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Small Molecule Control of Virulence Gene Expression in Francisella tularensis

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Abstract

In Francisella tularensis, the SspA protein family members MglA and SspA form a complex that associates with RNA polymerase (RNAP) to positively control the expression of virulence genes critical for the intramacrophage growth and survival of the organism. Although the association of the MglA-SspA complex with RNAP is evidently central to its role in controlling gene expression, the molecular details of how MglA and SspA exert their effects are not known. Here we show that in the live vaccine strain of F. tularensis (LVS), the MglA-SspA complex works in concert with a putative DNA-binding protein we have called PigR, together with the alarmone guanosine tetraphosphate (ppGpp), to regulate the expression of target genes. In particular, we present evidence that MglA, SspA, PigR and ppGpp regulate expression of the same set of genes, and show that mglA, sspa, pigR and ppGpp null mutants exhibit similar intramacrophage growth defects and are strongly attenuated for virulence in mice. We show further that PigR interacts directly with the MglA-SspA complex, suggesting that the central role of the MglA and SspA proteins in the control of virulence gene expression is to serve as a target for a transcription activator. Finally, we present evidence that ppGpp exerts its effects by promoting the interaction between PigR and the RNAP-associated MglA-SspA complex. Through its responsiveness to ppGpp, the contact between PigR and the MglA-SspA complex allows the integration of nutritional cues into the regulatory network governing virulence gene expression.

Introduction

Francisella tularensis, the aetiological agent of tularemia, is one of the most infectious bacterial pathogens currently known and a potential bioweapon. Although relatively little is known about the molecular mechanisms underlying F. tularensis pathogenesis [1], it is clear that genes present on the Francisella pathogenicity island (FPI) are essential for the intramacrophage growth and virulence of the organism [2–9]. These genes are thought to encode a novel protein secretion system related to the recently identified type VI secretion system [8,10–13].

Prominent amongst those regulators of virulence gene expression in F. tularensis [14–17] are the related global regulators MglA and SspA [4,18–20]. MglA and SspA work in concert with one another to positively regulate the expression of all of the genes on the FPI, and many genes outside of the FPI (~100 genes in total) [4,19,20]. Moreover, recent proteomic studies have revealed that MglA influences the abundance of proteins involved in responding to stress [21]. Although MglA and SspA control the expression of many genes implicated in virulence [4,19,20], they also control the expression of many others whose roles in virulence are not currently known.

MglA and SspA are members of the stringent starvation protein A family [18,22,23] and interact directly with one another to form a complex [20]. The MglA-SspA complex in turn associates with RNA polymerase (RNAP) to positively control virulence gene expression in F. tularensis [20]. Although the association of the MglA-SspA complex with RNAP is evidently central to its role in controlling gene expression, the molecular details of how MglA and SspA exert their effects on transcription are not known. Members of the SspA protein family also appear to play important roles in the control of virulence gene expression in other pathogenic bacteria by unknown mechanisms [24–27]. Although a putative DNA-binding protein called FevR was recently shown to regulate the same set of genes that is regulated by MglA and SspA in F. novicida, the mechanism by which FevR influences the expression of target genes was not determined [15].

Guanosine tetraphosphate, otherwise known as ppGpp (or magic spot), is a small molecule that in some Gram-negative bacteria binds directly to RNAP and inhibits transcription initiating from certain particularly strong promoters, such as the ribosomal RNA (rRNA) promoters (reviewed in [28–30]). ppGpp can also directly activate transcription from other promoters by...
Virulence Gene Control in F. tularensis by ppGpp

Author Summary

Guanosine tetraphosphate (ppGpp) is a small molecule that is produced by many different bacteria in response to nutrient limitation. Although ppGpp has been shown to play an important role in controlling the expression of virulence genes in several pathogenic bacteria, few studies have addressed how this occurs. Here we show that in the intracellular pathogen F. tularensis, ppGpp plays a critical role in controlling the expression of genes required for intracellular replication and virulence, and we uncover the molecular basis for its effect. In particular, we show that ppGpp works in concert with three other essential regulators of virulence gene expression in F. tularensis—a putative DNA-binding protein that we have called PigR and the SspA protein family members MglA and SspA. Our study provides evidence that ppGpp functions to promote the interaction between PigR and a component of F. tularensis RNA polymerase (RNAP) comprising the MglA and SspA proteins. By influencing the interaction between PigR and the RNAP-associated MglA-SspA complex, ppGpp serves to tie the nutritional status of the cell to the expression of genes that are essential for survival in the host.

Results

The MglA-SspA complex and ppGpp positively regulate the same set of genes in F. tularensis

In the course of testing specific models for how the MglA-SspA complex controls virulence gene expression we uncovered a critical role for the alarmone ppGpp. To test whether ppGpp might influence the expression of MglA/SspA-regulated genes we constructed derivatives of the live vaccine strain of F. tularensis (LVS) (an attenuated derivative of an F. tularensis subspecies holarctica strain) carrying in-frame deletions of the relA gene (LVS ΔrelA), the relA and spoT genes (LVS ΔrelA ΔspoT), and the mglA, relC, and spoT genes (LVS ΔmglA ΔrelC ΔspoT); as in other Gram-negative bacteria, deletion of relA and spoT in LVS resulted in a ppGpp null mutant (ppGppD) that no longer makes detectable amounts of ppGpp (Figure 1A). To determine whether deletion of relC, or deletion of relA and spoT, had any effect on the expression of MglA/SspA-regulated genes, RNA was isolated from mid-log grown cells of wild-type LVS, LVS ΔmglA, LVS ΔrelC, LVS ΔrelA ΔspoT, and LVS ΔmglA ΔrelA ΔspoT, and expression of the MglA/SspA-regulated iglA, pdpA, and FTl_1219 genes was measured by quantitative RT-PCR (qRT-PCR).

Deletion of mglA or relA and spoT caused a similar drastic reduction in the amounts of the iglA, pdpA, and FTl_1219 transcripts when compared to LVS wild-type cells (Figure 1B). Furthermore, similar amounts of the iglA, pdpA, and FTl_1219 transcripts were seen in cells of the ΔmglA mutant, the ΔrelA ΔspoT mutant, and the ΔmglA ΔrelC ΔspoT mutant. Only slightly reduced amounts of the iglA, pdpA, and FTl_1219 transcripts were present in LVS ΔrelC mutant cells relative to wild-type cells. Complementation of LVS ΔrelA ΔspoT mutant cells with a plasmid expressing spoT restored the amounts of the iglA transcript close to wild-type or ΔrelC mutant levels (Figure 1C). Because the LVS ΔrelA ΔspoT mutant can no longer make ppGpp, we infer from our results that ppGpp positively regulates the expression of MglA/SspA-regulated genes located both within (iglA and pdpA), and outside (FTl_1219), the FPI. Moreover, MglA and ppGpp appear to exert similar effects on target gene expression. In the case of two of these target genes, deletion of mglA in a ΔrelC ΔspoT mutant background did not result in a further decrease in expression suggesting that the effects of MglA and ppGpp are dependent on one another.

To determine whether ppGpp and the MglA-SspA complex regulate expression of the same set of genes, we performed DNA microarray analyses to compare the global gene expression profiles of ΔmglA mutant cells, ΔrelA ΔspoT mutant cells, and LVS wild-type cells. The results of these analyses reveal that in LVS, ppGpp and MglA regulate expression of essentially the same set of genes (Figure 1D; Table S1).

A ΔrelA ΔspoT mutant is defective for intramacrophage growth and for virulence in mice

MglA is thought to be essential for intramacrophage growth at least in part because it positively regulates the expression of genes present on the FPI that are in turn required for intramacrophage growth or survival [4, 18–20]. If MglA, SspA, and ppGpp function together to positively regulate the same set of genes, including many required for virulence, then we would predict that like MglA [4, 18, 19, 47], SspA and ppGpp should be required for intramacrophage growth and for virulence in mice. To test this prediction, we compared the abilities of LVS ΔmglA, LVS ΔspsA, LVS ΔrelA, LVS ΔmglA ΔrelA ΔspoT mutant cells, and LVS wild-type cells to replicate within J774 murine macrophages and to cause lethality in BALB/cByJ mice. Approximately 104-fold fewer cells of the LVS ΔmglA mutant, the LVS ΔspsA mutant, and the LVS ΔrelA ΔspoT mutant...
were recovered from J774 cells 24 hours post-infection compared to wild-type cells (Figure 2A), and cells of the LVS \(\Delta mglA\), LVS \(\Delta sspA\), and LVS \(\Delta relA\ \Delta spoT\) mutants were essentially avirulent in BALB/cByJ mice (Figure 2B). Furthermore, in accordance with our finding that \(\Delta relA\) has only a modest effect on the expression of \(mglA\) and \(sspA\) in broth-grown cells, \(\Delta relA\) mutant cells were only marginally defective for intramacrophage growth and were highly virulent in our mouse model (Figure 2). These findings suggest that, like MglA and SspA, ppGpp is essential for intramacrophage growth and virulence in mice and are consistent with the idea that the MglA-SspA complex and ppGpp play important roles in positively regulating the expression of virulence genes in \(F.\ tularensis\).

ppGpp has no effect on the abundance of MglA or SspA, and does not influence the association of the MglA-SspA complex with RNAP

In \(E.\ coli\), \(relA\) is required for the synthesis of SspA in stationary phase cells, presumably because ppGpp is required for expression of the \(sspA\) gene [23]. This raises the possibility that in \(F.\ tularensis\) ppGpp might influence the expression of MglA/SspA-regulated genes through an effect on the expression of \(mglA\) or \(sspA\). However, microarray and qRT-PCR analyses revealed that there was a less than two-fold difference in the abundance of the \(mglA\) and \(sspA\) transcripts in cells of the wild-type strain relative to those of the \(\Delta relA\ \Delta spoT\) mutant, suggesting that ppGpp does not significantly influence expression of the \(sspA\) or

Figure 1. ppGpp controls the expression of MglA/SspA-regulated genes in \(F.\ tularensis\). (A) Analysis of ppGpp concentrations in cells of the indicated strains of LVS by thin layer chromatography. For these analyses ppGpp was isolated from cells following a shift to conditions of nutrient limitation (see Materials and Methods). (B) Quantitative RT-PCR analysis of \(iglA\), \(pdpA\), and \(FTL_1219\) transcript abundance in wild-type (WT), \(\Delta mglA\), \(\Delta relA\), \(\Delta relA\ \Delta spoT\), and \(\Delta mglA \Delta relA \Delta spoT\) mutant backgrounds. RNA was isolated from cells grown in MH to mid-log. Transcripts were normalized to those of \(tul4\), whose expression is not influenced by MglA or ppGpp. (C) Complementation of the effects of the \(\Delta relA\ \Delta spoT\) mutations on \(iglA\) expression by \(spoT\) provided in trans. Quantitative RT-PCR analysis of \(iglA\) transcript abundance in wild-type (WT), and \(\Delta relA \Delta spoT\) mutant cells harboring the indicated plasmids. Transcripts were normalized to \(tul4\). Plasmid pF2-SpoT directs the synthesis of \(F.\ tularensis\) SpoT, whereas plasmid pF2 served as an empty vector control. (D) Venn diagram representation of the overlap between genes controlled by MglA and ppGpp. Each circle represents those genes whose expression was decreased by a factor of 2.5 or more (\(p<0.05\)) in the indicated mutant background compared to wild-type and whose expression altered by a factor of 2 or more in the other mutant background, as determined by DNA-microarray.

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mglA genes (data not shown). To determine whether ppGpp influences the abundance of the MglA or SspA proteins we used derivatives of wild-type LVS and LVS ΔrelA ΔspoT in which the native chromosomal copies of mglA and spoT have been altered such that they specify forms of MglA or SspA with C-terminal TAP and LVS ΔrelA ΔspoT contain a TAP-tagged form of the MglA-SspA complex with RNAP. To test this possibility we used derivatives of wild-type LVS and LVS ΔrelA ΔspoT [20]. Comparison of the amounts of MglA-TAP and SspA-TAP in wild-type and ΔrelA ΔspoT mutant cells, as revealed by Western blotting, suggests that ppGpp does not have any positive effect on the abundance of MglA-TAP or SspA-TAP (Figure 3A).

Although ppGpp does not appear to influence the amount of MglA or SspA in the cell, ppGpp could control expression of MglA/SspA-regulated genes through an effect on the association of the MglA-SspA complex with RNAP. To test this possibility we used derivatives of wild-type LVS and LVS ΔrelA ΔspoT that contain a TAP-tagged form of the β' subunit of RNAP (LVS ΔrelA ΔspoT and LVS ΔrelA ΔspoT ΔmglA-TAP) [20]. Following purification of RNAP and associated proteins from these strains by TAP, proteins were separated by SDS-PAGE and stained with silver. These analyses revealed that ppGpp does not appear to influence the ability of the MglA-SspA complex to associate with RNAP, i.e. the MglA-SspA complex is associated with RNAP regardless of the presence or absence of ppGpp (Figure 3B). Because MglA does not associate with RNAP in the absence of SspA [20], these findings suggest furthermore that ppGpp does not prevent MglA from interacting with SspA. Although it was formally possible that MglA and SspA could be required for the synthesis of ppGpp, thin layer chromatography of 32P-labelled nucleotides isolated from LVS wild-type, ΔmglA mutant, and ΔspoT mutant cells suggests that this is not the case (Figure 1A).

Genetic screen identifies CaiC, TrmE, CphA, and FTL_0449 (PigR) as positive regulators of MglA/SspA-regulated genes in F. tularensis

Given that ppGpp does not influence the abundance of MglA or SspA, and does not prevent association of the MglA-SspA complex with RNAP, we postulated that ppGpp might exert its effects through another regulator, distinct from MglA and SspA, that in turn is required for expression of the MglA/SspA regulon. In an attempt to identify such a regulator we took an unbiased genetic approach. We constructed a reporter strain in which the iglA gene on one of the copies of the FPI was replaced with lacZ (LVS ΔmglA: lacZ) (Figure 4A); expression of iglA is strongly dependent on MglA and SspA [20]. We then mutagenized the LVS ΔmclA:: nadA reporter strain with a mariner transposon and screened for mutants that displayed decreased levels of lacZ expression on CHA agar plates containing X-Gal. Among the transposon mutants with decreased β-galactosidase activity relative to the non-mutagenized reporter strain, we identified mutants of LVS carrying in-frame deletions of the genes ΔiglA, ΔsspA, ΔcaiC (encoding a putative acyl-CoA synthetase), ΔtrmE (encoding a putative DNA-binding protein with similarity to members of the MerR family of transcription regulators) (Figure 4B). Because we originally identified FTL_0449 as a positive regulator of a gene present on the FPI, we have named FTL_0449 PigR, for pathogenicity island gene regulator.

To confirm that inactivation of each of the genes identified in the screen was responsible for the effect of the original transposon insertions on iglA expression, we made mutants of LVS carrying in-frame deletions of the genes ΔcaiC, ΔtrmE, ΔpigR, and ΔpigR (FTL_0449) genes. We then quantified iglA transcript levels in cells of each of these mutant strains, and in LVS wild-type cells by qRT-PCR.

Figure 2. Cells of a ΔrelA ΔspoT mutant are defective for intramacrophage growth and for virulence in mice. (A) Survival of wild-type (WT) and the indicated derivatives of F. tularensis strain LVS within J774 cells. J774 murine macrophages were infected with cells of the indicated bacterial strains at a multiplicity of infection of ~15. Cells were lysed and bacteria (colony forming units [CFU]) were plated for enumeration 24 hours post-infection. (B) Survival of BALB/cByJ mice following intradermal delivery of ~10^7 cells of each of the indicated strains of LVS. Eight mice were inoculated for each strain tested. Experiments were performed at least twice. doi:10.1371/journal.ppat.1000641.g002
Deletion of caiC, trmE, cphA, or pigR resulted in large decreases in the amount of the iglA transcripts (Figure 4C). Complementation of cells of the LVS ΔcaiC, LVS ΔtrmE, LVS ΔcphA, and LVS ΔpigR mutant strains with plasmids expressing either caiC, trmE, cphA, or pigR, respectively, restored the amounts of iglA transcripts close to wild-type levels (Figure S1). Thus, CaiC, TrmE, CphA, and PigR positively regulate expression of the FPI-encoded iglA gene. In addition, DNA microarray analyses revealed that CaiC, TrmE, CphA, and PigR control the expression of the entire MglA/SspA regulon (Table S1). Taken together, our findings suggest that a common set of target genes are positively regulated by CaiC, TrmE, CphA, PigR, ppGpp, and MglA/SspA.

PigR and TrmE are required for intramacrophage growth and for virulence in mice

We next compared the abilities of LVS ΔcaiC mutant cells, LVS ΔtrmE mutant cells, LVS ΔcphA mutant cells, LVS ΔpigR mutant cells, and LVS wild-type cells to replicate within macrophages and to cause virulence in mice. We infer from the results depicted in Figure 5A that in LVS, only TrmE and PigR are required for intramacrophage growth or survival. Furthermore, consistent with these findings, only the ΔtrmE mutant cells, and the ΔpigR mutant cells are avirulent in mice (Figure 5B). These findings suggest that both TrmE and PigR are critical regulators of virulence gene expression in vivo, and that although CaiC and CphA play a significant role in controlling the expression of MglA/SspA regulated genes in broth-grown cells, they may not significantly influence the expression of these genes in vivo, at least not in the infection models used here.
ppGpp positively regulates pigR expression and may function upstream of PigR and MglA/SspA

Amongst the regulators identified in our screen, only PigR appeared to be subject to control by ppGpp; pigR is amongst the genes that we identified as being under the control of ppGpp in our DNA microarray analyses (Table S1), a finding we confirmed by using qRT-PCR to quantify the abundance of the pigR transcript in wild-type and ΔrelA ΔspoT mutant cells (Figure 6A). However, pigR (FTL_0419) is also amongst the genes that we identified previously as being under the control of MglA and SspA in LVS [20] (and confirmed using qRT-PCR in Figure 6A). These observations raise the possibility that the MglA-SspA complex and ppGpp might serve simply to control pigR expression, and that PigR itself might be directly responsible for regulating the expression of target genes. This model, in which MglA/SspA and ppGpp function upstream of PigR, predicts that ectopic expression of pigR should complement the phenotypes of a ΔmglA mutant, and a ΔrelA ΔspoT mutant.

To determine where PigR operates in the regulatory hierarchy, we introduced a vector expressing pigR (pF-PigR) and an empty control vector (pF), into LVS ΔmglA, LVS ΔrelA ΔspoT, and LVS ΔpigR mutant cells. (Expression of pigR is under the control of the heterologous groEL promoter on pF-PigR.) As a control, we also introduced the empty vector into wild-type LVS. Cells containing these plasmids were grown to mid-log, RNA was isolated, and the expression of the iglA, pdpA, and FTL_1219 transcripts was quantified by qRT-PCR.

Ectopic expression of pigR (i) restored expression of the MglA/SspA-regulated genes to above wild-type levels in cells of the ΔpigR mutant strain, (ii) failed to restore expression of the MglA/SspA-regulated genes in ΔmglA mutant cells, and (iii) only partially restored expression of the MglA/SspA-regulated genes in cells of the ΔrelA ΔspoT mutant strain (Figure 6B). These findings suggest that PigR functions together with the MglA-SspA complex downstream of ppGpp.

PigR autoactivates

Expression of the pigR gene is positively controlled by the MglA-SspA complex. If, as is suggested by our findings, PigR functions together with the MglA-SspA complex then we would predict that the expression of the pigR gene is positively regulated by PigR. ppGpp also regulates expression of pigR. Conceivably, by regulating expression of pigR (independently of PigR and the MglA-SspA complex), ppGpp might drive expression of sufficient amounts of pigR to promote autoactivation. This led us to test the prediction that PigR positively regulates the expression of its own gene (in concert with MglA and SspA), and to test the idea that ppGpp serves simply to positively regulate the expression of the pigR gene.

To determine whether PigR positively regulates the expression of its own gene we constructed a strain of LVS that contains lacZ in place of pigR (LVS pigR::lacZ) (Figure 7A). Ectopic expression of pigR (from plasmid pF-PigR that has pigR situated downstream of the heterologous groEL promoter) increased β-galactosidase activity in LVS pigR::lacZ ~100-fold, indicating that PigR autoactivates (Figure 7B). This finding is consistent with the idea that PigR functions coordinately with the MglA-SspA complex to control the expression of all target genes.

Using a ΔrelA ΔspoT mutant derivative of the ΔpigR::lacZ reporter strain (LVS ΔrelA ΔspoT ΔpigR::lacZ) we found that ppGpp did not detectably influence the basal activity of the pigR promoter in the absence of PigR (Figure 7B), suggesting that the effect of ppGpp is dependent upon the presence of PigR. Furthermore, we found that although ectopic expression of pigR resulted in an ~30-fold increase in expression of the pigR reporter in the ΔrelA ΔspoT mutant strain, it resulted in an ~110-fold increase in expression of the reporter in the same ΔrelA ΔspoT background as that used for Figure 5 (ppGpp controls the expression of pigR).

Figure 5. PigR and TrmE are required for intramacrophage growth and for virulence in mice. (A) Survival of wild-type (WT) cells, and cells of the indicated mutant derivatives of F. tularensis strain LVS within J774 cells. J774 murine macrophages were infected with cells of the indicated bacterial strains at a multiplicity of infection of ~ 15. Cells were lysed and bacteria (colony forming units (CFU)) were plated for enumeration 24 hours post-infection. (B) Survival of BALB/cByJ mice following intradermal delivery of ~10⁷ cells of each of the indicated strains of LVS. Eight mice were inoculated for each strain tested. Experiments were performed at least twice.

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mutant strain when spoT was also provided ectopically (Figure 7B), indicating that ectopic expression of pigR from a heterologous promoter can only partially alleviate the defects of a ΔrelA ΔspoT mutant. Taken together, our findings suggest that expression of the pigR gene is subject to autoactivation, that PigR and the MglA-SspA complex function coordinately to regulate the expression of all target genes (including pigR), and either that ppGpp influences the activity of PigR, or that ppGpp and PigR act by independent mechanisms but are both needed for pigR expression.

**PigR interacts with the MglA-SspA complex**

Most classical transcription activators function by binding the DNA and contacting a particular subunit of RNAP [48]. Because PigR works in concert with the MglA-SspA complex, and because the MglA-SspA complex associates with RNAP [20], we hypothesized that PigR may activate transcription in *F. tularensis* through a direct contact with the RNAP-associated MglA-SspA complex.

In order to test whether PigR can interact directly with the MglA-SspA complex we used a bacterial two-hybrid assay. This two-hybrid assay is based on the finding that any sufficiently strong interaction between two proteins can activate transcription in *E. coli* provided one of the interacting proteins is tethered to the DNA by a DNA-binding protein and the other is tethered to a subunit of *E. coli* RNAP [49,50]. In the version of the assay used here, contact between a protein (or protein domain) fused to the v subunit of *E. coli* RNAP and another protein fused to a zinc-finger DNA-binding protein (referred to as Zif) activates transcription of a lacZ reporter gene situated downstream of an appropriate test promoter containing a Zif binding site [51].

Our strategy for detecting a putative interaction between PigR and the MglA-SspA complex required that we modify the two-hybrid assay to permit detection of an interaction between PigR and a heteromeric complex. We reasoned that if we made *F. tularensis* SspA in our *E. coli* reporter strain, together with a fusion

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**Figure 6. Ectopic expression of pigR partially complements a ΔrelA ΔspoT mutant but fails to complement a ΔmglA mutant.** (A) Expression of pigR is positively regulated by MglA and by ppGpp. Quantitative RT-PCR analysis of *iglA* transcript abundance in wild-type (WT), ΔmglA, and ΔrelA ΔspoT mutant backgrounds. Transcripts were normalized to those of *tul4*. (B) Quantitative RT-PCR analysis of *iglA*, *pdpA*, and FTL_1219 transcript abundance in wild-type (WT), ΔpigR, ΔmglA, and ΔrelA ΔspoT mutant cells containing the indicated plasmids. Plasmid pF-PigR directs the synthesis of PigR and plasmid pF serves as an empty vector control. Transcripts were normalized to those of *tul4*.

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protein in which PigR was fused to Zif (PigR-Zif), and another in which MglA was fused to ω (MglA-ω). SspA would interact with the MglA-ω fusion protein, presenting a heteromeric target on RNAP that could be bound by the PigR moiety of the PigR-Zif fusion protein (Figure 8A). Therefore, for these “bridge-hybrid” experiments, plasmids directing the synthesis of a PigR-Zif fusion protein, an MglA-ω fusion protein [20], and F. tularensis SspA were introduced into the E. coli reporter strain KDZifΔZ, which harbors the test promoter depicted in Figure 8A linked to lacZ on an F′ episome [20,31]. In support of the idea that PigR interacts with the MglA-SspA complex directly, the PigR-Zif fusion protein activated transcription from the test promoter only in the presence of both SspA and the MglA-ω fusion (Figure 8B).

Evidence that ppGpp promotes the interaction between PigR and the MglA-SspA complex

Our findings suggest that PigR and the MglA-SspA complex function together to regulate the expression of target genes through a direct contact and that ppGpp might be required for the optimal activity of PigR. We therefore wondered whether ppGpp might exert its effects by modulating the interaction between PigR and the MglA-SspA complex. To determine whether ppGpp might influence the ability of PigR to interact with the MglA-SspA complex we took an in vivo crosslinking approach. First we constructed an epitope-tagged version of PigR that contained a vesicular stomatitis virus glycoprotein (VSV-G) epitope-tag fused to its C-terminus. The resulting fusion protein (PigR-V) was functional as it could complement a ΔpigR mutant strain (see Figure S2). We then ectopically expressed pigR-V from a heterologous promoter in cells of LVS, LVS MglA-TAP, and LVS ΔrelA ΔspoT MglA-TAP. Cells of the LVS MglA-TAP strain that did not express pigR-V served as an additional control. Formaldehyde was added to cells in order to crosslink PigR-V to the MglA-SspA complex. Following TAP, reversal of the crosslinks, and separation of proteins by SDS-PAGE, the amount of PigR-V associated with MglA was assessed by Western blotting. The results depicted in Figure 9 show that considerably less PigR-V was found associated with MglA-TAP in cells of the ΔrelA ΔspoT mutant, than in cells of the corresponding wild-type strain, despite there being similar amounts of PigR-V or MglA-TAP present in each cell type. Furthermore, the observed association between PigR and MglA was resistant to DNase treatment (data not shown). These findings suggest that ppGpp stimulates the protein-protein interaction between PigR and the MglA-SspA complex. To address whether ppGpp influences the interaction between PigR and MglA-SspA complexes that are associated with RNAP we performed a similar set of in vivo crosslinking experiments with cells of our LVS β′-TAP and LVS ΔrelA ΔspoT β′-TAP strains. The results depicted in Figure 9 suggest that PigR associates with RNAP and that ppGpp influences this association. Thus, in Francisella ppGpp appears to exert its effects on gene expression by promoting the interaction between PigR and the RNAP-associated MglA-SspA complex.

Discussion

The SspA protein family members MglA and SspA are key regulators of virulence gene expression in F. tularensis. We have found that the alarmone ppGpp and the putative DNA-binding protein, PigR, function in concert with MglA and SspA to regulate a common set of genes. Consistent with this idea, we have shown that cells of both a ΔpigR mutant strain and a ΔrelA ΔspoT mutant strain fail to replicate inside macrophages and are severely attenuated in a mouse model of infection, just like cells of a ΔmglA or a ΔsspA mutant. Furthermore, we have uncovered the molecular basis for the coordinate activities of PigR, ppGpp, and the MglA-SspA complex; we have shown that PigR interacts directly with the MglA-SspA complex, and presented evidence that ppGpp modulates this interaction.
PigR functions coordinately with the MglA-SspA complex

We identified PigR through a genetic screen for positive regulators of MglA/SspA-controlled genes in LVS. PigR resembles a classical transcription regulator as it may contain a helix-turn-helix (HTH) motif (residues 33–54), and shares a limited degree of homology with members of the MerR family of transcription regulators. Note that the ligand-binding or coactivator-binding C-terminal domain, which is often found in MerR family members [52], does not appear to be present in PigR. PigR was also recently identified in a screen for regulators of MglA/SspA-regulated genes in *F. novicida*, where it is called FevR [15]. As is the case for PigR in LVS, FevR in *F. novicida* is essential for replication in macrophages and for virulence in mice [13]. Our demonstration that PigR interacts with the MglA-SspA complex provides a molecular explanation for the findings that PigR in LVS, and FevR in *F. novicida* [15], control the expression of all MglA/SspA-regulated genes.

Our model for how ppGpp, the MglA-SspA complex, and PigR collaborate to control the expression of target genes is depicted schematically in Figure 10. The model specifies that PigR is a transcription activator whose activation target on RNAP is the MglA-SspA complex. Target promoters would contain specific binding sites for PigR, and contact between PigR and the RNAP- associated MglA-SspA complex would stabilize the binding of RNAP to the promoter, thereby activating transcription. Thus, in this model, promoter specificity is determined by the DNA sequence-specific binding of PigR, and the MglA-SspA complex effectively becomes a subunit of RNAP that serves as a contact site for DNA-bound PigR (Figure 10). The model further specifies that ppGpp exerts its effects by positively influencing the interaction between PigR and the MglA-SspA complex (Figure 10), through a mechanism that has yet to be elucidated. Note that in this model, PigR and MglA/SspA-regulated promoters are depicted as being recognized by RNAP holoenzyme containing σ70. Consistent with the idea that these promoters are σ70-dependent, the RNAP that copurifies with MglA appears to contain σ70, as determined by reaction with antibodies recognizing specific sites at target promoters.

Our studies of PigR are the first to demonstrate a direct interaction between a transcription activator and members of the SspA protein family. However, the sequence-specific DNA-binding protein Lpa from bacteriophage P1 may function analogously to PigR and activate transcription from target promoters by making
simultaneous contact with the DNA and with the E. coli RNA-P-
associated SspA protein. Indeed, E. coli SspA can function as a co-
activator of bacteriophage P1 late gene expression [53].

The pigR gene is positively autoregulated

Expression of the pigR gene is itself positively regulated by PigR (in addition to MglA/SspA and ppGpp). This positive feedback
loop may serve to amplify the effect of ppGpp on virulence gene
expression. The interaction between PigR and the MglA-SspA
complex therefore appears to provide an important regulatory
checkpoint for the control of virulence gene expression in F. tularensis. By being responsive to the signaling molecule ppGpp,
this checkpoint presumably serves to relay signals of cellular
nutrition and stress to the regulatory network governing virulence
gene expression.

TrmE influences the expression of MglA/SspA-regulated genes

Through a genetic screen for potential regulators of MglA/
SspA-regulated genes we identified TrmE, CphA, and CaiC in
addition to PigR. CaiC was identified recently in a similar genetic
screen in LVS as a regulator of MglA/SspA-controlled genes
present on the FPI, and has been renamed MigR [17]. We found
that although PigR, CaiC (MigR), CphA, and TrmE influence the
expression of all MglA/SspA-regulated genes in broth grown cells,
only PigR and TrmE were required for growth in macrophages
and for virulence in mice, suggesting that only PigR and TrmE
control the expression of MglA/SspA-regulated genes in vivo, at
least in the particular infection models used here.

We have yet to determine how TrmE exerts its effects on gene
expression. TrmE is a putative GTPase that in other organisms is
thought to influence translation through its RNA methyltransfer-
ase activity [56,57]. In E. coli, TrmE has been linked to the
regulation of gene expression [56], and in Streptococcus pyogenes
the TrmE homolog has been shown to regulate virulence gene
expression through an effect on translation of the transcription
activator RopB [58]. Whether TrmE might mediate its effects on
gene expression in F. tularensis by influencing the translation of
PigR, MglA, SspA, or some other positive regulator of transcription,
remains to be seen.

Materials and Methods

Ethics statement

All animals were handled in accordance with good animal
practice and all work involving animals was approved by the
Harvard Institutional Animal Care and Use Committee.

Plasmids, strains, and growth conditions

F. tularensis subspecies holarctica strain LVS was provided by
Karen Elkins (U.S. Food and Drug Administration, Rockville,
MD). Strains LVS ΔmglA, LVS ΔspsA, LVS ΔmglA-TAP, and LVS
β-TAP have been described previously [20]. All mutant strains
and wild-type LVS were grown with aeration at 37 °C in modified
Mueller-Hinton broth (Difco) supplemented with glucose (0.1%),
ferric pyrophosphate (0.025%), and Isovitalex (2%) or on cysteine
heart agar (Difco) supplemented with 1% hemoglobin solution
(VWR) (CHAH). Under these conditions cells of the wild-type
LVS strain had a culture doubling time of ~162 minutes whereas
cells of the LVS ΔmglA ΔspsA mutant strain (see below) had a
culture doubling time of ~168 minutes. When appropriate,
akamycin or nourseothricin (Werner BioAgents) were used for
selection at 5 μg/ml. E. coli strains XL1-blue (Stratagene) and
DH5α F′IQ (Invitrogen) were used as recipients for all plasmid
constructions. E. coli strain KDZif1ΔZ was used as the reporter
strain for the bacterial two-hybrid experiments. KDZif1ΔZ
harbors an F′ episome containing the lac promoter derivative
placZ if1-61 driving expression of a linked lacZ reporter gene and
has been described previously [51].

Deletion constructs and strains. The allelic replacement
vectors used to create in-frame deletion constructs for relA
(pEX2-relA), spoT (pEX2-spoT), caiC (pEX2-caiC), trmE (pEX2-trmE), pigR
(pEX2-pigRΔ), and cphA (pEX2-cphA) were generated as described
[20]. These vectors were used to create strains LVS ΔrelA, LVS
ΔaucC, LVS ΔtmE, LVS ΔpigR, and LVS ΔgldA, respectively, by allelic exchange. Strain LVS ΔrelA ΔspoT was created by using pEX2-spoT to delete the spoT gene from strain LVS ΔrelA, and strain LVS ΔmglA ΔrelA ΔspoT was created by using pEX2-mglA [20] to delete the mglA gene from strain LVS ΔrelA ΔspoT. Deletions were confirmed by the PCR.

Complementation vectors. Plasmid pF-PigR directs the synthesis of PigR under the control of the groEL promoter and was constructed by replacing gfp in the plasmid pFNLTP6 gro-gfp with full-length pigR [59]; plasmids pF-PigR-V, pF-PigR+SpoT, and pF-PigR+MglA were made in a similar manner, and express the PigR protein with a VSG-G epitope-tag fused to its C-terminus, the PigR protein which has been described previously [20], carries the groEL promoter and was used as an empty control vector for complementation experiments. Plasmids pF2-SpoT and pF2-TmrE were used for complementation of the LVS ΔrelA ΔspoT and LVS ΔtmE mutant strains, respectively, and were constructed by inserting full-length spoT and tmrE in place of ΔgldA in the plasmid pF2-SpaA [20]. Expression of the spoT and tmrE genes are therefore under the control of a weakened groEL promoter lacking its putative UP-element. Plasmid pF2 [20] contains the same weakened groEL promoter that is present on plasmids pF2-SpoT and pF2-TmrE, and was used as an empty control vector. Plasmids pCaC and pCphA were used for complementation of the LVS ΔaucC and LVS ΔgldA mutant strains, respectively, and were constructed by inserting full-length aucC and gldA, respectively, together with approximately 100 bp of upstream DNA sequence into plasmid pFNLTP6 [59]; plasmid pFNLTP6 was used as an empty control vector.

Plasmids and strains for TAP-tag experiments. Plasmid pEX-MglA-TAP was used to create strains that synthesized a TAP-tagged form of MglA (with a C-terminal TAP-tag) from the native mglA locus and has been described previously [20]. Plasmid pEX-RpoC-TAP was used to create strains that synthesized a TAP-tagged form of the subunit of RNAP from the native rpoC locus and has been described previously [59].

Plasmids for bacterial bridge two-hybrid assays. Plasmid pBRGPla directs the synthesis of the Gal11P-o fusion protein and has been described before [51]. Plasmid pBRGPla confers resistance to carbenicillin, harbors a ColE1 origin of replication, and carries an IPTG-inducible lacUV5 promoter that drives expression of the o fusion protein [51]. Plasmid pBRMgLa-α directs the synthesis of full-length MgLa fused to residues 2–90 of E. coli o, is a derivative of plasmid pBRGPla, and has been described previously [20]. The MgLa-o fusion protein is under the control of an IPTG-inducible lacUV5 promoter.

Plasmid pACL1920 [61] directs the synthesis of the LacZ fusion protein under the control of the lac promoter, confers resistance to spectinomycin, and harbors the pSC101 origin of replication. Plasmid pACL-SspA directs the synthesis of full-length, unmodified F. tularensis SspA under the control of the IPTG-inducible lacUV5 promoter, and was constructed by inserting full-length sspA together with the lacUV5 promoter into pACL1920.

Francisella reporter strains. Reporter strains LVS ΔaucC ΔtmE lacZ and LVS ΔaucC ΔtmE lacZ contain the E. coli lacZ gene in place of the LVS ΔaucC and ΔtmE genes, respectively. The allelic replacement vectors used to create these strains were generated by first amplifying regions flanking the iglA and pigR genes by the PCR and then splicing the flanking regions to the E. coli lacZ gene by overlap-extension PCR. The resulting PCR products were cloned into the suicide plasmid pEX18km [20], yielding plasmids pEX-iglA-lacZ and pEX-pigR-lacZ. The constructs were designed so that the iglA and pigR ORFs were replaced exactly with the lacZ ORF. Because the sacB gene on pEX18km is insufficient to mediate efficient sucrose counterselection in LVS, a second copy of sacB was subcloned from plasmid pPV [3] on a PstI fragment to create plasmids pEX2-iglA-lacZ and pEX2-pigR-lacZ, which were then used with wild-type LVS to create strains LVS ΔaucC-lacZ and LVS ΔaucC ΔtmE-lacZ by allelic exchange. Strains LVS ΔaucC ΔtmE ΔtmE-lacZ and LVS ΔaucC ΔtmE ΔaucC-lacZ were made using plasmid pEX2-pigR-lacZ together with strains LVS ΔaucC and LVS ΔaucC ΔtmE.

Transposon mutagenesis.

One microgram of the mariner transposon delivery plasmid pSD [62] was used to transform the reporter strain LVS ΔaucC-lacZ, and the resulting kanamycin-resistant isolates were patched onto CHAH supplemented with 100 μg/mL X-gal (Qiagen); approximately 9,000 mutants were screened for β-galactosidase activity. Chromosomal DNA was purified from isolates with decreased β-galactosidase activity relative to the non-mutagenized reporter strain, and the location of the transposon was determined by sequencing the chromosomal DNA as described previously [63].

TAP

TAP was performed as described previously [20].

Bacterial bridge two-hybrid assays.

Cells were grown with aeration at 37°C in LB supplemented with carbenicillin (100 μg/mL), tetracycline (10 μg/mL), spectinomycin.
(100 µg/mL), and IPTG at the concentration indicated. Cells were permeabilized with SDS-CHCl₃ and assayed for β-galactosidase activity as described previously [20]. Assays were performed at least three times in duplicate on separate occasions. Representative data sets are shown. Values are averages based on one experiment; duplicate measurements differed by less than 10%.

Protein crosslinking and co-purification assays
Plasmid pF3 is a derivative of plasmid pF that confers resistance to nourseothricin. pF3 was constructed by first amplifying the nourseothricin resistance gene from plasmid pJK795 (provided by Julia Kohler) by the PCR and then cloning the corresponding PstI-SphI-digested PCR product into PstI-SphI-digested plasmid pF. Plasmid pF3-PigR-V directs the synthesis of the PigR protein with a VSV-G epitope-tag fused to its C-terminus, and was constructed by amplifying pigR from pF-PigR by the PCR using an oligo encoding the VSV-G tag and then inserting the corresponding BamHI-Xmal-digested PCR product into BamHI-Xmal-digested pF3. For the experiments described in Figure 9, plasmids pF3-PigR-V and pF3 were transformed into the indicated strains. Cells were grown to mid-log phase and proteins were crosslinked in cells from 3 mL of culture using 1% formaldehyde for 30 minutes followed by quenching for 15 minutes with 250 mM glycine. Cells were washed three times with PBS, sonicated extensively, and TAP was performed on the crosslinked proteins essentially as described earlier [20]. Following TAP, proteins were resuspended in NuPAGE LDS sample buffer (Invitrogen), incubated at 100° for 30 minutes to reverse the crosslinks, and subjected to SDS-PAGE and immunoblotting.

Francisella β-galactosidase assays
Cells were grown to mid-log phase, permeabilized with SDS-CHCl₃, and assayed for β-galactosidase activity essentially as described above for the bacterial two-hybrid assays.

Immunoblots
Cell lysates and eluted proteins were separated by SDS-PAGE on 10% or 4%–12% Bis-Tris NuPAGE gels in MES or MOPS running buffer (Invitrogen). Membranes were blocked with 25 mL of SuperBlock the iBlot dry blotting system or the XCell II Blot Module running buffer (Invitrogen) and transferred to nitrocellulose using PAP; resolved on a PEI cellulose thin layer chromatography plate (J.T. Baker) using 1.5 M KH₂PO₄ (pH 3.4) running buffer. Images were then extracted by lysis of cells in 1 M formic acid and were then probed with monoclonal antibodies against the VSV-G-tag (Sigma), the CBP portion of the TAP-tag (Open Biosystems), or against the VSV-G epitope-tag fused to its C-terminus, and was constructed by amplifying pigR from pF-PigR by the PCR using an oligo encoding the VSV-G tag and then inserting the corresponding BamHI-Xmal-digested PCR product into BamHI-Xmal-digested pF3. For the experiments described in Figure 9, plasmids pF3-PigR-V and pF3 were transformed into the indicated strains. Cells were grown to mid-log phase and proteins were crosslinked in cells from 3 mL of culture using 1% formaldehyde for 30 minutes followed by quenching for 15 minutes with 250 mM glycine. Cells were washed three times with PBS, sonicated extensively, and TAP was performed on the crosslinked proteins essentially as described earlier [20]. Following TAP, proteins were resuspended in NuPAGE LDS sample buffer (Invitrogen), incubated at 100° for 30 minutes to reverse the crosslinks, and subjected to SDS-PAGE and immunoblotting.

Microarray experiments
Cells were grown to mid-log phase and RNA was isolated using Qiagen RNeasy Mini columns. RNA was purified from three separate cultures for each strain and DNase-treated using RQ1 DNase (Promega). cDNA was synthesized and prepared for hybridization as described previously [20]. Labeled probes in hybridization buffer (5x SSC, 50% formamide, 0.1% SDS, 0.6 µg/µL Salmon Sperm DNA) were applied to oligonucleotide arrays (provided by the NIAID Pathogen Functional Genomics Resource Center) using a Tecan HS400 Hybridization Station. Arrays were scanned using a GenePix 4000B microarray scanner (Molecular Devices) and data were analyzed with GeneSpring GX.

ppGpp assays
Intracellular levels of ppGpp were determined essentially as described [64]. Strains were grown with aeration at 37°C in modified Mueller-Hinton broth to an OD₆₀₀~0.2. Cells from 1 mL of culture were then pelleted and re-suspended in 250 µl MOPS-MGS [65] containing 55 mM mannose in place of mannitol, and incubated with aeration at 37°C for one hour in the presence of 25 µCi ³²P KH₂PO₄ (Perkin Elmer). Nucleotides were then extracted by lysis of cells in 1 M formic acid and were resolved on a PEI cellulose thin layer chromatography plate (J.T. Baker) using 1.5 M KH₂PO₄ (pH 3.4) running buffer. Images were acquired using a GE Storm Phosphoimager.

Supporting Information

Figure S1 Complementation of the effects of the ΔcaiC, ΔtmE, ΔphkA, and ΔpigR mutations on virulence gene expression in F. tularensis. Quantitative RT-PCR analysis of iglA transcript abundance in wild-type (WT), ΔtmE, ΔcaiC, ΔphkA, and ΔpigR mutant cells containing the indicated plasmids. Plasmid pF2-TmA directs the synthesis of TrmA and plasmid pF2 serves as an empty vector control. Plasmids pCaC and pCphA direct the synthesis of CaIC and CphA, respectively, and plasmid pFNLP6 serves as an empty vector control. Plasmid pF-PigR directs the synthesis of PigR and plasmid pF serves as an empty vector control. Transcripts were normalized to those of tufA. 

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Figure S2 Complementation of the effects of the ΔpigR mutation by PigR-V. Quantification of lacZ expression in strain LVS pigR::lacZ containing the indicated plasmids. Plasmids pF-PigR and pF-PigR-V direct the synthesis of PigR and PigR-V, respectively. Plasmid pF served as the empty control vector. 

Found at: doi:10.1371/journal.ppat.1000641.s002 (0.75 MB EPS)

Macropherases
J774 murine macrophages were seeded in DMEM (Mediatech) supplemented with 10% fetal bovine serum (Invitrogen) [DMEM-F] in a 96-well plate at a density of ~10⁶ macrophages per well and incubated at 37° with 5% CO₂ overnight. Macrophages were then infected with mutant strains or wild-type LVS (in triplicate; three wells per strain) at a multiplicity of infection of ~15 and incubated at 37° with 5% CO₂ for two hours. Macrophages were washed twice with PBS (Pierce) and then covered with DMEM-F containing gentamicin at 10 µg/mL. After 24 hours of incubation at 37° with 5% CO₂, intracellular bacteria were enumerated by lysing the macrophage in PBS containing 1% saponin (Calbiochem) for five minutes at room temperature, diluting the lysate in PBS, and then plating onto CHAH.

Mice challenge studies
Cultures were grown to early log phase (an OD₆₂₀ of 0.2) harvested, and resuspended to the appropriate concentration in sterile PBS (Gibco). Inoculums were verified by direct plating on CHAH plates. Male BALB/cByJ mice (6–8 weeks old; Jackson Laboratory, Bar Harbor, ME) were caged in a microisolator in a pathogen-free environment in the animal facility at Harvard Medical School. Mice received the appropriate bacterial inoculum in sterile PBS via intradermal injection into the midbelly. Survival was monitored for 21 days after challenge.
Table S1: Microarray analysis of genes whose expression changes by a factor of 2.5 or more with a p-value < 0.05 in either a ΔmgA, ΔsspA ΔtpS, ΔppGR, ΔacrC, ΔtmeE, or ΔppGR mutant background compared to wild-type. Negative values indicate genes that are upregulated by MglA, ppGpp, PgbR, CphA, TimE, or CphA, whereas positive values indicate genes that are negatively regulated. LVS ORFs are referred to by the LVS (FTL number) and Schu S4 (FTT number) locus tags for convenience, and gene names are included when available. “a” indicates those genes that belong to the MglA/SspA regulon [20]; “b” indicates that the p-value is between 0.05 and 0.1; and “c” indicates that the p-value is greater than 0.1. For all other fold changes the p-value is <0.05.

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References

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Author Contributions
Conceived and designed the experiments: JCC LTB SLD. Performed the experiments: JCC LTB MCH. Analyzed the data: JCC LTB DLK SLD. Wrote the paper: JCC SLD.


