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RESEARCH ARTICLE

Polyphosphate Kinase Antagonizes Virulence Gene Expression in *Francisella tularensis*

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ABSTRACT The alarmone ppGpp is a critical regulator of virulence gene expression in *Francisella tularensis*. In this intracellular pathogen, ppGpp is thought to work in concert with the putative DNA-binding protein PigR and the SspA protein family members MglA and SspA to control a common set of genes. MglA and SspA form a complex that interacts with RNA polymerase (RNAP), and PigR functions by interacting with the RNAP-associated MglA-SspA complex. Prior work suggested that ppGpp indirectly exerts its regulatory effects in *F. tularensis* by promoting the accumulation of polyphosphate in the cell, which in turn was required for formation of the MglA-SspA complex. Here we show that in *Escherichia coli*, neither polyphosphate nor ppGpp is required for formation of the MglA-SspA complex but that ppGpp promotes the interaction between PigR and the MglA-SspA complex. Moreover, we show that polyphosphate kinase, the enzyme responsible for the synthesis of polyphosphate, antagonizes virulence gene expression in *F. tularensis*, a finding that is inconsistent with the notion that polyphosphate accumulation promotes virulence gene expression in this organism. Our findings identify polyphosphate kinase as a novel negative regulator of virulence gene expression in *F. tularensis* and support a model in which ppGpp exerts its positive regulatory effects by promoting the interaction between PigR and the MglA-SspA complex.

IMPORTANCE In *Francisella tularensis*, MglA and SspA form a complex that associates with RNA polymerase to positively control the expression of key virulence genes. The MglA-SspA complex works together with the putative DNA-binding protein PigR and the alarmone ppGpp. PigR functions by interacting directly with the MglA-SspA complex, but how ppGpp exerts its effects was unclear. Prior work indicated that ppGpp acts by promoting the accumulation of polyphosphate, which is required for MglA and SspA to interact. Here we show that formation of the MglA-SspA complex does not require polyphosphate. Furthermore, we find that polyphosphate antagonizes the expression of virulence genes in *F. tularensis*. Thus, ppGpp does not promote virulence gene expression in this organism through an effect on polyphosphate.

KEYWORDS MglA, SspA, gene regulation, ppGpp

Francisella tularensis is a Gram-negative, intracellular pathogen and the causative agent of tularemia, a potentially fatal disease. *F. tularensis* mostly infects rodents and other small mammals but can also infect humans. Humans can become infected via multiple routes, including through an arthropod vector and through ingestion, but the most severe form of the disease occurs following inhalation of aerosolized bacteria (1). *F. tularensis* is a highly infectious pathogen, with the most virulent strains having an infectious dose of as few as 10 bacteria (2). Due to its highly infectious nature, its ability to cause severe disease, and its ability to be easily aerosolized, *F. tularensis* had been

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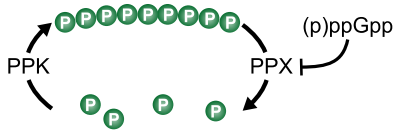

FIG 1 Control of polyphosphate abundance in *E. coli*. Polyphosphate is synthesized by polyphosphate kinase (PPK) and broken down by polyphosphate phosphatase (PPX). (p)ppGpp influences the abundance of polyphosphate by inhibiting PPX activity.

developed by several countries as a bioweapon, leading the CDC to list *F. tularensis* as a category A select agent (3).

The ability of *F. tularensis* to replicate within macrophages is key to its ability to cause disease. Prominent among those factors required for intramacrophage growth and virulence are the components of a type VI secretion system that are encoded on the so-called *Francisella* pathogenicity island (FPI) (4–8). All of the virulence genes on the FPI, as well as many other genes in *F. tularensis*, are positively regulated by three key regulators called MglA, SspA, and PigR (also known as FevR) (9–13). MglA and SspA are members of the stringent starvation protein A (SspA) family of proteins and form a heteromeric complex that binds to RNA polymerase (RNAP) (11, 14, 15). Interaction between the MglA-SspA complex and RNAP is thought to be essential for these proteins to exert their regulatory effects (11). Furthermore, interaction between PigR and the RNAP-associated MglA-SspA complex is necessary in order for PigR, MglA, and SspA to function as positive regulators (16). Although PigR, MglA, and SspA have been shown to occupy the promoters of both regulated and nonregulated genes, a small 7-bp sequence element is both necessary and sufficient for conferring control by PigR (and thus presumably by MglA and SspA as well) (17). This sequence, which is referred to as the PigR response element, is found ~6 bp upstream of the −35 element of target promoters and could function as a binding site for PigR (17).

The small molecules guanosine pentaphosphate (pppGpp) and guanosine tetraphosphate (ppGpp) (referred to collectively here as [p]ppGpp), also appear to be critical for virulence gene expression in *F. tularensis* (13). (p)ppGpp, which is referred to as an alarmone, is produced in response to a variety of stress signals (18). In *Escherichia coli*, (p)ppGpp is produced by the proteins RelA and SpoT (reviewed in reference 18). RelA is a monofunctional enzyme that synthesizes (p)ppGpp in response to amino acid starvation and thus mediates the so-called stringent response, a process in which the increase in (p)ppGpp leads to inhibition of rRNA transcription, increased expression of amino acid biosynthesis genes, and a reduction in protein synthesis (19–21). SpoT is a bifunctional enzyme, capable of both degrading and synthesizing (p)ppGpp, which responds to conditions of carbon, phosphate, and fatty acid limitation (20). The effects of ppGpp on transcription in *E. coli* are potentiated by the small RNAP-associated protein DksA (22, 23). Recently, ppGpp has been shown to bind to two distinct sites on *E. coli* RNAP, referred to as sites 1 and 2 (24–26). At high concentrations of ppGpp, binding to site 2, which is at the RNAP-DksA interface, is thought to account for most of the effects of ppGpp on transcription initiation (26). (p)ppGpp exerts regulatory effects on hundreds of genes in *E. coli* (27, 28) and has also been shown to regulate virulence gene expression in several pathogens, including *Legionella pneumophila* (29), *Salmonella* spp. (30, 31), *Vibrio cholerae* (32), *Mycobacterium tuberculosis* (33), *Pseudomonas aeruginosa* (34), and enterohemorrhagic *E. coli* (35) (reviewed in reference 36).

In addition to its effects on transcription, (p)ppGpp is also known to directly bind to target enzymes to influence their activity, allowing (p)ppGpp to directly control many cellular processes (reviewed in reference 37). In *E. coli* it is well established that (p)ppGpp inhibits the activity of polyphosphate phosphatase (PPX), the enzyme responsible for breaking down polyphosphate (reviewed in reference 38), and that *relA spoT* mutant cells that can no longer synthesize (p)ppGpp do not contain detectable amounts of polyphosphate (Fig. 1) (39, 40). Polyphosphate is a chain of tens to hundreds of inorganic phosphate molecules that is synthesized in response to stress

conditions, including starvation (38), by the enzyme polyphosphate kinase (PPK) (40). Polyphosphate has been shown to be important for the production of many virulence factors, including those involved in motility and biofilm formation in *P. aeruginosa* (41–43). Moreover, *ppk*, the gene encoding PPK, has also been found to be important for the virulence of several pathogens, including *Mycobacterium tuberculosis* (44, 45), *Salmonella* spp. (46), *Helicobacter pylori* (47), *Vibrio cholerae* (48), *Shigella flexneri* (46), and *F. tularensis* (49). Thus, at least in principle, the effects of (p)ppGpp on the virulence of a particular organism could be accounted for solely by its effects on the abundance of polyphosphate in the cell.

In *F. tularensis*, (p)ppGpp controls the expression of many of the same genes that are controlled by PigR, MglA, and SspA (13). Studies in *F. tularensis* suggest that (p)ppGpp promotes the interaction between PigR and the RNAP-associated MglA-SspA complex (13), and recent work has revealed that ppGpp can bind directly to the MglA-SspA complex to mediate its effects (15). However, other recent findings suggested that (p)ppGpp might exert its effects on virulence gene expression indirectly by promoting the accumulation of polyphosphate, which in turn was required for MglA and SspA to form a complex (50). Here we present evidence that polyphosphate is not required in order for MglA and SspA to interact. Moreover, we show that polyphosphate antagonizes the expression of genes that are positively regulated by (p)ppGpp, PigR, MglA, and SspA. Our findings have important implications for how (p)ppGpp exerts its regulatory effects in *F. tularensis*.

RESULTS

(p)ppGpp promotes the interaction between PigR and the MglA-SspA complex in *E. coli*. Prior work suggested that in *F. tularensis* (p)ppGpp exerts its effects on virulence gene expression by promoting the accumulation of polyphosphate, which in turn, is required for MglA and SspA to form a complex (50). To test whether or not the interaction between MglA and SspA depends (directly or indirectly) on the ability of cells to synthesize (p)ppGpp, we took advantage of a two-hybrid assay we had used previously to detect the interaction between MglA and SspA in *E. coli* (11, 16, 51). In this assay, MglA from *F. tularensis* is fused to a monomeric DNA-binding protein called Zif and SspA from *F. tularensis* is fused to the ω subunit of *E. coli* RNAP (Fig. 2A). The MglA-Zif and SspA-ω fusion proteins are then synthesized in an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible fashion in cells of an *E. coli* reporter strain in which a Zif binding site is positioned upstream of a test promoter that drives expression of *lacZ*. Interaction between the RNAP-bound SspA-ω fusion protein and the DNA-bound MglA-Zif fusion protein activates transcription from the test promoter, resulting in an increase in *lacZ* expression (11, 16). To determine whether (p)ppGpp modulates the interaction between MglA and SspA, we first constructed a *relA spoT* mutant derivative of our *E. coli* reporter strain that can no longer synthesize (p)ppGpp (indicated as ppGpp^0) (39). The results depicted in Fig. 2B show that the MglA-Zif fusion protein interacted with the RNAP-tethered SspA-ω fusion protein in both wild-type (WT) and ppGpp^0 cells of the reporter strain. Note that although the absolute amount of reporter gene expression was slightly higher in WT cells than in ppGpp^0 cells, the magnitude of MglA-Zif dependent activation from the test promoter was identical (~18-fold) in both WT and ppGpp^0 cells of the reporter strain (Fig. 2B). These findings indicate that in *E. coli*, formation of the MglA-SspA complex does not require ppGpp.

Because in *F. tularensis* (p)ppGpp promotes the interaction between PigR and the RNAP-associated MglA-SspA complex (13), we sought to determine whether we could recapitulate the effect of (p)ppGpp on this interaction in *E. coli*. To do this, we used a modified version of our two-hybrid assay that permits the detection of interactions among three proteins (13, 16). In the version of this bridge-hybrid assay used here, PigR from *F. tularensis* is fused to Zif and SspA from *F. tularensis* is fused to the ω subunit of *E. coli* RNAP (Fig. 2C). MglA from *F. tularensis* is then synthesized, together with the SspA-ω and PigR-Zif fusion proteins, in cells of the same *E. coli* reporter strain employed in our two-hybrid assay. In these cells, the complex formed between MglA and the

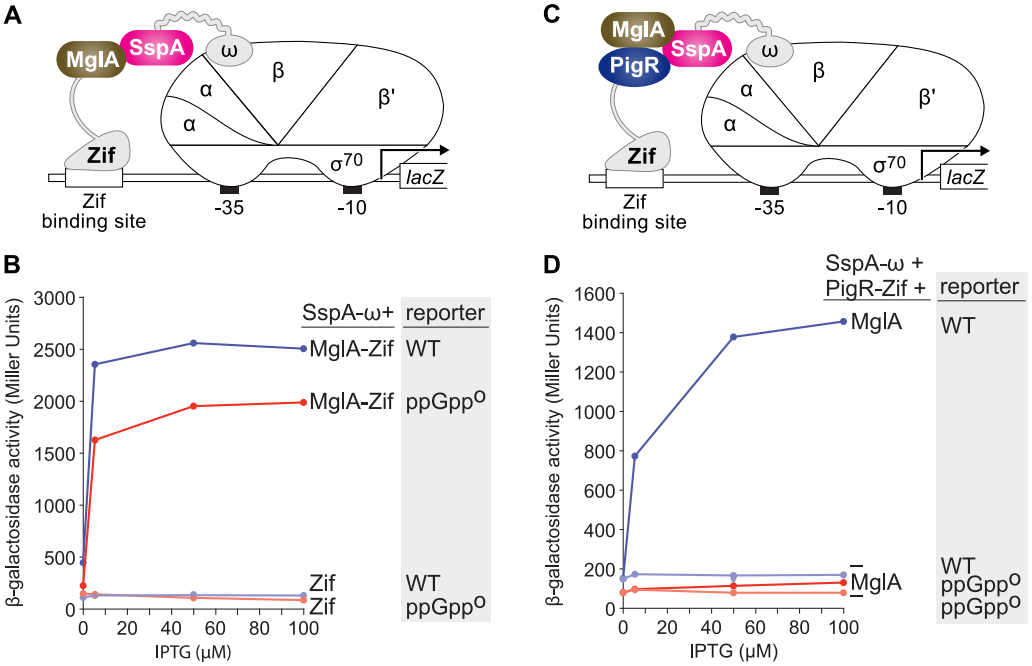

FIG 2 (p)ppGpp does not influence formation of the MglA-SspA complex but does promote the interaction between PigR and the MglA-SspA complex in *E. coli*. (A) Schematic representation of *E. coli* two-hybrid assay used in panel B. Interaction between the MglA-Zif and SspA-ω fusion proteins stimulates transcription from the test promoter driving expression of *lacZ*. (B) Bacterial two-hybrid assay of the ability of MglA and SspA to interact with one another in cells of the reporter strain that synthesizes ppGpp (indicated WT) and in cells of the reporter strain that cannot synthesize ppGpp (indicated ppGpp^0). (C) Schematic representation of *E. coli* bridge-hybrid assay used in D. Interaction between the PigR-Zif fusion protein and the complex formed between MglA and the SspA-ω fusion protein stimulates transcription from the test promoter that drives expression of *lacZ*. (D) Bacterial bridge-hybrid assay of the ability of PigR to interact with the MglA-SspA complex in cells of the reporter strain that synthesizes ppGpp (indicated as WT) and in cells of the reporter strain that cannot synthesize ppGpp (indicated as ppGpp^0). Assays in panels B and D were performed with cells of the *E. coli* reporter strain KDZif1ΔZ (indicated as WT) or cells of the *E. coli* reporter strain ARZif1ΔAZT (indicated as ppGpp^0). Cells containing compatible plasmids directing the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible synthesis of the specified proteins were grown in the presence of IPTG at the indicated concentration and then assayed for β-galactosidase activity.

SspA-ω fusion protein becomes tethered to RNAP through the ω moiety of the SspA-ω fusion. Interaction between the RNAP-bound MglA-SspA complex and the DNA-bound PigR-Zif fusion protein then activates transcription from the test promoter in the *E. coli* reporter strain (13, 16). In WT cells of the reporter strain, the PigR-Zif fusion protein interacted with the RNAP-tethered complex formed between MglA and the SspA-ω fusion protein to activate transcription from the test promoter (Fig. 2D). In the ppGpp^0 cells of the reporter strain, PigR did not detectably interact with the MglA-SspA complex (Fig. 2D). Taken together, our findings indicate that in *E. coli*, just as in *F. tularensis*, (p)ppGpp promotes the interaction between PigR and the MglA-SspA complex but is not required for MglA and SspA to interact with one another.

Polyphosphate is not required for formation of the MglA-SspA complex or the PigR-MglA-SspA complex in *E. coli*. Our findings with our *E. coli* two-hybrid and bridge-hybrid assays do not support a model in which (p)ppGpp modulates the interaction between MglA and SspA indirectly through an effect on polyphosphate accumulation, as had previously been suggested (50). In order to explicitly test whether polyphosphate influences the ability of MglA and SspA to interact with one another, we made a version of our *E. coli* two-hybrid reporter strain that could no longer synthesize polyphosphate (50). This strain harbors a deletion of *ppk* and thus lacks PPK, the enzyme responsible for the synthesis of polyphosphate in *E. coli* (reviewed in reference 38). Using a version of our two-hybrid system in which SspA is fused to Zif and MglA is fused to ω (Fig. 3A), we found that MglA and SspA interact with one another equally well in WT cells and in Δ*ppk* mutant cells (Fig. 3B). These findings indicate that

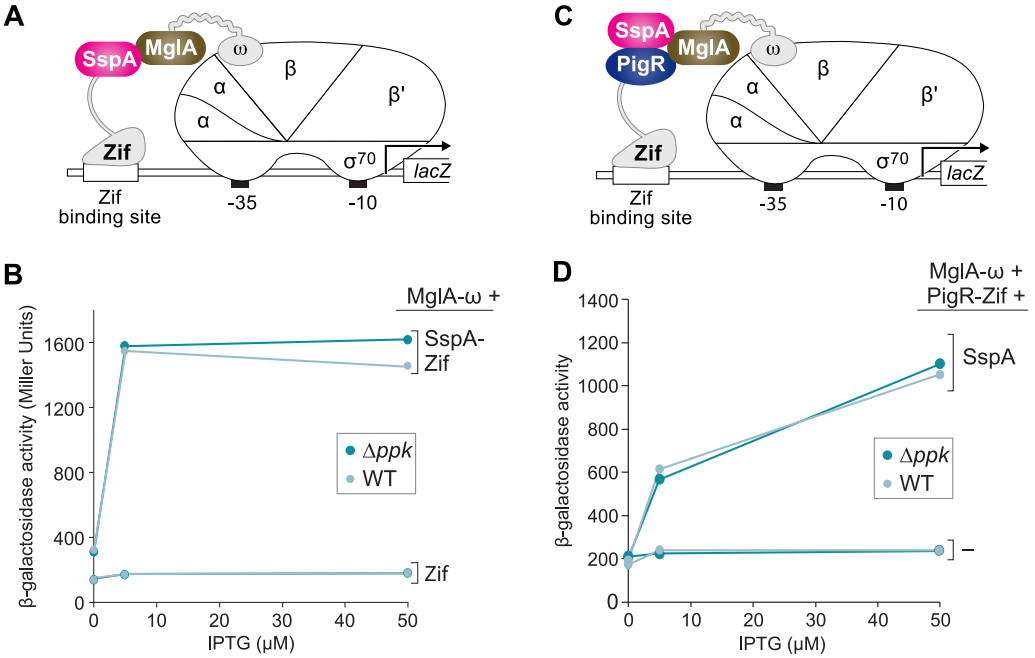

FIG 3 Polyphosphate does not influence formation of the MglA-SspA complex or the PigR-MglA-SspA complex in *E. coli*. (A) Schematic representation of *E. coli* two-hybrid assay used in panel B. Interaction between the SspA-Zif and MglA-ω fusion proteins stimulates transcription from the test promoter that drives expression of *lacZ*. (B) Bacterial two-hybrid assay of the ability of MglA and SspA to interact with one another in cells of the reporter strain that can synthesize polyphosphate (indicated as WT) and in cells of the reporter strain that cannot synthesize polyphosphate (indicated as Δ*ppk*). (C) Schematic representation of bacterial bridge-hybrid assay used in panel D. Interaction between the PigR-Zif fusion protein and the complex formed between SspA and the MglA-ω fusion protein stimulates transcription from the test promoter that drives expression of *lacZ*. (D) Bacterial bridge-hybrid assay of the ability of PigR to interact with the MglA-SspA complex in cells of the reporter strain that can synthesize polyphosphate (indicated as WT) and in cells of the reporter strain that cannot synthesize polyphosphate (indicated as Δ*ppk*). Assays in panels B and D were performed with cells of the *E. coli* reporter strain KDZif1ΔZ (indicated as WT) or cells of the *E. coli* reporter strain ARZif1ΔKZ (indicated as Δ*ppk*). Cells containing compatible plasmids directing the IPTG-inducible synthesis of the specified proteins were grown in the presence of IPTG at the indicated concentration and then assayed for β-galactosidase activity.

polyphosphate is not required in order for MglA and SspA to interact with one another in *E. coli*.

We next asked whether polyphosphate influences the ability of PigR to interact with the MglA-SspA complex in *E. coli*. To do this we employed a version of our bridge-hybrid assay in which PigR is fused to Zif, MglA is fused to ω, and SspA is provided in its native form (Fig. 3C). The results shown in Fig. 3D reveal that PigR interacts with the MglA-SspA complex equally well in WT cells and in Δ*ppk* mutant cells. Altogether, our findings indicate that polyphosphate is not required for formation of the MglA-SspA complex, nor is it required for PigR to interact with the MglA-SspA complex in cells of *E. coli*.

Polyphosphate kinase functions to represses virulence gene expression in *F. tularensis*. If formation of the MglA-SspA complex does not require polyphosphate and if polyphosphate does not promote the interaction between PigR and the MglA-SspA complex, then polyphosphate would not be expected to be required for the expression of genes that are positively regulated by (p)ppGpp/PigR/MglA/SspA in *F. tularensis*. To test this prediction, we constructed a mutant of the live vaccine strain of *F. tularensis* (LVS) in which the *ppk* gene (*FTL_0554*) (52) was deleted (LVS Δ*ppk*). We then measured the expression of the MglA-regulated *iglA* virulence gene in LVS WT cells, in LVS Δ*pigR* mutant cells (used as a negative control), and in LVS Δ*ppk* mutant cells that contained the empty vector pF2. We also measured the expression of the *iglA* gene in LVS Δ*ppk* mutant cells that contained the plasmid pF2-PPK-V, encoding PPK with a C-terminal vesicular stomatitis virus glycoprotein (VSV-G) epitope tag. The results depicted in Fig. 4A show that, consistent with previous findings, the abundance of the *iglA* transcript

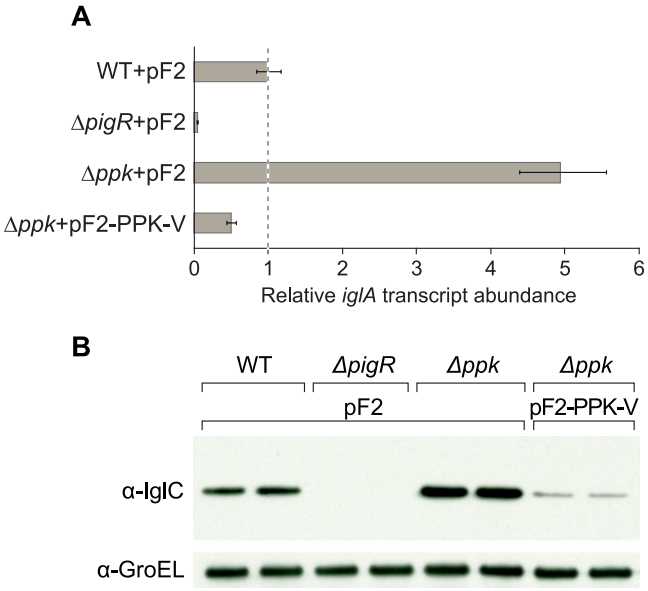

FIG 4 Polyphosphate kinase antagonizes virulence gene expression in *F. tularensis*. (A) Polyphosphate kinase functions to repress expression of the FPI *iglA* gene. The relative abundance of the *iglA* transcript was determined in cells of the indicated strains containing the specified plasmids by quantitative reverse transcriptase PCR (qRT-PCR). pF2 is an empty vector control, whereas pF2-PPK-V directs the synthesis of an epitope-tagged version of PPK. The figure shows data from a representative experiment with biological duplicates. Transcripts were normalized to *tul4*, and error bars represent ±1 standard deviation from the value (calculated using the mean threshold cycle). (B) Effect of polyphosphate kinase on the abundance of IglC. The abundance of IglC in the indicated strains containing the specified plasmids was determined by Western blotting with an antibody against IglC. (Note that the gene encoding IglC is in the same operon as *iglA*.) An antibody against GroEL was used as a loading control. Duplicate biological samples were tested for each strain and a representative data set is shown. Plasmids are as in panel A.

was ~20-fold lower in cells of the LVS Δ*pigR* mutant strain than in WT cells. Surprisingly, the abundance of the *iglA* transcript was ~5-fold higher in cells of the LVS Δ*ppk* mutant strain than in WT cells. Furthermore, ectopic expression of *ppk* in cells of the LVS Δ*ppk* mutant strain resulted in an ~10-fold decrease in expression of the *iglA* gene (Fig. 4A). Consistent with these findings, Western blotting revealed that the abundance of the product of the (p)ppGpp/PigR/MglA/SspA-regulated *iglC* virulence gene (which is in the same operon as *iglA*) was higher in cells of the LVS Δ*ppk* mutant strain than in WT cells and that the effect of the Δ*ppk* mutation on IglC abundance could be complemented by providing *ppk* in *trans* (Fig. 4B). Thus, consistent with our prediction, these findings indicate that polyphosphate kinase is not required for the expression of genes that are positively regulated by (p)ppGpp/PigR/MglA/SspA in *F. tularensis*. Furthermore, they suggest that under the conditions of our experiments, polyphosphate antagonizes virulence gene expression in *F. tularensis*.

Polyphosphate does not appear to exert its regulatory effects through substrate competition. RelA in *F. tularensis* is unusual in that it can only synthesize guanosine pentaphosphate (pppGpp) from ATP and GTP, unlike its counterpart in *E. coli* that can synthesize both pppGpp and ppGpp from ATP and GTP or ATP and GDP, respectively (53). Furthermore, *F. tularensis* is distinct from *E. coli* in that it does not appear to encode pppGpp phosphohydrolase, the enzyme principally involved in converting pppGpp to ppGpp in *E. coli* (54). However, PPX from *E. coli* has been shown to be capable of converting pppGpp to ppGpp (54). We therefore reasoned that in *F. tularensis*, PPX might be the enzyme chiefly responsible for converting any pppGpp that is made to ppGpp. If ppGpp were the only species that could influence gene expression in *F. tularensis* (see for example 55), then polyphosphate might effectively repress the expression of ppGpp/PigR/MglA/SspA-regulated genes by competing with pppGpp for the available PPX in the cell, thus reducing the intracellular concentration of active ppGpp (Fig. 5A). According to this model, in cells of the LVS Δ*ppk* mutant

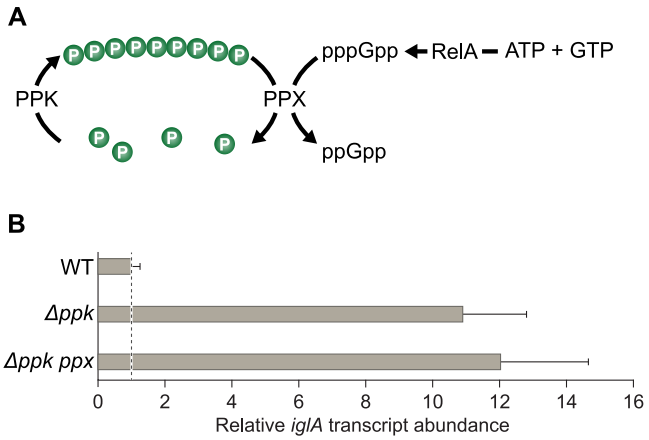

FIG 5 Polyphosphate does not exert its negative regulatory effects through substrate competition. (A) Substrate competition model to account for the negative regulatory effects of polyphosphate. (B) Inactivation of *ppx* in LVS Δ*ppk* mutant cells has no effect on the expression of the FPI *iglA* gene. The relative abundance of the *iglA* transcript was determined in cells of the indicated strains by qRT-PCR. The figure shows data from a representative experiment with biological duplicates. Transcripts were normalized to *tul4*, and error bars represent ±1 standard deviation from the value (calculated using the mean threshold cycle).

strain, ppGpp would be more abundant than in WT cells because there would be no polyphosphate available to compete with pppGpp for the available PPX. To test this possibility, we insertionally inactivated *ppx* in cells of our LVS Δ*ppk* mutant strain. The results depicted in Fig. 5B indicate that the abundances of the *iglA* transcript were similar in cells of the LVS Δ*ppk* and LVS Δ*ppk ppx* mutant strains, being ~11-fold and ~12-fold higher, respectively, than that found in WT cells. (Note that we typically find that the effect of the Δ*ppk* mutation on *iglA* expression is 2- to 4-fold greater in cells that do not contain plasmids than in cells that do [cf. Fig. 4B and 5B].) These findings suggest that polyphosphate does not antagonize virulence gene expression in *F. tularensis* through an effect on PPX substrate competition.

Polyphosphate may exert its regulatory effects through the Lon protease. It is well established that in *E. coli* the activity of the Lon protease is dependent upon polyphosphate (56). To test whether polyphosphate might repress virulence gene expression in *F. tularensis* by influencing the activity of the Lon protease, we created a mutant of LVS in which the *lon* gene was deleted (LVS Δ*lon*) and asked whether the effects of a *lon* deletion phenocopied those of a *ppk* deletion. The results depicted in Fig. 6A show that the abundance of the *iglA* transcript was ~20-fold higher in LVS Δ*ppk* mutant cells and ~11-fold higher in LVS Δ*lon* mutant cells, compared to that in WT LVS cells. We also found that the abundance of the (p)ppGpp/PigR/MglA/SspA-regulated *FTL_1219* transcript was ~10-fold higher in LVS Δ*ppk* mutant cells and ~7-fold higher in LVS Δ*lon* mutant cells, compared to that in WT LVS cells (Fig. 6A). In support of the idea that polyphosphate exerts a portion of its regulatory effects through Lon, the abundance of the *iglA* and *FTL_1219* transcripts was similar in LVS Δ*ppk* mutant cells and in cells of an LVS Δ*ppk* Δ*lon* double mutant (Fig. 6A). Consistent with the results of our analyses of the *iglA* transcript, Western blotting revealed that the product of the *iglC* gene (IglC) was higher in LVS Δ*ppk* mutant cells, in LVS Δ*lon* mutant cells, and in cells of an LVS Δ*ppk* Δ*lon* double mutant, than in WT cells (Fig. 6B). Furthermore, IglC was slightly less abundant in LVS Δ*lon* mutant cells than in LVS Δ*ppk* mutant cells, or in cells of the LVS Δ*ppk* Δ*lon* double mutant (Fig. 6B). These findings suggest that, like polyphosphate, the Lon protease can antagonize the expression of (p)ppGpp/PigR/MglA/SspA-regulated genes (although its effect appears somewhat reduced compared to that of polyphosphate). These findings support the possibility that in *F. tularensis*, polyphosphate exerts its negative effects on the expression of (p)ppGpp/PigR/MglA/SspA-regulated genes at least in part by stimulating the activity of the Lon protease.

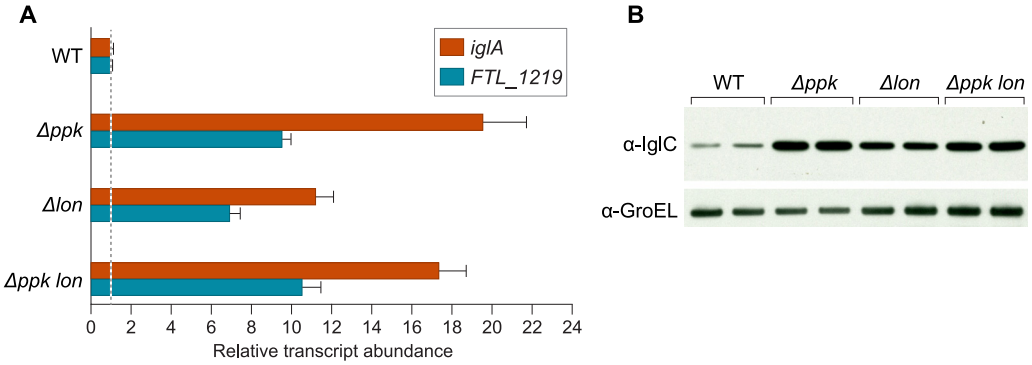

FIG 6 Both polyphosphate kinase and the Lon protease antagonize virulence gene expression in *F. tularansis*. (A) Effect of polyphosphate kinase and the Lon protease on expression of the *iglA* and *FTL_1219* genes. The relative abundance of the *iglA* and *FTL_1219* transcripts was determined in cells of the indicated strains by qRT-PCR. The figure shows data from a representative experiment with biological duplicates. Transcripts were normalized to *tul4*, and error bars represent ±1 standard deviation from the value (calculated using the mean threshold cycle) (B) Effect of polyphosphate kinase and the Lon protease on IglC abundance. The abundance of IglC in cells of the indicated strains was determined by Western blotting with an antibody against IglC. An antibody against GroEL was used as a loading control. Duplicate biological samples were tested for each strain, and a representative data set is shown.

DISCUSSION

The alarmone (p)ppGpp is a critical regulator of virulence gene expression in *F. tularensis* and is required for the expression of those genes that are positively regulated by PigR, MglA, and SspA (13). A prior study suggested that (p)ppGpp might exert its regulatory effects in *F. tularensis* indirectly by promoting the accumulation of polyphosphate in the cell, which in turn was required for formation of the MglA-SspA complex (50). We find that neither (p)ppGpp nor polyphosphate is required for formation of the MglA-SspA complex in *E. coli*. Furthermore, we find that (p)ppGpp promotes the interaction between PigR and the MglA-SspA complex in *E. coli*. Finally, we have obtained evidence that in *F. tularensis*, polyphosphate antagonizes the expression of genes that are positively regulated by (p)ppGpp, PigR, MglA, and SspA. Our findings thus establish that (p)ppGpp does not exert its positive regulatory effects in *F. tularensis* by promoting the accumulation of polyphosphate.

Our findings that neither (p)ppGpp nor PPK (and thus polyphosphate) are required in order for MglA and SspA to interact with one another were obtained using an *E. coli*-based two-hybrid assay. These findings refute the notion that (p)ppGpp exerts its regulatory effects in *F. tularensis* by influencing the formation of the MglA-SspA complex and run counter to those findings recently obtained using essentially the same two-hybrid system (50). Although we cannot readily explain the discrepancy between the findings reported here and those published in the competing study (50), we note that our findings in *E. coli* are consistent with our previous finding in *F. tularensis* that (p)ppGpp does not influence the amount of RNAP-associated MglA-SspA complex in the cell (13). Indeed, if (p)ppGpp were required for the accumulation of polyphosphate (as it is in *E. coli*) and if polyphosphate were strictly required for formation of the MglA-SspA complex, then cells that cannot synthesize (p)ppGpp would not be expected to contain any RNAP-associated MglA-SspA complex. Furthermore, our discovery that polyphosphate actually functions to repress genes that are positively regulated by (p)ppGpp shows that (p)ppGpp cannot exert its stimulatory effect on virulence gene expression in *F. tularensis* by promoting the accumulation of polyphosphate.

We have found that (p)ppGpp promotes the interaction between PigR and the MglA-SspA complex in both *F. tularensis* (13) and in *E. coli* (Fig. 2D). These findings are consistent with the recent discovery that ppGpp interacts directly with the MglA-SspA complex, with residues of MglA and SspA that are important for ppGpp binding being critical to their regulatory activities (15, 16). We note that if (p)ppGpp promotes the accumulation of polyphosphate in *F. tularensis*, as it does in *E. coli* (40), then (p)ppGpp would be expected to exert both positive and negative effects on virulence gene expres-

sion. Any effect of (p)ppGpp on the accumulation of polyphosphate in *F. tularensis* would serve to dampen the expression of virulence genes, possibly fine-tuning their expression.

Our findings indicate that PPK functions to antagonize virulence gene expression in *F. tularensis*, at least under the conditions of our experiments. Polyphosphate can bind directly to Lon from *E. coli* and stimulate its proteolytic activity (56), and we speculate that in *F. tularensis* polyphosphate produced by PPK exerts a portion of its negative effects on the expression of virulence genes by influencing the activity of the Lon protease. Prior work in *E. coli* and *Pseudomonas protegens* suggests that Lon degrades at least one important transcription regulator in each of these organisms (57, 58). In LVS, Lon is thought to degrade several virulence-associated factors and has been shown to be important for tolerance to certain stresses (59). Further work will be required to determine whether any of the key regulators of virulence gene expression in *F. tularensis*, including PigR, MglA, and SspA, serve as the substrates for Lon, or whether Lon might exert regulatory effects in *F. tularensis* through its ability to interact with the DNA (60, 61).

MATERIALS AND METHODS

Plasmids, strains, and growth conditions. *Francisella tularensis* subsp. *holarctica* strain LVS and the strain LVS Δ*pigR* have been previously described (11, 13). All *F. tularensis* strains were grown with aeration at 37°C in modified Mueller-Hinton (MH) broth (Difco) supplemented with 0.1% glucose, 0.025% ferric pyrophosphate, and 2% IsoVitaleX (BD Biosciences), or on cysteine heart agar (CHA; Difco) supplemented with 1% hemoglobin solution (BD Biosciences). When appropriate, 5 μg/ml kanamycin was used for selection. The *E. coli* strain XL1-Blue (Stratagene) was used for plasmid construction.

Construction of LVS deletion constructs and mutant strains. The strains LVS Δ*ppk*, LVS Δ*lon*, and LVS Δ*ppk* Δ*lon* contain an in-frame deletion of the *ppk* locus (*FTL_0544*), the *lon* locus (*FTL_0894*), or both the *ppk* and *lon* loci, respectively. The plasmids pEX-*ppk* and pEX-*lon* were constructed as described previously (11) and were used to generate the strains LVS Δ*ppk* and LVS Δ*lon* using allelic exchange (62). Note that pEX-*ppk* and pEX-*lon* are derivatives of the pEX plasmid (not the pEX2 plasmid) and contain a single copy of the *sacB* gene (11). The strain LVS Δ*ppk* Δ*lon* was generated from the strain LVS Δ*ppk* using allelic exchange and the plasmid pEX-*lon*. The strain LVS Δ*ppk ppx* was generated by insertional inactivation of the *ppx* locus (*FTL_0612*) in the strain LVS Δ*ppk*. The 5′ end of the *ppx* locus overlaps with the 5′ end of an *istfu2* repetitive element on the opposite strand, making it difficult to obtain a deletion of *ppx* using allelic exchange. The plasmid pEX-*ppx-frag* was used to generate the strain LVS Δ*ppk ppx*. The plasmid pEX-*ppx-frag* is a derivative of the pEX plasmid (11) that confers resistance to kanamycin and contains a 423-bp fragment of the *ppx* gene. This fragment corresponds to amino acids 51 to 191 of the product of *ppx* and includes a stop codon after the codon for amino acid 191. The suicide plasmid pEX-*ppx-frag* was electroporated into cells of strain LVS Δ*ppk*. Cells were plated on CHA supplemented with hemoglobin and 5 μg/ml kanamycin to select those in which the pEX-*ppx-frag* plasmid had integrated into the chromosome through a single homologous recombination event. All LVS strains were confirmed by PCR and/or Southern blotting. Similar to what has been found previously with cells of a *ppk* mutant of *F. tularensis* strain SchuS4 (49), cells of our LVS Δ*ppk* mutant exhibited an ~10-fold reduction in intracellular growth within J774 cells compared to those of WT LVS (see Fig. S1 in the supplemental material).

Plasmids for complementation analyses. Plasmid pF2-PPK-V was used for complementation of the LVS Δ*ppk* strain, and plasmid pF2 was used as the corresponding empty vector control. pF2-PPK-V confers resistance to kanamycin and directs the synthesis of full-length LVS PPK with a vesicular stomatitis virus glycoprotein (VSV-G) epitope tag fused to its C terminus and driven from a modified *groEL* promoter lacking a putative upstream promoter (UP) element. The empty vector pF2 containing the modified *groEL* promoter and lacking a putative UP element has been previously described (11). Plasmid pF2-PPK-V was generated by cloning an EcoRI- and BamHI-digested PCR product into EcoRI-BamHI-digested plasmid pF2. The PCR product for this vector was amplified from the full-length *F. tularensis ppk* gene using an appropriate template, a forward primer that introduced an EcoRI cleavage site and Shine-Dalgarno sequence to the 5′ end of the LVS *ppk* gene, and a reverse primer which added the sequence for the VSV-G epitope tag and a BamHI site to the 3′ end of the LVS *ppk* gene.

Plasmids for bacterial two-hybrid and bridge-hybrid assays. The plasmids pBR-MglA-ω, pBR-SspA-ω, pACTR-SspA-Zif, pACTR-MglA-Zif, pACTR-PigR-Zif, pACTR-AP-Zif, pCL-SspA, pCL-MglA, and pCL have been previously described (11, 13, 16).

Construction of *E. coli* strains for two-hybrid and bridge-hybrid assays. The *E. coli* strain KDZif1ΔZ was used as the wild-type reporter strain for the two-hybrid and bridge-hybrid assays and was previously described (51). The Δ*relA spoT* and Δ*ppk* derivatives of the *E. coli* reporter strain for the two-hybrid and bridge-hybrid assays were constructed from the strain FW102, which is the KDZif1ΔZ parent strain (51, 63). The Δ*relA spoT E. coli* reporter strain contains an in-frame deletion of the *E. coli relA* gene, a chloramphenicol resistance cassette inserted into the *spoST* locus (*spoS* is also referred to as *rpoZ* and encodes the ω-subunit of RNAP), and harbors an F′ episome containing the *lac* promoter derivative p*lacZ*if1-61, which drives expression of a linked *lacZ* reporter gene. This F′ episome has been previously described (51). To generate the Δ*relA spoT E. coli* reporter strain, the strain *relA*::*kan* was first generated through P1-mediated transduction of the allele *relA*::*kan* from the *relA* mutant strain of the Keio collection (64) to the recipient strain FW102. The kanamycin resistance cassette in the *relA*::*kan* allele is flanked by FLP recognition target (FRT) sites.

FLP recombinase was expressed from the plasmid pCP20 (65) in the strain FW102 *relA*::*kan* to excise the kanamycin resistance cassette and generate the Δ*relA* strain, which contains an in-frame deletion of the *E. coli relA* gene. The λ Red recombinase system (65) was used to generate the Δ*relA rpoZ-spoT*::*cat* strain. Specifically, PCR was used to amplify the *cat* gene from the pKD3 plasmid (65) using a forward primer that included the 40-bp sequence upstream of the 5′ end of *rpoZ* and a reverse primer that included the 3′ end of *spoT* and the 40 bp sequence downstream. The resulting PCR product, which contained the *cat* gene flanked by FRT sites and regions of homology to the 5′ end of *rpoZ* and the 3′ end of *spoT*, was electroporated into cells of the *E. coli* Δ*relA* strain containing λ Red helper plasmids, as described in reference 65. The desired Δ*relA rpoZ-spoT*::*cat* mutants were selected for as previously described (65). The previously described F′ episome containing the *lac* promoter derivative p*lacZ*if1-61, which drives expression of a linked *lacZ* reporter gene (51), was mated into the strain Δ*relA rpoZ-spoT*::*cat* to generate the strain ARZif1ΔAZT (referred to as the Δ*relA spoT* or ppGpp^0 *E. coli* reporter strain in the text).

The Δ*ppk E. coli* reporter strain for the two-hybrid and bridge-hybrid assays contains an in-frame deletion of the *E. coli ppk* locus and a chloramphenicol resistance cassette replacing the *E. coli rpoZ* gene (also referred to as *spoS*). The *rpoZ*::*cat* strain was first generated by P1-mediated transduction of the *spoS3*::*cat* allele from strain KDZif1ΔZ (51) into the recipient strain FW102 (63). P1-mediated transduction of the *ppk*::*kan* allele from the *ppk* mutant strain of the Keio collection (64) into the *rpoZ*::*cat* recipient strain generated the *ppk*::*kan rpoZ*::*cat* strain. The kanamycin resistance cassette in the *ppk*::*kan* allele is flanked by FRT sites. FLP recombinase was expressed in the *ppk*::*kan rpoZ*::*cat* strain to excise the kanamycin resistance cassette and generate the Δ*ppk rpoZ*::*cat* strain. The F′ reporter construct (51) was mated into the Δ*ppk rpoZ*::*cat* strain to generate the strain ARZif1ΔKZ (referred to in the text as the Δ*ppk E. coli* reporter strain). The strains ARZif1ΔKZ and ARZif1ΔAZT were confirmed by Southern blotting.

Bacterial two-hybrid and bridge-hybrid assays. The bacterial two-hybrid and bridge-hybrid assays were performed as previously described (11, 13, 16). Cells were grown with aeration at 37°C in LB supplemented with carbenicillin (100 μg/ml), tetracycline (10 μg/ml), and IPTG at the indicated concentration for the two-hybrid assay and with carbenicillin, spectinomycin (100 μg/ml), tetracycline, and IPTG at the indicated concentration for the bridge-hybrid assay. Cells were permeabilized with $CHCl_3$ and assayed for β-galactosidase activity as previously described (16). Assays were performed at least twice in duplicate. Duplicate measurements differed by less than 10%. Results shown are averages from a single representative experiment.

RNA isolation and qRT-PCR. LVS cells were grown in liquid culture (50 ml) in the presence of kanamycin with aeration at 37°C until cultures reached an optical density at 600 nm (OD_{600}) of ~0.25 to ~0.4. Ten milliliters of cells was harvested by centrifugation at 4,000 rpm for 20 min at 4°C. RNA was isolated using Tri-Reagent (Molecular Research Center, Inc.) as previously described (16). RNA quality was determined by gel electrophoresis. cDNA synthesis using Superscript III reverse transcriptase (Invitrogen) and quantitative reverse transcriptase PCR (qRT-PCR) were performed essentially as described previously (16, 66). The abundances of the *iglA* and *FTL_1219* transcripts were measured relative to that of the *tul4* transcript (11). qRT-PCR was performed at least twice on sets of biological triplicates. Data shown are from representative experiments.

Immunoblots. Cell lysates were separated by SDS-PAGE on 4 to 12% Bis-Tris NuPAGE gels in MES running buffer (Life Technologies). The XCell II Blot Module (Life Technologies) was used to transfer proteins to polyvinylidene difluoride (PVDF). Membranes were probed as described previously (66), using anti-GroEL (diluted 1:30,000) or anti-IglC (diluted 1:2,000) antibodies and subsequently incubated with polyclonal goat anti-rabbit antibody conjugated to horseradish peroxidase (diluted 1:5,000; Pierce) or polyclonal goat anti-mouse antibody conjugated to horseradish peroxidase (1:5,000; Pierce), respectively. Immunoblots were visualized using chemiluminescent detection as described previously (66).

Intramacrophage growth assays. Assays were performed with J774 murine macrophage-like cells essentially as described previously (66).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00460-17.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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