction), facilitating its increased virulence, while also preventing increased activation of the host innate immune system. This provides a parsimonious explanation for the

apparent paradox between the striking importance of the CRISPR/Cas system as a critical virulence factor in the pathogenic lifestyle of *F. novicida,* and the non-functionality of the system in the most virulent *Francisella* species.

Here, we correlate CRISPR/Cas system degradation and the subsequent increase in HGT, with the coincident loss of a CRISPR/Cas-regulated locus. Loss of CRISPR/Cas systems may provide a fitness advantage to organisms that undergo frequent and beneficial genetic exchange, particularly during patho-adaptation. For example, *Streptococcus pneumoniae* is unable to acquire critical virulence factors when a functional CRISPR/Cas system is present (and the system is engineered to contain spacers targeting those genes), demonstrating that CRISPR/Cas systems can directly restrict DNA acquisition and the emergence of virulence during *in vivo* infection (6). Further, many *S. pyogenes* CRISPR arrays contain targets against lysogenic bacteriophages, suggesting that they may prevent acquisition of phage-encoded virulence factors or act as regulators of virulence traits (2). It has also been suggested that a functional CRISPR/Cas system prevents HGT in *Staphylococcus epidermidis*, but that the more virulent *S. aureus* is able to acquire genes horizontally due to a lack of a functional CRISPR/Cas system (21). Similarly, antibiotic sensitive strains of *Enterococcus faecalis* often encode CRISPR/Cas systems whereas highly antibiotic resistant strains are less likely to encode these loci, suggesting that CRISPR/Cas systems prevent the acquisition of antibiotic resistance (22).

In the event that CRISPR/Cas systems play additional roles in bacterial physiology beyond their action in defense against foreign DNA, as we have demonstrated for the

Cas9 system in *F. novicida* (8), the loss or degradation might have more complex effects on bacterial physiology. For example, loss of *cas9* in *Neisseria meningitidis* or *Campylobacter jejuni* results in a decreased ability to attach, invade, and replicate within host cells (8, 23). Further, in *C. jejuni,* increased degeneration of the CRISPR/Cas system correlates with loss of a specific gene encoding a sialyltransferase, suggesting that the Cas9 system may be a regulator of this specific gene, and potentially providing another example of coincident evolution between a CRISPR/Cas system and a regulatory target (23). Furthermore, loss of *cas2* in *Legionella pneumophila* results in an inability to replicate within amoeba (24). Loss of the Type-I *cas* genes in *Pseudomonas aeruginosa* results in dysfunctional biofilm formation (an important virulence trait), suggestive of a broader CRISPR/Cas function in regulation beyond Cas9 and the Type II systems (25). The data presented here suggest that in those bacteria in which Cas9 or other CRISPR/Cas components play a role in gene regulation or other alternative functions, loss of CRISPR/Cas functionality would not only facilitate HGT, but would have a disruptive effect on gene regulation. Since these regulatory changes might negatively impact bacterial fitness, compensatory changes may be required to prevent this loss of fitness (as we describe here for *FTN_1103* deletion in highly virulent *Francisella* species). Thus, we propose that coincident loss of regulatory targets or other compensatory genomic changes may be a common and necessary occurrence in the face of CRISPR/Cas loss in diverse bacteria.

Chapter 5 Acknowledgements

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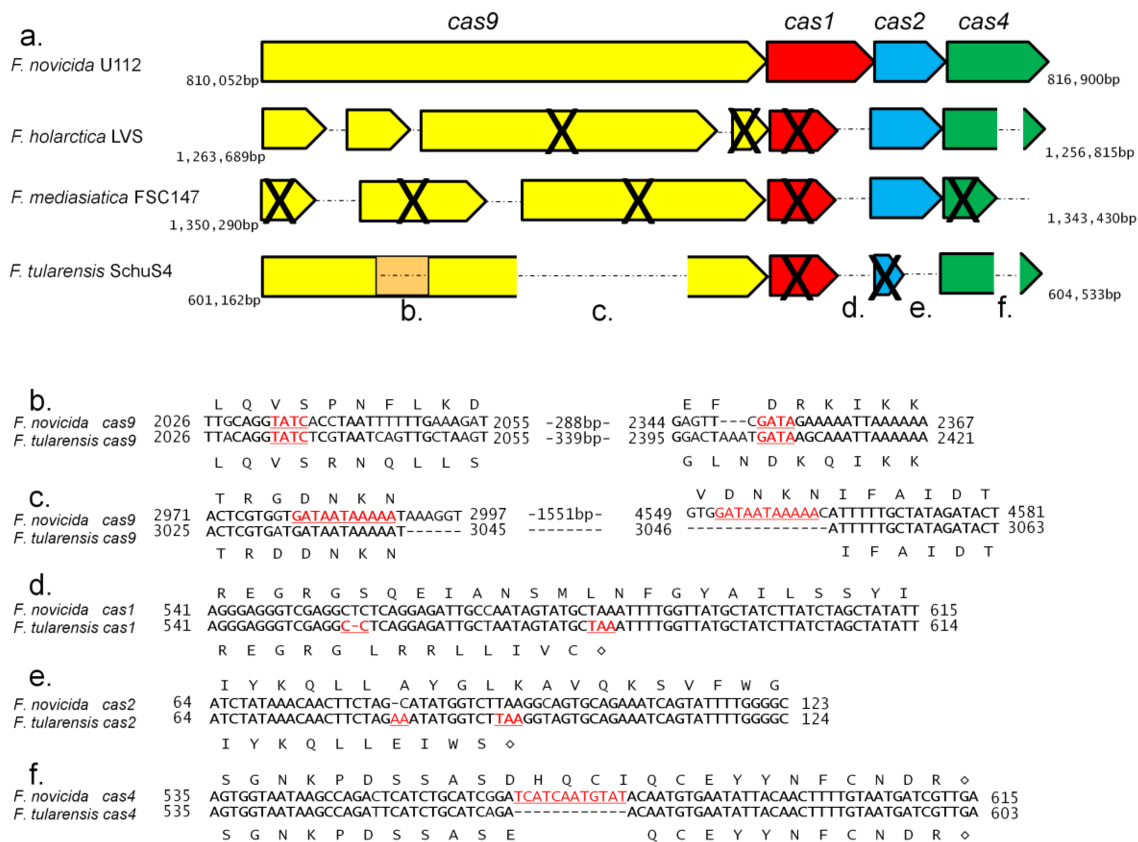

Figure 1. Analysis of the Type II CRISPR/Cas locus of *Francisella* species. (**A**) Operon alignment of the complete set of *cas* genes shared between four species, with the regions of significant difference between *F. novicida* and *F. tularensis* (insertions, deletions) indicated by letters. Bold X's indicate predicted pseudogenes or gene fragments. The brown box depicted within *cas9* of *F. tularensis* SchuS4 represents a region of significant dissimilarity with *cas9* of *F. novicida* U112. Operons are adapted from NCBI, *F. novicida* U112 (Accession #: NC_008601), *F. holarctica* LVS (NC_007880), *F. mediasiatica* FSC147 (NC_010677), and *F. tularensis* SchuS4 (NC_006570). (**B**) Highly dissimilar nucleotide sequence between *cas9* genes of *F. novicida* and *F. tularensis*. Four bp inverted repeats flanking the region highlighted in red (TATC/GATA). (**C**) Region of predicted intramolecular recombination, leading to excision within *cas9* of *F. tularensis*. GATAATAAAAA direct repeats highlighted by red bars. (**D**) *cas1*

nucleotide alignment demonstrating the -1 frameshift within *F. tularensis* (C-C, red) leading to an early stop codon (TAA, red, diamond). (**E**) *cas2* nucleotide alignment, demonstrating the +1 frameshift within *F. tularensis* (highlighted in red) leading to an early stop codon (TAA, red, diamond). (**F**) *cas4* nucleotide alignment showing in-frame loss of 12 nucleotides of the *F. novicida* sequence (red) in *F. tularensis*. For all alignments, bold text indicates identical nucleotides within the alignment. Amino acid sequences are above and below for *F. novicida* and *F. tularensis,* respectively, and diamonds indicate stop codons.

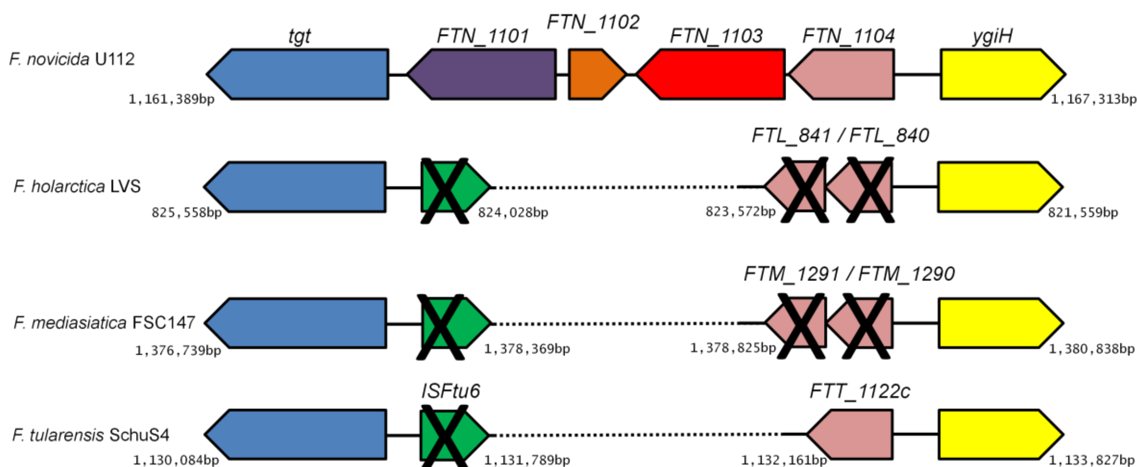

Figure 2. Operon alignment of the *FTN_1103* region between *F. novicida* and more virulent species. Operon alignments, with each color corresponding to orthologous genes between species, black X's representing predicted pseudogenes or gene fragments, and dashed lines indicating nucleotide deletions. ISFtu6 (green) represents a transposon insertion.

Chapter 6. Discussion of CRISPR-Cas –mediated gene regulation

Part 1. Summary and Predicted Mechanism of RNA Targeting

Through this project, we have demonstrated that specific components of the Type II CRISPR-Cas system in the Gram-negative intracellular pathogen *Francisella novicida* (one of two CRISPR-Cas systems present in this species (1)) regulate the expression of an endogenous transcript encoding a bacterial lipoprotein (BLP) (FTN_1103)(2, 3). Cas9, together with tracrRNA as well as a small RNA currently unique to the *F. novicida* system, termed scaRNA (small, CRISPR-Cas-associated RNA), form a dual RNA:protein complex capable of targeting the BLP transcript (Figure 1)(2). Interestingly, in contrast to its accessory role in canonical DNA targeting by Cas9, tracrRNA displays significant sequence complementarity to the BLP mRNA, and is thought to function in a targeting role (Figure 1)(2).

We predict that the scaRNA is capable of hybridizing to the tracrRNA at a sequence nearly identical to the CRISPR repeat, forming a dsRNA structure that may interact with Cas9 in a similar fashion as the crRNA:tracrRNA complex within the canonical DNA-targeting CRISPR-Cas system (Figure 1)(2, 4, 5). While the exact function of the scaRNA is unknown, the current hypothesis is that it may serve to stabilize the tracrRNA in such a way that tracrRNA can subsequently interact with the BLP transcript. This could be through direct changes in the structure of the RNA after hybridization, or by altering the way in which the RNAs interact with Cas9. Further structural and

stoichiometric studies will help to elucidate how these RNAs interact with Cas9 to target the BLP mRNA.

Targeting by the Cas9:tracrRNA:scaRNA machinery results in drastically lowered levels of BLP mRNA, through a process that alters the stability of the transcript (2). Exactly how the stability of the BLP mRNA is altered is unknown. Surprisingly, this regulation does not require the amino acid residues essential to the endonuclease activity of Cas9 proteins (2, 6, 7). This may suggest that Cas9 has redundant endonuclease motifs, each capable of acting on the targeted BLP transcript. Alternatively, an accessory RNase may be involved (Figure 1A). While a number of different RNases were tested for their function in regulation of this BLP, no single RNase mutant had an apparent regulatory defect (2). One specific RNase, RNase E, could not be analyzed using this approach since it is an essential gene (8). Other studies have shown that RNase E can be involved in the modulation of mRNA stability, and it is therefore possible that it may also act as an accessory for CRISPR-Cas-mediated regulation (8).

The predicted requirements for targeting the Cas9:tracrRNA:scaRNA system to the BLP transcript also differ considerably with those for targeting of foreign DNA by the canonical CRISPR-Cas system. Targeting of the Cas9 endonuclease to foreign DNA requires a crRNA with near 100% sequence identity to the target (9). Surprisingly, crRNAs are not required for targeting of BLP mRNA (2), and there does not appear to be a spacer sequence within the crRNA array that has sequence complementarity to this transcript, making this regulatory process distinct from the canonical DNA targeting

action of Cas9. Instead, the BLP mRNA is targeted by the tracrRNA, which only displays partial sequence complementarity to the transcript. This may suggest that only small stretches of sequence complementarity, or seed sequences, are important for initiating and establishing interaction of the tracrRNA and BLP mRNA, as has been demonstrated for nucleic acid targeting by other CRISPR-Cas systems (10, 11).

An interesting question is why BLP mRNA is apparently targeted, yet the BLP gene encoded in the chromosome is not targeted for disruption. It has been observed that targeting of the bacterial chromosome results in loss of either that chromosomal sequence or the CRISPR-Cas system itself (12-14). Since this is not the case in *F. novicida*, it strongly suggests that the DNA is not targeted for cleavage). The imperfect complementarity between the tracrRNA and the BLP mRNA would not be predicted to mediate targeting of the chromosomal DNA, based on canonical CRISPR-Cas targeting. Therefore, this lack of 100% complementarity could be an important safeguard to effectively prevent DNA targeting while nonetheless promoting targeting of mRNA. Understanding the structural and sequence requirements of these interactions will be important for elucidating how prokaryotes control the different activities of CRISPR-Cas systems.

While the data discussed support a model whereby *F. novicida* Cas9 post-transcriptionally modulates the stability of the BLP transcript, other potential models exist as well. It is theoretically possible that *F. novicida* Cas9 binds DNA but does not cleave it, and thereby physically blocks transcription. There is precedence for this, at least

in synthetically mutated Cas9 proteins (dCas9), which lack endonuclease activity, but retain the ability to bind DNA (15-20). Upon being guided to a target gene or its promoter, dCas9 can effectively prevent transcription by blocking access of RNA polymerase (RNAP) to the promoter and/or preventing RNAP elongation. This system has been successfully utilized as a tool in order repress target genes in numerous biological systems (15-20). A lack of *F. novicida* Cas9 endonuclease activity would be consistent with the observation that none of the conserved residues within any of the predicted endonuclease motifs of the protein were necessary for repression of the BLP transcript (2). However, recent data demonstrates that, in fact, *F. novicida* Cas9 is fully capable of targeting and cleaving DNA substrates, at least *in vitro* (7). The finding that *F. novicida* Cas9 can cleave DNA, yet the BLP gene is nonetheless present in the genome and the protein is produced (2, 3, 7, 21), supports the hypothesis that the DNA is not targeted while BLP mRNA is. Furthermore, this alternative transcription inhibition model would not explain the observed changes in the BLP transcript's stability after treatment of cells with rifampin which blocks transcription, nor the presence of the BLP transcript in association with Cas9 (2).

Assuming that *F. novicida* Cas9 does target RNA, it is possible that this is not a feature common to all Cas9 proteins. While similar to other Cas9 proteins, *F. novicida* Cas9 does have regions of significant sequence dissimilarity which may alter its function in targeting DNA or RNA (2, 5, 7, 9). There is precedence for such a scenario in Type III CRISPR-Cas systems. The Type III-A and III-B systems have slight differences in the structures of their targeting complexes (Cascade) which may account for their differential

ability to target DNA substrates (III-A) or RNA substrates (III-B)(22-25). Continued dissection of the molecular mechanism of Cas9 function will provide answers to these and other critical remaining questions, in particular focusing on structure and function comparisons between Cas9 variants (7, 26, 27).

Part 2. Role of CRISPR-Cas-mediated gene regulation in pathogenesis

The bacterial pathogen *Francisella novicida*, a relatively rare cause of disease in humans, evades detection by the host innate immune system and replicates within host cells (28). *F. novicida* has numerous mechanisms by which to subvert the function of host macrophages as well as other cells. Once taken up by macrophages, this pathogen enters the phagosome, a compartment containing numerous antimicrobials as well as innate immune recognition receptors (28). One such receptor is Toll-like Receptor 2 (TLR2), which detects BLPs (29, 30). Activation of TLR2 results in a pro-inflammatory response which recruits and activates immune cells, and acts to combat and clear the bacterial pathogen.

Utilizing Cas9, tracrRNA, and scaRNA as regulators, *F. novicida* represses the expression of the targeted BLP, significantly lowering overall BLP levels in its envelope by roughly 2-fold (Figure 2)(2, 3). This allows the pathogen to effectively dampen TLR2 activation, facilitating its survival within the host. In the absence of this CRISPR-Cas-mediated regulation, *F. novicida* elicits a significant TLR2-dependent inflammatory response (Figure 2), as revealed by the fact that *cas9*, tracrRNA, and scaRNA deletion mutants induce a much greater inflammatory response than wild-type bacteria (2). Not

only is this inflammatory response dependent on TLR2, but it is also dependent on the over-expression of BLP, as strains lacking either the regulatory components or the BLP elicit a response that is limited to near wild-type levels (2, 3). Furthermore, deletion mutants lacking these CRISPR-Cas components are highly attenuated (over 1,000 fold) (2). In addition, *cas9*, tracrRNA, and scaRNA deletion mutants are unable to induce a lethal infection of mice, further emphasizing their importance as regulators of virulence in *F. novicida* (2).

While the *F. novicida* CRISPR-Cas system is currently the only known example of a Cas9 system acting naturally in a regulatory capacity, there have been observations of other species utilizing Cas9 as a virulence factor. In a human lung epithelial cell model, Cas9 is essential for attachment of *Neisseria meningitidis* to the host cell surface, as well as both invasion and intracellular replication (2). Additionally, Cas9 is essential for attachment and invasion of *Campylobacter jejuni* in a colorectal epithelial cell model (31). The precise mechanism by which Cas9 functions as a virulence factor in these organisms is not yet known. However, based on the established role of Cas9 as a regulator of gene expression in *F. novicida,* it is likely that Cas9 acts in combination with tracrRNA or an alternative, unidentified small RNA, to regulate the levels of specific transcripts, ultimately leading to the control of virulence properties.

Additionally, the role of Cas9 as a *Campylobacter* virulence factor correlated with specific strains encoding the Cst-II sialyltransferase, and which produce a sialylated lipooligosaccharide (31). It is interesting to hypothesize that CRISPR-Cas-mediated

regulation may act not only to allow *C. jejuni* to efficiently attach to host cells, but also to mask its surface from detection by host receptors, and prevent activation of host defenses, such as the complement system. Since the known regulatory target of Cas9 in *Francisella* is a membrane BLP, and these additional examples of a contribution of Cas9 to virulence traits involve attachment of the bacterial cell to the host cell surface, it is interesting to speculate that CRISPR-Cas systems may generally act as regulators of envelope composition and structure.

Part 3. Role of CRISPR-Cas systems in the response to envelope stress

The regulation of the bacterial envelope is especially important during times of membrane stress, in order to resist and combat this stress, and to promote bacterial survival. Interestingly, it has been observed that expression of CRISPR-Cas components in several bacterial species can be induced following envelope stress. For instance, in *E. coli* when a membrane-targeted GFP is overexpressed, the downstream envelope stress response triggers the upregulation of CRISPR-Cas system expression (32). Additionally, CRISPR-Cas systems in other bacterial and archaeal species, including *Streptococcus thermophilus* and *Sulfolobus islandicus*, have been shown to be induced in the presence of bacteriophage (33, 34), suggesting that the envelope stress which occurs during attachment and entry of bacteriophage may be a signal to activate CRISPR-Cas systems. Envelope stress may further serve as a signal of increased cell permeability, a condition that would likely increase the chance of foreign nucleic acid uptake. Thus, induction of CRISPR-Cas systems at times of envelope stress might act to prepare the cell for incoming foreign nucleic acid and prevent acquisition of harmful genetic elements. In

addition, since CRISPR-Cas systems have been shown to regulate envelope components, and these systems are induced in response to membrane stress, it is tempting to speculate that the regulatory roles of these machineries may also serve to combat this stress. For example, the regulation of membrane BLP composition, as observed in *F. novicida* (2, 3), in addition to promoting evasion of the host innate immune response, may act to alter or enhance the integrity of the bacterial envelope.

Part 4. Role of other CRISPR-Cas components and systems in bacterial physiology

In addition to Cas9's role as a virulence factor in the bacterial pathogens *F. novicida, N. meningitidis,* and *C. jejuni,* CRISPR-Cas systems in other bacteria have been identified as having potential roles in virulence as well. Cas2, present within a Type II CRISPR-Cas system, is important for the ability of *Legionella pneumophila* to replicate within amoebae (35). Since amoebae are thought to be important for *L. pneumophila* survival in the environment (36-38), the role of Cas2 in intracellular amoebic survival may play a role in its survival in the environment. In addition, in strains encoding the Type II system, it may even promote subsequent transmission to human hosts (35). Exactly how Cas2 functions to mediate *Legionella* intracellular survival in amoebae is unknown. It is hypothesized to have an alternative function in conjunction with currently unidentified small RNAs, either in their processing or in the alteration of mRNA stability (35). Interestingly, Cas9 has no observed role in *L. pneumophila* survival in amoebae (35). Conversely, Cas2 has no observed role in the ability of *F. novicida* to modulate BLP expression, nor intracellular survival or virulence (2), demonstrating that while Type II CRISPR-Cas systems have similar genetic architectures, different species may have co-

opted alternative components for functions distinct from defense against foreign nucleic acids.

Type I CRISPR-Cas systems have also been implicated in aspects of bacterial physiology beyond their now canonical function in foreign nucleic acid defense. The Type I CRISPR-Cas system in *Pseudomonas aeruginosa* has been shown to play a role in modulating the production of biofilms (39, 40). While the exact regulatory mechanism has not been elucidated, the data suggest that the CRISPR-Cas system interacts with a specific gene within a chromosomally integrated prophage, inhibiting biofilm formation (39, 40). It is unclear if the CRISPR-Cas system targets the chromosomal DNA or the prophage transcript, but it is known that this regulation requires the Cas proteins involved in crRNA maturation, as well as those involved in targeting/degradation. Further, this regulatory activity depends on a specific crRNA with sequence identity to the prophage gene (39, 40). Interestingly, this crRNA does not exhibit 100% complementarity to its regulatory target. Similar to the *F. novicida* targeting of RNA, this is a non-identity interaction, perhaps providing a reason for why chromosomal targeting by the *Pseudomonas* CRISPR-Cas system would not result in a lethal event. Given that biofilm formation is a critical aspect of the pathogenic life cycle of *P. aeruginosa* (41), it is likely that this example of CRISPR-Cas-mediated regulation plays a vital role in infection.

Another Type I CRISPR-Cas system with regulatory attributes is found in the soil bacterium *Myxococcus xanthus*. Three genes, *devT, devR,* and *devS*, corresponding to *cas8, cas7,* and *cas5* respectively, have been shown to be necessary for sporulation and

fruiting body development (42-44). Specifically, it was observed that *devT* (*cas8*) mutants had significant delays in aggregation, sporulation, and chemotaxis. This correlated with low levels of transcript for a necessary activator of fruiting body formation (42). It is not known if the crRNA array is necessary for fruiting body formation, however it does encode two spacers with complementarity to chromosomal loci, one of which could hybridize to an integrase of a *Myxococcus* bacteriophage and the other that could hybridize to a *cas* gene in an exogenous CRISPR-Cas locus (44). How these CRISPR-Cas components interact to ultimately perform this regulatory function remains to be elucidated.

Additionally, the Archaeal Type I system encoded by *Pelobacter* sp. has been demonstrated to play a role in the regulation of gene expression as well (45). These species contain a spacer within the crRNA array with sequence identity to the gene encoding a histidyl-tRNA (45). Upon expression of this self-targeting spacer within a species related to *Pelobacter* encoding a similar CRISPR-Cas system (but lacking the self-targeting spacer), it was observed that histidyl-tRNA transcript levels were reduced, and that the bacteria exhibited a growth defect (as expected if protein synthesis is slowed by lower levels of a critical tRNA) (45). The precise mechanism and how the *cas* genes are involved in this process is yet unknown. In addition to these examples of self-targeting spacers involved in gene regulation through unknown mechanisms, there are numerous examples of self-targeting crRNA spacers (12). However, it is unclear if they are indeed involved in regulation. For example, *Aggregatibacter actinomycetemcomitans* encodes a spacer putatively targeting the important metabolic enzyme *glgP* (46). While

this crRNA is transcribed and processed, it is not known if it acts as a regulator of *glgP* production (46). Future study of such spacers will likely reveal a plethora of regulatory functions for CRISPR-Cas systems in diverse bacteria.

There also exist examples in which crRNAs may have regulatory roles, even in the absence of Cas proteins. *Listeria monocytogenes* encodes an isolated crRNA locus, consisting of five identical repeats, and four unique spacer sequences (47, 48). This locus, termed rliB, is not adjacent to any known *cas* genes, and is present even within *L. monocytogenes* strains that are devoid of any *cas* genes (47, 48). rliB is processed by the bifunctional polynucleotide phosphorylase (PNPase), which has exoribonuclease activity (48). The rliB crRNA has significant sequence complementarity to the transcripts for a two component system, a transcriptional regulator, and the *feoAB* iron transport system (47). In fact, rliB is capable of hybridizing to and repressing production of these transcripts (47). Since *feoAB* is an important virulence factor in numerous organisms, it is likely that rliB plays an important role in the virulence of *L. monocytogenes*. Interestingly, this orphaned system is still capable of acting canonically against plasmid transformation, provided there are *cas* genes produced in an exogenous locus. However it is unknown if this occurs through targeting of DNA or RNA substrates (48).

While the aforementioned examples of alternative CRISPR-Cas function focus on regulatory roles, there may be more indirect mechanisms by which CRISPR-Cas systems contribute to virulence. For example, it has been observed that both Cas1 and the crRNA array in the Type I CRISPR-Cas system of *E. coli* play a role in DNA repair (49). Given

that Cas1 is present in all known CRISPR-Cas systems, it is interesting to think that this gene may have a broad function in DNA repair in other species as well. Furthermore, bacterial DNA damage is thought to occur due to the action of specific host defenses during infection, in particular the production of radical nitrogen and oxygen species (50). It is therefore interesting to consider that Cas1 may be able to provide bacterial pathogens some redundancy in their capability to repair DNA damage incurred during infection.

Part 5. Conclusions

While now very well established to play roles in bacteriophage and foreign genetic element defense, the alternative functions that CRISPR-Cas systems play in the ability of bacterial pathogens to evade and dampen host defenses, and ultimately survive and replicate within the host, have only recently begun to be appreciated. Furthermore, with the continued observations that some CRISPR-Cas systems can target RNA substrates (24, 51, 52), this raises the strong possibility that regulation of endogenous genes by CRISPR-Cas systems can occur without the negative consequences of targeting the bacterial chromosome (12-14). Given that CRISPR-Cas systems are encoded in the genomes of numerous prokaryotes (including ~45% of bacteria and ~83% of Archaea) (CRISPRdb, 23 Jan 2014) (53), it is likely that numerous examples of alternative functions in gene regulation, virulence and physiology will be uncovered in the future.

Chapter 6 References

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Chapter 6 Figures

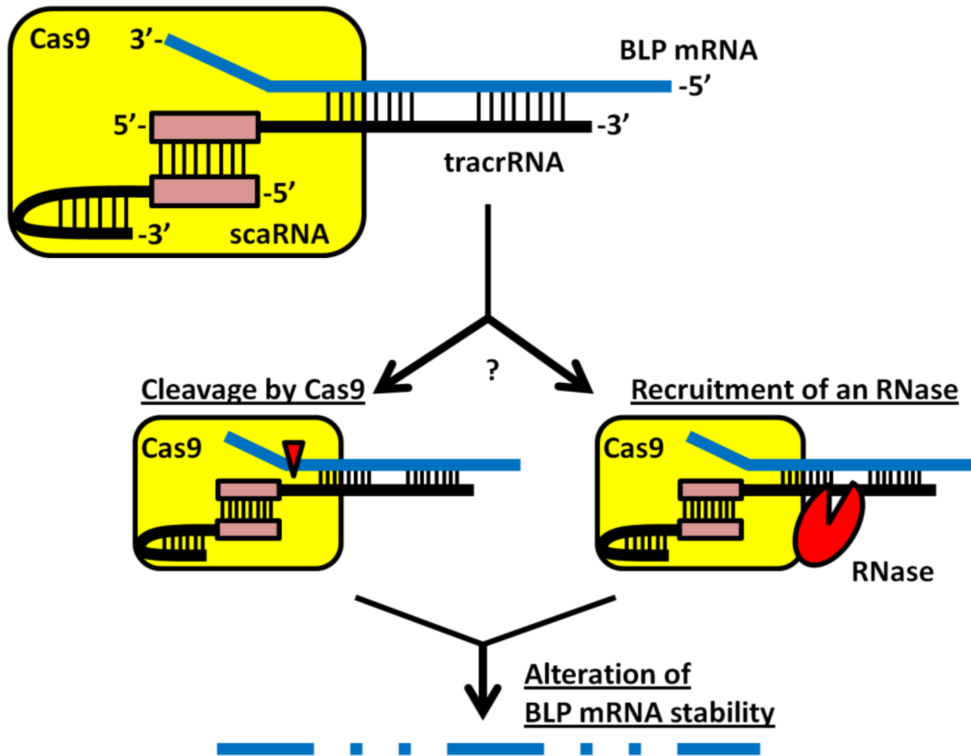

Figure 1. CRISPR-Cas-mediated gene regulation by *F. novicida*. A dual-RNA complex consisting of the tracrRNA and scaRNA forms through interaction of a sequence identical to the CRISPR repeat (pink box). This dsRNA structure is associated with *F. novicida* Cas9 and allows the free portion of the tracrRNA to interact through a non-identity interaction with mRNA encoding the BLP (*FTN_1103*; blue). Subsequently, the stability of the BLP mRNA is altered, possibly via catalytic activity of Cas9 (cleavage event indicated by red triangle) or by an unknown RNase (red sector).

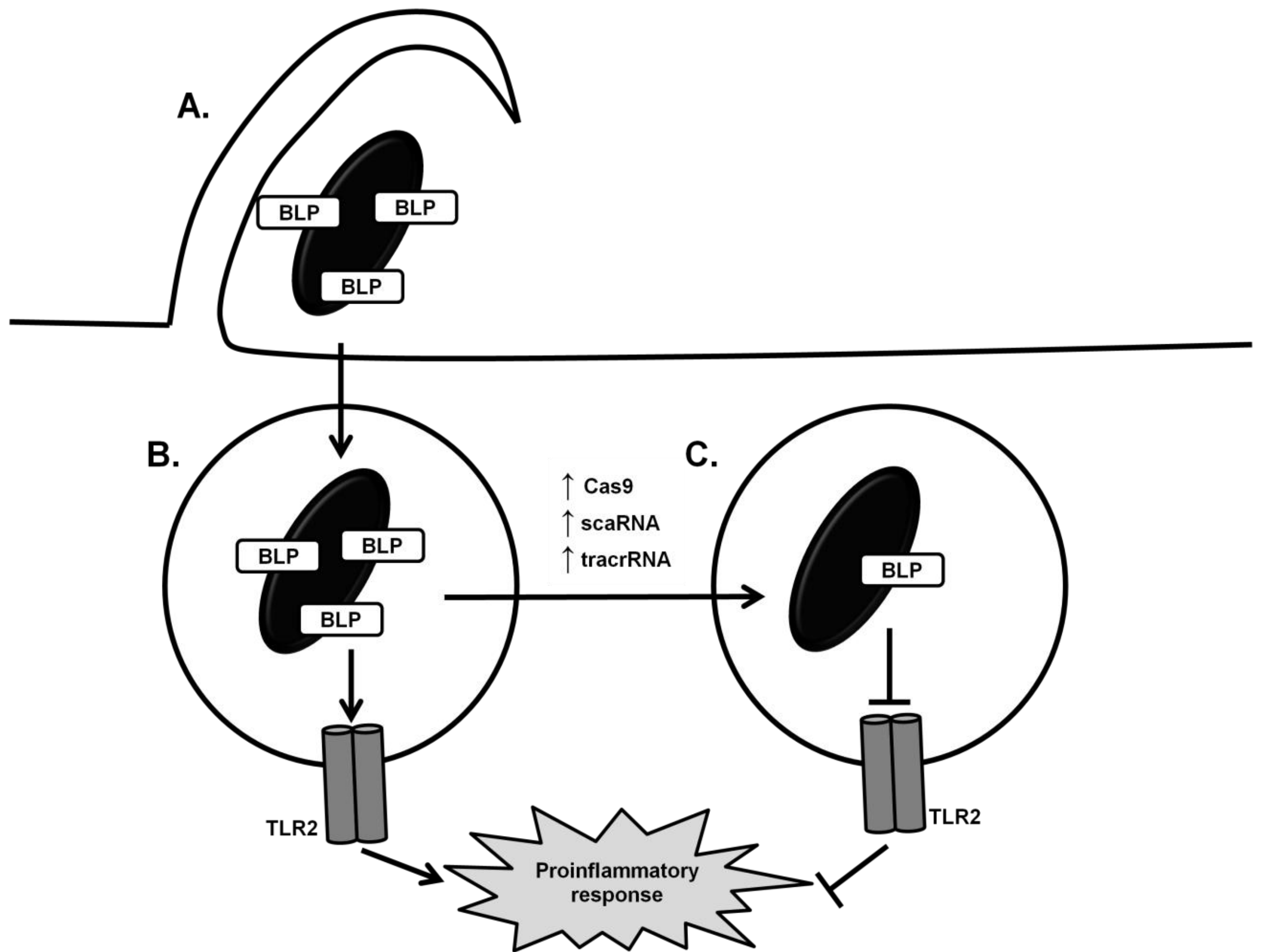

Figure 2. The role of the Cas9 regulatory system in *F. novicida* pathogenesis. (**A**) *F. novicida* is phagocytosed by a macrophage. (**B**) In the phagosome, *F. novicida* BLP can activate the host sensor protein TLR2 which signals for a pro-inflammatory response. (**C**) Cas9, scaRNA and tracrRNA are induced when the bacterium is in the phagosome, 30 min–1 h after infection, resulting in down-regulation of BLP and dampening of signalling through TLR2.

Chapter 7. Cas9 as a platform for genome engineering and implications of Cas9-mediated RNA targeting.

Part 1. Role for Cas9 in Genome engineering

Due to its specificity, coupled with the short sequence requirements for Cas9:crRNA targeting, it was hypothesized that Cas9 could be programmed to target and cleave any DNA sequence of interest within the bacterial cell. In fact, work by Jinek et al clearly demonstrated that introduction of a synthetic crRNA sequence capable of hybridizing to the DNA of a gene of interest, allows Cas9 to cleave that region (1). Targeting and subsequent cleavage requires a short (3 to 9 bp) sequence motif directly adjacent to the hybridized region, termed the Proto-spacer Adjacent Motif, or PAM, which is a necessary prerequisite for the Cas9:dual RNA complex to recognize the target sequence and prevent self-targeting.

Perhaps most importantly, the requirement for tracrRNA-mediated maturation could be abrogated when a synthetic double-stranded targeting RNA (a guide RNA or gRNA) was engineered (Figure 1A) (1). This specific RNA combines the targeting features of the crRNA with the dsRNA structure formed by the tracrRNA:crRNA complex, allowing this single RNA to fulfill the requirements of both small RNAs (sRNA) (1). This development increases the portability of the Cas9 system between both prokaryotic and eukaryotic models, by simplifying the components needed to initiate cleavage of targets

by Cas9 (e.g. there is no longer a need for an accessory RNA [tracrRNA], nor the need for RNase III to mature the crRNA:tracrRNA complex) (1).

These advances paved the way for multiple groups to port the Cas9 system into a number of different experimental models. To date, Cas9 genome editing has been successfully performed in bacterial cells, yeast, plants, nematodes, fruitflies, zebrafish, rodents, and human cells (both transformed cell lines and embryonic stem cells), solidifying its use as a convenient tool to site-specifically edit the genomes of multiple species (1-9). Editing by Cas9 can occur in one of two ways (Figure 1A). When both strands of the DNA target are cleaved by Cas9, the cell can undergo non-homologous end joining (NHEJ) to repair this double strand break. NHEJ often results in the loss or addition of nucleotides, causing frame-shift mutations or early stop codons and ultimately, the loss of function of the targeted gene (Figure 1A) (8, 10). Alternatively, following the cleavage of DNA, a donor construct containing a selectable (or non-selectable) marker flanked by sequences adjacent to the cleavage site can be introduced. This donor construct acts as a template for homology-directed repair (HDR) and results in the insertion of the marker into the targeted site (Figure 1A) (8, 10).

In order to decrease the frequency of NHEJ and subsequently increase the relative frequency of HDR, a specific Cas9 point mutant was engineered (8, 10). Generation of a single point mutant in the active site of one of the two critical Cas9 endonuclease domains (RuvC-I) renders Cas9 unable to create double strand breaks (1). Rather, the remaining intact endonuclease domain cleaves only one targeted strand, resulting in

nicked DNA (1). In the presence of a donor construct, these nicks are preferentially repaired by HDR. Use of this form of Cas9 significantly increases the frequency of marked mutations (8, 10). Together, these recent developments have increased the ease of generating site specific mutations in many organisms, and enhanced the ability to do so in systems that have been recalcitrant to genetic manipulation, hence opening numerous avenues for genetic research.

Part 2. Engineering Cas9 to Control Transcription

While the aforementioned applications of Cas9 allow manipulation of genomic content, there are cases in which gene disruption is not necessarily possible or desirable. Instead, altering the expression of the gene (or multiple genes) of interest could provide more useful information, without permanently altering the cell's genome. Because the ability of Cas9 to associate with targeted DNA does not depend on its endonuclease activities, an engineered Cas9 mutant that cannot cleave DNA can be employed (11, 12). Termed dCas9, this protein completely lacks catalytic activity against DNA, due to a point mutation within each of its critical endonuclease domains (both RuvC-I and HNH) (1, 11, 12). When guided to the target DNA by a gRNA, dCas9 binds, but does not cleave the target and instead prevents transcription by blocking RNA polymerase binding or elongation (Figure 1B) (11, 12). By guiding dCas9 to different locations along the gene sequence, or by targeting multiple sites within the same gene simultaneously, dCas9 can be used to modulate the level of repression (11, 12). Thus, Cas9 has the capability to not only edit genomes, but also to act as a transcriptional repressor.

The ability of dCas9 to site specifically bind DNA without causing cleavage can be exploited even further. dCas9 can be tethered to a transcriptional activator, such as the omega subunit of RNA polymerase within a bacterial system or VP64 in a mammalian system (12-15). Utilizing a gRNA to program the dCas9-activator fusions to a region upstream of the transcriptional promoter, these systems can successfully recruit RNA polymerase resulting in an increase in transcription of the gene of interest (Figure 1C). Similar to the ability of dCas9 to repress expression, this transcriptional activation can be modulated and tuned to differentially express a specific gene (12-15). The ability to control both activation and repression of genes of interest with Cas9 allows a number of experimental systems to be probed. For instance, bacterial systems currently rely on inducible and repressible promoters, requiring both the introduction of a new promoter to the gene of interest, and subsequently the addition of a small molecule. The dCas9-activation or repression systems would successfully bypass many of these steps and allow experimental questions to be addressed more quickly and in both prokaryotic and eukaryotic systems that currently lack controllable expression systems.

Part 3. Engineering of *Francisella* Cas9 to target viral RNA in eukaryotic cells

Cas9 targeting of DNA has been exploited to allow genome editing and transcriptional repression and activation. While extremely useful, targeting of DNA alone has some potential pitfalls. For instance, in a eukaryotic system, repression of one transcript may result in the loss of multiple splice variants, when targeting of only one is warranted. Alternatively, binding of Cas9 to DNA may be inhibited by DNA structure or chromosomally-bound proteins, preventing successful access to the targeted gene. It

would therefore be a significant enhancement of Cas9 technology if differential targeting of either RNA or DNA targets could occur.

Herein, we demonstrated that the Cas9 endonuclease encoded by the bacterial pathogen *Francisella novicida* is involved in the repression of a specific transcript (16). Our data suggest that *F. novicida* Cas9 (FnCas9) is guided to this mRNA by the tracrRNA and a novel small RNA termed scaRNA (small, CRISPR/Cas-associated RNA). Subsequently, the Cas9:scaRNA:tracrRNA:mRNA interaction results in decreased mRNA stability and low levels of expression of the target (Figure 2A) (16). It is therefore tempting to speculate that FnCas9, and possibly other Cas9s, may facilitate programmable RNA targeting (Figure 2B). Furthermore, the FnCas9 system likely represents a tool that can be harnessed to understand both the structural and sequence requirements that determine how Cas9 can preferentially target RNA or DNA.

We hypothesized that, in fact, the FnCas9 could be reprogrammed to target a distinct RNA in eukaryotic cells. Therefore, in collaboration with Aryn Price and Arash Grakoui (Emory University), we designed a synthetic RNA targeting system utilizing FnCas9. In order to eliminate the possibility of a confounding interaction of FnCas9 with a DNA substrate, we tested whether this protein could be specifically targeted to the genome of the +ssRNA virus, hepatitis C virus (HCV), which has no DNA stage in its lifecycle. To target the RNA genome of HCV, we engineered a small RNA (which we term an RNA-targeting guide RNA; rgRNA) consisting of a dsRNA region (necessary for associating with Cas9) and a ssRNA targeting sequence complementary to a portion of the HCV 5’

untranslated region (UTR)(Figure 3). Vectors encoding either this rgRNA or FnCas9 were transfected into human hepatocellular carcinoma cells (Huh-7.5), which were subsequently infected with HCVcc genotype 2a, a previously described recombinant virus encoding *Renilla* luciferase (17). Expression of both the 5' UTR-targeting rgRNA and FnCas9 together led to a striking reduction in the levels of viral proteins, as measured by quantification of luciferase production (Figure 4). In contrast, expression of either the rgRNA or FnCas9 alone had no significant effect (Figure 4). Similarly, expression of a non-specific rgRNA and FnCas9 did not lead to a reduction in the level of viral proteins (Figure 4), demonstrating the specificity of this system. To show that this inhibitory phenotype was not restricted to targeting a single region of the HCV genome (5' UTR), we generated an rgRNA complementary to a portion of the 3' UTR and again observed a similar decrease in viral protein levels as that seen with the combination of the 5' UTR-targeting rgRNA and FnCas9 (Figure 4). This preliminary data demonstrate the ability of FnCas9 to be utilized in the cytosol of a eukaryotic cell, and to be reprogrammed to specifically inhibit a major human pathogenic RNA virus.

Overall, these data suggest that FnCas9 can be reprogrammed to target a specific RNA in a eukaryotic cell, and that CRISPR-Cas systems could potentially be adapted for use as a eukaryotic antiviral defense. Since the infectious cycles of viruses with both RNA and DNA genomes require an RNA stage (generated during transcription, replication, or both), it is likely that the FnCas9:rgRNA machinery can be programmed to broadly target diverse viruses of interest. Furthermore, some eukaryotic viruses have developed mechanisms to circumvent antiviral defenses that target RNA, such as classical RNA

interference systems(18-20); however, since eukaryotic viruses have not evolved in the presence of Cas9, it is unlikely that they have mechanisms to evade this system. The FnCas9/rgRNA machinery could facilitate the targeting of emerging viruses as soon as their genome sequences are available, without a requirement for knowledge of the virus lifecycle or host receptors. Such a system could also theoretically be used to generate transgenic organisms that are resistant to specific viral infections. More broadly, it is likely that the FnCas9:rgRNA system can be used to target endogenous host cell RNAs, potentially functioning as a programmable RNA silencing machinery that would be useful in numerous biotechnological and pharmaceutical applications. While other CRISPR-Cas systems have successfully demonstrated the ability to target and cleave RNA substrates (21, 22), an RNA-targeting Cas9 system is less complex and may be more flexible in application. Given the vast success of Cas9 as a mediator of genome engineering in a multitude of species including yeast, fruit flies, zebrafish, mice, as well as in human cells (1, 2, 8-15, 23-29), this preliminary data suggest that FnCas9 could be used broadly in a similar range of species and systems, representing a new paradigm in Cas9-mediated genetic engineering. Overall, this work demonstrates the potential for a portable, inter-domain, antiviral defense machinery, which is likely just one of myriad potential RNA-targeting biotechnological and medical applications of Cas9.

Thesis Conclusions

In conclusion, this thesis work began with the results of an unbiased screen for virulence factors of the intracellular bacterial pathogen, *Francisella novicida*. In understanding the contribution of one particular virulence factor (*FTN_0757*) we found a previously

unappreciated form of innate immune evasion, whereby the bacterial pathogens actively limit the amount of a ligand that is capable of stimulating the host innate immune system. In seeking to understand the molecular mechanism behind this regulation, we uncovered an unexpected form of prokaryotic gene regulation. This regulation utilized the activity of CRISPR-Cas systems, which had previously not been thought to act in this fashion. Our work has laid the foundation for understanding such alternative regulatory activity of CRISPR-Cas systems in numerous other bacterial species. Finally, the study of this CRISPR-Cas mediated regulation has lead to the beginnings of development for a new form of RNA interference. By understanding how CRISPR-Cas systems are working as regulators in *Francisella novicida* future studies will shape the engineering of a plethora of new biotechnological applications.

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Chapter 7 Figures

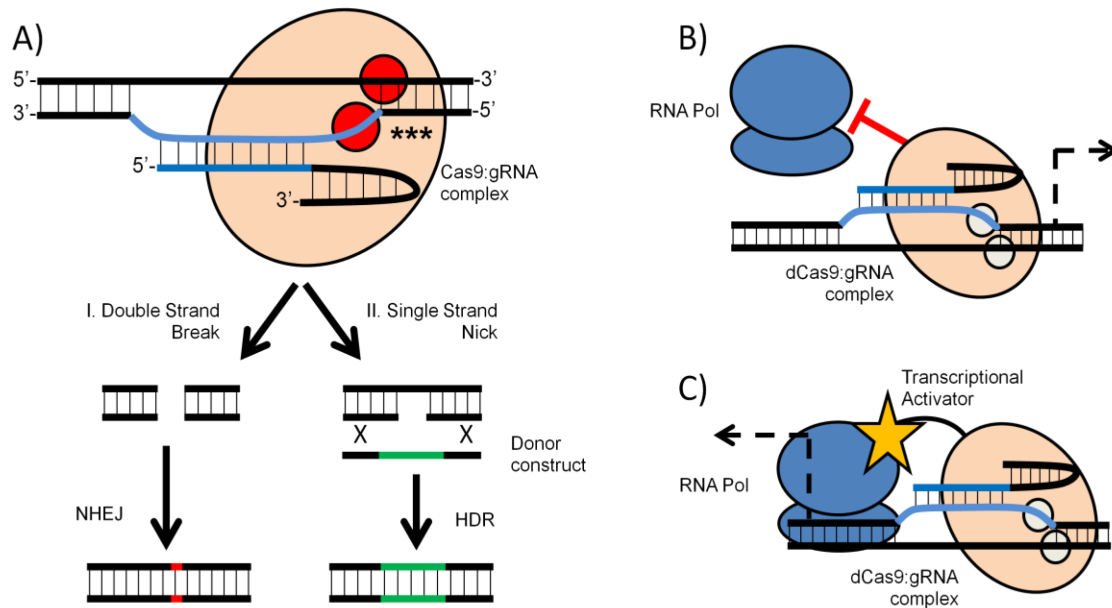

Figure 1. Diagram of Current Cas9 Genome Technologies. **(A)** Interaction of the Cas9:gRNA complex with its dsDNA target. Targeting gRNA sequence shown in dark blue, with the targeted sequence shown in light blue. Cas9 endonuclease motifs (shown as red circles) cleave either strand adjacent to the proto-spacer adjacent motif (PAM, stars). The subsequent repair by (I) non-homologous end joining (NHEJ) when both strands are cleaved can result in mutations (red). Alternatively, (II) homology directed repair (HDR) can occur when a single target strand is cleaved and a repair donor is present (green). (**B)** Cas9 with non-functional endonuclease motifs (grey circles, dCas9) can act as a transcriptional repressor by targeting a promoter region (light blue, dashed arrow) and preventing RNA polymerase association. (**C)** dCas9 fused to a transcriptional activator (yellow star) can act as a transcriptional activator by associating upstream of a promoter (light blue, dashed arrow) and recruiting RNA polymerase.

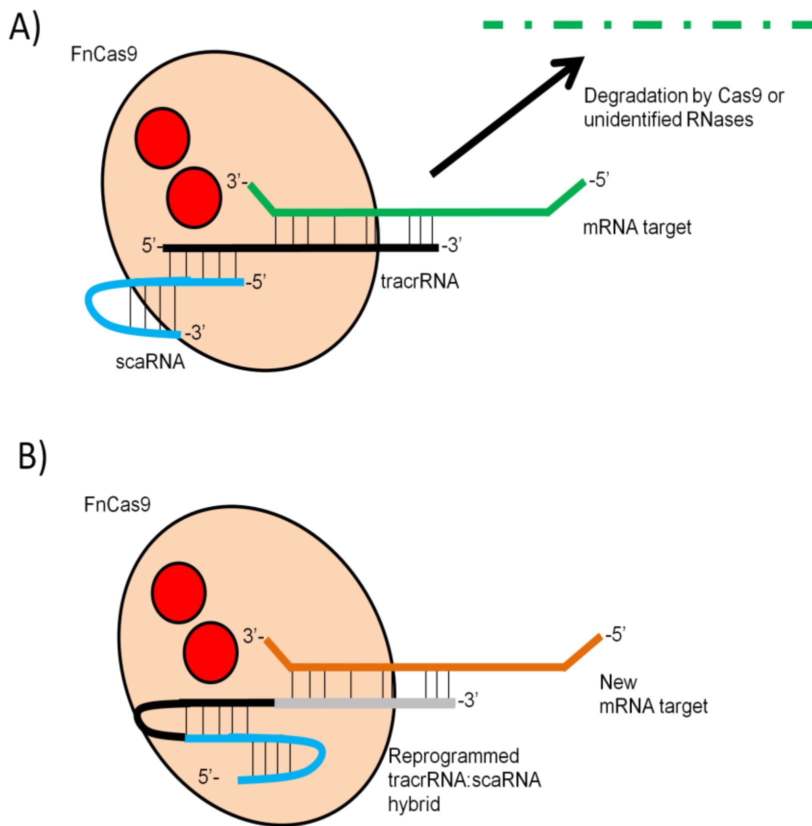

Figure 2. Schematic of FnCas9 Interaction with an RNA target. A) FnCas9 associates with a dsRNA complex formed by two small RNAs, tracrRNA (black) and the scaRNA (blue). Together, this allows tracrRNA to target an mRNA transcript (green). Subsequently, the mRNA target's stability is reduced and the transcript lost. This occurs by either currently unidentified FnCas9 activity or by the action of endogenous RNases. **B)** Schematic representative of a hypothetical tracrRNA:scaRNA hybrid which has been reprogrammed (grey) to target a new mRNA (orange).

HCV 5' UTR rgRNA

```
     HCV RNA
  269nt 5'-UGCGAAAGGCCUUGUGGUACUGCCUGAUAGGGCGCUUGC-3'   307nt
                          ||||||||||||||||||||
A-GUACCAAAUAAU-UAAUGCUCGAACACCAUGACGGACUAUG-5'
A   ||||||||||||| |||:: :
 A UAUGGUUUAUUAGAUUGUUGA
  G                    A
                        GGCUAGUCCGUUAUCAACUUG-3'      rgRNA
```

HCV 3' UTR rgRNA

```
     HCV RNA
  9576nt 5'-UUCUUGGUGGCUCCAUCUUAGCCCUAGUCACGGCUAGCUG-3'   9615nt
                          ||||||||||||||||||||
A-GUACCAAAUAAU-UAAUGCUCAGGTAGAATCGGGATCAGTG-5'
A   ||||||||||||| |||:: :
 A UAUGGUUUAUUAGAUUGUUGA
  G                    A
                        GGCUAGUCCGUUAUCAACUUG-3'      rgRNA
```

Figure 3. Design of chimeric targeting RNAs for action with FnCas9. Schematic of synthetic RNA-targeting guide RNAs (rgRNA) with targeting sequences (grey highlight) to portions of the 5'UTR or the 3'UTR of HCV genomic RNA, with predicted proto-spacer adjacent motif (PAM) in bold and underlined.

Figure 4. FnCas9 can be reprogrammed to inhibit viral protein production in eukaryotic cells. Huh-7.5 cells were transfected with the indicated combinations of FnCas9 and rgRNA and subsequently infected with hepatitis C virus (HCV). At 72 hours, cells were lysed and viral luciferase production quantified. Displayed as percent inhibition compared to the vector control. (n=3, bars the SEM, data is representative of at least 5 experiments) [Contributed by Aryn Price and Arash Grakoui]