

Title: Investigating the structure and function of a novel bacterial anti-virulence factor

Statement of the problem:

Francisella tularensis is a Gram-negative, facultative intracellular bacterial pathogen and is the causative agent of the potentially fatal human disease tularemia (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). During infection, *F. tularensis* evades the host immune system in part by replicating inside host cells; macrophage are thought to be a key niche. *F. tularensis* encodes virulence factors essential for intramacrophage growth, such as the transcription factor PmrA (Mohapatra *et al.*, 2007; Sammons-Jackson *et al.*, 2008). The primary function of PmrA is to repress expression of a novel anti-virulence factor called PriM (Ramsey & Dove, 2016). Other pathogens encode anti-virulence factors, but the role of anti-virulence factors in pathogenesis is still incompletely understood (Brown *et al.*, 2016). The anti-virulence factor PriM does not have clear sequence or structural homology to other studied proteins and how PriM functions to prevent intramacrophage replication is unknown. To elucidate the molecular mechanism by which PriM inhibits intramacrophage growth, we will take several approaches. By creating targeted mutations guided by the structure of PriM, we will assess how specific structural features contribute to intramacrophage replication. We will also identify genes that are critical for the function of PriM by performing a genetic selection. By increasing our understanding of bacterially-encoded anti-virulence pathways, we may be able to identify novel targets for anti-microbial therapeutics.

Overall goal: The overall goal of this research is to identify the molecular mechanism by which PriM functions to prevent intramacrophage growth.

Specific Aim #1: Identify critical structural features of PriM. Using a crystal structure solved by our collaborators, we have identified structural features of PriM that may be important for its function. We will use an allelic exchange protocol to create mutations targeting these structural features in both the wildtype strain as well as the $\Delta pmrA$ background and test the ability of these mutants to replicate in macrophage.

Specific Aim #2: Identify factors critical to the function of PriM. We will create a transposon mutant library in cells lacking *pmrA* that contain an additional copy of the *priM* gene. We will use this library in an intramacrophage growth assay and select for mutants that can replicate in macrophage, despite the production of PriM, to identify a list of genes critical to the function of PriM.

Justification for the Study:

The molecular mechanisms used by *F. tularensis* to cause disease are still incompletely understood. However, it is clear that survival and replication in macrophage is critical for infection. Many genes are necessary to ensure successful infection and replication of *F. tularensis* in macrophage. One gene identified as essential for virulence is *pmrA*, which encodes a transcription factor. When PmrA is absent, cells no longer have the ability to replicate inside

macrophage, and therefore cannot cause disease (Mohapatra *et al.*, 2007; Sammons-Jackson *et al.*, 2008). Cells lacking *pmrA* are unable to replicate in murine macrophage models as well as in mouse models (Mohapatra *et al.*, 2007; Sammons-Jackson *et al.*, 2008; Ramsey and Dove, 2016). PmrA is essential for virulence primarily because it acts as a repressor of the anti-virulence factor PriM (Ramsey & Dove, 2016). Transcript levels of *priM* increase approximately 300-fold in the absence of PmrA (Figure 1) (Ramsey & Dove, 2016).

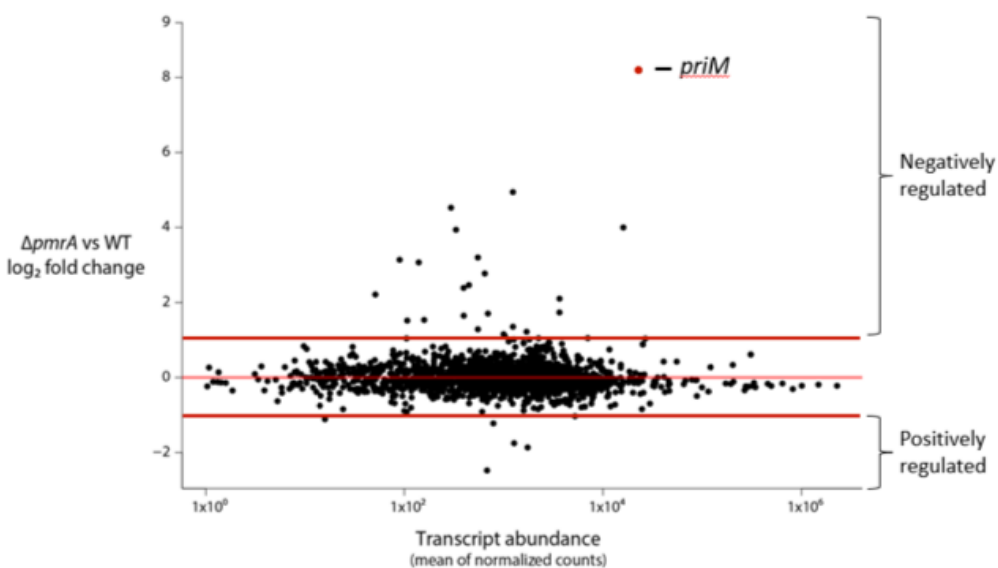


Figure 1.

Transcriptomic analysis comparing wild-type cells and cells lacking *pmrA* using RNA-Seq. Points represent individual genes (Ramsey & Dove, 2016).

ChIP-Seq analysis of PmrA identified enrichment at the *priM* promoter, suggesting that PmrA is directly repressing transcription of *priM* (Ramsey & Dove, 2016). Additionally, *F. tularensis* cells lacking both *pmrA* and *priM* regain the ability to replicate within macrophage (Figure 2) (Ramsey & Dove, 2016).

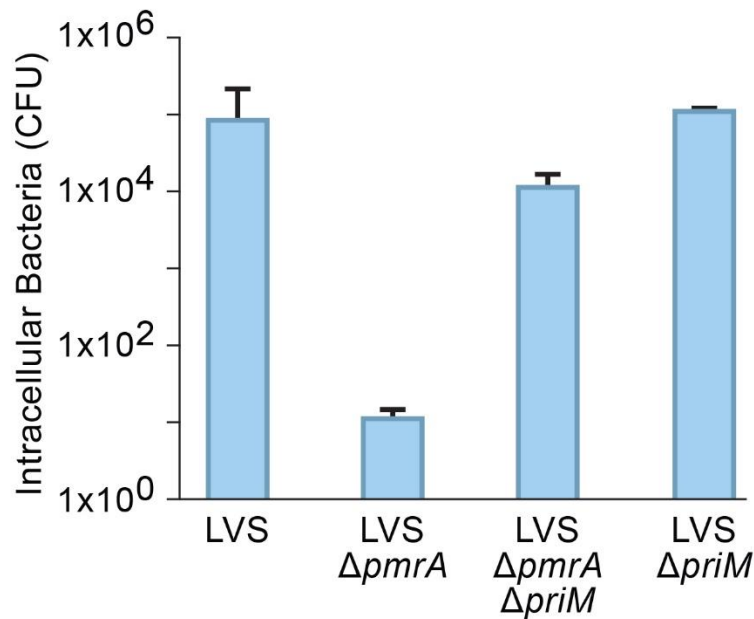


Figure 2.

Survival of wild-type Live Vaccine Strain (LVS) or indicated *F. tularensis* mutant cells 24 hours after infection of J774A cells (Ramsey & Dove, 2016).

Finally, production of full-length PriM is necessary to prevent intramacrophage replication and in cells that contain PmrA, ectopic production of PriM inhibits intramacrophage replication (Ramsey & Dove, 2016). These results suggest that the production of the anti-virulence factor PriM is the primary reason that cells lacking *pmrA* are unable to replicate within macrophage, although the mechanism is unknown.

While many studies investigate virulence factors, it is also important to consider anti-virulence factors to fully understand bacterial pathogenesis (Brown *et al.*, 2016). Anti-virulence factors can play a key role in pathogenesis of some species. Some anti-virulence factors have been found to regulate production or release of virulence factors in the context of infection

(Yeom *et al.*, 2018). PriM is the first anti-virulence factor identified in *F. tularensis* and our collaborators have solved a structure of PriM using X-ray crystallography (Figure 3).

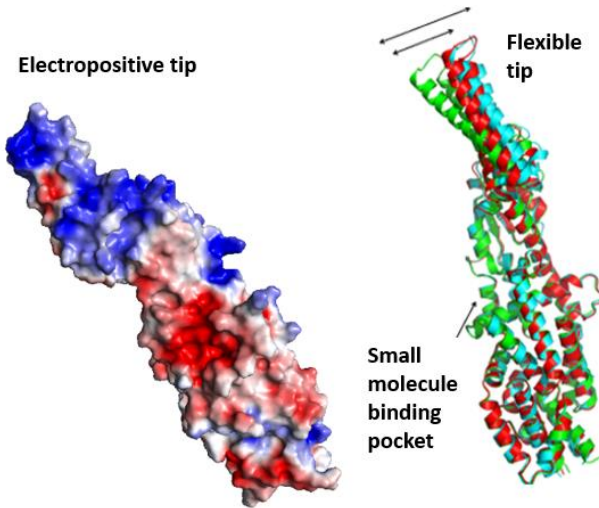


Figure 3.

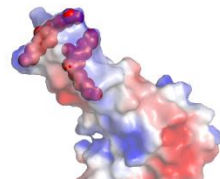
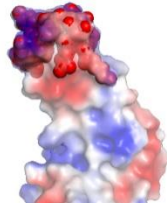
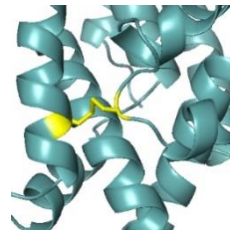
Representation of the crystal structure of the PriM protein. This structure has been described as a novel fold and highlights several key features. (Unpublished data, Dr. Maria Schumacher, Duke University Department of Biochemistry)

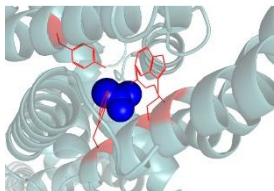
The structural model of PriM reveals a novel fold with no known structural homology currently found in the Protein Data Bank. A PriM monomer is approximately 116 angstroms in length with a long coil-coil domain and flexible, electropositive tip. Additional features include a disulfide bond between two cysteines and bound acetate, which co-crystallized with PriM. The bound acetate may indicate the presence of a binding pocket. To examine which of these features are key to the function of PriM as an anti-virulence factor, we will engineer mutations targeting these features. These mutations, presented in Table 1, will be constructed in both wild-type LVS cells and cells lacking the repressor *pmrA* (i.e., cells producing PriM) and then

evaluated in a macrophage infection assay. Cells expressing PriM mutant proteins that are able to replicate in macrophage will contain a mutation in a region of PriM that is essential for its function as an anti-virulence factor.

Table 1.

Overview of the PriM mutants to be created and tested.

Name	Purpose	Description	Illustration
PriM(mtip1)	Changes the tip region from an electropositive charge to electronegative	Mutations in the tip region: two lysine doublets (colored as red spheres) mutated to glutamic acid doublets	
PriM(mtip2)	Changes the tip region from an electropositive charge to neutral charge	Mutations in the tip region: all amino acids in tip region (colored as red spheres) mutated to glycines	
PriM(noC1/C2)	Removes 1 of the 2 cysteines to prevent disulfide bond production	Mutations changing the cysteines (colored in yellow) to serine.	

PriM(mpk1)	Prevents small molecules from binding in the pocket region	Mutations in the small molecule binding pocket region: arginine, two tryptophans and one tyrosine to alanines (acetate molecule in the potential binding pocket colored in blue)	
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To identify pathways critical for the function of PriM or potential interaction partners, we will take a genetic approach. Specifically, we will perform transposon mutagenesis to randomly inactivate genes in the genome using a strain that contains an extra copy of *priM*. Resulting mutants will be pooled and screened in the intramacrophage replication assay. Using a strain that contains the extra copy of *priM* is useful for the mutagenesis because it will eliminate any mutants that rescue virulence due to a transposon insertion in *priM*. Mutants that regain the ability to replicate in macrophage will be identified by arbitrary PCR and direct sequencing of loci flanking the transposon insertion site, thereby identifying the gene that was inactivated. We expect to identify factors necessary for the function of PriM as an anti-virulence protein that will help to elucidate the These genes that are identified may encode proteins with known functions or even display sequence/structural homology that could allow us to identify part of the molecular mechanism PriM is using during intramacrophage replication.

Methodology & Procedures:

Research to be conducted for this project using *Francisella tularensis* subspecies *holarctica* live vaccine strain (LVS) has been approved by The University of Rhode Island Institutional Biosafety Committee (IBC), number BI1920-001.

Bacterial Growth Conditions

F. tularensis LVS will be grown at 37°C aerobically in Mueller-Hinton Broth supplemented with 0.025% iron pyrophosphate, 0.1% glucose, and 2% Isovitalex or on cysteine heart agar plates containing 1% hemoglobin (CHAH) for 24 hours or until single colonies appear. When appropriate, kanamycin will be added to the CHAH plates at a concentration of 5 µg/mL. *E. coli* strain XL-1 Blue will be grown aerobically in lysogeny broth (LB) at 37°C or on LB agar plates. When appropriate, kanamycin will be added to the LB or LB agar at a concentration of 50 µg/mL.

Plasmid Design and Construction

Plasmids and primers will be designed using SnapGene (GSL Biotech LLC). Primers (ThermoFisher) will be used in PCR to amplify the desired product. PCR products will be digested using restriction enzymes and ligated into the pEX18Kan vector (Charity *et al.*, 2007). Plasmids will be transformed into XL-1 Blue chemically competent *E. coli* and purified using Qiagen QIAprep Spin Miniprep kits. Plasmids sequences will be confirmed by Sanger sequencing at the URI Genomic Sequencing Center.

Strain Construction

An allelic exchange protocol will be used to create markerless mutations in LVS. Plasmids will be electroporated into electrocompetent LVS cells (both wild-type and $\Delta pmrA$ cells) using the

following settings in a 2mm cuvette: 2.5 kV, 25 μ F, and 600 Ω . After electroporation the cells will be allowed to recover for 4-8 hours and cells containing a homologous recombination event between the integration vector and the plasmid (primary integrants) will be selected for by plating transformations on CHAH plates with 5 μ g/mL kanamycin. Primary integrants will subsequently be grown overnight on CHAH plates without antibiotic, resuspended in 1x PBS, and serial dilutions will be plated onto CHAH plates containing 10% sucrose. The sucrose-resistant colonies, which should lack the *sacB* gene encoded on the pEX18kan plasmid backbone, will be cross patched on CHAH plates and CHAH plates with kanamycin to validate the loss of the kanamycin resistance. Colony PCR, potentially followed by a diagnostic digest, will be used to confirm the mutation and patches containing validated mutants will be purified to single colonies on CHAH plates. Isolated single colonies will be cross patched to CHAH plates and CHAH plates with kanamycin to confirm loss of kanamycin resistance. The same colony PCR will be used to verify the mutation. Strains will be validated by isolating gDNA and the region including the desired mutation will be sequenced by Sanger sequencing at the URI Genomic Sequencing Center.

Macrophage Infection Assay

Cells of LVS derivatives will be tested in a macrophage infection assay to assess their ability to survive and replicate inside macrophage. J774A.1 murine macrophage-like cells, cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum grown at 37°C with 5% CO₂, will be seeded into two 96-well tissue culture-treated plate, plating

approximately 2×10^4 cells/well. In each assay, four strains will be tested, specifically the wild-type strain (LVS), the negative control ($\Delta pmrA$), and the two strains with the same *PriM* mutation in both the LVS background and the $\Delta pmrA$ background. 16-18 hours after seeding the macrophage, bacteria will be added to triplicate wells at a MOI (multiplicity of infection) of approximately 5. After two hours, any remaining bacteria will be removed, the wells will be washed twice with sterile 1x PBS, and DMEM with 10 $\mu\text{g/mL}$ gentamicin will be added to ensure that no bacteria outside the macrophage remain. Two hours after washing, one of the 96-well plates will be removed from the incubator, washed twice with PBS, and 200 μL of 1% saponin in 1x PBS will be added. Plates will be incubated at room temperature for 30 minutes to allow lysis of the macrophage and 50 μL will be plated on CHA plates in duplicate for enumeration. Twenty-four hours after washing, the remaining 96-well plate will be removed from the incubator and undergo the same lysis protocol. The saponin solution containing bacteria will be diluted in 1x PBS and the dilutions will be plated onto CHAH plates in duplicate for enumeration. The resulting colonies will be counted to assess intramacrophage replication.

Transposon Mutagenesis

An additional copy of the *priM* gene will be introduced into cells lacking *pmrA* background (*priM*(+) cells), such that inactivation of a single copy will not allow intramacrophage replication. The pSD26 plasmid, containing the gene encoding a mariner transposase and transposon with a kanamycin resistance gene (Charity et al., 2009), will be electroporated into *priM*(+) cells. Bacteria containing a single transposon insertion will be selected for by plating onto CHAH containing 5 $\mu\text{g/mL}$ kanamycin. The resulting colonies will be pooled, creating a library of transposon mutants. This pool of transposon mutants will be used in a macrophage

infection assay to identify the mutants that are able to replicate in macrophage. These transposon mutants that are able to replicate in macrophage will be isolated. The location of the transposon insertions will be identified using arbitrary PCR and Southern blotting will be used to verify that there was only a single transposon insertion. The importance of a particular genetic region for survival in macrophage in the presence of PriM will be validated by creating clean deletion mutants. These regions would represent genes encoding potential interacting partners or components of pathways essential to the function of PriM.

Resources Required:

Resources required for the completion of this project will come from the laboratory of Dr. Kathryn Ramsey. Sequencing services will be provided by the URI Genomic Sequencing Center.

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