

INVESTIGATING THE STRUCTURE AND FUNCTION OF
A NOVEL ANTI-VIRULENCE FACTOR

BY

JAMIE WANDZILAK

UNIVERSITY OF RHODE ISLAND

2020

MASTER OF SCIENCE IN CELL AND MOLECULAR BIOLOGY THESIS

OF

JAMIE WANDZILAK

APPROVED:

Thesis Committee:

Major Professor Kathryn Ramsey

Jodi Camberg

Alison Roberts

Nasser H. Zawia
DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND
2020

ABSTRACT

Francisella tularensis is a Gram-negative, facultative intracellular bacterial pathogen and the causative agent of the potentially fatal human disease tularemia (Sjöstedt, 2007). Like many pathogens, *F. tularensis* contains a number of proteins encoded by a variety of genes that are critical to its ability to cause disease including virulence factors and anti-virulence factors. *F. tularensis* encodes an anti-virulence factor called PriM that inhibits replication inside macrophage, one of its key host cell types. The goal of this study was to elucidate the molecular mechanism for how PriM functions as anti-virulence factor in *F. tularensis* subspecies *holarctica* live vaccine strain (LVS). Using information about the structure of PriM, we created strains producing mutant PriM proteins to test the importance of specific structural features to PriM's function as an anti-virulence factor. We also took a genetic approach, initiating study of mutant *F. tularensis* cells that re-gained the ability to grow in macrophage despite a genetic background that should permit PriM production and thereby prevent intramacrophage growth. We found that a putative binding pocket in PriM may be key to its ability to prevent intramacrophage growth. We also identified a gene previously unlinked to *F. tularensis* virulence whose modification promotes intramacrophage replication and determined that PriM regulation is more complex than previously appreciated. Continuing our work to determine how PriM functions as an anti-virulence factor may allow us to identify bacterially-encoded anti-virulence pathways to exploit in the development of future anti-microbial therapeutics for *F. tularensis* and potentially other pathogenic organisms.

ACKNOWLEDGMENTS

I would like to express my gratitude and thanks to my major professor Dr. Kathryn Ramsey. Her advice, support, and encouragement were immensely appreciated and helped shape my graduate career. I am honored to have been her first graduate student at the University of Rhode Island and am sure she will continue to be a wonderful mentor in the coming years.

I would like to thank the members of my committee Dr. Jodi Camberg and Dr. Alison Roberts as well as my defense chair Dr. Marta Gomez-Chiarri for their willingness to participate in the process of my Master's thesis. Their suggestions and interest in my project have been much appreciated. I would also like to thank Dr. Steven Gregory as the graduate director for his advice and support during my graduate career.

Thank you to the current and former lab members who were all a pleasure to work with including: Hannah Trautmann, John Church, Joseph Paquette, Tala Allababidi, Daniel Ruggiero, Carly Sautter, and Daniel Rosario. Thank you all for making me look forward to working in the lab every-day.

I would like to thank Janet Atoyan for her helping with sequencing for my project and help with working in the URI Genomics and Sequencing Center. I would also like to thank the URI INBRE core for allowing me to use their equipment for my experiments.

Thank you to our protein structure collaborators at Duke University, especially Dr. Maria Schumacher for her expertise and advice.

Lastly, I would like to thank the faculty and graduate students in the Cell and Molecular Biology department. Everyone who I got to know was always happy to provide suggestions and help with any problems that arose in the course of my studies.

Thank you for providing a welcoming and supporting community throughout my time at
URI.

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CHAPTER 1

INTRODUCTION & REVIEW OF LITERATURE

Francisella tularensis is a Gram-negative, facultative intracellular bacterial pathogen and the causative agent of the potentially fatal human disease tularemia (Sjöstedt, 2007). *F. tularensis* was first identified as an infectious pathogen in 1911 in a population of ground squirrels in Tulare County, California and was referred to as *Bacterium tularense*, named after the county in which it was discovered (McCoy, 1911). The pathogen was not identified as a human pathogen until 1913 when the bacterium was isolated from an ocular infection of a butcher (Wherry & Lamb, 1914). Dr. Edward Francis led much of the early research into this organism, categorizing some of the clinical manifestations of the disease and giving it the name of tularemia. Later the pathogen was renamed *Francisella tularensis* after Dr. Francis (Sjöstedt, 2007). Due to its highly infectious nature and its ability to be easily aerosolized, *F. tularensis* is considered a potential bioweapon (Dennis *et al.*, 2001; Oyston *et al.*, 2004; Sjöstedt, 2007). The United States, Japan, and the Soviet Union conducted research on the utilization of *F. tularensis* in biological warfare in the 1950's and 1960's (Dennis *et al.*, 2001). In 1999 *F. tularensis* was named as a Category A Select Agent by the Centers for Disease Control and Prevention along with five other pathogens (Rotz *et al.*, 2002).

There are several subspecies of *F. tularensis* which differ in their ability to cause disease, all of which are found primarily in the Northern Hemisphere (Sjöstedt, 2007). *F. tularensis* subspecies *tularensis* is highly infectious (as few as 10 organisms can cause

disease in humans), has the potential to cause lethal disease, and is only found in North America (Keim *et al.*, 2007). *F. tularensis* subspecies *holarctica* also causes disease in humans, but has a higher infectious dose and causes a milder form of the disease. An attenuated form of *F. tularensis* subspecies *holarctica* was created as a vaccine strain in Russia in 1942 (referred to as the live vaccine strain or LVS) by serial passage through mice and is commonly used as a model in laboratory research because it does not cause disease in humans but is still lethal in animal models (Sjöstedt, 2007). *F. tularensis* subspecies *novicida* is closely related to *F. tularensis*, sharing about 97% nucleotide identity, but despite its lethality in mice it is not known to cause disease in immunocompetent humans (Sjöstedt, 2007; Kingry & Petersen, 2014). *F. novicida* has been found in salt and brackish water, which may serve as an environmental niche, as it has not been identified in animals or arthropod vectors (Kingry & Petersen, 2014). Because *F. novicida* shares a high sequence identity with the more virulent *F. tularensis* strains, *F. novicida* is another commonly used model for the study of tularemia (Kingry & Petersen, 2014).

F. tularensis has a wide variety of transmission vectors including small mammals, insects, and can even be water borne. Insect vectors include blood-feeding arthropods such as ticks, flies, mites, and mosquitoes. These arthropod vectors can spread the pathogen to small mammals, particularly rabbits, hares, and multiple rodent species (Keim *et al.*, 2007). Coming into contact with infected animals, such as during hunting or agricultural activities, allows spread of the disease (Maurin & Gyuranecz, 2016). In some cases outbreaks have occurred from contaminated water sources that became infected by animal feces or carcasses (Maurin & Gyuranecz, 2016). In North America, ticks are

recognized as an important vector, although the key vector for transmission varies by geographic region (Keim *et al.*, 2007).

F. tularensis causes tularemia when an infection is established within the host. Tularemia progresses in different ways depending on the mode of transmission but the initial symptoms of the disease typically include fever, headache, chills and fatigue. The ulceroglandular form of the disease is typically caused by infection by arthropod bites, contact with or ingestion of infected animals, and ingestion of contaminated water sources (Sjöstedt, 2007). Patients with the ulceroglandular form may develop an ulcer at the site of infection followed by enlargement of the lymph nodes (Tärnvik & Chu, 2007). The pneumonic form of the disease is caused by inhalation of aerosolized organisms and subsequent respiratory infection. This form of the infection, which is also referred to as the respiratory form, has the potential to be fatal.

The *Francisella* pathogen is often detected using PCR based assays of clinical samples taken from the site of infection (Maurin & Gyuranecz, 2016). Once confirmed, the infection is typically treated using an antibiotic regimen that may include streptomycin, gentamicin, doxycycline, or ciprofloxacin (Dennis *et al.*, 2001). Major antibiotic resistance among *Francisella* species has not been observed, but there are reports of some biovars of subspecies *holarctica* that are resistant to erythromycin and *F. tularensis* is naturally resistant to β -lactam antibiotics (Maurin & Gyuranecz, 2016). Prior to the antibiotic era, anywhere from 5-60% of cases were fatal, depending on the mode of transmission (Tärnvik & Chu, 2007). Fatalities from tularemia have decreased with the use of antibiotic treatments. Currently, the United States has only a few hundred cases per year, resulting in less than 2% fatality (Sjöstedt, 2007).

***F. tularensis* replication in host cells**

As a facultative intracellular pathogen, *F. tularensis* replicates inside host cells. *F. tularensis* can replicate inside a variety of cell types including phagocytes, epithelial cells, and hepatocytes (Ramond *et al.*, 2012). In particular, macrophage are thought to be a key niche for *F. tularensis* replication. Once a bacterial cell is phagocytized by a macrophage, it must escape the maturing phagosome to avoid being destroyed by the host cell. While the exact mechanism for phagosomal escape is unknown, it is clear that a functional Type VI Secretion System is required (Barker *et al.*, 2009; Ledvina *et al.*, 2018). In order to successfully survive and replicate within host cells, *F. tularensis* must adapt to the host environment, prevent recognition by the host immune system, scavenge nutrients it is incapable of synthesizing, and generate nutrients that are unavailable within the host (Ramond *et al.*, 2012).

Virulence factors in *F. tularensis*

The intracellular environment of the host introduces a variety of stressors that *F. tularensis* has evolved to overcome. In order to survive within the host cell, *F. tularensis* must avoid detection by the host immune system and escape the phagosome, while surviving acidic environments, host antimicrobials, and amino acid starvation (Jones *et al.*, 2012). To withstand these stresses and cause disease, pathogens produce virulence factors, which we define as factors necessary for survival, proliferation, and pathogenesis

during infection of a host. There have been many genetic screens performed in different *Francisella* strains using different model systems to identify putative virulence factors (reviewed in Meibom & Charbit, 2010; Alkhuder *et al.*, 2010; Ireland *et al.*, 2019; Ramsey *et al.*, 2020). These screens have identified a number of virulence factors critical necessary for *F. tularensis* to survive and replicate within host cells. Some of the more important classes of virulence factors in *F. tularensis* are discussed below; however, this is not an all-inclusive list.

Cell Surface Structures

Some virulence factors involve modifications of the cell surface of the bacteria, including lipopolysaccharides (LPS) and production of capsule. Capsule is produced to envelope the LPS and outer membrane of the pathogen and can serve to prevent the pathogen from being recognized by the host immune system, specifically inhibiting antibody opsonization and complement-mediated lysis. *F. tularensis* was found to create two types of extracellular compounds as part of its capsule, O-antigen (a polysaccharide) and capsule-like-complexes (a group of glycosylated proteins; CLC) (Freudenberger Catanzaro & Inzana, 2020). Cells lacking these components experienced growth defects and are attenuated for virulence in mice (Freudenberger Catanzaro & Inzana, 2020). Under the capsule, lipopolysaccharides (LPS) makes up the outermost layer of the outer membrane. LPS is highly immunostimulatory and pathogens often modify their LPS to avoid immune detection. LPS in *F. tularensis* has specific structural modifications to avoid recognition by classical LPS recognition molecules (Rowe & Huntley, 2015).

Metabolic Enzymes and Protein Maturation Factors

Once *F. tularensis* enters the host, it must be able to produce or acquire essential nutrients. Thus, *F. tularensis* encodes biosynthesis pathways that are required during host infection. Among the pathways essential during infection is the purine biosynthesis pathway, as purines are a limiting metabolite in the host (Pechous *et al.*, 2008; Ramsey *et al.*, 2020). Cysteine is another limiting nutrient in macrophage during infection. Because *F. tularensis* is a cysteine auxotroph, it must obtain this metabolite from the extracellular environment (Alkhuder *et al.*, 2009). Specifically, *F. tularensis* utilizes a pathway to break down host-derived glutathione in the periplasm and imports it into the cytosol to obtain cysteine (Alkhuder *et al.*, 2009; Ramsey *et al.*, 2020). Mutants that disrupt this pathway are attenuated for virulence in tissue culture and animal models of infection (Alkhuder *et al.*, 2009; Ireland *et al.*, 2019; Ramsey *et al.*, 2020).

Some critical virulence factors require modification by protein maturation factors such as the *F. tularensis* DsbA homolog, FipB (Qin *et al.*, 2016; Qin *et al.*, 2009; Straskova *et al.*, 2009; Ren *et al.*, 2014). FipB catalyzes disulfide bond formation and can refold misfolded proteins, with many of the known substrates being critical virulence factors (Qin *et al.*, 2016; Schmidt *et al.*, 2013). Because it is necessary for production of active virulence factors, FipB itself is considered a critical virulence factor (Qin *et al.*, 2009; Schmidt *et al.*, 2013; Qin *et al.*, 2014; Ren *et al.*, 2014).

The Francisella Pathogenicity Island

F. tularensis subspecies *tularensis* and *holarctica* contain two copies of a genetic island known as the Francisella Pathogenicity Island (FPI), which encodes a type VI

secretion system subtype 2 (T6SSii; Nano *et al.*, 2004; Russell *et al.*, 2014). Type VI secretion systems are used to secrete effector molecules into the extracellular environment or into the membranes of surrounding cells (Bingle *et al.*, 2008). In *F. tularensis*, the T6SS secretes effector proteins into the host cell. The *F. tularensis* T6SS, and thus the FPI, is absolutely critical for virulence; *F. tularensis* cannot escape the phagosome when the T6SS is disabled (Nano *et al.*, 2004). Studies have shown that *F. novicida* secretes at least eight proteins through its T6SS, several of which are encoded outside of the FPI, although few have been well-characterized (Eshraghi *et al.*, 2016; Ledvina *et al.*, 2018).

Transcription Factors

Several transcription factors are essential for virulence in *F. tularensis* to control expression of genes essential for virulence. Possibly the best-studied virulence regulators specific to *F. tularensis* are the RNA polymerase-associated transcription factors MglA, SspA, and PigR (also referred to as FevR in *F. novicida*), which are key to positively regulate expression of the FPI (Brotcke *et al.*, 2006; Charity *et al.*, 2007; Brotcke *et al.*, 2008; Charity *et al.*, 2009; Rohlfing and Dove, 2014; Ramsey *et al.*, 2015; Cuthbert *et al.*, 2017). MglA and SspA directly interact with RNA polymerase, while PigR interacts with the MglA-SspA complex and positively regulates those genes which promoters contain a small sequence motif referred to as the PigR response element (Charity *et al.*, 2007; Charity *et al.*, 2009; Rohlfing *et al.*, 2014; Ramsey *et al.*, 2015; Cuthbert *et al.*, 2017). This complex of transcription factors has been shown to control expression of about 100

genes, both within and outside the FPI (Brotcke *et al.*, 2006; Charity *et al.*, 2007; Charity *et al.*, 2009).

Another transcription factor essential for virulence is the response regulator PmrA. Cells lacking this transcription factor are not able to replicate in macrophage or establish an infection in macrophage or animal models and multiple models have been proposed to explain how PmrA exerts its effects as a positive regulator of virulence (Mohapatra *et al.*, 2007; Sammons-Jackson *et al.*, 2008; Ramsey & Dove, 2016).

The role of PmrA in regulating gene expression and virulence

PmrA was first investigated in *F. novicida* due to its high similarity to the PmrA response regulator in *Salmonella*, which functions as part of a two component system (Mohapatra *et al.*, 2007). Two component systems are commonly used in bacteria to regulate gene expression; *E. coli* encodes about 30 systems, yet *F. tularensis* encodes very few (Yoshida *et al.*, 2015; van Hoek *et al.*, 2019). Two component systems consist of a sensor kinase that responds to a particular environmental signal by phosphorylating the DNA-binding transcription factor referred to as the response regulator. The phosphorylated response regulator then binds DNA to control expression of the genes important for response to the environmental signal (Stock *et al.*, 2000). PmrA in *F. tularensis* is referred to as an orphan response regulator because there is no sensor kinase encoded nearby, as is typical in two component systems.

In *F. novicida*, PmrA, also referred to as QseB, controls biofilm formation (Durham-Colleran *et al.*, 2010). However, the genes encoding the reported biofilm

formation pathway controlled by QseB are not present in *F. tularensis* (Durham-Colleran *et al.*, 2010).

Both *F. novicida* and *F. tularensis* LVS cells lacking PmrA are attenuated for virulence in macrophage and mouse infection models, although how PmrA exerts its effects to promote virulence has not always been clear (Mohapatra *et al.*, 2007; Sammons-Jackson *et al.*, 2008; Bell *et al.*, 2009; Ramsey & Dove, 2016). Microarray analyses of *F. novicida* cells revealed approximately 60 PmrA-regulated genes, some of which are encoded on the FPI (Mohapatra *et al.*, 2007). Another study performed in *F. tularensis* LVS identified 148 genes controlled by PmrA using microarray analyses. Similarly to what was found in *F. novicida*, some genes positively controlled by PmrA were encoded on the FPI (Sammons-Jackson *et al.*, 2008). Additional experimentation in *F. novicida* determined that PmrA can coprecipitate with the transcription factors MglA and SspA *in vitro*. These data led to a model proposing that PmrA works together with MglA and SspA in a complex to control FPI gene expression (Mohapatra *et al.*, 2007; Bell *et al.*, 2010).

This model, proposing that PmrA works with MglA and SspA to positively regulate FPI gene expression, has been brought into question by later results. In particular, a combination of ChIP-Seq and RNA-Seq experiments were used to identify the genetic locations where PmrA is associated and the genes regulated by PmrA in *F. tularensis* LVS (Ramsey & Dove, 2016). These studies found very little overlap between where PmrA associates with DNA compared to where MglA and SspA associate with DNA. Furthermore, PmrA was not found at FPI promoters or found to control expression of FPI genes. Instead, PmrA was found to tightly repress expression of a hypothetical

gene, which was named *priM* (PmrA repressed inhibitor of intramacrophage growth) (Ramsey & Dove, 2016).

It was hypothesized that PmrA is critical for intramacrophage growth because it is necessary to repress expression of *priM* (Ramsey & Dove, 2016). To confirm that it is the presence of *priM* that is responsible for the attenuation in virulence, cells in which both *pmrA* and *priM* were deleted were tested for their ability to replicate in macrophage. Consistent with the need for PmrA to repress *priM* transcription, cells without *pmrA* and *priM* were able to replicate nearly as well as wild-type cells, while deleting *priM* alone (i.e., in cells containing PmrA) had no effect on intramacrophage replication (Ramsey & Dove, 2016). To investigate if repression of *priM* was necessary to prevent RNA or protein production, an early stop codon was introduced into *priM*, to prevent production of PriM. Cells lacking PmrA with the early stop mutant of *priM* were also able to replicate to wild-type levels, indicating that production of PriM prevents intramacrophage growth in cells lacking PmrA. PriM was also expressed ectopically in wild-type cells under the control of a promoter not regulated by PmrA and was still found to decrease intramacrophage replication (Ramsey & Dove, 2016). These results are consistent with the presence of the PriM protein preventing *F. tularensis* intramacrophage replication.

These studies led to a new model proposing that the primary function of PmrA is to repress expression of the novel anti-virulence factor PriM (Ramsey & Dove, 2016). However, the molecular mechanism that allows PriM to exert its effects as an anti-virulence factor is unclear.

Anti-Virulence Factors

While many studies investigate virulence factors, it is also important to consider anti-virulence factors to fully understand bacterial pathogenesis (Brown *et al.*, 2016). An anti-virulence factor functions to decrease or inhibit virulence of a pathogen and have been discovered in at least 17 bacterial species (Brown *et al.*, 2016). In some cases anti-virulence factors play a key role in pathogenesis, but frequently their role is still incompletely understood. Some anti-virulence factors have been found to regulate production or release of virulence factors in the context of infection. One example from *Salmonella enterica* is the anti-virulence factor CigR. CigR functions by creating a threshold that the regulatory protein MgtC must overcome in order to become active. When active, MgtC prevents degradation of PhoP, a master regulator of *Salmonella* pathogenesis, and induces cellular changes that lower ATP levels, which effectively increases tolerance to acid pH and antibiotics (Yeom *et al.*, 2018). Other anti-virulence factors have been discovered in *Mycobacterium tuberculosis* and *Xylella fastidiosa*. These identified anti-virulence factors are required to prevent hypervirulence, which could increase detection by the host immune system and be detrimental to continued disease progression (Ionescu *et al.*, 2013; Shimono *et al.*, 2003).

PriM Functions as an Anti-Virulence Factor

The current model specifies that the major role of PmrA in virulence is to repress expression of the *priM* gene; *F. tularensis* cells lacking PmrA are unable to replicate inside macrophage due to the production of the anti-virulence factor PriM. Yet little is

known about this protein, including how PriM functions to prevent intramacrophage replication is unknown.

PriM has no clear sequence or protein domain homology with other proteins. However, PriM does contain a canonical N-terminal secretion signal, which can be readily identified using secretion signal prediction software like SignalIP 5.0 (Armenteros *et al.*, 2019). The PriM secretion signal is likely functional to secrete PriM through the inner membrane; PriM can be detected in the membrane fraction by western blotting (Figure 2). PriM does not contain a predicted transmembrane domain, so we hypothesize that it is found in the periplasm or is further secreted through the outer membrane.

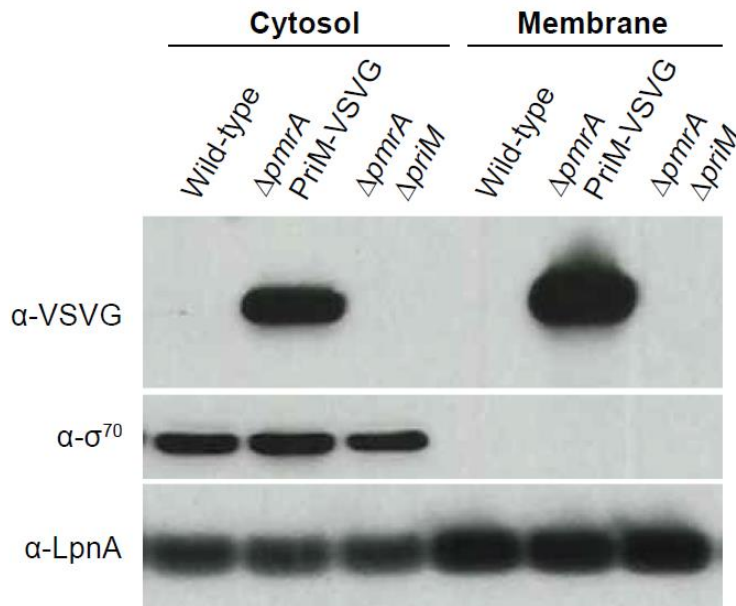


Figure 1. PriM is found in the membrane fraction. Immunoblot analysis of cytosolic and membrane fractions comparing VSV-G tagged PriM with wildtype and strains lacking PriM. Sigma 70 is used as a cytosolic control and LpnA is an abundant membrane protein in *F. tularensis*. (Unpublished data, Ramsey & Dove).

PriM was identified as the most abundant protein associated with outer-membrane vesicles produced in log-phase *F. novicida*, though the significance of this is not clear (McCaig *et al.*, 2013). Another study in *F. novicida* found that PriM is one of the 95 proteins detected in a secretome analysis, providing further evidence that PriM may be secreted in *F. novicida* (Eshraghi *et al.*, 2016). In *F. tularensis* LVS, PriM was also identified as being one of 31 proteins that coimmunoprecipitates with MoxR, a protein that is important for stress response and intramacrophage survival (Dieppedale *et al.*, 2013). The interaction between MoxR and PriM has not yet been validated and notably, MoxR is predicted to be cytoplasmic.

Given its key role in virulence and lack of sequence homology to other proteins, we were interested in obtaining information about PriM's structure, which might provide insight into function. Our collaborators used X-ray crystallography to solve a structure of PriM (Figure 1; Dr. M.A. Schumacher, personal communication). Because the PriM protein used for crystallography was heterologously produced in *E. coli* without its canonical N-terminal secretion signal, the crystalized PriM does not include the first 20 amino acids.

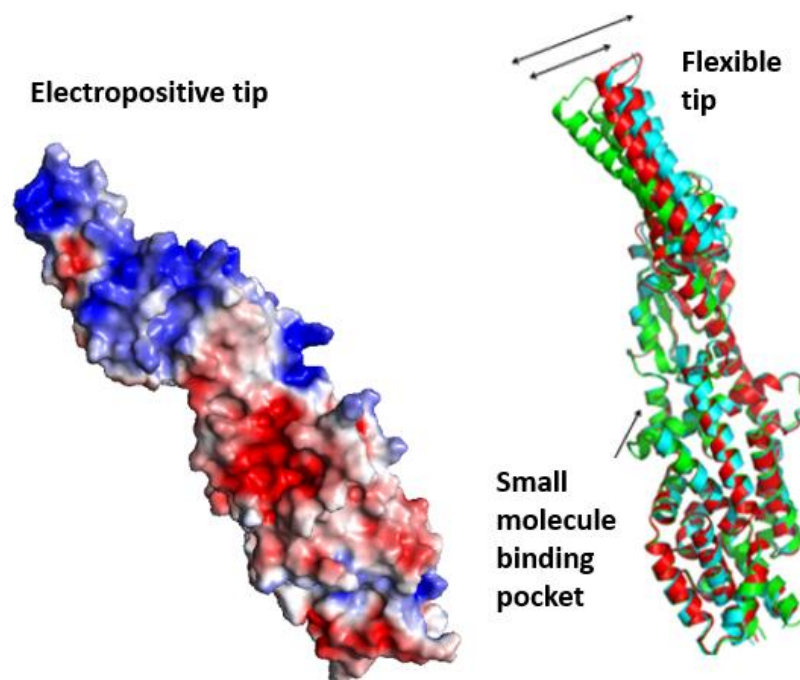


Figure 2. Representation of the crystal structure of the PriM protein. This structure has been described as a novel fold and highlights several key features. The model on the left displays a surface view with coloration based on the overall charge of each region of the protein; blue represents an electropositive charge and red represents an electronegative charge. The model on the right displays a ribbon diagram of the secondary structure with three overlapping molecules found in three different conformations, each depicted in a different color (Red, green, and cyan). Not shown in this model is an acetate molecule that co-crystallized with PriM in region indicated as the small molecule binding pocket. (Unpublished data, Dr. Maria Schumacher, Duke University Department of Biochemistry)

The structure of PriM reveals a novel fold with no known structural homology currently found in the Protein Data Bank. A PriM monomer is approximately 116 angstroms in length, with a long coil-coil domain and flexible, electropositive tip. The tip region was described as flexible because multiple structures were solved with the tip region in multiple conformations (Figure 1). Additional features include a disulfide bond between two cysteines, located near the C-terminus end of the protein, and a bound

acetate, which co-crystallized with PriM. The bound acetate may indicate the presence of a binding pocket.

Aims of Study

The mechanism through which PriM exerts its effect as an anti-virulence factor is still not understood. Using the *F. tularensis* LVS strain, which is attenuated for virulence in humans but retains pathogenicity in animal models, we can begin to address this question using structural and genetic approaches. To identify which structural features are key to PriM's anti-virulence function, we engineered mutations targeting specific structural features. We then assessed the impact of these mutations on the function of PriM using intramacrophage infection assays. We predicted that mutations in a region of PriM essential for its function as an anti-virulence factor will allow cells producing the mutant to replicate in macrophage, in contrast to wild-type PriM, which is inhibitory to intramacrophage growth.

We have also taken a genetic approach to attempt to identify genes critical for the function of PriM. In the course of our study we identified a strain that permits intramacrophage growth despite the absence of PmrA and the presence of *priM*. We hypothesized that mutations in this strain interfere with the anti-virulence function of PriM. Studying the genetic basis for this suppressor strain has increased our knowledge of *F. tularensis* virulence and regulation of PriM. Our goal has been to increase our understanding of bacterially-encoded anti-virulence pathways, which may lead to the identification of novel targets for anti-microbial therapeutics by exploiting these anti-virulence factors to limit bacterial pathogenesis.

CHAPTER 2

METHODOLOGY

Bacterial Growth Conditions

Francisella tularensis subsp. *holarctica* LVS was grown at 37° C aerobically, shaking in Mueller-Hinton Broth supplemented with 0.025% iron pyrophosphate, 0.1% glucose, and 2% Isovitalex (S-MHB) or on cystine heart agar plates containing 1% hemoglobin (CHAH) at 37° C for 24 hours or until single colonies appeared. When appropriate, kanamycin was added to the CHAH plates at a concentration of 5 µg/mL. *Escherichia coli* strain XL-1 Blue was grown aerobically in lysogeny broth (LB) or on LB agar plates at 30° or 37° C, as indicated. When appropriate, kanamycin was added to the LB or LB agar at a concentration of 50 µg/mL.

Plasmid Design and Construction

Plasmid pKL117 pEX_PriM_mtip2 was created to change the codons in *priM* (FTL_0702) specifying amino acids L121-I133, which correspond to the tip region. Primers containing the modified sequence were used to amplifying flanking regions of homology of 1000 base pairs on each side of the modified region of *priM* (FTL_0702). The DNA included on the primers changed the codons specifying amino acids 121 – 133 from AAA CTT GAA AGT CAA AAG AAA TTA GGC TGG AGA ATT to GGT GGA

GGT GGA GGT GGA GGT GGC GGT. The two PCR products containing the modified region were spliced together using overlap extension PCR, utilizing a 5' primer including DNA specifying a BamHI site on its 5' end and a 3' primer including DNA specifying a KpnI site at its 5' end. PCR products and pEX18Kan were digested with BamHI and KpnI and ligated together to create pKL117.

The plasmid pKR4 pEX_PriM_noC1 was created by amplifying two flanking regions of homology of about 500 base pairs on each side of the codon specifying cysteine 303 in *priM* (FTL_0702) by PCR. Primers included DNA which changed cysteine 303 to alanine by replacing the codon TTG with TGC, and created an MfeI cut site. The two PCR products with the modified codon were spliced together using overlap extension PCR, utilizing a 5' primer including DNA specifying a BamHI site on its 5' end and a 3' primer including DNA specifying a KpnI site at its 5' end. PCR products and pEX18Kan were digested with BamHI and KpnI and ligated together to create pKR4.

The intermediate plasmid pKL114 modifies the *priM* codons specifying tryptophan 219, arginine 222, and tryptophan 226 to encode alanines. To create pKL114, PCR was used to amplify flanking regions of homology including 800 - 1000 base pairs on each side of the modified region. The modified region altered DNA specifying amino acids 219 – 226 from TGG GGT GCA AGA GTA GTT CTT TGG to GCA GGT GCA GTA GTT CTT GCA. The two PCR products with the modified region were spliced

together using overlap extension PCR, utilizing a 5' primer including DNA specifying a BamHI site on its 5' end and a 3' primer including DNA specifying a KpnI site at its 5' end. PCR products and pEX18Kan were digested with BamHI and KpnI and ligated together to create pKL114. The plasmid pKL115 pEX_PriM_mpk1 was created from pKL114 and further modifies *prim* such that the codon specifying tyrosine 270 is modified to encode alanine. To create pKL115, PCR was used to amplify approximately 600 base pairs flanking the codon specifying tyrosine 270 and using primers modifying the codon TAT to CGT. The two PCR products with the modified codon were spliced together using overlap extension PCR, utilizing a 5' primer including DNA specifying a BamHI site on its 5' end and a 3' primer including DNA specifying a KpnI site at its 5' end. PCR products and pEX18Kan were digested with BamHI and KpnI and ligated together to create pKL115.

Plasmid pKR58 pEX_FTL_0146_SNP modifies the FLT_0146 codon specifying phenylalanine 315 to leucine. The plasmid was created by amplifying flanking regions of homology of 900 – 1000 base pairs on either side of the modified codon by PCR, using primers that change the codon specifying amino acid 315 from TCC to TAC. The two PCR products with the modified codon were spliced together using overlap extension PCR, utilizing a 5' primer including DNA specifying a BamHI site on its 5' end and a 3'

primer including DNA specifying a KpnI site at its 5' end. PCR products and pEX18Kan were digested with BamHI and KpnI and ligated together to create pKR58.

Plasmid pKL75 pEX_FTL_0702-VSVG was created using the pKL02 vector, which contains a multiple cloning site including a NotI site and one extra base pair, which encodes a 3 amino acid alanine linker, followed by DNA specifying the 11 amino acid vesicular stomatitis virus-glycoprotein (VSV-G) epitope tag, and a stop codon (Ramsey *et al.*, 2015). DNA specifying the *prim* gene was amplified from LVS genomic DNA using a 3' primer that includes a KpnI site and a 5' primer with a NotI site. The pKL02 plasmid and PCR product were digested with KpnI and NotI and the *prim* fragment was ligated into the digested pKL02, resulting in pKL75.

Plasmids were transformed into XL-1 Blue chemically competent *E. coli* and purified using Qiagen QIAprep Spin Miniprep kits. Plasmid sequences were confirmed by Sanger sequencing at the URI Genomics and Sequencing Center.

Strain Construction

An allelic exchange protocol was used to create markerless mutations in LVS essentially as previously described (Maier *et al.*, 2004). Cells were made electrocompetent by performing 3-5 washes in 10% sucrose. Electroporations using desired cells and plasmid were performed using the following settings in a 2 mm cuvette: 2.5 kV, 25 μ F, and 600 Ω . After electroporation, cells recovered for 4-8 hours shaking

aerobically in S-MHB. Cells containing a homologous recombination event between the integration vector and the genomic DNA were selected for by plating transformations on CHAH plates with kanamycin. Kanamycin-resistant cells were subsequently grown overnight on CHAH plates without antibiotic, resuspended in 1X PBS, and serial dilutions were plated onto CHAH plates containing 10% sucrose. Sucrose-resistant colonies, which should lack the *sacB* gene encoded on the pEX18Kan plasmid backbone, were patched on CHAH with and without kanamycin to validate the loss of the kanamycin resistance gene and plasmid backbone. Colony PCR (and diagnostic digest as necessary) was used to confirm the presence of the mutation of interest in kanamycin sensitive cells. Cells with confirmed mutations were purified to single colony and re-validated by PCR and antibiotic sensitivity.

A single integration event was used to add DNA specifying the VSV-G epitope. Cells were made electrocompetent by performing 3-5 washes in 10% sucrose. Electroporations using desired cells and plasmid were performed using the following settings in a 2 mm cuvette: 2.5 kV, 25 μ F, and 600 Ω . After electroporation, cells recovered for 4-8 hours shaking aerobically in S-MHB. Cells containing a homologous recombination event between the integration vector and the genomic DNA were selected for by plating transformations on CHAH plates with kanamycin. Kanamycin-resistant cells were grown overnight on CHAH-Kan plates and assessed by colony PCR for integration of the desired portion of the plasmid. Strains were validated by isolating genomic DNA via the MasterPure Complete DNA and RNA Purification Kit (Lucigen) and the region including the desired mutation was amplified by PCR and sequenced by Sanger sequencing at the URI Genomics and Sequencing Center.

LVS PriM(mtip) and LVS $\Delta pmrA$ PriM(mtip) were created using plasmid pKL117 pEX_PriM_mtip2 and LVS and LVS $\Delta pmrA$ cells, respectively. LVS PriM(C303A) and LVS $\Delta pmrA$ PriM(C303A) were created using plasmid pKR4 pEX_PriM_noC1 and LVS and LVS $\Delta pmrA$ cells, respectively. LVS PriM(mpk) and LVS $\Delta pmrA$ PriM(mpk) were created using plasmid pKL115 pEX_PriM_mpk1 and LVS and LVS $\Delta pmrA$ cells, respectively. LVS FTL_0146 F315L and LVS $\Delta pmrA$ FTL_0146 F315L were created using plasmid pKR58 pEX_FTL_0146_SNP and LVS and LVS $\Delta pmrA$ cells, respectively. The plasmid LVS pKL75 pEX_FTL_0702-VSVG was used to add DNA specifying a VSG-G tag to the *prim* gene of cells indicated above, producing LVS $\Delta pmrA$ PriM(mtip)-V, LVS $\Delta pmrA$ PriM(C303A)-V, and LVS $\Delta pmrA$ PriM(mpk)-V, as well as to wild-type LVS, producing LVS $\Delta pmrA$ PriM-V.

Macrophage Infection Assays

Cells of LVS and derivatives were tested in a macrophage infection assay to assess their ability to survive and replicate inside macrophage. Throughout the assay, J774A.1 murine macrophage-like cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (DMEM-F) grown at 37°C with 5% CO₂. Macrophage were seeded into wells of two 96-well tissue culture-treated plates, plating approximately 2×10^4 cells/well. Approximately 16-18 hours after seeding the macrophage, bacteria were added to wells in triplicate, at a multiplicity of infection (MOI) of approximately 5. After bacteria were added to the wells, the remainder of the inoculum was serially diluted in 1X PBS and 10 μ L from each dilution was plated onto CHAH plates in duplicate for enumeration to experimentally determine the MOI for each

strain. In each 96-well plate, two wells served as controls, ensuring there was no cross-contamination (macrophage only) or survival of extracellular bacteria (bacteria only). After two hours, the media was removed, the wells were washed twice with sterile 1X PBS, and DMEM-F with 10 $\mu\text{g/mL}$ gentamicin was added to remove remaining extracellular bacteria. Two hours after washing, media was removed from the wells of one plate, wells were washed twice with PBS, and 1% saponin in 1X PBS was added. Plates were incubated at room temperature for 30 minutes to allow lysis of the macrophage and 50 μL of the lysate was plated on CHAH plates in duplicate for enumeration. Twenty-four hours after washing, the remaining 96-well plate was removed from the incubator and the macrophage were washed and lysed as during the 2-hour timepoint. The lysates were serially diluted in 1X PBS and 10 μL from each dilution was plated onto CHAH plates in duplicate for enumeration. The resulting colonies were counted to assess intramacrophage replication. All intramacrophage growth experiments were performed at least twice.

Immunoblots

LVS cells and indicated derivatives were grown in 5 mL of S-MHB to mid-log ($\text{OD}_{600} \sim 0.3\text{-}0.4$) in biological triplicate. Cells were pelleted by centrifugation and resuspended in 1X sample loading buffer (NuPAGE) normalized to measured optical density. Samples were boiled at 98°C for 10 minutes prior to separation by SDS-PAGE on NuPAGE 4 to 12% Bis-Tris protein gels (ThermoFisher) with NuPAGE morpholinoethanesulfonic acid (MES) running buffer (ThermoFisher). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked

with Li-Cor Odyssey Blocking Buffer and washed four times with PBS with NP-40, and two times with PBS without NP-40. Membranes were incubated with primary antibodies, either polyclonal rabbit anti-VSV-G (Sigma) or mouse monoclonal anti-LpnA (BEI Resources, used as a loading control), and then secondary antibodies donkey anti-rabbit IgG IRDye 800CW and donkey anti-mouse IRDye 680LT. Proteins were visualized using the Odyssey Infrared Imager at the Rhode Island INBRE Core Facility. All immunoblots were performed at least twice on biological replicates unless otherwise noted.

RNA Isolation

LVS cells and indicated derivatives were grown in 5 mL of S-MHB to mid-log ($OD_{600} \sim 0.3-0.4$) in biological triplicate. Cells were pelleted by centrifugation and resuspended in TRI-Reagent. Samples were incubated at 60°C for 10 minutes and pelleted by centrifugation at 4°C. Direct-zol RNA isolation kits (ZYMO) were used to isolate nucleic acids, following manufacturer's guidelines. Nucleic acids were eluted in RNase-free molecular grade water and treated with DNaseI for 1 hour at 37°C to degrade DNA. Samples were re-purified with the Direct-zol RNA isolation kit and RNA samples were eluted in RNase-free molecular grade water. Purity was assessed by Nanodrop and gel electrophoresis.

cDNA Synthesis and qRT-PCR

RNA was converted into single-stranded cDNA using the NS₅ semi-random primer (5'-NSNSNSNSNS-3') and Superscript III Reverse Transcriptase (ThermoFisher). RNA was removed from cDNA samples by incubation with 1N NaOH (20% final

concentration) at 65°C, samples were neutralized with 1N HCl (20% final concentration), and cDNA was purified using the Qiagen QIAquick PCR purification kit. Abundance of *prim* transcripts were determined relative to *tul4* transcript abundance by quantitative real-time PCR (qRT-PCR). PowerUp SYBR Green master mix (ThermoFisher) and primers designed via IDT were used, and samples were run on the Roche LightCycler at the URI Genomics and Sequencing Center.

gDNA Isolation and Next Generation Sequencing

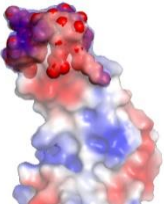
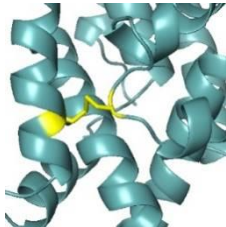
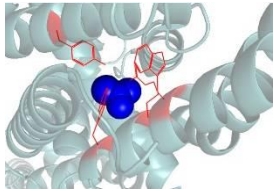
Total genomic DNA was extracted for use in next-generation sequencing using the MasterPure Complete DNA and RNA Purification Kit (Lucigen) and following manufacturer's instructions. Library preparation and next-generation sequencing was performed by the Microbial Genome Sequencing Center (University of Pittsburgh). Data was returned as 150 bp paired-end reads with about 8 million reads. Sequencing reads were aligned to *F. tularensis* subspecies *holarctica* LVS reference genome using the alignment program bowtie2 (Langmead & Salzberg, 2012). Resulting alignments were outputted as Sequence Alignment Maps or SAM files. The SAM files were converted to BAM files, the binary version of SAM files, and indexed and sorted using SAMtools (Li *et al.*, 2009). Variant files were generated using BCFtools to identify differences between the two alignments. IGV (Robinson *et al.*, 2011) was used to analyze the SAM alignment files and identify mutations between the two samples.

CHAPTER 3

FINDINGS

Production of the PriM protein prevents intramacrophage replication in *F. tularensis* (Ramsey & Dove, 2016). However, the molecular mechanism underlying the anti-virulence function of PriM is unknown. To investigate this mechanism, we first examined the relationship between PriM structure and function. We generated PriM mutants in regions corresponding to distinct structures (for PriM structure, see Figure 1), to see if any of these changes disrupt the anti-virulence functions of PriM. (Table 1). One mutant allowed us to investigate the importance of the charged tip region, as we altered the charge from electropositive to neutral by replacing all thirteen amino with glycines [which we refer to as PriM(mtip), Table 1]. In another PriM mutant, we probed the importance of PriM's disulfide bond by replacing one of the cysteines, cysteine 303, to alanine [which we refer to as PriM(C303A), Table 1]. Lastly, we examined the contribution of the PriM small-molecule binding pocket by altering the charged residues that line the potential pocket region to alanines [which we refer to as PriM(mpk), Table 1].

Table 1. PriM mutants investigated in this study

Name	Purpose	Description	Illustration
PriM(mtip)	Changes the tip region from an electropositive charge to neutral charge	Mutations in the tip region: L121-I133 (colored as red spheres in illustration) changed to glycines	
PriM(C303A)	Removes one of the two cysteines to prevent disulfide bond production	Mutation changing C303 to alanine (disulfide bond formed by cysteines 303 and 424 is colored in yellow in illustration)	
PriM(mpk)	Modifies “pocket” region, which potentially binds small molecules	Mutations in the small molecule binding pocket region: R222, W219, W226, and Y270 (colored in red in illustration; acetate molecule in the potential binding pocket is colored in blue) changed to alanine.	

We modified *priM* to encode each mutant in wild-type cells as well as in cells that lack the repressor PmrA. In wild-type cells, the transcription factor PmrA represses expression of *priM*, such that not enough PriM is produced to influence intramacrophage growth (Ramsey & Dove, 2016). Thus, PriM mutations in the wild-type background should not affect virulence and serves as a control of any unexpected changes in gene expression caused by the modifications made in *priM*. In the background that lacks

PmrA, *prim* is no longer being repressed and we can assess the anti-virulence function of the PriM protein and mutants.

To test the significance of each structural region of PriM on its function as an anti-virulence factor, we examined the ability of cells with PriM mutants to replicate inside a murine macrophage-like cell line, J774A.1 cells. These results allow us to assess the significance of each structural element to the anti-virulence function of PriM. Specifically, cells that produce PriM (i.e., cells lacking *pmrA*) are unable to replicate in macrophage. Therefore, if cells producing the mutant PriM proteins are able to replicate in macrophage at or near wild-type levels, it would suggest that the modified structural region is essential for the anti-virulence function of PriM.

Changes in the tip region do not affect PriM's anti-virulence function

Using the PriM(mtip) mutant (Table 1), we tested the contribution of the electropositive charge of the PriM tip region to its function as an anti-virulence factor in the context of intramacrophage growth. When performing intramacrophage growth assays, we include several controls. Wild-type LVS cells are able to survive and replicate during the 24-hour infection. Cells lacking PmrA (which containing a wild-type PriM; $\Delta pmrA$) do not replicate well in macrophage (Mohapatra *et al.*, 2007). We compare the intramacrophage growth phenotype of two test strains to these controls. The first test strain is the PriM mutation in the wild-type background [WT PriM(mtip)]; we expect that the presence of PmrA will repress mutant PriM production, allowing wild-type levels of intramacrophage replication. The second strain tested is the PriM mutation in the $\Delta pmrA$ background [$\Delta pmrA$ PriM(mtip)]; the absence of PmrA will allow production of the mutant PriM. We compare the ability of cells producing the mutant PriM to the cells with

wild-type PriM ($\Delta pmrA$) to replicate within macrophage to determine the effect the mutant PriM has on virulence. We found that the PriM(mtip) mutant cells are not able to regain the ability to replicate to wildtype levels inside macrophage cells (Figure 3). These results demonstrate that PriM is still able to function as an anti-virulence factor if the charge of the tip region is modified from electropositive to neutral. This suggests that the electropositive charge of the tip region of PriM is not required for its anti-virulence function.

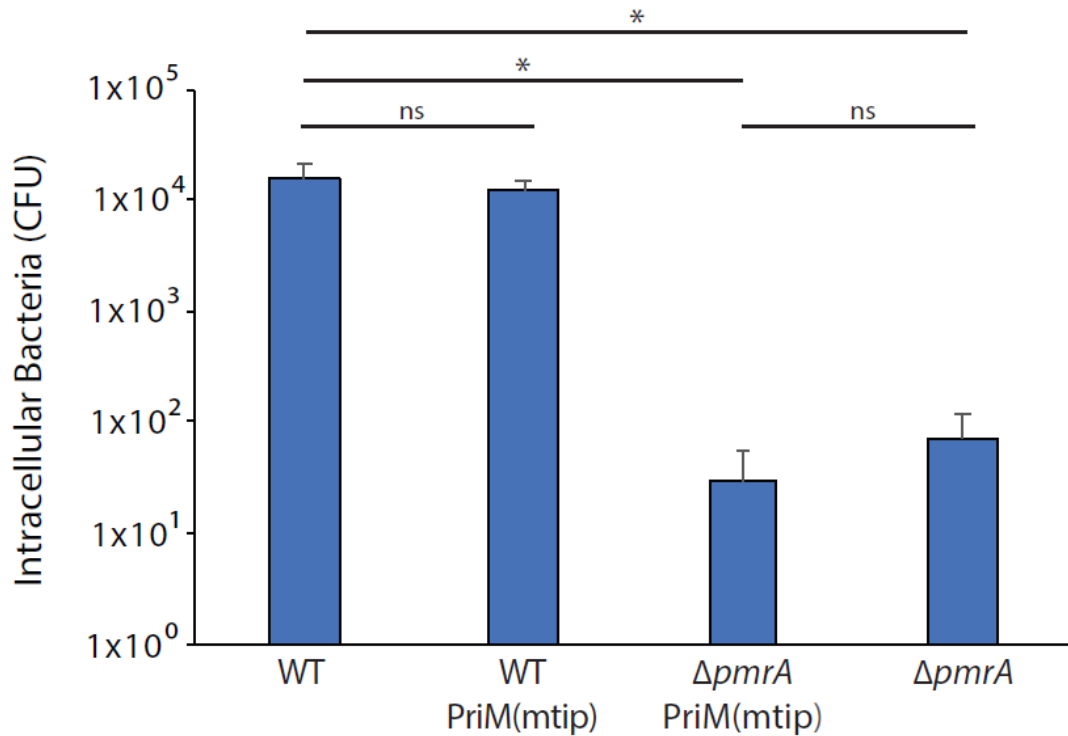


Figure 3. Intramacrophage growth assay testing PriM(mtip) mutants. Survival of wild-type *F. tularensis* LVS or indicated mutants 24 hours after infection of macrophage. Data represents the number of bacteria recovered after 24 hours of intracellular growth. Significance of $p \leq 0.01$ is indicated by a (*) between bars where a 2-tailed *t* test was performed and otherwise was labeled as not significant (ns). Error bars represent standard deviation. Assays were performed in duplicate with representative data shown.

Loss of the disulfide bond does not affect PriM function

Periplasmic disulfide bond formation is key for maturation of critical *F. tularensis* virulence factors. Disulfide bond formation is catalyzed by the enzyme FipB, the *F. tularensis* DsbA homolog (Qin *et al.*, 2016). PriM is also a substrate of FipB (Qin *et al.*, 2016), which is consistent with our finding that PriM contains a single disulfide bond (Figure 1, Table 1). We hypothesized that the production of PriM prevents virulence because high levels of PriM may titrate FipB away from critical virulence factors, preventing virulence factor maturation. Without functional virulence factors, the PriM-producing bacterial cells would no longer be able to replicate in macrophage. To test this hypothesis, we assessed cells producing a PriM variant that is unable to create an intramolecular disulfide bond. Specifically, these cells lack PmrA and contain PriM with the mutation C303A, changing one of the two cysteines in PriM to an alanine [$\Delta pmrA$ PriM(C303A)]. We found that cells producing PriM with the C303A mutation were still unable to replicate to wildtype levels inside macrophage cells (Figure 4). These results suggest that PriM does not function as an anti-virulence factor by titrating FipB activity away from critical virulence factors.

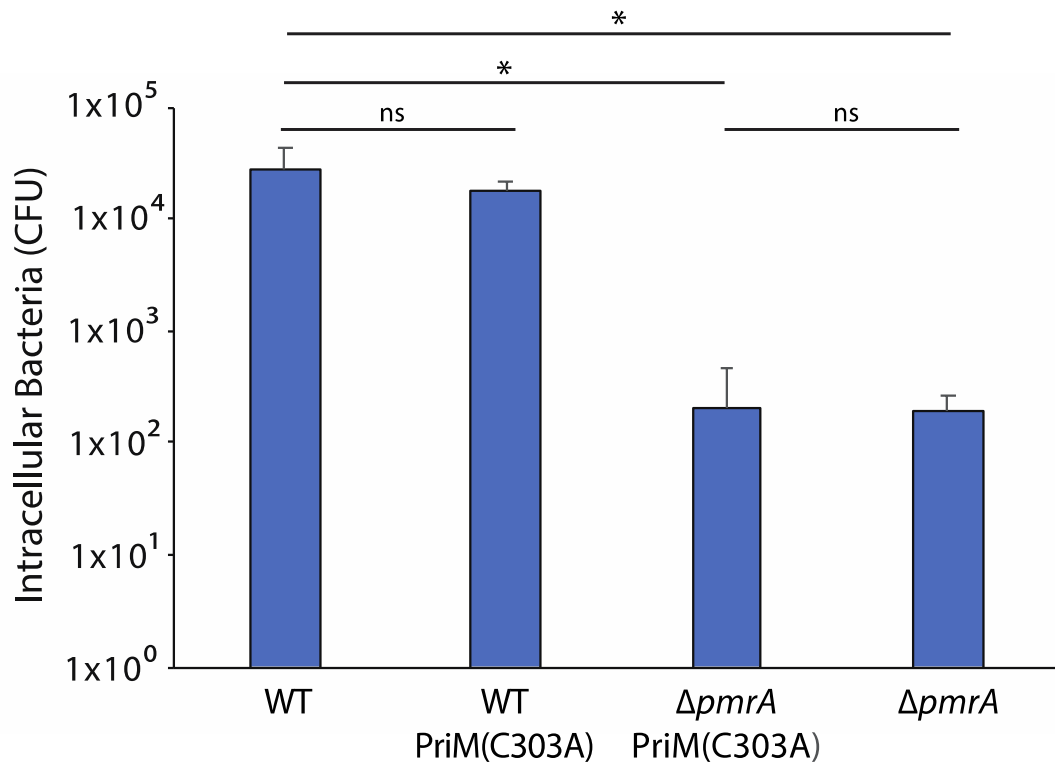


Figure 4. Intramacrophage growth assay testing the PriM(C303A) mutants. Survival of wild-type LVS or indicated *F. tularensis* mutant cells 24 hours after infection of macrophage. Data represents the number of bacteria recovered after 24 hours of intracellular growth. Significance of $p \leq 0.05$ is indicated by a (*) between bars where a 2-tailed *t* test was performed and otherwise was labeled as not significant (ns). Error bars represent standard deviation. Assays were performed in duplicate with representative data shown.

Disrupting the small molecule binding pocket inhibits PriM's anti-virulence function

Finally, we assessed the contribution of the potential small molecule binding pocket to PriM's anti-virulence activity. We tested the ability of cells producing PriM with mutations in residues key to forming the potential small molecule binding pocket [$\Delta pmrA$ PriM(mpk)] to replicate in macrophage. We found that *F. tularensis* cells producing PriM(mpk) are able to replicate to levels closer to wildtype inside macrophage (Figure 5). These results are consistent with the small molecule binding pocket being

involved in PriM's anti-virulence function. Yet it is also possible that the disruption of the potential binding pocket could also affect proper folding, localization, or abundance of PriM and thus prevent its anti-virulence function indirectly; we next sought to address this concern.

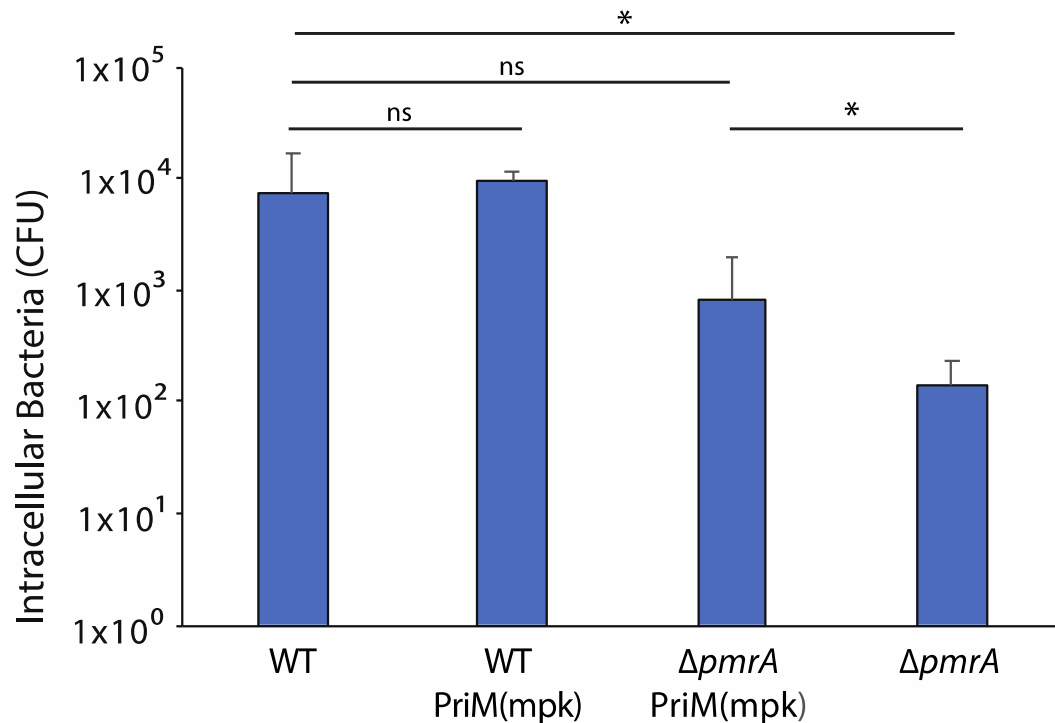


Figure 5. Intramacrophage growth assay testing PriM(mpk) mutants. Survival of wild-type LVS or indicated *F. tularensis* mutant cells 24 hours after infection of macrophage. Data represents the number of bacteria recovered after 24 hours of intracellular growth. Significance of $p \leq 0.05$ is indicated by a (*) between bars where a 2-tailed *t* test was performed and otherwise was labeled as not significant (ns). Error bars represent standard deviation. Assays were performed in duplicate with representative data shown.

Addition of a VSV-G epitope tag does not affect PriM function

Having determined how mutations in key structures of PriM influence intramacrophage growth, we needed to ascertain if our results are due to changes in specific structures or overall destabilization of the PriM protein. As there is no

commercial antibody for PriM, we used an epitope-tagging approach. Because the C-terminal region of PriM was found to be solvent-exposed in the PriM crystal and the N-terminus of PriM encodes a Sec secretion signal, we modified the *priM* gene to encode a C-terminal vesicular stomatitis virus-glycoprotein (VSV-G) tag. Given that the VSV-G tag is small, only 11 amino acids in size, we reasoned that when added to the unstructured C-terminal region it would be unlikely to interfere with PriM function. We then assessed the effect of the epitope tag on the anti-virulence function of PriM by testing cells producing PriM with a C-terminal VSV-G tag ($\Delta pmrA$ PriM-V) in an intramacrophage growth assay. We determined that addition of the C-terminal VSV-G tag does not affect the anti-virulence function of PriM (Figure 6).

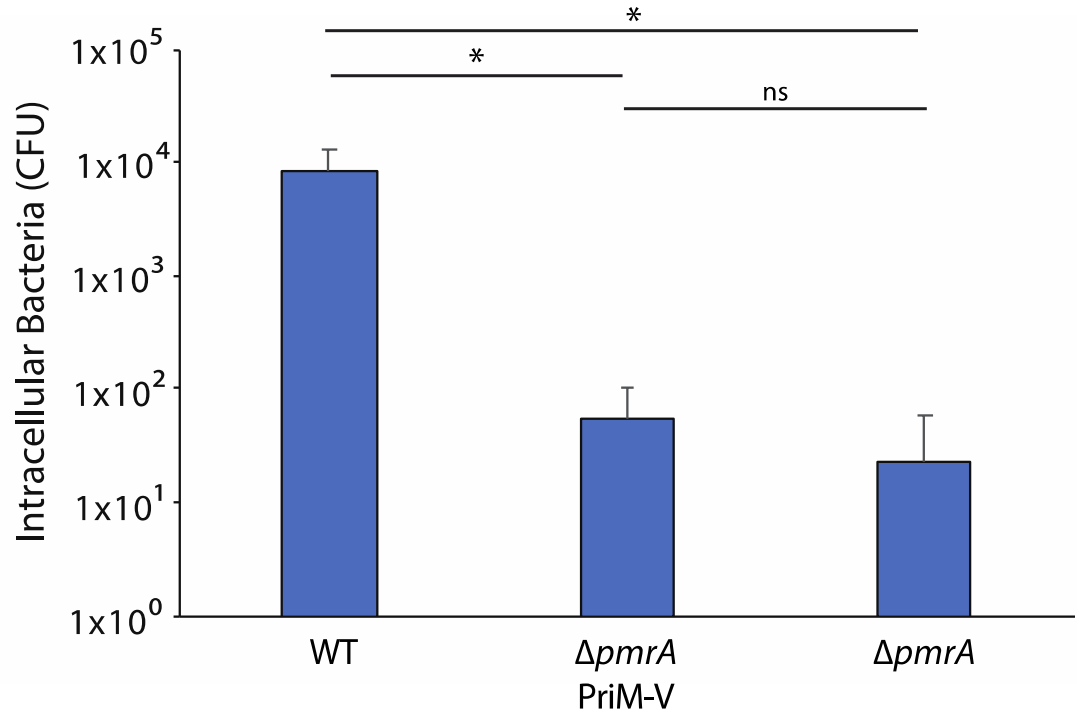


Figure 6. Intramacrophage growth assay testing PriM-VSV-G mutant. Survival of wild-type LVS or indicated *F. tularensis* mutant cells 24 hours after infection of macrophage. Strain referred to as $\Delta pmrA$ PriM-V specifies a C-terminal VSV-G tag. Data represents the number of bacteria recovered after 24 hours of intracellular growth. Significance of $p \leq 0.05$ is indicated by a (*) between bars where a 2-tailed *t* test was performed and otherwise was labeled as not significant (ns). Error bars represent standard deviation. Assays were performed in duplicate with representative data shown.

PriM mutant proteins are produced

Having confirmed that the addition of a VSV-G tag to PriM has no apparent effect on its function, we modified strains to produce epitope-tagged versions of PriM mutants. Using whole-cell lysates, we used immunoblotting to quantify the abundance of each PriM-V variant in comparison to the wild-type PriM-V in $\Delta pmrA$ cells, using an antibody to the *F. tularensis* lipoprotein LpnA as a loading control (PmrA does not control expression of LpnA [Ramsey and Dove 2016]; Figure 7). Because wild-type PriM is extremely abundant in $\Delta pmrA$ cells, the PriM-V samples were diluted fifty-fold relative

to other samples to prevent signal saturation. We are able to detect all of the PriM mutants, although they were all less abundant than wild-type PriM. We detected the least amount of PriM in the $\Delta pmrA$ PriM(mtip)-V cells. Given that $\Delta pmrA$ PriM(mtip) cells still retain PriM's anti-virulence function, our results suggest that the amount of PriM necessary to exert its function as an anti-virulence factor is much lower than the amount present in the $\Delta pmrA$ strain and can be as low as the amount of PriM present in the $\Delta pmrA$ PriM(mtip) cells. There is still more PriM present in the $\Delta pmrA$ PriM(mpk)-V cells than in $\Delta pmrA$ PriM(mtip)-V cells, suggesting that the increase in virulence in cells with PriM(mpk) is not due to lack of protein (Figure 7). However, it is important to note that the majority of PriM produced in the $\Delta pmrA$ PriM(mpk)-V cells appears to have a shift in mobility.

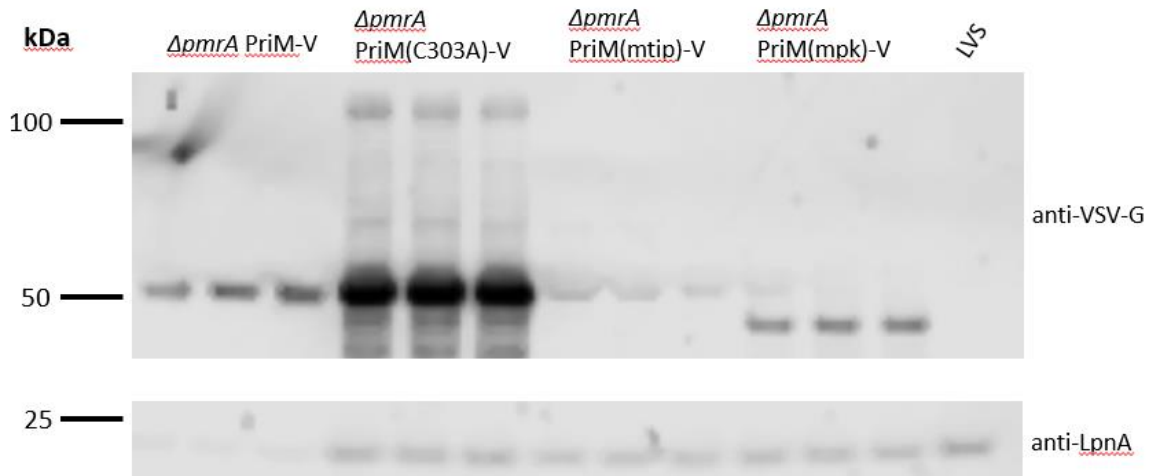


Figure 7. Relative abundance of wild-type and mutant PriM proteins. The abundance of specified PriM proteins present in whole-cell lysates was determined by immunoblot using an anti-VSV-G antibody. The LpnA protein, detected with an anti-LpnA antibody, serves as a loading control. Note that the LVS $\Delta pmrA$ PriM-V strain was diluted 50-fold to prevent signal saturation. Triplicate biological samples from a representative experiment are shown.

Cells lacking *pmrA* with restoration of virulence: investigating a suppressor mutant

To study the role of PriM, we utilize cells that lack *pmrA*, the repressor that prevents PriM production. Unexpectedly, we identified cells that lack *pmrA* (and contain *priM*), that are as virulent as wildtype cells (Figure 8). We refer to these cells as the PmrA suppressor strain [$\Delta pmrA$ (sup)] because PmrA is no longer essential for intramacrophage survival of these cells.

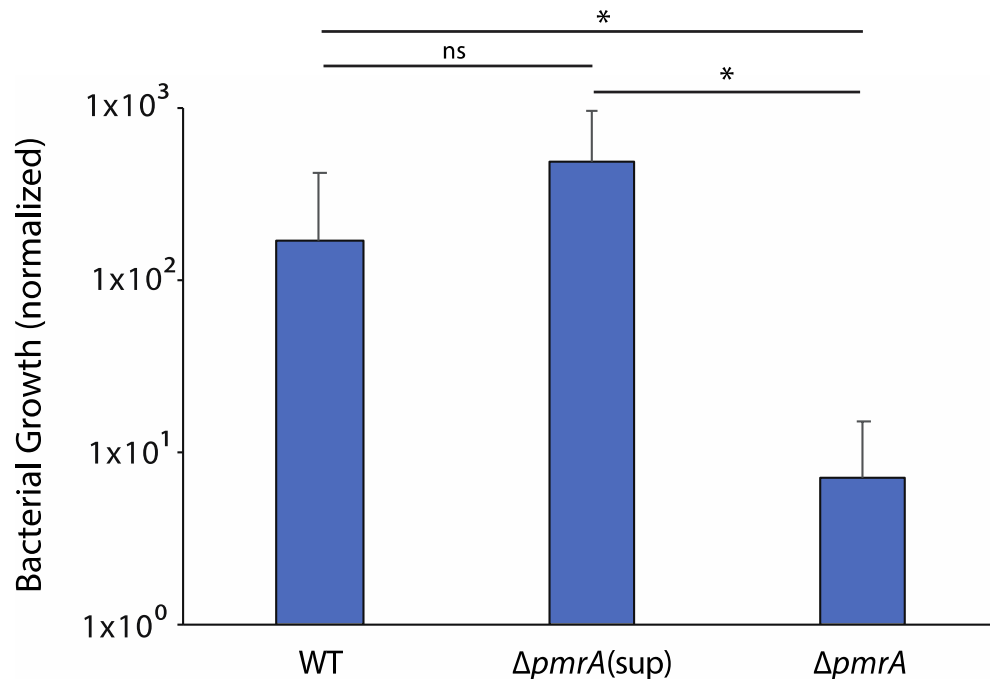


Figure 8. Intramacrophage growth assay testing $\Delta pmrA$ suppressor mutant. Survival of wild-type LVS or indicated *F. tularensis* mutant cells 24 hours after infection of macrophage. Assays were performed in duplicate with representative data shown. Data represents the fold change from 2 hours to 24 hours with error bars representing standard deviation. Significance of $p \leq 0.01$ is indicated by a (*) between bars where a 2-tailed *t* test was performed and otherwise was labeled as not significant (ns). Statistical tests were performed on data representing cells recovered after 24 hours of infection. Error bars represent standard deviation.

Since there is a difference in the intramacrophage growth phenotype between the $\Delta pmrA$ strain and the $\Delta pmrA$ suppressor cells, we reasoned that there must be some genetic difference between the two strains. After verifying by Sanger sequencing that *pmrA* is absent and *priM* is identical in both strains, we hypothesized that there must be other mutations somewhere in the $\Delta pmrA$ suppressor genome that results in this phenotypic change. We used whole-genome resequencing to test this hypothesis. After isolating genomic DNA (gDNA) from wild-type, $\Delta pmrA$, and $\Delta pmrA$ (sup) cells, we sent the gDNA to the Microbial Genome Sequencing Center (University of Pittsburg) for next generation library preparation and sequencing. We aligned the resulting sequencing data to the reference *F. tularensis* LVS genome to identify polymorphisms between the sequences of the three strains and the reference sequence.

Our results identified three single nucleotide polymorphisms (SNPs) present only in the $\Delta pmrA$ suppressor genome (Table 2). One mutation is in FTL_0146, which is annotated as an ATP binding protein. This mutation changed an C to A, a nonsynonymous mutation changing a phenylalanine to a leucine at amino acid 315 in FTL_0146. Another mutation is present in FTL_1339, which is annotated as a proton-dependent oligopeptide transport. This second mutation changed a C to an A, but it is a synonymous mutation and the encoded amino acid remains a glycine. The last mutation was found in a non-coding region upstream of FTL_0869 (annotated as a hypothetical protein) that changes a G to a T.

Table 2. Mutations unique to PmrA suppressor cells

Mutation Type	Gene or Region	Locus Number	Nucleotide Change	Mutations
SNP	ATP Binding Protein	FTL_0146	C152592A	F315L
SNP	Proton-dependent oligopeptide transport	FTL_1339	C1274708A	G421G
SNP	Upstream of hypothetical protein	Upstream of FTL_0869	G849877T	N/A

Mutation of an ATP binding protein increases virulence of cells producing PriM

Because it is the single polymorphism present in only the $\Delta pmrA$ suppressor cells that alters a protein, we hypothesized that the change in FTL_0146 may allow cells lacking *pmrA* to replicate in macrophage. FTL_0146 encodes an ATP binding protein that is typically found as part of an ATP-Binding Cassette (ABC) transport system (Atkins *et al.*, 2006). ABC transport systems are common in bacteria and are used for the import and export of a wide variety of molecules, including; amino acids, short peptides, ions, iron complexes, metals, mono- and oligo- saccharides (Saurin & Dassa, 1994). These systems typically consist of three parts: a transmembrane component, an ATP binding component and a substrate binding protein (Figure 9A; Atkins *et al.*, 2006). The protein encoded by FTL_0146 is predicted to have two domains. One domain is predicted to bind ATP (amino acids 26-136) and the other is frequently found associated with proteins with ATPase domains (C-terminal AAA-associated domain, amino acids 301-419). To the best of our knowledge, the function of the C-terminal AAA-associated domain, which includes the amino acid altered by the mutation identified in $\Delta pmrA$ suppressor cells, is unknown (Figure 9B). This gene has not previously been identified as

being important for intramacrophage growth, but its ATP binding domain and the upstream ABC transporter membrane protein (FTL_0145) are essential for *in vitro* growth (Ramsey *et al.*, 2020).

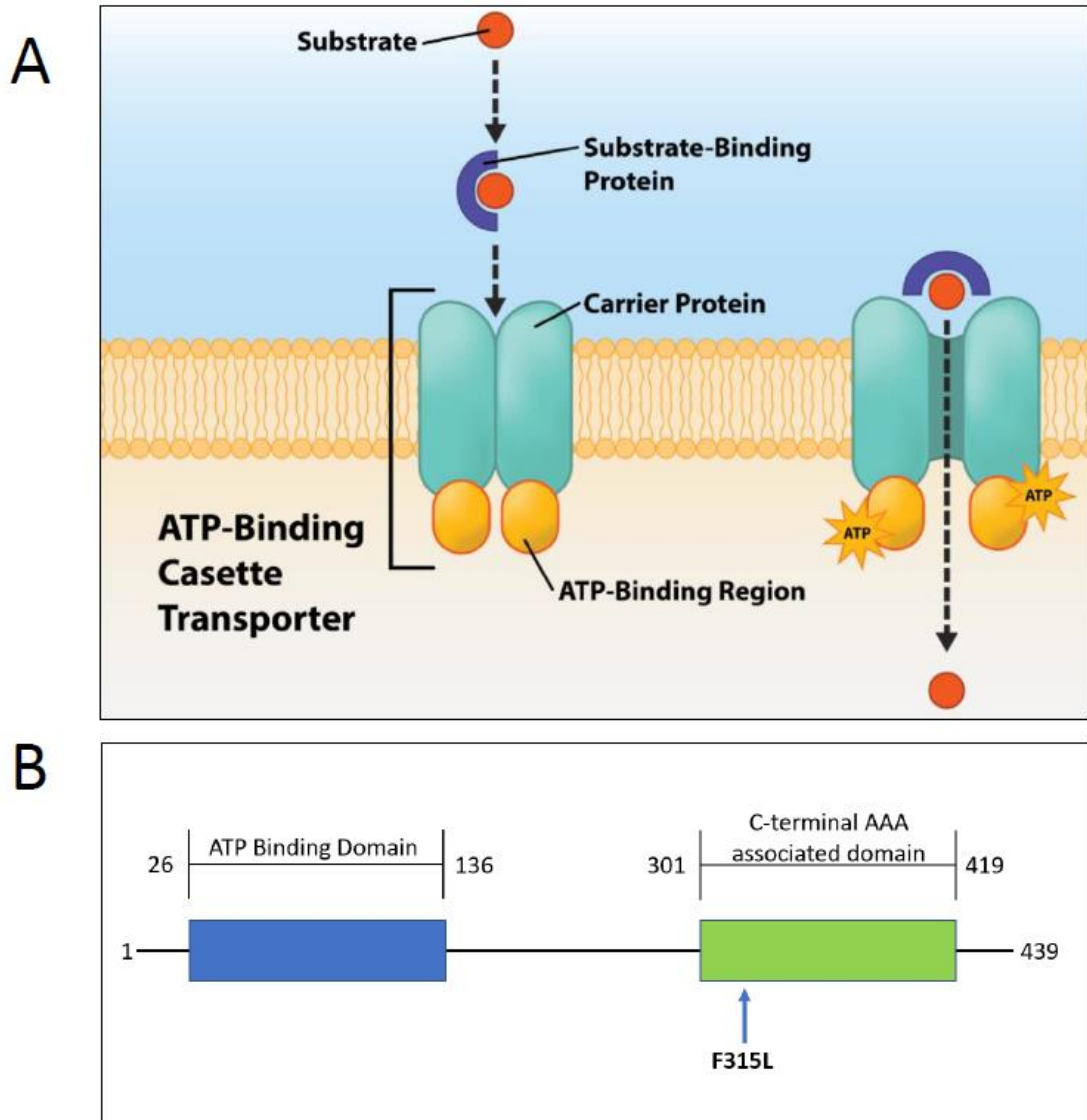


Figure 9. ABC transport systems and an ATP-binding protein from *F. tularensis*. **A.** Typical example of the process of an ABC transport system. Figure from Bruslind, General Microbiology. **B.** Predicted protein domains of the ABC transport protein encoded by FTL_0146. The location of the mutation is indicated by the blue arrow.

To evaluate the contribution of the mutation in FTL_0146 to intramacrophage growth, we recreated the F315L mutation in FTL_0146 in wildtype and $\Delta pmrA$ cells and tested the ability of the resulting cells [WT FTL_0146(F315L) and $\Delta pmrA$ FTL_0146(F315L)] to grow in intramacrophage growth assays. We found that the FTL_0146(F315L) mutation increases the ability of cells lacking *pmrA* to replicate in macrophage cells, although not quite to wild-type or $\Delta pmrA$ suppressor levels (Figure 10). It is possible that the ability of the $\Delta pmrA$ suppressor cells to replicate to wild-type levels in macrophage is due to a combination of the mutation in FTL_0146 and one or both of the other identified mutations. It is notable that the FTL_0146(F315L) mutation results in increased intramacrophage growth when present in wild-type cells. It seems that the FTL_0146(F315L) mutation contributes an intramacrophage growth advantage to the $\Delta pmrA$ suppressor cells but this advantage may not be specific to cells that lack *pmrA*.

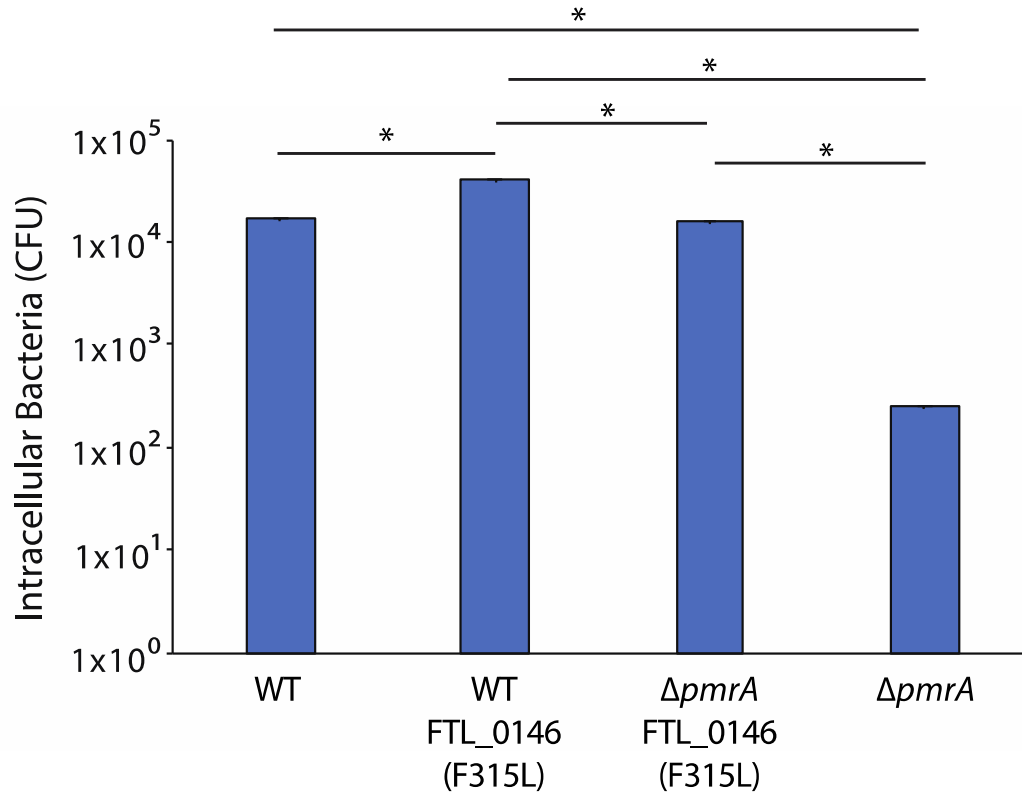


Figure 10. Intramacrophage growth assay testing FTL_0146(F315L) mutants. Survival of wild-type LVS or indicated *F. tularensis* mutant cells 24 hours after infection of macrophage. Data represents the number of bacteria recovered after 24 hours of intracellular growth. Significance of $p \leq 0.05$ is indicated by a (*) between bars where a 2-tailed *t* test was performed and otherwise was labeled as not significant (ns). Error bars represent standard deviation. Assays were performed in duplicate with representative data shown.

Transcript abundance of *pruM* is decreased in suppressor mutant strain

Having determined that the intramacrophage growth phenotype of *ΔpmrA* suppressor cells may be the result of multiple mutations, we sought to understand the molecular mechanism that allows the *ΔpmrA* suppressor cells to grow in macrophage. A simple reason for the increased ability of the *ΔpmrA* suppressor cells to grow in macrophage could be that they produce less PruM. To explore this possibility, we

examined *priM* transcript levels in $\Delta pmrA$ suppressor cells compared with $\Delta pmrA$ cells. Specifically, we used qRT-PCR to assess *priM* transcript abundance in LVS, $\Delta pmrA$, and $\Delta pmrA$ suppressor cells. Consistent with previous reports, we found that $\Delta pmrA$ cells have a 300-fold increase in *priM* transcript abundance compared to wild-type cells (Ramsey & Dove, 2016). Our comparison of *priM* transcript abundance in the $\Delta pmrA$ suppressor cells revealed that they have about 7 times less *priM* transcript than the $\Delta pmrA$ cells and only about 40-fold more *priM* transcript than wild-type cells

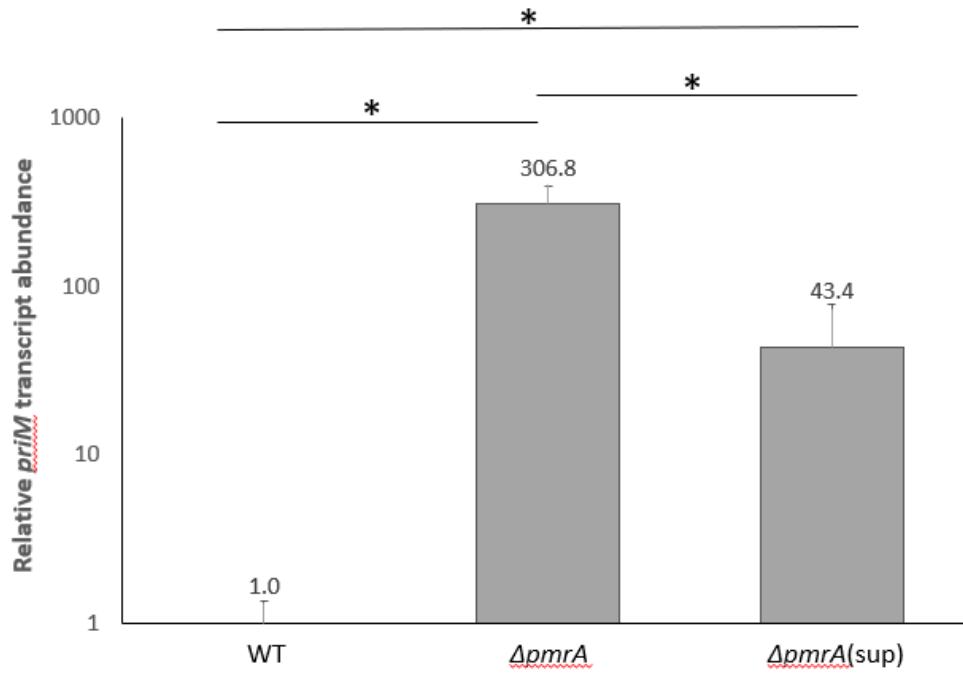


Figure 11. Quantification of *priM* transcript abundance in wildtype and indicated mutant strains by quantitative RT-PCR (qRT-PCR). Transcripts were normalized to *tul4*, whose expression is not influenced by PmrA, with wildtype (LVS) set to a value of 1. Error bars represent one standard deviation from the value, calculated using the mean threshold value. Significance of $p \leq 0.05$ is indicated by a (*) between bars where a 2-tailed T-test was performed and otherwise was labeled as not significant (ns). Data are displayed on a logarithmic scale. Experiments were performed in duplicate using triplicate biological samples; a representative data set is shown.

Protein abundance of PriM is decreased in suppressor mutant strain

After determining that levels of *prim* transcript are decreased in the $\Delta pmrA$ suppressor, we wanted to assess the impact of this lowered transcript abundance on abundance of the PriM protein. Similarly to the experiments with the PriM mutants described above, we added DNA specifying the VSV-G epitope tag to the 5' end of the *prim* gene in the $\Delta pmrA$ (sup) cells. Because PriM is extremely abundant in $\Delta pmrA$ cells, we diluted $\Delta pmrA$ PriM-V lysates fifty-fold relative to other samples to prevent signal saturation; $\Delta pmrA$ (sup) PriM-V lysates were not diluted. We found that PriM is in fact less abundant in the $\Delta pmrA$ suppressor strain than in wild-type cells lacking *pmrA* (Figure 12). This decrease in PriM protein abundance may completely or partially explain the ability of the $\Delta pmrA$ suppressor strain to replicate in macrophage to levels similar to wild-type.

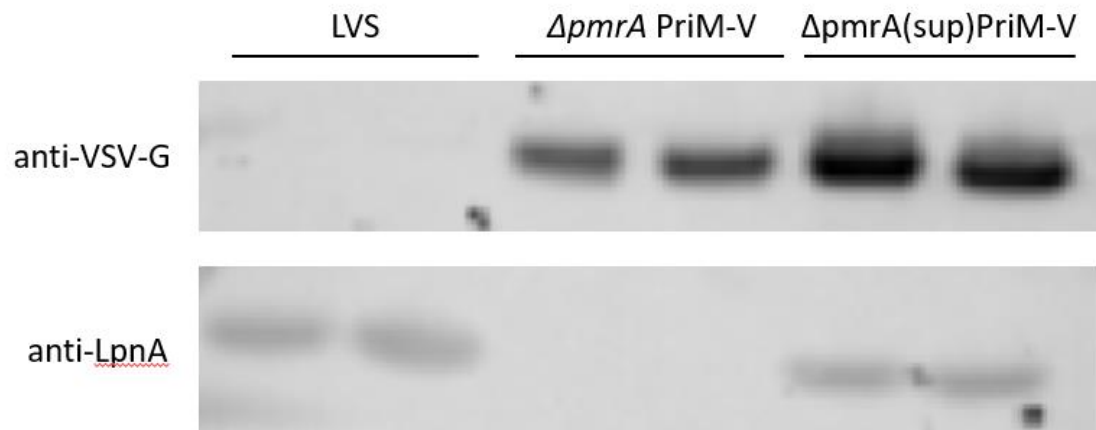


Figure 12. Relative abundance of PriM in $\Delta pmrA$ and $\Delta pmrA$ suppressor cells. The abundance of specified PriM proteins present in whole-cell lysates was determined by immunoblot using an anti-VSV-G antibody. The LpnA protein, detected with an anti-LpnA antibody, serves as a loading control. The LVS $\Delta pmrA$ PriM-V strain was diluted 50-fold. Duplicate samples are shown. Data shown is from a single experiment.

CHAPTER 4

DISCUSSION

Summary

Francisella tularensis is a highly infectious bacterial pathogen that has been classified as a potential bioweapon. Like many other bacterial pathogens, *F. tularensis* encodes an uncharacterized anti-virulence factor. Anti-virulence factors provide a unique target for anti-microbial research, as we may be able to exploit these systems to reduce or halt the virulence programs of pathogens. In understanding the role of this anti-virulence factor in *F. tularensis* we can further our knowledge of virulence regulation in this pathogen, which may apply to other pathogens as well.

While the presence of the novel *F. tularensis* anti-virulence factor PriM prevents intramacrophage replication, the exact molecular mechanism that results in this attenuation of virulence is unknown. Because the structure of the protein has been solved, we have been able to investigate specific structural elements that might provide insight into how PriM functions. We also took a genetic approach, investigating mutant cells that allow intramacrophage growth despite the presence of PriM, which we hypothesized contained mutations suppressing PriM's anti-virulence function. Our work led us to discover that the pocket region of PriM is important for its function as an anti-virulence factor, that there are additional levels of regulation of *priM*, and that modification of a

gene previously unknown to play a role in virulence allows for increased intramacrophage growth.

Results of PriM structure function analyses

In collaboration with the protein structure experts who solved the PriM structure, we identified elements of the PriM structure that we hypothesized may be key to its function as an anti-virulence factor. In particular, we identified the electropositive “tip” region, a putative small molecule binding pocket, and a disulfide bond as structural elements which might be key to PriM’s function. After studying mutations in these key features in PriM, we were able to conclude that the charged tip region of the protein and the disulfide bond are likely not essential for PriM to function as an anti-virulence factor. Modification of the potential small molecule binding pocket of PriM resulted in a partial restoration of intramacrophage replication, suggesting that this region is important for PriM as an anti-virulence factor. Although we found that the modified PriM proteins are less abundant than unmodified PriM, our results are consistent with changes in intramacrophage growth phenotypes due to specific modifications rather than loss of PriM (Figure 7).

The tip region of PriM was chosen as potentially being important for anti-virulence because it has an overall electropositive charge. This charged region could be important for an interaction with another protein or complex that aids in causing anti-virulence. Interaction sites in protein-protein interactions can rely on specific electrostatic charges and altering these charges can weaken or prevent these interactions (Reichmann *et al.*, 2007). However, after testing a mutant that altered the tip region of PriM to a

neutral charge, we found no increase in intramacrophage replication, meaning that PriM was still able to function as anti-virulence factor (Figure 3). These results suggest that an electropositively-charged tip region of PriM is not essential for a protein-protein interaction that leads to attenuated intramacrophage replication. While verifying production of the PriM tip region mutant, we noticed a decrease in the abundance of PriM as compared to wild-type PriM (Figure 7). This trend was observed for each of the PriM mutants, but the tip region mutant is the least abundant PriM mutant. One explanation for the decrease in PriM abundance in the tip mutant could be that there is decreased translation of the *priM* tip mutant mRNA due to ribosome stalling. To create the tip region mutant, we changed 13 amino acids all glycines, resulting in a 14 amino acid stretch of glycines (Table 1). Ribosome stalling can occur when the ribosome encounters a region with the same amino acid many times in a row, due to needing the same tRNA, which can slow down the rate of translation and decrease the abundance of the protein (Buskirk & Green, 2017). Although three distinct codons were used to code the stretch of 14 glycines and *F. tularensis* LVS encodes three tRNAs for glycine, it is possible that the high demand for glycine-containing tRNAs might cause ribosome stalling. Alternately, the amino acid changes made in the PriM mutants may have decreased the stability of the protein, leading to overall less abundant PriM.

Cells producing PriM (i.e., cells lacking the repressor PmrA) produce high levels of PriM. Because PriM contains an intramolecular disulfide bond, we hypothesized that PriM may function by titrating the enzyme responsible for disulfide bond creation, FipB, away from critical virulence factors that contain disulfide bonds; FipB activity is known to be essential for *F. tularensis* virulence (Qin *et al.*, 2016). We altered one of PriM's two

cysteines to an alanine, preventing the formation of intramolecular disulfide bonds (Table 1). After testing this PriM variant in intramacrophage growth assays, we did not find any recovery in intramacrophage growth, suggesting that the presence of a disulfide bond in PriM is not essential for its anti-virulence function and PriM does not function by titrating disulfide bond formation activity away from virulence factors (Figure 4). One caveat is that the mutation made in PriM targeted the formation of intramolecular disulfide bonds; it does not exclude the possibility that intermolecular disulfide bonds could be formed. Specifically, each modified PriM molecule still contains one of its two cysteines available for disulfide bond formation and it is possible that a disulfide bond would form between cysteines of two separate PriM molecules. We raise this possibility because in our immunoblots detecting mutant PriM-V (Figure 7), a band that may correspond to dimerization between PriM molecules is evident in lanes with lysates from the $\Delta pmrA$ PriM(C303A)-V cells (Figure 7). PriM(C303A)-V is expected to have a molecular mass of 56 kDa and the most intense signal corresponds to a band of that size. A dimer of PriM(C303A)-V would have a molecular mass of 112 kDa and we detect a fainter band corresponding to that molecular weight (Figure 7). Furthermore, this higher molecular weight band is not detected in any other lysate samples, suggesting it is specific to lysates containing PriM(C303A)-V. Due to the possibility of intermolecular disulfide bond formation, we cannot completely exclude our hypothesis that PriM functions by titrating away disulfide bond formation activity from other virulence factors.

The final PriM mutant examined the contribution of the potential small molecule binding pocket to PriM's anti-virulence function. Testing this PriM mutant in macrophage lead to a partial recovery of intramacrophage growth (Figure 5). Although

ΔpmrA PriM(mpk) cells do not replicate to the same level as wild-type cells in macrophage, they replicate nearly 15-fold more than cells producing wild-type PriM (*ΔpmrA* cells). These results are consistent with the idea that the changes made to the potential small molecule binding pocket in PriM are affecting its anti-virulence function. One important note to make is that we have been referring to the pocket region of PriM as a potential small molecule binding pocket because a small molecule, acetate, co-crystallized in this location. However, it is also possible that this so-called pocket region is an interaction site for a protein-protein interaction. Additionally, in verifying production of the mutant versions of PriM, we noticed that the majority of the signal corresponding to the PriM(mpk) mutant migrates about a 3 kDa lower when compared to wild-type and other PriM mutants (Figure 7). There are multiple explanations that could account for this apparent change in molecular weight. One is that there is a molecule complexed with PriM in the pocket region that can no longer bind the PriM(mpk) mutant, although this interaction would have to be strong enough to withstand the denaturing and reducing environment of SDS-PAGE. Another possibility is that the mutations that we made in this particular mutant caused mis-folding or mis-localization of the PriM protein, and a portion of the protein is degraded (Too *et al.*, 2013).

Characterizing cells that grow in macrophage despite loss of PmrA

Previous work determined that PmrA is essential for intramacrophage growth because it is necessary to repress production of *priM* (Ramsey *et al.*, 2016). During the course of these studies, we identified cells that lack the repressor PmrA and contain *priM* yet replicate to wildtype levels in macrophage (Figure 8). We refer to these cells as the

$\Delta pmrA$ suppressor strain because the intramacrophage growth defect of cells lacking PmrA is suppressed. We hypothesized that these cells regained their ability to replicate in macrophage due to a mutation in a potential PriM interaction partner essential for PriM's anti-virulence effects. Whole genome re-sequencing of wild-type, $\Delta pmrA$ and the $\Delta pmrA$ suppressor cells identified three single nucleotide polymorphisms unique to the $\Delta pmrA$ suppressor cells (Table 2). As there was only a single mutation predicted to result in a change at the protein level, we considered it the most likely mutation to cause the observed phenotypic change. This mutation changes the phenylalanine at position 315 to leucine in the protein encoded by locus FTL_0146.

We tested the contribution of the FTL_0146 F315L mutation to suppression of the $\Delta pmrA$ intramacrophage growth phenotype by re-creating the mutation in both wildtype and $\Delta pmrA$ cells. Cells lacking PmrA with the single FTL_0146 mutation [$\Delta pmrA$ FTL_0146(F315L)] had an increased ability to replicate in macrophage compared to cells lacking PmrA (more than 50-fold) but did not replicate as well as either wild-type cells or the $\Delta pmrA$ suppressor cells (Figure 10). Additionally, we found that the FTL_0146(F315L) mutation increased intramacrophage replication in not only the cells lacking PmrA, but also when present in wild-type cells (Figure 10). These results suggest that other mutations in the $\Delta pmrA$ suppressor strain contribute to the restoration of intramacrophage growth and that the mutation in FTL_0146 results in better intramacrophage replication overall, irrespective of the presence of PmrA.

The protein encoded by FTL_0146 is an ATP binding protein predicted to function as a component of an ATP-binding cassette (ABC) transport system. ABC transport systems are found in eukaryotes, archaea, and bacteria and are common in

bacteria for both import and export of a variety of molecules (Saurin & Dassa, 1994). In *F. tularensis* subspecies *tularensis*, approximately 15 functional ABC transport systems have been identified (Atkins *et al.*, 2006). The homolog of FTL_0146 in subspecies *tularensis* (FTT_0266) was predicted to be involved in nitrate transport but was also identified as being part of a nonfunctional ABC transport system (Atkins *et al.*, 2006). However, results from a transposon insertion sequencing (Tn-Seq) experiment identified FTL_0145, the predicted inner membrane transport protein associated with FTL_0146, and the ATP binding domain of FTL_0146 as essential for *in vitro* growth, suggesting that this system may be functional in LVS (Ramsey *et al.*, 2020). In addition to its ATP binding domain, FTL_0146 is predicted to have a C-terminal AAA-associated domain, the function of which is unknown. The mutation in FTL_0146 that permits increased intramacrophage growth occurs in this C-terminal AAA-associated domain (Figure 9). It is possible that the mutation in FTL_0146 provides an intramacrophage growth benefit by altering the function of the transport system. This could either directly improve intramacrophage growth or the modified function could decrease PriM production and indirectly improve intramacrophage growth.

Because it seems that multiple mutations influence the ability of the $\Delta pmrA$ suppressor strain to replicate in macrophage, we sought to further understand the molecular mechanism that permits intramacrophage growth of the $\Delta pmrA$ suppressor cells despite the presence of *priM*. We reasoned that the enhanced intramacrophage growth of these cells could be due to either a change in a pathway or interaction partner necessary for PriM function, rendering PriM ineffective at functioning as an anti-virulence factor, or a change in the amount of PriM produced. To assess the latter

possibility, we examined PriM transcript and protein abundance in the $\Delta pmrA$ suppressor cells. We found that although there is still more *priM* transcript in the $\Delta pmrA$ suppressor strain compared to wild-type, there is significantly less *priM* transcript than in the $\Delta pmrA$ strain (almost 10-fold; Figure 11). We next sought to confirm that the lower *priM* transcript abundance in $\Delta pmrA$ suppressor cells results in less PriM at the protein level. Preliminary immunoblot analysis confirmed that there is less PriM protein present in the $\Delta pmrA$ suppressor strain, is consistent with the observed lower *priM* transcript abundance (Figure 12). We have not yet determined if the observed decrease in PriM protein is sufficient to permit the increased intramacrophage growth observed in the $\Delta pmrA$ suppressor mutant. It is possible that the lower PriM abundance combined with the intramacrophage growth benefit provided by the FTL_0146 mutation could account for the large increase in intramacrophage growth observed by the $\Delta pmrA$ suppressor mutant. At this point we do not know why the levels of *priM* transcription are lower in the $\Delta pmrA$ suppressor, but it does indicate that *priM* regulation may be more complex than previously appreciated; it seems there are other factors that can control production of *priM* other than PmrA.

Future Experiments

Although we have made significant progress towards understanding PriM, many questions remain regarding the function of this anti-virulence factor. Here we will propose a number of experiments to answer some of the original questions we had as well as some new questions that surfaced during the course of this study.

Does PriM prevent disulfide bond formation in virulence factors?

We had hypothesized that PriM functions as an anti-virulence factor by preventing maturation of virulence factors, monopolizing the activity of the enzyme responsible for disulfide bond formation. We tested this hypothesis by creating a mutant that is unable to form an intramolecular disulfide bond [PriM(C303A)]. This PriM mutant would, in principle, not interact with FipB, the enzyme necessary for disulfide bond formation. However, immunoblot analyses suggest that the PriM(C303A) mutant is capable of forming intermolecular disulfide bonds. Thus, the PriM(C303A) mutant may still be interacting with FipB and potentially preventing FipB interaction with virulence factors. To address this possibility, we will create a mutant that replaces both cysteines with serines (serine is more structurally similar to cysteine than alanine) to prevent formation of both intramolecular and intermolecular disulfide bonds. By testing this mutant in intramacrophage growth assays we will be able to come to a more conclusive answer about the role of disulfide bond formation in PriM's function as an anti-virulence factor.

What proteins interact with PriM?

During the course of this study we were unable to identify any PriM interaction partners, but we did identify a region of PriM that is necessary for virulence. The mutant that we used to discover this region could provide an important tool for identifying potential interactions in that region. We would like to take a biochemical approach to address this question and immunoprecipitate direct interaction partners of PriM and PriM(mpk). If we find proteins that IP with PriM and not the pocket mutant, they would

be candidates for interaction partners that are important for anti-virulence. One reason this experiment may cause some difficulties is that PriM is found in the membrane fraction of the cell and must be immunoprecipitated from that fraction, which is insoluble, however this type of experiment has been successfully performed (Srivastava *et al.*, 2017).

How do the mutations in the $\Delta pmrA$ suppressor strain allow replication in macrophage?

We observed a decrease in PriM transcript abundance in the $\Delta pmrA$ suppressor strain but have not yet identified what is causing this decrease. One way to answer this question is to examine the levels of PriM transcript in the $\Delta pmrA$ FTL_0146(F315L) mutant to determine if the mutation in FTL_0146 plays a role in decreasing the amount of PriM being produced, or if another change in the $\Delta pmrA$ suppressor strain causes this decrease. Furthermore, it is still not clear which mutations in the $\Delta pmrA$ suppressor strain are responsible for the increase in intramacrophage growth. After recreating the FTL_0146 mutation in cells lacking PmrA, we found increased intramacrophage growth, but the increase did not reach the level of the $\Delta pmrA$ suppressor strain. To examine the role of the other identified mutations in the $\Delta pmrA$ suppressor strain, we will recreate the other two mutations identified in the $\Delta pmrA$ suppressor and test their contribution to increased survival in intramacrophage growth assays.

Can we identify direct interaction partners or pathways important for PriM function genetically?

One of our goals was to identify genes important for PriM to function as an anti-virulence factor. Originally we planned to take a genetic approach, creating a strain with an extra copy of *priM*, mutagenize this strain with a transposon, and identifying mutants able to replicate in macrophage, presumably due to inactivation of a PriM interaction partner. This approach requires starting with cells containing an extra copy of *priM*, as otherwise the majority of recovered mutants would contain a transposon insertion in *priM*. Unfortunately, we were unable to create the strain with two copies of *priM*. Given these technical difficulties, we will take an alternate approach, performing Tn-Seq on the $\Delta pmrA$ cells grown *in vitro* in comparison with those grown in macrophage, analogous to a recently-published experiment (Ramsey *et al.*, 2020). Tn-Seq would allow us to identify all mutants that permit intramacrophage growth in cells lacking PmrA (including those in PriM) and quantify their relative abundance, providing a genome-wide screen of factors necessary for PriM to function as an anti-virulence factor.

Why does the mutation in the ATP-binding protein FTL_0146 allow for increased intramacrophage growth?

Finally, we would like to determine why the mutation of the ATP-binding protein FTL_0146 permits increased intramacrophage growth. We found that the mutation in FTL_0146 present in the $\Delta pmrA$ suppressor strain provided an intramacrophage growth benefit when recreated in both the wildtype background as well as the $\Delta pmrA$ background, suggesting that it provides a growth advantage to cells growing in

macrophage regardless of the presence or absence of PmrA. It is possible that the mutation in FTL_0146 alters the efficiency of substrate transport; however, the substrate for the predicted ABC transport system comprised of FTL_0145 and FTL_0146 is unknown. To investigate the function of this transport system, we will identify interaction partners of this putative ABC transport system by immunoprecipitating each known component (the proteins encoded by FTL_0145 and FTL_0146) and identify co-purifying proteins by mass spectrometry. Ideally this experiment will shed light on the role of this ABC transport system in LVS.

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