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A response regulator promotes *Francisella tularensis* intramacrophage growth by repressing an anti-virulence factor

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Summary

The orphan response regulator PmrA is essential for the intramacrophage growth and survival of *Francisella tularensis*. PmrA was thought to promote intramacrophage growth by binding directly to promoters on the Francisella Pathogenicity Island (FPI) and positively regulating the expression of FPI genes, which encode a Type VI secretion system required for intramacrophage growth. Using both ChIP-Seq and RNA-Seq we identify those regions of the *F. tularensis* chromosome occupied by PmrA and those genes that are regulated by PmrA. We find that PmrA associates with 252 distinct regions of the *F. tularensis* chromosome, but exerts regulatory effects at only a few of these locations. Rather than by functioning directly as an activator of FPI gene expression we present evidence that PmrA promotes intramacrophage growth by repressing the expression of a single target gene we refer to as *priM* (PmrA-repressed inhibitor of intramacrophage growth). Our findings thus indicate that the role of PmrA in facilitating intracellular growth is to repress a previously unknown anti-virulence factor. PriM is the first bacterially-encoded factor to be described that can interfere with the intramacrophage growth and survival of *F. tularensis*.

Graphical Abstract

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Author Contributions

Conceived and designed the experiments: KMR SLD. Performed the experiments: KMR. Analyzed the data: KMR SLD. Wrote the paper: KMR SLD.

Introduction

Francisella tularensis is a Gram-negative facultative intracellular bacterium and the causative agent of the potentially fatal disease tularemia (Sjöstedt, 2007). The ability of *F. tularensis* to cause disease is strictly dependent on its ability to survive and replicate within macrophage (Gray *et al.*, 2002; Nano *et al.*, 2004; Sjöstedt, 2007; Barel and Charbit, 2013). A locus comprising the Francisella Pathogenicity Island (FPI) is critical to the ability of *F. tularensis* to grow within macrophage (Nano *et al.*, 2004; Larsson *et al.*, 2005; Nano and Schmerk, 2007). Recent studies have demonstrated that the FPI encodes a secretion system related to the type VI secretion system found in *Pseudomonas*, *Vibrio*, and *Bacteroidetes* species (Barker *et al.*, 2009; Bröms *et al.*, 2010; Russell *et al.*, 2014; Clemens *et al.*, 2015).

Expression of genes on the FPI is positively regulated by the transcription regulators MglA, SspA, and PigR (Lauriano *et al.*, 2004; Brotcke *et al.*, 2006; Charity *et al.*, 2007; Brotcke and Monack, 2008; Charity *et al.*, 2009; Rohlfing and Dove, 2014; Ramsey *et al.*, 2015). MglA and SspA are homologs of *E. coli* SspA and form a heteromeric complex that associates with RNA polymerase (RNAP) (Baron and Nano, 1998; Lauriano *et al.*, 2004; Charity *et al.*, 2007). PigR is a putative DNA-binding protein that interacts with the RNAP-associated MglA-SspA complex; interaction between PigR and the MglA-SspA complex is necessary for these three regulators to coordinately control the expression of approximately 100 genes, including those found on the FPI (Brotcke and Monack, 2008; Charity *et al.*, 2009; Rohlfing and Dove, 2014). While MglA, SspA, and PigR are found at essentially all promoters in *F. tularensis*, they exert positive effects only at those that contain a specific sequence motif referred to as the PigR response element, or PRE (Ramsey *et al.*, 2015). Examples of promoters that contain a PRE include the *iglA* and *pdpA* promoters present on the FPI (Ramsey *et al.*, 2015). Cells lacking PigR, MglA, or SspA are avirulent, highlighting the importance of these three transcription regulators in the coordinate control of virulence gene expression (Brotcke and Monack, 2008; Wehrly *et al.*, 2009; Charity *et al.*, 2009).

The ability of *F. tularensis* and the closely related *F. novicida* to survive and replicate within macrophage is also dependent on the response regulator PmrA (Mohapatra *et al.*, 2007; Sammons-Jackson *et al.*, 2008). PmrA is considered an orphan response regulator as there is no sensor kinase encoded in the same putative operon as *pmrA*. Initial studies in both *F. novicida* and in the *F. tularensis* live vaccine strain (LVS), indicated that PmrA influences the expression of 64–148 genes, exerting both positive and negative effects (Mohapatra *et al.*, 2007; Sammons-Jackson *et al.*, 2008). Subsequent studies in *F. novicida* also showed that PmrA (otherwise known as QseB) can positively regulate the expression of genes encoding a diguanylate cyclase and a phosphodiesterase that influence biofilm formation by modulating the concentration of cyclic di-GMP (Durham-Colleran *et al.*, 2010; Zogaj *et al.*, 2012).

Although it is not known which of PmrA's regulatory activities are required for intramacrophage growth, it has been suggested that the ability of PmrA to positively regulate the expression of genes on the FPI is responsible (Mohapatra *et al.*, 2007; Bell *et al.*, 2010; Dai *et al.*, 2011). Indeed, one model that has been proposed to account for the regulatory effects of both PmrA and the MglA-SspA complex on FPI gene expression specifies that PmrA binds directly to FPI promoters and stimulates transcription by interacting with the

indicative of PmrA functioning as a repressor at these locations by occluding RNAP. However, of the PmrA-associated regions not associated with promoters, approximately 47% are found entirely within coding regions, locations which are less likely to contain promoters than intergenic regions. We note that 32.5% of all PmrA ChIP-Seq peaks are within genes, and of these intragenic peaks, 18.2% are associated with promoters.

PmrA is not located at the FPI promoters in LVS

It has been suggested that PmrA positively regulates the expression of genes on the FPI by binding immediately upstream of promoters on the FPI and interacting directly with the RNAP-associated MglA-SspA complex (Mohapatra *et al.*, 2007; Bell *et al.*, 2010; Dai *et al.*, 2011). ChIP-Seq with PmrA revealed that PmrA associates with three regions within the FPI, one immediately upstream of *pdpE*, one in the middle of *pdpC*, and one upstream of *pdpB* (Fig. 2). We do not detect any association of PmrA with the *iglA* or *pdpA* promoters (Fig. 2), suggesting that in *F. tularensis* LVS PmrA does not bind directly to either of the two principal promoters on the FPI.

To identify the locations at which PmrA and the MglA-SspA complex may interact on a genome-wide basis, we compared the locations of PmrA (as determined by ChIP-Seq) with the previously published locations of MglA and SspA, as well as with the locations of PigR, a transcription regulator that has been shown to directly interact with the MglA-SspA complex to control gene expression (Brotcke and Monack, 2008; Charity *et al.*, 2009; Rohlfing and Dove, 2014; Ramsey *et al.*, 2015).

As illustrated in Figure 1A, MglA and PigR are found ubiquitously at essentially all promoters across the genome (Ramsey *et al.*, 2015). PmrA is frequently found at locations which are not identified as promoters (Fig. 1B and 1C). PmrA is rarely found at the same locations as MglA and PigR (Fig. 1B and 1C). Indeed, only at 26% of those regions that are occupied by PmrA do we detect MglA and/or SspA at the same location on the chromosome (Fig. 1B and 1D). Additionally, MglA and PigR are found at the *iglA* and *pdpA* promoters but PmrA is not (Fig. 2) (Ramsey *et al.*, 2015). Thus, under the conditions of our experiments, PmrA does not occupy either of the principal promoters on the FPI.

Few PmrA sites are associated with direct regulatory effects

In order to determine if all of the locations at which we detect PmrA by ChIP-Seq represent sites at which PmrA controls gene expression, we performed a transcriptomic analysis on cells lacking PmrA. We generated a strain with an in-frame deletion of *pmrA* (LVS *pmrA*) and used RNA sequencing (RNA-Seq) to compare transcript abundance in LVS *pmrA* mutant cells to wild-type cells. We identified 14 annotated genes that are positively regulated by PmrA by a factor of 2 or more (Table 1), and identified 24 annotated genes that are repressed by PmrA by a factor of 2 or more (Table 2). Combining our ChIP-Seq and RNA-Seq data, we find that of the 252 locations at which we detect PmrA, the transcript abundance of associated genes is affected by the loss of PmrA at only 18 (7%) of these sites. (Genes are considered to be associated with a ChIP-Seq peak if the translation start site is within 600 bp upstream to 1 kb downstream of the position of maximal enrichment.) These

findings indicate that at most of the regions that are occupied by PmrA, PmrA is not functioning to directly influence gene expression, at least under our experimental conditions.

PmrA mainly functions as a repressor

Of the 24 genes negatively regulated by PmrA, 21 are either associated with a PmrA site or are in a putative operon associated with a PmrA site (Table 2). Indeed, at the majority of sites (16 of 18) where PmrA appears to exert a regulatory effect on the expression of associated genes, PmrA functions as a negative regulator. This indicates that when PmrA directly controls the expression of a particular gene, it generally functions as a repressor. It is notable that two genes repressed by PmrA, FTL_1219 and FTL_0881, are known to be positively regulated by MglA and SspA (Table 2) (Brotcke *et al.*, 2006; Charity *et al.*, 2007). PmrA and the MglA-SspA complex therefore appear to exert opposite effects on the expression of some genes.

PmrA exerts its largest effect as a negative regulator of the hypothetical gene FTL_0702, which we have named *priM* (PmrA-repressed inhibitor of intramacrophage growth). The loss of PmrA results in an approximately 300-fold increase in *priM* transcript abundance (Table 2; Fig. 3A). In LVS *pmrA* mutant cells, ectopic expression of *pmrA* from a heterologous promoter resulted in repression of *priM* expression as analyzed by qRT-PCR (Fig. 3B). Furthermore, of the 252 regions found to be occupied by PmrA using ChIP-Seq, the putative promoter of *priM* is the region with the highest degree of enrichment of PmrA (over 500-fold) (Fig. 3A). Taken together, the significant occupancy of PmrA we observe at the putative *priM* promoter and the large increase in *priM* transcript abundance we observe in the absence of PmrA suggests that PmrA directly represses the expression of *priM*.

In our transcriptomic analyses, we did not detect changes in FPI gene expression in cells lacking PmrA, indicating that PmrA does not appreciably regulate FPI gene expression under the conditions of our experiment. Of the 14 genes we found to be positively regulated by PmrA, those most strongly influenced are found directly downstream of *pmrA* (FTL_0552) in a putative operon. Specifically, transcripts of the *lepB* (FTL_0553) and *rnc* (FTL_0554) genes exhibit the largest decrease in abundance in LVS *pmrA* mutant cells when compared to wild-type cells (3.9- and 6-fold, respectively) (Table 1). Although it has been reported that purified PmrA binds the *pmrA* promoter *in vitro* (Bell *et al.*, 2010), we did not detect any association between PmrA and the *pmrA* promoter *in vivo* by ChIP-Seq (Supporting Information Fig. S1). Moreover, unlike the effects of a *pmrA* deletion on *priM* expression, we found that the effects of a *pmrA* deletion on *rnc* transcript abundance could not be complemented by ectopic expression of *pmrA* (Supporting Information Fig. S2). These findings suggest that PmrA does not directly control the expression of the *pmrA* operon (which includes *lepB* and *rnc*), and that the decrease in expression of *lepB* and *rnc* that we observe in cells of the *pmrA* mutant are likely the result of polar effects of the *pmrA* deletion.

Of the remaining 12 genes which we found to be positively regulated by PmrA, we find only three genes which are associated with regions of PmrA enrichment (Table 1). These findings suggest that in LVS PmrA does not function directly to control expression of the majority of positively-regulated genes.

Repression of *priM* by PmrA is required for intramacrophage growth

It has been proposed that PmrA is required for intramacrophage growth because it directly positively regulates the expression of genes on the FPI (Mohapatra *et al.*, 2007; Bell *et al.*, 2010; Dai *et al.*, 2011). However, our ChIP-Seq studies indicate that PmrA does not occupy either of the two principal promoters on the FPI, and our RNA-Seq studies indicate that PmrA does not influence the expression of FPI genes. We therefore wondered whether cells lacking PmrA might be unable to replicate within macrophages due to the de-repression of *priM* expression that occurs in these cells. In order to test this hypothesis, we used a macrophage infection model. The results depicted in Figure 4A show that cells of the LVS *pmrA* mutant strain are attenuated for intramacrophage growth by about four orders of magnitude compared to wild-type LVS cells in murine macrophage-like J774A.1 cells. Cells of a LVS *pmrA priM* double mutant strain were attenuated only by one order of magnitude, indicating that deletion of *priM* in cells of a *pmrA* mutant enhances intramacrophage survival by three orders of magnitude (Fig. 4A). The loss of *priM* alone (i.e. in *pmrA*⁺ cells) did not affect the ability of bacteria to survive and replicate within macrophage (Fig. 4A). Our results are not due to differences in bacterial uptake, as equivalent numbers of cells of LVS wild-type, *pmrA* mutant, *pmrA priM* double mutant, and *priM* mutant were recovered from infected macrophage after 2 hours of infection (Supporting Information Fig. S3A). Cells of the LVS wild-type, *pmrA* mutant, *pmrA priM* double mutant, and *priM* mutant grew indistinguishably from one another *in vitro*, indicating that any differences in intramacrophage growth could not be attributed to differences in growth *in vitro*. These findings suggest that the attenuation of cells lacking PmrA is attributable, for the most part, to *priM*, which we propose is an anti-virulence factor.

We considered the possibility that deletion of *priM* might confer a non-specific growth advantage to attenuated strains in the macrophage infection model. However, we found that the rescue of intramacrophage growth after deletion of *priM* is specific to cells of a *pmrA* mutant; deletion of *priM* does not result in an increase in the ability of cells of a *pigR* mutant to replicate within macrophage (Fig. 4B).

Our RNA-Seq studies reveal that expression of *mreA* (FTL_0701) is increased in cells lacking *pmrA* (Table 2, Fig. 3A). The gene encoding MreA, which is on the plus strand, is located upstream of *priM*, which is encoded on the minus strand; the two genes are transcribed convergently (Fig. 3A). The increase in *mreA* transcript abundance in cells of a *pmrA* mutant appears to be largely due to reads mapping to its 3' end (Fig. 3A). To test whether the derepression of *priM* expression that occurs in the absence of PmrA might influence intramacrophage growth through an effect on the expression of *mreA*, we generated a mutant of *mreA* in which we introduced a stop codon at position 32 (LVS *mreA(S)*), which should prevent production of the full length MreA. Introduction of a stop codon into *mreA* had no effect on the ability of cells to infect or survive and replicate within macrophage, and did not rescue the attenuation of a *pmrA* mutant (Fig. 4C; Supporting Information Fig. S3B). These findings suggest that PmrA does not influence intramacrophage growth through MreA.

We next asked if it is the production of PriM, rather than the RNA encoded by *priM*, that is responsible for the intramacrophage growth defect of *pmrA* mutant cells. In order to answer this question, we generated a mutant of *priM* with a stop codon at position 17, which should prevent production of the full-length PriM protein. Cells with the stop codon at position 17 in *priM* (LVS *priM*(S)) infect and grow as well in macrophage as wild-type cells (Fig 4D; Supporting Information Fig. S3C), similar to cells of a *priM* mutant. However, cells of a *pmrA* mutant that contain a stop codon at position 17 in *priM* behave indistinguishably from cells of the LVS *pmrA priM* double mutant (Fig. 4D; Supporting Information Fig. S3C). These findings are consistent with the idea that it is the protein product of *priM* that is inhibitory to intramacrophage growth.

We next asked if production of PriM alone is sufficient to prevent the intramacrophage growth of *F. tularensis*. Wild-type cells expressing *priM* from the heterologous promoter on plasmid pF2 infect macrophage as well as wild-type cells, but are attenuated for intramacrophage growth approximately 10-fold in comparison to wild-type cells containing an empty vector (Fig. 4E; Supporting Information Fig. S3D). These wild-type cells in which PriM is synthesized ectopically grow indistinguishably from wild-type cells containing the empty vector *in vitro*, indicating that the effect of ectopic synthesis of PriM on intramacrophage growth cannot be attributed to any differences in growth rate observed *in vitro*. These findings indicate that production of PriM, in the absence of any other changes which may occur as a result of deletion of *pmrA*, is sufficient to inhibit intramacrophage growth.

PriM does not generally interfere with the activity of the Type VI Secretion System

The de-repression of *priM* that occurs in cells of a *pmrA* mutant results in a large decrease in intramacrophage growth. One system that is known to be essential for intramacrophage growth is the type VI secretion system (T6SS). We therefore asked whether PriM might inhibit intramacrophage growth by interfering with the function of the type VI secretion system. To do this we modified a recently established protocol (Clemens *et al.*, 2015) for use in LVS to examine the amount of IgIC secreted in the presence of 5% potassium chloride by cells lacking PigR, PmrA, PriM, or PmrA and PriM, compared to wild-type (Supporting Information Fig. S4). While cells lacking PigR do not synthesize as much IgIC as wild-type cells (as expected), the loss of PmrA and/or PriM appears to have little effect on IgIC synthesis or secretion (Supporting Information Fig. S4). From these results we conclude that it is unlikely that PriM inhibits intramacrophage growth by preventing secretion of IgIC or interfering with the assembly of the T6SS. We cannot, however, rule out the possibility that PriM might exert its effect by interfering with the secretion of a specific T6SS effector.

Discussion

Using ChIP-Seq we have found that PmrA occupies 252 different regions of the LVS chromosome. We find that the ability of PmrA to function as a repressor at only one of these locations explains why PmrA is required for intramacrophage growth. Specifically, we have obtained evidence that de-repression of a gene we refer to as *priM* is detrimental to the intramacrophage growth of cells that lack PmrA. Our findings demonstrate that the

repression of an anti-virulence factor by PmrA is essential for the intramacrophage growth and survival of *F. tularensis*.

It has been proposed that the ability of PmrA to positively regulate the expression of genes on the FPI accounts for the intramacrophage growth defect of cells lacking PmrA (Fig. 5A) (Mohapatra *et al.*, 2007; Sammons-Jackson *et al.*, 2008; Bell *et al.*, 2010; Dai *et al.*, 2011). Our results suggest that the major contribution of PmrA to intramacrophage survival is not as a positive regulator of the FPI genes but rather as a negative regulator of *priM* (FTL_0702) which encodes a novel anti-virulence factor. Based on our results, we propose a model to account for the role of PmrA in intramacrophage growth in which PmrA directly binds the *priM* promoter and represses gene expression by occluding RNAP (Fig. 5B). Our model specifies that RNAP (together with the RNAP-associated factors MglA, SspA, and PigR), can bind the *priM* promoter and initiate transcription only when PmrA is not bound to this region (Fig. 5C).

Using RNA-Seq we identified 38 genes that are subject to control by PmrA, the majority of which, including *priM*, are repressed by this regulator. In contrast to previous studies in both *F. novicida* and LVS (Mohapatra *et al.*, 2007; Sammons-Jackson *et al.*, 2008), we did not find that PmrA regulates the expression of FPI genes, at least under the conditions of our experiments (Tables 1 and 2). We note that our study was performed using a mutant of LVS that contains an unmarked in-frame deletion of *pmrA*, whereas prior studies used mutants in which the *pmrA* gene was replaced or interrupted with a kanamycin resistance cassette (Mohapatra *et al.*, 2007; Sammons-Jackson *et al.*, 2008). Consistent with the idea that in LVS PmrA does not directly regulate the expression of FPI genes, we did not detect any association between PmrA and the two key FPI promoters in this organism by ChIP-Seq (Fig. 2). However, studies with purified PmrA indicate that PmrA can bind the *pdpD* promoter from the *F. novicida* FPI *in vitro* (Bell *et al.*, 2010). Unlike *F. novicida*, LVS does not contain the DNA sequence corresponding to the *pdpD* promoter and so our studies do not address whether PmrA occupies this promoter *in vivo*. The presence of a PmrA binding site at the *pdpD* promoter on the *F. novicida* FPI could explain some of the differences between our study and the prior study in *F. novicida* (Mohapatra *et al.*, 2007). We note, however, that in agreement with the findings of our study, *priM* (FTL_0702) was identified previously as being repressed by PmrA in both LVS and in *F. novicida* (Mohapatra *et al.*, 2007; Sammons-Jackson *et al.*, 2008); *priM* is duplicated in *F. novicida* and encoded by the genes FTL_1260 and FTL_1261.

Several different models have been proposed to account for how MglA and SspA contribute directly to the expression of genes on the FPI. One of these specified that the RNAP-associated MglA-SspA complex serves as a contact site for PmrA bound at all of the FPI promoters (Fig. 5A) (Mohapatra *et al.*, 2007; Bell *et al.*, 2010; Dai *et al.*, 2011). Given that we do not detect PmrA at either the *igla* or *pdpA* promoter on the FPIs in LVS, and given that we do not find any evidence that PmrA positively regulates the expression of FPI genes, we think it unlikely that this model can account for the involvement of MglA and SspA in the direct control of these promoters. Our findings are consistent with an alternative model in which the RNAP-associated MglA-SspA complex serves as a contact site for the putative

DNA-binding protein PigR at the *iglA* and *pdpA* promoters (Charity *et al.*, 2009; Rohlfing and Dove, 2014; Ramsey *et al.*, 2015).

Although PmrA associates with 252 distinct regions of the LVS chromosome, our RNA-Seq analyses indicate that PmrA exerts effects on gene expression directly at few of these locations. It is possible that the occupancy of these sites, including those that are intragenic and not associated with promoters, may play more pronounced regulatory roles at other points of the growth curve, or under growth conditions that are different from the one we tested (i.e. reflect situations where regulation is cryptic). It is also possible that, rather than influence gene expression, the occupancy of these sites may influence chromosome structure and reflect a NAP function for PmrA. Indeed, a growing body of evidence stemming from genome-scale studies suggests that other transcription regulators can occupy many sites but only exert regulatory effects at a few (Shimada *et al.*, 2008; Minch *et al.*, 2015; Visweswariah and Busby, 2015). Additionally, it has been suggested that transcription factor binding may influence the accessibility of DNA to both mutagenic agents and repair enzymes (Warnecke *et al.*, 2012). Thus, transcription factor binding within coding regions by proteins such as PmrA may shape the evolution of a gene (Warnecke *et al.*, 2012).

Although many genes are known to be required for the intramacrophage growth and survival of *Francisella*, to the best of our knowledge *priM* is the first example of a gene from *F. tularensis* whose expression is inhibitory to intramacrophage growth. PriM is annotated as a hypothetical protein of unknown function that is predicted to contain a signal sequence at its N-terminus. Although we do not currently know the mechanism by which PriM inhibits the intramacrophage growth of *F. tularensis*, there is at least one precedent from a study in *Salmonella* for an anti-virulence factor that functions by inhibiting intramacrophage growth (Pontes *et al.*, 2015). Moreover, in *F. novicida*, Cas9 of the CRISPR-Cas system promotes virulence by inhibiting the expression of the FTN_1103 gene encoding a lipoprotein (Sampson *et al.*, 2013). In this situation, FTN_1103 functions as an anti-virulence factor not by inhibiting intramacrophage growth (Weiss *et al.*, 2007), but rather by enhancing the inflammatory response (Jones *et al.*, 2012). Because Cas9 encoded by *F. tularensis* strains may not be functional it is unclear whether Cas9 promotes the virulence of these strains by repressing the synthesis of bacterial lipoproteins (Sampson *et al.*, 2013). PriM and PmrA are conserved amongst species of *F. tularensis* and *F. novicida*, suggesting that inhibition of *priM* expression by PmrA may be a conserved mechanism for promoting the intramacrophage growth and survival of *Francisella* species.

Experimental Procedures

Growth conditions

F. tularensis subsp. *holarctica* LVS and its derivatives were grown aerobically at 37°C in Mueller Hinton (MH) broth (Difco), supplemented with glucose (0.1%), ferric pyrophosphate (0.025%), and Isovitalex (2%), or on cystine heart agar (Difco) medium supplemented with 1% hemoglobin solution (VWR) (CHAH). When appropriate, kanamycin was used for selection at 5 µg ml⁻¹. Plasmid construction was performed using *Escherichia coli* strain XL1-blue (Stratagene) and, when appropriate, 50 µg ml⁻¹ kanamycin was used to select for resistance. *E. coli* containing plasmid pF2-PriM were grown at 30°C.

Vector construction

The plasmid pKL13 (Ramsey *et al.*, 2015) was modified to generate the vector for the PmrA VSV-G tagging integration construct. The last 527 bp of *pmrA* was amplified using a 5' primer containing a KpnI site and a 3' primer containing a NotI site, allowing the fragment to be fused with DNA specifying an alanine linker and the VSV-G epitope tag. The fragment was subcloned into KpnI/NotI digested pKL13. The resulting plasmid, pKL15, contains the DNA specifying the 3' end of *pmrA* and was used to generate strain LVS PmrA-V.

The vector pEX18Kan was modified to make the in-frame deletion constructs for pEX *pmrA* and pEX *priM* as described previously (Charity *et al.*, 2007).

Plasmid pEX18Kan was also modified to make mutant genes by allelic replacement. Plasmid pEX-*mreA(S)* was generated by amplification of regions that flank and overlap base pairs 91–96 of *mreA* (FTL_0701) by PCR, using primers that integrate a PsiI restriction site (TTATAA; coding for leucine followed by a stop codon) in place of base pairs 91–96 (TTAAAA). Plasmid pEX-*priM(S)* was generated by amplification of regions that flank and overlap base pairs 46–54 of *priM* by PCR, using primers that integrate an AflIII site (AflIII site underlined: ACTTAAGTT; coding for threonine, a stop codon, and valine) in place of base pairs 46–54 (ACTTCAGTT). PCR products were ligated together and cloned into pEX18Kan.

Plasmid pF2-PriM was made by replacing *sspA* in the plasmid pF2-SspA (Charity *et al.*, 2007), which allows for production of PriM under the control of a weakened *groES* promoter; the pF2 plasmid (Charity *et al.*, 2007) used as a control does not contain DNA encoding PriM. Plasmid pF-PmrA, which synthesizes PmrA under the control of the full *groES* promoter, was made by cloning the *pmrA* gene into pFNLTP7 *gro-gfp* as described (Charity *et al.*, 2009).

Strain construction

Plasmids were integrated into LVS as previously described (Maier *et al.*, 2004). Cells in which a single homologous recombination event occurred between the integration vector and the chromosome were selected on CHAH containing 5 µg ml⁻¹ kanamycin. Strains containing the modified genetic region of interest were confirmed by colony PCR, Western blot, Southern blot, and/or sequencing PCR products made from genomic DNA. Strain LVS PmrA-V, which synthesizes PmrA with a C-terminal VSV-G tag, was generated by electroporation and subsequent integration of plasmid pKL15 into LVS. Strains containing deletions or mutations were generated by allelic exchange. LVS *pmrA*, which lacks the *pmrA* gene, was generated from LVS using plasmid pEX *pmrA*. LVS *priM*, which lacks the *priM* gene, was generated from LVS using plasmid pEX *priM*. LVS *pmrA priM*, which lacks both the *pmrA* and *priM* genes, was generated from LVS *priM* using plasmid pEX *pmrA*. LVS *mreA(S)*, in which a stop codon is incorporated at position 32 of *mreA*, was generated from LVS using plasmid pEX-*mreA(S)*. LVS *pmrA mreA(S)*, which lacks the *pmrA* gene and contains a stop codon at position 32 of *mreA*, was generated from LVS *mreA(S)* using plasmid pEX *pmrA*. LVS *priM(S)*, in which a stop codon is incorporated at position 17 of *priM*, was generated from LVS using plasmid pEX-*priM(S)*. LVS *pmrA*

prim(S), which lacks the *pmrA* gene and contains a stop codon at position 17 of *prim*, was generated from LVS *prim(S)* using plasmid pEX *pmrA*.

ChIP-Seq and data analysis

ChIP-Seq was performed in triplicate with LVS PmrA-V cells as previously described (Ramsey *et al.*, 2015). Data analysis was performed as described previously (Ramsey *et al.*, 2015). Sequencing reads have been submitted to the NCBI Sequence Read Archive (SRA, <http://www.ncbi.nlm.nih.gov/Traces/sra/>) with the accession number SRP074325.

RNA isolation

LVS cells and derivatives were grown in liquid culture until cultures reached an optical density at 600 nm (OD₆₀₀) of approximately 0.35. RNA was isolated as described previously (Rohlfing and Dove, 2014).

RNA sequencing and data analysis

Sequencing libraries were made from RNA samples essentially as described previously (Jorth *et al.*, 2014). Briefly, ribosomal RNAs were depleted from 5 µg of total RNA using the Ribo-Zero Magnetic Kit (Bacteria) (Epicentre) according to the manufacturer's specifications. The remaining RNA was ethanol-precipitated with 10 µg linear acrylamide and resuspended in RNase-free water. RNA was fragmented using the NEBNext Magnesium Fragmentation Module (New England Biolabs) according to the manufacturer's protocol and ethanol-precipitated. The resulting RNA was used to generate RNA-Seq libraries using the NEBNext Multiplex Small RNA Library Prep Kit for Illumina (New England Biolabs) according to the manufacturer's protocol. Libraries were sized-selected by PAGE using a 5% gel with TBE (Biorad), stained with SYBR gold nucleic acid stain (Life Technologies), and visualized using a blue-light transillumination system. Fragments corresponding to ~150–300 bp were gel purified, ethanol-precipitated, and resuspended in TE buffer according to the NEBNext Multiplex Small RNA Library Prep Kit for Illumina (New England Biolabs) protocol. Libraries were sequenced by Elim Biopharmaceuticals, Inc. (Hayward, CA), using an Illumina HiSeq 2500 which generated 50 bp single-end reads. Sequencing reads have been submitted to the NCBI Sequence Read Archive (SRA, <http://www.ncbi.nlm.nih.gov/Traces/sra/>) with the accession number SRP074325.

Sequencing reads were trimmed with Flexbar (version 2.4) (Dodt *et al.*, 2012) to remove adapter sequences incorporated during cDNA library preparation. Rockhopper (McClure *et al.*, 2013) was used to map reads to the *F. tularensis* subsp. *holarctica* LVS genome (NCBI locus AM233362), generate files to visualize read alignment, and perform differential gene expression analysis. We further considered genes which are more than 2-fold altered in transcript abundance and have q-values less than or equal to 0.05. Read alignment tracks were visualized using the Integrative Genomics Viewer (IGV), version 2.3.59 (Thorvaldsdóttir *et al.*, 2013).

cDNA synthesis and qRT-PCR

cDNA synthesis using Superscript III reverse transcriptase (Life Technologies) and qRT-PCR using FastStart Essential DNA Green Master (Roche) with the Roche LightCycler 96

detection system were performed essentially as described (Charity *et al.*, 2007). Abundance of the *priM* and *rnc* transcripts were measured relative to the *tul4* transcript. Experiments were performed twice in biological triplicate.

Macrophage replication assays

Approximately 2×10^4 cells of the murine macrophage-like J774A.1 cells were seeded in wells of a 96-well plate in DMEM (ATCC) supplemented with 10% bovine fetal serum (Gemini Bio-Products) and incubated overnight at 37°C with 5% CO₂. Macrophage were infected with LVS or derivative strains at an MOI of 5–10, using 3 wells per strain. After 2 hours, cells were washed twice with PBS and covered with DMEM containing 10 µg mL⁻¹ gentamycin. Cells were incubated at 37°C with 5% CO₂ until enumeration, for either 2 or 24 hours after addition of gentamycin. To identify the number of intracellular bacteria, macrophage were washed twice with PBS, lysed for 5 minutes with 1% saponin in PBS at room temperature, diluted in PBS, and plated on CHAH. Each experiment was performed at least twice.

Type VI Secretion Assay

Assays were performed as described (Clemens *et al.*, 2015) with some modifications. Briefly, LVS or derivative strains were grown in supplemented brain heart infusion broth with cysteine (BHlc) (Mc Gann *et al.*, 2010) with or without 5% KCl. Cells were pelleted by centrifugation and a 0.2 micron filter was used to sterilize the supernatant; resulting samples were analyzed by SDS-PAGE and immunoblotting.

Immunoblots

Proteins were separated by SDS-PAGE on 12% Bis-Tris NuPAGE gels in MOPS running buffer (Life Technologies). The XCell II Blot Module (Life Technologies) was used to transfer proteins to PVDF. Membranes were probed as described (Ramsey *et al.*, 2015), using anti-GroEL (diluted 1:160,000; provided by Karsten Hazlett, Albany Medical College, Albany, New York, United States) or anti-IgIC (diluted 1:1,000; BEI resources) and subsequently incubated with polyclonal goat anti-rabbit (diluted 1:5,000; Pierce) or polyclonal goat anti-mouse (1:5,000; Pierce), respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PmrA is found widely across the genome and is found at promoter and non-promoter locations

A representative illustration of the density of normalized sequencing reads after ChIP-Seq of PmrA (green), MglA (brown), PigR (blue), σ^{70} (orange), and HipB (dark pink) (A) at across a representative 400 kb region of the *F. tularensis* LVS chromosome (normalized reads are displayed on a log scale); (B) at the FTL_1279–*hemL* region, where PmrA associates with the FTL_1279 promoter and is found at the 3' ends of the convergently-transcribed FTL_1282 and *hemL* genes (normalized reads are displayed on a linear scale); (C) at the *surA*–*ostA* region, where PmrA associates with intragenic regions of *surA* and *ostA* but is not found at the *ostA* promoter (normalized reads are displayed on a linear scale). Gray

boxes below the read density plots indicate regions of significantly enriched reads with red lines indicating the point of maximum enrichment. Promoter regions, defined as areas with significant enrichment of RNAP with the chromosome (Ramsey *et al.*, 2015), are indicated by the purple boxes below the gene annotations. PmrA is found at promoter regions and non-promoter regions, at both intra- and intergenic locations. HipB is not significantly enriched in these regions. (D) Chart representing the percentage of PmrA locations that overlap with MglA and/or SspA locations.

Figure 2. PmrA is not found at the *iglA* or *pdpA* promoters of the Francisella Pathogenicity Island

A representative illustration of the density of normalized sequencing reads after ChIP-Seq of PmrA (green), MglA (brown), PigR (blue), σ^{70} (orange), $\beta' + \text{rif}$ (purple), and HipB (dark pink) across the FPI (normalized reads are displayed on a linear scale). MglA, PigR, σ^{70} , and the β' subunit of RNAP (in cells grown in the presence of rifampicin to trap RNAP at promoters [$\beta' + \text{rif}$]) are found at the *iglA* and *pdpA* promoters, while PmrA is not. PmrA is found at three locations within the FPI.

Figure 3. PmrA directly represses the expression of *priM*

(A) A representative illustration of normalized sequencing reads after ChIP-Seq of HipB (dark pink) and PmrA (green), RNA-Seq of LVS and LVS $\Delta pmrA$ (minus strand in gold, plus strand in blue), with all normalized reads displayed on a linear scale. The minus strand RNA-Seq data are truncated at 2000 normalized reads. The location with the most PmrA enrichment is found at the *priM* promoter and *priM* transcript levels increase dramatically in the absence of PmrA. Transcript levels of *mreA* also increase, due to the increase in reads mapping to the 3' end of *mreA*. (B) Quantitative RT-PCR analysis shows that in cells lacking PmrA, *priM* transcript abundance increases approximately 300-fold (LVS $\Delta pmrA$

pF), validating RNA-Seq data. Ectopic expression of PmrA using the vector pF-PmrA can restore the repression of *priM* to wild-type levels (LVS *pmrA* pF-PmrA compared to LVS pF). The figure depicts data from a representative experiment performed with biological triplicates. Transcripts were normalized to *tul4*, whose expression is not influenced by PmrA. Error bars represent ± 1 standard deviation from the value (calculated using the mean threshold cycle).

Figure 4. Production of PriM is inhibitory to intramacrophage growth and survival

Growth and survival of wild-type LVS (LVS) and cells of the indicated mutant derivatives within J774A.1 cells. Murine macrophage-like J774A.1 cells were infected with cells of the indicated strains at a multiplicity of infection of approximately 5–10. Cells were lysed and bacteria were plated for enumeration (colony forming units [CFU]) 24 hours post-infection. (A) Deletion of *priM* (LVS *pmrA priM*) rescues the intramacrophage growth defect of *pmrA* mutant cells (LVS *pmrA*). Cells lacking only PriM (LVS *priM*) grow within macrophage as well as wild-type cells (LVS). (B) Deletion of *priM* (LVS *pigR priM*) does not rescue the intramacrophage growth defect of *pigR* mutant cells (LVS *pigR*). (C) Introduction of a stop codon into *mreA* (LVS *pmrA mreA(S)*) does not rescue intramacrophage growth defect of *pmrA* mutant cells (LVS *pmrA*). Cells with a stop codon in *mreA* (LVS *mreA(S)*) grow within macrophage as well as wild-type cells (LVS). (D) Introduction of a stop codon into *priM* (LVS *pmrA priM(S)*) rescues the intramacrophage growth defect of *pmrA* mutant cells (LVS *pmrA*). Cells with a stop codon in *priM* (LVS *priM(S)*) grow within macrophage as well as wild-type cells (LVS). (E) Ectopic expression of *priM* (LVS pF2-PriM) results in decreased intramacrophage growth, compared to wild-type cells with empty vector (LVS pF2).

Figure 5. PmrA promotes intramacrophage growth by functioning as a repressor

(A) Prior model suggesting PmrA directly activates expression of FPI genes to promote intramacrophage growth (Mohapatra *et al.*, 2007; Bell *et al.*, 2010; Dai *et al.*, 2011). PmrA binds to a specific site (in green) at FPI gene promoters and interacts with the RNAP-associated MglA-SspA complex to positively regulate expression of FPI genes. (B) and (C) current model describing how PmrA functions to promote intramacrophage survival. (B) PmrA binds to the *priM* promoter, occluding RNAP and preventing production of PriM, which allows cells to grow within macrophage. In this model, we have illustrated PmrA binding to a site between the -10 and -35 elements of the *priM* promoter although the actual position of the binding site is not known. (C) In the absence of PmrA, RNAP (together with PigR and the MglA-SspA complex) can bind the *priM* promoter, resulting in production of PriM and inhibition of intramacrophage growth.

Table 1

Genes positively regulated by PmrA.

Locus number	Gene name	Product	Fold change ^a	PmrA location ^b
FTL_0554	<i>mnc</i>	ribonuclease III	-6.0	-
FTL_0553	<i>lepB</i>	signal peptidase I	-3.9	-
FTL_1190	<i>grpE</i>	heat shock protein GrpE	-3.6	-507
FTL_1264	<i>folB</i>	dihydroneopterin aldolase	-2.6	-
FTL_1697	-	metal ion transporter	-2.1	-
FTL_0531	-	hypothetical protein, pseudogene	-2.0	-
FTL_0560	-	hypothetical protein	-2.0	-
FTL_0633	-	rhodanese-related sulfurtransferase	-2.0	-1
FTL_0634	-	NADH oxidase	-2.0	-544
FTL_0709	<i>lpcC</i>	glycosyl transferases group 1 family protein	-2.0	-
FTL_0777	-	hypothetical protein	-2.0	-
FTL_0997	-	hypothetical protein	-2.0	-
FTL_1200	<i>cobS</i>	cobalamin (vitamin B12) synthesis protein/P47K family protein	-2.0	-
FTL_1682	-	hypothetical protein	-2.0	-

^aFold change represents ratio of gene expression level in LVS *pmrA* compared to LVS, $p < 0.01$. Only those genes whose expression changed by a factor of 2 or more are listed.

^bPmrA location is reported if the position of maximal enrichment of a PmrA peak is found within 1000 bp upstream to 600 bp downstream of the translation start site of the gene. Longer distances are indicated only if the gene is part of a putative operon with a gene reported in this table.

Table 2

Genes negatively regulated by PmrA.

Locus number	Gene name	Product	Fold change ^a	PmrA location ^b
FTL_0702	<i>prim</i>	PmrA-repressed inhibitor of intramacrophage growth	298.0	-58
FTL_0815	-	PRC-barrel protein, gene fragment	69.0	-520
FTL_0816	-	hypothetical protein, gene fragment	40.4	-268
FTL_0814	-	hypothetical protein, gene fragment	16.5	-934
FTL_0701	<i>mreA</i>	FAD binding family protein	7.9	-908
FTL_1401	-	hypothetical protein	7.7	-1059
FTL_0152	-	hypothetical protein	4.8	75
FTL_0810	-	cation transport regulator, gene fragment	4.3	-1339
FTL_0841	-	lipoprotein	3.5	-405
FTL_1635	-	chitinase, fragment	3.3	-31
FTL_1636	-	hypothetical protein	2.7	-2120
FTL_0655	-	hypothetical protein	2.7	-
FTL_0840	-	hypothetical protein	2.5	-73
FTL_1114	-	isochorismatase hydrolase family protein	2.5	9
FTL_0881	-	hypothetical protein	2.3	-270
FTL_0049	<i>dtd</i>	D-tyrosyl-tRNA(Tyr) deacylase	2.2	565
FTL_0943	-	Sodium/hydrogen exchanger family protein	2.2	-157
FTL_0447	-	hypothetical protein	2.2	48
FTL_0470	-	hypothetical protein	2.2	-149
FTL_0857	<i>lytB</i>	hypothetical membrane protein	2.1	21
FTL_1219	-	hypothetical protein	2.1	-2
FTL_0935	-	hypothetical protein	2.1	-
FTL_1402	<i>isf1</i>	transposase	2.0	-207
FTL_0299	<i>murQ</i>	N-acetylmuramic acid 6-phosphate etherase	2.0	-

^aFold change represents ratio of gene expression level in LVS *pmrA* compared to LVS, $p < 0.01$. Only those genes whose expression changed by a factor of 2 or more are listed.

^bPmrA location is reported if the position of maximal enrichment of a PmrA peak is found within 1000 bp upstream to 600 bp downstream of the translation start site of the gene. Longer distances are indicated only if the gene is part of a putative operon with a gene reported in this table.